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Epigenomic Disruption: The Effects of Early Developmental Exposures

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Abstract

Through DNA methylation, histone modifications, and small regulatory RNAs the epigenome systematically controls gene expression during development-- both *in utero* and throughout life. The epigenome is also a very reactionary system; its labile nature allows it to sense and respond to environmental perturbations to ensure survival during fetal growth. This pliability can lead to aberrant epigenetic modifications that persist into later life and induce numerous disease states. Endocrine disrupting compounds (EDCs) are ubiquitous chemicals that interfere with growth and development. Several EDCs also interfere with epigenetic programming. The investigation of the epigenotoxic effects of bisphenol A (BPA), an EDC used in the production of plastics and resins, has further raised concern for the impact of EDCs on the epigenome. Using the *Agouti viable yellow* (A^{vy}) mouse model, dietary BPA exposure was shown to hypomethylate both the A^{vy} and the $Cabp^{IAP}$ metastable epialleles. This hypomethylating effect was counteracted with dietary supplementation of methyl donors or genistein. These results are consistent with reports of BPA and other EDCs causing epigenetic effects. Epigenotoxicity could lead to numerous developmental, metabolic, and behavioral disorders in exposed populations. The heritable nature of epigenetic changes also increases the risk for transgenerational inheritance of phenotypes. Thus, epigenotoxicity must be considered when assessing these compounds for safety.

Keywords

epigenetics; bisphenol a (BPA); metastable epialleles; endocrine disrupting compounds (EDCs); *Agouti viable yellow* (A^{vy})

INTRODUCTION

Endocrine disrupting compounds (EDCs) are chemicals that interfere with endogenous hormone function in the endocrine system. Exposures can lead to reproductive abnormalities, altered development, brain and behavior defects, impaired immune function, and cancer (reviewed in (Diamanti-Kandarakis and others, 2009). The most commonly studied EDCs are DDT, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), phthalates, and bisphenol A (BPA). In addition to their endocrine active properties, some EDCs have been shown to disrupt epigenomic programming (Anway and Skinner, 2008; Dolinoy and others, 2007; Kang and Lee, 2005; Li and others, 1997). Here, following a brief summary of epigenetics, we will describe the epigenome's sensitivity to

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environmental exposures during development and two types of particularly sensitive loci: imprinted genes and metastable epialleles. We will then summarize the research using the *Agouti viable yellow* (A^{vy}) mouse model to detect the epigenotoxicity of BPA and discuss this model's potential for screening other EDCs. The transgenerational effects of EDCs are becoming more apparent. To understand the biological mechanisms behind the inheritance of phenotypes, epigenetic alterations across the genome and at imprinted genes must be closely examined.

EPIGENETIC PROGRAMMING: AN ORCHESTRATED SYSTEM

The “fetal origins of disease” hypothesis explains phenomena in which early developmental exposures influence disease onset later in life. The hypothesis, first proposed by David J. P. Barker, initially postulated that early nutritional exposures affect cardiac disease (Barker and Clark, 1997). This theory now encompasses the effects of numerous exposures on cancer initiation, developmental disorders, neurological diseases, and metabolic syndromes (Barker and others, 2002).

The biological mechanisms linking fetal exposures to disease progression are becoming clearer (Wadhwa and others, 2009). Epigenetic programming plays an important role in an organism's reaction to environmental stresses during critical developmental periods (Hanson and Gluckman, 2007; Mathers and McKay, 2009). Epigenetics literally means “above genetics”. Epigenetic modifications are heritable changes in gene expression that occur without a change in DNA sequence. Although our genetic code is relatively static throughout our lives, our epigenetic code must change dramatically during development to initiate differential gene expression amongst developing tissues. The epigenetic code consists of chemical modifications to DNA and to the histone proteins that package and compact our DNA in the nucleus. Gene expression is regulated in part by chromatin conformation, which is determined by post-translational modifications of histone tails, such as methylation, acetylation, and ubiquitylation. These chemical modifications induce a closed or open chromatin conformation that either suppresses or activates transcription, respectively. It is thought that histone modifications and DNA methylation are coordinately regulated. DNA methylation of cytosines at CpG sites works to repress transcription by sitting in the DNA major groove and interfering with transcription elements. In addition to histone modifications and DNA methylation, small RNAs also modify DNA to regulate gene expression.

During development, the epigenome cycles through a series of precisely timed methylation changes designed to ensure proper development (Figure 1). The appropriate timing and extraordinary accuracy of methylation in the gametes and following fertilization makes this highly concerted system particularly vulnerable to interference from environmental exposures (Murphy and Jirtle, 2003). Briefly, the paternal genome is actively demethylated and the maternal genome is passively demethylated following fertilization. At the morula stage, *de novo* methylation of the genome occurs to establish proper methylation patterns during embryonic development.

Throughout this process epigenetic marks are laid down to maintain proper expression of imprinted genes. Genomic imprinting results in monoallelic, parent-of-origin dependent expression of a small but significant subset of autosomal genes in Therian mammals (Murphy and Jirtle, 2003). This unique regulation of gene expression is governed by DNA methylation, histone modifications, and noncoding RNAs. Aberrant methylation or loss of methylation can either shut down these critical genes or lead to over-expression of the gene product. Due to their monoallelic expression, imprinted genes are particularly susceptible to deregulated expression resulting from epigenetic aberrations. During the demethylation

process, parent-of-origin methylation marks are maintained on imprinted genes and during *de novo* methylation secondary imprints are established. As the embryo grows, these parent-of-origin imprints are maintained in somatic tissues, but erased in primordial germ cells so that they can be reestablished in a sex-specific manner during gametogenesis. Histone modifications are thought to play a role in this sex-specific mark establishment as there is an extensive loss of histone methylation and acetylation along with the DNA methylation loss (Weaver and Susiarjo, 2009). The methylation marks are then sustained throughout the individual's lifetime until they are erased and reestablished following fertilization of the next generation (Jirtle and Skinner, 2007; Weaver and Susiarjo, 2009) (Figure 1).

Imprinting is vulnerable to deregulation at the time of primary imprint mark erasure and establishment during gametogenesis. These imprints also have to be protected from inappropriate erasure soon after fertilization when the DNA undergoes global demethylation (Jirtle and Skinner, 2007). Thus, environmental agents that alter the imprinting of these genes will lead to severe developmental disorders and enhanced disease susceptibility (Das and others, 2009).

THE EPIGENOME REACTS AND CONSEQUENCES ENSUE

The epigenome's labile nature allows it to respond and adapt to environmental stressors. These epigenetic modifications can also be detrimental-- both later in life and to future generations. Even amongst genetically identical individuals, epigenetic alterations can profoundly affect phenotype. For example, monozygotic twins raised in different environments have significantly contrasting methylation levels by adulthood (Fraga and others, 2005). This phenomenon is thought to be responsible for discordant incidences of cancer, asthma, and cardiovascular disease often seen amongst monozygotic twins. Most recently, systemic lupus erithematosus (SLE) was also found to vary amongst monozygotic twins (Javierre and others, 2010). Twins discordant for the disease also showed significant differences in DNA methylation and expression of genes relevant to SLE pathogenesis. In one rare instance, a monozygotic twin pair was found discordant for a caudal duplication syndrome. No genetic mutations were found and hypermethylation at the *AXIN1* gene was shown to lead to the disorder (Oates and others, 2006). These discoveries signify the extent to which epigenetic dysregulation governs disease onset and progression.

Epigenetic changes are not only heritable in somatic cells, but can also be maintained during meiosis. As a result, the epigenetic information can be inherited across generations. Examples of this arise in plants, insects, and mammals. In plants, environmental exposures can change epigenetic regulation of gene expression that persists through successive generations (Boyko and others, 2010; Molinier and others, 2006). In *drosophila melanogaster*, transgenerational epigenetic inheritance is governed by chromatin remodeling (Ruden and Lu, 2008). Finally, in the *Agouti viable yellow* mouse, inheritance of coat color phenotype in successive generations is controlled by epigenetic mechanisms at the *Agouti* allele (Blewitt and others, 2006; Morgan and others, 1999).

Human epigenomic responses to famine appear to also be consistent with transgenerational inheritance. Methylation at imprinted genes is altered in those exposed to famine *in utero* (Heijmans and others, 2008; Tobi and others, 2009). This response is thought to be adaptive to promote survival in a malnourished environment; however epigenomic dysregulation sustains through adulthood and correlates with increased disease states such as cancer, schizophrenia, and cardiovascular disease (Barker and others, 2009; Song and others, 2009). Additionally, increased neonatal adiposity, cancer, atopic and autoimmune diseases were seen in children of exposed parents, indicating the potential for transgenerational inheritance of epigenetic defects (Painter and others, 2008). Since alterations in DNA methylation in

imprint regulatory elements are present in people exposed *in utero* to famine conditions decades earlier, they have been proposed to be potentially useful as biosensors for developmental exposures to agents that alter the epigenome (Hoyo and others, 2009).

METASTABLE EPIALLELES: ENVIRONMENTAL BIOSENSORS

Metastable epialleles are alleles that are variably expressed in genetically identical individuals due to epigenetic modifications that are established during early development. These epigenetic marks have a number of important characteristics: 1) they are established in a stochastic manner (Morgan and others, 1999), 2) they are potentially inherited transgenerationally (Rakyan and others, 2003), and 3) their establishment can be altered by environmental agents, such as food supplements (Waterland and Jirtle, 2003). The Agouti viable yellow (A^{vy}) and Axin Fused ($Axin^{Fu}$) mice are unique animal models that carry the A^{vy} and $Axin^{Fu}$ metastable epialleles, respectively. Although metastable epialleles have not yet been identified in humans, these mouse biosensors are useful models for determining whether maternal nutritional and toxicant exposures influence epigenetic programming in the offspring.

The A^{vy} and $Axin^{Fu}$ metastable epialleles contain intracisternal A-particle (IAP) insertions. IAP retrotransposons are prevalent in the mouse genome at approximately 1000 copies per cell and consist of elements up to 7 kb in full length (Kuff and Lueders, 1988; Maksakova and others, 2006). IAP elements, along with several others, comprise Class II endogenous retroviruses, which make up 3% of the mouse genome, but only 0.3% of the human genome (Consortium, 2002). Although IAP elements are non-existent in the human genome, other transposable elements including retroviral like elements are present (Consortium, 2002). The long terminal repeats (LTRs) flanking IAPs carry promoters that initiate IAP transcription and-- in the case of these metastable epialleles-- the adjacent host sequences (Falzon and Kuff, 1988; Lewin, 2000; Mietz and Kuff, 1990).

The A^{vy} mouse carries an IAP retrotransposable insert into the murine *Agouti* gene, upstream of the normal transcription start site (Dickies, 1962) (Figure 2A). The murine *Agouti* gene encodes for a paracrine-signaling molecule that promotes follicular melanocytes to produce yellow pigment instead of black pigment (Miltenberger and others, 1999). Normally, transcription initiates from a hair cycle-specific promoter in skin. Transient expression in hair follicles results in a sub-apical yellow band on each black hair, causing the brown (agouti) coat color of wild-type mice.

In A^{vy} mice, a cryptic promoter in the proximal end of the IAP induces constitutive *Agouti* transcription in all cells and throughout the lifetime of the mouse (Waterland and Jirtle, 2003). This ectopic expression leads to yellow fur and to the binding of agouti protein to the melanocortin 4 receptor in all tissues including the satiation center in the hypothalamus (Miltenberger and others, 1999). The resultant signaling increases obesity, diabetes, and cancer in the yellow mice (Morgan and others, 1999). CpG methylation at the IAP is established during embryonic development and the levels correlate inversely with ectopic *Agouti* expression. Methylation levels vary amongst isogenic mice, causing phenotypes to range from yellow and obese (unmethylated) to pseudoagouti and healthy (methylated) (Figure 2B). This spectrum of potential phenotypes makes the A^{vy} mouse a unique biosensor model for determining the epigenetic effects of environmental and nutritional exposures (Dolinoy, 2008; Waterland and Jirtle, 2003).

Like A^{vy}, the $Axin^{Fu}$ allele contains an IAP insertion within the murine *Axin* gene. The axin protein is involved in mammalian embryonic axis formation. The IAP insertion results in expression of a truncated but biologically active *Axin* transcript, resulting in axial duplications and tail kinks that form during development (Rakyan and others, 2003). $Axin^{Fu}$

mice have kinked tails of varying severity; the extent of tail kink is inversely related to the degree of IAP methylation at the *Axin*^{Fu} locus (Rakyan and others, 2003). Just as in the *A*^{vy} model, the *Axin*^{Fu} model also provides a powerful tool for analyzing the ability of developmental exposures to affect genomic methylation and phenotype (Waterland and Jirtle, 2003).

Another metastable epiallele is the *Cabp*^{IAP}. The *A*^{vy} and *Axin*^{Fu} mice also carry the *Cabp*^{IAP} gene due to its presence in the C57BL/6 background mouse strain. The CDK5 activator binding protein *(Cabp)* gene is located on mouse chromosome 2. Interestingly, *Cabp*^{IAP} contains an IAP retrotransposon (Druker and others, 2004), and gene expression is inversely correlated to cytosine methylation at the 5′ LTR of the IAP element. The *Cabp* IAP insert is specific to the C57BL/6 mouse strain, indicating a recent retrotransposition. Due to *Cabp*^{IAP}'s presence in both *A*^{vy} and *Axin*^{Fu} mice, the effects of environmental exposures can be examined at more than one metastable locus within a single animal.

Using these mice, researchers showed that several nutritional and environmental exposures can alter epigenetic programming during gestation. For instance, exposure to methyl donors such as folic acid can hypermethylate the *A*^{vy} and *Axin*^{Fu} alleles, leading to a population shift in the offspring to brown (pseudoagouti) mice or mice with straightened tails, respectively (Cooney and others, 2002; Waterland and others, 2006a; Waterland and others, 2007). Genistein, a phytoestrogen found in soy, also has hypermethylating effects on the *A*^{vy} mouse epigenome (Dolinoy and others, 2006). In addition to nutritional supplementation, gestational alcohol exposure can alter DNA methyation in *A*^{vy} mice, leading to increased methylation levels at the *A*^{vy} allele and craniofacial abnormalities consistent with human fetal alcohol syndrome. These results coincide with another mouse study demonstrating genome wide dysregulation of methylation patterns in response to fetal alcohol exposure (Liu and others, 2009). Furthermore, environmental conditions such as *in vitro* fertilization also alter epigenetic programming. Culturing of *A*^{vy} mouse zygotes with *in vitro* fertilization medium prior to implantation led to a significant population coat color shift towards yellow (Morgan and others, 2008). These results coincide with an increase in imprinting disorders observed in humans conceived through assisted reproductive technologies (Manipalviratn and others, 2009). Although it remains unknown if the methylation changes at these IAP elements in the *A*^{vy} and *Axin*^{Fu} mice occur with the same sensitivity and rate across the entire genome, the observed epigenetic alterations are consistent with other mammalian studies, indicating that these mice are effective biosensors of developmental exposures that alter the epigenome (Baqir and Smith, 2003; Liu and others, 2009; Manipalviratn and others, 2009; Waterland and others, 2006b).

THE *A*^{vy} MOUSE MODEL AND BISPHENOL A: DETECTING EPIGENOTOXICANTS

The first environmental toxicant screened for its ability to alter the epigenome with the *A*^{vy} mouse model was bisphenol A (BPA) (Dolinoy and others, 2007). BPA is an industrial chemical used in polycarbonate plastic production and in epoxy resin linings in metal-based food and beverage cans. The primary route of exposure to BPA results from the leaching of the chemical into the food supply (Cao and others, 2009; EFSA, 2006). Exposure to BPA is concerning because it can act as an endocrine disrupting compound (EDC), binding to nuclear estrogen receptors ERϑ and ERß and potentially membrane bound ER and estrogen-related receptor gamma (FDA, 2008). Based on the results from standardized toxicity tests (NOAEL = 5mg/kg body weight), current low exposure levels (< 11 μg/person/day) are considered safe. Nevertheless, recent studies raise concern for BPA's effects on the brain, behavior, and prostate gland in fetuses, infants, and young children (FDA, 2008; 2010; NTP, 2008). As a result, the FDA recently aligned itself with the National Toxicology Program

(NTP) program of the National Institute of Health to support further research and to take steps to reduce human exposure to BPA (FDA, 2010; NTP, 2008).

The effect of BPA on endocrine organs may be mediated in part by its ability to alter the epigenome. Recently, in a study using A^{vy} mice, female animals were exposed perinatally to one of four diets: a modified control diet with corn oil substituted for soy bean oil, a modified diet containing 50 mg/kg BPA, a modified diet containing 50 mg/kg BPA and 250 mg/kg genistein, and a modified diet containing 50 mg/kg BPA and methyl donors (Dolinoy and others, 2006) (Figure 3A). DNA Methylation was quantified at the A^{vy} and $Cabp^{IAP}$ metastable epiallele loci by DNA bisulfite treatment, sequencing, PAGE, and phosphor imaging. The offspring population exposed to BPA displayed a significant shift in coat color towards yellow (Figure 3B) with a concomitant marked reduction in DNA methylation at the CpG sites in both the A^{vy} and $Cabp^{IAP}$ metastable epiallele loci. Thus, BPA exposure during pregnancy had a hypomethylating effect on the epigenome of the offspring. These epigenetic effects are thought to have occurred before germ layer differentiation in the embryonic stem cells since the methylation levels from tissue from three germ layers (i.e. brain, liver, and kidney) were not significantly different. Moreover, the negative effect of BPA on the epigenome was negated by supplementing the food with a mixture of methyl donors (i.e. folic acid, vitamin B_{12}, betaine, and choline chloride) or 250mg/kg of genistein (Figure 3B). Thus, food is medicine.

Although the biochemical mechanisms by which BPA alters epigenetic programming remain unclear, the results of other studies support the finding that BPA alters epigenetic programming. For example, neonatal exposure of rats to low-dose BPA (10μg/kg body weight) epigenetically regulates phosphodiesterase type 4 variant 4 (PDE4D4) expression and increases susceptibility to prostate cancer (Ho and others, 2006; Prins and others, 2008). Most recently, *in utero* expsure of mice to a high dose of BPA (5mg/kg) was shown to hypomethylate the *Hoxa10* gene in the uterus leading to abnormal expression and increased binding of ERϑ to the gene (Bromer and others, 2010). BPA also increases the expression of histone proteins during estrogen–mediated cell proliferation (Zhu and others, 2009). Whether this increase is correlative or causative of cell proliferation warrants further investigation. Additionally, low doses of BPA (20 μg/kg body weight) administered to pregnant mice throughout gestation altered the epigenome in the forebrain of the offspring (Yaoi and others, 2008). Exposure to the chemical led to hypomethylation at NotI loci and deregulation of gene expression. These alterations could help to explain the defects in brain development and behavior observed in response to BPA exposure in mice (Tando and others, 2007).

Epigenetic programming is a novel endpoint not yet examined in standard toxicity testing. Several endocrine disrupters, in addition to BPA, can alter epigenetic programming. Diethylstilbestrol (DES) was the first EDC shown to disrupt normal methylation patterns (Li and others, 1997). Since then, DDT, arsenic, phthalates, methoxychlor and vinclozolin have also been found to induce epigenetic alterations (Anway and others, 2005; Kang and Lee, 2005; Nilsson and others, 2008; Shutoh and others, 2009; Vahter, 2008). The ubiquitous nature of endocrine disruptors and other environmental toxicants that alter the epigenome causes high concern for early developmental exposures and demonstrates the need for preventative or therapeutic strategies to combat their negative epigenetic effects. Additionally, the effects of multiple mixed exposures and supplementations on the epigenome have yet to be fully determined. The results from this BPA study indicate that nutritional supplementation can improve the net epigenetic affect at metastable alleles; however, other mixtures could very well show additive or synergistic negative effects. The A^{vy} model provides a unique way to examine the potential effects of EDCs and other exposures.

TRANSGENERATIONAL EFFECTS of EDCs

Disruption of epigenetic programming likely contributes to transgenerational inheritance of phenotypes (Anway and Skinner, 2008; Chiam and others, 2009). Recently, low levels of BPA were shown to induce transgenerational effects in rats at environmentally relevant levels (1.2 and 2.4 μg/kg body weight). Male offspring perinatally exposed to BPA had reduced sperm counts and sperm motility and these phenotypes persisted through the F_3 population (Sallan and others, 2009). Methoxychlor and vinclozolin exposure *in utero* also result in transgenerational inheritance of abnormal reproductive phenotypes (Skinner and others, 2009). Although the pathologies observed in the methoxychlor and vinclozolin studies occurred at doses above which humans are exposed, the studies provide a model to examine the mechanisms behind transgenerational epigenetic inheritance. Interestingly, vinclozolin recently was shown to disrupt methylation patterns of several imprinted genes in mouse sperm up to three generation following exposure (Stouder and Paoloni-Giacobino, 2010), indicating that transgenerational inheritance of aberrant phenotypes could also have an epigenetic basis. It has been suggested that low exposure levels of EDCs could lead to less overt phenotypes in humans, such as type 2 diabetes, adipogenesis, and behavioral effects (Braun and others, 2009; Ling and Groop, 2009; Sargis and others, 2009; Somm and others, 2009). Thus, future studies should also examine the potential for low dose EDC exposure to disrupt the establishment of epigenetic marks, particularly in imprinted genes.

CONCLUSION

The sensitivity of the human epigenome to low levels of EDCs will directly influence the health of current and future populations. If EDCs are shown to interfere with epigenetic programming at current exposure levels, researchers speculate that in addition to altering disease susceptibility, they could also be contributing to the documented increase in human infertility (Price and others, 2007). Thus, as the regulatory bodies struggle to determine the best way to incorporate epigenetic endpoints into toxicological risk assessment, scientists must continue to develop better ways to increase the breadth of doses that can be studied and the scale of the epigenome interrogated in our quest to identify epigenotoxic agents that harm us not by mutating the genome, but by altering the epigenome.

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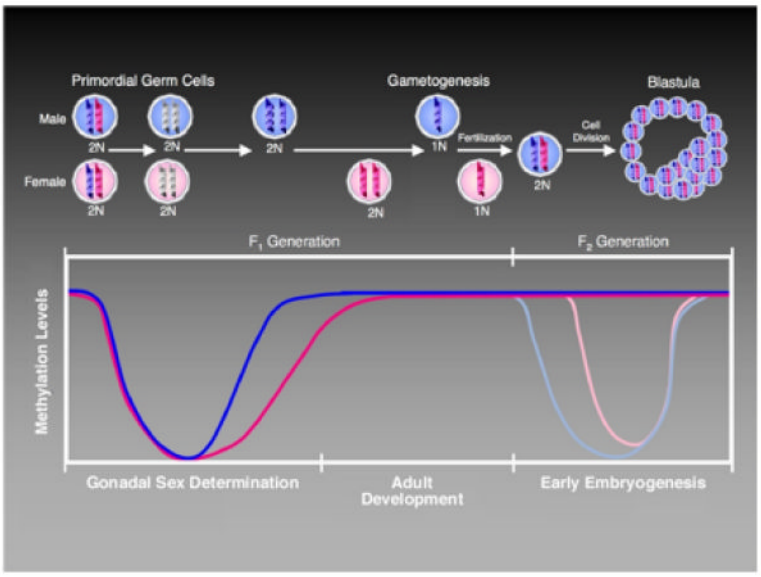

Figure 1.

Alterations in methylation status during development (Jirtle and Skinner, 2007). In primordial germ cells, genome wide demethylation erases previous parental specific methylation marks that regulate imprinted gene expression. Following this erasure, methylation patterns in imprinted genes are reestablished in a sex-specific manner, first in the developing gonocytes (male, colored purple), and later in the female (colored pink) germ line Imprinted genes maintain their primary methylation marks throughout life and during the epigenomic reprogramming that follows fertilization of the next generation. In the F_2 generation, epigenetic reprogramming reestablishes totipotency of the zygote. The paternal genome is actively demethylated (indicated by the lighter purple line in the graph), whereas the maternal genome undergoes passive demethylation (indicated by the lighter pink line in the graph) (Weaver and Susiarjo, 2009). Following implantation, remethylation of the genome occurs to regulate the differentiation of various cell types. Secondary imprints are also set at this time and, along with the primary imprints, are maintained throughout the individual's lifespan. This maintenance allows for the inheritance of parental specific monoallelic expression in somatic tissues throughout adulthood.

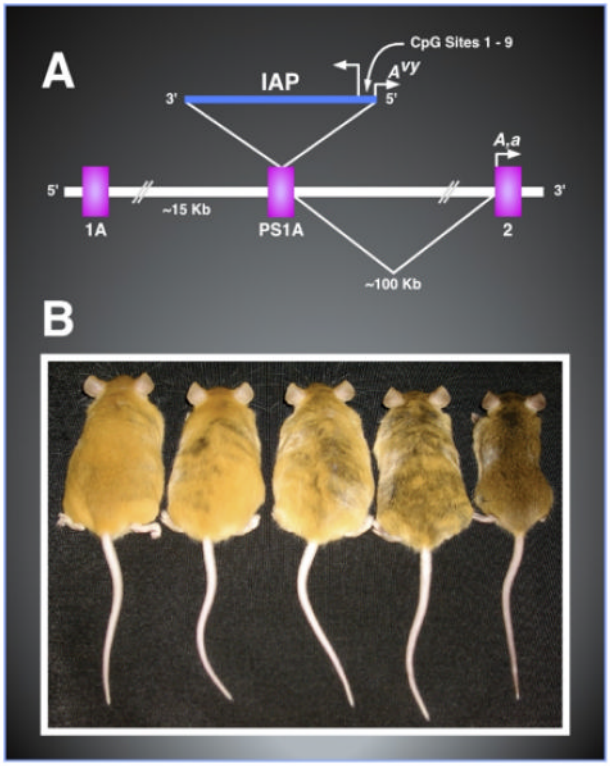

Figure 2.

Epigenetic gene regulation at the A^{vy} locus. **(A)** The A^{vy} metastable epiallele contains an intracisternal A particle insertion within pseudoexon 1A. Normal transcription occurs from a hair cycle specific promoter in exon 2 and leads to brown mice. The IAP insertion upstream of the wild type promoter leads to constitutive expression of *Agouti* from the IAP cryptic promoter and yellow mice. Stochastic Methylation of CpG sites upstream of the cryptic promoter correlates inversely with A^{vy} expression. **(B)** Fifteen-week old, genetically identical, A^{vy} mice with varying coat colors. Yellow mice (left) are hypomethylated upstream of the A^{vy} promoter while pseudoagouti mice (right) are hypermethylated at these CpG sites, recapitulating normal *Agouti* expression. Increasing levels of ectopic expression of *Agouti* in 15-week old A^{vy} mice (from right to left) leads to obesity, tumorigenesis, and diabetes.

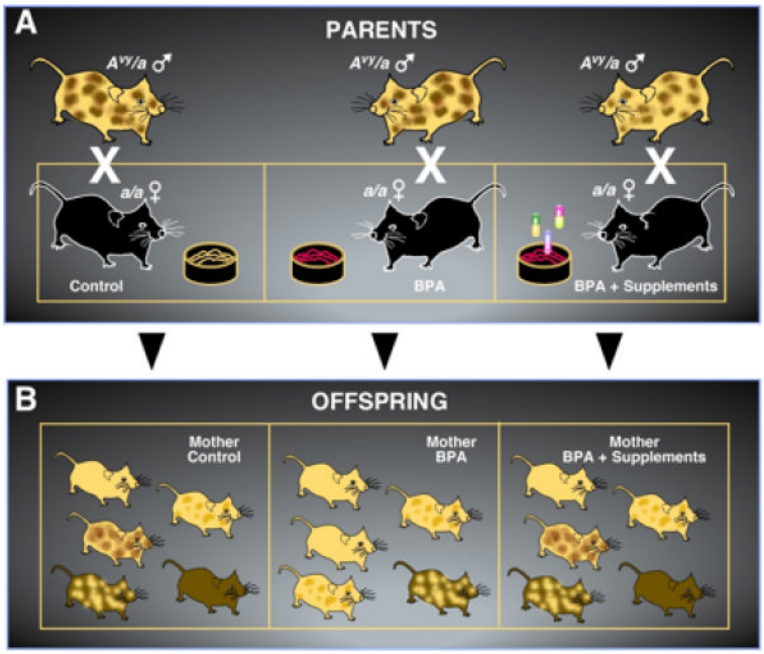

Figure 3.

Effect of bisphenol A (BPA) and maternal dietary supplementation on the phenotype and epigenotype of *Avy*/*a* offspring. **(A)** Female mice were exposed to a modified control diet with corn oil substituted for soy bean oil, a modified diet containing 50 mg/kg BPA, or modified diets containing 50 mg/kg BPA and supplemented with 250 mg/kg genistein or methyl donors. **(B)** Offspring exposed to BPA *in utero* and during lactation were hypomethylated at the A^{vy} allele and of a higher proportion yellow than control mice. Offspring that were exposed to BPA and supplemented with methyl donors and genistein returned to the methylation levels and coat color proportions of control.