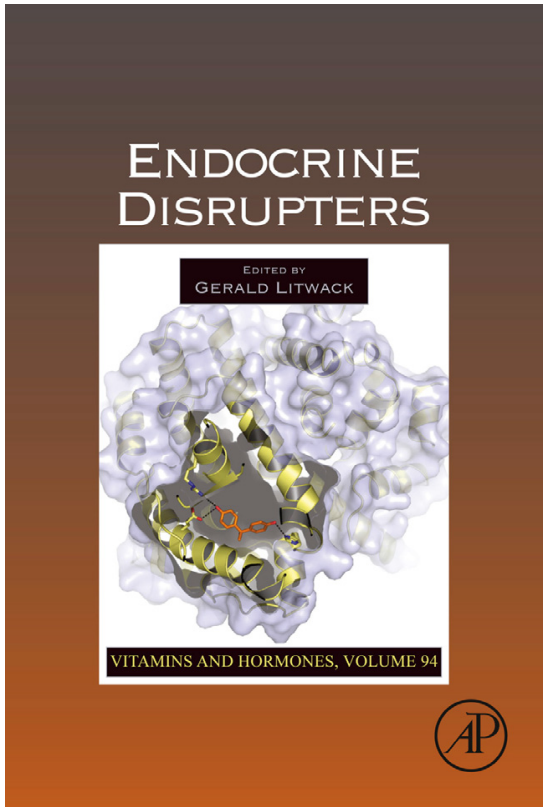

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CHAPTER TEN

Long-Lasting Effects of Neonatal Bisphenol A Exposure on the Implantation Process

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Abstract

Successful implantation is the result of complex molecular interactions between the hormonally primed uterus and a mature blastocyst. This very carefully synchronized interplay of hormonal signals and feedback loops is potentially vulnerable to chemicals such as endocrine disruptors that may disrupt endocrine signaling. Bisphenol A (BPA) is one of the highest-volume chemicals produced worldwide. This chapter describes the effects of brief postnatal exposure to BPA on female reproductive performance and specifically on the uterine adaptations during the preimplantation period. We propose that an early alteration in *Hoxa10* gene expression affects the functional differentiation of the preimplantation uterus as part of an altered endocrine signal transduction pathway. These molecular alterations could explain, at least in part,

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the adverse effects of BPA on uterine implantation. Exposure to endocrine disruptors, such as BPA, could contribute to the impaired female fertility noted over the past decades.

1. INTRODUCTION

During the past several decades, infertility rates and early pregnancy loss have increased worldwide and currently affect 10–15% of couples in the United States and other developed countries (Hayashi, Saitou, & Yamanaka, 2012). An important determinant of reduced fertility is failed implantation, which is thought to account for 50–70% of preclinical pregnancy losses in humans (Macklon et al., 2002; Norwitz, Schust, & Fisher, 2001). Although both the society and families have benefited from advancements in the field of assisted reproductive technology (ART), the overall success rate of ART still remains low. In the United States, 1% of births can currently be attributed to ART (Ola & Li, 2006). Many of the unsuccessful ART pregnancies can likely be attributed to the transfer of embryos into a nonreceptive uterus. In fact, recurrent implantation failure is considered to be an important limiting factor in the establishment of pregnancy by either ART or natural means (Norwitz et al., 2001).

Successful implantation is the result of complex molecular interactions between the hormonally primed uterus and a mature blastocyst. This very carefully synchronized interplay of hormonal signals and feedback loops is potentially vulnerable to chemicals such as endocrine disruptors that may disrupt endocrine signaling (Crain et al., 2008; Varayoud et al., 2011). Although many losses involve genetic abnormalities, there is often no known cause. Hormonal factors, endometrial or uterine gland changes, cytokines, and growth factors, among others, are part of the complex process that leads to implantation and the early stages of placental development (Norwitz et al., 2001).

Bisphenol A (BPA) is one of the highest-volume chemicals produced worldwide. Current estimates indicate that more than eight billion pounds of BPA is produced annually and that approximately 100 tons may be released into the atmosphere each year (Rubin, 2011; Vanderberg et al., 2010). BPA is used in the manufacture of plastics often used for food and beverage storage and is also a component of epoxy resins that are used to line food and beverage containers. Studies have shown that BPA can leach

from these and other products in contact with food and drink and, as a result, be routinely ingested. BPA can also be used in the manufacture of some dental sealants and composites and of thermal receipt paper (Biedermann, Tschudin, & Grob, 2010). Thus, given the prevalence of BPA in our environment, almost all the population seems to be exposed. BPA has been detected in more than 90% of urine samples obtained from a representative sample of US residents (Calafat, Ye, Wong, Reidy, & Needham, 2008). Detectable concentrations of BPA have also been measured in human follicular fluid and amniotic fluid, suggesting that exposure may occur as early as the periconception period.

This chapter deals with the long-term effects of early postnatal exposure to low doses of BPA and focuses on the lasting consequences in female fertility and the endocrine pathways that regulate the preparation of the endometrium for embryo implantation.

2. UTERINE IMPLANTATION OF THE EMBRYO

Different molecular events are essential to the implantation process. Various tissue compartments within the uterus, including the luminal epithelium, glandular epithelium, and stroma, undergo sequential proliferation and differentiation as the embryo attaches to the luminal epithelium and invades the stroma. However, our current understanding of the molecular signaling pathways that link these tissue compartments to achieve a functional state of the uterus conducive to implantation is still limited (Das, 2010).

The cascade of signaling events that occur in both fetal and maternal tissues at the time of implantation establishes an appropriate milieu critical to the development and survival of the fetus. Defects in the formation of this network and the inability to sustain this cross talk are believed to result in various pregnancy-associated complications that may manifest throughout pregnancy.

Although numerous of the molecules involved in the implantation process have been identified, the understanding of this process is still far from complete. For example, many of the genes that are expressed in an implantation-specific manner and appear to be important for implantation cannot be studied in depth because deletion of these genes results in embryonic lethality. One additional difficulty in identifying the critical roles of signaling molecules within a gene family is the redundant or compensatory functions of the gene products within the family.

2.1. Implantation-associated gene expression

Hoxa10 is one of the implantation-associated genes. This gene is known to be upregulated by progesterone in the uterine stroma during preimplantation period and its levels are sustained during decidualization (Daikoku et al., 2004; Satokata, Benson, & Maas, 1995; Wang & Dey, 2006). These characteristics have made *Hoxa10* an obvious marker for experimentation in an attempt to elucidate the molecular mechanisms of implantation. The severe infertility phenotype observed in *Hoxa10*-deficient female mice is multifactorial, suggesting that *Hoxa10* has pleiotropic effects and regulates multiple pathways. *Hoxa10*-deficient mice exhibit female infertility, with the proximal region of the uterus showing partial homeosis into an oviduct-like structure. However, the cause of infertility in these females seems to be impaired stromal cell proliferation and decidualization (Lim, Ma, Ma, Maas, & Dey, 1999). Furthermore, embryo transfer experiments have conclusively shown that *Hoxa10* is required for both implantation and decidualization (Bagot, Troy, & Taylor, 2000, Benson et al., 1996). Stromal cell proliferation in *Hoxa10*$^{-/-}$ mice in response to ovarian progesterone and estrogen is severely compromised, while epithelial cell proliferation remains normal in response to estrogen (Yao et al., 2003). This impaired stromal cell responsiveness to progesterone suggests that *Hoxa10* induces genes that are vital for stromal cell proliferation and differentiation and behaves as a mediator of progesterone effects on implantation.

B3 integrin (*ITGB3*) and *empty spiracles homolog 2* (*EMX-2*) are *Hoxa10*-target genes, which are known to be operative downstream in endocrine hormone-regulated *Hox* gene pathways. *ITGB3* has been proposed to be a bridging molecule between the endometrium and the trophoblast, thereby constituting an early link between maternal and fetal tissues (Sueoka et al., 1997). Endometrial *ITGB3* expression coincides with a systemic peak of progesterone, and high endometrial *Hoxa10* levels occur in the midsecretory phase of the menstrual cycle, around the time of embryo implantation (Taylor, Arici, Olive, & Igarashi, 1998). *ITGB3* expression has been shown to be directly regulated by *Hoxa10* through a consensus Abd-B-type *HOX* binding site located 5′ of the *ITGB3* gene within its regulatory region (Daftary, Troy, Bagot, Young, & Taylor, 2002). *EMX-2*, on the other hand, is a homeobox gene located outside the *HOX* cluster, orthologous to the *Drosophila empty spiracles* gene (Kastury et al., 1994), which is negatively regulated by *Hoxa10* in reproductive tissue (Troy, Daftary, Bagot, & Taylor, 2003). In contrast, at the same time, *Hoxa10* directly binds a regulatory

element in an enhancer region of the *EMX-2* gene and transcriptionally represses its expression (Troy et al., 2003). In baboons with experimental endometriosis, it has been found that *Hoxa10* expression is lower than that of disease-free animals and that this decreased expression is accompanied by abnormal expression of *EMX-2* and *ITGB3* (Kim et al., 2007).

Another important event associated with the preparation of the uterus for embryo implantation is mediated by the action of vascular endothelial growth factor (VEGF). VEGF constitutes the main factor responsible for the control of endothelial proliferation and vascular permeability. Immunoneutralization of VEGF inhibits pregnancy establishment in rhesus monkeys (Sengupta et al., 2007) and rats (Rabbani & Rogers, 2001; Rockwell, Pillai, Olson, & Koos, 2002). This protein is present in different uterine cellular compartments, and its differential expression is associated with ovarian steroid levels. Studies in three different rodent species have shown that the luminal epithelium shows increased VEGF expression in the uterus in response to estradiol (Karuri, Kumar, & Mukhopadhyay, 1998; Shweiki, Itin, Neufeld, Gitay-Goren, & Keshet, 1993; Yi et al., 1999). In addition, these studies have shown little or no VEGF expression in stromal tissue until after progesterone administration. Different results have shown that progesterone- and estradiol-induced uterine VEGF expression does not involve their nuclear receptor interactions with their classic consensus hormone response element (Kazi & Koos, 2007; Mueller et al., 2003). Thus, in the rat uterus, estradiol induces the recruitment of estrogen receptor α (ERα) to the proximal GC-rich region of the *VEGF* promoter, probably via interaction with specificity proteins (Kazi & Koos, 2007). Therefore, downstream VEGF regulation by ovarian hormones depends on the expression profiles and recruitment of different transcription factors to the *VEGF* promoter region.

2.2. Ovarian steroids and their nuclear receptors

One would be remiss to discuss the molecular mediators of implantation without first emphasizing the paramount roles of the ovarian steroids estradiol and progesterone. The nuclear ER and progesterone receptor (PR) are expressed in all compartments of the uterus, and embryo implantation has been shown to be critically dependent on their intricate balance and interaction (Rubel, Jeong, Tsai, Lydon, & Demayo, 2010; Tan, Paria, Dey, & Das, 1999; Tibbetts, Mendoza-Meneses, O'Malley, & Conneely, 1998).

The preimplantation period shows different changes in steroid levels and steroid hormone receptor expression in the uterus. In pregnant rodents, estradiol levels and ERα activity in the endometrium decrease temporarily on gestation day 2 and then increase near the moment of implantation. Transcription of targeted genes may be estrogen-sensitive and thus down-regulated during this time. By GD3, corpus luteum-synthesized progesterone initiates stromal cell proliferation and its increasing presence may cause indirect regulation of gene expression through direct *Hoxa10* regulation. It has been established that homeobox genes are pleiotropic transcriptional modulators and that *Hoxa10*, specifically, can act as a repressor and an activator of transcription (Dey et al., 2004).

Estradiol acts mainly through one of two ERs, ERα or ERβ, which are encoded by two separate genes. Progesterone acts through the PR, which consists of two isoforms, PR-A and PR-B. These two isoforms arise from differential transcription of the *PR* gene. Gene ablation studies have demonstrated that ERα and PR-A are primary regulators of the uterine function (Curtis Hewitt, Goulding, Eddy, & Korach, 2002). These studies have also shown that while ERα plays a permissive role in the regulation of uterine function, PR-A is critical for the uterus to support pregnancy (Tan et al., 1999; Tibbetts et al., 1998). These receptors do not act alone in the regulation of gene transcription but are aided by coregulator proteins. Activated steroid receptors recruit coactivators or corepressors to target gene promoters. Coactivators such as steroid receptor coactivator-3 (SRC-3) enhance the transcriptional activity of steroid hormone receptors via their intrinsic histone acetyltransferase activity by bridging nuclear receptors with the basal transcription machinery or by interacting with other histone acetyltransferases such as CBP/p300. On the other hand, corepressors, such as the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), are limiting factors that inhibit the transcriptional activity of steroid hormone receptors by recruiting histone deacetylases and disrupting receptor dimer interactions (Privalsky, 2004). Different coregulators regulate the transcriptional activity of a variety of nuclear receptors, including ERα, ERβ, and PR, and are expressed in several hormone-responsive tissues including the uterus (Jeong et al., 2007). Previous results have shown that these coregulators play a necessary role in coordinating steroid hormone regulation of normal reproductive uterine function.

2.3. Cell proliferation in the stroma and the endothelium

Stromal proliferation and endothelial proliferation are critical events associated with the preparation of the endometrium for embryo implantation.

Previous results have shown that an adequate proliferation rate of these compartments is a requisite for successful implantation because, as it has been shown in several mouse models, the failure of these processes affects the implantation rate. Progesterone, which is the essential stimulus needed for *in vivo* proliferation of stromal cells, acts as the switch for their specific decidual program (Huet-Hudson, Andrews, & Dey, 1989). In rats, progesterone switches proliferation from the epithelial to the stromal compartment on day 4 of pregnancy as a prerequisite for implantation and decidualization (reviewed in Carson et al., 2000). Although different genes have been associated with the control of stromal proliferation, *Hoxa10* is defined as a key molecule during the preimplantation period.

Endothelial proliferation is one of the most studied events associated with the uterine angiogenesis during the preimplantation period. Like stromal proliferation, endothelial proliferation is known to be influenced by estradiol and progesterone through the activation of their respective nuclear receptors. The sex steroid receptor complex exerts some biological effects indirectly via a variety of growth factors (Weitlauf, 1994). Among them, VEGF is likely the main factor responsible for the control of endothelial proliferation.

3. ENDOCRINE DISRUPTORS AND THE DEVELOPMENTAL PROGRAMMING HYPOTHESIS

The developmental programming hypothesis suggests that aberrant stimuli encountered during critical periods of development can permanently reprogram normal physiological responses and, thus, give rise to reproductive consequences later in life (Bartol, Wiley, & Bagnell, 2006). These acquired changes can persist transgenerationally and a possible explanation of such outcome is the epigenetic regulation of the genome. Exposure to environmental endocrine disruptors can affect the normal development of reproductive tissues and may contribute to declining conception rates and increased incidence of female reproductive disorders such as oocyte aneuploidy, polycystic ovarian syndrome, and altered cyclicity, as well as endometriosis, uterine fibroids, fetal growth retardation, and pregnancy loss (Crain et al., 2008).

As already mentioned, BPA is an estrogenic compound produced in large quantities and a study carried out by the Center for Disease Control and Prevention in the United States detected BPA in the urine of >90% of the Americans sampled (Calafat et al., 2008). However, few epidemiological studies have examined the effects of BPA in humans. For instance,

toddlers exposed to high maternal levels of BPA during pregnancy have shown externalizing behaviors (Braun et al., 2009). Also, urinary BPA levels have been shown to be associated with increased cardiovascular disease and diabetes in adults (Lang et al., 2008) and sexual dysfunction in men (Li et al., 2010). Because the true impact of endocrine disruptors on human health is difficult to assess, it is important to test their effect under controlled exposure conditions in animal models (Maffini, Rubin, Sonnenschein, & Soto, 2006; Tharp et al., 2012). The model of the endocrine disruptor diethylstilbestrol (DES)-induced reproductive tract malformations and cancers serves as a novel paradigm to study the pathological consequences that arise in adults exposed early in life to hormonally active substances (Mericskay, Carta, & Sassoon, 2005). There is no clear sign that any gene has undergone a permanent mutation in response to DES exposure. Instead, a transient disruption of normal gene expression, which impacts all subsequent and normal development, occurs during a critical period. It has been shown that DES potently represses a number of developmental control genes, including several *Hox* and *Wnt* genes, during critical periods of reproductive tract patterning (Couse et al., 2001).

To evaluate the effects of endocrine-disrupting compounds with estrogenic activity such as BPA, endosulfan, and DES, we have used a rat model with a brief postnatal treatment on days 1, 3, 5, and 7 of life by subcutaneous injections in the nape of the neck (Bosquiazzo, Varayoud, Muñoz-de-Toro, Luque, & Ramos, 2010; Milesi, Varayoud, Bosquiazzo, Muñoz-de-Toro, & Luque, 2012; Monje, Varayoud, Luque, & Ramos, 2007; Monje, Varayoud, Muñoz-de-Toro, Luque, & Ramos, 2009, 2010; Rodriguez, Santambrosio, Santamaría, Muñoz-de-Toro, & Luque, 2010; Varayoud et al., 2011; Varayoud, Ramos, Bosquiazzo, Muñoz-de-Toro, & Luque, 2008). In the rat, the uterine development and differentiation is completed during the first postnatal week; thus, the early postnatal period is critical to evaluate the effects of endocrine disruptor exposure.

The route of BPA administration and the doses of BPA administered are important issues to determine BPA health risks in animal models (CERHR, 2007). In fetuses and neonates, the low expression of the enzyme that conjugates BPA (uridine diphosphate-glucuronosyl transferase) implies that oral and nonoral administration of BPA during neonatal life results in the same internal active dose (Taylor, Welshons, & vom Saal, 2008). Since the Society of the Plastics Industry and the US Environmental Protection Agency have recommended using the current accepted lowest-observed-adverse-effect level dose (50 mg/kg/day) of BPA to calculate a maximum acceptable or

reference dose, thus, 0.05 mg/kg/day was chosen (U.S. Environmental Protection Agency, 1993). In our studies (Bosquiazzo et al., 2010; Varayoud et al., 2011, 2008), we examined the effects of two different doses of BPA: one identical to the reference dose (0.05 mg/kg/day; BPA.05) and the other 400-fold higher (20 mg/kg/day; BPA20), although 2.5-fold lower than the declared lowest-observed-adverse-effect level. The aim was to investigate whether a brief postnatal exposure to BPA disrupts transcriptional control of the development-related genes *Hoxa10* and *Hoxa11* in the uterus of rats on postnatal day 8. *Hoxa10* and its neighbor in the *Hoxa* gene cluster, *Hoxa11*, are abdominal B type homeobox genes, which normally regulate differentiation of the Müllerian duct (Taylor, 2000). Downregulation of both *Hox* genes has been detected using mice as a model of postnatal exposure to DES (Couse et al., 2001). Methoxychlor, a pesticide that has adverse effects on the reproductive capability of mice, decreases *Hoxa10* mRNA levels in Ishikawa cells *in vitro* and decreases uterine *Hoxa10* expression in mice treated *in vivo* (Fei, Chung, & Taylor, 2005). Little is known about BPA effects on Hox gene expression. A study has shown a dose–response increase in *Hoxa10* levels in the uterus of 2- and 6-week-old mice exposed *in utero* to BPA (Smith & Taylor, 2007). We detected that the two studied doses of BPA during early postnatal days decrease *Hoxa10* and *Hoxa11* expression in the prepubertal rat uterus (Varayoud et al., 2008). Taken together, the previously mentioned results show that *Hox* genes are a common target of endocrine disruption (Fei et al., 2005) and suggest that exposure during different developmental periods could lead to a different characteristic effect.

3.1. BPA effects on reproductive performance

The BPA effects on reproductive performance can be evaluated using different models of exposure. In laboratory animals, the exposure to components leached from dental sealants containing BPA alters reproductive end points (Darmani & Al-Hiyasat, 2006). While no effects on the number of implantation sites have been observed, an increased number of embryo resorptions (postimplantation loss) have been reported (Darmani & Al-Hiyasat, 2006). Other authors have shown that BPA exposure during the sensitive period of blastocyst implantation disrupts pregnancy (Berger, Hancock, & deCatanzaro, 2007). In our experimental design, we used a brief postnatal exposure and evaluated the long-lasting effects on reproductive performance. After early postnatal exposure to both doses of BPA, we

observed a trend to a decreased percentage of rats that became pregnant, although all females were sexually receptive. The number of corpora lutea (CL) in the ovaries from all BPA-treated pregnant rats was similar to that of controls (12–13 CL/rat). This result suggests that the ovulation rate and CL "activation" are not altered in most of the females after neonatal exposure to the xenoestrogen BPA. In contrast, the number of implantation sites was significantly lower in BPA-treated rats, suggesting an intrinsic uterine defect that preceded embryo arrival (Varayoud et al., 2011). Therefore, BPA effects on reproductive performance could be different depending on the animal model used, developmental stage at exposure, and/or the dose administered.

3.2. BPA alters endocrine signaling in the preimplantation uterus

The effects of BPA on reproductive performance can be associated with the disruption of different markers of uterine implantation mainly those regulated by ovarian steroid hormones. To evaluate whether the endocrine pathway is affected, we established the serum levels of sex steroids and uterine steroid hormone receptor expression (ERα and PR) on GD 5 (Varayoud et al., 2011). The uteri of control animals showed a high expression of ERα and PR in subepithelial stromal cells, whereas those of animals neonatally treated with BPA showed a lower expression of both receptors, without changes in serum levels of estradiol and progesterone. In addition, we found that neonatal exposure to BPA caused different effects on PR expression according with the BPA dose, suggesting that the uterine response to the hormonal milieu on GD 5 of pregnancy is differentially affected. The lower dose of BPA (BPA.05) diminished *PR* mRNA, but not PR protein, whereas the higher dose (BPA20) affected both mRNA and protein expression. In this context, we propose that BPA.05 could affect the ubiquitination of PR protein and consequent degradation. Other previous studies have shown that a reduced ubiquitination of PR contributes to its stabilization and is correlated with an increased response to progesterone (Lee, 2008). The estrogen influence on the ubiquitination system has been extensively shown (Ito et al., 2010). ERα is commonly associated with ubiquitin ligases (like Smurf) forming protein complexes that regulate the ubiquitination rate of many targets in an estrogen-dependent manner. In particular, PR is a substrate for the ubiquitin/proteasome pathway (Qiu & Lange, 2003). Ubiquitination of PR depends on S294 phosphorylation. This has been demonstrated using an S294A mutant, which does not undergo ubiquitination and degradation by the proteasome pathway after

progesterone binding (Qiu & Lange, 2003). These (and other) results provide some empirical data to speculate about the possible influence of xenoestrogens on the control of the ubiquitination process.

3.3. BPA and the expression of implantation-associated genes

With the intention to evaluate whether the effects of BPA exposure on reproductive performance are associated with modifications of implantation-associated genes, we have also assessed the expression of *Hoxa10*, *ITGB3*, and *EMX-2* (Varayoud et al., 2011). *Hoxa10* is an ovarian steroid downstream target gene, with a key role during implantation. In our work, we detected that *Hoxa10* uterine expression decreased in BPA-treated animals on GD 5 and that this decrease was further evidenced in the absence of variation in circulating steroid hormone levels and appeared to be due to a decrease in PR and ERα expression in the subepithelial stroma (Varayoud et al., 2011). Another previous report demonstrated that both steroid hormone receptors are implicated in the regulation of *Hoxa10* expression, using *in vitro* and *in vivo* models (Daftary & Taylor, 2006). Estradiol regulation of *Hoxa10* is associated with the detection of ER binding of two putative estrogen response elements in the 5′ regulatory region of *Hoxa10* (Taylor et al., 1998). Progestational regulation of *Hoxa10* occurs via PR and is therefore blocked by RU486 (Ma, Benson, Lim, Dey, & Maas, 1998).

As already mentioned, *EMX-2* and *ITGB3* are two *Hoxa10* target genes that are known to be operative downstream in endocrine hormone-regulated *Hox* gene pathways. Alterations in both genes are associated with subfertility in different species (Daftary & Taylor, 2006). Our results have shown that BPA-exposed animals exhibiting a lower number of implantation sites and a disruption of *Hoxa10* additionally showed a lower expression of *ITGB3* and a higher expression of *EMX-2*. Alterations in the endocrine-regulated *Hoxa10* gene pathways (steroid receptors–*Hoxa10*–*ITGB3*/*EMX-2*) could explain, at least in part, the BPA effects on the implantation process. Further evidence demonstrating that *Hoxa10*, *ITBG3*, and *EMX-2* function in a common pathway has been found in diseases with implantation defects, such as endometriosis, where simultaneous misregulation of these three genes has been documented (Lessey et al., 1994; Taylor, Bagot, Kardana, Olive, & Arici, 1999). A schematic representation showing the steroid receptors–*Hoxa10*–*ITGB3*/*EMX-2* network during the preimplantation period and the effects of early postnatal BPA exposure on this network is shown in Fig. 10.1.

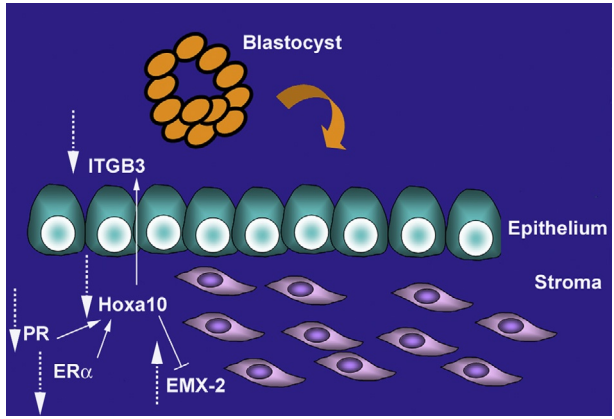

Figure 10.1 Schematic representation showing long-lasting effects of early postnatal bisphenol A (BPA) exposure on essential molecular events of the implantation process. In control conditions, the sex steroids estradiol and progesterone and their cognate receptors PR and ERα upregulate *Hoxa10* in the subepithelial uterine stroma, which in turn represses *EMX-2* expression. In addition, *Hoxa10* acting downstream of sex steroids upregulates *ITGB3* expression by endometrial epithelial cells, suggesting terminal differentiation in this cell type in preparation for embryo implantation. Dashed arrows indicate the neonatal BPA effects on implantation-associated gene expression evaluated in the rat uterine tissue on gestation day 5. Alterations in the endocrine-regulated *Hoxa10* gene pathways (steroid receptors–*Hoxa10*–*ITGB3*/*EMX-2*) could explain, at least in part, the BPA effects on the implantation process. - - - -▶ indicates induction and —┤ indicates repression. PR (progesterone receptor), ERα (estrogen receptor alpha), *ITGB3* (*Beta 3 integrin*), and *EMX-2* (*empty spiracles homolog 2*).

3.4. Early postnatal BPA exposure impairs the uterine response to ovarian steroids in the adult

The hormonal control of endometrial proliferation can be affected as a consequence of endocrine disruptor exposure. To evaluate whether a brief postnatal BPA exposure could adversely affect the uterine response to hormonal stimuli, we used a model of ovariectomized (OVX) adult rats with exogenous steroid hormone replacement with the intention to eliminate the variability in hormone levels characteristic of cycling animals (Varayoud et al., 2008). Progesterone pretreatment followed by a physiological dose of estradiol increased the number of synchronously proliferating subepithelial stromal cells (Rider, Thomson, & Seifert, 2003). In addition, mutant mice lacking normal *Hoxa10* expression showed defective progesterone-dependent uterine stromal proliferation (Yao et al., 2003). Figure 10.2A shows that rats neonatally exposed to BPA had an impaired response to normal ovarian steroid-mediated induction

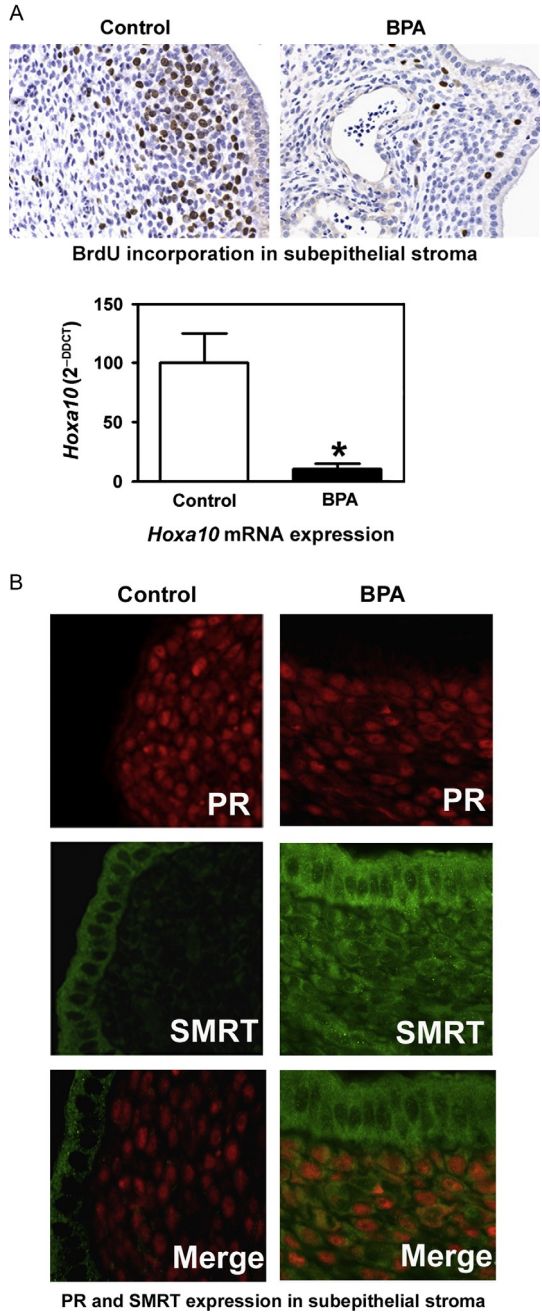

Figure 10.2 Long-term effects of postnatal exposure to BPA in the uterus of adult ovariectomized rats in response to steroid hormone replacement. (A) Subepithelial stromal

(*Continued*)

of uterine stromal cell proliferation. In addition, steroid-mediated activation of *Hoxa10* failed in adult females postnatally exposed to BPA. It is notable that the increased cell number and endometrial tissue volume seen in controls may provide the necessary conditions for optimal embryo implantation. In support of this, increasing endometrial thickness correlates with higher implantation rates in humans (Zhang et al., 2005). Adult CD-1 mice exposed *in utero* to low doses of BPA show a decreased volume of the endometrial lamina propria (Markey, Wadia, Rubin, Sonnenschein, & Soto, 2005). Based on our results, we propose that alterations in the proliferative status of uterine stromal cells in response to steroid hormones could affect the synchrony between the endometrium and the embryo, likely leading to decreased fertility. These alterations have been demonstrated in mice in which the progesterone pathway is affected with clear consequences in fertility (Velarde, Geng, Eason, Simmen, & Simmen, 2005). Furthermore, several studies have supplied evidence about the possible relationship between this impaired proliferative response and the development of endocrine-related diseases such as endometriosis and endometrial tumors (Gargett & Chan, 2006).

Progesterone + estradiol treatment in OVX rats increases not only subepithelial proliferation but also endothelial proliferation. Previous works have shown that the uterine vascularization is another key process in the preparation of the endometrium for embryo implantation (Weitlauf, 1994). We found that the proliferative response of the uterine endothelial cells to ovarian steroids during adulthood is altered by neonatal exposure to BPA (Bosquiazzo et al., 2010). Moreover, BPA exposure downregulates *VEGF* expression in the subepithelial uterine stroma, another event closely related to the control of the vascular compartment (Fig. 10.3A). It is well known that VEGF is a secreted growth factor that operates by binding to specific receptors and that the VEGF/receptor signaling system is mainly

Figure 10.2—Cont'd proliferation detected by bromodeoxyuridine (BrdU) incorporation. Relative *Hoxa10* mRNA expression was measured via real-time quantitative PCR and fold expression from control values was calculated by the equation $2^{-\Delta\Delta CT}$. Control group was assigned to a reference level of 100 and values are given as mean ± SEM. Ribosomal protein L19 was used as internal control. *Asterisk* indicates $p < 0.05$. ($n = 8$/group). Note that BrdU incorporation and *Hoxa10* mRNA expression decrease in uterine tissue of rats postnatally exposed to BPA. Magnification: ×400. (B) Dual immunofluorescence staining for progesterone receptor (PR, red) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT, green). An increase in SMRT expression is observed in the subepithelial stroma of postnatally exposed BPA rats. The merge images show that SMRT colocalizes with PR in the subepithelial stroma. Magnification: ×1000. *For methodological details, see Varayoud et al. (2008).*

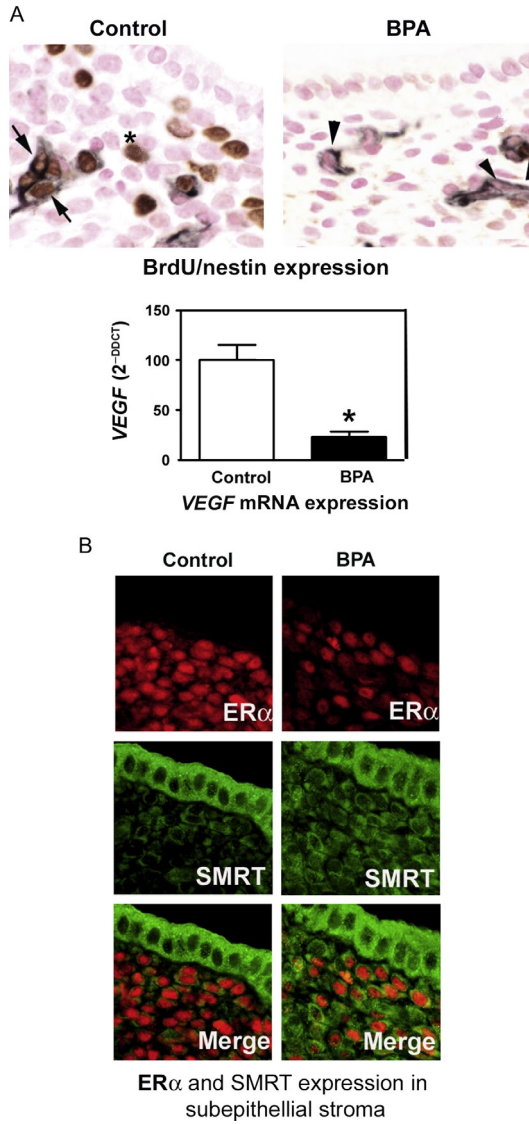

Figure 10.3 Effect of neonatal exposure to BPA on steroid regulation of vascular endothelial growth factor expression (*VEGF*) and endothelial cell proliferation. (A) Representative photomicrographs of uterine sections double-immunostained for BrdU (brown nuclei) and nestin (black cytoplasms) to evaluate endothelial proliferation. Arrows indicate double-positive cells (proliferating endothelial cells), and arrowheads indicate non-proliferating endothelial cells (positive for nestin in the cytoplasms with pink nuclei counterstained with nuclear fast red). Asterisk in the control photomicrograph indicates BrdU-positive fibroblastic stromal cell. Magnification: ×1000. Relative *VEGF* mRNA levels were measured by real-time quantitative PCR and fold expression from control values

(*Continued*)

involved in the regulation of endothelial cell proliferation (Ferrara, Gerber, & LeCouter, 2003). Therefore, our results suggest that the alteration in steroid-mediated activation of *VEGF* could be responsible for the impaired endothelial proliferative response found in BPA-exposed rats. The lack of changes in the processing of the *VEGF* mRNA splice variants suggests that xenoestrogens could modify *VEGF* mRNA expression by acting at the primary transcript level. Bredhult, Bäcklin, and Olovsson (2007) studied whether endocrine-disrupting chemicals affect the proliferation and viability of human endometrial endothelial cells *in vitro* and, in accordance with our results, observed that after treatment with 0.01, 1, or 100 μM BPA, endothelial proliferation was lower than in controls.

Taking into account that the steroid hormone responsiveness of the uterine stroma is affected in BPA-exposed animals, we determined stromal expression of PR and ERα in an OVX+progesterone+estradiol rat model and found no changes; however, we detected a decreased expression of both receptors in BPA-treated animals on GD 5. This indicates that results observed with regard to steroid hormone receptors are different between pregnant rats and the model of OVX rats with an exogenous ovarian steroid treatment that mimics the hormonal milieu during implantation. In addition, we determined the expression of coregulator proteins, because these proteins serve as partners for nuclear receptors, orchestrating the molecular events required for receptor-dependent transcriptional regulation (Hall & McDonnell, 2005). Therefore, the sensitivity to steroid hormones also depends on the availability of steroid receptor coregulators (Durrer, Maerkel, Schlumpf, & Lichtensteiger, 2005). The expression of the coactivator SRC-3 was similar in animals exposed to BPA and controls. However, our data demonstrate that neonatal exposure to BPA affects uterine stromal SMRT expression in response to progesterone+estradiol treatment, showing a clear upregulation of this corepressor in the subepithelial

Figure 10.3—Cont'd was calculated by the equation $2^{-\Delta\Delta CT}$. Control group was assigned to a reference level of 100 and values are given as mean ± SEM. Ribosomal protein L19 was used as internal control. *Asterisk* indicates $p < 0.05$. ($n = 8$/group). BrdU endothelial incorporation and *Vegf* mRNA expression decreased in rats neonatally exposed to BPA. (B) Dual immunofluorescence staining for estrogen receptor alpha (ERα, red) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT, green). An increase in SMRT expression is observed in the subepithelial stroma of BPA neonatally exposed rats. The merge images show that SMRT colocalizes with ERα in the subepithelial stroma. Magnification: ×1000. *For methodological details, see Bosquiazzo et al. (2010).*

stroma (Figs. 10.2B and 10.3B). Moreover, immunofluorescence results showed the coexpression of SMRT/ERα and SMRT/PR in the uterine stromal cells (Figs. 10.2B and 10.3D). Nuclear receptor coregulators were revealed as targets for endocrine disruptors, as shown for SMRT in the prostate and SRC-1 in the uterus (Durrer et al., 2005, 2007). The increase in SMRT described here may have implications for gene expression on a broad scale because SMRT is recruited by many transcription factors, including steroid receptors (Hall & McDonnell, 2005), and is also a limiting factor inhibiting transcriptional activity of steroid hormone receptors (Privalsky, 2004). The fact that the abnormal overexpression of SMRT was found in the same subepithelial stromal cells where VEGF and Hoxa10 induction failed suggests that the high levels of SMRT could interfere with different steroid-dependent genes in BPA-exposed animals. Our results show that the exposure to xenoestrogen chemicals during critical periods of perinatal life changes the uterine hormonal response during adulthood by disrupting the assembly of the transcription machinery of PR- and ER-dependent genes. Future studies will address this issue by studying whether neonatal exposure to BPA affects the transcription factor assembly in the *Hoxa10* promoter region.

4. ENDOCRINE DISRUPTION AND FEMALE FERTILITY

The combination of data showing reduced conception rates in humans and the common occurrence of female reproductive disease raises concerns that environmental factors may be having a negative impact on female reproductive health. However, the effects of endocrine disruptors are difficult to determine, because the consequences are evident long after exposure has ended. In addition, it is becoming increasingly clear from epidemiological studies in humans (Christiansen et al., 2005), and from genetic studies in rodents (Wang & Dey, 2006), that failed pregnancies are largely due to faulty uterine function or miscommunication between the embryo and the mother before placentation. Our results corroborate this paradigm and should serve to alert on the fact that reproductive performance and altered uterine function due to neonatal exposure to BPA are probably related. Results obtained after DES or endosulfan exposure using the same animal model (Varayoud et al., 2011, 2012) show that different endocrine-disrupting compounds impair fertility by altering signaling pathways similar to those described for BPA.

5. CONCLUSIONS

BPA effects on reproduction and specifically on the uterus physiology have been the focus of numerous studies. Using a rat model of a brief early postnatal exposure, we detected that low doses of BPA affect the course of uterine development with lasting consequences. Subfertility was evidenced by a lower number of implantation sites and different alterations in the endocrine pathways that regulate the preparation of the endometrium for embryo implantation. According to our and other results, *Hoxa10* is a common target of endocrine disruptors in uterine tissue and is affected during the early postnatal period. BPA effects observed during the prepubertal period remained during adulthood, and some *Hoxa10*-downstream events were fully disrupted. We propose that an early alteration in *Hoxa10* gene expression affects functional differentiation of the uterus during pregnancy as part of an altered endocrine signal transduction pathway.

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