The penetrance of an epigenetic trait in mice is progressively yet reversibly increased by selection and environment

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Natural selection acts on variation that is typically assumed to be genetic in origin. But epigenetic mechanisms, which are interposed between the genome and its environment, can create diversity independently of genetic variation. Epigenetic states can respond to environmental cues, and can be heritable, thus providing a means by which environmentally responsive phenotypes might be selectable independent of genotype. Here, we have tested the possibility that environment and selection can act together to increase the penetrance of an epigenetically determined phenotype. We used isogenic \(A^{\text{v}}\) mice, in which the epigenetic state of the \(A^{\text{v}}\) allele is sensitive to dietary methyl donors. By combining methyl donor supplementation with selection for a silent \(A^{\text{v}}\) allele, we progressively increased the prevalence of the associated phenotype in the population over five generations. After withdrawal of the dietary supplement, the shift persisted for one generation but was lost in subsequent generations. Our data provide the first demonstration that selection for a purely epigenetic trait can result in cumulative germline effects in mammals. These results present an alternative to the paradigm that natural selection acts only on genetic variation, and suggest that epigenetic changes could underlie rapid adaptation of species in response to natural environmental fluctuations.

\textbf{Keywords:} epigenetic inheritance; environmental epigenetics; agouti viable yellow; DNA methylation; piRNA; adaptive evolution

1. INTRODUCTION

Epigenetic control of gene expression is fundamental to eukaryotic biology, creating somatically stable states of gene expression that underlie the diversity of cell types within a multicellular organism. Epigenetics not only creates phenotypic diversity within an individual, but also within populations, and can do so without genetic variation [1]. An epigenetic variant could thus be a novel, non-genetic substrate for natural selection [2], but this would require that the epigenetic state of the responsible locus be heritable from one generation to the next.

Epigenetic states can be meiotically heritable, resulting in transgenerational epigenetic inheritance. This can occur independently of the underlying genotype, and so the pattern of inheritance rarely follows Mendel’s laws. Epigenetic inheritance is broadly distributed in nature: it is best described in plants and fungi (reviewed in [3,4]), but also occurs in species that segregate a germ-line, such as flies, mice and humans [5–11]. It is not known how many loci might exhibit epigenetic inheritance, and its molecular mechanism is also mysterious. There may be no one conserved carrier of intergenerational epigenetic marks across all species; candidates in mammals include cytosine methylation, chromatin proteins and small RNA molecules [4].

One of the best-studied models of epigenetic inheritance is the mouse strain \textit{agouti viable yellow} (\(A^{\text{v}}\)). This inbred strain displays spontaneous phenotypic variation among genetically identical mice. Littermates display a characteristic spectrum of phenotypes, from yellow fur with accompanying obesity and diabetes, through various degrees of mottling to agouti fur and lean body mass (figure 1a). The basis for the variation is variable expressivity of the \(A^{\text{v}}\) allele, a mutant allele of the \textit{agouti} coat-colour gene that arose spontaneously almost 50 years ago [12]. \(A^{\text{v}}\) was created by the insertion of an intracisternal A-particle (IAP) retrotransposon into an upstream non-coding exon of \textit{agouti} [13,14]. The IAP is epigenetically regulated, but shows variable activity between mice and between cells in an individual mouse. When the IAP is active, transcription from a cryptic promoter in its 5\textsuperscript{-}long terminal repeat (LTR) produces an ectopic \textit{agouti} transcript; constitutive \textit{agouti} expression produces the obese yellow syndrome. When the IAP is epigenetically silenced, \textit{agouti} is expressed in its wild-type pattern (called pseudoagouti) [15]. The activity state of \(A^{\text{v}}\) is inversely correlated with DNA methylation at the IAP cryptic promoter [7,16], but the factors responsible for setting its epigenetic state in each mouse are as yet unknown.

While the epigenetic state of the \(A^{\text{v}}\) allele varies widely among isogenic mice, it is stable in somatic cells, so that the phenotype of an individual mouse will remain the same throughout its lifetime. It is also partially stable in the female germline, leading to transgenerational...
epigenetic inheritance, so that offspring phenotypes are biased towards that of the dam [7]. The epigenetic state of the allele is unstable in the male germline and is completely reset between generations, such that sires of all coat colours produce offspring with the same spectrum of phenotypes.

Epigenetic states can be susceptible to environmental influence, altering phenotypes within a population [17]. The epigenetic state of $A^{ap}$ is influenced by the addition of excess methyl donor pathway molecules to the maternal diet, which shifts the spectrum of offspring phenotypes towards pseudoagouti [14,18–20]. We have previously shown that the epigenetic effects of methyl donor supplementation on $A^{ap}$ are heritable [20]. This heritability provides a means by which an environmentally induced phenotype might be selected for, and rapidly pervade a population without the need for genetic change.

Here, we asked whether selection applied over multiple generations of exposure to methyl donors could fix the epigenetic state of $A^{ap}$ in the germline. We found that multigenerational methyl donor supplementation, coupled with selection for the pseudoagouti phenotype, steadily increased the prevalence of the pseudoagouti phenotype in the population. Our result provides the first evidence that epigenetic traits can be substrates for selection in mammals, which suggests that they may contribute to adaptive evolution.

2. MATERIAL AND METHODS

(a) Ethics statement

All animals were handled in strict accordance with good practice as defined by the NHMRC’s (Australia) Statement on Animal Experimentation and the requirements of NSW State Government legislation.

(b) Mice and diets

The mice used in this study are descended from the C57BL/6 colony maintained at Oak Ridge National Laboratories (originally obtained from the Jackson Laboratories, stock no. 000017). The $A^{ap}$ mutation arose spontaneously on a C3H background and the allele was backcrossed into C57BL/6. The strain, called $A^{ap}$, has been maintained by brother-sister matings for over 40 years, and is thus isogenic. Mice were fed ad libitum on control (NIH-31) or supplemented (NIH-31 plus per kilogram), 15 g choline, 15 g betaine, 7.5 g L-methionine, 150 mg zinc, 15 mg folic acid and 1.5 mg vitamin B12 diet (Specialty Feeds, WA, Australia).

(c) Breeding strategy

The breeding strategy is summarized in figure 1b. Founder breeding pairs (pseudoagouti or heavily mottled $A^{ap}$/a males mated to a/a females) were supplemented from two weeks prior to mating. In generations F1–F4, male pseudoagouti $A^{ap}$/a offspring were weaned onto the supplemented diet and mated with female a/a mice; supplementation was continued for the duration of breeding. We passed the $A^{ap}$ allele only through the sire during this part of the experiment, because the $A^{ap}$ allele is sensitive to methyl donors only when inherited from the sire [20]. In generation F5, female pseudoagouti $A^{ap}$/a offspring were weaned onto control diet and mated with male a/a mice. Generations F6 and F7 female pseudoagouti $A^{ap}$/a offspring were again selected for breeding with a/a males; mice were maintained on control diet until the end of the study. We passed the $A^{ap}$ allele only through the dam through this part of the experiment to maximize our chances of observing inheritance of the cumulative effect (we have previously shown that the effect of methyl donors is inherited through the dam [20], and in the absence of methyl donors, the epigenetic state of $A^{ap}$ is heritable only through the dam [7]). Control populations consisted of mice bred with selection for the pseudoagouti phenotype but without any methyl donor supplementation. In both supplemented and control populations, all genotypes were observed in the expected Mendelian ratios; there was no difference in litter size or breeding performance between the two groups.
but no methyl donor supplementation. The founder population is the same as the founder population in (a). Proportions are expressed as a percentage of the control group (the phenotypic proportions in offspring of unsupplemented A\textsuperscript{av} A\textsuperscript{av} sires). (b) Proportions of phenotypes in each generation during continual methyl donor supplementation. Proportions are expressed as a percentage of the control group (the phenotypic proportions in offspring of unsupplemented A\textsuperscript{av} A\textsuperscript{av} pseudoagouti dams). n, number of A\textsuperscript{av} A\textsuperscript{av} offspring; *p < 0.05, **p < 0.005 versus control; *p < 0.05 versus F1; *p < 0.05 versus F2.

(d) **Phenotype scoring**

Coat colour in A\textsuperscript{av} mice is tightly linked to the epigenetic state of the A\textsuperscript{av} allele. Coat colour in A\textsuperscript{av} A\textsuperscript{av} offspring was assessed at mouse weaning age (21 days) by two trained, independent observers. We used a ranking system whereby coat-colour phenotypes were divided into five categories: full yellow (5–30% agouti fur), half mottled (30–70% agouti fur), heavily mottled (70–95% agouti fur), and full pseudo-agouti; representative phenotypes are illustrated in figure 1a. For data presentation and statistical analysis, phenotypes were divided into two groups: yellow/slightly mottled/half mottled, and heavily mottled/pseudoagouti. The five-category scores from the two observers correlated more than 82 per cent of the time, and the converted two-category scores from the two observers correlated more than 82 per cent of the time. For comparison of proportions of phenotypes between treatment groups, we used a chi-square test (\( \alpha = 0.05 \)).

(e) **DNA methylation analysis**

Genomic DNA was extracted from tail or fresh frozen whole testis by digestion with Proteinase K and extraction with phenol/chloroform. Two microgram DNA was treated with sodium bisulphite (Epitect kit, Qiagen), and 10 per cent of the sample was used in bisulphite PCR. Bisulphite allelic sequencing of the A\textsuperscript{av} promoter region (the U3 region of the 5\textsuperscript{LTR} of the inserted IAP retrotransposon) was performed as previously described [16].

(f) **Small RNA sequencing and analysis**

Libraries for deep sequencing were prepared from testis total RNA using the small RNA expression kit with barcoded primers (Ambion, as per the Applied Biosystems protocol). A single-emulsion PCR was used to couple the barcoded libraries to P1-coated beads as per the Applied Biosystems protocol. After emulsion PCR, templated beads were enriched in a glycerol gradient and deposited onto the surface of glass slides; sequencing was performed using 35 bp chemistry on a SOLID machine (version 3.0). Small RNA reads were mapped to the A\textsuperscript{av} IAP 5\textsuperscript{LTR} (GenBank AF540972, verified by independent sequencing of the LTR in our laboratory) using CoronaLite allowing a maximum of two colourspace errors. The number of mapped reads from each library was normalized according to the total number of reads from that library that were mappable to the mouse genome (mm9).

3. RESULTS

(a) **Multi-generational dietary change combined with selection increases the germline stability of A\textsuperscript{av}**

We exposed A\textsuperscript{av} mice to excess methyl donors for multiple generations, and coupled this with a selective breeding strategy that favoured those mice that responded to the supplement: in each generation after F\textsubscript{5}, only pseudoagouti mice were selected for further breeding (figure 1b). We chose male A\textsuperscript{av} A\textsuperscript{av} mice for breeding (with congenic a/a females) as the A\textsuperscript{av} allele is sensitive to methyl donors only when inherited from the sire [20].

Under these conditions (environmental stimulus, phenotypic selection and genetic homogeneity), we observe that over five generations a progressively larger proportion of mice are pseudoagouti and a smaller proportion are yellow (figure 2a). Selective breeding of pseudoagouti males without supplementation does not lead to any change in the spectrum of offspring phenotypes (figure 2b); conversely, supplementation of an unselected population has previously been found to have no cumulative phenotypic effect [21,22]. Thus, a combination of diet and selection causes the silent state of A\textsuperscript{av} to become significantly more prevalent in the population.
(b) The cumulative effect of dietary methyl donors is heritable for only one generation after withdrawal

To determine whether this induced germline stability is maintained when the dietary stimulus is withdrawn, at the fifth generation, we weaned mice onto an unsupplemented diet, and bred pseudoagouti mice for three further generations (figure 1b). Here, we chose A^po/a females for breeding (with a/a males) because we have previously shown that the effect of methyl donors is inherited through the dam [20] and, in the absence of methyl donors, the epigenetic state of A^po is heritable only through the dam [7]. Thus, our strategy maximized the chance of observing sustained inheritance of the cumulative effect. We observed that the phenotypic effects were maintained from F5 to F6, but in subsequent generations (F7 and F8) the phenotypes returned to baseline (figure 2c). Thus, germline stabilization of A^po appears reversible after withdrawal of the supplement, at least after exposure for a limited number of generations.

(c) The cumulative effect of dietary methyl donors is not dependent on DNA methylation

The cumulative and progressive silencing of A^po that we observe with multi-generational methyl donor exposure implies that some factor that promotes A^po silencing is accumulating in the germline over generations. This factor is either an epigenetic modification of the allele, or a molecule that interacts with it to induce an epigenetic change; cytosine methylation is an obvious candidate. We measured methylation around the A^po transcription start site (figure 3a) in unsupplemented pseudoagouti mice and in pseudoagouti mice that had been supplemented for five generations. Consistent with our previous findings [16], we find that DNA from the tails of supplemented pseudoagouti mice carries no more methylation than that from unsupplemented pseudoagouti mice (figure 3b(i)). We then examined A^po methylation in the testis to investigate whether increased DNA methylation in germ cells might be responsible for the increased stability of the A^po epigenotype. However, we found that the A^po promoter is already fully methylated in testes of control mice, and supplementation does not alter this (figure 3b(ii)).

(d) The A^po allele produces primary piRNAs

piRNAs are 24–30 nt RNAs that complex with members of the Piwi clade of Argonaute proteins to mediate retrotransposon silencing in the animal germline [23]. We considered the possibility that they may be the agent responsible for the cumulative germline stabilization of A^po. We used the SOLiD system (Applied Biosystems) to sequence small RNA libraries from the testes of eight pseudoagouti A^po mice (four supplemented and four unsupplemented), four unsupplemented yellow A^po mice, and an a/a mouse (a/a mice are congenic with A^po/a but do not carry the A^po allele). We obtained between 1.3 and 7.6 million mappable reads from each library (electronic supplementary material, table S1).

We found small RNAs that mapped to the A^po IAP 5’LTR in both the sense and antisense directions relative to A^po transcription (an average of 37 reads per million mappable reads in A^po/a mice; figure 4a). Far fewer small RNAs from the a/a mouse mapped to the A^po IAP 5’LTR (1.3 reads per million mappable reads).

Figure 3. DNA methylation at the A^po promoter is unchanged by multi-generational methyl donor supplementation. (a) Schematic of the A^po locus showing the region interrogated by allelic bisulphite sequencing, which includes portions of the IAP 5’LTR and pseudoexon 1A. CpG dinucleotides are represented by boxes; the A^po cryptic promoter is marked by an arrow. (b) Bisulphite allelic sequencing profiles from tail (i) and testis (ii) of unsupplemented mice and mice supplemented for five generations. Each line represents an allele, and each box a CpG dinucleotide (white, unmethylated; black, methylated). Each block represents sequences derived from a single mouse.
Figure 4. piRNAs are generated in the germline by the \( A^\circ \) locus but are unchanged by multigenerational methyl donor supplementation. Small RNA reads generated from the testes of \( a/a \) and \( A^\circ/a \) male mice were mapped to the U3 region (131–348 bp) of the 348bp \( A^\circ \) IAP 5’LTR; reads mapping in a sense direction relative to the \( A^\circ \) transcript (antisense to the LTR) are shown in red, and those mapping in an antisense direction relative to the \( A^\circ \) transcript (sense to the LTR) are shown in blue. (a) Alignment maps showing the numbers of reads that map across U3. The \( A^\circ \) cryptic promoter is indicated by an arrow. (b) Base composition of the small RNA reads. piRNAs are characterized by a 5’U; piRNAs generated by the ping–pong mechanism are characterized by a 10 nt A. The dotted line represents the proportion expected by chance. (c) \( \Delta \) yellow (Y el) and pseudoagouti (Ag), and from fifth-generation supplemented pseudoagouti males (Ag + methyl donor supplementation (MDS)); three independently sequenced in each group. Error bars represent standard error.

4. DISCUSSION

Our results indicate that multi-generational exposure to a dietary stimulus coupled with selection for a phenotype can progressively increase the prevalence of that phenotype in the population. The effects we observed are not genetic in origin, as all the mice in our experiment were genetically identical. The unlikely possibility that methyl donors created genetic variation that was selected is ruled out by the rapid reversion to the original spectrum of phenotypes upon withdrawal of the dietary stimulus. Thus, the effect we observed is due solely to the interaction of environment and epigenotype, demonstrating that purely epigenetic traits can provide a substrate for selection. This is completely compatible with Darwinian theory, but does not have a genetic basis. Natural selection acts on a
phenotype, and an evolutionary response to selection requires that the phenotype is heritable; the $A^{\text{ey}}$ allele creates a heritable phenotype, which happens to be determined not by genotype, but by epigenotype.

The $A^{\text{ey}}$ epigenotype is not normally heritable when the allele is passed through the male germline [7]; consequently, multi-generational selection alone would not be expected to have an effect on the frequency of phenotypes in the population, consistent with our observations. However, in the presence of the environmental stimulus (methyl donors) the proportions of offspring with a pseudo-agouti phenotype steadily increased. This implies that in each generation, the effect of methyl donors was both inherited and augmented. Thus, methyl donors induce epigenetic inheritance of $A^{\text{ey}}$ through the male, allowing selection to progressively propagate the silent epigenetic state of $A^{\text{ey}}$ through the population.

By selecting pseudo-agouti mice for breeding in each generation, we selected mice that carried a silent $A^{\text{ey}}$ allele and hence were potential ‘responders’ to the methyl donors. Had we instead bred the whole population without selection, any cumulative effect would necessarily have been diluted, as non-responsive mice would also have been included in the breeding population; indeed, multi-generational methyl donor supplementation without selection has no cumulative effect in laboratory-sized $A^{\text{ey}}$ populations [21,22].

Inheritance of the cumulative effect for one generation after withdrawal is consistent with our previous findings [20] and indicates that germ cells exposed to excess methyl donors within the developing $F_2$ females retained a memory of the methyl donor effect, manifested in $F_6$ mice. However, the germ cells that produced $F_5$ mice developed in $F_3$ embryos after methyl supplement withdrawal and displayed no memory of supplementation, indicating that the epigenetic memory was erased at some stage during the life cycle. Thus, the cumulative effect we observe is dependent on the continued presence of excess methyl donors. The observed reversion to baseline may reflect an epigenetic plasticity that could provide an advantageous ability to revert quickly to a former phenotype if environmental conditions were relatively transitory and the new phenotype no longer beneficial, something that could not be accomplished if the basis of the shift were selection for a genetic variant. It is also possible, given the selected mice were those that had potentially responded to the supplement, that the population now has an altered threshold for responding to future supplementation, so that a once-exposed lineage may respond more rapidly to renewed supplementation than a never-exposed lineage.

What is the molecular basis of the cumulative methyl donor effect, and of epigenetic inheritance itself? The increasing penetrance of the pseudo-agouti phenotype in each generation indicates a gradual strengthening of epigenetic inheritance over generations, and there must be some physical basis for this effect. We found no changes in DNA methylation at the $A^{\text{ey}}$ promoter, nor in the abundance of $A^{\text{ey}}$-derived piRNAs after five generations of supplementation. The lack of change in DNA methylation is consistent with our previous findings in the soma of supplemented mice [16] and is inconsistent with DNA methylation driving either the somatic or germline effects of methyl donors. That the abundance of piRNAs does not change with supplementation indicates that there is probably no simple relationship between the number of piRNAs and the strength of $A^{\text{ey}}$ inheritance. However, it may be that intergenerational differences in piRNA abundance are subtle and, given the small number of reads mapping to the $A^{\text{ey}}$ locus in all mice, it may be necessary to sequence much more deeply to discern them. Alternatively, given the many biological pathways and biochemical reactions involving methyl donors, there are many other potential molecular mediators of their epigenetic effects. In contrast to the genome, which is a stable molecule that is faithfully replicated and transmitted to succeeding generations, the ‘epigenome’ consists of an extremely complex assortment of proteins and chemical modifications that control the expression of information encoded by the genome.

Supplementary methyl donors increase the pool of S-adenosyl methionine, which can donate methyl groups to proteins and other molecules as well as DNA [25,26]. The effects of dietary methyl donors on the $A^{\text{ey}}$ allele may well be indirect, mediated by changes in the behaviour of one or more proteins that influence the probability of silencing the allele in the germline or at an early stage of development.

Our data provide evidence for a non-genetic process that can drive adaptive evolution. The idea that epigenetic states might act as a substrate for adaptive evolution has been presented by several authors [2,27–29]. Our data provide probably the first direct evidence that it is possible to select for a trait with a purely epigenetic basis (certainly the first in a mammalian system), and thereby increase its frequency in a population. Our findings are reminiscent of those of Sollars et al. [30] in Drosophila Kruppel mutants, where an environmentally induced trait was selectable; that the trait could be reversed using epigenetically modifying drugs implies that it, too, had an epigenetic basis, although no responsible epiallele was identified.

Are epigenetic processes likely to contribute to adaptive evolution in nature? Although we subjected our mice to ‘artificial’ selection, a wild population of $A^{\text{ey}}$ mice would probably be under similar selective pressure, as the yellow obese phenotype would almost certainly be disadvantageous (yellow mice develop type II diabetes and breed poorly). The type of environment we applied in our experiment—a persistent environmental change with selective effects—is common in evolutionary history, with climate change a prominent example. Pervasive climate-associated changes in behaviour have been observed in many species [31], with some fungi exhibiting an entire additional fruiting season [32]; these changes have occurred over time frames that are consistent with an epigenetic, but not a genetic, explanation. Adaptive evolution driven by epigenetic changes may be not only more rapid, but also more flexible than that driven by genetic change. Epigenetic changes in response to an environmental stimulus can occur in multiple individuals at once, facilitating population-wide responses to environmental change with an option for reversion if the environmental stressors do not persist. But epigenetically determined phenotypes may alternatively become fixed in a population via subsequent genetic mutation of methylated cytosines [33]. It remains to be seen whether stably heritable epigenetic processes alone can drive long-term population changes that are maintained independently of the inducing stimulus and without the need for a subsequent genetic change.
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