REVIEW ARTICLE

The marks, mechanisms and memory of epigenetic states in mammals

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It is well recognized that there is a surprising degree of phenotypic variation among genetically identical individuals, even when the environmental influences, in the strict sense of the word, are identical. Genetic textbooks acknowledge this fact and use different terms, such as 'intangible variation' or 'developmental noise', to describe it. We believe that this intangible variation results from the stochastic establishment of epigenetic modifications to the DNA nucleotide sequence. These modifications, which may involve cytosine methylation and chromatin remodelling, result in alterations in gene expression which, in turn, affects the phenotype of the organism. Recent evidence, from our work and that of others in mice, suggests that these epigenetic modifications, which in the past were thought to be cleared and reset on passage through the germline, may sometimes be inherited to the next generation. This is termed epigenetic inheritance, and while this process has been well recognized in plants, the recent findings in mice force us to consider the implications of this type of inheritance in mammals. At this stage we do not know how extensive this phenomenon is in humans, but it may well turn out to be the explanation for some diseases which appear to be sporadic or show only weak genetic linkage.

Key words: chromatin, inheritance, methylation.

INTRODUCTION

The various cell types in a multicellular organism are genotypically identical and yet phenotypically different. This is due to differences in the patterns of gene expression that exist between the different cell groups. The stable maintenance of these differences is thought to be due to epigenetic control of gene expression. This involves physically 'marking' the DNA, without altering the nucleotide sequence, either by the addition of methyl groups to certain cytosine bases and/or the packaging of the DNA into a highly condensed state. These modifications interfere with the DNA–protein interactions that facilitate transcription, resulting in transcriptional silencing of the epigenetically modified allele. Epigenetic modifications can, therefore, cause phenotypic variation in the absence of genetic differences.

It is well recognized that 'silenced' alleles can be inherited through many rounds of DNA replication, and therefore epigenetic modifications or 'marks' can be maintained through mitotic cell divisions. Generally, however, it has been assumed that these marks are erased and reset at some stage during gametogenesis or early embryogenesis to reinstate the totipotency of the developing embryo. There is now an increasing body of evidence which suggests that epigenetic marks at some mammalian alleles are not completely erased from one generation to the next, resulting in complex patterns of inheritance that do not conform to Mendelian principles. Therefore not only can phenotype vary in the absence of genetic and environmental factors, described by some as 'intangible variation' [1] or 'developmental noise' [2], but these phenotypic differences can also be inherited by the offspring.

This review will present a brief overview of the role of methylation and chromatin remodelling in epigenetic regulation of gene expression, followed by examples of classic epigenetic phenomena in mammals. We will then discuss the evidence available for epigenetic inheritance through the germline, with an emphasis on murine models, which suggest that this form of inheritance may be occurring at a number of mammalian loci.

EPIGENETIC MODIFICATIONS OF DNA

The two mechanisms by which DNA is epigenetically marked, although there may be others yet to be discovered, are methylation and chromatin condensation. Both of these mechanisms are associated with gene silencing, and recent evidence, discussed below, suggests that these two mechanisms are not mutually exclusive, but instead act in concert to silence gene expression in mammalian cells.

DNA methylation

Methylation involves the enzymic transfer of a methyl group to the 5-position of the pyrimidine ring of a cytosine residue [3–5]. This usually occurs at cytosine bases that are immediately followed by a guanine, known as CpG dinucleotides [6,7]. In mammalian genomes, the CpG dinucleotide is greatly underrepresented due to the increased spontaneous deamination rate of 5-methylcytosine into thymine. Of the CpGs present, approx. 70% are methylated [8], whereas the majority of unmethylated CpGs occur in small clusters known as CpG islands, which are ordinarily found within or near promoters or first exons of 'housekeeping' genes [9,10].

Methylation is catalysed by DNA methyltransferases (Dnmts) and four mammalian Dnmts have been identified so far, Dnmt1

Abbreviations used: Dnmt, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; MECP2, methyl-CpG binding protein 2; MBD, methyl-CpG binding domain; X_i, inactived X chromosome; Xist, X-inactivation-specific transcript; IGF2, insulin-like growth factor 2; A^{vy}, agouti viable yellow allele; $Axin^{Fu}$, axin-fused allele; IAP, intracisternal A-particle; LTR, long terminal repeat.

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[11], Dnmt2 [12], Dnmt3A and Dnmt3B [13], although our understanding of how these enzymes function is sketchy at best. Dnmt1 is probably involved in maintaining methylation patterns through mitosis [14]. Following DNA replication, the two double-stranded daughter molecules initially contain a hemi-methylated CpG pattern, which is recognized and converted into the fully methylated parental pattern by Dnmt1 [15]. However, it has been found that the error rate of replication of methylation patterns of an artificially methylated DNA sequence transfected into cell lines is significantly higher than that observed for DNA replication [16,17]. In addition, a later study [18] showed that clonal populations of histologically homogenous cells did not have homologous methylation patterns. These findings have been confirmed by more recent work, using the highly sensitive bisulphite conversion method to analyse methylation patterns *in vivo* [19,20]. Therefore the infidelity of replication of methylation patterns has the potential to generate phenotypic diversity among genetically identical cells of the same lineage.

Dnmt2 may play a role in epigenetic control of centromere function [21], and Dnmt3A and 3B are thought to be *de novo* methylases which set up the initial patterns of methylation during embryogenesis [22]. However, data suggests that Dnmts have overlapping functions [23,24], and the precise role of any particular Dnmt is determined by the cellular context. During mammalian development, there are 'waves' of extensive demethylation of the genome in the primordial germ cell stage and pre-implanatation embryo [25–28]. A mammalian protein with specific demethylase activity for CpG dinucleotides has been reported [29,30], although it remains to be fully characterized biochemically.

Chromatin packaging

In the nucleus, DNA exists as a nucleoprotein complex termed chromatin. Chromatin is assembled from arrays of nucleosomes, each of which is approx. 200 bp of linear DNA wrapped around an octamer of histone proteins. Two distinct types of chromatin are known, heterochromatin and euchromatin. Heterochromatin is believed to represent regions of DNA–protein complexes that are in a tightly packed conformation [31,32]. Constitutive heterochromatin is usually found at the centromeric and subtelomeric regions of chromosomes and tends to be transcriptionally silent [33]. Unlike constitutive heterochromatin, which is relatively uniform in all cells, facultative heterochromatic regions adopt a condensed conformation only in certain cells. Euchromatin contains less condensed regions of chromosomal DNA and is generally associated with transcriptional activity.

The overall euchromatic and heterochromatic content of the genome can vary widely between different cell types [34]. The dynamic nature of chromatin structure is made possible by modification of histones and the association of a number of non-histone proteins with specific regions of the DNA. Histones can be modified in various ways, including acetylation, methylation, ubiquitinylation, phosphorylation and ADP-ribosylation [35]. Acetylation, the most intensively studied modification of histone proteins, is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [36]. Acetylation reduces the affinity of histone protein H4 for DNA [37], with a subsequent relaxation of chromatin packaging into a more transcriptionally active state [38,39], whereas deacetylation of H4 correlates with the recruitment of H1 and the packaging of DNA into a more condensed conformation [40].

Several mechanisms have been suggested for the stable transmission of pre-existing acetylation patterns to newly assembled chromatin [41]. These include HATs and HDACs remaining in the vicinity and reassociating with the newly assembled chromatin following DNA replication. Evidence for this mechanism comes from the observation that some HATs form part of a complex that remains associated with its target DNA throughout the cell cycle [42–44]. A second mechanism may involve targeting the HATs and HDACs to regions of methylated DNA, so that pre-existing acetylation patterns are propagated along with methylation patterns during DNA replication. Indeed, it has recently been discovered that the maintenance methylase, Dnmt1, can interact with a histone deacetylase [45–47].

Epigenetic regulation of transcription

The precise mechanisms by which methylation and chromatin compaction regulate transcription are unclear, although several studies suggest that these two mechanisms are linked. MECP2 (methyl-CpG binding protein 2) is a transcriptional repressor that selectively recognizes methylated CpG dinucleotides [48,49]. MECP2, and other methyl-CpG binding proteins, associate with co-repressor complexes that include HDACs [50–53]. This directs the formation of stable repressive chromatin structures [54]. Recent findings [51,52] link the four different methyl-CpG binding domain (MBD) proteins, MECP2, MBD1, MBD2 and MBD3, with the chromatin-remodelling machinery, providing further evidence for the association between methylation and chromatin remodelling. Therefore it seems that methylation acts through histone deacetylation to establish a repressive chromatin state that blocks the access of the transcription machinery, although at present we do not know how the initial patterns of methylation are set up *de novo*. However, for certain organisms, e.g. *Drosophila*, methylation is observed only in very early embryogenesis [55] (for decades it was believed that DNA methylation was non-existent in *Drosophila*), and others like the yeast *Schizosaccharomyces pombe*, do not methylate their DNA at all. Therefore in some eukaryotic organisms chromatin-mediated mechanisms alone may be sufficient to mediate epigenetic regulation of gene expression.

EPIGENETIC PHENOMENA IN MAMMALIAN SYSTEMS

Epigenetic systems in mammals may have evolved from a host-genome defence system that exists in bacteria [56]. Bacteria have restriction-modification enzymes which cleave foreign DNA at specific sites. The recognition sites for these enzymes in the bacterial chromosome are methylated, which prevents the enzymes from cleaving the host DNA. There is also evidence to suggest that bacteria may use epigenetic modifications to control gene expression; a protoplast fusion between strains of *Bacillus subtilis* produces heterodiploid cells which results in the inactivation of one of the chromosomes, probably due to the modification in the structure of the bacterial chromatin [57].

It has been suggested that epigenetic mechanisms may have been a prerequisite for the evolution of multicellularity [58]. The vast phenotypic diversity that exists between different cells in a multicellular organism, even though they are all genetically identical, is thought to be due to the stable (mitotically heritable) repression of genes not required in specific cell types during development. Epigenetic modifications are ideally suited to being one of the mechanisms by which these organisms could stably repress different subsets of genes in the different tissues, thus allowing tissue differentiation, a hallmark of higher eukaryotes. Indeed, for human haematopoietic cells, it has been shown that the amount and distribution of condensed chromatin is similar in terminally differentiated cells of the same lineage, but it varies between different cell types [59]. Whatever the origins of epi-

genetic processes may be, they obviously offered substantial advantages during evolution, since these processes are observed in all the existing phyla in one form or another. In this section we briefly describe the two most widely studied examples of epigenetic effects in mammals: X inactivation and parental imprinting.

X chromosome inactivation

In female mammals one of the X chromosomes is silenced to compensate for the gene dosage difference between males (XY) and females (XX) [60]. X inactivation occurs early in female embryogenesis when one of the X chromosomes, the choice being random, undergoes heterochromatinization. The inactivated X chromosome (X_i) is then stably inherited through mitotic divisions. The inactivation is controlled by a region on the X chromosome termed the X-inactivation centre [61–63]. The X-inactivation-specific transcript (*Xist*) gene [64,65] and its antisense gene *Tsix* [66] are located within the X-inactivation centre. Upon initiation of inactivation, Xist RNA spreads to coat the chromosome that will become X_i [64,67]. *Tsix* expression is repressed from the X_i, but on the active X chromosome it is thought that Tsix RNA directly blocks Xist RNA action [66]. Although the X_i is also associated with hypermethylation of CpG islands [68,69] and underacetylation of histone H4 proteins [70], the exact interplay between these modifications and Xist RNA is unknown.

Parental imprinting

Certain genes are expressed from one allele only, for some it is the maternal allele which is expressed, and for others it is the paternal allele. This epigenetic phenomenon is termed parental imprinting [71]. A number of endogenous murine genes, including insulin-like growth factor 2 (*IGF2*) [72], *IGF2* receptor [73], *H19* [74], *Snrpn* [75] and their human homologues, and numerous murine transgenes [76–80], have been identified which display these effects.

Both DNA methylation and chromatin packaging have been implicated as being the imprinting marks. How these marks are established and maintained is poorly understood, although it is believed that parental imprints are erased in primordial germ cells and new sex-specific imprints are initiated during late gametogenesis [81]. Alternatively, it is possible that the sex-specific marks are established after fertilization, and indeed it has been found that in the zygote, the paternal genome is demethylated prior to demethylation of the maternal genome [82]. For IGF2 receptor, it is the maternal allele that is methylated [83], whereas the paternal allele is methylated at the H19 [84] and Snrpn [26] loci. In Dnmt1 mutant mouse embryos, the functional difference between alleles of imprinted genes is lost; for example, the normally silenced paternal H19 allele is activated, whereas the normally active paternal allele of the IGF2 is repressed [85]. Importantly, the differential methylation patterns of imprinted loci are conserved during the genomic demethylation that occurs during early embryogenesis [83,84].

Histone deacetylation may also influence the establishment of some imprints, since mouse embryos treated with an HDAC inhibitor express the normally silent paternal H19 allele [86]. Interestingly, sperm-specific histone-like proteins called protamines have been shown to associate with only some genes [87], raising the possibility that these protamines could mark the paternal alleles prior to the first cell division in the zygote. However, differential chromatin states do not always correlate with gene expression patterns as the silent IGF2 allele is in a potentially active chromatin state [88].

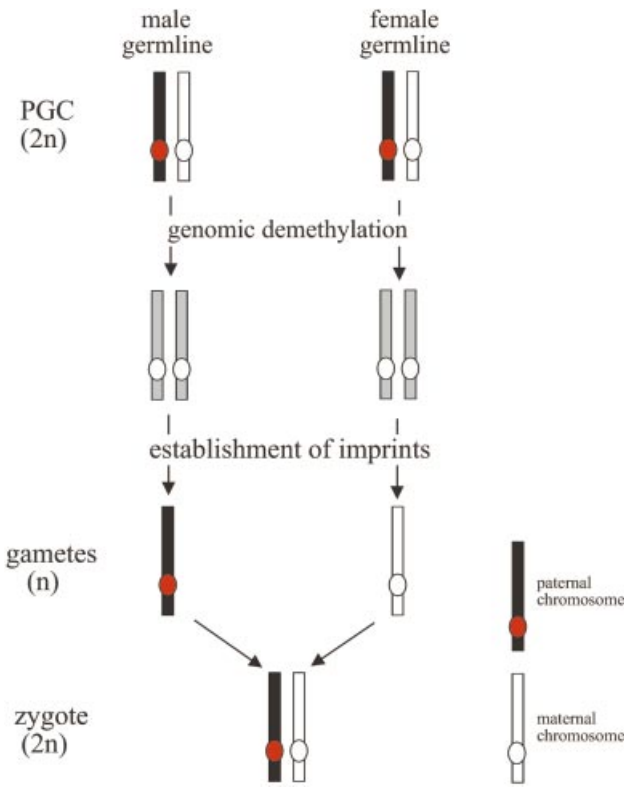

Figure 1 Reprogramming of the parental imprints in the germline

A detailed description of the model is provided in the text. The model described is for a hypothetical paternally imprinted gene. Gametes contain a haploid number of chromosomes. Abbreviation: PGC, primordial germ cells.

The mechanisms by which parental imprints are erased and established in the germline are described in Figure 1. The initial events in both male and female primordial germ cells include genome-wide demethylation and reactivation of the silenced X chromosome [26,28]. Following erasure of the imprint, the imprinted locus acquires an epigenetic state specific for the sex of the gamete. For example, for a paternally imprinted gene, the allele will be methylated through the male germline, whereas in the female germline, the allele will be unmethylated.

EPIGENETIC INHERITANCE

Reports of inheritance patterns that are not transient or predictably reversed like parental imprints, but not as stable as classical DNA sequence mutations, date back to the early part of the last century, although unfortunately a lot of this work has been neglected. In the 1920s, Jollos [89] studied inheritance patterns of this type in the protozoan, *Paramecium*. He observed that exposure to increased temperature or salt concentrations induced a specific change in resistance to these stimuli. Remarkably, these changes faded away only after hundreds of generations of asexual reproduction, and in a few cases these changes were inherited through sexual reproduction as well.

An especially interesting example is that of paramutation in plants, first observed in 1960 [90]. Paramutation is a directed, meiotically heritable alteration in the expression of one allele induced by another specific allele, and has been most extensively studied at the R locus (which influences pigment intensity in various parts of the plant) in maize [91]. The phenotypic

expression of a paramutable allele (an allele sensitive to paramutation) is decreased upon exposure to a paramutagenic allele (an allele which can induce paramutation) on the homologous chromosome. Following meiotic segregation, the paramutant alleles retain the reduced expression and become paramutagenic themselves. Mendel's first law states that genetic factors segregate unchanged from a heterozygote. This is clearly not true for paramutable alleles.

More recently, epigenetic inheritance has been demonstrated in the fission yeast, *S. pombe* [92,93], and in *Drosophila* [94]. Importantly, these studies have also revealed some of the molecular processes that may underlie the inheritance of epigenetic states in eukaryotic cells. *S. pombe* has the ability to switch mating type with a recurring pattern, so that one in four granddaughter cells swap *P*lus for *M*inus haploid mating type information or vice versa. The subsequent matings of cells of opposite mating type enable these organisms to become diploid. Efficient mating-type switching is dependent on epigenetic silencing of a region which contains the *mat2* and *mat3* loci [92]. Crippling the elements required for silencing at the *mat2/3* loci leads to variegated ON/OFF expression from the locus. In diploids, ON and OFF alleles can coexist for at least 30 generations and through meiosis as well [93]. Nakayama et al. [93] showed that a transient increase in the Swi6 protein, which is involved in chromatin remodelling, can switch the ON allele to an OFF state, which can be inherited through mitosis and meiosis, without the need for additional Swi6. The switch to the OFF allele is accompanied by hypoacetylation of chromatin at *mat2/3*. Their study [93] also showed that Swi6 acts as a dosage-critical factor, whose recruitment to the *mat2/3* region is the limiting step in establishment of the imprint, and remains associated with the mating-type region throughout the cell cycle, implying that Swi6 might itself be a component of the imprint.

The Fab-7 regulatory element derives from *Drosophila*, where it acts as a developmentally regulated enhancer and silencer, depending on the actions of the polycomb group and trithorax group proteins respectively [95]. In one of a number of transgenic *Drosophila* lines created by Cavalli and Paro [94], the Fab-7 element induced extensive silencing on a flanking GAL4-driven *lacZ* reporter and mini-white gene. However, a short single pulse of GAL4 during early embryogenesis was sufficient to release polycomb group protein-dependent silencing of the transgene. Such an activated state of Fab-7 was mitotically heritable through development and was also transmitted in a GAL4-independent manner to the subsequent generations through female meiosis, implying that Fab-7 is a switchable chromosomal element which can convey memory of epigenetically determined active and repressed chromatin states. Subsequent experiments [96] strongly suggested that changes in H4 acetylation are involved in the inheritance of the epigenetic state through mitosis and meiosis.

Epigenetic inheritance in mammals

Most geneticists, for the greater part of the last century, were convinced that, although epigenetic modifications may be important in maintaining homoeostasis during the life of mammalian species, these modifications had no role in transgenerational inheritance. It was believed that the only source of hereditary information passed on to the offspring is the DNA nucleotide sequence. Sexual reproduction in mammals results in the formation of a zygote, a single cell which contains all the necessary information to produce an entire organism comprised of billions of cells grouped into multitudinous cell types. Therefore epigenetic marks from the previous generation must be erased at some stage to ensure the totipotency of the cells in the early embryo, and indeed large-scale epigenetic reprogramming does occur during gametogenesis and again in early embryogenesis. However, the evidence we present in this section supports the argument that at some mammalian loci inefficient erasure of the epigenetic marks results in transgenerational epigenetic inheritance.

It has long been known that for certain mammalian alleles, the associated phenotype will sometimes manifest itself only partially or not at all. This phenomenon, known as variable expressivity or incomplete penetrance, has traditionally been attributed to differences in quantitative trait loci (i.e. the multiple genes affecting a phenotype) or environmental influences between individuals. These explanations are inadequate for understanding phenomena such as discordance in monozygotic twins. Extensive breeding studies performed with various endogenous [97–100] and transgenic [101–105] murine alleles have shown that variable expressivity can occur even when genotypic and environmental differences can be discounted. More importantly, in some cases the inheritance patterns of these alleles do not conform to Mendelian principles of inheritance.

Epigenetic inheritance at the endogenous agouti viable yellow (A^{vy}) and axin-fused ($Axin^{Fu}$) alleles

The product of the *agouti* gene causes hair follicle melanocytes to switch from the synthesis of eumelanin (black) to phaeomelanin (yellow). In wild-type mice, transcription during the mid-portion of the hair growth-cycle produces a sub-apical yellow band on a black hair, giving the characteristic agouti coat colour seen on most mice in the wild. The dominant A^{vy} allele has an intracisternal A-particle (IAP) (a retrotransposon) upstream of the *agouti* gene [106,107]. The insertion places the gene under the control of a promoter in the IAP long terminal repeat (LTR), which causes constitutive expression of the *agouti* gene, resulting in mice with completely yellow fur [106]. However, not all mice carrying an A^{vy} allele are yellow. Some of the mice have a mottled coat with patches of dark hair interrupting the yellow coat, whereas others, termed pseudoagouti, exhibit complete somatic reversion for coat colour. This range of phenotypes is observed even among isogenic littermates (Figure 2a) [100]. The coat colour phenotype correlates with methylation of the IAP [100]. Methylation of the LTR is associated with silencing of the IAP promoter and there is no ectopic expression of *agouti*, resulting in a pseudoagouti coat colour. Mottled mice have patches of yellow and pseudoagouti, which is probably due to the silencing of the LTRs in some cells, but not others, early during development and the subsequent mitotic inheritance of the epigenetic state.

Variable expressivity is also observed for the $Axin^{Fu}$ allele (Figure 2b) [98]. Wild-type axin protein regulates an early step in mammalian embryonic axis formation [108]. The $Axin^{Fu}$ allele contains an IAP within intron 6 of the gene, which results in the production of several aberrant RNA molecules [109]. The predominant phenotype associated with this allele, a kinked tail, is due to axial duplications during embryogenesis [98,108]. Again, the phenotype can vary from truncated and deformed tails (these mice are termed 'penetrant') to phenotypically normal tails (i.e. 'silent' mice) [98].

In the 1970s, breeding studies performed with the A^{vy} and $Axin^{Fu}$ alleles revealed some very interesting findings. Wolff [99] performed reciprocal crosses between A^{vy}/a (a is the null agouti allele) and a/a mice (a/a mice are uniformly black). He found that pseudoagouti A^{vy}/a females produced significantly greater pseudoagouti offspring than yellow A^{vy}/a females, i.e. there was some inheritance of phenotype. He suggested that these differ-

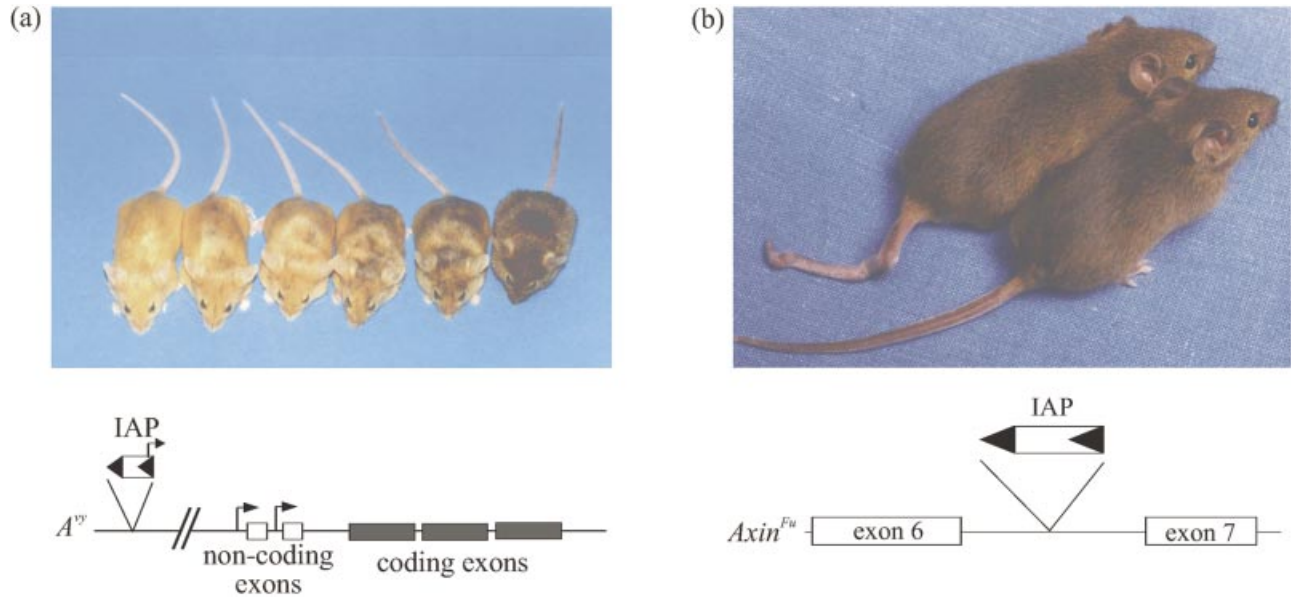

Figure 2 The A^{vy} and $Axin^{Fu}$ alleles

(**a**) Genetically identical A^{vy}/a mice show a range of coat colour phenotypes from yellow through to mottled and pseudoagouti. The A^{vy} allele has an IAP inserted approx. 100 kb upstream of the *agouti* gene. Transcription originating in a cryptic promoter in the LTR (black triangle) results in de-regulated *agouti* expression. (**b**) Genetically identical $Axin^{Fu}/+$ mice show a range of kinked tail phenotypes. The $Axin^{Fu}$ allele has an IAP insertion in intron 6 of the *axin* gene. This results in the production of several aberrant RNA transcripts. The A^{vy} and $Axin^{Fu}$ loci are not shown to scale.

ences were due to variations in the metabolic characteristics of the oviductal and uterine environments in yellow versus pseudoagouti A^{vy}/a females.

Similar studies were performed by Belyaev and Ruvinsky [97] to investigate the inheritance of the murine $Axin^{Fu}$ allele. When they performed reciprocal crosses between heterozygous $Axin^{Fu}$ mice and wild-type mice they found that, upon comparison of the heterozygous offspring from penetrant and silent parents, penetrant mice had a higher proportion of penetrant offspring than silent offspring, and likewise silent mice consistently gave more silent than penetrant offspring. It must be stressed that both the A^{vy} and $Axin^{Fu}$ alleles are genetically very stable, and strains that carry these alleles have been in existence for decades.

The studies of A^{vy} by Wolff [99] and $Axin^{Fu}$ by Belyaev and Ruvinsky [97] employed outbred murine strains only. The penetrance of both A^{vy} and $Axin^{Fu}$ is highly dependent on the genetic background. For example, in the case of the A^{vy} allele, it was found that yellow mice produced significantly more pseudoagouti offspring when they were crossed into the YS/ChWf strain compared with the AT/Wf strain [99]. Similarly, penetrance of the kinked tail phenotype is greatly reduced when $Axin^{Fu}$ mice are crossed into the C57BL/6 background [97]. Therefore it was possible that the inheritance patterns discussed above were due to unlinked modifier genes (i.e. genetic background effects) present in only a subset of the mice, so that phenotypically 'silent' mice have a set of modifiers that reduce penetrance, but these modifiers are absent in penetrant mice. More recently, we have performed extensive breeding studies in our lab with both the A^{vy} and $Axin^{Fu}$ alleles in inbred murine strains ([100]; V. K. Rakyan, unpublished work). An inbred mouse strain is defined as the descendants of a single brother–sister pair of mice produced by full-sibling inbreeding, so that the probability of homozygosity at any locus is at least 99.98 % [110]. It is highly unlikely that modifier genes that affect epigenetic inheritance would be present in only a subset of mice in an inbred strain.

We have characterized inheritance of coat colour phenotypes associated with the A^{vy} allele using the inbred C57BL/6 strain exclusively [100]. The phenotype of a female contributing an A^{vy} allele was related to the phenotypes of the offspring: yellow females produced yellow and mottled offspring only, whereas pseudoagouti females produced 20 % pseudoagouti offspring. However, the phenotype of a male contributing an A^{vy} allele was not related to the phenotypes of the offspring. Since the mice in this study were isogenic, genetic differences could not explain the effect of maternal phenotype on the offspring.

To discount the possibility that the maternal effect was due to metabolic differences in the intrauterine environments of yellow and pseudoagouti females, as suggested by Wolff [99], fertilized oocytes from yellow females (having pancellular *agouti* expression) were transferred to black females (with no *agouti* expression). The proportions of phenotypes in the resulting offspring was not different from the proportions born to yellow females, and was significantly different from the proportions born to black or pseudoagouti females mated to A^{vy}/a males. The embryo-transfer experiment could not exclude an effect of maternal environment occurring at some stage earlier than the embryo transfer. Yellow A^{vy}/a females were mated to pseudoagouti A^{vy}/a males, and some A^{vy}/a offspring from this cross were pseudoagouti, demonstrating that oocyte cytoplasm contributed by a yellow female does not prevent development of pseudoagouti offspring.

More recently (V. K. Rakyan, unpublished work), we have performed similar breeding studies with the $Axin^{Fu}$ allele using the inbred 129 Rr/J strain exclusively, and inheritance of phenotype has been observed through the male germline. The possibility that the inheritance effect in this case is due to cytoplasmic factors can be ruled out because the sperm contributes very little

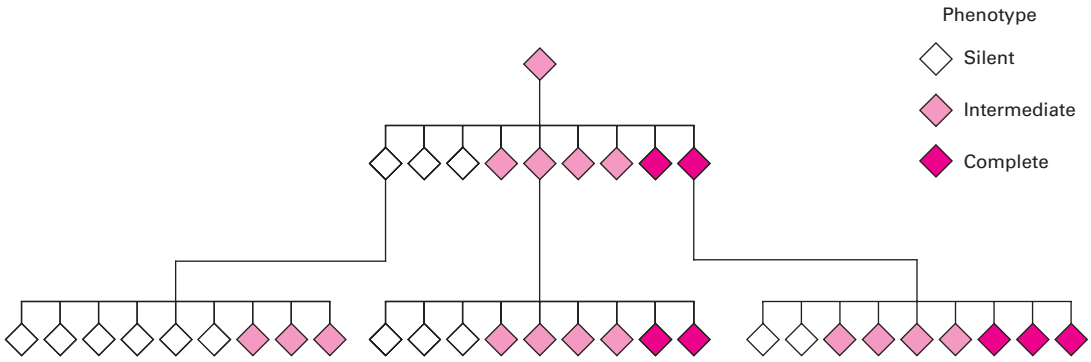

Figure 3 Schematic pedigree showing epigenetic inheritance of a transgenic allele

The pedigree shows how a transgenic mouse, expressing the transgene at an intermediate level, can produce offspring with a range of phenotype states that reflect the activity of the transgene, i.e. the transgene is variably expressed among genetically identical individuals. The activity of the transgene is inversely related to the extent of the epigenetic modification at the transgenic allele. The pedigree also shows how the range of phenotypes in a litter is dependent upon the phenotype of the parent, i.e. there is inheritance of the phenotype.

(if any) cytoplasm to the zygote. Therefore the inheritance is likely to be due to an epigenetic modification, and although this mark has yet to be determined, it probably involves modification of the IAP LTR, similar to the A^{vy} allele.

Epigenetic inheritance at transgenic murine loci

The studies described above demonstrate that phenotypic heterogeneity can exist even in the absence of environmental and genetic differences, but more importantly, the phenotype can also be inherited to an extent. Examples of variable expressivity have also been well documented for transgenic murine alleles [101–105], and not surprisingly, in many cases the transgenic alleles also displayed epigenetic inheritance, resulting in complex pedigrees (Figure 3).

One of the first extensive studies of a transgenic mouse line that displayed meiotic epigenetic inheritance at the transgene locus was by Allen et al. [101]. They created a transgenic line using a *lacZ* transgene and observed variable *lacZ* expression among littermates, which was correlated with differential methylation of the transgene. However, this effect was attributed to the presence of genotype-specific modifiers, as the studies were performed in a mixed genetic background. The effect of modifiers was demonstrated by backcrossing into the DBA/2 or 129 backgrounds, which resulted in demethylation of the transgene locus. On the other hand, backcrossing into the BALB/C background increased methylation of the transgene, although this effect was observed only when the transgene was inherited through the female (i.e. the transgene was maternally imprinted). More importantly, they also observed that the epigenetic modification of the transgene locus was cumulative over successive generations.

In one of the *lacZ* transgenic lines (containing an erythrocyte-specific enhancer) made in our laboratory, littermates had proportions of β-Gal-positive erythrocytes that varied over at least four orders of magnitude [104]. In some mice, transgene expression was completely silenced, and the silent state could be inherited for multiple generations irrespective of the sex of the parent. Furthermore, the silencing correlated with methylation of the transgene as well as an inaccessible chromatin structure, although these changes were reversed when the mice were crossed into another strain. We also found that the transgene had integrated near an L1 retroviral element, reminiscent of the IAP retrotransposon associated with the A^{vy} and $Axin^{Fu}$ alleles.

In another *lacZ* transgenic line, transmission of the transgene through the female germline resulted in hypermethylation of the transgene, with reduced expression in some offspring and complete transcriptional silencing in others [105]. The transgene was generally reactivated when inherited from the father. In this case it was found that the transgene had integrated into a region not known to carry imprinted genes or allele-specific differential methylation patterns.

It is also interesting to note that for the A^{vy} allele the penetrance of the phenotype (i.e. yellow fur) is much greater when the allele is inherited from the mother than from the father. On the other hand, for $Axin^{Fu}$ the phenotype is more penetrant through the male. Is it coincidental that the A^{vy}, $Axin^{Fu}$ and the transgenic alleles described above are all parentally imprinted? It has been noted that the CpG-rich differentially methylated sequences in the imprinted *IGF2* receptor, *Xist* and *H19* genes contain, or are immediately adjacent to, regions rich in direct repeats [111]. The IAP LTRs and tandem transgenic arrays (transgenes usually integrate in tandem arrays [112]) introduce directly repeated DNA sequences into regions that may not have had this feature previously. In addition, large non-coding antisense RNA transcripts are also thought to be associated with imprinted regions [113–117]. Read-through transcripts initiated from promoters within the IAPs or transgenes could produce such RNA molecules that attract the cellular imprinting machinery.

WHY DOES EPIGENETIC INHERITANCE OCCUR?

The common theme that emerges from the murine studies seems to be that these events are associated with the introduction of foreign DNA, either through retrotransposition or transgenesis, into the genome. In most cases, these events would be harmful for the host genome, as they could lead to insertional mutations or dysregulated expression of endogenous genes. In addition, due to the abundance of IAPs in the genome, transposition could create sites of homology that may lead to illegitimate recombination during meiosis. There is emerging evidence from several eukaryotic organisms that there are mechanisms that recognize and epigenetically silence repeated DNA structures. There are thousands of copies of IAP elements within mammalian genomes [118], and transgenes integrate in multiple-copy arrays [112]. Based on evidence from lower eukaryotes [119–122], it has been suggested that multiple copies of certain DNA elements can 'pair', either through DNA–DNA interactions or through RNA

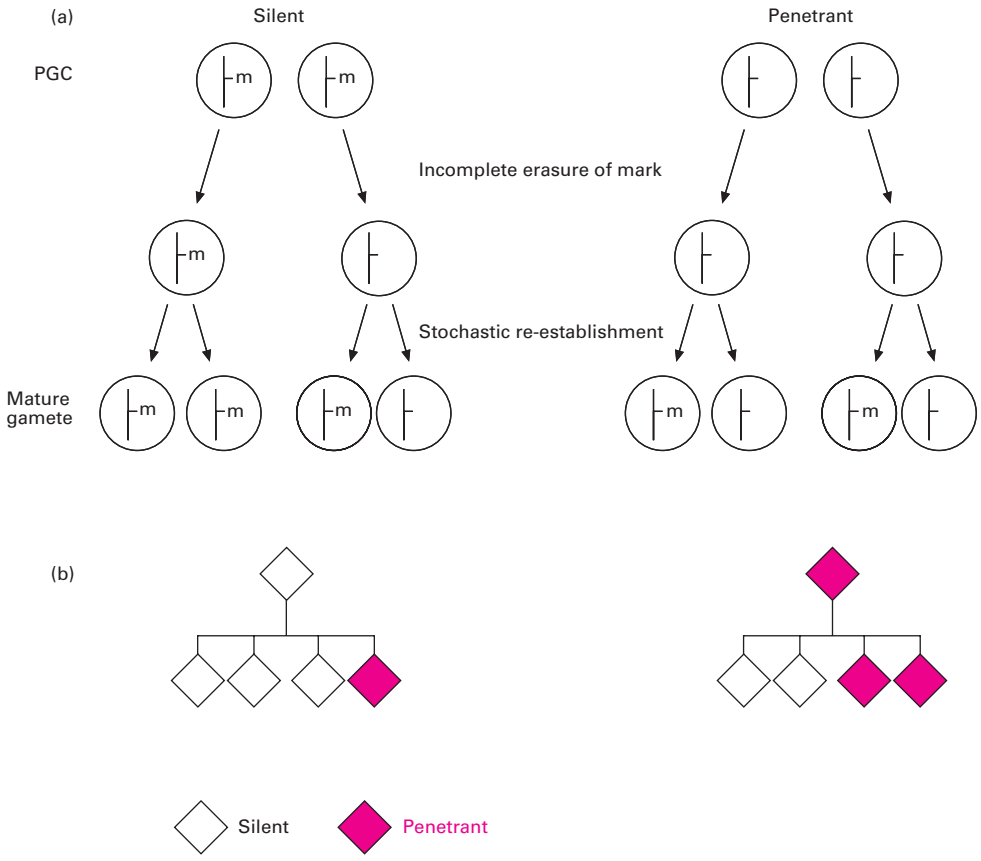

Figure 4 Model for epigenetic inheritance

A detailed description of the model is provided in the text. The model shows how incomplete erasure of an epigenetic mark (**a**), e.g. methylation, at a hypothetical allele results in inheritance of phenotype (**b**). In this model, methylation at the allele correlates with a 'silent' phenotype. Methylation is indicated by an 'm'.

intermediates, resulting in changes in localized chromatin conformation [123]. This is recognized by the cell, and a silenced state is established and stabilized by mechanisms that may include DNA methylation and association of proteins involved in the maintenance of heterochromatin. In fact, it has been suggested that DNA methylation serves primarily to silence retrotransposons [124]. However, there are hundreds of copies of rRNA genes in tandem arrays in eukaryotic genomes, and yet they have the ability to escape, to a large extent, the putative silencing mechanisms that recognize repeated DNA structures [125,126]. This implies that these mechanisms have some way of distinguishing 'foreign' DNA from other genes such as the rRNA genes.

Epigenetic inheritance in mice appears to result from the inefficient erasure of the epigenetic modifications at certain IAPs or transgenes during development. Based on evidence described above, we can postulate two models for how incomplete erasure and stochastic re-establishment of epigenetic marks could generate phenotypic diversity in the absence of genetic or environmental influences, and how phenotype can be inherited to the next generation (Figure 4). We can consider a hypothetical murine allele that displays epigenetic inheritance and is marked at a single site by methylation. The 'silent' phenotype corresponds to a methylated state and a 'penetrant' phenotype corresponds to the non-methylated state. In primordial germ cells of silent mice, the allele is initially methylated. In early gametogenesis, incomplete erasure of the mark results in some immature gametes having an allele that is still methylated. For penetrant mice, the allele was initially unmethylated, and therefore the inability of the cell to erase the mark is inconsequential. Re-establishment of the mark is stochastic, but because some cells in the 'silent' germline carry alleles that are already methylated, the overall proportion of mature gametes containing methylated alleles will be greater in silent mice. Genome-wide epigenetic reprogramming during early embryogenesis would faithfully replicate the original mark that was present in the mature gamete. In the second model, the original mark present in the primordial germ cell might be unchanged in epigenetic reprogramming during gametogenesis, and incomplete erasure of the mark and subsequent stochastic re-establishment would then occur in the preimplantation embryo. For both models, the silent mice will have a higher percentage of silent offspring compared with litters from penetrant mice. Epigenetic inheritance through one sex only (e.g. for A^{vy} and $Axin^{Fu}$) could occur because the germline of the other sex efficiently erases the mark or, for the second model, the developing embryo has proteins that specifically (but ineffeciently) erase marks on one of the parental chromosomes. As mentioned above, it has been found that during very early embryogenesis, the paternal genome is demethylated prior to demethylation of the maternal genome [82], so parent-of-origin-specific erasure does exist.

Inheritance of phenotype, has sometimes been associated with Lamarckian inheritance [58]. This involves the acquisition of phenotypes that have an adaptive function during the lifetime

of an organism and are also transmitted to the next generation. For example, according to Lamarckian theory, present-day giraffes have long necks because their ancestors stretched their necks when reaching for leaves on high branches. Therefore, in contrast to Darwinian theory, Lamarckism suggests that evolution is directed and not random. The epigenetic inheritance we have described here is not Lamarckian, since it is due to the random failure to completely erase marks at certain alleles during development. Nevertheless, recently [127] it has been shown that a diet rich in methyl donors induces a shift in proportions of phenotytpes of A^{vy} mice, providing an exciting link between an environmental factor (i.e. diet) and inheritance in mammals.

CONCLUSIONS

In this review we have discussed how epigenetic modifications, i.e. methylation and chromatin packaging, of DNA are involved in cellular function and how, in some cases, failure to erase the mark in early development can result in transgenerational inheritance of epigenetic states. But what implications does epigenetic inheritance have for human disease and evolution? The association between improper functioning of the epigenetic regulatory mechanisms and human disease is well established, and several reviews have been published on such diseases, including Fragile X, Beckwith Wiedmann, Angelman and Prader–Willi syndromes [128–130], to name a few. To date, no human alleles have been shown to display epigenetic inheritance. However, a couple of interesting reports have suggested these effects. Silva and White [131] showed that the methylation state at a few random sites in the human genome varied between individuals, and these states showed some degree of inheritance to the next generation. Recently [132], a paramutation-like effect has been associated with susceptibility to diabetes in humans. The inheritance of alternative epigenetic states may explain the variable expressivity and predisposition to some inherited diseases that cannot be explained by genetic or environmental influences [133]. Indeed, several researchers now recognize the inheritance of an epimutation (an aberrant epigenetic state) as a possible predisposing factor in tumour progression [129].

As both the A^{vy} and $Axin^{Fu}$ alleles are associated with IAPs, one may well ask if there is a correlation between epigenetic inheritance and retrotransposons. It has been estimated that transposons inhabit over 42% of euchromatic DNA [134], and if epigenetic inheritance is common at these DNA elements, then non-Mendelian inheritance could be occurring at a large number of loci in humans. The field of epigenetic inheritance is in its infancy and we know very little about the underlying mechanisms. However, we must seriously consider the possibility that genotype and environment (in its strictest sense) are not the only factors that influence the manifestation and evolution of human characteristics, epigenetic influences may play an important role as well.

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