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Endocrine disruptors, gene deregulation and male germ cell tumors

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ABSTRACT Endocrine disruptors (EDs) belong to a large group of compounds, usually present as environmental pollutants, which can alter the homeostasis of living organisms by modifying hormonal balance and changing the normal patterns of gene regulation during development and cell differentiation. Hence, the development of male gonads and their functionality may be affected by exposure to specific EDs or their mixtures. The molecular mechanisms of action of these reprotoxicants leading to pathologies of the reproductive system such as testicular cancer, are complex and not well characterized. It is likely, however, that these compounds alter the interaction between the mechanisms of gene regulation and functional gene networks in windows of risk, mainly during embryonic development. Moreover, such changes could be transmitted through generations by epigenetic mechanisms. There are examples of the action of EDs on the expression of mRNAs, small non-coding RNAs and epigenetic marks in the developing testis associated with cellular and molecular alterations found in germ cell tumors. In the present review, we will discuss various aspects of genetic, transcriptomic and epigenetic changes related to testicular development, exposure to EDs and the occurrence of germ cell tumors.

KEY WORDS: *testis, tumor, primordial germ cell, miRNA, piRNA, epigenetic, DNA methylation, histone modification*

Introduction

Testis development involves a series of processes of differentiation from early embryogenesis, mainly based in mammals on genetic determinants of male on chromosome Y. Mechanisms of germ and Sertoli cell differentiation, lead to the formation of seminiferous tubules that along with the intertubular Leydig cells form the cellular network of spermatogenesis. Autocrine, paracrine and endocrine regulations and precise specific gene expression allow setting the appearance of male characters and the continuous production of sperm in adult life (Brennan and Capel, 2004; Park and Jameson, 2005).

Spermatogenesis involves complex processes of proliferation, differentiation, cell interactions and morphogenetic changes to produce highly differentiated and haploid cells such as in spermatozoa. Although the time course of cell differentiation can be very variable among different mammals, basic traits of spermatogenesis at the cellular and molecular levels, as well as the developmental progression are comparable; this enables predictions using animal models of study. The exclusive nature of some of these processes requires developmentally orchestrated control of gene expression. It has been estimated that about 4% of the mouse genome, representing above 2300 genes, is specifically expressed in testis (Schultz *et al.*, 2003). In addition, alternative forms of post-transcriptional

Abbreviations used in this paper: BPA, bisphenol A; CIS, carcinoma *in situ*; DEHP di-(2-ethylhexyl) phthalate; dpc, days *post coitum*; E2, 17beta-estradiol; EC, embryonal carcinoma; EG, embryonal germ; ED, endocrine disruptor; ES, embryonal stem; HR, hormone receptor; MEHP, mono-(2-ethylhexyl) phthalate; miRNA, microRNA; NSGCT, non-seminoma germ cell tumor; PCB, polychlorinated biphenyl; PGC, primordial germ cell; piRNA, piwi-interacting RNA; POP, persistent organochlorine pesticide; SSC, spermatogonial stem cell; TDS, testicular dysgenesis syndrome; TE, transposable elements; TGCT, testicular germ cell tumor; UTR untranslated region; ZEA, zearalenone.

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regulation of gene expression such as alternative splicing (Yeo *et al.*, 2004), different polyadenilation sites and thus 3'-UTRs (Parraga and del Mazo, 2000; Wang *et al.*, 2006), and differences in the length of poly(A) tails of mRNAs (de Luis and del Mazo, 1998), are frequent in testicular germ cell differentiation.

The recent developments in the "world of small RNAs" are causing a revolution in our knowledge of gene regulation. RNA interference mediated by small non-coding RNAs is a powerful mechanism in the control of gene expression associated with cell differentiation and pathologies. In germ cells, three types of endogenous small RNAs, microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) (initially considered specific of germ cells) and endogenous small interfering RNAs (endo-siRNAs), with cell-type regulated biogenesis (Gonzalez-Gonzalez *et al.*, 2008; Garcia-Lopez and del Mazo, 2012), are being studied to determine their role in the differentiation of germ cells and its pathologies, including infertility and testicular cancers (He *et al.*, 2009).

The cells and biological systems can be affected by environmental contaminants through alterations of genetic systems, by multiple mechanisms of action also interacting with the genetic backgrounds (Edwards and Myers, 2007). The adverse effect of environmental pollutants on reproductive health is well documented (Woodruff *et al.*, 2008). An important and extensive group of chemical contaminants, widespread in the environment, is called endocrine disruptors (EDs). These are wide groups of chemical compounds such as: pesticides and herbicides, organic compounds, pharmacological substances, heavy metals, persistent organic pollutants, air contaminants that interfere with endocrine pathways and can induce gene deregulation in exposed organisms. To date, hundreds of compounds considered EDs and thousands of others are suspected of having similar properties have been identified. The testis has been clearly identified as a significant target for the deleterious action of these environmental toxicants. This knowledge generates worldwide concern as to consequences for reproductive health both in humans and wild species (Colborn and Clement, 1992). The causal relationship between endocrine disruption by environmental chemicals and disorders such as cancer and infertility has been the subject of numerous scientific reports. A classical, albeit contested, report (Toppari *et al.*, 1996) reached the conclusion that there has been a general, although not necessarily global decline in semen quality during the last fifty years as was previously reported (Carlsen *et al.*, 1992). The report also showed that 2-4% of the annual increase in testicular cancer was diagnosed in men under 50 years old in Britain, Scandinavia, Australia and the USA. Epidemiological and experimental data indicate that developmental exposure to EDs could induce, at the level of male gonad development and reproduction, the so-called "Testicular Dysgenesis Syndrome" (TDS) (Sharpe and Skakkebaek, 1993; Skakkebaek *et al.*, 2001). TDS includes four clinical and etiologically related traits: hypospadias, cryptorchidism, low sperm counts and testicular tumors (Asklund *et al.*, 2004). The impact of EDs, however, is still unclear due to the wide range of possible mechanisms for ED action, levels of ED exposure, mixture of chemicals potentially acting as EDs and the genetic sensibility of individuals or populations to the compounds.

The effects in germ cells not only may affect the exposed individual but can also be inherited and potentially influence the phenotype of subsequent generations by epigenetic mechanisms. The term "epigenetic", introduced by C.H. Waddington (1905-75) to describe interactions of genes with their environment (Waddington, 1942), is now used to define heritable changes in gene expression that are not coded in the DNA sequence. Main epigenetic mechanisms include DNA methylation and histone modifications (Kim *et al.*, 2009). More recently, additional mechanisms have been identified, among them microRNAs (miRNAs) can act on the epigenetic machinery, and in turn miRNA expression can also be controlled by epigenetic mechanisms (Sato *et al.*, 2011). The epigenetic patterns can be transmitted to the daughter cell, and possibly also through generations. In turn, deregulation of miRNAs can affect the regulation of expression of mRNA targets, generating complex mechanisms of alterations with pathological consequences in testis development and function.

Developmental origin of testicular germ cell tumors

Testicular germ cell tumors (TGCTs) are the most common solid cancers in men aged 15–40 years in developed countries (Huyghe *et al.*, 2003; Hussain *et al.*, 2008), constituting 2% of all human malignancies (Richiardi *et al.*, 2004; Motzer *et al.*, 2012) and representing the most frequent cause of death as a consequence of solid tumors in this age group (Oosterhuis and Looijenga, 2005). Germ cell tumors represent about 95% of malignant tumors arising in the testes whereas only 1-5% of the testicular cancers have their origin on somatic components of the testis: Sertoli and Leydig cells (Mostofi, 1973; Giglio *et al.*, 2003). During the last five decades the prevalence of these tumors increased 2-3 times and each year about 9,000 new cases of TGCTs are diagnosed in USA (Siegel *et al.*, 2012).

TGCTs consist of a heterogeneous group of neoplasms, established at different anatomical locations in the testis. Simplistically, TGCTs can be classified in seminomas (SGCTs) and non-seminomas (NSGCTs). Seminomas have features of the primordial germ cells (PGCs) or gonocytes, the ancestors of spermatogonia while non-seminomas include mixed germ cell tumors (the most common), embryonal carcinoma, teratoma, choriocarcinoma, and yolk sac tumors.

The fetal origin of some types of TGCTs is widely supported (Rajpert-De Meyts *et al.*, 1998; Looijenga *et al.*, 2007b; Kristensen *et al.*, 2008; Wohlfahrt-Veje *et al.*, 2009). Due to their physiological and genetic similarities, PGCs are considered the cell origin of the TGCTs (Rajpert-De Meyts, 2006). Indeed, seminomas have clear features of PGCs (see below) (Jiang and Nadeau, 2001; Oosterhuis and Looijenga, 2005; Gilbert *et al.*, 2011). Both seminomas and teratocarcinomas derived from abnormal germ cells that initiate within the seminiferous epithelium as carcinoma *in situ* (Skakkebaek, 1972). Carcinoma *in situ* and embryonic stem cell display similar profiles of gene expression (Almstrup *et al.*, 2004).

Although controversial (Vidaeff and Sever, 2005), epidemiological studies in humans associate the exposure to different environmental toxicants with the development of TGCTs. Epidemiological studies support the hypothesis that testicular cancer is associated with exposure to some EDs, at least to estrogens (Storgaard *et al.*, 2006), during the fetal or early postnatal life. Exposures to persistent organochlorine pesticides (POPs) have been associated to risk increase of seminomas and non-seminomas testicular cancers (McGlynn *et al.*, 2008). However, no association with exposure to polychlorinated biphenyls (PCBs) (even, inverted association) was detected (McGlynn *et al.*, 2009), speculatively explained by the

wide range of effects of the different PCBs and mixes (estrogenic, antiestrogenic, androgenic, and antiandrogenic).

The Sertoli cells as somatic components of the seminiferous epithelium play a pivotal role in the development of a functional testis, and as consequence in the development of the male phenotype (Skinner and Griswold, 2005). Their direct interaction with male germ cells into the seminiferous epithelium is crucial during spermatogenesis. Disorders in the correct maturation or numbers of Sertoli cells thus may be the cause of male reproductive disorders (reviewed by Sharpe *et al.*, 2003). Similarly, the Leydig cell represents a crucial element of testis functions, basically through the secretion of androgens (Payne *et al.*, 1996). The effects of EDs on such cells are considered of special relevance due to hormonal regulation of Sertoli and Leydig cells functions and by the hypothesis that the TDS could be mainly caused by functional disorders in Sertoli and Leydig cells (Sharpe *et al.*, 2003). However, dysregulation of gene expression in these cell types as consequence of ED exposures seems to cause dysfunctions in traits of TDS other than testicular cancer. Therefore, Sertoli or Leydig cells tumors will not be discussed in this review.

Genetic background of testicular germ cell tumors

From the genetic point of view, chromosomal unbalance is usually found in diverse forms of TGCT. The most common cytogenetic alteration is the amplification of human chromosome 12p region (Looijenga *et al.*, 2003b; Rodriguez *et al.*, 2003; Zafarana *et al.*, 2003; von Eyben, 2004). Different TGCTs show particular chromosome abnormality pattern. While in non-seminoma germ cell tumors gain of proximal 17q and loss of 10q have been usually detected (Mohamed *et al.*, 2012), seminomas show high-level amplification of 12p (Zafarana *et al.*, 2003; Mohamed *et al.*, 2012). Interestingly, this chromosome region contains multiple genes postulated to be involved in TGCT, such as *CCND2*, *STELLAR* and *NANOG*, expressing in PGCs and playing important roles in stem cell maintenance (Houldsworth *et al.*, 2006).

Compared to chromosomal abnormalities, specific gene mutations in TGCT are less frequent. The most frequent single genes affected in TGCTs are: *KIT*, *TP53*, *K-RAS*, *N-RAS*, and *B-RAF* (reviewed bySheikine *et al.*, 2012). Genetic susceptibility to TGCTs conditioning the familial testicular germ cell tumors has been established, confirming mutations or single nucleotide polymorphisms (SNPs) affecting some genes involved in the normal germ cell differentiation such as *KITLG, SPRY4*, *PDE11A* and *BAK1* (Greene *et al.*, 2010).

Genetic susceptibility to TGCTs by exposure to some types of EDs is currently being studied more intensively. Genetic polymorphism in humans, such as those found on hormone-metabolizing genes including *CYP17A1* and *HSD17B1*, can also modified the association between TGCT risk and exposure to specific EDs such as POPs (Chia *et al.*, 2010). The combination of genotyping and transcriptome analysis (named *genetical genomics*) could differentiate the genetic from the environmental components of gene expression variations (Gibson, 2008). After genome-wide association (GWA) studies, new approaches based on gene–environment (G×E) interactions methodology (Thomas, 2010; van Ijzendoorn *et al.*, 2011) will probably contribute to clarify the complex pathways involving multiple genes and exposures.

Comparing gene expression in PGCs and testicular germ cell neoplasias

In mammals, PGCs are the embryonic precursors of both female and male germ cells (De Felici, 2001). As we are going to discuss below, in the mouse embryo, PGCs are determined in the extraembryonic mesoderm of the yolk sac wall around the gastrulation period (6.5 days post coitum, dpc; and around the third week of gestation in humans) (McLaren, 2003; Saitou *et al.*, 2003; De Felici, 2012). From this region, they migrate within the developing gonadal ridges where they differentiate into female or male germ cells. Within the fetal testis, PGCs after a proliferation period exit temporarily from the cell cycle and are arrested in G0. Such quiescent cells included inside seminiferous cords are now called prespermatogonia or gonocytes. The sexual dimorphism of the mouse gonads is evident at 12.5-13.5 dpc (Fig. 1). Spermatogonial stem cells (SSCs) of the newborn and prepuberal testis derive from gonocytes although the processes of their differentiation from these cells are unknown. SSCs can self-renew and generate a large number of differentiated germ cells. In particular, they give rise to type A spermatogonia which after some rounds of proliferation differentiate into type B spermatogonia; these latter entering into meiosis become spermatocytes. After meiotic divisions, haploid spermatids are produced, suffering morphogenetic changes until their subsequent differentiation into spermatozoa.

The transcriptional repressor BLIMP1 (B-lymphocyte-induced maturation protein 1) participates in the initial specification of PGCs repressing their somatic program (Saitou *et al.*, 2003; McLaren and Lawson, 2005; Ohinata *et al.*, 2005; Vincent *et al.*, 2005). Subsequently, PGCs move from the proximal epiblast to the extraembryonic mesoderm of the yolk sac wall and then through the hindgut and dorsal mesentery to the gonadal ridges (11.5 dpc in mice and the 6th week in humans) (Godin *et al.*, 1991; Wylie, 1993; Donovan, 1994; Runyan *et al.*, 2006; Sheikine *et al.*, 2012).

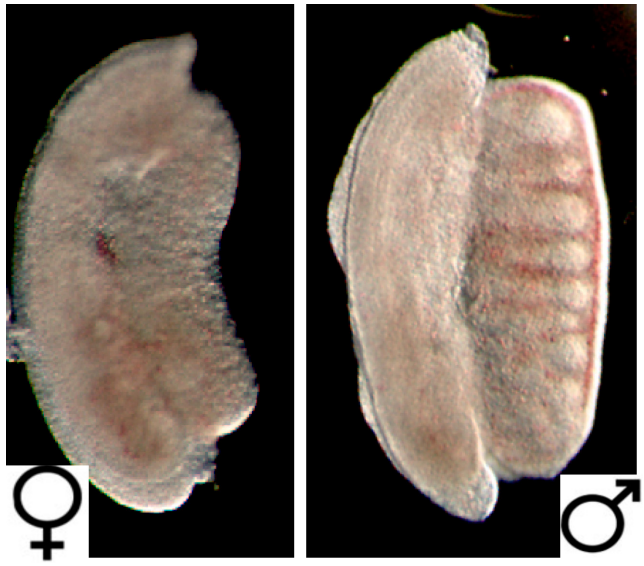

Fig. 1. In mice, the sexual dimorphism of the fetal gonads is clearly evident at 13.5 dpc. *The mesonephros, with an important role in sexual differentiation, is associated with the left of each gonad.*

During this period, PGC development is regulated by a variety of growth factors and cytokines including BMPs (bone morphogenic proteins), Kit ligand (KITLG), also known as the stem cell factor (SCF), and SDF1 (stromal cell derived-1) (for a review, see (De Felici and Farini, 2012). PGCs and gonocytes express several markers typical of stem cells such as *TNAP* (tissue not specific alkaline phosphatase), *NANOG*, *ESG1*, *SOX2*, *POU5F1* (also known as *OCT3/4*), and *SALL4* (Saitou *et al.*, 2002; Saitou *et al.*, 2003; Gashaw *et al.*, 2007; Niwa, 2007), as well as germ cell specific such as *MVH*, *NOBOX*, *DAZL*, *NANOS3* (reviewed by De Felici, 2009). PGCs and in part gonocytes have their original genomic imprinting pattern erased, which allows development of gender-specific germ cell lineages. PGCs maintain intrinsic pluripotency up to their differentiation into oocytes and gonocytes as shown by their capability to produce teratomas/teratocarcinoma or embryonal germ (EG) cells (for a review, see(Donovan and de Miguel, 2003).

It is likely that at least in part PGC intrinsic pluripotency depends on the methylation status of imprinted genes such as *H19*, *IGF2*, *IGF2R* and *SNRPM* (Szabo and Mann, 1995). The loss of imprinting in PGCs could be considered as one of the pathways implied in their differentiation and loss of pluripotency. In the presence of the Y chromosome, the gonadal stromal cells of the gonadal ridges express the transcription factor *SRY* and its target gene *SOX9* driving Sertoli cell differentiation (Polanco and Koopman, 2007). The Sertoli cells create a microenvironment that allows differentiation of gonocytes into SSCs. During the differentiation process, germ cells gradually lose expression of *NANOG*, *PLAP*, and *POU5F1*, partially of *KIT* and *SALL4*, and acquire expression of other genes including *MAGE4A*, and *TSPY* (Cao *et al.*, 2009). Gene signature of deregulation in TGCTs respect to normal male germ cells has been reported based on array analysis in different histological entities of TGCTs (Okada *et al.*, 2003; Gashaw *et al.*, 2005). These studies have associated patterns of deregulation of specific genes as potential molecular markers of gene expression that define types of TGCTs and prognosis of such tumors (Gashaw *et al.*, 2005). One feature that could define the fetal origin of TGCTs is the similarity of the patterns of gene expression between PGCs/gonocytes and neoplasic cells of testicular tumors (Almstrup *et al.*, 2004). Analysis by cDNA microarrays defined expression profile of 895 genes that were upregulated in human embryonal stem (ES) cells and carcinoma lines respect to the controls, showing highest expression the *OCT3/4* gene and confirming the hypothesis that human seminomas most closely resemble transformed PGCs (Sperger *et al.*, 2003). Interestingly, high representations of deregulated genes are localized on the 12p chromosome region.

In humans, seminomas, embryonal carcinoma, carcinoma *in situ* (CIS) and yolk sac tumors show expression of *OCT3/4* (Palumbo *et al.*, 2002; Looijenga *et al.*, 2003a; de Jong *et al.*, 2005; Richie, 2005; Jung *et al.*, 2006), *NANOG* (Hart *et al.*, 2005; Hoei-Hansen *et al.*, 2005) and *LIN28* (West *et al.*, 2009; Gillis *et al.*, 2011) which are not detectable in either normal testicular tissue or in teratomas. During development of human germ cell, the genes *OCT3/4*, *NANOG* and *LIN28* are expressed in PGCs and gonocytes being *LIN28* extended to prespermatogonia (Yeom *et al.*, 1996; Brehm *et al.*, 1998; Gillis *et al.*, 2011), amongst others involved in pluripotency (Nichols *et al.*, 1998). In mouse, LIN28 regulates the expression of members of the family of miRNA: *miR-let-7* (Hagan *et al.*, 2009), which potentially regulates other multiple potential mRNA targets. The regulation of specific gene expression during early development is also mediated by DNA methylation as occurs in the expression of *OCT3/4* (Gidekel and Bergman, 2002) whose expression levels appear a crucial key in the neoplasic process (Gidekel *et al.*, 2003). Other functional markers of gene expression in TGCTs common to early differentiated germ cells and CIS, are *PLAP* (Hustin *et al.*, 1987) and *c-KIT* (Rajpert-De Meyts and Skakkebaek, 1994; Strohmeyer *et al.*, 1995; Honecker *et al.*, 2004; Motzer *et al.*, 2012).

In contrast to other cancers, there are few animal models of TGCTs. In mouse, the only strain that developed TGCTs, and which also proved to originate from PGCs is the 129/SvJ strain, discovered by more than 50 years ago by Stevens (Stevens and Little, 1954; Stevens and Hummel, 1957; Stevens, 1962; Stevens, 1964; Stevens, 1967; Stevens, 1973; Stevens, 1984). More recently, it has identified mutations in the mouse homologous gene of zebrafish (Weidinger *et al.*, 2003): dead end homolog 1 (*Dnd1*) gene as an inducer of this phenotype (Matin and Nadeau, 2005; Youngren *et al.*, 2005). *Dnd1* encodes a protein participating in the "editosome" with activity of RNA-binding protein (Youngren *et al.*, 2005). RNA editing is a post transcriptional key regulator of gene expression (Keegan *et al.*, 2001) that generate alternative RNAs including mRNAs and double stranded RNAs such as are the miRNAs precursors (Nishikura, 2010). Recently, it has been reported the binding of Dnd1 to transcripts encoding negative regulators of the cell-cycle, involved in developing male germ cell mitotic arrest (Western *et al.*, 2008), such as: *p21Cip1*, *p27Kip1*, *Lats2*, *pRB*, *p53* and *Pten* promoting translation regulation (Cook *et al.*, 2011) as was previously suggested (Western, 2009). Experimental deletion of *Pten* in PGCs drives to testicular teratomas and abnormal germ cell proliferation (Kimura *et al.*, 2003; Moe-Behrens *et al.*, 2003). It has been proposed that a regulatory mechanism of Dnd1 is based on the protection of specific transcripts, as those mentioned, to the negative post-transcriptional regulation of miRNAs. In this view, the binding of the transcripts to 3' UTR regions protects for the binding and activity of miRNA competing for the same mRNA regions, as was observed in zebrafish where Dnd1 protein is able to protect *Nanos* from the negative translation regulatory action of miR-430 during PGC development (Kedde *et al.*, 2007). Combining both potential activities of Dnd1, a dual action of Dnd1 can be hypothesized: modification the sequence of miRNAs in their biogenesis and therefore divert them from their initial target, or alternatively competition with the target miRNAs. Consequently, complex regulatory mechanisms at postranscriptional level could also be involved in the genetic program of germ cells development in the embryo being their unbalance involved in testicular tumorogenesis.

Effects of endocrine disruptors on gene expression in PGCs and developing testis

Studies of transcriptome changes in testicular cells after chemical exposure have been carried out in mouse models using both *in vitro* and *in vivo* approaches. *In vitro* analysis allows to define changes of gene expression in specific cell types. Yet, the *in vitro* approaches create a handicap due to the removal in isolating cells from their natural physiological conditions. This method also is valid for cell types present in the precise time frame during testis development such as PGCs. For example, the level of AKT kinase activity, that is induce by phosphorylation of the KIT-ligand, crucial for PGC survival/proliferation, significantly decreased in mouse PGCs exposed to lindane (gamma-HCH) *in vitro* along with the

TABLE 1

GENES DEREGULATED IN PGCs AFTER *IN VITRO* EXPOSURE TO DIFFERENT TOXICANTS

Compound	Gene Symbol	Gene Name	Regulation
MEPH	*Rpl8*	ribosomal protein L8	UP
MEPH	*Rpl24*	ribosomal protein L24	UP
MEPH	*mt-Nd1*	ND1 NADH dehydrogenase subunit 1	DOWN
MEPH	*Rpl10a*	ribosomal protein L10A	DOWN
MEPH	*Eif2s2*	Eukaryotic translation initiation factor 2, subunit 2 (beta)	DOWN
MEPH	*Fau*	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived)	DOWN
MEPH	*mt-Co3*	Cytochrome Oxidase III, COX3	DOWN
ENU	*Rplp1*	Ribosomal protein, large, P1	UP
ENU	*Rpl31*	ribosomal protein L31	UP
ENU	*Rpl28*	ribosomal protein L28	UP
ENU	*Rpl35*	ribosomal protein L35	UP
ENU	*Rps17*	ribosomal protein S17	UP
ENU	*mt-Co3*	Cytochrome Oxidase III, COX3	DOWN
ENU	*Rps8*	ribosomal protein S8	DOWN
ENU	*mt-Co1*	cytochrome c oxidase subunit I	DOWN
ENU	*mt-Nd2*	NADH dehydrogenase subunit 2, mitochondrial	DOWN
ENU	*Sod1*	superoxide dismutase 1, soluble	DOWN
ADR	*Hbb-Y*	hemoglobin Y, beta-like embryonic chain	UP
ADR	*Rpl41*	ribosomal protein L41	DOWN

Deregulated genes were identified by differential screening of gene expression from cDNA libraries MEHP (mono-2-ethylhexyl phthalate), ADR (adriamycin) ENU (N-ethyl-N-nitrosourea)

increase in the number of apoptotic PGCs induced by lindane either in culture and in the embryo (La Sala *et al.*, 2009). Moreover, in a similar *in vitro* assay 17-beta-estradiol (E2) was able to rapid stimulation of AKT, KIT ERK2 and SRC phosphorylation in mouse PGCs (La Sala *et al.*, 2010).

Others *in vitro* studies have shown differential response of PGCs to toxicants with different mechanisms of action. *In vitro* cultured PGCs exposed to N-ethyl-N-nitrosourea (ENU) (a classical toxicant and mutagen, with effects on spermatogenic cells) (Lessard *et al.*, 2004) or Doxorubicin (trade name Adramycin, ADR) an anthracycline widely used in cancer therapy, with apoptotic effects on spermatogenic cells (Sjoblom *et al.*, 1998) demonstrated growth inhibition and apoptosis induction, whereas exposure to mono (2-ethylhexyl) phthalate (MEHP) (the direct metabolite of the di (2-ethylhexyl) phthalate or DEHP), a widespread plasticizer, ubiquitously found as environmental pollutant affected PGC adhesion to cell monolayers (Iona *et al.*, 2002).

In order to identify gene deregulation as early response to different toxicants cDNA libraries prepared from limited amount of cells followed by differential screening showed highly altered gene expression in PGCs after exposure *in vitro* to EDs (Table 1) (our own data). Most of deregulated genes detected encode proteins involved in pathways basic for cell survival: respiratory chain and oxidative stress, ribosomal proteins, metabolism of the cell and translation factors. The relationship between mitochondrial reactive oxygen species (ROS) and survival/cell death is well established (Orrenius, 2007) and was classically related to fertility impairment (Sikka *et al.*, 1995). The survival of mammalian cells exposed to adverse environmental conditions requires a radical reprogramming of protein translation (Yamasaki and Anderson, 2008).

Studies of gene expression deregulation caused by environmental reprotoxicant exposure on testis, including EDs, have focused on the analysis of specific genes and defined genetic pathways (Richburg *et al.*, 2002; Edwards and Myers, 2007). The use of DNA microarray technology has implemented the understanding of the molecular basis of the effects EDs and other toxicants (Francois *et al.*, 2003; Iguchi *et al.*, 2006; Iguchi *et al.*, 2007). Vinclozolin is a known ED, used as fungicide, with antiandrogenic activity (Kelce *et al.*, 1994). Analysis by microarrays of changes in the transcriptome of embryo testes from rats exposed during embryonic period to vinclozolin showed altered expression of 576 genes in embryo at 13-16 dpc (Clement *et al.*, 2010). Gene expression deregulation can be based on epigenetic mechanisms as was reported by the same research team (Anway *et al.*, 2005; Anway *et al.*, 2006). Some of the deregulated genes are close related with cancer development such as the tumor suppressor *HIC2* (hypermethylated in cancer 2).

Recently, we also reported a comparative analysis of the effect of different EDs on gene expression in developing testis, assessing at the same time dosage and developmental periods of exposure (Lopez-Casas *et al.*, 2012). Five compounds of different nature, but all considered as ED, were analyzed. 17beta-estradiol (E2), as a natural estrogen; lindane, as one of the oldest synthetic pesticides still in use worldwide; mono-(2-ethylhexyl) phthalate (MEHP), bisphenol A (BPA) as a worldwide environmental contaminant and zearalenone (ZEA) a non steroidal estrogenic mycotoxin. Experimental deleterious effects of each of these compounds have been widely reported. The experimental plan included *in vivo* exposure of mice to several doses of EDs following a defined protocol: mothers were exposed two weeks before mating; the same exposure and dose were maintained during pregnancy and four weeks after birth. The results indicated that the different EDs act during testis development and germ cell differentiation with different mechanisms and diverse molecular pathways, as shown by their patterns of gene expression deregulation. MEHP and ZEA exposures define specific gene expression signatures after unsupervised hierarchical clustering analysis of 2670 genes. The pattern of deregulation was irrespective of the concentration of the toxicant or the developmental period during which exposure occurred, which strongly suggested that the mechanisms of action at the level of deregulation of gene expression occurred in the early stages of development, since in an experimental group of mice the exposure period was only for two weeks in pre-matting mothers. Maternal accumulation of the EDs or epigenetic effects could explain the pattern of altered gene expression in adult testis (Lopez-Casas *et al.*, 2012). Interestingly, the most relevant gene network of the deregulated genes is involved in pathologies affecting: cancer, developmental and endocrine system disorders. In agreement with previous studies of toxicity of 309 chemicals, mostly pesticides, analyzed in ES cells *in vitro* (Chandler *et al.*, 2011), we detected that genes involved in oxidative stress response pathways such as *Nrf2* are highly deregulated by exposure to the different EDs analyzed. Genes involved in testicular embryonal carcinoma progression, including matrix metalloproteinase 2 (*Mmp2*) have also been recently reported as overexpressed in testicular carcinoma cells exposed to MEHP (Yao *et al.*, 2012).

Epigenetics

Epigenetics is referred to changes in gene expression transmitted mitotically and meiotically without altering the DNA sequence. Consequently, epigenetic changes are key process both in cell

differentiation and as result of environmental impact on biological processes (Youngson and Whitelaw, 2008; Feil and Fraga, 2012).

The most relevant epigenetic processes are: methylation of cytosine residues on DNA (Bird and Wolffe, 1999), post-translational modification of histone tails (Jenuwein and Allis, 2001), and regulation by noncoding RNAs (especially miRNAs) (Chuang and Jones, 2007; Costa, 2008). All this epigenetic processes contribute to define the condensed or decondensed chromatin state and consequently gene expression. DNA methylation is the most studied mechanism of epigenetic regulation. Cytosine methylation primarily occurs in CpG dinucleotides in CpG-rich sequences, known as CpG islands that is often found near or in the gene promoter regions and usually unmethylated in somatic cells but differentially methylated in genes of the germline (Bird *et al.*, 1985; Cross and Bird, 1995; Weber *et al.*, 2007; Borgel *et al.*, 2010; Popp *et al.*, 2010; Hackett *et al.*, 2012). DNA methylation, maintained by DNA methyltransferases, is a crucial mechanism of epigenetic gene silencing of cell-specific genes and transposons, cell differentiation and development, parental genomic imprinting, X chromosome inactivation in mammals and maintenance of cellular pluripotency (De Carvalho *et al.*, 2010; Meissner, 2010; Portela and Esteller, 2010).

The epigenetic reprogramming of PGCs development is necessary to provide these cells pluripotency (De Felici, 2011). Global demethylation of PGCs appears to be a key mechanism in this reprogramming (Popp *et al.*, 2010; Surani and Hajkova, 2010). However, some genes potentially germline determinants, escape the global demethylation, suggesting transgenerational transmission of DNA methylation pattern for these specific genes (Borgel *et al.*, 2010).

Epigenetic alteration, mainly changes in DNA methylation, is a common trait in cancer (Esteller, 2007; Sharma *et al.*, 2010). In TGCTs, the pattern of imprinted genes is similar to that observed in normal embryonic cells, indicating again the embryonic origin of the TGC and the possible role of epigenetic changes in TGCTs in adults (van Gurp *et al.*, 1994; Schneider *et al.*, 2001; Sievers *et al.*, 2005). The analysis of global methylation in TGCTs showed a profile distinct to other types of cancer affecting somatic cells (Ushida *et al.*, 2012), mimicking the pattern obtained in PGCs (Okamoto and Kawakami, 2007). However, differences are also founds between seminomas and non-seminomas. Seminomas show hypomethylation at the CpG islands while non-seminomas that show CpG island methylation at a level similar to other solid tumors (Smiraglia *et al.*, 2002), which can be related with the differentiation state of PGCs in the timing of the transformation to tumorogenic cells as has been proposed (Rajpert-De Meyts *et al.*, 1998).

Analyses of methylation levels in specific relevant genes also show alterations in TGCTs. In this sense, the serine protease testisin (*PRSS21*) expressing in premeiotic testicular cells and considered as tumor suppressor gene is also down-regulated in TGCTs (Kempkensteffen *et al.*, 2006) probably due to the high level of hypermethylation at the promoter region as was detected in TGCTs (Manton *et al.*, 2005).

The fact that methylation patterns, and its alterations can be transmitted transgenerationally encourage the study of specific epigenetic changes in DNA methylation as a result of exposure to toxicants compounds during gonadal development and their potential pathological consequences. Limited studies have been carried out in this respect. MGMT (*O*6-methylguanine-DNA methyltransferase) is a DNA repair enzyme participating in the natural defence against cytotoxics. Loss of MGMT function can facilitates mutagenesis in oncogenes and tumor suppressors genes (Gerson, 2004). Down-regulation of *MGMT* due to hypermethylation of its promoter has been associated to different types of cancers, including testicular cancer showing higher hypermethylation levels in non-seminomas respect to seminomas (Smith-Sorensen *et al.*, 2002; Honorio *et al.*, 2003).

Due to the multiple potential mechanisms of action of the EDs, diverse environmental epigenetic inferences have been reported (Zhang and Ho, 2011). Altered patterns of methylation domains of parental imprinted genes were reported in the sperm of mice exposed to the antiandrogen vinclozolin (Stouder and Paoloni-Giacobino, 2010). BPA alters the coat colour in the *agouti* mice (Dolinoy *et al.*, 2007b), character highly sensitive to changes in methylation (Dolinoy *et al.*, 2007a). BPA also induced in the mouse hypomethylation of the *Hoxa10* gene in uterine cells of females exposed in embryonic life (Bromer *et al.*, 2010). Similarly, low dosage of BPA induces in neonatal rats hypomethylation of the phosphodiesterase *Pde4d4* promoter (Ho *et al.*, 2006). The exposure of mice to DEHP results in global increase of genome methylation level in the testis in addition to significantly increase of transcript expression of *Dmnt1*, *Dmnt3a* and *Dmnt3b* methyltransferase genes (Wu *et al.*, 2010) which are essentials for the establishment of cytosine residue methylation (Li *et al.*, 2003). Transgenerational effects of specific ED have been reported. The studies, contested by other reported by the chemical industry (Schneider *et al.*, 2008), based on the effect on epigenetic mechanisms transmitted by germ cells were basically carried out by the exposure to vinclozolin (Anway *et al.*, 2005; Skinner and Anway, 2005; Anway *et al.*, 2006; Skinner, 2007; Skinner *et al.*, 2011). The window of exposure during development is crucial for the phenotypic manifestation in adults and in successive generations. Only exposure *in utero*, during the period of formation of embryonic germ cells and their DNA methylation changes, affected later in adult germ cells (Cupp *et al.*, 2003; Uzumcu *et al.*, 2004).

Histone methylation is a post-translational modification of these key proteins involved in the dynamic and stability of chromatin conformation, and potentially in gene expression (Greer and Shi, 2012). Recent bioinformatics approach, based on previously reported data of gene expression in testis in different mammals exposed to vinclozolin and dibutyl-phthalate *versus* unexposed, suggests that histone methylation states, and particularly the regulation of demethylase *Kdm1,* could participate in the phenotypes observed as consequence to EDs exposures and their potential transgenerational epigenetic inheritance (Anderson *et al.*, 2012). Although still incipient, the most studied mechanism concerning histone modification is the histone H3 lysine 9 trimethylation (H3K9me3) but hundreds of modifications with potential effects on epigenetic modifications should occurs considering all components of the core histones of the chromatin (Kubicek *et al.*, 2006; Bannister and Kouzarides, 2011). Recently, the histone demethylase (H3K27) UTX has been reported as the safeguard of H3K27me3 demethylation observed in PGCs (Sansam *et al.*, 2003). However, histone modifications potentially due to EDs and effects on testis development are rarely reported.

Based in the concept of epigenetic adaptation to exposure to adverse conditions during fetal development (Heijmans *et al.*, 2009; Tobi *et al.*, 2009), it is suggestive to hypothesize that the exposure to a particular ED could modify the epigenetic pattern

of cells destined to be germ cells, such as PGCs, conditioning an "adaptive" response later for not differentiate, as a "hibernation", in response to the "adverse environment". Such circumstances might condition the maintenance of the undifferentiated state throughout the development and be the niche of *carcinoma in situ*. In adulthood, these cells "epigenetically misplaced" and under conditions that no longer exists: the environmental element that induced such a change, could enter into proliferative processes and be the source of TGCS.

The lack of an unequivocal cause-effect relationship or "epigenetic gambling" in terms of sensitivity or risk of these events could be due to both the genetic background of individuals and the ability to "adaptive resistance" of some cells from other (Martin, 2009), even in the same genetic background as in monozygotic twins where there are different responses and patterns of DNA methylation (Fraga *et al.*, 2005).

Small non-coding RNAs

From a biological point of view, the transcriptome and the post-transcriptional regulation is far more complex than originally thought. Small non-coding regulatory RNAs have emerged as pivotal post-transcriptional modulators of gene expression and are involved in diverse processes of cell differentiation and development. In particular, microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) are increasingly seen as important elements in both gonadal development and spermatogenesis and their pathologies (Yu *et al.*, 2005; Looijenga *et al.*, 2007a; Ro *et al.*, 2007; Hayashi *et al.*, 2008).

miRNAs are small non-coding RNAs (≈ 22 nt long) that act as potent modulators of gene expression by targeting 3' UTR regions of mRNAs inducing their cleavage or translational repression (Ambros, 2001; Ross *et al.*, 2007; Guarnieri and DiLeone, 2008; Chua *et al.*, 2009). The majority of miRNA genes are transcribed from intergenic regions of the genome and the primary transcripts are known as pri-miRNAs (≈ 200-100 nt long). These pri-miRNAs are double stranded RNA molecules fold into hairpins, which undergoes two processing steps before to render a mature and functional molecule. In these processing steps are involved two members of the RNase III family of enzymes: DROSHA and DICER. The first processing step occurs in the nucleus, and the product of Drosha cleavage is named pre-miRNA (≈ 70 nt long). The pre-microRNAs are exported to the cytoplasm where DICER processed it, generating a double stranded structure without hairpins, known as miRNA duplex. One or both strands could be incorporated into the miRNA-induced complex (miRISC or simply RISC), whose function is to mediate the translational repression of mRNAs by base-pair complementarity among miRNA and mRNA sequences (Lau and MacRae, 2009; Snead and Rossi, 2010; Ladomery *et al.*, 2011). Thousands of human genes, representing about 30% of the human gene set, are miRNA targets (Bentwich, 2005; Bentwich *et al.*, 2005; Lewis *et al.*, 2005); individual miRNAs can suppress the production of hundreds of proteins (Selbach *et al.*, 2008); more than 1000 miRNA have been predicted as well as functional polymorphisms within miRNA-binding sites from mRNAs (Mu and Zhang, 2012). Additionally, the double stranded nature of the primary miRNAs allow potential editing mechanisms (adenosine to inosine) enhancing the diversity of alternative miRNAs from the same precursors and therefore increasing their potential in the post-transcription regulatory modulation of mRNA expression (Blow *et al.*, 2006). Our recent studies suggest differential and active dynamics of miRNA edition and degradation associated to fertilization (García-López *et al.*, 2013).

piRNAs are short RNA molecules (but larger than miRNAs) (24-32 nt long) that are processed in a DICER/DROSHA-independent manner and associated to PIWI proteins (Aravin *et al.*, 2006). They have a role in transposable elements (TEs) silencing (Siomi *et al.*, 2011) and interacting with DNA methylation during spermatogenesis, being basically detected in male germ cells (Aravin *et al.*, 2006; Girard *et al.*, 2006; Grivna *et al.*, 2006; Kim, 2006), although recently they have also been identified in brain cells (Rajasethupathy *et al.*, 2012).

Small non-coding RNAs and TGCTs

Recent studies reported the alteration of miRNA expression in different cells and tissues induced by diverse environmental pollutants, including some considered EDs (Fig.2) (Izzotti *et al.*, 2009a; Izzotti *et al.*, 2009b; Avissar-Whiting *et al.*, 2010; Hou *et al.*, 2011; Hou *et al.*, 2012). Differential expression of miRNAs in human cancer compared with normal cells showed defined signatures associated to diagnosis, progression and prognosis (Calin and Croce, 2006).

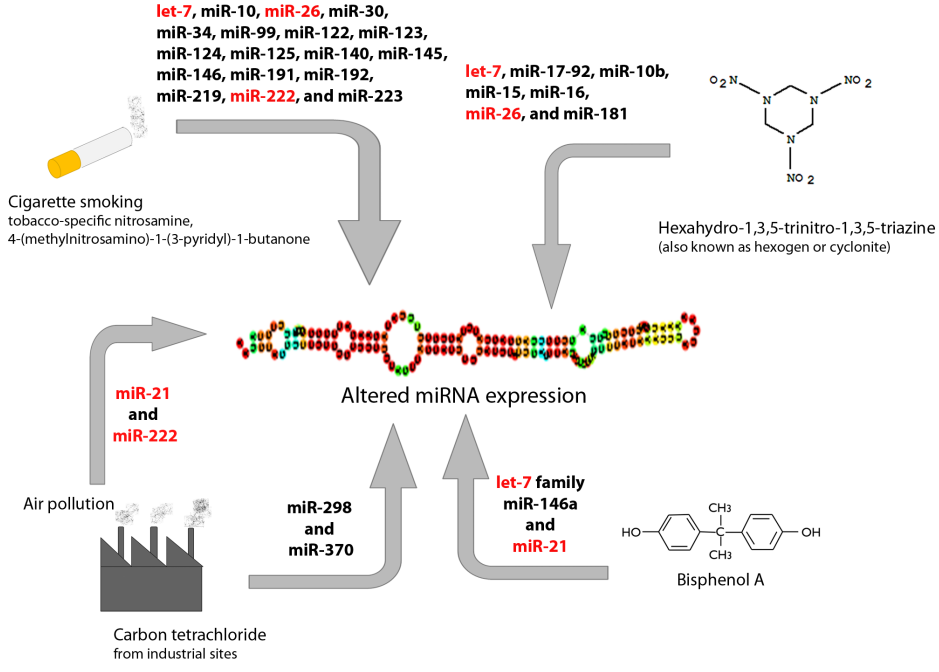

Fig. 2. miRNAs deregulated in different tissues after exposure of various contaminant agents including endocrine disruptors, such as bisphenol A (BPA). *The expression of some miRNAs was altered regardless of the inducer chemical agent or environmental factor (in red).*

As mentioned, the origins of testicular germ cell cancer have been associated to dysfunctional gonocytes from fetal development. The expression of *PRDM1* (also known as *BLIMP1*) is essential for PGC commitment (Ohinata *et al.*, 2005). *PRDM1* can be modulated by miR-*let-7* family of miRNAs. In turn, LIN28 is a miRNA-binding protein that controls the maturation of the precursor of miR-*let-7* (Heo *et al.*, 2008; Newman *et al.*, 2008; Piskounova *et al.*, 2008; Rybak *et al.*, 2008; Viswanathan *et al.*, 2008; Hagan *et al.*, 2009). Consequently, increased level of *let-7* can block the PGGs development. In fact, *in vitro* knockdown of *LIN28* in the mouse reduce the number of PGC colonies (West *et al.*, 2009) and also alters the expression of *Prdm14*, which is essential for germline specification. Moreover, downregulation of *let-7*, that is considered as tumor suppressor (Johnson *et al.*, 2005; Lee and Dutta, 2007), and overexpression of *LIN28* were linked to tumorogenesis (Chang *et al.*, 2009; Lu *et al.*, 2009). Consequently, the reported association of the overexpression of *LIN28* and its homologue *LIN28b* with human germ cell tumors is consistent (West *et al.*, 2009). Interestingly, the deregulation of *LIN28* is only associate to germ cell malignant tumors including seminomas, choriocarcinomas, embryonal carcinomas and yolk-sac tumours, but not to teratomas and normal testis (West *et al.*, 2009).

As in other types of cancer, TGCTs present particular miRNA expression profiles but related with the developmental origin (McIver *et al.*, 2012). The *hsa-miR-302* and the *hsa-miR-371~373* clusters are overexpressed in seminomas malignant tumors. However, the expression of miRNAs of these cluster are not altered in teratomas and non-malignant tumors (Palmer *et al.*, 2010). These clusters have been involved in the maintenance of pluripotency and their altered expression profile could be related with the differentiation grade of the tumor. The miRNA expression profile is even different among the types of testicular tumors (Gillis *et al.*, 2007). For example, *hsa-miR-21*, *miR-155*, *miR-19a* and *miR-29a* are overexpressed in type III tumors and seminomas, while *hsa-miR-145*, *miR-146* and *miR-133a* are under-expressed in both types of carcinomas in comparison with control samples (Gillis *et al.*, 2007). In general, it has been observed lower expression levels of miRNAs in cancers compared with control samples (Zhang *et al.*, 2007; Pan *et al.*, 2011). The relationship between the miRNA decay and cancer could be explained by the role of miRNA in cell differentiation and cell survival. But not only the miRNA expression decay was related to cancer disease, in other cases the up-regulation has awful consequences. In this sense, Voorhoeve *et al.,* (Voorhoeve *et al.*, 2007) found increased levels of *hsa-miR-371*, *miR-372* and *miR-373* in TGCTs. Over-expression of these miRNAs down-regulate a tumor suppressor gene expression involved in *RAS* oncogene pathway. Besides, *hsa-miR-373* has been identified as a cell migration factor and together with *hsa-miR-520c* promoted tumor invasion and metastasis (Huang *et al.*, 2008; Negrini and Calin, 2008).

Further studies will be necessaries to identify the exact role of all these miRNAs in testicular cancer, but their expression profiles could be informative checkpoint for diagnosis. Serum biomarker monitoring is employed for diagnosis of TGCTs. More than a 50% of TGCT patients have increased levels of α-fetoprotein, human chorionic gonadotropin and lactate dehydrogenase, however the lack of an increase does not exclude TGCT (Albers *et al.*, 2011). It is necessary for a good prognosis to found specific biomarkers that permit a best diagnosis for TGCTs. As miRNAs presents a high stability in blood and other body fluids, could be good candidates to be used as biomarkers (Gilad *et al.*, 2008; Brase *et al.*, 2010; Wang *et al.*, 2012; Zhang *et al.*, 2012).

Approximately 45% of the human genome is composed of repetitive elements, which consist of interspersed repeats and tandem repeats (Jordan *et al.*, 2003). LINE-1 and Alu are two major DNA repetitive elements: LINE-1 is a long group of interspersed nucleotide elements that constitutes at least 18% of the human genome (Kazazian, 2004). Alu and LINE1 elements are normally heavily methylated, and contain much of the CpG methylation found in normal human tissues (Kazazian, 2004) both LINE-1 and Alu repeats were extensively unmethylated in seminomatous TGCTs, whereas in non-seminomatous TGCTs, including two EC cell lines, the LINE-1 sequence was extensively unmethylated, but Alu elements were methylated (Ushida *et al.*, 2012). Therefore, as piRNAs are key molecules involved in the regulation of the transposable elements in germ line, the altered patterns of piRNAs in germ cells as consequence of EDs exposure represents an intriguing avenue of exploration.

Effects of EDs on miRNA expression profiles

As response to other external input, such as chemical drugs, EDs can deregulate the expression and physiological pathways of small non-coding RNAs (Hudder and Novak, 2008; Zhang and Dolan, 2010; Majumder and Jacob, 2011). Besides the direct action of EDs on the transcriptional regulation of multiple genes encoding proteins, the EDs could alter the balance of the fine post-transcriptional regulation of mRNAs mediated by non-coding small RNAs such as miRNAs. The action of deregulation of expression and accumulation of miRNAs by EDs can be carried out through by direct agonistic or antagonistic interactions with hormone receptors (HRs), which in turn regulate the expression of miRNAs or their biogenesis. In this sense, hormone sensitive cells exposed *in vitro* to estrogens (E2) shown deregulation of miRNAs. Some miRNAs are transcribed from polycistronic genes such as *hsa-miR-17-92* cluster encodes 6 miRNAs (*hsa-miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1*, and *miR-92–1*). The pri-miRNA of this cluster and the *mir-106a-363* paralogue cluster are upregulated by the ERα in breast cancer cells. Interestingly, the regulation occurs at the processing of primary forms to mature forms of miRNAs, as the *pre-miR-18a* is accumulated in ERα-positive and not ERβ-positive breast cancers (Castellano *et al.*, 2009). Furthermore, *hsa-miR-18a* regulates negatively *ERa* due to targeting of this miRNA in the *ERa* mRNA. In this context, previous studies reported that human *miR-206* regulates the post-transcriptional expression of specific HRs as the *ERa* but not the *ERb*, inhibiting the expression of *hsa-miR-206* the presence of ERα agonists, also indicating a feed-back regulation pathway (Adams *et al.*, 2007). In addition to the interaction between ERs and miRNAs in breast cancer, new studies show the association of miRNAs with other steroid receptors such as androgen receptor, progesterone receptor, glucocorticoid receptor in non-breast cancers (see review by (Tessel *et al.*, 2010).

Independently of the interaction between HRs and specific miRNAs, impaired biogenesis of miRNAs can be hormonally mediated. The processing of miRNAs can be repressed by the binding of DROSHA to ERα (Yamagata *et al.*, 2009). In some hormone-dependent cancers the global deregulation of miRNAs has been associated to the alterations in the expression of two key enzymes: DROSHA and DICER, involved in the miRNA processing. Down-

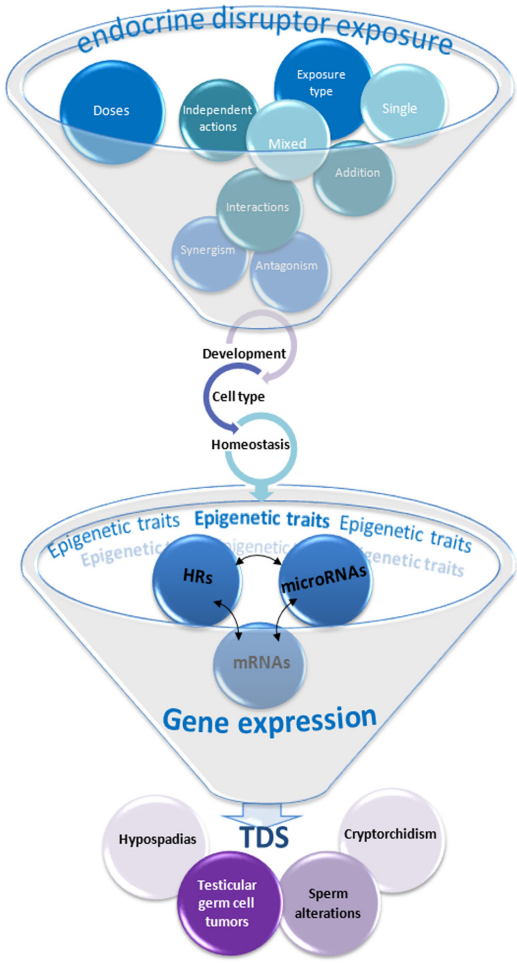

Fig. 3. Complex interactions between different factors and mechanisms of action of endocrine disruptors *can lead to various pathologies in the development of testis, including germ cell tumors.*

regulated *DICER* has been found in breast cancers (Grelier *et al.*, 2009) or both *DROSHA* and *DICER* in ovarian cancers (Merritt *et al.*, 2008) and subtypes of breast cancers (Dedes *et al.*, 2011). However, opposite regulation of *DICER* was detected in prostate adenocarcinoma (Chiosea *et al.*, 2006).

Recent studies reported that two well known EDs such as DDT and BPA alter the pattern of expression of multiple miRNAs of human MCF-7 including the estrogen–regulated *hsa-miR-21* considered as onco-miR in breast cancer (Tilghman *et al.*, 2012). Altered expression profiles of miRNAs linked to EDs exposure were also observed (Ficociello *et al.*, 2010; Choi *et al.*, 2011; Zhang and Ho, 2011). Specifically, it was observed overexpression of *hsa-miR-146a* in two placental cell lines in response to BPA exposure (Avissar-Whiting *et al.*, 2010). Such overexpression, leads slower proliferation and increase the sensitivity to other damaging compounds (Avissar-Whiting *et al.*, 2010). Furthermore, other miRNAs seem to be affected by BPA exposure. Three members of *let-7* miRNA family increased their expression levels after the BPA exposure (Avissar-Whiting *et al.*, 2010). As mentioned above, the equilibrium among *let-7* family members and LIN-28 is critical for regulating the differentiated cell state. Changes in this equilibrium, may originate altered rates of cell proliferation (Melton *et al.*, 2010; Newman and Hammond, 2010; Viswanathan and Daley, 2010; Wang *et al.*, 2010; Chen *et al.*, 2011; Pan *et al.*, 2011). However, *let-7* is considered as tumor suppressor (Zhang *et al.*, 2007) and consequently the possible effect of deregulation of *let-7* BPA-mediated in testis could have been involved in other pathologies of TDS different to TGCTs. Similar alteration in miRNA expression has been reported in Sertoli cells exposed to nonylphenol. A third of miRNAs analyzed showed altered expression after exposure to this ED (Choi *et al.*, 2011).

Recent reports show that epigenomic alterations may be parallel to those found in miRNA profiles and explained by crosstalk between epigenetic regulation and regulation of miRNA expression (Chuang and Jones, 2007; Fabbri, 2008), including specific patterns in testis (Schilling and Rehli, 2007).

All these studies clearly indicate a correlation between miRNA expression, and as a result on the corresponding products of the mRNA targets, and the hormone regulation pathways. Consequently, external factors with potential effects on hormonal balance, such as EDs, can alter the expression of miRNAs and hence the levels of mRNAs and the corresponding coded proteins. The harmful effects of EDs in miRNA expression profiles may be additional cause of altered proliferation cell rates on the reproductive system and potential source of TGCTs.

Concluding remarks

In mammals, testis development and germ cell fate is a complex and highly regulated genetically from early embryonic stages to adulthood (Kimble and Page, 2007). Consequently, disturbances in any of the regulatory pathways involved functional changes mediated by deregulation of gene expression, leading to reproductive dysfunctions and pathologies such as testicular cancer (Fig 3). In turn, the different regulatory mechanisms, including hormones, operate in a variable way in different stages of development. What is evident is that from the beginning of the primordial germ cell formation and differentiation in embryonic life there are key windows in the process, and therefore there are points of high risk susceptible of deleterious disruption by environmental signals.

The EDs and other potential reprotoxicants can alter the homeostasis necessary for correct testicular development and normal differentiation of germ cells but at different levels and developmental windows. Moreover, the synergistic effects of combinations of these compounds broaden the landscape of potential risk. The mechanisms of action of these compounds, leading to various pathologies in male gonadal development and function are multiple, but with a common core as deregulation of the expression of multiple genes. In these dysfunctional processes, different pathways could be affected: by direct action on transcriptional regulators, potentially mediated by hormones, and/or acting as post-transcriptional regulators such as those that affect miRNAs and piRNAs. Moreover, epigenetic deregulation may generate changes that in some cases may be inherited and transmitted transgenerationally (Youngson and Whitelaw, 2008). All these factors could contribute to the aetiology of pathologies manifested as germ cell tumors, along with intrinsic risk genomic factors in individuals or populations.

All this complex network of cause-effect can be evidenced by new high throughput approaches at molecular, cellular and developmental levels. However, considering the difficulties in the

implementation of *in vitro* systems in germ cells development, the abundance and increased risk factors in our environment and their additive or synergistic effects, the goal is foreseen a long way but as a great challenge.

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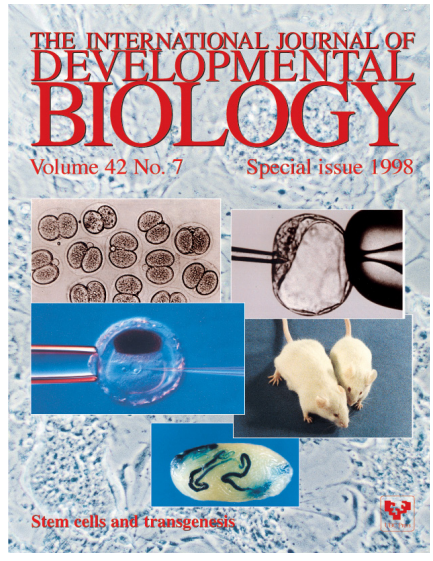

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