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Environmental signaling and evolutionary change: can exposure of pregnant mammals to environmental estrogens lead to epigenetically induced evolutionary changes in embryos?

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SUMMARY DNA methylation is one of the epigenetic and hereditary mechanisms regulating genetic expression in mammalian cells. In this review, we propose how certain natural agents, through their dietary consumption, could induce changes in physiological aspects in mammalian mothers, leading to alterations in DNA methylation patterns of the developing fetus and to the emergence of new phenotypes and evolutionary change. Nevertheless, we hypothesize that this process would require (i) certain key periods in the ontogeny of the organism where the environmental stimuli could produce effects, (ii) particular environmental agents as such stimuli, and (iii) that a genomic persistent change be consequently produced in a population. Depending on the persistence of the environmental stimuli and on whether the affected genes are imprinted genes, induced changes in DNA methylation patterns could become persistent. Moreover, some fragments could be more frequently methylated than others over several generations, leading to biased base change and evolutionary consequences.

INTRODUCTION

An old question in evolutionary biology is "how does variation originate?" No matter how old this question is, the controversy remains regarding whether (i) variability in populations appears exclusively by random mutations, a position defended by neo-Darwinism, or (ii) the formation of novel characters can, in some way, be induced by external environmental forces. Current understanding of epigenetic modification of DNA shows that such controversy still exists. In this sense, Jaenisch and Bird (2003) suggested that future lines of investigation should place emphasis on the identification of the stimuli that can initiate evolutionary changes. They proposed that it is possible for external factors, such as dietary compounds, to lead to the accumulation of epigenetic changes over the years within populations. Given the recent evidences on mechanisms of epigenesis, here we propose that under certain conditions, such epigenetic changes could become persistent over generations and this could have evolutionary genetic consequences in a lineage.

We believe that a first approach toward evaluating this problem requires separating the phenomenon of the emergence of an evolutionary novelty into two processes: (i) that responsible for the origin of a new character and (ii) that maintaining such a character over generations (i.e., fixation). Such separation has been previously proposed by authors such as Futuyma and Moreno (1988) and West-Eberhard (1998).

As Darwin, most evolutionary biologists have concentrated almost exclusively on the second process, that is the form in which an evolutionary novelty can be fixed, not inquiring into the problem of how evolutionary novelties originate. Variation among individuals and correlated differences in fitness became a central topic in Darwin's theory (Endler 1986) and thereafter, Neo-Darwinian theory interpreted changes in allelic frequencies of populations instead of studying the origin of new phenotypes (Nijhout et al. 1986).

In accordance with the separation between origin and fixation of an evolutionary novelty, some authors state that evolution is always a two-step process, first involving

developmentally mediated variation, and then selection, whose operation results in gene frequency changes (Wake and Larson 1987; West-Eberhard 1998). In this sense, changes arising because of alterations in early developmental processes, which, furthermore, could, in some cases, be environmentally induced, can appear whether or not such changes could become fixed and prosper in a population. Hence, in our opinion, the diversity and evolution of species should be explained not only by those selective processes imposed by the environment but also by the action of the environment as an inductor of genotypic and phenotypic variation, which is the material basis for selection.

Regarding the persistence of such epigenetic changes through generations, long ago, Weismann (1893) stated that external influences may produce hereditary variations when they are capable of modifying the determinants of the germ plasm. Nevertheless, this could be only one of the ways through which environmental factors induce transgenerational epigenetic changes. We recognize two ways for this to occur: one is by dramatically modifying DNA aspects in the germ line with transgenerational consequences, that is by means of producing mutations or transgenerationally persistent epigenetic modifications in the genome, and the other is through inducing ontogenetical variation at every generation, although not producing inheritance through the germ line. From our perspective, inductive environmental forces can act to create, through one or both of these forms, new conformation of organisms, which also implies new possibilities within its surrounding environment. Jablonka and Lamb (1995) have named the range of the possible responses of individuals to new environmental challenges as the "reaction range" of individuals.

Based on his experiments in *Drosophila*, Waddington proposed two new concepts related to the capacity of environmental influences to induce the appearance of new characters in organisms and their maintenance over generations. First, in the face of disturbing and external stressing influences, there are counteracting tendencies in development toward normal adult conditions (i.e., canalization; Waddington 1959). Second, whereas these counteracting tendencies exist, if a stressing stimulus is capable of developmentally modifying a strain of organisms, the derived population may evolve exhibiting the modification even in the absence of the stress (Waddington 1952). He termed this process "genetic assimilation."

An important fact to notice is that, through these concepts, Waddington distinguished particular environmental stimuli capable of inducing epigenetic changes, which are the "stressing" ones. McClintock (1984) also stated that a particular kind of stimuli producing stress lead to a genome's reaction to it, whose response may underlie formation of new species. Furthermore, she stated that genome produces programmed responses, although it is necessary to subject the genome repeatedly to the same challenge in order to observe the nature of the induced changes.

At present, it is widely known that DNA methylation is one of the epigenetic and hereditary aspects that regulate genetic expression in mammalian cells (Khosla et al. 2001). Furthermore, DNA methylation is capable of being modified by the action of externally applied agents (Mac Phee 1998). Not all, but particular compounds found in nature could act as such agents. Moreover, they could be capable of affecting the evolution of organisms, inducing profound changes in individuals and populations, perhaps with transgenerational consequences. We hypothesize that, whereas certain conditions are required for this process to occur, it is a feasible phenomenon. The task is to identify the conditions constraining such a process.

Experimental evidences concerning alterations of methylation patterns, at least in mammals, are generally restricted to studies of the effects of synthetic compounds or dietary restrictions of food items containing the methyl group (see Laird and Jaenish 1996; Singal and Ginder 1999). Although this is very important for understanding the mechanisms of DNA methylation, from an evolutionary perspective, it is of greater relevance to find compounds that are naturally in contact with organisms; for example, those available for dietary consumption, which, in addition, could produce alterations in patterns of DNA methylation in organisms.

In this article, focusing exclusively on the phenomenon of how evolutionary novelties originate, we describe how in mammals, certain natural agents could induce alterations in particular mechanisms of regulation of gene expression in individuals, such as methylation patterns, and the further arising of new, specific phenotypes in subsequent generations, leading to evolutionary change. Nevertheless, we hypothesize that this process would require (i) certain key periods in the ontogeny of the organism where the environmental stimuli could produce effects, (ii) particular environmental agents as such stimuli, moreover, acting persistently, and (iii) that a persistent genomic change be consequently produced in a population.

The first requirement emerges because not all compounds are capable of producing an effect on mothers that will have consequences on the fetus; the second emerges from the fact that an organism is not equally sensitive to outer stimuli throughout ontogeny; and the third because transgenerational persistency of characters is ensured when it reaches the genomic level. Each of the three requirements presented will be more extensively treated later in the text.

DNA METHYLATION: EPIGENETIC IMPRINTING ON THE GENOME

Experimentation on the problem of how evolutionary novelties arise and the consequences on the genetic system of exposition to an environmental stimulus have been the

focus of epigenetic studies in a variety of organisms, including *Drosophila* (Rutherford and Lindquist 1998), bacteria (Cairns et al. 1988), and yeast (Steele and Jinks-Robertson 1992).

Several types of epigenetic inheritance have been described to date. Jablonka and Lamb (1995) have proposed three systems of epigenetic inheritance: (i) *steady-state systems*, such as Wright's (1945) persistence of alternative cellular states as a result of changes in nuclear genes or in cytoplasmic constituents of the cell, (ii) *structural inheritance systems*, such as the maintenance through generations of the ciliary patterns in protozoa, albeit of the genetic constitution of the cells involved (Nanney 1985), and (iii) *chromatin-marking systems*, or those related to the transmission of specific patterns of the chromatin structure (Holliday 1987; Jablonka et al. 1987). Specifically, the latter refers to non-DNA parts of the chromosomes that are capable of binding proteins or additional chemical groups attached to DNA bases, which affect the nature and stability of gene expression, now commonly named genomic imprinting. DNA methylation describes a postreplicative modification, in which a methyl group is added to a DNA residue in a covalent manner (Laird and Jaenish 1996); for this reason, it is a form of genomic imprinting. The DNA methylation reaction is enzymatically catalyzed by DNA methyltransferases (Dnmts) and takes place in 5′ to 3′-oriented CG dinucleotides, which are known as CpG sites, at the carbon 5 of the cytosine ring (Singal and Ginder 1999). CpG islands are regions with a high frequency of CpG sites; these islands are often associated with genes, and are usually found in promoter zones (Gardiner-Garden and Frommer 1987). CpG sites are not evenly distributed within the genome, and are preferentially unmethylated, regardless of the transcriptional activity of the associated gene (Bird 1986). As other regions are normally methylated, patterns of genomic DNA methylation can be distinguished along the genome (Singal and Ginder 1999; Bestor 2000; Jones and Takai 2001). Nevertheless, there is controversial information regarding whether methylation patterns are established because of the enzymatic activity of one or more Dnmts (Bestor 2000; Yokochi and Robertson 2002).

There are at least three families of Dnmts described to date: Dnmt1, Dnmt2, and Dnmt3. However, there is no agreement regarding whether each one plays a specific, differential role in the process of DNA methylation (Bestor 2000). It has been speculated that Dnmt3A and Dnmt3B are responsible for the establishment of methylation patterns during early development, whereas Dnmt1 is responsible for the further maintenance of such patterns. Experiments conducted in vitro support this model, revealing that Dnmt1 has a preference for hemimethylated DNA as a substrate (Yoder et al. 1997), whereas Dnmt3A and Dnmt3B act as a de novo methyltranferase, preferring unmethylated DNA (Yokochi and Robertson 2002).

There are multiple isoforms of Dnmts, but all are encoded by the same cytosine–Dnmt gene (Deng and Szyf 1998). Among these isoforms, Dnmt1o is a variant of Dnmt1 that accumulates in oocyte nuclei during the follicular growth phase, and Dnmt3L is an isoform of Dnmt3a and Dnmt3b, but that lacks Dnmt enzymatic activity and interacts with Dnmt2a and Dnmt3b (Kierzenbaum 2002). Dnmt3L acts as a cofactor for *de novo* methylation of imprinted genes in the female gametes and for the establishment of methylation imprints in oocytes (Hata et al. 2002).

It is worth noting that Dnmt1 is localized principally in somatic cell nuclei, but it is cytoplasmatic in the oocyte and in the preimplantation embryo (Bestor 2000). However, the variant Dnmt1o has transient nuclear localization in the eight-cell stage, corresponding to the time when genomic imprints are established (Howell et al. 2001). On the other hand, Dnmt3L co-localizes with Dnmt3a and Dnmt3b in mammalian cell nuclei (Hata et al. 2002).

Given the crucial role of the diverse Dnmts in the epigenetic modification of DNA, it is of great interest to know whether there are environmental substances capable of modifying the intracellular levels of such enzymes or their patterns of gene expression. Nevertheless, no studies have reported this kind of interaction, which we suspect may have a role in relating environmental stimuli to DNA modification. However, the recent finding that individual Dnmts can be tracked, and that their binding to genomic DNA can be quantified in vivo in mammalian cells (Liu et al. 2003) can be enormously helpful for determining the link between environmental compounds and the process of DNA methylation.

IMPRINTED GENES: DNA METHYLATION AND PERSISTENCE OF MARKS THROUGH GENERATIONS

Roemer et al. (1997) were the first to show reappearance in the progeny of modified characters in parents. In their experiments on rodents, the adult phenotype produced because of the fusion of pronuclei with eggs of different genotypes was also observed in the offspring. Furthermore, such transgenerational persistence of the modified characters was related to altered methylation patterns that were, in turn, transmitted through male gametogenesis. However, not all genes are equally capable of passing on changes in patterns of methylation. There is a particular class of genes, crucial for understanding the mechanisms of epigenetic inheritance, that are known to have relatively unchanged methylation patterns over generations. These genes, named "imprinted genes", do not seem to be affected by overall alterations in methylation patterns that take place early in development (Constância et al. 1998). Such genes carry a molecular memory of their parental origin that is acquired early in the germ line (Surani

2001). This molecular memory is associated with specific methylation patterns in CpG islands of each allele, which consequently affect further genic expression (Costello and Plass 2001).

Once the allelic differences in methylation of imprinted genes are defined (during the establishment of germinal line in the developing embryo), such differences generally remain stable in the somatic tissues (Constância et al. 1998). The marking process of these genes appears to involve three stages: (i) the establishment of marks in gametes; (ii) the permanence of these marks during embryogenesis and in the adult somatic tissues; and (iii) the erasure of marks in the early germ line (Razin and Cedar 1994). Conclusive information on the way in which methylation in imprinted genes is initiated from an unmethylated state during gametogenesis is still elusive (Ferguson-Smith and Surani 2001). However, recent investigations indicate that primordial germ cells are substantially methylated (which corresponds to the same pattern in somatic cells) before they colonize gonads and become demethylated around the time of entry into the gonads (Hajkova 2002). An incomplete deletion of marks during gametogenesis would explain the inheritance of the parental epigenotype (Reik et al. 2001).

CHANGING DNA METHYLATION AND ITS IMPLICATIONS

Imprinted genes may be susceptible to undergoing changes in methylation patterns during preimplantational development (Khosla et al. 2001). As imprinted genes tend to conserve methylation patterns from one generation to the next, changing methylation patterns in these genes could lead to the appearance of the derived alterations in the future generations. Therefore, if external agents are capable of inducing particular changes in methylation patterns in these genes, such changes could flourish transgenerationally. Moreover, this could take place in the absence of the stimuli that initially changed its methylation pattern, generating a process that would be a kind of Waddington's "genetic assimilation" but in imprinted genes.

Changes of methylation patterns in certain imprinted genes can generate associated specific phenotypes (see Morison et al. 2001 for examples). Particularly interesting, from our perspective, is the Beckwith–Wiedemann syndrome. Researchers suspect that this syndrome is related to the loss of imprinting in *Igf2*, and is characterized by somatic overgrowth, macroglossia, abdominal wall defects, visceromegaly, and an increased susceptibility to childhood tumors (Caspary et al. 1999). Therefore, in this case, a change in methylation patterns in a single gene can lead to phenotypic changes in several characters.

However, even if no one imprinted gene is affected when altered by an environmental signal, environmentally induced changes in methylation patterns could also become persistent if such changes, and the environmental conditions allowing the establishment of such changes, are both conserved throughout generations. This could occur whenever there is a concordance, an association between the environmental stimuli, the established DNA methylation patterns, and the resulting phenotype of an organism. For instance, if some natural agent can induce the loss of methylation in genes and produce phenotypic alterations (e.g., those modifications emerging from the loss of methylation in *Igf2*), a standard phenotypic pattern will arise every time the specific environmental stimuli lead to the establishment of particular patterns of methylation. Still, it is important to consider that this could be a broader phenomenon, and environmentally induced changes in methylation patterns could affect several other imprinted genes as well. As a result, an environmental stimulus would bias the phenotypic change toward certain types of phenotypes.

Nevertheless, the consequences of altering DNA methylation toward specific persistent patterns could imply mutation in those specific segments of the genome. For instance, it is known that a methylated cytosine is half-way to the substitution of a cytosine for a thymidine. The completion of conversion requires only a hydrolytic deamination reaction (Singal and Ginder 1999). Therefore, if some methylated sites are frequently methylated over several generations, it is possible that an eventual base change from cytosine to thymidine will occur more frequently than any other substitution. In fact, CpG sites are hotspots for transitions from cytosine to thymidine, generated by a spontaneous deamination of 5-methyl cytosine to thymidine (Coulondre et al. 1978). The result would be, as mentioned by West-Eberhard (2003), that "evolved sensitivity to environmental influence during gene expression could influence susceptibility to certain kinds of structural change during evolution."

EARLY DEVELOPMENT: A KEY STAGE DURING ONTOGENY

The first condition for our statement on environmentally induced evolution is that the process must occur early in ontogeny, before or during the establishment of the germ line in metazoa. This is important for two main reasons: first, because eventual reprogramming of methylation patterns in the germ line can be transmitted to the progeny (Surani 2001), and second, because during development, there is an enhanced susceptibility of the organism to the action of outer compounds, with greater consequences in the adult than when the same stimulus occurs later in ontogeny (Amzallag 2000). With regard to the latter statement, Gould and Lewontin

(1979) have emphasized that during the early ontogenetic stages of complex organisms, "differentiation of organ systems and their integration into a functioning body is such a delicate process so easily derailed by early errors, with accumulating effects."

The morphogenic process of an organism is basically the product of a three-way interaction between the environment, genetic factors, and those characteristics that emerge from a self-organized dimension created by development itself (Amzallag 2000). The establishment of methylation patterns during early development (as well as other processes in morphogenesis) also depends on the immediate environment experienced by the embryo. These methylation patterns will guide the formation of particular cell types by controlling gene expression (Holliday 1998), therefore biasing further morphogenesis.

In mammals, patterns of methylation are established for the entire genome at least three times during development. The periods in which reprogramming of methylation patterns takes place are: (i) before the implantation of the embryo, (ii) during the development of the germ line (Reik et al. 2001), and (iii) during the period beginning soon after blastocyst implantation (Constância et al. 1998) until gastrulation (Mac Phee 1998). Before blastocyst implantation, a great part of the DNA is demethylated (Dean et al. 1998); thus, the DNA of blastocysts hardly shows methylation (Mac Phee 1998). Between blastocyst implantation and gastrulation, there is a wave of de novo methylations that restore the overall methylation patterns, which is retained in the somatic cells of animal for the rest of its life (Mac Phee 1998). In the germ line, reprogramming takes place by overall demethylations and methylations of the genome (Constância et al. 1998). In mice, primordial germ cells undergo an overall demethylation process in early development until day 13 or 14 (Reik et al. 2001). Later, during gametogenesis, there is a de novo methylation event until the previously observed high levels of methylation in the zygote (Mac Phee 1998), oocyte, and sperm genomes (Reik et al. 2001) are reached. It is likely that both demethylations taking place during the first stages of postzygotic cleavage, and methylations occurring after implantation, are important in removing acquired epigenetic modifications, especially those acquired during gametogenesis (Reik et al. 2001).

ROLE OF REPRODUCTION IN TRANSMITTING ENVIRONMENTALLY INDUCED ALTERATIONS IN METHYLATION PATTERNS

Reproduction involves the conservation in the progeny not only of the structure required to carry out the self-conserved organization represented by the organism but also the preservation of the structural characteristics of the environment that allow such organization to take place (Maturana-Romesín and Mpodozis 2000). An experimental approach to such a statement comes from Clark and Galef (1995), who proposed that daughters tend to resemble their mothers not only because both share a relatively large proportion of their genes but also because they tend to have similar histories of fetal exposure to steroids.

Applying this view to DNA methylation, reproduction plays a key role in passing on those changes in patterns of methylation that could eventually arise during early stages of the ontogeny. Reproduction, in addition to conserving the pattern of DNA methylation of an organism's genome throughout generations in a lineage, will also conserve the conditions allowing such patterns of methylation to be established in every generation. Hence, for a mammal to be formed from a zygote, and for development to take place generating a phenotype similar to the parental phenotypic pattern, the process requires not only the genetic content that provides a zygote with the potential to become an adult but also a surrounding environment for the embryo, which ensures the occurrence of appropriate methylations, at key periods of time during the embryological process.

Nevertheless, in mammals, despite the fact that the uterus acts as a buffer for either mechanical or chemical perturbations on the developing embryo, making the developmental process more isolated from environmental perturbations than in other taxa, the development is still susceptible to particular perturbations. Maternal effects such as variations in the hormonal status of a mother are capable of affecting the microenvironment in which the fetus develops (Clark and Galef 1998) and, consequently, its later ontogenetic processes (Bernardo 1996). For example, studies have shown that differential exposition to hormones can affect characters of the embryo. Clark et al. (1993) and Vandenbergh and Huggett (1994) demonstrated that the intrauterine position of female rodents affects the sex ratio of their litters, which is because of differential prenatal exposure to steroidal hormones, which in turn depends on the gender of neighboring embryos.

Besides, the hormonal state of the mammalian female can be strongly influenced by the environment through compounds that are naturally found in her diet (Nagao et al. 2001). In this sense, it has been reported that feed toxicants, or dietary imbalances of specific nutrients, can alter the composition of oviductal and uterine secretions (McEvoy et al. 2001).

Thus, the establishment of methylation patterns in the embryo is a process that depends directly on the environment in which it takes place, that is the intrauterine environment, but also indirectly on the surrounding environmental signaling, which, in some way, alters such an intrauterine environment. Accordingly, perturbing the intrauterine environment while early development takes place could bring about

consequences in the establishment of methylation patterns, with the corresponding phenotypic repercussions.

ENVIRONMENTAL AGENTS PRODUCING MOTHER TO FETUS INDIRECT EPIGENETIC EFFECTS

Our second condition is that only particular compounds in nature could act as environmental inputs for environmentally induced evolution to take place. The early embryo is exquisitely sensitive to alterations in its environment (McEvoy et al. 2001). Nevertheless, not every compound with which a mammalian mother has contact in nature is capable of altering the embryo environment, although some compounds could lead to alterations in mammalian hormonal features. Furthermore, we believe that some environmental compounds can, in addition to altering the hormonal status of a mammalian mother, be in turn capable of affecting important processes during the early development, including the establishment of methylation patterns in the embryo. Among those environmentally available compounds capable of affecting the hormonal status of a mammalian mother, there are some of synthetic origin, or xenobiotics (Danzo 1998) and of natural origin, such as phytoestrogens. The latter refers to secondary metabolites produced by plants (Croteau et al. 2000; Yu et al. 2000) that produce estrogenic action at a variety of levels in animals (McLachlan 2001). Phytoestrogens are readily available in the environment for animal consumption and their physiological, hormonal, and nonhormonal effects in animals have been studied to some extent (Levy et al. 1995; Santell et al. 1997; Boettger-Tong et al. 1998; Milligan et al. 1998; Gallo et al. 1999). Some phytoestrogens such as genistein and daidzein belong to a class of flavonoids, the so-called isoflavones (Liggins et al. 2000). The consumption of isoflavones can elicit uterotrophic and mammatrophic effects in mice and on the hypothalamic/pituitary axis as well (Santell et al. 1997). In humans, it has been reported that the consumption of phytoestrogens affects levels of the sex hormone-binding globulin, which regulates the bioavailability of steroidal sex hormones (Pino et al. 2000).

Changing the hormonal status in mammals could have consequences beyond the physiologic level. McLachlan (2001) suggested that estrogens could play a role in programming or imprinting those genes involved in cell proliferation, differentiation, or survival, either directly or through related signaling pathways. He also proposed that an estrogenic chemical may directly imprint a gene through a process leading to persistent genetic change, probably at the level of DNA methylation. In this sense, Barrett et al. (1981) suggested that diethylstilbestrol (DES), a powerful estrogenic synthetic compound, could transform cells by mechanisms other than punctual mutations, frameshift mutations, or small deletions. Currently, one could also interpret this cell transformation as alterations in methylation patterns. Some evidence for this phenomenon comes from studies in chicken liver, where estrogens appear to act in the regulation of expression of the vitellogenin I and II, and VLDL II genes, through changes in patterns of methylation of estrogen-responsive element sites (Edinger et al. 1997). It has also been shown that neonatal exposure to DES and adult ovary hormones produces abnormalities in the demethylation of the lactoferrine promoter, which shows that either hormonal xenobiotics or natural hormones are capable of triggering impairments during the development of organs (Li et al. 1997).

It has been reported that environmental estrogens can also produce direct effects on DNA methylation patterns. For example, administration of the phytoestrogens cumestrol and equal to newborn mice can enhance methylation and produce inactivation in the proto-oncogene *H-ras* (Lyn-Cook et al. 1995). In addition, Day et al. (2002) demonstrated that methylation patterns can be altered in 8-week-old mice that consumed high quantities of genistein.

With respect to hormonal effects early in development, Holliday (1998) was the first to envisage a possible link between hormone action and establishment of DNA methylation in mammalian embryos. He proposed that the effect of teratogens on a mother might disrupt the normal distribution of DNA methylation in a developing fetus, producing developmental abnormalities or defects that can appear in the subsequent generations. Newbold et al. (2000) reported that after administering DES to pregnant rats during early postimplantational development and neonatality, a greater susceptibility for specific tumor formation in rete testis and reproductive tract tissues occurred in F1 and appeared further in the non-DES exposed F2. These authors speculated that this transgenerational phenomenon could implicate epigenetic alterations that were transmitted through germ line, including changes in methylation patterns. Although this finding strongly suggests alteration and further transmission of a genomic change through germ line across more than one generation in response to an early exposition to an estrogenic compound, there is still missing evidence on the mechanism behind this process and whether it implies changes in DNA methylation patterns.

In the experiments of Newbold et al. (2000), the transgenerational persistence of the enhanced susceptibility to tumor formation takes place when mothers are exposed to DES after embryo implantation; however, estrogens play an important role even before implantation occurs. The implantation process involves complex interactions between the blastocyst and the uterus (Paria et al. 1993). Uterine preimplantational estrogen secretions are essential for activating the blastocyst of *Mus musculus* for further implantation, which is not possible if estrogen secretions are prevented by ovariectomization (Paria et al. 1998). Nevertheless, just as

estrogenic stimuli are needed for normal development, preimplantational exposure to synthetic estrogenic compounds can lead to phenotypic alterations. For instance, Takai et al. (2000) reported that in utero preimplantational exposure of rodent embryos to the synthetic estrogen bisphenol-A leads to an increased body mass of the animals at weaning. Furthermore, Wu et al. (2004) have recently shown that in vitro early exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin can indeed alter DNA methylation patterns in preimplantational embryos. Interestingly, those genes changed, *H19* and *IGF-2*, were imprinted genes.

Although it is not known whether compounds with estrogenic action (CEA) inside the uterus could act directly upon the developing embryo, or via intermediaries, it is possible that the relationship between estrogenic stimuli and methylation in the preimplantational embryo is mediated by the expression of *c-fos*. While on the one hand it is known that *c-fos* directly regulates the *dnmt1* transcription, increasing Dnmt1 levels (Bakin and Curran 1999), on the other, the induction of *c-fos* is a response attributed to membrane-mediated estrogen actions (Das et al. 2000). Through this mechanism, which provides an alternative pathway to the classical estrogen receptors α and β, CEA could trigger responses, as has been observed in pancreatic β cells (Nadal et al. 2000). In summary, the membrane-mediated estrogenic actions would first induce *c-fos* and then trigger the activation of the Dnmt1 enzyme.

Furthermore, in blastocysts, this indirect and membrane-mediated relationship between estrogenic stimuli and *c-fos* activation could also occur. In preimplantational blastocysts, Paria et al. (1998) demonstrated that latent blastocysts can be activated if they are incubated in vitro with 4-OH-17β-estradiol, a catecholestrogen synthesized from 17β-estradiol in uterine luminal epithelia by the action of the hydrogen-2/hidroxilase-4 enzyme. This response to 4-OH-17β-estradiol could also occur via a pathway distinct from the classical nuclear estrogen receptors (Paria et al. 1998). In addition, Paria et al. (1998) found that 4-OH-17β-estradiol increases with the epithelial growth factor (EGF) receptor. Interestingly, other studies have demonstrated that an increase in the EGF receptor may also be related to activation of *c-fos* (Kamiya et al. 1996). On the other hand, a direct induction of *c-fos* by estrogen has also been shown in different cell types (Allen et al. 1997; Garcia et al. 2000), which occurs via an estrogen receptor element present in this gene (Hyder et al. 1992). Thus, estrogenic stimuli could induce *c-fos*, either directly, through a gene receptor, or indirectly through membrane-mediated reactions.

Furthermore, phytoestrogens could also induce *c-fos* and consequently alter methylation patterns in cells. A study supporting this view demonstrated that the intake of genistein in ovariectomized female rodents induced the expression of the RNA messenger of *c-fos* in the uterus (Santell et al. 1997). Hence, we suspect that phytoestrogens can also act on blastocysts, which could occur through membrane-mediated estrogen actions, directly induced by isoflavones in uterine secretions, or mediated by other compounds secreted in the uterine epithelia such as 4-OH-17β-estradiol. The formation of this compound in uterine epithelia could be related to plasmatic isoflavone content, although no studies have attempted to detect such compounds in uterine secretions, or showed that its high consumption can alter the production of cathecolestrogens in the uterine epithelia.

Although there is strong evidence suggesting that the hormonal status of mammalian mothers can be an important feature related to the establishment of methylation patterns in early embryos, so far, there is no concluding evidence of this. We believe that an investigation on this subject should be performed in order to uncover the aspects behind an eventual epigenetic role of estrogenic compounds (both animal produced, plant produced, and synthetic) on developmental processes, in particular, on the establishment of methylation patterns in the early embryo.

THE "GENOMIC CHANGE" REQUIREMENT FOR A PROCESS TO BE CONSIDERED EVOLUTIONARY

The third requirement that we propose for the environmental and hormonal induction to become an evolutionary process is that genomic change should be achieved. Evolutionary change in the morphogenetic process must arise from changes in patterns of regulation and interaction during ontogeny (see discussion by Atchley 1987). Such a connection gains special importance when considering that the patterns of regulation and interaction occurring at early stages in ontogeny could, even in mammals, be susceptible to environmental changes.

Nevertheless, the question arising at this point goes beyond the relationship between the environmental stimuli and eventual epigenetic consequences on DNA methylation. The challenge is to know how an eventual change in DNA methylation patterns could become persistent and evolutionary. Besides, another question arises, regarding the definition of evolutionary change. Is persistence in the conditions allowing the establishment of changed methylation patterns across lineages a sufficient attribute for such changes to be considered as evolutionary, or do such changes need to reach the threshold of mutation at the genomic level?

It is true that genomic mutational change ensures a great degree of persistence through generations. However, persistence can also be the result of two processes, as previously mentioned: (i) the environment could persistently trigger, generation after generation, a specific change in methylation patterns, or (ii) persistence could be present in intrinsic features of the organisms as, for instance, the stable nature of the DNA.

Given the special feature of imprinted genes regarding possessing methylation patterns that are more stable across generations than other genes, persistence could be achieved through changing methylation patterns of imprinted genes. In this view, such changes in imprinted genes could have the same evolutionary value of mutations, given that there is an associated character variation with the changes, and because of the persistence of these changes throughout generations. Thus, the definition of "evolutionary change" at this point becomes blurred. What is true is that persistence through generations could be achieved in alternative ways to genomic mutation. Nevertheless, speaking in terms of genomic mutation, this could be achieved when the persistent change in methylated cytosines bias to specific mutations, as previously mentioned.

Regarding the frequency of eventual mutations derived from changes in methylation patterns, given that such changes can be environmentally induced, they cannot be considered to be at random. Therefore, we can expect that in these cases, the appearance of mutation will be in greater frequency than when mutation is considered to be at random. In fact, there is a 12-fold higher than normal mutation rate for the conversion of the methylated form of CpG to TpG and CpA, which reduces the occurrence of CpG to about 20% of its expected frequency in vertebrate genomes (Sved and Bird 1990).

Despite the evidence suggesting that the environment, through the action of naturally consumed agents, can alter the developmental process to the point that the emerging alterations can be inherited as evolutionary change, conclusive information is still elusive. Evidence in the direction of genomic change derived from alterations in methylation patterns is needed for our hypothesis to be plausible in the classic view of the meaning of evolutionary change.

SPECULATIONS ON THE EVOLUTIONARY IMPLICATIONS OF EARLY EXPOSURE TO ENVIRONMENTAL ESTROGENS

Holliday (1998) proposed that teratogens could target mechanisms that control patterns of DNA methylation in particular regions of the genome of developing embryos, modifying methylation patterns of the same DNA sequence in somatic cells, leading to a developmental alteration, and subsequently producing changes in germ line cells. Moreover, if such altered methylation patterns are eventually transmitted to a subsequent generation, the same type of defect might be seen (Holliday 1998). Phytoestrogens could act in the same manner, but with the peculiarity that they are naturally available for consumption by many organisms. Phytoestrogens are present in high quantities in food items commonly included in the natural dietary composition of rodents, such as fruits, nuts, seeds (Liggins et al. 2000) and especially wheat, oats, and soy (Thigpen et al. 1999).

In this sense, if a natural population of rodents is suddenly subjected to a high intake of phytoestrogens, it is feasible to hypothesize that such a high intake by pregnant rodents could influence the normal reproductive process, altering the mother's hormonal status, the intrauterine signaling, and, consequently, the establishment of DNA methylation patterns in embryos. The resulting phenotypes will be in accordance with the particular pattern of DNA methylation achieved as a consequence of the environmental stimuli, represented in this case by phytoestrogens. As a result, such changes in methylation patterns will persist in the population if the organisms are constantly subjected to this same environmental input and consequently, the achieved phenotypes will also persist throughout generations. Nevertheless, it is important to point out that such a newly formed phenotype must not be considered to be associated with any adaptive goal; on the contrary, the new forms of organisms will fit within the environment they live, resulting from an environmental input that leads to standardized phenotypes in concordance with the environmental stimuli that produced them. In the particular case of imprinted genes, changing methylation patterns on those genes could imply transgenerational persistence of epigenetic changes in the absence of the environmental input that initially produced them.

Because the early stages of ontogeny play key roles in the establishment of phenotypic variation, it is important to determine how environmental signals (particularly CEA) are involved in the developmental process. Nevertheless, a complete understanding of this involvement is difficult at this time. One of the complications is that the mechanisms through which estrogen and CEA bring about physiological actions are not yet clearly understood (Nilsson et al. 2001). Further studies on the effects of CEA on organisms, especially during early stages of ontogeny, are needed to provide new insights, and to help in the understanding of the impact of this class of compounds on ecosystems in general (McLachlan 2001), and, particularly, on the physiologically relevant evolutionary processes that guide the formation of organisms and lineages.

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