Disease Markers 23 (2007) 121–137
IOS Press

Epigenetics – DNA-based mirror of our environment?

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Abstract. Epigenetics affects health, appearance and behavior and propagates mammalian phenotypes across generations. Nutrients, drugs and behavior can all direct changes in epigenetics. In at least some cases, these directed changes are propagated across generations. This range of influences on epigenetics suggests that epigenetics is highly interactive with the environment. Changes in the environment may regularly change epigenetics and influence our future responses to the environment. The current research challenge is to understand these influences and use them to direct epigenetics toward improved health and longevity.

Keywords: Maternal nutrition, epigenetics, imprinting, methylation, acetylation, DNA, histones, expression, behavior, stress, glucocorticoid, hypothalamic-pituitary-adrenal, glucose, diabetes, metabolomics, reproduction, development, diet, mouse, rat, fox, human, agouti, axin, star, phenotype, genotype, GATACCA

1. Introduction

Epigenetics affects health, appearance and behavior and propagates phenotypes across generations. Epigenetics and phenotypes can be changed by diet and drugs and recent studies show clearly that epigenetics is affected by behavior and can propagate behavior patterns across generations. This range of influences on epigenetics, from diet to behavior, suggests that epigenetics is highly interactive with numerous environmental variables and that changes in the environment may regularly change epigenetics and influence our future responses to the environment.

1.1. Epigenetic mechanisms

While genes and numerous other functional sequences (replication origins, centromeres etc.) are contained in the genome, gene *activity,* and possibly the activity of numerous other functional DNA sequences, is determined by epigenetic mechanisms. These mechanisms use chromatin structure and DNA methylation to determine whether genes and other sequences are accessible or sequestered and to what degree. The chromatin structure and DNA methylation on the genome is called the epigenome. The epigenome varies between different cell types and, possibly, between any two cells, even of the same type. Along with the genome, the epigenome is duplicated during cell growth and division such that both the genome and the activities of the genome are duplicated in daughter cells. In many instances, such cellular differentiation, the epigenome may change to establish a new pattern of gene expression. While much of the epigenome is established in embryonic and fetal development some normal epigenetic changes clearly occur in postnatal development. Some *Hox* developmental genes are methylated postnatally [52] as is at least one gene controlling adult behavior [123].

Methylation of cytosines at the 5-position affects the interaction of DNA with numerous DNA binding proteins in chromatin. Generally, DNA methylation sequesters DNA making it less available for transcription. Mammals, higher plants, birds, reptiles, fish, and some fungi, all use DNA methylation as a means

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of genome control. This control includes suppression of the expression of intragenomic parasitic sequences such as endogenous retroviruses (ERVs) [15,67,78]; the inactivation of X-chromosomes [99,102]; and silencing of some, possibly many, genes including many showing genomic imprinting and epigenetic inheritance [28, 85,96,123]. At least three DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) and at least one methylated DNA binding protein, MeCP2, are necessary for mammalian development [68,93,115]. At the DNA replication fork, the newly synthesized daughter strand is methylated, duplicating the parental pattern [14,53,98]. However, this process is imperfect and leads to changes in DNA methylation patterns.

Genomic imprinting is inheritance of parental *germline* DNA methylation patterns by offspring [54, 77,97] such as the maternal and paternal allele specific methylation at the imprinted *Igf2* and *H19* loci [74]. Inheritance of *tissue specific* somatic DNA methylation patterns from parents to offspring has been termed methylation "blueprinting" [107] and is necessarily an indirect form of epigenetic inheritance. Inheritance of general *somatic* DNA methylation patterns from parents to offspring is transgenerational epigenetic inheritance [48,85,100] as occurs in the inheritance of epigenetically determined mouse coat color phenotypes between generations of mice [16,85,125,129].

Along with DNA methylation, histone modifications help determine the activity of chromatin [59, 65]. These modifications include enzymatic methylation and acetylation of specific sites on histone tails. Histone acetylation promotes active chromatin. Methylation of some sites promotes active chromatin, while methylation of other sites promotes inactive chromatin. For example, methylation of lysine 9 on histone H3 promotes gene silencing whereas acetylation of this lysine is found in transcriptionally active chromatin [65]. Acetyl and methyl groups for these reactions come from metabolism and may therefore be influenced by metabolic state and diet.

1.2. Methyl and acetyl metabolism

Epigenetic regulation relies heavily on enzymatic methylation of DNA and histones. The methyl donor for these reactions is S-adenosylmethionine (SAM), which is a product of methyl metabolism. Dnmts and histone methyltransferases use SAM to methylate cytosines in DNA and lysines and arginines in histones. Dnmt1 is inhibited by the reaction product S-adenosylhomocysteine (SAH) and is a zinc-finger enzyme [2,13,24]. Histone methyltransferases are also likely inhibited by SAH [59]. The methyl groups for SAM come from methyl metabolism and are either newly synthesized in one-carbon metabolism or are preformed in the diet.

Methyl metabolism uses dietary folates (or folic acid), dietary methionine, and dietary or endogenous betaine and choline (preformed methyl groups). Folate, methionine, zinc and vitamin B_{12} (cobalamin) are used as intermediates and enzymatic cofactors to transport and transfer methyl groups in methyl metabolism (Fig. 1 [26,83,86,119].) Choline and betaine are widespread in foods and are important sources of preformed methyl groups from the diet [136]. All of these components, except for betaine, are dietary essentials.

Epigenetic regulation also relies on acetylation of histones. The acetyl donor is acetyl-coenzyme A (acetyl-CoA) which is a common intermediate in fat and carbohydrate catabolism where it provides acetyl groups from both of these pathways to the citric acid cycle. Although acetyl groups are often abundant, they nevertheless are a source of available energy and could signal the potential for growth. Numerous regulatory molecules, cofactors and vitamins including insulin, epinephrine, carnitine and pantothenate are involved in production and transport of acetyl groups. The disposition and availability of acetate also depend on macronutrient levels and balance (carbohydrate, protein, fat) and on physiological factors such as fasting and exercise [60]. In addition to histones, acetylation of some transcription factors can increase their activity [22,58].

It has been proposed that gene regulation by methylation of DNA [26], methylation of histones [56] and acetylation of chromatin proteins [23] respond to levels of dietary and metabolic precursors and cofactors for methylation and acetylation. Interactions between diet, metabolism, gene regulation and epigenetics may well be carefully tuned, evolved responses to environmental variation.

1.3. Evolution, metabolism and epigenetics

There is little *a priori* reason to think that metabolism or the allocation of nutrients from the diet will contribute to the long-term health of adults or that early development and maternal metabolism will be geared toward the long-term health of the offspring. Instead, natural selection for reproductive fitness makes animals that are good early reproducers. Most animals (nearly all individuals of many species) will be killed by predators (macro- and microscopic) before they be-

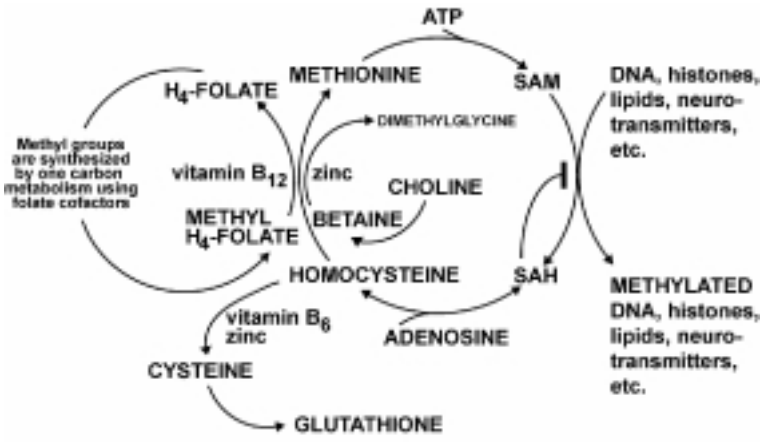

Fig. 1. Methyl metabolism showing some of the major intermediates, cofactors and dietary sources of methyl groups. Methyl metabolism intersects with the metabolism used by antioxidant defenses through homocysteine, cysteine and glutathione.

come aged. Animals allocate valuable resources such as nutrients and metabolites not for the long-term maintenance of the individual but for early reproduction [26–28]. Likewise, maternal metabolism is aimed at providing nutrients to assure that offspring will be efficient early reproducers. Nutrients will be allocated, and epigenetics and the epigenome will be developed and maintained, as suits short-term survival and reproduction.

Many geneticists and much popular culture promote the idea that genetics is the major or sole determinant of health and lifespan. In the science fiction movie, "GATACCA" DNA sequence was used to predict lifespan and future health with unrealistic precision. Genetics does determine that mice have roughly two-year lifespans compared to humans' roughly 70-year lifespans (although epigenetics in the two species are also different). However, within a species, epigenetics and other nongenetic influences can have huge effects. For example these influences are probably responsible for 50 versus 90-year lifespans in humans, and for 18 versus 33-month lifespans in inbred strains of mice and rats [28]. Importantly, many of the dietary, metabolic and behavioral variables that would be expected to contribute to such differences have not yet been studied at the epigenetic level.

2. Dietary and metabolic effects on epigenetic regulation

Some studies of epigenetics and nongenetic inheritance have used mice with specific natural mutations caused by ERVs. Some other studies use mice or rats in which diabetes is induced by nutrients or drugs that affect metabolism.

2.1. Yellow-agouti mice

Animals that vary in coat color are easily identified and categorized and several natural mutations in mouse coat color due to variations at the *agouti* locus have been identified. The *agouti* locus normally (wild type, A allele) produces agouti coat (brownish) mice. If overexpressed, the *agouti* locus (e.g. viable yellow, A^{vy} allele) produces yellow mice. If null, the agouti locus (a allele) produces black mice. In some cases, epigenetic modification of a long terminal repeat (LTR) of an ERV-like sequence regulates *agouti* expression and coat color. The resulting yellow-agouti mice are a clear example of epigenetics modulating genetics. The epigenetic phenotype in a genetically homogeneous, inbred, background affects the long-term health characteristics of mice. Epigenetics silences an otherwise deleterious, hypermorphic allele (A^{vy}) rendering it nearly harmless. This silencing provides A^{vy}/a mice with an agouti (brownish) coat and with normal health similar to the health of mice homozygous for a null agouti allele (a/a genotype, black coat) (Fig. 2).

Epigenetic suppression of the A^{vy} allele produces normalized *agouti* gene expression similar to that of some wild-type alleles and yields *pseudoagouti* mice (Y0), with agouti coats (Figs 2 and 3). Without DNA methylation, an LTR drives *agouti* gene expression and induces the yellow coat and yellow phenotype. The majority of A^{vy}/a mice constitute a continuous spectrum of variegated patterns of agouti areas (mottling) on yellow backgrounds. The degree of mottling defines

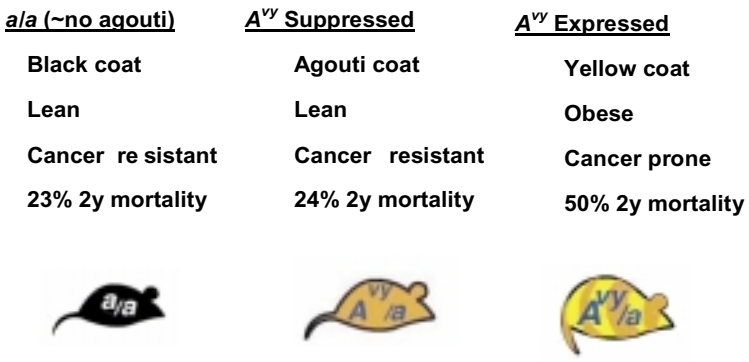

Fig. 2. Mice of three different types are generated by breeding a/a X A^{vy}/a mice. Left) Normal health is achieved by having "normal genetics" with only null *agouti* alleles (a/a). Middle) Normal health in most respects is achieved by having abnormal genetics (A^{y}/a) that is corrected by epigenetics. Right) Mice with abnormal genetics (A^{vy}/a) expressed have a variety of long term health problems.

Fig. 3. Examples of mice from the viable yellow mouse model. Strain VY mice showing A^{vy}/a mice, top, a heavily mottled, Y2, mouse, and bottom, left to right, a slightly mottled, Y4 mouse, a pseudoagouti, Y0 mouse, a clear yellow, Y5 mouse and a mottled, Y2 mouse. These five mice are genetically identical. Coat color patterns are due to the degree of A^{y} expression.

their Y0-Y5 phenotypes. A Y5 "clear yellow" mouse (Fig. 3) is not mottled and is at one extreme of this spectrum, whereas a Y0 mouse (Fig. 3) has an agouti coat and occupies the other extreme of the spectrum. In A^{vy} and related alleles the degree of agouti mottling and the degree of agouti IAP methylation are correlated [6,28,81,85,122]. For the A^{vy} allele the degree of agouti mottling and the level of methylation specifically within the *agouti* IAP LTR are very highly correlated ($r = 0.98$, $P < 0.03$, ref. [28]).

In A^{vy}/a mice, the level and pattern of *agouti* expression can be determined by each animal's coat-color pattern which appears just 7 days after birth [129,134]. Thus, the coat color is an early marker of long-term health and disease in these mice.

2.1.1. *Agouti overexpression causes obesity, diabetes, cancer and low 2-year survival*

The intermediate steps between ectopic *agouti* overexpression and many gross biological endpoints have been studied in some detail [88]. The agouti protein antagonizes melanocortin receptors and the endpoint of the signaling affects different events in different cell types. For example, in hair follicles, agouti effects yellow pigment deposition in the hair while in adipocytes, *agouti* promotes pathways associated with adipocyte differentiation [88].

Table 1
The composition of MS and 3SZM supplements

MS diet supplement	3SZM diet supplement
5 g Choline	15 g Choline
5 g Betaine	15 g Betaine
5 mg Folic acid	15 mg Folic acid
0.5 mg Vitamin B_{12}	1.5 mg Vitamin B_{12}
	7.5 g L-methionine
	150 mg Zinc

The above are added to NIH-31 diet to give 1000 g of the respective final diet. The final total amounts in these diets are substantial increases over the amounts in the base NIH-31 diet [28,129].

Ectopic *agouti* expression leads to obesity and type 2 diabetes [80,131,132,134]. Mice with ectopic *agouti* overexpression due to an active A^{vy} allele convert food calories to fat stores more efficiently (they have increased metabolic efficiency) compared to mice with the a agouti "null" allele [128]. Mice differing in these two alleles and calorie intakes also show different patterns of hepatic gene expression including expression differences in genes likely important in diabetes [63]. Agouti overexpression also increases susceptibility to several types of cancer [126,127,130,132] and lowers 2-year survival. The mortality at 24 months of age is twice as high for yellow A^{vy}/a mice (Y2-Y5) as it is for pseudoagouti A^{vy}/a(Y0) or black a/a mice [129, 132].

2.1.2. *Maternal diet affects the epigenetics of offspring*

Methyl metabolism and DNA methylation are dependent on numerous dietary components including betaine, choline, folic acid, methionine, vitamin B12 and zinc (Fig. 1 [26–28]). When dams were fed before and during pregnancy with control diet or with methyl-supplemented diets (Table 1) they produced offspring with different proportions of epigenetic phenotypes (Table 2 [28,129]). Phenotypes with more agouti (brownish) coats increase in proportion of the population as increasing levels of methyl supplement are added to the maternal diet. The highest level of supplementation was effective on two different strains of A^{vy}/a mice. The proportion of mice with majority agouti coat increased from 43% for mice fed control diet to 66% for mice on the high methyl (3SZM) diet (P <0.001, Table 2 [28,129]). Methyl supplement increased agouti pigmentation in the predicted direction and shifted the distribution of epigenetic phenotype. A new phenotype, Y1, was found *only* in litters from dams fed the 3SZM diet [28,129]. These Y1 mice, unique to the 3SZM diet, have a high degree of DNA methylation on their *agouti* proximal LTR commensurate with their high degree of agouti coat color [28]. Despite very high supplement levels (Table 1), no diet exerted any detectable adverse effects on litter size, neonatal mortality, health, etc. [129].

Table 2
The proportion of offspring from dams consuming control and 3SZM diets

Offspring epigenetic phenotype	Control diet dams offspring percentage	High methyl (3SZM) diet dams offspring percentage
Y0	19	18
Y1	0	13
Y2	18	21
Y3	11	29
Y4	32	15
Y5	20	4

2.1.3. *Parental epigenetics partially determine offspring epigenetics*

All epigenetic phenotypes of A^{vy}/a mice and a/a mice produce viable, fertile offspring and can contribute genetically and epigenetically to each new generation. Maternal epigenetic phenotype is *partially passed to the next generation*. Y0 dams are more likely to produce Y0 offspring than are Y2-Y5 dams [125, 129] and Y0 grandmothers are more likely to produce Y0 grandchildren (through Y0 daughters) than are Y2-Y5 grandmothers through Y0 daughters [85]. Whitelaw and coworkers [16] recently showed that haplo-insufficiency of the polycomb locus Mel18 introduces *paternal* transmission of the somatic epigenetic phenotype in the A^{vy} yellow-agouti mouse. Without this haplo-insufficiency of Mel18, they observed only maternal transmission of somatic epigenetic phenotype. Thus, whether an epigenetic trait is passed maternally, paternally or both depend not only on the gene(s) determining the trait (metastable epialleles) but also on genes that affect epigenetic modification.

Paternal epigenetic inheritance is also seen in another mouse epigenetics model where epigenetic modification of a LTR of an ERV-like sequence regulates *axin* expression [11,96]. Parental epigenetic inheritance suggests that maternal (and paternal) effects at A^{vy} may be heritable to subsequent generations and have multigenerational effects.

Changes in maternal diet and maternal epigenetics that change epigenetic phenotypes in this model have, for the most part, not been reported to directly change the long term health of offspring. It seems likely that they would have some long term effects as the normal variation in offspring epigenetic phenotypes presumably result from the combined influence of multiple factors including maternal diet and maternal epigenetics.

2.2. Induction of multigenerational diabetes

In strains of rats and mice not considered to have a genetic susceptibility to diabetes the disease can nevertheless be induced with drugs or glucose loading. In many cases, diabetes or related disorders are passed to one or more subsequent generations.

Maternal diabetes in rats has long been known to cause hyperglycemia in the offspring (see [33]). Numerous studies have shown that diabetes in female rats, well prior to pregnancy, causes diabetes in the offspring. Spergel et al. [110] used a single treatment with the drug alloxan to weanling rats to induce latent diabetes. Early descendants of these alloxan-treated rats have high blood insulin (hyperinsulinemia) which progresses to abnormally low blood insulin in later generations. Later, Van Assche and Aerts [120] treated first generation rats with streptozotocin and reported that some hallmarks of diabetes (such as pancreatic islet hyperlasia and beta cell degranulation) were found in the fetuses of third generation rats (F2) from mothers (second generation, F1) born to grandmothers (first generation, P1). These effects were not found in F2 control fetuses from mothers (F1) born to normal, untreated, grandmothers (P1).

Most effects observed in the above studies are maternal, and not paternal, even when the father was the offspring of a diabetic mother. Other groups have reported similar results where diabetes induced in one generation of rats is passed to subsequent generations [8, 33].

Transgenerational diabetes can also be induced nutritionally. Gauguier et al. [42,43] induced mild hyperglycemia by continuously infusing pregnant rats with glucose during the last week of pregnancy (third trimester). The adult offspring were compared with adult offspring from control dams (infused with a glucose-free solution). Compared to controls, adult offspring (F1) from hyperglycemic mothers had mild glucose intolerance and impaired insulin secretion which worsened with age to basal hyperglycemia and severe glucose intolerance. F2 newborns of these F1 hyperglycemic dams were also hyperglycemic, hyperinsulinemic, and macrosomic (showed fetal overgrowth) and later developed basal hyperglycemia and defective glucose tolerance and insulin secretion. These results show that maternal glucose intake in pregnancy can produce heritable diabetic states in the offspring.

Aerts and Van Assche [3] studied inheritance of induced *gestational* diabetes i.e. diabetes in the mother that occurs mainly during pregnancy. Aerts and Van Assche [3] produced mild diabetes in rats by treating them with streptozotocin. Two generations later, rats had mild diabetic symptoms during pregnancy (increased non-fasting blood glucose and no adaptation of pancreatic beta cells to pregnancy). Effects extended to at least the third generation. Van Assche and Aerts [120] later showed that these effects are mainly maternally transmitted. Gauguier et al. [44] also found higher maternal than paternal inheritance of diabetes in rats.

Multigenerational diabetes has also been induced in mice. Descendants of streptozotocin-induced diabetic mice were diabetic and this effect extended over several generations. Glucose tolerance was impaired in these mice, especially after the F6 generation [106].

Women with gestational diabetes are significantly more likely to have mothers with non-insulin-dependent diabetes (NIDDM) than to have fathers with NIDDM. Also, these women are more likely to have grandmothers with NIDDM than to have grandfathers with NIDDM. Maternal transgenerational inheritance of NIDDM due to gestational diabetes was suggested as an explanation for this trend [50].

Multigenerational inheritance and progression of diabetes in rats, mice or humans are not yet defined at the level of gene specific expression or epigenetic modification. Models do not need to necessarily, invoke DNA or chromatin modification but could rely on forms of metabolic imprinting by each mother on her offspring during pregnancy. However, recent studies with behavior, discussed below, provide a model for transmission of effects that includes an essential epigenetic modification step. The cause and mechanisms of diabetes are of great interest because of its potential direct relevance to the current rise in childhood and adult diabetes in the United States. Massively parallel "omic" methods [1, 63,65,76] should be useful on models such as these where the molecular mechanisms are undetermined.

3. Xenobiotic and endocrine disruptor effects on epigenetic regulation

Although many studies show only maternal transgenerational effects, others show important paternal transgenerational effects. Endocrine disruptors can have transgenerational effects through both sexes. Paternal and sometimes maternal effects have been observed with diethylstilbestrol (DES), methoxychlor and vinclozolin [5,90,118,121].

Anway et al. [5] studied changes in the rat male reproductive system after maternal exposure to the fungicide vinclozolin or the pesticide methoxychlor. In fetal development, during gonadal sex determination, the testes contain androgen receptor and estrogen receptor beta and are sensitive to exogenous androgens and estrogens. Anway et al. exposed pregnant rats (F0) from E8 to E15 with vinclozolin. Male offspring had increased spermatogenic cell apoptosis, decreased sperm number and decreased sperm motility. These characteristics were passed to male offspring for 4 generations (F1-F4) without further treatment. These characteristics were passed thru the male line as evidenced by transmission from males of the treatment group after mating with control females. In contrast, there was no transmission of these characteristics when females of the treatment group were mated with control males. Some similar effects were observed after maternal treatment with methoxychlor although the experiment was only carried to the F2 generation.

The degree of effects on spermatogenic cell apoptosis, decreased sperm number and decreased sperm motility were similar in each generation indicating that the effect did not diminish with generations. Additionally, about 8% of males in each generation of the treatment group were infertile after 3 months of age, an effect not observed in the control group.

DNA methylation changes, both hypo- and hypermethylation, were found in numerous sequences. Although two specific genes were identified these were not known loci involved in genomic imprinting or epigenetic inheritance. Methylation of such loci may nevertheless be causal in epigenetic inheritance of phenotypes or may be useful markers for phenotypes [5].

Paternal exposure can also affect epigenetic mechanisms. Preconceptual paternal treatment of rats with the chemotherapeutic cyclophosphamide causes increased neonatal and early adult mortality in F1, F2 and F3 offspring. Offspring of F1-F3 also have learning deficits [7]. After exposing male rats to cyclophosphamide and mating with control females, Barton et al. [9] collected one- and two-cell stage embryos and analyzed these by immunofluorescence for DNA methylation and histone H4 acetylation. At each pronuclear stage, there were some significant differences in DNA methylation or histone H4 acetylation between pronuclei of zygotes from mating of exposed males versus those from mating of control males. This indicates that early epigenetic events in development are altered by preconceptual paternal treatment with cyclophosphamide. Interestingly, this treatment, that was only paternal, affected both male and female pronuclei. Potentially, this preconceptual paternal treatment could affect epigenetic regulation of both paternal and maternal alleles in offspring.

4. Epigenetic determinants of behavior

Nongenetic multigenerational inheritance of behavior has been known in mice for almost 40 years [32] however mechanisms for nongenetic transmission of behavior have only recently been described.

In rats, dams show a continuum in behavior between a high degree of licking and grooming of pups and an arched back posture while nursing (high LG-ABN) and a low degree of licking and grooming of pups and a passive posture (lying on her side or back) while nursing (low LG-ABN). The degree of LG-ABN in the population is normally distributed. High and low LG-ABN mothers represent opposite ends of this behavioral continuum [19]. These behaviors, acquired through a mother's care of her pups (high or low LG-ABN) during the first week of postnatal life, are passed maternally to the next generation by adult female offspring who care for their pups in a similar manner (high or low LG-ABN). Further, pups receiving these behaviors maintain certain responses to stress and other behaviors throughout much or all of their lifetimes [39].

During the first week (postnatal), the degree of LG-ABN affects the epigenetic modification of the hippocampal glucocorticoid receptor (GR) gene promoter of the offspring. High LG-ABN probably affects epigenetics via increased serotonin binding to serotonin receptors and subsequent intracellular signaling. High LG-ABN results in histone acetylation, a hypomethylated GR gene exon 1–7, and NGFI-A transcription factor binding in the GR gene promoter and a high level of GR gene expression. Low LG-ABN results in less histone acetylation, a hypermethylated GR gene exon 1–7, blocked NGFI-A binding and a low level of GR gene expression [123].

The level of GR expression determines the density of GR in the hippocampus, the degree of feedback inhibition in the hypothalamic-pituitary-adrenal (HPA) system and the degree of stress response of the adult offspring. A high level of GR in the hippocampus results in more negative feedback relayed to the HPA and a lower response to stress. Rats with higher levels of hippocampal GR are less responsive to stresses, less fearful and show behavior that is more exploratory in a

novel environment such as during the open field test [18, 39].

Thus, the range of behavior by dams produces adult offspring with a range of response to stress. In particular, offspring who received high LG-ABN are less susceptible to stress, less fearful, show higher GR density of their hippocampus and show more exploratory behavior in open field tests. Offspring who received low LG-ABN are more susceptible to stress, more fearful, show lower GR density in their hippocampus and show less exploratory behavior in open field tests.

This system is heritable between generations because rats receive a level of LG-ABN from their mothers and later demonstrate similar behavior as dams nursing their pups. Thus, a dam's (F0) maternal behavior produces an epigenetic modification in the offspring (F1) that affects the offspring's adult behavior. The maternal behavior of these offspring (F1) then produces an epigenetic modification in their offspring (F2) that affects adult behavior. In other words, maternal behavior affects offspring epigenetics which affects that offspring's adult and maternal behavior. A model of this epigenetic transmission of behavior is illustrated in Fig. 4.

These effects of maternal care on epigenetics and on propagation to the next generation are manipulable at multiple levels. These include behavioral manipulations of cross-fostering and daily handling and nutrient or drug infusions to affect epigenetics.

When pups born to high LG-ABN dams are fostered by low LG-ABN dams they adopt the low LG-ABN behavior pattern. Likewise, when pups born to low LG-ABN dams are fostered by high LG-ABN dams they adopt the high LG-ABN behavior pattern. This behavior includes care of pups. In other words, the foster mother's (F0) behavior toward offspring (F1), determined how the adult female offspring (F1) would treat their pups (F2) [39]. Subsequent cross fostering studies showed that the foster mother's behavior toward offspring determined the offspring's pattern of epigenetic modification in the GR gene [123]. This demonstration clearly affirms that behavior establishes aspects of epigenetics.

Rats born to low LG-ABN dams will develop the high LG-AGN behavior pattern as well as high LG-ABN pattern of gene expression (of hippocampal GR and some other genes) if they are handled daily in the first 10 postnatal days by laboratory personnel. Handling of rats born to high LG-ABN dams does not change their high LG-AGN behavior pattern or gene expression. This shows that handling by laboratory personnel can substitute for low maternal care [39].

These two studies of behavior modification through maternal behavior or a surrogate (handling) show that changing the way pups are treated early in postnatal life can affect epigenetics. Although some epigenetics can change by drift or dysregulation later in life, it is important to know if epigenetics can be changed in a directed way in adults. Epigenetic marks established by postnatal maternal treatment of pups can be changed later in life by histone deacetylase inhibitors [123]. Rats who received high LG-ABN or low LG-ABN as pups received small volume intracerebroventricular infusions of the histone deacetylase inhibitor trichostatin or vehicle as adults (3 months of age). Histone acetylation promotes a transcriptionally active chromatin structure and is facilitated by deacetylase inhibitors such as trichostatin. This treatment in low LG-ABN adults resulted in histone acetylation (measured on histone H3 lysine 9) and loss of DNA methylation (on several CpGs in the GR gene exon 1_7) and increased hippocampal GR expression. Low LG-ABN rats treated in this way were also less susceptible to stress as measured by plasma corticosterone concentrations during restraint stress. By most measures, high LG-ABN adult rats were not significantly affected by trichostatin, although these rats already had high histone acetylation and low DNA methylation in the GR gene exon 1–7, and low plasma corticosterone concentrations during restraint stress. Trichostatin treatment completely eliminated the stress response programmed postnatally by low LG-ABN.

Methyl supplementation can direct changes in epigenetic marks established by postnatal maternal programming [124]. In experiments analogous to those above, rats received high LG-ABN or low LG-ABN as pups and later received small volume intracerebroventricular infusions of L-methionine or vehicle as adults (3 months of age). In high LG-ABN rats, methionine infusion caused increased DNA methylation in the GR gene exon 1_7 and decreased hippocampal GR expression. However, no affect was observed on histone acetylation. Such rats were more susceptible to stress as measured by plasma corticosterone concentrations during restraint stress and by immobility in a forced swimming test. By most measures, low LG-ABN adult rats were not significantly affected by methionine, although these rats already had low histone acetylation and high DNA methylation in the GR gene exon 1–7, and high plasma corticosterone concentrations during restraint stress. Methionine infusion treatment completely eliminated the stress resistance programmed postnatally by high LG-ABN.

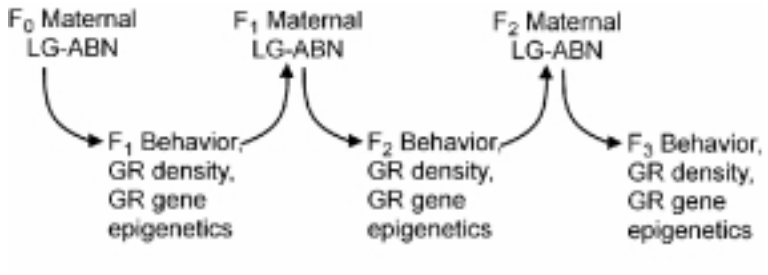

Fig. 4. A model for epigenetic transmission of behavior. In rats, maternal (F0) care of pups (F1) in the first week of postnatal life affects the pup's epigenetics and in turn affects downstream events including the pup's later adult behavior. Offspring's (F1) behavior includes a dam's (F1) maternal care of her pups (F2) which affects the pup's epigenetics and in turn affects downstream events including the pup's later adult behavior, and so on. This propagation of behavior patterns may occur over many generations. Based on Francis et al. [39], Weaver et al. [123].

In most cases, the treatments affected those groups whose behavior and local GR chromatin state had the "most room to change" relative to the treatment used. Although histone deacetylase inhibitors often affected DNA methylation, methionine did not significantly affect histone acetylation. Apparently, DNA methylation changes can bypass histone acetylation and nevertheless change gene expression. An important question is whether effects on histone acetylation or methyl metabolism will have mainly specific effects or will have broad, general effects on epigenetics. While the later might seem most likely, certain genes may be poised for environmental regulation in specific cells by virtue of DNA methyltransferases, demethylases, histone acetyltransferases and other enzymes located in their chromatin domains [124]. Genes not useful for response to metabolism, behavior etc. would not be so poised and would presumably be highly resistant to change in their epigenetics and activities.

Other models of behavior also indicate significant epigenetic effects. In mice, adult behavioral differences between strains, could be genetic but may instead, or also, be due to environmental differences during development. Behavioral tests in mice of different strains, including exploration of an open field, demonstrate that some behavioral effects are nongenetic and maternal [40]. In these experiments, mice of one strain were transferred as embryos to mice of another strain and/or were cross-fostered to mice of another strain. Adult behavior of offspring was only changed when both prenatal (embryo transfer) and postnatal (cross-fostering) maternal strain influences were combined. No effect was seen when transferring or fostering to same strain dams. For example, when C57BL/6J mice were embryo transferred to *and* fostered by BALB/cJ dams the adult offspring behaved as BALB/cJ mice in three of four tests. However, when C57BL/6J mice were transferred as embryos to *or* fostered by BALB/cJ dams or when C57BL/6J mice were transferred as embryos to *and/or* fostered by C57BL/6J dams, the adult offspring still behaved in all tests as C57BL/6J strain.

These pre- and postnatal effects in strain specific behavior indicate that these effects have been passed through many generations, at least in part, as nongenetic, apparently epigenetic, maternal effects [40]. These authors also suggested that the prenatal environment may prime the pup to respond to postnatal care to establish strain-specific behavior patterns independent of genotype. Other studies provide at least two potential mechanisms for these effects, one of producing effects through maternal behaviors [39] and another of producing effects through maternal environment [28,129]. Mice show a range of maternal licking and grooming behavior [40], and a mechanism similar to that described for rats may work in mice. Whether established mechanisms or new mechanisms are involved, remain to be determined. Figure 5 is a summary composite of some of the main, long-term maternal effects seen in offspring in the above studies.

Studies of transgenerational epigenetic inheritance and epigenetic pedigrees are not restricted to rodents but are also observed in foxes. Silver-black foxes are used for their fur and their coat colors are of great interest to fox breeders and to some geneticists. These foxes have been repeatedly domesticated by selecting for friendliness with humans and for behavior resembling that of domestic dogs. Belyaev and coworkers [10,12, 117] observed that domesticated foxes often had white spots or "stars" on the tops of their heads between the ears as well as modified ear and tail carriage. However, their ancestors did not have the star phenotype. Systematic breeding, pedigree mapping and classical genetic analysis were used to determine that star has dominant, S, and recessive, s, alleles. Homozygotes

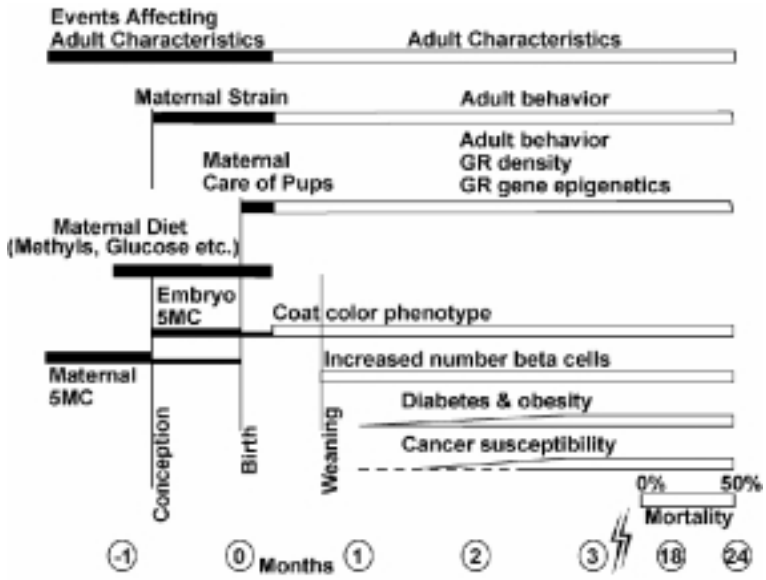

Fig. 5. Timeline of maternal effects and the phenotypic consequences. Prenatal and neonatal factors including maternal nutrition, maternal epigenetics and maternal behavior affect epigenetic features of the embryo/fetus/neonate between conception (-3 weeks) and one week ($+1$ week) after birth. These early events can have lifelong effects on behavior, response to stress, obesity, diabetes and mortality. Additional factors involved in epigenetics, such as histone methylation and histone acetylation and the respective enzyme levels, may have similar effects as those shown for DNA methylation (5MC). Note the break in time scale between 3 and 18 months.

for inactive star (*ss*) are silver black, have no spotting and look like wild-type, undomesticated foxes. Heterozygotes (*Ss*) usually have one or more spots of white hair between the ears with occasional spotting of the lower jaw, breast and belly. Homozygotes for active star (*SS*) have extensive spotting with a blaze between the ears that spreads along the nose sometimes making the face, chest, belly, navel, feet, legs and/or tails white. *SS* foxes always have variable eye color.

Unlike classical alleles, multiple tests show that S and s do not segregate as normal Mendelian alleles. For example, some *ss* foxes are produced in *SS* $\times$ *Ss* and *SS* $\times$ *ss* crosses. Star behaves as an autosomal, monogenic locus but S is not fully dominant and its expression fails to penetrate 100% [12]. Belyaev et al. [12] do not use the term epigenetics, although star expression appears to be inherited epigenetically in domesticated foxes [27]. The inheritance patterns of *star* (S) in foxes are reminiscent of A^{vy} inheritance in mice (variable penetrance, transgenerational epigenetic inheritance, imprinting). As with A^{vy} in mice, expression of star varies within fox litters. The exact environmental and molecular control mechanisms that suppress the activity of the dominant *star* allele S remain to be determined. The environmental differences could include diet, physical activity and human handling to name a few.

5. Monozygotic twins may offer unique insights into human epigenetics

Although studies of epigenetic differences between genetically homozygous mice have been available for some time, the epigenetics of homozygous humans (monozygotic twins) has only recently been studied. Monozygotic twins often differ in various ways (phenotypic discordance) including disease. Most notably, psychiatric disorders show substantial discordance in monozygotic twins [64,133].

Fraga et al. [38] tested monozygotic twins extensively using assays for gene-specific DNA methylation, global DNA methylation, histone acetylation, gene expression and others. They found that younger twins had substantial concordance whereas older twins had significantly less concordance. The main cells studied were lymphocytes but some tests were also done with epithelial skin (buccal) cells and muscle biopsies. Their results indicate that environmental factors and/or endogenous "epigenetic drift" result in these epigenetic differences over time.

The level at which epigenetic drift occurs remains to be determined. Are the same cell types being measured? Are the cells of the same "age"? Different cell populations may be made up of different proportions of cell types (e.g. among lymphocytes). Possibly many

cells of a particular type will pass through certain epigenetic patterns as they age, but they may have "aged" or proliferated more in one twin than the other. Even if cells now differ between twins but are derived from equivalent earlier progenitors, they still clearly represent a type of epigenetic change. The homozygosity of MZ twins provides important research opportunities. These include the study of environmental factors that may promote epigenetic drift, study of disease discordance to identify diseases that may have a large epigenetic component and the study of epigenetic markers for these diseases.

In a study of young MZ twins (twelve pairs of 5 year olds), Mill et al. [82] measured catechol-O-methyltransferase (COMT) gene DNA methylation in buccal cells. COMT gene methylation was concordant in half of these twins but in some, two in particular, differences were substantial. Although Mill et al. studied a different gene than Fraga et al. [38] it is nevertheless interesting to note that some young twins already appear to show epigenetic differences (discordance).

6. Candidates for epigenetic effects: Examples from prostate cancer

In addition to nutrients involved directly in methyl metabolism such as betaine, folate and methionine, a number of compounds that affect methyl metabolism, Dnmts or DNA methylation in adult animals have the potential to also cause maternal effects on early development and offspring epigenetics. Human cancers show an enormous number of epigenetic changes when compared to normal tissues. The development and progression of prostate cancer, in particular, may provide nutrient and drug candidates for directing epigenetic change in other systems.

As in other human cancers studied, the development and progression of prostate cancer results from a complex series of genetic, epigenetic, and cellular events [17,69]. Of all human cancers, prostate cancer has the perhaps the largest number of nutrient, drug and metabolite candidates reported for cancer prevention and control. These include some known to affect epigenetics or epigenetic mechanisms and others that are candidates for epigenetic effects.

All cancers studied show altered DNA methylation in the form of global hypomethylation and gene specific hypermethylation [34,35,62]. In some cases, cancers also show gene specific hypomethylation [20,73]. Certain methylation changes are characteristic of particular types of cancer or characteristic of cancer compared to the corresponding normal tissue. Hypermethylation of the promoter region of a glutathione S-transferase pi-class gene (*GSTP1*) is characteristic of prostate cancer and is not found in normal prostate [69]. The expression and hypomethylation of the synuclein gamma gene (*SNCG*) is associated with all advanced prostate cancer cases (14 of 14 cases stages II-IV) but only 10 to 20% of stage 1 or normal tissues [73]. In prostate cancer, a number of other genes show DNA methylation changes, hypomethylation [21] or hypermethylation [69].

Histone modification is another epigenetic mechanism affecting gene expression and cancer progression. Most PCa is slow growing, nonmetastatic and a low health risk. In a small proportion of patients, PCa is fast growing, likely to become metastatic and has high mortality [101]. Recently, immunohistochemistry measuring histone acetylation has been demonstrated for the early identification of these high-risk patients from the much larger number of low risk patients [104].

A large number of low toxicity agents have been reported to control the growth of PCa, PCa cell lines, or benign prostatic hyperplasia (BPH). Many of these agents are specific food components or drugs.

Procainamide is a long established antiarrhythmia drug that has been used to reverse CpG island hypermethylation of the GSTP1 gene and reactivate its expression in the prostate cancer cell line LNCaP [72]. Similarly, hydralazine, an antihypertensive drug, has been used to reverse CpG island hypermethylation of several genes including the GSTP1 and MGMT genes in human cervical cancer *in vivo* [135]. Procainamide and hydralazine each cause DNA hypomethylation in a variety of cell types [92]. These drugs lower Dnmt activity by competitive inhibition or by decreased expression, respectively [75].

The major polyphenol from green tea, (-)-epigallocatechin-3-gallate (EGCG), is both a target of *in vivo* methylation [94] and an inhibitor of nuclear DNMT activity. EGCG treatment of some cancer cell lines, including prostate cancer PC3 cells, resulted in hypomethylation and transcriptional activation of previously hypermethylated genes [37]. In addition to affecting prostate cancer cell lines, green tea reduces the growth of PCa in the TRAMP mouse model [47].

Genistein is an isoflavone component of soy that has been shown to reverse DNA hypermethylation of the *RAR-beta* gene in prostate cancer LNCaP and PC3 cells [36]. In other experiments Fang et al. showed that DNMT activity (from an esophageal squamous cell

carcinoma cell line) and recombinant DNMT1 activity, were inhibited by genistein. In addition to affecting PCa cell lines, genistein reduces the growth of PCa in the TRAMP mouse model [79].

Many other, mainly low toxicity agents, have been reported to control the growth of PCa, PCa cell lines, or BPH. These include allyl isothiocyanate [111], apigenin, baicalein and curcumin [105], diallyl disulfide [46], docetaxel and estramustine [70], histone deacetylase inhibitors [41], ibuprofen [45], inositol hexaphosphate (IP6 [108]), isosilybin [30], lycopene [49,57,114], nitroxide tempo (2, 2, 6, 6-tetramethyl-piperidine-1-oxyl) [113], pomegranate extracts [4], quercetin, resveratrol [105], selenium [25], silibinin [109], alpha-tocopherol [51], gamma-tocopherol [61], valproic acid [116], vitamin D (1,25[OH]2 D3) [45], and zinc [29,71]. All of these substances are candidates for changing epigenetics and in some cases are known to affect epigenetic processes such as histone acetylation or DNA methylation (e.g. histone deacetylase inhibitors, resveratrol [55], valproic acid [84]).

Lifestyle choices, including diet and smoking, strongly affect the occurrence and the progression of PCa [91,95,112] suggesting that these parameters can be modulated to reduce risk and outcome. Ornish et al. [95] used serum from men practicing "healthy" and control lifestyles to show that LNCaP cell growth was significantly lower when cells were grown using serum from "healthy" lifestyle men than when grown using serum from control lifestyle men.

7. Epigenetic markers for profiling

Throughout this review a variety of examples have been used to illustrate directed epigenetic effects. In order to test any directed effect at the molecular level it is necessary to have markers characteristic of the effect. Some markers are highly specific while others are only weakly correlated with a phenotype. In many cases, a group or profile of markers is necessary to distinguish phenotypes.

While there are a seemingly infinite number of methylation patterns on a genome, in many cases a finite number of genes, loci or sites can be an effective marker. In a few cases a single gene or even a single CpG site can be an effective marker. For example, GSTP1 gene hypermethylation is found in about 90% of prostate cancers but is not found in normal tissues [31,89]. Just one site, one CpG, is enough to profile the epigenetic coat color phenotype of Avy mice (CpG in the proximal Avy LTR [28]) and enough to profile certain epigenetically determined behavior patterns in rats (CpG in the NGFI-A binding site of the GR receptor exon 1–7 [123]).

Multiple approaches can be used to choose genes for epigenetic profiling. A few specific genes reported to be important in a specific cancer or phenotype can be assayed or a greater number of genes known to be important in a number of cancers or phenotypes can be assayed. We have used both of these methods to characterize tumors with respect to tumor types differing in patient survival [66,103]. In addition, proteomics or microarrays can be used to determine expression differences that may be epigenetically based (e.g. [65, 70]).

The use of DNA-methylation-based markers has several advantages. It represents a heritable state, it can be assayed from a variety of sources, including serum and archival tissue (paraffin embedded), and, if needed, it can be done on a huge number of genes and thus provide an extensive profile [87,103]. In most cases, no one gene methylation or other single measure fully characterizes the risk or type of a cancer or other phenotype, thus broad profiles are desirable.

8. Conclusion

Much of the literature on epigenetics thus far describes epigenetic changes as cancers develop or describe the natural range of epigenetic variation in animal models. The data on directing epigenetics are few. Epigenetic effects based substantially in DNA and chromatin structure clearly mirror some aspects of the environment. Future research will determine if this is a limited reflection or a very broad reflection. Given the effects observed so far, after relatively few inquires, it seems likely that much DNA and chromatin modification and much of development may mirror the environment. Targeted experiments designed to direct epigenetics, as well as massively parallel screens, are needed to help define the range of interaction between the environment and epigenetics.

Epigenetic effects are clearly important with respect to appearance, diabetes, obesity, behavior and stress in animal models. Additional effects on health including cancer susceptibility can be inferred from human data. Epigenetics that influences much of adult health and behavior may be in flux in embryonic, fetal and early postnatal development.

The available data about epigenetics and environment in mammals raises numerous possibilities and basic questions. Rather few compounds have been tested for their effects on epigenetics. In particular very few have been tested in normal animals or in development. In most cases we do not know the ranges of normal dietary constituents that affect epigenetics. Nor do we know the effects of most phytochemicals or drugs. Likewise the roles of signaling pathways and behavior are just now being explored [92,123,124].

Some maternal behaviors affect epigenetics. What other behaviors affect epigenetics? If responses to stress are epigentically determined are other behaviors also? Are habits and other well developed behavior patterns (e.g. addictions) rooted partly in epigenetics? Infusion of compounds into the brain can affect epigenetics. It will be important to know if other, less invasive, routes such as oral administration of nutrients and drugs can direct changes in adult epigenetics or maintain certain epigenetic patterns or profiles. What markers accessible by low to moderately invasive means (blood plasma, PET scanning, psychological testing etc.) can predict epigenetic effects? Can effects on epigenetics be predicted by metabolomics or from effects on signaling pathways?

Maternal behavior clearly affects epigenetics [39, 123]. Can adult behavior affect epigenetics? If stress is managed psychologically (e.g. meditation) does this feedback to change epigenetics?

Epigenetics appears to have evolved in part to allow for an adaptation to last for one or a few generations while preserving the potential for other epigenetic phenotypes should conditions change. How do epigenetic systems evolved over millions of years respond when encountering new environmental variables such as refined foods, drugs, xenobiotics, etc? Do once adaptive epigenetic responses within a natural range of nutrient balances become maladaptive when responding to extreme nutrient imbalances in refined foods and lead to diabetes and other chronic diseases? Do certain concentrated nutrients, e.g. from nutritional supplements or engineered crops, benefit or dysregulate epigenetics and long-term health? Do drugs designed to affect serotonin levels in the synapse affect epigenetic responses linked to serotonin signaling?

Human diets vary greatly in nutrient content including nutrients for methyl metabolism. For example, refined food diets can supply levels of folate and other nutrients that are deficient and that are several fold lower than levels supplied by whole food diets [27]. The current challenge is to identify environmental factors that influence or direct epigenetics to the benefit and maintenance of health as well as those that damage or misdirect epigenetics to cause disease and dysfunction.

Aknowledgements

I thank Martin Widschwendter for many insightful comments and for suggesting the inclusion of topics that helped balance this review. I thank Tonya Rafferty for mouse photographs and Kimberly Cooney for drawing and organizing most of the figures. Supported by grant P01AG20641 from the NIA/NIH and by the Arkansas Tobacco Settlement Fund.

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