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**Developmental Origins of Epigenetic Transgenerational Inheritance**

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**ABSTRACT**

Environmental factors can induce epigenetic alterations in the germ cells that can be transmitted transgenerationally. This non-genetic form of inheritance is termed epigenetic transgenerational inheritance and has been shown in a variety of species including plants, flies, worms, fish, rodents, pigs and humans. This phenomenon operates during specific critical windows of exposure, linked to the developmental biology of the germ cells (sperm and eggs). Therefore, concepts of the developmental origins of transgenerational inheritance of phenotypic variation and subsequent disease risk need to include epigenetic processes affecting the developmental biology of the germ cell. These developmental impacts on epigenetic transgenerational inheritance are the focus of this Perspective.

**INTRODUCTION**

The vast majority of environmental factors and toxicants do not have the ability to alter DNA sequence or promote genetic mutations directly [1]. However, many environmental factors can promote abnormal phenotypes or increase the risk of disease. Early life exposures during critical windows of development are one of the most important aspects of this process [2]. These environmental impacts on phenotype and disease risk are often not directly mediated through classical genetic mechanisms, even over the course of many generations; instead epigenetic mechanisms which can affect phenotype from one generation to the next are important [3]. The epigenetic mechanisms involved include DNA methylation, histone modifications, non-coding RNAs, and chromatin structure [3]. The environmental exposures that directly influence these epigenetic processes can range from nutrition, temperature and stress to large numbers of environmental toxicants [1, 3], Table 1. The vast majority of environmental exposures result in direct actions on the somatic cells of specific tissues during critical windows of development, for example to affect the numbers of cardiomyocytes or nephrons which affect the later risk of the exposed individuals to adult onset disease [2], Table 2A. Whilst these effects on somatic cells can have dramatic consequences for the individual, in classical thinking they do not have the ability to pass this phenotype transgenerationally: this is known as Weissman’s barrier that require the germline transmission of information between generations [4], Table 2A. Therefore, germ cell alterations are required to transmit molecular information to the next generation.

More recently, studies showing the ability of environmental exposures to alter the epigenetics of the germline have revealed the potential to promote a transgenerational phenotype [5]. The heritable transmission of environmentally induced phenotypes is referred to as epigenetic transgenerational inheritance [3, 5-7] (Table 2B) and is of particular interest as it may transmit risk of disease across generations in the absence of continued environmental exposures. This non-genetic or non-Mendelian form of inheritance requires epigenetic alterations of a germ cell (sperm or egg) to transmit the environmentally induced phenotypes between generations [1, 3, 8]. The focus of the current Perspective is on the developmental origins of these germline changes and role in epigenetic transgenerational inheritance, not the direct effects involved in multigenerational exposures. The ability to directly expose a germ cell to promote effects in the offspring (i.e. multigenerational exposure) are important, but the ability to promote a permanent epigenetic alteration in the germ cells to be maintained in the absence of the continued environmental exposure suggests a novel form of inheritance and a much greater impact on biology, disease etiology and evolution. Therefore, the literature reviewed will focus on epigenetic transgenerational inheritance.

Although direct exposure multigenerational observations have been made, the initial observation of an environmental factor promoting epigenetic transgenerational inheritance of disease involved an agricultural fungicide, vinclozolin [5, 9]. A wide variety of environmental factors from nutrition to toxicants have now been shown to promote the epigenetic transgenerational inheritance of disease or phenotypic variation [3] (Table 1). The largest group of environmental exposures are toxicants including vinclozolin [5, 9], methoxychlor [5, 10], dioxin [11, 12], the plasticizer compound bisphenol A (BPA) [6, 13], the pesticide dichlorodiphenyltrichloroethane (DDT) [14], phthalates [13, 15] and tributyltin [16]. Unbalanced nutrition, ranging from calorie or protein restriction [17-20] to high fat diets [21, 22], as well as manipulation of micronutrients such as folate [23], have also been shown to promote the epigenetic transgenerational inheritance of disease. Other environmental factors such as temperature [24-27], stress [28, 29], smoking or nicotine [30, 31], and alcohol [32] have also been studied (Table 1). Therefore, a wide variety of different environmental exposures involving different signal transduction mechanisms can promote the epigenetic transgenerational inheritance of disease and phenotypic variation.

Environmental factors have also been shown to promote the epigenetic transgenerational inheritance of disease or phenotypic variation in a wide variety of different species (Table 1). Extremes of temperature, salinity and drought promote abnormal transgenerational phenotypes in plants [24-27]. Nutritional challenges in worms (c. elegans) [33, 34], flies (drosophila) [35, 36], fish [37], pigs [38], rodents [5, 39] and humans [17, 18] have all been shown to promote transgenerational phenotypes. Smoking or nicotine [30, 31] and alcohol [32] induced transgenerational phenotypes in rodents or humans have been observed. Environmental stress has also been shown to influence transgenerational phenotypes in rodents [28, 29] and humans [40-42]. Environmental toxicants have been shown to promote transgenerational phenotypes in rodents [5, 6, 9-16, 43-46], fish [37], plants [24-27] and humans [31], Table 1. In all the species investigated environmental factors have been shown to promote the epigenetic transgenerational inheritance of disease or phenotypic variation. Therefore, the phenomenon is highly conserved among species, supporting an important role for this form of epigenetic transgenerational inheritance.

The ability of environmental factors to promote the epigenetic transgenerational inheritance of diseases and phenotypic variation has significant impact on concepts of the etiology of disease, especially the non-communicable diseases (NCDs) – diabetes, cardiovascular and chronic lung disease and some forms of cancer, and obesity. Epidemiological and molecular studies have shown that fixed genetic variations such as single nucleotide polymorphisms do not explain a substantial fraction of the risk for these diseases at the population level (Table 3A). Nor can such variations explain the dramatic increase seen in the prevalence of NCDs over a few decades globally, or the different patterns of disease between homozygotic twins [47], Table 3A. The environmental exposures associated with disease listed above have not been shown to promote genetic mutations causing disease [3]. Environmentally-induced epigenetic effects can help explain many of these observations.

Evolutionary biology studies have also demonstrated a number of observations that cannot easily be explained by classical genetics alone. These include rapid evolutionary events or microevolution involving disparate phenotypic variations within a species [48-50]. The fusion of classical Darwinian concepts, neo-Darwinian genetic concepts, and neo-Lamarckian environmental epigenetic concepts has suggested a more holistic theory for evolutionary molecular mechanisms [50]. For example, recently the ability of environmental exposures to promote the epigenetic transgenerational inheritance of sperm epimutations has been shown to promote the development of genetic mutations in later generations [51]. Therefore, a combination of epigenetics and genetics will likely influence the long term transgenerational phenotype [51]. Previously it has been shown that altered epigenetics can increase genome instability to promote nearly all forms of genetic mutations [51, 52]. This suggests many evolutionary processes such as genetic assimilation may be in part a function of earlier alterations in epigenetic processes [50, 51]. Therefore, environmentally-induced epigenetic transgenerational inheritance is a non-genetic inheritance mechanism that has dramatic impacts on a wide range of areas of science and medicine [1-3, 50, 53]. Critical elements that need to be considered include the developmental impacts and experimental limitations of epigenetic transgenerational inheritance studies.

**Developmental Biology**

Understanding of developmental biology has now moved on substantially from the concept of a genetic ‘programme’ for development, which protected the embryo and fetus from the influences of environmental factors [54]. In retrospect, it is hard to understand why this idea took so long to be revised to include epigenetic processes. The concept of Waddington (who coined the word ‘epigenetic’ in about 1942) of canalization emphasized that, whilst it protected development from extraneous influences, it was nonetheless a mutable process which provided a degree of developmental plasticity [55]. Today, the fields of evolutionary developmental biology and ecological developmental biology are well established [56, 57]. The transmission of phenotypic variation to offspring via developmental plasticity induced through parental cues forms the basis for parental effects reported in many species [58]. An area in which these concepts has been particularly influential in medicine is the Developmental Origins of Health and Disease (DOHaD). DOHaD research has shown how a range of aspects of the developmental environment, especially those mediated via the parents such as maternal (and to some extent paternal) diet, body composition and health-related behaviors, can affect the development of the offspring. These processes operate within the normal range, not only of diets and lifestyle in contemporary societies, but also in terms of prenatal development. They are not therefore necessarily accompanied by overt differences in phenotype visible at birth, even though epigenetic changes to organs, systems and control mechanisms may have occurred. These physiological phenotype changes are then associated with differences in responses to later environmental challenges such as living in an obesogenic environment which affects the individual’s risk of NCDs (for review see [2]). Environmental and parental stimuli inducing developmental plasticity operate over critical periods, and these have been shown to commence even before conception on the germline [59] and to involve the early embryo [60], the fetus [61] and the newborn [62]. During each of these periods, epigenetic processes have been invoked [63].

 Epigenetic transgenerational inheritance requires the germ-line transmission of altered epigenetic information between generations [1, 3, 5]. Therefore, the cell types and critical windows of exposure to consider involve sperm and egg development and differentiation. The onset of gonadal sex determination corresponds to cell fate determination when a primordial germ cell differentiates into an egg or sperm cell lineage [1, 3, 64]. This occurs during embryonic or fetal day E8 to E14 in the rodent or week 6-18 of gestation in the human [1, 3]. This fetal development of sex determination period in mammals is the initial critical window of exposure, and for other species there is a comparable time of embryonic development when germ cell differentiation is initiated. The other critical window for germ cell development is gametogenesis when differentiated sperm or egg develop. The egg develops later in development when it is arrested in meiosis and differentiates during follicle development [65]. The oogonia that are arrested in the adult female are not actively developing, but do provide a potential target for environmental factors. The susceptibility of the egg to epigenetic alterations at this adult stage of development needs to be further investigated. In contrast, the sperm actively undergo cell differentiation during spermatogenesis in the adult, and so they are potential targets for epigenetic change [1, 3, 65]. The majority of studies have demonstrated that the fetal period of gonadal sex determination is a critical window for environmentally induced epigenetic transgenerational inheritance [5, 6, 9-16, 43-46]. However, recent studies have also demonstrated that effects on the adult male’s spermatogenesis can promote epigenetic transgenerational inheritance of altered phenotypes [42]. They include stress-induced behavioral effects and nutritionally-induced metabolic conditions in rodents [17, 29]. Therefore, these critical developmental periods for germ cell differentiation are the windows of sensitivity for environmental factors to promote epigenetic transgenerational inheritance.

 The sperm and egg periods of development directly correspond to the germ cell epigenetic programming windows [1, 3, 65]. The stem cells for the germ cells are primordial germ cells (PGC) that early in development migrate to colonize the fetal gonad prior to gonadal sex determination [64, 65]. During this migration and colonization the DNA methylation in the PGC is erased for the most part to negligible levels, then, at the onset of gonadal sex determination the germ-line DNA initiates a re-methylation of the DNA in a sex specific manner [1, 3, 64] (Figure 1). The completion of the male germ cell DNA re-methylation is later in fetal gonadal development and after birth in the female germ cell, but the initiation of re-methylation is at gonadal sex determination for both sexes [1, 3, 64], Figure 1. Therefore, environmental exposures at this period of gonadal sex determination has the capacity to alter the epigenetic programming of the germ cells [3]. Although the female germ cell (oocyte) does not have a dramatic regulation of epigenetic processes in the adult, the male germ cell does have epigenetic programming during spermatogenesis as spermatogonia develop into spermatozoa in the adult testis [3]. Environmental exposures have been shown to alter this epigenetic programming [6, 7, 51] and to promote the epigenetic transgenerational inheritance of abnormal physiology [5, 6, 9-16, 43-46]. Therefore, alterations in epigenetic programming of the germ cells at these critical developmental windows promote transgenerational phenotypes and alteration in the epigenetics of the germ cells.

 Environmental factors can readily alter epigenetic processes, but not readily alter DNA sequence [1]. However, the genetic background will directly impact the influence of altered epigenetics. Therefore, any cellular, physiological or biological phenomena will involve a cascade of genetic and epigenetic events to produce a differentiated cell or tissue [66] (Figure 2). A cascade of genetic events leading to a progression of gene expression profiles will interact with the corresponding cascade of epigenetic alterations that occur during cellular differentiation. Although the genetic cascade is less sensitive to environmental factors, the epigenetic processes in an early critical window can be modified such that the subsequent interactions between the epigenetic and genetic events lead to altered differentiation (Figure 2). This in turn leads to an altered gene expression profile and changed susceptibility for disease [66, 67]. The integration of epigenetics and genetics during the early stages of development provides a molecular mechanism for the disease susceptibility and phenotypic variation observed [67]. In addition, these molecular events provide a mechanism for the developmental origins of disease and phenotypic variation [2, 68]. Although most exposures will directly act on somatic cell development and alter the exposed individual’s later life physiology and disease [2], in the event the exposure affects the germ-line and does not get erased at fertilization, the development of an epigenetic transgenerational inheritance phenotype occurs.

 To test this hypothesis several transgenerational disease models have been used to determine if the somatic cells critical to the disease have an altered epigenome and transcriptome. The first is the transgenerational male fertility effects involving spermatogenic cell apoptosis in the adult testis [5]. The adult testis somatic cell that supports the developing spermatogenic cells is the Sertoli cell forming the seminiferous tubule. Sertoli cells from control versus the vinclozolin F3 generation lineage (great grand-offspring) males were found to have a dramatic alteration in both gene expression and epigenetic DNA methylation profiles [69]. The gene expression profile identified could lead to altered pyruvate production which could explain the spermatogenic cell apoptosis observed [69]. A second example involved a female polycystic ovarian follicles (PCO) transgenerational model [70]. Many environmental toxicants promote this PCO transgenerational disease [70]. The granulosa cells within the ovarian follicles prior to the development of the PCO were found to have altered epigenetic DNA methylation profiles and transcriptomes in exposed lineage animals [70]. Many of the genes with altered expression had been previously shown to be associated with PCO [70]. These experiments demonstrate the importance of direct measurements in the germ cells and relevant somatic cell types themselves. Observations support the hypothesis that transgenerational germ-line epigenetic alterations acting through the embryonic stem cells, can promote disease susceptibility in a wide variety of cells and tissues [71].

**Critical Experimental Limitations**

 A number of experimental considerations need to be made in the design of epigenetic transgenerational inheritance studies. The first is to consider the critical windows of exposure discussed above in regards to germ cell development. Studies have demonstrated that the critical window of gestation and fetal development identified in many studies [5, 6, 9-16, 43-46] needs to be considered in the experimental design of transgenerational studies. Exposure to a stimulus during a window that preceded or followed the critical window of gonadal sex determination may produce effects other than those on germ cell epigenetic programming. For example, a recent study of vinclozolin actions used an exposure window that did not include the entire gonadal sex determination window (embryonic day E7-13 mouse and E8-14 in rat) and found no transgenerational effect [72]. In contrast to statements in a recent review [73], this does not constitute a negative result, but simply an experimental design that was not suitable.

 Another critical experimental consideration is the impact of inbreeding within the experimental model used. A number of studies that did not induce epigenetic transgenerational inheritance were performed using inbred strains of rodents [72, 74, 75]. Previously literature has demonstrated inbreeding depression of environmentally induced phenotypes, particularly toxicant actions [76] and suppression of epigenetic processes has been shown to be involved [77-79]. A study that compared inbred and outbred lines of mice found transgenerational phenotypes in the outbred, but not the inbred strains [39]. Therefore, recent studies that have used inbred strains and obtained negative observations may in part be confounded by such inbreeding [72, 73]. The molecular nature of this inbreeding depression on epigenetics remains to be established.

 In defining epigenetic transgenerational inheritance it is critical to consider the absence of direct environmental exposures in the transgenerational generations [80] (Table 4). The direct exposure of a cell or individual to an environmental exposure does not constitute a transgenerational phenotype (Figure 3). The exposure of an F0 generation pregnant female directly exposes her, the F1 generation fetus and the F2 generation germ-line within the F1 generation fetus [3, 80]. Therefore, the F3 generation is the first generation that is truly a test of transgenerational inheritance, the F0, F1 and F2 generations being due rather to a multigenerational exposure [3, 80] (Figure 3). In contrast, an adult male or non-pregnant female when exposed has direct exposure of the F0 generation adult and germ-line that will generate the F1 generation, so it is the F2 generation (grand-offspring) which are the first recipients of transgenerational inheritance [3, 80] (Figure 3), the F0 and F1 generations again being multigenerational exposures. Many studies have been published that have claimed transgenerational inheritance of phenotype, but instead have been multigenerational exposures not involving germ-line transmission of epigenetic information in the absence of direct exposure.

 As shown in Table 1, a large number of different laboratories, with a wide variety of environmental exposures in a number of different species have demonstrated environmental induction of epigenetic transgenerational inheritance [5, 6, 9-16, 43-46]. Several studies have reported an inability to induce transgenerational phenotypes [72, 74, 75, 81], but did not consider all the experimental limitations described above. A question of the bioinformatics used [72] has also been raised [82]. The suggestion that these are negative studies [73] neglects a consideration of these critical aspects of experimental design and interpretation, and therefore needs to bemade with caution.

**Conclusions**

 Developmental considerations in environmentally-induced epigenetic transgenerational inheritance of disease and phenotypic variation include the critical windows of exposure being linked to germ cell development. The time of gonadal sex determination when germ cells are undergoing epigenetic programming is a developmental period susceptible to induction of transgenerational phenotypes. The adult stage for males will be critical due to the epigenetic reprogramming during spermatogenesis. Other developmental stages likely exist but require further investigation. Studies not finding transgenerational phenotypes need to consider the need to affect a critical window of exposure in development.

 A number of studies have more recently suggested a role for different epigenetic processes in the germline transmission of epigenetic transgenerational inheritance. Previous studies have focused on DNA methylation due to the link with epigenetic programming and DNA methylation, table 3B. Genome wide effects on sperm DNA methylation profiles and the identification of differential DNA methylation regions (DMRs) have been found with a wide variety of environmental exposures [5-7, 10, 12-14, 43], Table 1 and 3B. More recently non-coding RNA (ncRNA) have also been shown to be altered in sperm transgenerationally [29, 83-85], Table 3B. Interesting studies have also used ncRNA injection into eggs to promote a transgenerational phenotype [29]. Histone retention in sperm has also shown to be altered, so alteration in histone modifications and retention have been shown to be involved [86-88](Rando et al 2012 A J Androl?). Although no studies extensively, the role of chromatin structure has also been suggested [88]. Therefore, several reviews have suggested the combined roles of different epigenetic processes in epigenetic transgenerational inheritance [3, 83, 89]. Although some reports suggest one molecular process may be more important than another, a combination of them all (Table 3) will likely regulate the epigenetic transgenerational inheritance phenomenon [3, 89].

 Epigenetic transgenerational inheritance provides a non-genetic form of inheritance. The impact on biology of environmental factors which can promote transgenerational disease and phenotypic variation is significant. For evolutionary biology the ability of environmental factors to promote phenotypic change is a neo-Lamarckian concept that can impact neo-Darwinian theory. Integration of environmental epigenetics and classical genetics provides a more robust molecular mechanism underlying evolution [50]. The combined mechanism helps explain topics such as genetic assimilation. Ancestral exposures that will have an impact on transgenerational disease susceptibility can play a critical role in disease etiology [3, 6]. The impact of environment on biology is significantly enhanced when epigenetic transgenerational inheritance is considered.

 Genetics and genetic inheritance is absolutely critical for biology. An additional consideration of epigenetic transgenerational inheritance as a non-genetic form of inheritance does not reduce the importance of genetics, but rather expands the repertoire of molecular mechanisms which underlie many aspects of biology that cannot be easily explained with classical genetics alone (Table 3A). Therefore, complementary roles for non-genetic and genetic inheritance exist and need to be considered. This includes the regulation of any cellular, physiological or biological system. No system will involve only genetics or epigenetics as these molecular mechanisms are so integrated that they depend on each other [3, 66] (Figure 2). Future elucidation of molecular and biological processes will need to consider both to understand the function of biological systems adequately.

 These considerations have far reaching implications, because they indicate that environmental or lifestyle challenges not only produce effects on the individuals exposed themselves, but may also be transmitted in potentially unmodified form to their offspring over several subsequent generations. The protection of future unborn generations from such risk must be a paramount consideration, raising a range of ethical as well as practical considerations. The situation is made more acute by the consideration that even if the inducing stimulus is removed, for example by reducing the level of an environmental toxicant, the transgenerational phenotype and associated effects on disease risk may still be transmitted. Transgenerational epigenetic inheritance thus has a range of implications for sustainable health and economic development in many situations.

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**FIGURE LEGENDS**

**Figure 1.** Epigenetic (DNA methylation) programming in the germline during various developmental periods.

**Figure 2.** Epigenetic and genetic cascade of events involved in development (Modified from [66]).

**Figure 3.** Environmentally induced transgenerational epigenetic inheritance: Schematic of environmental exposure and affected generations (Modified from [90]).

Table 1

|  |
| --- |
| **Exposure Induced Epigenetic Transgenerational Inheritance** |
| **Toxicants** | **Species** | **Generation** |  |
| Vinclozolin (Agricultural Fungicide)  | Rat and Mouse | F4 | [5, 9] |
| Methoxychlor (Agricultural Pesticide)  | Rat | F4 | [5, 10] |
| TCDD/Dioxin (Industrial Contaminant) | Rat, Mouse, Fish | F3 | [11, 12, 37] |
| Plastics (Bisphenol-A, Phthalate-DEHP & DBP)  | Rat | F3 | [6, 13] |
| Jet Fuel [JP8] (Hydrocarbon Mixture)  | Rat | F3 | [43] |
| Permethrin & DEET Pesticide & Insect Repellent  | Rat | F3 | [44] |
| DDT (Pesticide) | Rat | F4 | [14] |
| Bisphenol A (BPA) (Plastic Toxicant)  | Rat, Mouse, Fish | F3 | [45, 46, 91] |
| Phthalates (Plastic Toxicant)  | Rat | F3 | [15] |
| Tributyltin (Industrial Toxicant) | Rat | F3 | [16] |
| **Nutrition** |  |  |  |
| Folate (Nutrition)   | Mouse | F2, F3 | [23] |
| High Fat Diet (Nutrition) | Mouse and Rat | F2, F3 | [21, 22] |
| Caloric Restriction (Nutrition)  | Rat, Mouse, Pig, Worm, Flies | F2, F3 | [17-20, 33, 34, 36, 38] |
| **Other Types Exposures**  |  |  |  |
| Temperature & Drought (Plant Flowering and Health) | Plant | F2, F3 | [24-27] |
| Stress (Behavioral)  | Mouse, Rat, Human | F2, F3 | [28, 29, 40-42] |
| Smoking and Nicotine (Health) | Rat and Human | F3 | [30, 31] |
| Alcohol (Health) | Rat | F3 | [32] |

Table 2

**A**

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| --- |
| **Sites of Action and Phenotypes of Environmental Factors**  |
| **Site of Action** | **Biological Response and Toxicology** |
| Somatic Cells | Allows tissue specific toxicology and critical for adult onset disease in the individual exposed, but not capable of transmitting a transgenerational phenotype. |
| Germ Cells | Allows transmission between generations and in the absence of direct exposure to promote a transgenerational phenotype. |

**B**

|  |
| --- |
| **Transgenerational Versus Multigenerational Phenotypes**  |
| **Phenotype** | **Exposure** | **Definition** |
| Multigenerational | Direct | Coincident direct exposure of multiple generations to an environmental factor promoting alterations in the multiple generations exposed. |
| Transgenerational | None, except the initial generation | After the initial exposure the transgenerational phenotype is transmitted through the germ line in the absence of direct exposure. |

Table 3

**A**

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| --- |
| **Environmental Epigenetic Impacts on Biology & Disease** |
| * Worldwide Differences in Regional Disease Frequencies
 |
| * Low Frequency of Genetic Component of Disease as Determined with Genome Wide Association Studies (GWAS)
 |
| * Dramatic Increases in Disease Frequencies Over Past Decades
 |
| * Identical Twins with Variable and Discordant Disease Frequency
 |
| * Environmental Exposures Associated with Disease
 |
| * Regional Differences and Rapid Induction Events in Evolution
 |

**B**

|  |
| --- |
| **Transgenerational Germline Epigenetic Processes** |
| * DNA Methylation
 | [5-7, 10, 12-14, 43], |
| * Non-Coding RNA (ncRNA)
 | [29, 83-85] |
| * Histone Modifications
 | [86-88] (Rando et al 2012 A J Androl Ref?) |
| * Chromatin Structure
 | [88] |