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Mono-(2-Ethylhexyl) Phthalate-Induced Disruption of Junctional Complexes in the Seminiferous Epithelium of the Rodent Testis Is Mediated by MMP2[1]

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ABSTRACT

Tight junctions between Sertoli cells of the testicular seminiferous epithelium establishes the blood-testis barrier (BTB) and creates a specialized adluminal microenvironment above the BTB that is required for the development of the germ cells that reside there. Actin filament-based anchoring junctions between Sertoli cells and germ cells are important for maintaining close physical contact between these cells as well as regulating the release of mature spermatids into the lumen. Previously, we reported that Sertoli cell injury in rodents after mono-(2-ethylhexyl) phthalate (MEHP) exposure results in the activation of matrix metalloproteinase 2 (MMP2) and increases the sensitivity of germ cells to undergo apoptosis. A disruption in the physical association between Sertoli cells and germ cells and premature loss of germ cells from the seminiferous epithelium has been widely described after phthalate treatment. In this study, we investigate the functional participation of MMP2 in the mechanism underlying MEHP-induced disruption of junction complexes and the resultant loss of germ cells. Exposure of C57BL/6J mice to MEHP (1 g/kg, oral gavage) decreased the expression of occludin in the tight junctions between Sertoli cells and caused gaps between adjacent Sertoli cells as observed by transmission electron microscopy. A reduced expression of laminin-gamma3 and beta1-integrin in apical ectoplasmic specializations between Sertoli cells and germ cells in a time-dependent manner was also observed. Treatment with specific MMP2 inhibitors (TIMP2 and SB-3CT) both in vitro and in vivo significantly suppressed MEHP-induced germ cell sloughing and changes in the expression of these junctional proteins, indicating that MMP-2 plays a primary role in this process. Furthermore, the detachment of germ cells from Sertoli cells appears to be independent of the apoptotic signaling process since MEHP-induced germ cell detachment from Sertoli cells could not be prevented by the addition of a pan-caspase inhibitor (z-VAD-FMK).

apoptosis, blood-testis barrier, ectoplasmic specialization, germ cell, junction, MEHP, MMP2, paracrine signaling, Sertoli cells, signal transduction, testis

INTRODUCTION

During spermatogenesis the integrity of different cell to cell junctions and the regulation of dynamic junction proteins are critical in controlling the movement of germ cells across the seminiferous epithelium. Disruption of the adhesion between Sertoli cells and germ cells can cause premature loss of germ cells and possibly infertility [1]. It is important to recognize that the junctions between Sertoli cells and germ cells are dynamic and highly regulated. In the testis, tight junctions between adjacent Sertoli cells form the blood-testis barrier (BTB), allowing for the creation of distinct basal and adluminal compartments. The adluminal compartment is a highly specialized microenvironment that is dependent on Sertoli cell-secreted factors [1]. When differentiating germ cells (preleptotene and leptotene spermatocytes) move across the BTB, tight junctions are rapidly disassembled and subsequently reassembled [2]. Through this process, germ cells continue to be tightly anchored to Sertoli cells via anchoring junctions. The ectoplasmic specialization (ES) is a testis-specific junction associated with actin filaments that displays characteristics of adherens junctions, tight junctions, and focal adhesions [3, 4]. Therefore, disruption of junctions can be predicted to disrupt the movement of germ cells within the seminiferous epithelium and lead to the premature release of immature germ cells, a phenotype commonly observed after phthalate injury [5].

Sertoli cells are the primary target of di-(2-ethylhexyl) phthalate (DEHP) and its active metabolite mono-(2-ethylhexy) phthalate (MEHP) in rodents, with peripubertal animals being particularly sensitive to phthalate-induced injury [6, 7]. Phthalate-induced injury to Sertoli cells leads to a decrease in their ability to support the developing germ cells [8, 9]. Many reports document the detachment of germ cells from the seminiferous epithelium and an increase in the incidence in germ cell apoptosis in young peripubertal rodents after exposure to MEHP [8, 10]. Decreases in tight junction proteins and F-actin expression have been observed in primary cultured Sertoli cells following monophthalate exposure [11]. Therefore, it seems plausible that disruption of junctional complexes in the seminiferous epithelium may be an initial response to MEHP-induced Sertoli cell injury and account for the observed detachment and loss of germ cells from the testis.

Cytokine-mediated down-regulation of junction proteins results in an increase in BTB permeability [12]. Several studies have shown that transforming growth factor-β 3 (TGFB) and tumor necrosis factor α (TNF) are involved in regulating the transcription of claudin11, occludin, and zonula occluden 1 (ZO1), which are major components of tight junctions [13, 14]. In addition, TNF serves as an negative regulator of occludin expression in astrocytes through the tumor necrosis factor receptor superfamily member 1A (TNFRSF1A)-activated nuclear factor kappa-light-chain-enhancer of activated B cells 1 (NFKB1) pathway [15]. Therefore, TNF may be involved in the physical and metabolic cooperation between Sertoli cells

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and germ cells as well as in the impairment of Sertoli cell-germ cell adhesion. On the other hand, protease-mediated junctional regulation also has a biological role in restructuring cell junctions. Recent studies indicate that matrix metalloproteinases (MMPs) can disrupt the blood-brain barrier by degrading tight junction proteins [16, 17]. MMPs also regulate the permeability of the blood-retinal barrier through the proteolytic degradation of cadherin in diabetes patients [18].

Our previous studies indicate that MEHP reduces the expression of tissue inhibitor of metalloproteinase-2 (TIMP2) expression in Sertoli cells, which allows for the activation of MMP2 [8]. MMP2-mediated processing of TNF occurs in the seminiferous epithelium in response to MEHP exposure in peripubertal rodents, and, further, TNF is critical for triggering germ cell apoptosis [8, 19]. Importantly, the observed germ cell detachment that occurs following MEHP exposure in vivo could be inhibited by pretreatment with a MMP2 inhibitor [8]. These observations suggest a possible mechanism by which MMP2 activation may be responsible for modulating the dynamic physical interaction between Sertoli cells and germ cells. Here we report that MEHP-induced MMP2 activation in the testis contributes to the decreased supportive capacity of Sertoli cells following MEHP exposure by altering junctional connections. These results provide distinctive insights into the complex regulation of the interaction between Sertoli cells and germ cells, which may be useful for predicting and preventing male reproductive disorders.

MATERIALS AND METHODS

Animals, Primary Sertoli Cell-Germ Cell Cocultures, and MEHP Exposure

For in vivo experiments, 21-day-old C57BL/6J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The use of C57BL/6J mice was based on previous publications from our laboratory [8, 19, 20]. The climate of the animal room was kept at a constant temperature (22°C ± 0.5°C) at 35%–70% humidity with a 12L:12D photoperiod. Animals were given standard lab chow and water ad libitum and allowed to acclimate for 1 wk before experimental challenge. All procedures involving animals were performed in accordance with the guidelines of the University of Texas at Austin's Institutional Animal Care and Use Committee. Twenty-eight-day-old male mice were given a single dose of MEHP (1 g/kg, in corn oil; TCI America, Portland, OR) by oral gavage, a well-established dosing paradigm used for MEHP-induced Sertoli cell injury that results in a testicular concentration of ~100 μM [21]. Control animals received a similar volume of corn oil vehicle. Vehicle- and MEHP-treated animals were killed by CO_2 inhalation, and the testis was removed and either immediately frozen in liquid N_2 for protein analysis or fixed in Bouin solution (Polysciences, Inc., Warrington, PA) for histology analysis.

Primary cocultures of rat Sertoli cells and germ cells were isolated from 21-day-old Fisher rats (Harlan Sprague Dawley, Inc. Indianapolis, IN) according to previous publications from our laboratory and from other groups [8, 22, 23]. Primary rat cocultures also have a high yield, and they, therefore, can minimize the number of animals needed. Purified mixed populations of Sertoli cells and germ cells were plated on 35-mm laminin-coated culture dishes at a density of 2×10^6 cells/3 ml media containing Dulbecco modified Eagle medium/Ham F12 media (Invitrogen, Gaithersburg, MD) with 1 ng/ml epidermal growth factor (Sigma, St. Louis, MO), a mix of insulin, transferrin, selenious acid, bovine serum albumin, and linoleic acid, 10 μg/ml (BD Biosciences, San Jose, CA), gentamicin (50 μg/ml, Invitrogen), and 1% penicillin-streptomycin (Invitrogen). The cells were then incubated at 37°C for 48 h. Primary cocultures were treated with 200 μM MEHP diluted in dimethyl sulfoxide (DMSO, 0.004% final concentration in media) for various time periods as previously described [19].

Testicular Histopathology

Bouin solution-fixed testes were washed in 70% ethyl alcohol-Li_2CO_3-saturated solution and embedded in paraffin for histological analysis. Testicular cross sections (5 μm) were evaluated by using periodic acid-Schiff-hematoxylin (PAS-H) staining [24], and all the sections were imaged using a Nikon E800 microscope and captured with a Canon-5D digital camera.

Transmission Electron Microscope

Testes from vehicle- and MEHP-treated mice were fixed in 2.5% glutaradehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate, and postfixed with 2% OsO_4 (all reagents from Electron Microscopy Science, Hatfield, PA). After dehydration through a series of ethanol washes, the testes were embedded in Epon 812 (Electron Microscopy Science). Thin sections (~60 nm) were cut using a Leica Ultracut UCT microtome and transferred to 200-μm mesh copper grids, followed by double-staining with uranyl acetate and lead citrate. The testicular ultrastructure was examined using a Philips EM 208 Transmission Electron Microscope (Philips, Eindhoven, The Netherlands). The pictures were processed and analyzed using the Metamorph Imaging Software v6.1, and the average length of the opening in tight junctions was determined using its digital measurement tools.

Gelatinase Inhibitor SB-3CT Treatment In Vivo and In Vitro

SB-3CT (Chemicon, Temecula, CA) is a gelatinase-specific inactivator that inhibits MMP2 and MMP9 by binding to the active site of the enzymes [25]. In vitro, SB-3CT at a concentration of 0, 5, or 10 μM was added to media of primary Sertoli cell-germ cell cocultures for 12 h in the presence of 200 μM MEHP. Primary coculture cells were harvested for protein analysis. In vivo, various doses of SB-3CT (0, 5, 10, and 25 mg/kg in 10% DMSO in normal saline) were injected intraperitoneally into male 28-day-old C57BL/6J mice. After 6 h, MEHP (1g/kg) was administrated to SB-3CT-pretreated mice by oral gavage. Mice were killed by $C0_2$ inhalation 12 h after MEHP treatment, and the testes were collected. One testis was prepared for paraffin embedding, and the other testis was immediately frozen at −80°C for protein analysis.

Total Protein Preparation and Western Blot Analysis

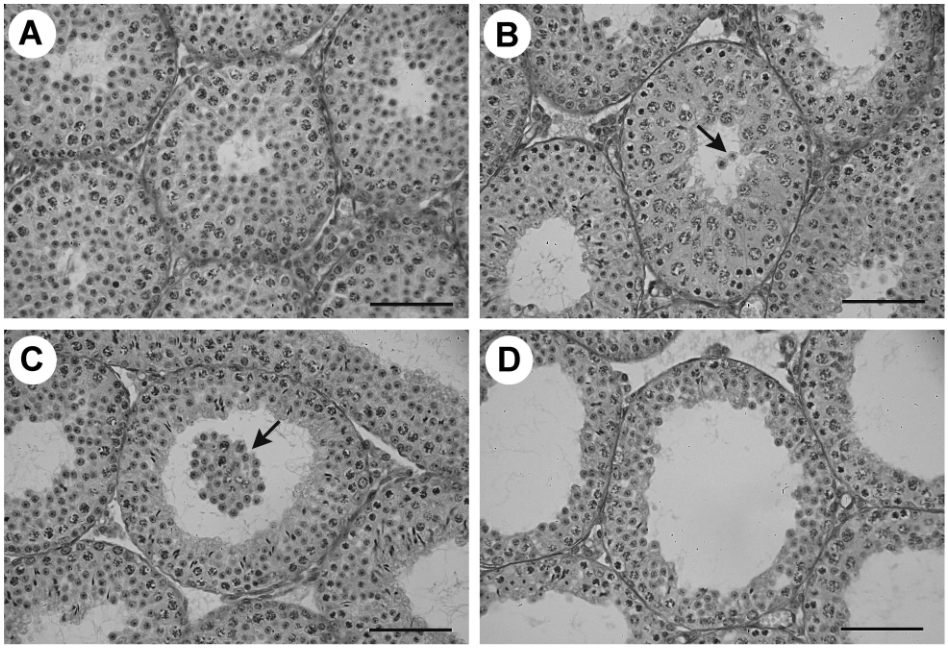
A detailed description for total protein preparation from primary cell cocultures and from mice testes and the subsequent Western blot analysis have been described in detail previously [19]. Primary antibodies used include those that detect claudin11 (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, sc-25711), occludin (1:1000; Abcam Inc., Cambridge, MA, ab31721), ZO1 (1:1000; Zymed, Gaithersburg, MD, #61–7300), laminin-γ3 (1:250; Santa Cruz Biotechnology Inc., sc-16601), β1-integrin (1:250; Santa Cruz Biotechnology Inc., sc-6622), and β-actin (ACTB) (1:500; Santa Cruz Biotechnology Inc., sc-1616). The ECL chemiluminescent substrate (Amersham Bioscicence, Piscataway, NJ) was used as the detection reagent. The inclusion of ACTB was used to ensure equal loading of samples.

Immunohistochemistry

Expression and localization of claudin11, occludin, and ZO1 in the seminiferous epithelium were determined by immunohistochemistry. Cross sections (5 μm) of paraffin-embedded testes were deparaffinized and rehydrated, and antigens were unmasked by heating in 10 mM sodium citrate solution. Sections were incubated with 3% H_2O_2 to block endogenous peroxidase activity and then incubated in blocking buffer containing 10% horse serum. Primary antibodies used in this study were rabbit-anti-ZO1 (1:100; Zymed, #61–7300), rabbit anti-occludin (1:100; Abcam Inc., ab31721), and rabbit anti-claudin11 (1:100; Santa Cruz Biotechnology Inc., sc-25711). Sections were incubated overnight in primary antibodies at 4°C. Immunodetection was performed by standard procedure using VectaStain ABC kit (Vector Labs, Burlingame, CA) and 3,3′-diaminobenzidine substrate (Vector Labs). All the sections were imaged using a Nikon E800 microscope and captured with a Canon-5D digital camera.

Immunofluorescence

Expression and localization of laminin-γ3 and β1-integrin in the seminiferous epithelium were determined by immunofluorescence staining. Cross sections (5 μm) of paraffin-embedded testes were deparaffinized and rehydrated, and antigens were unmasked in heated 10 mM sodium citrate. Sections were incubated in blocking buffer containing 10% horse serum, followed by overnight incubation in primary antibodies at 4°C. Primary antibodies used in this study were goat-anti-laminin-γ3 (1:50; Santa Cruz



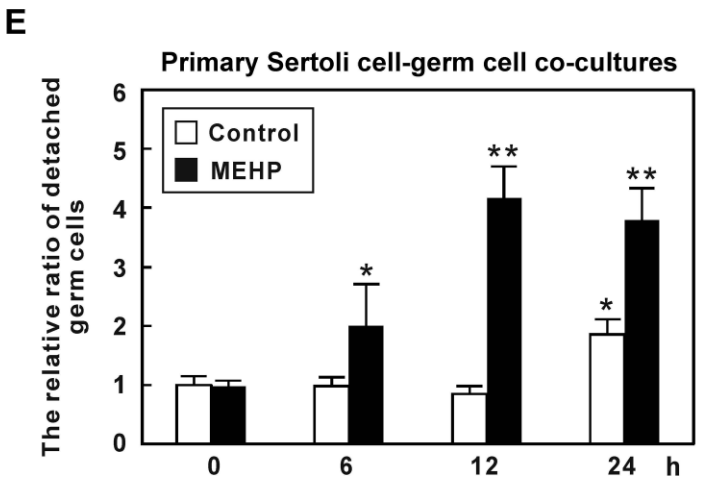


FIG. 1. MEHP exposure causes germ cell detachment in vivo and in vitro. **A–D**) Testicular morphology in 28-day-old C57BL/6J male mice is shown in cross sections from paraffin-embedded testes with PAS-H staining. Detached germ cells are indicated by arrows. Control (**A**), MEHP 6 h (**B**), MEHP 12 h (**C**), and MEHP 24 h (**D**). Bar = 50 μm. **E**) Primary rat Sertoli cell-germ cell cocultures treated with or without 200 μM MEHP for 0, 6, 12, and 24 h, and detached cells were quantified. The open bar represents control cells and the solid bar represents MEHP-treated cells. Values represent the mean ± SEM. Asterisks denote significant differences between the treatments and the control (*$P < 0.05$, ** $P < 0.01$, Student *t*-test).

Biotechnology Inc., sc-16601) and goat-anti-β1-integrin (1:50; Santa Cruz Biotechnology Inc., sc-6622). Sections were then incubated in Alexa Fluor conjugated anti-goat antibody (1:500; Molecular Probes, Gaithersburg, MD) for 1 h and mounted in Vectashield Mounting Medium (Vector Labs). Fluorescent signals were detected using excitation/emission wavelengths of 495 nm/521 nm, respectively. All sections were imaged using a Nikon E800 microscope and captured with a Nikon Cool-SNAP digital camera, and processed using MetaMorph Imaging software (v. 4.1) (Downingtown, PA). Images were captured as TIFF formats and adjusted by Adobe Photoshop (version 7.0.1, Adobe Systems, San Jose, CA) using the same brightness/contrast.

Germ Cell Detachment

Primary rat Sertoli cell-germ cell cocultures were seeded in 6-well culture plates and treated with 200 μM MEHP. In order to determine the number of germ cells detached from Sertoli cells after MEHP exposure, cells in media were collected and stained with 0.4% trypan-blue (Invitrogen). The number of detached cells was counted using a hemocytometer. The specific gelatinase inhibitor, SB-3CT (10 μM), MMP2 recombinant protein (50 ng/ml; R&D System Inc., Minneapolis, MN), or TIMP2 recombinant protein (50 ng/ml; R&D System Inc.) was added to primary rat cocultures in the presence of MEHP to suppress MEHP-induced MMP2 activation, and detached germ cells were counted after 6 h of incubation. In order to determine the relationship between germ cell apoptosis and germ cell detachment by MEHP exposure, primary rat coculture cells were pretreated with the caspase inhibitor z-VAD-FMK (20 μM, Sigma) for 2 h and then exposed to 200 μM MEHP for 3 and 6 h. At the same time, z-VAD-FMK was further added into primary rat coculture cells. Detached germ cells were counted. All the experiments were performed in triplicate.

Terminal Deoxynucleotidyl Transferase-Mediated Digoxigenin-dUTP Nick End Labeling (TUNEL) Assay

Apoptotic fragmentation of DNA in mouse paraffin-embedded testis cross sections was determined by TUNEL analysis using the ApopTag kit (Chemicon). The apoptotic index was calculated as the percentage of seminiferous tubules containing more than three TUNEL-positive germ cells in each cross section. At least two testicular cross sections per mouse and at least three mice in each treatment group were analyzed.

Statistical Analysis

All experimental groups were performed in triplicate and repeated at least three times using different animals and different sets of primary cells. The data were subjected to Student *t*-test or a parametric one-way analysis of variance (ANOVA) followed by Tukey test for post hoc comparisons. Statistical significance was considered to be achieved when $P < 0.05$.

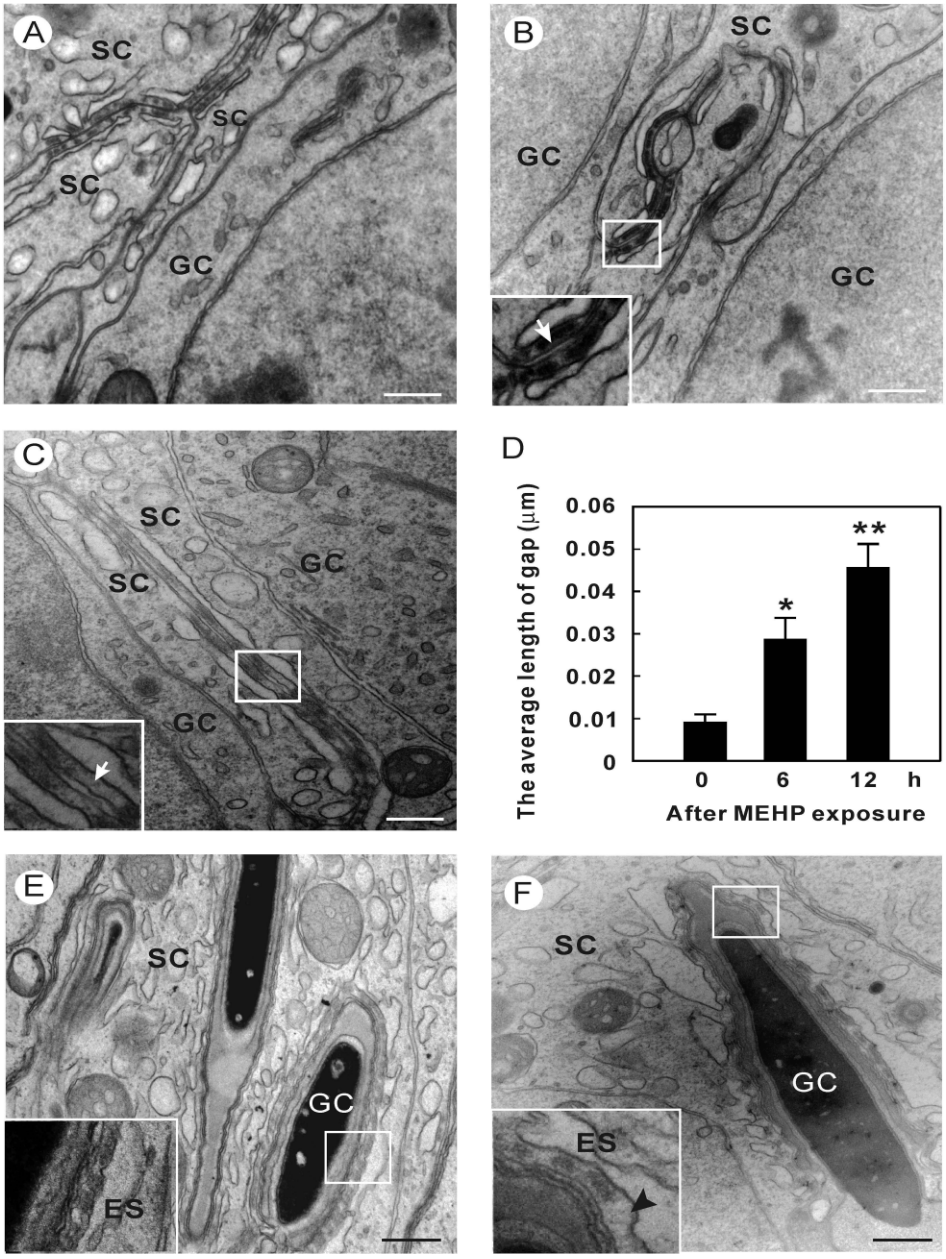


FIG. 2. Transmission electron microscope reveals the changes in the ultrastructure of junctions in the seminiferous epithelium after MEHP exposure. **A**–**C**) The blood-testis barrier (BTB) in 28-day-old control and MEHP-treated mice (6 and 12 h) are examined. The white arrows indicate openings in the junctions between adjacent Sertoli cells after MEHP exposure. **D**) The average length of opening in tight junctions is measured. After 12 h exposure, the gap in tight junctions is significantly wider than control (*$P < 0.05$, **$P < 0.01$, Student *t*-test). Values represent the mean ± SEM. **E** and **F**) The apical ectoplasmic specialization (ES) between Sertoli cells and elongate spermatids in 28-day-old control and MEHP-treated mice (12 h) are examined. The black arrowhead indicates the area of disorganized actin filaments. SC = Sertoli cell; GC = germ cell. Small boxed image represents the close view of disrupted junctions. Bar = 500 nm with magnification ×22 000.

RESULTS

MEHP-Induced Testicular Injury In Vivo and In Vitro

Twenty-eight-day-old C57BL/6J male mice were treated with MEHP (1 g/kg), and changes in testicular morphology within the seminiferous tubule were observed. The lumen of the seminiferous tubule gradually increased after MEHP exposure (Fig. 1, A–D), reflecting the retraction of Sertoli cell cytoplasm and germ cell loss. After 12 h of exposure, clusters of detached pachytene spermatocytes and spermatids were observed in the lumen (Fig. 1C). After exposure for 24 h, the lumen size in MEHP-treated seminiferous tubules became twice as large as controls (41.33 ± 3.09 μm and 88.00 ± 2.49 μm in control and MEHP-exposed mice, respectively) (Fig. 1D). In some MEHP-treated tubules, no round spermatids were found, and the germ cell population consisted of only spermatogonia and preleptotene and pachytene spermatocytes. These observations are consistent with those previously reported by other groups [8, 10]. In primary rat cocultures, the number of detached germ cells detected in the media was observed by 6 h after MEHP addition. The number of detached germ cells measured in the media dramatically increased (~4-fold) 12 h after MEHP addition as compared with controls (Fig. 1E).

MEHP Exposure Instigates the Opening of the Blood-Testis Barrier and the Remodeling of Apical Ectoplasmic Specializations

Transmission electron microscopy was performed to examine the ultrastructure of the BTB between adjacent Sertoli cells and anchoring junctions between Sertoli cells and germ cells. Tight junctions associated with dense electron signals that represent actin filament bundles appeared intact in 28-day-old control C57BL/6J mice (Fig. 2A). After 6 h of MEHP exposure, a slight gap in the BTB was observed (Fig. 2B), and

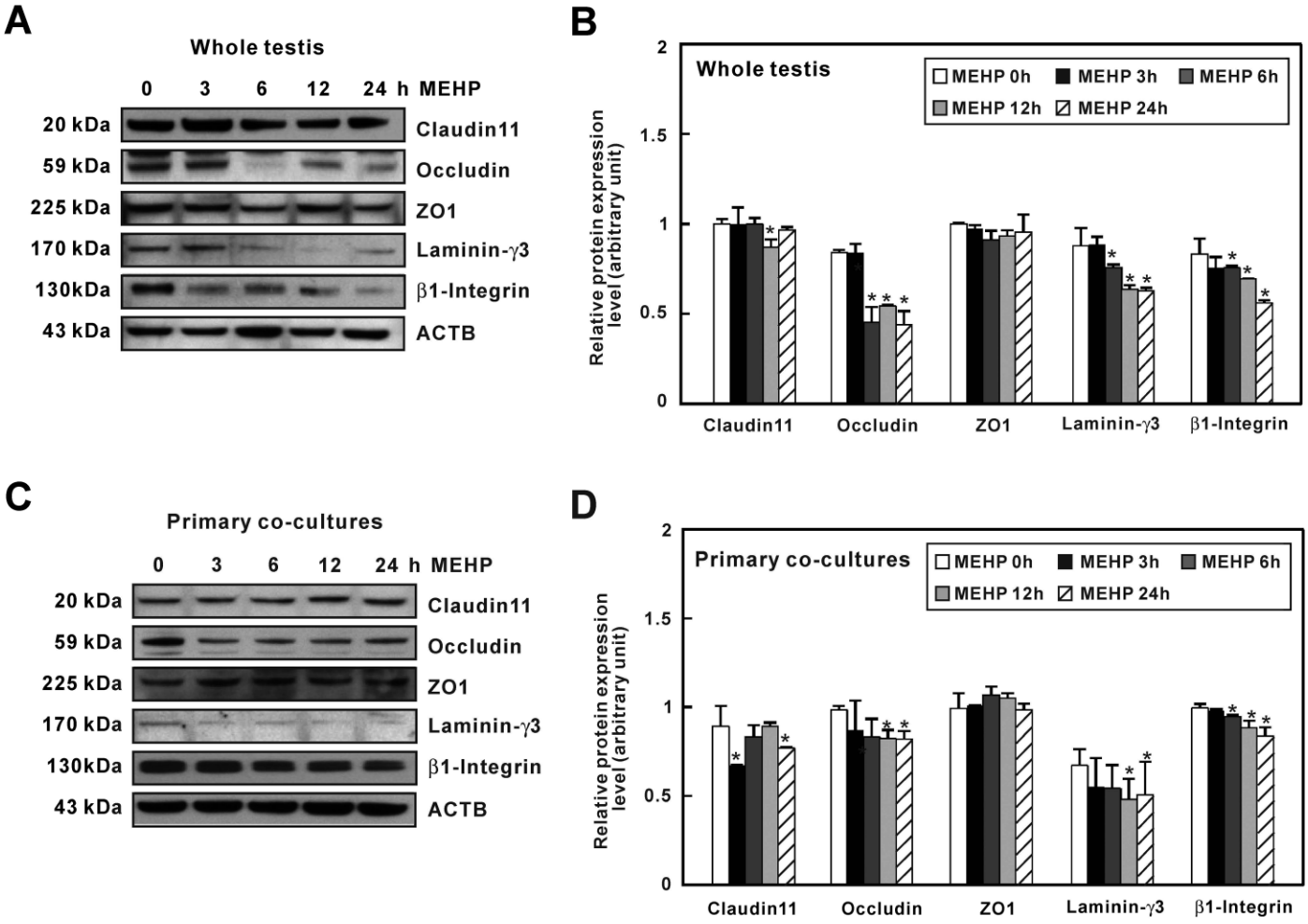


FIG. 3. MEHP exposure decreases expression levels of junction proteins in vitro and in vivo. The protein levels of claudin11, occludin, ZO1, laminin-γ3, and β1-integrin in whole testis homogenates (**A** and **B**) from 28-day-old C57BL/6J mice and in primary rat Sertoli cell-germ cell cocultures (**C** and **D**) are determined by Western blot analysis in response to MEHP exposure. ACTB serves as the loading control. **B**) The quantified relative protein level of **A**. **D**) The quantified relative protein level of **C**. Values represent the mean ± SEM. Asterisks denote significant differences between the treatments and the control (*$P < 0.05$, Student *t*-test).

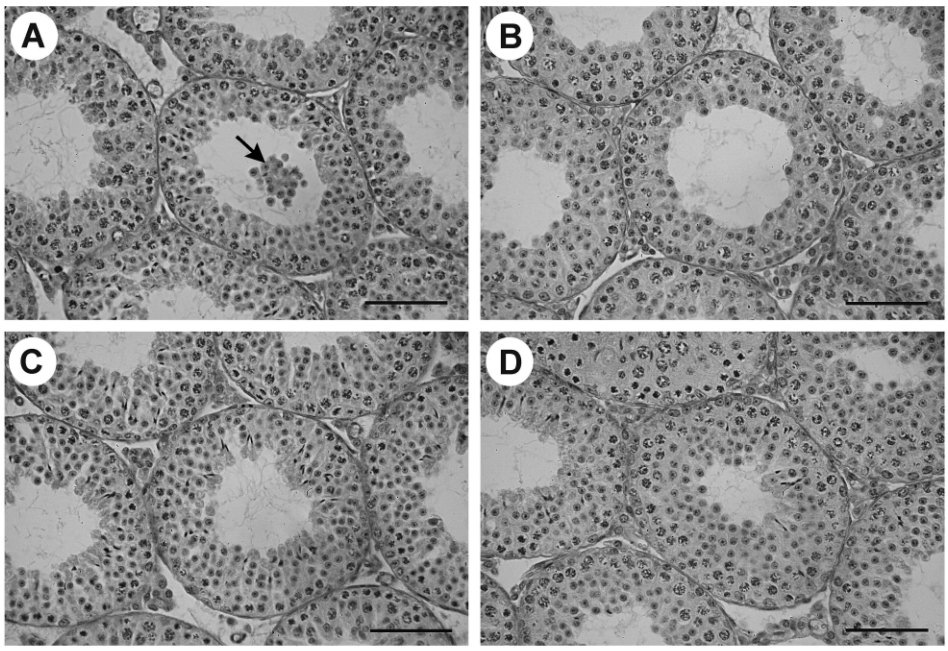
by 12 h after exposure, significantly wider gaps were observed (0.0457 μm wide compared with 0.0093 μm in controls) (Fig. 2C). The distribution of dense electron signals at the BTB were disorganized and less dense in MEHP-treated mice than in controls, suggesting that the actin filament bundles may be disorganized. A quantitative assessment of the gap width is shown in Figure 2D. The apical ectoplasmic specializations between elongate spermatids and Sertoli cells were defuse by 12 h after MEHP exposure and showed scattered electron dense signals, reflecting fewer associated actin filament bundles (Fig. 2F) than in the control (Fig. 2E).

Occludin, Laminin-γ3, and β1-Integrin Protein Expression Levels Are Decreased following MEHP Exposure

In order to understand whether MEHP exposure altered the organization of junctions within the seminiferous epithelium, the expression levels of five junction proteins (claudin11, occludin, ZO1, laminin-γ3, and β1-integrin) were determined by Western blot analysis of mouse testes homogenates (Fig. 3, A and B) and of primary rat Sertoli cell-germ cell cocultures (Fig. 3, C and D). The expression of the tight junction protein occludin was decreased in response to MEHP exposure in vivo and in vitro. No significant change in the levels of the ZO1 protein was detected, while claudin11 protein levels were slightly decreased after MEHP exposure (down to 87% in vivo and 89% in vitro at 12 h of exposure compared with controls). As for apical ES proteins, both laminin-γ3 and β1-integrin expression decreased in a time-dependent manner in vivo and in vitro after MEHP exposure.

SB-3CT Pretreatment Protects against MEHP-Associated Germ Cell Detachment from the Seminiferous Epithelium

Analysis of testicular cross sections revealed that MEHP-induced germ cell detachment was significantly inhibited in mice that were pretreated with a low (5 and 10 mg/kg) dose of SB-3CT (Fig. 4, B and C). Mice pretreated with the higher dose (25 mg/kg) of SB-3CT retained the normal diameter of testicular lumen (Fig. 4D; 83.88 ± 3.56, 113.32 ± 5.09, and 78.15 ± 2.89 μm in control, MEHP-treated, and SB-3CT-pretreated mice, respectively), suggesting that the suppression of MMP2 led to the protection of germ cells from MEHP-induced depletion and preservation of the interaction between Sertoli cells and germ cells in vivo.



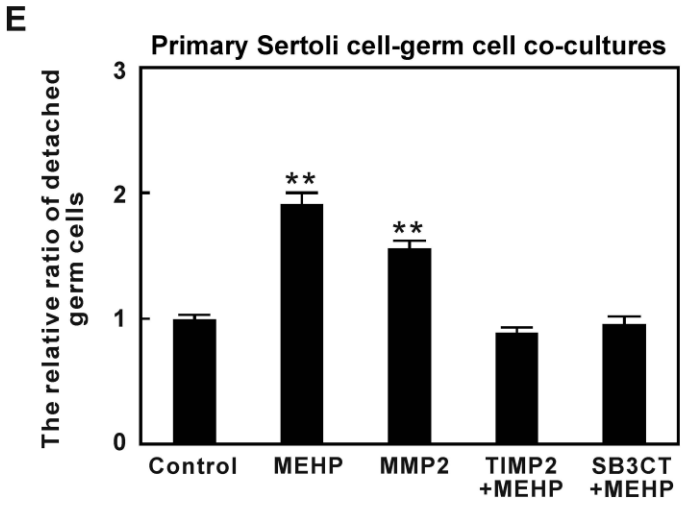


FIG. 4. Inhibition of endogenous MMP2 leads to the protection of germ cells from MEHP-induced depletion in vivo and in vitro. **A**–**D**) Testicular morphology in 28-day-old C57BL/6J male mice is shown by cross sections from paraffin-embedded testes with PAS-H staining. SB-3CT-pretreated mice (0, 5, 10, and 25 mg/kg) are post-exposed to MEHP (1 g/kg) for 12 h (**A**–**D**). The testicular lumen is decreased in SB-3CT-pretreated mice. No germ cell detachment is observed in high-dose SB-3CT-pretreated mice testes. Detached germ cells are indicated by an arrow. Bar = 50 μm. **E**) Primary rat Sertoli cell-germ cell cocultures are seeded in 6-well culture plates. After 48 h, cocultures are treated with MMP2 (50 ng/ml), TIMP2 (50 ng/ml), or SB-3CT (10 μM) in the presence or absence of MEHP for 6 h. Detached germ cells in conditioned media are counted. The inhibition of MMP2 by either TIMP2 or SB-3CT significantly decreases MEHP-induced germ cell detachment. Values represent the mean ± SEM. Asterisks denote significant differences between the treatments and the control (**$P < 0.01$, Student *t*-test).

Germ Cell Detachment Is Increased in Response to MEHP Through MMP2 Activation In Vitro

In vitro, the addition of recombinant MMP2 (50 ng/ml) to primary rat coculture cells resulted in a similar increase in detached germ cells to that found after MEHP exposure (1.72- and 1.79-fold, respectively; Fig. 4E). Conversely, the addition of either TIMP2 or SB-3CT was able to significantly reduce the amount of germ cell detachment after MEHP treatment (46.31% and 50.33% compared with MEHP treatment, respectively; Fig. 4E).

SB-3CT Administration Suppresses MEHP-Inhibited Junction Protein Expression In Vivo and In Vitro

To further investigate the effect of MMP2 on restructuring junctional complexes, the protein levels of junction proteins following SB-3CT treatment in vivo and in vitro were measured. A low dose of SB-3CT treatment both in vivo (Fig. 5, A and B) and in vitro (Fig. 5, C and D) was able to reduce the MEHP-induced decreases in the levels of occludin. SB-3CT pretreatment of mice did not significantly modify the MEHP-induced changes in the testicular levels of claudin11 and ZO1 (Fig. 5, A and B). The addition of SB-3CT to the media of primary cocultures of Sertoli cells and germ cells was able to somewhat suppress the MEHP-stimulated decrease of claudin11 protein levels (Fig. 5, C and D). Laminin-γ3 and β1-integrin were significantly decreased by MEHP exposure while their expression levels were restored by inhibiting MMP2 activation both in vivo (Fig. 5, A and B) and in vitro (Fig. 5, C and D).

Effects of SB-3CT Pretreatment and MEHP Exposure on the Immunohistochemical Localization of Junctional Proteins

The immunohistochemical localization of claudin11 and occludin in control mouse testis cross sections showed that they were mainly localized in the basal compartment, especially in the areas between the Sertoli cells and preleptotene/leptotene spermatocytes, an area that corresponds to the BTB (Fig. 6, A and B). The immunochemical detection of these two proteins is significantly reduced in testicular cross sections from mice 12 h

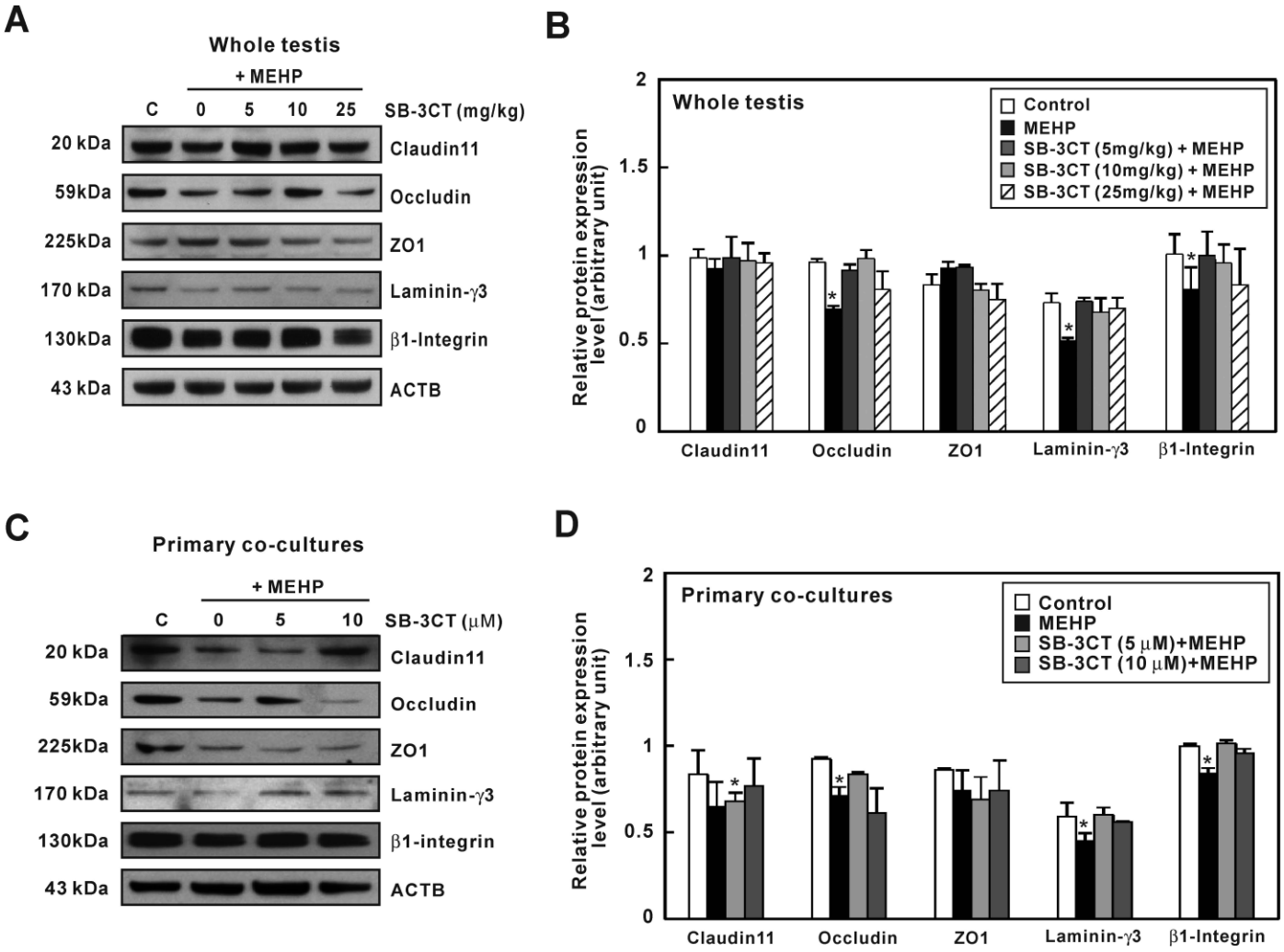


FIG. 5. SB-3CT administration causes the changes in expression levels of junction proteins in vivo and in vitro. In vivo, 28-day-old C57BL/6J mice are pretreated with SB-3CT (0, 5, 10, and 25 mg/kg) for 6 h and then posttreated with 1 g/kg of MEHP for another 12 h. In vitro, primary rat Sertoli cell-germ cell cocultures are treated with SB-3CT (0, 5, and 10 μM) in the presence of MEHP for 12 h. The protein levels of claudin11, occludin, ZO1, laminin-γ3, and β1-integrin in whole testis homogenates (**A**) and in primary rat Sertoli cell-germ cell cocultures (**C**) are determined by Western blot analysis. ACTB serves as the loading control. **B**) The quantified relative protein levels of **A**. **D**) The quantified relative protein levels of **C**. Values represent the mean ± SEM. Asterisks denote significant differences between the treatments and the control (*$P < 0.05$, Student *t*-test).

after MEHP exposure (Fig. 6, D and E). However, the pretreatment of mice with SB-3CT prevented this loss in the immunohistochemical detection of these proteins (Fig. 6, G and H). Interestingly, occludin was detected in the adluminal compartment above the BTB in both control and SB-3CT-pretreated mice testes but not in MEHP-treated samples. The ZO1 protein was immunolocalized in both the basal and the adluminal compartments in control mice (Fig. 6C), and its localization remained unchanged after MEHP treatment and/or SB-3CT pretreatment (Fig. 6, F and I).

Immunofluorescent staining revealed that laminin-γ3 was restricted to apical ESs between Sertoli cells and spermatids (Fig. 7B) and that its levels were decreased after MEHP exposure (Fig. 7D). The β1 integrin was also localized to apical ESs and along the basement membrane (Fig. 7A), its levels were also suppressed by MEHP exposure (Fig. 7C). However, in the testis of mice that were pretreated with SB-3CT, MEHP exposure did not result in a decreased immunodetection of either laminin-γ3 or β1-integrin (Fig. 7, E–J).

MEHP-Induced Germ Cell Detachment Is Independent of Germ Cell Apoptosis

MEHP-induced germ cell apoptosis occurred mainly in spermatocytes, but none of the detached germ cells in the lumen were TUNEL-positive cells (Figure 8A). In order to assess the relationship between germ cell apoptosis and germ cell detachment as a result of MEHP exposure, primary rat Sertoli cell-germ cell cocultures were pretreated with a general caspase inhibitor z-VAD-FMK (20 μM) for 2 h and then exposed to 200 μM of MEHP for various periods of time. The addition of z-VAD-FMK did not prevent the MEHP-triggered increase of germ cells released into the culture media (1.5-fold compared with control; Fig. 8B).

DISCUSSION

The premature sloughing of germ cells into the lumen has been widely described in many mammalian species after MEHP exposure [10, 26, 27]. Although this observation has long been appreciated to reflect a disruption in the physical

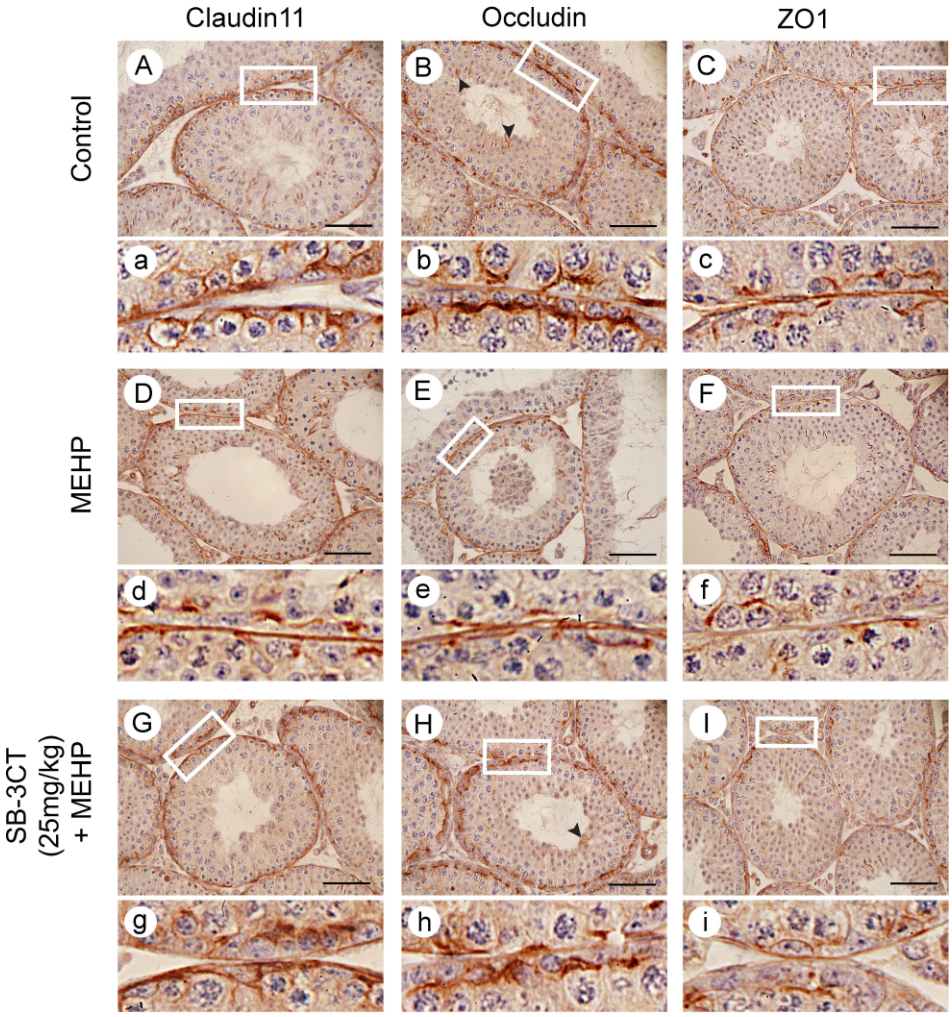


FIG. 6. Alterations in the expression and localization of claudin11, occludin, and ZO1 in the seminiferous epithelium are observed by immunohistochemical analysis. Twenty-eight-day-old C57BL/6J mice are pretreated with SB-3CT (0 and 25 mg/kg) for 6 h and then posttreated with 1 g/kg of MEHP for another 12 h. Control (**A–C**); MEHP treatment only (**D–F**); 25 mg/kg of SB-3CT pretreatment with MEHP posttreatment (**G–I**). **a–i**) The magnified view of the BTB (boxed area). Testis cross sections show that claudin11, occludin, and ZO1 strongly express along the BTB. The expression of both claudin11 and occludin is decreased after MEHP exposure, while they are increased in 25 mg/kg SB-3CT-pretread mice testes. The expression of occludin in the adluminal compartment is indicated by an arrow head. The expression of ZO1 is not significantly influenced by MEHP or SB-3CT treatment. Bar = 50 μm.

connection between Sertoli cells and germ cells, the cellular mechanism to account for this effect has not been resolved. Interestingly, in reports of other testicular toxicants, such as DDT, dinitrobenezene, and cisplatin, alterations in junctional structures have been reported in vitro as evidenced by changes in the levels of specific junction proteins or alterations in the localization of these proteins, including occludin, ZO1, N-cadherin, and Cx43 [28]. Therefore, these basic findings logically led us to investigate whether MEHP exposure can specifically result in the disruption of testicular junctional complexes and account for the observed detachment of germ cells. In this study, we focus on an investigation of the changes in structure and components of the BTB and apical ESs in MEHP-treated peripubertal rodents.

The BTB in rats has previously been reported to exhibit interspaces of 0.01–0.02 μm between adjacent Sertoli cells [29]. However, our results indicate the formation of a wider and longer gap of the BTB in the mouse testis after MEHP exposure (Fig. 2, A–D). A gap in the BTB could disrupt the tightly regulated microenvironment of the adluminal compartment of the seminiferous epithelium as well as interfere with the direct movement of preleptotene spermatocytes across the BTB.

It is well-known that TNF and TGFB are responsible for the remodeling of the BTB in adult rats [14, 30, 31]. Recent studies indicate that TNF facilitates the disassembly of tight junctions at the BTB by targeting occludin expression, allowing for the translocation of preleptotene/leptotene spermatocytes through the opening barrier [13, 14]. The addition of TNF to the media of astrocytes results in the activation of NFKB1 and subsequent down-regulation of occludin expression [15]. These studies illustrate the contribution of the TNF-activated NFKB1-signaling pathway on regulating the junctional structure. Our previous findings indicate that MEHP stimulates soluble TNF production by germ cells and further activates the NFKB1-signaling pathway in Sertoli cells [19]. Here, occludin protein expression is significantly decreased after MEHP exposure both in vivo and in vitro (Fig. 3). Collectively, these observations suggest that MEHP-enhanced production of soluble TNF may be responsible for the suppression of occludin expression and contribute to the disorganization of the BTB structure between adjacent Sertoli cells.

The ability of TNF in regulating ZO1 expression is dependent upon cell types. TNF-activated NFKB1-signaling pathway suppresses ZO1 expression in intestinal epithelial cells [32], but no changes are detected in astrocytes [15]. TNF also affects the localization of ZO1, resulting in disruption of barrier functions in human corneal epithelial cells [33]. In the testis, MEHP exposure causes no changes in the expression level and the localization of ZO1 (Figs. 3 and 6), suggesting that tight junction-associated protein may not be the primary target of MEHP. Even though it has been reported that MEHP treatment (600 μM) causes a disorganization of ZO1 in primary rat Sertoli cells [11], the dissimilar observations may be explained by different doses of MEHP exposure. In our system, a single dose of MEHP (1 g/kg) is given in vivo that results in a

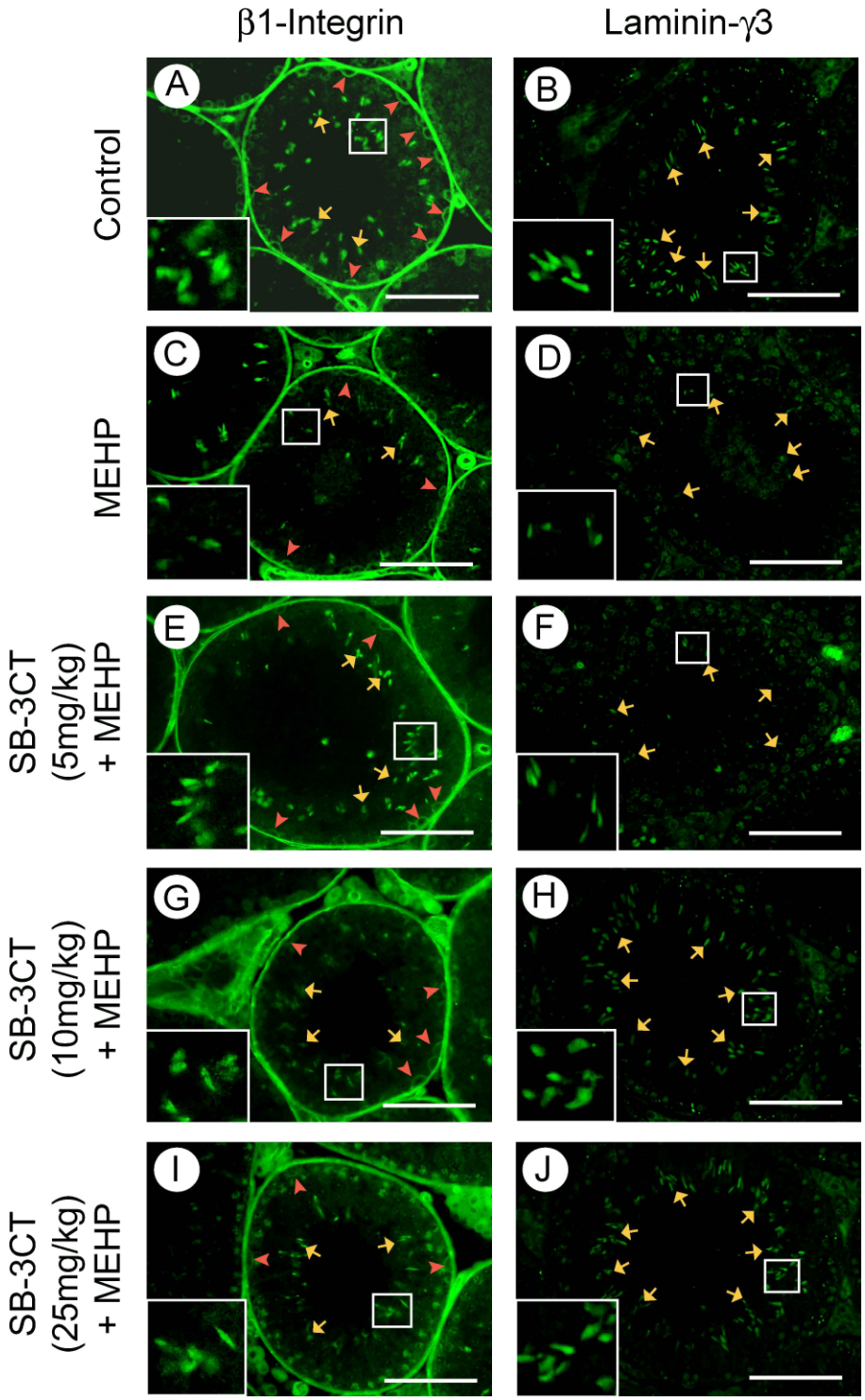


FIG. 7. Dynamic changes in the expression and localization of β1-integrin and laminin-γ3 in the seminiferous epithelium are revealed by immunofluorescence analysis. Twenty-eight-day-old C57BL/6J mice are pretreated with SB-3CT (0, 5, 10, and 25 mg/kg) for 6 h and then posttreated with 1 g/kg of MEHP for another 12 h. Control (**A** and **B**); MEHP only (**C** and **D**); 5 mg/kg of SB-3CT pretreatment (**E** and **F**); 10 mg/kg of SB-3CT pretreatment (**G** and **H**); 25 mg/kg of SB-3CT pretreatment (**I** and **J**). Testis cross sections show that β1-integrin and laminin-γ3 express close to the acrosome of spermatids, where the ectoplasmic specialization is located (orange arrows). β1-integrin also strongly expresses along the basement membrane as well as close to spermatogonia (red arrow heads). The expression of both β1-integrin and laminin-γ3 is decreased after MEHP exposure while increased in SB-3CT-pretreated mice testes. Bar = 50 μm.

testicular concentration of ~100 μM and causes Sertoli cell injury [21]. In vitro, 200 μM of MEHP is applied to primary rat Sertoli cell-germ cell cocultures in accordance with our previous studies [8, 19]. Therefore, the effect of Sertoli cell toxicants on the regulation of scaffold protein associated with tight junctions may be dose-dependent.

The effect of MEHP exposure on the structure of anchoring junctions in the seminiferous epithelium is also determined in this study. Apical ESs are testis-specific junctions composed of tight junctions, adherens junctions, focal contacts, and gap junctions [4]. The ultrastructure data shows a dramatic disruption in apical ESs in between Sertoli cells and elongate spermatids after MEHP exposure (Fig. 2, E and F). Scattered electron density is also observed at apical ESs in MEHP-treated mice testes, reflecting disconnected actin filament bundles (Fig. 2F). Laminin-333 in germ cells and α6β1-integrin in Sertoli cells form a focal adhesion complex to link two types of cells together at apical ESs [34, 35]. The disruption of laminin/integrin complexes during spermiation is necessary for the release of mature spermatozoa into the testicular lumen [3]. Recent studies showed that MMP2 is mainly localized in apical ESs associated with the heads of elongate spermatids [34, 36].

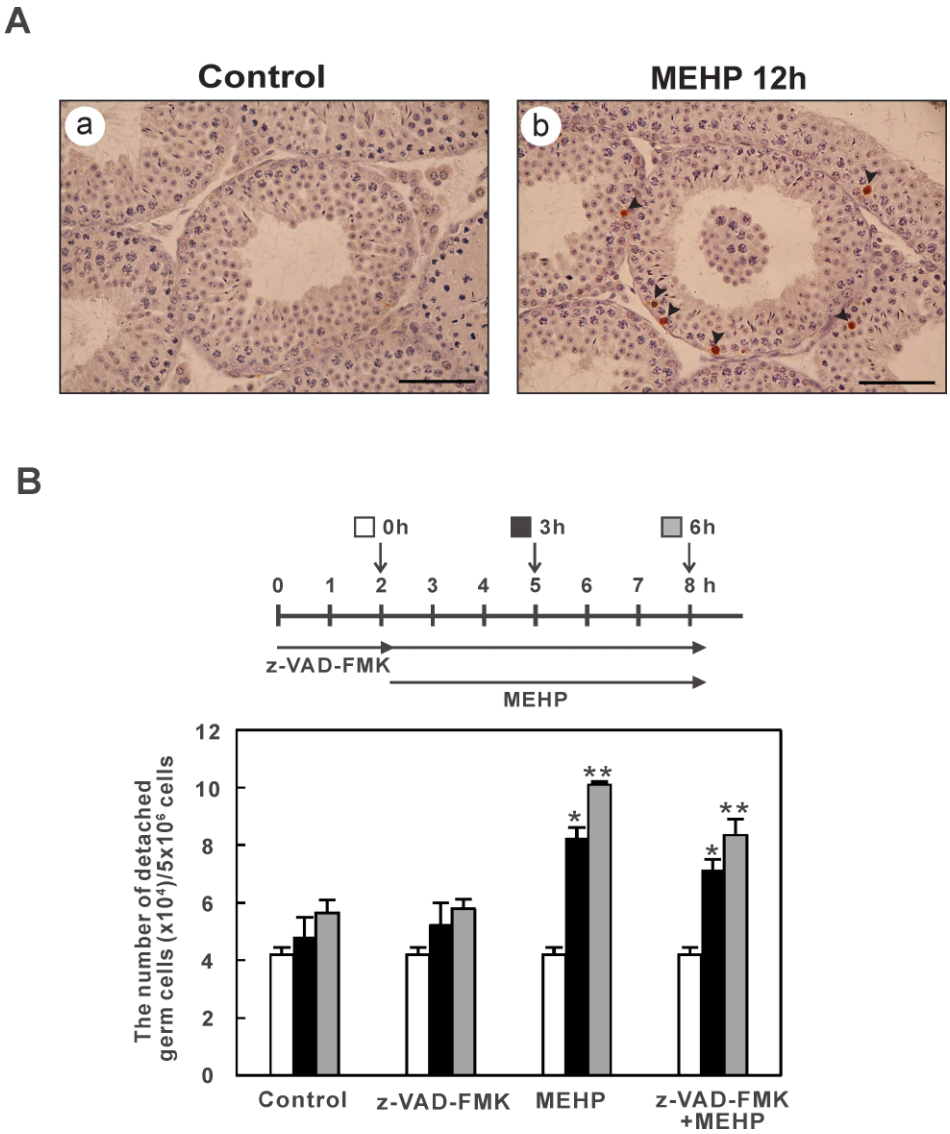


FIG. 8. MEHP-induced germ cell detachment is independent of germ cell apoptosis. **A**) Twenty-eight-day-old C5L/6J male mice are treated with 1g/kg MEHP, and germ cell apoptosis rate is determined by TUNEL assay. Control (**a**); MEHP 12 h (**b**). Testicular cross sections show TUNEL-positive germ cells as brown spots (arrow heads). **B**) Primary rat Sertoli cell-germ cell cocultures are pretreated with caspase inhibitor z-VAD-FMK (20 μM) for 2 h and then exposed to 200 μM MEHP for 0, 3, and 6 h in the presence or absence of additional z-VAD-FMK administration, as described in upper part of the figure. Detached germ cells are counted. Values represent the mean ± SEM. Asterisks denote significant differences between the treatments and the control (*$P < 0.05$, **$P < 0.01$, Student *t*-test).

Laminin-γ3 is a MMP2 and membrane type 1-MMP substrate at apical ESs [37], and the activation of MMP2 disrupts the laminin/integrin complex [34]. Here, time-dependent decreases in laminin-γ3 and β1-integrin expression after MEHP exposure in vivo and in vitro are observed (Figs. 3 and 7). Therefore, decreases in laminin-γ3 and β1-integrin may cause the disruption of apical ESs after MEHP exposure, resulting in premature germ cell loss.

Our present results also show that fewer germ cell detachment events are observed in SB-3CT-pretreated mice due to the inhibition of endogenous MMP2 (Fig. 4, B–D). The administration of MMP2 inhibitors, TIMP2 or SB-3CT, also significantly suppresses the detachment of germ cells in the presence of MEHP in vitro (Fig. 4E). These observations strongly indicate that MEHP-activated MMP2 is responsible for disrupting the interaction between Sertoli cells and germ cells, and initiating germ cell detachment. In addition, SB-3CT-pretreated mice show protection against decreases in laminin-γ3 and β1-integrin expression in response to MEHP exposure (Fig. 5, A and B), and similar results are detected in primary rat cocultures (Fig. 5, C and D). Immunofluorescent staining further shows a dramatic decrease in laminin-γ3 expression at apical ESs after MEHP exposure, while SB-3CT rescues the laminin-γ3 expression from MEHP treatment (Fig. 7). Collectively, these results provide the first evidence showing that MEHP may influence the structure of apical ESs, in part, by a mechanism involving the dissolution of laminin/integrin complexes via the activation of MMP2.

The proteolysis of laminin-333 at the apical ES has been reported to further influence the BTB permeability by reducing the level of occludin in the rat testis [22, 34]. In fact, the changes in the restructuring of apical ESs and the maintenance of the BTB reflect a coordinated regulation among junctional complexes that is crucial to normal spermatogenesis [22, 38]. Our current results indicate that germ cell depletion is the consequence of MEHP-triggered remodeling of both the tight

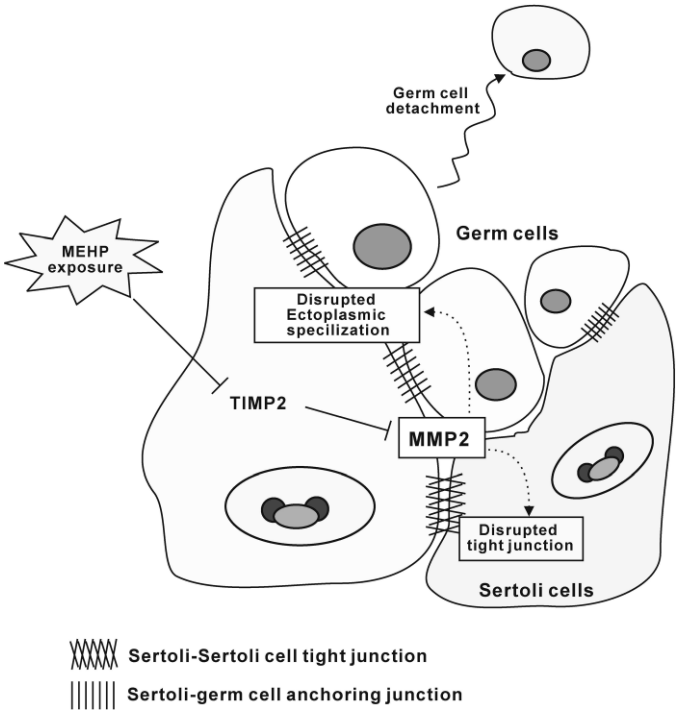


FIG. 9. A working model of MEHP-induced MMP2 activation and its influence on junctional complexes in the seminiferous epithelium. First, MEHP exposure causes a decrease in Sertoli cell TIMP2 expression, which allows for MMP2 activation in the seminiferous tubules. Second, activated MMP2 leads to the remodeling of tight junctions between adjacent Sertoli cells by multiple mechanisms. Third, activated MMP2 directly cleaves laminin-γ3 in order to break laminin/integrin complex at the ectoplasmic specialization between Sertoli cells and spermatids. Disruption of ectoplasmic specialization as well as remodeling of tight junctions allows for the premature detachment of germ cells in the seminiferous epithelium in response to MEHP exposure.

junction and the apical ES. Interestingly, occludin and claudin11 expression are increased in SB-3CT-pretreated groups (Fig. 5), and immunohistochemistry displays their continuous distribution along the BTB in contrast to MEHP-treated groups (Fig. 6). These observations suggest that the protection from MEHP-induced disruption of tight junctions is achieved by suppressing the activity of MMP2. We have previously shown that MEHP exposure results in the activation of MMP2, which can further enhance the cleavage of TNF to yield its active soluble form [8]. Therefore, the activation of MMP2 following MEHP exposure may either enhance TNF-mediated disruption of tight junctions or act directly to impair junctional proteins, especially apical ESs. The detachment of premature spermatocytes and round spermatids found in the lumen may be due to the breakdown of the BTB secondary to the disruption of apical ESs.

Our previous published findings demonstrate that a low-dose of SB-3CT protects germ cells from MEHP-initiated apoptosis [8], while the present study indicates that higher-doses of SB-3CT prevents germ cells from MEHP-induced detachment, maintaining the structure of seminiferous tubules. It appears that MEHP-induced MMP2 activation, that is, the alteration of the TIMP/MMP ratio, is a critical determinant in maintaining germ cell homeostasis through two different mechanisms: germ cell apoptosis and germ cell detachment. Testicular histology reveals that detached germ cells found within the lumen are not TUNEL-positive cells (Fig. 8A), suggesting that those detached germ cells are either non-apoptotic cells or early apoptotic cells with TUNEL-negative signals. Interestingly, our results show that the addition of the general caspase inhibitor, z-VAD-FMK, has no effect on reducing the number of detached germ cells from Sertoli cells in the presence of MEHP in vitro (Fig. 8B). This suggests that MEHP-induced germ cell sloughing is independent of MEHP-induced germ cell apoptosis and may be an initial response to MEHP-inhibited Sertoli cell supports. Similar results reveal that testosterone withdrawal-induced germ cell detachment appears to be independent of germ cell apoptosis in adult rats [39].

The observations of this report provide unique insights into the molecular mechanism by which MEHP-induced Sertoli cell injury leads to germ cell detachment (Fig. 9). MEHP exposure causes a decrease in Sertoli cell TIMP2 expression, which allows for MMP2 activation. Activated MMP2 may alter the microenvironment in the adluminal compartment by increasing the processing of TNF and further lead to the remodeling of tight junctions at the BTB between adjacent Sertoli cells. Finally, activated MMP2 may directly break laminin/integrin complexes at apical ESs between Sertoli cells and spermatids and further contribute to the release of these cells into the lumen. Taken together, the findings reported here indicate that both the disruption of apical ESs as well as the remodeling of the BTB combine to account for the mechanism of the observed detachment and premature depletion of germ cells in the seminiferous epithelium in response to MEHP exposure.

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