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Perfluorooctanoic acid disrupts the blood–testis barrier and activates the TNFα/p38 MAPK signaling pathway in vivo and in vitro

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Abstract Perfluorooctanoic acid (PFOA) is correlated with male reproductive dysfunction in animals and humans, but the underlying mechanisms for this remain unknown. To explore the potential reproductive toxicity of PFOA, we studied blood–testis barrier (BTB) damage using in vivo and in vitro models. Male mice were gavage-administered PFOA (0–20 mg/kg/d) for 28 consecutive days, and breeding capacity and permeability of the Sertoli cell-based BTB were estimated. Primary Sertoli cells (SCs) were exposed to PFOA (0–500 μM) for 48 h, and transepithelial electrical resistance (TER) was assessed. Furthermore, BTB-associated protein expression, TNFα content, and phosphorylation and protein levels of the mitogen-activated protein kinase (MAPK) pathway were detected. An apparent decrease in mated and pregnant females per male mouse as well as litter weight was observed. Marked BTB damage was evidenced by increased red biotin fluorescence in the lumen tubular of the testes and the decrease in TER in SCs in vitro. The protein levels of claudin-11, connexin-43, *N*-cadherin, *β*-catenin, and occludin were significantly decreased in the testes and also in the SCs in vitro except for *N*-cadherin and *β*-catenin. TNFα content showed a dose-dependent increase in the testes and a dose- and time-dependent increase in the SCs, with the p-p38/p38 MAPK ratio also increasing in testes and SCs after PFOA exposure. Moreover, PFOA altered expressions of claudin-11, connexin-43, TNFα, and p-p38 MAPK were recovered 48 h after PFOA removal in the SCs. The SCs appeared to be target to PFOA, and the disruption of the BTB may be crucial to PFOA-induced reproductive dysfunction in mice.

Keywords Perfluorooctanoic acid · Male infertility · p38 MAPK inhibitor · JNK · Transforming growth factor β3

Introduction

Perfluorooctanoic acid (PFOA) and other perfluoroalkyl substances (PFASs) are man-made chemicals, which have been produced and used in commercial products and industrial processes for over 60 years (Lindstrom et al. 2011). These chemicals are widely used as surfactants in textile, paints, waxes, polishes, electronics, adhesives, and food packaging due to their unique properties and strong hydrophobic and oleophobic carbon–fluorine bonds (Pico et al. 2011). These substances are highly persistent and ubiquitously found in the environment of global air, water, soil, wildlife pollutants, and even found in remote polar areas (Castiglioni et al. 2014). Unlike most other persistent and bioaccumulative organic toxicants, PFOA is water-soluble and does not bind well to soil, allowing for easy transportation through and contamination of human drinking water. PFOA has an exceedingly long half-life in humans and male rodents, posing harmful effects due to accumulation in organs (Hundley et al. 2006). Consistent median PFOA serum levels of 2–8 ng/ml have been found in various industrial countries around the world (Vestergren and

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Cousins 2009). Serum levels in communities with high environmental PFOA levels are much higher, with median levels of 28.2 ng/ml reported from 70,000 individuals in Ohio and West Virginia compared to about 4 ng/ml in the general population due to contaminated drinking water (Steenland et al. 2009). The maximum serum level (114,100 ng/ml) reported thus far was in a worker exposed to air with high concentrations of PFOA (Olsen et al. 2000).

Exposure to PFOA can cause tumor and nontumor effects on the immune and nervous systems and adversely affect hepatic function, reproduction, and development (Post et al. 2012; Shi et al. 2013; Yan et al. 2014). Research has demonstrated that maternal PFOA concentrations are significantly inversely associated with birth weight, birth length, and abdominal circumference of their offspring (Fei et al. 2007, 2008), with a negative relationship also found between testosterone level and serum perfluorooctane sulfonate (PFOS) in 247 healthy men (Joensen et al. 2014). Epidemiological studies have further attempted to characterize human PFOA exposure and determine potential correlations between internal PFOA levels and reproductive and developmental outcomes in adults and children (White et al. 2011). Little is known, however, about the mechanisms of these correlations and the modes of PFOA activity on mammal reproduction.

The blood–testis barrier (BTB) is one of the tightest blood–tissue barriers and physically divides the seminiferous epithelium into basal and apical compartments, which is crucial to male fertility and where different stages of germinal cell development occur (Saunders 2003). Sertoli cells (SCs) nurse steps of developing germinal cells and form the BTB between opposing SCs and adjacent Sertoligerm cells (Liu et al. 2012; Gunzel and Yu 2013; Runkle and Mu 2013). The BTB prevents exogenous substrates from entering apical compartments and creates an immunological barrier that sequesters post-mitotic antigens (Steinberger and Klinefelter 1993; Siu et al. 2009). The BTB periodically reconstructs to facilitate spermiogenesis and spermiation during the epithelial cycle, which is promoted by cytokines and chemicals (He et al. 2009; Alves et al. 2013). Among these cytokines, tumor necrosis factor α (TNFα) and transforming growth factor β3 (TGFβ3) act as signal factors to modulate junction restructuring and regulate diverse cellular processes pertinent to spermatogenesis by binding to their receptors, which are mostly confined to the SCs in testes (Xia et al. 2005, 2009; Li et al. 2006). Mitogen-activated protein kinase (MAPK) signals are reportedly downstream of TNFα and TGFβ3 and are activated in the process of the BTB reconstruction (Lie et al. 2013). Studies on PFOA reproductive toxicity have mainly focused on disrupting the Leydig cell function of sterol hormone synthesis; however, the effect of PFOA on BTB integrity remains unknown. In this study, BABL/c mice and primary SCs were used as a toxicological model for PFOA exposure. The BTB integrity and associated protein expressions were assessed. In addition, TNFα, TGFβ3, and downstream MAPK signaling pathways were also explored.

Materials and methods

Mice and PFOA treatment

BABL/c male mice (6 to 8 weeks old, 20–27 g in weight) were housed in a temperature (23 ± 1 °C)- and humidity (60 ± 5 %)-controlled room at a constant 12-/12-h light/dark cycle. All procedures were performed in accordance with the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. After 1 week of adaptation, 80 mice were randomly divided into four groups of equal size and dosed by oral gavage with either vehicle (Milli-Q water) or 1.25, 5, or 20 mg/kg/d PFOA (dissolved in Milli-Q water) for 28 consecutive days. After treatment, all mice and testes were sampled for analysis. The right testes of three mice were fixed in 4 % paraformaldehyde for immunofluorescence microscopy, while the remaining testes were immediately frozen in liquid nitrogen and stored at −80 °C for later analysis.

Breeding experiment

To obtain credible data, we reduced the experiment to the control group and the 5 mg/kg/d group for the breeding experiment. Male mice (6 to 8 weeks old, BABL/c; 15 for each group) mated with virgin females (8 to 10 weeks old, ICR mice) at the rate of 1:3 (45 female mice for each group, 90 female mice in total) after 28-day treatment. Male and female mice cohabitated at night and were separated during the day. Successful mating was indicated by finding a vaginal plug in the morning, with pregnant mice caged alone till parturition. Vaginal plug examine was conducted for 5 days, female mice without a vaginal plug were considered to have no mating behavior. Plugged females and pregnant females per male mice were counted, and average litter size and birth litter weight were later calculated.

In vivo BTB integrity assay

The integrity of the BTB assay was performed using a biotin tracer, as described previously (Meng et al. 2005). In short, after 28-day treatment, three mice from each group were anesthetized and their testes exposed. The interstitium testes were injected with 50 μl EZ-Link Sulfo-NHS-LC-Biotin (10 mg/ml freshly dissolved in physiological saline containing 1 mM $CaCl_2$, Pierce-Invitrogen Carlsbad, CA). After 30 min, the animals was euthanized, and their testes were embedded in Tissue-Tek OCT (Sakura Finetek, Japan) in preparation for

Table 1 Reproductive data of PFOA-treated males mate with ICR females

Male		Female number	Mated females (per male)	Pregnant females (per male)	Average litter size (pups/litters)	Average litter weight (g)
Group	Number					
Control	15	45	2.67 ± 0.16	1.80 ± 0.20	11.89 ± 0.54	19.95 ± 0.80
PFOA	15	45	1.47 ± 0.27**	0.7 ± 0.28**	10.27 ± 0.72	16.17 ± 1.63$

Males (6 to 8 weeks old, BABL/c) orally administered vehicle or 5 mg/kg/day PFOA for 28 days were mated with three virgin females (8 to 10 weeks old, ICR) at night and housed separately during the day. Successful mating was indicated by a vaginal plug in 5 days. Results are presented as mean ± SE

** $P < 0.01$, Mann–Whitney U test of plugged and pregnant females

$ $P < 0.05$, Student's t test of litter weight

cryosection. The sections (10 μm) were fixed in 4 % paraformaldehyde (PFA) for 20 min, washed with phosphate-buffered saline with 0.1 % Tween 20 (PBST), blocked in 0.01 M phosphate buffer solution (PBS) containing 15 % goat serum and 1 % bovine serum albumin (BSA, wt/vol), and incubated with Alexa Fluor® 568-conjugated streptavidin (Life-Invitrogen, Carlsbad, CA) for 2 h at room temperature. After mounting with mounting medium (Sigma-Aldrich, St Louis, MO), the sections were analyzed by fluorescence microscopy.

Sertoli cell isolation and treatment

With slight modifications made in the protocol, primary SCs were isolated from 14-day or 6- to 8-week-old BABL/c male mice that have not undergone any treatment according to the methods from previous studies (Feng et al. 2010), and the isolated SCs from 14-day-old male mice were used to measure transepithelial electrical resistance (TER). Mice were euthanized by CO_2 asphyxiation, and testes were immediately removed and placed in ice-cold PBS (pH 7.4) containing 200 IU/ml of penicillin and 200 g/ml of streptomycin (Invitrogen, Carlsbad, CA) and washed three times. Five testes were decapsulated once, and the seminiferous tubules were squeezed into 5 ml 0.1 % collagenase IV (17104-019, Gibco-Invitrogen, Carlsbad, CA) in 2.5-mm culture dishes (BD Biosciences, San Diego, CA). After 15-min incubation at 37 °C with occasional gentle shaking, loose tubules were washed three times with 0.15 M PBS, further digested with 0.1 % collagenase IV and 0.1 % hyaluronidase type I–S (H3506, Sigma-Aldrich, St Louis, MO) at 37 °C for 20 min, followed by continued digestion with 0.1 % DNase I (D5024, Sigma-Aldrich, St Louis, MO) and 0.25 % trypsin (Invitrogen, Carlsbad, CA) for 30 min at 37 °C with gentle shaking. Digestion was terminated by fetal bovine serum (FBS, Gibco, Grand Island, NY) and washing with PBS three times. The SCs were incubated in a CO_2 incubator at 35 °C in a humidified atmosphere with 95 % air/5 % CO_2, with the medium replaced daily. After 48 h, SC cultures were subjected to hypotonic solution (20 mM Tris, pH 7.4) for 3 min to remove germ cells. On day 3, SCs were exposed to PFOA.

Isolated SCs were seeded at 1.8×10^6 cells/cm^2 on Matrigel-coated plates (BD Biosciences, San Diego, CA) in serum-free Ham's F12 nutrient mixture and Dulbecco modified eagle medium (DMEM/F12) with 10 % FBS (Gibco-Invitrogen, Carlsbad, CA) on day 0 and exposed to 0, 300, 400, and 500 μM PFOA for 48 h from day 3. To perform cell viability assay, SCs were exposed to 0 to 700 μM PFOA for 48 h. To perform dynamic expression of cytokines analysis, SCs were subject to 0 (control) or 400 μM PFOA and terminated at 0, 3, 6, 12, 24, and 48 h after exposure. To study the recovery of cytokine expression, 0- and 400-μM PFOA-treated cells were washed and replenished with fresh DMEM/F12 medium. These cell cultures were terminated at 0, 3, 6, 12, 24, and 48 h.

In vitro BTB integrity assay

In vitro BTB integrity was assessed by measuring transepithelial electrical resistance with a Millicell ERS system (Millipore Corp., Bedford, MA) as described previously (Mruk and Cheng 2011). Briefly, SCs were isolated from 14-day-old BABL/c mice and seeded on Matrigel-coated Millicell bicameral units (Millipore Corp., Bedford, MA) at a density of 1×10^6 cells/cm^2. The SCs were maintained as a compact monolayer to mimic the BTB in vitro. PFOA (0, 300, 400, and 500 μM) was added to cells on day 4. After 24 h, the TER was detected in each unit at four different areas (12-, 3-, 6-, and 9-o'clock positions), which were averaged into a single value and presented as "R." Three wells in each group were detected. The blank control was conducted in the unit without SCs. The true TER value of each sample was calculated as: $\mathrm{TER}_{\mathrm{sample}}$ (Ω cm^2) = ($R_{\mathrm{sample}} - R_{\mathrm{blank}}$) (Ω) × Effective Membrane Area (cm^2).

Cytokines TNFα and TGFβ3 levels

Testes homogenates in 0.01 M PBS (control, 1.25, 5, and 20 mg/kg/day groups) were used to detect TNFα and TGFβ3. In vitro, both SCs protein lysates and spent media were subjected to cytokines detection. Enzyme-linked

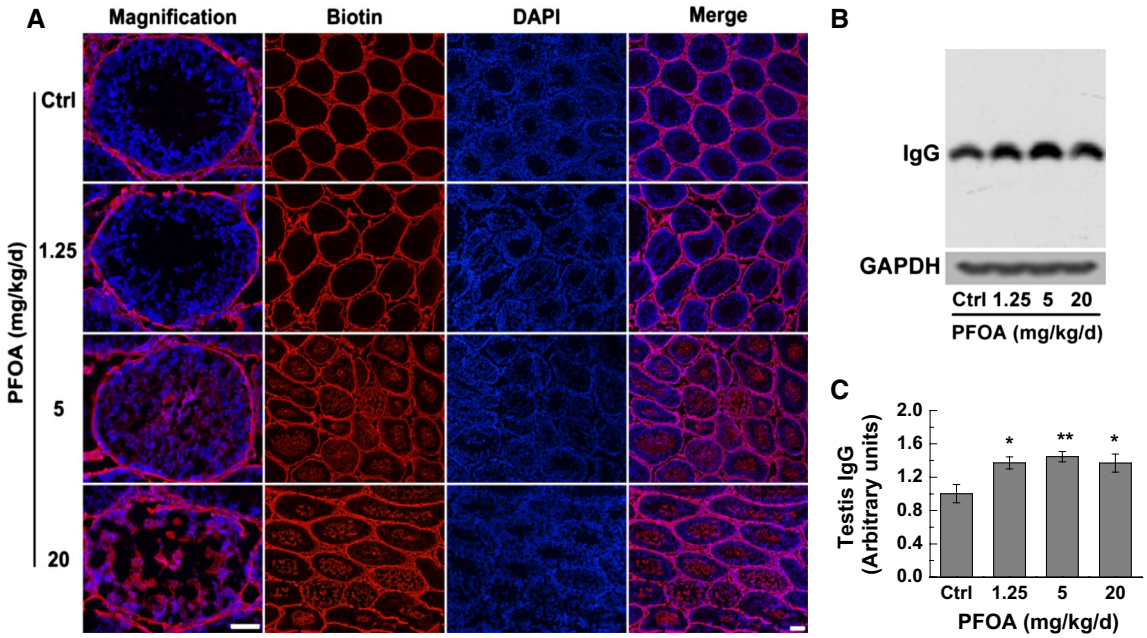

Fig. 1 PFOA disrupts BTB integrity in adult mice. **a** In vivo BTB integrity assay. Testes were injected with 50 μl EZ-Link Sulfo-NHS-LC-Biotin (*red*) in the interstitium testes, and cell nuclei were dyed by 4′-6-diamidino-2-phenylindole (DPAI, *blue*). **b** Western blot analysis of IgG level in testes. Testis extracts were prepared and labeled by anti-mouse IgG-HPR, and IgG was visualized by chemiluminescence. **c** Band densities of Western blot result in (**b**). GAPDH served as a loading control. Results are presented as mean ± SE ($n = 3$), $^{*}P < 0.05$; $^{**}P < 0.01$ (color figure online)

immunosorbent assay (ELISA) kits for tissue TNFα and TGFβ3 were purchased from Lichen Commercial and Trade Co., Ltd. (Shanghai, China). The minimum detectable concentration was less than 1.0 pg/ml, and the coefficient of variation between plates was less than 15 %. The procedures were performed according to the kit instructions. All data of both testes and SC lysates were normalized to total protein concentrations in corresponding lysates, and data of spent media were normalized to corresponding SC lysates protein concentration, which were detected using BCA protein assay kits (Beyotime Biotechnology, Zhejiang, China).

Western blot analysis

Preparation of the protein extracts from the testes and primary SCs, and Western blot analysis were performed as described previously (Feng et al. 2009, 2010). Phosphorylation protein extracts were prepared by RIPA buffer and supplemented with protease inhibitor (PMSF) and protein phosphatase inhibitors (Applygen Technologies, Beijing, China). *N*-cadherin, *β*-catenin, occludin, claudin-11, connexin-43, p-p38 (T180/Y182), p38, p-JNK (T183/Y185), JNK, p-ERK (T202/Y204), and ERK primary antibodies were used. All antibodies used were obtained commercially, and their sources and dilutions used for different experiments are listed in SI Table 1. The protein bands were visualized by enhanced chemiluminescence (superECL, Tigen, Beijing, China) on X-ray films and analyzed with Quantity One software (version 4.6.3, Bio-Rad). Data were normalized to protein expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the control data were set as 1. Results were presented as mean ± SE for each group of at least three individual samples.

Immunofluorescence microscopy

Deparaffinized testis sections (5 μm) and harvested SCs, which were fixed in 4 % paraformaldehyde and penetrated in 0.5 % Triton-100, were incubated in a blocking solution (0.01 M PBS containing 10 % normal goat serum and 5 % BSA) for 30 min; then incubated in occludin, claudin-11, and connexin-43 or WT1 antibodies at 4 °C overnight in a moist chamber; and finally washed three times with 0.01 M PBS. The slides were incubated in goat anti-rabbit IgG Alexa Fluor 488 or 594 (ZSGB-BIO, Beijing, China) at 37 °C for 1 h, washed three times with 0.01 M PBS, and mounted with Vectashield Mounting medium with 4′,6′-diamidino-2-phenylindole (DAPI, ZSGB-BIO, Beijing, China). Fluorescence images were captured using a Nikon DS-Ris digital camera interface to Nikon Eclipse 90i Fluorescence Microscope at 12.5 Megapixel (Mpx) with Nikon NIS Elements Advanced Research Imaging software (version 3.2) (Nikon Instruments Inc., Japan). Images were

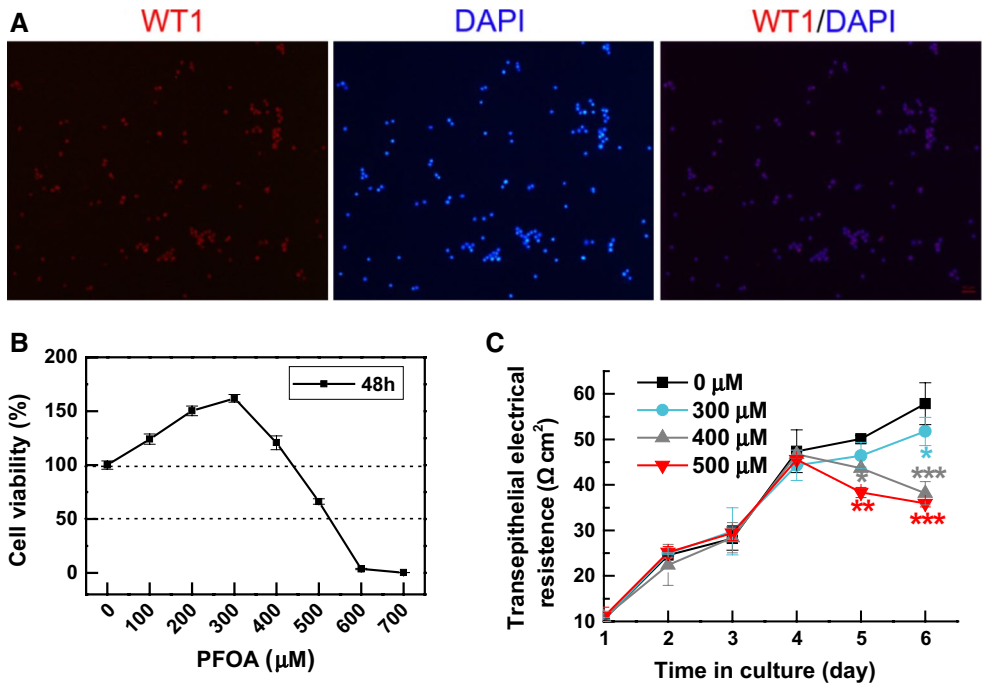

Fig. 2 PFOA disrupts SCs junction barrier. **a** Immunofluorescence staining of WT1 in SCs. Harvested SCs were fixed to coverslips by 4 % paraformaldehyde, penetrated, and stained by WT1 antibody. WT1 (*red*)-positive cells are SCs, and cells stained blue fluorescence were counted to total cell number. **b** Cytotoxicity of PFOA on primary SCs. Primary SCs at 1.8 × 10^6 cells/cm^2 cultured for 3 days on Matrigel-coated plates were treated without or with PFOA (100, 200, 300, 400, 500, 600, and 700 μM) for 48 h. MTT assay was used, and absorbance at 490 nm was recorded to measure cell viability of PFOA-treated SCs. **c** Transepithelial electrical resistance (TER) in SCs. Integrity of SCs junction was measured by TER. Primary SCs derived from 14-day-old BABL/c mice were cultured at 1 × 10^6 cells/cm^2. PFOA (0, 300, 400, and 500 μM) was added to the cells at day 4. The TER values were measured following the Millicell ERS system instructions ($n = 3$). The true TER value of each sample was calculated as: TER $_{sample}$ (Ω cm^2) = ($R_{sample} - R_{blank}$) (Ω) × Effective Membrane Area (cm^2). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (color figure online)

exported to TIFF format and analyzed in Photoshop using Adobe Creative Suite (version CS6).

Statistical analysis

Comparisons between groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference test, or using the independent samples *t* test using SPSS software (version 18, SPSS, Inc., Chicago, IL). The plugged females and pregnant females between control and treatment were compared using the Mann–Whitney *U* nonparametric test (SPSS, version 18 software). All data are presented as mean ± SE. Probability levels of $P < 0.05$ were considered significant.

Results

PFOA impaired male mice fertility

Male fertility was tested through mating virgin females with similar weight (23–31 g) after 28 days of either no treatment or PFOA (5 mg/kg/day) treatment. Our previous study has shown that no significant testis weight loss was found in 5 mg/kg/day PFOA mice (Zhang et al. 2014). Herein, 28 days of consecutive gavage of 5 mg/kg/day PFOA led to a decrease in the number of mated and pregnant females per male mouse compared to that in the control group. Although significant changes in average litter size were not observed, litter weight was shown to be significantly reduced after PFOA exposure (Table 1).

PFOA disrupted the BTB and caused immune privilege in mouse testes

The integrated BTB structure is related to male fertility. We explored the effect of PFOA on the BTB integrity. In normal testes, junctions between the Sertoli–Sertoli and Sertoli–spermatid interfaces formed tight barriers that prevent large molecules from passing through (control, Fig. 1a). However, the BTB barrier was opened after 28-day treatment with 1.25 and 5 mg/kg/d PFOA. Red fluorescent dye injected in the interstitium testes diffused dose-dependently

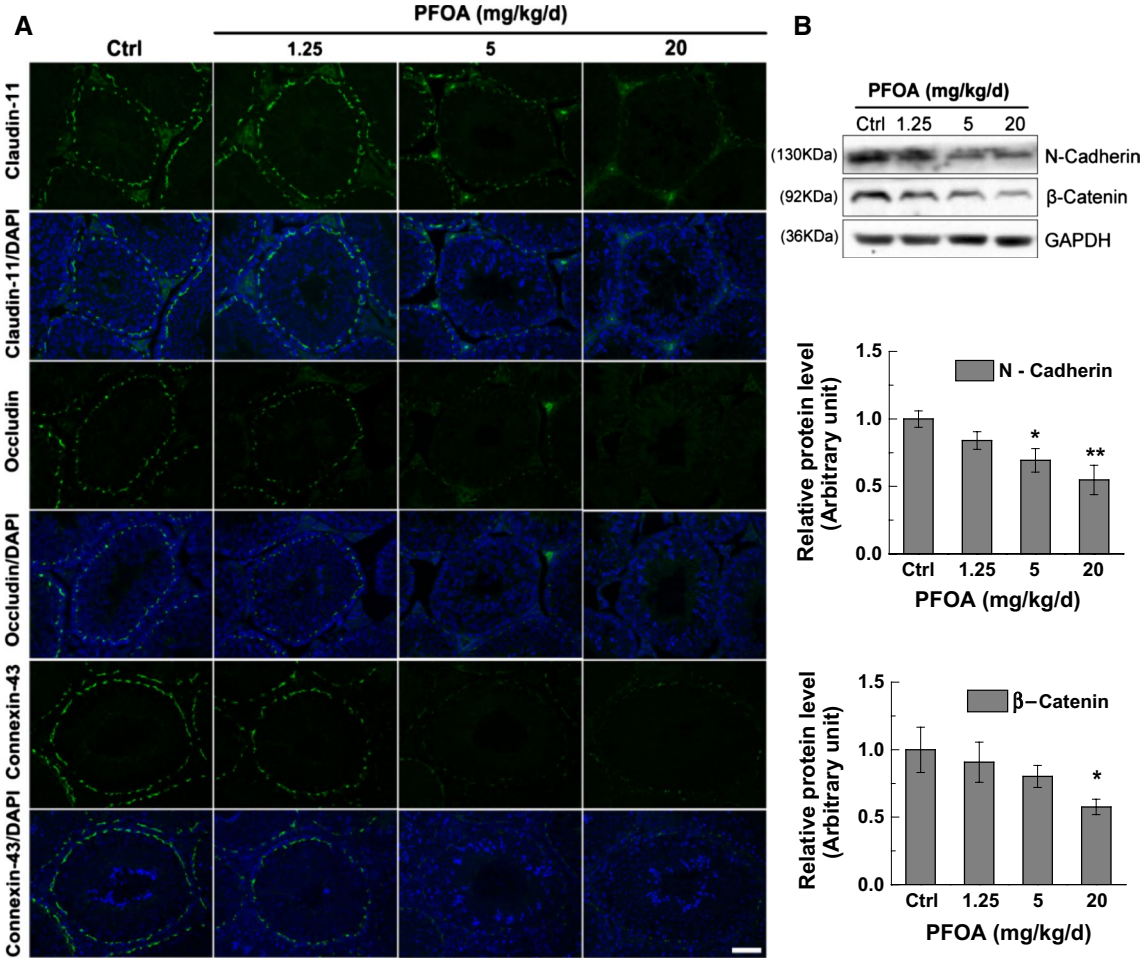

Fig. 3 Changes in BTB-associated proteins induced by PFOA in testes. **a** Immunofluorescence analysis of BTB-associated proteins. Fluorescent micrographs using cross sections of deparaffinized testes from mice received vehicle control and doses of PFOA (0, 1.25, 5, and 20 mg/kg/day) for 28 day. *Green* fluorescence represents claudin-11, occludin, and connexin-43, and DAPI stained for nuclei. **b** Western blot analysis of *N*-cadherin and *β*-catenin in mouse testes. Testis lysates (20 μg/well) were loaded onto gel and reacted with primary and later secondary antibodies; GAPDH was set as loading control. The *down panel* is a densitometrically scanned histogram of protein bands, and the control was arbitrarily set as 1 with $n = 3$, $^{*}P < 0.05$; $^{**}P < 0.01$ (color figure online)

in the BTB and appeared in the adluminal compartment (Fig. 1a). Additionally, PFOA induced damage of the BTB, which helps maintain tolerance to germ cell antigens, and this may have facilitated immune privilege, as evidenced by the higher IgG levels of testicular protein in the PFOA-treated mice (Fig. 1b).

PFOA disrupted the SCs junction barrier in vitro

To further study the effects of PFOA on the BTB, isolated primary SCs were treated with PFOA. The purity of the SCs was more than 98 % and was identified using WT1 staining. These cell cultures were considered appropriate for use in the following experiments (Fig. 2a). Cell viability was assessed following 48 h of PFOA treatment, and the IC_{50} was over 500 μM for the SCs (Fig. 2b). 400-μM PFOA exposure resulted in a disruption of the SCs junction barrier, as manifested by decreased TER without detective decrease cell viability. Below the IC_{50}, the TER decrease in 500-μM PFOA groups may have been due to the damaging effect of PFOA on the SCs junction barrier or on cell viability (Fig. 2c).

PFOA perturbed BTB protein expression in vivo and in vitro

The integrity of the BTB is based on various junction proteins that form coexisting tight junctions (TJs), ectoplasmic specializations (basal ESs), and gap junctions (GJs). After 28-day PFOA gavage, the following proteins were down-regulated: (1) TJ integral membrane proteins: occludin and claudin-11 (Fig. 3a); (2) basal ES proteins: *N*-cadherin and

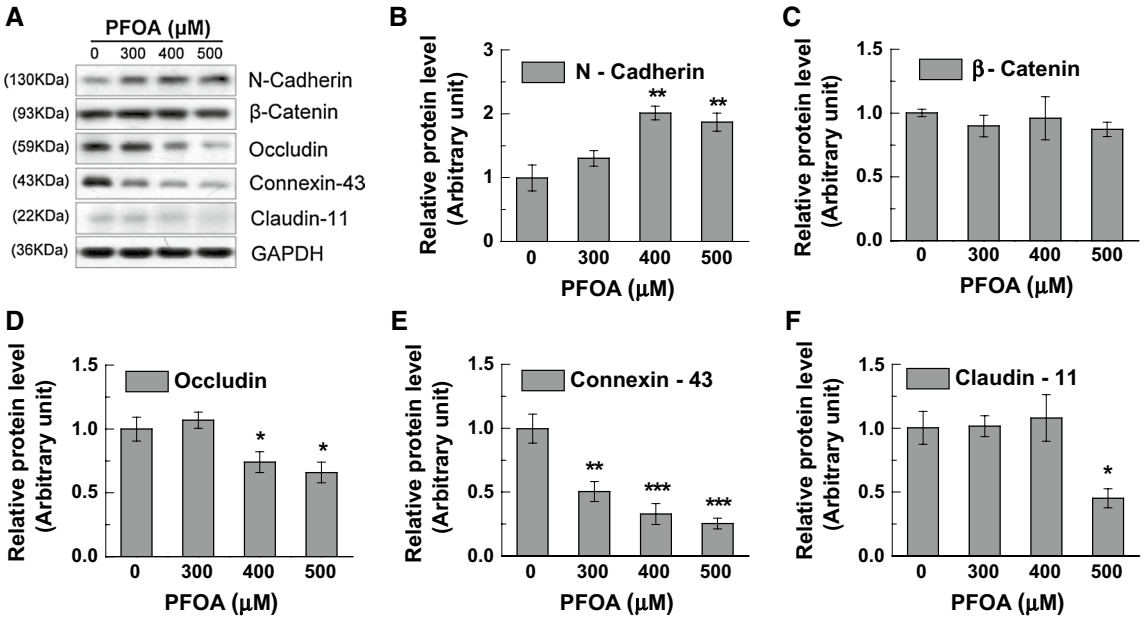

Fig. 4 Changes in BTB-associated proteins induced by PFOA in SCs. **a** Western blot analysis of BTB-associated proteins from PFOA-treated SCs. Primary SCs were cultured at 0.5 × 10^6 cells/cm^2 for 3 days on Matrigel-coated plates, followed by 48-h PFOA treatment. Therefore, cells were harvested using Western blot analysis. **b–f** Band densities of Western blot result in (**a**). GAPDH was set as the loading control, with the control (0 μM PFOA) arbitrarily set at 1 with $n = 3$, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

β-catenin (only in the 20 mg/kg/day group, Fig. 3b); and (3) GJ integral membrane proteins: connexin-43 (Fig. 3a). Taken together, PFOA was found to suppress BTB protein expression in the testes.

Similar to the in vivo study, connexin-43 expression was significantly down-regulated by PFOA in a dose-dependent manner. The expression levels of other junction proteins also decreased, including occludin and claudin-11. Furthermore, the level of *β*-catenin did not change, and *N*-cadherin levels in the SCs increased after PFOA treatment (Fig. 4a–f).

PFOA induced high TNFα content in vivo and in vitro

TNFα content in adult mouse testes was increased after 1.25- and 5-mg/kg/d PFOA treatment for 28 days. These effects were in a dose-dependent manner (Fig. 5a). However, testicular TGFβ3 expression was not affected (Fig. S1).

In primary SCs, the TNFα levels were elevated in a dose-dependent manner both in SCs and in spent media when cells were treated with PFOA for 48 h and were significant in the 300- and 400-μM PFOA groups (Fig. 5b). Moreover, TNFα also exhibited a time-dependent increase in the SCs exposed to 400 μM PFOA and started to increase at the 12-h point after PFOA exposure (Fig. 5c). Similar dose-dependent trends were observed for TGFβ3; however, these elevations appeared to decline after 12 h of exposure in the 400-μM PFOA group (Fig. S2).

PFOA activated p38 MAPK signaling in vivo and in vitro

The downstream MAPK signaling pathways of TNFα and TGFβ3 were assessed by Western blot analysis. The p-p38 MAPK/p38 MAPK ratio showed a dose-dependent increase in the testes after PFOA administration and was significant in the 20-mg/kg/d PFOA group compared to that of the control. The p-p38 MAPK content was high in 1.25- and 5-mg/kg/day groups, though p38 MAPK, p-JNK, JNK, p-ERK, and ERK did not significantly change (Fig. 6a–c).

Similarly, two pathways (p-p38/p38 MAPK and p-JNK/JNK) were involved in the 500-μM PFOA-induced junction damage in SCs, though only p-p38/p38 MAPK in the 400-μM PFOA-treated cells. It is worth noting that the p38 MAPK signaling pathway was sharply stimulated as manifested by the fourfold increase in p-p38 MAPK and twofold reduction in p38 in the 400-μM and/or 500-μM PFOA-treated SCs versus the control cells. The expression of p-ERK and ERK did not significantly change (Fig. 7a–d).

We further treated SCs with PFOA (500 μM) and a p38 MAPK inhibitor (SB203580, 10 μM). After treating SCs with 500 μM PFOA for 48 h, the TNFα level in SCs increased compared to that in the control cells, but not in the spent media. With both 500-μM PFOA and 10-μM SB203580 treatment, the TNFα level was lower than that in 500-μM PFOA-treated SCs. These results indicate that the elevation of TNFα induced by PFOA exposure can

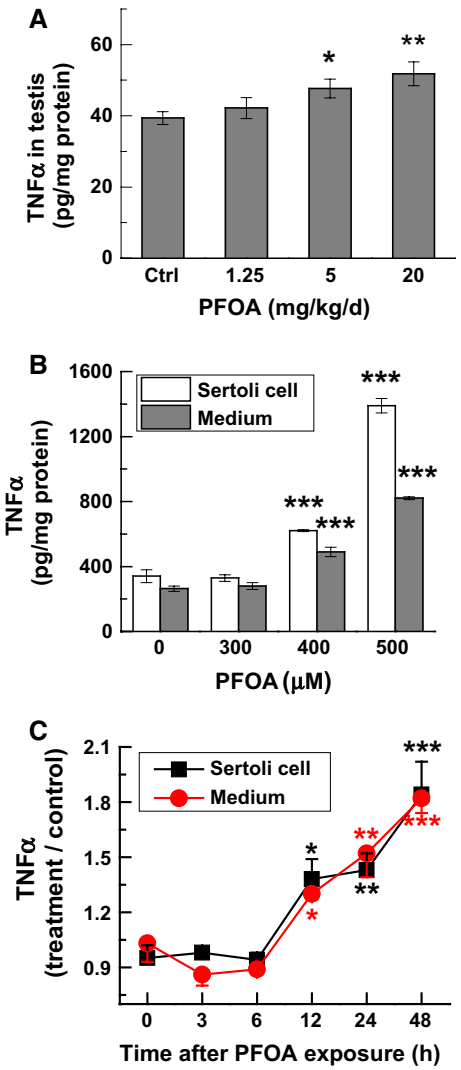

Fig. 5 Changes in TNFα levels in PFOA-treated mouse testes and SCs. **a** TNFα content in mouse testes. Testicular TNFα content was detected in testis lysates from mice that received vehicle control and doses of PFOA (1.25, 5, and 20 mg/kg/day) for 28 consecutive days. **b** TNFα content after exposure to PFOA at various concentrations. SCs were exposed to PFOA (0, 300, 400, and 500 μM) for 48 h. **c** TNFα content after exposure to PFOA at various time points. SCs cultured in plates for 2 days were exposed to 400 μM PFOA, which was set as 0 h, and terminated at various end points (0, 3, 6, 12, 24, and 48 h). Testis lysates, cell lysates, and spent media were collected for ELISA. The ratio between the 400-μM PFOA group and the control group was used in the diagram (**c**). All data of both testes and SCs lysates were normalized to total protein concentrations in corresponding lysates, and data of spent media were normalized to corresponding SCs lysates protein concentration, which were detected using BCA protein assay kits. The final results were shown as pg/mg protein or ratio of treated group versus the control group ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$, and $^{***}P < 0.001$

be partly blocked by the p38 MAPK inhibitor (Fig. 8), whereas the level of TGFβ3 in both SCs and spent media did not change (Fig. S3).

Recovery of BTB proteins, TNFα and MAPK signaling molecules after PFOA removal

To validate the disruptive effect of PFOA on the BTB, we studied the BTB protein expression and TER in SCs after PFOA removal. Forty-eight hours after PFOA removal, the contents of various claudin-11, connexin-43, and *N*-cadherin were recovered, which were found to decrease or increase in cells treated with 400 μM PFOA for 48 h (Fig. 9a, b). The junction barriers of the disrupted cells were reassembled 24 h after PFOA removal, as evidenced by the recovery in TER in 400- and 500-μM PFOA groups (Fig. 9c).

TNFα, MAPK signaling molecules, and TGFβ3 levels were detected in the same SC samples. TNFα content and its downstream protein p38 MAPK were recovered, and their cellular levels 48 h after PFOA removal were significantly different compared to those in continued PFOA-treated cells (Fig. 9d, f). There was a decline in the TNFα level in synchronicity with PFOA removal time, while TGFβ3 maintained a high level with only a small decrease in both the SCs and spent media (Fig. S4).

Discussion

The BTB prevents the entry of harmful endogenous substrates and exogenous contaminants, thereby providing a suitable environment for spermatogenesis. It is sensitive to a variety of environmental toxicants, such as PFOS (Zhang et al. 2008; Wan et al. 2014), which has been shown to damage the BTB both in vivo and in vitro (Qiu et al. 2013). After its detection in human cord blood, PFOA has also been proven to be capable of traversing the placental barrier (Apelberg et al. 2007). Our previous study using gavage administration concluded that PFOA accumulation in the mouse testes was dose-dependent, and 1.25- and 5-mg/kg/d PFOA exposures did not cause testis weight loss (Zhang et al. 2014). Here, we chose 1.25, 5, and 20 mg/kg/d PFOA and found 5- and 20-mg/kg/d PFOA exposures disrupted BTB integrity in adult mouse testes after PFOA exposure, and that impermeable biotin passed through the BTB and entered the lumen of tubules, which was also supported by TER in vitro study using a PFOA dose of above IC_{50}. The effects of PFOA are consistent with that of PFOS on rat primary SCs (Wan et al. 2014).

The disruptive effect of PFOA appeared to be mediated initially by changes in the expression of TJ-, GJ-, and basal ES-associated proteins in the SCs. The TJs are the major junction in many types of epithelial and endothelial cells, and they create a boundary that defines cell polarity and also contributes to the immunological barrier of host animals (Tsukita et al. 2001). Herein, claudin-11, a TJ protein, was down-regulated by PFOA in the testes and SCs, and

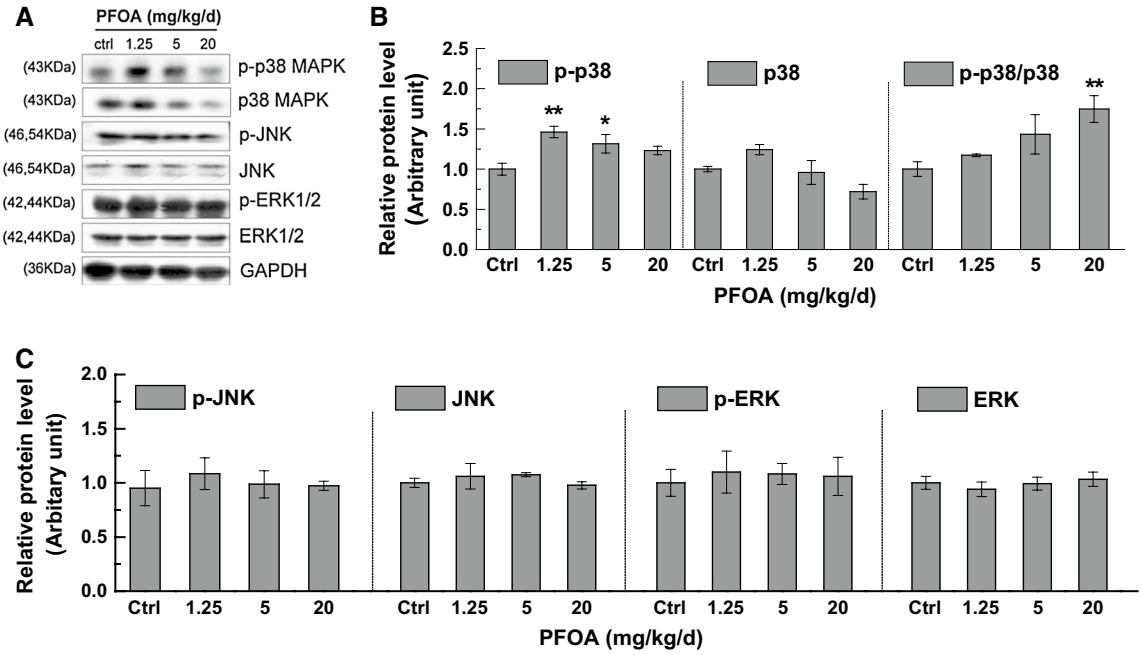

Fig. 6 Changes in MAPK signaling molecule expressions in mouse testes. **a** Western blot analysis of MAPK signaling pathway proteins. Testis lysates (20 μg/well) were loaded onto gel and reacted with primary and later secondary antibodies. **b**, **c** Band densities of Western blot result in (**a**). GAPDH was set as the loading control, with the control arbitrarily set at 1, with $n = 3$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

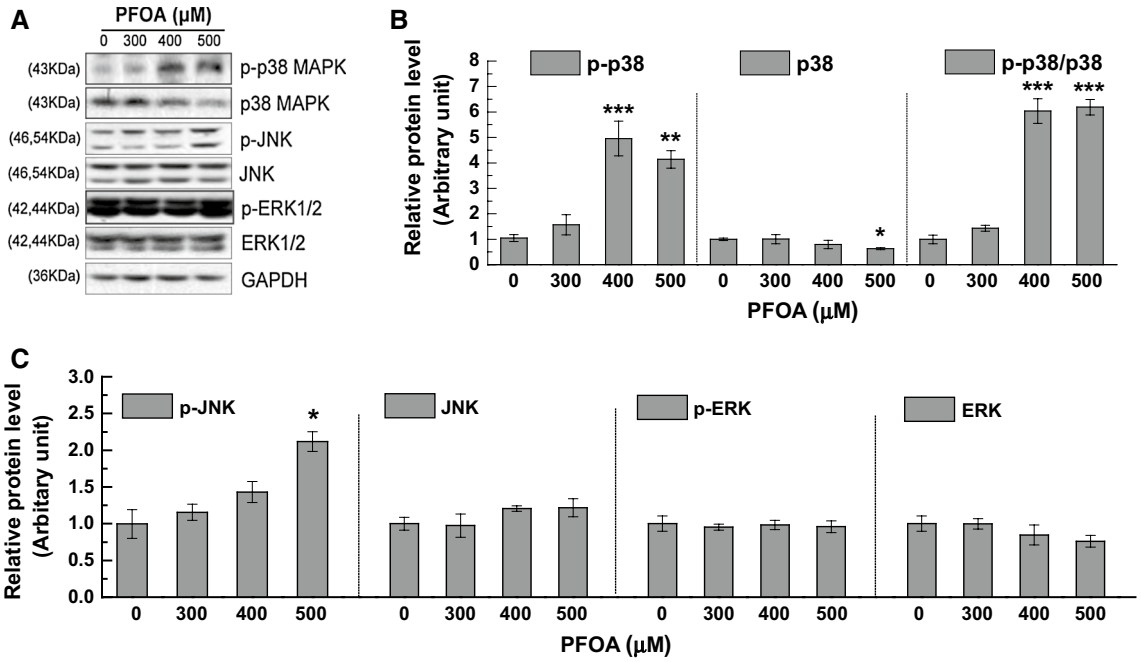

Fig. 7 Changes in MAPK signaling molecule expressions in SCs. **a** Western blot analysis of MAPK signaling pathway proteins. Primary SCs cultured at 1.8×10^6 cells/cm^2 for 3 days on Matrigel-coated plates were treated without or with PFOA (0, 300 400, and 500 μM) for 48 h. **b**, **c** Band densities of Western blot result in (**a**). GAPDH was set as the loading control, with the control arbitrarily set at 1, with $n = 3$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

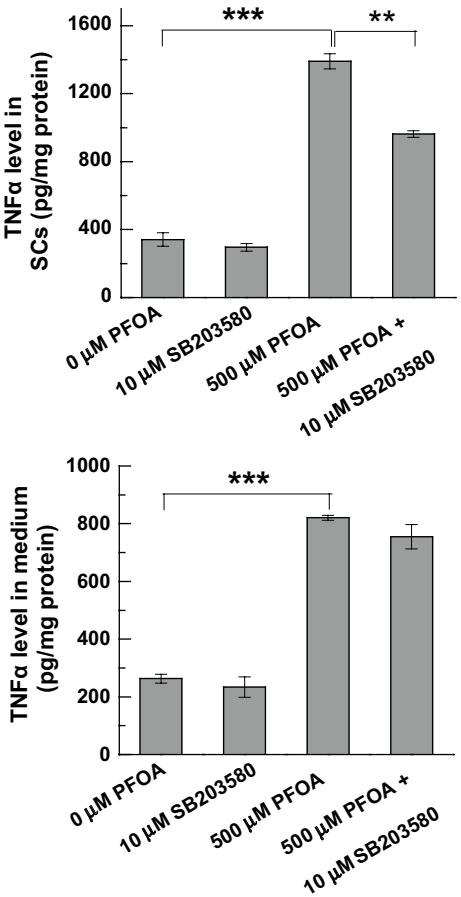

Fig. 8 TNFα levels with p38 MAPK inhibitor SB203580 treatment in SCs. Primary SCs were exposed to 500 μM PFOA and/or 10 μM SB203580 for 48 h. TNFα content was detected by ELISA. TNFα data were normalized to total protein concentrations ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

occludin expression also decreased in the testes. The GJs, including connexin-43, facilitate cross talk between various junction types to maintain BTB homeostasis through the rapid disassembly and reassembly of junctions, which facilitates transport of preleptotene spermatocytes across the barrier at stage VIII of the epithelial cycle (Li et al. 2009). General knockout of connexin-43 in mice can cause perinatal death, and SC-specific knockout of connexin-43 can result in infertility in male mice by preventing the initiation of spermatogenesis (Gunther et al. 2013). Knockdown of connexin-43 by RNA interference in SCs has been shown to impede the ability of the cell epithelium to reseal the TJs disrupted by calcium depletion and bisphenol A treatment (Li et al. 2010). In this study, the expression of connexin-43 was highly suppressed both in vivo and in vitro, indicating that compared to claudin-11, connexin-43 may be more susceptible to PFOA. Moreover, *N*-cadherin and *β*-catenin expression reduction in the testes indicated the disruption of basal ESs after PFOA exposure. However, in PFOA-exposed SCs, *N*-cadherin levels were elevated, while *β*-catenin and occludin levels remained unaltered. These inconsistent in vivo results may be caused by the simplified environment of the SC culture, which differs from the complex animal physiological environment in which BTB protein expression is also regulated by germ cell secretion factors and hormones such as testosterone. Moreover, there are studies reporting that *N*-cadherin increase may facilitate germ cell loss from the epithelium, and similar results have been obtained in experiments using adjudin-treated rats and androgen suppressed rat testes (Lee et al. 2003; Zhang et al. 2005).

TNFα mediates cross talk between Sertoli and germ cells, facilitates germ cell movement across the seminiferous epithelium, and perturbs BTB reconstruction by inducing a loss in the steady-state levels of integral membrane proteins when it is additionally supplied to animals (Lydka et al. 2012; Luo et al. 2013; Xiao et al. 2014). Administration of TNFα to testes at concentrations comparable to testis endogenous levels was shown to reversibly disrupt BTB integrity (Li et al. 2006). As for TGFβ3, it has been suggested to play a crucial role in regulating Sertoli cell TJs reconstruction (Hellani et al. 2000; Mankertz et al. 2000). In the present study, PFOA exposure resulted in elevated TNFα levels but not TGFβ3 levels in mouse testes, which was supported by in vitro studies concluding that PFOA induced both TNFα and TGFβ3 expressions in a dose- and time-dependent manner. This indicates that TNFα and TGFβ3 are probable mediators of PFOA-induced BTB disruption. The different responses of TGFβ3 levels between in vivo and in vitro PFOA exposure experiments may be the result of discrepancy between the microenvironment of the SCs, with the exact reasons requiring further investigation.

In the testes, three MAPK signaling pathways (p38 MAPK, JNK, and ERK) have been implicated in the regulation of junction dynamics and are activated by TNFα or TGFβ (Booth et al. 2000; Shaw et al. 2001; Coyne et al. 2002). Moreover, it has been suggested that MAPKs (p38, JNK, and ERK) are downstream molecules of cytokines and conduct TNFα-induced caspase-mediated apoptosis (Aggarwal 2003; Mocellin et al. 2005; Sabio and Davis 2014). A recent study demonstrated that PFOS exerts it effects on the SCs via p38 MAPK both in vivo and in vitro, and is partly blocked by a p38 MAPK inhibitor (Qiu, et al. 2013). Similar conclusions have been drawn by studies on other toxicants, such as cadmium, which perturbs the BTB dynamic through the p38 MAPK signaling pathway (Lui et al. 2003). In this study, the p-p38 MAPK/p38 MAPK ratio in mouse testes increased after PFOA administration, indicating that PFOA induced p38 MAPK signaling. Furthermore, in vitro study confirmed that p38 MAPK and p-JNK were activated by PFOA treatment in the primary SCs, and p38 MAPK was highly up-regulated after 400-μM PFOA exposure

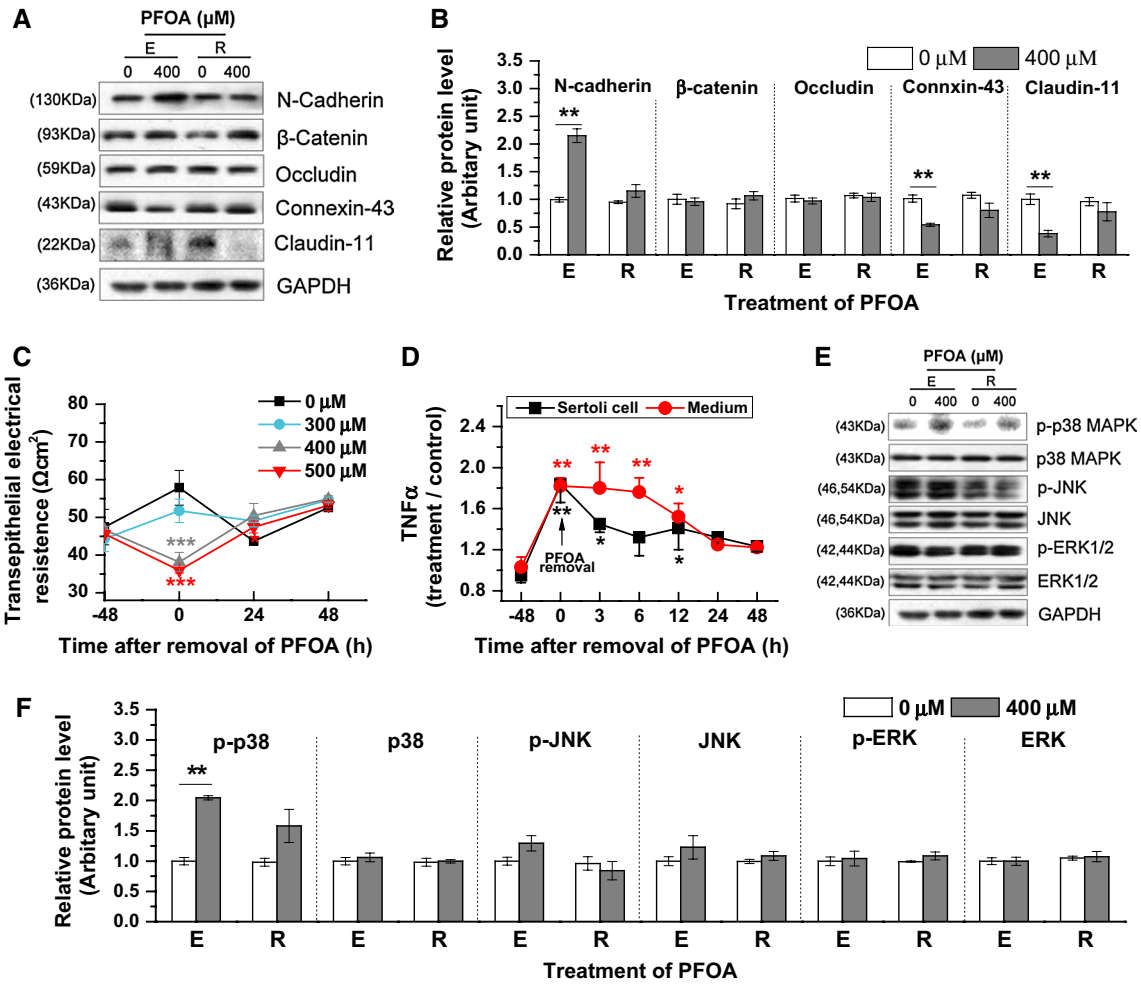

Fig. 9 Recovery of TNFα levels and the BTB-associated protein and MAPK signaling molecule expressions after PFOA removal. **a** Western blot analysis of the BTB-associated protein. **b** Bands densities of Western blot result in (**a**). **c** SCs junction reassembly measured by TER values. SCs were treated with PFOA (0, 300, 400, and 500 μM). After 48-h exposure, PFOA was removed, and TER values were measured in 0, 24, and 48 h. **d** Time-steady levels of TNFα after PFOA removal in SCs and spent media. Cells were cultured and treated with 400 μM for 48 h, followed by removal of PFOA, and terminated at various time points (0, 3, 6, 12, 24, and 48 h). "−48 h" represents the time in which PFOA treatment begins and "0" represents PFOA removal. All data were normalized to protein concentrations, and the ratio between the 400-μM PFOA group and the control group was used in the diagram. **e** Western blot analysis of the MAPK signaling molecules. **f** Band densities of Western blot result in (**e**). GAPDH was set as the loading control, with the control arbitrarily set as 1. *E* represents PFOA exposure for 48 h, and *R* represents replacement of the medium containing PFOA and continuous culture for 48 h. Comparisons were done between the data of each time point and 48 h using Student's *t* test ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

without detectable cell cytotoxicity. PFOA removal restored the expression of junction proteins, p38 MAPK and TNFα, indicating that PFOA-induced BTB damage could be recovered in animals and humans through clearing environmental PFOA. Interestingly, PFOA-induced TNFα elevation was suppressed or partially blocked by the use of a specific MAPK inhibitor (SB203580) in SCs, while the levels of TNFβ3 were not affected in SCs or spent media.

The relationship between PFOA exposure and sperm function is controversial. Some epidemiological studies discovered that PFOA exposure was negatively associated with sperm concentration and total sperm count in men (Vested et al. 2013), while the correlation between PFOA levels and sperm parameters was nonsignificant in other cases (Joensen et al. 2009). A recent mouse model study from our laboratory supported this relationship, whereby 5-mg/kg/day PFOA exposure decreased total sperm count and sperm motility (Zhang et al. 2014). The integrity of the BTB is crucial for male fertility. Germ cells undergo mitosis and cross the BTB at stages VIII–XI of the epithelial cycle to gain entry into the adluminal compartment of the seminiferous epithelium to facilitate germ cell release (Lie et al. 2009). In this study, the BTB disruption could be a potential reason for PFOA-induced reproductive dysfunction in adult male mice,

thus led to less sperm, fewer mated females, fewer pregnant females per male mouse, and decreased offspring litter weight. Meanwhile, PFOA is a endocrine-disrupting chemical that affects the endocrine system at the hormone level, such as testicular testosterone via inhibiting 3 beta-hydroxysteroid dehydrogenase (3β-HSD) and 17 beta-hydroxysteroid dehydrogenase 3 (17β-HSD3) activities (Zhao et al. 2010; Dankers et al. 2013). The testosterone reduced after PFOA exposure for 28 days, which has been reported in our previous study, can be another reason for PFOA-induced reproductive dysfunction in adult male mice.

This study confirmed that PFOA exposure disrupted BTB integrity and caused immune privilege and harm to the reproductive system, resulting in reproductive dysfunction. The TJ-, GJ-, and basal ES-associated proteins between SCs may be the target of PFOA. The increased TNFα content and activated p38 MAPK signaling pathway might be contributors to PFOA-induced BTB damage.

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