

How lifetimes shape epigenotype within and across generations

Nadia C. Whitelaw and Emma Whitelaw*

Division of Population Studies and Human Genetics, The Queensland Institute of Medical Research, Brisbane 4006, Australia

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Despite our detailed characterization of the human genome at the level of the primary DNA sequence, we are still far from understanding the molecular events underlying phenotypic variation. Epigenetic modifications to the DNA sequence and associated chromatin are known to regulate gene expression and, as such, are a significant contributor to phenotype. Studies of inbred mice and monozygotic twins show that variation in the epigenotype can be seen even between genetically identical individuals and that this, in some cases at least, is associated with phenotypic differences. Moreover, recent evidence suggests that the epigenome can be influenced by the environment and these changes can last a lifetime. However, we also know that epigenetic states in real-time are in continual flux and, as a result, the epigenome exhibits instability both within and across generations. We still do not understand the rules governing the establishment and maintenance of the epigenotype at any particular locus. The underlying DNA sequence itself and the sequence at unlinked loci (modifier loci) are certainly involved. Recent support for the existence of transgenerational epigenetic inheritance in mammals suggests that the epigenetic state of the locus in the previous generation may also play a role. Over the next decade, many of these processes will be better understood, heralding a greater capacity for us to correlate measurable molecular marks with phenotype and providing the opportunity for improved diagnosis and presymptomatic healthcare.

INTRODUCTION

Work over the last 50 years has provided us with a good molecular understanding of genotype. We are able to trace inheritance and measure the rate of change of genotype across generations and we understand in a broad sense how the genetic component of an organism contributes to its phenotype, yet genetically identical individuals display considerable phenotypic differences. Monozygotic (MZ) twins, essentially genetic clones, show a surprisingly high degree of discordance for many traits and diseases, for example, height and schizophrenia (1,2). Additionally, in the outbred human population, two individuals are estimated to have a genetic difference approximately once every ~1500 bp, predominantly in non-coding sequence (3,4), and this is seemingly insufficient to account for the phenotypic diversity. The classic experiments of Gartner in the 1970s and 1980s revealed that an unchanging degree of random variation persists among inbred animals living in rigorously controlled environments and in populations living in a highly variable

environment (5). The molecular basis for phenotypic disparity not attributable to genotype or environment, i.e. Gartner's 'third component', remains a biological question that is yet to be satisfactorily answered, and recent work suggests that epigenetic factors may underpin this 'intangible variation' (6).

The epigenotype refers to mitotically heritable patterns of DNA methylation at CpG dinucleotides and modifications to chromatin proteins (e.g. histone acetylation) that package DNA. These modifications can regulate gene expression and are essential for normal cellular functioning (7,8). The epigenome is far less stable than the DNA sequence itself, as the marks are reversible and are established and propagated in a stochastic manner. Thus, in contrast to the inherited genetic information that remains a relatively static component of phenotype, epigenetic marks are in constant flux and, as such, are uniquely able to modify gene expression states.

The rules governing the establishment of these marks are not understood. In a new organism, at any particular locus, it remains unclear how much the epigenetic state is defined by the environment, by genotype (both in *cis* and in *trans*)

*To whom correspondence should be addressed. Tel: +61 738453600; Fax: +61 738453504; Email: emma.whitelaw@qimr.edu.au

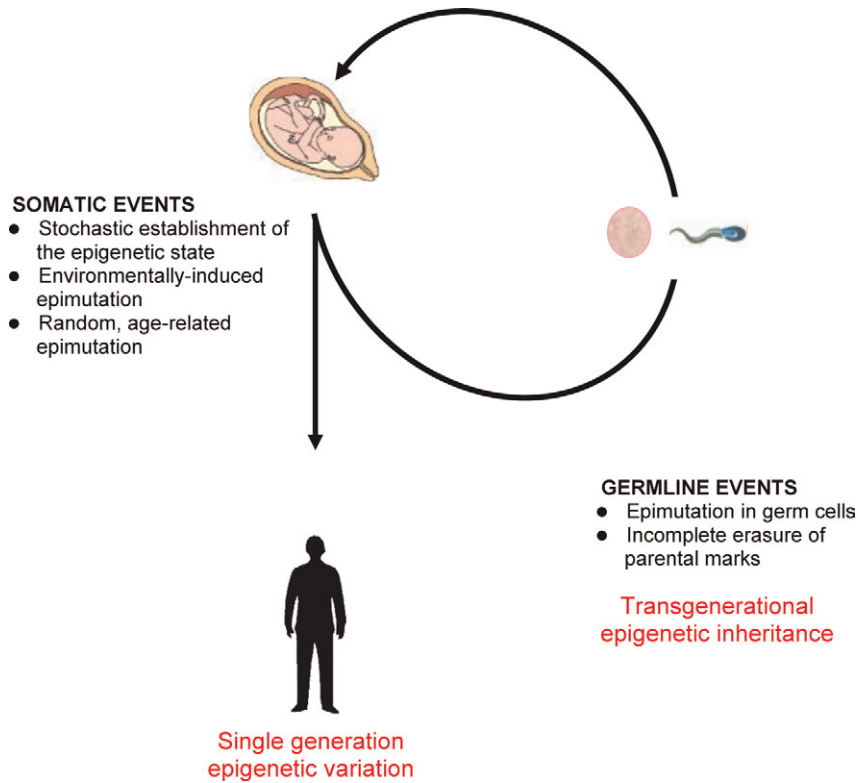


Figure 1. Mitotically heritable epigenetic variation can arise in somatic tissue within a lifetime and can alter phenotype. If epimutations occur in the germ line and are not cleared following fertilization, meiotically heritable epigenetic modifications may affect the phenotype of offspring.

and by the epigenetic state of the germ cells of the parents (Fig. 1). This review will discuss current efforts to resolve to what extent the epigenome is established and maintained in a DNA sequence-autonomous manner and to what extent epigenetic instability affects phenotype.

CHANGES WITHIN A LIFETIME: THE STOCHASTIC COMPONENT

The establishment of epigenetic patterns, like many biological processes, is inherently probabilistic. The underlying molecular events are likely to involve numerous sources of random variation. For example, the maintenance DNA methyltransferase, Dnmt1, which is involved in the propagation of the methylated mark at a CpG dinucleotide through rounds of replication, has an estimated error rate of up to 5% and also has a small *de novo* methylation rate (9,10). The clonal analysis permitted by bisulphite sequencing provides direct evidence for this; even among cells of the same type, single nuclei have distinct CpG methylation patterns at many loci (11,12).

Single-cell analysis of this type is not possible in chromatin studies but indirect evidence, again, supports the idea that stochastic events are involved. The classic studies of position effect variegation (PEV) in *Drosophila* are a good example. PEV is a phenomenon whereby heterochromatic regions randomly spread in *cis* to silence the adjacent *white* locus resulting in a variegated red and white eye colour (13). Evidence

that local changes in DNA sequence, e.g. point mutations, insertions, deletions, translocations, etc., can influence the extent of variegation is apparent from a number of systems and fits well with our understanding of transcriptional control. Extensive studies of PEV have shown that the process can also be influenced by genes in *trans*. Epigenetic modifiers (proteins that influence the probability of epigenetic gene silencing at a given locus) function in a dynamic equilibrium, and silencing events are sensitive to fluctuations in their cellular concentration (14). Further support for this idea comes from the finding that the presence or absence of whole chromosomes in *Drosophila* can alter expression states at autosomal loci (15) and there is some evidence that this also occurs in mammals (16). One explanation for dosage-dependent phenomena is that particular chromosome regions act as sinks that compete for epigenetic modifiers at unlinked loci and, conversely, may function as tanks that provide a ready source of modifier proteins (Fig. 2). Thus, the probability-based competition for a finite cellular pool of modifiers may form the basis for dosage-dependent shifts in variegation.

The intrinsic plasticity of the epigenetic state is apparent from ageing studies in mice. A normally silenced intronic intracisternal A particle (IAP) retrotransposon has been demonstrated to undergo stochastic activation and demethylation in older mice (17). In this case, demethylation is presumed to occur as a corollary of repeated activation of the upstream promoter. Similarly, normally silenced murine genes on the inactive X-chromosome and in imprinted

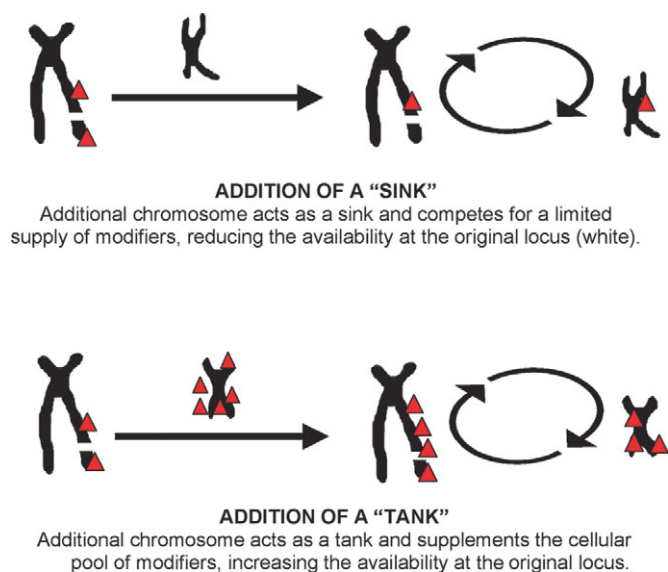


Figure 2. Changes in the cellular concentration of epigenetic modifier proteins (red triangles) affect gene silencing (14). This dosage-dependent phenomena may be a result of competition for a finite cellular pool of modifiers. In this model, chromosomes that are added to the cell (e.g. at fertilization) can act as sinks that compete for modifiers at unlinked loci or can act as tanks that enrich the pool of modifier proteins. In this way, gene loci can act in *trans* to alter patterns of variegation.

regions have been shown to undergo age-related, progressive epigenetic derepression (18). Such changes, in combination with rare somatic mutation, are likely to explain the increase in the heterogeneity of gene expression on a cell-to-cell basis, which has been reported to occur in the ageing mouse (19). Interestingly, loss of function of a chromatin protein has been found to accelerate degenerative ageing-like phenotypes in mice (20). A progressive loss of epigenetic integrity has been regarded as a major cause of cancer for some time (21,22); it is likely that other age-associated phenotypes will be found to have an underlying epigenetic basis, perhaps because of the chance acquisition of epimutations in some cell lineages.

Epigenetic silencing of foreign genetic elements such as IAPs occurs in the pre-implantation embryo (23) and the probability-based nature of this repression can give rise to cell populations that exhibit mosaic patterns of gene expression (24). When attempting to understand the role of epigenetics in shaping phenotype, inherited genetic characteristics and environmental factors must be discriminated. This is difficult in an outbred human population, and for this reason, inbred animal strains have proven valuable in examining epigenetic phenomena, as their genotype is completely controlled and their environment can be highly standardized. These studies have enabled the identification of a group of genes, known as metastable epialleles, that display variable expression among isogenic mice (25). The *agouti viable yellow* (A^{vy}) allele is one example. Mice carrying this gene exhibit a range of coat-colour phenotypes, from yellow to mottled to agouti. The degree of yellowness inversely correlates with DNA methylation levels at an IAP upstream of the A^{vy} gene, i.e. an unmethylated IAP is associated with

yellow coat colour (26). A second metastable epiallele, *axin fused* ($Axin^{Fu}$), manifests as a kinked tail of varying severity among offspring (27). Variation among $Axin^{Fu}$ mice, like A^{vy} , is dictated by the transcriptional activity of a neighbouring retrotransposon, which has a characteristic erratic methylation state as a result of stochastic epigenetic silencing events. As with PEV in *Drosophila*, the epigenetic state and transcriptional activity of metastable epialleles have been shown to be influenced by genes in *trans* (16).

CHANGES WITHIN A LIFETIME: ENVIRONMENTAL EFFECTS

The phenomena of epigenetic metastability, as evidenced by A^{vy} and $Axin^{Fu}$, provides a mechanism for the environment to act on heterocellular gene expression patterns in the developing embryo to shift adult phenotype. We now know that this natural epigenetic variation can be restricted or altered in response to a changed cellular environment (28,29). An early study showed that when non-agouti dams (a/a) are mated to sires carrying the A^{vy} allele (A^{vy}/a), the proportion of agouti offspring is increased if the pregnant dams are fed methyl-supplemented diets during pregnancy (30). A similar developmental effect has recently been observed if genistein (the major phytoestrogen in soy) is added to the diet of pregnant dams (31). *Agouti* transcription, driven by the IAP element, was shown to be suppressed in both of these cases (31,32). Thus, an environmental factor (dietary supplementation) is able to increase the probability of methylation upstream of the A^{vy} locus.

Arguably, the best-characterized example of environmental input guiding epigenetic gene regulation is the flowering response of *Arabidopsis* to vernalization. Following prolonged exposure to cold, or vernalization, concerted actions of histone methylation and acetylation, DNA methylation and the recruitment of repressors such as heterochromatin proteins act to epigenetically silence the FLOWERING LOCUS C, a repressor of flowering (33–36). The promotion of flowering is hence dependent on a sophisticated epigenetic detection of environmental exposure to cold in winter. The vernalized epigenetic state is maintained across many rounds of mitosis and coordinates flowering in the spring and summer months.

CHANGES WITHIN A LIFETIME: REPLICATION-INDEPENDENT EVENTS

The current model is that most epigenetic changes are coupled to DNA replication. Emerging evidence that DNA methylation and chromatin modifications can occur in a replication-independent manner has challenged this notion. Maintenance of the epigenotype is a dynamic process, for example, studies using FRAP (fluorescence recovery after photobleaching) in living cells have shown that a key protein involved in maintaining a transcriptionally silent state, HP1, exhibits transient binding to target chromatin domains (37). Remarkably, the turnover time for the entire cellular pool of HP1, at a given heterochromatin binding site, is a matter of seconds. This epigenetic plasticity

may facilitate gene expression changes in response to environmental events throughout all stages of cellular life.

Within a single cell cycle, changes in chromatin states have been linked to changes in gene expression levels in many cases. Dynamic chromatin states that cycle with 24 h periodicity are characteristic of some circadian patterns of gene activation and repression (38). Transcription of the mouse albumin D element-binding protein (*Dpb*) gene exhibits light-induced circadian oscillations (39), and analysis of the chromatin state at the promoter region has revealed that it fluctuates daily between euchromatin and heterochromatin (40). This experiment was performed on mouse liver cells, which are essentially non-replicative. In other words, these epigenetic changes occur in the absence of DNA replication. Similarly, the deposition of histone variants into nucleosomes can take place in the absence of cell division. In particular, the histone variant H3.3 can be incorporated in a replication-independent manner and is enriched at genes that are actively transcribed, thus serving as a molecular memory for recent transcriptional events (41,42).

In addition to the chromatin state, evidence for the programming of new CpG methylation patterns in predominantly non-replicating cells such as the brain provides another epigenetic mechanism for the shaping of phenotype in adult life. The response of rats to stress is altered by the extent to which, as a pup, they were licked and groomed by their mother. This effect is mediated by increased cytosine and histone methylation at the glucocorticoid receptor (*GR*) promoter in the hippocampus (43). The authors have recently shown a similar effect at the estrogen receptor alpha promoter (44). Of note, the *GR* promoter methylation can be abrogated in mature offspring by treatment with the histone acetylase inhibitor trichostatin A or by infusion with the methyl-donor L-methionine (43,45).

In summary, environment-mediated changes to gene expression can be correlated with epigenetic changes. This emerging evidence for epigenetic changes in non-dividing cells blurs the line between transient transcriptional events and molecular memory. Recent transcriptional activity can certainly leave an 'epigenetic' footprint in the absence of cell division. Nevertheless, the field of epigenetics has, until now, been defined by mitotic heritability and so perhaps these molecular events should be thought of as transcriptional and distinct from bona fide epigenetics. Of course, ultimately what is important is that the process occurs at all, not the semantics.

STUDIES IN HUMANS

As presented earlier, mounting evidence in animal models suggests that the labile nature of the epigenetic state may allow manipulation of adult phenotype during development. Current research in humans is now beginning to implicate reprogramming both before and after birth as a normal developmental process, and one likely to be influenced by environmental input. MZ twin discordance may be a natural consequence of these molecular events. A 2005 study examined DNA methylation and histone acetylation in 80 pairs of twins, ranging from 3 to 74 years of age, using a combination

of global and locus-specific methods (46). One-third of MZ twins were found to have a significantly dissimilar epigenetic profile. Of note, the most disparate pairs were older twins and those with a history of non-shared environments.

Another study has recently demonstrated that CpG methylation discordance is in fact present in MZ twins at an early age. Mill *et al.* (47) studied the methylation level of two CpG sites in the promoter of the catechol-*O*-methyltransferase gene, *COMT*, which has been loosely linked to psychiatric disease, in clinically unaffected 5-year-old MZ twin pairs. They showed that methylation levels between twin pairs are highly variable and that some twin pairs show clear discordance in methylation state. Thus young MZ twins, presumably with a highly shared environment, can display marked epigenetic differences. In these two studies, however, we do not know whether the epigenetic discordance is responsible for or associated with any phenotypic discordance.

A similar study, investigating an MZ twin pair discordant for a caudal duplication phenotype, has revealed a role for allele-specific stochastic methylation in disease (12). Analysis of a candidate gene for caudal duplication, the human homolog of mouse *Axin*^{Fu} (*AXINI*), showed that there to be no mutation in the coding region of the gene. DNA methylation analysis at the *AXINI* locus showed that the discordant pair had a significantly more methylated CpG island promoter compared with controls and compared with the unaffected twin. This study demonstrates that a divergence in the epigenetic state of genetically identical individuals can be associated with phenotypic differences and, as such, epigenetic events could be a significant source of variation in the human population as a whole. It has been argued that medical therapies could utilize the reversible nature of DNA methylation and covalent chromatin modification to repair epigenetic lesions acquired throughout life (48). Certainly, the potential benefits to human health are worth the scientific investment, but just as gene therapy has not yet fulfilled its promise, one must remain sceptical about how long this might take.

EPIGENETIC INHERITANCE

For over a century, DNA has been recognized as the sole inherited determinant of phenotype. New evidence, namely that the epigenetic state at some alleles in germ cells exhibit meiotic heritability, has challenged this dogma, and as a result, the epigenotype is becoming accepted as a second form of transgenerational information.

In plants, the transmission of epigenetic states to progeny at some alleles, and the molecular events underlying this process, termed paramutation, is well documented (49). The epigenetic state at these alleles is sensitive to environmental programming. This phenomenon was first observed 50 years ago and describes changes in the expression of the maize *R* allele if the allele has been in the presence of the *R-st* allele in previous generations (50). Exposure of heterozygote *R/R-lst* seedlings to different temperature and light conditions has been shown to alter the expression of the *R* allele in subsequent generations (51). These heritable changes occur in the absence of further environmental treatment, demonstrating a lasting programmed epigenetic event.

There are now several examples of transgenerational epigenetic inheritance in mammals. The A^{vy} and $Axin^{Fu}$ metastable epialleles both exhibit this mode of inheritance in mice (26,27). Yellow A^{vy} dams produce a greater proportion of yellow offspring than agouti dams and, similarly, $Axin^{Fu}$ parents with a more penetrant tail kink transmit this phenotype to a higher proportion of offspring. A question that remains to be answered is whether the dietary-induced changes to A^{vy} phenotypes discussed previously are transmitted to untreated offspring. Although this seems likely, it has not yet been shown and would provide evidence that the external environment can program meiotically heritable epialleles in mammals.

In the A^{vy} and $Axin^{Fu}$ examples, gene expression is governed by CpG methylation at an IAP retrotransposon. Retrotransposons are abundant in the human genome and, although epigenetic inheritance of a transcriptional state is yet to be demonstrated in humans, repetitive elements may be driving this process throughout all mammalian genomes. There is some evidence that these repetitive elements are largely resistant to the demethylation that occurs in gametogenesis and early development (52). In contrast, there is some evidence at the A^{vy} locus that DNA methylation is not the inherited epigenetic mark, suggesting that a transmitted chromatin or RNA state may dictate meiotic inheritance of epigenetic patterns (53).

The strongest evidence that RNA may be involved comes from a recent study of transgenerational epigenetic inheritance at the *Kit* locus in mice (54). Mutation in the mouse *Kit* gene causes several developmental defects, including characteristic white patching. When a heterozygous *Kit* mutant mouse was mated with a wild-type mouse, there was a greater than expected proportion of mice with white spots. A large proportion of these phenotypically mutant mice are, in fact, wild-type or "paramutant". The authors went on to show that heterozygous male *Kit* mutant mice, unlike wild-type mice, overexpress abnormal *Kit* RNA in postmeiotic spermatids and that the transcripts persist in mature sperm. Furthermore, RNA from heterozygous males (total RNA, sperm RNA or *Kit*-specific microRNAs) injected into one-cell wild-type embryos was sufficient to induce the paramutant phenotype. This study provides support for an involvement of inherited RNA transcripts in mediating transgenerational gene expression changes. Somewhat unexpectedly, paramutant mice are capable of transmitting the mutant phenotype to offspring for several generations. A mechanism for this, which relies solely on the persistence of grandparental mutant *Kit* RNA transcripts, seems unlikely.

In addition to directing transcriptional states, epigenetic modifications are also required at other genomic regions, e.g. centromeres, to maintain the structural integrity of chromosomes. Centromeric heterochromatin is essential for chromatid alignment and segregation during mitosis; however, the rules defining the site for the epigenetic assembly of centromeres are unknown. Although primate centromeres are typically associated with arrays of repetitive α -satellite DNA, ectopic centromeres (neocentromeres) can form at chromosomal sites devoid of α -satellite sequence (55). In an otherwise healthy human family, transgenerational paternal inheritance of a functional neocentromere on chromosome 4 has been described (56). There was no evidence of genetic

alteration to the original inactivated centromere or to the region which became the neocentromere. This case demonstrates the faithful meiotic transmission of a parental chromatin state, apparently independent of an underlying genetic defect, and may be considered the first reported case of epigenetic inheritance in humans.

CONCLUSIONS

There is strong evidence in plants for meiotic inheritance of the epigenetic state. Moreover, this inherited component can be modified by environmental influences. Certainly, in recent years, mammalian research has revealed unusual epigenetic phenomena, including transgenerational epigenetic inheritance. These findings have exposed an oversimplification in our current understanding of inheritance, which hinges on a solely genetic basis for heredity. Nonetheless, caution must be taken when extending these findings to a general model. The evidence so far has been limited to studies in transgenic strains, at transcriptionally inactive loci (such as the centromere) or, in the case of A^{vy} and $Axin^{Fu}$, at alleles that are rare in the normal mouse population. It remains to be seen whether these are the first examples of a widely occurring phenomenon in mammals.

Nonetheless, recent research has revealed that the epigenotype is remarkably pliable. This allows phenotype to be shaped by the environment throughout gestation and into adulthood. Thus, the epigenome provides a readout of an individual's recent, and possibly ancestral, environmental history. Technological advances are rapidly expanding our capacity to profile global epigenetic marks (57–59). In the future, the traditional field of epidemiology may extend to "epigenetic epidemiology", enabling a detailed epigenetic assessment of risk factors and disease susceptibility.

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