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The Interplay of Collagen IV, Tumor Necrosis Factor-α, Gelatinase B (Matrix Metalloprotease-9), and Tissue Inhibitor of Metalloproteases-1 in the Basal Lamina Regulates Sertoli Cell-Tight Junction Dynamics in the Rat Testis

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During spermatogenesis, preleptotene and leptotene spermatocytes must translocate across the blood-testis barrier formed by inter-Sertoli cell-tight junctions (TJs) from the basal compartment of the seminiferous epithelium adjacent to the basement membrane to the adluminal compartment at stages VIII–IX for further development. Because of the close proximity between extracellular matrix (ECM) that constitutes the basement membrane and the blood-testis barrier, we sought to investigate the role of ECM in Sertoli cell TJ dynamics. When Sertoli cells were cultured *in vitro* to initiate the assembly of the Sertoli cell TJ-permeability barrier, the presence of an anticollagen IV antibody indeed perturbed the barrier. Because ECM is known to maintain a pool of cytokines and TNFα has been shown to regulate TJ dynamics in other epithelia, we investigated whether TNFα can regulate Sertoli cell TJ function via its effects on collagen α3(IV) and other proteins that maintain the homeostasis of ECM. As expected, recombinant TNFα perturbed the Sertoli cell TJ-barrier assembly *in vitro* dose dependently. TNFα also inhibited the timely induction of occludin, which is known to associate with the Sertoli cell TJ-barrier assembly. Furthermore, TNFα induced the expression of Sertoli cell collagen α3(IV), gelatinase B (matrix metalloprotease-9, MMP-9) and tissue inhibitor of metalloproteases-1 but not gelatinase A (matrix metalloprotease-2), and promoted the activation of pro-MMP-9. These results thus suggest that the activated MMP-9 induced by TNFα is used to cleave the existing collagen network in the ECM, thereby perturbing the TJ-barrier. This in turn creates a negative feedback that causes TNFα to induce collagen α3(IV) and tissue inhibitor of metalloproteases-1 expression so as to replenish the collagen network in the disrupted TJ-barrier and limit the activity of MMP-9. Taken collectively, these observations strengthen the notion that ECM is involved in the regulation of junction dynamics in addition to its structural role in the testis. (*Endocrinology* 144: 371–387, 2003)

THROUGHOUT SPERMATOGENESIS, preleptotene and leptotene spermatocytes residing at the basal compartment of the seminiferous epithelium must translocate across the Sertoli cell-tight junctions (TJs) that constitute the blood-testis barrier (BTB) near the basal lamina, entering into the adluminal compartment for further development (for reviews, see Refs. 1–3). However, the mechanism(s) and molecules that regulate the opening and closing of the BTB to facilitate the timely passage of developing germ cells across the BTB are largely unknown (for a review, see Ref. 3). In other epithelia, such as those found in the small intestine and the collecting tubule in the kidney, TJs are located at the apical portion of adjacent cells, farthest away from the extracellular matrix (ECM). Below the TJ structures are the cell-cell actin-based adherens junctions (AJs), followed by cell-cell intermediate filament-based anchoring junctions called desmosomes. These structures in turn constitute the junctional complex, responsible for anchoring cells together forming an impermeable epithelium (for reviews, see Refs. 3 and 4). Gap junctions (GJs) are found behind this junctional complex, and the epithelial cells in turn attach to ECM via hemidesmosomes or focal contacts (for reviews, see Refs. 3 and 4). In contrast to these epithelia, Sertoli cell TJs that constitute the BTB in the testis are located adjacent to the basement membrane, a modified form of ECM in the testis (for a review, see Ref. 5) and are structurally present side by side with cell-cell actin-based AJs, cell-cell intermediate filament-based desmosomes, cell-matrix actin-based focal contacts, and cell-matrix intermediate filament-based hemidesmosomes (for a review, see Ref. 3). As such, the relative position of TJs and AJs in the testis is different from other epithelia (for reviews, see Refs. 2 and 3). Because of such morphological intimacy between TJs and the basal lamina, we sought to examine whether the ECM in the testis takes an active role in regulating TJ dynamics.

In the testis, the basal lamina is composed of: 1) the basement membrane, which is comprised of thin, sheetlike extracellular structures constituted by ECM proteins, such as

Abbreviations: AJ, Adherens junction; BTB, blood-testis barrier; Cas, Crk-associated substrate; ECM, extracellular matrix; ES, ectoplasmic specialization; FAK, focal adhesion kinase; GCCM, germ cell-conditioned medium; GJ, gap junction; GSK-3β, glycogen synthase kinase-3β; HRP, horseradish peroxidase; HSD, 3β-hydroxysteroid dehydrogenase; ILK, integrin-linked kinase; MMP, matrix metalloproteinase; SAPK/JNK, stress-activated protein kinase/Jun-terminal kinase; SCCM, Sertoli cell-enriched culture medium; STBM, seminiferous tubule basement membrane; TER, transepithelial electrical resistance; TIMP, tissue inhibitor of metalloproteinases; TJ, tight junction; uPA, urokinase plasminogen activator; ZO-1, zonula occludens-1.

type IV collagen, laminin, heparan sulfate proteoglycan (6), entactin (7), and other substances; 2) a thin collagen layer; and 3) a layer of peritubular myoid cells (for a review, see Ref. 5). ECM is known to play an important role in the regulation of Sertoli cell function by affecting its morphology, differentiation, and migration (for a review, see Ref. 5). Modifications of the basement membrane by passive transfer of antibodies raised against seminiferous tubule basement membranes (STBMs; Ref. 8) or noncollagenous fraction of STBMs have been shown to cause focal sloughing of the seminiferous epithelium in the rat (9). Yet it is not known whether ECM plays any role in the regulation of TJ dynamics in the testis. Previous studies have demonstrated the presence of type IV collagen in the rat testis (10). Also, the expression of collagen α3(IV) chain peaked at 10–20 d after birth coinciding with the assembly of the BTB in the mouse testis at approximately postnatal d 13 (11). As such, we thought it pertinent to examine whether both Sertoli and germ cells contribute to the pool of collagen α3(IV) in the basal lamina and whether collagen α3(IV) can affect Sertoli TJ assembly *in vitro*.

The homeostasis of ECM proteins are regulated, at least in part, by the coordinated activity between proteases and protease inhibitors (for reviews, see Refs. 12 and 13). For instance, matrix metalloproteases (MMPs), such as MMP-9 and MMP-2, and tissue inhibitors of metalloproteases (TIMPs), such as TIMP-1 and TIMP-2, produced by testicular cells under the hormonal and paracrine regulation (14), are believed to take part in these processes because collagen IV and laminin, the major components in STBMs, are putative substrates for MMP-2 and MMP-9 (for reviews, see Refs. 13 and 15). Moreover, abnormal thickening and multiple lamination of the STBM can induce infertility, further implicating the importance of ECM homeostasis for normal testis function (for a review, see Ref. 5).

There is accumulating evidence that the expression and action of MMPs and TIMPs can be regulated by cytokines, such as TNFα (for reviews, see Refs. 12 and 13). Indeed, rats administrated with TNFα displayed a massive loss of germ cells from the adluminal compartment (16). Other studies have shown that TNFα affects TJ dynamics in both epithelial and endothelial cells (for a review, see Ref. 17). Furthermore, TNFα can inhibit the expression of occludin, a TJ-integral membrane protein (for a review, see Ref. 3), in HT-29/B6 cell line (18). As such, we hypothesized that the role of TNFα on TJ dynamics may rely not only on its effects on TJ proteins, but it can also be mediated, at least in part, via the ECM proteins, such as collagen, MMPs, and TIMPs. To test this hypothesis, we have investigated: 1) the expression of TNFα by Sertoli cells during the assembly of the Sertoli cell TJ-permeability barrier; 2) the effects of recombinant TNFα on the Sertoli cell TJ-barrier; 3) its effects on the expression of collagen, MMPs, TIMPs, and TJ-associated proteins during Sertoli cell TJ-barrier assembly; and 4) the downstream signaling pathway mediating these effects.

Materials and Methods

Animals

Male Sprague Dawley rats were obtained from Charles River Laboratories, Inc. (Kingston, MA). The use of animals for this study was approved by the Rockefeller University Animal Care and Use Committee with protocol nos. 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 (19–21). At low cell density (5×10^4 cells/cm^2), Sertoli cell TJ barrier could not form, largely because of the lack of proximity between cells rendering them incapable of assuming the columnar shape, which is common in Sertoli cells when they form TJs *in vitro* (20, 22–25). Nonetheless, both AJs and GJs were present (20, 21, 26). Isolated cells were plated at 5×10^4 cells/cm^2 in 100-mm Petri dishes in 9-ml serum-free Ham's F12 nutrient mixture (F12) and DMEM (1:1, vol/vol) (~4.5×10^6 cells/9 ml per 100-mm dish) supplemented with 15 mM HEPES, 1.2 g/liter sodium bicarbonate, 10 μg/ml bovine insulin, 5 μg/ml human transferrin, 2.5 ng/ml epidermal growth factor, 20 mg/liter gentamicin, and 10 μg/ml bacitracin. At high cell density (0.75 or 1×10^6 cells/cm^2), cells were plated onto Matrigel (Collaborative Biochemical Products, Bedford, MA)-coated (diluted 1:7 with F12/DMEM, vol/vol) 12-well dishes (Corning, Inc., Corning, NY) as previously described (21, 26, 27); TJs, AJs, and GJs were formed. These cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 (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, cultures were hypotonically treated with 20 mM Tris, pH 7.4, at 22 C for 2.5 min to lyse residual germ cells (28). This was followed by two successive washes with F12/DMEM to remove germ cell debris. Media were replaced every 24 h. Cell cultures were terminated at specific time points by RNA STAT-60 for RNA extraction.

For the detection of collagen α3(IV) protein, Sertoli cells cultured at low cell density were lysed to prepare whole-cell lysates on d 3 (*i.e.* 24 h after hypotonic treatment) for immunoblotting. For the preparation of Sertoli cell-enriched culture medium (SCCM), cells were cultured for an additional 8 d after hypotonic treatment, spent media were collected on the following d 4 and 8 as previously described (19). For adult Sertoli cell cultures, Sertoli cells were isolated from rats at 45 and 90 d of age with a purity of about approximately 95% as previously described (29). The purity of these Sertoli cell cultures were analyzed microscopically (29, 30) and by RT-PCR using primer pairs specific to markers of Leydig cells [3β-hydroxysteroid dehydrogenase (HSD)], germ cells (c-Kit receptor), and peritubular myoid cells (fibronectin; Ref. 30) (PBS containing 0.1% BSA, wt/vol).

Sertoli cells cultured with recombinant human TNFα protein. Sertoli cells (0.75×10^6 cells/cm^2), isolated as described above, were cultured on Matrigel-coated 12-well dishes (effective surface area, ~3.8 cm^2/dish, containing 3 ml F12/DMEM). Recombinant human TNFα protein (Calbiochem, La Jolla, CA) at concentrations of either 10 ng/ml or 100 ng/ml was added to Sertoli cell cultures at time 0. TNFα was prepared as a stock solution of 10 μg/ml in PBS (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4, at 22 C) containing 0.1% BSA (wt/vol). Cultures were hypotonically treated 48 h thereafter to remove residual germ cells as described above. Media containing TNFα was replenished every 24 h. Cultures were terminated at specific time points for RNA extraction and whole-cell lysate preparation, and media were collected as SCCM. Controls included Sertoli cells cultured alone in F12/DMEM without TNFα or vehicle alone (PBS containing 0.1% BSA, wt/vol).

Germ cell isolation. Total germ cells were isolated from rat testes at 10, 15, 20, 45, 60, 90, and 200 d of age by a mechanical procedure (31). When the final preparation was analyzed by DNA flow cytometry (31), it consisted largely of spermatogonia, spermatocytes, and round spermatids with a relative percentage of 17%, 19%, and 64%, respectively, because elongate spermatids were removed in the glass wool filtration step (31). 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 (31–33); c-Kit receptor, a spermatogonium product (34); HSD, a Leydig cell product (35); and fibronectin, a peritubular myoid cell product (36). Cell number of freshly isolated germ cells was determined by a hemocytometer. Freshly isolated germ cells were terminated for RNA extraction and for cell lysate preparation. To obtain germ cell-conditioned medium (GCCM), freshly isolated germ cells from

90-d-old rat were cultured at 0.3×10^6 cells/cm^2 in 100-mm dish (22.5×10^6 cells/9 ml per 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 as described (37).

Effects of anticollagen antibody or human recombinant TNFα protein on the assembly of Sertoli cell TJ-barrier in vitro

Freshly isolated Sertoli cells were plated on Matrigel-coated bicameral units (Millicell HA-filters, effective surface area, ~0.6 cm^2, Millipore Corp., Bedford, MA) at a density of 0.75×10^6 cells/cm^2 (20) and treated as cultures at d 0. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 (vol/vol) at 35 C with 0.5 ml F12/DMEM each in the apical and basal chamber of the bicameral unit. Media were replaced every 24 h. The assembly of the Sertoli cell TJ-barrier was assessed by measuring the transepithelial electrical resistance (TER) across the cell epithelium using a Millicell electrical resistance system (Millipore Corp.) every 24 h as described (21, 25, 38, 39). In experiments targeted to assess the effects of anticollagen antibody on the assembly of the Sertoli cell TJ-barrier, 1 or 5 μg anticollagen IV IgG (rabbit antihuman collagen IV, catalog no. YMPS44, Accurate Chemical & Scientific Corp., Westbury, NY) was added to both the apical and basal compartments of bicameral units at the time of cell plating and to the daily replacement media. In control experiments, 0.1 or 1 μg rabbit γ-globulin (Sigma, St. Louis, MO) was used.

To assess the effects of TNFα on the Sertoli TJ-barrier, 10 or 100 ng/ml of recombinant human TNFα were added to the basal compartment of the bicameral unit immediately following cell plating. Preliminary experiments had shown that its inclusion in the apical compartment had no effects on the TJ-barrier, seemingly suggesting that TNFα receptors were recruited only to the basal region of polarized Sertoli cells after forming an epithelium. TNFα at desired concentrations was also included in the replacement medium. Sertoli cells cultured alone in F12/DMEM without TNFα or with vehicle (PBS containing 0.1% BSA, wt/vol) were used as controls. To assess the specificity of TNFα treatment, TNFα was removed by two successive washes in selected experiments on d 2 to examine whether the perturbed TJ-barrier was capable of resealing. Each time point had triplicate cultures, and each experiment was repeated three times using different batches of Sertoli cells.

Semiquantitative RT-PCR

Total RNA was isolated from tissues or cells by RNA STAT-60 (Tel-Test "B" Inc., Friendswood, TX) according to the manufacturer's protocol. Semiquantitative RT-PCR was performed essentially as earlier described (26, 27, 40). To enhance the detection sensitivity and yield semiquantitative data for analysis, trace amount of [γ-^{32}P]-labeled primers were included in the RT-PCR tubes. Briefly, the sense primers of a target gene and S16 were labeled at the 5′-end with [γ-^{32}P]-dATP (specific activity, 6000 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) using T_4 polynucleotide kinase (Promega Corp., Madison, WI). Approximately 1×10^6 cpm were used per PCR. The ratio of the [γ-^{32}P]-labeled sense primer of a target gene to [γ-^{32}P]-labeled S16 was the same as the unlabeled primers. To ensure the linearity of the target gene and S16 during their amplification, 10-μl aliquots of PCR products at 18, 20, 22, 24, and 26 cycles were withdrawn and resolved onto 5% *T* polyacrylamide gels using Tris-borate EDTA buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0, at 22 C) as a running buffer in preliminary experiments. Also, different concentrations of primer pairs and reverse transcription products and annealing temperatures were used in preliminary experiments to ensure that the production of each target gene and S16 in each PCR experiment reported herein 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. However, the linearity for the target gene was at its exponential phase in the PCR. Following gel electrophoresis, PCR products were visualized by ethidium bromide staining, and autoradiography was performed using X-OMAT AR film (Eastman Kodak Co., Rochester, NY). The authenticity of the PCR products for collagen α3(IV), MMP-9, MMP-2, TNFα, occludin, ZO-1, TIMP-1, and S-16 (Table 1) was confirmed by direct nucleotide sequencing as described (27, 41).

Immunoblotting analysis

For immunoblotting, total cell lysates were prepared as follows: 1 ml lysis buffer [0.125 M Tris, pH 6.8, at 22 C containing 1% SDS (wt/vol), 2 mM EDTA, 2 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, 1.6% 2-mercaptoethanol (vol/vol), 1 mM sodium orthovanadate (a protein tyrosine phosphatase inhibitor), and 0.1 μM sodium okadate (a protein Ser/Thr phosphatase inhibitor)] was added to the freshly isolated germ cells or to the Sertoli cells in either 100-mm dishes or each well of a 12-well dish. Samples were vortexed, incubated at room temperature for 5–10 min, centrifuged at $15{,}000 \times g$ at 4 C to remove cellular debris. The clear supernatant was used as whole-cell lysate. SCCM and GCCM were prepared as previously described (19, 37). Protein content was estimated by Coomassie blue dye-binding assay using BSA as a standard (42). Equal amounts of proteins from each sample (100 μg per lane for cell lysates and 40 μg per lane for SCCM and GCCM) were resolved onto 7.5%, 10%, or 12.5% *T* SDS-polyacrylamide gels by SDS-PAGE under reducing conditions (43) and electroblotted onto nitrocellulose membranes. 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% (vol/vol) Tween.

For immunoblottings, the following primary antibodies were used: rabbit anticollagen type IV, which also cross-reacted with α1, 2, 3, and 5 chains of type IV collagen (catalog no. sc-11360, lot no. B021), goat anti-TNFα (catalog no. sc-1351, lot no. K121), goat anti-TIMP-1 (catalog no. sc-6834), rabbit anti-β1-integrin (catalog no. sc-8978, lot no. B221),

TABLE 1. Primers used for semiquantitative RT-PCR to assess the steady-state mRNA levels of collagen α3 (IV), MMP-9, MMP-2, TNF-α, occludin, ZO-1, TIMP-1, and S16

Target gene	Primer sequence	Orientation	Position	Length (bp)	Annealing temp. used (C)	Reference
Collagen α3 (IV)	5′-AACACAGCCACTCTACAG-3′	Sense	69–86	148	58	79
	5′-GTTACACACGTTATCGAC-3′	Antisense	199–216			
MMP-9	5′-AGTTTGGTGTCGCGGAGCAC-3′	Sense	509–528	754	58	80
	5′-TACATGAGCGCTTCCGGCAC-3′	Antisense	1243–1262			
MMP-2	5′-ATCTGGTGTCTCCCTTACGG-3′	Sense	2413–2432	150	58	81
	5′-GTGCAGTGATGTCCGACAAC-3′	Antisense	2543–2562			
TNF-α	5′-CAAGGAGGAGAAGTTCCCAA-3′	Sense	177–196	500	58	82
	5′-CGGACTCCGTGATGTCTAAG-3′	Antisense	657–676			
Occludin	5′-CTGTCTATGCTCGTCATCG-3′	Sense	202–220	294	61	Genebank accession no. AB016425
	5′-CATTCCCGATCTAATGACGC-3′	Antisense	476–495			
ZO-1	5′-GCCTCTGCAGTTAAGCAT-3′	Sense	61–78	249	60	83
	5′-AAGAGCTGGCTGTTTTAA-3′	Antisense	292–309			
TIMP-1	5′-TGGTTATAAGGGCTAAAT-3′	Sense	137–154	212	58	84
	5′-GCCCGCGATGAGAAACTC-3′	Antisense	331–348			
S16	5′-TCCGCTGCAGTCCGTTCAAGTCTT-3′	Sense	15–38	385	58–61	85
	5′-GCCAAACTTCTTGGATTCGCAGCG-3′	Antisense	376–399			

goat antiintegrin-linked kinase (ILK) (catalog no. sc-7516, lot no. G111), mouse antiglycogen synthase kinase-3β (GSK-3β) (catalog no. sc-7291, lot no. E171), mouse antiphospho-c-Jun (catalog no. sc-822, lot no. G191), rabbit antifocal adhesion kinase (FAK) (catalog no. sc-558, lot no. J051) and mouse anti-c-Src (catalog no. sc-8056, lot no. C051) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-α3 chain of collagen IV (catalog no. MAB3, lot no. 97) was from Weislab (Lund, Sweden). Rabbit anti-MMP-9 (catalog no. AB805, lot no. 21100763) was from Chemicon (Temecula, CA). Rabbit antioccludin (catalog no. 71-1500) was from Zymed Laboratories, Inc. (Burlingame, CA). Rabbit antiphospho-GSK-3α/β (Ser21/9) (catalog no. 9331, lot no. 2A) and rabbit antiphospho-stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK) pathway kit (catalog no. 9912, lot no. 4), which included phospho-specific anti-SEK1/MKK4 antibody, were from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit antiphospho-FAK (Tyr397) (catalog no. 07-012, lot no. 21019) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse anti-p130 Crk-associated substrate (Cas) (catalog no. P27820, lot no. 7) was 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.

Depending on the source of the primary antibody, the following secondary antibodies were used. These include goat antirabbit IgG-horseradish peroxidase (HRP) (catalog no. sc-2004), bovine antigoat IgG-HRP (catalog no. sc-2350) and goat antimouse IgG-HRP (catalog no. sc-2005) (Santa Cruz Biotechnology, Inc.). Both the catalog and lot numbers for each antibody used in this report were listed because preliminary experiments had shown that several antibodies from other vendors failed to yield satisfactory results. The immunoreactive target protein in a blot was visualized with an enhanced chemiluminescence system from Amersham Pharmacia Biotech (catalog no. RPN2106) and Biomax MR films (Eastman Kodak Co.).

Immunohistochemistry

Immunohistochemistry was performed to localize TNFα and MMP-9 in the seminiferous epithelium of normal adult rat testes essentially as previously described (44–46) using a Histostain SP kit (catalog no. 95-6143, Zymed Laboratories, Inc.). Frozen sections (~8 μm thick) obtained with a microtome in a cryostat were air dried, fixed in Bouin's fixative, and treated with 3% hydrogen peroxide in methanal to block endogenous peroxidases. To minimize nonspecific antibody binding, sections were incubated with serum-blocking solution. Sections were then incubated with primary antibodies in a moist chamber at 35 C overnight. Primary antibodies were used with the following dilutions: goat anti-TNFα (1:50) and rabbit anti-MMP-9 (1:75). Sections were rinsed and incubated with biotinylated rabbit antigoat IgG or goat antirabbit IgG for 30 min and then with the streptavidin-peroxidase conjugate for 10 min. Sections were then treated with the aminoethylcarbazole mixture (a substrate-chromogen mixture) for 5–10 min, counterstained in hematoxylin, and mounted. Sections were examined and photographed in BX-40 (Olympus Corp., Melville, NY). Controls include sections incubated with normal rabbit serum by substituting the primary antibody, PBS by substituting the primary antibody, the primary antibody preabsorbed with the corresponding peptide used for antibody generation, and normal rabbit or normal goat serum instead of the biotinylated second antibody.

Statistical analysis

Multiple comparisons were performed using one-way ANOVA followed by Tukey's honest significant different test to compare selected pairs of experimental groups using the GB-STAT statistical analysis software package (version 3.0, Dynamic Microsystems, Inc., Silver Spring, MD). In some experiments, *t* test was also performed to compare a treatment group with its corresponding control.

Results

Purity of testicular cells

The purity of cells used in the studies described in this report was assessed by 1) direct microscopic examination; 2) RT-PCR analysis for testin, a putative Sertoli cell protein not expressed by normal germ cells (31–33), c-Kit receptor, a spermatogonium specific product (34), HSD, a Leydig cell product (35) and fibronectin, a peritubular myoid cell product (36); and/or 3) DNA flow cytometry (31, 37). For Sertoli cells isolated from testes using rats of different ages, microscopic analysis revealed that the cell purity was routinely greater than 95%. For germ cells, these preparations did not express testin, HSD, or fibronectin. These analyses also illustrate that these cell preparations were contaminated with a negligible number of other cell types. In selected experiments, germ cells were also subjected to DNA flow cytometry as described (31) to assess the relative percentages of spermatogonia, spermatocytes, and spermatids. For instance, germ cells isolated from adult rat testis had a relative percentages of 17%:19%:64% for spermatogonia (2 C, diploid):spermatocytes (S-phase and 4 C, tetraploid):spermatids (1 C, haploid). Elongated spermatids were not detected because they were removed in the glass wool filtration step (31).

Relative expression of collagen α3(IV) in Sertoli and germ cells

To determine whether Sertoli and germ cells express collagen α3(IV), RT-PCR and immunoblot analysis were performed. The steady-state mRNA level of collagen α3(IV) in germ cells was found to be comparable to Sertoli cells (Fig. 1, A and B). To confirm that the collagen mRNA detected in particular in germ cells could indeed be translated to a functional protein, immunoblotting analysis was performed (Fig. 1C) using both a rabbit anticollagen IV (Santa Cruz Biotechnology, Inc.) and mouse anti-α3 chain of collagen IV (Weislab) antibody. Because the rabbit polyclonal antibody from Santa Cruz is reactive with α1, 2, 3, and 5 chains of type IV collagen, two immunoreactive bands of approximately 170 kDa (type IV collagen α2 chain) and approximately 180 (type IV collagen α1/3 chain) kDa were detected in Sertoli and germ cell lysates (Fig. 1C). Only a single band of approximately 180 kDa was detected in GCCM but not SCCM; this immunoreactive band is likely the α1(IV) chain and/or α3(IV) chain (Fig. 1C). The approximately 180-kDa protein band was subsequently confirmed to be the α3(IV) chain using a mouse monoclonal antibody specific to the α3(IV) chain (data not shown). Only GCCM, but not SCCM, was shown to contain the α3(IV) chain (Fig. 1C). The lack of collagen α3(IV) chain in SCCM could be the result of collection time, which was d 8 *vs.* 16 h for GCCM because the expression of α3(IV) chain greatly diminished in Sertoli cells *in vitro* by d 5 (Fig. 2A).

Developmental regulation of collagen α3(IV) in Sertoli and germ cells and the testis

The steady-state mRNA level of collagen α3(IV) reduced significantly during Sertoli cell maturation (Fig. 1, D and E). In contrast, its expression increased significantly and peaked at 45–60 d of age in germ cells during maturation (Fig 1, F and G). During testis maturation, the steady-state mRNA level of collagen α3(IV) became detectable at 5 d of age and peaked at 10–20 d of age coinciding with the assembly of the BTB (Fig. 1, H and I). Because a decline of the α3(IV) chain in the aging testis at 90 d of age was detected (Fig. 1H) but the level of this collagen in germ cells at 90 d of age was relatively high (Fig. 1F), these data seemingly suggest that

the collagen α3(IV) chain expression by germ cells can be down-regulated by Sertoli cells in the testis *in vivo*. Another alternative yet possible explanation for the increases in collagen α3(IV) expression by germ cells at ages between 45 and 90 d shown in Fig. 1F may be that the collagen α3(IV) chain is up-regulated rapidly during germ germ isolation.

Changes in the expression of collagen α3(IV), MMP-9, and MMP-2 in Sertoli cell cultures during junction assembly in vitro

When Sertoli cells were cultured at low cell density (0.5×10^4 cells/cm^2) on Matrigel-coated dishes in serum-free F12/DMEM to allow the assembly of Sertoli cell AJs, it was associated with a significant but transient induction of collagen α3(IV) and MMP-9 (Fig. 2, A–D) and a steady rise in the expression of MMP-2 (Fig. 2, E and F) These results seemingly suggest that their expression correlates with Sertoli cell AJ assembly. At high cell density (1×10^6 cells/cm^2), a transient but significant increase in collagen α3(IV) and MMP-9 steady-state mRNA levels was detected between d 1 and 2 (Fig. 2, G–J) at the time Sertoli cell TJs were being assembled (see also Fig. 3 *vs.* Fig. 2), whereas MMP-2 was induced throughout the entire culture period (Fig. 2, K and L), implicating the involvement of collagen α3(IV), MMP-9, and MMP-2 in Sertoli TJ assembly and its maintenance.

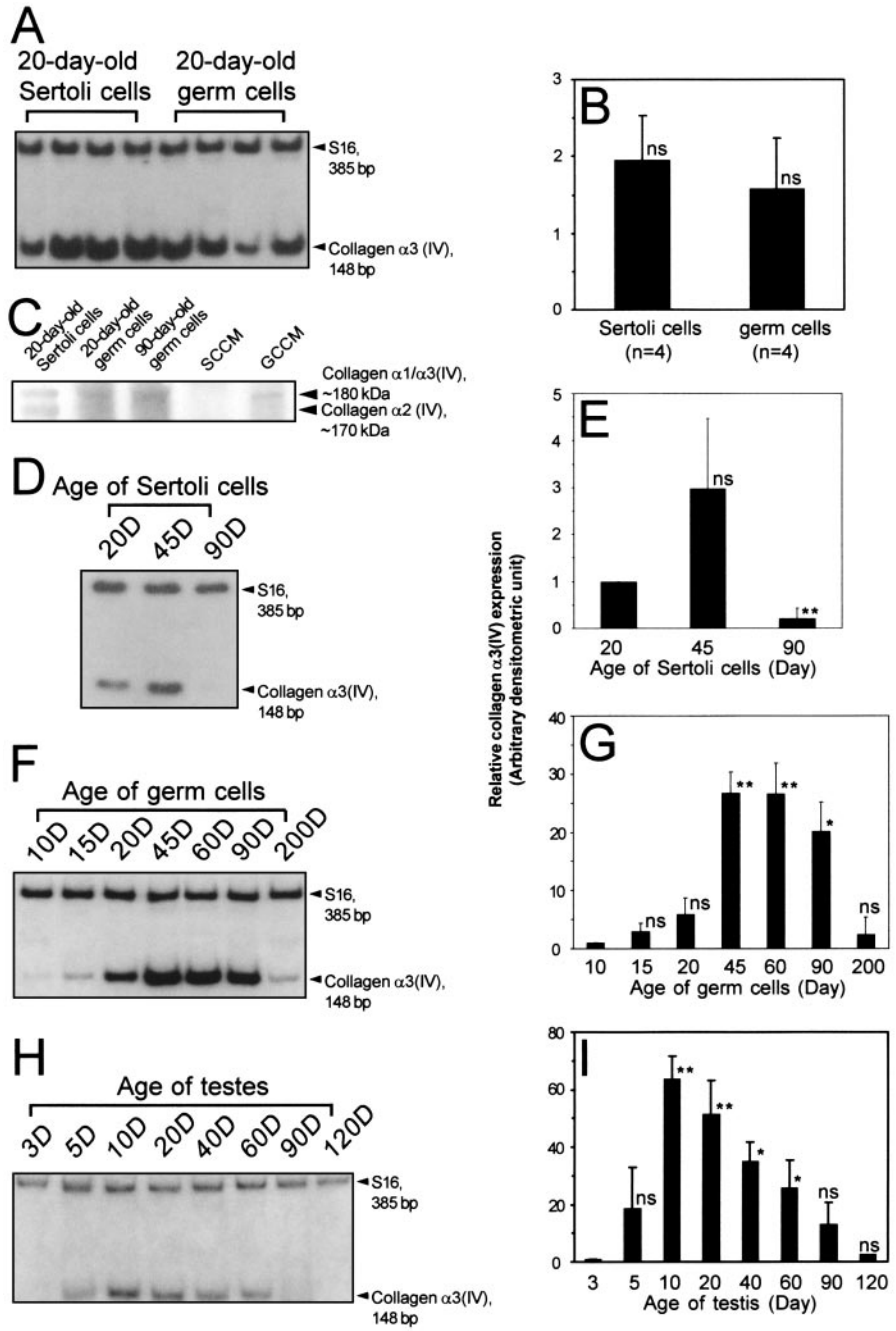
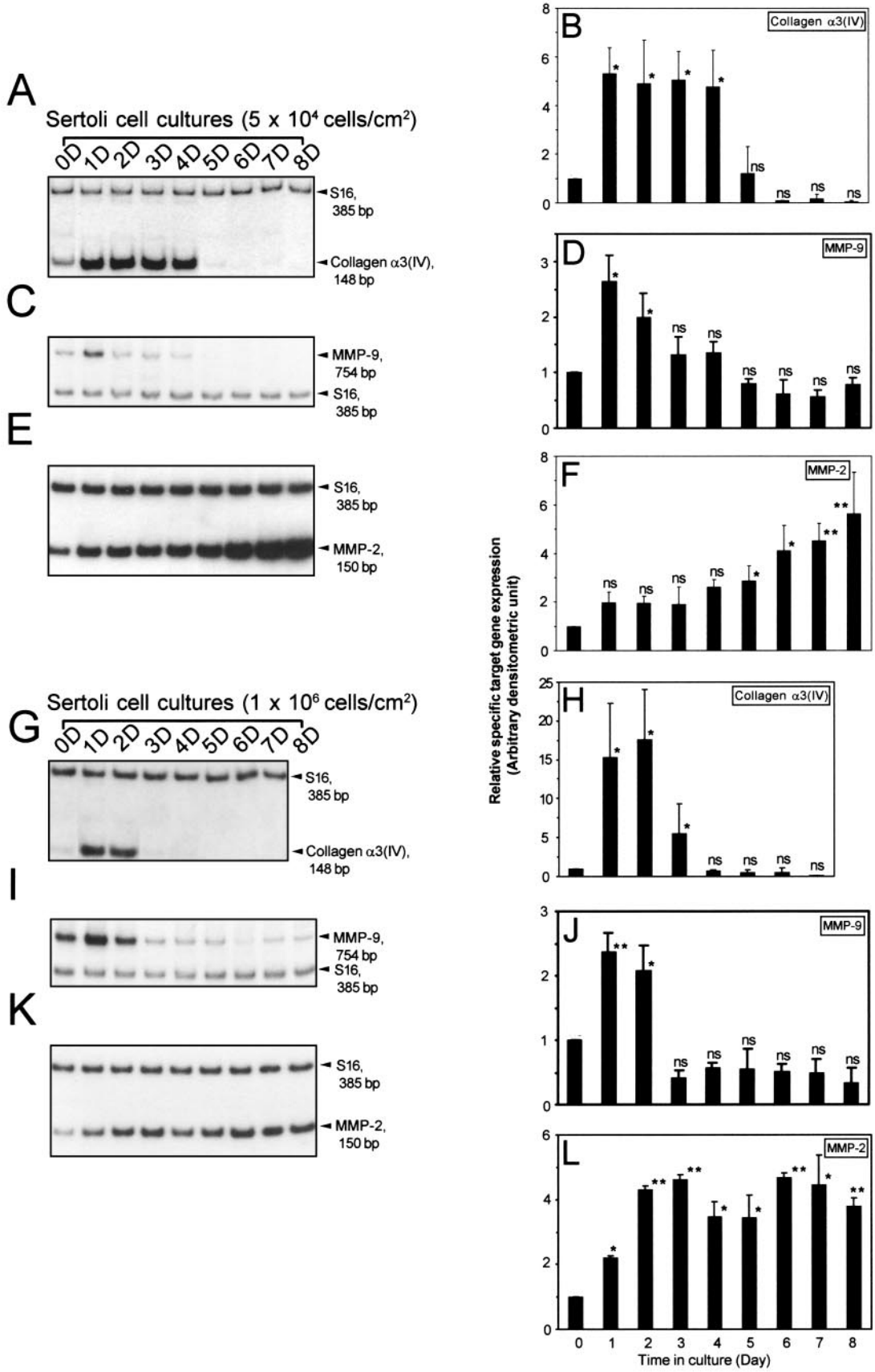


FIG. 1. A–I, Differential expression and protein level of collagen α3(IV) in Sertoli and germ cells, SCCM, and GCCM and changes of its expression in these cells and the testis during maturation. Total RNA and whole-cell lysates were extracted from Sertoli cells, germ cells, or whole testis as described in *Materials and Methods*. Sertoli cells (5×10^4 cells/cm^2) isolated from rats at specified ages were cultured *in vitro* for 3 d with hypotonic treatment on d 2 to remove contaminating germ cells before termination. Semiquantitative RT-PCR was performed to assess changes in the steady-state mRNA level of collagen α3(IV) using a primer pair specific to this target gene and coamplified with S16 (see Table 1). The purity of cells used in these studies was verified by microscopic and other analyses as described in *Materials and Methods.* A, An autoradiogram showing the relative steady-state mRNA level of collagen α3(IV) in Sertoli and germ cells isolated from 20-d-old rats. B, Corresponding densitometrically scanned results using autoradiograms, such as the one shown in A. C, Immunoblot analysis of collagen IV protein using whole-cell lysates from Sertoli and germ cells, SCCM, and GCCM with approximately 40 μg protein/lane. This analysis illustrates that the collagen α3(IV) mRNA detected in germ cells can translate into functional protein. D, F, and H, Autoradiograms showing changes in the steady-state collagen α3(IV) mRNA level in Sertoli and germ cells and testes during development, respectively. E, G, and I, Corresponding densitometrically scanned results using autoradiograms, such as those shown in D, F, and H and normalized against S-16. Each *bar* represents a mean ± 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 collagen α3(IV) in either cells or testes at other ages *vs.* d 20 (E), 10 (G), or 3 (I), 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.



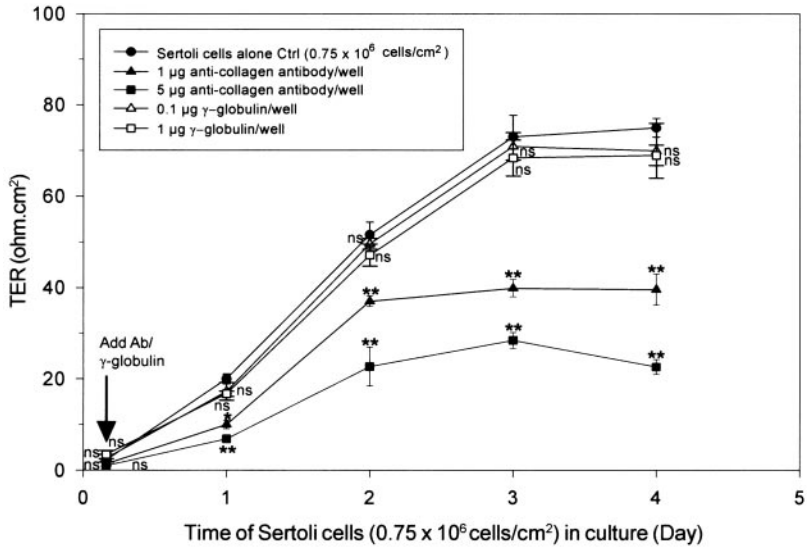


FIG. 3. Effects of anticollagen antibody on the assembly of inter-Sertoli tight junction-permeability barrier *in vitro*. Sertoli cells isolated from 20-d-old rats were cultured at a density of 0.75×10^6 cells/cm^2 on Matrigel-coated bicameral units for a period of 4 d to allow for the assembly of the inter-Sertoli tight junction-permeability barrier. The assembly of TJ-barrier was assessed by quantifying the TER across the Sertoli cell epithelium as described in *Materials and Methods*. Sertoli cell monolayers were exposed to either anticollagen antibody (DEAE affinity-purified IgG) or rabbit γ-globulin (control) by adding anticollagen IgG or γ-globulin at desired concentrations to both the apical and basal compartments of the bicameral units (see *arrow*). TER readings across the Sertoli cell epithelium were taken at specified time points. Anticollagen IgG or γ-globulin was also included in the daily replacement medium. Each time point represents triplicate cultures (mean ± SD) from three experiments, using different batches of Sertoli cells. *, Significantly different by *t* test, compared with the corresponding controls, $P < 0.05$; **, significantly different by *t* test, compared with the corresponding controls, $P < 0.01$; ns, not significantly different by *t* test, compared with the corresponding controls.

Effects of anticollagen antibody on the assembly of Sertoli TJ-permeability barrier in vitro

Because there was a transient but significant induction of collagen α3(IV) at the time Sertoli TJs were being assembled *in vitro,* it is logical to investigate whether a blockade of the collagen network by using an anticollagen IV antibody could perturb the assembly of Sertoli TJ-barrier. The assembly of the TJ-barrier was monitored by quantifying the TER across the Sertoli cell epithelium. It was noted that the TER across the cell epithelium soon after cells were plated *in vitro* rose steadily, which reached plateau by d 3–4 with a level of approximately 80 ohms·cm^2 (Fig. 3), consistent with results of earlier reports (25, 38, 39). The presence of an anticollagen IV antibody indeed perturbed the assembly of the Sertoli cell TJ-barrier *in vitro* manifested by a dose-dependent decline in TER, compared with Sertoli cells cultured alone (Fig. 3). These effects appeared to be specific because γ-globulin alone had no effects in perturbing the TER (Fig. 3). These results also suggest the potential significance of collagen IV in Sertoli cell TJ assembly.

Changes in the endogenous steady-state mRNA and protein levels of TNFα in Sertoli cell cultures during junction assembly in vitro

Previous studies have shown that an infusion of TNF to rats iv could perturb spermatogenesis by causing germ cell loss from the seminiferous epithelium (16). TNFα is also a known regulator of collagen and MMPs (47, 48). We sought to investigate whether the mechanism by which TNF-induced germ cell loss relates to its effects on ECM remodeling in the rat testis. The changes in the steady-state mRNA and protein levels of TNFα in Sertoli cells cultured at high and low cell density were first monitored (Fig. 4, A–D). However, during Sertoli cell TJ-barrier (Fig. 4, A and B, *vs.* Fig. 3) or AJ (Fig. 4, C and D) assembly, there was a time-dependent TNFα decline in Sertoli cell cultures (Fig. 4, A–D). These results seemingly suggest that the presence of TNFα may have a negative effect on the assembly and maintenance of Sertoli TJs and AJs. This trend of time-dependent plummeting in TNFα expression was verified by immunoblotting using an anti-TNFα antibody and 40 μg protein of SCCM

FIG. 2. A–L, Changes in the expression of collagen α3(IV), MMP-9, and MMP-2 by 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 (5×10^4 cells/cm^2) (A–F) or high cell density (1×10^6 cells/cm^2) (G–L). Cultures were terminated at specified time points for RNA extraction. Time 0 represents RNA STAT-60 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 collagen α3(IV) (A and G), MMP-2 (C and I), and MMP-9 (E and K) in Sertoli cells and were coamplified with S16 at the time of TJ assembly. B, D, F, H, J, and L are the corresponding densitometrically scanned results using autoradiograms, such as those shown in A, C, E, G, I, and K, and normalized against S-16 and cultures at time 0, which was arbitrarily set at 1. Each *bar* represents a mean ± SD of three experiments. Each experiment had replicate cultures. *, Significantly different from cultures at time 0 by *t* test, $P < 0.01$; **, significantly different from cultures at time 0 by *t* test, $P < 0.001$; ns, not significantly different by *t* test.

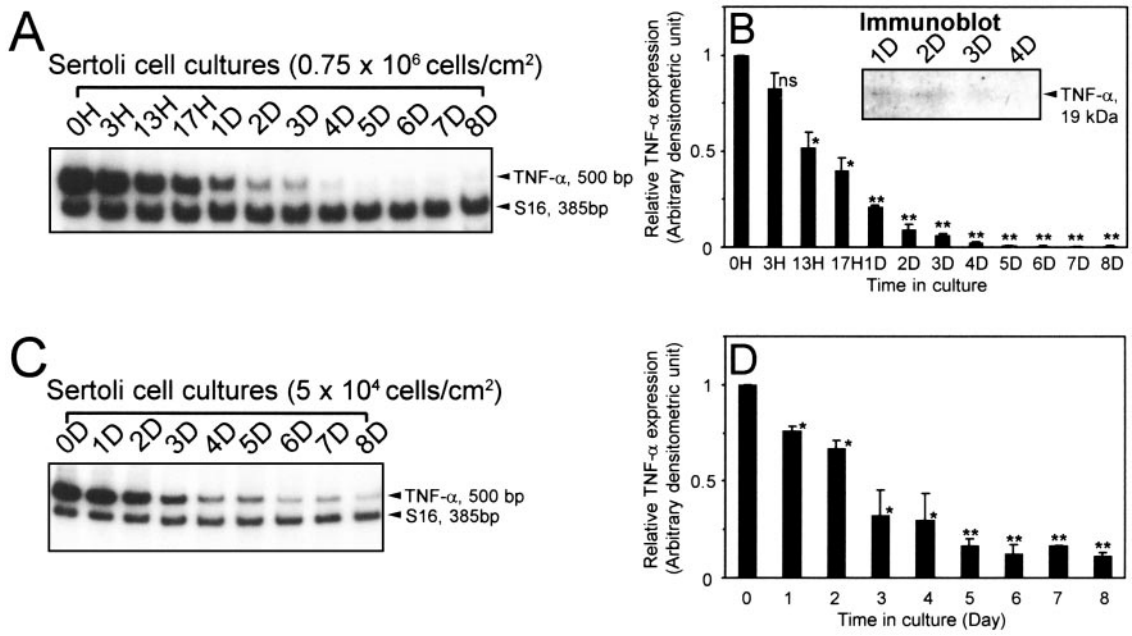


FIG. 4. A–D, Changes in the expression and protein level of TNFα during the assembly of inter-Sertoli cell TJs (A and B) and AJs (C and D) *in vitro*. Sertoli cells were cultured at either high (0.75×10^6 cells/cm^2) or low cell density (5×10^4 cells/cm^2). Cultures were terminated at specified time points for RNA extraction and the preparation of SCCM. Time 0 represents the lysis of Sertoli cells approximately 3 h after plating except in A and B in which 0H represents freshly isolated Sertoli cells terminated immediately after their isolation. Semiquantitative RT-PCR coamplified with S16 and immunoblotting were performed to assess changes in TNFα steady-state mRNA and protein levels, respectively. B and D, Corresponding densitometrically scanned results using autoradiograms, such as those shown in A and C, and normalized against S16 where cultures at time 0 were arbitrarily set at 1. Each *bar* represents a mean ± SD of three experiments. Each experiment had replicate cultures. *, Significantly different from cultures at 0H by *t* test, $P < 0.01$; **, significantly different from cultures at 0H by *t* test, $P < 0.001$; ns, not significantly different.

from the same experiment shown in Fig. 4A (see *inset* in Fig. 4B) for analysis, which clearly demonstrated a significant reduction in TNFα (19 kDa) protein during TJ assembly.

Effects of recombinant TNFα protein on the assembly of Sertoli cell TJ-barrier in vitro

To verify the data shown in Fig. 4, that the changes in TNFα during TJ assembly indeed relate to the event of junction dynamics instead of an endogenous cellular maintenance event, we sought to investigate whether TNFα can indeed perturb the assembly of Sertoli cell TJs. As expected, the presence of TNFα perturbed the assembly of Sertoli cell TJs *in vitro* dose dependently (Fig. 5). When TNFα was removed by two successive washes on d 2, the perturbed Sertoli cell TJ-barrier resealed as manifested by a rise in TER, illustrating the effects of TNFα on the assembly of Sertoli cell TJs were likely not the result of cell cytotoxicity (Fig. 5).

Effects of recombinant TNFα on the steady-state mRNA and/or protein levels of occludin and ZO-1 in cultured Sertoli cells during Sertoli cell TJ-barrier assembly in vitro

Recent studies have shown that there is a transient but significant induction of occludin (a TJ-integral membrane protein; Refs. 38 and 39; for a review, see Ref. 3) and zonula occludens-1 (ZO-1, a TJ-associated peripheral protein that has a signaling function and can also act as a scaffold protein at the site of TJs; Refs. 25 and 26; for a review, see Ref. 3) at the time of Sertoli TJ assembly *in vitro*. We thus sought to investigate whether the TNFα-induced perturbation on the Sertoli TJ barrier is mediated via its effects on these two TJ-proteins. In control cultures without TNFα, there was an 8-fold and 15-fold increase in occludin and ZO-1 expression, respectively, coinciding with the Sertoli cell TJ assembly (Fig. 6 *vs.* Fig. 5). However, TNFα at 10 and 100 ng/ml inhibited this transient induction of occludin expression, but not ZO-1, when Sertoli TJs were assembled (Fig. 6). These changes in the occludin mRNA level during Sertoli cell TJ assembly in the absence or presence of TNFα were confirmed by immunoblot analysis (see *lower panel* in Fig. 6A). These results thus suggest that the perturbing effects of TNFα on the Sertoli cell TJ assembly may be mediated, at least in part, via its inhibitory effects on occludin, a building block of TJs (for a review, see Ref. 3), but not via ZO-1, which is a TJ-associated signaling protein.

Effects of recombinant TNFα on the steady-state mRNA and/or protein levels of collagen α3(IV), MMP-9, MMP-2, and TIMP-1 in cultured Sertoli cells during TNFα-induced disruption of the Sertoli cell TJ-permeability barrier in vitro

We next sought to examine whether collagen α3(IV), MMP-9, MMP-2, and TIMP-1 were involved during the TNFα-induced disruption of the Sertoli cell TJ barrier (Fig. 5). It was noted that TNFα at 10 and 100 ng/ml was capable of inducing the steady-state mRNA of collagen α3(IV) and MMP-9; however, it had no effects on MMP-2 (Fig. 7, A, B,

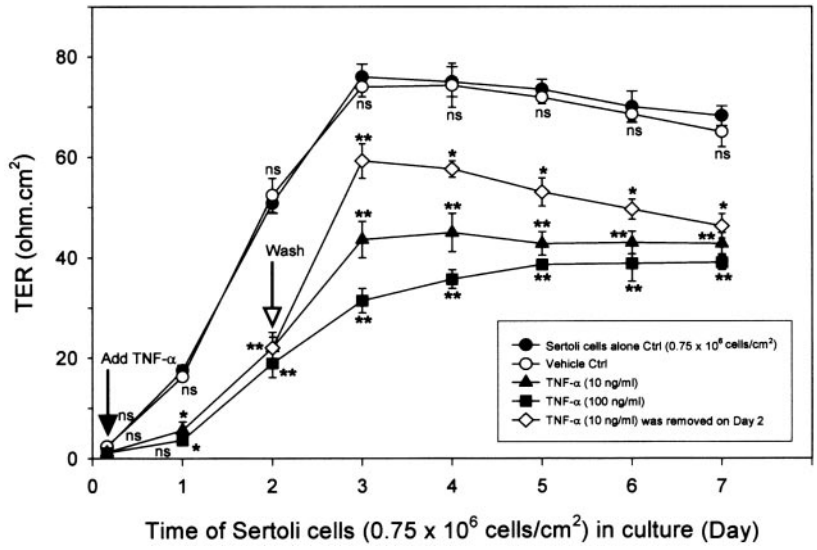


FIG. 5. Effects of recombinant TNFα on the assembly of the inter-Sertoli tight junction-permeability barrier *in vitro*. Sertoli cells were cultured at a density of 0.75×10^6 cells/cm^2 on Matrigel-coated bicameral units to allow the assembly of the TJ-permeability barrier. Different concentrations of TNFα were included in the basal compartments of bicameral units throughout the culture period. The effects of TNFα on the assembly of Sertoli cell TJs *in vitro* were assessed by quantifying TER across the cell epithelium at specified time points. In selected experiments, TNFα was removed by two successive washes with F12/DMEM on d 2, and the replacement media also did not contain TNFα (see *arrow*). It was noted that TNFα had a dose-dependent effect on perturbing the Sertoli cell TJ-barrier. These TNFα effects appear to be specific because removal of TNFα permitted resealing of the TJ-barrier. Each time point is a mean ± SD of triplicate cultures from two experiments. *, Significantly different by *t* test, compared with the corresponding controls, $P < 0.05$; **, significantly different by *t* test, compared with the corresponding controls, $P < 0.01$; ns, not significantly different by *t* test, compared with the corresponding controls.

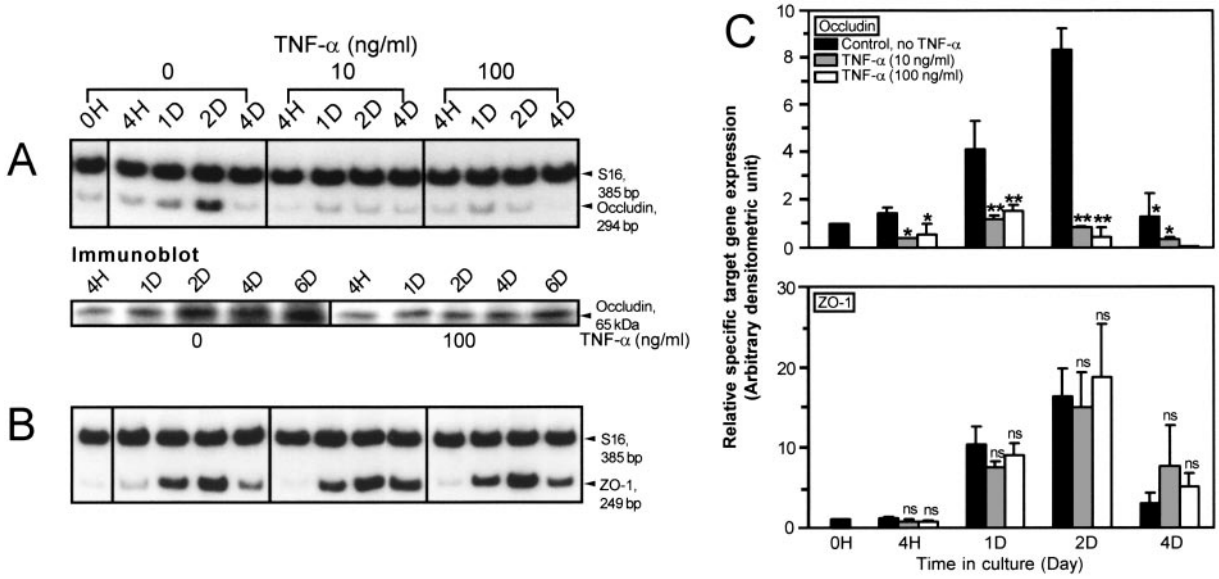


FIG. 6. A–C, Effects of recombinant TNFα on occludin and ZO-1 gene expression during Sertoli cell culture *in vitro*. Sertoli cells (0.75×10^6 cells/cm^2) isolated from 20-d-old rats were cultured with 10 or 100 ng/ml TNFα or without TNFα (control) immediately after their isolation as described in *Materials and Methods*. Cell cultures were terminated at specified time points for RNA extraction and protein analysis. Semiquantitative RT-PCR and immunoblot were performed to assess the changes in different target gene expression. A and B, Autoradiograms showing changes in the expression of occludin and ZO-1 in Sertoli cells during the assembly of TJ-permeability barrier, respectively, in the absence (control) or presence of either 10 or 100 ng/ml TNFα. The *lower panel* in A is the immunoblot to visualize occludin protein using Sertoli cell lysates treated with or without (control) 100 ng/ml TNFα (~100 μg protein/lane). C, Corresponding densitometrically scanned results using autoradiograms, such as those shown in A and B, and normalized against S16 where the level of the corresponding target gene at OH was arbitrarily set at 1. Each *bar* represents a mean ± SD using results from three experiments. Each experiment had replicate cultures. *, Significantly different by *t* test, compared with the corresponding controls, $P < 0.05$; **, significantly different by *t* test, compared with the corresponding controls, $P < 0.01$; ns, not significantly different by *t* test, compared with the corresponding controls.

C, and E). The protein level of the collagen α3(IV) chain in Sertoli cell lysates was also induced by TNFα at 100 ng/ml using a mouse anticollagen α3(IV) (Weislab) antibody consistent with the results of RT-PCR analysis (Fig. 7A, *lower panel vs. upper panel*). MMP-9 protein was also activated by TNFα in cultured Sertoli cells and both the pro-MMP-9 (92 kDa) and active-MMP-9 (84 kDa) were detected by immunoblots (Fig. 7B, *lower panel*). Also, its effects on the induction of steady-state mRNA and protein levels of TIMP-1 was transient (Fig. 7D) *vs.* its effects on collagen α3(IV) and MMP-9 (Fig. 7, D *vs.* A and B).

Immunohistochemistry localization of TNFα in the seminiferous epithelium of adult rat testis

In light of the changes of TNFα detected at the time of Sertoli cell TJ assembly, we next sought to localize TNFα in the adult rat testis. The localization of TNFα in the rat testis

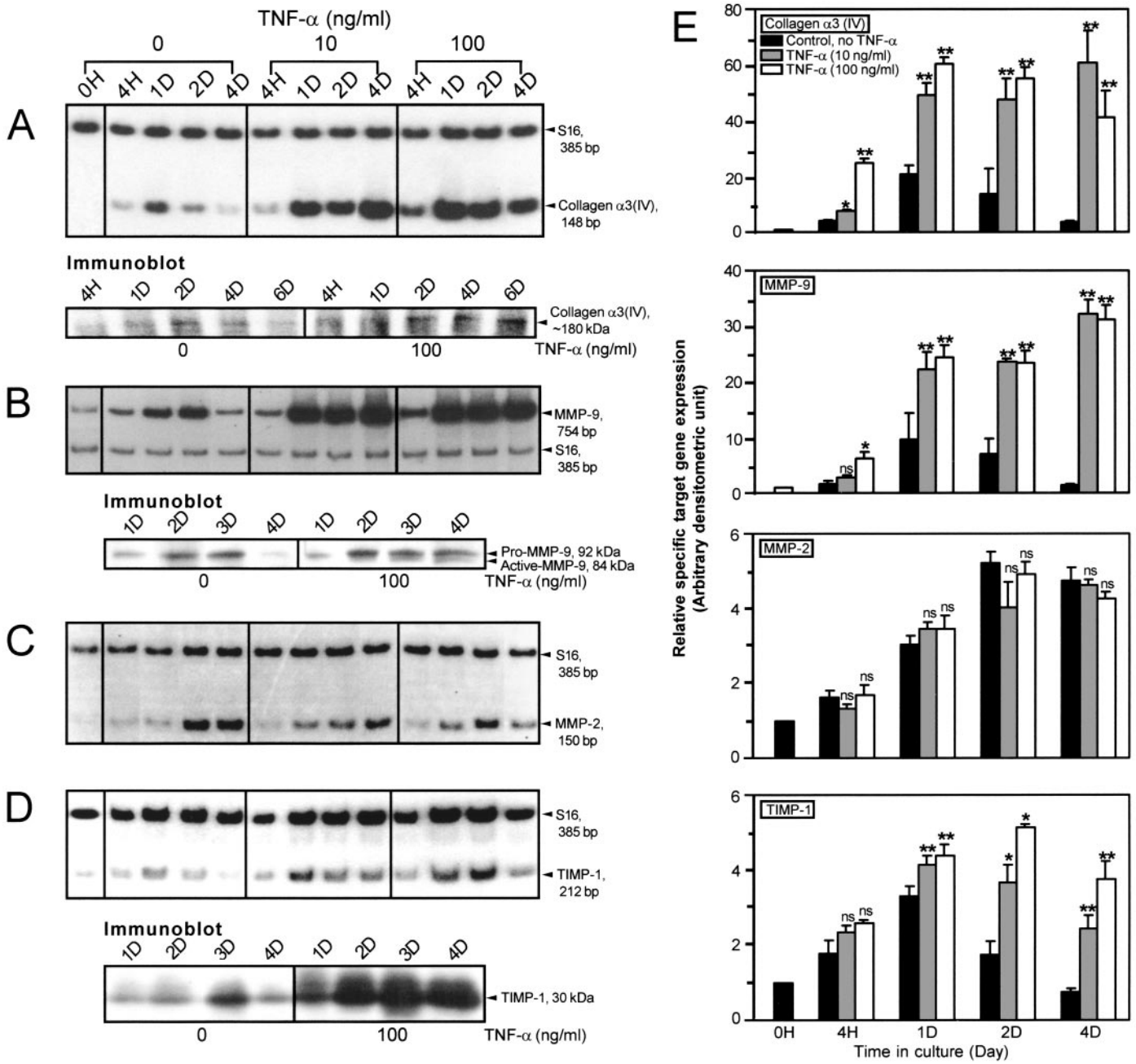


FIG. 7. A–E, Effects of recombinant TNFα on collagen α3(IV), MMP-9, MMP-2, and TIMP-1 gene expression during Sertoli cell culture *in vitro*. Sertoli cells (0.75×10^6 cells/cm^2) isolated from 20-d-old rats were cultured with TNFα at 10 ng/ml or 100 ng/ml or without TNFα (control) immediately after their isolation to allow TJ assembly as described in *Materials and Methods*. Cultures were terminated at specified time points for RNA extraction and protein analysis. Semiquantitative RT-PCR and immunoblot analysis were performed to assess the changes in different target genes expression. The *upper panels* in A–D are autoradiograms showing the changes in the expression of collagen α3(IV), MMP-9, MMP-2, and TIMP-1, respectively, in Sertoli cells during the assembly of the TJ-permeability barrier in the absence (control) or presence of either TNFα at 10 or 100 ng/ml. The *lower panels* in A, B, and D are the corresponding immunoblots to visualize collagen α3(IV), MMP-9, and TIMP-1 using whole-cell lysates [collagen α3(IV)] and the spent medium (MMP-9 and TIMP-1) from Sertoli cells treated with or without (control) 100 ng/ml TNFα. About 100 μg protein were used per lane for SDS-PAGE. E, Corresponding densitometrically scanned results using autoradiograms, such as those shown in A–D, normalized against S16 where the control cultures without TNFα at OH were arbitrarily set at 1. Each *bar* represents a mean ± SD using results from two experiments. Each experiment had replicate cultures. *, Significantly different by *t* test, compared with the corresponding controls, $P < 0.05$; **, significantly different by *t* test, compared with the corresponding controls, $P < 0.01$; ns, not significantly different by *t* test, compared with the corresponding controls.

was stage specific with the highest staining found in the seminiferous epithelium at stages VI–VII, largely around the heads of elongated spermatids adjacent to the seminiferous tubule lumen (Fig. 8, A and D *vs.* A–C and E–H). Figure 8B is the control cross-section of an adult rat testis in which the primary antibody was substituted by normal rabbit serum

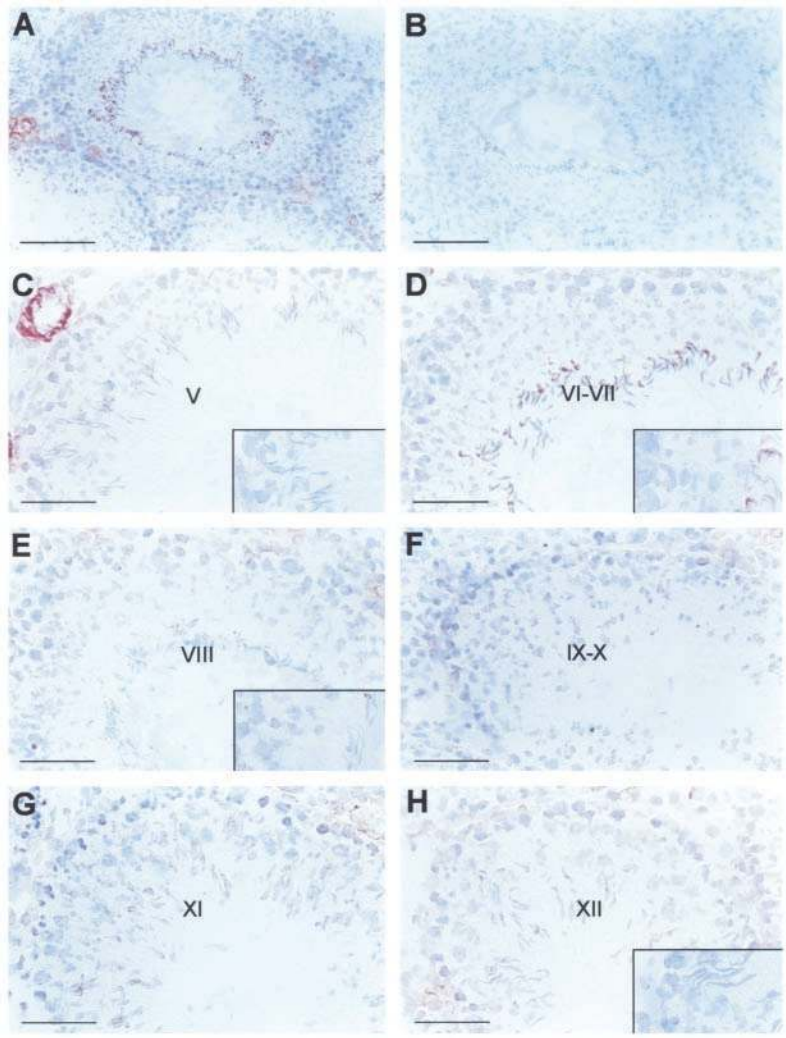


FIG. 8. A–H, Micrographs of cross-section of adult rat testis showing stage-specific localization of immunoreactive TNFα in the seminiferous epithelium. A, Cross-section of an adult rat testis at low magnification. B, Corresponding control using normal rabbit serum to substitute the primary antibody. Other controls yielded similar results (data not shown). C–H, Cross-sections of tubules at stages V, VI–VII, VIII, IX–X, XI, and XII, respectively. Immunoreactive TNFα appears as *reddish brown* precipitate. *Insets* are selected regions of the seminiferous epithelium at higher magnification illustrating the localization of TNFα and its cellular association. *Bar,* 120 μm for A, B; *bar,* 60 μm for C–H.

(other controls yielded virtually identical results) indicating the reddish brown precipitate of TNFα shown in Fig. 8A was specific. At stage VIII, this intense TNFα staining surrounding elongated spermatids virtually disappeared preceding spermiation (Fig. 8E). In stages IX–V (Fig. 8, F–H and A), light staining of TNFα was detected in pachytene spermatocytes and elongating spermatids. However, in virtually all stages of the cycle, very weak staining of TNFα was detected at the basal compartment (Fig. 8) confirming the observations (see Figs. 4 and 5) that TNFα is an important regulator of Sertoli cell TJ dynamics.

Immunohistochemical localization of MMP-9

MMP-9 was also localized to the rat testis by immunohistochemistry as shown in Fig. 9, A–F. MMP-9 was found in the seminiferous epithelium virtually in all stages of the cycle and was largely associated with spermatocytes and round spermatids but not elongated spermatids (Fig. 9, A–E; F is a control section in which the primary antibody was substituted by normal rabbit serum, other controls yielded similar results, data not shown). In contrast to TNFα, which is highest at stages VI–VII (see Fig. 8), MMP-9 was found largely associated with spermatocytes and round spermatids, peaked after spermiation at stage XIV (Fig. 9 *vs.* Fig. 8).

TNFα-induced signaling events at the contact sites of Sertoli cells and basement membrane

Because occludin, collagen α3(IV), MMP-9, and TIMP-1 were shown to be either up- or down-regulated by TNFα, we next sought to investigate the possible signaling pathways by which TNFα mediated its effects. Changes in the protein levels of signal transducers (β1-integrin, ILK, GSK-3β, phospho-GSK-3β, phospho-SEK1/MKK4, and phospho-c-Jun) and components of focal adhesion complex (FAK, phospho-FAK, c-Src, and p130 Cas) in Sertoli cells during TNFα-mediated disruption of the Sertoli cell TJ-barrier were detected. It was noted that TNFα was capable of inducing significant increase in the protein levels of β1-integrin, ILK, phospho-GSK-3α/β, phospho-SEK1/MKK4, and phospho-c-Jun, phospho-FAK, c-Src, and p130 Cas. However, it had no effects on the protein levels of the nonphosphorylated form of GSK-3α/β and FAK during the TNFα-induced Sertoli cell TJ-barrier disruption (Fig. 10). These results seemingly suggest that TNFα perturbed

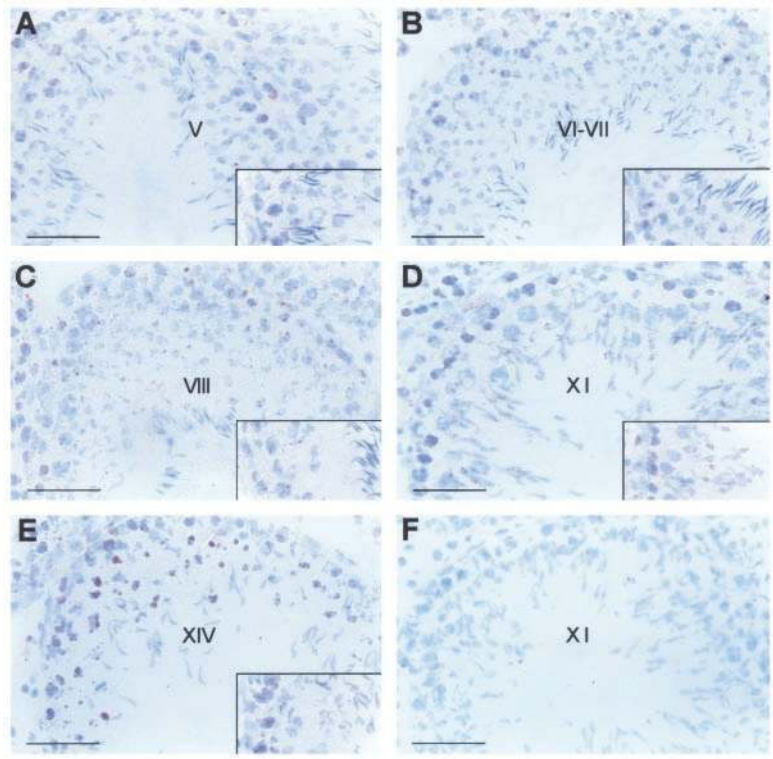


FIG. 9. A–F, Immunohistochemical localization of MMP-9 in the rat testis showing stage-specific staining in the seminiferous epithelium. A–E, Corresponding seminiferous epithelium at stages V, VI–VII, VIII, XI, and XIV, respectively. F, Control in which the primary antibody was substituted by normal rabbit serum. Immunoreactive MMP-9 appeared as *reddish brown* precipitates. *Insets* in A–E show selected portion of the corresponding staged epithelium at higher magnification illustrating the localization of MMP-9 and its cellular association. *Bar,* 60 μm.

the Sertoli cell-TJ barrier via the β1-integrin-ILK-GSK-3 pathway.

Discussion

Germ cells contribute to the pool of collagen α3(IV) chains in the basal lamina of the testis

Type IV collagen is a triple helical molecule composed of three α chains, which in turn constitutes a monomer. These monomers in turn become the building blocks of the type IV collagen network in the basal lamina (for reviews, see Refs. 49 and 50). To date, six genetically distinct α chains, designated α1 to α6, have been identified. Each monomer is characterized by a 7S domain near its N terminus, a middle collagenous domain and a carboxyl-terminal noncollagenous (NC1) domain (for reviews, see Refs. 49 and 50). The distribution of α1(IV) and α2(IV) chains is ubiquitous in tissues, whereas α3(IV), α4(IV), and α5(IV) chains have a more restricted tissue distribution (for a review, see Ref. 49). Studies by immunohistochemistry have localized α3(IV) and α4(IV) chains in the rat basement membrane of the testicular cords, seminiferous epithelium, myoid cell layer in the basal lamina, and tunica albuginea (51). In this report, we have demonstrated that germ cells also contribute significantly to the pool of the α3(IV) chain in the seminiferous epithelium behind the BTB, which in turn maintains the homeostasis of the ECM in the rat STBM. These results may also account for the high level of α3(IV) chain found in the bovine STBM (52). An increase in α3(IV) chain mRNA level during testis maturation between 5 and 60 d of age as reported herein is in agreement with an earlier immunohistochemistry study, which illustrates the strongest α3(IV) chain staining in 15-d-old rat testes (51), coinciding with the assembly of BTB. Likewise, these results support the notion that α3(IV) chain is involved in spermatogenesis because the appearance of α3(IV) chain mRNA coincides with the initiation of spermatogonial proliferation at 3–6 d after birth (11).

ECM remodeling by MMPs and TIMPs plays a crucial role in the regulation of Sertoli cell TJ dynamics

Collagen has been shown to play a role in TJ assembly in brain endothelial cells and A6 cells, a kidney epithelial cell line *in vitro* (53, 54). Data presented herein demonstrated that ECM, such as collagen IV, can affect Sertoli cell TJ dynamics because an anticollagen IV IgG can perturb the assembly of the TJ-barrier *in vitro.* Furthermore, there is a transient but significant induction of collagen α3(IV) at the time of Sertoli TJ assembly.

MMPs are synthesized as inactive zymogens (pro-MMPs), an initial proteolytic cleavage step is required to activate these proteases, and this activation process is largely regulated by TIMPs. The interplay of these molecules determines the rate and extent of ECM remodeling (for a review, see Ref. 13). In this report, a significant but transient induction of MMP-9 is detected at the time of Sertoli cell TJ assembly, suggesting that MMP-9 may play a crucial role in TJ formation by regulating the ECM dynamics. Because plasmin activated by urokinase plasminogen activator [uPA; the assembly of Sertoli cell TJ barrier *in vitro* is also associated with an induction of uPA (25)] can in turn activate pro-MMPs *in vivo* (55); thus, the induction of MMP-9 during Sertoli cell TJ assembly may be regulated, at least in part, by uPA via a yet-to-be-defined pathway or mechanism. In contrast to MMP-9, MMP-2 was induced throughout the entire culture period. The fact that the expression patterns of MMP-9 and MMP-2 in Sertoli cells cultured at both low and high cell

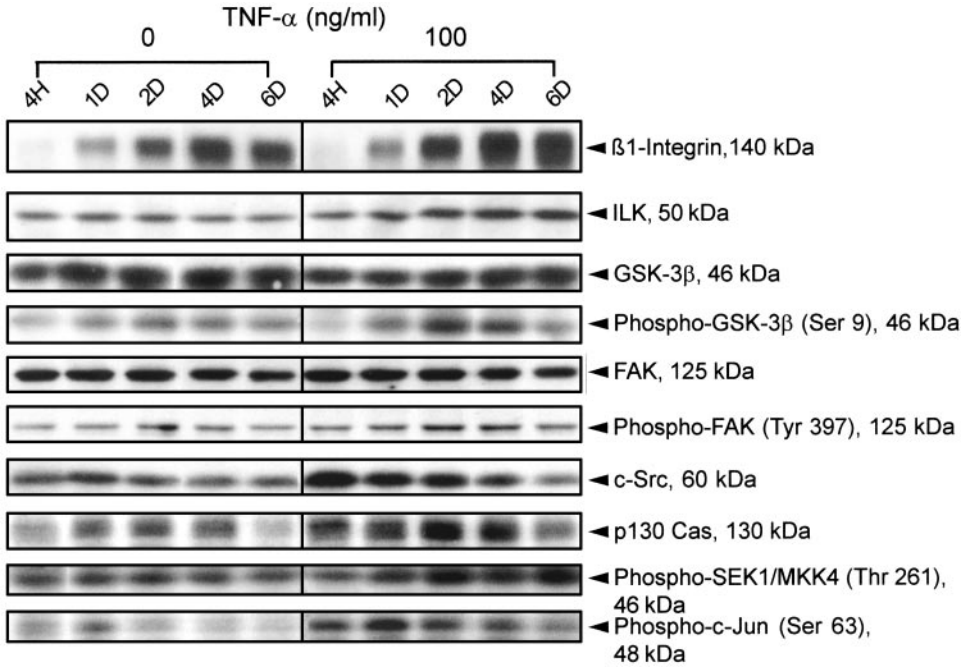


FIG. 10. A study to assess the possible signal pathway by which TNFα uses to regulate the Sertoli cell TJ-barrier *in vitro*. Studies were conducted by immunoblotting to examine changes in β1-integrin, ILK, GSK-3β, phospho-GSK-3β, FAK, phospho-FAK, c-Src, p130 Cas, phospho-SEK1/MKK4, and phospho-c-Jun in Sertoli cells cultured *in vitro* during TJ assembly. Sertoli cells (0.75×10^6 cells/cm^2) isolated from 20-d-old rats were cultured with TNFα at 100 ng/ml or without TNFα (controls) immediately after their isolation as described in *Materials and Methods*. Cell cultures were terminated at specified time points to obtain whole-cell lysates for immunoblotting. Equal amounts of cell lysates (~100 μg protein) per lane from different samples within an experimental group were resolved by SDS-PAGE using 10% *T* polyacrylamide gels under reducing conditions. Proteins on gels were transferred onto nitrocellulose papers and immunostained sequentially using the corresponding antibody against β1-integrin, ILK, GSK-3β, phospho-GSK-3β, FAK, phospho-FAK, c-Src, p130 Cas, phospho-SEK1/MKK4, and phospho-c-Jun. After probing the blot with a primary antibody specific to a target protein and following its visualization by a corresponding second antibody, the blot was incubated with a stripping buffer to remove these antibodies. It was then blocked and reprobed with a different primary antibody against another target protein. As such, the same blot was used for the analysis of up to four to six target proteins with minimal interexperimental variations. Each experiment had replicate cultures, and each experiment was repeated at least three times using different batches of cells. Only a representative set of blots were shown herein because two other experiments yielded virtually identical results.

density were somewhat similar seemingly implicate that they may take part in both TJ and AJ assembly. It is interesting to note that there are two different patterns of MMP and TIMP expression during Sertoli cell TJ assembly. First, MMP-9 and TIMP-1 were induced during Sertoli cell TJ assembly, which plummeted rapidly soon after TJs were assembled. Second, MMP-2 and TIMP-2 were induced throughout the entire culture period during TJ assembly (56).

TNFα perturbs the Sertoli cell TJ-permeability barrier in vitro possibly mediated via its effects on occludin and claudin-11

Studies to date have shown that germ cells and testicular macrophages are the major source of TNFα in the testis (57). In this report, freshly isolated Sertoli cells were also shown to express TNFα; however, its level tumbled rapidly during the assembly of Sertoli TJs, and by d 8 the level of TNFα was less than 10% of that detected in freshly isolated Sertoli cells. These observations seemingly suggest a regulatory role of TNFα in Sertoli cell TJ dynamics. Indeed, addition of recombinant TNFα to Sertoli cell cultures can perturb the Sertoli cell TJ-barrier assembly. Furthermore, TNFα also inhibits the timely induction of occludin, a TJ-integral membrane protein in Sertoli cells (58), known to be induced at the time of TJ assembly (38, 39). A recent study has also shown that the expression of claudin-11, also a TJ-integral membrane protein, can be inhibited by TNFα with an ED_{50} of 4.5 ng/ml in mouse Sertoli cells *in vitro* (59). Taken collectively, these results suggest that TNFα perturbs the Sertoli cell TJ-barrier possible via its effects on TJ-integral membrane proteins, such as occludin.

Unexpectedly, TNFα failed to inhibit ZO-1 (60), whose expression was induced during TJ assembly (25, 26, 39). It is possible that TNFα affects only the redistribution of ZO-1 in cultured Sertoli cells, but not its expression, which is supported by the findings that TNFα and IFN-γ when used together can induce redistribution of the junctional adhesion molecule, another TJ-integral membrane protein, in human endothelial cells (61). Furthermore, in occludin-deficient embryonic stem cells (62) and oligodendrocyte-specific protein/claudin-11 null mice testes (63), ZO-1 was found at the site of TJs regardless whether occludin or claudin was present. Also, the $CdCl_2$-induced Sertoli cell TJ disruption was associated with a loss of occludin induction but not ZO-1 (38, 64).

TNFα is known to induce dissociation of epithelial cells by perturbing the expression of cadherin and β-catenin (65). These observations further support the notion that a reduced TNFα expression in Sertoli cells cultured *in vitro* during AJ assembly is needed, permitting the timely expression of cad-

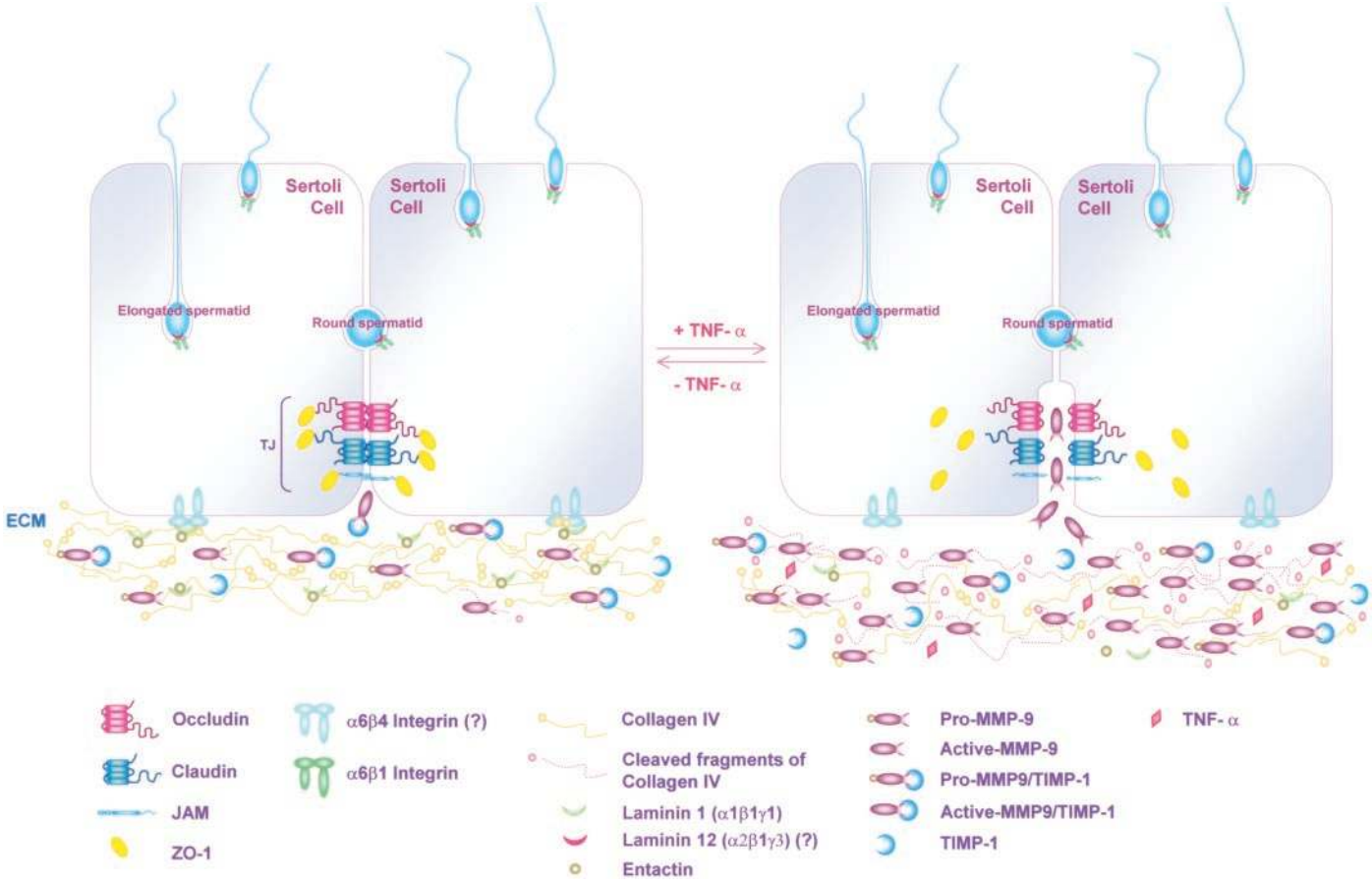


FIG. 11. A schematic drawing that illustrates Sertoli cell TJ dynamics are regulated by the intricate interactions among TNFα, MMP-9, TIMP-1, and collagen IV. This diagram was prepared based on earlier reports and reviews (3, 13, 49), illustrating the molecular architecture of tight junctions between adjacent Sertoli cells in the testis. The α6β1 integrin is localized at the site of ES with the proposed binding ligand, laminin γ3 chains, which is a novel, nonbasement membrane-associated laminin γ chain in the adluminal compartment of the seminiferous epithelium (78). The α6β4 integrin is suggested to localized at base of Sertoli cells (76). Based on the results presented herein, it is possible that TNFα perturbs the Sertoli cell-TJ barrier via its effects on MMP-9, TIMP-1, and collagen IV, which in turn affects the composition and homeostasis of ECM. For instance, the activation of MMP-9 induced by TNFα may facilitate the opening of the TJ-barrier permitting the preleptotene/leptotene spermatocytes to translocate across the BTB. Furthermore, cleavage of the collagen network in the basal lamina by the activated MMP-9 can also damage the integrity of the ECM affecting the TJ barrier. The role of TIMP-1 is to limit the action of MMP-9, maintaining the integrity of the BTB.

herin and β-catenin during junction assembly. Likewise, studies by immunohistochemistry have detected TNFα at the basal and adluminal compartment of the seminiferous epithelium throughout the epithelial cycle further strengthening the postulate that TNFα is actively involved in junction dynamics. The fact that there is a surge in TNFα accumulation surrounding elongated spermatids at the site of ectoplasmic specialization (ES) at stages VI–VII seemingly suggest that this cytokine may also be functionally significant at spermiation and an important player of ES dynamics.

TNFα-induced changes in Sertoli cell TJ dynamics are mediated via its effects on collagen α3(IV), MMP-9, and TIMP-1

The interactions of cytokines with ECM proteins are known to regulate the dynamics of ECM (for a review, see Ref. 66). As reported herein, TNFα induces collagen α3(IV), MMP-9, and TIMP-1 but not MMP-2 when it perturbs the Sertoli cell TJ-barrier. Unexpectedly, the assembly of Sertoli cell TJs *in vitro* was associated with an induction of collagen α3(IV), MMP-9, and TIMP-1. These results seemingly suggest that these molecules may have a biphasic effect on the Sertoli cell TJ assembly and disassembly. We offer the following rationale to explain the observation that an induction of collagen α3(IV), MMP-9, and TIMP-1 is associated with the events of Sertoli cell TJ disassembly. First, the assembly of type IV collagen networks depends not only on the chain availability, but also the NC1 domain in an intact collagen chain also plays a crucial role in chain selection and dimerization for network assembly (50, 67). As such, both the TNFα-induced expression and activation of MMP-9 [MMP-9 is known to cleave type IV collagen (68)] may break down the collagen network by cleaving the central triple helix of collagen IV, leaving the N-terminal 7S domains and COOH-terminal NC1 domains. Furthermore, this cleavage process may also induce a loss of other basement membrane proteins (such as laminin and entactin) and growth factors (such as TGFβ) because type IV collagen network has a scaffolding function for these proteins and growth factors (for reviews, see Refs.

13 and 49). This somewhat extensive proteolytic breakdown of ECM proteins may contribute to the TNFα-mediated disruption of the Sertoli cell TJ-barrier, possibly because cells can no longer attach themselves tightly to an intact ECM (Fig. 11).

Second, the NC1 domains cleaved from the collagen network by MMP-9 during its proteolytic breakdown may set off a chain reaction, further perturbing the type IV collagen network and ECM composition because of the fact that NC1 domains isolated from type IV collagen can bind to the central triple helical domain, which in turn perturbs the lateral association of native type IV collagen, inhibiting the assembly of intact type IV collagen network (69). This hypothesis is further strengthened by a recent study reporting that exogenous addition of NC1 domains can perturb ECM formation in *Hydra vulgaris* (70).

Third, proteolysis of ECM proteins by MMPs can also result in the release of biologically active ECM fragments (for reviews, see Refs. 12 and 13). For instance, type I collagen fragments are known to induce a rapid disassembly of smooth muscle focal adhesion complex, which is mediated via the integrin-dependent cleavage of FAK, paxillin, and talin (71). Also, these biological active fragments can have a negative feedback effect that inhibits the expression of collagenase and degradation of collagen (72). Thus, an induction of collagen α3(IV) and TIMP-1 at the time TNFα perturbs the Sertoli TJ-barrier may be a negative feedback mediated by the biologically active ECM fragments, which are the cleavage products of the TNFα-induced MMP-9.

Furthermore, studies by immunohistochemistry to localize MMP-9 in the seminiferous epithelium have shown that MMP-9 is detected in virtually all stages of the epithelial cycle and is associated with spermatids and spermatocytes largely in the basal and part of the adluminal compartment. Yet MMP-9 peaks at stage XIV and remains associated largely with spermatocytes, spermatids, and possibly spermatogonia in the seminiferous epithelium. In this context, it is of interest to note that the movement of preleptotene and leptotene spermatocytes from the basal to the adluminal compartment occurs in late stage VIII and early IX of the cycle (73) when MMP-9 staining is detected in the seminiferous epithelium near the basal compartment. This observation apparently is in agreement with an earlier report, which also demonstrated an induction of proteolytic activity during junction restructuring, such as the assembly of AJs between Sertoli and germ cells *in vitro* (24).

The TNFα-induced effects on Sertoli cell TJ dynamics are possibly mediated via the β1-integrin-ILK-GSK-3β-dependent signaling pathways

A recent study has shown that MMP-9 in intestinal epithelial cells is regulated by ILK via GSK-3 and activator protein-1 (AP-1) transcription factor (74). ILK also plays a crucial role in cell adhesion because its overexpression in normal epithelial cells can reduce E-cadherin expression as well as induce a redistribution of β-catenin, moving β-catenin from the cytoplasm to the nucleus (for a review, see Ref. 75). Interestingly, TNFα-induced reduction of cadherin and β-catenin expression also causes the loss of cell adhesiveness in epithelial cells (65). In the testis, ILK has been shown to colocalize with β1 integrin at the site of both apical and basal ES (76). Thus, the TNFα-induced effects on junction dynamics may be mediated via integrin β-subunit and ILK because the protein levels of these two signal transducers were shown to be stimulated by TNFα as reported herein. These changes are also coupled with an increase in phosphorylation and inhibition of GSK-3β activity induced by TNFα. These observations thus implicate the TNFα-induced up-regulation of MMP-9 and TJ dynamics is mediated via the β1-integrin-ILK-GSK-3β-dependent pathway.

Besides, several cytokines, including TNFα, are known to regulate the expression of MMPs via an induction and/or activation of c-Jun, which binds to the activator protein-1 site in the promoter of MMP genes (for a review, see Ref. 13). Based on the data presented herein, TNFα apparently activates SEK1/MKK4 and c-Jun, the signal transducers of the SAPK/JNK pathway, in Sertoli cells. And the SAPK/JNK pathway can be regulated, at least in part, by the autophosphorylation of FAK, which recruits c-Src to FAK (for a review, see Ref. 75). Other studies have shown that the TNFα-induced phosphorylation of proteins in focal adhesion complexes, such as paxillin and FAK, are involved in actin cytoskeleton reorganization (77). In this study, TNFα was shown to induce phosphorylation of FAK as well as inducing both p130 Cas and c-Src in Sertoli cells. These results further support the notion that the SAPK/JNK pathway is involved in the regulation of MMPs by TNFα. These data also strongly suggest that TNFα perturbs the Sertoli cell TJ-barrier by disrupting the actin cytoskeleton via changes in phosphorylation of selected proteins in the focal adhesion complex.

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References

1. **Dym M, Cavicchia JC** 1978 Functional morphology of the testis. Biol Reprod 18:1–5
2. **De Kretser DM, Kerr JB** 1988 The cytology of the testis. In: Knobil E, Neill J, eds. The physiology of reproduction. New York: Raven Press; 837–932
3. **Cheng CY, Mruk DD** 2002 Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. Physiol Rev 82:825–874
4. **Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD** 1994 Cell junctions, cell adhesion, and the extracellular matrix. In: Molecular biology of the cell. New York: Garland Publishing Inc.; 949–1009
5. **Dym M** 1994 Basement membrane regulation of Sertoli cells. Endocr Rev 15:102–115
6. **Hadley MA, Dym M** 1987 Immunocytochemistry of extracellular matrix in the lamina propria of the rat testis: electron microscopic localization. Biol Reprod 37:1283–1289

7. **Lian G, Miller KA, Enders GC** 1992 Localization and synthesis of entactin in seminiferous tubules of the mouse. Biol Reprod 47:316–325
8. **Lustig L, Denduchis B, González NN, Puig RP** 1978 Experimental orchitis induced in rats by passive transfer of an antiserum to seminiferous tubule basement membrane. Arch Androl 1:333–343
9. **Denduchis B, Satz ML, Sztein MB, Puig RP, Doncel JF, Lustig L** 1985 Multifocal damage to the testis induced in rats by passive transfer or antibodies prepared against non-collagenous fraction of basement membrane. J Reprod Immunol 7:59–75
10. **Davis CM, Papadopoulos V, Sommers CL, Kleinman HK, Dym M** 1990 Differential expression of extracellular matrix components in rat Sertoli cells. Biol Reprod 43:860–869
11. **Enders GC, Kahsai TZ, Lian G, Funabiki K, Killen PD, Hudson BG** 1995 Developmental changes in seminiferous tubule extracellular matrix components of the mouse testis: α3 (IV) collagen chain expressed at the initiation of spermatogenesis. Biol Reprod 53:1489–1499
12. **Werb Z** 1997 ECM and cell surface proteolysis: regulating cellular ecology. Cell 91:439–442
13. **Sternlicht MD, Werb Z** 2001 How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516
14. **Ulisse S, Farina AR, Piersanti D, Tiberio A, Cappabianca L, D'Orazi G, Jannini EA, Malykh O, Stetler-Stevenson WG, D'Armiento M, Gulino A, MacKay AR** 1994 Follicle-stimulating hormone increases the expression of tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 and induces TIMP-1 AP-1 site binding complex(es) in prepubertal rat Sertoli cells. Endocrinology 135:2479–2487
15. **Fritz IB, Tung PS, Ailenberg M** 1993 Proteases and antiproteases in the seminiferous tubule. In: Russell LD, Griswold MD, eds. The Sertoli cell. Clearwater, FL: Cache River Press; 217–235
16. **Mealy K, Robinson B, Millette CF, Majzoub J, Wilmore DW** 1990 The testicular effects of tumor necrosis factor. Ann Surg 211:470–475
17. **Walsh SV, Hopkins AM, Nusrat A** 2000 Modulation of tight junction structure and function by cytokines. Adv Drug Deliv Rev 41:303–313
18. **Mankertz J, Tavalali S, Schmitz H, Mankertz A, Riecken EO, Fromm M, Schulzke JD** 2000 Expression from the human occludin promoter is affected by tumor necrosis factor α and interferon γ. J Cell Sci 113:2085–2090
19. **Cheng CY, Mather JP, Byer AL, Bardin CW** 1986 Identification of hormonally responsive proteins in primary Sertoli cell culture medium by anion-exchange high performance liquid chromatography. Endocrinology 118:480–488
20. **Grima J, Pineau C, Bardin CW, Cheng CY** 1992 Rat Sertoli cell clusterin, α2-macroglobulin, and testins: biosynthesis and differential regulation by germ cells. Mol Cell Endocrinol 89:127–140
21. **Grima J, Wong CCS, Zhu LJ, Zong SD, Cheng CY** 1998 Testin secreted by Sertoli cells is associated with the cell surface, and its expressions correlates with the Sertoli-germ cell junctions but not the inter-Sertoli tight junction. J Biol Chem 273:21040–21053
22. **Byers SW, Hadley MA, Djakiew D, Dym M** 1986 Growth and characterization of polarized monolayers of epididymal epithelial cells and Sertoli cells in dual environment culture chambers. J Androl 7:59–68
23. **Janecki A, Steinberger A** 1986 Polarized Sertoli cell functions in a new two-compartment culture system. J Androl 7:69–71
24. **Mruk D, Zhu LJ, Silvestrini B, Lee WM, Cheng CY** 1997 Interactions of proteases and protease inhibitors in Sertoli-germ cell co-cultures preceding the formation of specialized Sertoli-germ cell junctions *in vitro.* J Androl 18: 612–622
25. **Wong CCS, Chung SSW, Grima J, Zhu LJ, Mruk D, Lee WM, Cheng CY** 2000 Changes in the expression of junctional and nonjunctional complex component genes when inter-Sertoli tight junctions are formed *in vitro.* J Androl 21:227–237
26. **Chung SSW, Lee WM, Cheng CY** 1999 A study on the formation of specialized inter-Sertoli cell junctions *in vitro.* J Cell Physiol 181:258–272
27. **Mruk D, Cheng CY** 1999 Sertolin is a novel gene marker of cell-cell interactions in the rat testis. J Biol Chem 274:27056–27068
28. **Galdieri M, Ziparo E, Palombi F, Russo MA, Stefanini M** 1981 Pure Sertoli cell cultures: a new model for the study of somatic-germ cell interactions. J Androl 5:249–259
29. **Li JCH, Lee WM, Mruk D, Cheng CY** 2001 Regulation of Sertoli cell myotubularin (rMTM) expression by germ cells *in vitro.* J Androl 22:266–277
30. **Lee NPY, Mruk D, Lee WM, Cheng CY,** Is the cadherin/catenein complex a functional unit of cell-cell actin-based adherens junctions (AJ) in the rat testis? Biol Reprod, in press
31. **Aravindan GR, Pineau C, Bardin CW, Cheng CY** 1996 Ability of trypsin in mimicking germ cell factors that affect Sertoli cell secretory function. J Cell Physiol 168:123–133
32. **Cheng CY, Grima J, Stahler MS, Lockshin RA, Bardin CW** 1989 Testin are structurally related Sertoli cell proteins whose secretion is tightly coupled to the presence of germ cells. J Biol Chem 264:21386–21393
33. **Zong SD, Zhu LJ, Grima J, Aravindan R, Bardin CW, Cheng CY** 1994 Cyclic and postnatal development changes of testin in the rat seminiferous epithelium-an immunohistochemical study. Biol Reprod 51:843–851
34. **Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, Fujimoto T, Nishikawa S** 1991 Role of c-Kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-Kit expression and function. Development 113:689–699
35. **Zwain IH, Morris PL, Cheng CY** 1991 Identification of an inhibitory factor from a Sertoli clonal cell line (TM4) that modulates adult rat Leydig cell steroidogenesis. Mol Cell Endocrinol 80:115–126
36. **Tung PS, Skinner MK, Fritz IB** 1984 Fibronectin synthesis is a marker for peritubular cell contaminations in Sertoli cell-enriched cultures. Biol Reprod 30:199–211
37. **Pineau C, Syed V, Bardin CW, Jegon B, Cheng CY** 1993 Germ cell-conditioned medium contains multiple biological factors that modulate the secretion of testins, clusterin, and transferrin by Sertoli cells. J Androl 14:87–98
38. **Chung NPY, Cheng CY** 2001 Is cadmium chloride-induced inter-Sertoli tight junction permeability barrier disruption a suitable *in vitro* model to study the events of junction disassembly during spermatogenesis in the rat testis? Endocrinology 142:1878–1888
39. **Lui WY, Lee WM, Cheng CY** 2001 Transforming growth factor-β3 perturbs the inter-Sertoli tight junction permeability barrier *in vitro* possibly mediated via its effects on occludin, zonula occludens1 and claudin-11. Endocrinology 142: 1865–1877
40. **Grima J, Zhu LJ, Cheng CY** 1997 Testin is tightly associated with testicular cell membrane upon its secretion by Sertoli cells whose steady-state mRNA level in the testis correlates with the turnover and integrity of inter-testicular cell junctions. J Biol Chem 272:6499–6509
41. **Chung SSW, Mo MY, Silvestrini B, Lee WM, Cheng CY** 1998 Rat testicular N-cadherin: its complementary deoxyribonucleic acid cloning and regulation. Endocrinology 139:1853–1862
42. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
43. **Laemmli UK** 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
44. **Zhu LJ, Cheng CY, Phillips DM, Bardin CW** 1994 The immunohistochemical localization of α2-macroglobulin in rat testis is consistent with its role in germ cell movement and spermiation. J Androl 15:575–572
45. **Zhu LJ, Moo-Yong A, Bardin CW, Cheng CY** 1997 Immunohistochemical localization of testin in the female reproductive system of the rat is consistent with its involvement in the turnover of specialized junctional complexes. Biol Reprod 56:1330–1335
46. **Li JCH, Sammy ET, Grima J, Chung SSW, Mruk D, Lee WM, Silvestrini B, Cheng CY** 2000 Rat testicular myotubularin, a protein tyrosine phosphatase expressed by Sertoli and germ cells, is a potential marker for studying cell-cell interactions in the rat testis. J Cell Physiol 185:366–385
47. **Li YY, Feng YQ, Kadokami T, McTiernan CF, Draviam R, Watkins SC, Feldman AM** 2000 Myocardial extracellular matrix remodeling in transgenic mice overexpressing tumor necrosis factor α can be modulated by anti-tumor necrosis factor α therapy. Proc Natl Acad Sci USA 97:12746–12751
48. **Siwik D, Chang DLF, Colucci WS** 2000 Interleukin-1β and tumor necrosis factor-α decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts *in vitro.* Circ Res 86:1259–1265
49. **Hudson BG, Reeders ST, Tryggvason K** 1993 Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. J Biol Chem 268:26033–26036
50. **Timpl R, Wiedemann H, van Delden V, Furthmayr H, Kuhn K** 1981 A network model for the organization of type IV collagen molecules in basement membranes. Eur J Biochem 120:203–211
51. **Fröjdman K, Pelliniemi LJ, Virtanen I** 1998 Differential distribution of type IV collagen chains in the developing rat testis and ovary. Differentiation 63:125–130
52. **Kahsai TZ, Enders GC, Gunwar S, Brunmark C, Wieslander J, Kalluri R, Zhou J, Noelken ME, Hudson BG** 1997 Seminiferous tubule basement membrane. Composition and organization of type IV collagen chains, and the linkage of α3(IV) and α5(IV) chains. J Biol Chem 272:17023–17032
53. **Jaeger MMM, Kalinec G, Dodane V, Kachar B** 1997 A collagen substrate enhances the sealing capacity of tight junctions of A6 cell monolayers. J Membr Biol 159:263–270
54. **Savettieri G, Liegro ID, Catania C, Licata L, Pitarresi GL, D'Agostino S, Schiera G, De Caro V, Giandalia G, Giannola LI, Cestelli A** 2000 Neurons and ECM regulate occludin localization in brain endothelial cells. Neuroreport 11:1081–1084
55. **Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D** 1997 Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. Nat Genet 17:439–444
56. **Longin J, Guillaumot P, Chauvin MA, Morera AM, Magueresse-Battistoni BL** 2001 MT1-MMP in rat testicular development and the control of Sertoli cell proMMP-2 activation. J Cell Sci 114:2125–2134
57. **De SK, Chen HL, Pace JL, Hunt JS, Terranova PF, Enders GC** 1993 Expression of tumor necrosis factor-α in mouse spermatogenic cells. Endocrinology 133: 389–396
58. **Moroi S, Saitou M, Fujimoto K, Sakakibara A, Furuse M, Yoshida O, Tsukita S** 1998 Occludin is concentrated at tight junctions of mouse/rat but not human/guinea pig Sertoli cells in testes. Am J Physiol 274:C1708–C1717

59. **Hellani A, Ji J, Mauduit C, Deschildre C, Tabone E, Benahmed M** 2000 Developmental and hormonal regulation of the expression of oligodendrocyte-specific protein/claudin 11 in mouse testis. Endocrinology 141:3012–3019
60. **Byers S, Graham R, Dai HN, Hoxter B** 1991 Development of Sertoli cell junctional specialization and the distribution of the tight-junction-associated protein ZO-1 in the mouse testis. Am J Anat 191:35–47
61. **Ozaki H, Ishii K, Horiuchi H, Arai H, Kawamoto T, Okawa K, Iwamatsu A, Kita T** 1999 Cutting edge: combined treatment of TNF-α and IFN-γ causes redistribution of junctional adhesion molecule in human endothelial cells. J Immunol 163:553–557
62. **Saitou M, Fujimoto K, Doi Y, Itoh M, Fujimoto T, Furuse M, Takano H, Noda T, Tsukita S** 1998 Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J Cell Biol 141:397–408
63. **Gow A, Southwood CM, Li JS, Pariali M, Riordan GP, Brodie SE, Danias J, Bronstein JM, Kachar B, Lazzarini RA** 1999 CNS myelin and Sertoli cell tight junction strands are absent in Osp/Claudin-11 null mice. Cell 99:649–659
64. **Grima J, Cheng CY** 2000 Testin induction: the role of cyclic 3′, 5′-adenosine monophosphate/protein kinase A signaling in the regulation of basal and lonidamine-induced testin expression by rat Sertoli cells. Biol Reprod 63:1648–1660
65. **Tabibzadeh T, Kong QF, Kapur S, Satyaswaroop PG, Aktories K** 1995 Tumour necrosis factor-α-mediated dysocohesion of epithelial cells is associated with disordered expression of cadherin/β-catenin and disassembly of actin filaments. Hum Reprod 10:994–1004
66. **Adams JC, Watt FM** 1993 Regulation of development and differentiation by the extracellular matrix. Development 117:1183–1198
67. **Ries A, Engel J, Lustig A, Kühn K** 1995 The function of the NC1 domains in type IV collagen. J Biol Chem 270:23790–23794
68. **Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He CS, Bauer EA, Goldberg GI** 1988 H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem 263:6579–6587
69. **Tsilibary EC, Charonis AS, Reger LA, Wohlhueter RM, Furcht LT** 1988 The effect of nonenzymatic glucosylation on the binding of the main noncollagenous NC1 domain to type IV collagen. J Biol Chem 263:4302–4308
70. **Zhang X, Hudson BG, Sarras Jr MP** 1994 Hydra cell aggregate development is blocked by selective fragments of fibronectin and type IV collagen. Dev Biol 164:10–23
71. **Carragher NO, Levkau B, Ross R, Raines EW** 1999 Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of $pp125^{FAK}$, paxillin and talin. J Cell Biol 147:619–629
72. **Vogel W, Gish GD, Alves F, Pawson T** 1997 The discoidin domain receptor tyrosine kinases are activated by collagen. Mol Cell 1:13–23
73. **Russell LD** 1977 Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Am J Anat 148:313–328
74. **Troussard AA, Costello P, Yoganathan TN, Kumagai S, Roskelley CD, Dedhar S** 2000 The integrin linked kinase (ILK) induces an invasive phenotype via AP-1 transcription factor-dependent upregulation of matrix metalloproteinase 9 (MMP-9). Oncogene 19:5444–5452
75. **Dedhar S** 2000 Cell-substrate interactions and signaling through ILK. Curr Opin Cell Biol 12:250–256
76. **Mulholland DJ, Dedhar S, Vogl AW** 2001 Rat seminiferous epithelium contains a unique junction (ectoplasmic specialization) with signaling properties both of cell/cell and cell/matrix junctions. Biol Reprod 64:396–407
77. **Koukouritaki SB, Vardaki EA, Papakonstanti EA, Lianos E, Stournaras C, Emmanouel DS** 1999 TNF-α induces actin cytoskeleton reorganization in glomerular epithelial cells involving tyrosine phosphorylation of paxillin and focal adhesion kinase. Mol Med 5:382–392
78. **Koch M, Olson PF, Albus A, Jin W, Hunter DD, Brunken WJ, Burgeson RE, Champliaud MF** 1999 Characterization and expression of the laminin γ3 chain: a novel, non-basement membrane-associated, laminin chain. J Cell Biol 145:605–617
79. **Ryan JJ, Katbamna I, Mason PJ, Pusey CD, Turner AN** 1998 Sequence analysis of the 'Goodpasture antigen' of mammals. Nephrol Dial Transplant 13:602–607
80. **Kim MH, Kitson RP, Albertsson P, Nannmark U, Basse PH, Kuppen PJK, Hokland ME, Goldfarb RH** 2000 Secreted and membrane-associated matrix metalloproteinases of IL-2-activated NK cells and their inhibitors. J Immunol 164:5883–5889
81. **Fang J, Shing Y, Wiederschain D, Li Y, Butterfield C, Jackson G, Harper J, Tamvakopoulos G, Moses MA** 2000 Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proc Natl Acad Sci USA 97:3884–3889
82. **Lutterová M, Szatmáry Z, Kukan M, Kuba D, Vajdová K** 2000 Marked difference in tumor necrosis factor-α expression in warm ischemia- and cold ischemia-reperfusion of the rat liver. Cryobiology 41:301–314
83. **Willott E, Balda MS, Fanning AS, Jameson B, Van Itallie C, Anderson JM** 1993 The tight junction protein ZO-1 is homologous to the *Drosophila* discs-large tumor suppressor protein of septate junctions. Proc Natl Acad Sci USA 90:7834–7838
84. **Okada A, Garnier JM, Vicaire S, Basset P** 1994 Cloning of the cDNA encoding rat tissue inhibitor of metalloproteinase 1 (TIMP-1), amino acid comparison with other TIMPs, and gene expression in rat tissues. Gene 147:301–302
85. **Chan YL, Paz V, Olvera J, Wool IG** 1990 The primary structure of rat ribosomal protein S16. FEBS Lett 263:85–88