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REVIEW

Epigenetic transgenerational toxicology and germ cell disease

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Summary

The ability of an environmental exposure (i.e. endocrine disruptor) during sex determination to reprogramme the male germline and promote an epigenetic transgenerational disease phenotype suggests that environmental factors and compounds may permanently alter the germline epigenome. This epigenetic transgenerational phenomenon will be discussed with respect to adult-onset germline disease (e.g. testicular cancer). A thorough literature review is not provided, rather a perspective is provided on how this epigenetic transgenerational toxicology should be considered in germ cell disease and testicular cancer.

Review

Epigenetics refers to factors around the genome that do not directly involve DNA sequence to regulate the genome. Epigenetic regulation of the genome involves factors such as chromatin structure, histone modifications and DNA methylation. The DNA sequence of the genome is the essential building block of the individual and species. Therefore, the stability of the genome sequence is critical and is not readily mutated, modified or altered. The majority of environmental factors and toxicants have not been shown to directly modify DNA sequence, but promote more complex molecular origins of disease (Cunniff, 2001; Zlotogora, 2003). Many environmental factors from nutrition to environmental toxicants can alter epigenetic factors such as DNA methylation or chromatin structure (Issa, 2002; Gluckman & Hanson, 2004). A consideration of this environment–genome interaction requires epigenetic regulation to be considered as a component of the molecular basis of these environmental interactions with the genome.

A transgenerational phenomenon is defined as the ability of an acquired physiological phenotype or disease to be transmitted to subsequent generations through the germline. A refinement of this definition is that the subsequent generation is not directly exposed to the environmental factor or toxicant. For example, the exposure of a gestating mother exposes the F0 generation mother, the F1 generation embryo and the germline of the F2 generation (Fig. 1). As multiple generations are exposed, the phenotypes in the F0, F1 and F2 generations could be due to the toxicology of the direct exposure and not necessarily transmitted through an alternate mechanism. Therefore, in the example above, the F3 generation would be the first unequivocal transgenerational generation. This does not rule out that the F2 generation does not involve a transgenerational phenotype, but simply points out a limitation to this conclusion due to the direct exposure. A multigenerational exposure can transmit a phenotype due to the toxicology of the direct exposure; however, a transgenerational phenotype requires the transmission of a phenotype independent of direct exposure.

Environmental exposures have been reported to promote several transgenerational disease states (Anway & Skinner, 2006). Generally, an embryonic or early postnatal exposure is required for these transgenerational phenotypes to develop. An example includes the ability of an embryonic diethylstibesterol exposure to promote F2 generation female and male reproductive tract defects (Newbold *et al.*, 1998). Another example is the ability of embryonic nutritional defects (i.e. caloric restriction) to promote an F2 generation diabetes phenotype (Zambrano

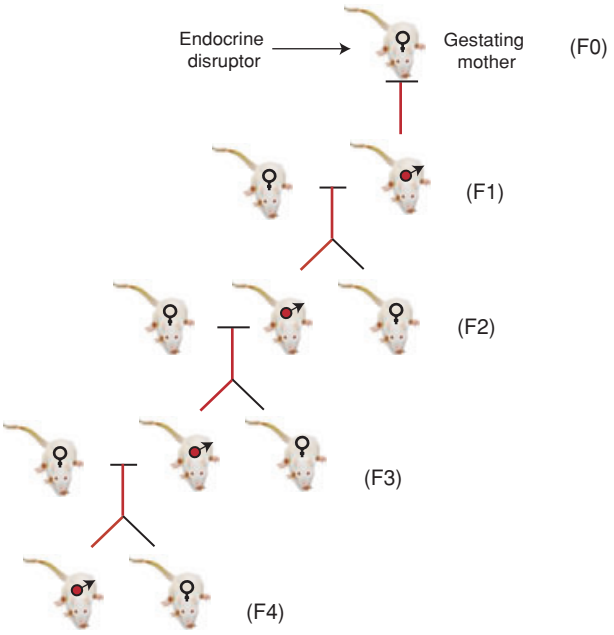

Figure 1 Epigenetic transgenerational adult onset disease transmitted through the male germline.

et al., 2005). The reproducibility and frequency of these disease phenotypes suggests that they are likely epigenetic and not the outcome of DNA sequence mutations. The potential that these exposure phenotypes are epigenetic transgenerational phenotypes remains to be directly demonstrated, but suggests such a phenomenon may exist.

Recently the observation was made that the transient exposure of an F0 generation gestating rat at the time of embryonic sex determination to an endocrine disruptor can promote an adult-onset disease of spermatogenic defects and male subfertility in 90% of all male progeny across four generations (F1–F4) (Anway *et al.*, 2005) (Fig. 1). This transgenerational phenotype was only transmitted through the male germline (sperm) and was not passed through the female germline (oocyte). Currently it is unknown why the phenotype is only transmitted through the male germline, but may involve a protection of the oocyte due to its arrest in meiosis or unique mechanisms found only in male testis development. Prior to 120 days of age the primary disease phenotype was a male testis and spermatogenic cell defect (Anway *et al.*, 2005, 2006b). When the animals were allowed to age up to 1 year the transgenerational disease phenotype included the development of numerous disease states involving a 20% frequency of tumour development, 50% frequency of prostate disease, 40% frequency in kidney disease, 30% immune abnormalities and 30% severe infertility in the males of F1–F4 generations (Anway *et al.*, 2006a). Females also developed transgenerational disease, but did not transmit it to subsequent generations. The clinical pathology and disease phentotypes have been thoroughly described (Anway *et al.*, 2006a), but no testicular cancer was observed. This transgenerational (F1–F4) phenotype was induced by exposure to the endocrine disruptor vinclozolin, which is an anti-androgenic compound used as a fungicide in the fruit industry (e.g. wineries) (Kelce *et al.*, 1994). The phenotype was also promoted by the pesticide methoxychlor which is a mixture of oestrogenic anti-oestrogenic and anti-androgenic metabolites (Anway *et al.*, 2005). The ability of endocrine disruptors to promote adult-onset disease has been previously reviewed (Gluckman & Hanson, 2004). This is a large class of environmental toxicants ranging from plastics to pesticides (Heindel, 2005). The impact of exposure to individual compound vs. a mixture of compounds on this or most phenotypes is unknown and remains to be elucidated. These environmental toxicants generally do not promote DNA sequence mutations. The frequency of the transgenerational phenotype described above (30–90%) also could not be attributed to DNA sequence mutations that occur at a frequency generally less than 0.01% (Barber *et al.*, 2002). The hypothesis was developed that the transgenerational phenotype described above was an epigenetic transgenerational phenotype (Anway *et al.*, 2005; Anway & Skinner, 2006).

The potential epigenetic mechanism involved in this transgenerational phenotype was investigated through the analysis of control and vinclozolin F3 generation sperm DNA (Chang *et al.*, 2006). A methylation-sensitive restriction enzyme analysis identified 25 DNA sequences/genes in the vinclozolin generation sperm that had an altered DNA methylation pattern and most were confirmed with bisulphite sequencing and shown to be transgenerational (Chang *et al.*, 2006). Therefore, the endocrine disruptor exposure during sex determination reprogrammed the epigenetic programming of the developing male germline and induced the presence of new imprinted-like genes/DNA sequences that are transmitted through the male germline (i.e. paternal allele) transgenerationally. This was associated with an alteration in the transcriptomes of different organs and induction of disease phenotypes (Anway *et al.*, 2005, 2006a). Previous studies have shown that the germ cells during sex determination undergo a de-methylation and re-methylation (Yamazaki *et al.*, 2003) such that a reprogramming of DNA methylation of the germline by endocrine disruptors is possible. Therefore, the epigenetic transgenerational phenotype appears to involve an endocrine disruptor (i.e. vinclozolin) exposure during embryonic sex determination to epigenetically reprogramme (i.e. DNA methylation) the male germline and induce new imprinted-like DNA sequences that are transmitted through the male germline to all subsequent

progeny (F1–F4) (Fig. 1) to then cause a dysregulation of the genome to alter transcriptomes in a variety of organs and appear to promote transgenerational disease (Anway *et al.*, 2005, 2006a; Chang *et al.*, 2006).

This epigenetic transgenerational toxicology provides a mechanism for environmental toxicants to promote transgenerational phenotypes and adult-onset disease. A large number of studies have demonstrated that embryonic or postnatal exposures can induce adult-onset disease (Gluckman & Hanson, 2004; Heindel, 2005). The mechanism for this fetal basis of adult-onset disease is largely unknown, but probably, in part, involves alterations in the epigenome. Many adult-onset disease phenotypes are not transgenerational, but manifest in the individual exposed. These individual disease exposures and phenotypes may also involve epigenetic mechanisms. A recent study demonstrated a pubertal exposure to bisphenol A promoted an alteration in DNA methylation of a number of genes and in the adult associated with a high frequency of prostate disease (Ho *et al.*, 2006). Therefore, either an embryonic, postnatal or adult exposure could cause an epigenetic event that alters the physiology of a tissue and promotes disease. It is likely that rapidly developing organs that have the ability to alter a critical developmental step will be more sensitive to environmental exposures and epigenetic modifications. This epigenetic mechanism may be important for individual exposures and disease phenotypes. Therefore, a critical mechanism in the ability of an environmental exposure to induce an abnormal phenotype or physiology will likely be an epigenetic mechanism. Understanding toxicology on a molecular level is essential for the identification of biomarkers and the development of therapies to treat exposures. Epigenetics will be an important process to consider in the investigation of environmental exposures, environment–genome interactions and the toxicology of specific compounds.

The epigenetic transgenerational phenotype previously observed involves the epigenetic reprogramming of the male germline that promotes a spermatogenic cell defect in the adult (Anway *et al.*, 2005, 2006b; Chang *et al.*, 2006). This germ cell defect was detected by an increased spermatogenic cell apoptosis in stage 11 tubules, reduced sperm number and decreased motility (Anway *et al.*, 2005, 2006b). The spermatogenic cell defect increased in frequency as the animals aged and was detected in F1–F4 generations (Anway *et al.*, 2006a). Therefore, an environmental compound (i.e. endocrine disruptor) appears to induce a permanent male germline epigenetic change through the induction of new imprinted-like genes (Chang *et al.*, 2006). The ability of environmental factors to epigenetically reprogramme the germline suggests fetal exposures can promote adult-onset defects and abnormalities in the germline. The possibility that the epigenetic changes in the germline may eventually promote germ cell tumours and testicular cancer now needs to be considered. The rat model used did not develop testicular cancer. Observations demonstrate that environmental exposures during embryonic sex determination can epigenetically reprogramme the male germline to then promote adult-onset spermatogenic cell defects transgenerationally. How this change in the germline epigenome may influence germ cell disease and testis cancer remains to be investigated.

The current hypothesis for germ cell tumours and testis cancer involves the development early in life of carcinoma in situ and subsequently testicular cancer (Rajpert-De Meyts, 2006). Observations suggest abnormal germ cell development and differentiation will eventually lead to germ cell tumour development. The epigenetic transgenerational phenotype described suggests environmental factors may promote an abnormal germline epigenome that can cause germline disease. The possibility that epigenetic abnormalities may be involved in germline tumours and testicular cancer now needs to be investigated. The current focus has been on germ cell chromosomal and DNA sequence abnormalities (Almstrup *et al.*, 2005), but epigenetic changes are also likely involved. Elucidation of the epigenetic mechanisms potentially involved in germ cell disease and tumours will provide insights into how environmental factors may influence the disease, develop a better understanding into the causal mechanism of the disease and develop novel molecular markers for testicular cancer.

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Panel discussion

S. Schwartz (Seattle, WA, USA)

What is the mechanism through which vinclozolin affects DNMT3A levels of expression? Is this an anti-androgenic effect, because we know that vinclozolin is an androgen receptor (AR) antagonist, or are there other downstream factors involved with an eventual effect on DNMT3A?

M. Skinner (Pullman, WA, USA)

During the time of embryonic development of the testis, germ cells and also somatic cells are AR-positive although androgens are not produced by the testis until later in development. The adrenal produces androgens at this time. Vinclozolin has the capacity to act on the AR but we do not know if this is the mechanism involved. There are 25 metabolites of vinclozolin and only a few metabolites are anti-androgenic whereas most are non-anti-androgenic although they may have a toxic effect. My speculation is that there is a toxicological effect interfering with somatic cell function causing subsequent delayed germ cell development, and I am not sure if this is an anti-androgenic effect. Methoxychlor has the same effect but acts through a different mechanism from vinclozolin. It is not known how the expression of DNMT3A is altered.

O. Lindegaard (Denmark)

Your diagram suggested that the demethylation process which occurs just after fertilization does not involve imprinted genes. Therefore, you imply that the imprinted genes are not demethylated during the time of either gametogenesis or formation of gonads.

M. Skinner

There are examples of imprinted genes which are not demethylated, but some imprinted genes are demethylated then remethylated to their original pattern. During demethylation of the germline we are not sure if imprinted genes are demethylated and remethylated, or if they retain their state of methylation. Imprinting is not a simple process and we have still much to learn.

O. Lindegaard

Other studies have suggested that imprinting has an adaptive function in the fetal environment. If there are no changes, it is difficult to imagine how it is adaptive at the same time.

M. Skinner

This is an adaptive evolutionary event which induces a permanent change in the germline and any advantage is

transferred to the developing animal. Methylation of the genome is a random event. We have studied 25 different lines of animal and we do not know yet if there has been a similar methylation pattern every time indicating that this is a very conserved mechanism.

D. Page (Cambridge, MA, USA)

How permanent are these epigenetic changes? Firstly, you demonstrated equal changes in second, third and fourth generations in male animals, but are they also propagated with equal fidelity in female animals? Secondly, the transcriptional profiles seen in second and third generations appear to be regressing toward the wild type, suggesting that they may not be permanent.

M. Skinner

The changes seen in succeeding generations are reproducible and occur with similar frequencies in all generations with no reduction in effect. This indicates that there is no segregation of the initial event from the genetic event. The changes are only propagated through male animals and not through females. This raises the question of how a paternal allele can communicate with a maternal allele resulting in non-segregation for the next generation. This may act through the Y chromosome. Another mechanism is a recently described phenomenon first identified in plants whereby one allele can communicate with another allele by a permutation event probably acting through small RNA molecules.

Secondly, exposing pregnant rats to endocrine disruptors has direct effects on three generations: the mother in the first generation (F0), the embryo in the second generation (F1) and the germline in the third generation (F2). It is only the next generation (F3) which has not been directly exposed and these animals must be examined in a truly transgenerational study. Analysing transcriptions in the testis following direct exposure, we see 1500 genes affected in F1 animals, 700 genes affected in F2 animals and 200 genes affected in F3 animals. These 200 affected genes in the F3 animals are the permanent changes, and are seen in all organ systems examined; whereas the majority of the affected genes normalize to the wild type and this phenomenon is not permanent. There is partial but not complete regression back to the control situation. The non-permanent changes are likely due to methylation of metastable alleles.