



The role of DNA methylation in epigenetics of aging

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ABSTRACT

Recent research suggests that epigenetics, especially DNA methylation, plays a mechanistic role in aging. Epigenetic clocks, which measure changes in a few hundred specific CpG sites, can accurately predict chronological age in a variety of species, including humans. These clocks are currently the best biomarkers for predicting mortality in humans. Additionally, several studies have characterized the effects of aging across the methylome in a wide variety of tissues from humans and mice. A small fraction (~2%) of the CpG sites show age-related changes, either hypermethylation or hypomethylation with aging. Evaluation of non-CpG site methylation has only been examined in a few studies, with about ~0.5% of these sites showing a change with age. Therefore, while only a small fraction of cytosines in the genome show changes in DNA methylation with age, this represents 2 to 3 million cytosines in the genome. Importantly, the only study to compare the effect of aging on DNA methylation in male and female mice and humans found that >95% of the age-related changes in DNA methylation in the hippocampus were sexually divergent, i.e., the methylation did not differ between males and females at young age but age-related changes occurred in one sex but not the other. The age-related changes in DNA methylation tend to be enriched and under-represented in specific genomic contexts, with some commonalities between tissues and species that require further investigation. The strongest evidence that the age-related changes in DNA methylation play a role in aging comes from studies of anti-aging interventions (e.g., caloric restriction, dwarfism, and rapamycin treatment) in mice. These anti-aging interventions decelerate the epigenetic clocks and reverse/prevent 20 to 40% of the age-related changes in DNA methylation. It will be important in the future to demonstrate that at least some of the age-related changes in DNA methylation directly lead to alterations in the transcriptome of cells/tissues that could potentially contribute to aging.

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Abbreviations: 5hmC, 5-hydroxymethyl cytosine; 5mC, 5-methyl cytosine; DNMT, DNA methyl transferase; Tet, Ten eleven translocation; WGBS, Whole Genome Bisulfite Sequencing.

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1. Introduction

Epigenetics is often defined as functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, e.g., DNA methylation or histone modification. These changes in DNA methylation and/or histone modification alter the availability of DNA to the binding of proteins that can either induce or repress gene transcription. All cytosines [CpGs and CpHs (H = A, T, C)] in DNA can potentially become methylated; however, 5-methyl cytosine (5mC) is enriched in CpG dinucleotides. In addition, these palindromic sites have the unique potential for methylation occurring on both strands of DNA, which can then be copied during cell replication from the parent to daughter strand. Therefore, most of the attention on DNA methylation has focused on CpG sites. However, there are more CpH sites in the genome than CpG sites, and CpH sites have been found to be associated with some cellular outcomes (He & Ecker, 2015). Functionally, 5mC can bidirectionally regulate chromatin status, e.g., methylated DNA co-localizes with heterochromatin (Mellen, Ayata, Dewell, Kriaucionis, & Heintz, 2012) and can directly affect the ability of transcription factors and other DNA binding proteins to access DNA. In the context of gene promoters, hypomethylated CpGs are generally associated with active, constitutively-expressed genes, while hypermethylated CpGs are associated with genes that are silenced or have low expression (Bird, 2002).

Fig. 1A shows the pathway involved in the methylation and demethylation of DNA. Cytosine methylation is catalyzed by three independently encoded DNA methyl transferases (DNMTs), which use S-adenosyl methionine as the methyl donor: DNMT1, DNMT3a and DNMT3b. DNMT1 is generally considered a maintenance methylase, because it methylates only hemi-methylated DNA at CpG sites (Groth, Rocha, Verreault, & Almouzni, 2007; Li, Bestor, & Jaenisch, 1992). Thus, DNMT1 plays an important role in the heritability of epigenetic information (Breiling & Lyko, 2015). In contrast to DNMT1, DNMT3a and DNMT3b are de novo methyl transferases, i.e., they are capable of recognizing and methylating both hemi-methylated and unmethylated DNA (Lim et al., 2010; Okano, Bell, Haber, & Li, 1999). These two methylases are also responsible for DNA methylation that occurs at CpH sites. While transcription factors and histones are known to direct DNMTs to specific genomic regions, these mechanisms are poorly understood (Marchal & Miotto, 2015). Knocking out any of these three DNMTs in mice is embryonically lethal because DNA methylation patterns are essential in embryonic development, (Li et al., 1992; Okano et al., 1999). Our understanding of DNA methylation has evolved from one in which

methylation patterns are set during development and generally immutable, to a broader perspective where DNA methylation is dynamically regulated in the cell (Smith & Meissner, 2013).

The 5mC residues in the genome can be lost spontaneously when the cytosines are not methylated during replication, from the deamination of 5mC to thymine, or from the repair of regions of the genome that contained 5mCs. In addition, the demethylation of 5mC sites can occur through an active pathway where the methyl group on cytosine is first modified and then removed and replaced by DNA repair as shown in Fig. 1A. Ten-eleven translocation (TET) proteins (also termed 5-mC dioxygenases) initiate the specific demethylation of 5mC residues in DNA. The TET family of proteins includes three proteins: TET 1, TET 2, and TET 3. All three TETs have similar activity and catalyze similar reactions (Ito et al., 2010). The three TET proteins catalyze the oxidation of the methyl group on cytosine to 5-hydroxymethyl cytosine, 5hmC (Tan & Shi, 2012). Because the increase in 5hmC levels coincides with the reduction of 5mC (Iqbal, Jin, Pfeifer, & Szabo, 2011; Wossidlo et al., 2011), the formation of 5hmC is believed to be the first step in the active demethylation of 5mC residues in DNA. However, it is now becoming apparent that 5hmC might be important in the epigenetic regulation of gene expression in its own right (Chen, Dzitoyeva, & Manev, 2012), especially in stem cells (Cheng, Bernstein, Chen, & Jin, 2015).

The TET proteins are also involved in the oxidation of 5hmC to 5-formylcytosine and 5-carboxylcytosine. In turn the 5-formylcytosine and 5-carboxylcytosine are directly excised and repaired by the base excision repair pathway (Pastor, Aravind, & Rao, 2013). 5hmC can also be deaminated by activation-induced cytosine deaminase to 5-hydroxymethyluracil; therefore, 5hmC is also removed and replaced with a non-methylated cytosine by the base excision repair pathway (Guo, Su, Zhong, Ming, & Song, 2011). Because changes in one or more of the enzymes involved in DNA methylation could have a global impact on DNA methylation, several groups have studied the effect of age on the expression of DNMTs and TETs. In a recent review, we showed there is currently no consensus on how the expression of any of the DNMTs or TETs changed with age; some studies show an increase, some a decrease, and others no change with age (Unnikrishnan et al., 2018).

The expression of the DNMTs and TETs vary greatly from tissue to tissue, and for example the TET proteins are generally highly expressed in brain (Ito et al., 2010; Khare et al., 2012). As shown in Fig. 1B, the relative levels of the the DNMTs and TETs differs greatly in the various cell types found in the central nervous system. The functional implications

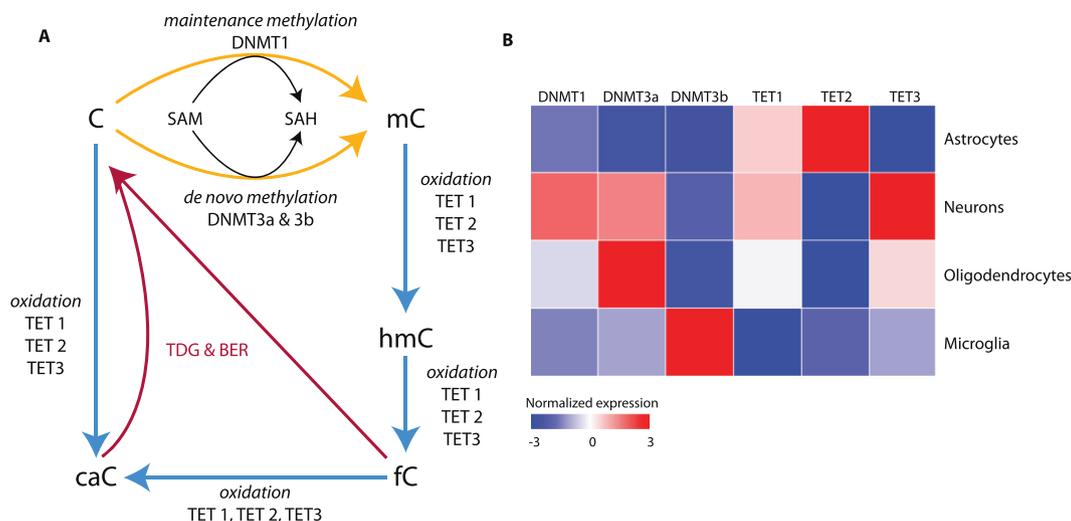


Fig. 1. The pathway for DNA methylation. A) The steps involved in the formation and elimination of 5-methyl cytosine (mC) in DNA is shown. Abbreviations: SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; hmC, 5-hydroxymethyl cytosine; fC, 5-formyl cytosine; caC, 5-carboxyl cytosine; BER, base excision repair; TDG, thymidine DNA glycosylase. B) Expression of genes involved in methylation varies between cell types and tissues. For example, different cell types in brain express various levels of the mRNA transcripts for DNMTs and TETs. Data taken from Zhang et al. (2014).

of these different patterns of expression of DNMTs and TETs remains to be determined.

The first evidence that aging might have an effect on DNA methylation came from studies using high-performance liquid chromatography (HPLC) to measure the 5mC content of hydrolyzed DNA. In 1973, Vanyushin et al. reported that the total levels of 5mC in DNA isolated from various tissues of rats decreased with age. Subsequently, Wilson, Smith, Ma, and Cutler (1987) reported that the levels of 5mC decreased with age in DNA isolated from various tissues or cells from mice, *Peromyscus*, and humans; this decrease was greatest in the shorter-lived mice. These reports lead to the hypomethylation hypothesis of aging, which proposed that the spontaneous, random loss of 5mCs with age leads to the abnormal expression of genes in cells/tissues of old animal that are normally repressed (Zs-Nagy, Cutler, & Semsei, 1988). However, investigators have not been able to demonstrate a relaxation in gene regulation with age. In addition, most of the current research reports no change in the total 5mC content of the genome occurs with increasing age (see review by Unnikrishnan et al., 2018).

Over the past three decades several assays for measuring DNA methylation have been developed, and Masser et al. (2018) have described the advantages and disadvantages of these assays. For example, antibodies have been generated that are specific for mC or hmC, which allow investigators to measure global levels of methylation in a DNA sample or in various tissues by immunochromatography. Using these antibodies combined with next-generation-sequencing assays such as methylated DNA immunoprecipitation (meDIP-Seq) (Weber et al., 2005), and methyl-binding domain sequencing (MBD-Seq) (Rauch & Pfeifer, 2005), assays have been developed that can identify the regions of the genome where changes in DNA methylation occur. However, these affinity-based approaches only give one a general view of what regions of the genome show a change in methylation; changes in the methylation at specific sites cannot be measured with these assays.

A major development in studying DNA methylation occurred when it was shown that treating DNA with bisulfite, which specifically deaminates unmethylated cytosine to uracil but not methylated cytosines, combined with DNA sequencing allowed investigators to measure DNA methylation in specific cytosine residues (Clark, Harrison, Paul, & Frommer, 1994; Frommer et al., 1992). Using bisulfite conversion, investigators can measure changes in DNA methylation at specific sites throughout the genome using either oligonucleotide-arrays or next-generation-sequencing. The current oligonucleotide-arrays commercially available allow investigators to measure DNA methylation at <2% of the CpG sites in the human genome. On the other hand, next-generation-sequencing theoretically allows investigators to measure methylation at all cytosines, both CpG and CpH sites, in any genome. Although the global amount of 5mC in a genome does not appear to change significantly with age (Unnikrishnan et al., 2018), it is becoming clear that DNA methylation at specific sites in the genome changes with age; they can become either hypo- or hypermethylated. Over the past five years, several reviews on the effect of age on DNA methylation have appeared (Horvath & Raj, 2018; Johnson et al., 2012; Jung & Pfeifer, 2015; Zampieri et al., 2015). In this review, we focus on the effect of age and anti-aging interventions on the methylation of cytosine residues in specific regions or at specific sites in the genome of humans and laboratory rodents. With the development of deep sequencing,

methylation of adenine (N^6 -methyl-adenine, 6 mA) in DNA has been observed, albeit at very low levels, in a variety of eukaryotes including humans (Xiao et al., 2018). While 6 mA has been recognized as an epigenetic signal in microorganisms (Wion & Casadesus, 2006), its role in eukaryotes is poorly understood; therefore, we have focused our review on cytosine methylation and hydroxymethylation.

2. Epigenetic clocks

With the availability of oligonucleotide-arrays to specific CpG sites in the human genome, investigators have studied how DNA methylation changes at specific CpGs in various age-related diseases and with aging. Reports by Bjornsson et al. (2008), Christensen et al. (2009), and Teschendorff et al. (2010) were some of the first to show that methylation at specific loci across the human genome changed with age. The report by Bocklandt et al. (2011) showing that 88 CpG sites in DNA from saliva were highly correlated with age stimulated the search for CpG sites that would be a biomarker of chronological age. Building from these initial observations, investigators used supervised machine learning methods (e.g., elastic net) on datasets containing both DNA methylation data and chronological age information to 'learn' which CpG sites in the human genome predict chronological age with high accuracy. The predictive accuracy of the trained models in estimating chronological age were then validated in independent cohorts of subjects of various ages. Using this methodology, several epigenetic clocks have been developed that predict chronological age based the rate of change in methylation in 3 to 513 CpG sites in DNA from a tissue or blood.

2.1. Human epigenetic clocks

Currently, four clocks have been developed to measure chronological age in tissues from humans, and they are described in Table 1. The most widely used epigenetic clock was developed by Horvath (2013). The Horvath clock used DNA methylation data obtained from publicly available datasets for various human tissues and cell types. Using 353 CpG sites, the Horvath clock predicts chronological age with a mean absolute deviance of 3.6 years. Interestingly, while many of the CpG sites individually show only a weak correlation with age, this subset of CpG sites predicts chronological age with great accuracy. Hannum et al. (2013) also developed an epigenetic clock using DNA methylation data from blood. The Hannum clock model is based on 71 CpG sites and when applied to an independent cohort shows high age correlation of with a mean absolute deviance of 4.9 years. Even though the Horvath and Hannum clocks are robust measures of chronological age (see review by Horvath & Raj, 2018) only 5 CpG sites are shared between the clocks. The predictive accuracy of the Hannum clock, which was developed using blood samples, declines when applied to other tissues. In contrast, the Horvath clock performs well with a variety of tissues; however, the accuracy of the Horvath clock in predicting age is less for skeletal muscle, heart, and breast (Horvath, 2013).

In 2014, Weidner et al. described an epigenetic clock derived from blood that could predict age using just 3 CpG sites. For site selection the authors first reduced their dataset to 102 CpG sites that were highly correlated with age (mean absolute deviance = 3.3 years), and from

Table 1
List of epigenetic clocks developed for humans.

Epigenetic clock	Platform used	Tissues used in training	CpG Sites identified	Accuracy in predicting age
Horvath Clock Horvath (2013)	27 k & 450 K arrays	Blood, brain, breast, buccal cells, colon, heart, liver, lung, placenta, saliva, CD4 cells, immortalized B cells, adipose, kidney, muscle, uterine tissue	353	$r^2 = 0.96$
Hannun Clock Hannun et al. (2013)	27 k & 450 K arrays	Blood	71	$r^2 = 0.91$
Weidner Clock Weidner et al. (2014)	27 k & 450 K arrays	Blood	3	$r^2 = 0.98$
PhenoAge Clock Levine et al. (2018)	27 k, 450 K, & EPIC arrays	Blood	513	$r^2 = 0.92$

Table 2
List of epigenetic clocks developed for animals.

Epigenetic Clock	Species	Platform Used	Tissues Used in Training	CpG Sites Identified	Accuracy in Predicting Age
Thompson et al. (2017)	Dogs & wolves	RRBS	Blood	115	$r^2 = 0.79$
Petkovic et al. (2017)	Mice (C57BL/6)	RRBS	Blood	90	$r^2 = 0.90$
Stubbs et al. (2017)	Mice (C57BL/6)	RRBS	Liver, lung, muscle, spleen, cortex, & cerebellum	329	$r^2 = 0.84$
Wang et al. (2017)	Mice (Ames wild type)	RRBS	Liver	148	$r^2 = 0.91$

these sites, 3 sites located on the ITGA2B, ASPA and PDE4C genes were able to predict chronological age in human blood samples.

Can the epigenetic clocks also predict biological age, i.e., the decline in function of an animal, as well as chronological age? Over the past 5 years, several reports suggest that the Horvath and Hannum clocks might be useful in measuring biological age in human tissues/cells, for example:

- Several age-related conditions are linked to epigenetic age acceleration, e.g., Alzheimer's disease, cancer, cardiovascular disease, and Huntington's disease (for a review see Horvath & Raj, 2018). However, the Horvath clock was not associated with cognitive decline in monozygotic twins (Starnawska et al., 2017).
- Frailty (Breitling et al., 2016) and frailty outcomes such as grip strength (Sillanpaa et al., 2018) were associated with epigenetic age acceleration; however, standing balance and chair-rise time were not correlated to accelerated epigenetic aging (Simpkin, Suderman, & Howe, 2017).
- Accelerated epigenetic aging is observed in individuals with Werner's syndrome who exhibit many signs of accelerated aging (Maierhofer et al., 2017); however, decelerated aging observed in Syndrome X patients is not correlated to changes in the epigenetic clock (Walker et al., 2015).
- HIV related changes that mimic accelerated aging are correlated with accelerated epigenetic aging (Horvath & Levine, 2015; Levine et al., 2016; Horvath et al., 2018). In addition, measures of obesity such as BMI and waist circumference are shown to be associated with accelerated epigenetic aging (Grant et al., 2017; Horvath et al., 2014). However, such a correlation was not observed in other studies (Nevalainen et al., 2017; Sillanpaa et al., 2018), and Grant et al. (2017) did not find a significant correlation to their 7 diabetes-related phenotypes.
- The epigenetic clocks predict all-cause mortality in a variety of human cohorts (Chen et al., 2016; Christensen et al., 2016; Perna et al., 2016; Levine et al., 2018; Marioni et al., 2018). However, Kim, Myers, Wyckoff, Cherry, and Jazwinski (2017) found that the Horvath clock did not predict mortality in blood from a cohort of 262 subjects ranging in age from 60 to 103.

Because the Horvath, Hannum, and Weidner clocks were trained primarily on chronological age, Horvath's group set out to develop an

epigenetic clock that would better predict biological aging by incorporating clinical measures (e.g., levels of albumin, creatinine, glucose, C-reactive protein, etc.) in addition to chronological age to capture changes in healthspan. The resulting PhenoAge clock described in Table 1 uses 513 CpG sites (Levine et al., 2018). Forty-one of the CpG sites were shared with the Horvath clock and 6 shared with the Hannun clock. The PhenoAge is highly correlated with chronological age ($r = 0.94$) and outperforms earlier iteration of the epigenetic clock in prediction of disease risk and mortality using various tissues and cells.

2.2. Animal epigenetic clocks

Due to the potential utility of the epigenetic clock in predicting accelerated aging in humans, investigators began developing epigenetic clocks to predict chronological age in other animal species. In 2017, four epigenetic clocks were developed for mice and dogs/wolves (Table 2). In contrast to the human clocks, which used oligonucleotide-arrays to identify subsets of CpG sites that predicted chronological age, these clocks were developed using reduced representation bisulfite sequencing (RRBS), which analyzes a greater number of CpG sites (< 1 million) compared to the array-based methods. Thompson, Vonholdt, Horvath, and Pellegrini (2017) used DNA from the blood of domestic dogs and gray wolves to generate an epigenetic clock of 115 CpG sites (41 CpGs for dogs and 67 CpGs for wolves). When comparing the syntenic locations of the 115 CpG sites to CpG sites generated for human clocks, they found there was a statistically significant (but small) conservation of sites, which is striking because the studies used different platforms to measure DNA methylation and the relatively small number of animals used by Thompson et al. (2017).

The epigenetic clocks for mice are of particular interest to the aging community because mice are the most studied mammalian model with respect to the biological pathways and processes involved in aging. Thus, epigenetic clocks for mice have the potential to provide new information on how changes in DNA methylation might impact aging. The three epigenetic clocks developed for mice were generated with tissues from male C57BL/6 mice (Table 3). The three clocks used the following number of CpG sites: 90 (Petkovich et al., 2017), 329 (Stubbs et al., 2017), and 148 (Wang et al., 2017). These CpG sites were distributed across the mouse genome. The Petkovich and Stubbs clocks predicted the chronological age in both male and female C57BL/6 mice. The

Table 3
The effect of age on methylation of specific CpG sites in the genome.

Age	Sex	Tissue/cell type	Method	CpG sites				References
				Total Sites Studied	Total changed	Hyper-methylated	Hypo-methylated	
Humans^a								
19–101	M & F	Blood	Array	485,577	70,387 (14.5%)	–	–	Hannum et al., 2013
34–61	M	PBMC	Array	463,228	10,625 (2.3%)	7081	3544	Steegega et al., 2014
28–64	M & F	Liver	Array	455,526	20,396 (4.4%)	19,548	848	Bysani et al., 2017
Mice^b								
4 & 24	M (C57Bl/6)	HSC	WGBS	~20 million	448,166 (2.2%)	275,622	172,543	Sun et al., 2014
3 & 24	M (C57Bl/6)	Hippocampus	BOCS	~3 million	36,170 (1.2%)	21,295	14,294	Masser et al., 2017
3 & 24	F (C57Bl/6)	Hippocampus	BOCS	~3 million	48,410 (1.6%)	27,987	19,842	Masser et al., 2017
5 & 27	F (C3B6F1)	Liver	BS-Seq	~29 million	79,400 (0.3%)	48,625	30,775	Hahn et al., 2017
2 & 22	M (UM-Het3)	Liver	WGBS	~21 million	49,899 *	14,365*	35,534*	Cole et al., 2017
3 & 24	M (C57Bl/6)	Hippocampus	BOCS	~2 million	41,585 (2.0%)	26,180	15,425	Hadad et al., 2018

M & F – samples from males and females combined for data generation; ^ahuman age in years; ^bmouse age in months; WGBS - Whole Genome Bisulfite Sequencing; BOCS - Bisulfite oligonucleotide Capture Sequencing; BS-Seq - Bisulfite Sequencing; Array- Illumina BeadChip; * - numbers represent differential methylated regions and not CpG sites.

Petkovich clock also accurately predicted the chronological age of B6D2F₁ mice, and the Wang clock predicted chronological age in Ames wild type mice and UM-HET3 mice. None of the CpG sites identified by Petkovich et al. (2017) matched the CpG sites used in either the Horvath and Hannum clocks.

A major advantage of studying mice is that several interventions have been shown to increase the lifespan and delay aging in mice (Unnikrishnan et al., 2018). Therefore, it is possible to determine if the mouse clocks able to predict reduced aging in these models. Petkovich et al. (2017) studied the mice fed a calorie-restricted (CR) diet, Snell dwarf mice, and growth hormone receptor knockout mice (GHR-KO). All of these manipulations increase lifespan, improve most physiological functions, and reduce most pathological lesions (for review see Unnikrishnan et al., 2017); therefore, it is well accepted that aging is delayed/retarded in these mice, i.e., they are biologically younger than their chronological age. Petkovich et al. (2017) found that the epigenetic age of mice fed a CR diet was 20% lower than the chronological age. The epigenetic age of the Snell dwarf mice and the GHR-KO dwarf mice was also lower than the chronological age of their littermate controls. Wang et al. (2017) also studied the effect of dwarfism and CR on their clock as well as rapamycin, which has been shown to increase the lifespan of a variety of mouse models (Richardson, Galvan, Lin, & Oddo, 2015) the epigenetic age was significantly less for all three of these mouse models of delayed aging. At 22 months of age, the Ames dwarf mice, CR mice, and rapamycin treated mice showed an average reduction in epigenetic age of 10.1, 9.4, and 6 months, respectively compared to their age-matched controls. Thus, these data suggest that the clocks developed by Petkovich et al. (2017) and Wang et al. (2017) appear to not only measure chronological age, but are a reflection of biological age. Stubbs et al. (2017) reported that ovariectomy accelerated their clock in female mice, and they suggested that this was due to accelerated aging. However, the effect of ovariectomy on aging in rodents is uncertain. For example, while Asdell, Doornenbal, Joshi, and Sperling (1967) reported that ovariectomy reduced the lifespan of rats, Iwasa et al. (2017) found that ovariectomy increased the lifespan of female rats.

Recently, Frobel et al. (2018) reported an interesting use of the epigenetic clock in determining the effect of the tissue micro-environment on the cellular aging of human hematopoietic stem cells. They used the Horvath and Hannum clocks to determine whether human stem cells transplanted into mice showed an increased rate of epigenetic aging because of the accelerated aging environment of the mouse. Nineteen weeks after transplanting the human stem cells into mice, they found that epigenetic aging was only modestly increased in mice, leading the authors to conclude that the accelerated aging xenogenetic environment of the mouse had minimal effect on the epigenetic clock of the human stem cells.

2.3. Summary

In less than five years, epigenetic clocks have become a well-accepted, robust measure of chronological age in humans, dogs/wolves, and mice that works effectively in both males and females. In addition, Ito, Udono, Hirata, and Inoue-Murayama (2018) recently reported that methylation of CpG sites within three genes predicted age in chimpanzees. It is clear from the current studies that there is no 'one' unique clock; rather, methylation at various combinations of CpG sites are able to predict chronological age. In addition, epigenetic clocks appear to be a more accurate measure of chronological age than other biomarkers that have been developed in the past. For example, telomere length has been the most used biomarker of aging in the past, with telomere length predicting mortality better than age (Bize, Crisculo, Metcalfe, Nasir, & Monaghan, 2009). However, telomere length's actual age prediction is very poor ($r^2 = 0.14$) compared to the epigenetic clocks.

While epigenetic clocks have been highly accurate in predicting chronological age, it is research is now focused on whether these clocks

can accurately measure biological age, i.e., the functional status of an individual. As discussed above, a large number of studies show that accelerated epigenetic aging is associated with various age-related diseases and conditions associated with aging. In addition, the clocks appear to predict mortality in humans. However, there are a few reports showing no significant correlation of the clock to conditions that are associated with aging. For example, Quach et al. (2017) reported that metformin treatment, which is used to treat type 2 diabetes and has some anti-aging properties (Martin-Montalvo et al., 2013) does not delay epigenetic aging, and heroin use results in younger epigenetic age (Kozlenkov et al., 2017). Currently, strongest evidence that the epigenetic clock can predict biological age comes from studies by Petkovich et al. (2017) and Wang et al. (2017), which showed that interventions that increased lifespan and delayed aging in mice decelerated epigenetic aging. Thus, epigenetic clocks are currently the best biomarker for predicting chronological age. In addition, these clocks appear to predict biological aging, which makes them the only biomarker available to investigators at the present time for assessing biological aging.

3. Effect of age on DNA methylation

3.1. Effect of Age on changes in 5mC at specific Sites in the Genome

The reports by Horvath (2013) and Hannum et al. (2013) showing that changes in a few hundred CpG sites were able to predict chronological age sparked a great deal of interest in the role DNA methylation might play in aging. Because the epigenetic clocks use only a small fraction of the ~40 million CpG sites and none of the ~430 million CpH sites in the genome to measure age, the epigenetic clocks are of limited value in understanding the mechanisms by which DNA methylation may regulate the genome and aging. With the ability to measure methylation at specific cytosines throughout the genome, investigators are now beginning to gain insight into how DNA methylation throughout the genome changes with age, which is the first step in determining the functional importance of changes in DNA methylation. In this section, we focus on those reports that have studied the effect of age on major portions of the genome (> 400 million cytosines); we have not included studies that have looked at the effect of age on a few promoters or a small region of the genome. Table 3 lists the nine studies that have been reported over the past five years that have interrogated the methylation at specific cytosines in a large portion of the genome. Depending on the amount of the genome interrogated, 10 thousand to several hundred thousand CpG sites show significant changes (increase or decrease) in methylation with age. In humans, where ~450 thousand CpG sites were analyzed using commercial arrays, 2 to 14% of the CpG sites studied showed a change in methylation. In mice, where a greater fraction of the genome was analyzed, 0.3 to 2% of the CpG sites studied showed a significant change with age. Except for the study by Hannum et al. (2013), all the studies showed that <5% of the CpG sites analyzed show a significant change in methylation with age in humans and mice, i.e., it appears that only a small fraction of the CpG sites in the genome is altered with age; however, even if 2% (the average of the studies showing a 0.3 to 4% change) of the CpG sites change with age, it would mean that the methylation of ~800 thousand CpG sites are likely to change with age in the genome of a tissue/cell.

The data in Table 3 also show that CpG sites become both hyper- and hypomethylated with age. Except for the study by Cole et al. (2017), all eight studies show more hypermethylation with age than hypomethylation. It will be interesting in the future, as more studies are reported, if there continues to be a predisposition for hypermethylation with age at specific sites and whether this is similar across tissues and cell types.

There have been two studies that have looked at the effect of age on the methylation of CpH sites in the genome. Both of these studies focused on the hippocampal genome of mice. Masser et al. (2017) reported that out of ~28 million CpHs sites analyzed, 150 and 191

thousand CpH sites changed with age (3 to 24 months) in male and female mice respectively. Of the CpH sites that changed significantly with age, 57% and 66% became hypermethylated in male and female mice, respectively. Subsequently, Hadad et al. (2018) observed a significant change in methylation at 79 thousand CpH sites in the ~25 million sites analyzed in male mice. They also observed that ~56% of the sites were hypermethylated. Based on these limited data, it would appear that only 0.3 to 0.7% of the CpH sites in the genome would show changes in methylation with age. However, this would correspond to over 2 million CpH sites in the entire genome, and thus the majority of age related changes in DNA methylation may actually be in the CpH context.

Because changes in DNA methylation with age are likely to occur in hundreds of thousands of cytosines, the question arises as to how many of the changes are spontaneous and random and how many are regulated responses directed to specific genomic locations. As described above, the robust nature of epigenetic clocks and the statistically significant changes described above, which were built on increases or decreases in methylation at specific CpG sites, strongly suggest the changes in methylation are regulated in some manner to specific genomic loci. That does not preclude the possibility that other sites in the genome experience random gains or losses of methylation with aging as these sites would be eliminated in the mathematical development of an aging clock or in statistical tests for age-related differences. To begin to address this question, we have measured the effect of age on the variance in DNA methylation in the mouse brain. As shown in Fig. 2, we observe sex differences in variance with age. Young and old females showed very little difference in variance in methylation patterns, while in male mice there was a significant increase in variance of methylation in old mice compared to young mice. Evaluating the contribution of stochastic changes in DNA methylation with aging would be best understood at the single cell level, which several investigators have attempted to do. While single cell bisulfite sequencing approaches still require refinement to provide base-specific data at the same sites across cells, there is some evidence that the patterns of methylation become more random with age (Vijg, Gravina, & Dong, 2018). Thus, more studies are required to determine the contribution of regulated and random changes to DNA methylation patterns that occur with age. This also leads to another issue in nomenclature – the term ‘epigenetic drift’, which has been utilized both in the context of regulated changes and stochastic changes thereby leading to confusion as to what is actually meant by this term (Martin, 2005, 2009). We suggest the term epigenetic drift should be used to define only those stochastic changes in methylation with age.

3.2. Effect of sex on the changes in DNA methylation that occur with aging

Because the epigenetic clocks for humans and mice work effectively in both males and females (see above section), it is generally assumed that the age-related changes in DNA methylation are largely similar in the two sexes. However, as can be seen from Table 3, all of the current studies to date, except one, only studied males. Therefore, except for the study by Masser et al. (2017), we have no information as to whether age-related changes in DNA are similar or differ in males and females. Masser et al. (2017) measured the methylation in both CpG and CpH sites in DNA isolated from the hippocampus of 3- and 24-month-old male and female C57BL/6 mice. The principle finding was that while males and females share common age-related changes in methylation, the majority (>95%) of the changes that occurred with age were sex specific as shown in Fig. 3. In other words, <5% of the changes in DNA methylation with age are common in males and females. It is important to reiterate that these are sex divergences with aging and not life-long sex differences. Thus, although the epigenetic clocks showed no sex differences in predicting chronological age, it is clear from the study by Masser et al. (2017) that male and female have quite different effects on what specific cytosines are altered by aging in the brains of mice. These findings demonstrate the complex interaction of sex and aging

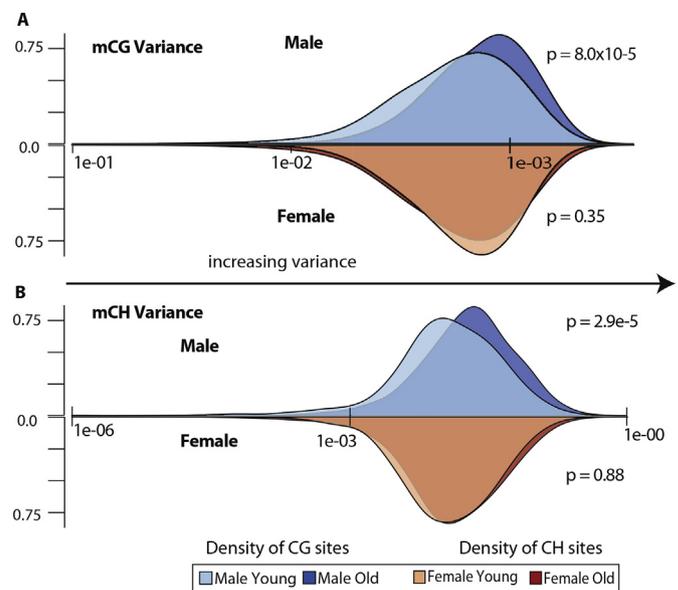


Fig. 2. DNA methylation variance with age. A) Mean inter-animal mCG variance of cytosines present at $\geq 20\times$ sequencing depth in every sample (354,522 CG sites) among young and old males (blue distributions) and females (red distributions) relative to density of sites at a given mean variance. mCG variance distribution in old males (mean variance = 0.157774) compared to young males (mean variance = 0.103581) was significantly higher ($p = 8.0 \times 10^{-5}$), while comparison of mCG variance in old females (mean variance = 0.112484) to young females (mean variance = 0.107045) was not significant ($p = .35$). B) Mean inter-animal mCH variance of cytosines present at $\geq 20\times$ sequencing depth in every sample (5,577,396 CH sites) among young and old males (blue distributions) and females (red distributions) relative to density of sites at a given mean variance. mCH variance distribution in old males (mean variance = 0.050142) compared to young males (mean variance = 0.034209) was significantly higher ($p = 2.9 \times 10^{-5}$), while comparison of mCH variance in old females (mean variance = 0.038597) to young females (mean variance = 0.033432) was not significant ($p = .88$).

that requires a careful and specific terminology because the term ‘sex differences’ is often used indiscriminately. A suggested nomenclature is that changes in only one or the other sex with aging should be described as ‘sex divergences’, i.e., methylation does not differ between sexes at young age but an age-related change occurs in one sex (McCarthy, Arnold, Ball, Blaustein, & De Vries, 2012). Masser et al. (2017) found that >95% of the CpG sites and ~99% of the CpH sites that showed age-related changes in methylation were sex divergent. A ‘sex difference’ would describe a lifelong differential methylation that is unaffected by aging. A few thousand autosomal CpG and CpH sites analyzed by Masser et al. (2017) fit this definition. Sex differences in epigenetic patterns of sex chromosomes would be expected given the different chromosomal content between males and females. Lastly, the often inappropriately used term sex dimorphism should not be used in the context of DNA methylation as this is best defined as a characteristic, such as specific gonads, which is only observed in one sex.

3.3. The genomic context of age-related changes in DNA methylation

As described above, age-related changes in DNA methylation have been observed to occur in tens to hundreds of thousands of CpG sites throughout the genome. Investigators have begun to determine if these changes are randomly distributed throughout the genome or if there are ‘hot spots’ where the changes in DNA methylation preferentially occur as well as regions where the changes do not occur. Therefore, CpGs that are differentially methylated with age (DMCGs) have been mapped to coordinates of known features of the genome (e.g., promoters (TSS sites), CG-islands, shores, and shelves) as well as histone marks (acetylated and methylated histones), which are associated with gene regulatory regions (e.g., activated genes, enhancers, and repressors). Table 4 lists the nine reports that have characterized distribution of DMCGs in the genome. These studies show that DMCGs

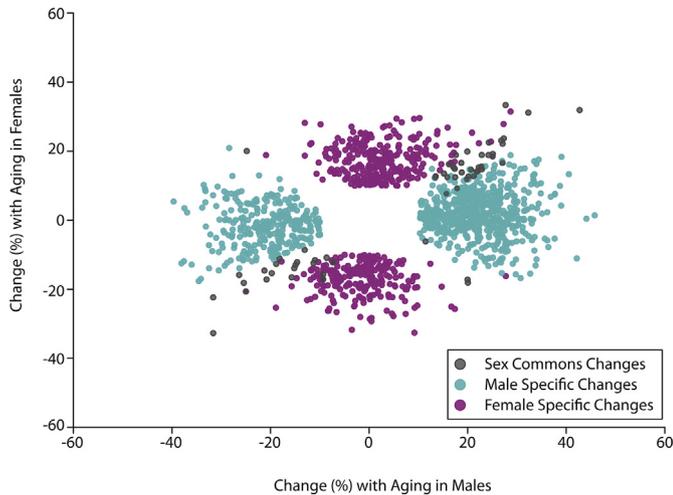


Fig. 3. – Sex divergence with aging. Hippocampal methylation changes (mCpG) with aging (young = 3 months, old = 24 months, $q < 0.01$, $|\Delta\%| > 10$) are primarily sex-specific. When viewed in this scatterplot there are common changes with age in both males and females (black circle) but the majority of changes occur in only males (blue) or females (pink). Data taken from Masser et al. (2017).

(or DMRs, differentially methylated regions) are enriched in some areas of the genome and under-represented in other regions. In other words, the age-related changes in CpG methylation are not totally random but the changes appear to occur preferentially in certain genomic contexts. However, the current studies do not show a clear trend as to the regions of the genome where DMCGs are enriched or under-represented. For example, several studies indicate that DMCGs are enriched in CG-islands (Cole et al., 2017; Day et al., 2013; Steegenga et al., 2014), while other studies report a depletion/under-representation of DMCGs in CG-islands (Hadad et al., 2018; Masser et al., 2017; Sun et al., 2014). These contradictory observations are likely due to the limited information currently available, e.g., some studies analyzed only ~25 thousand CpG sites while others analyzed 2 to 28 million sites. In addition, the studies listed in Table 4 have studied a variety of tissues, and it is likely that the distribution of DMCGs in genomic contexts varies from tissue to tissue. For example, the three studies with liver tissue (Bysani et al., 2017; Cole et al.,

2017; Hahn et al., 2017) observed that DMCGs (or DMRs) were enriched in areas of the genome involved in transcription (promoters and enhancers). On the other-hand, DMCGs were found to be under-represented in promoters in the hippocampus (Hadad et al., 2018; Masser et al., 2017). Two studies (Hadad et al., 2018; Masser et al., 2017) have also analyzed the genomic distribution of CpH sites that show changes in methylation with age in hippocampus. In general, the genomic distribution of the differentially methylated CpH sites appear to be similar to what was observed for CpG sites, e.g., they were under-represented in CG-islands, shelves, and promoters.

Masser et al. (2017) compared the genomic distribution of CpG and CpH sites that were differentially methylated with age in the hippocampus of male and female mice. As noted in the previous section, over 95% changes in methylation at specific CpG or CpH sites that occurred with age were sex specific (Fig. 3). However, when the changes in DNA methylation are mapped to genomic regions, they observed similar genomic distribution of DMCGs and DMCHs for males and females. For example, differential methylation with age for both CpG and CpH sites were enriched in intergenic and intronic regions and under-represented in promoters, CG-islands and specific enhance regions in both sexes. Thus, age-related changes in DNA methylation were occurring in the same genomic regions for males and females even though the exact CpG/CpH sites that were altered with age were sex specific. These data suggest that certain genomic elements are labile with age in both males and females even though the exact cytosines altered are sex-specific.

3.4. The relationship of the age-related changes in DNA methylation to changes in gene expression

The current data demonstrate that major changes in DNA methylation occur with age (Table 3); however, a major question is whether the age-related changes in DNA methylation have a functional significance. It is well accepted that DNA methylation is essential in mammalian development and promoter methylation tracks inversely with gene expression across genes or between tissues (Smith & Meissner, 2013). In the context of gene promoters, hypomethylated CpGs are generally associated with active, constitutively-expressed genes, while hypermethylated CpGs are associated with lowly expressed/silenced genes (Bird, 2002). It is not known whether this generalization will hold for changes in DNA methylation that occur with age. Currently,

Table 4
Genomic locations of age-related changes in DNA methylation.

Reference	Age	Tissue/cell	Genomic coordinates
Humans*			
Day et al. (2013)	30–90	Blood, brain, kidney, muscle	Hypermethylated CpGs are found primarily in CG-islands while hypomethylated sites are very rare in CG-islands in all tissues. For Brain and kidney, the trend was similar for shores. In blood and muscle, the shores were enriched in hypomethylated CpGs.
Raddatz et al. (2013)	18–75	Epidermis	Hypermethylated DMRs are enriched in promoters and hypomethylated DMRs are enriched in enhancers.
Steegenga et al. (2014)	30–43 & 52–66	PBMCs	Hypermethylated DMCGs predominantly present in CC-islands while hypomethylated DMCGs found in less dense CpG regions, e.g., shelves and single CpGs.
Bysani et al. (2017)	28–64	Liver	Enrichment of DMCGs observed at transcription start sites, 5'-untranslated regions, and first exons. Under-representation of DMCGs at the gene body, 3'-UTR, and intergenic regions. Significant enrichment of DMCGs observed at GC-islands and under-represented in shelves.
Mice*			
Sun et al. (2014)	4 & 24	Hematopoietic stem cells	The distribution of CpG sites that are hypo- and hypermethylated with age is similar in CG-islands, shores, and promoters. However, these sites are enriched in the shores and depleted in CG-islands.
Cole et al. (2017)	2 & 22	Liver	DMRs are most abundant in genes, introns, and enhancers and disproportionately enriched at genic super-enhancers in highly expressed genes involved in liver function.
Masser et al. (2017)	3 & 24	Hippocampus	Differential methylation for both CpG and CpH sites is enriched in intergenic and intronic regions and under-represented in promoters, CG islands and specific enhances in both male and female mice.
Hahn et al., 2017	5 & 27	Liver	DMRs are strongly enriched for open chromatin histone marks. Enrichment was more pronounced for hypomethylated DMRs. Hypomethylated DMRs are also strongly enriched for distal and intragenic active enhances and active promoters.
Hadad et al. (2018)	3 & 24	Hippocampus	DMCGs were enriched in introns and shores and under-represented in CG-islands and shelves, exons, and promoters. The depletion of DMCGs is most pronounced in promoters containing CGIs. Enrichment of DMCGs was associated with both repressive and activation histone marks.

there is very little information on how the age-related changes in DNA methylation effect the transcriptome or function of a tissue or cell. There is evidence that some genes that are associated with aging are also regulated epigenetically. For example, POLG mutant mice display accelerated aging (Bailey et al., 2009). The POLG gene's promoter is controlled by methylation (Tsai, Tyagi, & St John, 2018), and its methylation status has been observed to be influenced by factors such as inflammation (Maiuri, Li, Stein, Tennessen, & O'Hagan, 2018), which is known to increase with age (Franceschi & Campisi, 2014). Similarly, SOD3 mutants show premature aging (Kwon, Lee, Lee, Kim, & Kim, 2015), and this gene is epigenetically regulated by DNA methylation (Kamiya, Nakahara, Mori, Hara, & Adachi, 2017). CDKN2A (p16 ink4a/arf) is a cell-cycle inhibitor, and its expression is a well-established marker of aging (Krishnamurthy et al., 2004) and cellular senescence. Its expression is controlled by methylation and is often activated in various cancers (Matsuda, Ichida, Matsuzawa, Sugimura, & Asakura, 1999). BMAL1 is a circadian rhythm gene associated with cellular senescence (Khapre, Kondratova, Susova, & Kondratov, 2011) and is controlled epigenetically by both DNA methylation and histone modification (Sahar & Sassone-Corsi, 2013). Age-related changes in promoter and intragenic methylation of MHC1 genes, which are involved in immune function, have been observed to track with altered gene expression (Mangold et al., 2017).

Investigators are beginning to collect transcriptome data along with the DNA methylation profile to identify the genes whose expressions are potentially altered by DNA methylation, i.e., those changes in gene expression that are correlated to age-related changes in DNA methylation. Currently, there are two studies that have conducted a paired comparison of the changes in the transcriptome and methylome in humans. Steegenga et al. (2014) compared the changes they observed in CpG methylation to changes in gene expression in human peripheral blood mononucleated cells. Of the 470 probes that were highly methylated, 334 were associated with 168 genes that were downregulated and 136 probes were associated with 78 genes that were up-regulated. Similarly, 256 probes were found to be highly hypomethylated, out of which 130 were associated with 101 genes that were up-regulated and 126 probes associated with 95 genes that were down-regulated. Bysani et al. (2017) reported that the CpG sites that changed with age (~20 thousand) in liver were annotated to 6021 genes; 3852 of the genes harbored a significant number of CpG sites annotated to CpG islands. The expression of only 151 of these genes showed a significant correlation to DNA methylation, which was observed at 351 CpG sites. In other words, only ~2% of the CpG sites that changed with age were associated with a change in gene expression.

There have been three studies with mice comparing the age-related changes in DNA methylation to changes in the transcriptome. Using hematopoietic stem cells (HSCs) from 4- and 24-month-old mice, Sun et al. (2014) reported that of all the HSC specific genes analyzed 70% changed with age and were correlated to differential DNA methylation. Cole et al. (2017) evaluated the relationship between DMR's (hypo- or hypermethylation) and gene expression in the livers of 2- and 22-month-old mice. Out of the 4444 genes (3901 of which harbored hypomethylated enhancers and 543 genes with hypermethylated promoters), 168 genes were up-regulated and 71 were down-regulated. Hahn et al. (2017) studied whether age-related DMRs (which showed significant enrichment in specific genes) were associated with age-related changes in gene expression in the livers of 5- and 27-month-old mice. Of the 403 genes that showed differential methylation with age in their promoters, <10% (39) showed a correlation to expression, and this correlation was not statistically significant. These findings point to a relationship between DNA methylation and gene expression, but one that is not as simple as initially thought. It is also important to consider that most gene expression data collected above are from a single time point or condition. Gene expression is highly dynamic in response to feeding, circadian rhythms and other stimuli. Future studies

will need to examine the relationship of DNA methylation to stimulus-responsive gene expression.

3.5. Summary

The current studies clearly demonstrate that aging has a major effect on DNA methylation. Almost all the studies to date have focused on methylation at CpG sites, and the studies suggest that ~2% of the CpG sites show changes in DNA methylation with age. Currently, there are only three reports that have studied the effect of aging on methylation at CpH sites, and these studies show that ~0.5% of the CpH sites analyzed change significantly with age. Thus, the current data indicate that while only a small fraction of the cytosines in the genome show changes in methylation with age, this translates into a change in large number of sites in the genome, e.g., ~800,000 CpG sites and over 2 million CpH sites.

Currently, there is interest in other DNA modifications that could play a role in epigenetic regulation. One modification that is gaining a great deal of interest is 5-hydroxymethyl cytosine (5hmC). In contrast to 5mC, which is found mostly at intragenic regions, 5hmC is found primarily in promoter regions (Jin, Wu, Li, & Pfeifer, 2011). The presence of 5hmC is generally associated with increased gene expression (Chen et al., 2012; Jin et al., 2011), which coincides with the co-localization of 5hmC to euchromatin (Kubiura, Okano, Kimura, Kawamura, & Tada, 2012; Mellen et al., 2012). Using immunostaining, Szulwach et al. (2011) quantified the total level of 5hmC in the brains of 6 week and 1 year old mice. They found a significant increase in 5hmC levels in the cerebellum but not the hippocampus. Contrary to this finding, Gontier et al. (2018) reported an age related decrease in 5hmC using dot blot analysis to measure 5hmC levels. The traditional bisulfite sequencing technology cannot differentiate between 5mC and 5hmC, therefore, all the studies described above represent a sum of 5mC and 5hmC. The levels of 5hmC have been reported to vary from tissue to tissue (Li & Liu, 2011), and it is generally assumed that this not a major problem because the levels of 5hmC in most tissues is much <5mC. In analyzing ten tissues from mice, we have found that 5hmC levels are <5% of 5mC levels in most tissues. However, 5hmC in brain makes up 20–25% of methylated cytosines (Fig. 4). It is now possible to differentiate between 5mCs and 5hmCs at the nucleotide level by oxidative bisulfite sequencing where DNA is first oxidized with potassium peruthenate before bisulfite conversion. The oxidation step converts 5hmC to uracil, which results in a final sequence output of only 5mC (Masser et al., 2018). As Fig. 4 shows, the ability to differentiate 5mC and 5hmC can be important in interpreting DNA methylation data. Using oxidative bisulfite sequencing, Hadad et al. (2016) found no change in total levels of hippocampal 5hmC with aging in male or female mice. Because potential changes in 5hmC could be functionally important, especially in tissue from the central nervous system, future studies should analyze the effect of age on hydroxymethylation of DNA.

To determine if the age-related changes in methylation have functional consequences, investigators are pairing genome wide DNA methylation profiles to transcriptome data to identify genes whose expression changes with age and is correlated to changes in methylation. However, this analysis is difficult because methylation tends to affect regulatory interactions and is not a direct measurement (e.g., like transcription) of molecular species abundance. In addition, it is difficult to identify which methylation changes may affect long-range interactions (e.g., enhancers) with specific genes. Data from the current studies show that age-related changes in methylation can be correlated to some of the genes that show changes in expression with age. However, the simple relationship of increased expression correlating to hypomethylation and reduced expression correlating to hypermethylation does not appear to hold with aging. For example, several studies report that hypo- and hypermethylation were correlated with both increased and decreased expression of specific genes. However, it is not known if the

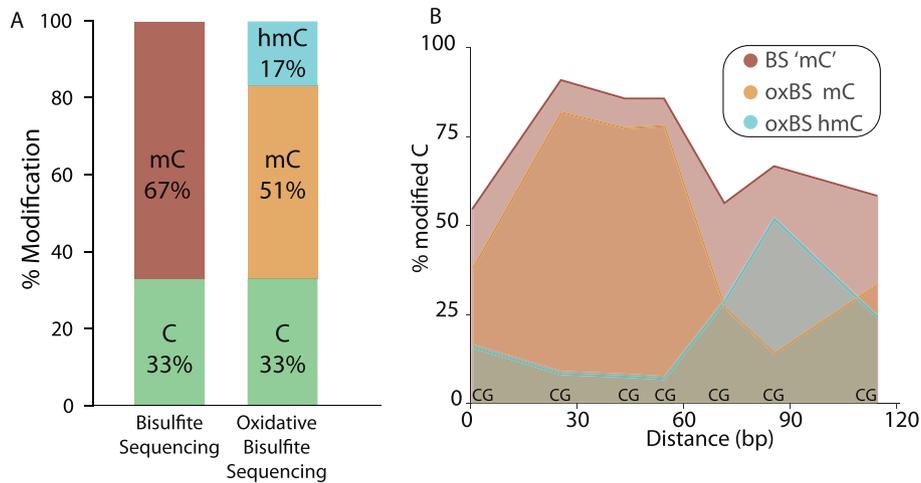


Fig. 4. Comparison of methylation and hydroxymethylation of cytosines in hippocampus. Graph A. The 5mC and 5hmC levels in DNA from the hippocampus is shown when measured by the traditional bisulfite sequencing assay versus the oxidative bisulfite assay. Approximately 25% of the cytosines that would be classified as 5mC by the bisulfite assay are 5hmC residues. Graph B. The distribution of 5mC and 5hmC over a small region (~120 bp of Fkbp6) of the hippocampus genome. Data taken from Messer et al. (2018).

changes in methylation that correlate to changes in expression are causative.

4. Effect of anti-aging interventions on DNA methylation

Manipulations that retard/delay aging are an essential tool in identifying the biological mechanism(s) or pathways involved in aging. By identifying the molecular process that are altered by these manipulations, investigators can begin to discover the molecular processes that are important in aging. Over the past two decades, a number of manipulations have been reported to increase the lifespan of mice (for review see Unnikrishnan et al., 2018). Currently, the most studied and robust anti-aging manipulations are caloric restriction (CR), dwarfism, and rapamycin treatment. These manipulations have been shown to increase lifespan by several laboratories, and the increase in lifespan is accompanied by improved physiological functions and reduced pathology. In other words, the mice appear younger and healthier than their littermate controls.

CR (also called dietary restriction) was the first and is the most studied anti-aging manipulation. In 1936, McCay, Crowell, & Maynard, 1989 showed that a severe restriction of food increased the lifespan of mice. Since this report, numerous laboratories have repeated McCay's observation with a variety of strains of laboratory rats and mice. In addition, CR has been shown to increase the lifespan of invertebrates (yeast, *Drosophila*, and nematodes), dogs, and non-human primates (Unnikrishnan et al., 2018). Therefore, CR has become the "gold standard" by which all anti-aging interventions are compared. The lifespan of rodents is increased 20 to 25% when the caloric intake is reduced 30 to 50%. In 1996, Bartke's laboratory published the first report showing that the lifespan of a mammal could be increased by a mutation in a specific gene (Brown-Borg, Borg, Meliska, & Bartke, 1996), *Prof1* resulting in Ames dwarf mice. Subsequently, increased lifespan has been shown in other dwarf mouse models: Snell dwarf mice, which have a mutation in *Pit 1* gene (Flurkey, Papaconstantinou, & Harrison, 2002), 'little'

mice with a mutation in the *Ghrhr(lit)* gene (Flurkey, Papaconstantinou, Miller, & Harrison, 2001), and growth hormone receptor knockout mice (Coschigano et al., 2003). The lifespan of the dwarf mice is increased 20 to 50% over that of their control littermates (Unnikrishnan et al., 2018). More recently, Harrison et al. (2009) showed that lifespan could be increased pharmacologically by feeding mice rapamycin (commercially known as Sirolimus). Subsequently, there have been a large number of studies showing rapamycin increases the lifespan of various strains of mice and various disease models of mice (Richardson et al., 2015). A unique aspect of rapamycin is that rapamycin can increase lifespan even when implemented late in life (Bitto et al., 2016).

As described above, the epigenetic clocks developed by Petkovich et al. (2017) and Wang et al. (2017) showed that CR decelerated epigenetic aging in mice. Recently, four studies have been published that compared the effect of age on DNA methylation in tissues of rats and mice fed ad libitum or a CR-diet. These studies are described in Table 5. All four studies show that CR attenuated many of the age-related changes in CpG methylation. As shown in Fig. 5, Hadad et al. (2018) found that CR prevented ~35% of the age-related changes in CpG methylation in the hippocampus of mice. While CR prevented a similar percentage of the age-related changes in CpH methylation, the effect of CR on hypo- and hypermethylation differed for CpG and CpH sites (Fig. 5). CR attenuated hypo- and hypermethylation of CpG sites similarly; however, CR had a much greater effect (5-fold) on hypermethylated CpH sites than hypomethylated CpH sites. Hahn et al. (2018) reported that ~19% of the CpG sites that change with age in liver were prevented by CR. However, they found that CR had a greater effect on hypomethylated CpG sites than hypermethylated CpG sites (14% vs 4%). A recent report from Maegawa et al. (2017) also suggests that CR in Rhesus monkeys prevents age-related changes in DNA methylation.

Cole et al. (2017) studied the effect of CR as well as dwarfism (Ames dwarf mice) and rapamycin treatment on DNA methylation in liver by

Table 5
List of studies investigating the effect of caloric restriction on DNA methylation.

Reference	Animal	Sex	Method	Tissue	Length of CR*	Age Studied*	CpG Sites Studied	% Sites Altered by CR
Kim et al. (2016)	Rat (Sprague Dawley)	Male	MeDIP-Seq	Kidney	1	25	Unknown	Unknown
Cole et al. (2017)	Mice (UM-HET3)	Female	WGBS	Liver	18	22	~42 million	~3%**
Hahn et al. (2017)	mice (c3b6f1)	Female	BS-Seq	Liver	24	27	~29 million	~18%
Hadad et al. (2018)	Mice (C57BL/6)	Male	BOCS	Hippocampus	21	24	~2 million	~35%

* Given in months. **This percentage is based on differentially methylated regions and not specific CpG sites.

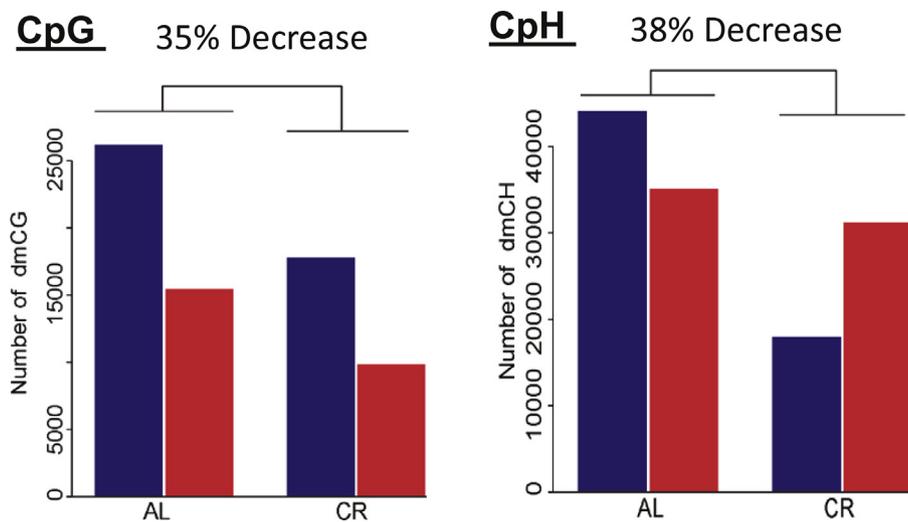


Fig. 5. Effect of age and CR on DNA methylation. DNA was obtained from the hippocampus of 3- and 24-month-old mice fed ad libitum and 24-month-old mice fed a CR diet. The number of CpG and CpH sites that changed with age are shown: blue bars are sites that become hypermethylated and red bars are sites that become hypomethylated. The methylation of ~120 K Cs changed with age (~41 K CpGs and 80 K CpHs). CR reduced the number of sites that changed with age from 35 to 38%. For CpG sites, CR reduced hyper- and hypomethylated sites equally (32 to 36%). However, for CpH sites, CR had had a much greater effect on hypermethylated sites (59%) compared to hypomethylated sites (11%). Data taken from [Hadad et al. \(2018\)](#).

identifying regions (500 bp) of the genome that were differentially methylated (DMRs) with age or the anti-aging manipulations. CR increased methylation in the age-associated hypomethylated DMRs (45% to 55%) and decreased methylation in the age-associated hypermethylated DMRs (62% to 58%) in the liver of female mice. [Cole et al. \(2017\)](#) also studied the effect of rapamycin treatment (42 ppm for 18 months) on DNA methylation in the liver of female mice. Similar to CR, rapamycin increased methylation in the hypomethylated DMRs but to a lesser extent (45% to 50%). However, rapamycin did not significantly alter the methylation of hypermethylated DMRs. [Cole et al. \(2017\)](#) also compared the age-related changes in CpG methylation in male Ames dwarf mice and wild type mice. Dwarf mice had 10-times more hypermethylated CpGs than the WT mice, which increased further with age, suggesting that the wild type and dwarf mice exhibit more epitype differences with age. In contrast, hypomethylated DMRs were 2-fold higher in wild type mice than the dwarf mice, but the dwarf mice were more resistant to age-related hypomethylation. Thus, dwarf mice exhibited more stability against age-associated hypomethylation than hypermethylation. When [Cole et al. \(2017\)](#) compared the CpG sites that were differentially methylated with age, they found 3-times more CpG sites changed with age in wild type mice than dwarf mice, suggesting that the methylome of dwarf mice is more stable with age.

The emphasis of the studies on CR have focused on how CR attenuates the age-related changes in DNA methylation as a way to identify sites of methylation that potentially play an important role in aging. However, it is clear from the study by [Hadad et al. \(2018\)](#) that CR has a greater effect on the methylome than just altering the age-related changes in DNA methylation. As shown in [Fig. 6](#), they found that 70% of the CpG sites that were differentially methylated in the hippocampus of old CR mice occurred at CpG sites that did not change with age. [Hahn et al. \(2017\)](#) reported that CR induced the methylation ~28 K CpG sites in liver that were independent of age; over 80% of these sites were hypermethylated. [Cole et al. \(2017\)](#) also observed that CR resulted in age-independent hypo- and hypermethylation (1301 hypomethylated and 3269 hypermethylated DMRs) in liver. Interestingly, they found that rapamycin induced substantially more age-independent changes in CpG methylation than CR.

[Hadad et al. \(2018\)](#) found that the genomic context of the CpG sites that changed with CR were markedly different from those CpG sites that changed with age. CR hypermethylated CpGs were over represented in CG-inlands and shores and under-represented in shelves. CR

hypomethylated CpGs were only enriched in shores. In addition, they found that CR hypermethylated CpGs were associated with repressive histone marks and hypomethylated CpGs were over-represented in both activational and repressive marks. [Cole et al. \(2017\)](#) found that CR suppressed the age-associated changes in genes, enhancers, and CG-islands. Interestingly, rapamycin suppressed the age-related changes in CpG methylation in a smaller number of genes, enhancers, and CG-islands than CR, i.e., CR appeared to be more efficient than rapamycin in suppression of methylation in specific genomic regions. [Hahn et al. \(2017\)](#) found an enrichment of CR DMRs in the genes involved in the expression of fatty acid, triglyceride, and ketone body metabolism. When differential DNA methylation was correlated to differential gene expression, DNA methylation was significantly correlated negatively to the expression of 69 genes out of the 149 genes that showed differential methylation.

An important facet of CR that has been largely overlooked is that CR can have early effects and can create a cellular memory that persists even when CR is discontinued. For example, 4.5 months of CR early in life ([Yu, Masoro, & McMahan, 1985](#)) or CR pre-weaning ([Sun, Sadighi Akha, Miller, & Harper, 2009](#)) significantly increased the lifespan of rats and mice, respectively, when they were fed ad libitum for the remainder of their life. Thus, CR can have early effects on longevity that create a molecular memory, which persists even when CR is discontinued, suggesting that the life-extending action of CR might occur, at least partially, through an epigenetic mechanism, such as DNA methylation. Three studies show that short-term CR can induce changes to DNA methylation. [Kim et al. \(2016\)](#) reported that one month of CR in old (25 months of age) rats reversed methylation in kidney for ~17 thousand DMRs that changed significantly with age. The short-term CR had no effect on the methylation profile in different genomic contexts. [Hahn et al. \(2017\)](#) reported that 2 months of CR in young mice (5 months of age) induced ~50 thousand DMRs in liver. Interestingly, short-term CR resulted in significantly more DMRs in 5-month-old mice than long-term (18 months) CR. [Unnikrishnan et al. \(2017\)](#) showed that 1 month of CR in 4-month-old male mice induced significant changes in the expression of hundreds of genes across five tissues, and 20 to 50% of the changes in gene expression persisted 2 months after CR was discontinued, suggesting the possibility of epigenetic regulation of the expression of these genes. [Unnikrishnan et al. \(2017\)](#) then measured DNA methylation in the promoter regions of several genes whose expression was found to be significantly altered by CR and remained altered when CR was discontinued. Short-term CR was

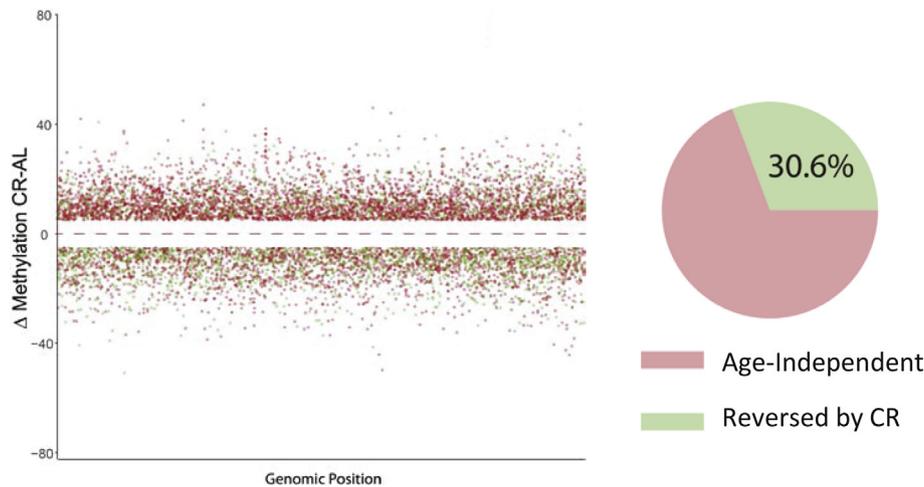


Fig. 6. Effect of CR on DNA methylation. The CpG sites differentially methylated between old mice fed ad libitum and CR is shown. The CpG sites that change with age in the ad libitum fed mice that were prevented by CR are shown in green. The changes in methylation induced by CR that did not change with age in ad libitum fed mice are shown in red. Approximately 70% of the changes in DNA methylation induced by CR are age independent, i.e., do not occur with age in mice fed ad libitum. Data taken from Hadad et al. (2018).

found to induce a significant decrease in methylation in each of the three CpG sites in the promoter of the *Nts1* gene, and the hypomethylation of these sites persisted even when CR was discontinued and was correlated with increased *Nts1* mRNA expression.

5. Conclusion

Epigenetic clocks, which measure the changes in the methylation of a few hundred CpG sites, accurately predict chronological age and are currently the best biomarkers for predicting mortality in humans. However, it is uncertain as to how well epigenetic clocks can predict biological age. The discovery of epigenetic clocks has led investigators to study the role DNA methylation plays in the molecular mechanism(s) underlying aging. Over the past five years, several laboratories have characterized the effect of aging on the methylation of specific cytosines throughout the genome. Most of these studies have focused on 5mCs at specific CpG sites and have shown that ~2% of the CpG sites change significantly with age. While both hypomethylation and hypermethylation occurs with age, the tendency is for more hypermethylation. Two studies have shown that ~0.5% of the CpH sites change with age. Therefore, it currently appears that only a small fraction of cytosines in the genome show changes in DNA methylation; however, these changes translate to 2 to 3 million cytosines. A crucial caveat to our current understanding is that the data have been collected at the tissue level. Tissues have heterogeneous cell populations, and it is well established that different cell types have different epigenetic profiles. Therefore, a critical goal of the field should be to collect cell-type specific data when studying the effect of aging on DNA methylation.

The only study that has compared the effect of aging on DNA methylation in male and females found that >95% of the age-related changes in DNA methylation in the hippocampus were sex divergent (Masser et al., 2017), i.e., methylation did not differ between the males and females when they were young but age-related changes occurred in methylation at specific sites in one sex but not the other. This observation is surprising in that the epigenetic clocks work equally well in predicting chronological age in male and female humans and mice. Therefore, future studies should measure DNA methylation in both males and females as they age to determine if the sex divergence in DNA methylation is common and found in other tissues. In future studies, it will be important that the effect of age on the methylation of CpH sites as well CpG sites be measured because (1) age-related changes in the methylation of specific CpH sites have been reported, (2) the total number of cytosines in CpH sites that can potentially become methylated are ~10-fold greater than CpG sites, and

(3) methylation at CpH sites has been associated with some cellular changes (He & Ecker, 2015). In addition, it will be important in the future that investigators also measure 5hmC because 5hmC is associated with changes in gene expression (Chen et al., 2012; Jin et al., 2011), and all the current studies reporting changes in 5mC at specific CpG or CpH sites are actually measuring both 5mC and 5hmC, i.e., one cannot determine if the changes in methylation are due to 5mC or 5hmC. While levels of 5hmC are minor in most tissues (e.g., <5% of 5mC levels), 5hmC is relatively high in some tissues, e.g., 5hmC makes up >20% of the methylated cytosines in brain (Fig. 4).

While the age-related changes in DNA methylation occur throughout the genome, these changes are enriched or under-represented in certain genomic contexts (e.g., promoters/gene bodies, GC-islands, shores, and shelves). However, there is currently no consensus as to what genomic contexts are more or less prone to change with age. Investigators have begun to pair genome wide DNA methylation profiles to transcriptome data to identify genes whose expression changes with age and is correlated to changes in methylation. Data from several studies show that hypo- and hypermethylation that occurs with aging in specific genes is correlated with both increased and decreased expression of some genes. Thus, the simple relationship of increased expression correlating to hypomethylation and reduced expression correlating to hypermethylation does not appear to hold with aging. However, it is not known if the changes in methylation that correlate to changes in expression are causative. Therefore, it will be important in the future to establish that the changes in gene expression are actually responsible for the changes in expression. Using genome editing technology, it may be possible to determine in the future if methylation at specific cytosines can directly alter the expression of a gene. For example, using CRISPR to drive fusion proteins with DNMTs or TETs to specific locations in the genome, it may be possible to alter methylation at specific sites and determine how these changes alter the transcriptome of the cell (Masser et al., 2018; Vojta et al., 2016).

Currently, the strongest data in support of DNA methylation playing a role in aging comes from studies that have evaluated the effect of anti-aging interventions on DNA methylation, especially CR. First, epigenetic aging is decelerated by CR, dwarfism, and rapamycin. Second, CR has been shown to attenuate many of the changes in CpG methylation that occurs with age. CR also induces changes in DNA methylation that are independent of age. Therefore, CR could have an impact on age by preventing change in DNA methylation that occur with age as well as inducing changes in DNA methylation that alters gene expression that have anti-aging effects. Third, CR may act through an epigenetic mechanism because it has been shown that CR has early effects and can create

a cellular memory that persists even when CR is discontinued. For example, CR early in life can significantly increase the lifespan of rats (Yu et al., 1985) and mice (Sun et al., 2009) and improve glucose/insulin tolerance when CR is discontinued (Matyi et al., 2018). Several studies have shown CR for 1 to 2 months can induce changes in DNA methylation, and the changes in DNA methylation in a specific gene has been shown to persist after CR is discontinued.

In summary, the current data clearly demonstrate that major changes in DNA methylation occur with age. The major hurdle facing researchers is to determine which, if any, of these changes in DNA methylation are functionally important in the aging phenotype. In other words, do the changes in methylation play a role in the molecular and physiological changes that lead to aging?

* Age for humans given in years and from mice in months. Abbreviations: PBMC = peripheral blood mononucleated cells; DMR = differentially methylated regions; DMGC = differentially methylated CpG sites. The methylation of these regions or CpG sites change (either increase or decrease) change significantly with increasing age.

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Conflict of interest

The authors declare no conflict of interest.

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