Available online at www.sciencedirect.com
ScienceDirect
Mutation Research 600 (2006) 46–57
Fundamental and Molecular Mechanisms of Mutagenesis
www.elsevier.com/locate/molmut
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Environmental and nutritional effects on the epigenetic regulation of genes

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Available online 18 July 2006

Abstract

Major efforts have been directed towards the identification of genetic mutations, their use as biomarkers, and the understanding of their consequences on human health and well-being. There is an emerging interest, however, in the possibility that environmentally-induced changes at levels other than the genetic information could have long-lasting consequences as well. This review summarises our current knowledge of how the environment, nutrition, and ageing affect the way mammalian genes are organised and transcribed, without changes in the underlying DNA sequence. Admittedly, the link between environment and epigenetics remains largely to be explored. However, recent studies indicate that environmental factors and diet can perturb the way genes are controlled by DNA methylation and covalent histone modifications. Unexpectedly, and not unlike genetic mutations, aberrant epigenetic alterations and their phenotypic effects can sometimes be passed on to the next generation.

Keywords: Epigenetic; Environment; Toxicology; Nutrition; Genomic imprinting; DNA methylation; Biomarker

1. Genotype and epigenotype

The term 'epigenetic' is used to refer to stably maintained patterns of gene expression that occur without changes in the DNA sequence. Epigenetic regulation plays an important role in animal and plant development, and throughout adult life, and is required to achieve stable expression, or repression, of genes in specific cell types or at defined developmental stages. There are many covalent epigenetic modifications involved in keeping genes stably repressed, or active. Possibly the best studied epigenetic modification is DNA methylation. In the genomes of mammals, this covalent modification occurs at many of the cytosine residues that are followed by a guanine residue. In most cases, the acquisition and somatic maintenance of such 'CpG methylation' induces gene repression. However, there are also examples where DNA methylation at specific sequence elements permits the expression of neighbouring genes. Additionally, gene expression is determined by the organisation of the histones in the nucleosomes around which the DNA is wrapped. In recent years, many covalent modifications have been discovered to occur at the amino acids that constitute the N-terminal tails of histones. Alone, or in combination, these histone modifications are thought to be indispensable for the regulation of the continued repression, or expression, of genes. From extensive recent work, it follows that in particular histone acetylation and histone methylation are essential for the somatic maintenance of gene regulation [1,2].

When considering how different kinds of environmental stress can influence epigenetic mechanisms, it should be important to emphasize that the epigenetic modifications on DNA and chromatin constitute the link

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doi:10.1016/j.mrfmmm.2006.05.029

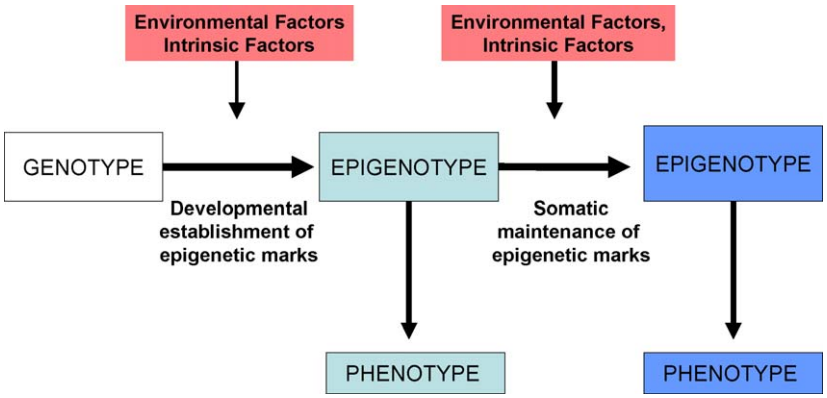

Fig. 1. The dynamic link between genotype, epigenotype, and phenotype. Heritable patterns of DNA methylation and other epigenetic modifications are established during development, in the different lineages of the embryo. This involves many different intrinsic mechanisms, and is influenced by the uterine environment as well. The resulting epigenotype(s) determines heritable gene expression, and thus the phenotype. Environmental, toxicological, and nutritional factors impact on the establishment and somatic maintenance of epigenetic patterns. This may alter the epigenotype, and can thus influences the phenotype.

between the genotype and the phenotype (Fig. 1). In specific cell lineages, and at defined developmental stages, chromatin at genes is modified in a way that leads to acquisition of constant gene repression, or activation. This developmental process is governed to a large extent by intrinsic factors, but it is now clear that environmental factors may affect epigenetic patterns as well [3]. The combination of the different epigenetic modifications at genes and non-coding sequences is commonly referred to as the epigenome, or the epigenotype. The epigenotype determines whether genes are maintained in a repressed state, or kept potentially active, and it influences the phenotype at birth. Importantly, epigenetic modifications need to be maintained throughout every cell cycle, in order not to alter the epigenotype(s). Intrinsic factors play important roles here, such as the methyltransferases that somatically maintain patterns of DNA methylation. However, environmental factors and nutrition could also have an impact on how faithfully patterns of epigenetic modifications are maintained throughout life. In case of aberrant environmental effects, or of stochastic shifts in intrinsic maintenance factors, the epigenotype may become altered. This may give rise to altered gene expression and, therefore, to an altered phenotype (Fig. 1). Thus, the phenotype is determined by the epigenotype, which may become altered during development, or in postnatal life, due to errors in intrinsic mechanisms, or due to environmental influences. So far, there are few studies addressing the environmental and toxicological effects on DNA methylation and histone modifications. Undoubtedly, this will be an important question for future research. The theme is elaborated in the current review, which focuses mostly on studies in the mouse, but gives human examples as well.

2. Genomic imprinting, an example of epigenetic regulation in mammals

In mammals, there are many examples of epigenetic repression or activation of genes [4]. These include: (a) X-chromosome inactivation, i.e. the inactivation of one of the two X chromosomes in female somatic cells [5]; (b) the allelic silencing occurring at imprinted genes, a group of key genes whose expression is dictated by whether they are inherited from the mother or the father; (c) the control of lineage-specific maintenance of gene expression at different loci; (d) the heritable repression of repeat elements of viral or retroviral origin [6].

Imprinted genes constitute a particularly attractive example of epigenetic regulation, since in the same cell, one of the two alleles is stably repressed by epigenetic modifications, whereas the other allele is maintained in an active state (Fig. 3). This allele-specific regulation is entirely determined by the parental origin of the allele, that is, by whether the gene is inherited from the mother or from the father. To date, some eighty genes have been found to be controlled by imprinting in humans and mice. Many of these play key roles in development, cellular proliferation and behaviour [7–9]. A characteristic feature of imprinted genes is that they are organised in clusters in the genome. These imprinting clusters are similar between humans and mice, and imprinting is evolutionarily conserved in other placental mammals as well [10–12]. Epigenetic deregulation of imprinted genes is

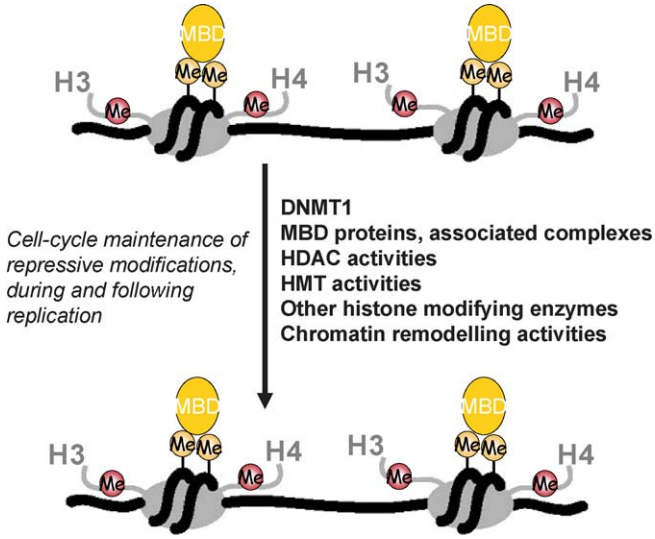

Fig. 2. Somatic maintenance of gene repression involving DNA methylation. On the repressed chromatin, besides methylation (Me) on the DNA, there are also associated methylation marks on the histones of the nucleosomes [4]. For simplicity, only histones H3 and H4 are shown, but repressive histone modifications occur on histones H2A and H2B as well. On H3, lysine-9 methylation is associated with DNA methylation; on H4 there are other potential methylation marks including methylation on lysine-20 [93]. The histones are maintained free of acetylation by histone deacetylation. The cell-cycle maintenance of this complex organisation of the chromatin involves DNMT1, to perpetuate the DNA methylation, methylated DNA binding proteins (MBDs) and their associated enzymatic complexes, HDAC activities, HMTs, and other chromatin modifying activities. Recent data suggest that ATP-dependent chromatin remodelling factors could be involved as well.

associated with different human disease syndromes, and is frequently observed in cancer as well [13–15].

One of the best-studied imprinted genes is the insulin-like growth factor-2 (*IGF2*) gene on human chromosome 11p15.5. *IGF2* encodes one of the main growth factors and is expressed from the paternally inherited allele only in most tissues, during fetal development and after birth [16,17]. The allelic repression of this gene is regulated by an essential sequence element, called an 'imprinting control region' (ICR). This ICR corresponds to a CpG island and is located close to a neighbouring imprinted gene, called *H19*. The ICR regulating *IGF2* is epigenetically marked by DNA methylation on its paternally inherited copy only. This paternal imprint is acquired during spermatogenesis and, after fertilisation, is maintained throughout development in all the lineages. It is because of this allelic DNA methylation at the ICR that the *IGF2* gene is expressed from the paternal chromosome only. In fact, it is thought that all clusters of imprinted genes have ICRs, which are differentially methylated [11,12]. However, DNA methylation is not the only epigenetic modification found at ICRs. Chromatin studies show that on the allele that is marked by DNA methylation, the chromatin is compacted and marked by repressive histone modifications. On the opposite parental allele, where there is no DNA methylation, there are histone modifications that are typical for an 'open chromatin' structure [18–20]. The way the differential DNA methylation and the associated chromatin features at ICRs convey imprinted gene expression, differs between imprinted gene clusters [11,15].

As outlined in more detail below, in several animal studies on environmental and nutritional effects, imprinted gene loci and X-linked loci were chosen as a model system, since at these loci even minor epigenetic changes are readily detectable [21].

3. Somatic maintenance of epigenetic patterns

How are the epigenetic patterns on the DNA and chromatin maintained from one cell generation to the next, for instance, at imprinting control regions, or at silenced repeat elements? And, to which extent is this process influenced by extrinsic factors? Even in a simple scenario, besides fluctuating external effects, there are multiple intrinsic factors involved in the somatic maintenance of epigenetic patterns. Let us consider, for instance, a hypothetical gene silenced by DNA methylation at its upstream promoter region (Fig. 2). Each cell cycle, this DNA methylation is perpetuated by the maintenance methyltransferase DNMT1, which puts methylation onto the newly replicated strand at each replication cycle [22]. The levels of expression of DNMT1 need to be stably controlled in the cell, since alterations

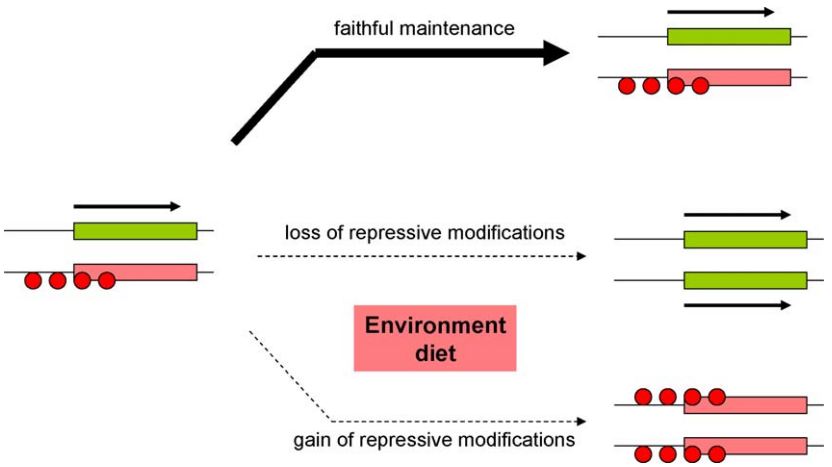

Fig. 3. Environmental perturbation of epigenetic patterns. As an example, a hypothetical imprinted gene (rectangles) is presented which is silenced by repressive DNA and histone modifications (red circles) on one of its two parental alleles. The opposite allele is marked by epigenetic modifications that allow transcription to occur. During development and after birth, there is usually faithful maintenance of the repressive modifications, with unaltered allele-specific expression. Different kinds of environmental stress may interfere with the highly complex maintenance of the opposite epigenetic patterns through the cell cycle. In case this leads to non-maintenance of repressive modifications, the imprinted gene becomes expressed from both its alleles, doubling the dose of expression. Environmental effects may also lead to acquisition of repressive modifications on the normally active allele. This brings about a heritable loss of gene expression.

can have developmental consequences and can lead to abnormal global DNA methylation and perturbed epigenetic regulation [23]. In normal circumstances, however, the somatic maintenance of DNA methylation is faithfully conserved throughout each cell cycle. Additionally, linked to the DNA methylation, on the chromatin of our gene are present repressive histone modifications, such as methylation on lysine 9 of histone H3 [2,4,19]. These also need to be faithfully maintained during each cell cycle. This involves specific histone methyltransferases (HMTs). Like DNMT1, these HMTs use *S*-adenosylmethionine as their donor of methyl groups. Furthermore, the chromatin associated with the methylated DNA is characterised by the absence of acetylation on histone H3, a status, which, presumably, requires recruitment of specific histone deacetylase (HDAC) activities to the locus [24]. In summary, the maintenance of repression entails numerous endogenous protein complexes that carry DNMT, HMT, and HDAC activities, and likely involves chromatin remodelling complexes as well [25,26]. By contrast, on a heritably active gene (such as at the stably active allele of an imprinted gene) the introduction of repressive DNA and histone modifications needs to be prevented at every cell division, whereas the somatic maintenance of an active chromatin that carries histone acetylation and other histone methylation marks, such as H3 lysine-4 methylation [11,19], is carried through each cell cycle.

Thus, the cell cycle maintenance of differential DNA methylation and its associated chromatin features is a highly complex process requiring many different enzymatic complexes (Fig. 2). Presumably, each of these factors will show some stochastic variation in its nuclear concentration, or in its recruitment to its sites of action [27]. In spite of this tremendous complexity, normally there is faithful maintenance of epigenetic patterns during development and adult life [28]. When the external conditions change, however, such as during embryo and cell culture (see below), frequently there is no longer faithful maintenance of epigenetic patterns. At imprinted genes, for example (Fig. 3), this may lead to loss of the repressive modifications (and biallelic gene expression), or to aberrant gain of repressive modifications (and loss of gene expression). Although still poorly understood, environmental perturbations of epigenetic patterns are thought to occur in different human conditions as well; for instance as a consequence of diet, or following application of assisted reproduction technologies [15].

4. Epigenetic drift during ageing

Using inbred mouse lines as their experimental model, several groups have explored the somatic maintenance of X-inactivation and genomic imprinting in ageing animals, based on the idea that epigenetic instability could be one of the contributing factors [28]. In one study on X-inactivation and imprinting [29], mice were analysed between 2 and 24 months of age. Upon ageing, during this 2-year period, the imprinted *Igf2* gene became re-activated on its normally-silent allele to an extent of

up to 7%. Then, X-inactivation was quantified in the female mice of this cohort. At one X-linked gene (*Atp7a*), there was an extent of relaxation of about 2% upon ageing. These findings are consistent with earlier work on translocation mouse lines, showing that there is partial re-activation of the inactive X chromosome during ageing [30,31]. Interestingly, the observed frequencies of 'loss of imprinting' and relaxation of X-inactivation in the mouse studies are much higher (about two magnitudes) than those reported for the occurrence of genetic mutations [32]. This indicates that epigenetic alterations occur more frequency than genetic mutations and could, thus, be particularly important in ageing-related phenotypes. Ageing-induced affects on expression have been reported also for the imprinted *CDKN1C* gene, a cell cycle gene located close to *IGF2* [33]; however, it is not clear whether this reflects perturbed epigenetic imprinting at the locus. Apart from its influence on X-linked and imprinted genes, ageing in mice has been linked to loss of DNA methylation at specific retrotransposon elements as well [34].

In humans, the effects of ageing on the maintenance of X-inactivation and imprinting have been more difficult to determine, in particular because of genetic differences between individuals. In one study on the human X-linked *HPRT* locus, however, a low degree of reactivation of the inactive allele was observed during the first year after birth [35]. Several studies were performed on monozygotic twins as well [36,37]. For instance, a recent study on a large cohort of monozygotic twin sisters reported a higher frequency of skewed X-inactivation (preferential inactivation of one of the two chromosomes) in twins of more than 50 years of age [38]. However, it had not been determined whether this correlated with epigenetic alterations.

The relaxation of gene repression in mice and humans, and its relatively high frequency of occurrence, suggest that epigenetic alterations accumulate during ageing. However, till recently, there was little direct evidence for this to be the case. This has now changed thanks to a large epigenetic study on monozygotic twins [39]. In this extensive work, global and locus-specific differences in DNA methylation and histone acetylation were examined using a battery of different experimental approaches. Although largely based on peripheral blood lymphocytes, comparable data were obtained from epithelial cells and skeletal muscle biopsies. Consistent with the idea that monozygotic twins are epigenetically comparable at birth in most cases (i.e. they shared the same uterine environment and are genetically identical), little or no epigenetic differences were detected between twins early in life. Older monozygotic twins (>28 years of age), in contrast, exhibited major differences in their overall content and distribution of DNA methylation and histone acetylation. This correlated with differential gene expression between the twins at a large number of genes. Remarkably, in some of the older monozygotic twin pairs analysed, there was no less than 20% difference between the twins in overall levels of DNA methylation and histone acetylation. Furthermore, the twin siblings, who showed the biggest global differences in methylation and acetylation, had also spent less of their lifetime together, or had a more highly diverging natural health-medical history, as compared to other twins. Based on these striking correlations, it was suggested that the epigenetic differences that accumulate during postnatal life are at least due in part to environmental influences [39]. Although these epigenetic drifts were detected in different cell types, it could be that organs, which have a high proliferative potential accumulate age-related epigenetic changes more readily than organs with lower proliferative potential [40].

An additional question relates to which extent these epigenetic differences between twins could be explained by stochastic intrinsic events [35,36]. As discussed above, such stochastic shifts could have an impact on the complex somatic maintenance of epigenetic patterns. Such intrinsic effects could explain also, why in many studies on monozygotic twins and on inbred animals, phenotypic differences were reported in the absence of discernable environmental differences [37,41–43].

Relative to the above-described global DNA methylation changes, it should be important to also note the rapidly expanding literature on altered methylation at specific genes in relation to cancer. Extensive studies have suggested that some of these methylation changes accumulate during life and may be influenced by lifestyle/environmental factors. They could thus be early events in the process of tumourigenesis. The theme of cancer and epigenetics is not covered in the current text, and is summarised in excellent reviews elsewhere [44–47].

Whatever the relative importance of intrinsic factors versus environment, the studies on monozygotic twins indicate that there is accumulation of epigenetic changes throughout life. This may influence gene expression and, consequently, phenotype (Fig. 1). For future research into the importance of environmental versus intrinsic effects, it should be interesting to explore global and locus-specific levels of epigenetic modifications between animals of the same inbred line (i.e. animals that are genetically identical). Here, comparison between animals that are kept under identical conditions, with those that are subjected to different environments, would

be particularly insightful. An early study showed that, indeed, also in mice there are global changes in DNA methylation that arise upon ageing, and it was estimated that postnatally there is about 0.01% loss of methylation per month [48].

The ageing studies indicate that stochastic events and the environment may perturb the epigenetic state of genes, including imprinted genes. Such epigenetic alterations alter the epigenotype and, hence, the phenotype (Figs. 1 and 3). However, once aberrant epigenetic alterations have arisen, how well are these maintained subsequently, and can they be transmitted to the next generation? Relative to this important questions, many novel insights have emerged from inbred mouse models [49]. Whereas the expectation is that all animals in an inbred mouse line are phenotypically identical, this is clearly not always the case. In an inbred strain carrying a particular allele of the Agouti coat-colour gene ('Agouti viable yellow', A^{vy}), for instance, some mice are yellow, whereas others are variegated yellow with bits of agouti, and yet others completely agouti (a brownish colour). The variable colour phenotype in these genetically identical animals is regulated by DNA methylation at an intra-cisternal-A particle (IAP) transposon close to the gene [50]. Agouti-coloured mice in the inbred mouse line have full methylation at this IAP element, and normal expression of the agouti gene. In contrast, yellow mice of the line have absence of methylation at the IAP, leading to the aberrant expression of the agouti gene, and hence, the yellow coat colour. Under normal nutritional conditions (see below), these different epigenetically patterns are somatically maintained and are relatively stable. Strikingly, the authors even observed trans-generational persistence of the colour phenotypes. In fact, the coat colour of the mother (and the grandmother) determined to a great extent the coat colour of the offspring. Yellow mothers had more frequently yellow offspring, whereas agouti mother had more frequently agouti offspring. This correlated with the inheritance of specific DNA methylation states at the IAP element close to the agouti gene, and provides one of the known examples of epigenetic inheritance in mammals [50].

Another, similar, example of epigenetic inheritance of aberrant methylation states is provided by the axin-fused ($Axin^{Fu}$) line. This allele of the axin gene contains an IAP that can be either methylated or not. This alternative methylation state determines whether the mice have the kinky tail characteristic of this allele. Precisely as in the A^{vy} mice, the methylation patterns in the mothers were frequently passed on to the next generation. Remarkably, however, such a trans-generational effect was observed upon transmission through the male germ line as well [51]. Since it is thought that there are hardly any histones in sperm (the DNA is organised around protamines), this may imply that the transmission through the male germ line is determined entirely by the aberrant DNA methylation patterns at the IAP element.

5. Long-lasting nutritional effects

The different phenotypes in the Agouti and Axin-fused inbred lines are dictated by DNA methylation. This raises the question as to which extent these phenotypes can be influenced by environmental factors, or by providing the mice with a specific diet. The latter was explored in the viable yellow agouti mice by dietary methyl supplementation. Specifically, the mouse food was complemented with extra folic acid (folate), vitamin B(12), choline, and betaine, which are thought to enhance the metabolism of methyl donors (*S*-adenosylmethionine) in the cell. This dietary supplementation led to clear shifts in the phenotypes related to a concomitant increase in DNA methylation at the A^{vy} locus [52]. This study represents a clear demonstration of how nutrition can influence the epigenetic organisation of genes, and as a consequence, can have long-term affects on gene expression and phenotype. These studies emphasize that dietary supplements may not always be beneficial, and can have aberrant effects on the epigenetic regulation of gene expression [52]. Conversely, when mice were fed a methyl-donor-deficient diet (lacking folic acid, vitamin B12 and choline), this led to down-regulation of the imprinted *Igf2* gene. This reduced expression correlated with altered DNA methylation at a differentially methylated region [53].

In several human population studies, it was reported that the nutritional conditions of grand-parents can have phenotypic consequences in their grandchildren. These trans-generational effects are not readily explained by genetic mutations, and could, thus, be related to epigenetic inheritance [54–58]. A first direct example in humans of the effects of the diet on the methylation status of DNA, and thereby on the phenotype, comes from a recent study on patients with hyper-homocysteinaemia [59]. This disorder is characterised by an increase in *S*-adenosylhomocysteine in the cell. This is a powerful inhibitor of *S*-adenosylmethionine-dependent methyltransferases (DNMT1, etc.), suggesting the possibility of unbalanced DNA methylation as a consequence. Indeed, the patients had reduced levels of total DNA methylation as compared to controls. Interestingly, complementation of their diet with folates restored normal methylation lev-

els in the patients, both globally, and locus-specifically, at the imprinted *IGF2-H19* locus [59]. Folates are indispensable in the methionine cycle, and therefore for the synthesis of *S*-adenosylmethionine, the methyl donor for DNA methylation. Several earlier studies had already indicated the developmental importance of folic acid as a dietary factor *in utero*, and how it modulates disease risks later in life. It remains to be determined whether, as in the case of hyper-homocysteinaemia, these phenotypic effects occur through altered DNA methylation [60].

The above examples in mice and humans emphasize that DNA methylation and other epigenetic modifications are not always stably maintained, and can be influenced by the environment and by dietary intake. Once aberrant epigenetic patterns arise, these may affect gene expression and phenotype. Intriguingly, such epigenetic effects care sometimes be transmitted to the next generation, or even to the grandchildren.

6. Epigenetic consequences of *in vitro* culture

The uterine environment is essential for the correct establishment and maintenance of epigenetic patterns [3]. Not surprisingly, therefore, when embryonic cells and embryos are transferred to an artificial environment, epigenetic patterns may become altered leading to aberrant phenotypes. Many studies have explored the epigenetic effects of *in vitro* culture and manipulation of early embryos and cells [61]. In several of these, altered DNA methylation patterns were detected at imprinted gene loci. Since DNA methylation is normally confined to one of the two parental alleles only, imprinted gene loci allow even minor changes to be readily detected. The general implication from these and other studies is that *in vitro* culture may perturb epigenetic gene regulation, and that this can have long-lasting consequences. Additionally, and not surprisingly, the chemical composition of the culture medium, and whether it is complemented with serum, has a determining influence on the occurrence of methylation changes [10,62–65].

Concerning the effects of cell culture on DNA methylation, these have not only been observed in undifferentiated stem cells [66–68], but were reported also in differentiated cell types, such as fibroblasts [69]. Thus, epigenetic patterns may become altered in vitro in both undifferentiated and differentiated cells, affecting gene expression.

Embryo culture and manipulation are part of procedures used in assisted reproduction clinics. This has raised the question of whether assisted reproduction could induce epigenetic alterations, and could, thereby, affect uterine and postnatal development [70]. There are some indications that this could be the case [15]. Several imprinting disorders, including a fetal overgrowth syndrome linked to the region comprising the imprinted *IGF2* and *CDKN1C* genes, were found to occur at significantly higher frequencies following assisted reproduction. It is not entirely clear, however, whether these epigenetic disease syndromes arise, indeed, because of manipulation and embryo culture, or whether they could somehow be explained by the compromised fertility of the treated couples [71].

7. Toxicological effects

Another important topic of discussion is whether human exposure to toxic components, in natural environments or linked to applications in agriculture or medicine, can have long-lasting consequences that are mediated by epigenetic alterations. Already, it is statistically a challenge to link a specific exposure to observed health problems, such as for the first-proven causal involvement of cigarette smoke in carcinogenesis. Given the frequently pleiotropic effects of toxic components, it is difficult to judge whether the health consequences are caused by epigenetic alterations rather than genetic ones, and, consequently, whether these could constitute useful biomarkers. Several recent studies on endocrine disrupters applied in agriculture, and on a related oestrogen-receptor agonist, however, suggest that there could be long-lasting effects mediated by aberrant epigenetic patterns. Although there are many outstanding questions, these recent studies provide possible paradigms of how epigenetic modifications might be used as markers to monitor the effects of specific toxicological exposures [72,73].

Till the early 1970s of the last century, the oestrogen receptor agonist diethylstilbestrol (DES) was used in the United States and other countries as a drug to reduce the risk of miscarriage in women. Additionally, before its use was forbidden, the drug was fed to farm animals to enhance meat production, which led to low level exposure of the general population. In later years, however, it was discovered that the daughters, whose pregnant mothers were treated with DES, presented abnormal development of the uterus, cervix or vagina, and increased risk of developing a rare and specific type of adenocarcinoma. So far, systematic studies have not yet been performed on the grand-daughters of DES treated women, to determine whether the phenotypic effects can be transmitted to yet-another generation. In mice exposed to DES, however, this is clearly the case [73,74]. Mouse studies suggest that these effects are

mediated by alterations in DNA methylation and chromatin organisation at specific genes [75,76]. Neonatal exposure of laboratory mice to DES increases expression of the c-fos gene and this correlates with loss of methylation at specific enhancer sequences [77]. There is also altered expression of specific homeotic genes, involving altered chromatin organisation rather than aberrant DNA methylation [75,78]. Given their similarities, it has been proposed that the phenotypic effects of DES are comparable to the heritable phenotypes mediated by altered expression of the stress-related chaperone protein HSP90 [73,79]. The latter phenotypes seem to be mediated by an epigenetic mechanism, involving aberrant histone acetylation at developmental genes of the WNT signalling pathway [73,80]. Further studies are now required that will allow the genome-wide analysis of the levels of DNA methylation at many different loci [39,81,82]. Such large-scale studies could pinpoint to which extent DES alters the epigenome in somatic cells and germ cells, and may thus affect phenotype in subsequent generations.

It has been reported that chronic exposure to a widely used anticancer agent, cyclophosphamide, may have effects on the epigenetic organisation of the male germ line. Earlier studies on rats had already shown that paternal exposure to cyclophosphamide leads to increased embryo loss, malformations, and behavioural deficits in the offspring. Intriguingly, these abnormalities were transmissible to subsequent generations [83]. In a more recent study on rats [84], it was explored whether this could be linked to epigenetic alterations in the germ cells and the early embryo. Zygotes sired by drug-treated males were found to be developmentally abnormal and showed disruption of the epigenetic programming of both parental genomes in the early embryo. Specifically, it was found that early after fertilisation, both pronuclei were hyper-acetylated on their histones, as compared to control zygotes. By mid-zygotic development, the male pronucleus had reduced DNA methylation, and at the two-cell stage, there was a disrupted distribution of histone H4 acetylation in the nucleus. Although it remains unclear how precisely these changes account for the heritable developmental abnormalities, these findings indicate that paternal exposure to the drug induces aberrant reprogramming in the early embryo [84].

In agriculture, the anti-androgenic compound ‘vinclozolin’ is commonly used as an antifungal agent, particularly for treatment on vineyards. The related, hormone-like chemical methoxychlor is an oestrogenic compound used as a pesticide. It was known that these endocrine disruptors can cause reproductive abnormalities in laboratory animals. Potentially, this group of chemicals might induce reproductive abnormalities and cancer in humans as well [72]. In an interesting recent study, pregnant rats were exposed to vinclozolin at mid-gestation, during the period of gonadal sex determination. Although the offspring of these females appeared to be generally healthy, the male offspring had reduced sperm counts and reduced sperm mobility. These were associated with reduced fertility. Surprisingly, the reduced fertility was inherited through the male germ line by almost all the males of the subsequent generations. Effects were noted even in the fourth generation. By performing a large-scale DNA methylation assay, it was found that multiple genes had altered patterns of DNA methylation [85]. Although these studies do not conclusively proof that an epigenetic mechanism is causally involved, the altered epigenotype at these genes correlated well with the heritable effects on reproduction.

Other toxic components in the environment are suspected to have a potential effect on DNA methylation and chromatin organisation as well, but so far, published data remain rather non-conclusive. For instance, pollution of drinking water with arsenite compounds in East-India and Bangladesh is associated with a strongly increased risk of skin and bladder cancer. Arsenite is methylated during its metabolism, and was therefore suspected to have potential effect on DNA methylation [86]. Indeed, amongst other effects on cultured cells, it was observed that arsenite induces DNA hypomethylation, but also a gain of methylation at specific sequences [87,88]. Since these methylation patterns are similar to those observed in cancer, it is not clear whether they are a consequence of cell transformation, or whether they are directly induced by arsenic, for instance because of methyl donor depletion [86]. There are also reports in the literature suggesting that nickel and zinc could have epigenetic effects as well, but no conclusive studies have been reported in the literature so far [89].

Although the above examples of toxicological exposures and linked methylation changes are most interesting, it is difficult to conclude whether these are truly epigenetic effects. It is not known, for instance, whether gain of DNA methylation at specific promoters and CpG islands causes loss of gene expression, or conversely, whether the DNA methylation is a consequence of the loss of expression. Another caveat relative to these examples is that toxicological exposures may have an impact on the cell cycle and on cellular proliferation, and that this in turn, could alter gene expression and chromatin. This question of cause and consequence constitutes one of the main challenges for future research.

8. Future outlook

We are at the beginning of addressing how the environment influences the establishment and maintenance of epigenetic states of gene regulation. Although the recent work on human monozygotic twins and inbred animals has pinpointed the substantial influence of the environment during ageing, the precise extent to which external and dietary factors affect epigenetics remains to be explored. Nevertheless, the studies performed so far clearly show that environmental effects can induce epigenetic alterations. Importantly, these alterations are not corrected subsequently, and affect phenotype, sometimes even in the next generations. One of the outstanding questions is, to which extent epigenetic drifts are caused by the intrinsic factors that are involved in the somatic maintenance of DNA methylation and covalent histone modifications.

To explore the importance of the environment and nutrition, versus stochastic intrinsic effects, it should be important to compare animals that are genetically identical and which are kept under identical conditions, or are subjected to different environments. Although technically challenging, and still rather costly, ideally one would perform large-scale and genome-wide analyses of DNA methylation and histone modifications (for recent examples, see [39,81,82]). An alternative approach would be to consider selected subsets of genes, based on what is known on the environmental or nutritional effect that is being studied. Such approaches have been most successful in epigenetic studies on cancer, and have pinpointed the combinations of genes at which DNA methylation becomes altered in specific tumour types [90]. These aberrant patterns of methylation can be applied as biomarkers to diagnose specific types of cancer, or to follow the efficiency of specific cancer treatments [14,91].

In future work on environmental and toxicological effects, it should be instructive to combine genetic data with the epigenetic information that will be obtained. Clearly, the genetic constitution of specific gene regions influences strongly the way their associated chromatin is organised. Inversely, epigenetic alterations across domains may have pronounced genetic consequences. For instance, it has been shown that loss of DNA methylation in mammalian cells strongly increases the frequency of chromosomal translocations [92]. Similarly as during the process of tumourigenesis [91], therefore, environmental and toxicological phenotypic effects most likely have both genetic and epigenetic components. After many years of emphasis on the genetic alterations, the future challenge will be to now unravel the relative importance of the epigenetic components, and to explore whether these can be applied as biomarkers.

Acknowledgements

The laboratory acknowledges grant funding from the Association pour la Recherche sur le Cancer (ARC), the Cancéropôle Grand Sud-Ouest, and the European Science Foundation EuroSTELLS programme. RF thanks members of the 'Genomic Imprinting and Development' group, Elisabetta Andermarcher, and the reviewers for helpful comments on the manuscript, and apologises to those whose work could not be cited because of space limitations.

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