Chapter 2

Definition of Epigenetic Transgenerational Inheritance and Biological Impacts

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Chapter Outline

INTRODUCTION AND HISTORICAL CONTEXT

For this review of epigenetic transgenerational inheritance, it will be useful to provide some historical context. To that end, the initial experiments from the authors' laboratory describing the phenomenon of epigenetic transgenerational inheritance will be outlined, and issues related to investigations in this field will be discussed using these studies as an example.[1]

Historically, the authors were investigating the actions of two environmental toxicants (the fungicide vinclozolin and the pesticide methoxychlor) following an exposure of an F0 generation gestating rat during fetal gonadal sex determination, and characterizing the effects of the toxicants on gonadal development and function.[2,3] A serendipitous observation was made when a postdoctoral fellow in the laboratory mistakenly bred the F1 generation to generate an F2 generation. Upon analysis of the F2 generation testis phenotype, over 90% of the males had a spermatogenic cell apoptosis defect. This prompted a multiple year study to further investigate this surprising observation and culminated in one of the first observations of the environmentally induced epigenetic transgenerational inheritance of disease.[1] This study demonstrated the transmission of testis spermatogenic cell apoptosis to the F1, F2, F3, and F4 generations, as well as to an outcrossed generation in which affected male rats were bred to wild-type females. This phenotype was transmitted through non-Mendelian genetic inheritance and affected 90% of the male population. The epigenetic effects on the sperm were identified as changes in DNA methylation at specific sites.[1] The impact of this study on our understanding of the molecular control of inheritance, disease etiology, and environmental toxicology is significant.

Over the past 10 years, a series of studies have further investigated this phenomenon in a number of different laboratories. Although an exhaustive review of the literature is not provided here, the major issues are discussed. There are specific issues that need to be addressed in order to better understand epigenetic transgenerational inheritance. These include environmental factor specificity, i.e., whether different environmental toxicants would produce similar phenomena. Another issue is germline epigenetics and the mode of transmission of disease transgenerationally. A final issue involves disease etiology, or how it is that an epigenetic change during fetal development can result in a disease phenotype in adult animals several generations later.[4,5]

EPIGENETIC TRANSGENERATIONAL INHERITANCE

The definition of epigenetic transgenerational inheritance is "germline-mediated inheritance of epigenetic information between generations in the *absence* of direct environmental influences, that leads to phenotypic variation."[6] The initial investigations described above meet these criteria, because after the initial environmental exposure, germline epigenetic effects persist transgenerationally in the absence

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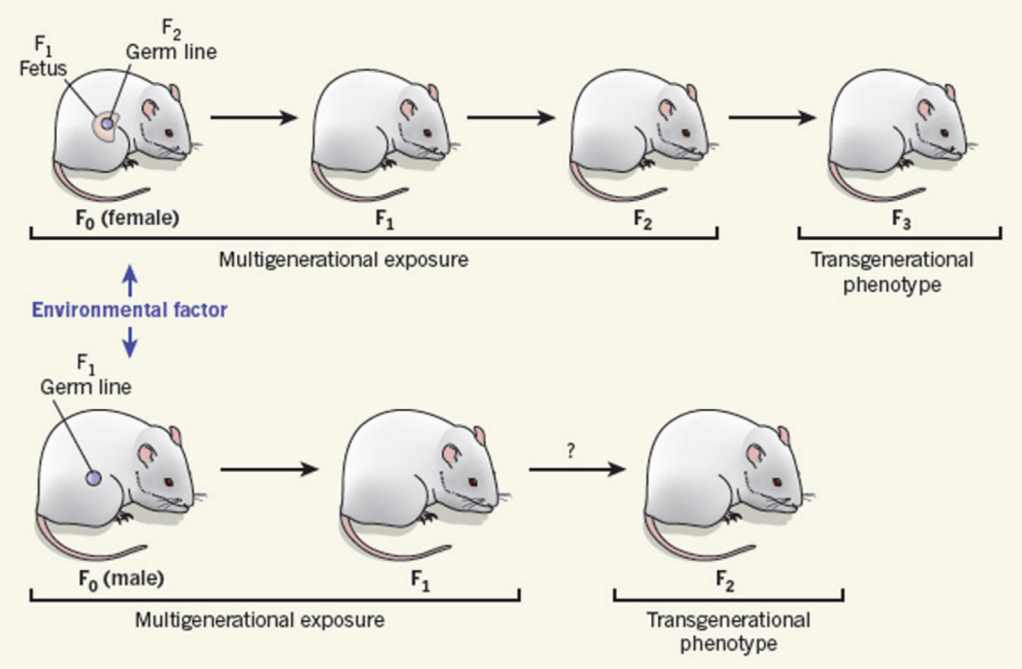

FIGURE 2.1 Environmental effects across generations. Whereas most environmental factors cannot alter an animal's DNA sequence, many promote epigenetic alterations that influence somatic cells and so the disease status of the individual exposed (F0 generation). In pregnant females, direct environmental exposure may also cause epigenetic modifications in the next two generations (F1 and F2) through the fetus and its germline. The effect of such multigenerational exposure in subsequent generations (F3 and beyond) is considered a transgenerational phenotype. By contrast, multigenerational exposure in males is limited to the F0 and F1 generations. *(Modified from* [48]*).*

of direct environmental influences. Epigenetic transgenerational inheritance is in contrast to epigenetic inheritance that can involve a direct environmental germline or somatic cell exposure leading to epigenetic responses during early development that subsequently influence later-life phenotypes. An example of (non-transgenerational) epigenetic inheritance may be seen in the agouti mouse model that can respond to an environmental exposure *in utero* to affect an epiallele and coat color of the offspring.[5,7] These environmental epigenetic inheritance responses are then corrected in subsequent generations during the normal epigenetic programming of the germline or early embryo such that the phenotype is lost.[8] Another example of epigenetic programming that is not transgenerational is the fetal actions of diethylstilbestul (DES): The children and grandchildren of women treated with DES during pregnancy show abnormalities or increased risk of disease for which no abnormalities have been shown for the F3 (great-grandchildren) generation.[9] A pregnant woman treated with DES would have directly exposed the fetus (F1 generation), as well as directly exposing the germ cells present in that developing fetus. These exposed germ cells would have become the F2 generation (grandchildren). In order to meet the definition of transgenerational epigenetic inheritance, germline-mediated inheritance of epigenetic information between generations must occur in the *absence* of direct environmental exposures. Therefore, in this example, the first unexposed generation is the F3 generation (Figure 2.1). There are multiple examples of multigenerational epigenetic inheritance in the literature that are not transgenerational,[9–11] including several studies in which the term transgenerational is used in a manner inconsistent with the current definitions.[12–18]

In the event the germline is epigenetically reprogrammed during fetal gonad development to become permanently programmed and transmit the phenotype in the absence of direct environmental exposures, then the phenomenon becomes epigenetic transgenerational inheritance.[1,4,5,19–22] Is it possible that a phenotype seen in the F2 generation after exposure of a gestating F0 generation female to a toxicant is transgenerational? If the phenotype was due to an abnormality generated in the germ cell due to direct toxicant exposure, then no; if the phenotype was due to a permanent reprogramming of the germline epigenome, then yes. However, definitive conclusions that the F2 phenotype is transgenerational requires that the F3 generation be produced and examined, to control for the variable of direct F2 generation germline exposure.

The best-characterized molecular mechanism involved in environmentally induced epigenetic transgenerational inheritance involves an environmental factor acting on a gestating female during fetal gonadal sex determination to influence the epigenetic (i.e., DNA methylation) programming so as to induce a permanent imprinted-like site in the fetal germline (Figure 2.2). Imprinted epigenetic sites

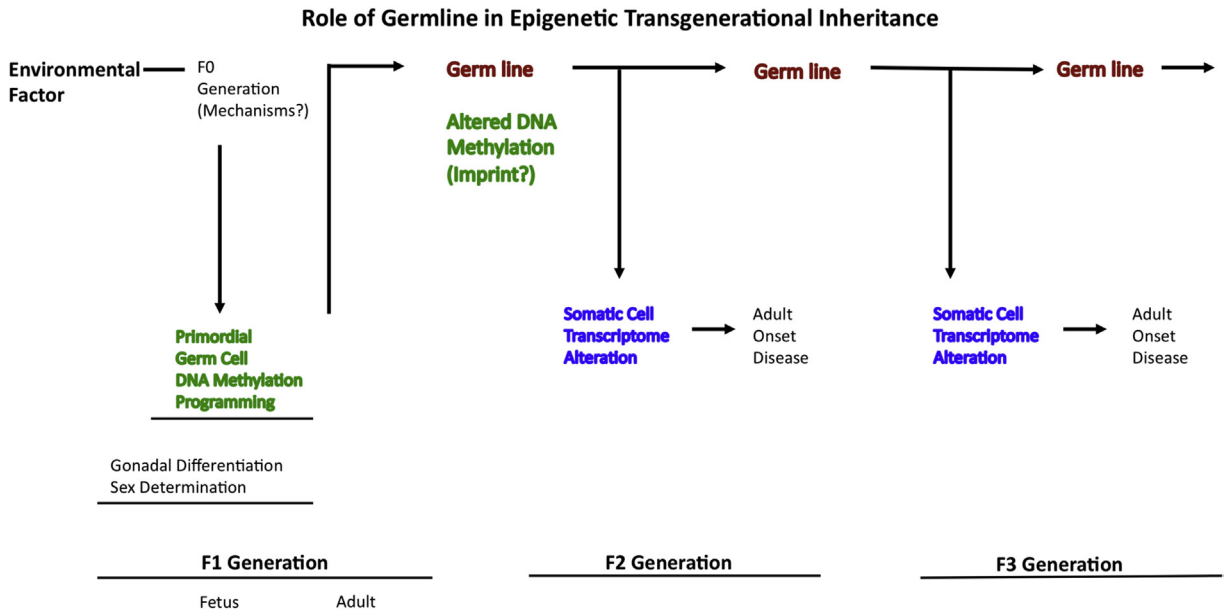

FIGURE 2.2 Role of the germline in epigenetic transgenerational inheritance. (i) An environmental factor acts on the F0 generation gestating female to influence (ii) the developing F1 generation fetus to alter gonadal development to reprogram the primordial germ cell DNA methylation. (iii) This altered DNA methylation in the germline becomes permanently programmed, similar to an imprinted-like gene, and is transferred through the germline to subsequent generations. The embryo generated from this germline starts with an altered epigenome that (iv) affects developing somatic cells and tissues to have an altered epigenome and transcriptome. This altered somatic cell transcriptome can then promote adult-onset disease transgenerationally. *(Modified from*[4]*).*

and genes are defined as "parent-of-origin allelic transmission with monoallelic gene expression." While the transgenerational epigenetic sites do involve a parent-of-origin allelic transmission, the monoallelic expression has not been investigated and may not be involved due to the distal regulation of gene expression. Therefore, the transgenerational epigenetic sites are termed "imprinted-like".[5] Although other epigenetic marks such as histone modifications[23] and non-coding RNA[24] will likely have important roles in transgenerational phenomena, these mechanisms remain to be elucidated. The transmission of this epigenetic information to the next generation affects the epigenome of the developing embryo and embryonic stem cells such that all subsequent somatic cells and tissues generated may have a transgenerational transcriptome change. Previous studies have shown all tissues and cell types investigated do have transgenerational changes in their transcriptomes.[25–27] Those tissues susceptible to an alteration in their transcriptome will have an increased susceptibility to development of disease. This will continue to be transgenerationally inherited due to the altered epigenetic programming of the germline (Figure 2.2).[1,4,5] The following studies described support this molecular etiology of epigenetic transgenerational inheritance.

GERMLINE EPIMUTATIONS

The initial environmental compound (toxicant) used to study epigenetic transgenerational inheritance in the authors' studies was vinclozolin, which is one of the most widely used agricultural fungicides.[1] The outcross information from the initial study indicated that the transgenerational phenotype was transmitted through the male sperm,[1] and so DNA modifications of the sperm were investigated. A genome-wide promoter analysis identified approximately 50 differential DNA methylation regions (DMRs) in the vinclozolin lineage F3 generation sperm in comparison to the control (vehicle-exposed) lineage F3 generation sperm.[28] Since histones and DNA can both be methylated, the term differential methylated regions is not precise, so differential DNA methylation region (DMR) is used instead. These DMRs are termed "epimutations".[4] Therefore, it was established that there was altered epigenetic information in the germline.

A critical issue to resolve was whether the phenomenon was unique to vinclozolin or was also induced by other toxicants. A series of studies investigated the actions of dioxin,[29] a pesticide and insect repellent mix (permethrin and *N,N*-diethyl-m-toluamide (DEET)),[30] plastics (bisphenol A (BPA) and phthalates),[31] and hydrocarbons (jet fuel JP8),[32] all of which were found to promote the transgenerational inheritance of disease and sperm epimutations.[33] Interestingly, each toxicant exposure promoted a unique set (i.e., signatures) of sperm epimutations with negligible overlap between the exposures (Figure 2.3).[33] All these exposures used a pharmacologic treatment to induce the transgenerational response and so were not reflective of risk assessment. The information available can now be used to design more efficient studies to determine the environmental hazards of

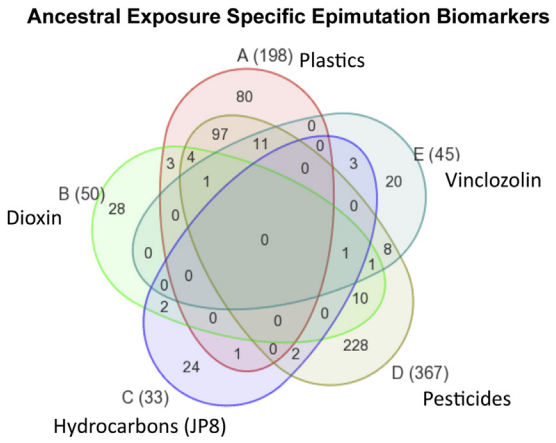

FIGURE 2.3 The transgenerational epimutations associated with each exposure group identified. Venn diagram of exposure epimutation lists of F3 generation rat genes with differential DNA methylation due to ancestral exposure of F0-generation gestating female with: dioxin; pesticide; plastics; or hydrocarbons/jet fuel. *(Modified from [33]).*

these exposures. A number of other laboratories have now shown transgenerational inheritance of disease induced by a variety of environmental factors including nutrition,[34] stress,[35] and other toxicants.[36] Combined observations suggest that epigenetic biomarkers for ancestral exposures and adult-onset disease exist and require future investigation.

TRANSGENERATIONAL INHERITANCE OF DISEASE AND PHENOTYPIC VARIATION

The transgenerational disease or abnormality observed in the initial study was a spermatogenic cell apoptosis defect.[1,37] Subsequently, a number of diseases and pathologies were observed,[38] including prostate disease,[29–33,38,39] kidney disease,[29–32,38] mammary tumor development,[38] immune abnormalities,[32,38] and behavioral effects related to anxiety.[40] Other laboratories have shown transgenerational effects on reproduction,[41,42] stress response,[43] and obesity.[32,44] Many of the transgenerational diseases were found to be induced by any of several different environmental exposures.[33] For example, ovarian diseases, including polycystic ovaries and reduction of primordial follicle pool size, were found in the majority of females from all the toxicant exposure groups examined.[25] Therefore, a wide variety of diseases and abnormalities have been observed to be inherited transgenerationally. The role of environmentally induced epigenetic transgenerational inheritance in the etiology of disease requires further investigation.

In addition to disease etiology, the ability of environmental factors to promote the epigenetic transgenerational inheritance of phenotypic variation certainly has a significant impact on areas of biology such as evolution. A study found that the vinclozolin lineage F3 generation animals have significantly altered mate preference behavior.[45] This variation in behavior phenotype is then available to be acted upon by natural selection. Since sexual selection is a major determinant in evolutionary biology, the observation that an environmental exposure can induce the epigenetic transgenerational inheritance of an altered mate preference[45] suggests transgenerational epigenetics may have a critical role in evolution.[4] Future studies are needed to help establish the role of epigenetics in evolution.

TRANSGENERATIONAL DISEASE ETIOLOGY

Since the epigenome regulates gene expression, transgenerational the germline transmission of epimutations will potentially lead to all somatic cells or tissues having transgenerational changes in their transcriptomes.[4,6,46] The initial study to investigate a transgenerational transcriptome change was performed on the fetal testis.[47] More recently, the authors examined the transgenerational transcriptome changes in 11 different tissues in male and female vinclozolin versus control lineage animals.[26] All tissues had a transgenerational transcriptome change that was unique to each specific tissue with negligible overlap between tissues. Consideration of how a relatively small number of epimutations can promote such a large number of specific transcriptome changes led to evaluation of the genomic locations of the epimutations and differentially expressed genes involved.[26] This led to the identification of "epigenetic control regions," involving regions of 2–5 megabases with statistically significant over-representation of regulated genes within the vicinity of both epimutations and long non-coding RNA. The long non-coding RNA is proposed to mediate the regional distal gene regulation (Figure 2.4).[26] Such observations suggest that epigenetic regulation of genomic activity may involve unique molecular mechanisms that require further investigation.

In researching how the epigenetic transgenerational inheritance of a germline epimutation can promote an adult-onset disease, investigations studied both testicular and ovarian disease. The ovarian diseases of polycystic ovary disease (PCO) and premature reduction of the primordial follicle pool size (i.e., primary ovarian insufficiency (POI)) were found to be transgenerationally induced by the majority of environmental toxicants examined.[25] To investigate the molecular etiology of this disease induction, the ovarian follicle somatic granulosa cells were isolated from younger animals prior to disease onset, and investigated for epigenome and transcriptome alterations. The granulosa cells were found to have an altered epigenome and transcriptome that suggested specific signaling pathways were affected, and a number of the differentially expressed genes were known to be involved in PCO and POI.[25] A similar approach was used to investigate the molecular etiology of the transgenerationally induced testis disease associated with male

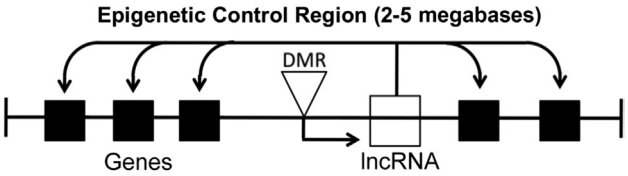

FIGURE 2.4 Schematic summary of an epigenetic control region (ECR). The ECR are 2–5 megabases in size with multiple distal gene regulation under the control of an epimutation (DMR) and long noncoding RNA (lncRNA). The arrows indicate potential distal regulation of expression.

infertility.[27] The somatic Sertoli cells in the testis were also found to have transgenerational alterations in their epigenomes and transcriptomes, with a number of the cellular processes and differentially regulated genes previously shown to be involved in male infertility.[27] Observations support a role for environmentally induced epigenetic transgenerational inheritance of disease via changes in somatic cell gene expression, which requires further investigation.

IMPACT AND FUTURE STUDIES

The novel impact of studies on epigenetic transgenerational inheritance involves the identification of: 1) a non-genetic (i.e. epigenetic) form of inheritance; 2) a non-genetic (i.e. epigenetic) etiology of disease; 3) a molecular mechanism of how environmental factors can indirectly influence genome activity and disease; and 4) the existence of the epigenetic transgenerational inheritance of phenotypic variation.[4,5] These novel observations conflict with the current major paradigm in science today: "genetic determinism." This dogma is that genetic DNA sequence variation is the mechanism behind all biological phenomena. Epigenetic mechanisms such as epigenetic transgenerational inheritance challenge this dogma and require a paradigm shift in our view of the molecular control of biology. It is crucial to understand that genetics and DNA sequence are essential, but they are simply not the whole story, and it is becoming increasingly clear that epigenetics is an equally important partner in regulating biological function.[4,6] Therefore, future studies need to investigate the role of epigenetics and transgenerational inheritance in disease etiology, evolutionary biology, and all areas of cell and developmental biology. The next steps in the research need to demonstrate the mechanisms of developmental and generational transmission of the germline epimutations, the molecular mechanisms and genomic features of why specific sites are susceptible to transgenerational programming, and the translation of these animal model studies to humans. The further investigation of environmentally induced epigenetic transgenerational inheritance will undoubtedly have significant impact on our understanding of normal biology and disease etiology.

GLOSSARY

Epigenetic transgenerational inheritance Germline-mediated inheritance of epigenetic information between generations in the absence of direct environmental influences that leads to phenotypic variation.

Epimutations Differential presence of epigenetic marks that lead to altered genome activity.

F0, F1, F2, F3 Various generations (parent, offspring, grand-offspring, great-grand-offspring).

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