Epigenetic Transgenerational Effects of Endocrine Disruptors on Male Reproduction

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ABSTRACT

Endocrine-disrupting chemicals generally function as steroid receptor signaling antagonists or agonists that influence development to promote adult-onset disease. Exposure to the endocrine disruptors during the initiation of male reproductive tract development interferes with the normal hormonal signaling and formation of male reproductive organs. In particular, exposure to the endocrine disruptor vinclozolin promotes transgenerational transmission of adult-onset disease states such as male infertility, increased frequencies of tumors, prostate disease, kidney diseases, and immune abnormalities that develop as males age. An epigenetic change in the germ line would be involved in the transgenerational transmission of these induced phenotypes. Nevertheless, other studies have also reported transgenerational transmission of induced epigenetic changes, without altering the germ line. Here we propose a nomenclature to help clarify both cases of transgenerational epigenetic transmission. An *intrinsic epigenetic transgenerational process* would require a germ-line involvement, a permanent alteration in the germ cell epigenome, and only one exposure to the environmental factor. An *extrinsic epigenetic transgenerational process* would involve an epigenetic alteration in a somatic tissue and require exposure at each generation to maintain the transgenerational phenotype.

KEYWORDS: Transgenerational, epigenetic, endocrine disruptors, germ line, DNA methylation

Several reports have shown in a variety of organisms that endocrine-disrupting chemicals common in the environment can induce changes in reproductive parameters. Endocrine-disrupting chemicals generally function as steroid receptor signaling antagonists or agonists that influence development to promote adult-onset disease.[1] Among the compounds producing these phenotypes are plant-produced endocrine disruptors such as phytoestrogens,[2,3] synthetic endocrine disruptors such as the pharmacological estrogenic substance diethylstilbestrol (DES),[4–6] the plastic components bisphenol A (BPA)[7,8] and phthalates,[9–11] and the fungicide vinclozolin.[12–17] A particular area of interest is the effect of endocrine-disrupting chemicals on male reproductive organ development. Exposure to the endocrine disruptors during the initiation of male reproductive tract development interferes with the normal hormonal signaling and formation of male reproductive organs. When a genetic male is exposed to antiandrogenic agents during this critical period of sexual differentiation, the androgen-dependent differentiation of the Wolffian-derived structures is disrupted to promote abnormal development of the male reproductive tract and genitalia.[18] This developmental

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period is also when the process of germ-line segregation from somatic cells takes place.

MALE GERM-LINE SEGREGATION AND ENDOCRINE DISRUPTORS

The process of germ-line segregation from somatic cells in different organisms occurs either through (1) "preformation," when germ cell differentiation is determined by the localization of maternally inherited determinant factors before or immediately after fertilization, or through (2) "epigenesis," when germ cells arise as a result of inductive signals from surrounding tissues.[19] In metazoans, epigenesis would be the main mechanism of germ cell specification,[19] suggesting the segregation of the germ line would be susceptible to environmental influences on these organisms. The functional activation of the male and female reproductive systems is an occasion during which environmental signals, including endocrine disruptor chemicals, could act to alter normal physiology.[18] In males, prenatal exposure to DES produces several developmental abnormalities in the male mouse reproductive tract and increases tumor incidence.[4] Embryonic exposure to methoxychlor during the period of sex determination affects embryonic testis cellular composition and germ cell number and survival.[20] Embryonic testicular cord formation is also affected when embryos are exposed in vitro to vinclozolin, and transient in utero exposure to vinclozolin increases apoptotic germ cell numbers in the testis of pubertal and adult animals, which correlates with reduced sperm motility and number in the adult.[21] In utero exposure to phthalates are also shown to disrupt differentiation of androgen-dependent tissues in male rat offspring.[9,10] In addition to in utero effects, endocrine disruptors are shown to act on the male reproductive tract having consequences not only in the directly exposed offspring but in the subsequent generations of offspring as well. Newbold et al[6] have shown that after administering DES to pregnant rats during early postimplantation development and also neonatally, a greater susceptibility for specific tumor formation in rete testis and reproductive tract tissues not only occurred in the F1 generation but reappeared in the nonexposed subsequent F2 generation. However, when the F1 generation embryo is exposed in the maternal uterus, the F2 generation germ line is also directly exposed. Therefore, a definitive confirmation of a transgenerational phenomenon would be to observe changes in the F3 generation.[22]

ENDOCRINE DISRUPTORS AND TRANGENERATIONAL PHENOTYPES IN MALES

Research in our laboratory has focused on epigenetic transgenerational effects of the endocrine disruptor vinclozolin in the male germ line of rats. Vinclozolin is a fungicide commonly used in agriculture that is known for its antiandrogenic endocrine-disrupting action.[23,24] Exposing a pregnant rat to either vinclozolin or methoxychlor during embryonic days 8 to 14, a critical period for testis sex differentiation and testis morphogenesis, produces transgenerational defects in spermatogenic capacity, which are transmitted through four generations (F1 to F4).[12] The transgenerational phenotypes observed in these animals also include adult-onset diseases such as male infertility,[12,14] increased frequencies of tumors, prostate disease, kidney diseases, and immune abnormalities that develop as males age.[13] Changes in behavior and learning capacity have also been observed following vinclozolin exposure,[25–29] including transgenerational changes in mate preference[26] and anxiety behavior.[29] Transgenerational effects on tissue transcriptomes have also been observed. For example, in the embryonic testis transcriptome a subset of genes have their expression altered in a consistent manner in males from the F1 through the F3 generation.[30] The transgenerational effects of vinclozolin are not mimicked by the antiandrogenic actions of flutamide, suggesting vinclozolin is acting through other mechanisms to promote the transgenerational phenomena.[15]

In considering the molecular mechanism(s) involved in the transgenerational transmission of this adult-onset disease phenotype, DNA sequence mutations need to be taken into account. If heritable damage occurred in the zygote at the beginning of embryonic development, it can be transmitted to the next generation through the altered germ line.[31] In some cases, environmental compounds have been shown to produce that kind of mutagenic response. For example, it has been shown that either chlorambucil or melphalan is capable of inducing heritable deletions and mutations in mouse germ cells.[32,33] Although some endogenous and exogenous agents are associated with DNA mutations, most endocrine disruptors or environmental factors do not promote DNA sequence mutation. These chemicals and factors induce modifications of DNA without altering nucleotide composition,[34] referred to as epigenetic changes.[35] The term *epigenetics* (epigenesis + genetics) was initially described by Waddington to describe "the branch of biology which studies the causal interactions between genes and their products which bring phenotypes into being."[36,37] The study of epigenetic regulation of development has provided significant insights into the molecular mechanisms of gene expression and developmental biology.[38]

EPIGENETIC MECHANISMS OF GENE REGULATION

The most established epigenetic modification shown to be transgenerationally transmitted through the germ line is DNA methylation. This process of DNA modification

constitutes a postreplicative modification, in which a methyl group is added covalently to a DNA residue.[39] The reaction of DNA methylation occurs at the carbon 5 of the cytosine ring in 5′ to 3′ oriented CG dinucleotides (known as CpGs) and is catalyzed by the action of DNA methyltransferases.[40] In addition to DNA methylation, other well-known epigenetic mechanisms are chromatin condensation and histone modifications. There are regions on the chromatin that can be transiently condensed or uncondensed during development, leading to variation in gene expression.[41] These chromatin states are susceptible to modification depending on specific stimuli, such as transcriptional repressors, functional RNAs, or accessory factors that interact with a variety of proteins.[42] Histones, in turn, are susceptible to several posttranslational modifications such as phosphorylation, acetylation, methylation, ubiquitination, sumoylation, adenosine diphosphate ribosylation, glycosylation biotinylation, and carbonylation.[43] Methylation provides an epigenetic mechanism that allows the stable transfer of gene expression profiles to progeny cells.[43] The proposal has been made that an "epigenetic conversation" exists between histones and DNA that involves cytosine methylation and histone modifications acting in synergy to generate a self-reinforcing epigenetic cycle.[44] Small RNAs are the newest epigenetic mechanism that has been described and deals with the action of several classes of small RNAs, ranging from 20 to 31 nucleotides, on regulating gene expression.[45] RNA factors, histone methylation, and chromatin remodeling enzymes appear to cooperate with DNA methyltransferases to establish and maintain methylation site-specific and tissue-specific patterns.[46]

The reprogramming of methylation patterns in mammals occurs during key periods of development, namely after fertilization and during fetal development of the germ line.[47] Major changes in global DNA methylation status occurs in differentiating germ cells.[35,47] Primordial germ cells are substantially methylated before they colonize gonads and then become demethylated around the time of entry into the gonads prior to sex determination.[48] Allelic differences in methylation, which is characteristic of imprinted genes, are also defined during the establishment of the germ line.[49] Imprinted genes conserve their methylation patterns through generations, but the molecular process involved remains to be elucidated.[35] Therefore, if external agents are capable of inducing methylation changes in these imprinted genes or induce new sites during the critical periods of establishment and erasing methylation marks, such changes could flourish and persist transgenerationally.[12,50]

EVIDENCE OF EPIGENETIC CHANGES IN MALE REPRODUCTIVE TISSUES

Exposure to the endocrine disruptor vinclozolin promotes transgenerational transmission of adult-onset disease states.[12,13] These observations have led to test the hypothesis that an epigenetic change in the germ line would be involved in the transgenerational transmission of the induced phenotypes. Changes in DNA methylation were found in epididymal sperm of F1 to F3 generation rats after F0 generation gestating females were exposed to vinclozolin.[12] Currently, we have evidence to support genomewide alterations in DNA methylation patterns of F3 vinclozolin generation sperm using Chip-Chip and Chip-Seq technologies (unpublished data). Other studies examining F1 generation epigenetic effects have been obtained as a consequence of exposure to BPA, although follow-up to the F3 generation has not been performed. Exposure to environmentally relevant doses of BPA during the neonatal developmental period in rats increases susceptibility to precancerous prostatic lesions as animals age and also sensitizes the prostate gland to adult-onset carcinogenesis.[51] Several genes exhibit methylation changes in response to neonatal BPA treatment, many of which appear permanent.[51] Changes in methylation would also explain reappearance of the increased susceptibility for tumor formation in F2 generation mice after developmental exposure to DES.[6,52]

CONCLUSIONS

The majority of environmental exposures and epigenetic effects will not involve transgenerational phenomena. An epigenetic transgenerational phenotype will generally involve a germ-line mediated process with a permanent alteration in the epigenome. This process would require a critical period of exposure and alteration in imprinted-like sites. However, the majority of exposures will involve somatic tissue effects rather than germ-line effects. Critical developmental periods for the somatic tissue and alteration in the epigenome will be involved. This somatic cell effect will influence disease states in the individual exposed but would not promote a transgenerational phenomena. Although exposure at each generation could promote a transgenerational phenotype, once the exposure is terminated the somatic cell effect would be lost. An example of such an extrinsic effect is the ability for good maternal care early postnatally to induce epigenetic alterations in brain development that then promotes a female to initiate the next generation to provide good maternal care.[53] Once the good maternal care is terminated, the phenotype is lost. Therefore, this phenomenon does generate a transgenerational epigenetic phenotype but requires continued extrinsic exposure at each generation. We propose to classify these two types of transgenerational phenomena (Table 1). The first is defined as an intrinsic transgenerational process that requires a germ-line involvement, permanent alteration in the germ cell epigenome, and only one exposure to the

Table 1 Definition of Intrinsic and Extrinsic Epigenetic Transgenerational Processes

Intrinsic epigenetic transgenerational process:
Epigenetic changes in the germ line triggered during embryonic germ cell development that become transgenerationally transmitted in the absence of any subsequent environmental stimuli.
Extrinsic epigenetic transgenerational process:
Epigenetic changes triggered during an organism's development (embryo, postnatal, puberty, adult) that become transgenerational only due to a persistent environmental exposure, generation after generation.

environmental factor. The second is defined as an extrinsic epigenetic transgenerational process that involves an epigenetic alteration in a somatic tissue and requires exposure at each generation to maintain the transgenerational phenotype. Therefore, the intrinsic and extrinsic epigenetic transgenerational phenomena are distinguished by the involvement of the germ line and an isolated exposure versus a somatic cell effect and continued generational exposures (Table 1).

Exposure to chemicals or other environmental agents will induce epigenetic changes in the genome primarily when the exposure is during a critical developmental period.[50,54–56] Intrinsic transgenerational persistence of epigenetic modifications depends on the germ-line epigenome being altered after environmental factors acting on the gonad during germ cell development (i.e., sex determination). In contrast to germ-line development, the sensitive periods for somatic cell development to endocrine disruptors to produce epigenetic chances are many.[57] All these times of exposure offer opportunities for extrinsic transgenerational epigenetic changes to occur. Several examples exist describing such sensitive periods in terms of altering DNA methylation patterns. The period between fertilization and blastocyst implantation is shown to be responsive to the endocrine disruptor 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which produces changes in methylation in the imprinted genes *Igf2* and *H19*.[58]

The pubertal period is critical for prostate development, which is when BPA has been shown to influence the prostate epigenome.[51] Another example involves changes in DNA methylation that are observed as a consequence of maternal care in the first week postpartum.[53] The majority of environmental exposures and effects on the epigenome will involve somatic tissues and not be intrinsically transgenerational. Nevertheless, depending on the transgenerational persistence of environmental stimuli, extrinsic transgenerational processes may also be important as a factor in adult-onset disease. The nomenclature proposed is provided to help clarify the molecular mechanisms involved.

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