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Review

The role of epigenetics in aging and age-related diseases

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ABSTRACT

The role of epigenetics in aging and age-related diseases is a key issue in molecular physiology and medicine because certain epigenetic factors are thought to mediate, at least in part, the relationship between the genome and the environment. An active role for epigenetics in aging must meet two prior conditions: there must be specific epigenetic changes during aging and they must be functionally associated with the aged phenotype. Assuming that specific epigenetic modifications can have a direct functional outcome in aging, it is also essential to establish whether they depend on genetic, environmental or stochastic factors, and if they can be transmitted from one generation to the next. Here we discuss current knowledge about these matters and future directions in the field.

1. Introduction

Epigenetics was originally defined as the study of how genotypes give rise to phenotypes through programmed changes during development (Waddington, 1942). Today, epigenetics refers to both heritable changes in gene activity and expression (in the progeny of cells or of individuals) and also stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable (http://nihroadmap.nih.gov/epigenomics/index.asp). In this sense epigenetics would include all mechanisms for the unfolding of the genetic programme for many processes in a cell's life, development, differentiation, stress response and pathological state among them. Indeed, epigenetic modifications are stable but, at the same time, can be modulated by many factors, including physiological and pathological circumstances and by the environment (for reviews, see Holliday, 2006; Rakyan and Beck, 2006; Whitelaw and Whitelaw, 2006).

The best known epigenetic modifications are DNA methylation and histone post-transcriptional modifications, including methylation, acetylation, ubiquitination and phosphorylation (Jenuwein and Allis, 2001). DNA methylation consists of the addition of a methyl group to the aromatic ring of a single DNA base. This is a widespread phenomenon in the genome of many organisms and, in mammals, is mostly restricted to the 5-carbon of the cytosine ring of a CpG dinucleotide. In normal human tissues, 5-methylcytosine accounts for 3–6% of the total cytosine (Callinan and Feinberg, 2006). The CpG dinucleotide is found at a very low frequency in the genome, but is concentrated in particular gene promoters (or their surrounding areas), where it can regulate gene expression, blocking transcription when the methyl group is present. Most of these regions, called CpG islands, are subjected to dynamic methylation modifications during development and cell differentiation. Promoter methylation accounts for only a small part of global genome methylation; the bulk of the CpG methylated in the genome is localized in repetitive sequences, most of which are derived from transposable elements. Methylation keeps these sequences silenced, hindering the events of amplification and new insertion in the genome (Callinan and Feinberg, 2006). Other important mechanisms that rely on DNA methylation are genomic imprinting and X-chromosome inactivation (Reik, 2007). Genomic imprinting occurs in some genes whose expression is restricted either to the maternal or to the paternal allele. It requires DNA methylation at one of the two parental alleles of a gene to ensure monoallelic expression. A similar gene-dosage reduction is involved in X-chromosome inactivation in females (Reik, 2007).

There are many pathologies associated with alterations of DNA methylation, the best studied of which is cancer (Esteller, 2008). Alterations of DNA methylation in cancer were first reported almost 30 years ago (Feinberg and Vogelstein, 1983), and since then epigenetics has come to be recognized as a crucial component in the study of cancer biology (Jones and Baylin, 2002). In cancer

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cells, the transcriptional silencing of tumour-suppressor genes by CpG island promoter hypermethylation is key to the tumourigenic process, and contributes to all of the typical hallmarks of a cancer cell that result from tumour-suppressor inactivation. By contrast, repetitive genomic sequences, which are normally heavily methylated, lose a substantial proportion of their methylation, resulting in a global loss of methylcytosine content in the cell (Feinberg and Tycko, 2004). Loss of imprinting (LOI) is also well established as a mechanism of gene activation in some types of cancer (Reik, 2007).

The other well-studied epigenetic mechanism is histone modification. The core histones (H2A, H2B, H3 and H4), together with the 147 base pairs of genomic DNA wrapped around them, comprise the nucleosomes, which are the basic units of chromatin. The interactions between DNA and histones determine the degree of chromatin condensation and, thus, of gene expression (Feinberg, 2007). Histone proteins have a number of positively charged aminoacids at their 5′ tails that protrude from the core structure of the nucleosome and can be chemically modified. Given that there are at least 30 sites of possible modification for each nucleosome and six types of modification (methylation, acetylation, phosphorylation, ubiquitylation, sumoylation and proline isomerization), the number of possible combinations is huge. It has been proposed that histone modifications operate as part of a predictive and heritable epigenetic code that specifies patterns of gene expression throughout differentiation and development (Turner, 2007). Nevertheless, it has never been experimentally demonstrated that histone marks are heritable through generations in multicellular organisms. On the other hand, experiments in yeast give some clue of how histone marks can be propagated through cell generations, identifying for example K12 in the H4 histone tail as the key memory mark for propagating epigenetic state of a telomeric gene (Smith et al., 2002). In a recent theoretical work, mathematical modelling has been applied to demonstrate that with a few assumptions related to the cooperativity and dynamicity of histone marks, good bistability and heritability through cell generations can be achieved (Dodd et al., 2007). Thus, epigenetic factors play a central role in gene regulation and, subsequently, in the control of most processes in cells, tissues and the whole organism. Whereas the epigenetic modifications in cancer and other diseases have been intensely studied for many years (Bjornsson et al., 2004), "aging epigenetics" is an emerging discipline that promises exciting revelations in the near future, such as the definition of a DNA methylome and a histone modification map that will help to distinguish between a "young" and an "old" cell and to characterize all the chromatin modifier enzymes involved in the process (Fraga and Esteller, 2007). The aging process consists of a complex of anatomical, physiological, biochemical and genetic changes that all organisms undergo during their lifetime. Epigenetics is probably only one of several components of aging, but its features make it a very strong candidate for explaining these changes. Although epigenetic factors are heritable (at least at the cellular level), they can be modulated by external factors and in this way represent a molecular link between environment and aging. An active role for epigenetics in aging must fulfil two prior conditions: there must be specific epigenetic changes during development and aging, and they must be functionally associated with the aged phenotype. Assuming that specific epigenetic modifications can have a direct functional outcome on aging, it is also essential to establish whether they depend on environmental factors and/or occur stochastically, and if they can be transmitted from one generation to the next. In the text that follows, we discuss the current knowledge about these matters and future directions in the field.

2. Epigenetic changes during ontogenic development and aging

The relationship between epigenetics and aging was proposed many years ago (Table 1). A pioneering study by Berdyshev et al. (1967) showed that genomic global DNA methylation decreases with age in spawning humpbacked salmon. Subsequently, Vanyushin et al. (1973) also detected a global loss of cytosine methylation during aging in rat brain and heart. More recently, Wilson et al. (1987) confirmed the gradual loss of DNA methylation with age in various mouse tissues and in human bronchial epithelial cells. Similarly, Fuke et al. (2004) recently found an age-dependent decrease in global methylation levels in human leukocytes. The definitive corroboration on intra-individual epigenetic variation over time in humans, was recently provided

Table 1
Epigenetic alteration associated with aging.

Epigenetic hit	Species	Tissue or cell type	References
Global hypomethylation			
	Spawning salmon	Many organs	Berdyshev et al. (1967)
	Rat	Brain, heart	Vanyushin et al. (1973)
	Mouse	Brain, liver, small intestine	Wilson et al. (1987)
	Human	Bronchial epithelial cells	Wilson et al. (1987)
	Human	Leukocytes	Fuke et al. (2004)
	Mouse, hamster and human	Primary cult. fibroblast	Wilson and Jones (1983)
Promoter-specific hypermethylation			
Ribosomal DNA	Rat	Liver and germ cells	Oakes et al. (2003)
ER	Human	Colon	Issa et al. (1994)
MYOD1, N33	Human	Colon	Ahuja et al. (1998)
IGF2	Human	Colon	Issa et al. (1996)
MLH1 and p14ARF	Human	Colon	Issa (2003)
LOX, p16INK4a, RUNX3, TIG1	Human	Gastric mucosa	So et al. (2006)
E-cadherin, c-fos and collagen alpha1	Human	Various tissues	Fraga and Esteller (2007)
Histone marks and histone-modifying enzymes			
Global histone acetylation	Human	Human diploid cells WI-38	Ryan and Cristofalo (1972)
Histone methylation	Rat	Brain and liver	Lee and Duerre (1974)
Senescence-associated heterochromatic foci (SAHF)	Human	Fibroblasts	Narita et al. (2003)
H3K9me, Suv39h1	Mouse	Splenocytes, lymphocytes	Braig et al. (2005)
H3K27me3 and EZH2	Human, mouse	HEF, MEF	Bracken et al. (2007)
H4K20, Suv4–20h	Rat	Liver, kidney	Sarg et al. (2002)
SIRT1	Human, mouse	Human lung, MEF	Sasaki et al. (2006)
SIRT1	Mouse	–	Sommer et al. (2006)

in a longitudinal study of DNA methylation patterns in which successive DNA samples were collected more than 10 years apart in more than 100 individuals (Bjornsson et al., 2008).

In addition to global hypomethylation, a number of specific loci have been described as becoming hypermethylated during aging. For instance, using restriction landmark genomic scanning Oakes et al. (2003) demonstrated an increase of methylation in ribosomal DNA hypermethylated clusters in liver and germ cells of senescent rats. This observation is important because it can be associated with the decrease of RNA levels during aging and because failure to maintain normal DNA methylation patterns in male germ cells could be one of the mechanisms underlying age-related abnormalities in fertility and progeny outcome. In addition to ribosomal DNA, CpG island promoter hypermethylation in non-tumourigenic tissues has been reported for several genes, including the estrogen receptor (ER) (Issa et al., 1994), myogenic differentiation antigen 1 (MYOD1), tumour-suppressor candidate 33 (N33) (Ahuja et al., 1998) and insulin-like growth factor II (IGF2) (Issa et al., 1996). In some cases, such as MLH1 and p14ARF, colon promoter hypermethylation was more common in aged tissues (reviewed in Issa, 2003). Similarly, a recent study found promoter hypermethylation of the tumour-suppressor genes lysyl oxidase (LOX), p16INK4a, runt-related transcription factor 3 (RUNX3) and TPA-inducible gene 1 (TIG1) in non-neoplastic gastric mucosa to be positively and significantly associated with aging (So et al., 2006). Other examples of genes with increased promoter methylation during aging include those encoding E-cadherin, c-fos and collagen alpha1 (reviewed in Fraga and Esteller, 2007).

Another group of studies concentrated on a classic *in vitro* model for aging: the replicative senescence of primary cultured cells. The process of cellular senescence was first described in a seminal study by Hayflick and Moorhead (1961), who observed that normal human fibroblasts were able to enter a state of irreversible growth arrest after serial cultivation *in vitro*, while cancer cells were able to proliferate indefinitely. They proposed that there were some factors whose gradual loss through cell proliferation limited the number of cell divisions and that this process could contribute to organismal aging. It is still not completely clear how the latter might occur, but two main processes have been suggested: the accumulation of senescent cells in tissues and the limitation of regenerative potential of adult stem cell pools (Fraga et al., 2007). Wilson and Jones (1983) first showed how global DNA methylation also decreased with the number of cell passages in cultures of diploid fibroblasts of mice, hamsters and humans, while immortal cell lines had stable levels of methylation. The greatest loss of methylation was observed in mouse cells, which survived the fewest divisions, implying that the rate of methylation loss may be correlated with functional senescence.

Thus, two specific alterations of DNA methylation occur during aging: a decrease in global 5-methylcytosine and hypermethylation of specific loci (primarily CpG island promoters). Intriguingly, global DNA hypomethylation and aberrant promoter hypermethylation are known epigenetic alterations in cancer (Esteller, 2008), which suggests that the accumulation of epigenetic alterations during aging may directly contribute to malignant transformation, although this hypothesis needs further evaluation.

Other epigenetic factors, such as histone modifications, are also known to change during aging. Early papers reported an age-associated decrease in the rate of histone acetylation in cultured human diploid cells (Ryan and Cristofalo, 1972) and changes with age in histone methylation have been found in rat brain and liver (Lee and Duerre, 1974). More recently, the accumulation of distinct heterochromatic structures has been reported in senescent human fibroblasts (Narita et al., 2003). These structures were termed senescence-associated heterochromatic foci (SAHF) and coincide with the accumulation of methylated histone H3 lysine 9 and the recruitment of heterochromatin proteins and the retinoblastoma (Rb) tumour suppressor to E2F-responsive promoters (Narita et al., 2003). Subsequently, this epigenetic phenomenon was shown to depend specifically on the histone methyltransferase Suv39h1 (Braig et al., 2005). *In vitro* senescence has also recently been associated with decreased levels of H3K27me3 and EZH2, the histone methyltransferase responsible for this epigenetic modification (Bracken et al., 2007). The methylation status of the lysine 20 of the histone H4 (H4K20), a marker of constitutive heterochromatin (Gonzalo et al., 2005), has also been found to increase in senescent cells (Sarg et al., 2002), which could be associated with the accumulation of heterochromatic structures in senescent human fibroblasts (Narita et al., 2003). The increase of trimethylated K20-H4 in senescent cells might also be associated with alterations of Suv4–20h, although it is not known whether this histone-modifying enzyme is altered with age. Interestingly, a recent study showed that the human premature aging disease Hutchinson–Gilford Progeria Syndrome (HGPS) recapitulates some of the epigenetic alterations observed in normal aging, such as a decrease of histone H3 trimethylated on lysine 27 (accompanied with the downregulation of EZH2) and an increase in the trimethylation of histone H4K20 (Shumaker et al., 2006). HGP originates from mutations in the nuclear Lamin proteins (Lamin A) but the link between these proteins and the histone-modifying machinery is still unclear. In this regard, a recent study in mice reported that disruption of FACE1, a metalloprotease involved in prelamin A proteolytic maturation, is associated with premature aging and with the disruption of the integrity of the nuclear envelope (Cadinanos et al., 2005) and alterations of DNA methylation and histone modification in adult stem cell niches (Espada et al., 2008).

Several histone-modifying enzymes are also known to have an important role in aging. Of these, the Sirtuin family of epigenetic enzymes, which have histone deacetylase (HDAC) activity, deserves special attention. Histone acetylation is essential for the control of chromatin structure and thereby the regulation of gene-expression regulation. Sirtuins are NAD+-dependent HDACs that are involved in a range of cellular events, including chromatin remodelling, transcriptional silencing, mitosis and the control of lifespan (reviewed in Guarente and Picard, 2005). The family's founder member, silent information regulator 2 (*Sir2*), was initially described in yeast (Rine and Herskowitz, 1987), in which its deletion shortened replicative lifespan, whereas an extra copy of the gene increased it, suggesting that the Sir2 family has an important role in aging (Kaeberlein et al., 1999). As *Sir2* can deacetylate histones, it has been suggested that *Sir2* proteins might link metabolic rate and aging through NAD-dependent gene regulation and chromatin remodelling (reviewed in Guarente, 2000). This evidence has raised considerable interest in the sirtuins, the mammalian orthologues of *Sir2*. Seven homologues of the yeast Sir2 protein, Sirtuin 1–7 (SIRT1-7), have been identified in mammals, where they are involved in the regulation of gene expression, stress responses, DNA repair, apoptosis, cell cycle, genomic stability and insulin regulation. Of these family members, SIRT1 is particularly relevant with respect to aging. SIRT1 features a NAD+-dependent deacetylase activity similar to that observed in yeast Sir2: in mammals, SIRT1 can act not only on histone tails (mainly K16-H4 and K9-H3 positions (Pruitt et al., 2006; Vaquero et al., 2004)), but also on key transcription factors, such as tumour protein p53 (p53), forkhead transcriptional factors (FOXO), p300 histone acetyltransferase, the tumour protein p73 (p73), E2F transcription factor 1 (E2F1), the DNA repair factor Ku antigen, 70-kD subunit (Ku70), the nuclear factor kappa-B inhibitor (NF-κB) and the androgen receptor (AR) (reviewed in Fraga et al., 2007). SIRT1 is expressed in most tissues, and is downregulated in

senescent cells (Sasaki et al., 2006) and during aging (Sommer et al., 2006). Recent evidence that small molecule activators of Sirtuins increase lifespan (Baur et al., 2006) reinforces the importance of these HDACs in aging.

3. The impact of epigenetic changes accumulated during aging on the aging phenotype

A key question about the role of epigenetics in aging is whether epigenetic changes accumulated during aging have a causal role in establishing the aging phenotype or if the two phenomena are unrelated. To settle this matter, it is important to consider the region in the genome/chromatin where these changes occur. Changes occurring in non-coding sequences will potentially have a smaller biological impact than those occurring in coding sequences as modifications of the latter type generally involve changes in gene expression. It is also important to consider the cells and tissues in which these occur because epigenetic patterns are cell- and tissue-specific so that changes occurring in a specific cell or tissue would not necessarily imply the same functional consequences in different cells or tissues.

The functional relationship between epigenetic modifications and aging is still unknown, although the relationship between specific epigenotypes and disease phenotypes has been thoroughly studied. One of the best examples relating epigenetics and disease phenotype is found in *agouti* mice. The *agouti* alleles regulate the production of pigment in individual hair follicles. The A allele is responsible for the black, wild-type coat colour of animals, whereas the A^{vy}, A^{iapy} and A^{hvy} alleles are responsible for a range of phenotypes in these mice. This spectrum includes coat colour, which varies from entirely yellow to fully *agouti*. Yellow and mottled mice are obese and prone to diabetes and cancer, in contrast to fully *agouti* mice, known as pseudo*agouti*s, which are lean and non-diabetic. Expression of the A^{vy}, A^{iapy} and A^{hvy} alleles is controlled by an intracisternal A particle (IAP) retrotransposon, through spontaneous insertions of single IAP sequences in different regions of the *agouti* gene (Duhl et al., 1994). Transcription originating in an IAP retrotransposon inserted upstream of the *agouti* gene (*A*) causes ectopic expression of *agouti* protein, resulting in yellow fur, obesity, diabetes and increased susceptibility to tumours. Isogenic A^{vy} mice are epigenetic mosaics and have coats that vary over a continuous spectrum from full yellow, through variegated yellow/*agouti*, to full *agouti* (pseudo*agouti*) due to IAP expression. What is particularly remarkable and important about this model is that the activity of the A^{vy} allele is associated with its promoter methylation status (Morgan et al., 1999), which makes the *agouti* mice one of the best examples of how a specific epigenetic modification results in a specific disease phenotype.

In humans, the most obvious link between epigenetic modifications and a disease phenotype are the epigenetic changes that occur in cancer (Table 2). The epigenetic patterns of cancer cells are considerably altered relative to normal cells (review in Esteller, 2007). Cancer cells undergo a global loss of DNA methylation and at the same time acquire specific patterns of hypermethylation at certain promoters of tumour-suppressor genes. The hypermethylation of tumour-suppressor genes, which is associated with their transcriptional silencing, is recognized as a key feature of cancer pathogenesis, while the global loss of DNA methylation is linked to chromosomal instability (Esteller, 2007). In addition, these DNA methylation changes are associated with the presence of altered patterns of histone modification. For example, the trimethylation of K20-H4, which is enriched in differentiated cells (Biron et al., 2004) and increases with age (Prokocimer et al., 2006; Sarg et al., 2002) and progeria syndromes (Shumaker et al., 2006), is commonly reduced in cancer cells (Fraga et al., 2005b; Olins and Olins, 2005; Pogribny et al., 2006; Sarg et al., 2002; Tryndyak et al., 2006; Van Den Broeck et al., 2008) (Table 2). The loss of trimethylated K20-H4 in cancer can be caused by the loss of expression of the K20-H4-specific methyltransferase Suv4–20h (Pogribny et al., 2006; Tryndyak et al., 2006; Van Den Broeck et al., 2008), loss of the tumour-suppressor RB (Isaac et al., 2006; Siddiqui et al., 2007), or deregulation of other histone-modifying enzymes. However, it is not known whether the expression of these histone-modifying enzymes changes with age. Interestingly, two histone modifications marks (monoacetylated K16-H4 and acetylated K9-H3) (Pruitt et al., 2006; Vaquero et al., 2004) associated with the "anti-aging" histone deacetylase Sirt1 have been shown to be altered in cancer. SIRT1 is upregulated in mouse lung carcinomas, lymphomas and soft-tissue sarcomas (Chen et al., 2005), and in human lung cancer (Yeung et al., 2004), prostate cancer (Kuzmichev et al., 2005) and leukaemia (Bradbury et al., 2005). Most important, the histone-targets of SIRT1, K16-H4 and K9-H3 (Vaquero et al., 2004) are known to be altered in various types of tumour (Fraga and Esteller, 2005; Seligson et al., 2005). Underacetylation of K9-H3 is associated with a higher risk of recurrence in prostate cancer (Seligson et al., 2005). Cancer cells

Table 2
Epigenetic alteration associated with age-related diseases.

Disease	Epigenetic hit	Species	Tissue, cell type	References
Cancer	Global hypomethylation	Various	Various	Review in Esteller (2007)
	Promoter-specific hypermethylation (tumour suppressors)	Various	Various	Review in Esteller (2007)
	Decrease of H4K20me3	Various	Various	Fraga et al. (2005b), Olins and Olins (2005), Pogribny et al. (2006), Sarg et al. (2002), Tryndyak et al. (2006), Van Den Broeck et al. (2008)
	Loss of expression of Suv4–20h	Various	Various	Pogribny et al. (2006), Tryndyak et al. (2006), Van Den Broeck et al. (2008)
	Altered patterns of acetylated K16-H4	Various	Various	Fraga et al. (2005b), Kapoor-Vazirani et al. (2008), Pfister et al. (2008), Pruitt et al. (2006)
	Hypoacetylation of K9-H3	Human	Prostate cancer	Seligson et al. (2005)
	Upregulation of SIRT1	Mouse	Lung carcinoma, lymphoma, and soft-tissue sarcoma	Chen et al. (2005)
	Upregulation of SIRT1	Human	Lung cancer, prostate cancer, leukaemia	Yeung et al. (2004), Kuzmichev et al. (2005), Bradbury et al. (2005)
	Downregulation hMOF	Human	Primary breast carcinoma, medulloblastoma	Pfister et al. (2008)
Type-2 diabetes	Hypermethylated COX7A1	Human	Skeletal muscle	Ronn et al. (2008)
Alzheimer disease	Demethylation of Tau promoter	Human	Cerebral cortex	Tohgi et al. (1999)

have a lower level of acetylated K16-H4 (Fraga et al., 2005b; Pfister et al., 2008) due to hypoacetylation at repetitive elements (Fraga et al., 2005b) and gene promoters (Kapoor-Vazirani et al., 2008; Pruitt et al., 2006). The decrease of acetylated K16-H4 in cancer can be due to the upregulation of SIRT1 in tumours (Bradbury et al., 2005; Chen et al., 2005; Kuzmichev et al., 2005; Yeung et al., 2004) or to the downregulation of the K16-H4-specific histone acetyltransferase hMOF (Pfister et al., 2008). The precise relationship between the upregulation of Sirt1 in cancer and its possible downregulation during aging (Sasaki et al., 2006; Sommer et al., 2006) is still poorly understood and represents a fascinating field of research of relevance to the understanding of age-related diseases such as cancer.

Interestingly and importantly, two non-oncogenic diseases strongly associated with aging, Alzheimer and Type-2 diabetes (TD2), have also been found to be associated with specific epigenetic alterations. Amyloid-β protein deposition in the aged brain in Alzheimer disease has recently been associated with cytosine demethylation (Tohgi et al., 1999), and the COX7A1 gene, which is involved in glucose metabolism, has been found to become increasingly methylated with age (Ronn et al., 2008). As the risk of TD2 increases with age, the authors of the latter work suggest that the increase of methylation of the COX7A1 promoter with aging could directly contribute to the development of TD2.

In addition to these age-related diseases, several epigenetic modifications have also been associated with disease phenotypes that are not necessarily linked to the aging process. Examples of this include the hypermethylation of the dopamine receptor D2 (DRD2) in schizophrenia (Petronis et al., 2003; Zhang et al., 2007), the methylation status of catechol-O-methyltransferase (COMT) in low birth weight (Mill et al., 2006), X-chromosome inactivation and reduced DNA methylation status in the PPIEL (peptidylprolyl isomerase E-like) promoter in bipolar disease (Kuratomi et al., 2008; Rosa et al., 2008) and the hypermethylation of the AXIN1 promoter and discordant caudal duplication (Oates et al., 2006).

4. Factors involved in the generation of epigenetic modifications during aging

One of the key issues in aging epigenetics is how epigenetic changes are generated during the aging process. For a long time it has been believed that epigenetic modifications occurring during aging may depend on environmental factors. This idea is attractive because, if true, epigenetics could provide a link between the environment, disease and aging. It also opens the possibility of targeted intervention aimed, for example, at improving healthspan or healthy aging. Thus, the first question is whether specific environmental factors can directly induce specific epigenetic modification. In this regard, one of the best examples is the promoter hypermethylation of tumour-suppressor genes that occur in non-tumourigenic lung tissues of smokers but not in the corresponding tissue of non-smokers (Belinsky et al., 2002). In addition it has been extensively described how maternal nutritional status and particular diet components can affect the epigenome depending on the tissue and developmental stage: for instance, dietary methyl donors such as folate, methionine, choline and vitamin B12 are capable of increasing DNA methylation levels at particular loci in animal models (Waterland, 2006; Waterland and Jirtle, 2003) as well as in humans. Another notable example is the effect of *in utero* exposure to a high-fat diet that is able to reduce natural ER promoter hypermethylation with aging in rats and to cause stronger expression of ER and a higher incidence of tumours in offspring (Yenbutr et al., 1998).

Other environmental factors have also been implicated in the modulation of DNA methylation, including metal ions. Chromium (Shiao et al., 2005), cadmium (Takiguchi et al., 2003), and nickel (Salnikow and Costa, 2000) are pollutants capable of reducing methylation levels at genetic loci by inhibiting the activity of DNA methyltransferases. In the case of nickel, carcinogenic nickel compounds have also been described to decrease global histone H4 acetylation and increase histone H3 lysine 9 dimethylation at the promoter level, repressing gene expression (Chen et al., 2006). Many chemical compounds and xenobiotics to which populations can be exposed are known to alter DNA methylation at a global and/or local level. These include diethylstilbestrol (DES) (Veurink et al., 2005), a drug previously used to prevent miscarriages, bisphenol A (Maffini et al., 2006), used in the plastics industry, and vinclozolin (Anway et al., 2006), a fungicide used in vineyards. These molecules are considered to be endocrine disruptors and have been convincingly related to DNA methylation alteration of specific promoters, developmental disorders and tumourigenesis. Another of the possible targets for dietary epigenetic modulation is histone acetylation. Recently, substances naturally occurring in some foods, like butyrate in cheeses, diallyl disulphide in garlic and sulphoraphane in broccoli, have been identified as HDAC inhibitors, and a putative role for some of these has been proposed in cancer chemoprevention through the disruption of the uncontrolled progression of cell cycle or by the induction of apoptosis via increased acetylation and de-repression of genes such as P21 and BAX (Dashwood and Ho, 2007). Despite the ability these substances to induce punctual epigenetic changes, it has to be clarified whether they should be considered as real epigenetic modifiers. For being considered real epigenetic modifiers, epigenetic changes should remain after the exposure to the compound.

Further evidence of the influence of non-genetic factors on the accumulation of epigenetic changes during aging is represented by the epigenetic variation overtime observed in monozygotic twins (Fraga et al., 2005). Twin studies are extremely useful in human genetics and medicine for estimating the relative importance of genetic and non-genetic components in any phenotype or disease (Poulsen et al., 2007). Despite being almost genetically identical, MZ twin pairs often feature numerous phenotypic differences, such as susceptibility to disease and a wide range of anthropomorphic features (Poulsen et al., 2007). It has recently been proposed that phenotypic discordance between MZ twins may depend on epigenetic factors that change over the lifetime of the individual. We recently analyzed global and locus-specific epigenetic differences in different-aged MZ twins and showed that elderly MZ twin pairs who lived apart from their own families and who exhibited numerous phenotypic differences had more epigenetic differences than young and phenotypically similar MZ twin pairs reared together (Fraga et al., 2005). We also found that although most of the epigenetic changes occurred in non-functional and repetitive DNA elements, MZ twins had significantly different gene-expression phenotypes (Fraga et al., 2005), as had been reported previously by other authors (Cheung et al., 2003; Choi and Kim, 2007; Sharma et al., 2005). Further evidence of the epigenetic differences between twin siblings was recently reported by Kaminsky et al. (2009), wherein a genome-wide analysis of DNA methylation patterns in MZ and dizygotic (DZ) twins revealed significant epigenetic differences in both groups. The intra-individual epigenetic variation overtime was further corroborated in a longitudinal study of DNA methylation patterns (Bjornsson et al., 2008). The demonstration of age-accumulating epigenetic modifications supports the idea of age-related loss of normal epigenetic patterns as a possible mechanism for the late onset of common human diseases (Bjornsson et al., 2004).

Other examples of epigenetic modulation in response to environmental factors include the epigenetic downregulation of genes involved in pancreatic β-cell function in abnormal intrauterine environments (Simmons, 2005) and the specific DNA methylation profiles of offspring associated with maternal

diet (Lillycrop et al., 2005) and even with maternal behaviour (Weaver et al., 2004). However, there are epigenetic changes that occur during ontogenic development and aging that cannot be explained solely by environmental effects. For example, an early study showed that isogenic laboratory animals maintained under identical environmental conditions presented significant phenotypic differences (Gartner, 1990). Such phenotypic variability was believed to depend on a so-called "third component" of epigenetic origin that was independent of the environment but significantly contributed to the creation of random biological variability (Gartner, 1990). More recent studies using parthenogenetic marbled crayfish as the model experimental animal showed DNA methylation-associated epigenetic differences in isogenic animals living under the same environmental conditions (Vogt et al., 2008). Therefore, epigenetic modifications are associated with phenotypic variation as a result of both external environmental factors and several still little-understood stochastic events. The role of noise is not new to biology and genetics. Some classical example are the variegation effect in Drosophila, first studied with the white gene, where different spreading of heterochromatin in each cell during development leads to variation in gene silencing generating an inter-individual variability independent of both genetics and environmental factors. X-inactivation is another classic example of a stochastic event in mammals that causes phenotype variability. In any case, stochastic epigenetic changes occurring during aging are likely to take place in individual cells and this makes extremely difficult to detect and to quantify this phenomenon with current experimental techniques.

In addition to environmental (reviewed in Feinberg, 2007) and stochastic (Vogt et al., 2008) factors, recent data indicate that intra-individual epigenetic variation over time also depends on hereditary factors (Bjornsson et al., 2008; Heijmans et al., 2007), but the relative contribution of the latter is not clear. One possibility is that the influence of one factor or another depends on which DNA region is involved. Consistent with this, most of the environment-related changes in phenotypic expression between MZ twins preferentially occur in heterochromatic, gene-poor regions (Choi and Kim, 2007; Sharma et al., 2005), which, interestingly, are also the regions where most epigenetic differences are found in environment-dependent phenotypically discordant MZ twins (Fraga et al., 2005). In addition, the IGF2/H19 locus, whose epigenetic variation primarily depends on genetic factors, is resistant to methylation changes over time (Heijmans et al., 2007). The DNA regions that accumulate epigenetic changes over time are important regarding the functionally of these molecular alterations. Although the epigenetic differences between twin pairs preferentially occur outside functional elements (Fraga et al., 2005; Kaminsky et al., 2009), the discordant expression of phenotypes of MZ twins (Choi and Kim, 2007) and the relationship between environment-dependent epigenetic alterations and cancer (Feinberg, 2007) – probably the best known age-related epigenetic disease (Esteller, 2008) – revealed in studies of large cohorts of MZ twins discordant for this disease (Lichtenstein et al., 2000) suggest that they could also have an important role (Hoover, 2000).

5. Transmission between generations of epigenetic changes accumulated during aging

As epigenetic factors can be affected by the environment, there has recently been debate about whether these alterations can be transmitted from generation to generation when they occur in germ cells. Most genomic DNA methylation is erased between fertilization and pre-implantation in sexual reproduction (Reik et al., 2001), which is difficult to reconcile with the massive heritability of epigenetic marks accumulated during aging. Nevertheless, there are several epigenetic modifications that can be transmitted down the generations. For specific epigenetic scenarios to be transmitted from generation to generation requires them to be inefficiently cleared in sexual reproduction by a phenomenon known as "epimutation". This term was first coined by Robin Holliday to describe heritable changes in gene activity due to DNA modification and to distinguish them from classical gene mutations, which are due to changes in DNA sequences (Holliday, 1987).

Probably one the best known examples of specific environmentally dependent epigenetic marks transmitted between generations is to be found in *agouti* mice. Morgan et al. (1999) studied epigenetic inheritance at the *agouti* locus and found that the phenotype of a mouse dam with the A^{vy} allele was related to the phenotypes of the offspring, whereby yellow dams produced yellow and mottled offspring, but not pseudo*agouti* offspring. Interestingly, the passage of the allele through two generations of pseudo*agouti* females produced significantly more pseudo*agouti* offspring than through only one generation of pseudo*agouti* dams. Wolff et al. (1998) described how the maternal diet enriched in methyl donors could affect the expression of the agouti gene in the offspring.

In humans, the first report of a possible epimutation was published by Buiting et al. (2003), who found that epimutations at the SNURF-SNRPN locus correlated with loss of imprinting in patients with Prader–Willi syndrome. Subsequently, Suter et al. (2004) reported that germ line epimutation at the DNA mismatch-repair gene *MLH1* was associated with a greater risk of nonpolyposis colorectal cancer. However, further work by the same group questioned the transmission of this epigenetic mark between generations (Hitchins and Ward, 2007). Chan et al. (2006) also reported a stably inherited, allele-specific, mosaic methylation in the promoter of another DNA mismatch-repair gene (MSH2) in a family affected with nonpolyposis colorectal cancer. However, the presence of the epimutation was not studied in germinal cells, which makes necessary further research to consider this observation as a phenomenon of trans-generational epigenetic inheritance. Importantly, two recent studies in monozygotic and dizygotic twins show that specific DNA methylation signatures can be trans-generationally heritable by either genetic (Heijmans et al., 2007) or epigenetic (Kaminsky et al., 2009) mechanisms. Taking everything together, it is evident that further research is needed to decipher the precise molecular mechanisms of epigenetic inheritance in mammals.

6. Perspectives and challenges

Epigenetics has emerged as an important subject area in aging biology and may be able to explain many of the phenotypic changes related to the aging process. Nevertheless, further research in this field is needed to elucidate the functional role of epigenetic modifications accumulated during aging in the establishment of the aging phenotype. For instance, although it is possible to associate the accumulation of methylation at the promoters of tumour-suppressor genes during aging with the predisposition to developing cancer, there is no experimental or mechanistic evidence of a direct relationship between these genes and aging. In the same way, although TD2 and Alzheimer are considered diseases of aging and are known to be associated with several specific epigenetic alterations, the functional link between specific epigenetic modifications and the aging process itself is far from being definitively demonstrated. One of the most important challenges is to find more appropriate model systems in which genetic or epigenetic manipulation is possible and to identify clear characteristics that will allow the aging phenotype to be measured. Studies on humans are of evident interest from the clinical and applied point of view but are extremely difficult because of the lack

of material. Moreover, inter-individual heterogeneity makes it hard to detect statistically significant differences in population studies and the long lifespan of the human being is an obstacle to following changes in single individuals. At the same time, there is intra-individual heterogeneity due to tissue- and cell-specific epigenetic mechanisms that cannot be overcome. Other models are not problem-free. So research is moving towards the study of different organisms in which it might be easier to associate a definite epigenetic change with the aged phenotype. Alternatively the comparison of data from different *in vitro* and *in vivo* models could provide the final answer. In recent years, technological innovations such as microarrays and ultra-high-throughput sequencing have enabled the global epigenetic landscape to be described, multiplying the amount of information that can be obtained from any given sample. Nevertheless, given the heterogeneity of epigenetic marks from cell to cell, further technical improvements are needed to improve the analysis of individual cells or homogeneous group of cells. Finally, new possibilities are opening up involving the use of imaging techniques in living (Yamagata, 2008) and whole organisms (Zinn et al., 2008). It is to be hoped that these will provide novel information about the epigenetic dynamics of the living cell.

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