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Transforming Growth Factor β 3 Regulates the Dynamics of Sertoli Cell Tight Junctions Via the p38 Mitogen-Activated Protein Kinase Pathway¹

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ABSTRACT

Earlier studies have implicated the significance of transforming growth factor- β 3 (TGF β 3) in the regulation of Sertoli cell tight junction (TJ) dynamics, possibly via its inhibitory effects on the expression of occludin, claudin-11, and zonula occludens-1 (ZO-1). Yet the mechanism by which TGF β 3 regulates the Sertoli cell TJ-permeability barrier is not known. Using techniques of semiquantitative reverse transcription-PCR (RT-PCR), immunoblotting, immunohistochemistry, and inhibitors against different kinases coupled with physiological techniques to assess the Sertoli cell TJ barrier function, it was shown that this TGF β 3-induced effect on Sertoli cell TJ dynamics is mediated via the p38 mitogen-activated protein (MAP) kinase pathway. First, the assembly of the Sertoli cell-TJ barrier was shown to be associated with a transient but significant decline in both the TGF β 3 production and expression by Sertoli cells. Furthermore, addition of TGF β 3 to Sertoli cell cultures during TJ assembly indeed perturbed the TJ barrier with an IC_{50} at ~ 9 pM. Second, the TGF β 3-induced disruption of the TJ barrier was associated with a transient induction in MEK2 but not the other upstream signaling molecules that mediate TGF β 3 action, such as Smad2, Cdc42, Rac2, and N-Ras, suggesting this effect might be mediated via the p38 MAP kinase pathway. This postulate was confirmed by the observation that TGF β 3 also induced the protein level of the activated and phosphorylated form of p38 MAP kinase at the time the TJ barrier was perturbed. Third, and perhaps the most important of all, this TGF β 3-mediated inhibitory effect on the TJ barrier and the TGF β 3-induced p-p38 MAP kinase production could be blocked by SB202190, a specific p38 MAP kinase inhibitor, but not U0126, a specific MEK1/2 kinase inhibitor. These results thus unequivocally demonstrate that TGF β 3 utilizes the p38 MAP kinase pathway to regulate Sertoli cell TJ dynamics.

Sertoli cells, signal transduction, spermatogenesis, testis, tight junction

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INTRODUCTION

During spermatogenesis, the Sertoli cell tight junctions (TJs) that constitute the blood-testis barrier (BTB) must disassemble and reassemble intermittently to facilitate the translocation of preleptotene and leptotene spermatocytes across this barrier at stages VIII and IX of the cycle (for reviews, see [1–3]). This thus permits the movement of spermatocytes from the basal to the adluminal compartment of the seminiferous epithelium while differentiating into haploid spermatids [3, 4]. Nonetheless, the biochemical and molecular bases of these events are not yet known. Recent studies, however, have shown that the event of germ cell movement across the seminiferous epithelium apparently requires the participation of proteases, protease inhibitors, and junctional complex components, which in turn are regulated by cytokines and signaling molecules (for reviews, see [1, 5–7]). For instance, previous studies have shown that, when the Sertoli cell TJ-permeability barrier is assembled *in vitro*, it associates with a timely induction of several TJ-associated proteins, such as occludin, zonula occludens-1 (ZO-1), and claudin-11 [8–11], which are the building blocks of TJs (for reviews, see [1, 12–14]). Furthermore, the assembly of Sertoli cell TJs *in vitro* is associated with a decline in the endogenous TGF (transforming growth factor) β 2 and TGF β 3 expression by Sertoli cells [10]. These results thus illustrate a reciprocal relationship exists between TGF β 3 and TJ-associated proteins. Needless to say, these studies also implicate the significance of TGF β in TJ dynamics. To confirm and expand these earlier studies, we have demonstrated that the addition of recombinant TGF β 3 to Sertoli cell cultures during TJ assembly can indeed trigger a plummeting in the expression of ZO-1 and occludin, perturbing the TJ barrier [10]. These results are also in agreement with earlier studies, which have shown that interferon- γ and hepatocyte growth factor are capable of perturbing the assembly of the TJ barrier in T84 and Madin Darby canine kidney (MDCK) cells, possibly by reducing ZO-1 expression or causing the redistribution of ZO-1, moving it away from the site of TJs to cytoplasm [15, 16]. However, the downstream signaling pathway(s) by which these cytokines, such as TGF β 3, are utilized to regulate TJ dynamics is entirely unknown. As part of our ongoing efforts to study the biology and regulation of TJ dynamics during spermatogenesis, we sought to identify the pathway(s) utilized by TGF β 3 to regulate these events. If this information is known, there will be several important ramifications. First, this will provide a clue to understanding the biology of TJ dynamics in the testis. Second, studies can be designed to compromise the integrity of the blood-testis barrier *in vivo* to perturb germ cell movement

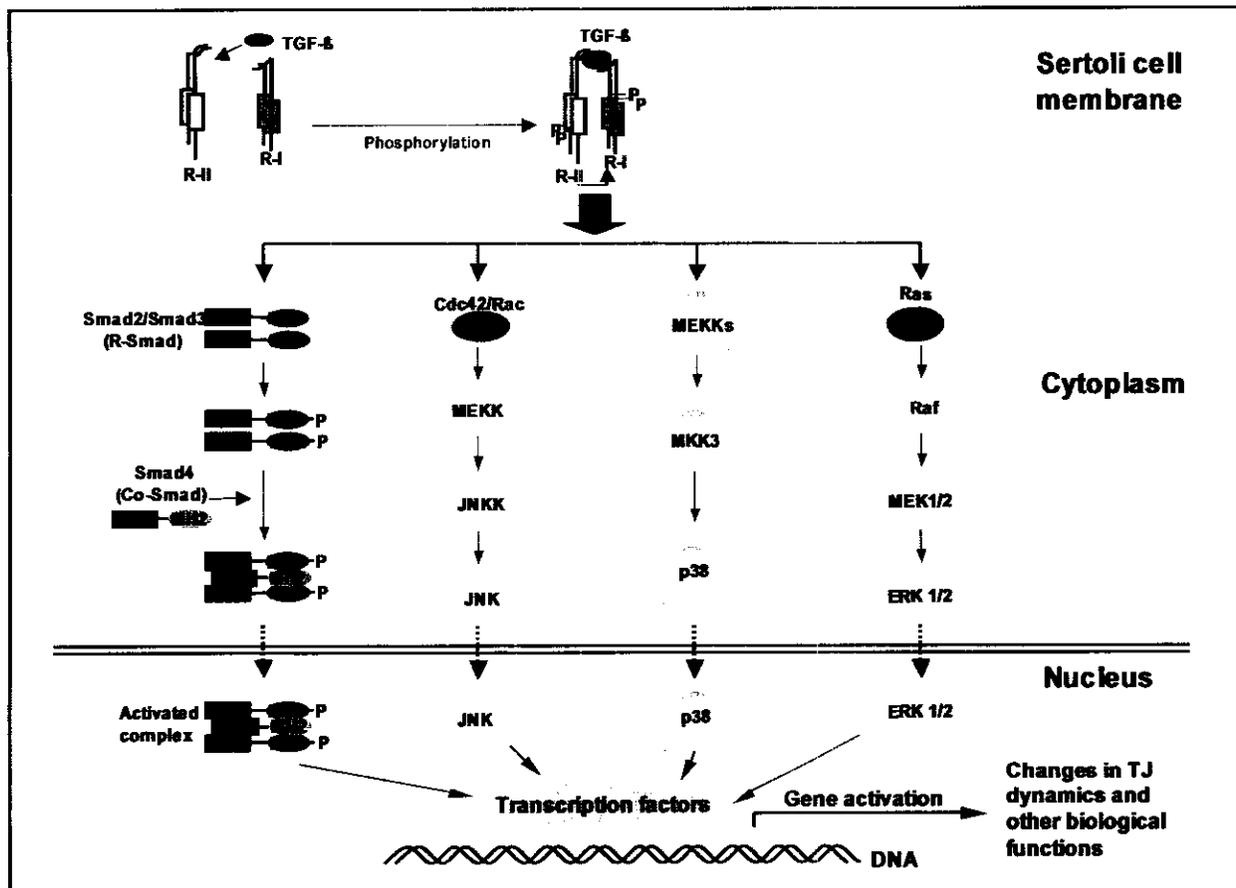


FIG. 1. A schematic drawing illustrates the four signaling pathways utilized by TGF β to affect cellular function. This diagram was prepared based on reviews [18, 19, 21]. Original articles that describe the discovery of the key molecules depicted in these pathways can be found in these reviews. MEKKs, MAP/ERK kinase kinases, which include MEKK1, MEKK2, MEKK3, and others; JNK, c-Jun N-terminal kinase also known as Jun kinase or stress-activated protein kinase, SAPK; JNKK, c-Jun N-terminal kinase kinase; MKK3, MAP kinase kinase 3; MEK1/2, MAP/ERK kinase 1 and MAP/ERK kinase 2; ERK1/2, extracellular signal-regulated kinase 1 and extracellular signal-regulated kinase 2.

across the Sertoli cell TJ-barrier, disrupting spermatogenesis, which may lead to the development of a novel male contraceptive. Third, this may even offer clues to understand the pathophysiology of certain unexplained male infertility.

Several studies have shown that TGF β regulates cellular function via its initial interaction with the TGF β type II receptor, which is a putative Ser/Thr protein kinase capable of undergoing autophosphorylation upon activation following coupling with its ligand (see Fig. 1) (for reviews, see [17–19]). This in turn causes the phosphorylation of the TGF β type I receptor, and this activated type I receptor induces signals via one of the four signaling pathways (see Fig. 1). These include SMAD (intracellular proteins that mediate signaling from receptors for TGF- β and its related factors, named after the first two proteins identified, Sma in *Caenorhabditis elegans* and Mad in *Drosophila*) proteins [20] and three distinct mitogen-activated protein kinases (MAPKs also known as ERKs, extracellular signal-regulated kinases), namely i) stress-activated protein kinase (SAPK) or c-Jun N-terminal protein kinase (JNK), ii) p38 MAPK (p38), and iii) extracellular signal-regulated kinase 1/2 (ERK1/2) (see Fig. 1). Their corresponding upstream transducers are Cdc42/Rac GTPases, MEKKs (MAP/ERK kinase kinases), and Ras GTPases, respectively (for

reviews, see [21, 22]). It is known that the TGF β -induced expression of matrix metalloproteinase 13 (MMP-13) is mediated via the activation of p38 in fibroblasts [23]. By activating ERK1/2 and JNK, TGF β is also known to up-regulate the expression of collagenase-1 in gingival fibroblasts [24]. Also, TGF β -induced phosphorylation of SMADs can form a complex with Co-SMAD, which in turn translocates the activated multiprotein complex to the nucleus to induce gene activation via specific transcription factors [17, 25, 26]. For example, the transcription of plasminogen activator inhibitor-1 (PAI-1) is induced by the synergistic cooperation of Smad proteins and transcription factor TEF3 (transcription enhancer factor 3) [27]. It is therefore possible that TGF β 3 perturbs the Sertoli cell TJ barrier via one of these four pathways (see Fig. 1).

In the present study, we sought to address the following issues. First, we expanded our earlier regulation study [10] to the protein level by using immunoblotting and to assess the IC₅₀ of TGF β 3. Second, we examined the cellular distribution and developmental regulation of four signal transducers and their changes during the TJ assembly in vitro. Third, the upstream signaling molecules and the signaling pathway by which TGF β 3 exerts its effects were investigated. These studies were in turn confirmed

by using different MAP kinase inhibitors and protein immunoblotting techniques.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were obtained from Charles River Laboratories (Kingston, MA). Rats were killed by CO₂ asphyxiation. The use of animals for this study was approved by the Rockefeller University Animal Care and Use Committee with Protocol Numbers 00111 and 95129-R.

Antibodies

The polyclonal antibodies used in studies reported herein were raised in rabbits against the corresponding proteins of human (TGF β 3, JNK, p-JNK, p-ERK1/2, p-38, p-p38, MEKK2, Rac2) or rat (ERK1/2) origin. The monoclonal antibodies of Smad2, Cdc42, N-Ras were from mouse myeloma cells using antigens of either human (Cdc42, N-Ras) or mouse (Smad2) origin. Antibodies against JNK (cat. no. 9252, lot. no. 4), p-JNK (cat. no. 9251S, lot. no. 3), ERK1/2 (cat. no. 9102, lot. no. 5), p-ERK1/2 (cat. no. 9101S, lot. no. 4), p38 (cat. no. 9212, lot. no. 3), and p-p38 (cat. no. 9211S, lot. no. 3) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against TGF β 3 (cat. no. sc-82, lot. no. H280), MEKK2 (cat. no. sc-1088, lot. no. A181), Rac2 (cat. no. sc-96, lot. no. G231), and N-Ras (cat. no. sc-31, lot. no. D111) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Cdc42 (cat. no. 610842, lot. no. 5) and Smad2 (cat. no. 610928, lot. no. 5) were from BD Transduction Laboratories (San Diego, CA). All antibodies used in this study cross-reacted with the corresponding rat protein as indicated by the manufacturer. Bovine anti-rabbit IgG (cat. no. sc-2370, lot. no. B051) and goat anti-mouse IgG (cat. no. A-2304, lot. no. 51K4849) conjugated to horseradish peroxidase (HRP) were from Santa Cruz Biotechnology and Sigma (St. Louis, MO), respectively. Both the catalog and lot numbers were indicated because selected antibodies from other vendors failed to yield satisfactory results as shown in preliminary experiments.

Preparation of Testicular Cells for Culture Experiments

Sertoli cell cultures. Primary Sertoli cells were isolated from 20-day-old Sprague-Dawley rats as previously described [28, 29]. Freshly isolated Sertoli cells were cultured at high cell density (0.5×10^6 cells/cm²) on Matrigel-coated 12-well dishes in serum-free Ham F12 Nutrient Mixture (F12) and Dulbecco modified Eagle medium (DMEM) (1:1, v/v) as described [10]. Cells were then incubated at 35°C in a humidified atmosphere of 95% air/5% CO₂ (v/v). To obtain Sertoli cell cultures with greater than 95% purity, cells were hypototically treated with 20 mM Tris, pH 7.4, for 2.5 min to lyse contaminating germ cells 36 h after plating [30]. Thereafter, the Sertoli cell epithelium was washed twice to remove cellular debris. Media were replaced every 24 h and cells were incubated for an additional 6–7 days. Cultures were terminated at specified time points for RNA extraction.

Isolation of Sertoli cells from adult rat testes. The isolation of Sertoli cells from 60- and 90-day-old Sprague-Dawley rat testes were performed essentially as earlier described [31].

Germ cells. Germ cells were isolated from 5-, 10-, 20-, 40-, 60-, 90-day-old male Sprague-Dawley rat testes by a mechanical procedure without the use of trypsin [32] as described [33].

Sertoli cells cultured with recombinant human TGF β 3 protein. Sertoli cells prepared as described above were cultured on Matrigel-coated 12-well dishes at a density of 0.5×10^6 cells/cm². To study the effects of TGF β 3 on cellular gene expression at the time of Sertoli TJ assembly, recombinant human TGF β 3 protein (3 ng/ml) (Calbiochem Corp., La Jolla, CA) was added to these cultures immediately following their isolation. Media containing TGF β 3 was replaced every 24 h.

Effects of human recombinant TGF β 3 and MAP kinase inhibitors apigenin, SB202190, and U0126 on the assembly of Sertoli cell TJ barrier *in vitro*. Sertoli cells isolated as described above were cultured at high cell density on Matrigel-coated bicameral units (Millicell HA filters; Millipore, Bedford, MA) to allow the assembly of TJs. These cultures were used to assess the effects of apigenin (4',5,7-trihydroxyflavone, an inhibitor for all MAP kinases) [34], SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, a specific p38 kinase inhibitor) [23], and U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene, a specific MEK1/2 kinase inhibitor) [35] on TJ-permeability barrier in the

absence (controls) or presence of recombinant TGF β 3. Apigenin and SB202190 were purchased from Calbiochem (La Jolla, CA) and U0126 was from Cell Signaling Biotechnology (Beverly, MA). To prepare the stock solution of these inhibitors, apigenin (1 mM), SB202190 (3 mM), and U0126 (10 mM) were dissolved in DMSO. Prior to use, these inhibitors were diluted in F12 DMEM to desired concentrations. Briefly, freshly isolated Sertoli cells were plated on Matrigel-coated HA filters in the apical compartment at 1.2×10^6 cells/cm² and treated as cultures at Day 0 [36]. To assess the assembly of the Sertoli cell TJ barrier, TER (trans-epithelial electrical resistance) across the Sertoli cell epithelium was quantified using a Millicell electrical-resistance system (Millipore Corp.) as described [10, 37]. To investigate the effects of different MAP kinase inhibitors on the TJ barrier, freshly isolated Sertoli cells were cultured for 3 h alone, cells were then pretreated with apigenin (1 μ M), SB202190 (0.1 nM–1 μ M), and U0126 (10 μ M) for 16 h, 20 min, and 1 h, respectively, followed by two successive washes with F12 DMEM to remove these inhibitors. TGF β 3 (3 ng/ml) was then included in the replacement media. Media containing TGF β 3 were replenished every 24 h in both the apical (0.5 ml) and basal (0.5 ml) compartments of the bicameral units. Two sets of controls were also included, which were i) Sertoli cells cultured alone without TGF β 3 or inhibitor and ii) Sertoli cells cultured with TGF β 3. TER across the Sertoli cell epithelium was determined at specified time points. Each time point had triplicate cultures and each experiment was repeated at least twice using different batches of Sertoli cells.

Preparation of Sertoli Cell-Enriched Culture Medium and Sertoli Cell Lysates

Sertoli cells, prepared as described above, were cultured on Matrigel-coated 12-well dishes at a density of 0.5×10^6 cells/cm² for a period of up to 7 days. Sertoli cell-enriched culture medium (SCCM) was collected from a set of two dishes each day as described [28]. To obtain cell lysates, cultures in 12-well dishes were briefly rinsed with F12 DMEM. Cells were resuspended in 1 ml SDS sample buffer (0.25 M Tris, pH 6.8, at 22°C, containing 1% SDS, 1.6% 2-mercaptoethanol, 2 mM PMSE, 1 mM EDTA) and incubated at room temperature for 5 min. In samples to be used for analysis of activated MAP kinase by immunoblotting, the above lysis buffer also contained 1 mM sodium orthovanadate (a protein tyrosine phosphatase inhibitor, PTPi) and 0.1 μ M sodium okadaate (a protein Ser/Thr phosphatase inhibitor, PPI). Samples were sonicated, vortexed, and centrifuged at 15,000 \times g for 10 min at 4°C. The supernatant was collected and used as total cell lysates. Protein estimation was performed by Coomassie blue-dye binding assay [38] using BSA as a standard.

Immunohistochemistry

Frozen sections (8 μ m thick) were obtained in a cryostat (Model HM 500M; Microm Lab GmbH, Walldorf, Germany) at -20°C using a disposable blade. Sections were placed on poly-L-lysine-coated slides, air dried at room temperature, and fixed in Bouin fixative for 5 min. The endogenous peroxidase activity in the sections was blocked by treatment with 1% hydrogen peroxide (v/v) for 20 min. Nonspecific sites were blocked by incubating with 10% normal goat serum for 1 h. Tissue sections were then incubated with a rabbit polyclonal anti-TGF β 3 polyclonal antibody at a dilution of 1:50 at 35°C overnight and then washed three times with PBS (5 min each). Thereafter, sections were incubated with a goat anti-rabbit IgG conjugate to horseradish peroxidase for 30 min and washed three times with PBS. The immunoreactive TGF β 3 protein was visualized using a 3,3'-diaminobenzidine reagent system (Zymed, South San Francisco, CA) for 5–10 min. The slides were then washed in water for 10 min to stop the reaction and counterstained with Mayer hematoxylin. Controls were performed by i) substituting the primary anti-TGF β 3 antibody with normal goat serum, ii) substituting the primary anti-TGF β 3 antibody with normal rabbit serum, iii) preabsorbing the primary antibody overnight with 0.1 μ g of recombinant TGF β 3 at 4°C before its use for incubation (1:50 in a sample volume of 200 μ l), and iv) substituting the second antibody with normal goat serum.

Detection of Target Gene Steady-State mRNA Levels and Their Changes During Assembly of Sertoli Cell TJ Barrier by Semiquantitative Reverse Transcription and Polymerase Chain Reaction

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed essentially as previously described [10, 37, 39, 40] to detect changes in the expression of selected target genes in dif-

TABLE 1. Primers used for RT-PCR for the analysis of mRNAs encoding for S16 and different target genes.

Target gene	Primer sequence	Orientation	Position	Length	Reference
S-16	5'-TCCGCTGCAGTCCGTTCAAGTCTTT-3'	Sense	15-38	385	[58]
	5'-GCCAAACTTCTTGGATCCGAGCC-3'	Antisense	376-399		
Smad2	5'-AGCCGGTGGGTCCGGAGGAC-3'	Sense	63-83	244	[59]
	5'-GTAAGGCCCTGTGTGTCTCCCA-3'	Antisense	286-306		
Cdc42	5'-GTGGTCAATGGTGGTGTGG-3'	Sense	25-44	528	[60]
	5'-GGGTGAGTTCCGGAGGCTC-3'	Antisense	532-552		
Rac2	5'-GAGGCCCGGACCAGACCCATC-3'	Sense	127-148	196	[61]
	5'-TGGCTACCATCTGGATAGCCCTT-3'	Antisense	299-322		
MEKK2	5'-AGCCAGTCCGCCAGCAITAT-3'	Sense	56-75	279	[62]
	5'-TGTGAATACTGCCAATCCAGG-3'	Antisense	315-334		
N-Ras	5'-AACTGGTGGTGGTGGAGCCAT-3'	Sense	13-35	518	[63]
	5'-CCATCGTCACTGCTGTTCAG-3'	Antisense	511-530		

ferent samples within an experimental group. All samples within an experimental group were processed simultaneously for RNA extraction and RT-PCR to eliminate inter-experimental variation. Briefly, about 2 µg of total RNA was reverse transcribed into cDNAs using 0.3 µg of oligo(dT)₁₅ with a Moloney murine leukemia virus reverse transcription kit (Promega, Madison, WI) in a final reaction volume of 25 µl. PCR was routinely performed by combining 2-3 µl of the RT product with 0.4 µg each of the sense and antisense of selected target gene primer pairs coamplified with the rat ribosomal S16 primer pair (~0.03 µg) (Table 1). Coamplification with S16 was included to ensure that equal amounts of RNA were reverse transcribed and amplified in each reaction tube. Preliminary experiments were performed to ensure both the PCR product of the target genes and S16 were still in the linear phase using various concentrations of primer pairs (i.e., target gene and S16), different ratios of target gene to S16, different concentrations of RT products, and different PCR cycles. It is noted however that, in most instances, the linearity of the S16 PCR product in the reaction was close to saturation whereas the target gene was at its exponential phase due to the disparity between the basal steady-state mRNA levels of S16 versus the target genes being investigated. These preliminary experiments, however, suggest that samples within a treatment group can be compared statistically after the target gene expression is normalized against S16. To vigorously confirm these results, most of the key experiments using RT-PCR were verified by immunoblotting using commercially available specific antibodies against the corresponding target genes. Nonetheless, these immunoblot analyses revealed that results of the semiquantitative RT-PCR were reliable. For PCR analysis, RT products were mixed with 5 µl 10× PCR buffer, 3 µl of MgCl₂ (25 mM), 8 µl of dNTPs (200 µM each of dATP, dGTP, dCTP, and dTTP), 1.25 U Taq DNA polymerase (Promega), and sterile double distilled water to a final volume of 50 µl. The cycling parameters for PCR reaction were as follows: denaturation at 94°C for 1 min, annealing at 58-59°C (temperature used was dependent on the target gene to be investigated as determined in preliminary experiments) for 2 min, and extension at 72°C for 3 min, for a total of 21-26 cycles, which were followed by an extension period of 15 min at 72°C. In order to enhance the detection limit and to yield data for densitometric scanning to permit semiquantitative analysis, PCR was performed by the inclusion of [³²P]-labeled primers. Briefly, the sense primer of target genes and S16 were labeled at the 5' end with [³²P]-dATP (specific activity, 6000 Ci/mmol; Amersham Pharmacia Biotech) using T₄ polynucleotide kinase (Promega). Approximately 0.5 × 10⁶ cpm were used per PCR reaction and the ratio of the [³²P]-labeled sense primer of target gene to S16 was similar to the ratio of the unlabeled primers. To ensure the linearity in the synthesis of both target genes, such as Smad2 and S16 in the PCR, 10-µl aliquots of PCR products at 18, 20, 22, 24, and 26 cycles were withdrawn and resolved onto 5% T polyacrylamide gels using 0.5× TBE (44.5 mM Tris-borate, 1 mM EDTA, pH 8.0) as a running buffer for visualization in preliminary experiments. Following gel electrophoresis, PCR products were visualized by ethidium bromide staining, and autoradiography was performed using Kodak X-OMAT AR s-ray film (Eastman Kodak, Rochester, NY). Autoradiograms from three separate experiments were densitometrically scanned at 600 nm using a Pharmacia Ultrascan XL Laser Densitometer (Model LKB 2222-020), normalized against S-16, and used for statistical analysis.

Detection of TGFβ3 Protein During Sertoli Cell TJ Assembly by Immunoblot Analysis

Spent media were collected at specified time points during the assembly of the Sertoli cell TJ barrier using cells cultured at 0.5 × 10⁶ cells/

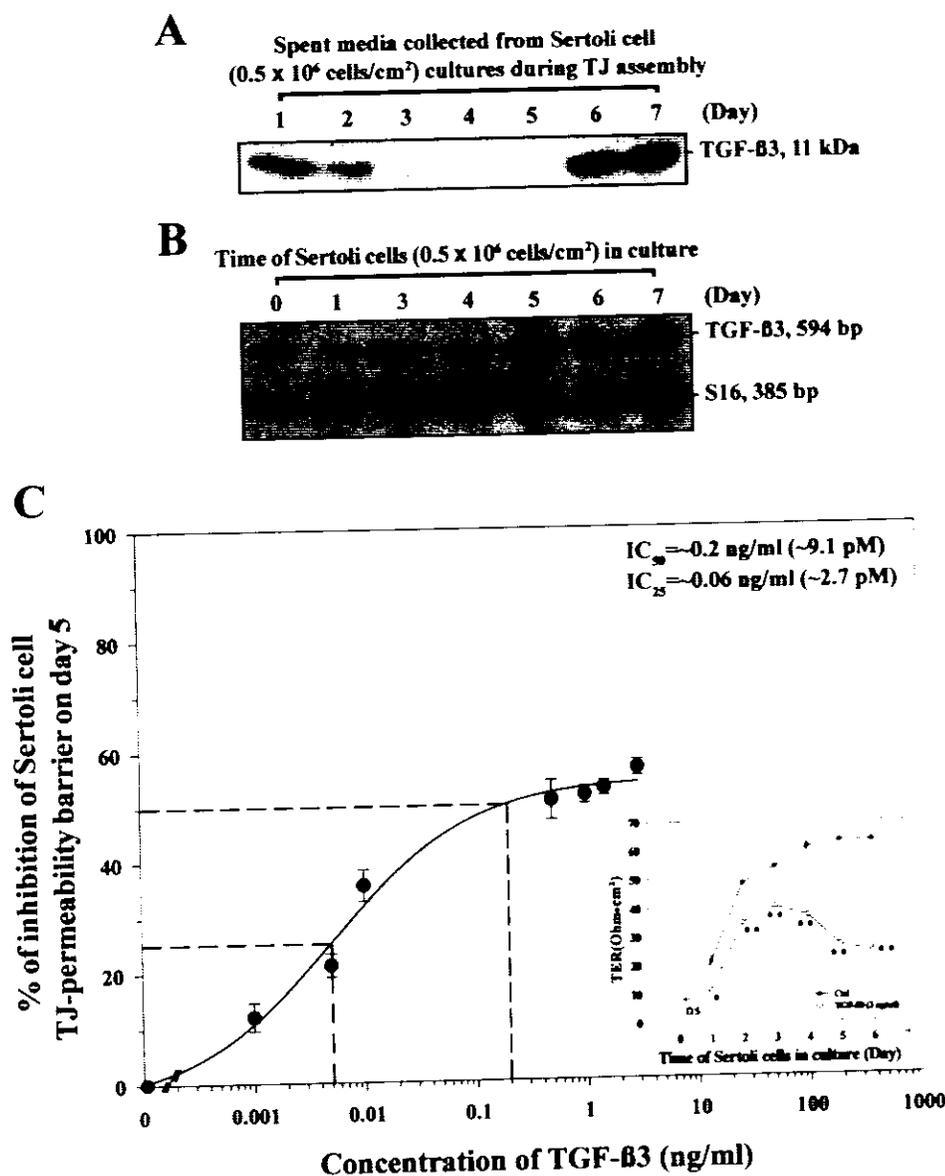
cm² on 12-well Matrigel-coated dishes. About 100 µg protein per sample at different time points was resolved on 15% T SDS-polyacrylamide gels by SDS-PAGE under reducing conditions. The presence of TGFβ3 protein in SCCM was detected by immunoblotting and TGFβ3 was visualized by the Enhanced Chemiluminescence (ECL) Detection System (Amersham Pharmacia Biotech) using Kodak BioMax films.

Detection of Activated p38 MAP Kinase During Sertoli Cell TJ Assembly

Proteins (200 µg) from Sertoli cell lysates derived from cultures with or without TGFβ3 treatment were resolved onto 7.5% T SDS-polyacrylamide gels. The activation of JNK, p38, and ERK1/2 following TGFβ3 treatment was determined by immunoblotting using antibodies specific for both the total and phosphorylated/activated forms of the corresponding MAPK. Briefly, following electrophoretic transfer of proteins onto nitrocellulose paper, blots were blocked with 0.1% Tween-20/5% nonfat dry milk in PBS-Tris (10 mM Tris, 10 mM sodium phosphate, 0.15 M sodium chloride, pH7.4, at 22°C) for 1 h, followed by three washes in 0.1% Tween-20/PBS-Tris (15 min once, 5 min twice). Soon after these washings, the blot was incubated in 0.1% primary antibody in PBS-Tris containing 0.1% BSA (w/v). The blot was then incubated with 0.05% bovine anti-rabbit IgG-HRP/0.1% BSA in PBS-Tris for 1 h and visualized by the ECL Detection System.

Statistical Analysis

In all the experiments reported herein, results were derived from three separate experiments using different batches of cells. And in each experiment, each time point or treatment group had duplicate or triplicate cultures. About 10-20 rats at 20 days of age were used in each culture experiment to obtain sufficient cell numbers to prepare the needed replicate/triplicate cultures and the different treatment groups. To complete the entire studies described in this report, a total of 400 rats were used over a period of 2 yr. Statistical analysis was performed using a statistical analysis software package from GB-STAT (Version 7.0) from Dynamic Microsystems Inc. (Silver Spring, MD). For results shown in Figures 2, 4, 5, 6, and 10, statistical analysis was performed by Student *t*-test. In Figures 2 and 10, each treatment group within an experiment was compared with the corresponding control cultures without TGFβ3 or TGFβ3 plus inhibitor. In Figure 4, the mRNA and protein levels of the corresponding TGFβ upstream signaling molecules in germ cells were compared with Sertoli cells, which were arbitrarily set to one. In Figures 5 and 6, the steady-state mRNA levels of the corresponding TGFβ upstream signaling molecules in Sertoli and germ cells (Fig. 5, B and D) or testes (Fig. 6B) at other ages were compared with the youngest age group, which was arbitrarily set to one. For data shown in Figures 8, G and H, and 9B, statistical analyses were performed by ANOVA using Tukey honestly significant difference test, where each sample group at a specified time point was compared with other sample groups within the same experiment. For instance, for data shown in Figure 9B (right panel), the protein level of p-p38 in Sertoli cells during TJ assembly on Days 1-3 after TGFβ3 treatment was shown to be significantly higher when compared with cells on Days 0, 4, 5, 6, and 7 by ANOVA, which is also different from cultures without TGFβ3 treatment (left panel).



RESULTS

Changes in TGF β 3 During Assembly of Sertoli Cell TJ-Permeability Barrier by Immunoblot and Semiquantitative RT-PCR Analysis

Previous studies have shown that there was a significant decline in Sertoli cell TGF β 3 steady-state mRNA level coinciding with the assembly of the Sertoli TJ barrier in vitro [10]. Due to the low detection limit of the conventional immunostaining technique, which requires ~10–30 ng of TGF β 3 to be visualized, changes in TGF β 3 protein during Sertoli cell TJ assembly were not visible [10]. When an enhanced chemiluminescent-based immunodetection system was used, which had a sensitivity >200-fold compared with the colorimetric-based technique, a plummeting in TGF β 3 protein in the spent media of Sertoli cells was detected coinciding with the assembly of the TJ barrier (Fig. 2A). These results were also consistent with the RT-PCR data (see Fig. 2B).

FIG. 2. Changes in the TGF β 3 protein level in Sertoli cell spent media (control and 3 ng/ml TGF β 3) and its steady-state mRNA level in Sertoli cell cultures during the assembly of the Sertoli cell TJ permeability barrier and the time course of TGF β 3 in the Sertoli cell TJ barrier in vitro. **A**: One hundred micrograms protein from Sertoli cell spent media cultures terminated at specified time points during the assembly of the Sertoli cell TJ barrier were subjected to a 15% SDS-polyacrylamide gel. SDS-PAGE was performed under reducing conditions and immunoblotting was performed using anti-TGF β 3 antibody. **B**: Semiquantitative RT-PCR was performed to assess the steady-state mRNA level of TGF β 3 in Sertoli cells during the assembly of the TJ barrier. **C**: Sertoli cells (1.2×10^6 cells/cm²) were cultured in Matrigel-coated bicameral units with increasing concentrations of TGF β 3 ranging between 0.001 and 3 ng/ml. TER across the cell epithelium was quantitated at specified time points to assess the magnitude of the TJ barrier assembly. TER was assessed by plotting the percentage of inhibition in the Sertoli cell TJ barrier against the TGF β 3 in Day 5 at the respective TGF β 3 concentration versus control values against different concentrations of TGF β 3, which was added to the cultures at the x-axis. To assess the percentage of inhibition, control media was used. TER, TER/TER_{control} $\times 100$, where TER_{control} represents TER readings in the control units in Day 5 and TER represents TER readings in the presence of TGF β 3 at a specified concentration. TER_{control} represents TER readings in the corresponding cultures in Day 5 and TER represents TER readings in the presence of TGF β 3. Error bars represent the mean \pm SD of three separate experiments being conducted in triplicate and each experiment containing three replicates. NS, No significant difference between the control and TGF β 3-treated units with respect to TER. Statistical significance was determined from the percentage inhibition with respect to TGF β 3 by Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Error bars represent the mean \pm SD of three separate experiments.

Determination of IC₅₀ of TGF β 3 on the Sertoli Cell TJ Barrier In Vitro

The addition of recombinant TGF β 3 at 3 ng/ml to Sertoli cells cultured in vitro at 1.2×10^6 cells/cm² on Matrigel-coated bicameral units to permit TJ assembly could indeed perturb the assembly of the Sertoli cell TJ-permeability barrier (see inset to Fig. 2C). The IC₅₀ of TGF β 3 on the assembly of Sertoli cell TJ barrier was assessed by quantifying the TER across the Sertoli cell epithelium in the presence of different concentrations of TGF β 3 between 0.001 and 3 ng/ml on Day 5 at the time Sertoli cells completed the TJ barrier assembly (Fig. 2C and inset), which is manifested by a steady TER across the cell epithelium. It is noted that TGF β 3 could only perturb, but not abolish, the event of TJ assembly because there was no difference in its magnitude of inhibition between 0.5 and 3 ng/ml. The percentage of inhibition induced by TGF β 3 was normalized against controls and plotted against different concentrations of TGF β 3 used for the experiment; the IC₅₀ was found to

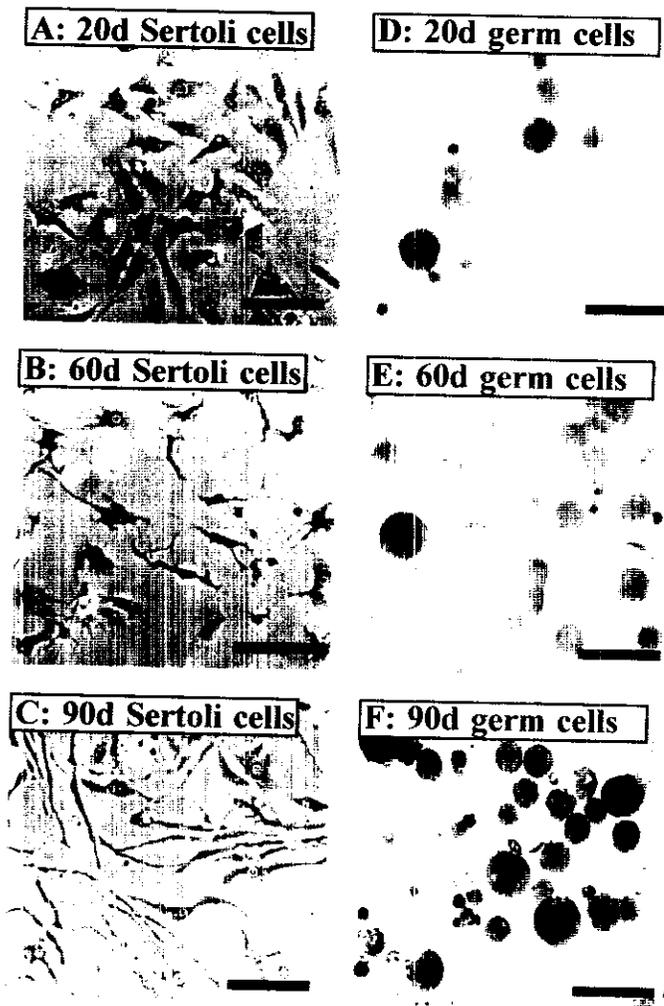


FIG. 3. Morphological analysis of Sertoli and germ cells isolated from rats of different ages to confirm their purity. Sertoli cells isolated from 20- (A), 60- (B), and 90-day-old (C) rats and germ cells isolated from 20- (D), 60- (E), and 90-day-old (F) rats. Cells were fixed in increasing methanol (from 20% to 100%) concentrations and stained with 1% toluidine blue-O (0.1%, w/v) in PBS. The purity of the cell preparations was greater than 95%. Bar = 100 μ m in A-C; bar = 20 μ m in D-F.

be at 9.1 pM, which is equivalent to \sim 0.2 ng/ml TGF β 3 (Fig. 2C).

Characterization of Sertoli and Germ Cells by Light Microscopy

Sertoli (Fig. 3, A-C) and germ (Fig. 3, D-F) cells were isolated from rats at different ages. The Sertoli cell preparations used in the studies described herein were greater than 90% pure (Fig. 3, A-C). The final germ cell preparation obtained from 60- and 90-day-old rat testes were devoid of elongated spermatids, which were removed by the glass wool filtration step (Fig. 3, E and F). Germ cells isolated from 20-day-old rats consisted primarily of spermatogonia and spermatocytes with a relative percentage of about 50%:50% (Fig. 3D). For germ cells isolated from 60- and 90-day-old rats, the relative percentages of spermatogonia, spermatocytes, and round spermatids were 16%:19%:65% (Fig. 3, E and F), consistent with earlier reports [41]. Germ cell preparations used in the studies described herein were basically free of somatic cell contamination since analysis by RT-PCR failed to detect testin, a putative

Sertoli cell protein [42], in these germ cell preparations (data not shown).

Relative Cellular Expression of the TGF β 3 Upstream Signal Transducers in the Seminiferous Epithelium Behind the Blood-Testis Barrier

If TGF β 3 indeed plays a crucial role in regulating the junction dynamics between Sertoli cells and/or between Sertoli and germ cells during spermatogenesis, it is conceivable that Sertoli and germ cells express the TGF β -associated signal transducers. As expected, RT-PCR and immunoblot analyses revealed that both Sertoli and germ cells expressed Smad2, Cdc42, Rac2, MEKK2, and N-Ras (Fig. 4, A-C), the four upstream TGF β transducers (see Fig. 1). However, Smad2, Cdc42, MEKK2, and N-Ras were more predominant in germ cells than Sertoli cells, with a Sertoli:germ cell ratio of 1:2, 1:8, 1:5, and 1:3.5 (Fig. 4, A and C), respectively. Yet Rac2 was more predominant in Sertoli cells, with a relative Sertoli:germ cell ratio of 2:1 (Fig. 4, A and C). By using immunoblot analysis, similar results were obtained (Fig. 4, B and C), confirming results of the RT-PCR (Fig. 4C versus Fig. 4A).

Expression of Upstream Signal Transducers in Sertoli and Germ Cells During Maturation

Due to the differences in the steady-state mRNA levels of these signal transducers between Sertoli and germ cells as shown in Figure 4, we thought it pertinent to examine whether there are any changes in these upstream signal transducers during maturation. It was noted that there was an increase in the expression of Smad2 and Cdc42 by Sertoli cells during maturation (Fig. 5, A and B). In contrast, a plummeting in Rac2 and MEKK2 was detected during maturation (Fig. 5, A and B). For N-Ras (Fig. 5, A and B), no significant changes in expression were detected during Sertoli cell maturation. With regard to the expression of these transducers in germ cells during maturation, it was noted that the steady-state mRNA levels of Smad2, MEKK2, and N-Ras increased during maturation (Fig. 5, C and D). However, the expression of Cdc42 and Rac2 increased during aging peaked at 20-40 days of age; thereafter, their expression tumbled at 60-90 days of age, returning to a level similar to immature rats (Fig. 5, C and D).

Developmental Regulation of Upstream TGF β Signal Transducers in the Testis

Because there is a significant increase in Sertoli-germ cell interactions and junction restructuring during testicular maturation, we have examined the steady-state mRNA levels of TGF β upstream signal transducers in developing testes (Fig. 6, A and B). The expression of Smad2, Rac2, and MEKK2 peaked at \sim 10-20 days of age at the time the blood-testis barrier was assembled (Fig. 6, A and B), suggesting these signal transducers may be involved in these events. However, significant increases in the steady-state mRNA levels of Cdc42 and N-Ras were detected at 40-90 days of age (Fig. 6, A and B).

Immunohistochemical Localization of TGF β 3 in the Seminiferous Epithelium

TGF β 3 was detected in the seminiferous epithelium and localized in a stage-specific fashion extending from

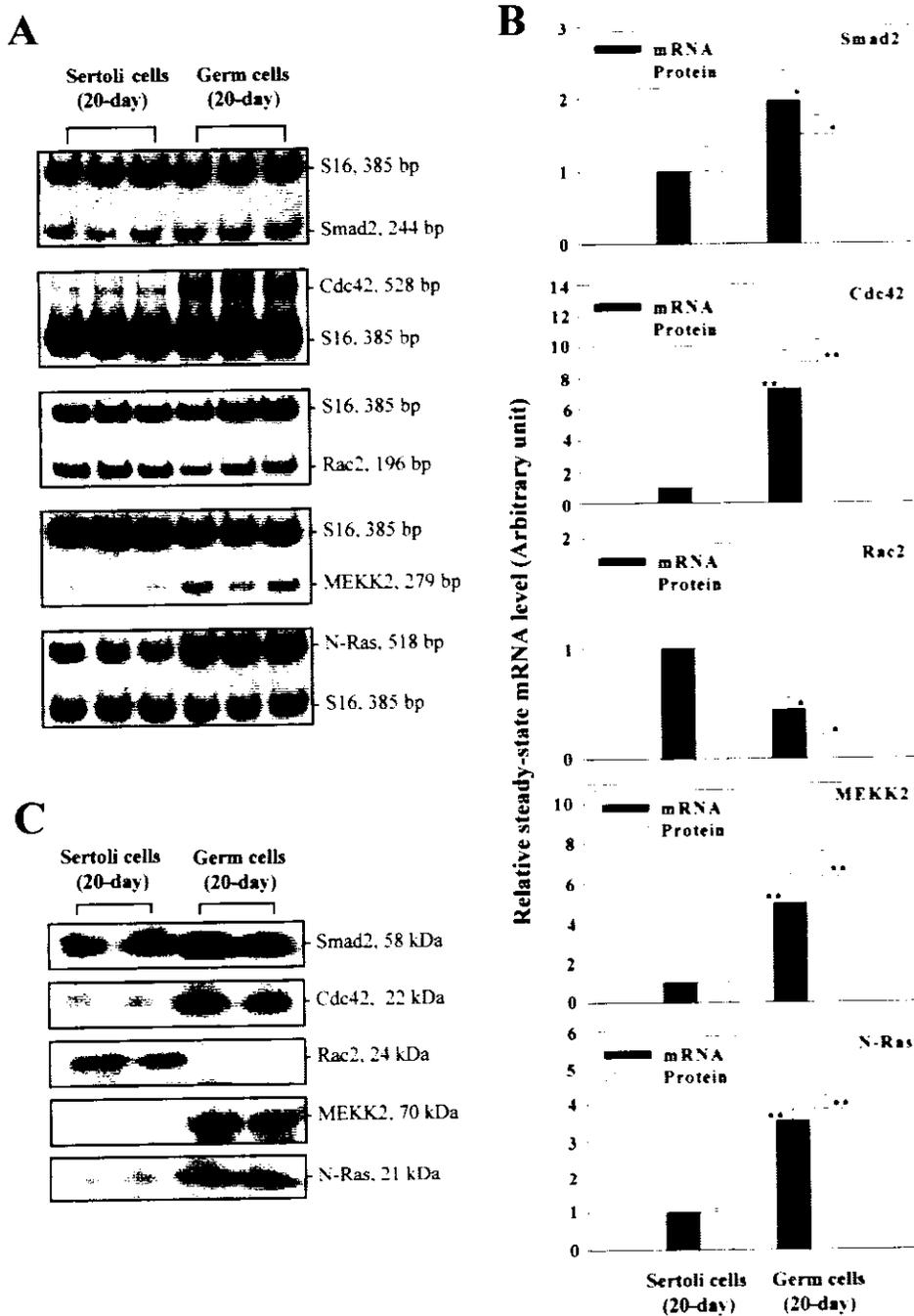


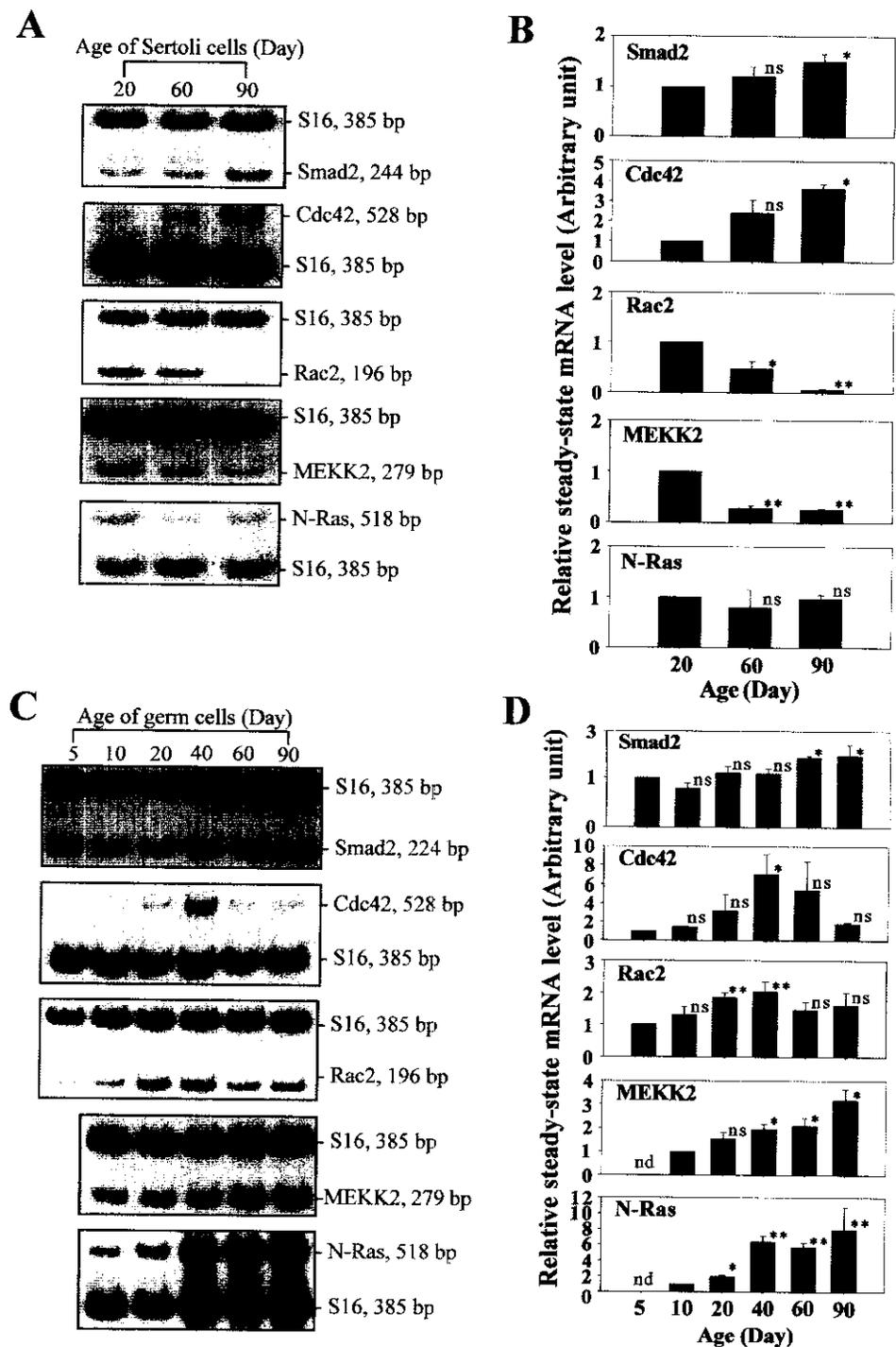
FIG. 4. Regulation of expression of the TGF β 3 upstream signal transducers in Sertoli and germ cells during the assembly of the TJ barrier in testes. **A:** Northern blot for TGF β 3 was performed to assess the steady-state mRNA levels of Smad2, Cdc42, Rac2, MEKK2, and N-Ras in Sertoli and germ cells. Immunoblots were performed in parallel experiments to assess the protein levels of each of these signaling transducers in Sertoli and germ cells using the corresponding specific antibodies (see Materials and Methods). **B:** This figure shows the corresponding immunoblots obtained using anti-rat antibodies in immunoprecipitates with anti-rat IgG. **A and C:** Results are expressed as mean \pm SD using three different experiments. Immunoprecipitation experiments in anti-rat IgG and anti-rat IgG α experiments had the same results. These analyses revealed that the steady-state mRNA and protein levels of the TGF β 3 signaling transducers were significantly higher in germ cells than in Sertoli cells. **C:** Relative steady-state mRNA levels of Smad2, Cdc42, Rac2, MEKK2, and N-Ras in Sertoli and germ cells. The values are the mean \pm SD of three different experiments.

the basal to the adluminal compartment (Fig. 7A). The localization shown in Figure 7A appears to be specific because the immunoreactive brown precipitates were not found in control sections (Fig. 7B versus Fig. 7A). TGF β 3 was found in stages I–X (Fig. 7, C–E) and at stages VII–VIII associated with spermatocytes, round spermatids, and Sertoli cells, but it was not associated with elongated spermatids (Fig. 7, C–E). Its localization appeared to be predominant in stages V–VII and became diminished in stages IX and X (Fig. 7E). Immunoreactive TGF β 3 was virtually undetectable in stages XII–XIV (Fig. 7F). Immunoreactive TGF β 3 was found to extend from the basal region of Sertoli cells to near the adluminal compartment.

Changes in Steady-State mRNA and Protein Levels of MEKK2 in Sertoli Cells During the TGF β 3-Mediated Disruption of Sertoli Cell TJ-Permeability Barrier

It was shown that there was a significant decline in the expression of TGF β 3 (Fig. 2, A and B) coinciding with the assembly of the Sertoli cell TJ barrier (Fig. 2C) and that the addition of recombinant TGF β 3 to Sertoli cells cultured *in vitro* during TJ assembly could indeed perturb the TJ barrier (Fig. 2C). We have examined the steady-state mRNA levels of the upstream TGF β 3 signal transducers during TJ assembly to assess if the pattern of their expression parallels that of TGF β 3. Yet no significant changes in the protein levels of Smad2, Cdc42,

FIG. 5. Developmental regulation of the steady-state mRNA levels of the TGF β upstream signal transducers in Sertoli (A, B) and germ (C, D) cells. RT-PCR was performed to assess the steady-state mRNA levels of Smad2, Cdc42, Rac2, MEKK2, and N-Ras in Sertoli cells (A) and germ cells (C) during maturation. B, D) The corresponding densitometrically scanned results using autoradiograms such as those shown in A and C. Results are expressed as mean \pm SD using two batches of cells normalized against S16 from two different experiments. Each experiment had triplicate cultures. ns, Not significantly different from cultures isolated from rats at 20 days of age in B and 5 or 10 days of age in D, which was arbitrarily set at one, by Student *t*-test; *, significantly different by Student *t*-test, $P < 0.05$; **, significantly different by Student *t*-test, $P < 0.01$; nd, not detectable.



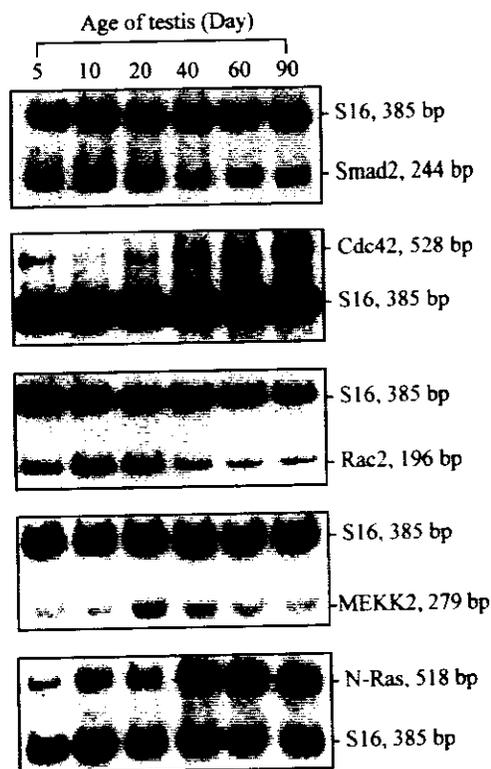
Rac2, and N-Ras were detected in the Sertoli cell cultures during the assembly of the TJ barrier (Fig. 8A), consistent with results of RT-PCR (data not shown). Similar results were obtained when Sertoli cells were cultured in the presence of TGF β 3 (Fig. 8B), which is known to perturb the Sertoli TJ barrier (see Fig. 2). These results seemingly suggest that Smad2, Cdc42, Rac2, and N-Ras are not likely the upstream signal transducers mediating the action of TGF β 3. While the Sertoli cell MEKK2 mRNA and protein levels remained steady during the assembly of the Sertoli cell TJ barrier (Fig. 8, C, E, and G), its pattern of expression was in sharp contrast with the other TGF β upstream transducers at the time of

TGF β 3-mediated TJ barrier disruption. Fourfold increases in MEKK2 mRNA and protein levels were detected when the TJ barrier was perturbed by TGF β 3 (Fig. 8, D, F, and H), suggesting the TGF β 3-induced disruptive effects on the TJ assembly are mediated via the MEKK2 MAP kinase (Fig. 1).

Inhibition of Sertoli Cell TJ Assembly by TGF β 3 Via the Activation of p38 MAPK

To confirm the above observation, we next assessed the activation of three distinct downstream MAPKs such

A



B

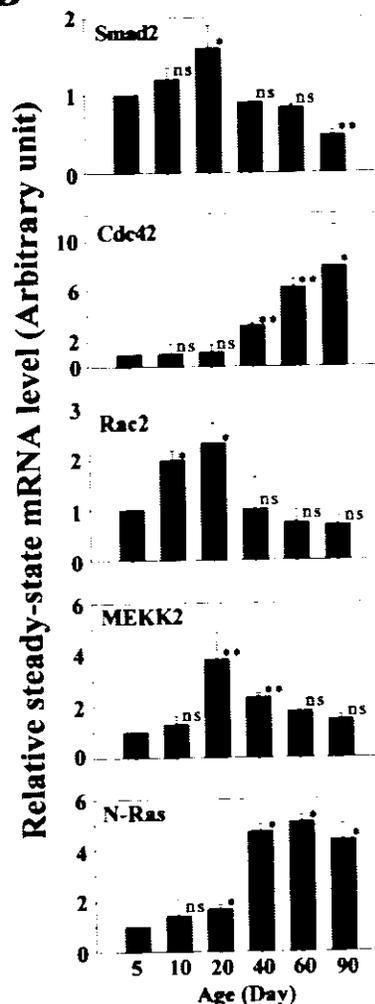


FIG. 2. Changes in the steady-state mRNA levels of TGF β 3 upstream's signaling molecules in the testis during development. **A**: Northern blotting for S16, S was performed to assess the steady-state mRNA levels of Smad2, Cdc42, Rac2, MEKK2 and N-Ras in testis during maturation. **B**: This panel shows the corresponding densitometric analysis of the results as indicated in **A**. Results are expressed as mean \pm SD of six testis from three different rats of 5, 10, and 20 days of age. N.S., not significant; ** P < 0.01; *** P < 0.001. Student's *t*-test was used for comparison. Scale bar represents 100% of the steady-state mRNA level at 5 days of age.

as JNK, p38, and ERK1/2 by immunoblotting using specific antibodies against both their total and phosphorylated/activated forms. Sertoli cells cultured at 0.5×10^6 cells/cm² on Matrigel-coated dishes in the absence (Fig. 9, A and B, left panel) or presence (Fig. 9, A and B, right panel) of TGF β 3 (3 ng/ml) were terminated at specified time points during the assembly of the TJ-permeability barrier. Cells were lysed in SDS sample buffer to obtain total lysates. As shown in Figure 9, A and B, the levels of both the total and phosphorylated/activated JNK (p-54 JNK and p-46 JNK) remained the same in both controls and TGF β 3-treated Sertoli cell lysates at the time of TJ assembly. For ERK1/2, a time-dependent decline in the protein level of its phosphorylated form, but not the total unphosphorylated form, was also detected during Sertoli cell-TJ barrier assembly, yet this pattern of changes during TJ assembly is not responsive to TGF β 3 (Fig. 9, A and B). In contrast, there was a mild decrease in the activated p38 level (p-p38) in Sertoli cell lysates (Fig. 9, A and B) at the time the Sertoli cell TJ-permeability barrier was formed, ~1–3 days (see Fig. 2). More important, the presence of TGF β 3 at 3 ng/ml not only abolished this transient decline in p-p38 level during the assembly of the Sertoli cell TJ-permeability barrier; instead, it in-

duced a transient but significant increase in the p-p38 protein level (Fig. 9, A and B).

Effects of Different MAP Kinase Inhibitors on the TGF β -Induced Disruption of the Sertoli Cell TJ-Permeability Barrier

To confirm TGF β 3 indeed mediates its inhibitory effects on the Sertoli cell TJ barrier via the p38 MAP kinase pathway, different inhibitors were tested on their ability to block the TGF β 3-induced effects. When the entire MAPK pathways were blocked by apigenin at 1 nM to 1 μ M, which was added to the Sertoli cell epithelium 16 h prior to TGF β 3 (3 ng/ml) treatment, it blocked the TGF β 3-mediated effects (Fig. 10A). This result clearly illustrates that TGF β 3 exerts its effects via MAP kinases. The addition of a specific p38 MAP kinase inhibitor, SB202190, at 0.1 nM to 1 μ M to the Sertoli cell epithelium 20 min before TGF β 3 treatment also dose dependently blocked the TGF β 3 effects (Fig. 10B). In contrast, when a MEK1/2 MAP kinase inhibitor, U0126 (10 μ M), which is known to block the Ras/ERK1/2 pathway [35] (see Fig. 1), was used, it failed to block the TGF β 3 effects (Fig. 10C), illustrating the specificity of the apigenin and SB202190 treatment shown in Figure 10, A and B.

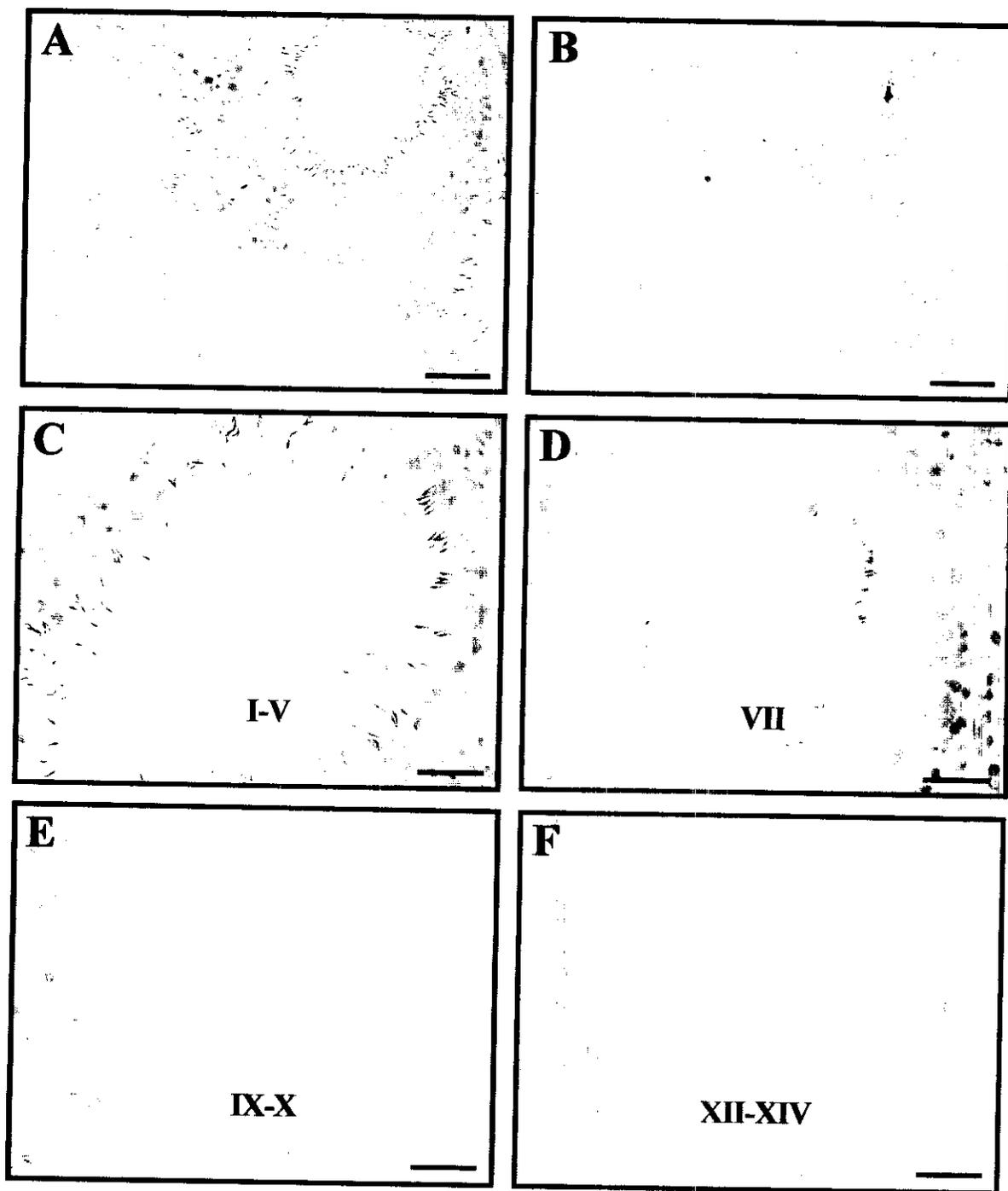
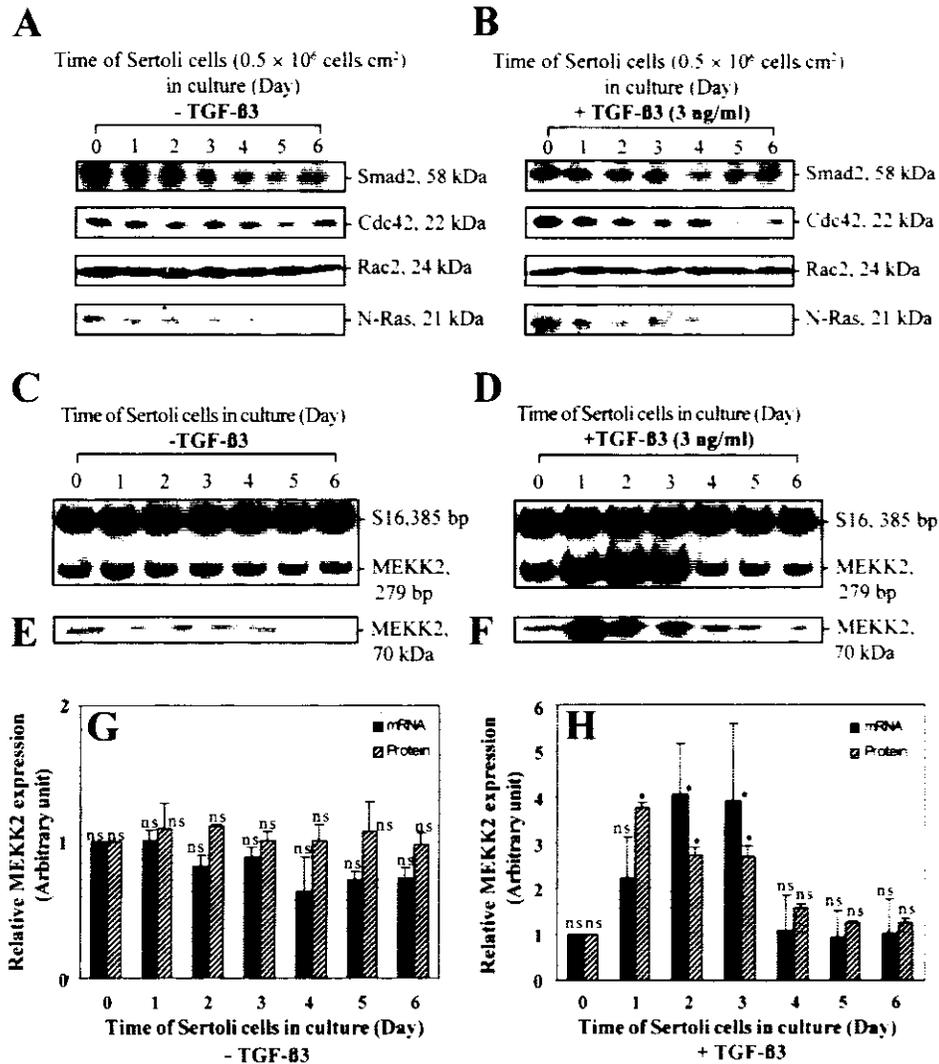


FIG. 7. Cross-sections of rat testes that show changes of TGF β 3 in the seminiferous epithelium in different stages of the spermatogenic cycle by immunohistochemical localization. Micrographs of adult testes showing the seminiferous epithelium immunostained with TGF β 3 (A, C-F) or normal rabbit serum that substituted the primary antibody (B). The specificity of this antibody was confirmed by immunoblotting shown in Figure 2. C) Seminiferous epithelium at stages I-V; immunoreactive TGF β 3 was found in the epithelium extending from the basal to the adluminal compartment. TGF β 3 was found to associate with elongated spermatids. D) Seminiferous epithelium at stage VII, with the most intense immunostaining. However, TGF β 3 was not associated with the elongated spermatids. E) Seminiferous epithelium at stages IX and X after spermiation and the level of immunoreactive TGF β 3 was reduced. F) No TGF β 3 immunostaining material was visible in the epithelium at stages XII-XIV. Bar = 120 μ m for A and B; bar = 50 μ m for C-F.

Changes in Sertoli Cell Total and Activated p38 MAP Kinase Protein Level During Assembly of TJ Barrier with and Without Pretreatment with SB202190 and TGF β 3

Because SB202190 could block the TGF β 3-mediated inhibitory effects on the Sertoli cell TJ barrier (Fig. 10), it is crucial to investigate if it also blocked the TGF β 3-mediated increase in p-p38 MAP kinase level. To verify this, Sertoli

cells cultured at 0.5×10^6 cells/cm² on Matrigel-coated dishes in the absence (Fig. 11A) and presence (Fig. 11B) of TGF β 3 (3 ng/ml) or with pretreatment of SB202190 (0.1 nM) before the addition of TGF β 3 (3 ng/ml) (Fig. 11C) were terminated at specified time points during TJ assembly to obtain total lysates for immunoblot analysis. As shown in Figure 11, the levels of the phosphorylated/activated p38



were stimulated when Sertoli cell TJ barrier was perturbed by TGF β 3 (Fig. 11B), similar to results shown in Figure 9. However, pretreatment of Sertoli cells with SB202190 followed by TGF β 3 could abolish the TGF β 3-induced p-p38 MAP kinase protein level (Fig. 11C versus Fig. 11B), making the pattern of p-p38 expression similar to cells cultured alone without TGF β 3 treatment (Fig. 11A versus Fig. 11, B and C). Thus, these results have unequivocally demonstrated that the TGF β 3-induced TJ disruption is mediated via the p38 MAP kinase pathway.

DISCUSSION

Is TGF β 3 the Only Determinant in the Regulation of Sertoli Cell TJ Dynamics?

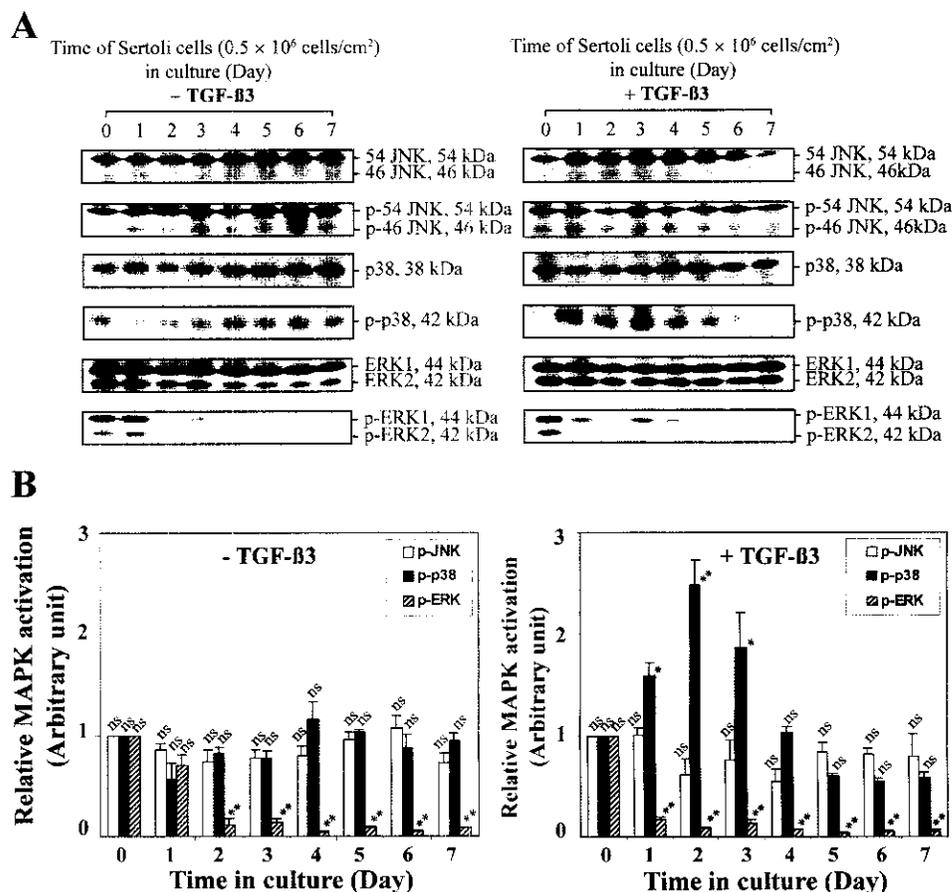
While the timely movement of preleptotene and leptotene spermatocytes across the blood-testis barrier to gain entry into the adluminal compartment for further development at stages VIII–XI in the rat has been known for more than two decades [43], the participating molecules and the biochemical basis that regulate this event are entirely unknown. Results of the studies presented herein have shown for the first time that TGF β 3 plays a crucial role in the regulation of the Sertoli cell TJ dynamics via the MEKK2/p38MAP kinase pathway. Yet TGF β 3 is unlikely to be the only player that dominates these events.

First, studies on the IC $_{50}$ of TGF β 3 reported herein have shown that while this cytokine is effective in perturbing the Sertoli cell TJ barrier, yet it cannot block the assembly of the Sertoli TJ-permeability barrier *in vitro*. Second, recent studies on the regulation of Sertoli cell TJ dynamics have shown that the interplay of kinases and phosphatases that determines the cellular phosphoprotein content also plays a crucial role in regulating TJ dynamics [44]. For instance, the use of either PTPi, such as sodium orthovanadate, or PPI, such as sodium okadaate, was shown to perturb the assembly of the Sertoli cell TJ barrier [45]. Still, these inhibitors, either used alone or in combination, while effectively and reversibly perturbing the Sertoli cell TJ-permeability barrier, they also failed to block the entire events of TJ assembly *in vitro* [44, 45]. Taking these findings collectively, it is clear that the TJ dynamics in the testis are regulated by an array of molecules and signaling pathways, which are intriguingly related but are also differentially regulated. Such complexity is also physiologically important because a failure in one of these signaling events will not cause a shut-down of germ cell movement, halting spermatogenesis.

Roles of Cytokines in the Regulation of TJ Dynamics

Recent studies using different epithelial and endothelial cells have demonstrated the physiological significance of

FIG. 9. Change in the levels of total and activated MAPK protein when Sertoli cell TJs were assembled *in vitro* in the absence and presence of TGF β 3. Sertoli cells (0.5×10^6 cells/cm 2) cultured on Matrigel-coated dishes in the absence (left panel, A) or presence (right panel, A) of TGF β 3 (3 ng/ml) were lysed at specified time points during the assembly of the Sertoli cell TJ barrier to obtain cell lysates. Two hundred micrograms of protein of whole cell lysates from each time point were loaded onto a 7.5% T SDS-polyacrylamide gel and SDS-PAGE was performed under reducing conditions. Immunoblotting was performed using specific antibodies against total JNK, p38, and ERK1/2 and their corresponding phosphorylated and activated forms: p-JNK, p-p38, and p-ERK1/2. B) Corresponding densitometrically scanned results using results of the ECL blots such as those shown in A illustrating the protein levels of activated MAPKs were normalized against total MAPK. This experiment was repeated three times using different batches of cells. Each time point had duplicate cultures. ns, Not significantly different by ANOVA, in which each sample at a given time point was compared with samples of all other time points within the same experimental group; *, significantly different by ANOVA, $P < 0.01$; **, significantly different by ANOVA, $P < 0.001$; D, days.



cytokines, such as hepatocyte growth factor (HGF), interleukin (IL), and interferon- γ (IFN- γ) in the regulation of TJ dynamics (for review, see [46]). For instance, HGF caused the redistribution of ZO-1, moving it away from the site of TJs to cytoplasm, and reduced the association of occludin with ZO-1, which in turn perturbed the TJ-permeability barrier in MDCK cells [15]. And IFN- γ has been shown to reduce the expression of occludin and ZO-1 in T84 cells [16]. In a previous study, we have also demonstrated that TGF β 3 perturbs the Sertoli cell TJ barrier possibly via its inhibitory effects on the timely expression of occludin and ZO-1 associated with TJ assembly. Yet no further investigation was done to identify the signaling pathways by which cytokines mediate their effects in the regulation of TJ dynamics. A more recent study has shown that TNF- α , another cytokine known to be produced by Sertoli and germ cells (for reviews, see [7, 47]), can regulate the homeostasis of the extracellular matrix via its effects on the level of metalloproteases and tissue inhibitors of metalloproteases (TIMPs), which in turn regulates the Sertoli cell dynamics [48]. Taken collectively, these data clearly illustrate the pivotal role of cytokines in TJ dynamics in the testis.

Molecular Mechanism Utilized by TGF β 3 to Perturb the Sertoli Cell TJ Barrier

In this report, we have provided compelling evidence that the TGF β -induced inhibitory effects on the Sertoli cell TJ-permeability barrier are mediated via the p38-MAP kinase pathway. First, it was shown that the TGF β -mediated inhibitory effect on TJ barrier was associated with an increase in MEKK2 expression, the p38

MAP kinase upstream transducers, but not Smad2, Cdc42, Rac2, and N-Ras (see Fig. 1). These results strongly suggest that the TGF β -induced disruptive effects on the TJ assembly are mediated via the MEKK2 MAP kinase signaling pathway. Second, a transient surge in p-p38, but not the p-JNK or the p-ERK, as demonstrated by immunoblotting techniques, was induced by TGF β 3. Third, perhaps the most important of all, the TGF β -induced effects on the Sertoli cell TJ barrier could be reversed by pretreatment of Sertoli cells with SB202190, a specific p38 MAP kinase inhibitor, but not U0126, which is a specific MEK1/2 inhibitor. Furthermore, SB202190 not only blocks the TGF β -induced effects to perturb the Sertoli cell TJ barrier, it also blocks the TGF β -induced transient induction in the p-p38 MAP kinase protein. In this connection, it is noteworthy to mention that the TGF β -mediated inhibitory effect on the Sertoli cell TJ barrier is associated only with an increase in the activated and phosphorylated form of p38-MAP kinase (p-p38) but not the overall p38 MAP kinase. These results seemingly suggest that this TGF β -induced transient p-p38 MAP kinase increase somehow triggers a yet-to-be defined transcriptional activation pathway. This in turn shuts down the production of occludin, claudin-11, and ZO-1, the building blocks needed to maintain the TJ functionality. Work is now in progress to investigate the involved transcription factor(s) that regulate the occludin promoter. Alternatively, it is possible that TGF β stimulates the phosphorylation of p38 MAP kinase, which in turn activates other molecules that perturb TJ assembly. For instance, activation of p38 MAP kinase is known to induce MMP-13 in fibroblasts [24]. Indeed,

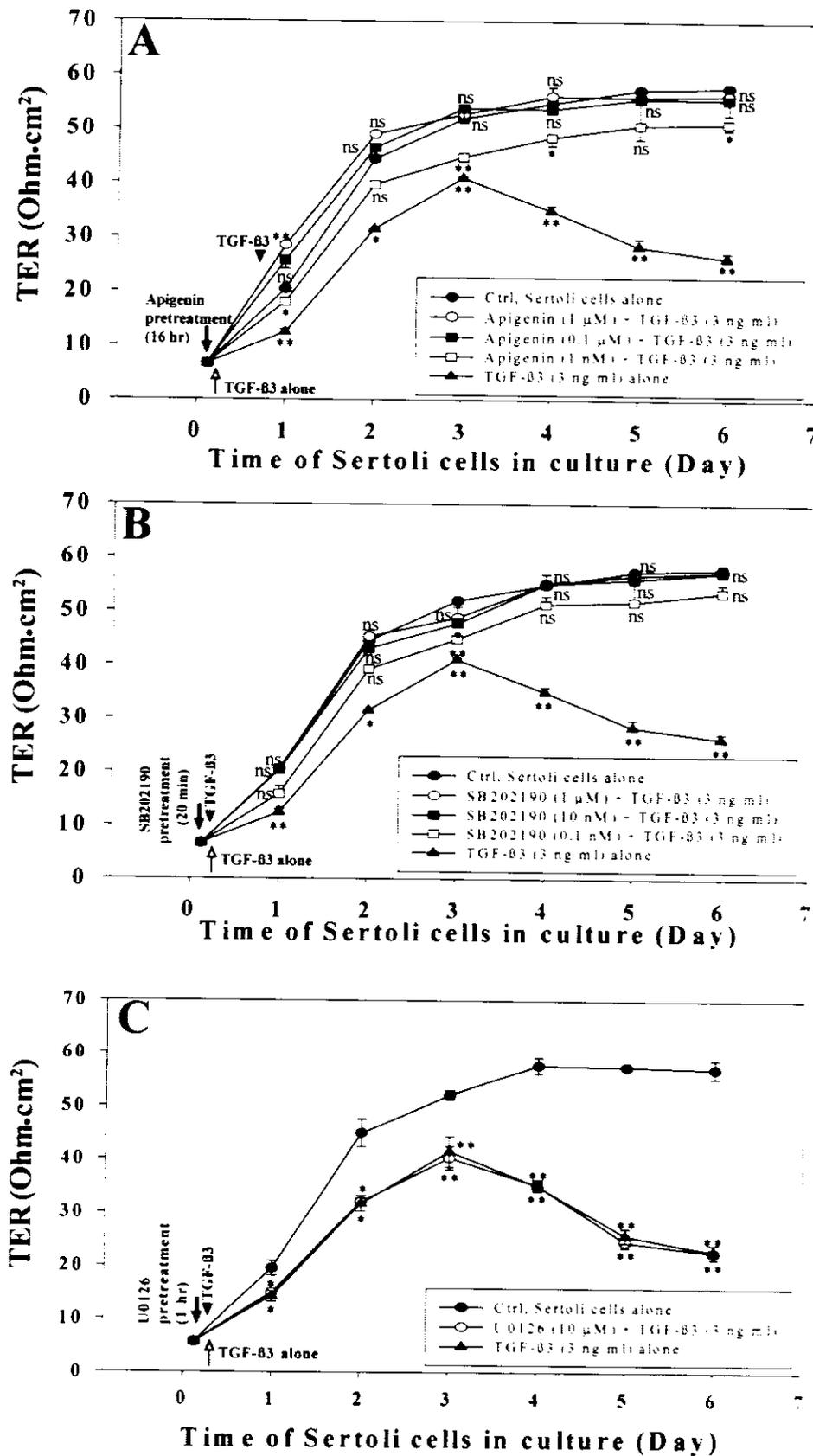


FIG. 7. Effects of TGF β 3 on the assembly of the Sertoli cell TJ barrier. Tight junctional barrier of the cell epithelium with MAPK inhibitors in the Sertoli cells (1:2:1:1) was simultaneously cultured in Matrigel-coated 6-well culture units were pretreated with different concentrations of apigenin (an inhibitor of a MAPKases; arrow **A**), SB202190 (a specific p38 kinase inhibitor; arrow **B**) and U0126 (a specific MEK1/2 kinase inhibitor; arrow **C**) for 16 h, 20 min, and 1 h, respectively, followed by two successive washes with F12/DMEM to remove any inhibitor. Thereafter, TGF β 3 (3 ng/ml) was added to the Sertoli cell epithelium (arrowhead **A-C**). The effects of TGF β 3 on the assembly of the Sertoli cell TJ barrier in vitro were assessed by quantifying TER at various time points up to the 6th specified time points. Each time point had triplicate cultures and each experiment was repeated three times using different batches of cells. Results are expressed as mean \pm SD. In cultures pretreated with each of the three inhibitors, they were also compared with cultures incubated with TGF β 3 alone (open arrowheads). Not significantly different from the corresponding control by Student test ($P > 0.05$). * significantly different from the corresponding control by Student test ($P < 0.05$). ** significantly different from the corresponding control by Student test ($P < 0.01$). (Ctrl, control).

other studies have shown that the dynamics of both TJs and AJs (cell-cell actin-based adherens junctions, such as ectoplasmic specialization, which is a testis-specific AJ type) are regulated, at least in part, by protease and pro-

tease inhibitors [9, 48, 49]. It is therefore possible that this TGF β 3-induced p38 activation can exert its effects via more than one pathway. It is also of interest to note that the assembly of Sertoli cell TJs is also associated

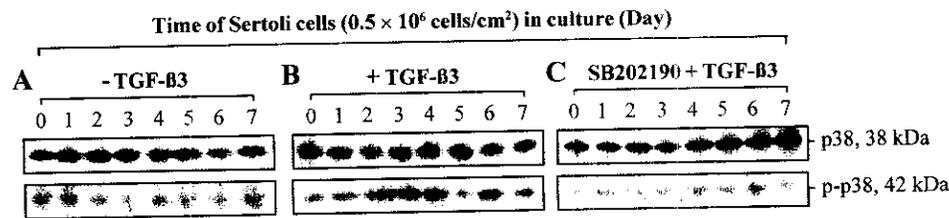


FIG. 11. Effects of recombinant TGF β 3 on the levels of total and activated p38 MAPK protein in Sertoli cells during TJ assembly with pretreatment of SB202190 for 20 min in vitro. Sertoli cells (0.5×10^6 cells/cm 2) were cultured in the absence (A) and presence (B) of TGF β 3 (3 ng/ml) or with pretreatment with SB202190 for 20 min before the addition of TGF β 3 (3 ng/ml) (C). At specified time points during the assembly of the TJ barrier, cells were lysed in SDS sample buffer. Two hundred micrograms of protein of whole cell lysates from each time point were loaded onto a 7.5% T SDS-polyacrylamide gel and SDS-PAGE was performed under reducing conditions. Immunoblotting was performed using specific antibodies against the total p38 and phosphorylated/activated form of p38 (p-p38).

with a decline in pERK1/pERK2 protein expression, which seemingly suggests that an induction of its expression may perturb the TJ-permeability barrier.

Other Functions of TGF β -Mediated Signaling Pathways in the Testis

Earlier studies have shown that the TGF β signaling mechanism consists of an elaborate network of signal transducers. These include Smad proteins and MAP kinases (for reviews, see [21, 22]; see also Fig. 1). For instance, Smad2, one of the TGF β signal transducers, is known to be expressed by mouse Sertoli and germ cells and was implicated in the regulation of spermatogenesis [50]. Surprisingly, there are no detailed studies on the cellular distribution and developmental regulation of these TGF β upstream signal transducers in the testis since their discovery in the testis. Herein, we reported that both Sertoli and germ cells express all of the known TGF β upstream and downstream signaling molecules, suggesting that these cells are equipped with all the needed transducers to mediate TGF β signals for a variety of biological functions. Furthermore, these results also clearly demonstrate that, while the TGF β -induced inhibitory effects on the Sertoli cell TJ barrier are mediated via the p38-MAPK kinase pathway, TGF β and/or other cytokines can utilize these other signal transducers to execute other biological effects in the testis essential to maintaining normal testicular function. More important, these results illustrate that germ cells are equipped with the same signaling counterparts found in Sertoli cells. The observation that the level of TGF β 3 remains elevated after the Sertoli cell TJ barrier is established (see Fig. 2A and B) also suggests that, while a transient reduction of TGF β 3 is needed for the TJ barrier to assemble, an elevated level may be required to maintain the well-being of the Sertoli cell epithelium.

TGF β 3 Is a Stage-Specific Protein in the Testis

Previous studies have shown that the localization of TGF β 1 and - β 2 in the rat testis are stage specific, that a decline of TGF β 1 is found at stages VIII-IX, and that an increase in TGF β 2 localization is detected at stages V and VI in the rat testis [51]. However, it is not known if TGF β 3 is stage specific. In this study, we have examined the localization of TGF β 3 in the rat seminiferous epithelium by immunohistochemistry. TGF β 3 in the rat seminiferous epithelium is a stage-specific protein, being highest in stages V-VII, declining thereafter, associated primarily with spermatocytes and early spermatids but not elongated sperma-

tids, yet becoming virtually undetectable in the epithelium at stages XII-XIV.

Current Molecular Models in the Regulation of TJ Dynamics

There are two currently available molecular models (for a review, see [1]) attempting to explain how small molecules, such as fatty acids, amino acids, glucose, and IgG, can traverse epithelial TJs to permit food absorption in small intestine and to allow the migration of neutrophils and macrophages across TJs in inflammatory responses. First, the Ca $^{2+}$ switch model (for a review, see [52]). This model is based on the observation that depletion of Ca $^{2+}$ from MDCK cells induces immediate disruption of the TJ barrier. Upon addition of [Ca $^{2+}$] to the media, the TJ barrier reseals. We have recently shown that the Sertoli TJ barrier can also be disrupted and resealed by manipulating [Ca $^{2+}$] in the culture media [37]. Second, the ATP depletion-repletion model [52] hypothesizes that, when ATP is depleted from the system, ZO-1 becomes associated with cytoskeletal proteins, fodrin. This, in turn, pulls ZO-1 laterally away from the sites of TJs, inducing TJ leakiness. Upon repletion of ATP, the association between ZO-1 and fodrin becomes disrupted, allowing ZO-1 molecules to move back to the TJ sites, resealing the TJ [53]. These models, however, apparently can only explain how TJs become leaky in vitro to allow the passage of small molecules and ions. This is in contrast with the dynamics of the BTB, which must disassemble and reassemble to allow for the passage of preleptotene and leptotene spermatocytes, which are in the micrometer size range. Recent studies have shown that cellular phosphoprotein content is an important determinant in regulating TJ assembly/disassembly. For instance, the assembly of TJs in the blood-brain barrier requires occludin. However, a high level of occludin is not sufficient to ensure a high resistance in these TJs; rather, it is regulated by the state of phosphorylation of occludin [54]. A recent study on the assembly of TJs in MDCK cells has shown that highly phosphorylated occludin is selectively concentrated at TJs whereas non- or less phosphorylated occludin is distributed on the basolateral membranes [55; for a review, see 1]. Also, increased tyrosine phosphorylation induces redistribution of AJ- and TJ-associated proteins. This in turn perturbs the TJ permeability barrier [56] and AJ dynamics [57]. Taking these results collectively, it is possible that TGF β in conjunction with other molecules operating synergistically yet under different paracrine and/or hormonal control regulate the intriguing event of cell movement across the BTB.

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