

# Impacts of environmental toxicants on male reproductive dysfunction

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**Male infertility caused by exposure to environmental toxicants such as cadmium, mercury, bisphenol A (BPA) and dioxin is a global problem, particularly in industrialized countries. Studies in the testis and other organs have illustrated the importance of environmental toxicant-induced oxidative stress in mediating disruption to cell junctions. This, in turn, is regulated by the activation of PI3K/c-Src/FAK and MAPK signaling pathways, with the involvement of polarity proteins. This leads to reproductive dysfunction such as reduced sperm count and reduced quality of semen. In this review, we discuss how these findings can improve understanding of the modes of action of environmental toxicants in testicular dysfunction. Thus, specific inhibitors and/or antagonists against signaling molecules in these pathways may be able to 'reverse' and/or 'block' the disruptive effects of toxicant-induced damage. Additional studies comparing high-level acute exposure versus low-level chronic exposure to environmental toxicants are also needed to fully elucidate the underlying molecular mechanism(s) by which these toxicants disrupt male reproductive function.**

## Introduction

Infertility is defined as the 'inability to conceive after frequent and unprotected sexual intercourse for more than a year', a condition that currently affects 15% of couples worldwide [1,2]. Approximately 50% of the cases are attributed to the male partner [1,3]. Pathophysiological conditions (e.g. varicocele and urogenital infection) are directly linked to only 23% of all cases of male infertility; environmental factors such as exposure to environmental toxicants are one of the major causes of the remaining cases [4]. Numerous environmental toxicants have been shown to adversely affect spermatogenesis in rodents and humans (Box 1), which can lead to low sperm count, abnormal sperm morphology and poor semen quality [5–7]. These include a vast variety of elements and different classes of compounds, such as heavy metals (e.g. mercury, lead and cadmium), organic polychlorinated dibenzodioxins (e.g. 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin), dicarboximide fungicides (e.g. vinclozolin) and environmental phenols [e.g. 2, 2-bis[4-hydroxyphenyl] propane or bisphenol A (BPA)], which are often released into the environment during industrial processes. In turn, humans take up these environmental toxicants by ingestion of contaminat-

ed food and water, usage of consumer products (e.g. plasticware and cosmetics) and inhalation of polluted air [5–9].

According to the National Health and Nutrition Examination Survey conducted by the USA Centers for Disease Control and Prevention, biologically active levels of BPA were detected in the urine of >90% of the general population of the USA [10,11]. In China, where there has been a rapid increase in the number of automobiles and industrial production, metabolites of polycyclic aromatic hydrocarbons were detected in 100% of test candidates in a recent study, and higher levels were associated with male infertility [12]. These results collectively indicate that environmental toxicants have infiltrated different aspects of human life and are affecting most people in 'developing' and 'developed' countries.

Environmental toxicants can disrupt male reproductive function by affecting the endocrine system (i.e. acting as endocrine disruptors) by altering gene expression that is pertinent to spermatogenesis, by altering steroidogenesis, and by exerting epigenetic effects, which can result in abnormalities in the reproductive system of male offspring up to four generations after *in utero* exposure [13–18]. For example, BPA is a known disruptor of the endocrine system and, at an environmentally relevant dose level, it can mediate its biological effects (e.g. increase proliferation of testicular seminoma cells) through putative membrane estrogen receptors (ERs), one of which may be G-protein coupled receptor 30 (GPR30) [19]. Indeed, various common environmental toxicants (e.g. polychlorinated biphenyl and methoxychlor) have been found to bind to the classical nuclear ER at an affinity 1000–2000-times lower than the endogenous 17 $\beta$ -estradiol [20]. This suggests that these toxicants can mediate their effects via non-genomic membrane ER-initiated pathways [21]. In addition, BPA was shown to cause defects in male and female reproductive systems after prenatal and neonatal exposure at <50  $\mu$ g/kg/day, (the current 'safe' dose acceptable for daily intake by humans recommended by the USA Food and Drug Administration (FDA) [22]). This suggests that environmental toxicants can affect reproductive systems via multiple pathways and different mechanisms.

Increasing evidence suggests that an induction of oxidative stress in the testis represents another common response after exposure to environmental toxicants. Increase in oxidative stress can be seen in  $\leq$ 80% of clinically proven infertile men, and exposure to environmental toxicants is a major factor contributing to such an increase [23–25]. Environmental toxicants that have been shown to induce

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### Box 1. Spermatogenesis in mammalian testes

Spermatogenesis is the generation of spermatozoa (haploid, 1n) from spermatogonia (diploid, 2n) via mitosis, meiosis and spermiogenesis [112,113]. Type A spermatogonia, which are located at the basal compartment of the seminiferous tubule, undergo mitosis and differentiation to form type-B spermatogonia, which are the germ cells that differentiate into primary spermatocytes. After meiosis, primary spermatocytes develop into spermatids (haploid, 1n), which undergo extensive morphological and molecular changes to develop into spermatozoa via spermiogenesis. The entire process of spermatogenesis is complicated, and the success of producing healthy and adequate spermatozoa for fertilization with the ovum is dependent upon at least three factors. First, similar to oogenesis, spermatogenesis is under the influence of hormones such as follicle-stimulating hormone (FSH), testosterone and estrogen [110,114]. Thus, tightly regulated hormonal levels by the pituitary gland and by Leydig cells and Sertoli cells are necessary for normal spermatogenesis. Second, the integrity of the blood–testis barrier (BTB) is essential to create a unique microenvironment for meiosis and the development of post-meiotic germ cells to isolate these events from the systemic circulation, which would otherwise develop anti-sperm antibodies. The BTB between Sertoli cells comprises a co-existing tight junction (TJ), desmosome, gap junction and a testis-specific adherens junction (AJ) called the basal ectoplasmic specialization (ES). The basal ES is typified by the presence of actin filament bundles ‘sandwiched’ between the plasma membrane and the cisternae of endoplasmic reticulum in two

neighboring Sertoli cells. It is believed that the presence of multiple junction types at the BTB contributes to the inherent tightness of this blood–tissue barrier, which is among the ‘tightest’ in mammals. However, recent studies show that the unique structural aspects of the BTB, such as the presence of focal adhesion protein FAK, also renders the testis highly susceptible to damage from environmental toxicants (see text for details). Third, during spermiogenesis when round spermatids differentiate into elongated spermatids, genetic material in the spermatid head condense to form the tightly packed nucleus with the formation of an acrosome above the head region and elongation of the spermatid tail. During this time, spermatids migrate towards the adluminal compartment of the seminiferous tubule until elongated spermatids are released into the tubule lumen via the disassembly of another ES, the apical ES, at spermiation. The apical ES is the only anchoring device surrounding the entire head region of step 8-19 spermatids (i.e. elongating and elongated spermatids) in rats (in humans, only six steps of spermatids are found during spermiogenesis versus 19 and 16 in rats and mice, respectively). In contrast to the basal ES, actin filament bundles are restricted to the Sertoli cell at the Sertoli cell–spermatid interface. The apical ES anchors developing spermatids in the seminiferous epithelium until they are fully developed. Thus, disruption of the apical ES (e.g. by environmental toxicants) causes the premature release of spermatids that are structurally defective (e.g. lack of acrosome and/or tail) and which are incapable of fertilizing the ovum.

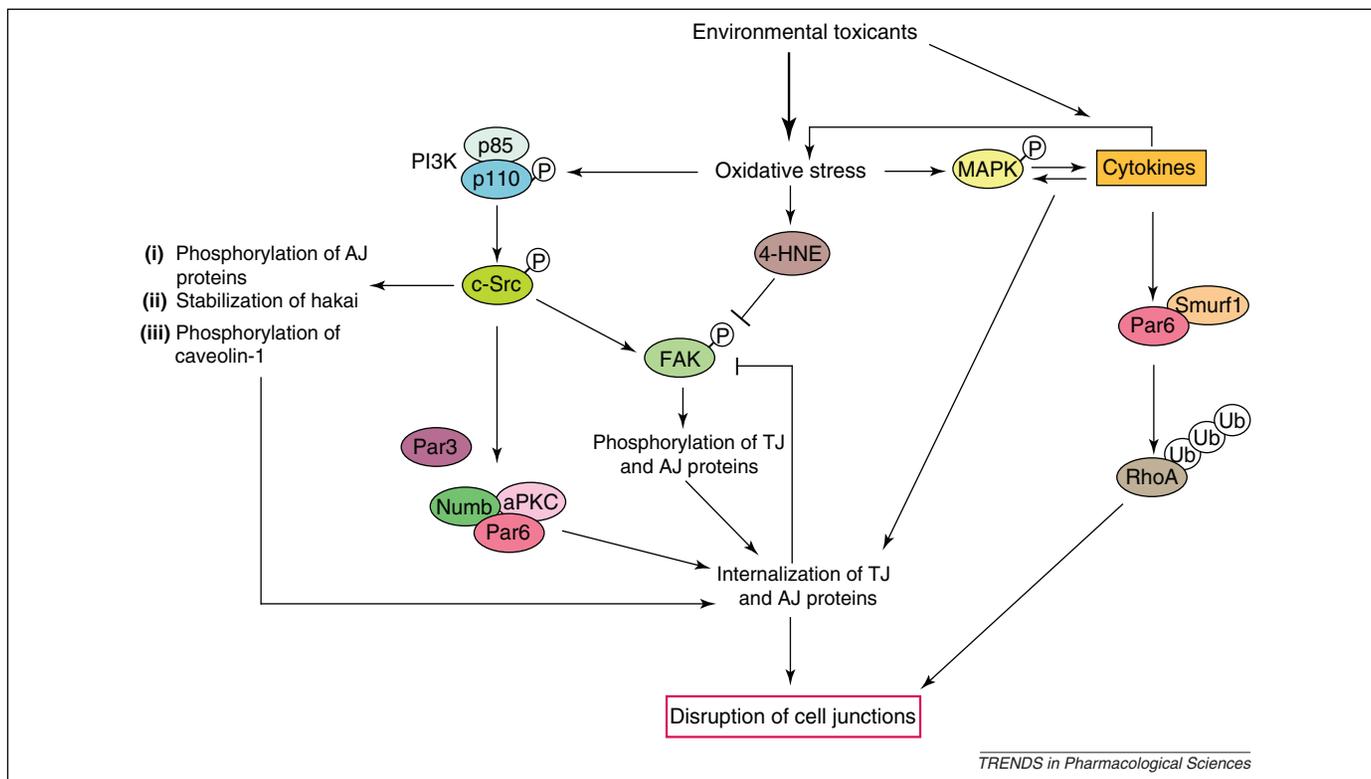
oxidative stress in the testis are highly heterogeneous, with different chemical structures, and include cadmium [26,27], bisphenol A [28] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [27,29,30]. Interestingly, these environmental toxicants commonly increase oxidative stress by downregulating the production of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase [26,28,30,31]. In turn, excessive amounts of reactive oxygen species (ROS) are produced. ROS damage the lipids, proteins, carbohydrates and DNA in cells. Importantly, these observations were confirmed in studies illustrating that co-administration of antioxidants such as vitamin E with environmental toxicants could alleviate the pathophysiological effects (e.g. reduction in sperm count) of toxicants in the testis [32,33]. These findings demonstrate that oxidative stress induced by environmental toxicants is one of the major contributing factors to male infertility. In fact, oxidative stress has long been linked to male infertility; although most studies have focused on its roles in causing abnormalities in germ cells and apoptosis [24,34–36].

Recent studies have shown that environmental toxicant-induced oxidative stress can cause male infertility by disrupting the cell junctions and adhesion between Sertoli–Sertoli cells and/or Sertoli–germ cells via the phosphatidylinositol 3-kinase (PI3K)/c-Src/focal adhesion kinase (FAK) signaling pathway. These findings are significant because human exposure to environmental toxicants is often below toxic dose levels that cause cell death [37]. In this review, we examine these findings and also discuss the pathophysiological roles of mitogen-activated protein kinase (MAPK) and cytokines (which are activated during exposure to environmental toxicants) in the testis. Significantly, these signaling pathways were found to mediate their disruptive effects on intercellular junctions via polarity proteins. We highlight recent studies that show how environmental toxicants induce male reproductive dysfunction through these signaling pathways in

response to an increase in oxidative stress in the testis. Conceptually, these studies can provide a framework to develop approaches to treat and/or manage the damaging effects of environmental toxicants to male reproductive health.

### Environmental toxicant-induced oxidative damage activates the PI3K/c-Src signaling pathway

Exposure to different classes of environmental toxicants commonly increases oxidative stress in the testis [26–31]. Oxidative stress is known to increase epithelial and endothelial permeability by disrupting tight junctions (TJ) and adherens junctions (AJ) between cells [38–40]. In fact, oxidative stress has been implicated in epithelial–mesenchymal transition, a process in which epithelial cells lose their junctional structures and polarity to acquire migratory capacity like mesenchymal cells [41]. Intensive research in the past decade revealed that PI3K plays a central part in mediating oxidative stress-induced junction disruption (Figure 1). Upon challenge by oxidative stress, the regulatory subunit of PI3K p85 translocates from the cytosol to near the cell–cell interface [42,43]. Such redistribution of p85 to the plasma membrane is associated with an increase in tyrosine phosphorylation of the catalytic subunit p110 [43]. Activation of PI3K in turn stimulates a non-receptor tyrosine kinase c-Src [44]. In the testis, c-Src is localized predominantly and stage-specifically at the blood–testis barrier (BTB) and apical ectoplasmic specialization (ES) (Box 1), associating with connexin43/plakofillin-2 and  $\beta$ 1-integrin/laminin $\alpha$ 3 $\beta$ 3 $\gamma$ 3 protein complexes at the corresponding cell junctions, respectively [45,46]. Activation of the PI3K/c-Src signaling pathway in response to oxidative stress induced by environmental toxicants could be a common mechanism by which the toxicants trigger damage to the testis. Early evidence shows that the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the testis are caused by an induction in c-Src kinase activity [47].



**Figure 1.** Signaling pathways activated by environmental toxicants via increase in oxidative stress. Oxidative stress induced by environmental toxicants activates the PI3K/c-Src/FAK pathway, which subsequently controls the phosphorylation of caveolin-1, TJ and/or AJ proteins, the stability of E3 ubiquitin ligase Hakai, and the interaction of polarity proteins with the endocytic adaptor Numb. This leads to the internalization of TJ and AJ proteins at the cell-cell interface. Disassembly of cell junctions induced by oxidative stress can occur concurrently with the down-regulation and/or inactivation of integrins (note: integrins are found at the apical ES, a testis-specific anchoring junction type [113]), which imposes a feedback mechanism to dephosphorylate FAK. Aldehydes such as 4-hydroxy-2-nonenal (4-HNE) are produced during oxidative stress to inactivate FAK. In addition, environmental toxicants induce the production of cytokines which are also regulated by the activation of MAPK via oxidative stress. Cytokines stimulate the production of reactive oxygen species (ROS) from leukocytes to further increase oxidative stress. Cytokines and the activation of MAPK together result in endocytic vesicle-mediated internalization of TJ and AJ proteins. Polarity proteins such as Par6 are also involved in mediating the action of cytokines to recruit the E3 ubiquitin ligase Smurf1 for the polyubiquitination and degradation of RhoA, which is important for the disruption of cell junctions. This illustrates that crosstalk exists between the PI3K/c-Src/FAK and cytokines/MAPK pathways via polarity proteins as their common downstream signaling mediators.

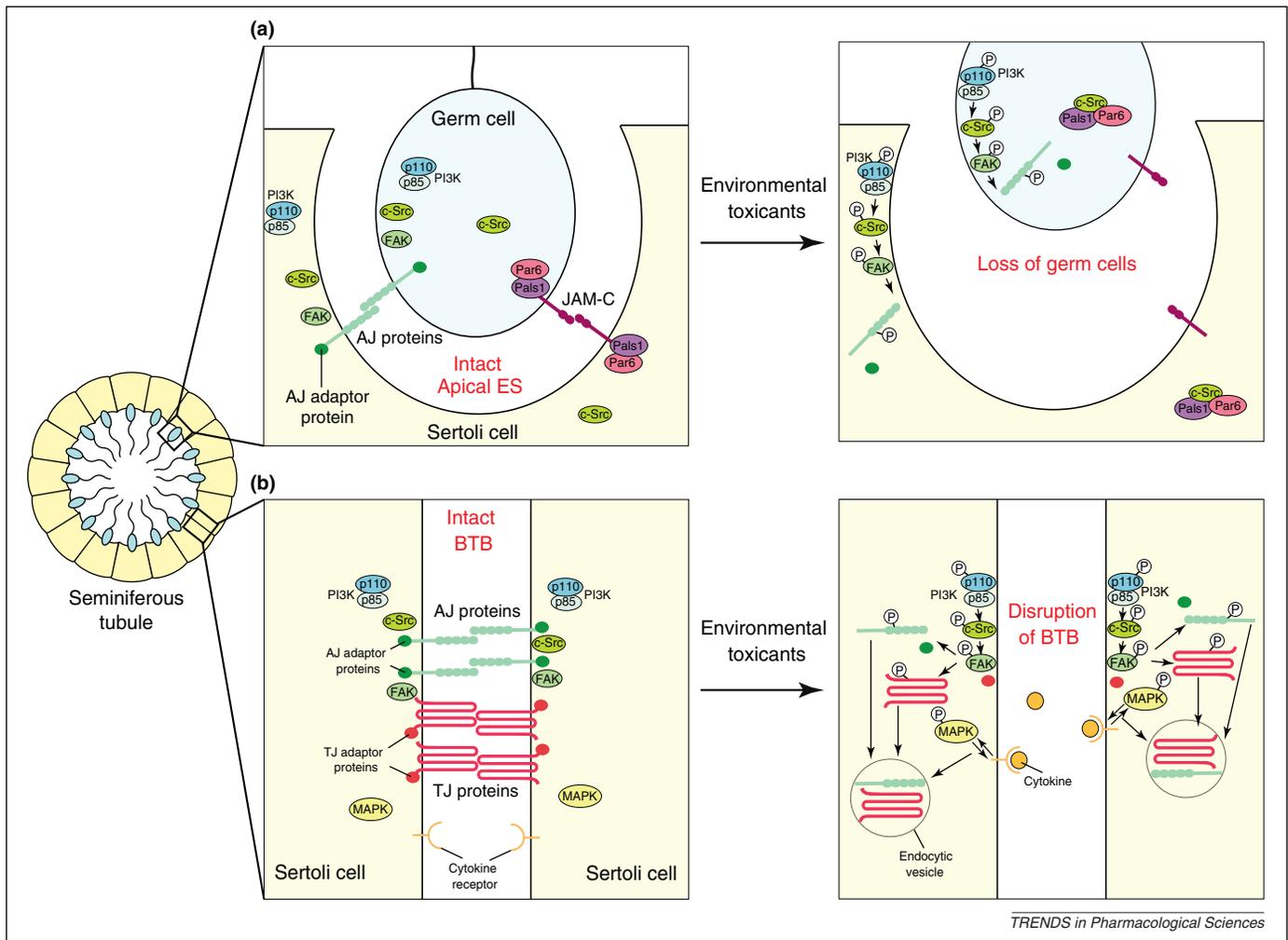
Furthermore, significant increase in the c-Src level has also been detected in the testis after cadmium exposure in rodents, indicating that c-Src is activated in response to multiple environmental toxicants [48,49].

#### FAK is a target of the PI3K/c-Src signaling pathway during environmental toxicant-induced junction disruption

In epithelial cells, FAK is a substrate of c-Src. The FAK-Src dual kinase complex is known to regulate multiple cellular events in physiological (e.g. cell migration) and pathological (e.g. tumor development) conditions [50,51]. Indeed, FAK is a downstream mediator of the PI3K/c-Src signaling pathway in oxidative-stress induced junction disruption [44,52,53]. It is believed that membrane translocation and activation of PI3K and c-Src by oxidative stress activates FAK via phosphorylation. This causes an increase in tyrosine phosphorylation of junction proteins (e.g. occludin, N-cadherin) by FAK to alter the adhesive function of protein complexes at TJ and/or AJ. Interestingly, depending on the cell type, the source of ROS and the duration of treatment, FAK can be dephosphorylated [54–57]. Altogether, it is likely that, at the initial phase during oxidative stress, FAK is activated by c-Src to cause unwanted phosphorylation of junction proteins at the cell-cell interface. This induces redistribution of proteins from the cell-cell interface to the cytosol, and

leads to the disruption of TJ and AJ [40]. In addition, cell adhesion is further compromised by the dissociation of integral membrane proteins (e.g. occludin and N-cadherin) with the corresponding cytoplasmic adaptors (e.g. ZO-1 and  $\beta$ -catenin) [40,42]. Disassembly of focal adhesion contact (e.g. by decreasing and/or inactivation of integrins [55]) and the formation of biologically active aldehydes during oxidative stress (e.g. 4-hydroxy-2-nonenal, 4-HNE [58]) inactivate FAK via dephosphorylation. Endocytosed integral membrane junction proteins eventually undergo endosome-mediated intracellular degradation, which further destabilizes junctions at the cell-cell interface. Furthermore, a biphasic effect on the phosphorylation of FAK has been reported, illustrating that the duration of oxidative stress is a critical factor in determining the activation or inactivation of FAK in the cell epithelium [54]. These results illustrate that FAK is a critical mediator of the disruption of TJ and AJ during oxidative stress. Therefore, the critical phosphorylation site of FAK (e.g. Tyr<sup>397</sup>) might provide a novel therapeutic target to protect the testis from oxidative stress.

This hypothesis is further supported by findings that illustrate FAK is a molecular target of cadmium in the testis [49]. FAK is one of the best studied non-receptor tyrosine kinases in the testis [49,59–61]. FAK structurally interacts with the occludin-ZO-1 protein complex between Sertoli cells at the BTB in the seminiferous epithelium



**Figure 2.** Pathophysiological effects of environmental toxicants in the seminiferous epithelium of mammalian testes. **(a)** At the apical ES, AJ proteins (e.g. N-cadherin and nectin) are present to adhere germ cells onto the Sertoli cells in the seminiferous epithelium. JAM-C (which is regarded as a TJ protein in epithelial cells) is also found at the apical ES, although no TJ ultrastructure is visible under transmission electron microscopy between elongating spermatids and Sertoli cells. After exposure to environmental toxicants, the PI3K/c-Src/FAK pathway is activated to phosphorylate AJ proteins. This causes the internalization of AJ proteins and dissociation from their corresponding adaptors. Adhesion of germ cells in the seminiferous epithelium is further weakened by the increase in association between the c-Src and Par6/Pals1 complex, which sequesters them from JAM-C, thereby destabilizing the JAM-C-based adhesion protein complexes. As a result, germ cells are eventually released from the seminiferous epithelium prematurely due to a disruption of adhesion complexes at the apical ES. **(b)** At the BTB, activation of the PI3K/c-Src/FAK pathway by environmental toxicants causes the phosphorylation of TJ and AJ proteins. Furthermore, induction of cytokines (e.g. TGF- $\beta$ 3) and activation of MAPK also leads to enhancement in endocytosis of junction proteins. The cell junctions at the BTB are disrupted, which destroys the microenvironment necessary for the normal development of germ cells. This reduces the sperm count and the number of normal sperm, leading to infertility or subfertility.

[49,59]. In addition, the activated and phosphorylated forms of FAK (e.g. phospho-FAK-Tyr<sup>397</sup> and phospho-FAK-Tyr<sup>576</sup>) are found to associate with  $\beta$ 1-integrin between elongating/elongated spermatids and Sertoli cells at the apical ES [61]. Cadmium is known to disrupt cell junctions in the testis, such as the Sertoli cell BTB, by accelerating endocytosis of integral membrane proteins (e.g. occludin, ZO-1 and N-cadherin) (Figure 2) [59]. A knockdown of FAK by RNAi in the Sertoli cell epithelium with an established functional BTB was shown to desensitize Sertoli cells to cadmium-induced TJ barrier disruption [49]. This, at least in part, is due to the loss of FAK to elicit redistribution of ZO-1 from the cell-cell interface to the cytosol to maintain cell junction integrity [49]. Furthermore, due to the physical 'intimacy' between FAK and junction proteins at the BTB, FAK mediates the disruption of the BTB (which occurs at  $\sim$  7–16 h post-cadmium treatment at 3 mg/kg b.w., i.p.) more rapidly and efficiently than that of the endothelial TJ barrier in the interstitial space

(which occurs at  $\sim$ 20–24 h) where FAK is restricted to the focal adhesion contact [48,59] and is not an integrated component of the occludin-ZO-1 protein complex in the TJ barrier of the microvessel [51,62]. The unique distribution of FAK at the BTB thus explains the unusual susceptibility of the testis to cadmium-induced damage. Although it remains unknown if FAK mediates other environmental toxicant-induced testicular dysfunction at the BTB, evidence gathered from oxidative stress studies [44,52,56] supports the notion that FAK is probably the common target in mediating the cell-junction damage caused by environmental toxicants.

#### Activation of c-Src enhances the endocytosis of junction proteins

In addition to activating FAK, c-Src participates directly in the regulation of cell junctions. Elevated expression and/or activity of c-Src is widely observed in multiple types of carcinoma. In particular, activation of c-Src is linked to

cancer progression, which is signified by the loss of cell–cell adhesion and cell polarity [62,63]. In agreement with the observations in tumor cells, overexpression and/or activation of c-Src always leads to disruption of cell junctions in normal epithelial and endothelial cells [64,65]. It is also commonly activated during environmental toxicant-induced junction disruption [44,66]. In response to oxidative stress, Tyr<sup>418</sup> of c-Src is known to be phosphorylated for its activation [67,68]. Thereafter, c-Src likely undergoes a conformational change that releases the auto-inhibitory C-terminal domain to allow access of substrates to the catalytic kinase domain [63].

Activation of c-Src leads to a disruption of cell junctions via three pathways. First, c-Src activation is known to induce phosphorylation of AJ proteins such as E-cadherin, N-cadherin and p120<sup>ctn</sup> [64,69]. p120<sup>ctn</sup> is a substrate of c-Src and a peripheral adaptor of cadherins. Its association with cadherins is required to maintain the localization of cadherins on the cell surface [70]. Tyrosine phosphorylation of p120<sup>ctn</sup> by oxidative stress-induced c-Src activation triggers the translocation of p120<sup>ctn</sup> from the plasma membrane to the early endosomal compartment. This leads to internalization and endosome-mediated degradation of N-cadherin and disassembly of the AJ. Interestingly, phosphorylation of p120<sup>ctn</sup> by c-Src is isoform-specific which demonstrates specificity in the kinase substrate [69]. Second, c-Src functions to stabilize Hakai, an E3 ubiquitin-ligase involved in the ubiquitination of E-cadherin. Activation of c-Src increases the association between E-cadherin and Hakai, which in turn causes the ubiquitination and internalization of E-cadherin [64]. Furthermore, binding of Hakai to E-cadherin competes with the interaction of p120<sup>ctn</sup> to E-cadherin, which further enhances the endocytosis of E-cadherin [64]. Third, activation of c-Src can induce the phosphorylation of the constituent protein of caveolae, caveolin-1, to interfere with the endocytosis and/or transcytosis of cell-junction proteins [67]. For example, c-Src phosphorylates Tyr<sup>14</sup> of caveolin-1 during oxidative damage to facilitate the dissociation of VE-cadherin and  $\beta$ -catenin [66]. Such disassembly of the AJ adhesion complex is accompanied by an increase in endocytosis and transcytosis of internalized proteins. These observations were supported by findings showing that caveolin-1<sup>-/-</sup> mice were resistant to oxidative stress-induced damage in vascular permeability [67]. In this context, environmental toxicants (e.g. BPA) that can mediate their actions through the non-genomic membrane ER-mediated pathway can exert their effects via PI3K/c-Src and/or MAPK (see below) [19,71,72]. However, it remains to be determined if changes in protein ubiquitination, endocytosis, recycling and/or transcytosis are involved.

#### Activation of MAPKs by environmental toxicants

The MAPK pathways have emerged as a common signaling platform for multiple environmental toxicants (Figure 1). Three MAPKs (extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38) have been shown to be activated in the testis after exposure to environmental toxicants (Table 1). MAPKs are involved in regulating normal reproductive functions in the testis, which include spermatogenesis (e.g. cell-cycle progression,

meiosis, BTB dynamics, cell adhesion dynamics and spermiogenesis), steroidogenesis, sperm hyperactivation and acrosome reaction [73,74]. As a result, unregulated activation of MAPKs by environmental toxicants imposes an array of pathophysiological effects on Sertoli cells, germ cells and Leydig cells in the testis. These include an increase in DNA damage and apoptosis, disruption of cell junctions and steroidogenesis [75,76]. MAPKs are activated by oxidative stress induced by environmental toxicants in cells and tissues. For example, blocking oxidative stress by free-radical scavengers (e.g. N-acetyl cysteine), reverses cadmium-induced MAPK activation [77,78]. This phenomenon is partly regulated by the inhibition of Ser/Thr protein phosphatases 2A (PP2A) and 5 (PP5) by oxidative stress, which results in an increase in phosphorylation of MAPK [78]. Unexpectedly, cadmium induces the expression of MAPK phosphatase-1 (MKP-1) [79], a major inhibitor of MAPK activation. Hence, the protective effect of MKP-1 to inactivate MAPK is superseded by inhibition of protein phosphatases PP2A and PP5, resulting in an overall increase in MAPK signaling after exposure to environmental toxicants. In addition, activation of ERK can lead to phosphorylation of c-Src, FAK and paxillin under oxidative stress, implying that MAPKs might be one of the upstream targets to activate these non-receptor tyrosine kinases [54].

Activation of MAPKs by environmental toxicants also upregulates the expression of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) in macrophages and monocytes [80,81], which can diffuse from microvessels in the interstitial space and disrupt the BTB because they are known to perturb the Sertoli cell TJ-permeability barrier (Figures 1 and 2) [82,83]. Similarly, cadmium and pollutants from motorcycle exhausts (e.g. polycyclic aromatic hydrocarbons) increase the expression of transforming growth factor- $\beta$  (TGF- $\beta$ ) and interleukin-6 (IL-6) in the testis, respectively [84,85]. TNF $\alpha$ , TGF- $\beta$  and IL-1 $\alpha$  are known to disrupt Sertoli–Sertoli and Sertoli–germ cell junctions via downregulation [82,86] and/or redistribution of junctional proteins [87–89] such as occludin, ZO-1 and N-cadherin in the seminiferous epithelium. Consequently, the loss of integral membrane proteins at the cell–cell interface causes disruption of the BTB and adhesion of germ cells in the seminiferous epithelium, which lead to the premature release of germ cells from the epithelium and hence infertility [82,86,87]. Furthermore, proinflammatory cytokines (e.g. IL-6 and TNF $\alpha$ ) activate leukocytes to produce ROS, which amplifies the deleterious effects of environmental toxicant-induced oxidative stress [24,36]. Intriguingly, further studies in the testis found that cytokines (e.g. TGF- $\beta$ 3) utilize the MAPK pathways to disrupt cell junctions [90].

Thus, MAPKs are the stimulator and mediator of cytokines induced by environmental toxicants. The involvement of MAPKs in environmental toxicant-induced signaling is further complicated by the self-protective program of the testis. This protective action is mediated by the JNK pathway to induce a non-specific protease inhibitor ( $\alpha_2$ -macroglobulin) to limit the damage caused by cadmium [91]. Although  $\alpha_2$ -macroglobulin cannot rescue the loss of germ cells from the seminiferous epithelium and

**Table 1. Pathophysiological effects of environmental toxicants in the testis via activation of MAPK and cytokines**

Chemical group	Environmental toxicant	Animal model/ cell type	MAPK activated	Cellular responses	Effects on cytokine production	Ref
Heavy metal	Cadmium	Primary pig Sertoli cells	p38	↓Cell proliferation, ↑DNA damage, ↑apoptosis	ND*	[75]
		Rat testis	p38, JNK	↓TJ and AJ protein levels but ↑ p120 <sup>ctn</sup> , ↑α <sub>2</sub> -macroglobulin, damage to the BTB, apical ES and endothelial TJ, ↑premature release of germ cells, ↑redistribution of TJ proteins from the cell–cell interface to the cytoplasm, ↑association between FAK, occludin and ZO-1	↑TGFβ2, TGFβ3	[85], [48, 49,59,91]
		Rat Sertoli cell-gonocyte coculture	JNK, p38	↑Apoptosis, ↑polyubiquitination of proteins, ↑detachment of gonocytes from Sertoli cells	ND	[115]
Environmental phenols	Bisphenol A	Rat testis, primary rat Sertoli cells	ERK	Disruption of the BTB in immature rats, ↓occludin and N-cadherin on Sertoli cell surface, ↓TJ, AJ and gap junction protein levels, ↑internalization of TJ and AJ proteins, delay gap-junction communication	ND	[116,117]
		Rat testis	ERK	↑Raf1 and phosphotyrosine proteins, ↓in gonocyte proliferation but transient ↑ in spermatogonia and Leydig cell number	ND	[118]
		Rat Leydig cells (R2C cells)	ERK, JNK, p38	↑Aromatase, cyclooxygenase-2, prostaglandin E2, ↓testosterone, activation of protein kinase A and B	ND	[76]
Organic polychlorinated dibenzodioxins	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin	Mouse testis	JNK, p38, ERK	↓Antioxidant enzymes, activation of Smad2, ↑c-Jun and ATF3	↑TGFβ1	[29]
Polycyclic aromatic hydrocarbons		Rat testis	N. D.	↓Spermatid number, testosterone and superoxide dismutase activity	↑IL-6	[84]

\*ND: not determined. AJ, adherens junction; BTB, blood–testis barrier; ERK, extracellular-signal-regulated kinase; ES, ectoplasmic specialization; FAK, focal adhesion kinase; IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor; TJ, tight junction.

irreversible BTB damage, it protects the testis from unwanted proteolysis during the cascade of events that leads to the loss of germ cells from the testis [48,49,91].

#### Endocytosis of junction proteins via Src signaling and cytokine signaling is mediated by polarity proteins

In addition to Hakai and caveolin-1, c-Src controls endocytosis of integral membrane proteins at cell junctions by regulating polarity proteins (e.g., Par3, Par6 and atypical PKC (aPKC)) via an endocytic adaptor protein, Numb (Figure 1) [92]. Studies in kidney and intestinal epithelial cells have shown that polarity proteins function as ‘spatial cues’ for the establishment and maintenance of intercellular junctions. It is known that loss of polarity proteins results in a delay in the formation or a complete loss of cell–cell junctions [93]. Hence, the precise positioning of polarity proteins in a cell epithelium is essential for the integrity of cell junctions. In contrast to most epithelial cells (in which polarity proteins are restricted to the TJ), Par6 is found at the apical ES (a testis-specific anchoring junction) between Sertoli cells and elongating spermatids in the testis (where no TJ ultrastructure is present when examined by electron microscopy (Figure 2)) [94]. Similarly, polarity proteins such as Par3, aPKC and 14-3-3 are found at the apical ES or expressed in germ cells [94,95]. Nevertheless, recent studies have shown that polarity proteins

also function as crucial molecules to regulate the integrity of cell junctions in the testis.

The first piece of evidence comes from the observation that Par6 is absent from the apical ES several hours before this ultrastructure undergoes disassembly to release mature spermatids from the seminiferous epithelium to the tubule lumen at spermiation [94]. To validate the notion that Par6 contributes to the maintenance of cell junctions at the apical ES, adjuvin [1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydraizde] was administered to adult rats *in vivo* to induce premature release of spermatids from the seminiferous epithelium [94]. In elongating spermatids that were about to deplete from the seminiferous epithelium prematurely, a loss of Par6 at the apical ES was detected. This was accompanied by the defragmentation of the actin filament bundles that are necessary to maintain the apical ES integrity. These results thus indicate the pivotal role of Par6 for the adhesion of elongated spermatids by maintaining the actin filament bundles at the apical ES [94]. In this context, Par6 probably coordinates with the actin nucleation protein Arp3, which is detected predominantly at the apical ES (alongside with Par6) at stage-VII to early stage-VIII tubules. Together, they regulate the highly organized hexagonally packed actin filament bundles and facilitate endocytosis of integral membrane proteins at the apical ES prior to spermiation [96]. This idea is

supported by findings showing that Arp3 is an effector downstream of the Par6/aPKC/Cdc42 complex that is necessary for the localized branching of the actin filament network and endocytosis of E-cadherin [97,98]. Subsequently, the expression of Par6 and Arp3 diminishes rapidly to allow disintegration of the actin filament bundles which, in turn, allows the apical ES to facilitate spermiation at late stage VIII of the seminiferous epithelial cycle.

In v-Src-transformed MDCK cells in which Src kinase is constitutively active, Numb binds preferentially to aPKC and Par6 instead of E-cadherin and Par3 [92]. The dissociation of Numb and E-cadherin appears to be required for the internalization of E-cadherin from the cell surface to the cytosol. This conclusion is further supported by the observation that Numb is important for the appropriate localization of E-cadherin at the cell–cell interface [92]. Intriguingly, phosphorylation of Numb by aPKC decreases the affinity of Numb for integrin and prevents the internalization of integrin in HeLa cells [99]. This discrepancy might be explained by the specificity of Numb to link selective transmembrane proteins to  $\alpha$ -adaptin, a subunit of the adaptor protein (AP) 2 complex that is essential for the assembly and sorting of cargoes into clathrin-coated endocytic-vesicular structures for endocytosis [100]. Furthermore, it is unclear if the dissociation of Numb from E-cadherin upon c-Src phosphorylation is essential to facilitate the binding of Hakai to E-cadherin for its endocytosis because Numb and Hakai bind to identical tyrosine residues of E-cadherin [64,92]. These findings suggest that distinct mechanisms are used to regulate cadherin- and integrin-mediated adhesion by c-Src via Numb and polarity proteins.

Future investigations will generate a clearer picture as more polarity proteins are found to be substrates for c-Src [101]. Although it remains to be determined if environmental toxicant-induced c-Src activation phosphorylates polarity proteins or Numb in the testis, the interaction between Src kinase and Par6 is known to be important for the integrity of apical ES for the adhesion of elongating spermatids (Figure 2) [94]. This is illustrated by the increase in affinity of Src kinase for Par6 via adaptor protein Pals1 before adjudin-induced disruption of the apical ES. Such enhanced interaction among Src kinase, Par6 and Pals1 is likely necessary to maintain the proper phosphorylation status of Par6 and/or Pals1 by Src kinase [94]. Nevertheless, the molecular targets (e.g. integral membrane proteins) downstream of this signaling pathway remain to be elucidated.

Unsurprisingly, cytokines that are induced after exposure to environmental toxicants also regulate cell junctions via polarity proteins, similar to c-Src (Figure 1). Par6 is a substrate of receptor tyrosine kinase TGF $\beta$  receptor II (T $\beta$ RII) [102]. TGF $\beta$  stimulation recruits T $\beta$ RII to phosphorylate Par6 at the cell surface, which constantly interacts with endogenous T $\beta$ RI. Non-phosphorylatable Par6 protects epithelial cells from TGF $\beta$ -induced junction disruption, illustrating that Par6 is essentially the mediator in this signaling pathway [102]. Ser-phosphorylated Par6 by T $\beta$ RII promotes the assembly of E3 ubiquitin ligase Smurf1 to the cell–cell interface [102]. Currently, it is unclear if Smurf1 directly facilitates the ubiquitination

of cell junction proteins, although the TJ protein occludin associates with the T $\beta$ RI/II/Par6 complex. Nevertheless, ubiquitination of the Ras homolog gene family, member A (RhoA), by Smurf1 certainly plays a pivotal part in this process [102]. Cytokines (e.g. TGF $\beta$ 3 and TNF $\alpha$ ) have recently been shown to regulate junction dynamics in the testis via an endocytic vesicle-mediated pathway [88,89,103,104]. Significantly, TGF $\beta$ 3-enhanced endocytosis is mediated by the activation of Cdc42 in the Sertoli cell epithelium [88]. Activated Cdc42 is part of the Par6 complex, and both have been shown to be required for endocytic vesicle-mediated protein trafficking in epithelial cells [105]. Together, these data strongly suggest that polarity proteins are involved in cytokine-mediated cell junction dynamics in the testis, and that they are critical components of the environmental toxicant-mediated damage to cells and tissues via oxidative stress.

### Concluding remarks

The male reproductive system has emerged as one of the major targets of environmental toxicants. Although acute exposure to toxicants contributes to apoptosis and the necrosis of testicular cells, chronic and sub-lethal exposure is prevailing in the general public [37]. Due to the unusually long half-lives of some of these toxicants in the mammalian body (e.g. cadmium has a mean half-life of >15 years [106]), chronic and low-level exposure to humans could cause long-term unwanted health effects. We highlighted the disruptive effects of environmental toxicants on cell junctions mediated by non-receptor tyrosine kinases (e.g. c-Src and FAK) and cytokines through oxidative stress because such damage is often observed in low-level exposure before apoptosis occurs [107]. Significantly, these signaling pathways converge to utilize polarity proteins to regulate intercellular junctions. Polarity proteins (which are known to control cell adhesion in the testis) thus emerge as novel targets for therapeutic intervention to limit environmental toxicant-induced infertility. Although it is equally important to study the epigenetic (e.g. vinclozolin) and endocrine-disruptive (e.g. BPA, dioxin, cadmium) effects of environmental toxicants, it is increasingly clear that these toxicants are imposing an immediate deleterious effect in the testis via disruption of cell junctions between testicular cells due to increase in oxidative stress. In addition, endocrine-disrupting toxicants that affect estrogen levels might cause a disturbed balance of ROS and oxidative stress because estrogen is an important free-radical scavenger in humans [108,109], besides being essential for spermatogenesis [110].

Recent studies have emphasized the importance of assessing the effects of a mixture of environmental toxicants on male reproductive function because humans are exposed to an array of chemicals that might antagonize or agonize each other [37,107]. Although this type of study is inherently difficult to undertake, it is crucial for a full understanding of the impact of environmental toxicants on the reproductive system. Studies showing antioxidant (e.g. vitamin E and selenium) supplementation therapy has some success in term of improving sperm qualities (e.g. sperm motility) [24,111] offer potential treatment. However, much work is needed to understand the precise

molecular events and mechanism(s) regulated by environmental toxicants to target c-Src, FAK, MAPK and polarity proteins in the testis. Only then can we identify specific phosphorylation targets or isoforms so that small-molecule agonists and/or antagonists can be designed to limit systemic toxicity *in vivo*.

## Disclosure

The authors declare no conflict of interest.

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