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ORIGINAL ARTICLE ***Reproductive biology***

Environmental toxicants perturb human Sertoli cell adhesive function via changes in F-actin organization mediated by actin regulatory proteins

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STUDY QUESTION: Can human Sertoli cells cultured *in vitro* and that have formed an epithelium be used as a model to monitor toxicant-induced junction disruption and to better understand the mechanism(s) by which toxicants disrupt cell adhesion at the Sertoli cell blood–testis barrier (BTB)?

SUMMARY ANSWER: Our findings illustrate that human Sertoli cells cultured *in vitro* serve as a reliable system to monitor the impact of environmental toxicants on the BTB function.

WHAT IS KNOWN ALREADY: Suspicions of a declining trend in semen quality and a concomitant increase in exposures to environmental toxicants over the past decades reveal the need of an *in vitro* system that efficiently and reliably monitors the impact of toxicants on male reproductive function. Furthermore, studies in rodents have confirmed that environmental toxicants impede Sertoli cell BTB function *in vitro* and *in vivo*.

STUDY DESIGN, SIZE AND DURATION: We examined the effects of two environmental toxicants: cadmium chloride (0.5–20 μM) and bisphenol A (0.4–200 μM) on human Sertoli cell function. Cultured Sertoli cells from three men were used in this study, which spanned an 18-month period.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human Sertoli cells from three subjects were cultured in F12/DMEM containing 5% fetal bovine serum. Changes in protein expression were monitored by immunoblotting using specific antibodies. Immunofluorescence analyses were used to assess changes in the distribution of adhesion proteins, F-actin and actin regulatory proteins following exposure to two toxicants: cadmium chloride and bisphenol A (BPA).

MAIN RESULTS AND THE ROLE OF CHANCE: Human Sertoli cells were sensitive to cadmium and BPA toxicity. Changes in the localization of cell adhesion proteins were mediated by an alteration of the actin-based cytoskeleton. This alteration of F-actin network in Sertoli cells as manifested by truncation and depolymerization of actin microfilaments at the Sertoli cell BTB was caused by mislocalization of actin filament barbed end capping and bundling protein Eps8, and branched actin polymerization protein Arp3. Besides impeding actin dynamics, endocytic vesicle-mediated trafficking and the proper localization of actin regulatory proteins c-Src and annexin II in Sertoli cells were also affected. Results of statistical analysis demonstrate that these findings were not obtained by chance.

LIMITATIONS, REASONS FOR CAUTION: (i) This study was done *in vitro* and might not extrapolate to the *in vivo* state, (ii) conclusions are based on the use of Sertoli cell samples from three men and (iii) it is uncertain if the concentrations of toxicants used in the experiments are reached *in vivo*.

WIDER IMPLICATIONS OF THE FINDINGS: Human Sertoli cells cultured *in vitro* provide a robust model to monitor environmental toxicant-mediated disruption of Sertoli cell BTB function and to study the mechanism(s) of toxicant-induced testicular dysfunction.

Key words: human Sertoli cell / testis / F-actin / cell adhesion protein / spermatogenesis

Introduction

About 15% of couples in the USA are infertile (http://www.mayoclinic.com/health/infertility/DS00310), and about 48.5 million couples worldwide are also infertile (Mascarenhas *et al.*, 2012). Among these couples, men are the sole or contributory infertility factor in 50% of cases (Smith *et al.*, 2010). It is increasingly clear that environmental toxicants could be one of the major causes of infertility in both men and women (Hunt *et al.*, 2009; Cheng *et al.*, 2011; Marques-Pinto and Carvalho, 2013) and are implicated in declining male fertility (Benoff *et al.*, 2000; Cheng *et al.*, 2011; Mruk and Cheng, 2011b; Marques-Pinto and Carvalho, 2013). Recent reports that track semen quality (e.g. sperm concentrations) among men in multiple countries for decades and/or years have demonstrated a declining trend in both developing and developed countries (Nordkap *et al.*, 2012; Rolland *et al.*, 2013), suggesting that toxicants may be one of the contributing factors. However, public concern about the disruptive effects of environmental chemicals (e.g. pesticides, polychlorinated biphenyls, food additives) on male reproductive health is not supported by available data in humans (Swan *et al.*, 2000; Bonde *et al.*, 2008; Sharpe, 2010; Toft *et al.*, 2012). It has also been shown that environmental toxicants that induce male reproductive dysfunction exert their effects at multiple levels (Heindel, 2006; Hauser, 2008; Hunt *et al.*, 2009; Siu *et al.*, 2009a; Mruk and Cheng, 2011b; Wong and Cheng, 2011; Wan *et al.*, 2013e). Indeed, Sertoli cells, in particular testis-specific cell junctions at the Sertoli-Sertoli and Sertoli-germ cell interface, are an emerging target for toxicant effects (Wong *et al.*, 2010; Mruk and Cheng, 2011b; Wong and Cheng, 2011; Qiu *et al.*, 2013; Wan *et al.*, 2013c). Since virtually all earlier studies that examined the impact of environmental toxicants on male reproductive functions were based on *in vivo* or *in vitro* rodent models (Siu *et al.*, 2009a; Skinner *et al.*, 2010; Cheng *et al.*, 2011; Vandenberg *et al.*, 2012; Sengupta, 2013; Wan *et al.*, 2013c), these findings may not be directly applicable to humans. In fact, a number of significant differences between species have been documented including variation in the timing of Sertoli cell proliferation, the effects of androgen suppression, seasonal variability and the responses to toxins (Brown *et al.*, 1995; Apostoli *et al.*, 1998; Young and Nelson, 2001; Sharpe *et al.*, 2003). Sertoli cell proliferation is thought to occur in two periods in all species, one during fetal and neonatal life, and a second in the peripubertal period. However, in some species, one period is most important, such as in rhesus monkeys where proliferation mainly occurs in the peripubertal period and in the rat where proliferation in the neonatal period (the timing of which overlaps with the peripubertal period) predominates. Both periods are important in humans where they are separated by more than a decade (Sharpe *et al.*, 2003). In mammals, the efficacy of spermatogenesis is vastly different between species. For instance, only ~3 spermatids are formed per differentiated A1 spermatogonium in humans versus 97, 51 and 39 in rats, dogs and rabbits, respectively (Ehmcke and Schlatt, 2006; Hess and de Franca, 2008). Also, there are data showing that the correlation of male reproductive toxicity in animals and in humans is often poor. Moreover, widely varying sensitivities to male reproductive toxins have been reported even within the same species, for example, in different strains of rats to effects of lead exposure (Apostoli *et al.*, 1998). No mixture of cultured cells can replicate the biochemical and physiologic processes of a whole body but due to the ethical limitations of testing on human volunteers, animals are the only option for testing in whole organisms. To increase the accuracy of toxicology testing the use of *ex-vivo* or 3-dimensional *in vitro* model systems composed of normal human cells, particularly human stem cells, has been proposed (Trosko, 2010). Thus, there is a need to develop *in vitro* methods, such as a reliable human Sertoli cell *in vitro* culture system, to study testicular toxicity and to detect Sertoli cell toxicants in a way that is relevant to their effects *in vivo*.

A population of human Sertoli cells isolated from deceased normal males remains proliferative when cultured *in vitro* if fetal bovine serum (FBS) is included in the medium (Ahmed *et al.*, 2009; Chui *et al.*, 2011). Importantly, this population of cells has been extensively characterized with specific Sertoli cell markers to positively identify putative and highly purified human Sertoli cells via flow cytometry, electron microscopy and fluorescence microscopy. Functionally, the ability of Sertoli cells to form tight junction (TJ)-permeability barriers that mimic the Sertoli cell blood–testis barrier (BTB) *in vivo* has also been reported (Chui *et al.*, 2011). We sought to examine whether human Sertoli cells cultured *in vitro* can be used to assess the effects of two environmental toxicants cadmium and bisphenol A (BPA) on the integrity of cell junctions at the Sertoli cell–cell interface. We also provide a mechanistic basis for how these two toxicants impede Sertoli cell junction integrity. Since a similar *in vitro* system using rodent Sertoli cells has been extensively characterized (Byers *et al.*, 1986; Janecki *et al.*, 1991, 1992; Grima *et al.*, 1992; Lui *et al.*, 2001; Kaitu'u-Lino *et al.*, 2007; Yan *et al.*, 2008; Nicholls *et al.*, 2009; Siu *et al.*, 2009b; Lie *et al.*, 2012; Su *et al.*, 2012c), and reproduces studies performed *in vivo* (Su *et al.*, 2012b,c), we reasoned that this model is a physiologically relevant one.

Materials and Methods

Human Sertoli cell cultures

Human Sertoli cells obtained from 3 different deceased donors (12-, 23- and 36-year old) as previously described (Chui *et al.*, 2011) were from MandalMed (San Francisco, CA, USA), which also offers the cells for research through Lonza (Walkersville, MD, USA). Human Sertoli cells were shipped on dry ice and stored in liquid nitrogen upon arrival. Cryopreserved cells were thawed in a 37°C water bath and transferred immediately to 100 mm culture dishes. All culture dishes were coated with 2 μg/cm^2 human fibronectin (BD Biosciences). Fibronectin was prepared as a 1 mg/ml stock in sterile deionized water and diluted in PBS [10 mM NaH_2PO_4 containing 0.15 M NaCl, pH 7.4 at 22°C] according to the instructions supplied by the manufacturer, as described previously (Chui *et al.*, 2011). Sertoli cells were propagated in Dulbecco's Modified Eagle's Medium/Ham's F12 Nutrient Mixture (DMEM/F12) (Sigma-Aldrich) supplemented with 5% (v/v) fetal bovine serum (FBS, Invitrogen) and penicillin (100 units/ml)/streptomycin (100 μg/ml) (Sigma-Aldrich), and incubated at 35°C in a CO_2-incubator with 5% CO_2–95% air (v/v) in a humidified atmosphere. The growth medium was changed every 3–4 days. When required for further use, such as subculture or cryopreservation, cells about to reach 70–80% confluence were removed from the culture substrate by treatment with trypsin (0.05%)-EDTA (0.02%) solution (Sigma-Aldrich) for 2 min so that human Sertoli cells were re-suspended in medium and were collected (100 g, 5 min, room temperature). Human Sertoli cells cultured in the presence of 5% FBS in F12/DMEM remained mitotically active as described (Ahmed *et al.*, 2009; Chui *et al.*, 2011), and the cells used for all the experiments reported herein were from the fourth or fifth passage to ensure reproducibility. Sertoli cells were plated at an initial density at about 25% of the targeted cell density on fibronectin-coated coverslips (targeted cell density 5×10^4 cells/cm^2) or 12- or 24-well dishes (targeted cell density 1×10^5 cells/cm^2), respectively, to be used for immunofluorescence analysis or immunoblotting. Cell density

was monitored and assessed by cell counts using a hematocytometer following trypsinization in a control well or coverslip in duplicates. Under these culture conditions, the doubling time of human Sertoli cells is ~4 days in F12/DMEM with 5% FBS and penicillin/streptomycin. It routinely took ~10 days to reach subculture readiness for specific experiments. For cryopreservation, human Sertoli cells cultured in a 100-mm dish to ~70% confluence were trypsinized, suspended in 9-ml F12/DMEM, washed three times (100 g, 5 min each at room temperature) and pelleted at 100 g (5 min). Next, the cells were resuspended in 1 ml of freezing medium (5% DMSO, 25% F12/DMEM, 70% FBS, no antibiotics) in a cryovial. Cryovials were placed immediately in an isopropanol chamber and stored at −80°C overnight, before transfer to liquid nitrogen for long-term storage.

Treatment of human Sertoli cell cultures with environmental toxicants

When Sertoli cells reached 70–80% confluence, cells were serum-starved for 5 h to quench signaling pathways before treatment. Thereafter, cells were treated with vehicle control (0.1% ethanol), 0.5–20 μM $CdCl_2$ (i.e. 0.09–3.7 μg/ml) or 0.4–200 μM BPA (i.e. 0.09–46 μg/ml) in F12/DMEM supplemented with 5% FBS and antibiotics. These selected concentrations of $CdCl_2$ and BPA were based on initial pilot experiments in which a phenotype was detected after exposure. Observed changes were: (i) localization and/or distribution of integral membrane proteins at the Sertoli cell–cell interface, (ii) organization of F-actin in Sertoli cells and/or (iii) steady-state levels of proteins of the adhesion protein complexes. It is noted that human serum/plasma levels of cadmium (Pollack *et al.*, 2011; Chen *et al.*, 2013) and BPA (Sprague *et al.*, 2013; Wan *et al.*, 2013a) are <1 ng/ml and ~1 ng/ml, respectively. It is estimated that human daily intake (orally, via food and drink) of BPA and cadmium is 34 ng/kg body weight and 1.06 μg/kg body weight, respectively (Wan *et al.*, 2013e). While the doses used herein are acute so that phenotypes were detected based on pilot experiments for this mechanistic study, however, the half-life of cadmium and BPA in humans are >20 years and 2 h, respectively (Wan *et al.*, 2013e). As such, high and toxic level of toxicants, in particular cadmium, can be accumulated in specific organs (e.g. the testis) over an extended period (Siu *et al.*, 2009a; Wan *et al.*, 2013e). Furthermore, evidence is emerging that challenges the assumption that humans metabolize BPA with a half-life of under 2 h, quickly enough to render it undetectable in blood and therefore decrease its harmful effects (Vandenberg *et al.*, 2013). For instance, a high serum level of BPA has been reported to be associated with elevated breast density, an index of breast cancer risk, in postmenopausal women (Sprague *et al.*, 2013).

RT–PCR and immunoblot analysis

For RNA extraction and protein lysate preparation, human Sertoli cell cultures (density ~1×10^5 cells/cm^2) on fibronectin-coated 12- or 24-well dishes (each well contained 2.5- or 1.5-ml medium, respectively) were terminated following exposure to a specified toxicant for 2 days. For RNA extraction, cells were harvested in TRIzol® Reagent (Invitrogen) according to manufacturer's instructions. For protein lysate preparation, media were removed and cells were treated with lysis buffer (10 mM Tris, pH 7.4 at 22°C containing 0.15 M NaCl, 1% NP-40 [v/v] and 10% glycerol [v/v]. Protease and phosphatase inhibitors were added immediately before use at a 1:100 dilution in a final volume of ~200–300 μl. RT–PCR was carried out as described earlier (Xiao *et al.*, 2011). Two primer pairs (Gene Link) were used for detection of the occludin gene in cultured human Sertoli cells (Table I). For immunoblotting, total protein concentration was determined using the Bio-Rad DC protein assay kit. Immunoblot analysis was performed using ~15–25 μg protein per lane as described previously (Xiao *et al.*, 2011, 2013b) using a Fujifilm LAS-4000 mini imaging system, and a chemiluminescence detection kit prepared in our laboratory (Mruk and Cheng, 2011a). Table II lists the antibodies and conditions used for immunoblotting experiments in this study.

Immunofluorescence analysis

For immunofluorescence analysis, Sertoli cells were plated at a density of ~$2.5–5 \times 10^4$ cells/cm^2 on fibronectin-coated round glass coverslips (18-mm, diameter) in 12-well dishes with each well containing 2-ml F12/DMEM with 5% FBS. After 2 days of exposure to a specified toxicant, immunofluorescence analysis was performed (Xiao *et al.*, 2011, 2012a) by using Alexa Fluor® Dyes 488- or 555-conjugated secondary antibodies (Invitrogen). Sertoli cells at ~$0.025–0.05 \times 10^6$ cells/cm^2 on fibronectin-coated coverslips were fixed in methanol at −20°C for 5 min. For F-actin staining, cells were fixed in 4% paraformaldehyde (w/v) in PBS at room temperature for 10 min, followed by incubation with rhodamine-phalloidin (Invitrogen, Eugene, OR, USA). Images were captured using a Nikon Ds-Qi1Mc-U2 camera in a Nikon 90i motorized fluorescence microscope, and acquired using the NIS-Elements AR software (v3.2; Nikon Instruments, Inc.), and compiled in Adobe Photoshop in Adobe Creative Suite CS 3.0 (Adobe Systems). Table II lists the antibodies and conditions used for all immunofluorescence-
staining experiments in the study. The immunofluorescence analysis was repeated at least three times using different batches of human Sertoli cells. Results were similar each time and data from a representative experiment are presented. Pilot experiments that were used to establish the necessary experimental conditions including changes in phenotypes were excluded from this analysis. All the samples used to compare the treatment groups versus the corresponding controls were processed in a single experimental session to avoid inter-experimental variation.

Cytotoxicity assay

Cells were seeded at a density of ~$0.5–1 \times 10^5$ cells/cm^2 in 96-well plates and treated with the corresponding toxicant at specified concentrations for 1, 2 or 3 day(s). Cell proliferation kit II (XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (Roche) was used for quantification of cytotoxicity as described (Li *et al.*, 2009). The XTT solution with a final concentration 0.3 mg/ml was added to

Table I Human occludin-specific primers used for RT–PCR[a].

Primer sequence	Orientation	Position	Size (bp)	Tm (°C)	Reference
5′-GACCCAAGAGCAGCAA-3′	Sense	833–848	688	50.5	NC_018921.2/NM_001205254.1
5′-CATCCACAGGCGAAGT-3′	Antisense	1505–1520			
5′-TTTGTGGGACAAGGAACACA-3′	Sense	1273–1292	312	53.8	NC_018921.2/NM_001205255.1
5′-GCAGGTGCTCTTTTTGAAGG-3′	Antisense	1565–1584			

[a]RT–PCR was performed by using a GeneAmp 2400 PCR system (Applied Biosystems, Inc.), Tm (°C), annealing temperature and 30 cycles.

Table II Antibodies used for different experiments in this report.

Antibody	Host species	Vendor	Catalog number	Application(s)/dilution(s)
ZO-1	Rabbit	Invitrogen	61-7300	IB (1:250), IF (1:100)
N-Cadherin	Rabbit	Santa Cruz Biotechnology	sc-7939	IB (1:200), IF (1:100)
	Mouse	Invitrogen	33-3900	IB (1:250)
β-Catenin	Rabbit	Invitrogen	71-2700	IB (1:250), IF (1:100)
Actin	Goat	Santa Cruz Biotechnology	sc-1616	IB (1:200)
Arp3	Mouse	Sigma-Aldrich	A5979	IB (1:3000), IF (1:200)
Src	Mouse	Santa Cruz Biotechnology	sc-8056	IB (1:200), IF (1:100)
Annexin II	Rabbit	Santa Cruz Biotechnology	sc-9061	IB (1:200)
	Mouse	BD Transduction laboratories	610068	IB (1:1000), IF (1:100)

IB, immunoblotting; IF, immunofluorescence analysis.
It is noted from the manufacturers that these antibodies cross-reacted with the corresponding proteins in humans.

each well 24 h prior to absorption measurements, which were done with microplate reader (Model 680, Bio-Rad) at a wavelength of 450 nm with the reference wavelength set at 655 nm.

Image analysis

Immunoblot data were quantified by using Scion Image (v4.0.3.2; Scion Corporation; http://scion-image.software.informer.com/) as described (Xiao *et al.*, 2011). To obtain semi-quantitative data from immunofluorescence images for analysis, fluorescence signals: (i) in human Sertoli cells such as red fluorescence for F-actin visualized by rhodamine-phalloidin, or (ii) at the Sertoli cell–cell interface such as tight junction adaptor protein ZO-1 and basal ectoplasmic specialization (ES) proteins N-cadherin and β-catenin, were quantified by ImageJ (Version 1.45, U.S. National Institutes of Health, Bethesda, MD, USA; http://rsbweb.nih.gov/ij) in micrographs without the DAPI overlay to avoid interference. The fluorescence intensity of a target protein in control cells was arbitrarily set at one against which statistical analysis was performed. Comparisons among multiple experimental groups were performed by two-way analysis of variance (ANOVA), using the repeated measures model followed by Dunnett's test to compare changes between treatment groups and their corresponding controls, and *P*-value of <0.05 was taken as statistically significant. About 50 cells were randomly selected in each coverslip, and at least two coverslips were examined in each experiment; and data from three experiments were pooled and analyzed with $n = 300$ cells.

Statistical analysis

Each experiment used human Sertoli cells from a specific donor (Table III). In each experiment, triplicate dishes (e.g. for immunoblotting, XTT cytotoxicity assay) or microscopic slides (e.g. for immunofluorescence analysis) were used to collect data. Each data point expressed as a mean ± SD of $n = 3$ independent experiments, and each experiment equates to a different Sertoli cell donor (see Table III). It is noted that pilot experiments performed to optimize the experimental conditions were not included in our statistical analysis. For image analysis of fluorescence signals, at least 300 cells were scored, and for cell adhesion proteins (e.g. N-cadherin, β-catenin, ZO-1), at least two panels per pair of adjacent cells were analyzed to assess changes in protein localization as illustrated by the white rectangles shown in Fig. 1B. For each experiment, data in treatment groups were normalized against the corresponding control, which was arbitrarily set at 1. As such, no error bars were present in controls. Two-way analysis of variance (ANOVA) using the repeated measures model followed by Dunnett's test was performed to compare changes between treatment groups and their corresponding controls using the GB-STAT statistical analysis software package (Version 7.0; Dynamic Microsystems, Silver Spring, MD, USA). This thus assessed within-experiment effects which were the focus of the analysis. *P*-value of <0.05 was taken as statistically significant. A bar without any annotation indicated that treatment group was not statistically significant different from its corresponding control.

Table III Typical conditions used for culturing the three human Sertoli cell samples for experiments reported in this study.

Donor age (years)	Thawing of cells	Time needed to reach ~70–80% confluency (days)					
		P1	P2	P3	P4	P5	
36	Time 0	16	24	8	63	14	No FBS
23		8	9	16	24		With FBS
12		11	14	21	53		

P, passage number. For all the experiments reported herein, human Sertoli cells were used on the fourth or fifth passage. In the absence of FBS, human Sertoli cells were also proliferative, but it took longer culture for them to reach ~70–80% confluence. Sertoli cells obtained from the 12-year-old subject are differentiated since human Sertoli cells cease to divide *in vivo* by puberty at 12 years of age (Sharpe *et al.*, 2003) without exposure to serum, and Sertoli cells from this subject displayed similar morphological characteristics as of the other two subjects, and the responses of Sertoli cells from this subject to Cd and BPA were also similar to the other two subjects as reported herein (see Figs 1, 3 and 4).

Results

Maintenance of human Sertoli cells *in vitro*

Nineteen experiments were performed using human Sertoli cells from three subjects (see Table III which listed 13 experiments), including 6 pilot experiments to optimize Sertoli cell culture conditions. The initial cell density in each subculture was at ~0.01–0.2 × 10^5 cells/cm^2, and when cells reached confluence of ~70–80% via proliferation in ~8–63 days, a subculture was performed, such as in P1 (Table III). In all experiments reported herein, we used cells from the fourth pass (P4) for the 23- and 12-year-old donors and fifth pass (P5) for the 36-year-old donor.

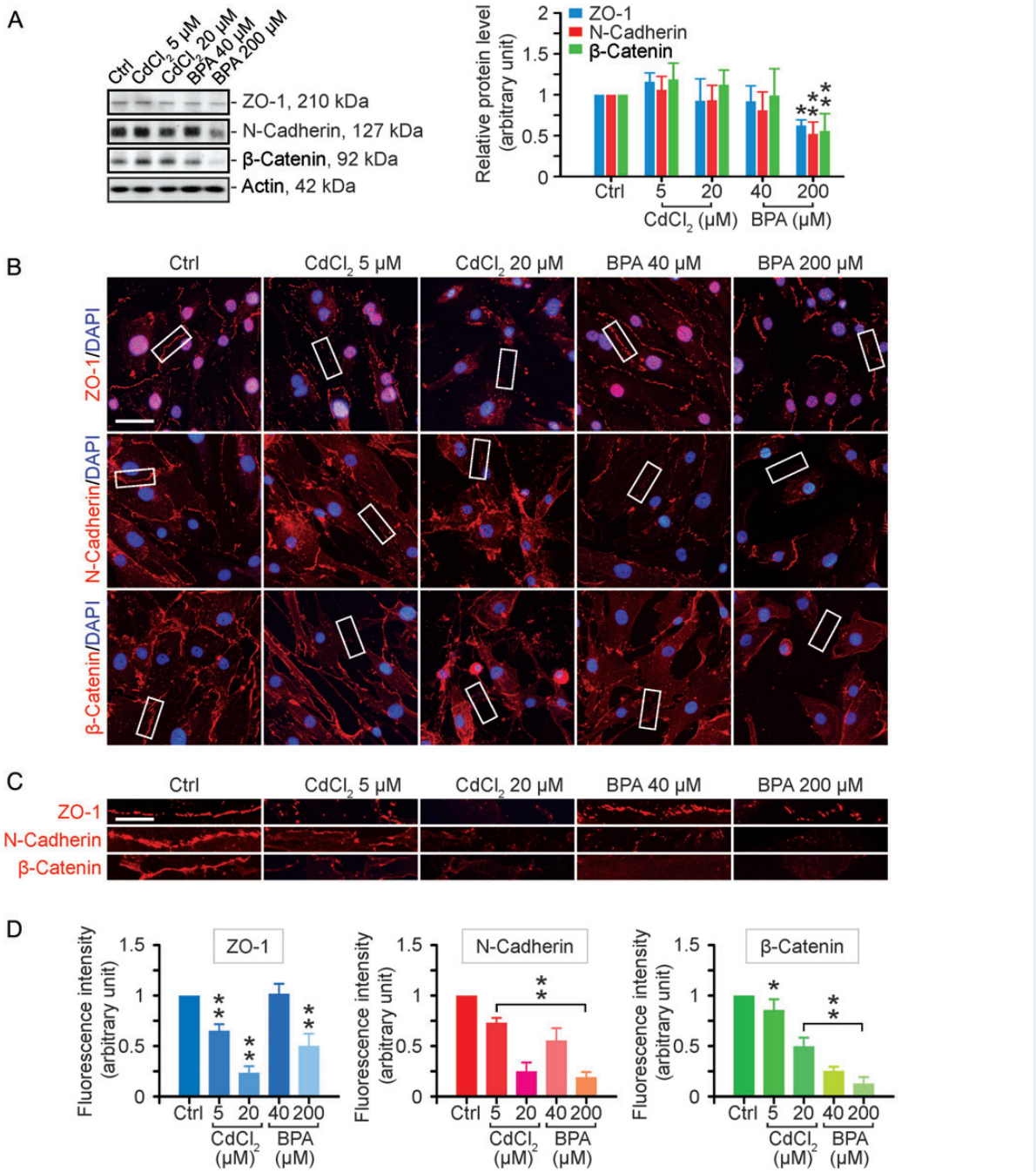

Figure 1 A study to assess the effects of cadmium chloride ($CdCl_2$) and bisphenol A (BPA) on the expression and localization of cell adhesion proteins in human Sertoli cells. (**A**) Human Sertoli cells were cultured on fibronectin-coated dishes and exposed to $CdCl_2$ (5 and 20 μM) or BPA (40 and 200 μM) for 2 days. Thereafter, cells were harvested for lysate preparation and for immunoblotting (left panel). The histogram in the right panel summarizes the immunoblotting data. Protein bands were densitometrically scanned, and normalized against β-actin; each bar is a mean ± SD of $n = 3$ independent experiments. Controls were normalized to 1.0. *$P < 0.05$; **$P < 0.01$ with treatment versus corresponding control (Ctrl) group. (**B**) Immunofluorescence analysis of some blood–testis barrier (BTB)-associated proteins (all in red fluorescence): zonula occludens-1 (ZO-1, a tight junction adaptor), N-cadherin (a basal ectoplasmic specialization (ES) integral membrane protein) and β-catenin (a basal ES adaptor). Cell nuclei were visualized by DAPI (4′,6-diamidino-2-phenylindole) staining. Scale bar = 40 μm. (**C**) Enlarged images corresponding to the white boxes in (B) of ZO-1, N-cadherin and β-catenin at the human Sertoli cell–cell interface, illustrating changes in their localization following exposure to toxicants. Scale bar = 15 μm. (**D**) Image analysis of the changes in the fluorescence intensity of cell adhesion proteins at the human Sertoli cell–cell interface. Each bar is a mean ± SD of three experiments including ~100 cells per experiment. Controls were normalized to 1.0 in each experiment. *$P < 0.05$; **$P < 0.01$ with treatment versus corresponding control (Ctrl) group.

Notably, even in the absence of FBS in F12/DMEM, human Sertoli cells remained proliferative, but required more time to reach ~70–80% confluence versus cells cultured in medium with 5% FBS (Table III).

Cadmium and BPA perturb human Sertoli cell junction integrity

When human Sertoli cells were exposed to $CdCl_2$ for 2 days, no apparent effects were found on the expression of several BTB-associated proteins in cell lysates examined by immunoblot analysis: ZO-1 (a tight junction (TJ) adaptor), N-cadherin (a basal ES integral membrane protein) and β-catenin (a basal ES adaptor protein). On the contrary, BPA at higher doses down-regulated the expression of these proteins (Fig. 1A). Since these BTB-associated proteins usually localize to the Sertoli cell–cell interface and constitute the Sertoli BTB (Cheng and Mruk, 2009, 2010), we next examined the localization pattern and/or distribution at the human Sertoli cell–cell interface following exposure to toxicants (Fig. 1B–D). While these toxicants did not perturb the expression of these proteins, they significantly altered protein localization at the Sertoli cell BTB (Fig. 1B), which can be better visualized in the enlarged images shown in Fig. 1C. In control cells, ZO-1, N-cadherin and β-catenin were localized distinctively to the human Sertoli cell–cell interface, but treatment of these cells with $CdCl_2$ or BPA, even at low doses, induced their redistribution, mostly via re-localization from the cell surface to the cell cytosol (Fig. 1B and C). This loss of fluorescence at the cell–cell interface was semi-quantified and is shown in Fig. 1D. These findings support the notion that there are considerable changes in the localization and/or distribution of these proteins at the human Sertoli cell BTB following exposure to two environmental toxicants. Data shown in Fig. 1B suggest that cells exposed to a high dose of either toxicant appeared to be retracting their cytoplasmic processes, and thus perturbing Sertoli cell barrier function. Also, while occludin has been detected at the tight junction in human blastocysts (Ghassemifar *et al.*, 2003), human intestinal epithelial T84 cells (Ando-Akatsuka *et al.*, 1996) and cervical epithelial cells (Zeng *et al.*, 2004), it was not detected in human Sertoli cells when two different primer pairs (see Table I) were used for RT–PCR amplification. The lack of occludin in human Sertoli cells reported herein is consistent with an earlier report that human occludin was not found at the Sertoli cell tight junction in human testes (Moroi *et al.*, 1998), thus occludin was not included in our analysis.

Cytotoxicity of cadmium and BPA in human Sertoli cells

To assess whether changes observed in the expression, localization or distribution of BTB-associated proteins in the human Sertoli cell epithelium following exposure to toxicants were caused simply by cell cytotoxicity, we assessed cytotoxicity using an XTT assay. As shown in Fig. 2, following exposure of human Sertoli cells to high doses of $CdCl_2$ and BPA at 20 μM and 200 μM, respectively, for 2 or 3 days, (but not for 1 day), mild but statistically significant cytotoxicity was noted.

Cadmium and BPA induce truncation and depolymerization of F-actin in human Sertoli cell epithelium

We examined changes in the distribution of the F-actin network, following exposure of human Sertoli cells to $CdCl_2$ or BPA, by using rhodamine-phalloidin to visualize the microfilaments. In human Sertoli cells under control conditions, microfilaments of F-actin were properly aligned along the cell cytosol (Fig. 3), similar to rat Sertoli cells (Siu *et al.*, 2009c; Xiao *et al.*, 2011). Following exposure to 0.5 and 5 μM $CdCl_2$ or 0.4 and 40 μM BPA, doses where no cytotoxicity was detected (Fig. 2), truncation and/or de-polymerization of actin microfilaments was clearly noted (Fig. 3). Also more F-actin was observed near the Sertoli cell surface, and localized more closely to the cell nuclei (Fig. 3), despite the fact that overall fluorescence signals of F-actin were similar in all treatment groups versus controls (Fig. 3). These findings support the notion that disorganization of F-actin in Sertoli cells in the epithelium impedes the attachment of the adhesion protein complex (e.g. N-cadherin- β-catenin) to the underlying actin-based cytoskeleton (Fig. 1B). Through this perturbation in proper localization of F-actin, an impairment of Sertoli cell BTB integrity could result.

Cadmium and BPA induce changes in the localization and/or distribution of F-actin regulatory proteins in Sertoli cell epithelium

We examined the underlying mechanism by which these toxicants impede the actin-based cytoskeleton in Sertoli cells. Both ZO-1- and β-catenin-based integral membrane proteins at the cell–cell interface

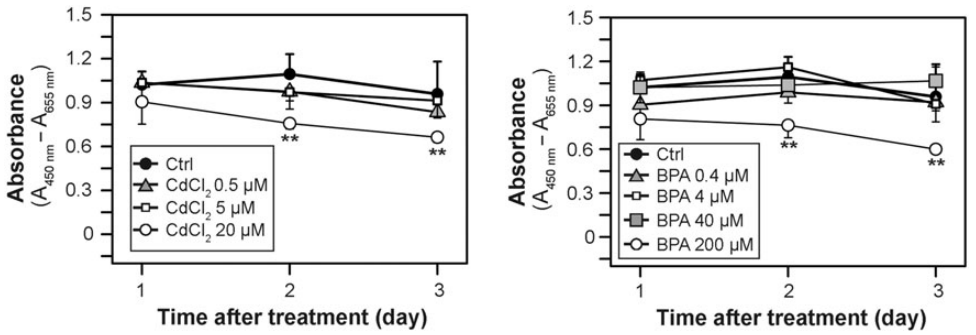

Figure 2 Cytotoxicity assay. Human Sertoli cells cultured for 1, 2 and 3 days in the presence of either $CdCl_2$ (0.5, 5 and 20 μM) or BPA (0.4, 4 40, and 200 μM) or in the absence of both toxicants (control, Ctrl) were subjected to a cytotoxicity assay based on the reduction of the tetrazolium dye XTT. Each data point is a mean ± SD of $n = 3$ experiments.**$P < 0.01$.

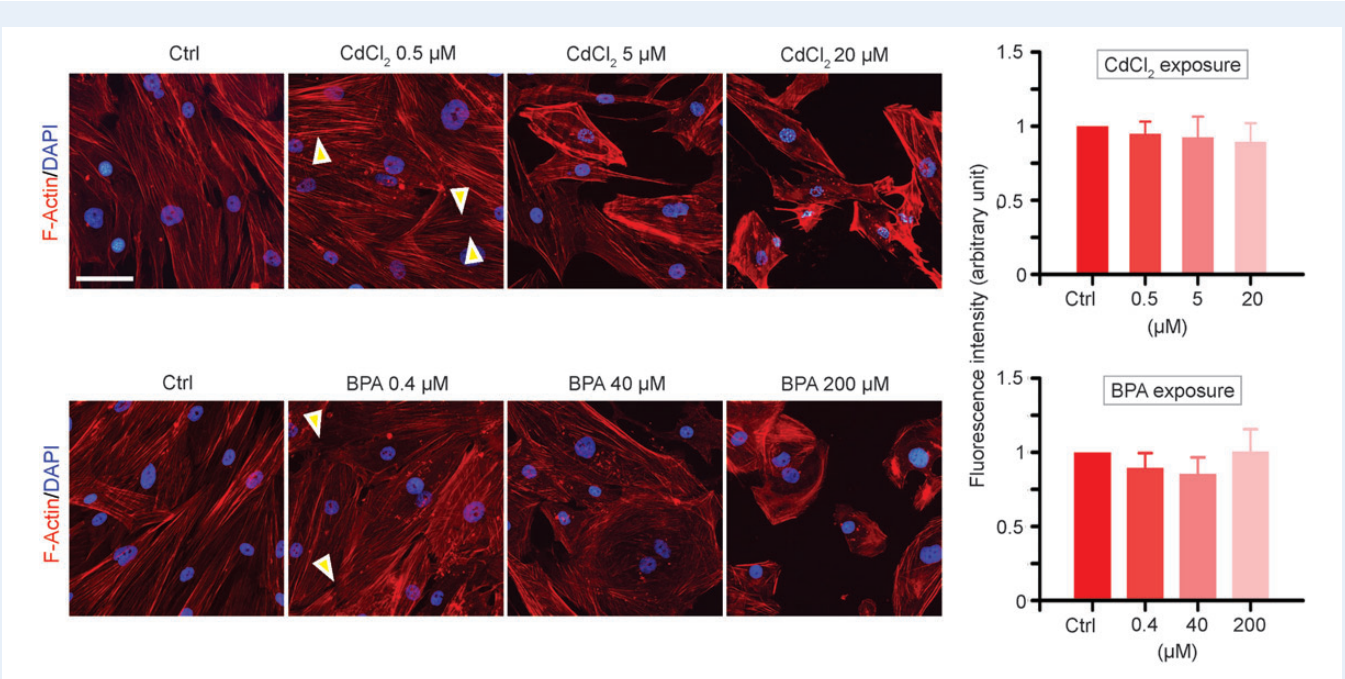

Figure 3 A study to assess changes in the F-actin organization in human Sertoli cells following exposure to $CdCl_2$ or BPA. Cells were exposed to each toxicant at specified concentrations for 2 days, fixed and stained for F-actin using rhodamine-phalloidin (red fluorescence) as shown on the left panel. Following exposure to toxicants, actin microfilaments were found to be truncated and depolymerized (annotated by 'yellow' arrowheads). Furthermore, actin microfilaments were shown to be retrieved into the cell cytosol instead of stretching out across the cell cytosol as shown in normal control cells. At these doses, no cytotoxicity was detected at up to 3 days as shown in Fig. 2. Cell nuclei were visualized by DAPI. On the right panel based on image analysis of fluorescence signals, the overall levels of F-actin in the cells in both treatment groups versus controls were not significantly different except there were changes in distribution and/or localization as illustrated on the left panel. Scale bar = 40 μm, which applies to all other micrographs.

adhere to actin for their attachment. Earlier studies have shown that an extensive network of microfilaments creates arrays of actin bundles at the basal ES, which confer unusually high adhesive strength to the BTB. In addition, the conversion of 'bundled' to 'un-bundled/branched' configurations that likely facilitate the transport of preleptotene spermatocytes across the BTB at stage VIII of the epithelial cycle is a result of the concerted activity of the actin barbed end capping and bundling protein Eps8 (epidermal growth factor receptor pathway substrate 8) (Lie *et al.*, 2009) and actin barbed end polymerization protein Arp3 (actin-related protein 3) (Lie *et al.*, 2010, 2012). Furthermore, c-Src (a nonreceptor protein tyrosine kinase) and annexin II (also known as annexin A2, a putative substrate of c-Src) that are known to be involved in F-actin dynamics in mammalian epithelial cells and tissues (Frame, 2004; Tobe, 2010; Grieve *et al.*, 2012; Xiao *et al.*, 2012b) are also found in the testis and expressed predominantly by Sertoli cells (Lee and Cheng, 2005; Xiao *et al.*, 2013a). Treatment of human Sertoli cells with both toxicants for 2 days had relatively little effect on the expression of these proteins (except at high doses) (Fig. 4A). However, $CdCl_2$ at 5 and 20 μM and BPA at 200 μM caused the proteins to be re-distributed in human Sertoli cells from the cell periphery to the nuclei, with associated retraction of cytoplasmic processes (Fig. 4B) clearly illustrated in Arp3 immunostained cells dual-labeled with rhodamine-phalloidin (Fig. 4B). It is likely that the mislocalization and/or re-distribution of actin regulatory proteins perturbed the organization of F-actin in Sertoli cells (Fig. 3), thereby impeding the proper localization of cell adhesion protein complexes at the cell–cell interface (Fig. 1). Based on these findings, we propose a hypothetical model shown in Fig. 5, illustrating the likely events that lead to disruption of cell adhesion in human Sertoli cells following exposure to $CdCl_2$ or BPA. In brief, toxicants induce disorganization of actin microfilaments, making them incapable of assuming a 'bundled' configuration via changes in the localization of actin bundling protein Eps8 and branched actin polymerization protein Arp3 (Fig. 5). As such, adhesion protein complexes can no longer confer proper cell adhesion, leading to BTB disruption.

Discussion

Human Sertoli cells, when cultured *in vitro*, establish a functional epithelium that is associated with a physiological, tight junction-permeability barrier that mimics the Sertoli cell BTB *in vivo* (Chui *et al.*, 2011). These findings are analogous to primary rat or mouse Sertoli cells cultured *in vitro* which also establish a functional tight junction barrier that mimics the BTB *in vivo*, and this system has been widely used to study Sertoli cell BTB function *in vitro* (Janecki *et al.*, 1991, 1992; Grima *et al.*, 1992; Gye, 2003; Lui *et al.*, 2003a; Siu *et al.*, 2003; Chen *et al.*, 2012; Cheng and Mruk, 2012; Du *et al.*, 2013; Puri and Walker, 2013; Qiu *et al.*, 2013). More important, many earlier data obtained from this system have now been expanded and confirmed in subsequent studies *in vivo* that ravel the biology of the BTB (Lui *et al.*, 2003b; Wong *et al.*, 2004; Su *et al.*, 2012b; Wan *et al.*, 2013b). Herein, we have shown that BTB-associated proteins, such as ZO-1, N-cadherin and β-catenin, also assemble at the human Sertoli cell–cell interface to confer cell

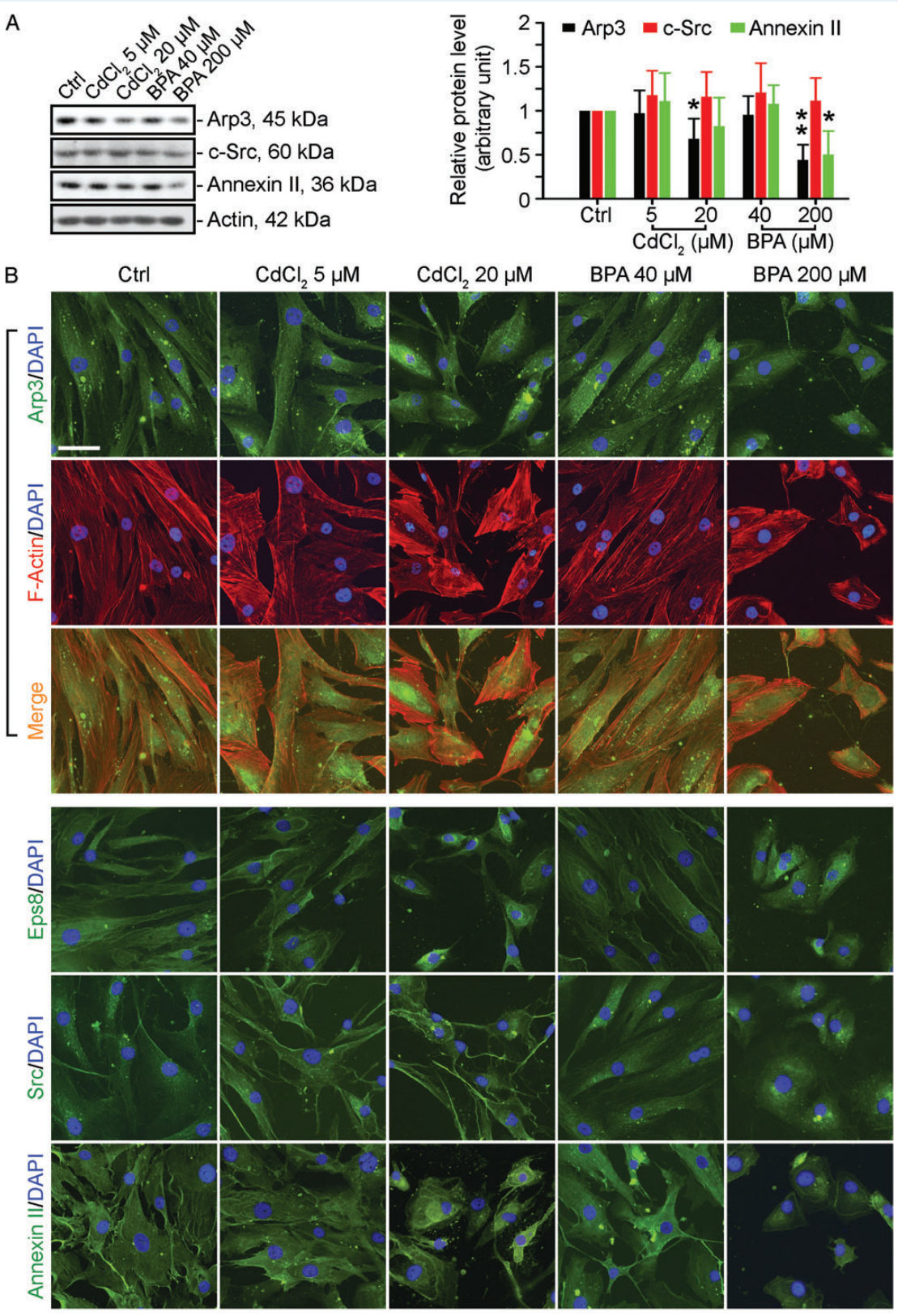

Figure 4 A study to assess changes in the localization and/or distribution of actin regulatory proteins in human Sertoli cell following exposure to $CdCl_2$ or BPA. (**A**) The expression of several actin regulatory proteins, such as Apr3, c-Src and annexin II, examined by immunoblotting of lysates of human Sertoli cells was not significantly affected by $CdCl_2$ or BPA unless a high dose was used (see left panel). These immunoblotting data are summarized in the histogram shown on the right panel. Each bar is a mean $\pm$ SD of $n = 3$ independent experiments. *$P < 0.05$; **$P < 0.01$ with treatment versus corresponding control (Ctrl) group. (**B**) Sertoli cells were exposed to either $CdCl_2$ (5 or 20 μM) or BPA (40 or 200 μM) for 2 days before being fixed and processed for immunofluorescence analysis to visualize the distribution and/or localization of Arp3, F-actin, Eps8, Src and annexin II. Cell nuclei were visualized by DAPI staining. Scale bar = 40 μm, which applies to all other micrographs.

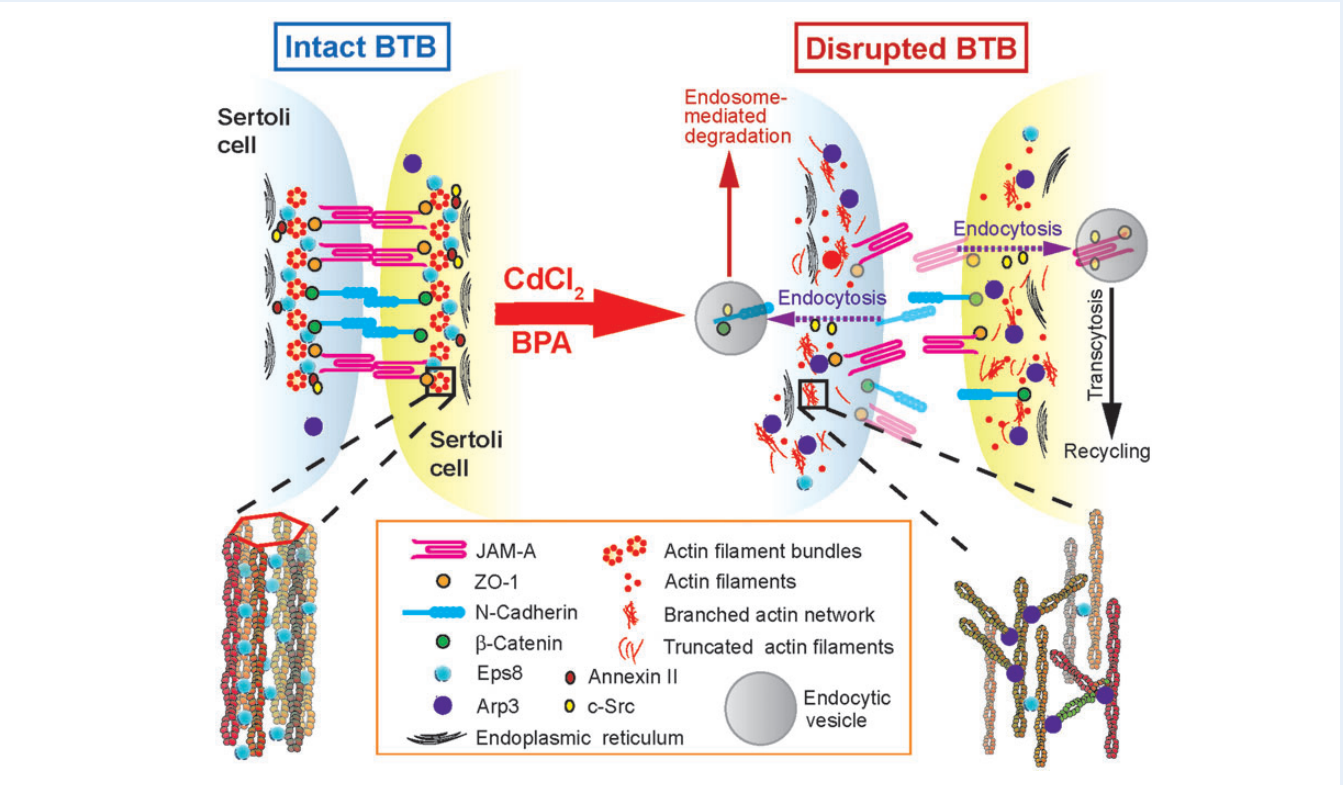

Figure 5 A schematic drawing illustrating a cascade of events leading to the mislocalization of adhesion protein complexes at the human Sertoli cell–cell interface following exposure to the environmental toxicants $CdCl_2$ or BPA. On the left panel, the intact BTB between adjacent Sertoli cells is maintained by a network of actin filament bundles, which is created by the actin barbed end capping and bundling protein Eps8; both c-Src and annexin II also promote the integrity of actin filament bundles, so that adhesion protein complexes such as JAM-A-ZO-1 and N-cadherin-β-catenin can properly localize to the Sertoli cell–cell interface (Fig. 1). However, following the exposure of human Sertoli cells to toxicants such as $CdCl_2$ or BPA, there are changes in the localization and/or distribution of the actin regulating proteins Arp3, Eps8, c-Src and annexin II. These changes promote the conversion of F-actin from a 'bundled' to a 'de-bundled/branched' configuration in which microfilaments are retrieved from near the cell surface, become truncated and depolymerized, and are no longer capable of supporting the proper localization of ZO-1- and β-catenin-based adhesion protein complexes that normally confers cell adhesion between Sertoli cells, and BTB disruption results. The precise mechanism(s) and/or pathway(s) by which c-Src and/or annexin II regulates actin microfilaments remain unknown. Recent studies have shown that they may be involved in regulating the events of endocytic vesicle-mediated protein trafficking (Xiao *et al.*, 2011) as hypothesized here (right panel).

adhesion, analogous to rodent Sertoli cells cultured *in vitro* (Su *et al.*, 2011; Lie *et al.*, 2012; Xiao *et al.*, 2012a, 2013b). As found in the rat in which environmental toxicants, such as $CdCl_2$, BPA and PFOS (perfluorooctanesulfonate), at non-cytotoxic concentrations were shown to induce reversible Sertoli cell tight junction disruption (Janecki *et al.*, 1992; Siu *et al.*, 2009c; Su *et al.*, 2012a; Qiu *et al.*, 2013; Wan *et al.*, 2013d), human Sertoli cells are also susceptible to treatment with either $CdCl_2$ or BPA.

It was long thought that mammalian Sertoli cells do not divide after puberty. For instance, Sertoli cells in rats are differentiated by ~15–17-day post-partum (dpp) (Orth, 1982) when they cease to divide at the time the BTB begins to assemble a functional immunological barrier, coinciding with the occurrence of the first meiosis I (Bergmann and Dierichs, 1983; Russell *et al.*, 1989; Mok *et al.*, 2011). In humans, Sertoli cells are differentiated and cease to divide at puberty at ~12 years of age (Sharpe *et al.*, 2003). Thus, Sertoli cells isolated from 20-day-old rat testes are differentiated and cease to divide when cultured in chemically defined serum-free medium (Mather and Sato, 1979; Mruk and Cheng, 2011c). Recently this view has been challenged by results from several studies. Proliferation was observed in Sertoli cells in adult Djungarian hamsters that were exposed to short day photoperiod to suppress gonadotrophins and then treated with FSH, suggesting that the cells were not terminally differentiated but existed in a transitional state, exhibiting both undifferentiated and differentiated features (Tarulli *et al.*, 2006). In mice, the proliferation of adult Sertoli cells *in vitro* in the absence of specific hormonal supplementation was found to be associated with a 70% decrease in expression of the cell cycle inhibitor CDKN1B (P27kip1), and a 2-fold increase in the levels of the proliferation inducer ID2 (inhibitor of DNA binding/differentiation) (Ahmed *et al.*, 2009). We are able to isolate routinely a small number of human adult Sertoli cells from normal adult testis from cadaveric tissue that actively divide in culture in the absence of specific hormone treatment, possess immunoregulatory activity, and are able to form tight junctions (a property unique to Sertoli cells in the testes) (Chui *et al.*, 2011). However, it is noted that fetal bovine serum was included in the culture medium used in these earlier studies (Ahmed *et al.*, 2009; Chui *et al.*, 2011). Nonetheless, the fact that we can isolate only a relatively small number of cells that possess this ability from the testes suggests

that they represent a subpopulation of Sertoli cell-progenitor cells as has been suggested (Hayrabedyan *et al.*, 2012). However, it is not clear if this is the case or if adult Sertoli cells in general can resume robust proliferation *in vitro* in the absence of specific hormone supplementation but with fetal bovine serum in the culture medium. Fluorescence-assisted cell sorting (FACS) revealed that proliferative human Sertoli cells and mesenchymal stem cells express a number of the same cell surface antigens, suggesting that the ability of this population of adult human Sertoli cells to actively divide may be another characteristic that is shared with mesenchymal stem cells (Chui *et al.*, 2011). Recently, it was shown that knockdown of the expression of c-myc reduced WNT/β-catenin-mediated stimulation of the proliferation of adult human Sertoli cells (Li *et al.*, 2012). Interestingly, proliferation of human umbilical cord blood-derived mesenchymal stem cells by treatment with prostaglandin E(2) was also induced by β-catenin-mediated c-Myc and VEGF expression (Jang *et al.*, 2012). Needless to say, these findings illustrate that it may be feasible to use human Sertoli cells for mechanistic and functional studies without the need for fresh primary cultures from human testes. Herein, we also show that even in the absence of FBS, human adult Sertoli cells, unlike rat neonatal Sertoli cells, remain proliferative, but require twice the time for plated cells to reach ∼70–80% confluence. This observation is also consistent with a recent study that reported that differentiated and non-dividing post-pubertal rat Sertoli cells resumed proliferation after transplantation (Mital *et al.*, 2013), illustrating differentiated Sertoli cells from both humans and rodents can be de-differentiated and mitotically active, and can even proliferate slowly, perhaps in response to 'stress' conditions which may be a necessary physiological response for species survival. Collectively, these findings illustrate that the human Sertoli cell *in vitro* system as characterized herein is a useful system to study BTB function, and it is physiologically relevant to the BTB in humans *in vivo*.

Using Sertoli cells obtained from three different human donors, we observed that exposure to $CdCl_2$ or BPA at doses considered non-cytotoxic induces mislocalization of adhesion proteins at the cell–cell interface. This in turn is mediated via changes in F-actin organization in which microfilaments became truncated and defragmented, retracting from the cell–cell interface. As a consequence, cell adhesion protein complexes such as N-cadherin- β-catenin fail to anchor onto the actin-based cytoskeleton, redistribute from the cell–cell interface into the cell cytosol and perturb Sertoli cell adhesion. These toxicant-induced changes in actin microfilament organization at the Sertoli cell BTB are mediated, in part, by changes in the localization and/or distribution of actin barbed end capping and bundling protein Eps8 and branched actin polymerization protein Arp3. *In vivo* rat studies have shown that the restrictive spatiotemporal expression of these two proteins during the epithelial cycle plays a crucial role in regulating the conversion between the 'bundled' and 'de-bundled/branched' configuration of the actin microfilaments at the Sertoli cell BTB (Lie *et al.*, 2009, 2010). For instance, Eps8 is highly expressed at the Sertoli cell BTB at stage V–VII of the epithelial cycle to maintain the integrity of the actin filament bundles, but Eps8 is considerably diminished at stage VIII of the cycle to allow actin microfilament restructuring to accommodate the transport of preleptotene spermatocytes at the BTB (Lie *et al.*, 2009). In contrast, Arp3 that induces barbed end branched actin nucleation and polymerization (Cheng and Mruk, 2011; Rotty *et al.*, 2013), effectively converting bundled actins to a branched network, and destabilizing the basal ES at the BTB, is weakly expressed at the basal ES/BTB at stage V-VII (Lie *et al.*, 2010). But Arp3 is intensively expressed at the basal ES/BTB in stage VIII of the epithelial cycle (Lie *et al.*, 2010). Thus, the concerted efforts of Eps8 and the Arp2/3 complex confer plasticity to the actin cytoskeleton in Sertoli cells during the epithelial cycle, which in turn facilitate BTB restructuring and to accommodate the transport of preleptotene spermatocytes at the BTB at stage VIII of the epithelial cycle (Cheng *et al.*, 2013).

In brief, changes in the localization and/or distribution of Arp3 and Eps8 perturb the homeostasis of actin microfilament organization. In turn, there is perturbation of the anchorage of adhesion protein complexes to the underlying actin-based cytoskeleton, causing junction disruption at the Sertoli cell–cell interface. These changes are accompanied by redistribution of the c-Src and annexin II which are the proteins that play a role in actin dynamics and endocytic vesicle-mediated trafficking in epithelial cells (Grieve *et al.*, 2012; Xiao *et al.*, 2012b). For instance, it is known that annexin 2, a putative substrate of c-Src, is involved in remodeling of actin microfilaments via its effects on barbed end capping (Hayes *et al.*, 2006), and also activation of cofilin (de Graauw *et al.*, 2008), which is an actin-binding and depolymerization protein that induces actin filament disassembly by severing microfilament at its minus end (Bravo-Cordero *et al.*, 2013). c-Src, on the other hand, is a crucial regulator of endocytic vesicle-mediated trafficking between the plasma membrane and late endosomes and lysosomes (Tsutsumi *et al.*, 2008; Sato *et al.*, 2009). It is also increasingly clear that annexin II is involving in Rab11a-mediated protein recycling (Lock and Stow, 2005; Bryant *et al.*, 2010). Thus, it is tempting to speculate that the toxicant-induced changes in the localization and/or distribution of c-Src and annexin II may impede actin microfilament dynamics by enhancing cofilin-mediated actin cleavage and also by disrupting endocytic vesicle-mediated protein trafficking. As noted in Fig. 5, we provide a hypothetic model based on these data, which could form the basis of future functional studies of human Sertoli cells cultured *in vitro* to examine the mechanisms by which environmental toxicants perturb human reproductive function.

Authors' roles

C.Y.C. conceptualized and designed research; X.X. and E.I.T. performed research and acquired data; C.Y.C., X.X. and D.D.M. analyzed and interpreted data; X.X. and C.Y.C. drafted the article; C.M.J., P.J.T., C.K.C.W., W.M.L., B.S. and C.Y.C. revised article critically for its intellectual content; X.X., D.D.M., E.I.T., C.K.C.W., W.M.L., C.M.J., P.J.T., B.S. and C.Y.C. were involved in final approval of the version to be published.

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Conflict of interest

C.M.J. has equity in MandalMed which has a pending patent on proliferative human Sertoli cells. All other authors have no competing interest.

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