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Revisiting the genomic hypomethylation hypothesis of aging

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Abstract

The genomic hypomethylation hypothesis of aging proposes that an overall decrease in global DNA methylation occurs with age, and it has been argued that the decrease in global DNA methylation could be an important factor in aging, resulting in the relaxation of gene expression regulation and abnormal gene expression. Since it was initially observed that DNA methylation decreased with age in 1974, 16 articles have been published describing the effect of age on global DNA methylation in various tissues from rodents and humans. We critically reviewed the publications on the effect of age on DNA methylation and the expression of the enzymes involved in DNA methylation to evaluate the validity of the hypomethylation hypothesis of aging. On the basis of the current scientific literature, we conclude that a decrease in the global methylation of the genome occurs in most if not all tissues/cells as an animal ages. However, age-related changes in DNA methylation in specific regions or at specific sites in the genome occur even though the global DNA methylation does not change.

Keywords

DNA Methylation; gene expression; DNA methylation enzymes; dietary restriction; hypomethylation; aging

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Competing interests

The authors declare no competing interests.

Introduction

The presence of 5-methylcytosine (5mC) in DNA was first reported by Johnson and Coghill in 1925 as one of the hydrolysis products of tuberculinic acid.¹ However, it was not until 1948 that Hotchkiss conclusively identified 5mC in calf thymus DNA using paper chromatography.² Because of its relatively high abundance (~ 4% of the cytosines in the human genome are methylated³), 5mC is often referred as the fifth base. One of the interesting features of cytosine methylation is its enrichment in CpG dinucleotides. These palindromic sites have the unique potential for the symmetric methylation of cytosines (i.e., methylation can occur on both strands of DNA), which can then be copied during cell replication from the parent to daughter strand. Although CpG sites are enriched in 5mC, cytosines at CpH (H = A, T, or C) sites are also methylated, and because CpH sites are much more abundant than CpG sites, the majority of 5mC in the genome is found in CpH sites in some tissues, such as brain.⁴

Cytosine methylation is catalyzed by a complex interplay of three independently encoded DNA methyltransferases (DNMTs) that use *S*-adenosyl methionine as the methyl donor: DNMT1, DNMT3a, and DNMT3b.^{5–7} DNMT1 is a maintenance methyltransferase capable of recognizing and functioning only on hemimethylated DNA^{8–10} through ubiquitin-like PHD and RING finger domains (UHRF1), which interact with DNMT1 and hemimethylated CpG.^{11,12} Thus, DNMT1 plays an important role in the heritability of epigenetic information.¹³ In contrast, DNMT3a and DNMT3b are *de novo* methyltransferases capable of recognizing and methylating both hemimethylated and unmethylated DNA,^{14,15} and these methyltransferases are responsible for changes in DNA methylation that occur at CpH sites. Because DNA methylation patterns are important in embryonic development, knocking out any of these three DNMTs in mice is embryonically lethal.^{10,15}

The 5mC residues in the genome can be lost through passive and active mechanisms. The passive mechanism involves the loss of 5mC that occurs when the cytosines are not methylated during replication or from the repair of regions of the genome that contained 5mCs.¹⁶ The active mechanism involves DNA demethylation whereby the methyl group is modified and the resulting modified cytosine residue is removed and replaced. The process of demethylation of 5mC residues involves ten-eleven translocation (TET) proteins, also termed 5mC dioxygenases. The TET family of proteins includes the TET1, TET2, and TET3 proteins. These proteins catalyze the oxidation of the methyl group on 5mC to 5-hydroxymethyl cytosine (5hmC).¹⁷ Because the increase in 5hmC levels coincides with the reduction of 5mC,^{18,19} formation of 5hmC is believed to be the first step in the active demethylation of 5mC residues. However, 5hmC has also been shown to play an epigenetic role in gene regulation,^{20,21} especially in stem cells;²² therefore, 5hmC might have functional consequences in addition to being the first step in the demethylation of 5mC. Once 5hmC is formed, there are several pathways by which the 5hmC is removed. For example, 5hmC is more susceptible to deamination by activation-induced deaminase than 5mC,²³ and the resulting product, 5-hydroxymethyluracil, is removed via the base-excision repair pathway and replaced with a non-methylated cytosine by the base-excision repair pathway via thymine DNA glycosylase.²³ In addition, the TET family of proteins also catalyze the subsequent oxidation of 5hmC to 5-formylcytosine and 5-carboxylcytosine,

which are also excised by the base-excision repair pathway.²⁴ A critical area for further investigation is the targeting mechanisms by which 5mC is added or removed from specific sites. While transcription factors and histones are known to direct DNMTs to specific genomic regions, these mechanisms are poorly understood.²⁵

Research over the past 2 decades has shown that changes in DNA methylation are an important component of epigenetic regulation in mammals, leading to alterations in gene expression and the maintenance of the differentiated state in cell lineages.²⁶ The first report that DNA methylation changed with age was published in 1973, when Vanyushin *et al.*²⁷ reported that the total levels of 5mC in the DNA isolated from various tissues of rats decreased with age. Subsequently, Wilson *et al.*²⁸ reported that the levels of 5mC decreased with age in DNA isolated from various tissues or cells from mice, *Peromyscus*, and humans. These reports generated a great deal of interest, because they provided a mechanistic explanation for the relaxation of gene expression with age, a process termed *dysdifferentiation*.²⁹ This concept was largely based on a study by Ono and Cutler,³⁰ which reported that expression of globin genes in nonerythroid tissues, such as liver and brain, increased with age.³⁰ It was argued that the loss of 5mC in the genome with increasing age was responsible for the abnormal expression of genes that are normally repressed in cells/tissues.²⁹ However, in a recent study, White *et al.*³¹ did not observe any major relaxation of gene repression at old age, although some minor effect cannot be ruled out. Although investigators have not been able to replicate the study by Ono and Cutler³⁰ showing a relaxation in the regulation of genes that are not normally expressed in differentiated cells, there have been several studies reporting a decrease, no change, or an increase in global methylation of the genome of tissues from animals as they age. Here, we critically evaluate the effect of age on the DNA methylating and demethylating enzymes and the global content of 5mC in the genomes of animals to determine the validity of the genomic hypomethylation hypothesis of aging.

Effect of age on the levels of 5mC

We have listed in Table 1 the published reports that have studied the effect of age on global DNA methylation (i.e., the content of 5mC in total DNA). We did not include studies that measured DNA methylation during development/maturation (e.g., the study by Romanov *et al.*⁴⁵ showing that DNA methylation was lower in heart and thymus of 10- to 12-year-old cows compared with embryos;⁴⁵ the study by Stubbs *et al.*⁴⁶ showing that hypermethylation in DNA occurred between birth and 10 month of age in mouse liver, lung, heart and cortex; and the study by Heyn *et al.*⁴⁷ that compared DNA methylation in blood from a newborn infant and a centenarian. In addition, we did not include reports in Table 1 where methylation was studied in only a portion of the genome. The initial study by Vanyushin *et al.*²⁷ used thin-layer chromatography (TLC) to separate 5mC from other bases in formic acid hydrolysates of DNA and measured the level of 5mC eluting from the TLC by UV absorbance. They reported that the 5mC content of DNA hydrolysates from brain, heart, and spleen decreased over a range of ages between 1 and 28 months in male rats. However, they did not observe a significant change in 5mC content in DNA isolated from liver, lung, and kidney. In 1987, Wilson's group developed a ³²P-postlabeling assay that was at the time the most sensitive and accurate method for measuring 5mC levels in DNA.²⁸ After digesting

DNA with micrococcal nuclease and calf spleen phosphodiesterase, the deoxynucleosides were radioactively labeled using T4 polynucleotide kinase and ^{32}P -ATP. The hydrolysates were then separated by 2-dimensional TLC, and the 5mC content was calculated based on the percent of radioactivity in 5-methyl-deoxycytidine compared with the radioactivity in deoxycytidine. In 1983, Wilson and Jones published a seminal paper in the area of DNA methylation and age showing that the levels of 5mC decreased with increasing population doublings in DNA isolated from skin fibroblasts from mice, hamsters, and humans.⁴⁸ Although this paper is often cited as showing that hypomethylation of the genome occurred with age, this report only measured the effect of population doubling (i.e., replicative senescence) on DNA methylation, not aging. In 1987, Wilson *et al.* studied the effects of aging on DNA methylation when they measured the levels of 5mC in DNA from various tissues of laboratory mice and *Peromyscus leucopus*.²⁸ As shown in Figure 1, they observed a decrease in the 5mC content of DNA isolated from the brain, liver, and small intestine of mice. They also observed a decrease in the 5mC content of DNA from the liver and small intestine of *Peromyscus* as well as a decrease in 5mC content in DNA from bronchial epithelial cells isolated from human donors of various ages. Interestingly, they observed that the rate of 5mC decrease with age was lower in *Peromyscus*, which live about twice as long as laboratory mice, and was the lowest in humans. Thus, they argued that the rate of 5mC loss from the genome was inversely correlated to life span—the genomes of shorter-lived animals had higher rates of 5mC loss. Subsequently, several investigators used high-performance liquid chromatography (HPLC) to analyze the 5mC content of DNA hydrolysates from cells/tissues from mice and humans of various ages. These studies, which used UV light to detect and quantify 5mC, reported a decrease in DNA methylation (Table 1). However, the decrease reported by Fuke *et al.*³³ was very small. The fourth study, by Fasolino *et al.*³⁵ used HPLC coupled with tandem mass spectrometry to identify and quantify 5mC levels in brain, and they failed to observe any significant change in DNA methylation with age.

Over the past 5 years, investigators have used antibodies that specifically bind to 5mC sites in the genome to measure the effects of aging on DNA methylation. However, antibody-based detection of 5mC preferentially detects regions with few CG sites, and its reactivity with mCH is unclear.⁴⁹ In addition, antibody-based approaches only provide a relative quantification, and standards used in most 5mC enzyme-linked immunosorbent assays provide an inaccurate methylation level, as we have described previously.⁴² Five studies have used antibodies against 5mC to measure DNA methylation in the hippocampi of young and old mice and rats. The two studies with rats showed decrease in methylation with age, while the three studies with mice showed either an increase or no change in DNA methylation with age. Chouliaras *et al.*³⁶ also measured the effect of dietary restriction on DNA methylation in the hippocampus, because dietary restriction has been shown to increase life span by delaying/retarding aging. They reported that lifelong dietary restriction prevented the age-related increase in DNA methylation, which they observed in the hippocampi of mice. Armstrong *et al.*³⁷ and Brown-Borg *et al.*⁵⁰ measured DNA methylation in Ames dwarf mice, which live ~ 50% longer than wild-type littermates. The level of global DNA methylation in the Ames dwarf mice was similar to wild-type mice and did not change with age from 3 to 24 months of age.

More recently, investigators have begun using bisulfite treatment, which converts unmethylated cytosines to uracil but not methylated cytosines, and next-generation-sequencing to quantify specific cytosines that are methylated in the genome. In a highly cited paper using this method, Heyn *et al.*⁴⁷ reported that the genome of T cells from humans becomes hypomethylated with age. However, these data were generated with cord blood from one infant and blood from one centenarian. Recently, five groups, including ours, measured the effects of age on the 5mC content (percent of cytosines methylated) using next-generation sequencing/bisulfite, as shown in Table 1. Both Lister *et al.*⁴ and Hadad *et al.*⁴² measured 5mC in CpG and CpH sites, while Raddatz *et al.*,⁴⁰ Sun *et al.*,⁴¹ Cole *et al.*,⁴³ and Hahn *et al.*⁴⁴ measured 5mC at only CpG sites. Lister *et al.*⁴ found no change in global DNA methylation with age in the genome of the cortex from humans or male mice. Our group (Hadad *et al.*⁴²) also found no age-related change in 5mC in either CpG or CpH in the hippocampus from male and female mice. This observation was replicated in a subsequent experiment where we used high-depth whole-genome bisulfite sequencing to measure the 5mC content in ~ 20 million CpG sites and ~ 300 million CpH sites in DNA isolated from the hippocampi of male and female mice. As Figure 2 shows, the content of 5mC in both the CpG and CpH sites is similar in the hippocampus genomes of male and female mice and does not change with age. Raddatz *et al.*⁴⁰ reported no change with age in the 5mC content at CpG sites in the genome of human epidermis. Sun *et al.*⁴¹ also reported that DNA methylation at CpG sites was similar in hematopoietic stem cells from 4- and 24-month-old mice (83.5% versus 84.6% methylation, respectively). Recently, Cole *et al.*⁴³ and Hahn *et al.*⁴⁴ found no change with age in the methylation of CpG sites in the liver genome from male and female mice, respectively. They also found that dietary restriction had no effect on the global methylation of CpG sites in the liver. It should be noted that Cruickshanks *et al.*⁵¹ reported a slight decrease (~ 7%) in the methylation of CpG sites in genome of senescent human lung fibroblasts (IMR90) compared with proliferating fibroblasts using next-generation sequencing/bisulfite.⁵¹

In addition to the studies listed in Table 1 where DNA methylation was measured in the whole genome using next-generation sequencing/bisulfite, there are two studies using this technology where the 5mC content of specific regions and sites throughout the genome was measured as a function of age. Beerman *et al.*⁵² measured DNA methylation primarily in CpG islands and annotated gene promoters in hematopoietic stem cells from 3.5- and 25-month-old C57BL/6 male mice. They observed a slight but significant increase in global DNA methylation in these regions. More recently, our group measured 5mC content in ~ 120 Mb of regions across the hippocampus genome containing the most important gene regulatory regions (e.g., all gene promoters; all of the non-repeat region CpG islands, shores, and shelves; enhancers; and intragenic regions).⁵³ We found no significant change in the 5mC content across the ~ 120 Mb of the hippocampal genome of 3- and 24-month-old male and female C57BL/6 mice. However, there were major sex and age differences in DNA methylation at thousands of specific CpG and CpH sites (both increases and decreases in methylation).

Effect of age on enzymes involved in DNA methylation

Because one would predict that age-related changes in one or more of the enzymes involved in DNA methylation could have a global impact on DNA methylation, we have also reviewed the scientific literature in this area. Table 2 lists the published articles that have studied the effects of age on the expression (either mRNA or protein levels) of the DNMT and TET families. The studies measuring the levels of DNMT and TET proteins are somewhat limited by the poor specificity of most of the antibodies that are currently available.⁴² As can be seen from Table 2, there is currently no consensus on how the expression of any of the DNMT proteins change with age; some studies show an increase, some a decrease, and others no change with age. This variability does not appear to be due to differences between tissues, because many of the studies using the same tissue reported different results. The effect of age on the expression of the TET proteins is not quite as variable, with the studies showing either no change or a decrease in expression. All of the current studies on TET2 show no change with age.

As noted above, knocking out genes involved in DNA methylation is lethal; however, there is a study that has looked at the effect of reduced levels of DNMT1 on life span using heterozygous *Dnmt1* knockout mice (*Dnmt1*^{+/-}). The *Dnmt1*^{+/-} mice showed a 33% decrease in DNMT1 expression and no change in the expression of DNMT3a or DNMT3b; however, the life span of the *Dnmt1*^{+/-} mice similar to wild-type mice.⁶³

There has been only one published study that examined the effects of dietary restriction (DR) on some of the proteins involved in DNA methylation. Chouliaras *et al.*⁵⁹ reported that the age-related increase in the level of DNMT3a protein in the hippocampus was attenuated by DR. We recently studied the effects of aging and DR on the expression (transcript levels) of the DNMT and TET genes in colon mucosa isolated from mice. As shown in Figure 3, we observed an age-related decrease in the expression of *Dnmt1*, *Dnmt3b*, *Tet1*, and *Tet3*, but no change in the expression of *Dnmt3a1* or *Tet2*. Interestingly, DR prevented the age-related decline in the expression of all of the six genes we studied compared with old mice fed *ad libitum*; however, the increase was not statistically significant for *Tet2*. Armstrong *et al.*³⁷ measured the expression of the DNMT genes in male Ames dwarf and wild-type mice from 3 to 24 months of age. They observed that the transcripts for DNMT1 and DNMT3a were significantly higher in young dwarf mice compared with young wild-type mice; however, with age this difference disappeared.

Summary

A great deal of interest in the role of DNA methylation in aging was recently sparked by Horvath and others when they showed that DNA methylation at a few hundred CpG sites in the human genome was a quantitative biomarker of chronological age.^{64,65} Similar epigenetic clocks have been developed for 90–329 CpG sites in mice.^{46,66,67} These observations have led to the re-emergence of the hypomethylation hypothesis of aging. However, a critical examination of the current scientific literature calls into question this hypothesis, as shown in Table 1. There are currently 17 publications that have reported on the effects of aging on global DNA methylation in tissues from rodents and cells from

humans. Seven of the 17 studies reported a decrease in DNA methylation with age, while nine reported no change in global DNA methylation and one study reported a significant increase in methylation. The most likely explanation for the varied results is improved technology and increased sensitivity currently available to measure the 5mC content of DNA isolated from the tissues/cells. For example, all of the recent studies, which have used next-generation sequencing/bisulfite to measure the 5mC content of DNA, have consistently failed to observe significant age-related changes in global DNA methylation. These observations have been made in the cortex (Lister *et al.*⁴), hematopoietic stem cells (Lister *et al.*⁴), hippocampus (Hadad *et al.*⁴²), and liver (Cole *et al.*⁴³ Hahn *et al.*⁴⁴) from mice, and the cortex (Lister *et al.*⁴) and skin (Raddatz *et al.*⁴⁰) from humans. A review of the current literature with respect to the effect of age on the enzymes involved in DNA methylation also demonstrates no obvious overall effect of age on the expression of the DNMT and TET proteins, as shown in Table 2.

On the basis of the current literature, we conclude that a decrease in methylation is not a universal process that occurs in the genomes of all tissues/cells as an animal ages. While the studies reviewed above have been of methylation levels in tissues, the possibility exists that specific cells (e.g., certain cell types, or senescent cells) could be undergoing hypomethylation. With new methods to analyze single-cell methylation profiles, it is now possible to gain single-cell resolution.^{68,69} We re-analyzed the data from Gravina *et al.*⁷⁰ and examined genome-wide methylation levels from young and old hepatocytes and found no differences in genome-wide methylation levels in this specific cell type (Fig. 4). Thus, the current data do not support the hypothesis that a global, random loss of 5mC from the genome is a hallmark of aging and contributes to some of the phenotypes that occur with age. However, it is important to emphasize that all of the studies using next-generation sequencing/bisulfite that did not observe a change in global DNA methylation did observe age-related changes in DNA methylation in specific regions or at specific sites in the genome. For example, we observed major age differences in DNA methylation at thousands of specific CpG and CpH sites (both increases and decreases in methylation) in the ~ 120 Mb of the hippocampal genome analyzed, even though there was no change in the total 5mC content of the genome.⁵³ Raddatz *et al.*⁴⁰ found age-related changes in DNA methylation in promoter and enhancer regions of the genome of human epidermis that were correlated with changes in transcription, and Lister *et al.*⁴ also observed age-related changes in methylation in specific CpG and CpH sites in the cortex of mice and humans. Sun *et al.*⁴¹ observed a decrease in methylation with age in CpG islands and promoters and increases in CpG sites in gene bodies and repetitive elements at CpG sites in hematopoietic stem cells, even though global methylation of the genome of these cells did not occur with age. Both Cole *et al.*⁴³ and Hahn *et al.*⁴⁴ found hypo- and hypermethylation events occurring with age and DR as distinct regulatory features in the liver genomes of mice. even though the global content of 5mC did not change with either age or DR.

In conclusion, it is clear from the current literature that aging has a major effect on the patterns of methylation at specific sites in the genome; however, these changes in DNA methylation are not due to a general loss of methylation in the genome leading to the relaxation of gene expression with age and dysdifferentiation, as proposed 40 years ago by Nagy *et al.*²⁹ Rather, the changes in DNA methylation appear to occur at specific CpG and

CpH sites and regions in the genome, indicating that the changes in methylation that occur with age are regulated and may be functionally important.^{4,42,53} Future studies examining methylation changes with aging will need to utilize these new methodologies to fully describe the patterns of differential methylation across the genome with aging. These patterns, including CpH methylation and other cytosine modifications (5hmC, 5-formylcytosine, and 5-carboxylcytosine) need to be fully described and integrated with chromatin/histone marks to understand the network of epigenomic changes occurring with aging. Through these types of systems analyses, the role of the epigenome in altered gene expression with aging can be understood and interventional approaches to maintain a youthful epigenome tested.

Materials and methods

Animals

Male C57BL/6 mice were obtained from the NIA aging and caloric-restricted colony at 9 and 24 months of age, housed on arrival in the animal facility at the University of Oklahoma Health Sciences Center, and maintained under SPF conditions in a HEPA barrier environment until sacrifice. The animals were fed irradiated NIH-31 mouse/rat diet from Teklad (Envigo, Madison, WI). Both DR and AL mice were individually housed. DR was initiated at 14 weeks of age at 10% restriction and increased to 25% restriction at 15 weeks and to 40% restriction at 16 weeks, where it is maintained throughout the life of the animal. Mice were then sacrificed and colon mucosa was harvested, snap frozen in liquid nitrogen, and stored at $-80 \pm C$ until used. For the methylation data, 3- and 24-month-old male and female C57BL/6 mice were obtained from the NIA aging colony. All animal experiments were performed according to protocols approved by the OUHSC Institutional Animal Care and Use Committee.

Gene expression

The levels of mRNA transcripts of genes involved in DNA methylation (*Dnmt1*, *Dnmt3a1*, *Dnmt3b*, *Tet1*, *Tet2*, and *Tet3*) were measured in the individual samples ($n = 5/\text{group}$) with three technical replicates using real-time PCR. Briefly, RNA was isolated using RNeasy RNA isolation kit from Qiagen (Germantown, MD, USA). The first strand cDNA was synthesized from 1 μg RNA using random primers (Promega, Madison, WI) and purified using the QIAquick PCR purification kit (Qiagen, Germantown, MD). Expression of the candidate genes were quantified using real-time PCR with pre-designed Taqman probes (Thermo Fisher, Waltham, MA). Relative gene expression was quantified as comparative ct analysis using the $2^{-\text{ct}}$ analysis method with β -actin as endogenous control. One-way ANOVA design with Tukey's multiple test correction was used to statistically analyze individual samples.

Whole-genome methylation analysis

Whole-genome bisulfite sequencing was performed as previously described in Hadad *et al.*⁴² in the hippocampi of 3- and 24-month-old animals. In this instance, bisulfite conversion and library construction were performed according to the manufacturer's protocol (Accel-NGS Methyl-Seq, Swift Biosciences, Ann Arbor, MI). Libraries were sequenced (HiSeq 2500) to

an average coverage depth of $> 10\times$ across the entire genome. Alignment and quantitation was as previously described by Hadad *et al.*⁴² Quantitation at each CG and CH site covered at $10\times$ was then computed, and an average level of methylation across the genome for each mouse was calculated.

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Figure 1.
Effect of age on DNA methylation in mouse tissues. Data for male C57BL/6 mice taken from Ref. 28.

Figure 2.

Effect of age on the global methylation of the hippocampus genome. The 5mC content in CpG and CpH sites was measured in hippocampal DNA from young (3 months) and old (24 months) male (blue) and female (red) C57BL/6 mice by whole-genome bisulfite sequencing. Each value represents the mean $\pm$ SEM of three mice per group. There was no significant difference with age or sex in global DNA methylation at either the CpG or CpH sites.

Figure 3. Effect of age and dietary restriction on the expression of genes involved in DNA methylation. The levels of the various transcripts were measured by qPCR for colon mucosa isolated from young (9 months, light blue) and old (24 months, dark blue) mice fed *ad libitum* and old (24 months, red) DR mice. Each bar represents the mean $\pm$ SEM of five mice per group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 4. Effect of age on the genome-wide methylation profiles of single hepatocytes. Analysis done on the data repostited by Gravina et al.⁷⁰

Table 1

Effect of age on global methylation of the genome

Changes in methylation	Animal model	Tissue or cell type	Age	References
Thin-layer chromatography				
Decrease	Rat–albino	Brain, heart, spleen	1–28 months	26
No change	Rat–albino	Liver, lung, kidney	1–28 months	26
Decrease	Mouse–C57BL/6	Brain, liver, intestine	2–35 months	27
Decrease	<i>Peromyscus</i>	Liver, intestine	2–60 months	27
Decrease	Human	Lung fibroblasts	Various ages	27
High-pressure liquid chromatography				
Decrease	Mouse–C57BL/6	Liver	6 & 31 months	30
Decrease	Human	Leukocytes	4–94 years	31
Decrease	Mouse–C57BL/6	Hippocampus	7–19 months	32
No change	Mouse–C57BL/6	Striatum, substantia nigra	3–18 months	33
Antibody based				
Increase	Mouse–C57BL/6	Hippocampus	12 & 24 months	34
No change	Mouse–C57BL/6	Hippocampus	2–24 months	20
No change	Mouse–unknown	Liver	3–24 months	35
Decrease	Rat–SD	Hippocampus	2–30 months	36
Decrease	Rat–SD	Hippocampus	3–30 months	37
Next-generation sequencing				
No change	Human	Cortex	16–64 years	4
No change	Mouse–C57BL/6	Cortex	1.5–22 months	4
No change	Human	Skin–epidermis	~ 22 & ~ 72 years	38
No change	Mouse–C57BL/6	Hematopoietic stem cells	4 & 24 months	39
No change	Mouse–C57BL/6	Hippocampus	3–24 months	40
No change	Mouse–UMHET3	Liver	2 & 22 months	41
No change	Mouse–C3B6F1	Liver	5 & 27 months	42

Table 2

Effects of age on enzymes involved in DNA methylation

Species	Sex	Age ^d (months/ years)	Tissue	Level of expression	Reference
<i>Dnmt1</i>					
Mouse (C57BL/6)	M	3 – 18	Hippocampus	nc-mRNA	52
Rat (Wistar)	M	3 – 20	Hippocampus	↑-protein	53
Mouse (Swiss albino)	M	2.5 – 20	Hippocampus & Cortex	nc-mRNA ↓-Protein	54
Mouse (C57BL/6)	M	4–24	BM-HSCs	↓-mRNA	39
Rat (Sprague-Dawley)	?	3 – 30	Hippocampus	↓-Protein	37
Humans	M/F	35 – 75	PMCs	↓-mRNA	55
Mouse (C57BL/6)	M	3–18	Striatum	↑-mRNA	33
Mouse (C57BL/6)	M	3–18	Substantia Nigra	nc-mRNA	33
Mouse (C57BL/6)	M/F	3–24	Hippocampus	nc-mRNA	40
Mouse	?	3–24	Liver	nc-mRNA	35
<i>Dnmt3a</i>					
Humans	M/F	16 – 78	Liver	↑-mRNA	56
Mouse (C57BL/6)	M	12 – 24	Hippocampus	↑-protein	57
Mouse (C57BL/6)	M	3 – 18	Hippocampus	↓-mRNA	52
Mouse (Swiss albino)	M	2.5 – 20	Hippocampus & Cortex	nc-mRNA nc-protein	54
Mouse (C57BL/6)	M	4–24	BM-HSCs	↓-mRNA	39
Rat (Sprague-Dawley)	?	3 – 30	Hippocampus	↓-protein	37
Mouse (C57BL/6)	M	3 – 18	Striatum	↑-mRNA	33
Mouse (C57BL/6)	M	3 – 18	Substantia Nigra	nc-mRNA	33
Mouse (C57BL/6)	M/F	3 – 24	Hippocampus	nc-mRNA	40 ^c
Mouse	?	3–24	Liver	nc-mRNA	35
<i>Dnmt3b</i>					
Humans	M/F	16–78	Liver	↑-mRNA	56
Mouse (C57BL/6)	M	3–18	Hippocampus	nc-mRNA	52

Species	Sex	Age ^a (months/ years)	Tissue	Level of expression	Reference
Rat	M	3–20	Hippocampus	nc-protein	53
Mouse (Swiss albino)	M	2.5–20	Hippocampus & Cortex	nc-mRNA nc-protein	54
Mouse (C57BL/6)	M	4–24	BM-HSCs	↓ mRNA	39
Humans	M/F	35–75	Blood-PMCs	↓ mRNA	55
Mouse (C57BL/6)	M/F	3–24	Hippocampus	nc-mRNA	40
Mouse	?	3–24	Liver	nc-mRNA	35
<i>Tet1</i>					
Mouse (C57BL/6)	M	2–24	Hippocampus	nc-mRNA	20
Mouse (C57BL/6)	M	4–24	BM-HSCs	↓ mRNA	39
Mouse (C57BL/6)	?	3–30	Hippocampus	↓ mRNA	58
Humans	?	20–83	Blood-T cells	↓ mRNA	59
Humans	M/F	34–74	Blood-MNCs	↓ mRNA	60
Mouse (C57BL/6)	M/F	3–24	Hippocampus	nc-mRNA	40
<i>Tet2</i>					
Mouse (C57BL/6)	M	2–24	Hippocampus	nc-mRNA	20
Humans	?	20–83	Blood-T cells	nc-mRNA	59
Mouse (C57BL/6)	M	3–18	Striatum	nc-mRNA	33
Mouse (C57BL/6)	M	3–18	Substantia Nigra	nc-mRNA	33
Humans	M/F	34–74	Blood-MNCs	nc-mRNA	60
Mouse (C57BL/6)	M/F	3–24	Hippocampus	nc-mRNA	40
<i>Tet3</i>					
Mouse (C57BL/6)	M	2–24	Hippocampus	nc-mRNA	20
Mouse (C57BL/6)	M	4–24	BM-HSCs	↓ mRNA	39
Humans	?	20–83	T cells	↓ mRNA	59
Humans	M/F	34–74	Blood-MNCs	↓ mRNA	60
Mouse (C57BL/6)	M	3–18	Striatum	nc-mRNA	33
Mouse (C57BL/6)	M	3–18	Substantia nigra	nc-mRNA	33
Mouse (C57BL/6)	M/F	3–24	Hippocampus	nc-mRNA	40

Hadad *et al.* measured both *Dnmt3a1* and *Dnmt3a2*; however, *Dnmt3a2* was undetectable by qPCR.

Article did not give sex of animals studied

Age of mice in months, age of humans in years

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