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The effects of endocrine disruptors on the male germline: an intergenerational health risk

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ABSTRACT

Environmental pollution is becoming one of the major concerns of society. Among the emerging contaminants, endocrine-disrupting chemicals (EDCs), a large group of toxicants, have been the subject of many scientific studies. Besides the capacity of these compounds to interfere with the endocrine system, they have also been reported to exert both genotoxic and epigenotoxic effects. Given that spermatogenesis is a coordinated process that requires the involvement of several steroid hormones and that entails deep changes in the chromatin, such as DNA compaction and epigenetic remodelling, it could be affected by male exposure to EDCs. A great deal of evidence highlights that these compounds have detrimental effects on male reproductive health, including alterations to sperm motility, sexual function, and gonad development. This review focuses on the consequences of paternal exposure to such chemicals for future generations, which still remain poorly known. Historically, spermatozoa have long been considered as mere vectors delivering the paternal haploid genome to the oocyte. Only recently have they been understood to harbour genetic and epigenetic information that plays a remarkable role during offspring early development and long-term health. This review examines the different modes of action by which the spermatozoa represent a key target for EDCs, and analyses the consequences of environmentally induced changes in sperm genetic and epigenetic information for subsequent generations.

Key words: endocrine disruptor, paternal exposure, spermatozoa, epigenetics, DNA damage, intergenerational effects

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I. INTRODUCTION

Industrial and technological progress, as well as the exponential population growth experienced during the last few centuries, have led to unprecedented environmental changes. These alterations, caused by the release of many chemical substances and contaminants, are now endangering the health of ecosystems and living organisms. In 2011, 347 million tons of anthropogenic chemicals were produced, of which more than 50% were considered environmentally harmful and around 10% were classified as having a severe chronic environmental impact (Gavrilescu *et al.*, 2015). Among these, particular attention has been paid to emerging contaminants: synthetic or naturally occurring chemicals present in the environment whose emission, degradation, or effects often remain unknown. Despite the fact that emerging contaminants may have been present in the environment for years, only recently have they become subject to investigation (Kümmerer, 2010; Petrie, Barden & Kasprzyk-Hordern, 2014). In 2005 the European Commission created a network (the NORMAN association) of reference laboratories, industries, public institutions, and non-governmental organisations to compile data and knowledge regarding emerging contaminants (Dulio *et al.*, 2018).

Current research is mainly focused on compounds described as biologically active or even toxic, especially those affecting the endocrine system since it plays a crucial role in animal homeostasis, reproduction, development, and behaviour (Thomaidis, Asimakopoulos & Bletsou, 2012). Agents able to interfere with the synthesis, secretion, transport, binding or elimination of endogenous hormones are known as endocrine-disrupting chemicals (EDCs) (Kavlock *et al.*, 1996). In 1992 a consensus of specialists from several disciplines reached the conclusion that endocrine disruptors threaten both wildlife and human survival (Colborn & Clement, 1992). Since then there has been burgeoning scientific evidence from animal studies providing insights into the mechanisms by which EDCs alter hormonal function and thereby lead to biological changes (Schug *et al.*, 2011; Sifakis *et al.*, 2017; Combarnous & Nguyen, 2019). EDCs have been claimed to interfere with the endocrine system by at least nine different mechanisms. Only two of these imply that the EDC binds to the hormone receptor, stimulating or inhibiting its signalling pathway, whereas the rest of them involve interference with the synthesis or availability of endogenous hormones, with the synthesis and stability of their receptors or with any component of the hormone signalling pathway downstream of its receptor (World Health Organization, 2017; Combarnous & Nguyen, 2019). As far as the hormone-receptor complex is concerned, EDCs can act as agonists by imitating natural hormones and leading to overstimulation; as antagonists, when they bind to the receptors of endogenous hormones and no response occurs; or as blocking substances for natural hormones and/or their receptors (Kabir, Rahman & Rahman, 2015). Depending on their modes of action, three categories of effects triggered by EDCs have been identified: low dose reversible, low dose irreversible and chronic cumulative irreversible (White *et al.*, 2009). Besides their modes of action, the exposure concentration to one or several compounds and the developmental period during which organisms are exposed may also determine the risk of disease (Kortenkamp *et al.*, 2012; Diamante *et al.*, 2017).

To facilitate the regulation of EDCs, they have been classified according to their nature (Diamante *et al.*, 2017), their origins (Caliman & Gavrilescu, 2009), their main uses (Gore *et al.*, 2015) and/or their effects (Kortenkamp *et al.*, 2012). The huge number of known EDCs (1484 of the 85000 manufactured chemicals) encompass a variety of chemicals including pesticides, herbicides, perfluorochemicals and plasticisers (TEDX List; endocrinedisruption.org/interactive-tools/tedx-list-of-potential-endocrine-disruptors).

While it is well known that maternal exposure to EDCs during the periconception period or pregnancy can have deleterious effects for the progeny, the modifications produced in spermatozoa resulting from paternal exposure have received less attention, but can also have long-term intergenerational effects. Taking into account that spermatogenesis is a complex process that requires a proper hormonal balance and involves substantial changes to chromatin structure, EDCs have been increasingly reported to disrupt male reproduction, even at low doses. However, previous reviews have focused only on the consequences of EDC exposure for male breeding capacity, and have neglected any impacts of paternal exposure on future generations. Herein, we explain the impact of EDCs on the information contained in the sperm cells of both humans and other animals, including non-mammalian species that, despite having different strategies of epigenetic remodelling, are useful in the context of understanding the transgenerational impact of EDCs. We provide insight into how these changes affect the development of subsequent generations, thus correlating the direct impact of EDCs on sperm cells with their derived intergenerational effects.

II. MODES OF ACTION

The EDCs do not induce single specific effects, but they rather trigger pleiotropic responses, thus displaying wide-ranging effects. They have been reported to alter gene expression not only due to their interference with hormone signalling but also as a result of their genotoxicity and/or their ability to modify epigenetic patterns (Combarnous & Nguyen, 2019).

(1) Endocrine-disruptive effects

EDCs interfere with endocrine signalling through multiple mechanisms, which have been extensively reviewed elsewhere (Sifakis *et al.*, 2017; Combarnous & Nguyen, 2019). They impact both well-known hormone receptors (androgen, oestrogen, thyroid and glucocorticoid receptors) and also other less-known receptors such as orphan or aryl hydrocarbon receptors (Lauretta *et al.*, 2019). In this review, we focus on the mechanisms through which EDCs have been confirmed to affect male reproductive health. As summarised by Di Nisio & Foresta (2019) and Sifakis *et al.* (2017), *in vivo* and *in vitro* studies have shown that EDCs affect hormone-dependent pathways responsible for male gonadal development, either through direct interaction with hormone receptors or *via* epigenetic and cell-cycle regulatory modes of action. Interference of EDCs with hormone binding mostly involves oestrogen receptor (ERs) and androgen receptor (ARs), but G-protein-coupled oestrogen receptors (GPERs) and aryl hydrocarbon receptors (AhRs), which function in male reproduction, may also be involved. In addition to their effects on hormone-related receptors, EDCs affect the expression and/or activity of enzymes involved in steroidogenesis as well as the metabolism of these and other hormones crucial for male reproduction (Sifakis *et al.*, 2017).

(*a*) Interference of EDCs with hormone receptors

(i) Oestrogen receptors. Oestrogen plays an important role in testicular development and spermatogenesis (Delbès, Levacher & Habert, 2006). Several EDCs are able to bind to ERs, acting either as agonists or antagonists of oestrogens, with this activity depending on both the ER subtype and the tissue involved (Kurosawa *et al.*, 2002). Upon binding to the ligand, the cytosolic forms of ERs undergo dimerization and then migrate into the nucleus where they can regulate gene expression through two different mechanisms (Acconcia, Pallottini & Marino, 2015). In the canonical model, the ligand–ER complex can bind directly to specific palindromic sequences of gene promoters known as oestrogen response elements (EREs), thereby recruiting co-activators or other components of RNA polymerase II to enhance gene transcription (Gruber *et al.*, 2004). This complex is also able to promote the transcription of genes lacking EREs by protein–protein interaction with other transcription factors; a process called the tethering pathway (Heldring *et al.*, 2007; Li *et al.*, 2013). Aside from this nuclear translocation, ERs located in the plasma membrane of some cells also mediate rapid genomic responses, such as activation of phosphatidyl-inositol-3-kinase/protein kinase B (PI3K/AKT) or extracellular signal-regulated kinases (ERK) pathways, which have short-term effects on gene expression (Bolli *et al.*, 2008; Le & Belcher, 2010). The binding of EDCs to ERs located in the plasma membrane can trigger non-genomic effects as well, including increased ion fluxes and activation of kinases and phosphatases (Rosenfeld & Cooke, 2019) (Fig. 1A).

EDCs can also affect genomic responses. Agonistic actions following binding to ERα and/or ERβ have been described for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Boverhof *et al.*, 2006), bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane] (Moreman *et al.*, 2018; Brown *et al.*, 2019), bisphenol AF (BPAF), 2-2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Table 1 provides a list of EDC abbreviations for easy reference), genistein, kaempferol, coumestrol, and daidzein (Li *et al.*, 2013), the latter of which is known to modulate the activity of transcription factors such as activator protein-1 (AP-1) and specificity protein 1 (Sp1) *via* the tethering pathway. The interference of BPA with ERs can also have antagonistic effects on the testis, preventing 17β-estradiol from binding to these receptors, so that the steroidogenic genes can no longer be transcribed (Rehman *et al.*, 2018). The oestrogenic effects of EDCs also might trigger DNA damage throughout spermatogenesis by dysregulating the expression of genes involved in DNA repair (see Section II.2).

Oestrogenic pathways can also be affected by the effects of EDCs on ER expression levels, as demonstrated in mice testes exposed to BPA, which has been linked to impaired spermatogenesis (Takao *et al.*, 2003). Doshi *et al.* (2011) identified that the altered expression of ERα and ERβ observed in rat testes following neonatal exposure to BPA was mediated by an epigenetic mechanism: the hypermethylation of ER genes.

(ii) Androgen receptors. ARs belong to the steroid hormone group of nuclear receptors and thus share a similar cellular location and mechanisms of action to that of canonical nuclear ERs (Tan *et al.*, 2015). Similarly, ARs joined to their ligand are translocated to the nucleus where they bind to androgen response elements (AREs), promoting gene transcription (MacKay & Abizaid, 2018) (Fig. 1B).

The plasticisers di(2-ethylhexyl)phthalate (DEHP) and BPA have the ability to bind to ARs, competing with testosterone and hindering their androgen-induced nuclear translocation (Borch *et al.*, 2006; Wang *et al.*, 2017). Based on these data, ARs may require higher concentrations of androgens or a longer time to exert their genomic effects in the presence of some EDCs.

(iii) G-protein-coupled oestrogen receptors. EDCs can interfere with GPER pathways. This receptor was first discovered as an orphan G-protein coupled receptor predominantly located in the membrane of the endoplasmic reticulum (Gaudet *et al.*, 2015; MacKay & Abizaid, 2018). It was later demonstrated that the oestrogen–GPER complex was able to induce rapid intracellular signalling (Filardo

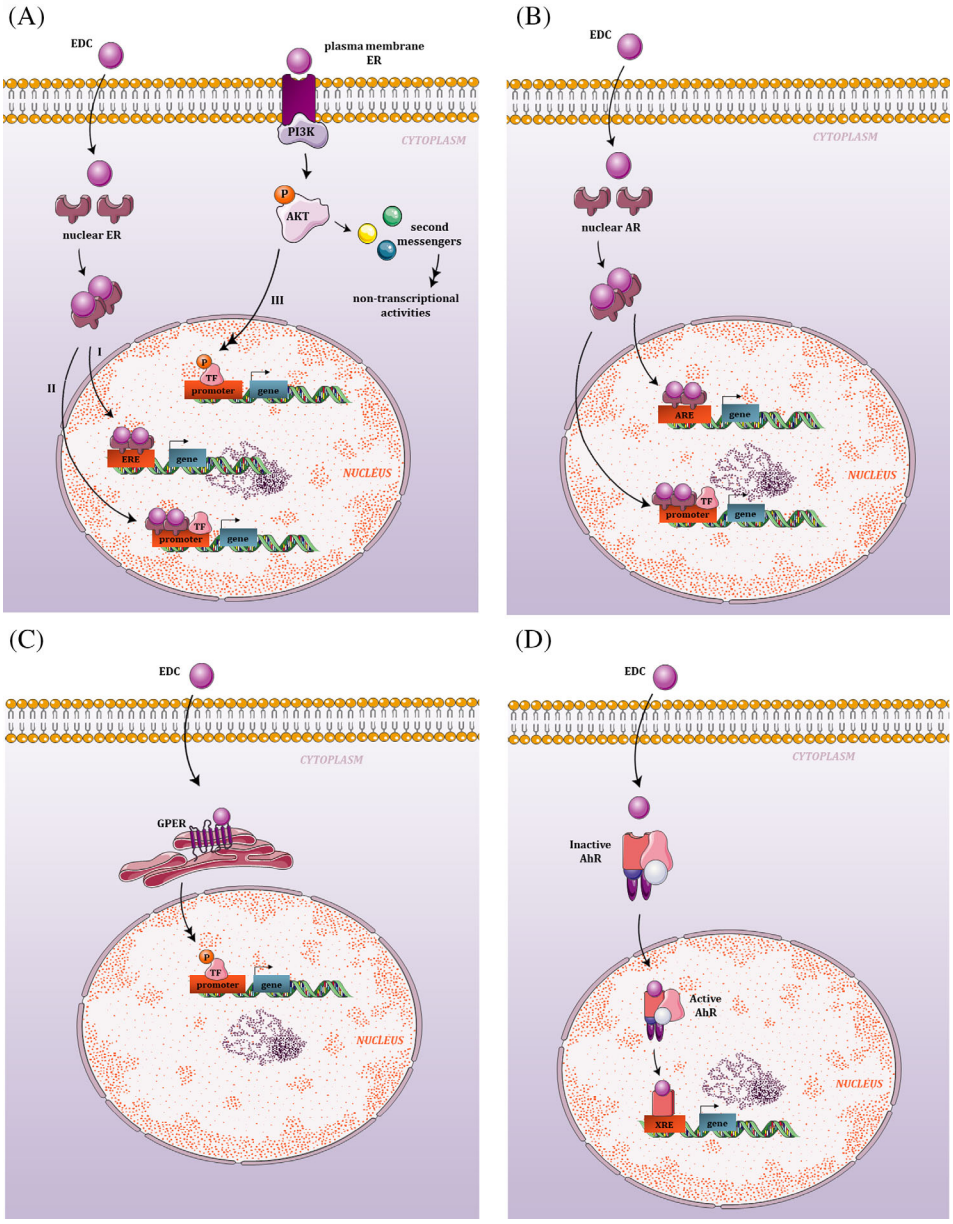

Fig 1. Intracellular endocrine-mediated responses exerted by endocrine-disrupting chemicals (EDCs). (A) Molecular mechanisms mediated by oestrogen receptors (ERs). I, the canonical pathway: binding of EDCs to cytoplasmic ERs triggers dimerization and nuclear translocation, where they bind to oestrogen response elements (EREs), enhancing gene transcription. II, the tethering pathway: the EDC–ERs complex migrates to the nucleus where it interacts with transcription factors (TFs), modulating gene expression. III, the rapid endocrine response: EDCs bind to palmitoylated ERs located in the plasma membrane activating different downstream pathways, such as phosphatidyl-inositol-3-kinase/protein kinase B (PI3K/AKT), also affecting gene transcription. P, phosphate group. (B) Canonical and tethering pathways mediated by androgen receptors (ARs). ARE, androgen response element. (C) Rapid endocrine response mediated by G-protein-coupled oestrogen receptors (GPERs) located in the membrane of the endoplasmic reticulum. (D) Genomic response exerted by aryl hydrocarbon receptor (AhR). XRE, xenobiotic response elements.

et al., 2014). Binding of GPER to oestradiol switches on many pathways within the cell: adenylyl cyclase activity is increased, intracellular Ca^{2+} is mobilised, and PI3K and mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) signalling pathways and epidermal growth factor receptors (EGFRs) are activated (Revankar *et al.*, 2005). Due to its capacity to promote rapid intracellular responses, GPER can also modulate gene expression (Prossnitz *et al.*, 2008) (Fig. 1C).

BPA interferes with the GPER signalling pathway *via* several mechanisms. Although the affinity of oestradiol for GPER is 10-fold lower than for ERα (Revankar

Table 1. List of abbreviations used for endocrine-disrupting chemicals in this review

Abbreviation	Chemical name
BPA	Bisphenol A; 2,2-bis(4-hydroxyphenyl) propane
DBP	Dibutyl phthalate
DDT	Dichlorodiphenyltrichloroethane
DEHP	di(2-ethylhexyl)phthalate
DES	Diethylstilbestrol
EE2	17α-ethynylestradiol
HPTE	2-2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane
MEHP	Mono-(2-ethylhexyl) phthalate
MEHHP	Mono-(2-ethyl-5-hydroxyhexyl) phthalate
MEOHP	Mono-(2-ethyl-5-oxohexyl) phthalate
MXC	Methoxychlor
PCB	Polychlorinated biphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
VCZ	Vinclozolin

et al., 2007), BPA has a relative binding affinity of 2.83% with GPER (Thomas & Dong, 2006). It has been shown that both *in vivo* male exposure and *in vitro* testicular exposure to BPA lead to increased *gper* expression and Gper protein levels in zebrafish (*Danio rerio*) (González-Rojo *et al.*, 2019).

(iv) Aryl hydrocarbon receptors. AhRs belong to the basic helix-loop-helix/PAS transcription factors known to mediate the toxic effects of dioxins, polyaromatic hydrocarbons and related compounds (Abel & Haarmann-Stemmann, 2010). In the cytoplasm, the Ahr forms a complex with chaperone proteins that keeps it inactive. Upon binding to the ligand, AhR is activated and translocated to the nucleus where it promotes genomic responses by modulating xenobiotic-responsive elements (XREs) or by interacting with other transcription factors (Rothhammer & Quintana, 2019) (Fig. 1D).TCDD and polychlorinated biphenyl (PCBs) are thought to affect spermatogenesis by altering the transcription of steroids and growth factors since they are able to bind to AhRs (Rehman *et al.*, 2018). Moreover, since sperm possess AhRs, perinatal exposure to TCDD has been reported to impair capacitation, the acrosome reaction, sperm–egg binding, and fertilisation in humans (Mocarelli *et al.*, 2011). BPA is able to affect male reproduction by AhR inactivation, leading to inhibition of aromatase, the enzyme controlling steroid biosynthesis and metabolism (Bonefeld-Jørgensen *et al.*, 2007).

(*b*) Interference of EDCs with steroidogenesis and hormone metabolism

Steroidogenesis is a complex process that can be seriously affected by EDCs. Many different studies have reported altered levels of hormones, enzymes, transporters or transcriptional factors related to the steroid pathway as a result of exposure to a variety of environmental contaminants, either alone or as complex mixtures (Doshi *et al.*, 2011; Lan *et al.*, 2017; Buñay *et al.*, 2017, 2018; Singh & Singh, 2019).

Steroid dysregulation can occur at the transcriptional level by activation of the genomic pathway or by epigenetic changes at specific promoters. Some genes encoding steroidogenic enzymes are targets of nuclear receptors that bind to EDCs, affecting their transcription and leading to sex hormone imbalance. In that regard, exposure to phthalates, alkylphenols and diethylstilbestrol (DES) has been reported to decrease the messenger RNA (mRNA) levels of the enzyme hydroxysteroid dehydrogenase in rat testis (Kim *et al.*, 2007). The upregulation of *Cyp11a1* and *Cyp17a1* gene expression in rats exposed pre- and postnatally to flutamide, either alone or in combination with dienestrol or linuron, resulted in the feminisation of male pups (Katsanou *et al.*, 2020). *CYP* genes were also dysregulated by BPA in mouse testis through the activation of the c-Jun N-terminal kinases (JNK/c-Jun) signalling pathway and probably also of ERK1/2 and AMP-response element binding protein (CREB), resulting in an approximately 70% decrease in the testosterone/oestradiol ratio (Lan *et al.*, 2017).

An additional mechanism by which EDCs interfere with the steroid pathway is related to their effect on activities of enzymes involved in hormone metabolism. Phthalates inhibit cytochrome P450 17alpha-hydroxylase (CYP17) activity, decreasing the synthesis of testosterone in Leydig cells (Foster, 2005), whereas thiophosphates inhibit CYP3A4 and CYP1A2 which both take part in the metabolism of oestrone and testosterone in the liver (Usmani, Rose & Hodgson, 2003; Usmani *et al.*, 2006).

(2) DNA-damaging potential of EDCs

Much of the information required for the development and homeostasis of living organisms and their subsequent generations is contained in the genome, so it is extremely important to protect the DNA from damage. However, some endogenous (metabolites) and exogenous (ionising and ultraviolet radiation or chemical mutagens) factors can threaten DNA integrity (Yoshiyama, Sakaguchi & Kimura, 2013). Chronic exposure to EDCs has been shown to cause meiotic arrest, to induce meiotic aneuploidy and chromosome aberrations, and to inhibit meiotic double-strand break (DSB) repair (Brieño-Enríquez *et al.*, 2011; Prusinski Fernung *et al.*, 2018; Samarasinghe *et al.*, 2018). Some studies have shown that DNA damage caused by EDCs is due to their endocrine-disruptive activity. Liu *et al.* (2013) reported that BPA can induce persistent DSBs in pachytene spermatocytes by upregulating two proteins involved in DNA repair, phosphorylated ataxia telangiectasia mutated (pATM) and phosphorylated H2A.X Variant Histone (γH2AX), through ER binding. Moreover, oestrogen levels increases the activity of cellular tumor antigen p53, thus both oestrogenic and anti-oestrogenic effects of BPA could lead to an improper DNA damage response (Fernández-Cuesta *et al.*, 2011). Additionally, BPA can cause DNA damage *via* ER-independent pathways (Aghajanpour-Mir *et al.*, 2016). The ability of bisphenols, phthalates and parabens to generate reactive oxygen species (ROS) means they have been widely

characterised as genotoxic agents (Gassman, 2017; Samarasinghe *et al.*, 2018; Song *et al.*, 2019). An increase in ROS levels causes oxidative stress, leading to DNA damage that results in phosphorylation of several proteins involved in the DNA damage response, such as ATM and H2AX. Moreover, an increase in ROS levels can induce caspase-3-mediated apoptosis (George & Rupasinghe, 2018).

Apoptosis is a type of programmed cell death, described as a homeostatic mechanism that takes place throughout development and ageing (Elmore, 2007). However, there are some pathological conditions that can trigger apoptosis activation; for example, genotoxic damage can induce p53-mediated apoptosis (Fernández-Cuesta *et al.*, 2011). Apoptotic pathways are highly sophisticated and are commonly divided into two main groups: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Albeit different, both pathways converge on the activation of caspase-3, leading to the degradation of cytoskeletal and nuclear proteins (cell shrinkage), chromatin condensation (pyknosis), plasma membrane blebbing, the formation of apoptotic bodies and, eventually, uptake by phagocytes (Igney & Krammer, 2002). Concerning apoptosis, DEHP has been reported to induce cell death through the intrinsic mitochondrial pathway in mouse spermatocytes by increasing the expression of the pro-apoptotic protein Bcl-2 Associated X-protein (Bax) and decreasing the expression of the anti-apoptotic protein Apoptosis regulator Bcl-2(Bcl-2) (Fu *et al.*, 2017). Moreover, a significant increment of caspase-3 after exposure to BPA, nonylphenol (NP) and DEHP has been shown in breast cells (Ibrahim, Elbakry & Bayomy, 2016), testicular cells (Fu *et al.*, 2017; Srivastava & Gupta, 2018), bronchial epithelial cells (George & Rupasinghe, 2018) and reproductive tract cancer cells (Urriola-Muñoz *et al.*, 2018).

(3) Epigenetic toxicity

Environmental factors are well known to promote the development of several diseases. However, although genome integrity plays a crucial role in health, some deleterious effects caused by environmental factors can not be explained solely by alterations to the DNA sequence (Skinner, 2014). In the mid-20th century, the Scottish embryologist Conrad Waddington coined the term 'epigenetics' to describe all genetic and developmental changes occurring from fertilisation to the formation of mature organisms (Wadington, 1957). Nowadays, epigenetics is defined as the study of mitotically and/or meiotically inherited changes in gene expression that are not produced by alterations of the DNA sequence (Felsenfeld, 2014). Epigenetic mechanisms include DNA methylation, histone modifications and the presence of coding and non-coding RNAs (Feil & Fraga, 2012).

(*a*) DNA methylation

DNA methylation was the first epigenetic mechanism to be studied, and it represents the only covalent modification directly attached to the DNA. Methyl groups are mostly found at cytosines bound to guanines by phosphate residues: the CpG sites (Gruenbaum *et al.*, 1981). Although in animals the most common methylation occurs in the 5th carbon of cytosine (5mC), methylation in other positions such as 4-methylcytosine (4mC) and 6-methyladenine (6mA) has been confirmed in plants and fungi (Seidl, 2017; Liu *et al.*, 2019). CpG-rich regions of the genome are known as CpG islands (CGIs), and much attention has been paid to those present in transcription start sites (TSSs), given that demethylated CpG at these locations is generally associated with active gene transcription (Smith & Meissner, 2013) (Fig. 2A). Although most CGIs are demethylated when located at the TSS, CGI methylation at these sites is commonly associated with long-term silencing (in X chromosome inactivation, genomic imprinting, silencing of retroviral elements, and tissue-specific gene expression) (Jones, 2012). By contrast, away from the TSS regions in the gene bodies of dividing cells, higher percentages of methylation are associated with higher levels of gene expression. However, methylation in both the first exon and in the rest of the gene bodies of slowly dividing and non-dividing cells is often related to gene repression (Moore, Le & Fan, 2013). DNA methylation is catalysed by DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenyl methionine (SAM) to a cytosine (Feil & Fraga, 2012) (Fig. 2A). In mammals, there are four enzymatically active DNMTs: DNMT1, DNMT3a, DNMT3b, and DNMT3c. DNMT1 is responsible for maintaining DNA methylation after each cell division: during replication, DNMT1 recognises hemimethylated DNA, and it methylates the new strand according to the original epigenetic pattern (Jones, 2012). On the contrary, DNMT3a and DNMT3b are responsible for *de novo* methylation during development and differentiation, and are essential during the earliest stages of development (Lyko, 2018) (Fig. 2B). DNMT3c, and its cofactor DNMT3L (a protein that shares homology with Dnmt3a and Dnmt3b, but lacks enzymatic activity) are involved in male reproduction. In mice, *Dnmt3l*-deficient and *DNMT3c* mutant males are both sterile and exhibit abnormal differentiation of spermatogonia and spermatocyte arrest (Hata *et al.*, 2002; Jain *et al.*, 2017).

Passive DNA demethylation implies inactivity of DNMT1 during genome replication, thus diluting overall levels of DNA methylation in each cell division (Moore *et al.*, 2013). By contrast, active DNA demethylation is mediated by ten-eleven translocation enzymes (TETs) that mediate the iterative oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) and other intermediate molecules eventually to restore cytosine levels (Wu & Zhang, 2017).

(*b*) Histone modifications

The histones are proteins that pack the DNA to form the nucleosomes. They usually undergo post-translational modifications in their protruding tails that allow them to regulate chromatin structure and to recruit non-histone proteins that can also bind to chromatin (Lawrence, Daujat & Schneider, 2016). An astonishing number of histone

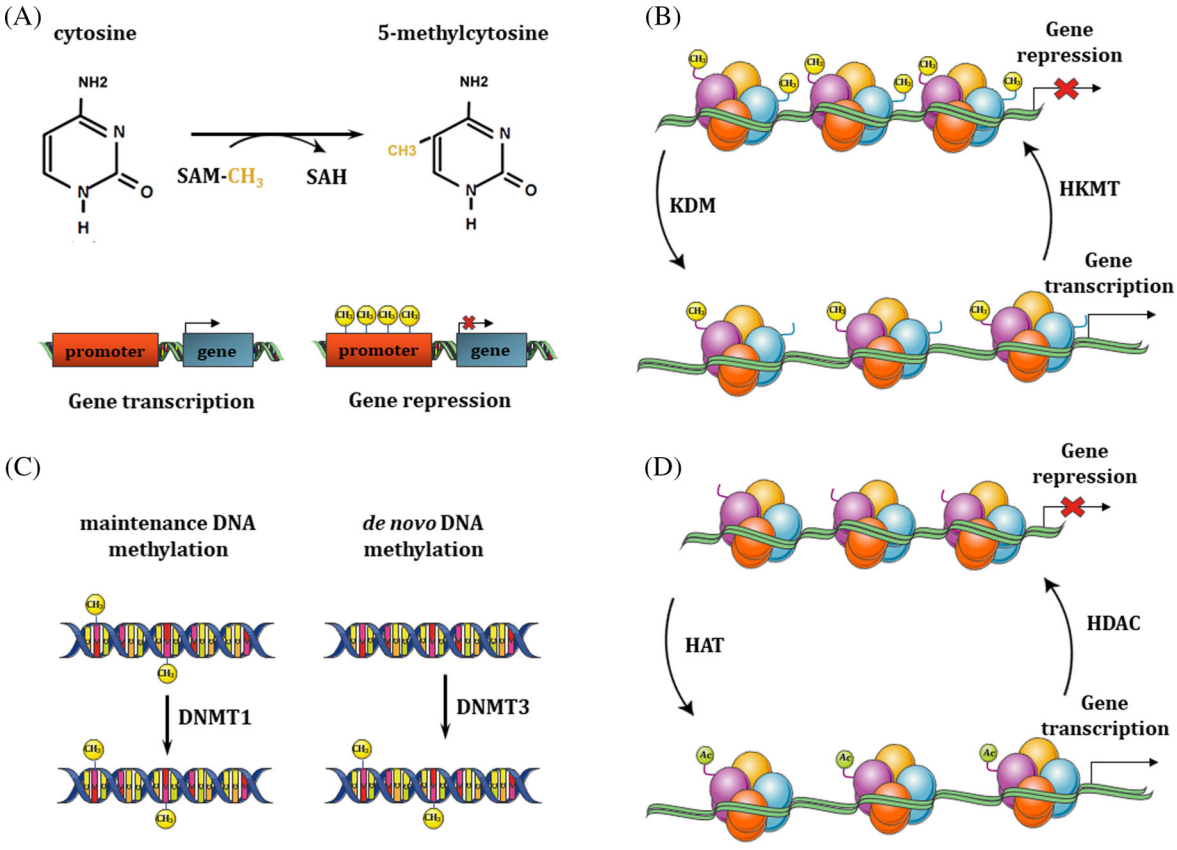

Fig 2. DNA methylation and histone post-translational modifications. (A) Methyl groups are transferred from S-adenosylmethionine (SAM) to the 5th carbon of cytosine, converting it into 5-methylcytosine (5mC). Such DNA methylation of gene promoters is commonly associated with gene repression. SAH, S-adenosylhomocysteine. (B) DNA methyltransferases (DNMTs) are enzymes catalysing both maintenance DNA methylation according to the original pattern (DNMT1) and *de novo* DNA methylation (DNMT3a/b/c/L). (C) The addition of methyl groups to lysine residues of histones is catalysed by histone lysine methyltransferases (HKMTs), whereas the reverse reaction is catalysed by lysine demethylases (KDMs). Depending on the number and location of methyl groups, histone methylation can either activate or repress gene expression. (D) Histone acetyltransferases (HATs) are enzymes catalysing histone acetylation (linked to enhanced gene transcription), whereas histone deacetylases (HDACs) deacetylate lysine residues in histone tails. Ac, acetyl group.

modifications have been identified, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, propionylation and butyrylation (Kebede, Schneider & Daujat, 2015). The available information is mainly focused on deciphering the mechanisms and functions of methylation and acetylation, since their discovery by Allfrey, Faulkner & Mirsky (1964). Both lysine and arginine residues of histones can be subject to methylation. Lysines can be mono-, di- or trimethylated in their amino groups. The enzymes mediating this process are the histone lysine methyltransferases (HKMTs), whereas those catalysing histone demethylations are the lysine demethylases (KDMs) (Hyun *et al.*, 2017). In contrast to the role of DNA methylation in gene regulation, methylation of lysine residues in histones can be related to either gene transcription activation or repression, depending on the number and location of the methyl groups (Zhang, Cooper & Brockdorff, 2015) (Fig. 2C). Monomethylated H3K27 and H3K9 are linked to gene activation, while trimethylated H3K27 and H3K9 are associated with gene repression (Dong & Weng, 2013). Furthermore, activating and silencing histone modifications can coexist in the same gene promoter, generating 'bivalent domains'. In particular, the coexistence of H3K4me3 (enhancing mark) and H3K27me3 (repressing mark) in gene promoters has been stated to play a crucial role in embryo development (Brykczynska *et al.*, 2010). Histone acetylation is also important in chromatin remodelling since it neutralises the positive charges of lysine residues, decreasing the affinity of histones for DNA and making histone acetylation frequently associated with a transcriptionally active state (Gates *et al.*, 2017). Histone acetylation is mediated by histone acetyltransferases (HATs) and the erasure of acetyl groups is catalysed by histone deacetylases (HDACs) (Eberharter & Becker, 2002) (Fig. 2D).

(*c*) RNAs

Other changes in gene expression, which can be mitotically and/or meiotically inherited, result from allelic interactions:

paramutations. As these alterations do not modify the DNA sequence, they are also considered epigenetic phenomena. Both coding and non-coding RNAs are known to be involved in paramutations (Hamatani, 2012; Hollick, 2016). Rassoulzadegan *et al.* (2006) showed that abnormal accumulation of *Kit* mRNAs led to changes in mouse skin pigmentation: homozygote $Kit^{+/+}$ mice showed a normal pigmentation but when these homozygotes were obtained from heterozygotes $Kit^{-/+}$, paramutations in the *Kit* gene transmitted through sperm made them display a white tail and feet, the pigmentation pattern characteristic of heterozygotes.

Non-coding RNAs are a set of RNAs that do not encode functional proteins, but represent an essential mechanism of gene expression and chromatin structure regulation (Wei *et al.*, 2017). These RNAs can be grouped according to their size into long (lncRNAs) or small non-coding RNAs (sncRNAs), which comprise micro-RNAs (miRNAs) and piwi-interacting RNAs (piRNAs) (Stefani & Slack, 2008). miRNAs are approximately 22-nucleotide-long sncRNAs that bind to the 3′-untranslated region of mRNAs (Cannell, Kong & Bushell, 2008). Due to their ability to regulate gene expression at transcriptional and post-transcriptional levels, miRNAs are involved in reproductive processes such as germline development, spermatogenesis and oogenesis (Robles, Valcarce & Riesco, 2019). Furthermore, miRNAs play a crucial role in transgenerational transmission of environmentally induced epimutations, as reviewed by Champroux *et al.* (2018).

(*d*) Epimutations caused by EDCs

While most environmental toxins do not appear to promote genome modifications, they can drastically influence the epigenome, thereby altering gene function and phenotype (McCarrey, 2012). Environmentally induced alterations of epigenetic marks on DNA or histone-associated proteins are known as epimutations (McCarrey, 2014). Since epimutations modify one or more epigenetic mechanisms in a particular cell type, they can potentially be inherited from one cell to its mitotic daughter cells or between generations when germ cells are affected (Anway *et al.*, 2005). Two types of epimutation were described by Whitelaw & Whitelaw (2008): primary epimutations which are epigenetic changes independent of genetic defects; and secondary epimutations which represent genetic alterations (usually in genes coding for epigenetic enzymes) that lead to epigenetic alterations. More recently, tertiary epimutations were characterised as initially epigenetic alterations that trigger genetic changes, being propagated *via* epigenetic or genetic inheritance (McCarrey, 2012). Besides their oestrogenic activity, EDCs can alter the epigenetic pattern by modifying epigenetic regulators and their cofactors or by directly interfering with the epigenetic properties of specific genes (Alavian-Ghavanini & Rüegg, 2018). Epigenetic toxicity of EDCs was first reported in the yellow agouti (A^{vy}) mouse model when maternal exposure to BPA led to a decrease in CpG methylation in an intracisternal A particle (IAP) upstream *Agouti* gene. This epigenetic modification caused an altered coat colour distribution in the offspring, which was successfully counteracted by maternal dietary supplementation with methyl donors like folic acid (Dolinoy, Huang & Jirtle, 2007). It is noteworthy that a more recent study was not able to reproduce this shift towards yellow in the F1 generation of *a/a* (non-agouti) females mated with A^{vy}/a males and exposed during pregnancy to the same doses of BPA (Rosenfeld *et al.*, 2013), raising doubts regarding the effects of maternal BPA exposure on offspring phenotype. Nonetheless, BPA has been widely claimed to alter global and gene promoter DNA methylation (Doshi *et al.*, 2011; Miao *et al.*, 2014; Yin *et al.*, 2016) as well as the expression of DNMTs in several model species (Kundakovic *et al.*, 2013; Laing *et al.*, 2016; Santangeli *et al.*, 2016). These results were not conclusive, some showing that BPA triggers DNA hypomethylation and others that it leads to hypermethylation, depending on the timing of exposure, species, sex, type of cell, and genomic context of the specific genes involved, among other variables. Interestingly, many studies have focused on determining whether there is a link between epigenetic and endocrine disruptive effects. For BPA, two reports provide evidence of such a relationship. In one of these, exposure of newborn rats to BPA was reported to cause the downregulation of AR expression due to an increase in DNA methylation of its promoter (Doshi *et al.*, 2011). In the second, Santangeli *et al.* (2019) found that maternal exposure to BPA in zebrafish triggered DNA hypermethylation in the promoter of *amh* (which encodes anti-Mullerian hormone), leading to repression of its transcript across three generations. Likewise, foetal and neonatal exposure to methoxychlor (MXC) were reported to alter adult ovarian function by inducing significant hypermethylation in ERβ promoter regions (Zama & Uzumcu, 2009). In addition, hormonal toxicity reported in mouse seminal vesicles after neonatal exposure to DES was thought to result from changes in DNA methylation of a set of genes mediated by ERα (Li *et al.*, 2014).

Both embryonic and adult exposure to BPA have been reported to affect histone acetylation (Kumar & Thakur, 2017; González-Rojo *et al.*, 2019; Lombó *et al.*, 2019*c*) and the expression of enzymes catalysing histone acetylation/deacetylation (Chen *et al.*, 2017; Lombó *et al.*, 2019*a*). Similarly, neonatal exposure to DES in the mouse has been claimed to decrease expression levels of histone methyltransferase enhancer of zeste homolog 2 (Ezh2), histone lysine acetyltransferase 2A (Kat2a), and the histone deacetylases Hdac1, Hdac2, and Hdac3, leading to alteration of histone modifications (H3K9ac, H3K4me3, H4K5ac) in specific genes (Jefferson *et al.*, 2013). Studies carried out in different cell lines (hepatocytes, prostate and breast cancer cells) have shown that treatment with TCDD induces epigenetic histone modifications of target genes, as summarised by Patrizi & Siciliani de Cumis (2018).

Recent research has investigated the impact of EDCs on miRNAs. Exposure of breast cancer cells to high concentrations of BPA and low levels of dichlorodiphenyltrichloroethane

(DDT) resulted in decreased expression of some miRNAs due to their oestrogenic disruptive abilities (Tilghman *et al.*, 2012). This capacity of BPA to modify oestrogen-regulated miRNAs has been confirmed in other mammalian cell lines, such as endometrial stromal cells (Reed *et al.*, 2018). In female chicks, treatment with DES altered a set of miRNAs in the oviduct that regulate a key protein in the outer layer of the vitelline membrane of eggs (Lim & Song, 2015). Exposure of human Sertoli cells to TCDD led to dysregulation of several miRNAs related to cell proliferation, growth and development (Ribeiro *et al.*, 2018). Exposure of pregnant rats to vinclozolin (VCZ) was reported to change the expression of several sncRNAs and lncRNAs in sperm of at least three generations (Ben Maamar *et al.*, 2018*a*).

Given the ubiquitous presence of EDCs in the environment, that they are able to interfere with the endocrine system, and that they display genotoxic and epigenotoxic potential, the molecular mechanisms by which EDCs affect the health of both humans and wildlife, and how these effects are transmitted to subsequent generations is a pressing research topic.

III. EDCS AND THE PATERNAL CONTRIBUTION TO EMBRYO DEVELOPMENT

Fertilisation encompasses many coordinated molecular events involved in the fusion of egg and sperm haploid pronuclei to form a diploid zygote. It has long been considered that the only function of a spermatozoon is to deliver the paternal genome to the oocyte (Georgadaki *et al.*, 2016). Nevertheless, many studies support a paternal contribution to development beyond simply the transmission of spermatic nuclear DNA. In fact, many sperm mRNAs and ncRNAs transferred to the oocyte are involved in early embryonic development (Miller & Ostermeier, 2006; Chen & Chan, 2016). The epigenetic landscape of spermatozoa is also transmitted and, therefore, it may have an impact on offspring health (Carrell & Hammoud, 2010). Most surprisingly, a recent study reported that in some exceptional cases paternal mitochondrial DNA can be passed to the progeny (Luo *et al.*, 2018).

(1) Impact of EDCs on the information contained by the spermatozoa

Spermatozoa are highly specialised cells formed in the testes through spermatogenesis from spermatogonial stem cells (Morais *et al.*, 2013). Spermatozoa have a highly compacted nucleus, to ensure protection of the paternal genome, and a flagellum that allows them to move towards the egg. Spermatogenesis begins with mitotic phases, allowing diploid spermatogonia to proliferate; next, a meiotic phase occurs in primary and secondary spermatocytes and, finally, spermiogenesis takes place turning haploid spermatids into motile and flagellated spermatozoa (Champroux *et al.*, 2016).

(*a*) Sperm chromatin

The sperm nucleus represents an extreme form of chromatin compaction. In somatic cells, chromatin is formed by the association of DNA with histone proteins. The basic unit of chromatin organisation is the nucleosome: 146 base pairs (bp) of DNA wrapped in a histone octamer consisting of two copies each of the core histones H2A, H2B, H3 and H4 (Luger *et al.*, 1997). In many species, spermatogenesis involves replacement of these histones with protamines. The association of DNA with protamines generates the toroids – regions of high chromatin compaction. However, the percentage of histone replacement depends on the species: there is total replacement in sea bass (*Dicentrarchus labrax*) and trout (*Oncorhynchus* spp.), partial replacement in humans (5–10% of the paternal genome is still packed into nucleosomes); whereas in other species such as zebrafish, the nucleosomal architecture persists (i.e. there is no histone–protamine transition) (Hammoud *et al.*, 2009; Herráez *et al.*, 2017). In zebrafish, the greater compaction in sperm DNA likely results from a higher ratio of H1 linker histone to core histones (Ausió, González-Romero & Woodcock, 2014). Epigenetic marks have been described to play an important role in sperm genome condensation (Wu, Zhang & Cairns, 2011). Strand DNA breaks occur naturally in spermatozoa during meiosis, in order to allow chromosome recombination and nuclear condensation (Rathke *et al.*, 2014). Nonetheless, these cells are particularly sensitive to DNA damage produced by oxidative stress, since they lack DNA repairing machinery and display limited antioxidant protection (Herráez *et al.*, 2017). Indeed, several ROS-generating chemicals are known to affect sperm DNA integrity in mammals and fish (Russo *et al.*, 2006; Sipinen *et al.*, 2010; Santos *et al.*, 2013).

(*b*) EDCs and sperm DNA damage

In epidemiological studies, urinary concentrations of BPA in humans have been associated with increased levels of sperm DNA damage (Meeker *et al.*, 2010). Urinary concentrations of DEHP and its metabolites [MEHP, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP)] have also been correlated with sperm DNA damage in different human populations (Hauser *et al.*, 2007; Pant *et al.*, 2008; Wang *et al.*, 2016). The presence of DEHP metabolites in the seminal plasma of Chinese men revealed that MEHP was associated with increased sperm apoptosis (You *et al.*, 2015).

From toxicological studies, in zebrafish levels of DNA damage were very high in spermatozoa from males treated with two doses of BPA (100 and 2000 μg/l) during different periods of spermatogenesis, especially when meiosis and spermiogenesis was involved (Lombó *et al.*, 2019*a*). *In vitro* exposure of human and dog spermatozoa to low doses of

DEHP and PCB 153 (37.32 μg/ml and 1.32 ng/ml, respectively), which negatively impacted their motility, was linked to greater levels of sperm DNA fragmentation (Sumner *et al.*, 2019). While most studies are focused on the genotoxic effects of EDCs on testicular cells, some have considered specific sperm cell death: *in vivo* exposure of male mice to low doses of TCDD induced sperm apoptosis and cytotoxicity (Elsayed *et al.*, 2019). Furthermore, *in vitro* exposure of motile human spermatozoa to increasing doses of BPA (from 300 to 800 μM) also had prooxidative/apoptotic effects, leading to mitochondrial dysfunction (Barbonetti *et al.*, 2016).

(*c*) Sperm epigenetic alterations

(i) Sperm epigenetic remodelling. Throughout gametogenesis, germ cells undergo intense epigenetic remodelling that involves the establishment of sex-specific patterns in both the spermatozoa and oocyte (Fig. 3). In mammals, the mitotic period of spermatogenesis is characterised by a decrease in repressive marks (DNA methylation, H3K27me3 and H3K9me3) and an increase in activating marks (H3ac, H4ac and H3K4me2/3) (Hammoud *et al.*, 2009; Dada *et al.*, 2012). Notwithstanding these modifications, in spermatogonia, a process of *de novo* DNA methylation takes places in imprinted genes, whose epigenetic marking results in monoallelic expression (Falls *et al.*, 1999). During meiosis, progressive gene silencing has been reported: the permissive mark H3K4me3 decreases and the silencing marks H3K27me3 and H3K9me3 increase in spermatocytes (Carrell & Hammoud, 2010). As a result, spermatozoa arise as highly methylated cells, especially in zebrafish where 91–95% of CpGs are methylated (Potok *et al.*, 2013). Still, several hypomethylated regions corresponding to genes expressed during early development as well as some permissive histone marks (H3K4me3, H3K4me2 and H4K16ac) associated with genes involved in meiosis persist in sperm (Fig. 3). Wu *et al.* (2011) showed that permissive histone marks are present in genes expressed in zebrafish embryos before mid-blastula transition (MBT), when embryonic transcription is activated. Moreover, they demonstrated that key developmental genes are packaged in bivalent or multivalent marks, in which activating and repressing histone marks and DNA hypomethylation co-exist. In summary, the repressive marks might avoid the expression of certain genes in the male germline, whereas the activating marks may prevent DNA methylation in the promoters of genes necessary for development, allowing their activation in the embryo when required (Carrell, 2011).

During the last stages of spermiogenesis, Sertoli cells phagocytose most of the cytoplasm and its RNAs, generating a cytoplasmic residue known as the chromatoid body (Parvinen, 2005). Thus, spermatozoa were thought to lack essential components of the cytoplasmic ribosomes involved in the translational machinery, although the presence of 18S ribosomal RNA (rRNA) has been confirmed in mature human spermatozoa (Cappallo-Obermann *et al.*, 2011).

Despite being transcriptionally inactive, sperm cells have been reported to harbour both coding (mRNAs) and non-coding RNAs (miRNAs, piRNAs and lncRNAs) (Jodar *et al.*, 2013; Robles *et al.*, 2019); Ostermeier *et al.* (2002) reported that normal human sperm contain around 3000–7000 types of coding transcripts. Since their original discovery (Pessot *et al.*, 1989), sperm-borne RNAs have been been identified in multiple organisms, and a database of all known sperm transcripts of the mouse, rat, rabbit and human is now available [SpermBase; www.spermbase.org (Schuster *et al.*, 2016*b*)]. Thus, spermatic RNAs are undoubtedly delivered into the oocyte during fertilisation and, although initially considered to be only remnant transcripts of spermatogenesis, they may play an important role in mammalian early embryo development (Ostermeier *et al.*, 2004; Fang *et al.*, 2014; Guo *et al.*, 2017), as well as in offspring phenotype (Rassoulzadegan *et al.*, 2006). Sperm RNAs also have been suggested to facilitate communication and cooperation among spermatozoa within the same ejaculate, thus functioning as signals of relatedness (Hosken & Hodgson, 2014).

(ii) Spermatic epimutations induced by EDCs. Given the large-scale epigenetic changes that take place in sperm cells, many studies have the potential for epimutations induced by exposure to environmental toxicants (Table 2). Most of these focus on sperm DNA methylation. As far as the effects of EDCs on sperm DNA methylation are concerned, epidemiological studies using long interspersed nuclear elements (LINE-1) as a marker of genome-wide methylation status demonstrated that occupational exposure to BPA alters the global levels of 5mC and 5hmC in human sperm: the BPA-exposed group had significantly lower spermatic LINE-1 methylation (median 0.74) than the non-exposed group (median 0.79) (Miao *et al.*, 2014), but higher LINE-1 hydroxymethylation (median 12.97%) than the non-exposed group (9.68%) (Tian *et al.*, 2018). Whole-genome bisulfite sequencing (WGBS) in sperm of young Russian adults revealed 52 differentially methylated regions between the lowest and the highest peripubertal serum TCDD concentrations (Pilsner *et al.*, 2018).

In the toxicological studies, *in utero* exposure to BPA, DEHP, dibutyl phthalate (DBP) (Manikkam *et al.*, 2013), VCZ (Guerrero-Bosagna *et al.*, 2010; Ben Maamar *et al.*, 2018*a*; Nilsson *et al.*, 2018), and TCDD in rats (Manikkam *et al.*, 2012), exposure to VCZ and to DEHP in mice (Stouder & Paoloni-Giacobino, 2010; Prados *et al.*, 2015) and embryonic exposure to MEHP in zebrafish (Kamstra *et al.*, 2017) have all been shown to alter the DNA methylation pattern of specific regions or genes in spermatozoa of exposed males and, in certain cases, of their future generations (see Section III.3).

Regarding histone modifications, most toxicological studies have focused on how exposure to EDCs changes the histone marks of testicular cells (Chen *et al.*, 2017; González-Rojo *et al.*, 2019), with the effects on the spermatozoa being less explored. Exposure of adult male zebrafish to BPA has been reported to increase the levels of H3K9ac and H3K27ac in sperm (Lombó *et al.*, 2019*a*), whereas embryonic exposure to BPA in this species promotes a decrease of H3K9ac levels in spermatozoa during adulthood (Lombó *et al.*, 2019*b*). Using the same experimental model, H3K9ac enrichment specifically

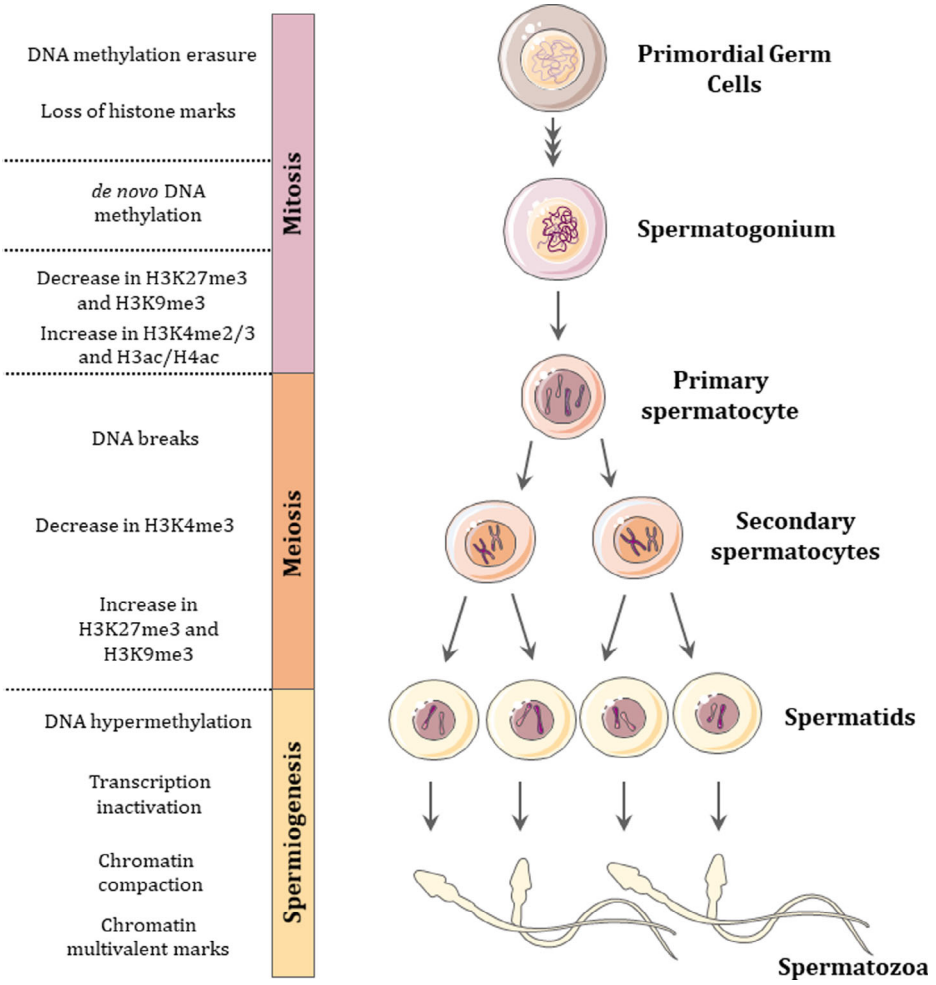

Fig 3. Modifications occurring during male germline formation. Primordial germ cells undergo epigenetic remodelling that consists of erasure of DNA methylation and loss of activating histone marks. From this 'epigenetic ground state', male germ cells are subject several epigenetic and other molecular changes during spermatogenesis. The mitotic proliferation of spermatogonia is characterised by *de novo* DNA methylation and the acquisition of permissive histone marks. During meiosis, diploid cells (spermatocytes) become haploid cells (spermatids), a process when the number of histone repressive marks and DNA breaks increases to facilitate chromatin compaction. Finally, spermatids differentiate into spermatozoa in a process known as spermiogenesis that involves transcription inactivation due to DNA hypermethylation. Some multivalent domains remain present in the sperm genome to ensure proper embryo development.

affected the promoters of some genes crucial for embryo development, such as *hand2*, *kat6a* and *esr2b* (M. Lombó & M.P. Herráez, in preparation).

Information regarding the effects of EDC exposure on sperm RNAs remains scarce. Two studies found that *in utero* exposure to VCZ in rats was correlated with changes in miRNA, piRNA and small temporal RNA (stRNA) of spermatozoa (Schuster, Skinner & Yan, 2016*a*; Ben Maamar *et al.*, 2018*a*) and another demonstrated that *in utero* exposure to DEHP in mice led to an increase in miRNA expression and decrease in miRNA promoter methylation of sperm (Stenz *et al.*, 2017). In addition, exposure of male zebrafish to BPA and 17-α-ethinylestradiol (EE2) triggered alterations in specific sperm mRNAs [*insrb* and *esr2b*, respectively (Lombó *et al.*, 2015; Valcarce *et al.*, 2017)].

(2) Epigenetic landscape during embryo development

Environmental factors are able to promote genotoxic and epigenotoxic effects in sperm, thus disrupting male reproduction and affecting the development of future generations. Maternal transmission of epigenetic alterations induced by maternal lifestyle and/or by environmental exposure during pregnancy have been studied in several species (Dolinoy *et al.*, 2007; Manikkam *et al.*, 2013; Stenz *et al.*, 2017; Bansal *et al.*, 2019; Santangeli *et al.*, 2019). Recently, attention has focused on the paternal inheritance of epigenetic alterations, since this may also impact offspring development. Exposure to EDCs in the workplace has been linked to changes in the DNA methylation profile of human spermatozoa (Miao *et al.*, 2014; Zheng *et al.*, 2017; Tian *et al.*, 2018).

Table 2. Sperm epimutations triggered by exposure to endocrine-disrupting chemicals (EDCs)

Type of study	Type of EDC and exposure	Dose	Timing of exposure	Epigenetic effects on sperm	Method used	Species	Reference
Epidemiological studies	Occupational exposure to BPA	Urinary concentrations		LINE-1 hypomethylation	RT-qPCR	Human	Miao *et al.* (2014)
	Occupational exposure to BPA	Urinary concentrations		Increase in LINE-1 hydroxymethylation	RT-qPCR	Human	Tian *et al.* (2018)
	Occupational exposure to BPA	Urinary concentrations		Increase in 5hmC	hMeDIP	Human	Zheng *et al.* (2017)
	Peripubertal exposure to TCDD	Serum concentrations		Changes in DNA methylation of specific regions	WGBS and RRBS	Human	Pilsner *et al.* (2018)
Toxicological *in vivo* studies	Pregnant female exposure to VCZ	100 mg/kg BW/day	E14–E18	Altered DNA methylation in specific genes	MeDIP	Rat	Guerrero-Bosagna *et al.* (2010)
	Pregnant female exposure to VCZ	50 mg/kg BW/day	E10–E18	Altered DNA methylation in specific genes	Pyrosequencing	Mouse	Stouder & Paoloni-Giacobino (2010)
	Pregnant female exposure to TCDD	100 ng/kg BW/day	E14–E18	Changes in DNA methylation of specific regions	MeDIP-ChIP and MeDIP-PCR	Rat	Manikkam *et al.* (2012)
	Pregnant female exposure to BPA, DEHP and DBP	50, 750 and 66 mg/kg BW/day, respectively	E14–E18	Changes in DNA methylation of specific regions	MeDIP-ChIP and MeDIP-PCR	Rat	Manikkam *et al.* (2013)
	Pregnant female exposure to DEHP	300 mg/kg BW/day	E9–E19	Changes in DNA methylation of specific regions	MBD-seq	Mouse	Prados *et al.* (2015)
	Pregnant female exposure to VCZ	100 mg/kg BW/day	E14–E18	Changes in DNA methylation of specific regions	MeDIP	Rat	Nilsson *et al.* (2018)
	Pregnant female exposure to VCZ	100 mg/kg BW/day	E14–E18	Changes in DNA methylation of specific regions and ncRNAs expression	sncRNA-Seq	Rat	Schuster *et al.* (2016*a*); Ben Maamar *et al.* (2018*a*)
	Pregnant female exposure to DEHP	300 mg/kg BW/day	E9–E19	Increase in miRNA expression and decrease in miRNA promoter methylation	RNA-Seq and MBD-Seq	Mouse	Stenz *et al.* (2017)
	Embryonic exposure to MEHP	30 mM	0hpf–6dpf	DNA methylation of specific regions	LC/MS and RRBS	Zebrafish	Kamstra *et al.* (2017)
	Embryonic exposure to BPA	4000 µg/l	0hpf–1dpf	Decrease in H3K9ac	Whole mount immunostaining	Zebrafish	Lombó *et al.* (2019*b*)
	Male exposure to BPA	2000 µg/l	14 days	Decrease in specific mRNAs	RT-qPCR	Zebrafish	Lombó *et al.* (2015)
	Male exposure to EE2	5 ng/l	14 days	Increase in specific mRNAs	RT-qPCR	Zebrafish	Valcarce *et al.* (2017)
	Male exposure to BPA	2000 µg/l	21 days	Increase in H3K9 and H3K27ac	Cell immunostaining	Zebrafish	Lombó *et al.* (2019*a*)

BW, body mass; dpf, days post fertilisation; 5hmC, 5-hydroxymethylcytosine; hMeDIP, hydroxymethyl-DNA immunoprecipitation; hpf, hours post fertilisation; LC/MS, liquid chromatography/mass spectrometry; LINE-1, long interspersed nuclear elements; MBD-seq, methyl binding domain sequencing; MeDIP, methyl-DNA immunoprecipitation; MeDIP-Chip, methyl-DNA immunoprecipitation-chromatin immunoprecipitation; MeDIP-PCR, methyl-DNA immunoprecipitation-polymerase chain replication; miRNA, microRNA; mRNA, messenger RNA; ncRNA, non-coding RNA; RNA-seq, RNA sequencing; RRBS, reduced representation bisulfite sequencing; RT-qPCR, real-time quantitative polymerase chain replication; sncRNA-seq, small non-coding RNAs sequencing; WGBS, whole-genome bisulfite sequencing. For abbreviations of EDCs see Table 1.

Table 3. Intergenerational effects of parental exposure to endocrine-disrupting chemicals (EDCs)

Type of EDC and exposure	Dose	Timing of exposure	Effects for the descendants	Species	Reference
Pregnant female exposure to TCDD	100 ng/kg BW/day	E14–E18	Changes in sperm DNA methylation of specific regions up to F3	Rat	Manikkam *et al.* (2012)
Pregnant female exposure to BPA, DEHP and DBP	50, 750 and 66 mg/kg BW/day, respectively	E14–E18	Changes in sperm DNA methylation of specific regions up to F3	Rat	Manikkam *et al.* (2013)
Pregnant female exposure to VCZ	100 mg/kg BW/day	E14–E18	Over 200 differentially expressed sncRNAs in sperm of F3	Rat	Nilsson *et al.* (2018)
Pregnant female exposure to VCZ	100 mg/kg BW/day	E14–E18	Changes in sperm DNA methylation of specific regions up to F3	Rat	Beck *et al.* (2017)
Pregnant female exposure to VCZ	100 mg/kg BW/day	E8–E18	Changes in sperm and brain DNA methylation of specific regions up to F3	Rat	Nilsson *et al.* (2018)
Pregnant female exposure to VCZ	100 mg/kg BW/day	E14–E18	Changes in sperm DNA methylation of specific regions up to F3	Rat	Nilsson *et al.* (2018)
Pregnant female exposure to VCZ	100 mg/kg BW/day	E14–E18	Changes in DNA and differentially expressed sncRNAs in sperm of F2	Rat	Ben Maamar *et al.* (2018*a*)
Pregnant female exposure to VCZ and DDT	100 and 25 mg/kg BW/day, respectively	E14–E18	Differential histone retention sites in the F3 sperm	Rat	Ben Maamar *et al.* (2018*b*)
Pregnant female exposure to glyphosate	25 mg/kg BW/day	E14–E18	Changes in sperm DNA methylation of specific regions and multiple pathologies up to F3	Rat	Kubsad *et al.* (2019)
Pregnant female exposure to VCZ and DDT	100 and 25 mg/kg BW/day, respectively	E14–E18	Changes in sperm DNA methylation of specific regions and testis and ovarian pathologies up to F4	Rat	Ben Maamar *et al.* (2020)
Pregnant female exposure to BPA, VCZ and DEHP	0.2, 100 and 750 mg/kg BW/day, respectively	8.5–12.5 dpf	No transmission of epimutations to subsequent generations	Mouse	Iqbal *et al.* (2015)
Male exposure to dioxin-like PCBs	0.006–0.011 mg/kg BW/day	6 months	Higher embryo mortality	Fathead minnows	Coulter *et al.* (2019)
Male exposure to BPA	50 µg/kg BW/day	21 days	Anxiety and depression in F1	Rat	Fan *et al.* (2018)
Male exposure to BPA	2000 µg/l	14 days	Cardiac malformations in F1 and F2	Zebrafish	Lombó *et al.* (2015)
Male exposure to EE2	5 ng/l	14 days	Increase in specific mRNAs	Zebrafish	Valcarce *et al.* (2017)
Male exposure to BPA	2000 µg/l	21 days	Impairment of F1 development	Zebrafish	Lombó *et al.* (2019*a*)

BW, body mass; dpf, days post fertilisation; mRNA, messenger RNA; sncRNA, short non-coding RNA. For abbreviations of EDCs see Table 1.

Transgenerational inheritance of epigenetic changes requires that germline cells are affected so that epigenetic alterations are transmitted to subsequent generations even if they have never been in contact with the disrupting agent (McCarrey, 2014). Maternal exposure during pregnancy implies that the F0 female, F1 foetus and the germ cells of the foetus, which will eventually generate the F2 generation, are all exposed. Therefore, any effects found in the F0, F1 and F2 generations may be correlated with direct exposure to the toxin; thus an assessment of F3 progeny would be needed to establish the transgenerational inheritance of such effects. By contrast, in paternal exposure, only the F0 male and its germ cells which give rise the F1 generation are affected, so in this case observed effects on the F2 generation would be sufficient to show transgenerational transmission (Nilsson & Skinner, 2015).The inheritance of epimutations by subsequent generations is far from straightforward. To avoid the transmission of altered epigenetic marks established during gametogenesis, extensive epigenetic reprogramming occurs in early embryos soon after fertilisation (Smallwood & Kelsey, 2012). In mammals, passive loss of global DNA methylation, mainly affecting the male pronucleus, occurs from fertilisation to the blastocyst stage. Additionally, the paternal pronucleus undergoes active DNA methylation *via* the enzyme TET3 (Gu *et al.*, 2011). At the onset of gastrulation, global genome methylation begins, to allow the loss of cellular pluripotency and thus to enable cellular lineage determination (Reik, Dean & Walter, 2001). The highly methylated pattern of spermatozoa is diluted in the zygote, especially in retroelements, since the hypomethylated status of the oocyte is reflected in the zygote (Smith *et al.*, 2012). In the zebrafish, the DNA suffers moderate demethylation after fertilisation and remethylation of the DNA begins sooner than in mammals. Although the DNA of the oocyte is also hypomethylated in zebrafish, this maternal pattern is only maintained in embryos until the 16-cell stage. Jiang *et al.* (2013) demonstrated that the global methylation level at this stage of development overlaps the mean DNA methylation values of the oocyte and sperm (80 and 91%, respectively). The embryonic epigenome is progressively methylated and, by the MBT stage, the methylome of zebrafish embryos is almost identical to that of sperm (Potok *et al.*, 2013). Due to the gradual resetting of the maternal DNA methylation pattern, the methylation profile for most gene promoters of MBT embryos is also very similar to that of sperm (Lindeman *et al.*, 2010). For example, genes involved in embryo development (*hox* clusters) or germline function (*vasa*, *piwi* and *dazl*) are hypermethylated in oocytes and hypomethylated in both sperm and MBT embryos (Potok *et al.*, 2013). From the MBT stage to 24 h post fertilisation, thousands of differentially methylated regions were identified by Lee *et al.* (2015), most of which were located in intergenic regions (outside gene promoters, CpG islands and island shores), where they surprisingly function as developmental enhancers.

Histone modifications also vary throughout embryo development. In mammals, in which protamines are the predominant sperm nuclear proteins, the paternal histones are highly hyperacetylated; however, there is a dramatic increase in histone methylation (H3K4me1, H3K9me1 and H3K27me1) immediately following histone incorporation that leads to an epigenetic state more similar to maternal chromatin (Morgan *et al.*, 2005). In zebrafish, the histone modifications also depend on the sperm pattern. Murphy *et al.* (2018) described in this species the existence of 'placeholder' nucleosomes containing histone H2A.Z and H3K4me1, which occupy all hypomethylated DNA in both sperm and early embryos. Upon genome activation, placeholders either become marked as active (in housekeeping genes) or repressed (in developmental genes).

(3) Inheritance of deleterious effects through the male germline

The establishment of epigenetic signatures specific to a cellular lineage is of utmost importance during embryogenesis. Thus, when epimutations carried by the gametes escape epigenetic erasure and are transmitted to the zygote, embryonic development can be affected.

To date, only a few studies have investigated the inheritance of effects triggered by male exposure to EDCs; most investigations that include data on the impacts of EDC exposure on the progeny involve a combination of both maternal and paternal exposure (Guo *et al.*, 2019; Dabeer *et al.*, 2020; Huang *et al.*, 2020). In mammals, when only the fathers were treated with BPA there was an increase in anxiety behaviours in F1 female rats and depression behaviours in F1 rats of both sexes (Fan *et al.*, 2018); whereas in fishes, paternal exposure to a mixture of PCBs or BPA led to impairment of F1 embryo development (Coulter *et al.*, 2019; Lombó *et al.*, 2019*a*).

Despite the fact that few data are available, the transmission of deleterious effects caused by EDCs through the male germline has become of great interest. Table 3 summarises results indicating that the transmission of EDC effects can be paternally mediated. Manikkam *et al.* (2012, 2013) reported that exposure of pregnant female rats to TCDD and a mixture of plastic-derived EDCs (BPA, DEHP and DBP) was correlated with a different pattern of DNA methylation of 50 and 197 regions, respectively, in the sperm up to the F3 generation. Other studies have demonstrated that maternal exposure during pregnancy to VCZ, DDT, PCBs and glyphosate during pregnancy was linked with specific changes in sperm DNA methylation that are transgenerationally inherited (Beck, Sadler-Riggleman & Skinner, 2017; Gillette *et al.*, 2018; Nilsson *et al.*, 2018; Kubsad *et al.*, 2019; Ben Maamar *et al.*, 2020). In addition to modifications in sperm DNA methylation, *in utero* exposure to VCZ and DDT in rats has been associated with altered histone H3 retention sites in sperm of F3 males (Ben Maamar *et al.*, 2018*b*) as well as with transgenerational alterations in both spermatic sncRNAs and lncRNAs (Schuster *et al.*, 2016*a*; Ben Maamar *et al.*, 2018*a*). Future studies should therefore focus on the effects of alterations of the epigenetic pattern and of RNAs of sperm beyond the immediate effects

on reproduction, given their potential to affect the health of subsequent generations. In many cases, any effects triggered by *in utero* exposure to EDCs (BPA, VCZ and DEHP) on the DNA methylation pattern of germ cells will not be carried through to subsequent generations, due to the important corrective role of the epigenetic remodelling processes (Iqbal *et al.*, 2015).

Clearly, changes to germ cells can elicit a transgenerational phenotype and such multigenerational effects should not be underestimated (Xin, Susiarjo & Bartolomei, 2015). Alterations of histone epigenetic marks in mature spermatozoa promoted by non-endocrine disruptive factors in mice have been associated with abnormal embryonic gene expression and phenotype (Siklenka *et al.*, 2015; Pérez-Cerezales *et al.*, 2017). In *Caenorhabditis elegans*, changes in gene expression triggered by temperature-induced epimutations have been demonstrated to be inherited over at least 14 generations through both oocyte and sperm (Klosin *et al.*, 2017). Additionally, metabolic alterations caused by paternal obesity were inherited up to the F2 generation due to changes in spermatic non-coding RNAs (Cropley *et al.*, 2016).

Given that alterations to sperm epigenetic patterning can affect the phenotype of the progeny, and that EDCs can trigger epimutations, several studies have focused on the impact of these compounds on the phenotype of subsequent generations. In rats, exposure to glyphosate or DDT and VCZ during pregnancy led to prostate disease, obesity, kidney disease, ovarian and testis disease, and birth abnormalities in F3 descendants (Kubsad *et al.*, 2019; Ben Maamar *et al.*, 2020). In zebrafish, paternal exposure to EDCs induces both multigenerational and transgenerational phenotypes: male treatment with EE2 was correlated with an increased percentage of lymphoedema and otolith areas of F1 larvae (Valcarce *et al.*, 2017), whereas male exposure to BPA during early spermatogenesis was related to a decrease in remnant mRNAs in the spermatozoa, and to cardiac disorders in the F2 progeny (Lombó *et al.*, 2015). Also in this model species, an altered cardiac phenotype of embryos obtained from zebrafish males exposed to 2000 μg/l BPA during early spermatogenesis, which showed the same hyperacetylation pattern as the sperm, was successfully rescued by treatment of the embryos with an inhibitor of histone acetyl transferases (epigallocatechin gallate) during embryo epigenetic remodelling (3.3 h after fertilisation) (M. Lombó & M.P. Herráez, in preparation).

IV. CONCLUSIONS

(1) Exposure to EDCs is increasingly reported to disrupt male reproduction, affecting the survival of germ cells and the sperm count even at low doses. While most experiments have focused on understanding the consequences of EDC exposure on aspects of male breeding capacity, few have considered the impact of such exposure on the information provided by the spermatozoa and, therefore, on the health of subsequent generations.

(2) Considering the importance of information carried by sperm for embryonic development, future studies should focus on the deleterious effects triggered by EDCs on sperm DNA integrity, epigenetic marks and RNAs, given the possible synergy between genetic and epigenetic effects.

(3) A better understanding of how EDCs could impact the paternal contribution to embryo development will be very helpful in terms of arriving at better regulation of the presence and concentrations of these compounds to which humans are exposed.

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