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Signaling Pathways in Spermatogonial Stem Cells and Their Disruption by Toxicants

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Abstract

Spermatogenesis is a complex biological process that is particularly sensitive to environmental insults such as chemicals and physical stressors. Exposure to specific chemicals has been shown to inhibit fertility through a negative impact on germ cell proliferation and differentiation that can lower sperm count. In addition, toxicants might produce DNA damages that could have negative consequences on the development of the offspring. This review describes spermatogonial stem cell development in the testis, signaling pathways that are crucial for self-renewal, and possible target molecules for environmental toxicants such as phthalate esters and nanoparticles.

Keywords

spermatogonial stem cells; phthalates; nanoparticles; signaling pathways

Introduction

Mammalian gametogenesis requires balancing proliferation, differentiation, and apoptosis of the male and female germ cells. In the male, spermatogenesis starts shortly after birth with a small population of stem cells called spermatogonial stem cells (SSCs), which are a subpopulation of type A spermatogonia. At puberty, mitotically dividing spermatogonial cell cohorts maintain the ability of self-renewal and occupy niches in the seminiferous tubule. Without a healthy population of stem cells, spermatogenesis would be durably compromised. Therefore, studying the effects of toxicants on that particular germ cell population may be important to understand the increase of reproductive disorders. The concept of testicular dysgenesis syndrome (TDS), developed by NE Skakkebaek (Boisen et al., 2001), includes abnormalities such as hypospadias, cryptorchidism, testicular cancer, and low sperm production in adulthood. These defects are possibly caused by events that have taken place in the intrauterine and perinatal periods (Skakkebaek, 2003; Sharpe, 2006). The incidence of TDS is increasing, but there are striking variations between countries, indicating environmental as well as genetic causes to this syndrome. Until recently, research on the effects of reproductive toxicants in the testis focused on Sertoli and Leydig cells due to their ease of isolation. It has often been assumed that the effects of reproductive toxicants on SSCs and their progeny, such as delayed differentiation and apoptosis, are exclusively the consequence of somatic cell alterations. However, more recently it has become apparent that other mechanisms might be involved. Reproductive toxicants targeting the germ line have the potential to cause alterations that can be passed to the next generations via genetic or epigenetic mechanisms. For example, both the fungicide, vinclozolin, and the pesticide,

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methoxychlor, induce epigenetic changes in male germ cells. These changes involve alterations of DNA methylation that cause spermatogenic defects, prostate disease, kidney disease, and cancer in the next generations (Anway and Skinner, 2008). Genetic changes can also be caused by paternal smoking, since men who smoke heavily generate spermatozoa that suffer from high levels of DNA damage, largely as a result of oxidative stress (Ji et al., 1997). In this study, paternal preconception smoking was related to a significantly elevated risk of childhood cancers, particularly acute leukemia and lymphoma.

Phenols (including estrogen-like compounds) and phthalate esters are among toxicants that might cause male infertility and possibly TDS. Both groups are heavily represented in the environments (including food) of industrialized countries. Another concern is the increased use of nanomaterials, in particular nanoparticles, which have the ability to penetrate tissues, epithelia, and even cellular membranes. These particles have been shown to pass equally easily through the blood-testis barrier (Kim et al., 2006). The focus of this review is to describe the first steps of spermatogenesis, including signaling pathways crucial for the maintenance, self-renewal, and differentiation of SSCs, and to give an overview of the mechanisms by which some toxicants might alter the biology of these cells by interfering with signaling pathways.

Development of Premeiotic Germ Cells

Primordial germ cells (PGCs) are the progenitors of the germ cell lineage in both ovary and testis. In the mouse embryo at E6.25, germline-competent cells or PGC precursors can be identified by the expression of B-lymphocyte-induced maturation protein 1 (Blimp1) in a founder population of epiblast cells (Ohinata et al., 2005). Between E7.0 and E8.5, a cluster of ~50 PGCs, which are also acquiring alkaline phosphatase positivity, are identified at the posterior end of the primitive streak in the extra-embryonic mesoderm, near the allantois (Donovan, 1998). During gastrulation, PGCs are shifted into the embryo and reach the hindgut by E9. They then migrate through the hindgut and dorsal mesentery, and eventually enter the gonadal primordia by E10.5. On their way to the gonads, PGCs proliferate and the number of germ cells increases from about 50 cells at E7 to more than 35,000 cells by E13.5 (Donovan, 1998). PGCs in male and female gonads are indistinguishable until days after they settle in the genital ridges and are then called gonocytes. In the male gonads, these cells proliferate until around E15.5 and then arrest in the G0 phase of the cell cycle until shortly after birth. By this time, the sex cords (future seminiferous tubules) are composed of pre-Sertoli cells at the periphery and gonocytes in the center. Just after birth the gonocytes resume mitosis, migrate to the basement membrane at the outer edge of the cords, and are then called A_{single} spermatogonia (Oakberg, 1971). A_{single} spermatogonia are considered the putative SSCs and have been widely studied morphologically (Clermont, 1966; Dym and Fawcett, 1971; Huckins, 1971) and functionally (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Oatley and Brinster, 2008). In the adult mouse testis, these cells represent a very small percentage (0.03%) of the total population of germ cells (Tegelenbosch and de Rooij, 1993). A_{single} spermatogonia can either self-renew or produce two daughter cells linked by an intercellular bridge, called A_{paired} spermatogonia. A_{paired} spermatogonia further divide to form chains of A_{aligned} spermatogonia, which further divide to form A1 to A4 spermatogonia, type B spermatogonia, and finally spermatocytes that will complete meiosis and further become haploid spermatids and sperm. A_{single}, A_{paired}, and A_{aligned} spermatogonia are also collectively referred to as undifferentiated spermatogonia, while type A1 to A4 spermatogonia are called differentiating spermatogonia (de Rooij and Russell, 2000). Recently, it has been demonstrated that the first wave of mouse spermatogenesis originates from gonocytes rather than SSCs, shunting the undifferentiated spermatogonia stages and proceeding directly with differentiating spermatogonia onward,

while the subsequent rounds of spermatogenesis are derived from SSCs and their undifferentiated progeny (Yoshida et al., 2006).

Isolation and study of SSCs have been hampered by the lack of markers specific for these cells. Specific proteins are expressed by gonocytes and undifferentiated spermatogonia, such as *GFR α -1*, *Ret*, *Oct-4*, *Plzf*, and *Etv5* (*Erm*). These membrane receptors and transcription factors play crucial roles in the establishment and/or maintenance of the stem cell state (Meng et al., 2000; Pesce and Scholer, 2000; Buaas et al., 2004; Costoya et al., 2004; Naughton et al., 2006; Oatley et al., 2007). The expression of another transcription factor, Neurogenin 3 (*Ngn3*), is restricted to undifferentiated spermatogonia (Yoshida et al., 2004). The exact function of *Ngn3* is still unknown. Upon transition into differentiating spermatogonia, *Ngn3* expression is downregulated and *c-Kit*, the membrane receptor for Kit ligand (stem cell factor, SCF), is up-regulated (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999). SCF signaling through the c-kit receptor is essential for survival and proliferation of differentiating spermatogonia (Feng et al., 2000).

Brinster and Zimmerman (1994) were the first to demonstrate that the undifferentiated spermatogonia population contains stem cells. They isolated cells from the seminiferous tubules of neonatal and prepubertal mice carrying a LacZ transgene, and microinjected them into adult wild type recipient testes devoid of germ cells. The donor cells colonized the recipient seminiferous tubules and restored spermatogenesis, producing mature sperms capable of fertilizing oocytes and producing offspring (Brinster and Avarbock, 1994). It was later demonstrated that spermatogonia expressing the membrane receptor, *GFR α -1*, such as *A_{single}* and some *A_{paired}* spermatogonia, are more likely to colonize seminiferous tubules and restore spermatogenesis (Buageaw et al., 2005). In addition, transgenic mice over-expressing glial cell line-derived neurotrophic factor (GDNF), the ligand for *GFR α -1*, show an increase in self-renewal of SSCs in their testes (Meng et al., 2000). Therefore, *GFR α -1* is considered the most specific marker for SSCs.

The Spermatogonial Stem Cell Niche

In the tissues of most model organisms, the stem cell niche is defined as the microenvironment that supports stem cell behavior (Schofield, 1978; Xie and Spradling, 2000). The niche regulates specific stem cell properties, including self-renewal, pluripotency, quiescence, and the ability to differentiate into single or multiple lineages (Adams and Scadden, 2006). The niche can be defined as a complex interplay of short- and long-range stimuli between the stem cells, their differentiating daughters, supporting cells, and the extracellular matrix that controls stem cell behavior. In the mammalian testis, the somatic Sertoli cell, the basement membrane, and cellular components of the interstitial space between the seminiferous tubules are major components of the niche. Sertoli cells provide growth factors necessary for self-renewal such as GDNF and basic fibroblast growth factor (bFGF) (Meng et al., 2000; Hofmann et al., 2005b), the basement membrane provides for anchoring through integrins (Shinohara et al., 1999; Chiarini-Garcia and Russell, 2002), and stimuli from the vascular network and interstitial cells are crucial for the localization of undifferentiated spermatogonia along specific portions of the basement membrane (Chiarini-Garcia et al., 2003; Yoshida et al., 2007). The integration of these signals provides the cues necessary for self-renewal and retention of the SSCs in their undifferentiated state.

GDNF Signaling Pathways

Maintenance and self-renewal of SSCs are partly controlled by the GDNF signaling pathway (Meng et al., 2000), and its activity is critical in maintaining permanent spermatogenesis (Naughton et al., 2006). GDNF is a small peptide produced by the nursing Sertoli cells in the seminiferous epithelium. It was first discovered in the brain (Lin et al., 1993), then later in

other organs including kidneys and testis (Choi-Lundberg and Bohn, 1995). GDNF is a peptide belonging to the transforming growth factor- β (TGF- β) family, which acts upon cells after binding to its receptor, c-Ret, and its co-receptor, GFR α -1, to form a “ménage à trois” (Robertson and Mason, 1997). Binding of GDNF to the receptor complex promotes the activation of different secondary messengers involved in survival and self-renewal of undifferentiated spermatogonia, mainly SSCs and A_{paired} spermatogonia. Knocking out *GDNF*, *GFR α -1*, or *c-Ret* triggers SSC depletion (Naughton et al., 2006). Conversely, *GDNF* over-expression leads to the development of germ cell tumors in transgenic mice (Meng et al., 2001). Furthermore, several groups of investigators reported that GDNF promotes SSC proliferation and enrichment both in mice and bovine germ cell cultures (Kanatsu-Shinohara et al., 2003; Hofmann et al., 2005b; Aponte et al., 2006). In undifferentiated spermatogonia, GDNF regulates self-renewal/proliferation through phosphorylation of Ret and Src-kinase family proteins (SFKs) (Braydich-Stolle et al., 2007). The activation of SFKs plays a significant role in SSCs self-renewal (Oatley et al., 2007). Further, SFK activation triggers the phosphorylation of PI3K, which activates AKT (Braydich-Stolle et al., 2007; Lee et al., 2007). Finally this signaling cascade leads to an increase of expression of N-Myc, a transcription factor first identified as an inducer of tumor progression (Nesbit et al., 1999), and now recognized as crucial to the maintenance of the stem cell state (Laurenti et al., 2008). Binding of GDNF to GFR α -1 and Ret also leads to the activation of the canonical Ras-Erk1/2 pathway and regulates SSC proliferation (He et al., 2008). Through the successive activation of Shc/Grb2 and Ras, phosphorylated Erk1/2 triggers the activation of three important transcription factors: Creb, ATF1, and Crem. Ultimately, this leads to the activation of c-Fos expression. C-Fos is another transcription factor that controls the expression of Cyclin A and regulates the cell cycle.

Effects of Environmental Toxicants on SSCs

Exposure to chemical substances from the environment causes adverse effects on the male and female reproductive systems. These environmental toxicants are usually organic chemicals whose metabolites persist in the environment and accumulate in the food chain. They can be divided into five distinct groups (Table 1). Toxicants that target the male reproductive system can affect sperm count or shape, alter sexual behavior, and/or decrease fertility. Some of these substances are called endocrine disruptors (endocrine disrupting compounds, or EDCs) because they are able to mimic endogenous hormones, or to act as hormone antagonists. Some EDCs also modulate hormone receptor activity or alter steroidogenic pathways. Therefore, EDCs have the ability to adversely affect developmental and reproductive processes. The effects of EDCs depend on the timing, duration, and dose of exposure, which can occur at very low concentrations. As mentioned above, in males, exposure to EDCs has been suggested as one of the cause of TDS, and to play a role in the increase in the incidence of this syndrome. A class of toxicants that might cause male infertility and possibly TDS are phthalate esters, which are believed to have anti-androgenic activity, as well as toxic effects on Sertoli cells (Fisher, 2004). Another group of toxicants that can affect reproductive health includes air pollutants containing metal micro- and nanoparticles (Utell and Frampton, 2000; Mohallem et al., 2005; Warheit et al., 2008). For example, semen quality is affected by air pollution and smoking due to epigenetic alterations (Selevan et al., 2000; Elshal et al., 2008; Yauk et al., 2008). In addition, in utero exposure to nanoparticles contained in Diesel exhaust affect testicular function by suppressing testicular production of testosterone after inhibition of StAR and 17 β -hydroxysteroid dehydrogenase (Li et al., 2009).

Effects of Phthalate Esters on SSCs

Phthalates are chemicals widely used worldwide as plasticizers for PVC and other plastics. Chemically, the molecule is an ortho-dibenzoic acid that is mono- or diesterified by different

alkyl chains. Addition of phthalates to plastics, up to 40% of their volume, improves their mechanical properties by softening the final products and increasing their flexibility. Dibutyl-phthalate (DBP), di(2-ethylhexyl)-phthalate (DEHP), and dimethyl-phthalate (DMP) are the phthalate esters most commonly utilized in industry. Phthalates are included in the polymer network but are not chemically coupled to it. This non-covalent binding allows phthalates to leak from the polymer and spread into the environment, where they are ubiquitously found, from streambeds to household dust. In Japanese rivers, DEHP was found in 90% of the sampled locations, up to 2000 $\mu\text{g}/\text{kg}$ in sediment and 25 $\mu\text{g}/\text{L}$ in river water (Yuwatani et al., 2006). Phthalates are not only found in plastics, but also in other consumer products, such as cosmetics, paints, and lubricants. Because of their properties, they are widely used in the fabrication of dishware, medical instruments, and children toys. Environmental pollutants like phthalates might be linked to TDS in humans after exposure in utero or perinatally (Fisher et al., 2003; Skakkebaek et al., 2003; Mahood et al., 2007). Pathways of exposure include oral intake (foods and leaks from packaging devices) and inhalation. Since phthalates are highly lipophilic, fatty foods are the major source of contamination, and breastfeeding is a significant source of exposure in infants (Main et al., 2006; Hines et al., 2009).

The molecular mechanism(s) responsible for the effects of phthalate esters in the testis are not completely elucidated. Exposure to these substances is associated with testicular atrophy, and germ cell alteration and loss. Until now, the majority of investigators have focused their efforts on understanding the effects of phthalates on Sertoli cells, and have shown that these effects result in vacuolization, destruction of tight junctions, collapse of vimentin filaments, lack of response to follicle stimulating hormone (FSH) and activation of the Fas-FasL pathway (Creasy et al., 1983; Gray and Beamand, 1984; Heindel and Chapin, 1989; Richburg and Boekelheide, 1996; Lee et al., 1997; Richburg et al., 2000; Zhang et al., 2008). When administered in fetal life, phthalate esters are also anti-androgenic by reducing the production of testosterone and causing Leydig cell aggregation (Akingbemi et al., 2001; Mylchreest et al., 2002; Joensen et al., 2008; Svechnikov et al., 2008). Until now, these deleterious effects on somatic cells were thought to be the cause of germ cell disruption, by producing increased incidence of multinucleated gonocytes and eliminating spermatogonia and spermatocytes by apoptosis in the malformed seminiferous tubules.

The role of stem cells as target for phthalates has not been extensively examined. This might be because the vast interest that stem cells have generated is quite recent. It might also be because stem cells divide slowly and, in the case of SSCs, are less susceptible to common toxicants, which tend to affect later germ cell stages. Another reason might be that these cells are difficult to define and isolate, and testing their functionality is laborious. However, recent work indicates that phthalate esters, in particular the metabolite of DEHP, mono-2-ethylhexyl phthalate (MEHP), can directly affect germ cells perinatally. MEHP impairs human gonocyte development in organ cultures without altering steroidogenesis (Lambrot et al., 2009) and disrupts gonocyte adhesion to Sertoli cells in mouse co-cultures (Li et al., 1998). In addition, type A spermatogonia respond to MEHP by increasing Akt1 phosphorylation and activity. This allows the germ cells to counteract MEHP-induced apoptosis mediated by the Fas/FasL pathway (Rogers et al., 2008). We and others (Braydich-Stolle et al., 2007; Lee et al., 2007; Oatley et al., 2007) have shown that Akt signaling is essential for self-renewal and proliferation of SSCs and their progeny. Therefore, undifferentiated spermatogonia might initiate a mechanism of defense against the effects of phthalate esters, which does not exist in more mature germ cells.

Because MEHP also affects Erk1/2 phosphorylation in the rat testis (Ryu et al., 2008), and because Erk1/2 mediates in part GDNF signaling in SSCs, we have studied the effects of MEHP on proliferation and Erk1/2 phosphorylation in these cells. We used a cell line that

we recently established, C18-4, as an in vitro system to mimic SSCs (Hofmann et al., 2005a). C18-4 cells express the GFR α -1 and Ret receptors, and are responsive to GDNF. Further, they express germ cell nuclear antigen (GCNA), Dazl, and Vasa, which are germline markers, and Oct-3, which is a marker for undifferentiated spermatogonia. Our results indicate that in the presence of GDNF, MEHP inhibits C18-4 cell proliferation in a time- and dose-dependent manner. This decrease in proliferation parallels a decrease of Erk1/2 phosphorylation. Therefore, the overall effect of MEHP on SSCs and more differentiated germ cells might depend on the balance between Akt1 and Erk1/2 signaling.

Effects of Nanoparticles on SSCs

Nanoparticles, which are by definition in the 1–100 nm range, are substances showing novel physical/chemical properties and functions due to their miniscule size (Cui and Gao, 2003). They are now widely used in the fabrication of electronic devices, sensors, and munitions. If formulated properly with other materials, nanoparticles may provide greater stability and efficiency for propellant systems. Nanoparticles are also found in consumer products. For example, zinc oxide nanoparticles are now used in sunscreen lotions since they have superior UV blocking properties. Other nanoparticles are incorporated in plastics and clothing to improve strength and tear resistance. If applied to medicine, nanotechnology has the potential to significantly advance the diagnosis and treatment of diseases. Anticipated applications in medicine include drug delivery, in vitro and in vivo diagnostics, and production of improved biocompatible materials. Nanoparticles are attractive for medical purposes because of their unique features, such as their surface to mass ratio that is much larger than that of other particles, their quantum properties and their ability to adsorb and carry other compounds. Therefore, nanoparticles can transport drugs, probes, proteins, oligonucleotides, and plasmids to specific cell types while protecting these macromolecules from enzymatic degradation (Chavany et al., 1994; Janes et al., 2001; Kaul and Amiji, 2002; Zhu et al., 2004). In addition, antineoplastic agents have been encapsulated into nanoparticles to be selectively carried at the tumor site, thus circumventing adverse side effects of the drugs (Chawla and Amiji, 2002; Sahoo et al., 2004).

Nanoparticles and their non-manufactured counterpart, the ultrafine particles, might also be a concern for reproductive health. The number of reports suggesting that these particles are toxic to many organs, including the testis, is increasing (De Jong and Borm, 2008; Yauk et al., 2008; Li et al., 2009). Most effects are due to the high surface area to volume ratio, which can make the particles very reactive or catalytic. Following systemic administration, nanoparticles easily penetrate very small capillaries throughout the body, therefore offering the most effective distribution to certain tissues. More importantly, nanoparticles can pass through epithelia and biological membranes and thus can affect the physiology of any cell in an animal body (Kim et al., 2006; Kashiwada, 2006). In addition to passing through the blood-brain barrier, nanoparticles penetrate the blood-testis barrier and distribute in the gonads (Kim et al., 2006). However, there are still limited toxicology data available on the effect of nanoparticles on the biology of adult stem cells, including SSCs of the testis.

We recently tested the effects of nanoparticles on the C18-4 cell line (Braydich-Stolle et al., 2005). Data obtained indicated that molybdenum, aluminum, and silver nanoparticles have a dose-dependent effect on metabolic activity and cell viability, and that silver nanoparticles were the most toxic. These results led us to investigate whether silver nanoparticles are able to interfere with signaling pathways controlling SSC self-renewal/proliferation, such as GDNF signaling. Independent of its level of oxidation, silver as a bulk chemical is known for its antimicrobial activity on a broad range of organisms (Hardman et al., 2004; Lansdown, 2006). Because of this interesting property, nanosized silver has found antimicrobial uses in bandages, coatings on clothing and other surfaces, and in paints (Samuel and Guggenbichler, 2004; Cioffi et al., 2005; Percival et al., 2007; Vigneshwaran et al.,

2007). In addition, a number of studies demonstrated that surface treatment of nanoparticles can modify their biological effects and decrease their toxicity (Wilhelm et al., 2003; Chen et al., 2005; Gupta and Gupta, 2005). Therefore, we investigated the effect of silver nanoparticles, including the role of different surface coatings, on the GDNF-Src signaling pathway. Our data indicated that independent of coating and size, silver nanoparticles decreased cell proliferation at concentrations $>10 \mu\text{g/mL}$. At a concentration of $10 \mu\text{g/mL}$, the cells did not show any apoptosis or induction of reactive oxygen species (ROS), which would be indicative of cytotoxic stress. However, GDNF signaling was already impaired. While Ret expression and activation triggered by GDNF were normal, activation of Fyn, a Src family member expressed in germ cells, was decreased in the presence of nanoparticles. As a consequence, Akt1 activity declined and *N-myc* expression was down-regulated, therefore explaining the inhibition of cell proliferation.

Conclusions

Exposure to chemical substances from the environment causes adverse effects on the male and female reproductive systems. These environmental toxicants are usually organic chemicals whose metabolites persist in the environment and accumulate in the food chain. Two of these pollutants, phthalates and nanoparticles, are likely to have direct effects on SSCs and their progeny in addition to somatic testicular cells. Because these substances inhibit SSC proliferation, at least in vitro, we assessed their effects on signaling pathways triggered by the growth factor, GDNF. Our data show that both toxicants can inhibit the activity of key kinases of the Src and Ras pathways in SSCs. Because little is known about the direct effects of environmental toxicants on SSCs, it is likely that this area will be the subject of intensive research in the decade to come.

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TABLE 1
Groups of Environmental Toxicants*

Industrial chemicals	Polychlorinated biphenyls (PCBs) and dioxins
Pesticides	Organochlorines, organophosphates, pyrethroids
Fungicides	Vinclozolin
Common consumer products	Phthalates (plasticizers, soap, paints) Benzo-a-pyrene (cigarettes) Bisphenol-A (food wrap, dental fillings, linings of canned food)
“Emerging” toxicants of concern	Polybrominated diethyl ethers Perfluorinated compounds Ultrafine particles Nanomaterials, nanoparticles

* Adapted from Younglai et al., 2007. *Curr Pharm Des* 13:3005–3019.