Spermatogenesis 2:4, 294-303; October/November/December 2012; © 2012 Landes Bioscience

Tumor necrosis factor α-mediated restructuring of the Sertoli cell barrier in vitro involves matrix metalloprotease 9 (MMP9), membrane-bound intercellular adhesion molecule-1 (ICAM-1) and the actin cytoskeleton

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Keywords: testis, Sertoli cell, germ cell, TNFα, cell junction

Abbreviations: BTB, blood-testis barrier; DAPI, 4',6-diamidino-2-phenylindole; FSH, follicle stimulating hormone; GC, germ cell; ICAM-1, intercellular adhesion molecule-1; IF, immunofluorescent staining; IHC, immunohistochemical staining; LBRC, lateral border recycling compartment; LH, luteinizing hormone; mICAM-1, membrane-bound ICAM-1; MMP, matrix metalloprotease; MT1-MMP, membrane type-1 matrix metalloprotease; PECAM, platelet/endothelial cell adhesion molecule; SC, Sertoli cell; sICAM-1, soluble ICAM-1; ST, seminiferous tubule; T, testis; TBS, Tris-buffered saline; TNFα, tumor necrosis factor α

The mammalian blood-testis barrier (BTB) restructures throughout spermatogenesis, thereby allowing developing germ cells to enter the adluminal compartment of the seminiferous epithelium. Previous studies have shown pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1α to be important regulators of Sertoli cell barrier/BTB function in vitro and in vivo. In this study, the effects of TNFα on Sertoli cell barrier function were assessed, with emphasis on changes in proteases and cell adhesion molecules following treatment. By immunoblotting and immunohistochemistry, MMP9 was found to be present in germ cells, localizing by and large to spermatocytes and spermatids in the adult rat testis. Following treatment of Sertoli cells with physiologically relevant consecutive doses of recombinant human TNFα (25 ng/ml), the steady-state levels of active-matrix metalloprotease 9 (MMP9), membrane-bound intercellular adhesion molecule (mICAM-1) and androgen receptor increased significantly. TNFα also downregulated the steady-state level of occludin, in agreement with earlier results that showed TNFα to disrupt Sertoli cell barrier/BTB function. In addition, TNFα affected the filamentous actin cytoskeleton in Sertoli cells, which appeared to be mediated by cortactin, a regulator of actin dynamics. Taken collectively, these findings imply that germ cells may be involved in BTB restructuring via the localized production of TNFα. These results also illustrate that barrier restructuring correlated with an increase in Sertoli cell mICAM-1, suggesting that it may be critical for adhesion as germ cells traverse the "opened" BTB.

Introduction

Spermatogenesis is an extremely complex cellular process that produces approximately 150 million sperm daily in an average healthy male, and it takes place within the seminiferous epithelium under the regulation of several factors, including testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol 17β.[1-3] To appreciate the process of spermatogenesis, the biology of possibly the most important ultrastructure within the seminiferous epithelium—the blood-testis barrier (BTB, also known as the Sertoli cell barrier)—has to be first understood.[1,4-7] For instance, the BTB is known to be constituted by different types of co-existing junctions (i.e., tight junctions, basal ectoplasmic specializations, desmosomes and gap junctions) that localize basally between adjacent Sertoli cells, and it creates a highly specialized microenvironment that separates developing germ cells into two distinct yet inter-related compartments: (1) the basal compartment where spermatogonia and primary spermatocytes exist and (2) the adluminal compartment where secondary spermatocytes, haploid spermatids and spermatozoa reside. As germ cells transition through different developmental stages, their movement across the seminiferous epithelium is coordinated with restructuring of the BTB, which allows zygotene spermatocytes to gain entry into the adluminal

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Submitted: 09/03/12; Revised: 10/10/12; Accepted: 10/16/12
http://dx.doi.org/10.4161/spmg.22602

compartment.[8-10] Thus, unique mechanisms are believed to be in place to facilitate the timely movement of preleptotene and leptotene spermatocytes across the BTB without compromising the homeostasis of the seminiferous epithelium and without affecting spermatogenesis. Indeed, morphological studies have shown there to be a brief moment during the seminiferous epithelial cycle when a preleptotene or leptotene spermatocyte can be observed as being trapped between Sertoli cell barriers, thereby creating an intermediate compartment.[9,11,12] The only imaginable way that spermatocytes can transit upwards without affecting the integrity of the BTB is if disassembly of old junctions is synchronized with assembly of new junctions that are present above and below migrating spermatocytes, respectively. In this way, the integrity of the seminiferous epithelium is maintained.

To get a better idea of which additional molecules and mechanisms might be involved in BTB restructuring, we looked carefully at studies from other in vitro and in vivo systems. From these studies, we identified membrane-bound ICAM-1 (mICAM-1) as an important protein involved in junction dynamics and cell movement. mICAM-1 is best studied with respect to the movement of leukocytes across the endothelium during inflammation,[13] and its expression in Sertoli and germ cells has been previously reported.[14] Soluble forms of ICAM-1 (sICAM-1) generated from the proteolytic cleavage of mICAM-1 have also been shown to have important biological functions in Sertoli cells as well as in other epithelia.[15-17] In a recently published study, we showed mICAM-1 and sICAM-1 to have critical but opposing roles in BTB function,[14] which led us to conclude that mICAM-1 participates in the assembly of the new BTB below migrating spermatocytes and conversely that sICAM-1 participates in the disassembly of the old BTB above migrating spermatocytes. Earlier studies by flow cytometry using mouse Sertoli cells showed ICAM-1 to be upregulated by tumor necrosis factor α (TNFα),[18-20] a pro-inflammatory cytokine produced by spermatocytes and spermatids.[21] Interestingly, TNFα negatively affected Sertoli cell barrier/BTB function by upregulating proteases [e.g., matrix metalloprotease 9 (MMP9)], downregulating transmembrane protein levels [e.g., occludin, junctional adhesion molecule-A (JAM-A), and coxsackie and adenovirus receptor (CAR)] and triggering protein internalization (e.g., occludin and JAM-A).[22-25] In this in vitro study, we continue our investigation on the role of mICAM-1 and sICAM-1 in Sertoli cell barrier dynamics by determining if their steady-state levels are indeed affected by TNFα since early studies relied largely on cytometric analysis. Our results support the role of mICAM-1 in BTB dynamics as well as contribute to a new mechanism of BTB regulation by TNFα. Our results illustrate that BTB restructuring involves many proteins. They also suggest that germ cells may trigger BTB restructuring through localized secretion of TNFα, thereby upregulating mICAM-1, which may be critical for adhesion as germ cells traverse the barrier.

Results

MMP9 and MT1-MMP are present in Sertoli and germ cells, localizing predominantly to spermatocytes and spermatids in the adult rat testis. This study was initiated by investigating the presence of MMP9 in the adult rat testis, Sertoli and germ cells by immunoblotting, followed by immunohistochemistry (IHC) and immunofluorescent (IF) staining experiments where MMP9 was localized to the adult rat testis. By immunoblotting, MMP9 (both pro and active forms) was found to be present in the testis, Sertoli and germ cells (**Fig. 1A and B**). The lowest and highest levels of active-MMP9 were detected in Sertoli and germ cells, respectively (**Fig. 1B**). To assess germ cell purity, lysates were screened by using a testin antibody. Testin, a Sertoli and Leydig cell protein,[34-36] was not detected in germ cell lysates, illustrating negligible contamination. The monospecificity of another MMP9 antibody was also assessed by immunoblotting (**Fig. 1C**), and 92 and 84 kDa proteins corresponding to pro- and active-MMP9, respectively, were observed in seminiferous tubule lysate. This specific antibody was used for subsequent IHC and IF staining experiments as it yielded a cleaner immunoblot than the antibody used in **Figure 1A and B**. By IHC and IF staining, MMP9 was found to localize predominantly to spermatocytes, round and elongating spermatids (**Fig. 1D**), consistent with a previously published report.[24] Of these, pachytene spermatocytes were most immunoreactive for MMP9. Weak MMP9 immunoreactivity was also noted with Sertoli cells (**Fig. 1D**).

By immunoblotting, MT1-MMP was also found to be present in the testis, Sertoli and germ cells (**Fig. 2A and B**). Likewise, the lowest level of MT1-MMP was observed in Sertoli cells (**Fig. 2A and B**). When this antibody's monospecificity was assessed, a 65 kDa protein corresponding to inactive MT1-MMP was observed in testis lysate (**Fig. 2C**). IHC and IF staining showed that total MT1-MMP surrounded elongating/elongated spermatids, consistent with previously published reports.[37,38] In agreement with MMP9 localization, weak MT1-MMP immunoreactivity was also detected in pachytene spermatocytes and round spermatids (**Fig. 2D**). On the other hand, very strong immunoreactivity was observed in residual bodies (**Fig. 2D**), structures containing excess cytoplasm and organelles.[39,40] It should be noted that this antibody recognized the hinge region of MT1-MMP, which links the C-terminus with the catalytic domain and may be important for MT1-MMP proteolytic processing.[41]

TNFα regulates Sertoli cell barrier function in vitro by upregulating active-MMP9, mICAM-1 and AR, and by downregulating occludin. Previous studies have shown TNFα to affect the levels of several proteins, including MMP2 and MMP9.[24,42] To better understand the effects of TNFα on Sertoli cell barrier function, we treated Sertoli cells with physiologically relevant consecutive doses of recombinant human TNFα and investigated the steady-state levels of different integral membrane, scaffolding and regulatory proteins known to be involved directly or indirectly in Sertoli cell barrier/BTB function. TNFα was added to Sertoli cells on day 4 in vitro, shortly after the formation of a functional Sertoli cell barrier. Immunoblotting revealed no significant changes in the steady-state levels of pro-MMP9, sICAM-1, N-cadherin, β-catenin, ARP3, cortactin, *p*-Cortactin-Y421, c-SRC, FAK and *p*-FAK-Y407 when compared with time-matched Veh-treated Sertoli cells (**Fig. 3A and B**). On the other hand, TNFα treatment brought about an increase in

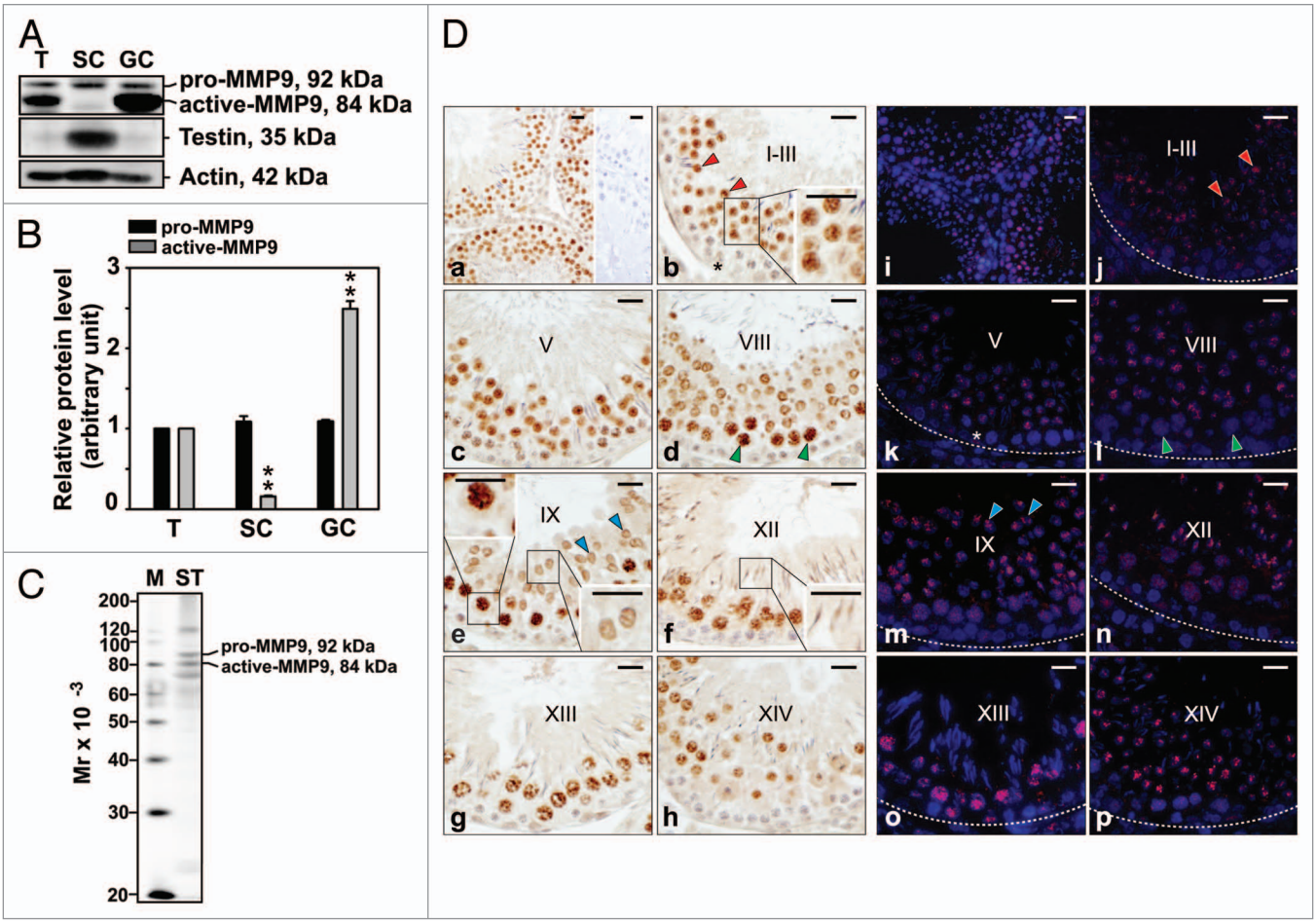

Figure 1. Cellular distribution and localization of MMP9 in the seminiferous epithelium of the adult rat testis during the epithelial cycle of spermatogenesis. (**A**) Presence of MMP9 in testis (T), Sertoli (SC, isolated from 20-d-old testes and cultured at high density for 4 d) and germ cell (GC, isolated form 90-d-old testes and used immediately for lysate preparation) lysates (50 μg protein/lane) by immunoblotting. Testin, a Sertoli and Leydig cell protein, was used to assess the purity of germ cells. Actin served as an indicator of equal protein loading. (**B**) Histogram summarizing results shown in (**A**). The relative levels of pro- and active-MMP9 detected in Sertoli and germ cell lysates were compared with pro- and active-MMP9 in the testis, whose levels were arbitrarily set at 1. Each bar represents the mean ± SD of n = 3 experiments. **p < 0.01 (ANOVA followed by Dunnett's post-hoc test). (**C**) Immunoblot showing the specificity of the anti-MMP9 polyclonal antibody (Table 1) in seminiferous tubule (ST) lysate (50 μg protein). This antibody was used subsequently for IHC and IF staining. Mr, molecular weight; M, MagicMark™ XP Western Protein Standard (Invitrogen). (**D**) IHC and IF staining of MMP9 in paraffin-embedded (5 μm) and frozen (7 μm) adult testis cross-sections, respectively. MMP9 immunoreactivity appeared as a brownish precipitate in IHC experiments (a–h) and as a reddish precipitate in IF experiments (i–p). Nuclei were visualized with DAPI (blue, i–p). Stages of the seminiferous epithelial cycle are denoted as Roman numerals (b–h and j–p). Insets (b, e and f) are magnified images corresponding to boxed areas within the same panel. Specificity was assessed when 10% normal goat serum (vol/vol) was used instead of anti-MMP9 IgG (a, right panel). Dashed lines (j–p) mark the periphery of seminiferous tubules. Scale bars, 25 μm. MMP9 immunoreactivity associated intensely with round spermatids at stages I-VII (red arrowheads in b and j, and insert in b) and moderately/weakly with elongating spermatids at stages VIII-XII (blue arrowheads in e and m, right-most inset in e, inset in f). MMP9 also associated strongly with pachytene and diplotene spermatocytes at stages VIII–XIII (green arrowheads in d and l, left-most insert in e). Weak staining was also detected in Sertoli cells (asterisks in b and k).

the levels of active-MMP9, ICAM-1 and AR, but a decrease in the level of occludin (**Fig. 3A and B**). These results are in agreement with previously published results from this and other laboratories.[18,20,23,24,42,43]

Next, we investigated the cellular localization of MMP9 and MT1-MMP in Sertoli cells following TNFα treatment. MMP9 localized to the cytoplasm in Veh-treated Sertoli cells (**Fig. 4A**); however, an increase in MMP9 was observed, not only throughout the cytoplasm, but also between adjacent Sertoli cells following TNFα treatment (**Fig. 4B**). On the other hand, the immunofluorescent localization of MT1-MMP was different from that of MMP9. In Veh-treated Sertoli cells, MT1-MMP localized to the plasma membrane, in agreement with its role as a type I membrane-anchored protease.[44,45] Following TNFα treatment, MT1-MMP increased appreciably, suggesting that there may be an increase in proteolysis. In support of immunoblotting results (**Fig. 3A and B**), there was a loss in occludin from the Sertoli cell surface compared with Veh-treated cells (**Fig. 5A**). However, no changes were observed in ZO-1 at the Sertoli cell surface (**Fig. 5A**). These results are in agreement with previously published results, which showed Sertoli cell barrier function to be regulated negatively by TNFα.[24]

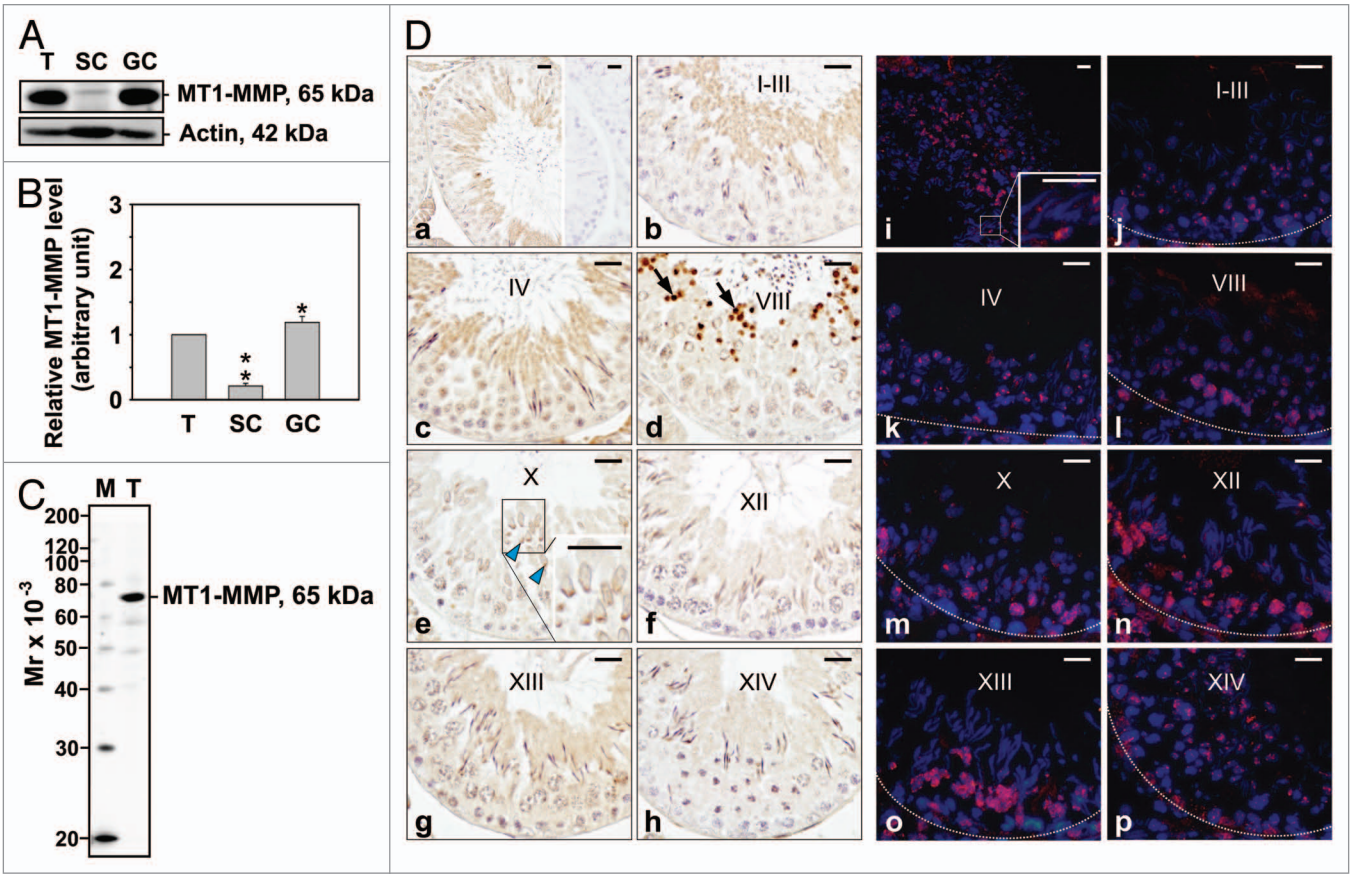

Figure 2. Cellular localization and stage-specific expression of MT1-MMP in the seminiferous epithelium of the adult rat testis during the epithelial cycle of spermatogenesis. (**A**) Presence of MT1-MMP in testis (T), Sertoli (SC, see legend to Fig. 1 for details) and germ cell (GC, see legend to Fig. 1 for details) lysates (50 μg protein/lane) by immunoblotting. Actin served as an indicator of equal protein loading. (**B**) Histogram summarizing results shown in (**A**). The relative level of MT1-MMP detected in Sertoli and germ cell lysates was compared with MT1-MMP in the testis, whose level was arbitrarily set at 1. Each bar represents the mean ± SD of n = 3 experiments. *p < 0.05; **p < 0.01 (ANOVA followed by Dunnett's post-hoc test). (**C**) Immunoblot showing the specificity of an anti-MT1-MMP polyclonal antibody (Table 1) in testis lysate (50 μg protein). This antibody was used subsequently for IHC and IF staining. Mr, molecular weight; M, MagicMark™ XP Western Protein Standard (Invitrogen). (**D**) IHC and IF staining of MT1-MMP in paraffin-embedded (5 μm) and frozen (7 μm) adult testis cross-sections, respectively. MT1-MMP immunoreactivity appeared as a brownish precipitate in IHC experiments (a–h) and as a reddish precipitate in IF experiments (i–p). Nuclei were visualized with DAPI (blue, i–p). Stages of the seminiferous epithelial cycle are denoted as Roman numerals (b–h, and j–p). Insets (e and i) are magnified images corresponding to boxed areas within the same panel. Specificity was assessed when 10% normal goat serum (vol/vol) was used instead of MT1-MMP IgG (a, right panel). Dashed lines (j–p) mark the periphery of seminiferous tubules. Scale bars, 25 μm. Immunoreactive MT1-MMP immunoreactivity associated intensely with residual bodies (arrows in d) and weakly with pachytene spermatocytes, and round and elongating/elongated spermatids (blue arrowheads in e).

TNFα affects the actin cytoskeleton in Sertoli cells in vitro. The effects of TNFα on the Sertoli cell actin cytoskeleton were investigated by staining F-actin with fluorescently-tagged phalloidin or cortactin with a cortactin antibody. In Veh-treated Sertoli cells, actin stress fibers ran parallel across cells (**Fig. 5B**). The localization of cortactin, an actin-binding protein known to regulate branched actin assembly,[46,47] in control Sertoli cells was similar to that of F-actin (**Fig. 5B**). Cortactin-positive dot-like structures were also observed in Veh-treated cells. Following TNFα treatment, both F-actin and cortactin were found to aggregate at the periphery of Sertoli cells (**Fig. 5B**).

Discussion

BTB restructuring, one of the most complicated cellular events to occur during spermatogenesis, is orchestrated by multiple players, including proteases/protease inhibitors, kinases/phosphatases, cytokines, structural proteins and their associated adaptor proteins.[7,8] For instance, numerous studies have shown cytokines such as TNFα and interleukin-1α (IL-1α) to disrupt Sertoli cell barrier/BTB function,[24,25,48-50] except that a complete understanding of the mechanisms involved is still lacking. TNFα is a pro-inflammatory cytokine produced by pachytene spermatocytes and round spermatids as well as by activated monocytes and macrophages in the interstitium.[21,51,52] In the mammalian testis, TNFα has pleiotropic roles; at low concentrations it acts as a survival factor, but at high concentrations

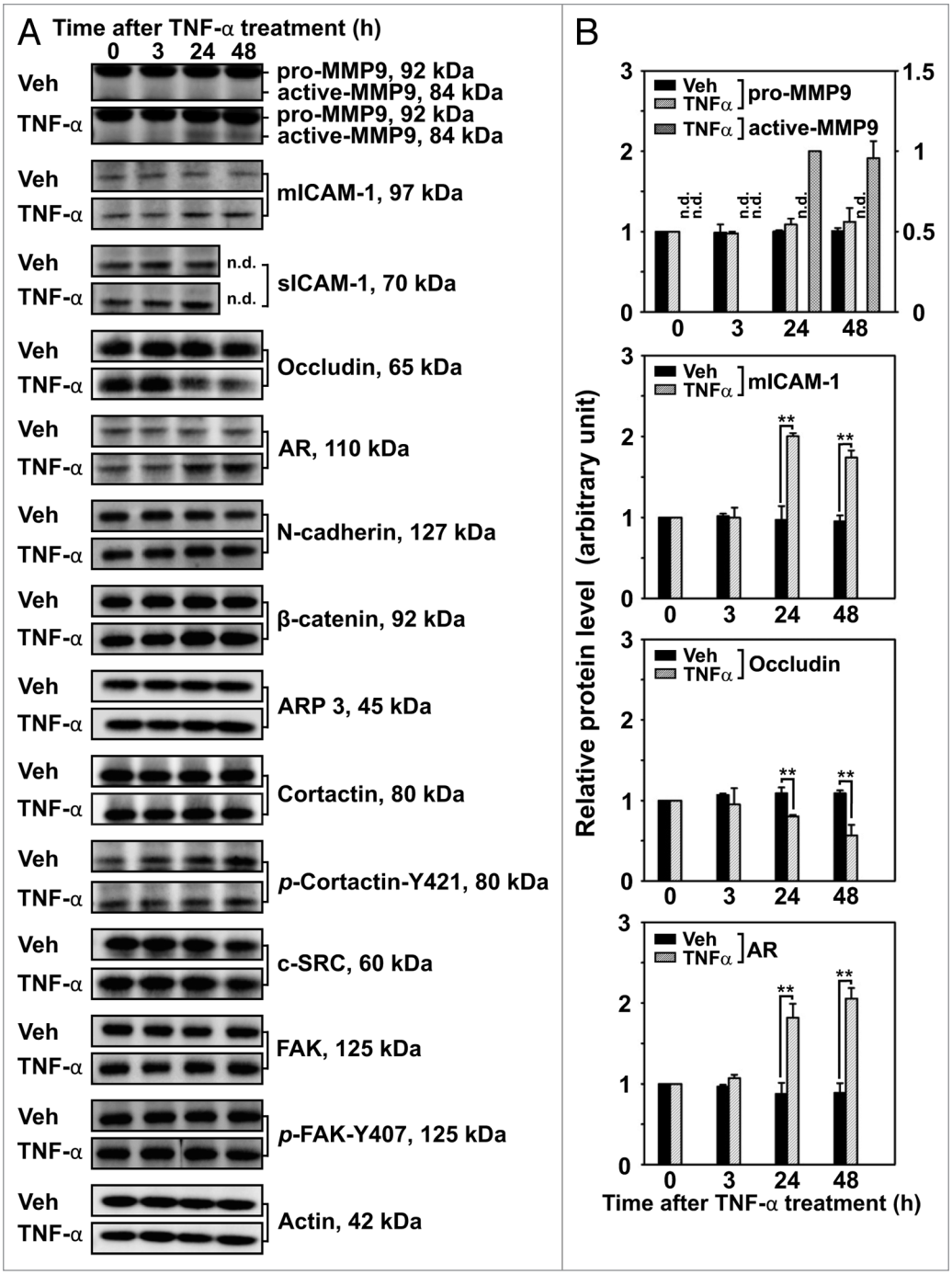

Figure 3. Effects of TNFα on the steady-state levels of integral membrane, scaffolding and regulatory proteins in Sertoli cells in vitro. Sertoli cells were cultured at high density on Matrigel™-coated 12-well dishes as described in Materials and Methods. On day 4 (designated as 0 h in this figure), TNFα (25 ng/ml) was added into Sertoli cell cultures, and cells were terminated at specific time points thereafter for lysate preparation. TNFα was dissolved in 10 mM NaH_2PO_4 pH 7.4 at 22°C containing 0.15 M NaCl and 0.1% BSA (wt/vol). The control consisted of culturing Sertoli cells in media containing an equivalent amount of BSA. (**A**) Immunoblots of selected proteins involved in the regulation and in the maintenance of Sertoli cell barrier function. Actin served as an indicator of equal protein loading. (**B**) Histograms summarizing results shown in (**A**) from at least three independent experiments. Histograms are not shown for proteins whose levels did not change significantly. Each data point was normalized against its corresponding actin data point and then against the protein level at 0 h, which was arbitrarily set as 1. Each bar represents the mean ± SD of n = 3–4 experiments. **p < 0.01 (ANOVA followed by Dunnett's post-hoc test). n.d., not determined.

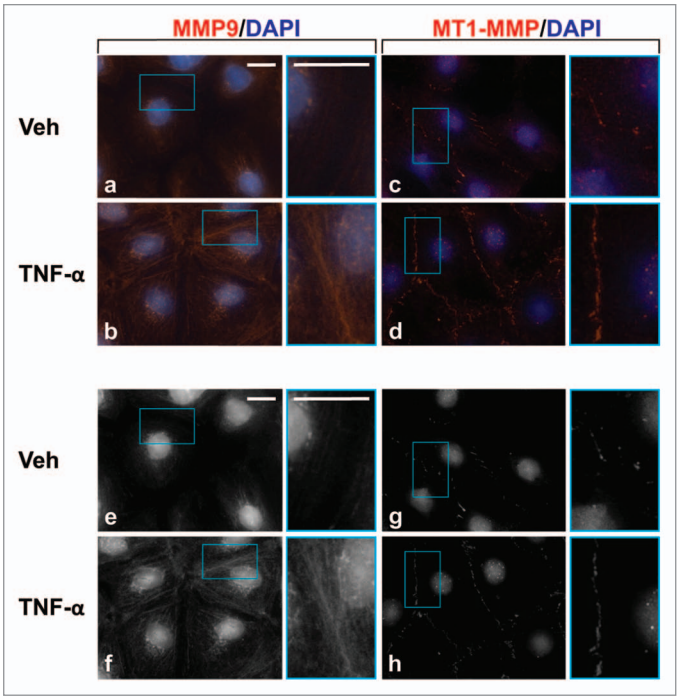

Figure 4. Localization of MMP9 and MT1-MMP in Sertoli cells cultured in the presence of TNFα. Sertoli cells (0.04×10^6 cells/cm^2) were cultured on Matrigel™-coated micro cover glasses for 4 d and incubated with TNFα (25 ng/ml) for 24 h as described in Materials and Methods. Thereafter, cells were processed for the immunofluorescent visualization of MMP9 (red, a and b) and MT1-MMP (red, c and d). Nuclei were visualized with DAPI (blue, a–d). Images encircled in blue boxes and shown to the immediate right of a–h are enlarged images corresponding to boxed areas within a–h. Gray scale images (e–h) are included to better depict changes in MMP9 and MT1-MMP. Scale bars, 20 μm.

it triggers cell death. The study by Boussouar et al. provides a good example of how TNFα behaves as a survival factor for post-meiotic germ cells. Here, low doses of the cytokine (ED_{50} = 2.5 ng/ml) were found to stimulate lactate dehydrogenase A, a critical enzyme involved in lactate production, in primary Sertoli cells.[53] TNFα also functions in inflammation and immunoregulation, reinforcing its role as a multifunctional molecule in the testis.

Previous studies have shown TNFα to upregulate proteases/protease inhibitors [e.g., MMP9 and tissue inhibitor of metalloproteases 1 (TIMP 1)], downregulate transmembrane protein levels [e.g., occludin, JAM-A and CAR] and trigger protein internalization (e.g., occludin and JAM-A) in Sertoli cells.[22-25] Equally important, these events culminated in Sertoli cell barrier/BTB disruption in vitro and in vivo.[24,25] At the in vivo level, we wonder why and how pachytene spermatocytes and round spermatids residing in the adluminal compartment of the seminiferous epithelium modulate BTB function. It is possible that germ cells residing near the BTB (i.e., preleptotene and leptotene spermatocytes) do not produce TNFα because they are not equipped with inherent mechanisms to control precisely the concentration of TNFα throughout spermatogenesis. This is critical since misregulation of TNFα by preleptotene and leptotene spermatocytes (that is, if they were to produce the cytokine) would be detrimental to BTB function and fertility. The production of TNFα by germ cells residing above the BTB may be linked to the disassembly of the old BTB, which is supported by its cyclic production during the seminiferous epithelial cycle in the rat.[51] While activation of MAPK signaling appears to be involved in these cellular events,[19,25,54] additional studies are needed to expand the role of TNFα in the testis.

In agreement with a previously published report from this laboratory,[24] TNFα increased the steady-state level of MMP9 (alternatively known as gelatinase-B) in Sertoli cells in vitro. MMPs are zinc-dependent soluble endopeptidases capable of degrading several biomolecules, including extracellular matrix proteins (e.g., collagen), growth factors, growth factor receptors, cytokines, proteases, protease inhibitors and cell adhesion proteins at physiological pH.[55-57] They contain at least three conserved domains: the pro-peptide domain, the catalytic domain and the hemopexin-like C-terminal domain, which is linked to the catalytic domain by a hinge region. MMPs are synthesized as inactive zymogens (i.e., pro-enzymes) but activated by proteases that remove the pro-peptide domain. Once activated, they are inhibited by TIMPs.[58] However, important differences are known to exist across different MMP family members. For instance, MT-MMPs are anchored to the plasma membrane by a transmembrane domain.[59] At the cell surface, MT1-MMP activity is tightly controlled, with localization to lipid rafts/caveolae and internalization playing major regulatory roles.[60] Active MT1-MMP (~54 kDa) can also be auto-catalytically cleaved into a ~44 kDa membrane-tethered fragment lacking the catalytic domain (44-MT1) and a ~20 kDa soluble fragment containing the catalytic domain via ectodomain shedding.[61-63] Even though both fragments were reported to be inactive, 44-MT1 was subsequently found to regulate indirectly the amount of active MT1-MMP at the cell surface by controlling its rate of internalization.[64] Results from IHC and IF staining experiments described herein illustrate that germ cells were highly immunoreactive for MMP (but less so for MT1-MMP). While staining experiments could not distinguish between active and inactive forms, these results clearly illustrate the involvement of germ cells in the breakdown of the extracellular milieu during cell movement.

In this study, TNFα also increased the steady-state level of mICAM-1 in Sertoli cells, consistent with previously published reports.[18-20,54] mICAM-1 is an immunoglobulin-like adhesion and signaling protein that is indispensable for the movement of leukocytes across the endothelium during inflammation.[13,15,65,66] In an earlier study, we reported on the role of mICAM-1 in BTB dynamics.[14] Specifically, we showed transient mICAM-1 overexpression to upregulate Sertoli cell barrier function, indicating its importance in BTB integrity. Since mICAM-1 was previously shown to localize to the Sertoli cell barrier/BTB, its upregulation in Sertoli cells by TNFα may be critical for transient states of (weak?) adhesion as preleptotene/leptotene spermatocytes cross the BTB during spermatogenesis. While germ cells were not included in this in vitro study, restructuring of the Sertoli cell barrier alone is likely to involve important signaling events that underlie germ cell movement in vivo. Connecting these in

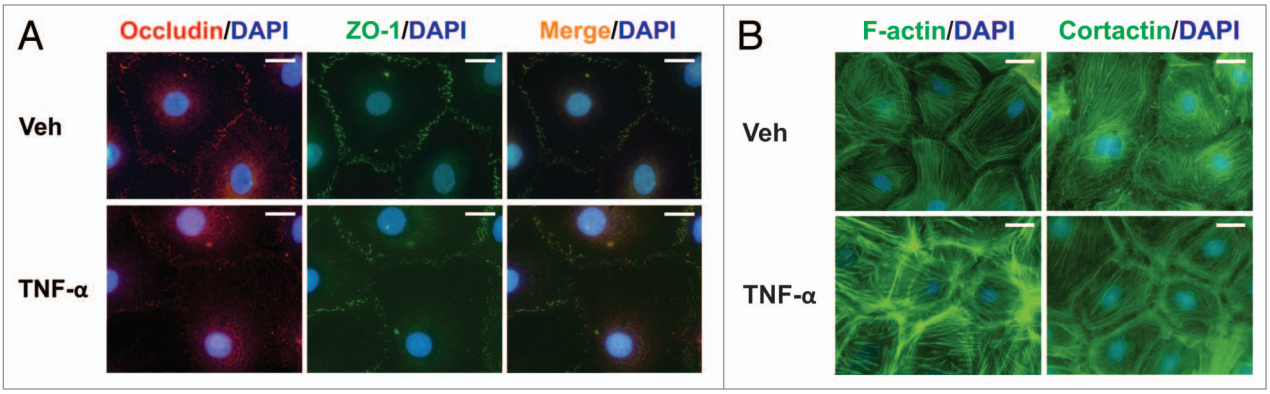

Figure 5. Effects of TNFα on protein distribution within Sertoli cells. Sertoli cells (0.04×10^6 cells/cm^2) were cultured on Matrigel™-coated micro cover glasses for 4 d and treated with TNFα (25 ng/ml) for 24 h as described in Materials and Methods. Sertoli cells were then dual-labeled for occludin (red)/ZO-1 (green), or labeled for F-actin or cortactin (both green). Corresponding images were merged to show areas of co-localization (orange, **A**). Nuclei were visualized with DAPI (blue, **A and B**). Scale bars, 20 μm.

vitro findings with in vivo consequences will require additional research. For instance, additional experiments are needed to better define, for instance, the ligand for mICAM-1 in germ cells (note: germ cells also express mICAM-1[14]) since transient protein-proteins interactions are likely to be established during germ cell movement. It would also be interesting to investigate if mICAM-1 is an endpoint of androgen signaling since AR was upregulated following TNFα treatment.[43] In other systems, mICAM-1 was found to bind MMP9 as well as Tyr phosphorylated cortactin,[67-69] suggesting that these proteins may also be part of a functional complex in the seminiferous epithelium. Cortactin, an actin-binding protein, is usually found at the edge of migrating cells where it co-localizes with ARP2/3.[46,47] With roles in cell adhesion, cell migration and endocytosis, we speculate that cortactin is one of many proteins involved in restructuring of the Sertoli cell actin cytoskeleton following TNFα treatment. Interestingly, cortactin has been shown to regulate the secretion and cell surface localization of MMPs.[70,71] It would be interesting to investigate if cortactin has a similar role in the seminiferous epithelium.

Herein, we have investigated the effects of TNFα on Sertoli cell barrier dynamics. While many more important studies remain to be performed, our findings have demonstrated the importance of MMPs, mICAM-1 and the actin cytoskeleton in TNFα-mediated Sertoli cell barrier restructuring.

Materials and Methods

Animals. Male Sprague-Dawley rats at 90 (~300 g b.w.) and 20 d of age were purchased from Charles River Laboratories. The Institutional Animal Care and Use Committee of The Rockefeller University approved the use of rats (protocol numbers 09016 and 12506). All experiments involving rats were performed by following ethical guidelines outlined in the Guide for the Care and Use of Laboratory Animals.

Sertoli cell cultures and treatment of cells with TNFα. Sertoli cells were isolated from 20-d-old rat testes and cultured in serum-free DMEM/F12 (Sigma-Aldrich) supplemented with growth factors and an antibiotic.[26-28] Cells were plated onto Matrigel™- (BD Biosciences) coated 12-well plates at 0.5×10^6 cells/cm^2 for lysate preparation or onto Matrigel™-coated micro cover glasses (18 mm, Thomas Scientific) at 0.04×10^6 cells/cm^2 for immunofluorescent staining. Cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 (vol/vol) at 35°C. At 48 h, cultures were treated with a hypotonic buffer (20 mM Tris pH 7.4 at 22°C) to lyse contaminating germ cells, yielding Sertoli cells with a relative purity of ~98%.[29] Recombinant human TNFα (R&D Systems, Inc.) at 25 ng/ml (~1.4 nM) was added daily into Sertoli cell cultures from day 4 onwards. Thereafter, Sertoli cells were terminated at specific time points for lysate preparation. TNFα was prepared at a stock concentration of 100 μg/ml in PBS (10 mM NaH_2PO_4 pH 7.4 at 22°C containing 0.15 M NaCl) containing 0.1% BSA (wt/vol). The control consisted of culturing Sertoli cells in DMEM/F12 containing an equivalent amount of BSA (wt/vol).

Immunohistochemistry and immunofluorescent staining. Immunohistochemistry was performed as previously described.[30] Testes were fixed in Bouin's fixative (Polysciences, Inc.) for 24 h, dehydrated using increasing concentrations of ethanol, cleared in xylene, embedded in paraffin and sectioned at 5 μm. For antigen retrieval, sections were submerged in 10 mM citrate buffer pH 6.0 at 22°C and heated in a microwave (750 W) twice for 5 min. Endogenous peroxidase activity was inhibited with 3% H_2O_2 (vol/vol) in methanol for 10 min, and non-specific binding sites were blocked with 10% non-immune goat serum (vol/vol) for 30 min at R.T. Thereafter, sections were incubated with primary antibody at 4°C overnight in a humidified chamber (**Table 1**). On the next day, biotinylated goat anti-rabbit IgG (1:400; Vector Laboratories) was applied for 60 min, followed by an avidin-biotinylated horseradish peroxidase complex (Vectastain® ABC Reagent, Vector Laboratories). Sections were carefully washed with Tris-buffered saline (TBS; 0.05 M Tris-HCl pH 7.6 at 22°C containing 0.15 M NaCl) after each step. The primary antibody was also diluted in TBS. Bound antibody was visualized by using 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, wt/vol), 0.01% H_2O_2 (vol/vol) and 0.07% imidazole (wt/

Table 1. Antibodies used in this study

Antibody	Host species	Vendor	Catalog number	Application(s)/dilution(s)
MMP9	Rabbit	Millipore	AB19016	IB* (1:750), IHC (1:800), IF (1:800, testis cross-sections), IF (1:50, cultured Sertoli cells)
MT1-MMP	Rabbit	Millipore	AB6004	IHC (1:200), IF (1:100, testis cross-sections), IF (1:50, cultured Sertoli cells)
mICAM-1	Rabbit	Novus Biologicals	NB100–81977	IB (1:500)
sICAM-1	Rabbit	(Xiao, et al. 2012)	-	IB (1:50)
Cortactin	Rabbit	Millipore	05–180	IB (1:200), IF (1:50)
p-Cortactin-Y421	Rabbit	Invitrogen	44–854G	IB (1:250)
ARP-3	Mouse	Sigma-Aldrich	A5979	IB (1:3000)
Occludin	Rabbit	Invitrogen	71–1500	IB (1:200), IF (1:50)
ZO-1	Mouse	Invitrogen	33–9111	IF (1:100)
N-Cadherin	Rabbit	Santa Cruz Biotechnology	sc-7939	IB (1:200)
β-Catenin	Rabbit	Invitrogen	71–2700	IB (1:250)
c-SRC	Mouse	Santa Cruz Biotechnology	sc-8056	IB (1:200)
FAK	Rabbit	Santa Cruz Biotechnology	sc-558	IB (1:200)
p-FAK-Y407	Rabbit	Invitrogen	44–650G	IB (1:1000)
AR	Rabbit	Santa Cruz Biotechnology	sc-816	IB (1:200)
Testin	Rabbit	(Cheng and Bardin 1987; Cheng, et al. 1989)	-	IB (1:200)
Actin	Goat	Santa Cruz Biotechnology	sc-1616	IB (1:200)

*IB, immunoblotting; IHC, immunohistochemistry; IF, immunofluorescence staining. Cheng CY and Bardin CW 1987 Identification of two testosterone-responsive testicular proteins in Sertoli cell-enriched culture medium whose secretion is suppressed by cells of the intact seminiferous tubule. *J Biol Chem*; 262:12768–12779. Cheng CY, Grima J, Stahler MS, Lockshin RA and Bardin CW 1989 Testins are structurally related Sertoli cell proteins whose secretion is tightly coupled to the presence of germ cells. *J Biol Chem*; 264:21386–21393. Xiao X, Cheng CY and Mruk DD 2012 Intercellular adhesion molecule-1 is a regulator of blood-testis barrier function. *J Cell Sci* doi:10.1242/jcs.107987.

vol) in TBS. Sections were counterstained lightly with Mayer's hematoxylin. Finally, sections were dehydrated, cleared in xylene and mounted with Poly-Mount medium (Polysciences, Inc.). The control consisted of incubating sections with 10% normal goat serum (vol/vol) instead of the primary antibody.

For immunofluorescent (IF) staining of testes, frozen tissues were sectioned at 7 μm, adhered to poly-L-lysine-coated microscope slides (Polysciences, Inc.) and fixed in Bouin's fixative or ice-cold methanol. Thereafter, sections were pretreated with 0.1% Triton X-100 (vol/vol) in TBS, and non-specific binding sites were blocked with 10% non-immune goat serum (vol/vol) or 1% bovine serum albumin (wt/vol, BSA). Following an overnight incubation with primary antibody at R.T. (**Table 1**), sections were saturated with Alexa Fluor® secondary antibodies (1:200; Invitrogen). Sections were subsequently mounted with ProLong® antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). For IF staining of Sertoli cells, cells were cultured at 0.04×10^6 cells/cm^2 on Matrigel™-coated micro cover glasses and treated with TNFα (25 ng/ml) for 24 h. They were then fixed with 4% paraformaldehyde (wt/vol) in PBS for 10 min, permeabilized with 0.1% Triton X-100 (vol/vol) and blocked with 10% non-immune goat serum (vol/vol) or 1% BSA (wt/vol). Primary antibodies from two species were used for an overnight incubation at R.T. [**Table 1**; 1:50–1:100 in 1% BSA (wt/vol)/PBS], followed by a 30 min incubation with Alexa Fluor® secondary antibodies [1:100 in 1% BSA (wt/vol)/PBS]. For F-actin staining, cells were fixed with 4% paraformaldehyde (wt/vol) in PBS at R.T. for 10 min, followed by incubation with Oregon Green® 514 phalloidin (Invitrogen). Images were captured by using an Olympus BX61 fluorescent microscope and MicroSuite™ FIVE software (v. 2.7; Olympus America). Images were compiled in Adobe Photoshop CS5 Extended software (v. 10.0.1; Adobe Systems Inc.). Images were adjusted for brightness and/or contrast, if needed, and identical adjustments were applied to all images within a single experiment.

General methods. Adult testes, germ and Sertoli cells were used for the preparation of lysates in lysis buffer. Germ cells were isolated as previously described.[31] Filtration of germ cells through glass wool was omitted. Seminiferous tubules were isolated as previously described.[14,32,33] Protein concentration was determined by using the D_C protein assay and a model 680 microplate reader (BIO-RAD Laboratories). Immunoblotting was performed by using a routine protocol. Chemiluminescent images were captured and analyzed by using a LAS-4000 mini imaging system and MultiGauge software (v. 3.1; FujiFilm Life Science USA), respectively. **Table 1** lists the antibodies and conditions that were used in this study. The sICAM-1 antibody was produced in-house and characterized as previously described.[14] By

immunoblotting, this antibody cross-reacted strongly with a ~70 kDa Sertoli and germ cell protein that corresponded to sICAM-1, and weakly with a ~97 kDa Sertoli and germ cell protein that corresponded to mICAM-1.[14] However, this antibody was not used for mICAM-1 detection. Instead, a commercially available antibody was used (**Table 1**).

Statistical analyses. Comparisons were performed by one-way ANOVA, followed by Dunnett's post-hoc test (GB-STAT software, v. 7.0; Dynamic Microsystems). Each experiment was repeated at least three times by using different batches of Sertoli cells. Within a single experiment, each treatment/time point consisted of Sertoli cells cultured in 12-well plates or on micro cover glasses in triplicate. $p < 0.05$ was taken as statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported in part by NICHD, NIH (R03 HD061401 to D.D.M.; R01 HD056034 and U54 HD029990 Project 5 to C.Y.C.) and by the Ministry of Science and Higher Education (K/ZDS/002590 to B.B.).

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