Cryptorchidism-induced CFTR down-regulation results in disruption of testicular tight junctions through up-regulation of NF-κB/COX-2/PGE₂

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Submitted on December 21, 2011; resubmitted on June 10, 2012; accepted on June 13, 2012

STUDY QUESTION: Does elevated temperature-induced cystic fibrosis transmembrane conductance regulator (CFTR) down-regulation in Sertoli cells in cryptorchid testis disrupt testicular tight junctions (TJs) through the nuclear factor kappa B (NF-κB)/cyclooxygenase-2 (COX-2)/prostaglandin E₂ (PGE₂) pathway?

SUMMARY ANSWER: Our results suggest that CFTR may be involved in regulating testicular TJs and the blood-testis barrier (BTB) through its negative regulation of the NF-κB/COX-2/PGE₂ pathway in Sertoli cells, a defect of which may result in the spermatogenesis defect in cryptorchidism.

WHAT IS KNOWN ALREADY: Cryptorchidism, or undescended testes, is known to result in defective spermatogenesis. Although an elevated testicular temperature is regarded as an important factor affecting spermatogenesis in cryptorchidism, the exact mechanism remains elusive. It is known that the expression of functional CFTR is temperature sensitive. Our previous study has demonstrated that CFTR negatively regulates NF-κB/COX-2/PGE₂ in bronchial epithelial cells. Disruption of TJs by COX-2/PGE₂ has been found in tumour cells.

STUDY DESIGN AND METHODS: Expression of CFTR, NF-κB, COX-2 and TJ proteins was examined in the testes of a surgical-induced cryptorchidism mouse model and a testicular hyperthermia mouse model, as well as in control or CFTR-inhibited/knocked down primary rat Sertoli cells. PGE₂ production was measured by ELISA. Sertoli cell barrier function was determined by transepithelial resistance (TER) measurements in rat Sertoli cell primary cultures. BTB integrity in the cryptorchidism model was monitored by examining tracker dye injected into seminiferous tubules.

MAIN RESULTS: Down-regulation of CFTR accompanied by activation of NF-κB, up-regulation of COX-2 and down-regulation of TJ proteins, including ZO-1 and occludin, was observed in a cryptorchidism mouse model. BTB leakage revealed impaired BTB integrity in cryptorchid testes, confirming the destruction of TJs. The inverse correlation of CFTR and COX-2 was further confirmed in a mouse testis hyperthermia model and CFTR knockout mouse model. Culturing primary Sertoli cells at 37°C, which mimics the pathological condition of cryptorchidism, led to a significant decrease in CFTR and COX-2 expression and PGE₂ production compared with the culture at the physiological 32°C. Inhibition or knockdown of CFTR led to increased COX-2 expression and PGE₂ production with compared with the culture at the physiological 32°C. Inhibition of CFTR or administration of PGE₂ significantly decreased Sertoli cell TER.

LIMITATIONS: This study has tested only the CFTR/NF-κB/COX-2/PGE₂ pathway in mouse testes in vivo and in rat Sertoli cells in vitro, and thus, it has some limitations. Further investigations in other species, especially humans, are needed.
INTRODUCTION

Cryptorchidism, the failure of the testis/testes to descend into the scrotum at birth, is one of the most common birth defects in the male genitalia. The incidence of cryptorchidism is ~1%, and is still rising (Toppari et al., 2001). The main consequences of cryptorchidism are infertility (Hadziselimovic, 2002) and increased incidence of testicular cancer (Mendis-Handagama et al., 1990).

Infertility in cryptorchidism is largely due to the elevated testicular temperature, which has deleterious effects on spermatogenesis (Hutson et al., 2010). However, the exact underlying mechanism is obscure. It has been recognized for a long time that germ cell loss occurs because of apoptosis in response to elevated temperature. However, some studies suggest that the Sertoli cell damage precedes germ cell damage and that germ cell damage could be caused at least to some extent by Sertoli cell malfunction in cryptorchidism (Bergh, 1983; Bergh et al., 1984). In particular, inter-Sertoli cell tight junctions (TJs), which constitute the blood–testis barrier (BTB), have been reported to be disrupted in cryptorchidism. Defective BTB development with fewer TJ fibrils and abnormal spermatogenesis are observed in cryptorchidism seminiferous epithelium (Aumuller et al., 1992; Maekawa et al., 1995; Pinart et al., 2000).

BTB defines the microenvironment for germ cell differentiation, and thus is critical to spermatogenesis. Apart from acting as a 'barrier' and 'fence', TJs also play a key role in spermatogenic waves. Disassembly and reassembly of the TJs, which are cyclically regulated by cytokines [e.g. transforming growth factor-α3 and tumour necrosis factor-α], are pivotal to germ cell differentiation across seminiferous tubules. Therefore, disruption of TJs may lead to failure of spermatogenesis (Cheng and Mruk, 2002).

The role of cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) in destruction of TJs was first found in tumour cells both in vitro (Attiga et al., 2000; Rozic et al., 2001; Nithipatikom et al., 2002) and in vivo (Kakiuchi et al., 2002). Although there is no direct evidence of COX-2/PGE2 damaging inter-Sertoli cells TJs, it has been reported that PGE2 interacts with multiple cytokines (Ishikawa and Morris, 2006), which are shown to regulate TJs and BTB (Li et al., 2006, 2009; Xia, et al., 2009; Lie et al., 2011; Catizone et al., 2012), suggesting that PGE2 is very likely to play a part in the network of cytokine-regulated TJs formation, as in the context of cryptorchidism.

Cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel located at the apical membrane of many epithelial tissues, is known to interact with junctional complex associated molecules (Short et al., 1998; Moyer et al., 1999; Castillon et al., 2002; Wang, et al., 2006; Chanson et al., 2007). Overexpression of CFTR is shown to enhance TJs (LeSimple et al., 2010), while inhibition or down-regulation of CFTR leads to destruction of TJs or affect epithelial polarity (LeSimple et al., 2010; Nilsson et al., 2010). CFTR has been reported to be expressed in Sertoli cells (Booc kfor et al., 1998) and play a critical role in regulating spermatogenesis (Xu et al., 2011). Our recent study has demonstrated that CFTR negatively regulates the NF-κB/COX-2/PGE2 pathway in bronchial epithelia (Chen et al., 2012). Interestingly, the processing of CFTR in endoplasmic reticulum and its trafficking to the apical membrane is temperature dependent, as elevated temperature leads to decreased CFTR expression on the cell membrane (Lukacs et al., 1994). Given the characteristics of CFTR, we hypothesized that elevated testicular temperature in cryptorchidism might lead to abnormal CFTR plasma membrane expression, resulting in disruption of TJs and impaired spermatogenesis due to defective CFTR-dependent regulation of the NF-κB/COX-2/PGE2 pathway. We undertook the present study to test this hypothesis.

MATERIALS AND METHODS

CELL CULTURE MATERIALS

Epidermal growth factor (EGF), insulin, human transferring, bovine insulin, bactercin, gentamycin, trypsin, soybean trypsin inhibitor, collagenase, hyaluronidase and DNase were all obtained from Sigma. Ham’s F12/Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma-Aldrich Co.

DRUGS AND REAGENTS

CFTRinh-172, PGE2, Curcumin and NS-398 were obtained from Sigma-Aldrich Co.

ANIMALS

Male C57BL/6 mice, male CFTR knockout (Cftrtm1Unc) mice and male SD rats were maintained in the Laboratory Animal Service Center, the Chinese University of Hong Kong (CUHK). All procedures were approved by the Animal Ethical Committee of the Chinese University of Hong Kong.

ARTIFICIAL CRYPTOCHORDISM MOUSE MODEL

Anaesthesia was prepared by mixing 0.75 ml of ketamine with 0.5 ml of xylazine in 0.75 ml of distilled water. At 8 weeks of age, the C57BL/6 male mice were anesthetized by injecting 0.075 mg/g body weight (i.p.). To induce right cryptorchidism, midline abdominal incisions were made. The right testis was gently brought up into the abdominal cavity and fixed on the upper abdominal wall with suture. The left testis was also manipulated into the abdomen and then returned to the scrotum to

WIDER IMPLICATIONS OF THE FINDINGS: Our study may shed more light on one of the aspects of the complicated underlying mechanisms of defective spermatogenesis induced by cryptorchidism.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the National Basic Research Program of China (2012 CB944900), Natural Sciences Foundation of China (30870933), Li Ka Shing Institute of Health Sciences, the Focused Investment Scheme of the Chinese University of Hong Kong and Morningside Foundation. The authors declare no conflict of interest.

Key words: cryptorchidism / CFTR / cyclooxygenase-2 / tight junction / blood-testis barrier
serve as a control. The animals were allowed to recover on a heated plate after treatment until conscious and were then returned to their cages, with food and water available ad libitum. The mice were sacrificed by exposure to absolute CO₂ 15 days after surgery. The testes were collected for further experiments.

Testicular hyperthermia mouse model

At 8 weeks of age, the C57BL/6 mice were subjected to anaesthesia, and the experimental group mice were placed with their scrotum in a water bath at 43°C for 30 min, whereas the control group mice underwent the same procedure using a 33°C water bath. The mice were sacrificed humanely at different time points after the treatment and the testes were collected for western blots.

BTB leakage

At Day 15 after the surgical induction of cryptorchidism, both the control and the cryptorchid testes were isolated. Microinjection of seminiferous tubules was performed as described (Yin et al., 2007). Around 5 μl of tracker dye (0.4% trypan blue) was injected into each testis via the efferent duct to fill up <50% of the surface area of the testis. Rapid diffusion of the tracker dye with reduced dye intensity was used to indicate disrupted BTB integrity.

Sertoli cell primary culture

Sertoli cells were isolated from 20-day-old male rats as described (Xu et al., 2011). The Sertoli cells were plated on Matrigel-coated (Collaborative Biochemical Products, Bedford, MA; diluted 1:7 with F12/DMEM, vol/vol) 12-well dishes (Corning, Inc., Corning, NY) in serum-free Ham's F12 nutrient mixture and DMEM, supplemented with 15 mm HEPES, 1.2 g/l sodium bicarbonate, 10 μg/ml bovine insulin, 5 μg/ml human transferrin, 2.5 ng/ml EGF, 20 mg/l gentamicin and 10 μg/ml bacitracin, at a density of 0.5 × 10⁶ cells/cm². It was reported that TJs were formed under this culturing condition (Siu et al., 2003). These cultures were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 32°C. Media were replaced every 24 h. Primary Sertoli cells were hypothetically treated with 20 mM Tris (pH 7.4) for 2.5 min to lyse contaminating cells, primary rat Sertoli cells were hypothermically treated with 20 mM Tris (pH 7.4) for 2.5 min to lyse contaminating germ cells 48 h after plating (Galdien, 1981).

siRNA against CFTR and transfection

The cells were incubated for an additional 3 days after the hypotropical treatment to allow formation of TJs. Then, 100 nM stealth siRNA targeting CFTR or the universal negative control, which was a scramble oligo (Thermo), was transfected into the Sertoli cells in a 6-well plate, using lipofectamine 2000 transfection reagent. Forty-eight hours after transfection, cells were collected for further studies.

Epithelial cell transepithelial resistance measurement

To examine the effects of CFTR inhibition or excessive PGE₂ on the assembly of the inter-Sertoli TJ permeable barrier in vitro, primary rat Sertoli cells were isolated and cultured at 3 × 10⁶ cells/cm² in medium with 10 μM dimethylsulphoxide (DMSO), CFTRinh-172 or PGE₂, respectively, for 2 days on Matrigel (1:7)–coated bicaneral units (Millipore Corp., Bedford, MA) (Grima et al., 1992). In order to test whether CFTRinh-172 or PGE₂ had an effect on the maintenance of inter-Sertoli TJs, cells with a seeding density of 1.5 × 10⁶ cells/cm² were cultured for 5 days after isolation in culture medium to allow formation of inter-Sertoli TJs, and then treated with 10 μM DMSO, CFTRinh-172 or PGE₂ for 2 days. The Sertoli cell TJ assembly was monitored by epithelial cell transepithelial resistance (TER) measurement using a Millicell-ERS (Electrical Resistance System) (Grima et al., 1998). The cultures were washed with F12/DMEM for three times and stabilized at room temperature for 20 min before TER measurement. The procedure was according to the manufacturer’s instructions. Each group contained triplicate cultures, and each experiment was repeated at least twice using different batches of cells.

Manipulation of RNA and real-time quantitative RT–PCR

The cultured cells were lysed directly by using TRIZOL Reagent (1 ml per 10 cm²). Tissue samples were homogenized in 1 ml of TRIZOL Reagent (Invitrogen, USA) per 50–100 mg of sample using Tissue-Tearor™ (BioSpec Products, USA), then 0.2 ml per 1 ml of TRIZOL Reagent of chloroform was added to the lysates and the tubes were shaken vigorously by hand for 15 s. After centrifugation at 14 000g for 10 min at 4°C, the upper aqueous phase was transferred to a 1.5-ml diethyl pyrocarbonate-treated microcentrifuge tube. To precipitate the RNA, 0.5 ml per 1 ml of TRIZOL Reagent of isopropyl alcohol was added to the aqueous phase and the samples were incubated at −20°C for 10–30 min. The RNA was pelleted by centrifuging at 14 000g for 10 min at 4°C and washed once with 1 ml 75% ethanol. The RNA was dissolved in an appropriate amount of RNase-free water. The RNA concentration was determined by S2000 UV-Vis spectrophotometer (Perkin-Elmer, USA), and 400 ng total RNA was transcribed to cDNA with random hexamers (Taqman Reverse Transcription Reagents, Perkin-Elmer). S18 rRNA was used as an endogenous control (S18 control kit, Perkin-Elmer). PCR conditions were 2 min 50°C, 10 min 95°C and 40 cycles of 15 s 95°C, 1 min 60°C. The PCR assays were performed in separate tubes and relative quantification of the mRNAs was performed using the standard curve method according to the manufacturer’s instructions (PE Applied Biosystems: ABI PRISM 7700 Sequence Detection System).

Manipulation of proteins and western blot

The cells were lysed and the tissues were cut into small pieces and diced by razor blade in radioimmunoprecipitation assay lysis buffer, supplemented with protease inhibitor cocktail and 1 mM phenylmethylsulphonyl fluoride. The lysates were analyzed by SDS–PAGE followed by immunoblotting. Membranes were blocked with 4% milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) for 1 h, and then incubated with primary antibodies (anti-CFTR 1:200 Alomone Lab, anti-COX-2 1:300 Cayman Chemical, anti-inhibitor of kappα B (kBα) 1:1000 Santa Cruz Biotechnology, anti-ZO-1 1:500 Invitrogen, anti-occludin 1:500 Zymed, anti-β-tubulin 1:2000 Santa Cruz Biotechnology) in 2% milk at 4°C overnight. Membranes were washed three times in TBS-T followed by incubation with anti-rabbit immunoglobulin G (IgG)-horse-radish peroxidase (1:10000) in 2% milk for 1 h at room temperature. Following three washes in TBS-T, proteins are detected using an enhanced chemiluminescence kit according to the manufacturer’s instructions.

Immunofluorescent staining in Sertoli cells

The Sertoli cells on coverslips were fixed in cold methanol and acetone (1:1) for 15 min. The cells were blocked with 1% bovine serum albumin (BSA) for 1 h and incubated for 1 h with anti-ZO-1, followed by incubation with Alexa Fluor 488-conjugated goat-anti-rabbit IgG (Invitrogen). Nuclei were counterstained by Hoechst 33342 for 10 min. Slides were mounted with Prolong™Gold Antifade Reagent (Invitrogen). Fluorescent images were captured on a fluorescence microscope.

Immunofluorescent staining in mouse testes

Specimens were fixed by 4% polyformaldehyde, embedded in paraffin and cut into 5 μm sections. The tissue sections were deparaffinized and
rehydrated. Then they were washed three times with phosphate-buffered saline (PBS) for 10 min and blocked with 1% BSA for 1 h at room temperature, followed by incubation with ZO-1 (1:50) or NF-κB (1:100) primary antibody overnight at 4°C. The sections were washed three times with PBST for 10 min and incubated with Alexa 488 goat anti-rabbit secondary antibody for 1 h at room temperature. Nuclei were counterstained by Hoechst 33342. The slides were mounted under glass coverslips, using antifade mounting media (Invitrogen, USA). Negative controls were performed by omission of the primary antibody and replacing it with dilution buffer.

Prostaglandin E2 ELISA

Primary Sertoli cells were plated on 24-well plates at a density of 0.5 × 10⁶ cells/cm². The cells were hypotonically treated to remove residual germ cells 48 h after plating. Twenty-four hours after hypotonic treatment, the cells were treated with different reagents (DMSO, 172, PGE₂, NS-398, etc.). Supernatants were collected for PGE₂ ELISA using a PGE₂ Enzyme immunoassay (EIA) Kit-Monoclonal (Cayman chemical).

Statistical analysis

Data are expressed as the mean ± SEM. Differences in measured variables between two groups were assessed by using Student’s t-tests. One-way analysis of variance was deployed when there were more than two groups. Results were considered statistically significant at P < 0.05.

Results

Down-regulation of CFTR is associated with up-regulation of COX-2 in cryptorchid testes and hyperthermia models

The experimental cryptorchidism model was established as described earlier. At Day 15 after the surgery, the size of the cryptorchid testes were strikingly smaller than the control testes as shown in Fig. 1A. H&E staining was carried out to check the morphological changes of cryptorchid testes (Fig. 1B). In cryptorchid testes (lower pannels), spermatagonia, spermatocyte, round spermatids and Sertoli cells were left in the seminiferous tubules, while more differentiated germ cells were absent. Vacuoles were formed between Sertoli cells. These results were consistent with the previous findings by Kerr et al. (1979) in a rat cryptorchidism model.

CFTR and COX-2 expression in the control testes and cryptorchid testes were examined by QRT-PCR (Fig. 1C and D) and western blot (Fig. 1E–G). The results revealed that the CFTR mRNA expression level in cryptorchid testes was significantly lower than that in control testes. On the contrary, a higher level of COX-2 expression was detected in cryptorchid testes. Western blotting results also showed down-regulation of CFTR and up-regulation of COX-2 in cryptorchid testes, which was consistent with the QRT-PCR results, indicating an inverse correlation between CFTR and COX-2 at the protein level in the testis.

Since elevated testicular temperature is considered to be the cause of defective spermatogenesis in cryptorchidism and CFTR protein is temperature-sensitive, we established an acute hyperthermia mouse model to confirm whether down-regulation of CFTR and up-regulation of COX-2 in cryptorchidism is due to elevated temperature. The result showed that the expression of CFTR and COX-2 protein at 33°C remained constant in the control group, but exhibited a dynamic time-dependent change in the 43°C group. The expression of CFTR decreased from 1 to 8 h, while the expression of COX-2 from 1 to 8 h indicated inverse expression levels (Fig. 1H and I).

To further confirm that down-regulation of CFTR can lead to up-regulation of COX-2, we took advantage of CFTR knockout mice. We examined the expression of COX-2 in the testis of CFTR knockout mice by western blot. As shown in Fig. 1J and K, significantly higher levels of COX-2 protein were detected in the testes of Cфtr−/− mice compared with that of wild-type controls (Cфtr+/+), indicating that defective CFTR expression might lead to up-regulation of COX-2.

Cryptorchidism-induced COX-2 up-regulation is mediated by NF-κB activation

Since accumulating evidence has indicated that a defect in CFTR results in endogenous activation of NF-κB in many cell types (Andersson et al., 1994; Bonfield et al., 1995; Vij et al., 2009), and NF-κB activation is also known to be transcriptionally activated COX-2 (Maier et al., 1990). The concomitant up-regulation of COX-2 in the two CFTR down-regulated animal models and the CFTR knockout model suggested possible involvement of NF-κB in mediating the CFTR down-regulation-induced COX-2 up-regulation. Immunofluorescent staining showed enhanced nuclear expression of NF-κB p65 in the Sertoli cells of cryptorchid testes compared with control testes (Fig. 2A), indicating nuclear translocation of p65 and enhanced NF-κB activity in the Sertoli cells of cryptorchid testes. Statistical analysis of the number of p65 nuclear positive Sertoli cells in each tubule cross section indicated a significantly increased percentage of p65 nuclear positive Sertoli cells in cryptorchid testes compared with control testes (Fig. 2B), which suggested an extensive Sertoli cell NF-κB activation in cryptorchidism. We also checked the expression of IкBα, which inhibits the activation of NF-κB in the cytoplasm, in both control and cryptorchid testes. The result showed consistently lower levels of IкBα expression in cryptorchid testes compared with control testes (Fig. 2C and D). The higher level of nuclear NF-κB p65 and lower level of IкBα expression suggested enhanced NF-κB activity, which was consistent with up-regulation of COX-2, in cryptorchid testes.

Decreased TJ proteins expression in cryptorchid testes

Since CFTR and COX-2/PGE₂ have been implicated in regulation of junctional complexes, we investigated the expression of TJ proteins, ZO-1 and occludin by western blot analysis in control and cryptorchid testes. As shown in Fig. 3A–C, decreased levels of ZO-1 and occludin were found in cryptorchid testes 15 days after surgically induced cryptorchidism, suggesting destruction of TJs.

Immunofluorescent staining was employed to localize TJ proteins. ZO-1 is an important interacting protein that exists in TJ fibrils. Previous studies have illustrated that the localization of ZO-1 in mouse testis is near the basement membrane of the seminiferous epithelium (Wong et al., 2004). In the present study, immunofluorescence microscopy showed that ZO-1 formed continuous belts of fluorescent staining at the BTB site in the control testes (Fig. 3D), in line with the localization reported by others. The intensity of
Figure 1 Down-regulation of CFTR is associated with up-regulation of COX-2 in testes of cryptorchidism and hyperthermia models. (A) Reduced size of the Cryptorchid testis (CR) compared with the control testis (Ctrl). (B) H&E staining of testicular cross sections of control (upper panel) and cryptorchid (lower panel) testes. (C and D) QRT-PCR results show decreased CFTR and increased COX-2 mRNA expression in cryorchid testes compared with the control testes. ***p < 0.001, n = 4. (E) Western blot results showed decreased CFTR and increased COX-2 protein expression in cryptorchid testes compared with that in the corresponding control testes. (F and G) Statistical analysis of the western blot result in (E). *p < 0.05, ***p < 0.001, n = 4. (H and I) Time-dependent down-regulation of CFTR and up-regulation of COX-2 in the mouse testis hyperthermia model. (H) Western blot results show that CFTR expression gradually decreased while COX-2 expression increased after heat shock treatment (43°C) of testes. (I) Western blot result shows a stable expression of CFTR and COX-2 after treatment at 33°C, which is the normal temperature of the testes. (J) Western blot result shows up-regulation of COX-2 in the testes of CFTR knockout (CFTR−/−) mice compared with wild-type controls (CFTR+/+). (K) Statistical analysis of western blot results in (J). *p < 0.05, n = 3.
immunofluorescence of ZO-1 was comparable at all other stages except for stage VII–VIII, during which the TJs were physiologically ‘opened’ and down-regulated to permit the pass of differentiating germ cells. However, in the cryptorchid testes, the immunofluorescent staining of ZO-1 in all seminiferous tubules was much weaker than in the control and the localization of the weak immuno signals was no longer restricted to the BTB (Fig. 3D), suggesting that the BTB was damaged.

Figure 2 Cryptorchidism-induced COX-2 up-regulation is mediated by NF-κB activation. (A) Immunofluorescent staining of NFκB p65 (green) in cryptorchid model testes. Nuclei (blue) were counterstained with Hoechst 33342. Enhanced nuclear immunofluorescent signals in Sertoli cells (arrow heads) and increased percentages of nuclear positive Sertoli cells in each tubule cross-section were found in the cryptorchid testes compared with the control testes. (B) Statistical analysis of p65 nuclear positive Sertoli cell percentages per tubule cross-section; 13–14 tubule cross-sections were analysed in each group. ***P < 0.001. (C) Western blot result showed consistently decreased expression of IκBα in cryptorchid testis compared with its control testis in four experimental cryptorchidism mice. ***P < 0.001, n = 4. (D) Statistical analysis of the western blot results.
Elevation of culture temperature results in down-regulation of CFTR and up-regulation of COX-2 in primary cultured rat Sertoli cells

To confirm the involvement of CFTR-regulated COX-2 pathway in cryptorchidism pathogenesis, primary cultures of rat Sertoli cells were established. One group of cells were originally cultured at 32°C for 2 days and placed at 37°C for further incubation for 48 h, while their control counterparts were still cultured at 32°C for the same duration. Western blot analysis revealed significantly decreased CFTR expression in Sertoli cells cultured at 37°C for 48 h compared with those maintained at 32°C (Fig. 4A and B), confirming the temperature-sensitive characteristic of CFTR expression. Consistent with the observation in the in vivo models, decreased CFTR expression was found to be accompanied by an increase in COX-2 expression in Sertoli cells cultured at 37°C for 48 h compared with those maintained at 32°C (Fig. 4A–C), further confirming the inverse relationship between CFTR and COX-2 in Sertoli cells.

To test whether inhibition of CFTR function may lead to up-regulation of COX-2 in Sertoli cells, we examined the effect of inhibition of CFTR on COX-2 expression. Primary Sertoli cells were treated with either DMSO or CFTR inh-172 for 48 h. Western blot results showed elevation of COX-2 expression after blocking CFTR channel function (Fig. 4D and E). ELISA results showed that inhibition of CFTR also resulted in an increase in PGE2 production (Fig. 4F), indicating the negative regulation of COX-2 expression and PGE2 production by CFTR in Sertoli cells.

Inhibition/knockdown of CFTR on the cell membrane results in decreased TJ proteins expression through increased PGE2

Since down-regulation of CFTR was accompanied by up-regulation of COX-2 and down-regulation of TJs in the cryptorchidism model, we further examined whether CFTR could regulate TJs through COX-2 and PGE2 in primary culture of Sertoli cells. Treatment of Sertoli cells with CFTR-inh-172 for 48 h resulted in significantly decreased expression of ZO-1 and occludin as detected by western blot analysis (Fig. 5A). Knockdown of CFTR protein expression by siRNA also led to a decrease in ZO-1 and occludin expression (Fig. 5B).

In order to investigate whether CFTR is involved in assembly and disassembly of TJs between Sertoli cells, Sertoli cells were treated either with DMSO or with CFTR-inh-172 for 48 h immediately after...
isolation or 5 days after plating and TJs were examined by immuno-fluorescent staining of ZO-1. The results showed strong immuno-signal of ZO-1 at the cell–cell contact sites between Sertoli cells treated with vehicle control for 48 h, while decreased intensity and breakdown of the ZO-1 signals were found in cells treated with CFTRinh-172 immediately after isolation (Fig. 5C) or 5 days after culture (Fig. 5D). The effect of CFTR-inh-172 could be mimicked by PGE2, suggesting that CFTR is involved in formation and maintenance of TJs through negative regulation of PGE2.

Effect of CFTR down-regulation on TJ protein expression can be reversed by inhibition of NF-κB and COX-2

We conducted further experiments to confirm that the effect of CFTR down-regulation on TJ protein expression was mediated by a NF-κB/COX-2 pathway. NS-398, a specific COX-2 inhibitor (Futaki et al., 1994), dose dependently reversed the effect of CFTR-inh-172 on PGE2 production (Fig. 6A). Curcumin, an NF-κB inhibitor, which also inhibits COX-2 (Plummer et al., 1999), also reversed the down-regulation of ZO-1 and occludin expression by CFTRinh-172 (Fig. 6B), suggesting that the CFTR-dependent regulation of TJ in Sertoli cells is mediated by the NF-κB/COX-2/PGE2 pathway.

Impaired barrier function of Sertoli cells by inhibiting CFTR and in cryptorchid testes

In order to investigate whether inhibition of CFTR led to impaired barrier function of Sertoli cells, TER across the Sertoli cell epithelia was measured to evaluate the barrier function. Treatment with CFTRinh-172 or PGE2 immediately after Sertoli cell isolation (Fig. 7A) or 5 days after culture (Fig. 7B) led to decreased TER, compared with the respective vehicle control group, suggesting destruction of inter-Sertoli cell junctions and barrier function.

To demonstrate the disruption of ‘BTB’ function in cryptorchidism, we established a BTB leakage model. Since the physiological function of the BTB is to maintain a specialized microenvironment in the luminal compartment for germ cell development by preventing the diffusion of substances across the BTB, disruption of the BTB would lead to a free diffusion from the luminal compartment to the interstitial...
space. Indeed, our results indicated that the tracker dye injected into the luminal compartment of the cryptorchid testis showed a diffused pale blue colour compared with the dark blue colour confined to the seminiferous tubules observed in the control testis (Fig. 7C), suggesting leakage of the dye into the interstitial space in the cryptorchid testis. These results indicated that cryptorchidism would lead to the breakdown of TJs and barrier function of the BTB.

**Discussion**

A previous study by Kerr et al. (1979) has indicated damage of inter-Sertoli cell TJs after surgically induced cryptorchidism in rats, suggesting that the permeability of the BTB is altered by the raised intra-abdominal temperature. However, the exact mechanism underlying the effect of elevated testicular temperature leading to disruption of BTB remains unknown. In the present study, we have demonstrated that inhibition or down-regulation of CFTR in the Sertoli cells due to elevated temperature results in up-regulation of NF-κB/COX-2/PGE2, leading to disruption of TJs and impaired barrier function of Sertoli cells, which may cause defective spermatogenesis in cryptorchidism. The present findings not only provide a possible mechanism for cryptorchidism-induced infertility, but also lend support to the previously demonstrated critical role of CFTR in spermatogenesis (Xu et al., 2011).

The present results suggest that alteration in TJs may precede germ cell apoptosis in cryptorchidism. A previous study of surgically induced cryptorchid testes showed positive staining for apoptotic DNA at Days 7 and 10, but not at Day 4 (Yin et al., 1997). In our study, elevating culture temperature of Sertoli cell for 48 h led to a significant decrease in CFTR expression. Either inhibition of CFTR with CFTRinh-172 or knockdown of CFTR in cultured Sertoli cells resulted in a quick decrease in occludin and ZO-1 expression in 48 h. Moreover, a time-dependent decrease in CFTR expression was observed from 1 to 8 h after testicular hyperthermia in the mouse model, with a concomitant increase in COX-2 expression, suggesting the early onset of the CFTR/NF-κB/COX-2/PGE2 pathway in response to hyperthermia.
to elevated temperature in the testis. Thus, in cryptorchidism, the high temperature-induced decrease in CFTR expression and subsequent reduction in expression of TJ proteins in Sertoli cells may precede germ cell apoptosis, contributing to defective spermatogenesis in cryptorchidism.

As described previously, CFTR is found to regulate junctional proteins; however, the underlying mechanisms are still obscure. Some studies advocate that this regulation is through protein–protein interactions between CFTR and TJ proteins. It has been found that CFTR interacts with a cytosolic protein network, including ezrin, actin and the TJ protein ZO-1 (Short et al., 1998; Moyer et al., 1999; Sun et al., 2000; Castillon et al., 2002; Chasan et al., 2002). ZO-1 is linked to CFTR by the actin cytoskeleton. Formation of the CFTR-ZO-1-ezrin complex has also been detected in the three-dimensional (3-D) organization of adult human airway epithelial cells (Castillon et al., 2002). The interaction between CFTR and ZO-1 may suggest a role of CFTR in the formation and/or maintenance of TJs. Alternatively, the present study suggests that CFTR can also regulate TJs through negative regulation of the NF-κB/COX-2/PGE2 pathway. Defects in CFTR result in over-activation of NF-κB, which induces up-regulation of COX-2 and elevation of PGE2 production, leading to down-regulation of TJ proteins and disruption of TJs. In fact, in a number of studies (Al-Sadi et al., 2010; Amasheh et al., 2010), NF-κB has been reported to down-regulate TJs by regulating the expression of TJ proteins. COX-2/PGE2 has also been found to regulate TJs either positively or negatively in different cell types (Basu et al., 2006; Flores-Benitez et al., 2009; Verma et al., 2010). The role of NF-κB and COX-2/PGE2 is further supported by the reversal of CFTR down-regulation-induced down-regulated TJ proteins by NF-κB and COX-2 inhibitors and the disruption of TJs by PGE2 in the present study. The signalling pathway of CFTR/NF-κB/COX-2/PGE2 identified in Sertoli cells may represent another possible mechanism underlying the regulation of TJs by CFTR, in addition to direct protein–protein interactions.

TJs are the most important component that constitutes the BTB, which defines the microenvironment for spermatogenesis. Testis is an immune-privileged site. Antigens derived from spermatogenic cells are tolerated without evoking detrimental inflammatory immune responses due to the presence of the BTB, which segregates the antigens in the seminiferous tubules from the immune cells in the interstitial space (Barker and Billingham, 1977). Therefore, the BTB is essential for protecting the germ cells from an immune response by creating an immune-privileged microenvironment. Our results have shown impaired barrier function in CFTR-inhibited Sertoli cells and cryptorchid testes, as demonstrated by a decreased TER in Sertoli cells treated with CFTR inhibitor and dye leakage in seminiferous tubules of the cryptorchid testes. Impaired BTB integrity in cryptorchidism may expose the antigens to immune cells, leading to an immune response in the testes that may interfere with spermatogenesis.

Our previous study has demonstrated that CFTR plays a role in normal spermatogenesis through its regulation of CREB, which is a key transcription factor in spermatogenesis; a defect in this CFTR-dependent CREB pathway is found in azoospermia patients (Xu et al., 2011). The presently observed down-regulation of CFTR in cryptorchidism suggests that defect in the CFTR-dependent CREB activation pathway is also likely to be involved in the cryptorchidism-induced defective spermatogenesis. Thus, it is possible that abnormal CFTR expression in cryptorchidism may lead to defective spermatogenesis also through a CREB-dependent pathway.

The study by Kerr et al. (1979) demonstrated disordered Sertoli cell function as shown by a decrease in androgen-binding protein (ABP)
production in a cryptorchidism model of rats. ABP is known to be important in spermatogenesis since it can bind to testosterone and dihydrotestosterone, thus concentrating androgens in the seminiferous tubules. Interestingly, we have recently found that androgens can up-regulate CFTR expression in the male reproductive system, such as in the prostate (Xie et al., unpublished results). Therefore, it is possible that the observed cryptorchidism-induced decrease in ABP may reduce androgen levels in the seminiferous tubules and down-regulate CFTR expression. Taken together, cryptorchidism may affect multiple pathways, with CFTR down-regulation as a key event leading to impaired spermatogenesis.

In conclusion, the present study has highlighted an important role of CFTR in negatively regulating COX-2/PGE2 in the testis and its involvement in regulating testicular junctional complexes and the BTB.

Figure 7. Impaired Sertoli barrier function by CFTR inhibition and disrupted BTB integrity in cryptorchid testes. (A) Primary rat Sertoli cells were treated with CFTRinh-172 (10 µM), PGE2 (10 µM) or DMSO (vehicle control) immediately after isolation for 48 h. TER across the Sertoli cell epithelia was significantly decreased in cells treated with CFTRinh-172 and PGE2 compared with vehicle control. (B) Primary rat Sertoli cells were treated with CFTRinh-172 (10 µM), PGE2 (10 µM) or DMSO (vehicle control) 5 days after culture for 48 h. TER across the Sertoli cell epithelia was significantly decreased in cells treated with CFTRinh-172 and PGE2 compared with vehicle control. (C) Disrupted BTB integrity was indicated by the leakage of tracker dye (blue) injected into the seminiferous tubules. Diffusion of tracker dye was indicated by decreased dye intensity. The tracker dye injected into the luminal compartment of the cryptorchid testis shows a diffused pale blue colour compared with the concentrated dark blue colour confined to the tubules observed in the control testis.
overexpression of COX-2 and excessive PGE₂ production, which leads to further damage of inter-Sertoli cell TJs and eventually defective spermatogenesis. These findings may explain the defective spermatogenesis in cryptorchidism and provide novel insights into cryptorchidism-induced infertility.

**Authors’ roles**

H.C.C. conceived the idea and designed and supervised the work. J.C., K.L.F., H.C., X.H.Z. and W.M.X. performed data acquisition. H.C.C., J.C. and H.C drafted and revised the manuscript.

**Funding**

This work was supported by the National Basic Research Program of China (2012CB944903), Natural Sciences Foundation of China (30870933), Li Ka Shing Institute of Health Sciences, the Focused Investment Scheme of the Chinese University of Hong Kong and Morningside Foundation.

**Conflict of interest**

None declared.

**References**

Al-Sadi R, Ye D, Said HM, Ma TY. IL-1 beta-induced increase in intestinal epithelial tight junction permeability is mediated by MEKK-1 activation of canonical NF-kappaB pathway. *Am J Pathol* 2010; **177**:2310–2322.


Bergh A. Early morphological changes in the abdominal tests in unilaterally cryptorchid rats. *Int J Androl* 1983; **6**:73–90.


Hadjiselimovic F. Cryptorchidism, its impact on male fertility. *Eur Urol* 2002; **41**:121–123.


