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Revised TRMOME-D-18-00128-R1 Mechanistic insights into PFOS-mediated Sertoli cell injury

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Abstract

Studies have proven that per- and polyfluoroalkyl substances are harmful to humans, most notably PFOS (perfluorooctanesulfonate). PFOS induces rapid disorganization of actin- and microtubule-based cytoskeletons in primary cultures of rodent and human Sertoli cells, perturbing Sertoli cell gap junction communication, thereby prohibiting Sertoli cells from maintaining cellular homeostasis in the seminiferous epithelium to support spermatogenesis. PFOS perturbed several signaling proteins/pathways such as FAK and mTORC1/rpS6/Akt1/2. The use of either an activator of Akt1/2 or overexpression of a phosphomimetic (and constitutively active) mutant of FAK or connexin 43 have demonstrated that such treatment blocked the PFOS-induced Sertoli cell injury by preventing the actin- and MT-based cytoskeletal disorganization. These findings thus illustrate an approach to manage PFOS-induced reproductive dysfunction.

Keywords

PFOS; Sertoli cell injury; environmental toxicant; mTORC1; rpS6; FAK; human Sertoli cell; cytoskeleton; actin; microtubule

Polyfluoroalkyl Chemicals have Toxic Properties in Humans and Wildlife

Growing evidence based on studies in mammals including wildlife and fish have shown that per- and polyfluoroalkyl chemicals such as perfluorooctanesulfonate (PFOS) pose serious health risks. PFOS and its related compound such as PFOA (perfluorooctanoate) were

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produced in 1949 by 3M Company as an active component in stain repellents by lowering surface tension of water [1]. Since then, PFOS and its related compounds have been widely used in consumer products including textiles, paper, leather, paints, general cleaning products, carpets and drapery for decades. Its toxic effects began to be noted initially in wildlife and then in humans. PFOS-induced health risks were shown to associate with defects in development, pregnancy loss, neonatal mortality, liver function, endocrine disruption tumorigenesis [2-5], and ADHD (attention deficit hyperactivity disorder) [6]. Furthermore, PFOS, PFOA and related perfluorinated compounds are developmental toxins in rodents. They are agonists of peroxisome proliferator-activated receptors (PPARs) [3], which are nuclear receptors/transcription factors involved in cell differentiation, metabolism, and growth [7, 8]. Human exposure to PFOS remains relatively low, and studies have established that the acceptable daily intake/tolerable daily intake (ADI/TDI) dose of PFOS is 150 ng/kg body weight/day or ~12 µg/person/day. The median blood concentration of PFOS in humans rose from 29.5 ng/ml in 1974 to 34.7 ng/ml in 1989 [9]. Since the health risks associated with PFOS/PFOA were identified, considerable efforts were made by PFOS/PFOA manufacturers to reduce their environmental contamination in the U.S., and a 25% and 32% reduction of human blood concentration of PFOA and PFOS, respectively, was noted from 2003-2004 vs. data from 1999-2000 [10]. While this information is encouraging, the fact that these compounds have a relatively long half-life remains a concern. For instance, the half-life of PFOS in humans is ~5.4 years and 3.8 years for PFOA [11]. Thus, a considerable concentration of PFOS and its related compounds can be accumulated in different organs in humans over an extended period of time. Due to the health risks associated with PFOS/PFOA, 3M phased out its production in 2000 (http://solutions.3m.com/wps/portal/3M/en_US/PFOS/PFOA/Information/Action/) [12]. The European Union also banned the use of PFOS in 2006, followed by Canada in 2009. The use of PFOS for industrial applications such as hydraulic fluids for aviation and photolithography continue in the U.S. In 2006, the U.S. Environmental Protection Agency (EPA) asked manufacturers to reduce their emissions and product content by 95% no later than 2010, and the use of the chemicals was eliminated by 2015. However, PFOS continues to be used in multiple industries in the U.S, such as hydraulic fluid in commercial aviation, in photolithographic chemicals including anti-reflective coatings and photoacid generators, metal plating and firefighting foams (<http://www.environment-switzerland.ch/uw-0922-e>). The only remaining producer of PFOS globally is China, where these chemicals remain widely used; however, China announced an effort to reduce and phase-out the use of PFOS in 2017 (<http://documents.worldbank.org/curated/en/420591485529131506/China-Reduction-and-Phase-out-of-PFOS-in-Priority-Sectors-Project-Environmental-and-Social-Management-Framework-ESMF-executive-summary>). Nonetheless, PFOS and its related compounds remain widely used in China, and PFOS remains in daily life, even at reduced quantities because it remains to be used in the U.S., Canada and Europe for specific industries as listed above. The risks of PFOS exposure to male reproductive health need to be better understood since high concentrations of PFOS are known to induce rat Sertoli cell injury, perturb human sperm morphology, and reduce testosterone production in men [13-15]. We focus our discussion on the signaling proteins and protein complexes, providing a mechanistic insight on the molecular targets of PFOS and the possible therapeutic approach to manage PFOS-induced Sertoli cell injury. This analysis may also be helpful to

provide clues to management of male reproductive dysfunction induced by other environmental toxicants, such as cadmium and phthalates.

Focal Adhesion Kinase (Fak) Is Important For Sertoli Cell Function

In the testis, Sertoli cells serve as the “mother cell” to support and nurture the development of different germ cell types during spermatogenesis (Figure 1A). In fact, studies have shown that each Sertoli cell nurtures ~30-50 germ cells in the seminiferous epithelium [16, 17] wherein Sertoli and germ cells are the only two cell types in the epithelium. FAK plays a crucial role to support Sertoli cell function in particular the **blood-testis barrier** (BTB) dynamics and spermatid adhesion [18-21]. **FAK** is a non-receptor protein tyrosine kinase and a signaling protein that relays integrin-mediated signals at the actin-based cell-extracellular matrix (ECM) anchoring junction known as focal adhesion complex (FAC). In short, FAK is the downstream signaling protein of integrin-based receptor at the FAC [22, 23]. The involvement of FAK in spermatogenesis and testis function was not known until 2003 when FAK was first shown to be localized in the rat testis by immunohistochemistry and immunofluorescence microscopy using a specific anti-FAK antibody [19]. Specifically, the two phosphorylated forms of FAK, p-FAK-Tyr397 and p-FAK-Tyr576, were localized at the apical ES (ectoplasmic specialization, a testis-specific anchoring junction type [24, 25]), beginning in stage VII through late stage VIII of the epithelial cycle until the release of sperm takes place at spermiation [19, 21]. These FAK isoforms are also supported by other associated signaling proteins including PI 3-kinase, PKB [26], p130Cas, DOCK180, and their interactions with $\beta 1$ -integrin at the apical ES [20]. Notably, neither FAK nor any of its phosphorylated forms were detected at the **Sertoli cell-basement membrane** (note: basement membrane is a modified form of ECM in the testis [27, 28]) interface (i.e., FAC) since there is no identifiable ultrastructure of FAC in the seminiferous epithelium of the testis by electron microscopy. Instead, p-FAK-Tyr407 is highly expressed at the basal ES/BTB, but unlike p-FAK-Tyr397 which exclusively expressed at the apical ES, p-FAK-Tyr407 is also expressed at the apical ES [18]. In short, FAK is an integrated component of the actin-based Sertoli-spermatid anchoring junction ES. p-FAK-Tyr397 is specifically expressed at the apical ES in the rat testis whereas p-FAK-Tyr407 is expressed both at the apical and basal ES [18]. Studies have shown that p-FAK-Tyr397 promotes apical ES function, maintaining apical ES integrity until its degeneration to facilitate the release of sperm at spermiation in the rat testis [19, 21, 29]. This concept has been subsequently confirmed in an in vivo study [30] by overexpressing a **phosphomimetic** and constitutively active mutant of p-FAK-Tyr397 designated p-FAK-Y397E (by mutating Tyr397 to Glu397) in the rat testis using a mammalian expression vector pCI-neo [18]. Overexpression p-FAK-Y397E mutant in the rat testis using a mammalian expression vector (pCI-neo) containing the p-FAK-Y397E cDNA clone was found to delay the degeneration of F-actin at the apical ES in late stage VIII tubules, by maintaining the spatiotemporal expression of the barbed end capping and actin bundling protein Eps8 (and also actin bundling protein palladin) and also barbed end nucleation protein Arp3, thereby retaining apical ES adhesion proteins (such as nectin 2, and nectin 3) at the site, delaying the occurrence of spermiation [30]. By contrast, p-FAK-Tyr407 promotes BTB integrity since overexpression of a phosphomimetic and constitutively active p-FAK-Tyr407 mutant designated p-FAK-Y407E (by mutating

Tyr407 to Glu407 by site-directed mutagenesis) in primary rat Sertoli cells was shown to promote the Sertoli cell TJ-permeability barrier, making it “tighter” [18], illustrating the possible “protective” role of this mutant on the Sertoli cell barrier function.

Role of FAK in PFOS-mediated Sertoli cell injury

Studies in rodent Sertoli cells

Studies have shown that the Sertoli cell is one of the primary targets of testis injury following exposure of rats to toxicants including PFOS, phthalates, bisphenol, cadmium and others [31-35]. As such, investigators have sought to use primary Sertoli cells isolated from rat testes and cultured in vitro as a model system to study the biology of toxicant-induced Sertoli cell injury [36-39] (Table 1).

The functional TJ-barrier established by Sertoli cells cultured in vitro mimics the Sertoli cell BTB in vivo based on studies in rats or mice as noted by multiple investigators both functionally and ultrastructurally when examined under electron microscopy. These findings are possibly similar to humans based on emerging evidence using human Sertoli cells for studies as noted below. For instance, both actin-based TJ, basal ES, and gap junction, as well as intermediate filament-based desmosome are noted at the Sertoli cell-cell interface when these cells are cultured in vitro. Since p-FAK-Tyr407 is one of the signaling proteins shown to promote Sertoli cell BTB integrity [18, 40], it is not surprising that PFOS-induced Sertoli cell TJ-permeability barrier disruption was associated with a considerably down-regulation of p-FAK-Tyr407 in rat Sertoli cells [13, 41]. PFOS was also shown to perturb the organization of the actin- and microtubule (MT)-based cytoskeletons in rat (and also human Sertoli cells (Figure 1B)), suggesting that FAK may also be involved in cytoskeletal organization. In fact, this possibility was supported by an earlier report by utilizing different phosphomimetic mutants of FAK, including both constitutively active mutants (e.g., p-FAK-Y407E, p-FAK-Y397E) vs. constitutively inactive (i.e., non-phosphorylatable) mutants (e.g., p-FAK-Y407F, p-FAK-Y397Y) and to examine their effects on the Sertoli cell TJ-permeability barrier function following their overexpression in Sertoli cells in vitro [18]. Overexpression of p-FAK-Y407E in Sertoli cells promoted Sertoli cell TJ-barrier function and was associated with an increase in actin nucleation activity, illustrating an increase in actin polymerization is necessary to make the Sertoli cell barrier “tighter” [18]. These observations are important, since these findings illustrate the possibility that PFOS-mediated Sertoli cell injury can be blocked by overexpressing p-FAK-Y407E mutant since exposure of primary rat Sertoli cells to PFOS was found to considerably down-regulate p-FAK-Y407 [13]. Indeed, overexpression of p-FAK-Y407E mutant in rat Sertoli cells in vitro was found to block the PFOS-induced Sertoli cell TJ-permeability barrier disruption [13]. Overexpression of p-FAK-Y407E in Sertoli cells also blocked the PFOS-induced F-actin disorganization so that the TJ adhesion protein complex occludin-ZO-1 that utilized actin for attachment could remain at the Sertoli cell-cell interface following PFOS treatment to support the Sertoli cell TJ-barrier function [13]. A subsequent study has shown that the PFOS-induced down-regulation of p-FAK-Tyr407 is associated with a considerable down-regulation of Akt1/2, notably p-Akt1-T308, p-Akt1-S473 and p-Akt2-S474 [41], illustrating

both FAK and Akt1/2 signaling proteins may be involved in PFOS-induced Sertoli cell injury.

Indeed, the use of a specific Akt1/2 activator SC79 (2-amino-6-chloro- α -cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester, Mr 364.78) was shown to block the PFOS-induced Sertoli cell TJ-permeability barrier function [41]. The disruptive effects of PFOS on the organization of actin- and MT-based cytoskeletons in Sertoli cells are mediated by changes in the spatial expression of **actin regulatory proteins** (e.g., branched actin polymerization protein Arp3, and actin bundling protein palladin) and **MT regulatory proteins** (e.g., EB1, a +TIP known to promote MT stability), respectively [41]. SC79 treatment was found to promote corrective expression of these regulatory proteins in the presence of PFOS, such that these proteins became properly expressed in Sertoli cells, so that the organization of actin- and MT-based cytoskeletons was not affected by PFOS [41]. These findings are important since they illustrate the possibility that toxicant-induced Sertoli cell injury might be prevented by manipulating a signal protein such as via overexpression of a p-FAK-Y407E mutant.

Studies in human Sertoli cells

Although the findings above are of interest regarding the protective effects of overexpressing p-FAK-Y407E in rat Sertoli cells against PFOS-mediated Sertoli cell injury, it remains to be established if these findings are relevant to humans since the expression of FAK by human Sertoli cells was not established until recently [42]. In 2011 it was shown that primary human Sertoli cells can be maintained in vitro when cultured in F12/DMEM in the presence of 5-10% fetal calf serum (FCS) [43]. In addition, these primary human Sertoli cells remain proliferative in the FCS-containing culture medium, with a replication time ranging between ~7-10 days [43, 44]. Since then, primary cultures of human Sertoli cells have been used by multiple investigators [45-47].

When human Sertoli cells cultured in vitro were treated with PFOS, similar to rat Sertoli cells, p-FAK-Tyr407 expression was also considerably down-regulated [42]. We prepared a phosphomimetic and constitutively active mutant of human FAK, namely p-hFAK-Y407E, and cloned into the mammalian expression vector pcDNA 3.1(+) [42]. Similar to rat Sertoli cells, PFOS induced human Sertoli cell injury as manifested by disruptive organization of actin- and MT-based cytoskeletons across human Sertoli cells (Figure 1B). This was shown to be the result of disruptive changes in the spatial expression of branched actin-nucleation protein Arp3 and actin barbed-end capping and bundling protein Eps8, and MT stability promoting protein EB1 [42]. The net result of these changes thus perturbed proper organization of cytoskeletons *versus* control human Sertoli cells [42]. F-actin and MTs no longer stretched across the Sertoli cell cytosol as noted in control cells, but were considerably truncated and mis-aligned. Overexpression of the human phosphomimetic mutant p-hFAK-Y407E in human Sertoli cells blocked the PFOS-induced human Sertoli cell TJ-permeability barrier disruption by inducing proper organization of actin- and MT-based cytoskeletons [42]. This was mediated, at least in part, by correcting the spatial expression of actin- and MT-regulatory proteins: the spatial expression of EB1 across the Sertoli cells was

similar to control cells following overexpression of p-hFAK-Y407E mutant in PFOS-treated cells [42].

Overall evidence of role for FAK in PFOS-mediated Sertoli cell injury

Taken together, these findings based on the use of rat and human Sertoli cells are physiologically significant since overexpression of p-FAK-Y407E can have protective effects by blocking PFOS-induced Sertoli cell injury. Figure 1C illustrates a hypothetical model based on results of the studies discussed herein to support the notion that p-FAK-Tyr407 is one of the targets of PFOS during PFOS-induced Sertoli cell injury. Two main questions remain to be addressed. First, do other environmental toxicants exert their disruptive effects on Sertoli cells via p-FAK-Tyr407? Second, does overexpression of p-FAK-Tyr407 in the testis in vivo exert similar protective effects to environmental toxicants, such as PFOS?

mTORC1/rpS6/Akt1/2 signaling pathway and Sertoli cell injury

mTOR (mammalian target of rapamycin) is a non-receptor Ser/Thr protein kinase found in virtually all mammalian (and non-mammalian) cells, crucial to energy metabolism, thereby regulating cellular energy status and cell growth and is essential to virtually all aspects of cellular function [48-50]. Due to the presence of functional domains along the sequence of mTOR, it associates with different adaptors and regulatory proteins (Figure 2). When mTOR binds to the adaptor protein Raptor (regulatory-associated protein of mTOR) or Rictor (rapamycin-insensitive companion of mTOR) through its HEAT repeat domains near its N-terminus, it creates either the mTORC1 (mammalian target of rapamycin complex 1, i.e., sensitive to rapamycin) or mTORC2 (i.e., insensitive to rapamycin), respectively (Figure 2). Recent studies including the use genetic models and RNAi approaches, however, have shown that mTORC1 and mTORC2 have distinctive functions in modulating spermatogenesis, in particular regarding their role on cytoskeletal organization, spermatogonial differentiation, gap junction and cell adhesive function that support spermatogenesis [51-57] (Table 2; Figure 2). Accumulating evidence has shown that the mTOR/Raptor signaling pathway (i.e., the mTORC1-dependent signaling pathway) is most notably involved in promoting Sertoli cell BTB remodeling [52, 54, 55] through its effects on the organization of cytoskeletons [56, 58], thereby facilitating the restructuring of the immunological barrier to support the transport of **preleptotene spermatocytes** across the barrier that occurs at stage VIII of the epithelial cycle of spermatogenesis in rodent testes [59]. In this context, the activated forms of rpS6 (namely p-rpS6-S235/S236 and p-rpS6-S240/S244) and Akt, in particular the p-Akt1/2, that are downstream of the mTORC1 are involved in this mTORC1-dependent signaling events [55, 56]. In short, the mTORC1 signaling complex regulates Sertoli cell homeostasis by inducing BTB “remodeling” to support BTB restructuring to facilitate the transport of preleptotene spermatocytes across the immunological barrier at stage VIII of the epithelial cycle. The mTORC1 exerts its disruptive effects through inducing expression of p-rpS6-S235/S236 and p-rpS6-S240/S244 and downregulating p-Akt1/2 expression [55, 56]. These observations are important, since they illustrate that manipulation of p-rpS6 (the activated form of rpS6) or p-Akt1/2 can either potentiate or alleviate the disruptive effects of mTORC1 on Sertoli cell function. In

fact, a recent study has shown that overexpression of a quadruple (and constitutively active) mutant of rpS6, namely p-rpS6-S235E/S236E and p-rpS6/S240E/S244E (i.e., converting Ser(S) to Glu(E) by site-directed mutagenesis) in the rat testis *in vivo* induces considerable BTB disruption, making the BTB “leaky” [58]. By contrast, the use of an Akt1/2 activator (e.g., SC79 [60]) promotes the Sertoli BTB integrity, making it “tighter” [41]. The mTOR/Rictor (mTORC2) signaling pathway (i.e., the mTORC2-dependent signaling pathway) also promotes the BTB integrity, which is in sharp contrast to the effects of the mTORC1 signaling pathway. mTORC2 exerts its effects by promoting the Sertoli cell gap junction communication, but also through the PKC α and Rac1 GTPase pathway to promote proper organization of the actin- and MT-based cytoskeletons to support Sertoli cell function in the rat testis [57]. Using the adjuvin model in rats following an acute dose of adjuvin (250 mg/kg b.w., via oral gavage), we have also shown that overexpression of Cx43 is capable of promoting F-actin organization in the testis to re-establish the adjuvin-induced BTB disruption *and* to re-initiate meiosis [61].

These contrasting effects of mTORC1 *vs.* mTORC2 on Sertoli cell function (e.g., TJ-permeability barrier, adhesion) mediated through changes in the cytoskeletal organization has largely been confirmed in studies using genetic models of deleting (knockout, KO) specific genes in the mTOR signaling pathways, involving Sertoli cell- or germ cell-specific KO of mTOR, Raptor and/or Rictor, illustrating cytoskeletons are one of the targets of these two signaling proteins (Table 2). Herein, we briefly summarize and critically evaluate some recent findings regarding the role of mTOR in particular its downstream signaling proteins in environmental toxicant-induced Sertoli cell injury.

Role of mTOR and downstream signaling proteins in PFOS-mediated Sertoli cell injury

Studies in rodent Sertoli cells.

In 2012 the first study showing that mTOR is involved in Sertoli cell function was published. Treatment of Sertoli cells with rapamycin (100 ng/ml) cultured *in vitro* with an established functional TJ-permeability barrier was shown to promote the TJ-barrier function [54]. This change was associated with a considerable down-regulation of p-rpS6 (the activated form of rpS6), but not total rpS6 [54], implicating rpS6 is one of the regulators of Sertoli cell BTB function. Indeed, knockdown of rpS6 by RNAi was found to promote the Sertoli cell TJ-barrier function, accompanied by a considerable reduction of p-rpS6 [54]. Since rpS6 is the downstream signaling complex of mTORC1 (i.e., mTOR binds to its adaptor Raptor) [62], these findings suggest that the mTORC1/rpS6 signaling complex promotes Sertoli cell BTB disruption, making the BTB “leaky” under physiological conditions. This concept was supported by findings that p-rpS6-S235/S236 and p-rpS6-S240/S244 was specifically expressed at the BTB in stage VIII-IX tubules, at the time of BTB remodeling to accommodate the transport of preleptotene spermatocytes across the immunological barrier at these stages [56].

The notion that mTORC1/rpS6 complex promotes Sertoli cell BTB disruption was further expanded by overexpressing a quadruple and constitutively active mutant p-rpS6 mutant

(i.e., p-rpS6-S235E/S236E/S240E/S244E) in rat Sertoli cells cultured in vitro with an established TJ-barrier in two studies [55, 56]. For instance, it was shown that overexpressing this p-rpS6 mutant was capable of inducing Sertoli cell TJ-barrier disruption, and the disruptive effects were considerably greater than those observed by overexpressing the rpS6 wild-type (WT, i.e., the full-length rpS6 cDNA encoding the entire open reading frame of rpS6) [55]. More importantly, this disruptive effect was mediated through changes in the actin-based cytoskeletal organization wherein overexpression of p-rpS6 mutant in Sertoli cells caused extensive truncation of actin filaments across the Sertoli cell cytosol, accompanied by a reduction of actin polymerization kinetics and actin bundling capability [56]. These changes thus perturbed the distribution of TJ-proteins (e.g., occludin, and claudin-11) at the Sertoli cell-cell interface as these proteins were rapidly internalized and moved to cell cytosol, away from the cell cortical zone to support Sertoli cell adhesion [55]. Additionally, a considerably down-regulation on the expression of p-Akt1/2 was also detected [56] following overexpression of p-rpS6 mutant in Sertoli cells, illustrating mTORC1/rpS6 signaling complex is working in concert with p-Akt1/2 downstream. Indeed, a knockdown of Akt1/2 was found to perturb the Sertoli cell TJ-barrier function, and accompanied by extensive defragmentation of actin filaments across the Sertoli cell [56].

In short, the mTORC1/rpS6/Akt1/2 signaling pathway is activated during Sertoli cell injury, which is accompanied by the following phenotypes, including disruption of the Sertoli cell TJ-permeability barrier function, distinctive disorganization of actin filaments across the cell (e.g., defragmentation), and re-distribution of proteins at the cell-cell interface (e.g., rapid internalization of cell adhesion proteins from the cell cortical zone into the cell cytosol). To test the possibility that PFOS-induced Sertoli cell injury is mediated through this mTORC1-rpS6/Akt1/2 signaling pathway, rat Sertoli cells were treated with PFOS at 10, 20 and 50 μ M, which was found to have dose-dependent effects to perturb the Sertoli cell TJ-barrier function [41]. Furthermore, PFOS (20 μ M) was found to induce considerable disruption on the organization of actin- and MT-based cytoskeletons [41]. Since both actin filaments and MT protofilaments were extensively truncated and these cytoskeletons no longer stretched across the cell cytosol to support cellular functions following PFOS treatment as noted in corresponding control Sertoli cells. These changes thus impeded the distribution of TJ- (e.g., CAR/ZO-1) and basal ES- (e.g., N-cadherin/ β -catenin) adhesion protein complexes at the cell cortical zone as these proteins were mostly internalized and randomly distributed in cell cytosol [41]. A considerable down-regulation on the expression of the activated forms of Akt1/2, namely Akt1-T308, pAkt1-S473 and p-Akt2-S474, was also detected [41]. To confirm the physiological relevancy of these findings, we used a known activator of Akts SC79 (2-amino-6-chloro- α -cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester, Mr 364.74 by enhancing phosphorylation and activation of p-Akt1-T308 and p-Akt1-S473 [60]) to block the PFOS-mediated inactivation of p-Akt1/2 [60]. As anticipated, while the use of SC79 alone had no effects on the rat Sertoli cell TJ-permeability barrier function, it effectively blocked the PFOS-induced TJ-barrier disruption [41]. SC79 also blocked the PFOS-induced disorganization of actin- and MT-based cytoskeleton by promoting the corrective spatial expression of actin regulatory proteins Arp3 (a branched actin nucleation protein [63]) and palladin (an actin bundling protein [64]), and also MT regulatory protein EB1 (a +TIP protein known to stabilize MTs [65, 66]) [41]. Perhaps the most important of

all, the spatial expression of p-FAK-Tyr407 that was perturbed by PFOS treatment was also properly corrected by localizing at the Sertoli cell-cell interface [41], suggests that the mTORC1/rpS6/Akt1/2 and p-FAK-Tyr407 signaling complex/protein may be working in concert to regulate PFOS-mediated Sertoli cell injury. We are working to expand this study in human Sertoli cells and these findings should provide significant physiological and mechanistic insights into the therapeutic treatment of toxicant-induced Sertoli cell injury and male reproductive dysfunction.

In short, findings obtained from studies based on the use of the rodent Sertoli cell in vitro model have shown that environmental toxicant PFOS-induced Sertoli cell injury is mediated through the mTORC1/p-rpS6/p-Akt1/2 signaling pathway. This involves an up-regulation of p-rpS6 expression, concomitant with a down-regulation of p-Akt1/2, thus, the use of an activator of Akt1/2 was effective to block the PFOS-mediated Sertoli cell injury.

Concluding remarks

While much work remains to be done to better understand the mechanism(s) by which environmental toxicants (e.g., PFOS) induce Sertoli cell injury that leads to male reproductive dysfunction (see Outstanding Questions), recent findings provide a viable path to explore possible therapeutic approach to manage toxicant-induced male infertility (see Clinician's corner, box 1). This could include the use of either activator(s) or inhibitor(s) of specific signaling pathway(s), or the use of an endogenously produced biomolecule to "tighten up" the Sertoli cell BTB to prevent entry of environmental toxicants to the testis to exert their disruptive effects. For instance, recent studies have illustrated that an 80 kDa fragment released from Iaminin- α 2 chain in the basement membrane is capable of promoting Sertoli cell BTB function [67, 68]. This biologically active peptide should be tested in future studies regarding its suitability to "tighten" the BTB to protect toxicant-induced Sertoli cell injury. More importantly, the use of human Sertoli cells should be better evaluated by more investigators in the field to examine its [69] suitability as an in vitro model to monitor toxicant-induced human reproductive dysfunction. In this context, it is of interest to note that the tightening of the BTB by using the p-FAK-Y407E mutant is not likely to perturb the timely opening of the immunological barrier to accommodate the transport of preleptotene spermatocytes across the BTB since studies have shown that germ cells are capable of modulating the Sertoli cell TJ-barrier function [70]. As such, males (possibility including men) treated with p-FAK-Y407E mutant via transfection may not impede the timely transport of preleptotene spermatocytes to sustain spermatogenesis and this possibility should be carefully evaluated in future studies.

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Glossary

Actin regulatory proteins.

These actin binding proteins (e.g., palladin, fascin, Eps8, Arp2/3 complex) play a role in regulating actin cytoskeletal dynamics, thereby conferring plasticity to the actin cytoskeleton at the ectoplasmic specialization (ES) to support spermatid and organelle transport across the seminiferous epithelium, and also spermatid or Sertoli cell adhesion, during spermatogenesis. These actin filaments continuously convert between bundled and unbundled/branched configurations, thus conferring plasticity to the actin cytoskeleton, which is necessary to support spermatid and Sertoli cell adhesion (and de-adhesion) and spermatid (and organelle) transport across the seminiferous epithelium to support spermatogenesis.

Basement membrane.

The basement membrane in the testis is located at the base of the seminiferous epithelium, which is a modified form of extracellular matrix (ECM) [27, 85], also known as basal lamina in other epithelia. The two major building blocks of the basement membrane is type IV collagen in particular collagen $\alpha 3$ (iv) chains and laminins (most notably laminin $\alpha 2$ chains).

Blood-testis barrier (BTB).

The BTB in rats, mice and humans is formed byt basal tight junctions between adjacent Sertoli cells, and it is located near the base of the seminiferous tubules, near the basement membrane. The BTB and the BBB (blood-brain barrier) are two of the tightest blood-tissue barriers in the mammalian body. At the ultrastructural level, the BTB is constituted by actin-based tight junction (TJ), reinforced and supported by basal ES and also the intermediate filament-based desmosome. The BTB also divides the seminiferous epithelium into the basal and the adluminal compartments.

Ectoplasmic specialization (ES).

ES is a testis-specific but atypical adherens junction (AJ) in the mammalian testis [86, 87]. It is restrictively expressed by Sertoli cells at the Sertoli-spermatid (step 8-19 vs. step 8-16 spermatids in the rat and mouse testis, respectively) interface known as the apical ES. It is also found at the Sertoli cell-cell interface but only at the BTB known as the basal ES. The most notably ultrastructural feature of the ES is the actin filament bundles that are sandwiched between cisternae of the endoplasmic reticulum and the apposing Sertoli-spermatid plasma membranes (apical ES) or Sertoli cell plasma membranes (basal ES). Once apical ES appears, it is the only anchoring junction at the Sertoli-spermatid interface, replacing desmosome and gap junction until the release of sperm at spermiation. However, basal ES coexists with actin-based tight junction and gap junction, which together with the intermediate filament-based desmosome, they constitute the BTB.

Focal adhesion kinase (FAK).

FAK is a major signaling protein of cell-extracellualr matrix anchoring junction and a component of the focal adhesion complex (FAC also known as focal contact). FAK is involved in multiple cellular function such as cell growth, cell death, and tumorigenesis [22, 23, 88-90]. However, there is no FAC ultrastructure in the testis based on studies by electron microscopy.

Microtubule (MT) regulatory proteins.

MTs are also an integrated component of the ES since MTs are located near the actin filament bundles at the ES [91, 92]. MTs organized as track-like structures undergo rapid growth through polymerization *versus* depolymerization and shrinkage (i.e., catastrophe). These changes thus confer plasticity to the ES to support cell adhesion and spermatid (and organelle) transport across the seminiferous epithelium during spermatogenesis.

Phosphomimetic mutant.

Proteins are phosphorylated (and activated) at either Tyr, Ser or Thr. When Tyr, Ser or Thr is mutated to Glu (E) by site-directed mutagenesis by PCR, the configuration of Glu (while not phosphorylated) appears as phosphorylated Tyr, Ser or Thr structurally at the molecular level, thereby mimicking phosphorylated Tyr, Ser or Thr. As such, phosphomimetic mutant, such as p-FAK-Y407E, is also constitutively active mutant, capable of activating FAK as if it is the phosphorylated FAK-Y407. It is a research tool widely used by biochemist to study protein kinases.

Sertoli cells.

Sertoli cells are also known as the nurse (or mother) cell in the testis. The cross-section of a seminiferous tubule reveals the seminiferous epithelium that lays on the tunica propria wherein spermatogenesis takes place. Sertoli cells and germ cells of different classes including spermatogonia, spermatocytes, and spermatids are the only cell types in the seminiferous epithelium. Each Sertoli cell takes care of ~30-50 developing germ cells, offering structural, nutrient, and paracrine supports since proteins from the systemic circulation are excluded from the seminiferous epithelium due to the BTB.

Preleptotene spermatocytes.

Preleptotene spermatocytes, derived from type B spermatogonia in the basal compartment, are the only germ cell type that are transported across the BTB in stage VIII tubules in both rat and mouse testes (vs. stage VI in human testes) while differentiating into leptotene spermatocytes to enter the adluminal (apical) compartment to differentiate into zygotene and pachytene spermatocytes to prepare for meiosis I and II.

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Highlights:

- The use of rat and human primary Sertoli cells cultured system in vitro are useful models to study toxicant-induced Sertoli cell injury.
- Using these two in vitro models, it was shown that environmental toxicants (e.g., PFOS) induced Sertoli cell injury by perturbing Sertoli cell tight junction-permeability barrier function, localization of Sertoli adhesion proteins, organization of Sertoli cell cytoskeletons, and/or kinetics of polymerization or bundling activity of actin- and/or microtubule-based cytoskeletons.
- PFOS-induced Sertoli cell injury is mediated through p-FAK-Tyr407-based and mTORC1/rpS6/Akt1/2-based signaling complexes/pathways.
- PFOS-induced Sertoli cell injury can be blocked by manipulating either p-FAK-Tyr407 or mTORC1/rpS6/Akt1/2 signaling proteins, illustrating a potential therapeutic approach to manage toxicant-induced male reproductive dysfunction.

Clinician Corner (Box 1)

1. Since PFOS induces human Sertoli cell injury through down-regulation on the expression of p-FAK-Tyr407, and an over-expression of the phosphomimetic and constitutive active human p-FAK-Y407E can block the PFOS-induced Sertoli cell injury, these findings should be expanded to investigate if PFOS-induced male reproductive dysfunction can be therapeutically intervened by using this human phosphomimetic mutant.
2. An in vitro human testis model using human testicular cells including human Sertoli and human germ cells, similar to the mouse or rat 3-D based testis model [69, 71-73], should be established. This could be used as an in vitro human testis model to study the effects of environmental toxicants on male reproductive dysfunction prior to clinical studies.
3. The use of Akt activators, such as SC79, should be explored for their use to rescue PFOS-induced testis injury using the in vitro 3D model of human testes.
4. Evidence from studies using PFOS evaluated herein may be applicable to other environmental toxicants, such as cadmium. However, specific studies using other environmental toxicants should be conducted and such evidence might be used to examine whether toxicant-induced human Sertoli cell injury is mediated through a common downstream signaling molecule or pathway.

Outstanding Questions:

1. Is toxicant-induced Sertoli cell injury, in particular cytoskeletal organization, mediated through specific signaling protein(s) and/or pathway(s)?
2. If these signaling proteins and/or pathways are known, can the toxicant-induced Sertoli cell injury be blocked (or reversed) by use of specific activators, inhibitors, or through overexpression of mutants (i.e., constitutively active or inactive mutants)?
3. Are there common signaling pathways utilized by distinctive groups of environmental toxicants?
4. Can these studies performed in vitro based on the use of primary Sertoli cells in rodents and humans be reproduced in studies in vivo?

Figure 1. Morphological features of the seminiferous epithelium and the ability of PFOS to induce Sertoli cell injury through disruptive changes on the cell cytoskeletons.

(A) Cross-section of an adult rat testis, illustrating the seminiferous epithelium a stage V seminiferous tubule. The BTB near the basement membrane divides the seminiferous epithelium into the adluminal (apical) and the basal compartments. Only Sertoli cells and germ cells at different stages of their development are found in the seminiferous epithelium. A Sertoli cell (yellow arrowhead), a spermatocyte (red arrowhead), round spermatid (green arrowhead) and elongated spermatid (blue arrowhead) are annotated. Scale bar, 40 μ m. (B)

Human Sertoli cells cultured for 8 days in F12/DMEM with 10% fetal calf serum (wt/vol) as earlier described [42, 44] were treated with PFOS at 20 μ M (dissolved in DMSO) for 24 hr. Microtubules (MT) were visualized by α -tubulin staining (α - and β -tubulin dimers are the building blocks of MTs) (red fluorescence) using Alexa Fluor-555 and F-actin (green fluorescence) was visualized by FITC-phalloidin as described [42]. Sertoli cell nuclei were visualized by DAPI (4',6-diamidino-2-phenylindole) (blue fluorescence). Scale bar, 10 μ m; and inset, 4 μ m; which apply to corresponding micrographs and insets. (C) A hypothetical model by which PFOS-induced Sertoli cell injury that perturbs the Sertoli cell TJ-barrier function through changes in the organization of actin- and MT-based cytoskeletons.

Figure 2. A schematic drawing that illustrates the signaling pathways by which mTOR regulates Sertoli cell BTB function through changes in actin- and MT-based cytoskeletons.

mTOR when binds to adaptor protein Raptor or Rictor creates the corresponding mTORC1 and mTORC2 signaling complex. Studies have shown that mTORC1 exerts its disruptive effects on the Sertoli cell BTB function via actin- and MT-based cytoskeletons, mediated through the downstream signaling proteins p-rpS6 and p-Akt1/2 to make the BTB “leaky”. On the other hand, mTORC2 exerts its promoting effects to “tighten” the Sertoli cell BTB via connexin 43 (Cx43)-based gap junction (GJ) to coordinate tight junction (TJ) and basal ES function to maintain BTB homeostasis. mTORC2 also likely exerts its promotive effects by making the Sertoli cell BTB “tighter” through PKCα and Rac1

Table 1.

Toxicant-induced Sertoli cell injury

Chemical group	Environmental Toxicant	In vitro and/or in vivo	Signaling pathway	Cellular responses	References
Perfluoroalkyl compounds (PFCs)	Perfluorooctanesulfonate or perfluorooctane sulfonic acid (PFOS)	Primary mouse Sertoli cells; male 8-wk-old mice	Activation of P38 MAPK pathway	Perturbs Sertoli cell TJ-barrier function; reduces junction protein expression in Sertoli cells; induces junction disruption.	[36, 74]
Phthalate esters	Mono-(2-Ethylhexyl) Phthalate (MEHP)	Primary cocultures of rat Sertoli cells and germ cells; 21-day-old C57BL/6J male mice.	MMP-2 activation; inhibition of FSH-stimulated cAMP signaling pathway; activates the MYC-based signaling pathway.	MEHP induces germ cell adhesion, leading to germ cell lost and down-regulation of cell adhesion protein expression; MEHP suppresses Sertoli cell TIMP2 levels and allows for the activation of MMP2 in seminiferous epithelium	[75-77]
Phthalate esters	DBP	Sprague-Dawley rats		Sertoli cells in DBP-exposed fetal testis had retracted apical cytoplasmic processes, altered organization of the vimentin cytoskeleton, and abnormal cell-cell contacts with gonocytes	[78]
Phthalate Esters	BBP, DBP, DEHP, DPP	Testicular coculture model from rodent testicular cell lines, including spermatogonial cells, Sertoli cells, and Leydig cells with specified cell density and extracellular matrix (ECM)		Dose-dependent decrease of cell viability	[37]
	BEN, BPA, BPS, CHL, DIA, ES, TCS			Dose-dependent decrease of cell viability	[37]
	BA, HU, TCEP, VIN, VPA			Dose-dependent decrease of cell viability	[37]
Commercial polychlorinated biphenyls mixture	Aroclor 1254		Activation of p38 MAPK pathway	Increases in: (i) junction protein (e.g., JAM-A, N-cadherin, and β -catenin) endocytosis, and (ii) occludin ubiquitination; promotes the caveolin-dependent endocytosis and ubiquitine-proteasome degradation of junction proteins	[38]
Heavy metals	Cadmium chloride (CdCl ₂)	Immature rat Sertoli cells	Activation of p38 MAPK	Induces Sertoli cell TJ-barrier disruption	[39]

Chemical group	Environmental Toxicant	In vitro and/or in vivo	Signaling pathway	Cellular responses	References
Heavy metals	Cadmium chloride (CdCl ₂)	Male Sprague-Dawley rats	Activation of p38 MAPK	CdCl ₂ induces BTB damage.	[79]
Aliphatic diketone/gasoline	2,5-hexanedione (HD)	Adult male Fischer 344 rats		Reduces germ cell proliferation, thereby reducing germ cell vulnerability to x-rays	[80, 81]

Abbreviations: BBP, benzyl butyl phthalate; DBP, dibutyl phthalate; DEHP, dioctyl phthalate; DPP, dipentyl phthalate; BEN, benzophenone-3,3', 4,4'-tetracarboxylic dianhydride; BPA, 2,2-bis(4-hydroxy-3-methylphenyl)propane; BPS, 4,4'-sulfonyldiphenol; CHL, chlorpyrifos; DIA, diazinon; ES, β -estradiol; TCS, 2,4,4'-trichloro-2'-methoxydiphenyl ether; BA, Boric acid; HU, hydroxyurea; TCEP, tri-(2-chloroethyl)phosphate; VIN, vinclozolin; VPA, valproic acid sodium salt.

Table 2.

Phenotypes in the mouse testis following specific knockout (KO) of mTOR, Raptor, Rictor in testicular cells or epithelial cells in the organs of the male reproductive tract in mice *

Gene KD	Phenotype	References
mTOR SC-cKO (<i>mTOR</i> ^{fllox/fllox} ; <i>Amhr2</i> ^{cre/+})	Progressive testicular atrophy during development and associated with disorganization of the seminiferous epithelium, loss of Sertoli cell polarity, increased germ cell apoptosis, premature germ cell release, reduced epididymal sperm count, increased sperm abnormalities, up-regulation of p-rpS6, mice were infertility	[52]
mTOR GC-cKO (<i>mTOR</i> ^{fllox} / <i>mTOR</i> ⁻ ; <i>Ddx4</i> - <i>Cre</i>)	Disruption of germ cell development and differentiation	[82]
Raptor Wolffian duct-cKO (<i>Raptor</i> ^{fllox} / <i>Raptor</i> ⁻ ; <i>Ksp</i> - <i>Cre</i>)	Male infertility with an overall reduction in sperm number, due to extensive organ regression with aging in the epididymis, vas deferens and seminal vesicles	[83]
Rictor ^{Germine} -cKO (<i>Rictor</i> ^{fllox} / <i>Rictor</i> ⁻ ; <i>Ngn3</i> - <i>Cre</i>)	Defects in spermatid polarity, complete loss of spermatocytes with tubules devoid of germ cells.	[84]
Rictor ^{SC} -cKO (<i>AMH</i> - <i>Cre</i> ⁺ ; <i>Rictor</i> ^{loxP/loxP})	Excessive germ cell loss, azoospermia	[53]

* Abbreviations used: *Amh*, anti-Mullerian hormone gene; *Ddx4*, DEAD-Box helicase 4 gene, also known as *Vasa*, GC, germ cells; KO, knockout; *Ksp*, also known as CDH16 gene or Ksp-cadherin gene; *Ngn3*, neurogenin 3 gene; SC, Sertoli cells;