Chapter 3
Endocrine Disruptors, Epigenetic Changes, and Transgenerational Transmission

Roberta Rizzo, Daria Bortolotti, Sabrina Rizzo, and Giovanna Schiuma

3.1 Effects of Epigenetics on Human Reproduction: An Introduction

Recent discoveries in the field of molecular biology are focused on phenomena like chromatin condensation, histone (H) modification, and deoxyribonucleic acid (DNA) methylation, as well as the action of small non-coding ribonucleic acid (RNA), which together belong to the branch of epigenetics. The term "epigenetics" was coined in 1940 by Conrad Waddington [1] who described it as "the branch of biology which studies the causal interactions between genes and their product which bring phenotypes into being." In fact, epigenetics includes all those mechanisms that are able to regulate DNA expression without modifying nucleotide sequence.

Among the main epigenetic mechanisms mentioned earlier, DNA methylation is the most widely known and most studied modification (Table 3.1). The process of DNA methylation constitutes a postreplicative modification, in which a methyl group is added covalently to a DNA residue [10]. The methylation occurs at the carbon 5 of the cytosine ring in 5′-3′-oriented CG dinucleotides (named as CpGs), and it is catalyzed by the action of DNA methyltransferases (DNMTs) [11]. Furthermore, recent evidences have shown that also RNA factors, such as small RNAs (small interfering RNA [siRNA] and microRNA [miRNA]), have the ability to direct DNA methylation through a mechanism called RNA-directed DNA methylation (RdDM), performed by double-stranded RNA (dsRNA), which may be produced after the transcription of inverted repeats [12].

R. Rizzo (✉) · D. Bortolotti · S. Rizzo · G. Schiuma
Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, Ferrara, Italy
e-mail: rbr@unife.it; daria.bortolotti@unife.it; sabrina.rizzo@unife.it; giovanna.schuima@unife.it

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Table 3.1 Main epigenetic mechanisms, molecular targets, and effects on gene expression

Epigenetic modification	Molecular target	Effect on gene expression
DNA methylation	Cytosine residue of DNA	Gene silencing
Small noncoding RNAs (miRNA and siRNA)	Messenger RNA (mRNA)	Gene silencing [2, 3]
Histone acetylation	Lysine of histone H2A, H2B, H3, and H4	Gene activation [4]
Histone methylation	Lysine and arginine of histone proteins	Gene activation/ silencing [5]
Histone phosphorylation	Serine and threonine of histone proteins	Gene activation/ silencing [6]
Histone sumoylation	Lysine of histone proteins	Gene silencing [7]
Histone ubiquitination	Lysine of histone proteins	Gene activation [8]
Histone ADP ribosylation	Lysine of histone proteins	Gene activation [9]

Furthermore, also the action of small noncoding RNA, transcribed from noncoding DNA, was identified as another epigenetic process involved in chromosome remodeling and transcriptional or posttranscriptional regulation, by influencing RNA stability and gene expression (Table 3.1) [13–16].

Besides the mentioned mechanisms, chromatin condensation and histone modification are also key processes involved in epigenetic modification, particularly acting on a chromosome structure. In fact, in eukaryotic organisms, genome is compacted by basic proteins named histones, which allow the organization of DNA into chromatin [17] that is susceptible to modification depending on specific stimuli such as transcriptional repressors, functional RNA, or other accessory factors [18]. For these reasons, epigenetic regulation of chromatin, and the consequent variation in gene expression, may be environmentally dependent. In particular, histones are susceptible to a large variety of posttranslational modifications such as phosphorylation, acetylation, methylation, ubiquitination, sumoylation, adenosine triphosphate (ADP) ribosylation, glycosylation, biotinylation, and carbonylation [17] that are involved in chromatin state alteration and consequently act on gene expression (Table 3.1). The combination of the different histone modifications mentioned above constitutes the histone code [19].

The data available in the literature suggest that epigenetic mechanisms, involving molecular regulators such as histone variant, histone posttranslational modifications, nucleosome positioning chromosome looping, DNA structural variations, and RNA-mediated regulation [20–25], are closely related to chromatin state and therefore affect normal gene expression, as shown in Table 3.1. The molecular explanation of the influence of these mechanisms on gene activation or silencing is represented exactly by their ability to modulate chromatin conformation, which can be condensed with the consequent inhibition of polymerase accessibility for gene expression that causes lack of gene transcription and translation, leading to gene silencing. Anyway, DNA methylation, noncoding RNAs, and histone modification,

with their consequences on chromatin state, are deeply interlinked to each other and represent a more integrated epigenetic system rather than disconnected events.

Epigenetic modifications, because of their effects on gene expression, play a central role in the regulation of gene expression during embryo development, from gametogenesis (oogenesis and spermatogenesis) to organogenesis, acting on chromatin state through DNA methylation and histone alterations and influencing later development [26].

During oogenesis and in particular during critical stages of oocyte growth and meiotic maturation, chromatin modifications control different key processes including gene expression, the establishment of maternal-specific DNA methylation marks, and chromosome stability [27]. The presence of a correct epigenome is responsible for proper chromosome segregation, for silencing of repetitive elements and potentially dangerous transposons and for meiotic centromere stability. The mammalian oocyte needs epigenetics, as dynamic chromatin alterations, to gain meiotic and developmental potential. Chromatin conformation is modified by chromatin remodeling proteins and histone modifications, which regulate heterochromatin formation and centromere function in the female germ line [28]. Differential methylation patterns are established during gametogenesis, both oogenesis and spermatogenesis, guaranteeing allele-specific parental identity by the process of genome imprinting [29]. The expression of these imprinted genes depends on regulatory sequences called imprinted control regions (ICRs) that are differentially methylated in the germ line [30]. Maternal methylation pattern at specific loci during oogenesis begins to be established during a critical period of postnatal oocyte growth starting from Day 5 of postnatal development, coincident with the transitional stage from primary to secondary follicles [31–33].

Epigenetics also regulates the biological process of spermatogenesis, in which the expression of several genes in the testes is controlled by modifications like DNA methylation, histone modifications, and chromatin remodeling. Testicular DNA has a unique pattern of methylation, which is eight times hypomethylated compared with somatic tissues. In particular, Sertoli cells had low levels of methylation in euchromatin and high levels in juxtacentromeric regions [34]. The characteristic methylation pattern of testis germ cells is established before meiosis, when these different demethylation/methylation processes act [35]. Testicular germ cells have distinct methylation patterns that depend on genomic sequence and usually occur on nonrepetitive genomic regions that are methylated de novo [35, 36]. Although the methylation status of certain genes may be changed during the different stages of spermatogenesis, it may or may not correspond to the gene's expression pattern [37]. Besides methylation, some variants of histones, called H2AL1, H2AL2, and H2BL1, have been identified in mature spermatozoa, and these probably take part in reprogramming pericentric heterochromatic regions during spermatogenesis [38]. In addition, the testes-specific linker histone variant H1T is normally exchanged for H1 during spermatogenesis [39], but H1T can also be replaced by the linker histone named HILS1, which may influence chromatin state and promote condensing spermatids [39, 40]. Thus, epigenetics is responsible for proper regulation of

spermatogenesis to ensure physiologic sperm function and embryonic development; in fact, epigenetic aberrations are linked with male infertility [36].

As well as ensuring the correct gametogenesis in both males and females, epigenetic mechanisms also take part in cellular specialization during embryo development [41], because this process consists of changing patterns of gene expression that allow the specification of the cells of the early embryo from totipotency (the potential to form any kind of cells of the body, the extraembryonic membranes, and the placenta) to a discrete cell population. The lineage-specific pattern of gene expression is based on modifications of chromatin structure and function, which do not involve any change in nucleotide sequence of DNA, at the same way of every other epigenetic modification. Now, it is well known that differentiation of pluripotent cells is related to the methylation of the promoter of the essential pluripotency transcription factor, Oct4 [42].

Several variations in gene expression related to the chromatin state (condensed or uncondensed) observed during embryogenesis have been studied. In fact, considering embryo development, chromatin state is crucial in determining whether some genomic regions can be transiently condensed and accordingly silenced or, on the contrary, expressed when uncondensed [43], providing a fine mechanism of control of gene expression that guarantees the correct development of tissues and organs [41, 44].

Epigenetic mechanisms are active players in many physiological processes of human life, and for this reason, they are strongly involved in reproduction and some related genetic diseases. The embryo inherits two copies of the same gene, one from the mother and the other from the father, but each one of these can be silenced by epigenetic modifications causing many illnesses and disorders. This phenomenon, that is, the "turning off" of one parent's gene during gametogenesis (oogenesis and spermatogenesis), is defined as genetic imprinting.

The alteration of the genetic imprinting due to epigenetic disorders leads to the arise of reproductive genetic diseases, including Angelman syndrome and Prader-Willi syndrome (PWS) [45]. These two syndromes are characterized by an aberrant chromosome silencing that causes the loss of expression of specific maternal or paternal genes: In Angelman syndrome, a small deletion in chromosome 15 [46] affects the gene UBE3A, leading to the expression of the maternal gene and the silencing of paternal one determining nervous system impairment, while Prader-Willi syndrome (PWS) is a genetic disorder characterized by extreme feeding problems including hyperphagia or insatiable appetite and obsession with food, as well as decreased muscle tone in affected children due to epigenetic repression of PWS genes on maternal chromosome [47].

During the entire embryo development, there are two major rounds of epigenetic reprogramming [48, 49] that include a global loss of DNA methylation in cells of the early embryo. A phase of demethylation acts on paternally inherited genome in the first cell cycle after fertilization, while the maternally inherited one loose methylation progressively, because of the failure of the maintenance of methylation pattern together with replication after each cell division. The result is the hypomethylation of the entire embryo by the blastocyst stage [49] that is followed

instead by a process of methylation during implantation [50]. Some earlier studies proposed that the formation of 5′-hydroxymethylcytosine, derived from the oxidation of 5-methylcytosine in the paternal pronucleus in fertilized oocyte, could represent a possible intermediate for global DNA demethylation of the paternal genome [51]. Moreover, it has been found that this modification is stable in both paternally and maternally derived genomes, and therefore, it may provide its own unique form of epigenetic information [52], even because 5′-hydroxymethylcystosine is quite a stable modification of the genome [53].

However, exposure of the embryo to a range of stresses during the period of its physiological epigenetic reprogramming can result in abnormal developmental outcomes. Moreover, external stimuli increase their own detrimental potential when act in the early stages of organism development. In fact, in these phases, the embryo is more sensitive to these stimuli because the alteration resulting from the stresses could be transmitted through the germ line and be hidden until much later in life [54, 55]. Therefore, any environmental stress during the early stages of development produces more systemic consequences than the same exposure in adulthood, which on the contrary has more local and limited consequences [56].

Recently, growing evidences suggest that the environment in which the embryo, fetus, and neonate develop seems to be involved in alteration of physiological epigenetic program. Nowadays, we are surrounded by thousands of compounds, chemicals and not, which daily interact with us and inevitably have effects on our state of health. Among all the different toxicants we are dealing with, there is a group of interest which is known as endocrine-disrupting chemicals (EDCs) that have the ability to act on human epigenome, affecting mostly reproduction [57]. In addition, the environmental effects on epigenetic settings during germ cell formation have the potential for the transgenerational inheritance of some of these induced modifications, and for these reasons, the epigenetic effect of EDCs on human reproduction should be taken into account.

3.2 Association Between EDCs and Epigenetics on Human Fertility

The study of epigenetic regulation during reproduction and development focused on the molecular mechanisms of gene expression related to developmental biology [58], in order to define the environmental mechanisms involved in alterations of gene expression patterns without affecting DNA sequence [59]. Environmental factors have a significant impact on biology, particularly referring to toxic compounds [57]. These environmental toxicants can modulate biological systems and influence physiology, even promoting disease states. Chemical compounds can be found in pharmaceutical drugs, personal care products, food additives, and food containers. These products could interfere with human endocrine systems and have the ability to induce diseases such as prostate and breast cancers or metabolic

diseases [60–62] or have effects on the human reproductive, thyroid, cardiovascular, and neuroendocrinology systems. However, the effect of external toxicants on human health mainly consists of epigenetic alteration of genome without any modification to DNA sequence, thanks to the fact that DNA developed a general resistance against external attacks in order to maintain genome stability during evolution.

Among the thousands of contaminants released in the environment with a clear impact on human health, there is a group of endocrine-active substances mentioned before, the endocrine-disrupting chemicals (EDCs), which can interact directly or indirectly with the endocrine system and subsequently result in an effect on the endocrine system, organs, and tissues and may affect reproductive function. The term EDCs itself, coined at the Wingspread Conference in Wisconsin (in the USA) in 1991 [63], already refers to exogenous chemical entities or mixtures of compounds that are capable of interfering with or mimicking endogenous hormones and other signaling molecules of the endocrine system. Among these molecules that are able to interact with EDCs, the family of nuclear receptors (NRs) that includes orphan receptors (whose ligand is not known [64, 65]) such as steroid and xenobiotic receptors (SXRs) can recognize and bind many classes of EDCs [56], inducing endocrine responses and accordingly underlining the association between nuclear receptors and endocrine disruption coming from the external environment [66]. Orphan NRs family includes the estrogen receptor-related receptors (ERRs) [67] that share target genes, coregulators, and promoters [68, 69] with estrogen receptors (ERs), but contrast the classic ER-mediated estrogen-responsive signal [70, 71]. In fact, also EERs, similarly to SXRs, have the ability to bind EDCs [56, 72]. In addition, some EDCs have been reported to probably bind other crucial receptors involved in the hormonal signaling, like aryl hydrocarbon receptor (AhR) [73] and thyroid hormone receptor [74].

This area of investigation has grown in the last years introducing as EDCs a variety of substances [63], including xenoestrogen [75], environmental hormones [76, 77], hormonally actives agents [78], and environmental agents [79]. All these chemicals are categorized in classes and are integral part of the world economy and commerce. The global, social, and economic importance of EDCs is confirmed by the attention paid to them by various international organizations, such as the United States Environmental Protection Agency (USEPA) and the Organization for Economic Cooperation and Development (OECD) that have set up a task force to identify, prioritize, and validate test methods for the detection of endocrine disruptors [56, 80].

EDCs are categorized to different classes. For example, they can be first classified according to their endocrine effect (Fig. 3.1) that could be related to antiandrogenic, androgenic, estrogenic, or aryl hydrocarbon receptor agonists; inhibitors of steroid hormone synthesis; antithyroid substances; and retinoid agonists. In addition, endocrine disruptors can be classified based on their usage in agriculture and daily life: For example, pesticides (dichlorodiphenyltrichloroethane [DDT] and methoxychlor [MTX]), fungicides (vinclozolin), herbicides (atrazine), industrial chemicals (polychlorinated biphenyls [PCBs] and dioxins), plastics (phthalates,

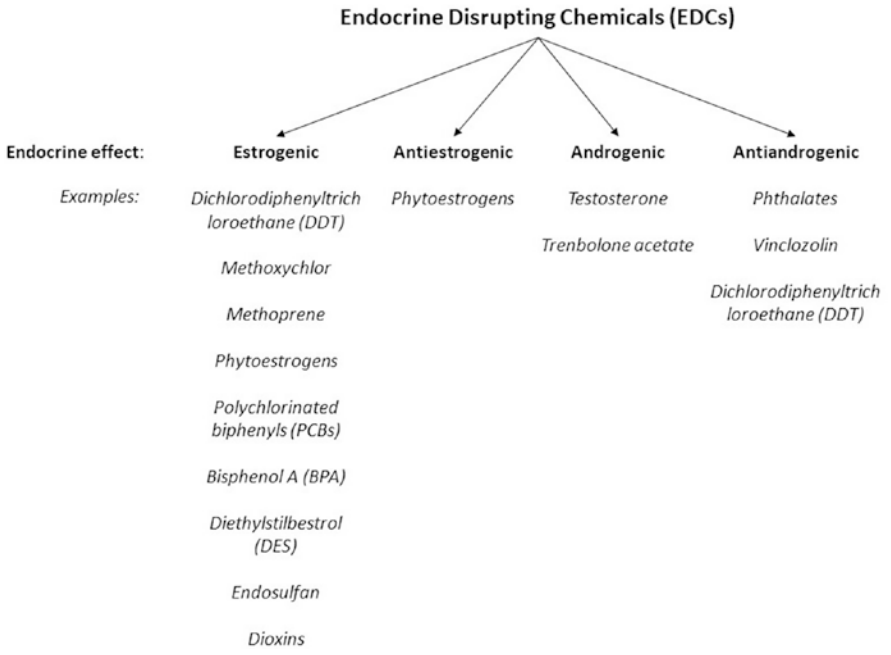

Fig. 3.1 Schematic representation of EDC classification based on their endocrine mechanisms

bisphenol A [BPA], and alkylphenols), and plant hormones (phytoestrogens). Lastly, some pharmaceuticals, personal care products, and nutraceuticals are also known as endocrine disruptors [81].

Considering pesticides, an increasing number of these substances have been recognized with androgen antagonist activity (antiandrogen), like dichlorodiphenyltrichloroethane (DDT) with its metabolites or other insecticides [82, 83]. Moreover, also linuron, another compound classified as an herbicide with a toxic effect specifically on human fertility, has been shown to compete with ligand for binding with the androgen receptor, resulting in the alteration of androgen-dependent gene expression [84, 85].

In addition to those synthetic EDCs already described, endocrine function can also be disrupted by chemicals originated in living organisms. Chemical compounds named phytochemicals or phytoestrogens are produced by plants and act as endogenous signals within the plant or are secreted for communications with other organisms, for example, to inhibit predatory herbivores [86]. Interestingly, phytoestrogens are isoflavones capable of binding to estrogen receptors alpha and beta (ERα and ERβ) and acting as weak agonists of estradiol [87, 88], partially exhibiting the estrogenic activity of the actual hormone [89, 90].

During the past decades, a particular attention has been given to the harmful effects of EDCs in the reproductive system as it has been reported that compounds with endocrine-disrupting mechanism of action can seriously affect human

reproduction with a negative influence on human fertility [91–94]. In fact, several studies have demonstrated a considerable decrease in fertility biomarkers, notably sperm counts, in human populations that have been exposed to EDCs [95–98]. For example, bisphenol A (BPA) has been shown to affect fertility in mouse model, [99] and studies on its effects and mechanisms are continuing. Fertility has also been shown to be affected by medical prescriptions and drugs, like diethylstilbestrol (DES), a synthetic drug whose effects were only detected in the offspring 20 years after its administration to pregnant women [100]. The synthetic estrogen DES, in fact, was inappropriately prescribed to pregnant women between 1940 and 1970 to prevent miscarriage, premature labor, and complications of pregnancy, but it has been identified as the trigger of a rare vaginal clear-cell adenocarcinoma [101]. Again, some of these drugs can also alter gonad quality and reduce subsequent fertility and effectiveness of reproduction [102].

Besides the direct effect of ECDs on the endocrine system, these compounds may also exert their harmful effects by inducing epigenetic changes in the genome, particularly when they act during critical periods of the ontogeny of the organism exposed [103, 104]. In this specific case, epigenetic modifications due to EDCs (Table 3.2) are capable of inducing adult onset diseases than can also be transmitted through multiple generations by the germ line [114]. Transgenerational epigenetic inheritance has been proposed to be mediated by DNA methylation, histone modifications, and specific miRNA expression [115, 116]. In practice, prenatal exposure to EDCs may affect human fertility altering primordial germ line differentiation and development, inducing transgenerational epigenetic disorders. However, in the early stage of development of mammals, uterus and placenta represent barriers against which external factors are strongly buffered in their concentration, but despite these important forms of protection during pregnancy, in some cases, EDCs can cross placental and brain barriers, interfering with normal embryo development and organ functions [63].

EDCs can reach the fetus through two principal ways: The first is via oviductal and uterine endometrial secretions [117] that together contribute to constitute the environment within the uterus where the embryogenesis happens. However,

Table 3.2 Epigenetic mechanisms induced by endocrine-disrupting chemicals on the human reproductive system and some of their effects

Epigenetic mechanism of EDCs	Adverse phenotypes
DNA methylation	• Decreased sperm quality and subfertility, decreased testes weight, and decreased testosterone and estradiol levels [105]; • Primary follicle depletion and inhibition of oocyte maturation [106–108]; • Abnormal testis development and increased spermatogenic cell apoptosis with decreased sperm concentration [109]; • Male infertility [109, 110]
Histone modifications	• Decreased sperm quality and testes testosterone [111]
Noncoding RNAs (miRNAs)	• Increased cell death and changes in cell function [112]; • Increased progesterone production [113]

maternal secretion of epithelial uterine steroids can be altered by endocrine disruptors acting on embryos even before its implantation, resulting in aberrant methylation in this latter [103]. The alteration of fetal methylation patterns, following the changes in preimplantation intrauterine environment, affects nonimprinted and imprinted genes too, as detected by Wu et al. [118]. The second way used by EDCs to reach the embryo is crossing placenta [119]. This possibility was reported after transplacental exposure to endocrine disruptors like 17α-ethinyl estradiol, bisphenol A, and genistein during the gestation days 11–20 in rat, which was associated with changes in several genes' expression.

3.3 Effect of EDC Epigenetics Modification on Gene Expression in Human Reproduction

EDCs could regulate gene expression in many different ways [120, 121], inducing alterations in DNA methylation patterns [122]. In fact, DNA methylation in key genes that occurs after EDC exposure can be followed by transcriptional changes, leading to cellular abnormalities that may cause functional perturbation of tissues or organs [104]. Barrett et al. first reported an association between EDC exposure and cell transformation [123], laying the bases to speculate, by applying the current knowledge of epigenetic mechanisms, that such transformations could be the result of an epigenetic process. This was also supported by the evidence that individuals exposed to a secondary environmental exposure presented an increased susceptibility in terms of aberrant DNA methylation, changes in the transcription of key genes, and the consequent tumorigenic processes [104]. The research group of Li et al. [124], after the neonatal administration of DES, observed abnormalities in the demethylation of the lactoferrin promoter. In addition, the administration to newborn mice of some phytoestrogens, such as coumestrol and equol, showed an increased methylation that implies the silencing of the proto-oncogene H-Ras [125]. Lastly, Day et al. [126] individuated alteration in methylation patterns in 8-week-old mice, caused by the consumption of genistein. The association between EDCs and methylation status of genes has been recently confirmed by new scientific findings, for example, the discovery that 2,3,7,8-tetrachlorodibenzodioxin (TCDD), DES, or polychlorinated biphenyl-153 (PCB153) influences DNMT activity in early embryos [127].

EDCs' epigenetic effect may also be due to histone modification. With regard to EDCs on histone acetylation, Hong et al. [128] revealed that the chemicals genistein and equol produce this kind of epigenetic modification through the stimulation of the histone acetyltransferase activity, mediated by either estrogen receptors alpha and beta (ERα and ERβ). Again, Singleton et al. [129] showed that treating breast cancer cells overexpressing ERα with bisphenol A (BPA) or estradiol leads to differential expression of a set of histone-related genes. Moreover, BPA upregulated histone H2B and downregulated histone H1, while it had no effect on histone

deacetylase, showing a completely opposite effect compared with estradiol [129]. Interestingly, from an epigenetic perspective, these histones have implications for chromatin condensation, which means gene silencing. Other findings about genetic expression regulation mediated by histone modifications demonstrate that gene silencing was associated with histone H3 trimethylation at lysine9 (H3K9me3) and with histone H3 acetylation at lysine 4 and di- or tri-lysine methylation (H3K4me2/3), and these were very common modifications related to changes in gene expression [130]. The gene expression alteration observed was due to the effect of these histone modifications, as well as others, on transcription regulation. However, no known histone code was related to the regulation processes mediated by hormones, and neither these modifications have been directly associated with EDCs.

In conclusion, changes in gene expressions due to epigenetics and without any modification in nucleotide sequence could be determined by both DNA methylation and chromatin state modifications. The epigenetic and epigenome regulation has been studied to identify the genes involved in the endocrine reproductive signaling and their relationship with emerging toxicants in the environment.

The theory that environmental factors can influence physiological phenotype and in particular the reproductive system was first derived from the observations of a wildlife biologist in the field [76], for example, the observation of reproductive dysfunction in many species (fish, birds, reptiles, mammals, etc.) living in areas contaminated with environmental toxicants [63, 131–133]. Then, chemical contaminant levels were increasingly being detected in humans in hormonally active tissues and in breast milk [57], which became a hot topic of discussion in the 1990s, highlighting the impact of environmental chemicals on human reproduction. After the discovery of the huge variety of EDCs, a growing body of literature confirmed the link between the increasing contamination in the environment and the parallel increasing incidence of breast cancer [134], decreasing sperm counts and increasing incidence of testicular cancer [135], which together had adverse effects including birth defects, reproductive failures, and sexual abnormalities. As the genetic background in human populations was essentially static, while disease disorders and infertility were dramatically increasing [136], it has been understood that environmental exposures must act primarily through epigenetic mechanisms to promote reproductive diseases [137, 138].

In fact, EDC exposure has the main effect of causing negative epigenetic changes, like alterations in DNA methylation or histone modification patterns, both inducing changes in normal gene expression that is associated with a wide range of diseases, including various reproductive disorders [139]. The reason for this type of EDCs' molecular mechanism of action on human health is that the majority of endocrine disruptors are not actually able to alter DNA sequence, but their action appears to be related to alterations in the epigenome, where they can affect normal reproductive physiological development and functions by acting as weak estrogenic, antiestrogenic, or antiandrogenic compounds. Females exposed to an excess of androgens early in gestation exhibit increased susceptibility to diseases such as polycystic ovaries in adult life [140], while, in adult male, perinatal or pubertal exposure to compounds such as estradiol and BPA alters the prostate epigenome [141].

Based on the findings supporting the close relationship between EDCs, epigenetic changes, and reproductive system, it is clear that the most vulnerable period for EDC exposure is embryogenesis, due to the high level of cell division characterized by specific epigenetic marks and critical modifications [142] that consequently can be transmitted over consecutive mitotic divisions and affect more cells than those occurring in adults during postnatal development. In addition, the placenta and its functions can be altered or influenced by the environment as well, which may result in pregnancy problems such as early pregnancy loss, preterm birth, intrauterine growth restriction (IUGR), congenital syndromes, and preeclampsia, which have all been linked again to epigenetic alterations [143].

Among the different epigenetic modifications induced by EDCs, DNA methylation is the most frequent and consequently the most studied one, due to its heritable nature, stability, and ease of measurement. Nevertheless, there are not yet many publications examining EDC effects on chromatin state, more precisely on chromatin condensation. However, DNA methylation has been studied extensively in particular in relation to reproductive biology, because the main methylation reprogramming occurs in germ cells formation (primordial germ cells) [48] and during the early stages of embryo development after fertilization [49]. Thus, the alteration of the methylation process has also been related to various disorders, such as those linked to imprinting [144], as mentioned above referring to Angelman syndrome and PWS. In these terms, a special attention has been paid, for example, to the chemicals vinclozolin and methoxychlor; the two pesticides that act as antiandrogenic endocrine and as estrogenic endocrine disruptors, respectively [145], reported to exert a specific effect on DNA methylation correlated to an aberrant phenotype of the reproductive tract.

3.4 Effect of EDC-Induced DNA Methylation on Male and Female Reproductive Tract

The endocrine-disrupting effects of many EDCs can be interpreted as interference with the normal regulation of reproductive processes by steroid hormones. Several evidences indicate that xenobiotics such as EDCs can bind to androgen and estrogen receptors on target tissues, to androgen-binding protein and to sex hormone–binding globulin [146]. Although environmental chemicals have a weak hormonal activity, their ability to interact with more than one steroid-sensitive pathway provides a mechanism by which their nature can be augmented. A given toxicant may be present in low concentration in the environment, and therefore, it can be harmless. However, we are not exposed to one toxicant at a time, but rather to all of the xenobiotics present in the environment. Therefore, numerous potential agonists/antagonists working together through several steroid-dependent signaling pathways could prove to be dangerous to human reproductive health.

During embryogenesis, the genital tract in males and females is first set up, but it is fully differentiated only after puberty, when sex hormone levels rise [147]. Normal physiology can be altered by EDCs exactly during the initiation of the functional activation of male/female reproductive system. Indeed, the most risky periods for xenobiotic exposure are represented by embryonic, neonatal, and pubertal periods, when the reproductive systems undergo to a finely tuned modulation by steroid hormones [76]. Physiological effects due to EDC exposure have been reported to occur in germ line in both males and females during the critical stages of development such as sex determination.

For example, embryo exposure to methoxychlor (MTX) or vinclozolin (Table 3.3) during sex determination period affects embryonic testis cellular composition and germ cell number and survival [109, 148]. In fact, the transient exposure to these EDCs can induce reprogram or imprint changes that show an effect in the adult reproductive physiology. The proof of concept that the effect observed on reproductive system was due to MTX or vinclozolin exposure came from the evidence that exposition of pregnant rats to both EDCs during the critical period for gonadal sex differentiation and testis morphogenesis (days 8–14 of pregnancy) produced transgenerational defects in spermatogenic capacity, which are transmitted through four generations (F1 to F4) [109]. This event was found to be due to an epigenetic mechanism involving altered DNA methylation that led to a permanent reprogramming of the male germ line. The causal effect of EDC involvement in reproductive tract morphogenesis derived from several findings demonstrates that a transient embryonic in utero exposure to an endocrine disruptor influences the embryonic testis transcriptome by epigenetic effects like DNA methylation. These epigenetic alterations resulted in abnormal testis development and in an increased adult spermatogenic cell apoptosis with decreased sperm concentration [109]. In addition to this alteration in the male reproductive tract, vinclozolin exposure has also been reported to induce transgenerational phenotypes in these animals, including adult onset diseases like male infertility [109, 110], increased frequencies of tumors, prostate disease, kidney diseases, and immune abnormalities [151]. Moreover, vinclozolin also induced changes in behavior and learning capacity [152–156], including transgenerational changes in mate preference [153] and anxiety behavior [156]. Transgenerational effects on tissue transcriptomes have also been observed. For example, in the embryonic testis transcriptome, a subset of genes presented a significantly altered expression in males from the F1 through the F3 generation, after vinclozolin exposure [165]. This transgenerational modified phenotype appears to be due to epigenetic changes, particularly due to alterations in DNA methylation of the male germ line [109, 166, 167]. After these first observations on MTX and vinclozolin, other agents that may promote transgenerational phenotypes associated with reproductive tract alterations have been identified.

In male testes, the expression of several genes is regulated via epigenetic modifications, underlining once again the direct influence of epigenetics on the process of spermatogenesis and how epigenetic aberrations (epimutations) can cause male infertility. Genes like MTHFR, PAX8, NTF3, SFN, HRAS, JHM2DA, IGF2, H19, RASGRF1, GTL2, PLAG1, D1RAS3, MEST, KCNQ1, LIT1, and SNRPN can be

Table 3.3 Some endocrine-disrupting chemicals and their effects of induced DNA methylation on male/female reproductive systems and germ line differentiation

Endocrine-disrupting chemicals	Class	Effect
Methoxychlor (MTX)	Pesticide, estrogenic	Transgenerational defects in spermatogenic capacity, increase in adult sperm cell apoptosis and decrease in concentration, abnormal testis development [109, 148], and aberrant folliculogenesis [149]
Vinclozolin (VCZ)	Fungicide, antiandrogenic	Transgenerational defects in spermatogenic capacity, increase in adult sperm cell apoptosis and decrease in concentration, abnormal testis development [109, 150], male infertility [109, 110], predisposition to tumors, prostate disease, kidney diseases and immune abnormalities [151], changes in behavior and learning capacity [152–156], transgenerational changes in mate preference [153] and anxiety behavior [156], and aberrant development of PGCs [150]
Bisphenol A (BPA)	Plastic, estrogenic	Female fertility problems, polycystic ovary syndrome and endometriosis, decrease in antral follicle counts, and decrease in oocytes number [157–159]
Genistein	Plant hormones, estrogenic/ antiestrogenic	Inhibition of oocytes maturation [108]
2,3,7,8-Tetrachlorodi benzodioxin (TCDD)	Dioxin, estrogenic	Aberrant folliculogenesis [149]
Phthalates	Plastic, antiandrogenic	Aberrant folliculogenesis [149]
Diethylstilbestrol (DES)	Drug, estrogenic	Predisposition to uterus epithelial tumors and to vaginal and cervical cancer, reproductive tract abnormalities [104], and susceptibility to tumor in testis and reproductive tract tissues [160]
Decitabine (5-aza-CdR)	Drug	Altered sperm morphology, decrease in sperm motility and capacity, and decrease in embryo survival [161, 162]
Chlorambucil	Drug	Transgenerational increase in deletions and other mutations in germ cells [163, 164]
Melphalan	Drug	Transgenerational increase in deletions and other mutations in germ cells [163, 164]

often hypermethylated by environmental toxins/drugs and lead to poor semen parameters and male infertility [36]. For example, the anticancer agent decitabine (5-aza-20-deoxycytidine or 5-aza-CdR) (Table 3.3) is able to reduce global DNA methylation [161, 162], causing altered sperm morphology, decreased sperm motility, decreased fertilization capacity, and decreased embryo survival, similar to the effect showed by the EDCs previously mentioned, methoxychlor and vinclozolin. Among all the endocrine toxicants that can induce aberration in the reproductive

tract, BPA has been reported to affect both male [168] and female [169] reproductive tracts.

Concerning EDC effect in female reproductive tract, beside BPA, other EDCs, such as genistein (Table 3.3), have shown to have an inhibitory effect on maturation of mammalian oocytes [108]. This evidence is crucial, since the main biological adverse effects of EDCs with regard to the development of female reproductive system are attributed to folliculogenesis [149]. The primordial follicles evolve to primary, preantral, and antral follicles. In particular, it has been reported that toxicity caused by EDCs to the antral follicles can lead to infertility. EDCs, such as BPA, MTX, TCDD, and phthalates (Table 3.3), can interfere with the development of the aforementioned types of follicles. For example, it was found that 3-month-old mice exposed in utero to 250 μg/kg BPA presented an increased percentage of ovarian tissue occupied by antral follicles. BPA has also been associated with female fertility problems, polycystic ovary syndrome, and endometriosis, whereas in women undergoing fertility treatments BPA levels have been associated with decreased antral follicle counts and a reduction in the number of oocytes [157–159].

Other findings reported the effects of female reproductive tract of perinatal exposure to diethylstilbestrol (DES) (Table 3.3). DES exhibits an estrogen agonist with an effect on the development of reproductive organs [37], supporting the epigenetic effect of DES exposure on the methylation pattern promoters controlling several estrogen-responsive genes associated with the development of reproductive tract. Perinatal DES exposure early in life has been found to increase predisposition to uterus epithelial tumors in adulthood, to several reproductive tract abnormalities, and to vaginal and cervical cancer risk in women [104]. Newbold et al. [160] administrated DES to pregnant rats during early postimplantation development and neonatal period, observing in males a grater susceptibility for tumor in rete testis and reproductive tract tissues in F1 and F2, due to epigenetic alterations like DNA methylation transmitted through the germ line.

Taken together, these evidences represent a clear example of how estrogenic xenobiotic exposure during a critical period of development can modify DNA sequence methylation status and consequently change the transcription of key genes involved in organ development, possibly increasing cancer risk later in life.

3.5 Effects of EDC-Induced DNA Methylation During Development and Germ Line Differentiation

In mammals, germ cell differentiation is initiated in the primordial germ cells (PGCs) during fetal development. PGCs are the embryonic precursors of the germ cell lineage (gametes, i.e., sperms and eggs), and their specification consists of global epigenetic reprograming, characterized by epigenetic phenomena such as the erasure of DNA methylation and histone modifications [170]. After the onset of gonadal sex determination, the PGC genome initiates the remethylation process of

DNA accompanied by remodeling of histone modifications in a sex-specific manner [170, 171]. Genetic and epigenetic changes during reprogramming of embryonic germ cell precursors make the prenatal period a sensitive window for potential adverse effects caused by environmental factors like EDCs [150]. In addition, the lack of any metabolic or excretion mechanism in the fetus highlights how much harmful chemicals can be if exposure occurs in this specific period of development.

However, the observation that PGCs and the developing germ line undergo major epigenetic programming, which can be transgenerationally altered by endocrine disruptors, was identified at first in 2015 [64].

During mammalian development, the primordial germ cells migrate down the genital ridge toward the newly formed gonad, prior to sex determination [172–174]. The germ cells develop into a male or female germ cell lineage at the initial stages of gonadal sex determination: The female germ line forms from oogenesis during follicle development that generate oocytes, and the male germ line, in turn, develops from spermatogonial stem cells and undergoes spermatogenesis, which originates spermatozoa in the testis. The critical period for epigenetic regulation of the germ line takes place during the phase of primordial germ cell migration and gonadal sex determination. Permanent alteration in the epigenetic programming of the germ line appears to be the mechanism involved in the transgenerational altered phenotype [109, 166, 167, 175].

Heritable damage can also occur in the zygote at the beginning of the embryonic development and can be transmitted to the next generation through modification occurred during germ line development [176]. Moreover, such heritable damage can be induced while germ line is developing. For example, chlorambucil and melphalan (Table 3.3) are able to induce a high frequency of heritable deletions and other mutations in mouse germ cells [163, 164], thereby producing a transgenerational mutation. Nevertheless, although some endogenous and exogenous agents are frequently associated with DNA mutations and transgenerational transmission, chemically induced epigenetic modifications of DNA may have the same net effect on the phenotype of newly altered cells and on their progeny [177]. Regarding this, Holliday [178] reported that teratogens could target mechanisms that control patterns of DNA methylation on genome of developing embryos, modifying methylation patterns that will be present on somatic cells, leading to a developmental alteration and subsequently to changes in germ line cells.

Modifications transmitted through germ line cells that occurred during the process of differentiation have been studied by Anway et al. [109], as already mentioned earlier. Later, the authors also detected 25 different genes that had altered methylation patterns in the F1 born to mothers subjected to the vinclozolin administration (Table 3.3) [109]. Therefore, the exposure of a gestating mother to EDC during critical periods of sex differentiation and testis morphogenesis triggers to decreased spermatogenic capacity and sperm viability that was transgenerationally transmitted in the male. This alteration appears to be associated with altered DNA methylation of the germ line [179].

Another evidence of ECDs' implication on germ line methylation alteration came from Brieno-Enriquez et al. [150]. In this study, gestating female mice were

exposed to vinclozolin (Table 3.3) with the aim to produce epigenetic transgenerational inheritance of testicular cell apoptosis and abnormalities. Then, the observations were extended to PGCs, allowing the identification of alterations in epigenetic programming and gene expression that were critical for PGC development (such as those in Blimp1) that promoted epigenetic PGC noncoding RNA programming. In fact, Blimp-1 pathway plays a critical role in determining DNA methylation reprogramming and gene expression alterations that occur during normal development of PGCs and the subsequent germ line, providing a major resource for epigenetic alterations during the development of the human germ line epigenome [180]. The importance of epigenomic control in PGCs was confirmed by the identification of specific DNA methylation sites that escaped DNA methylation erasure in PGCs specification, termed "escapees," supporting a role for altered germ line DNA methylation in epigenetic transgenerational inheritance [180].

3.6 Final Considerations

Epigenetics, first described by Waddington [1], involves all those molecular mechanisms that are able to modulate genome expression without modifying DNA nucleotide sequences. Among the main epigenetic processes, we can mention DNA methylation [10, 11], the action of small noncoding RNA like miRNA and siRNA [13–16], and a large variety of histone modifications [17]. In fact, in eukaryotic organism genome is compacted by basic proteins named histones that allow the organization of DNA into chromatin [17], whose conformation influences gene expression and can be modulated by these epigenetic mechanisms, which have the ability to induce chromatin condensation and/or chromatin relaxation to respectively silence and/or activate gene expression (Table 3.1). Because of their effect on gene expression related to the chromatin conformation (condensed or uncondensed), epigenetic modifications take part in many human biological processes, mostly involving reproductive system, and in some genetic diseases [45]. Their action appears to be crucial during embryo development, from gametogenesis to organogenesis, controlling gene expression to guarantee the correct development of tissues and organs [41, 44]. During oogenesis [27], epigenetics allows the establishment of the physiological epigenome and the gaining of meiotic and developmental potential of the oocyte via chromatin modifications. Differential methylation patterns are established during gametogenesis [31–33], and the processes of DNA methylation at specific loci during oogenesis have been investigated. During spermatogenesis, the expression of many genes in the testes is controlled in the same way by epigenetic mechanisms, like DNA methylation, histone modifications, and chromatin remodeling. Testicular DNA has a unique pattern of methylation established before meiosis [35], that is, much more hypomethylated than somatic tissues. Therefore, it is clear that methylation pattern and the consequent methylation phenomena occurring during both male and female germ line development are crucial [34].

Epigenetics is responsible not only for proper regulation of gametogenesis (oogenesis and spermatogenesis), but also for cellular specialization during embryo development [41]. In fact, cells of the early embryo must be specialized from totipotency to a specific cell population, depending on lineage-specific pattern of gene expression based on modifications of chromatin structure and function. During the entire embryo development, there are two major rounds of epigenetic reprogramming [48, 49]: a global DNA methylation loss in cells of the early embryo [49], followed instead by a process of methylation during implantation [50]. These events of reprogramming can be influenced by external stresses and represent a period of increased sensitivity of the embryo toward potential environmental toxicants, because this latter can transmit the alteration resulting from the stresses through the germ line, with the consequence of abnormal developmental outcomes in adulthood [54, 55].

Nowadays, it is well known that environmental factors have a significant impact on biology [57] since they have the capability to modulate biological systems and to influence physiology, even promoting disease states. Among all the different toxicants, there is a group of growing interest which is known as endocrine-disrupting chemicals (EDCs) that act on human epigenome without modifying DNA sequence. As the name itself suggests (coined in 1991 by Colborn T. [63]), endocrine disruptors are a set of endocrine-active substances that can interfere with human endocrine system mimicking endogenous hormones and other signaling molecules and affect the reproductive system [57]. EDCs can be classified according to their endocrine effect as shown in Fig. 3.1, and they can be found in a huge variety of sources. Therefore, EDCs may influence human health in two principal ways: acting directly on endocrine system as hormonal agonists/antagonists binding with hormonal receptors and inducing epigenetic changes in the genome. In fact, exposure to toxicants during critical periods of the ontogeny of the organism exposed [103, 104] can lead to epigenetic modification even transmissible through offspring by the germ line (transgenerational epigenetic disorders) [114].

After the first observations of how much environmental factors were linked to reproductive dysfunction in many species in wildlife [63, 76, 131–133], during the past decades, it has been reported that endocrine chemicals can seriously affect human reproduction (Table 3.2) [95–98], with a negative influence on fertility [91–94]. In human reproduction, EDCs may change gene expression through DNA methylation and histone modifications of key genes, both acting through the alteration of chromatin state, and it is associated with a wide range of diseases like various reproductive disorders [139].

However, between all the epigenetic mechanisms induced by EDCs, DNA methylation is the most frequent and consequently the most studied one, because of its heritable nature and stability. Normal physiology and subsequent phenotype can be altered by EDCs, and the most sensitive periods for exposure are represented by embryonic, neonatal, and pubertal periods, when there is a finely tuned modulation of hormones [76]. For example, the exposition of pregnant rats to chemicals, such as methoxychlor or vinclozolin (Table 3.3), during the critical period for gonadal sex differentiation and testis morphogenesis, produced transgenerational defects in

spermatogenic capacity [109] due to the alteration of DNA methylation in male germ line. Therefore, embryonic testis transcriptome is influenced by DNA methylation, resulting in abnormal reproductive tract morphogenesis and in a general loss of gametes [109]. After these first observations, other agents that may promote transgenerational phenotypes associated with reproductive tract alterations have been identified, like bisphenol A (Table 3.3), which has been reported to affect both male [168] and female [169] reproductive tracts. The main biological adverse effects of EDCs concerning female reproductive system are attributed to folliculogenesis [149]. Besides BPA; MXC; 2,3,7,8-tetrachlorodibenzodioxin; and phthalates (Table 3.3), the chemical genistein, for example, has shown to have an inhibitory effect on maturation of mammalian oocytes [108], [157–159]. Diethylstilbestrol (Table 3.3) exposure early in life is instead associated with several reproductive tract abnormalities and increased vaginal and cervical cancer risk in women [104], once again due to the alteration of methylation pattern controlling several estrogen-responsive genes.

In humans, germ cell differentiation is initiated in PGCs that are the embryonic precursors of the germ cell lineage. Their specification is characterized by a global epigenetic reprogramming [170], involving erasure of DNA methylation and histone modifications in a sex-specific manner [170, 171], which makes prenatal period a particular sensitive window for the harmful effects of EDCs [150]. Alteration in the epigenetic programming of the germ line appears to be the mechanism involved in the transgenerational altered phenotype [109, 166, 167, 175]. Holliday [178] reported the association between teratogens and modification of DNA methylation pattern in particular genomic regions of developing embryos, leading to a developmental alteration and thus to changes in germ line cells. Anway et al. [109] showed that the exposure of a gestating mother rat to vinclozolin or methoxychlor (Table 3.3) during the critical periods was related to transgenerational defects in the spermatogenic capacity as the result of altered DNA methylation of the germ line [179]. Again, the exposure of gestating female mice to vinclozolin (Table 3.3) promotes epigenetic transgenerational inheritance of abnormalities in male reproductive tract [150], confirming that PGC alterations in epigenetic programming and gene expression were critical for their development [180].

Therefore, we can assume that epigenetics could represent an innovative frontier of scientific investigation to identify the molecular basis of alterations of normal epigenome during the sensitive periods of embryo and germinal line development associated with diseases in adulthood. The increase of pollutants in the environment, including EDCs, with their direct effect on human endocrine and reproductive systems, as well as their epigenetic mechanisms of action that induce the abovementioned aberrant phenotypes and diseases, represents a growing health concern that underlines the need of decreasing the release of contaminants in the environment and searching for new therapies acting through epigenetic mechanisms too.

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