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Mitogen-activated protein kinases in male reproductive function

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Abstract

Recent studies have shown that male reproductive function is modulated via the mitogen-activated protein kinase (MAPK) cascade. The MAPK cascade is involved in numerous male reproductive processes, including spermatogenesis, sperm maturation and activation, capacitation and acrosome reaction, before fertilization of the oocyte. In this review, we discuss the latest findings in this rapidly developing field regarding the role of MAPK in male reproduction in animal models and in human spermatozoa *in vitro*. This research will facilitate the design of future studies in humans, although much work is needed before this information can be used to manage male infertility and environmental toxicant-induced testicular injury in men, such as blood–testis-barrier disruption.

Male reproductive health and the involvement of the mitogen-activated protein kinase pathway

Male infertility and subfertility have been attributed to poor semen quality and/or sexual dysfunction. Abnormal semen quality includes reduced sperm counts and defects in the morphology, genetic content or motility of spermatozoa, whereas sexual dysfunction refers to impotence or defects in ejaculation [1,2]. The causative agents for abnormal semen quality are numerous and include heat, physical insults to testicles, radiation, alcohol and/or drug abuse, cigarette smoking and environmental toxicants. Heavy metals (e.g. cadmium and lead) and some drugs, such as chemotherapeutic compounds and antibiotics, are also known to impair fertility [1]. Even though the direct association of the mitogen-activated protein kinase (MAPK) pathway and reproductive dysfunction in men remains to be established, studies in animal models have indicated that the MAPK pathway is often involved in disrupting spermatogenesis and other functions of germ cells or Sertoli cells (see Glossary). This, in turn, leads to a decline in semen quality and fertility.

In this article, the participation of the MAPK pathway in various processes leading to abnormal semen quality is explored, as well as its role in normal spermatogenesis and germ cell functions. We also suggest that targeting kinases in the MAPK pathway might have potential for treating or managing male reproductive dysfunction. To assist the conception of couples with fertility problems, *in vitro* fertilization and intrauterine insemination are commonly used. Members of the MAPK pathway might serve as therapeutic targets to reverse the decline in semen quality and fertility as a result of disruption of sperm function or after testicular injury such as a disruption of the blood–testis barrier (BTB) induced by environment toxicants (e.g. cadmium). Furthermore, the MAPK pathway has been shown to be involved in the differentiation, maturation, and function of male germ cells. Kinases in the MAPK pathway might therefore also have potential for investigation as candidate targets for male contraception.

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Overview of the MAPK signaling pathway

The MAPK pathway is known to have an important role in the signal transduction of many cellular responses. In the testis, it is involved in processes including the cell-cycle progression and differentiation of germ cells, germ cell apoptosis, BTB dynamics and Sertoli and germ cell adhesion. The core of the MAPK pathway involves the sequential phosphorylation and activation of three protein kinases, namely MAPK, MAP kinase kinase (MAP2K) and MAP kinase kinase kinase (MAP3K) (Figure 1). When under stress or induced by external stimuli, such as cytokines and growth factors or exposure of cells and/or testes to environmental toxicants (e.g. cadmium), MAP3Ks are activated by MAP kinase kinase kinases (MAP4Ks), which interact with small G proteins. The phosphorylated MAP3K will activate the MAP2K. Upon activation by MAP3Ks, dual specific MAP2Ks will in turn activate MAPKs directly through phosphorylation of both a Tyr and a Ser or Thr residue.

The downstream MAPK consists of three major families of protein kinases, including p38 MAPKs (α , β , γ and δ), extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinases 1–3 (JNK1–3) (Figure 1). p38 MAPKs and JNKs are also named stress-activated protein kinases. There are some other MAPKs, such as ERK3, ERK5 and ERK7, which have different regulations and functions. Activated MAPKs will then phosphorylate downstream effectors, such as membrane proteins, cytoplasmic proteins and nuclear proteins [3,4]. The net results of these events affect cell-cycle differentiation, cell death, cell movement, cytoskeletal status and intracellular trafficking [3].

Members of the MAPK or MAP2K families are from a single gene family, whereas members of the MAP4K and MAP3K families are from diverse gene families and are more numerous than MAPK or MAP2K family members [4,5]. This means that the activation of different MAP4Ks and MAP3Ks under different circumstances would converge on the MAP2K and MAPK level. MAPKs would then activate different substrates for context-specific outcomes. The signal integration and spatiotemporal regulation depends on various factors that modulate the rate and intensity of the signal [4]. These factors include the cellular localization of MAPKs and their substrates [4,6], the interactions of kinases with an MAPK module with scaffolding proteins [7,8] and the inactivation of MAPK by dual-specific MAPK phosphatases [9]. Besides phosphorylation, ubiquitination has also been suggested to be important in the regulation of these events [10]. Collectively, these factors determine the duration and magnitude of the kinase activation and thus the physiological outcome [4].

The MAPK pathway in development, maturation and function of germ cells

Overview of the involvement of MAPK in germ cell development and maturation

The MAPK pathway is involved in many stages of germ cell development. These include spermatogenesis, germ cell cycle progression, germ cell apoptosis, acquisition of motility in the epididymis, sperm capacitation and acrosome reaction before the fertilization of oocytes [11,12]. After spermatozoa enter the female reproductive tract, they need to acquire the hyperactivating motility and undergo capacitation and the acrosome reaction before a single spermatozoon can fertilize an ovum [13]. The roles of MAPK in these events will be illustrated below.

Some studies have demonstrated that MAPKs could also affect germ cell development indirectly through their effects on Sertoli cells. Any perturbation in Sertoli cell function would impair spermatogenesis and thus reduce the semen quality. It is known that Sertoli cells cease to divide and become fully differentiated, such as by days 15–18 post-partum in rats [14,15]. The differentiation or proliferation of Sertoli cells is partly modulated by the follicle stimulating hormone (FSH), and the action of FSH is dependent on the ERK signaling cascade [16]. Heat-

stress-induced de-differentiation of adult monkey Sertoli cells was also shown to be mediated by ERK1/2 [17]. Because the de-differentiation of Sertoli cells would render them incapable of supporting spermatogenesis and maintaining germ cell function in adult testes, these findings thus illustrate that the regulation of Sertoli cell functions by the MAPK pathway would also affect germ cell development.

MAPK and germ cell cycle progression

During spermatogenesis, haploid spermatids (1n) are produced from diploid (2n) primary spermatocytes after a single round of DNA replication, which is followed by two successive divisions in meiosis [18]. Preleptotene primary spermatocytes, which are derived from type B spermatogonia, are in transit at the BTB while they differentiate into leptotene and zygotene spermatocytes at stages VIII and IX of the seminiferous epithelial cycle in rats [19]. The lengthy prophase I in spermatocytes begins to take place during the transit of spermatocytes across the BTB and takes ~10 days in mice and 16 days in humans to complete. This involves the pairing and duplication of chromatin. This process prepares the germ cells for metaphase I, when the two meiotic divisions occur at stage XIV of the epithelial cycle [20]. It is known that the maintenance of condensed chromatin during the short interphase that separates metaphase I from metaphase II is crucial to prevent DNA duplication and the correct reduction of the genome from a diploid to a haploid state [21,22].

Recent studies have shown that the MAPK pathway is required for the maintenance of the condensed chromatin to avoid a second round of DNA duplication between the two meiotic divisions. For instance, the chromosome condensation in mouse primary spermatocytes to enter metaphase I was shown to be regulated by ERK1 in studies using the specific inhibitor PD98059, a selective inhibitor of MEK1/2/5 (MAPK/ERK kinase 1, 2 and 5) [23]. It has been postulated [24,25] that when primary spermatocytes enter the late prophase I, ERK1 is activated, which allows its translocation to the nucleus to phosphorylate and activate its downstream effector ribosomal protein S6 kinase α -2 (p90-Rsk2). The activated ERK1–p90-Rsk2 complex would bind to the chromatin and phosphorylate NimA-related protein kinase 2 (Nek2), a cell-cycle regulator. The activated Nek2 in turn phosphorylates the high mobility group protein HMGI-C (also known as HMGA2), a non-histone protein regulator in the transcriptional control and cell cycle progression. The phosphorylation of HMGA2 leads to its release from the chromatin due to a reduction of its affinity for the DNA [24], thus allowing the binding of condensation factors to the chromatin, triggering chromosome condensation. As such, MAPK was shown to regulate the progression of prophase I to metaphase I during meiosis in germ cells.

MAPK and germ cell apoptosis

In the testes, almost 75% of the developing germ cells must undergo spontaneous apoptosis during normal spermatogenesis [26,27] in mammals [28,29] to maintain a constant ratio of the number of germ cells to Sertoli cells. This is because the quantity of Sertoli cells is fixed in adult rat testes and each Sertoli cell can only sustain ~30–50 developing germ cells [14,15]. Apoptosis becomes accelerated when the testis is under stress, such as mild hyperthermia or a change in the level of sex hormones, including FSH, testosterone and estrogen [26]. Both testosterone and heat-induced stress are also known to regulate germ cell apoptosis through the mediation of the MAPK pathway. For instance, p38 MAPK α was shown to be activated during germ cell apoptosis induced by mild hyperthermia or via a suppression of testosterone in monkeys and rats [30]. JNK1 has also been implicated in the induction of the apoptosis of immature germ cells when they became detached from Sertoli cells [31].

In short, these findings indicate the participation of MAPK in germ cell apoptosis. Any alteration in the MAPK homeostasis in germ cell apoptosis, such as with specific inhibitors or

agonists, might potentially shift the ratio of germ cells to Sertoli cells and affect reproductive health in men.

MAPK and spermatozoan function

Prior to the fertilization of an oocyte, spermatozoa in the female reproductive tract have to become hyperactivated and undergo capacitation and acrosome reaction [11,12]. Capacitation is a biochemical event that involves changes to spermatozoa in the female reproductive tract, particularly the plasma membrane at the sperm head overlying the acrosome. This leads to the hyperactivation of sperm cell motility and an increase in permeability to calcium ions at the sperm head to prepare for the acrosome reaction [11]. Acrosome reaction occurs when a spermatozoon binds to the zona pellucida (ZP) of the oocyte, which is the glycoprotein coat surrounding the egg. The plasma membrane of the sperm fuses with that of the oocyte with the help of enzymes released from the sperm head [13]. It is known that capacitation is induced by cumulus oophorus, a few layers of cumulus cells that have a matrix composed of hyaluronic acid, surrounding the oocyte [13]. Capacitation is then followed by the acrosome reaction. It is generally accepted that the 'acrosome-reacting' spermatozoa cleaves the cumulus matrix of the cumulus oophorus via the action of hyaluronidase released from the acrosome. The acrosin of the 'acrosome-reacted' spermatozoa facilitates the penetration of the spermatozoa through the ZP, so that the plasma membrane of the sperm head fuses with that of the oocyte to allow the injection of the sperm nucleus into the oocyte [11,13].

ERK1/2 and p38 MAPK, but not JNK1/2, have been detected in the tail of ejaculated human spermatozoa [11]. There are many reports concerning the role of ERK in sperm motility; however, due to conflicting findings, it remains uncertain whether ERK promotes or inhibits motility associated with capacitation (for further details see the recent review by Almog [11]). A recent study reported the involvement of ERK1/2 and p38 MAPK in the regulation of forward and hyperactivated motility of sperm [32]. In this study, it was reported that ERK becomes phosphorylated to promote motility, whereas p38 MAPK is phosphorylated to inhibit sperm motility. The same report also implicated the positive role of ERK and p38 MAPK in acrosome reaction. ARHGAP6, a Rho GTPase-activating protein 6 and a putative ERK substrate, was found in mature human spermatozoa [32]. These findings seemingly suggest that the MAPK pathway is involved in regulating capacitation and the acrosome reaction in the female reproductive tract before fertilization [11], perhaps involving small GTPases, such as Rho. However, it is important to note that these studies were performed using isolated spermatozoa *in vitro* and involved the use of inhibitors, some of which might be toxic to sperm cells [11, 32]. Thus, these findings need to be carefully validated in future studies. Much work is needed to identify the downstream effectors of ERK1/2 and p38 MAPK that confer sperm capacitation and the acrosome reaction before fertilization.

MAPKs as regulators of cell adhesion in the seminiferous epithelium

The MAPK cascade and junction restructuring

During spermatogenesis, the movement of developing germ cells across the seminiferous epithelium is accompanied by extensive junction restructuring at the Sertoli–Sertoli and Sertoli–germ cell interfaces (Figure 2). At stages VIII and IX of the seminiferous epithelial cycle, primary preleptotene and leptotene spermatocytes are in transit at the BTB [19,33]. Thus, the BTB must undergo 'restructuring' (or 'open') to accommodate the passage of these primary spermatocytes of ~8–10 µm in diameter while maintaining the integrity of the immunological barrier at the same time. It was proposed that 'new' tight junction (TJ)-fibrils were formed below a migrating primary leptotene spermatocyte before the 'old' TJ-fibrils above the cell in transit were defragmented [34–36].

Cytokines and testosterone were postulated recently to regulate junction restructuring at the BTB. Cytokines, such as transforming growth factor (TGF)- β 2 and TGF- β 3, are known to induce the disruption of BTB integrity via the p38 MAPK signaling pathway [37,38]. Conversely, testosterone is known to promote BTB integrity [39–41]. TGF- β 2 and - β 3 and testosterone have all been shown to accelerate the endocytosis of integral membrane proteins (e.g. occludin, junctional adhesion molecule-A [JAM-A]) at the BTB [42,43]. The cytokine-induced and testosterone-induced endocytosed proteins are targeted to late endosomes for intracellular degradation or recycled back to the cell surface, respectively [42]. The combined actions of TGF- β 2/TGF- β 3 and testosterone would allow the relocation of integral membrane proteins at the BTB from the apical to the basal region of the spermatocyte in transit via transcytosis. This would result in the assembly of new TJ-fibrils at its basal region and the disruption of old TJ-fibrils at its apical region and hence its passage across the BTB. Because their effects on protein endocytosis and recycling occur within 5–30 min, based on published findings [42,43], it is plausible that testosterone exerts its effects via the ERK pathway instead of the classical genomic pathway. A recent study has demonstrated that the androgen receptor is capable of activating the ERK pathway through the mediation of Src for rapid physiological outcome [44].

During spermiogenesis, developing spermatids remain attached to Sertoli cells in the seminiferous epithelium via anchoring junctions, such as the desmosome-like junction. When acrosome begins to form above the condensed nucleus in step 8 spermatids (see spermatogenesis in Glossary), they are anchored to the Sertoli cell exclusively through the apical ectoplasmic specialization (apical ES, a testis-specific anchoring junction type) [45]. Unlike other adherens junctions (AJs), the apical ES utilizes cell-matrix actin-based anchoring junction proteins, such as integrin and focal adhesion kinase (FAK), to regulate its restructuring. These proteins are usually restricted to focal contacts, also known as focal adhesion complex, at the cell-matrix interface. In fact, one of the major adhesion protein complexes at the apical ES is α 6 β 1-integrin–laminin- α 3 β 3 γ 3, wherein the α 6 β 1-integrin is on the Sertoli cell side and the laminin- α 3 β 3 γ 3 is on the elongating spermatid side [46]. A recent study has shown that at late stage VIII of the epithelial cycle at the time of spermiation, the MMP-2 (matrix metalloprotease-2) localized at the apical ES is activated and is likely to be used to cleave the laminin chains [47]. This would generate the biologically active fragments that induce BTB restructuring, which in turn facilitates the transit of primary leptotene spermatocytes across the BTB. It was demonstrated that laminin fragments were capable of disrupting the function of the Sertoli cell TJ-permeability barrier, and an activation of ERK1/2 was also detected [42].

In short, these findings illustrate that two cellular events, namely spermiation and BTB restructuring, that occur simultaneously at the opposite ends of the Sertoli cell epithelium at stage VIII of the seminiferous epithelial cycle are being coordinated, at least in part, via ERK1/2. These findings also implicate that by manipulating ERK1/2 activity in the seminiferous epithelium, one can possibly disrupt spermatogenesis and impair fertility.

The importance of junction integrity in normal spermatogenesis

The maintenance of the integrity of the BTB and the adhesion of germ cells to Sertoli cells in the seminiferous epithelium are crucial for normal spermatogenesis [36,48]. A loss of contact of premature testicular germ cells with Sertoli cells would affect their development [49] and survival [31].

Studies have shown that environmental toxicants are able to disrupt the integrity of the BTB and anchoring junctions at the cell–cell interface in the seminiferous epithelium [50–52]. Premature release of germ cells from the seminiferous epithelium was detected after treatment of rodents with endocrine disruptors such as cadmium chloride [50,53]. Spermatogenesis was

thus impaired. The perturbation of the BTB would also facilitate the passage of harmful substances to the apical compartment of seminiferous epithelium, possibly affecting the health of developing spermatids. These together would result in a decline in the number of functional spermatozoa in the semen. Protecting the junction integrity against such damages induced by testicular insults might alleviate their adverse effects on male reproduction.

The role of MAPK in the maintenance of junction integrity in animal models

Besides having a functional role in normal junction restructuring, the MAPK signaling cascade was found to be employed by environmental toxicants to damage junction integrity, such as at the BTB. For instance, it was shown that cadmium induced a disruption of the BTB via an initial activation of the TGF- β 3–TGF- β receptor complex at the BTB. This in turn induced the activation of p38 MAPK and subsequently led to a disruption of the TJ barrier function at the BTB [50,54], possibly mediated via a loss of integral membrane proteins at the BTB site [54, 55]. Disruption of the TJ barrier resulted in an eventual disintegration of TJ fibrils and dislodgement of germ cells [53].

In cadmium-induced toxicity in the seminiferous epithelium, other proteins, such as cathepsin L (a cysteine protease) and α_2 -macroglobulin (a non-specific protease inhibitor), also took part in the events of BTB disruption and germ cell loss [50,56]. Proteases were proposed to induce the proteolysis of cell adhesion molecules at the Sertoli–Sertoli and Sertoli–germ cell interface, leading to BTB disruption and germ cell loss from the epithelium. Conversely, α_2 -macroglobulin limited excessive proteolysis in the epithelium so that the integrity of the testis could be maintained at the time the fertility of the males was compromised [50,56].

Interestingly, the induction of α_2 -macroglobulin after exposure of adult rats to cadmium is partly mediated by JNK instead of p38 MAPK [56]. For instance, administration of DMAP (6-dimethylaminopurine, a protein kinase inhibitor that can inhibit JNK) to the testis before cadmium treatment was shown to ‘worsen’ the cadmium-induced testicular damage [56]. A more extensive loss of germ cells from the epithelium and BTB function was observed [56]. Some of these findings have recently been confirmed using three-dimensional primary Sertoli cell–gonocyte cocultures. The toxicity of cadmium was shown to be mediated by the p38 and JNK pathways to alter intra-cellular protein degradation and apoptosis [57]. It was shown that the cadmium-induced BTB disruption and the premature release of germ cells associated with an induction in proteolysis could possibly be modulated with specific inhibitors against either p38 or JNK MAPK [54,56]. This implies that environmental toxicant-induced infertility can be therapeutically managed by specifically targeting p38 and/or JNK MAPKs in the seminiferous epithelium.

Apart from their role in BTB function, members of the MAPK family act as regulators of anchoring junctions at the Sertoli–germ cell interface that confers germ cell adhesion. An *in vivo* model was used in which rats are treated with adjuvin, formerly called AF-2364 (1-[2,4-dichlorobenzyl]-1H-indazole-3-carbohydrazide), by gavage to induce anchoring junction restructuring at the Sertoli–germ cell interface. In this model, the loss of germ cells from the epithelium is induced without perturbing BTB integrity [56]. It was shown that the events of anchoring junction restructuring are regulated partly by an activation of cytokines (e.g. TGF- β 3, tumor necrosis factor α [TNF α]) and the ERK signaling pathway [58–60]. The involvement of ERK in germ cell adhesion was confirmed with U0126, a specific inhibitor of MEK1/2/5. It was shown to delay the adjuvin-induced germ cell loss from the seminiferous epithelium [58]. In a study using *l*-CDB-4022, an indenopyridine and potential contraceptive, the loss of germ cells was also shown to be accompanied by an activation of ERK [61], analogous to adjuvin [58].

In this context, it should be noted that in the cadmium and adjuvant models used to study the BTB and germ cell adhesion, respectively, TGF- β 3 was shown to be transiently induced [50, 54,58]. A subsequent study has shown that in the testis, TAB1 (TGF- β -activated kinase 1 [TAK1]-binding protein 1, also known as MAP3K7IP1 [MAP3K7 interacting protein 1]) and CD2AP (cluster of differentiation antigen 2-associated protein), two different adaptors for TGF- β receptor 1 (T β R1), were used to distinguish which MAPKs should be activated to elicit the restructuring of the BTB or anchoring junction after exposure to cadmium or adjuvant [62]. After exposure of rats to cadmium, the activated TGF- β 3-T β R1 complex interacts with both TAB1 and CD2AP. The p38 and ERK MAPKs are both activated and induce restructuring of the BTB and anchoring junction, leading to BTB disruption and germ cell loss from the epithelium. However, after exposure of rats to adjuvant, the activated TGF- β 3-T β R1 complex interacts with CD2AP alone. Only the ERK MAPK was activated, leading to restructuring of the anchoring junction without perturbing the BTB [62]. Many reported effects of adjuvant on MAPKs have been confirmed using another study model utilizing testosterone/estradiol (T/E) implants to suppress the intratesticular androgen level. This would thereby lead to the loss of germ cells, most notably elongating spermatids, from the seminiferous epithelium because of a disruption of the apical ES [63]. In short, these findings have unequivocally demonstrated that MAPKs are potential therapeutic targets for managing male reproductive dysfunction, regarding both BTB function and germ cell adhesion in the seminiferous epithelium.

Development of inhibitors of kinases in the MAPK pathway to modulate reproductive success

MAPK pathway inhibitors for therapy of various diseases

Small molecule inhibitors of kinases in the MAPK pathway have been actively developed in the hope of treating various diseases, including inflammatory diseases [64], ischemic heart disease [65] and cancer [66,67] (Figure 1). There are inhibitors being used in the field that target p38 MAPKs and JNKs directly, whereas inhibitors against ERK1 and ERK2 exert their effects indirectly through an inhibition of their upstream activators, namely MEK1/2/5, and Raf. Sorafenib tosylate (Nexavar®; Onyx Pharmaceuticals, Inc.; Emeryville, CA), formerly known as Bay 43-9006, is a specific inhibitor of Raf and tyrosine kinases of growth factor receptors. It has been approved by the FDA and EU for treating patients with advanced liver cancer [68].

Many of these inhibitors have been widely used in cell cultures *in vitro* or animal models *in vivo* for the functional study of the MAPK pathway. Most of these 'specific' inhibitors, however, were subsequently shown to affect other protein kinases. For instance, most p38 MAPK inhibitors target the ATP binding pocket of p38 MAPKs and thus act as ATP competitors. This thus broadens their selectivity and limits their therapeutic usage. Clinical development of many small molecule inhibitors has similarly halted owing to systemic toxicity at the early stage of clinical trials.

The MAPK cascade as a therapeutic target to protect and/or restore BTB integrity and spermatogenesis

Small molecule inhibitors of the MAPK module could be used to protect the seminiferous epithelium, in particular the BTB, against testicular insults. Disruption of the BTB and impairment of spermatogenesis induced by toxicants such as cadmium could possibly be relieved by blocking their unwanted activation of the MAPK signaling pathways in Sertoli cells (Figure 2). This could perhaps improve the semen quality and thus male fertility. Due to the roles of the MAPK cascade in the development and function of normal germ cells, the introduction of small inhibitor molecules directly to germ cells to revive fertility should be carefully evaluated to prevent the occurrence of any defects on sperm cells.

The possibility of blocking the disruption of cell adhesion has been demonstrated in two *in vivo* studies (see Table 1). This is further illustrated by several selected examples on junction disruption based on studies in other epithelia (Table 1). For instance, in the MDCK cell line, the MEK1/2/5 inhibitor PD98059 has been shown to reverse damage in the TJ integrity resulting from an overexpression of Ras [69], an upstream activator of ERK1/2 and other cellular pathways [4].

Chemotherapeutic agents such as cisplatin are known to cause testicular toxicity in cancer patients [70]. These adverse effects on fertility could be transient or permanent. But MEK1/2/5 inhibitor PD98059 was able to prevent the cisplatin-induced increase in interleukins and nitric oxide synthases in Sertoli cells [71]. It thus indicates that these small molecule inhibitors could be used together with chemotherapeutic agents to reduce their testicular toxicity.

Recent advances in the delivery of male contraceptives might help to tackle the problem of systemic toxicity [42]. Utilizing specific receptors on the cell surface of Sertoli cells, these small molecule inhibitors could be directed to Sertoli cells selectively. For instance, the conjugation of a small molecule inhibitor with a mutant form of FSH, which lacks the intrinsic hormonal activity, would allow its selective targeting to the FSH receptor on Sertoli cells [72,73]. This reduces their systemic toxicity partly by reducing the duration of exposure to other organs.

In short, these findings suggest that targeting the inhibitors of kinases in the MAPK pathway to Sertoli cells selectively could provide a means to partially restore fertility in males with reproductive dysfunction caused by occupational exposure to endocrine disruptors [74], patients with poor sperm quality, or cancer patients.

Small molecule inhibitors of the MAPK cascade as a potential approach for male contraception

We have discussed the roles of the MAPK cascade in the life cycle of male germ cells, but small molecule inhibitors might also be worth considering as a potential approach for targeting sperm function for birth control. Inhibition of kinases in the MAPK pathway in germ cells might lead to formation of dysfunctional germ cells that are rendered incapable of fertilization. Early primary spermatocytes, such as preleptotene spermatocytes, would be the preferable target because they are located in the basal compartment. This means that the inhibitors could reach the primary preleptotene spermatocytes easily without the need to perturb the BTB. The other germ-cell type present in the basal compartment is spermatogonia. These cells are not a suitable target because DNA damage in spermatogonia might be passed onto spermatids, causing irreversible damage to embryos. Delivery of these inhibitors to early primary spermatocytes would be expected to lead to failure in the condensation of chromosomes for meiosis and thus could disrupt the progression of the cell cycle. For selective targeting of these inhibitors, cell surface receptors specific to primary spermatocytes could also be utilized.

Concluding remarks

Here we have summarized the significance of MAPK in normal male reproductive function and during environmental toxicant-induced reproductive dysfunction. In short, MAPK plays a part in virtually every step of spermatogenesis in the testis, including spermatogonial stem cell renewal (mitosis), germ cell cycle and meiosis, spermiogenesis, and spermiation. MAPK is also involved in the subsequent sperm maturation and motility, hyperactivation, capacitation and acrosome reaction in the female reproductive tract before fertilization with the ovum. Much work needs to be conducted to explore the direct association of MAPK activity and subfertility. However, limited evidence, including studies in the testes and other epithelia (Table 1), indicates that the use of specific inhibitors against members of the MAPK family is a potential

approach for managing the disruption of junction integrity in the seminiferous epithelium induced by testicular insults like environmental toxicants. This might help to reverse impairments in spermatogenesis and thus provide new therapeutic approaches for some cases of unexplained male infertility or subfertility. Table 2 also summarizes several clinical trials recently completed based on the use of systemic administration (such as oral tablets) of inhibitors of MAPK for treatment of rheumatoid arthritis and metastatic thyroid cancer. These studies suggest that the use of such specific inhibitors might have potential as a therapeutic approach, but this clearly needs to be further investigated for the management of the disruption of reproductive functions and/or testicular injury induced by toxicants (e.g. cadmium).

At present, the effect of inhibiting the activation of MAPKs on spermatogenesis is unclear due to a lack of cell-specific knockout models. Gene knockout rodent models have provided limited information on the role of different kinases in the MAPK pathway in male reproduction. For instance, many rodent knockout models with kinases in the MAPK pathway (MAPK, MAP2K or MAP3K) being targeted died prematurely at the embryonic stage [3]. As such, the impact of dysfunctional activation of MAPKs on spermatogenesis remains largely unknown. In some cases, the animals remain viable and fertile, such as in the knockout model of ERK1. This indicates that some kinases in the MAPK pathway can indeed be blocked without inhibiting reproductive function, and it is possible that their functions might be superseded by other kinases. To further examine the roles of the MAPK pathway in spermatogenesis, small molecule inhibitors against specific kinases of the MAPK cascade could be selectively targeted to Sertoli cells. Specific knockdown of a member of the MAPK family using an RNA interference approach in Sertoli cells cultured *in vitro*, with an established TJ-permeability barrier that mimics the BTB *in vivo*, is a powerful alternative for examining MAPK function in BTB regulation and biology.

It should be noted that inhibition of certain MAPKs might also block the beneficial effects mediated by those pathways in epithelial cells, including the seminiferous epithelium. For instance, the MEK1/2/5 inhibitors PD98059 and U0126 could completely block the activation of ERK1/2. This would be expected to impede the protective function on the TJ barrier against oxidative stress induced by the epidermal growth factor in MDCK cells [75]. Hence, the development of more selective inhibitors for therapeutic use for improving male reproductive health relies on further investigation of the roles of different MAPKs and their isoforms in spermatogenesis. In Box 1 we have listed some of the pressing questions that should be carefully evaluated by investigators in future studies regarding the roles of MAPKs in reproductive function.

Box 1

Outstanding questions

- Is MAPK involved in the disruption of cumulus oophorus, penetration of the zona pellucida, sperm–egg membrane fusion, or all three cellular events during fertilization? Which member(s) of the MAPK family are involved in these events and the cascade of signaling function?
- What is the optimal delivery approach for targeting specific inhibitor(s) of MAPK to improve fertility and/or to correct environmental toxicant induced-disruption of testicular function, such as BTB disruption?
- What is the precise molecular mechanism(s) by which cytokines and testosterone regulate the transit of primary preleptotene and leptotene spermatocytes at the BTB at stage VIII of the epithelial cycle, which is known to involve activation of MAPK?

- Spermatozoa are highly differentiated cells with relatively little transcriptional activity, so what mechanism(s) are involved in providing the necessary mRNAs of different MAPKs for regulating sperm function?

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Glossary

Anchoring junctions	junction complexes conferring adhesion to cells. These include adherens junction (AJ), desmosome, hemidesmosome and focal contact (or focal adhesion complex). They are usually present more basally than tight junctions. AJs and desmosomes are cell–cell adhesion complexes that are linked to the actin cytoskeleton and intermediate filament, respectively. Focal contacts and hemidesmosomes are responsible for cell adhesion to the cell matrix using actin and intermediate filament, respectively, as the attachment sites. In the testis, focal contact is absent. However, ectoplasmic specialization (ES), a testis-specific AJ type at the Sertoli–elongating spermatid interface (apical ES) and Sertoli–Sertoli cell interface (basal ES), possesses the properties of focal contact by having focal contact proteins at the site, such as focal adhesion kinase (FAK), vinculin, paxillin, integrins, integrin-linked kinase (ILK) and laminins
Blood–testis barrier (BTB)	a unique physiological barrier amidst the blood–tissue barriers. It is created by adjacent Sertoli cells near the basement membrane in the seminiferous epithelium instead of the specialized endothelial cell tight junction of the microvessels in the interstitium, such as those found in the blood–brain barrier and the blood–retina barrier. The BTB divides the seminiferous epithelium into the apical (or adluminal) and basal compartment and segregates the entire event of post-meiotic germ cell development from the systemic circulation. The BTB is composed of coexisting tight junction, gap junction, desmosome-like junction and basal ectoplasmic specialization, an atypical adherens junction type only found in the testis
Seminiferous epithelial cycle	a series of sequential changes in cellular association occurring in a given area of the seminiferous epithelium. The cellular associations were defined mainly by the stage of development of the spermatids. This can be identified easily after routine histological staining, such as the periodic acid-Schiff's reaction (PAS) for visualizing changes in the Golgi region of spermatids during the formation of the acrosome. The number of stages identified in different animals varies. In adult rats, each seminiferous epithelial cycle is composed of 14 stages and completes in ~12.9 days. In mice, each epithelial cycle is composed of 12 stages and completes in 8.6 days. Only six stages are defined in humans
Sertoli cells	Sertoli cells are nursery cells in the seminiferous epithelium located near the basement membrane (see Figure 2) of the seminiferous tubule that provide the structural and nutritional supports to the developing germ

cells in adult testes. In rats, Sertoli cells cease to divide by ~day 15–18 post-partum at the time they create the blood–testis barrier (BTB), and the Sertoli cell number remains relatively constant thereafter at ~40 million per testis throughout adulthood. Each Sertoli cell can only support ~50 germ cells at different stages of their development. Thus ~75% of germ cells undergo apoptosis and are phagocytosed by Sertoli cells so that a Sertoli:germ cell ratio of ~1:50 can be maintained in the seminiferous epithelium for proper germ cell development during spermatogenesis. Thus, Sertoli cells also maintain the proper germ cell number in the testis and are known as the scavenger in the seminiferous epithelium

Spermatogenesis

male gametogenesis, in which male spermatogonia (diploid, 2n) develop into spermatozoa (haploid, 1n). In mammals such as rats, spermatogenesis includes several steps as follows: (i) the renewal of spermatogonia via mitosis; (ii) the formation of haploid spermatids from tetraploid diplotene spermatocytes derived from diploid primary spermatocytes via meiosis (namely meiosis I and meiosis II); (iii) differentiation of step 1 spermatids (round spermatids) to step 19 elongated spermatids in spermiogenesis, which is typified by the formation of acrosome above the condensed nucleus in the spermatid head coupled with elongation of the tail and shedding of the cytoplasm as residual body; and (iv) spermiation, when fully developed elongated spermatids (spermatozoa) at the adluminal edge of the epithelium empty into the seminiferous tubule lumen. This is followed by the acquisition of motility of spermatozoa in the epididymis

Tight junction (TJ)

a junction complex present only in vertebrates. TJ strands are formed to join two adjacent epithelial or endothelial cells to form an impermeable barrier. TJ also restricts the flow of water, ions and biomolecules between epithelial cells, known as the ‘fence’ function. It also confers cell polarity by restricting the movement of other integral membrane proteins between apical and basolateral regions. TJ is composed of heterotypic or homotypic interaction of transmembrane proteins of TJ, such as occludins, claudins and junctional adhesion molecules (JAMs). These transmembrane proteins are anchored to the actin cytoskeleton through different adaptor proteins

Xenobiotics

substances that are foreign to a biological system or an organism. A xenobiotic substance could be natural or synthetic. The term xenobiotic is often used in reference to environmental toxicants, including cadmium, mercury, lead, bisphenols and vinclozolin

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Figure 1.

The core components of the MAPK signaling pathway. The MAPK pathway transduces signals from external stimuli (e.g. when testicular cells are exposed to environmental toxicants, such as cadmium, or cytokines, such as TGF-β3) to cells (e.g. Sertoli and/or germ cells) so that appropriate physiological responses can occur. When induced by external stimuli, as shown in the left-hand box, there is a sequential activation of MAP4Ks and MAP3Ks (e.g. MEKK, TAO, Mos, Raf); see the right-hand box, which shows examples of members of the MAPK cascade and some commonly used inhibitors. Phosphorylated MAP3Ks will activate MAP2Ks (e.g. MEK4/7, MEK3/6, MEK1/2, MEK5). Upon activation by MAP3Ks, dual-specificity MAP2Ks (e.g. MEK4/7, MEK3/6, MEK1/2, MEK5) will in turn activate MAPKs (e.g. JNK1-3, p38 MAPKα-δ, ERK1/2, ERK5) directly through phosphorylation of both a Tyr and a Ser or Thr residue. Different stimuli affect different components of the MAPK pathway, and different effectors of MAPK will be activated for different physiological responses. Some of the commonly used inhibitors that block specific MAPKs are shown. Inhibitors that are shown in red are used mostly for basic research because their therapeutic developments were not continued due to undesirable side effects. The chemical names of the inhibitors listed are as follows: SP600125, anthra[1-9-cd]pyrazol-6(2H)-one; SB202190, 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol; SB203580, 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine; SB242235, 5-[5-(4-fluorophenyl)-3-piperidin-4-yl-3H-imidazol-4-yl]-2-methoxy-pyrimidine; SCIO-469, 2-[6-chloro-5-[(2R, 5S)-4-(4-fluorobenzyl)-2,5-dimethylpiperazin-1-yl]carbonyl]-1-methyl-1H-indol-3-yl]-N,N-dimethyl-2-oxoacetamide; Sorafenib, 4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]phenoxy]-N-methyl-pyridine-2-carboxamide; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene; PD98059, 2'-amino-3'-methoxyflavone. Abbreviations: Mos, oocyte maturation factor; Raf, proto-oncogene serine/threonine protein kinase; MEKK, MAPK/ERK kinase kinase, also known as MAP3K, mitogen-activated protein kinase kinase kinase 1, a serine/threonine kinase; TAO, thousand and one amino acid protein.

Figure 2.

The role of MAPK in regulating cell adhesion function and BTB dynamics in the testis. **(a)** A schematic drawing of the cross-section of a seminiferous tubule showing the seminiferous epithelium. The seminiferous epithelium is composed of only Sertoli cells and germ cells (e.g. spermatogonia, spermatocytes, round spermatids, elongating spermatids, and elongated spermatids) at different stages of their development during spermatogenesis. The endothelium is encircled by the basement membrane, which is a modified form of the extracellular matrix. The BTB (blue-boxed area) divides the epithelium into the basal and adluminal compartments. Primary preleptotene spermatocytes (diploid, 2n) traverse the BTB at stages VIII and IX of the epithelial cycle (see Glossary) while differentiating into leptotene and zygotene spermatocytes. Once behind the BTB, meiosis occurs at stage XIV of the epithelial cycle in rat testes, wherein diplotene spermatocytes (tetraploid, 4n) undergo two cycles of reduction divisions (meiosis I and meiosis II) to form round spermatids (haploid, 1n). All the postmeiotic germ cell development, namely spermatids that undergo spermiogenesis via steps 1 through 19, is segregated from the systemic circulation by the immunological barrier conferred by the BTB. As such, the BTB is a unique and physiologically significant ultrastructure for the maintenance of spermatogenesis. **(b)** Testicular insults, including exposure to environmental toxicants (e.g. cadmium) or drugs, might induce a perturbation of germ cell adhesion via the p38 and ERK MAPK pathways. (Note, the anchoring junction between the Sertoli cell and germ cell is crucial

to maintain: (i) proper communication between these cells for germ cell development, because germ cells must rely on Sertoli cells for both structural and nourishment support; and (ii) proper orientation, because without the intact anchoring junction, germ cells either undergo apoptosis or detach from the epithelium prematurely.) (c) Testicular insults might also cause a disruption of the BTB via the p38 and ERK MAPK pathways. (Note, the BTB, unlike other blood–tissue barriers such as the blood–brain barrier, is composed of co-existing tight and anchoring junctions instead of only tight junctions, which is crucial to maintain the immunological barrier function at the BTB during anchoring junction restructuring through a yet-to-be defined mechanism.) The disruption of germ cell adhesion and BTB function might potentially be blocked or modulated with the use of small molecule inhibitors against kinases of the MAPK cascade (indicated with dashed arrows).

Table 1

A summary of recent studies using specific inhibitors against different MAPKs to manage the disruptive effects of toxicants, drugs and other causative agents on junction dynamics^a

Causative agents (e.g. drugs, toxicants, viruses, proteins and peptides)	Effects on junction dynamics	Inhibitor (s) used to block and/or reverse the phenotype	Kinase(s) blocked	Organ, tissue or cell line
Adjudin	Disruption of Sertoli-germ cell anchoring junction [58]	U0126	MEK1/2/5	Testes
Cadmium chloride	Disruption of the BTB (e.g. loss of occludin, ZO-1, and N-cadherin from the BTB) [38,50]	SB202190	p38 MAPK	Testes
Aspirin	Disruption of TJ-permeability barrier and a decline in the protein level of claudin-7 [76]	SB203580, but not PD98059 and U0126	p38 MAPK	MKN28 (human gastric adenocarcinoma cell line)
Group B coxsackievirus 3	Disruption of the organization of ZO-1 and F-actin [77]	SB203580	p38 MAPK	HUVEC (human umbilical vein endothelial cells)
HIV-1 Tat protein	Decline in tight junction barrier integrity and a decrease in level of claudins, integral membrane proteins of the tight junction [78]	PD98059	MEK1/2/5	D407 (human retinal pigment epithelial cell line)
Overexpression of Ras	Disruption of tight junction and adherens junction [69]	PD98059	MEK1/2/5	MDCK (Madin-Darby canine kidney cell line)

^a This Table is not intended to be exhaustive: only selected examples are shown to illustrate the use of specific inhibitors to block (or delay) damage to cell junctions (e.g. BTB) in the testes induced by toxicants and other causative agents in other epithelial cell junctions.

Table 2Selected clinical trials using MAPK inhibitors for different pathological conditions^a

Compound	Background	Targeted pathological conditions (patient number, <i>n</i>)	Clinicaltrials.gov identifier	Status
SB-681323 and methotrexate	SB-681323 is a p38 MAPK inhibitor and methotrexate is commonly used to treat rheumatoid arthritis. This trial sought to examine whether SB-681323 can have beneficial effects in inflammatory conditions. Because other p38 MAPK inhibitors were shown to cause liver damage, this study aimed to examine the effects of co-administration of the two compounds on liver function. This is a phase I study	Rheumatoid arthritis (<i>n</i> = 15)	NCT00419809	Completed but no study results posted
SB-681323	A phase II study to assess the safety and tolerability of SB-681323 and the levels of serum C-reactive protein (CRP) in patients with rheumatoid arthritis	Rheumatoid arthritis (<i>n</i> = 84)	NCT00320450	Completed but no study results posted
Sorafenib tosylate	Sorafenib (Bay 43-9006), a multi-kinase inhibitor that also blocks Raf, was investigated in this phase II study to examine whether it inhibited tumor growth by blocking enzymes needed for growth and by stopping	Metastatic thyroid carcinoma (<i>n</i> = 16)	NCT00095693	Completed but no study results posted

Compound	Background	Targeted pathological conditions (patient number, <i>n</i>)	Clinicaltrials.gov identifier	Status
	blood flow to the tumor			

^aThese are a few selected examples of clinical trials that are investigating MAPK inhibitors for various pathological conditions based on information obtained from www.ClinicalTrials.gov. At present, there are 63 ongoing clinical studies using inhibitors against different members of the MAPK family. Systemic administration of MAPK inhibitors is being used to treat various pathological conditions, such as rheumatoid arthritis, metastatic thyroid cancer, lung cancer and neurological conditions.