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Epigenetic Transgenerational Actions of Endocrine Disruptors

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Endocrine disruptors have recently been shown to promote an epigenetic transgenerational phenotype involving a number of disease states (*e.g.* male infertility). The anti-androgenic fungicide vinclozolin was found to act transiently at the time of embryonic sex determination to promote in the F1 generation a spermatogenic cell defect and subfertility in the male. When the animals were allowed to age up to 1 yr, a number of other disease states developed. This phenotype was transferred through the male germ line to all subsequent generations analyzed (F1–F4). The ability of an environmental factor (*i.e.* endocrine disruptor) to promote an epigenetic transgenerational phenotype impacts the potential hazards of environmental toxins, mechanisms of disease etiology, and evolutionary biology. The biological importance of the epigenetic actions of environmental agents is reviewed in the context of the primordial germ cell and development of epigenetic transgenerational phenotypes. (*Endocrinology* 147: S43–S49, 2006)

GENOMIC DNA CONTAINS the core of genetic information of the cell. There is a distinct pattern of gene expression throughout mammalian development that is heritable from parents to offspring. Epigenetics is defined as the molecular phenomena that regulate gene expression without alterations to the DNA sequence (1). The most studied epigenetic modification is DNA methylation of CpG nucleotides that are essential for mammalian development (2–5). DNA methylation of CpG sites is used by mammals to regulate transcription of genes, alter chromosomal positioning, influence X-chromosome inactivation, control imprinted genes, and repress parasitic DNAs (1, 5–9). Alterations in the DNA methylation state can lead to multiple disease states including cancers (10, 11), Rett syndrome, and Prader-Willi/Angelman syndrome (11–13), male infertility (14), autism (12), and Angelman and Beckwith-Wiedemann syndromes (13). Both chemical and environmental toxins have been shown to alter DNA methylation patterns resulting in epigenetic phenotypes (14, 15).

DNA methylation patterns are established at two times during development: the lineage-specific pattern during gastrulation and the germ-line-specific pattern in the gonad after sex determination (16). The lineage-specific pattern establishes the DNA methylation for somatic cell development after fertilization. This epigenetic reprogramming is based on the genetic material transferred from the egg and sperm. Alterations in the lineage-specific epigenetic reprogramming results in developmental defects or embryonic lethality (13, 16). The germ-line DNA methylation pattern is established during gonadal development and is sex specific (16–18). Epigenetic reprogramming of the germ line is critical for imprinting (19–22). Unlike the lineage-specific reprogramming, alterations in the germ-line epigenetic reprogramming can alter the heritable epigenetic information, resulting in a transgenerational phenotype (15) (Fig. 1). The embryonic period is the most sensitive for chemical and environmental effects on the epigenetics of the male germ line (15, 21, 22).

Recent investigations of the DNA methylation state of the primordial germ cells have indicated that as primordial germ cells migrate down the genital ridge, a demethylation (*i.e.* erasure of methylation) starts, and upon colonization in the early gonad, a complete demethylation is achieved (21–23). This has been primarily observed through the analysis of specific imprinted genes (24). During the period of sex determination in the gonad, the germ cells undergo a remethylation involving a sex-specific determination of the germ cells (Fig. 2). Although the demethylation may not require the gonad somatic cells (21), the remethylation of the germ line appears to be dependent on association with the somatic cells in the gonads (22, 23). Because of this unique property of the germ cells to undergo a demethylation and remethylation during the period of sex determination in the developing gonad, the ability of an environmental agent such as an endocrine disruptor to influence through an epigenetic process the germ line is postulated. This epigenetic effect on the germ line could reprogram the germ cell through an event such as altered DNA imprinting (25, 26). This epigenetic effect could cause a transgenerational effect on subsequent generations through the germ line. Because the remethylation of the germ line appears dependent upon the gonadal somatic cells, an alteration in somatic cell function by an agent such as an endocrine disruptor could indirectly influence the germ cell remethylation (Fig. 2). Epigenetic alterations that lead to transgenerational transmission of specific genetic traits or molecular events (*e.g.* imprinting) have recently been identified (6, 7, 27). These observations have led to the conclusion that a reprogramming through altered epigenetics of the male germ line is possible (15). The impact this has on human health and evolutionary biology is significant (6, 27).

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Abbreviation: AR, Androgen receptor.

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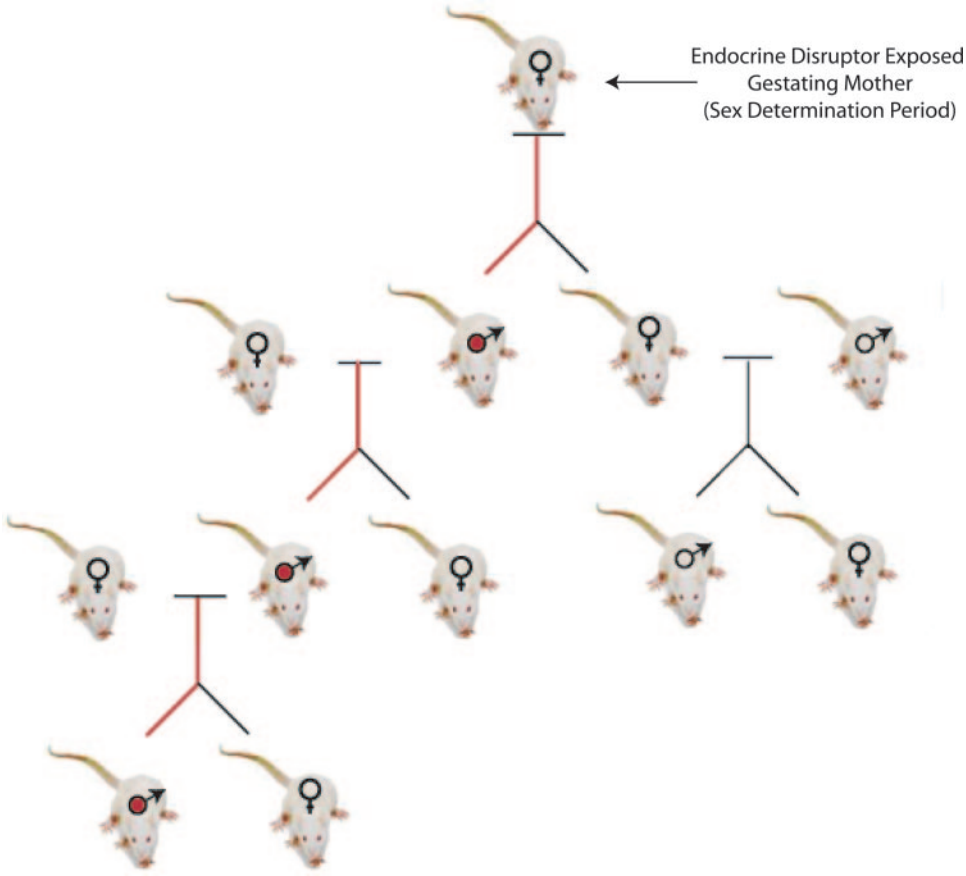

FIG. 1. Epigenetic transgenerational actions of endocrine disruptors through the male germ line.

Transgenerational Phenomena and Environmental Factors

Environmental effects of irradiation, chemical treatments (*e.g.* chemotherapy), and environmental toxins such as endocrine disruptors have been observed over the past decade. The majority of observations are simply the effects of the agent on the gestating mother (F0) and subsequent actions on the offspring associated with the F1 generation (28–30). Examples of environmental factors during embryonic development that influence the F1 generation include the effects of heavy metals causing cancer (31), abnormal nutrition that causes diabetic and uterine defects (32–34), chemical exposure (*i.e.* ethosuximide and benzpyrene) causing brain and endocrine defects (35, 36), and endocrine disruptors such as diethylstilbestrol (37, 38), phthalates, and dithiothreitol causing reproductive tract and endocrine defects (39–41). Environmental factors have effects on the F1 generation of a number of species including insects (42–44), fish (45, 46), birds (47), and other species (48). Therefore, exposure to a number of environmental factors *in utero* can cause abnormal phenotypes in the F1 generation in a number of different species. Because the F1 generation is exposed to the environmental factor, the F1 effect is not a transgenerational phenotype.

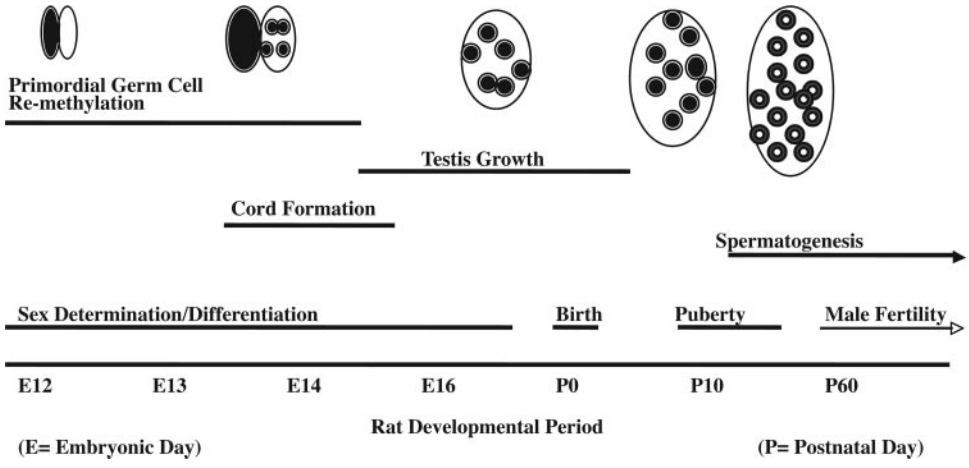

FIG. 2. Rat testis developmental timing and processes.

Transgenerational effects of environmental factors require effects minimally on the F3 generation (15, 49) (Fig. 1). This is because the F3 generation is the first generation not directly exposed to the environmental factor. The ability of an external agent to induce a transgenerational phenotype requires a genetic (*i.e.* DNA sequence) or an epigenetic (*i.e.* DNA methylation) phenomenon mediated through the germ line (50–53). Transgenerational inheritance of an epigenetic state has been shown to occur using several mouse genetic lines and markers (6, 27, 54) and more recently with the use of monozygotic twins with epigenetic differences (55). Irradiation exposure was one of the first transgenerational phenomena observed to be transmitted through the germ line to multiple generations, often associated with mutagenesis and tumor formation (50–53). The chemotherapeutic treatment of cancers has been shown to cause F1 generation effects (31, 35, 46), but the transmission to multiple generations has not been thoroughly investigated. Environmental factors do appear to promote a transgenerational susceptibility to cancer (56, 57). Gestating nutritional deficiency effects on the F1 generation have been observed (34), and recently these nutritional effects on a diabetic condition and growth defects have been shown to be transgenerational to the F2 generation (58–60). Several environmental chemical exposures have also been shown to transgenerationally affect the F2 generation including benzpyrene (36, 61), orthoaminoasotoluol (62), and dioxin (63). Environmental toxins such as endocrine disruptors have also been shown to influence the F1 generation after parental exposure (39, 46, 64–67), but few have demonstrated transgenerational effects on multiple generations (15). Some evidence that diethylstilbestrol has effects in the F2 generation have been reported (68).

Endocrine Disruptors and Reproductive Toxicology

Many reports have suggested that environmental endocrine disruptors, which act to mimic estrogens or act as antiestrogens or antiandrogens, are detrimental to reproduction and may promote abnormalities such as a decrease in sperm count, an increase in testicular cancer (69, 70), and an increase in abnormalities in sex determination for many species (71). Examples of environmental endocrine disruptors that have been targeted for adverse effects on reproductive systems in humans and other animals are pesticides [*e.g.* dichlorodiphenyltrichloroethane (DDT) and methoxychlor] (72), fungicides (*e.g.* vinclozolin) (15, 73), insecticides (*e.g.* trichlorfon) (74), herbicides (*e.g.* atrazine) (75), plastics (*e.g.* phthalates) (76), and a range of xenoestrogens (77). Most of these chemicals are ubiquitous in the environment, resulting in daily exposure for humans and other animals. Many of these compounds and endocrine disruptors can be metabolized into both estrogenic and antiandrogenic activities (78). Recently, methoxychlor and vinclozolin have been used (66, 67) as model endocrine disruptors (72) that have estrogenic, antiestrogenic, and antiandrogenic metabolites (78).

Many environmental endocrine disruptors are weakly estrogenic and elicit their actions through the estrogen receptors. The two mammalian receptors for estrogen (ER-α and ER-β) are widely distributed throughout the reproductive tract (79, 80). ER-β is present in higher concentrations within the fetal testis and ovary, whereas ER-α is present mainly within the uterus (81, 82). During fetal testis development, ER-β is expressed in Sertoli and myoid cells after seminiferous cord formation (83). In rats, ER-β has also been localized to prespermatogonia, which may explain the proliferative actions of estrogen on early postnatal gonocyte cultures (84). The importance of ER-α was delineated when knockout mice (85) and human males (86) lacking expression of this gene were found to be sterile. Fetal development of the testis in these experiments was not altered; however, fetal testis morphology in a double knockout remains to be examined (87). Neonatal exposure to estrogen alters the ER-α and ER-β expression during postnatal testis and hypothalamic/pituitary development (88, 89). Interestingly, neonatal exposure to the estrogenic compound diethylstilbestrol promotes abnormal testis and male reproductive tract development (90) and leads to changes in gene expression (91). Therefore, actions of estrogenic endocrine disruptors on estrogen receptors may impair normal fetal gonadal development and lead to infertility. Although the estrogen receptors are thought to have a role in testis development (92–94), the specific functions remain to be elucidated. Treatment of males with estrogens during early fetal life may alter responsiveness to androgens by changing androgen receptor (AR) expression patterns (95, 96) and/or Leydig cell function (91).

Antiandrogenic endocrine disruptors can also influence fetal gonad development. AR expression is very similar to ER-β expression in the developing testis (82, 97). AR is detected in Sertoli, myoid, and prespermatogonial cells just after cord formation (98) and in interstitial cells late in fetal development. It is proposed that AR is present in cells that migrate from the mesonephros and enables cord formation to occur (98). Therefore, inappropriate expression or actions of AR through treatment by endocrine disruptors may affect the process of morphological sex differentiation (*i.e.* cord formation). Antiandrogens such as flutamide (99) or cyproterone acetate (100) administered to pregnant rats at different ages of gestation impair fertility in the male offspring. Both flutamide and cyproterone acetate block the ability of androgens and epidermal growth factor to stabilize the Wolffian duct (101). Therefore, perturbation of AR may also cause inappropriate expression and action of growth factors in the testis. A commonly used antiandrogenic endocrine disruptor is vinclozolin, which is used as a fungicide in the wine industry (102, 103). Vinclozolin has been shown to act as an environmental antiandrogen and influence gonad development and fertility (15, 67).

Epigenetic Transgenerational Actions of Endocrine Disruptors

A recent observation demonstrated that the exposure of a pregnant rat transiently to endocrine disruptors caused a spermatogenic cell defect and subfertility in the F1 generation and all subsequent generations examined (F1–F4) (15) (Fig. 1). The endocrine disruptors used were the antiandrogenic fungicide vinclozolin used in the fruit (*e.g.* wine) industry (73) and the pesticide methoxychlor used to replace dichlorodiphenyltrichloroethane (DDT) (78). The critical exposure period was at the time of sex determination, and the

transgenerational phenotype was transmitted through the male germ line (15) (Fig. 1). The phenotype of increased spermatogenic cell apoptosis and decreased sperm numbers and sperm motility was observed in greater than 90% of all males of all the generations examined. When the animals were allowed to age up to 1 yr, additional diseases developed including cancer, prostate disease, kidney disease, and immune cell defects (Anway, M. D., and M. K. Skinner, submitted for publication). A high frequency of transmission was observed in all generations examined for all the disease states.

The frequency of the transgenerational phenotype was such that a DNA sequence mutational event could not be involved. The random nature of a DNA sequence mutation has a phenotype typically less than 1%, and this often declines in subsequent generations (50, 104). An epigenetic mechanism is involved because of the frequency of the phenotype. To support these conclusions, two genes were identified in the sperm that had altered methylation patterns associated with the transgenerational phenotype discussed (15). Therefore, the endocrine disruptors appear to induce an epigenetic transgenerational disease condition for four generations through the male germ line (15) (Fig. 1). The epigenetics appears to involve altered DNA methylation. Although most genes get reset in early embryonic development, a subset of genes called imprinted genes maintains their DNA methylation pattern that appears to be permanently programmed. In contrast to all somatic cells, the primordial germ cells undergo a demethylation during migration and early colonization of the embryonic gonad, followed by a remethylation starting at the time of sex determination in a sex-specific manner (23, 24, 105). The exposure of the pregnant mother at the time of sex determination appears to have altered the remethylation of the germ line and permanently reprogrammed the imprinted pattern of DNA methylation (15). This provides a unique epigenetic mechanism to promote a transgenerational phenotype induced by an environmental factor.

Summary

The observations that an environmental toxin (*e.g.* endocrine disruptor) can have an epigenetic effect on the germ line and cause a transgenerational effect on male reproduction significantly impacts our understanding of the potential hazards of these compounds to human health as well as all other mammalian species (15). These studies establish a novel mechanism of action not previously appreciated on how environmental toxins may act on a gestating mother to influence her grandchildren and subsequent generations. Elucidation of this phenomenon will allow us to better understand the true hazards of environmental toxins, identify the specific causal agents, and develop appropriate preventative and therapeutic approaches. Independent of the specific compound or agent of interest, the establishment of this potential mechanism of action is critical to our insight into the effects of environmental factors that influence embryonic development and adult reproduction.

The level of endocrine disruptors used in the recent studies (15, 66, 67, 106) (Anway, M. D., and M. K. Skinner, submitted for publication) is higher than anticipated in the environment, such that conclusions regarding the toxicology of these endocrine disruptors are not possible. However, the important factor is the identification of this novel phenomenon, that an environmental factor can promote an epigenetic transgenerational phenotype (15). Because of this observation, the potential hazards of environmental factors need to be carefully evaluated. If the exposure of your grandmother at midgestation to environmental toxins can cause a disease state in you with no exposure, and you will pass it on to your grandchildren, the potential hazards of environmental toxins need to be rigorously assessed. Transgenerational studies need to be performed in evaluating the toxicology of environmental compounds.

The epigenetic transgenerational phenotype also provides critical insights into disease etiology. Because a number of common disease states are induced (Anway, M. D., and M. K. Skinner, submitted for publication), an epigenetic component of disease now needs to be seriously considered. The fetal basis of adult-onset disease could be a result of epigenetic factors (107, 108). In the event a major epigenetic component exists, the epigenetic background of an individual may be a significant factor in susceptibility to disease development. Therefore, identification of the genes involved with altered methylation may provide essential new diagnostics to assess onset of disease. These epigenetic factors may influence the outcomes of current medical therapies such as assisted reproductive procedures (109, 110). Further analysis of the epigenetic transgenerational phenotypes and identification of specific epigenetic changes will allow new therapeutic targets and therapies to be developed to potentially prevent the onset of disease. This is a new paradigm in disease etiology that needs to be considered.

In a broader biological perspective, the ability of an environmental factor to cause a permanent genetic trait in all subsequent progeny of an effected individual can significantly impact our understanding of evolutionary biology. Currently, a DNA sequence mutation event that allows an adaptation and natural selection is considered the driving factor in evolutionary biology. However, the frequency of specific evolutionary events (110, 111) and regional influences on evolution suggest an additional epigenetic mechanism should be considered (112–115). Although a DNA sequence mutational event will be important for evolutionary biology, an epigenetic component influenced by an environmental factor needs to be considered as an alternate factor that will help explain some aspects of evolutionary biology. Epigenetics is the next layer of complexity beyond the DNA sequence.

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