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The potential role of epigenetic responses to diet in ageing

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Epigenetic changes may be causal in the ageing process and may be influenced by diet, providing opportunities to improve health in later life. The aim of this review is to provide an overview of several areas of research relevant to this topic and to explore a hypothesis relating to a possible role of epigenetic effects, mediated by sirtuin 1, in the beneficial effects of dietary restriction, including increased lifespan. Epigenetic features of ageing include changes in DNA methylation, both globally and at specific loci, which differ between individuals. A major focus of research on dietary influences on epigenetic status has been on nutrition *in utero*, because the epigenome is probably particularly malleable during this life-course window and because epigenetic marking by early exposures is a compelling mechanism underlying effects on life-long health. We explore the potential of diet during adulthood, including the practice of dietary restriction, to affect the epigenetic architecture. We report progress with respect to deriving data to support our hypothesis that sirtuin 1 may mediate some of the effects of dietary restriction through effects on DNA methylation and note observations that resveratrol affects DNA methylation and other epigenetic features. Disentangling cause and effect in the context of epigenetic change and ageing is a challenge and requires better understanding of the underlying mechanisms and also the development of more refined experimental tools to manipulate the epigenetic architecture, to facilitate hypothesis-driven research to elucidate these links and thus to exploit them to improve health across the full life-course through dietary measures.

Sirtuin 1: DNA methylation: Dietary restriction: Ageing

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A body of literature now robustly supports the premise that diet can affect the epigenetic architecture. Much of the research in this area stemmed initially from attempts to identify mechanisms underlying the links between nutrition *in utero* and health throughout the life-course. Such links invoke the idea of a form of 'cellular memory', the most intuitive type of which may be some form of 'stamp' applied to the heritable component of the cell – the DNA. Thus, researchers pursued the concept that 'stamping' or 'marking' the DNA through changes to the epigenetic architecture may be induced by early dietary exposures. Of the principal forms of epigenetic modification, DNA methylation is a compelling candidate mechanism for long-term cellular memory because it has been shown to be somewhat more stable than other epigenetic modifications[1] – notably modification of the histone proteins through processes including acetylation, methylation and phosphorylation. Hence, to date, evidence linking diet to epigenetic change is most extensive and robust in the specific instance of gestational exposure affecting DNA methylation. Studies in this area also demonstrate effects of diet *in utero* on health in later life. However, demonstrating causal links between observed changes in DNA methylation and phenotypic outcomes remains a challenge. Extending these ideas leads to the central theme of this paper, which is that diet-induced epigenetic effects

Abbreviations: DNMT, DNA methyltransferase; DR, dietary restriction; ERα, oestrogen receptor α; SAH, *S*-adenosylhomocysteine; Sirt1, sirtuin 1.
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throughout the whole life-course may affect in particular the process of ageing and thus lifespan. To draw together the threads of this theme, the literature reporting changes in the epigenetic architecture that accompany increasing age will be reviewed briefly and possible underlying mechanisms, as well as the mechanisms through which such effects may have phenotypic consequences relevant to ageing, will be explored. The evidence that diet influences ageing will then be considered. Evidence for effects of diet on epigenetic modification will be reviewed, focusing here on effects of diet beyond very early exposures. The paper will explore the idea that a specific dietary intervention that has been shown very robustly to protect against ageing-related disease and/or extend lifespan – that of dietary (or energy) restriction – may bring about some of the beneficial effects through changes in epigenetic markings, in particular DNA methylation, that oppose changes that accompany (and thus potentially cause) the ageing process. In this context, a major focus will be the protein sirtuin 1 (Sirt1), a protein deacetylase that appears to play a pivotal role in mediating the effects of dietary restriction (DR) and whose broad substrate specificity includes histone proteins, thus suggesting epigenetic actions. Finally, major gaps in research that addresses the role of epigenetic responses to diet in ageing will be identified and priorities for future research suggested.

Epigenetic changes observed in ageing

Research on epigenetic changes observed as organisms age has focused largely on DNA methylation. Initial observations were that the total methyl-cytosine content of the vertebrate genome decreases with age[2], with age-related DNA hypomethylation occurring predominantly in repeated sequences[3]. Recent cohort studies in human subjects, however, reveal considerable inter-individual differences in how global DNA methylation changes over time. A study by Bjornsson *et al*. is particularly illuminating because it reports individual longitudinal measures[4]. The measurements, made in cohorts from Iceland (over an average interval of 11 years) and Utah (over an average interval of 16 years), revealed both decreases and increases in global DNA methylation. Moreover, there was an indication of familial clustering of the tendency to lose or gain DNA methylation over time, which may indicate that genetic factors contribute to the process (and so potentially to ageing through such mechanisms). The investigators put forward an argument that environmental factors are unlikely to be responsible for this familial clustering, however, such a conclusion is somewhat tenuous so acceptance of a genetic component to ageing-related changes in DNA methylation would be premature at this point.

A slew of data reveals changes in methylation status at specific gene loci to be an epigenetic feature of ageing. For example, an increase in methylation of the oestrogen receptor alpha gene (*ERα*) in human colon from older compared with younger people has been noted in independent studies[5,6]. Other examples include *RARβ2, RASSF1A*, *GSTP1* and *NKX2-5*; along with *ERα* these genes were hypermethylated in prostate in older compared with younger subjects[7]. The recent emergence of microarray-based (and similar) approaches to measure DNA methylation at specific loci but simultaneously across the whole genome (the 'methylome') has lead to an enormous increase in knowledge about how DNA methylation changes as human subjects and other mammals age, including about the level of inter-individual variability. For example, changes in DNA methylation across 1240 genes were detected in DNA from intestinal mucosa of old compared with young mice[8]. Analysis of genome-wide data on DNA methylation with respect to the methylation status of gene targets of polycomb group proteins – transcriptional regulators whose repression in stem cells maintains stem cell phenotype and whose derepression allows cell differentiation – revealed hypermethylation with age of a subset of 69 CpG sites in seven independent data sets, including whole-blood DNA, ovarian-cancer tissue and bone-marrow mesenchymal stem cells[9]. In addition to identifying what may be considered a methylation signature of ageing, these findings support a model of age-related predisposal to neoplastic transformation in which stem cells are 'locked' in an undifferentiated state of self-renewal.

A recent study by Rakyan *et al*.[10] also reports observations consistent with the idea that some changes in DNA methylation status that accompany ageing may push cells into a 'stem-like' permanent state of self-renewal, predisposing to cancer. Here, 231 CpG sites hypermethylated with age and 147 sites hypomethylated with age were identified in DNA from whole blood; bivalent chromatin domains, which are sites that in ES cells show marks of both active and inactive chromatin and that are frequently hypermethylated in cancers, were over-represented among the sites of age-related DNA hypermethylation. We highlight this study here because it addresses a caveat to many investigations on ageing-associated epigenetic differences, which is that it may be difficult or impossible to dissociate changes in epigenetic profile of specific cell types/lineages from ageing-associated changes in tissue composition with respect to individual cell types (with different epigenetic profiles). Rakyan *et al*. provide evidence that many of the ageing-related DNA methylation changes they observed were not dependent on the composition of cells in the samples analysed since relationships between DNA methylation and age were retained in sorted CD14+ monocytes from the same cohort, particularly at hypermethylated sites. This observation also indicates that ageing-associated DNA hypermethylation arises in precursor/stem cells. Moreover, many of the same effects were observed in T-cells and buccal cells from independent samples. Since buccal cells arise from ectoderm and leucocytes from mesoderm, a further implication of the findings is that effects of ageing on DNA methylation are not germ-layer specific.

In another study of genome-wide DNA methylation, where analysis focused on the pertinent question of tissue-specificity of DNA methylation as well as age-related effects, investigators grouped data according to methylation 'patterns', which separated tissues according to type

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and also proved predictive with respect to tissue classification by type[11]. Age was also associated significantly with methylation class. Age-associated changes in DNA methylation, which were generally increased, included genes involved in epigenetic regulation (e.g. DNA methyltransferase 3 (DNMT3), where methylation was reduced), telomere maintenance and the locus (*WRN*) co-inherited with the genetic premature ageing syndrome, Werner disease. Overall, CpG sites in CpG islands showed a positive association between methylation and age whereas others showed a negative association.

Compared with DNA methylation, data on other changes to the epigenetic architecture that accompany ageing are limited. Examples include reports of an ageing-related progressive reduction in heterochromatin-like domains[12,13], increased H4-K20 trimethylation in rat kidney and liver in older animals[14] and a progressive dephosphorylation of histone H1 with age in human peripheral blood lymphocytes[15]. Late (P75) *v.* early (P30) fibroblasts showed a reduction in H3 and H4 expression, observed also in fibroblasts from an old (90 years) compared with a young (9 years) individual, and also an altered distribution of histone methylation marks[16]. In contrast to this observed ageing-related reduction in histone protein expression, our own observations have revealed increased histone protein expression in mouse intestine[17]. Reasons for the discordant observations may relate to differences in cell/tissue type studied, and it is also worth noting that fibroblast passage is a procedure that eventually leads to a state of cellular senescence, which, although accepted as relevant in some way to the ageing process, may have dichotomous roles. An exposition of the relationship between senescence and ageing is beyond the scope of the current article, but is covered by recent published opinion[18,19].

Possible mechanisms underlying ageing-associated epigenetic changes

The mechanisms underlying changes in the epigenetic profile with age are largely unknown. Compelling theoretical explanations include a loss in fidelity of the processes through which epigenetic marks are copied as cells divide and/or aberrant addition or removal of epigenetic marks either in differentiated cells (perhaps at particularly susceptible sites, to explain why such changes can be observed by sampling the epigenome across multiple cells) or in stem cells. With respect to DNA methylation, age-related changes in the levels of expression and/or activity of the DNMT or of enzymes responsible for active DNA demethylation – whose existence/identity remains a controversial topic[20] – would thus be reasonable hypotheses to pursue. Indeed, there is some evidence of ageing-related reductions in expression of DNMT1, the DNMT responsible for 'copying' the DNA methylation pattern to the nascent strand during DNA replication as cells divide[21,22]; such reductions may lead to a process of passive demethylation of the genome. Ageing-related increases in DNA methylation may be associated with an increase in the expression of DNMT3, the DNMT responsible for *de novo* methylation of DNA[21]. It is likely that a focus on ageing-related changes in expression/activity of enzymes involved in the establishment or maintenance of epigenetic marks is too narrow to reveal eventually the principal mechanisms through which the epigenome changes with ageing; for example, factors such as the involvement of small RNA species[23,24], which are beyond the scope of the current article, should not be overlooked.

Mechanisms through which epigenetic changes may influence ageing

Demonstrating causal links between epigenetic changes and phenotypic sequelae is a challenge. Loss of DNA methylation, particularly in repetitive regions, may contribute to an ageing cellular phenotype through promoting genomic instability[25,26] and loss of telomere integrity[27]. Site-specific changes in DNA methylation may potentially be causal in ageing or related diseases through changes in gene expression. There is a need to develop tools and techniques to replicate in experimental systems specific changes in epigenetic architecture that accompany ageing to allow the testing of specific hypotheses; perhaps in this respect transgenic mouse models with altered expression of enzymes that play a central role in establishing, maintaining or removing epigenetic markings may be illuminating. *In vitro* methylation of DNA constructs that can then be expressed in cell line models offers an approach to determining if methylation affects gene expression, and pharmacological tools to manipulate epigenetic status (such as the DNMT inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine, *Sss*I DNA-methylase and the histone deacetylase inhibitor trichostatin A) are available, but these approaches lack the level of refinement necessary to replicate the actual levels and CpG site specificity of DNA methylation or specific changes in histone modifications observed *in vivo*.

Evidence that diet influences ageing

Observational studies in human subjects support a view that quality of the diet affects length of life and a range of age-related diseases. For example, adherence to a 'modified Mediterranean diet' was associated with reduced mortality across nine European countries in healthy individuals aged ≥ 60 years at recruitment into EPIC cohort[28]. Of course, such studies are limited by the many associated confounding variables and the difficulties inherent in identifying and correcting for all such factors, so there is a need for rigorous intervention studies in human populations to establish unequivocally if diet can affect ageing and to identify those dietary practices that best support a healthy ageing trajectory. A focus on specific nutrients or other dietary factors may be the most realistic approach. To highlight possible avenues for further investigation in this context, it is notable that meta-analysis of randomised trials testing effects of vitamin D supplementation suggested reduced mortality rate associated with supplementation at ordinary doses[29]. Thus, vitamin D may be a prudent choice for intervention trials.

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Alternatively, meta-analyses based on other dietary factors for which suitable data sets can be identified may reveal other candidates.

Animal models offer the opportunity to study with greater control effects of dietary interventions on ageing and lifespan. Lifespan is often accepted as a readout of the rather nebulous concept of 'ageing' but has obvious limitations as an end point in longer-lived species, so (short-lived) rodent models have particular utility in this respect. Other than DR, as noted below, there is still a paucity of robust evidence relating to the efficacy of any specific dietary intervention to delay ageing/increase lifespan in animal models, perhaps highlighting a need for further research in this area. In this context, it is worth noting rapamicin as a pharmacological, rather than dietary, intervention that increased lifespan in mice[30]; the target of rapamicin TOR pathway appears to play a central role in ageing and lifespan determination[31], a view further substantiated by these findings.

Dietary restriction

Restricting food intake but without severe nutritional deprivation or similar interventions in simpler model systems including yeast (reduction of the glucose concentration of the medium[32]), flies and worms (dilution of the food source[33–35]), appears particularly robust as a dietary intervention that can extend lifespan. This practice, which will be referred to here as DR, was identified as a measure that extended lifespan in laboratory rodents as long ago as the 1930s[36] and the original observations have been substantiated by many later studies[37–39]. Evidence for effects of DR on longevity in human subjects is currently based largely on epidemiological data, and in this regard the unusually long-lived population of the Japanese Island of Okinawa has been particularly informative. Culturally, it was usual to eat very sparingly, such that recent analysis revealed the energy intake of individuals in their eighth decade of life to be 11% lower than recommended over the first half of adult life with survival curves revealing increases in average and maximum lifespan compared with Japan overall and with the USA[40]. Overall, though, it remains uncertain if DR increases lifespan in human subjects[41]. However, ongoing research in non-human primates is revealing positive responses to DR, including reduced incidence of diabetes, cancer, CVD and brain atrophy in Rhesus monkeys maintained since adulthood at a level of 30% food restriction[42]. Moreover, mortality from age-related diseases was reduced significantly in Rhesus monkeys under DR compared with controls and, at the time of reporting, although numbers were too small to reach statistical significance, a larger number of animals in the DR group compared with the control group had reached the age of 30 years[42].

A specific dietary polyphenol worthy of special note is the stilbene, resveratrol, which has been the topic of intense debate and controversy surrounding reports that it activates Sirt1. Sirt1, as discussed below, may be an essential mediator of the longevity response to DR. Several observations support a view that resveratrol can extend lifespan and/or (in mammals) protect against phenotypic features of ageing. For example, resveratrol increased activity of the Sirt1 homologue, Sir2, and increased lifespan in yeast[43]. Resveratrol also increased lifespan in *Caenorhabditis elegans* and *Drosophila* through a Sir2-mediated process[44]. In mice, dietary resveratrol protected against diet-induced obesity and insulin resistance and induced other metabolic and physiological effects associated with longer lifespan[45,46]. Several reports in the literature that resveratrol activates Sirt1 are based on measured effects of resveratrol on the activity of recombinant Sirt1 against a commercially available synthetic fluorescently tagged ('Fluor-de-Lys') substrate[43,47]. The debate concerning whether or not resveratrol is indeed an activator of Sirt1 is fuelled by reports that this assay may be unreliable, inasmuch as the synthetic substrate appears to be deacetylated by Sirt1 only when a florescent tag is included[47–49]. While it thus appears that investigators may have been misled into a view that resveratrol activates Sirt1 directly, there is certainly still robust evidence that it can have a beneficial effect in healthy ageing and that this effect may include through Sirt1-related mechanisms. Exogenously expressed Sirt1 was shown to deacetylate natural histone substrates in an NAD^+-dependent manner, specifically H4-K16 and H3-K9[50]. Also, resveratrol treatment increased deacetylation of the Sirt1 substrate PGC-1α in multiple mouse tissues, consistent with Sirt1 activation, and resveratrol failed to induce deacetylation of PGC-1α in Sirt1$^{-/-}$ mouse embryonic fibroblasts[46], further supporting the view that resveratrol acts through Sirt1 activation.

Evidence that diet influences epigenetic modification

Gametogenesis and early embryogenesis include waves of enormous epigenetic change. After fertilisation, both maternal and (more rapidly) paternal genomes are demethylated then patterns of methylation become re-established. In a second wave of demethylation, the DNA of germ cells is wiped almost clean of methylation once they reach the embryonic gonads then methylation occurs at imprinted loci[24,51]. For these reasons, the period surrounding fertilisation, implantation and development *in utero* has been considered a time of epigenetic plasticity and thus a window of exposure during which diet is likely to influence the epigenome. Moreover, epigenetic marking, and DNA methylation in particular, is a very attractive and plausible mechanism through which effects of nutrition *in utero* may be 'recorded' and thus influence health in later life. Effects of exposures *in utero* on DNA methylation thus provide a mechanistic hypothesis to explain the now well-established link between nutrition *in utero* and later health outcomes, notably the link between low birth weight and later susceptibility to features of the metabolic syndrome[52]. For such reasons, the effect of early life nutrition on epigenetic modification has been the subject of vigorous investigation. The topic has been covered in recent reviews[53–55] so will not be considered in detail here, other than to highlight two studies as examples, one because it provides evidence for DNA methylation being a mechanism that can explain the intriguing phenomenon of

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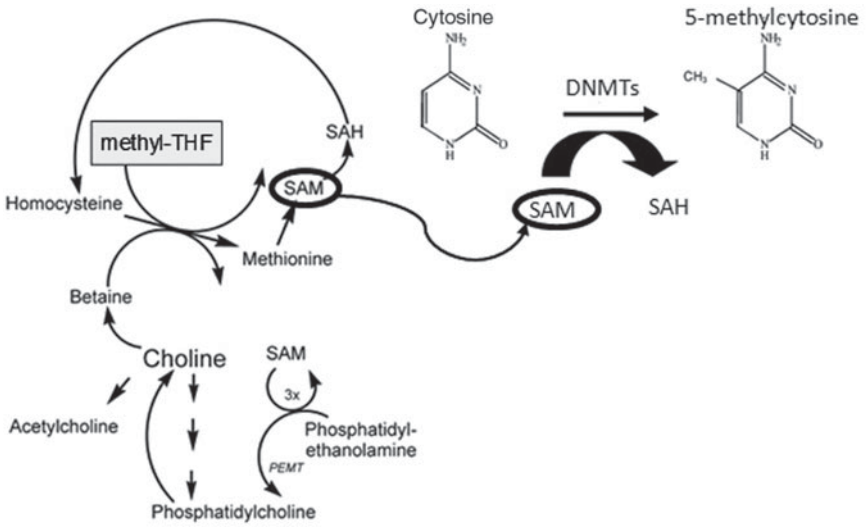

Fig. 1. Folate is linked, through the one-carbon cycle, to the process of DNA methylation. Folate enters the one-carbon cycle (left-hand side of the figure) through methyl-tetrahydrofolate (methyl-THF; highlighted). The one-carbon cycle re-generates *S*-adenosylmethionine (SAM; highlighted) from *S*-adenosylhomocysteine (SAH). The process of DNA methylation (top right of the figure), catalysed by the DNA methyltransferases (DNMT), requires SAM as the methyl donor, generating SAH.

transgenerational effects of nutrition *in utero* and a second because it provides data in exposed human subjects that sub-optimal nutrition *in utero* affects DNA methylation. The first study, by Burdge *et al.*, used a model of *in utero* protein restriction in the rat, which the group has shown in other related studies leads to effects on DNA methylation in the offspring[56]. Consumption by dams only of the F0 generation of the low-protein diet restricted to the period of pregnancy led to reduced DNA methylation at both the *PPARα* and glucocorticoid receptor loci in liver in male offspring of the F1 and, intriguingly, the F2 generation[57]. Transgenerational inheritance of DNA methylation patterns is a compelling, though as yet unproven, explanation for transgenerational effects on health of poor nutrition *in utero*, as now observed in the population of the Netherlands exposed at the end of the Second World War to a period of famine often referred to as the Dutch Hunger Winter[58]. The second study we highlight here revealed effects of this period of nutrient deprivation on DNA methylation; increased mean DNA methylation, compared with unexposed same-sex siblings, was measured at five loci in individuals exposed during the periconceptional period (one locus was affected only in men and an effect at a second locus more pronounced in women) and reduced mean methylation was observed at one of these same loci in a group exposed during late gestation[59].

A much smaller body of published literature addresses the topic of epigenetic remodelling by diet during later life. Some of the earliest evidence for epigenetic effects of diet, however, is based on adult rodents; more than 30 years ago Castro and Sevall reported effects of diet on the susceptibility of rat liver chromatin to digestion by micrococcal nuclease[60]. Studies in human monozygotic twins have great potential to disentangle epigenetic effects of *in utero* exposures and effects resulting from diet in later life (as well as genetic components of epigenetic responses). Groundbreaking research using such a model revealed that differences between monozygotic twins in DNA methylation and histone acetylation were more marked in older pairs and in pairs who had spent more of life separated, consistent with an impact beyond the very early life period of environmental influences, potentially including diet[61].

Folate has been the topic of much research on epigenetic actions. Folate is involved through the 1-carbon cycle in the generation of *S*-adenosylmethionine, the donor of methyl groups incorporated into DNA through the activity of the DNMT (Fig. 1), so is a prime candidate for influencing DNA methylation status. An additional focus of research on epigenetic effects of dietary components has been with respect to effects on tumour initiation and/or progression, based on robust observations made in numerous investigations over many years that the DNA methylation status of tumour tissue is highly abnormal, featuring in general global hypomethylation and regions of site-specific hypermethylation (e.g. within tumour suppressor genes)[62]. Folate supplementation decreased DNA hypomethylation in macroscopically normal rectal mucosa in some patients with single (but not multiple) polyps remaining after resection of colonic adenoma, and a parallel observation was that baseline methylation levels were inversely correlated with energy and fat intake[63]. Another study in patients with colorectal adenoma also revealed an increase in response to folate supplementation in global DNA methylation in rectal mucosa that appeared normal and the effect was observed also in leucocyte DNA[64]. A more recent and much larger scale study examined effects of folate supplementation on gene-specific DNA methylation – at the *ERα* and secreted frizzled related protein-1 (SFRP1) loci, selected for analysis on the basis of factors including increased methylation in colon cancer, ageing (*ERα*) and likely functional roles in tumour initiation (SFRP1)[5,6,65]. Whereas in this study there was

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Table 1. A summary of selected evidence for effects of specific dietary components on DNA methylation based on studies in cell culture models, rodents and human subjects

Dietary component/ intervention	Model	Observations	Reference
Methyl donors	Cell culture	Global and *p53*-specific DNA hypomethylation induced by folate-free medium in the colon adenocarcinoma cell line SW620; reversed by folic acid addition	(87)
		Global DNA hypomethylation induced by folate deficiency in NIH/3T3 and CHO-K1 (non-transformed) cell lines, but not in HCT116 and Caco-2 colon cancer (transformed) cell lines	(88)
	Rodent	DNA hypermethylation at A^{vy} allele, accompanied by shift in coat colour towards pseudoagouti, in pups of agouti mice fed methyl-supplemented diet	(89,90)
		Reversal of bisphenol A-induced DNA hypomethylation at A^{vy} allele in pups, and shift towards yellow coat colour, reversed by folate supplementation of agouti mice	(91)
	Human	Inverse correlation between DNA hypomethylation in colonic mucosa and erythrocyte/serum folate concentration in healthy subjects	(92,93)
		Global lymphocyte DNA hypomethylation induced in postmenopausal women by low folate diet; reversed by folate-supplemented diet	(94,95)
Bioactive polyphenols	Cell culture	Demethylation of *RAR*β locus by epigallocatechin-3-gallate in MCF-7 and MDA-MB-231 breast cancer cell lines	(96)
		Reversal of gene-specific DNA hypermethylation ($p16^{INK4a}$, *RAR*β and *MGMT*) in KYSE 510 cells by genistein; reversal of *RAR*β methylation and induction of corresponding mRNA expression by genistein in PC3 and LNCaP prostate cancer cell lines and by biochanin A and daidzein in KYSE 510 cells	(97)
	Rodent	Shift towards pseudoagouti coat colour, and corresponding increase in methylation at the A^{vy}locus, in agouti mice pups from dams fed genistein supplemented diet	(98)
		Reversal of bisphenol A-induced DNA hypomethylation at A^{vy} locus in agouti mouse pups by co-administration of dietary genistein *in utero*	(91)
	Human	Increased plasma homocysteine concentration and reduced folate concentration in subjects consuming coffee polyphenol chlorogenic acid (no direct measure of DNA methylation)	(99)
Zn	Rodent	Depressed immune function induced by gestational Zn deficiency in mice persisted for two generations, after Zn repletion, indicating epigenetic effect	(100)
		Global DNA hypomethylation measured in liver of rats in response to Zn-deficient diet	(101)
Selenium	Cell culture	Global DNA hypomethylation in Caco-2 and HT-29 (colon adenocarcinoma) cells induced by removal of selenium from culture medium, along with demethylation of *p53* promoter in Caco-2 cells	(102,103)
	Rodent	Global DNA hypomethylation in rat liver and colon in response to selenium-deficient diet	(104)
Vitamin A	Cell culture	Demethylation of *RAR*β*2* promoter in NB4 (promyelocytic leukaemia) cells, but not in T47D or MCF-7 (breast cancer) cells, induced by all-*trans* retinoic acid	(105,106)
	Rodent	Global hepatic DNA hypomethylation in rats in response to dietary all-*trans* retinoic acid, but not in response to retinyl-palmitate or 13-*cis*-retinoic acid	(107)
		No effect of dietary β-carotene or retinyl-palmitate on gene-specific methylation (hydroxmethylglutaryl coenzyme A reductase, *c-myc, c-Ha-ras*) in rat model of hepatocarcinogenesis	(108)

no significant effect of folate supplement compared with placebo, red blood cell folate levels were positively associated with higher levels of methylation at both loci, and a second observation of relevance in the context of the current discussion was a strong inverse correlation between protein intake and SFRP1 methylation[5].

A variety of dietary factors additional to folate, including specific micronutrients and, notably, dietary polyphenols, have been investigated as potential modulators of epigenetic status in various experimental models. Table 1 lists some selected examples of studies revealing positive effects. Use of cell line models offers a tractable approach to identifying dietary factors with the potential to affect epigenetic marking and also to probe potential underlying mechanisms. Such models have been used fairly widely to investigate effects on DNA methylation. Table 1 includes a selection of these studies. A more comprehensive summary of studies into potential epigenetic actions of specific dietary components, including studies using cell line models, is included in a recent review of the topic[66]. Details about the approaches and outcomes of this work will not be considered further here.

The mechanisms through which diet may affect epigenetic marking include, as alluded to above, effects on methyl group supply and also effects on the expression or activity of the raft of enzymes involved in epigenetic modification. Detailed exposition of these potential mechanisms, which remain to a large extent hypothetical, and the (limited) evidence for them is beyond the scope of this article, but a recent review provides a summary[66].

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The honeybee: effects of diet on lifespan mediated through epigenetic actions

The honeybee possibly offers to date the most compelling model for effects of diet on lifespan mediated through epigenetic actions. Intriguingly, larvae are not born genetically predetermined to take on the role of the queen bee but develop the phenotype as a result of the diet of royal jelly on which they are fed. Of particular relevance to the current discussion is that the lifespan of a queen bee is up to twenty times longer than that of a worker. Groundbreaking research revealed that these effects of royal jelly are mediated through its suppression of DNMT3 expression, leading to altered DNA methylation patterns; importantly it has been shown that RNA interference-mediated silencing of DNMT3 induces queen-like features, establishing a causal link[67]. Interestingly, supplementing the diet of mice with royal jelly was found to increase the age at which 50% of animals remained alive (although there was no effect on lifespan *per se*)[68]. An investigation of possible effects of a royal jelly supplement on DNA methylation in human subjects or a mammalian model may be worthwhile.

Do beneficial effects of dietary restriction include responses mediated through sirtuin 1-dependent actions on epigenetic status?

A hypothesis we are currently investigating is that some of the beneficial effects of DR may be mediated through epigenetic changes, in particular through effects on DNA methylation. This hypothesis is predicated on the spectrum of activity of Sirt1, a protein that appears to be pivotal in the longevity response to DR across the evolutionarily diverse species in which the practice appears effective[69,70], and invoking a reasonable assumption, as expounded above, that the changes in DNA methylation observed as mammals age are causal in the ageing process. Sirt1 (like its homologue Sir2 in yeast, *Drosophila* and *C. elegans*) is an NAD-dependent (class III) histone deacetylase. As noted below in more detail, Sirt1 deacetylates a broad range of substrates – not only histone proteins. However, the activity with respect to histone modifications, which are intricately linked with methylation of the associated DNA, suggest that Sirt1 activity may affect DNA methylation. Particularly compelling evidence that Sir2 is an essential mediator of increased lifespan in response to DR in simpler model organisms includes the abolition of the response in mutants of yeast[32], *C. elegans* [71] and *Drosophila* [72] that do not express the protein. The prevailing view is that DR increases Sirt1 expression in mammals; DR increased Sirt1 expression in several rodent tissues, including liver[73,74], and in human skeletal muscle[75]. Several metabolic and physiological effects of DR were mimicked in a transgenic mouse model in which Sirt1 expression was increased in several tissues, including brown and white adipose tissue and brain. These effects included reduced fasting levels of insulin, glucose and cholesterol and reduced adiposity[76]. In a different mouse model of Sirt1 gain-of-function, ubiquitous expression of the transgene at 2–3-fold above endogenous levels improved glucose tolerance due to increased hepatic insulin sensitivity on a background predisposing to diabetes (high fat diet or backcrossed on a *db*/*db* (leptin receptor-deficient) background)[77]. As noted above, Sirt1 is catalytically active with respect to a range of substrates (additional to histone proteins). Potential downstream targets of Sirt1 activation whose deacetylation may contribute to lifespan extension are numerous and include substrates such as p53 and the transcriptional co-activator PGC-1α [70] as well as many other transcription factors[78]. Other effects of Sirt1 relevant to effects on lifespan are unrelated to deacetylation activity, including regulation of insulin secretion through binding to the promoter of the mitochondrial uncoupling protein 2 gene (*UCP2*)[79]. The mechanisms through which effects of Sirt1 activation on such targets then translate into effects on ageing and/or lifespan remain unclear.

In spite of the compelling link between Sirt1 activity and epigenetic status, there appears to be a paucity of published observations relating either to epigenetic effects of DR or epigenetic actions of Sirt1, indicating a need for further research in both areas. With respect to the former, documented responses include transient global DNA hypomethylation in liver and suppression of age-dependent changes in methylation of the *c-myc* oncogene in mice under conditions of DR[80] and hypermethylation in response to DR of the *c-Ha-ras* oncogene in rat pancreatic acinar cells[81]. Epigenetic actions of Sirt1 in the broadest sense are indicated by a variety of published observations. For example, Sirt1 was found to be associated with promoters of tumour suppressor genes only in breast and colon cancer cell lines in which the corresponding genes were hypermethylated and silenced, and not in cell lines where the same genes showed more normal patterns of methylation and expression[82]. In another study, use of a selectable (through ganciclovir sensitivity) E-cadherin promoter-reporter construct integrated into the MB-MDA-231 breast cancer cell line demonstrated repression of reporter gene expression (and thus ganciclovir resistance) to result from methylation of the exogenous E-cadherin promoter co-incident with the accumulation at the methylation site of Sirt1 and DNMT1 and DNMT3[83]. As a final example, Sirt1 binding to specific genes in mouse ES cells was associated with repressed expression of the corresponding transcript and, relevant to epigenetic effects of Sirt1 in ageing, Sirt1 target genes were significantly over-represented among genes up-regulated in the ageing mouse brain[84].

A schematic figure that articulates our current hypothesis that some of the effects of DR that delay or reverse some of the physiological changes associated with ageing are through Sirt1-mediated effects on epigenetic status is presented as Fig. 2.

We are addressing this hypothesis through a number of approaches. *In silico* analysis of overlaps between groups of mouse genes that bind Sirt1, show a change in expression in response to DR or have been identified as undergoing a change in methylation status in older compared with younger animals revealed that all overlaps were greater than expected by chance, lending overall support to our hypothesis[85]. We have developed a cell line model of

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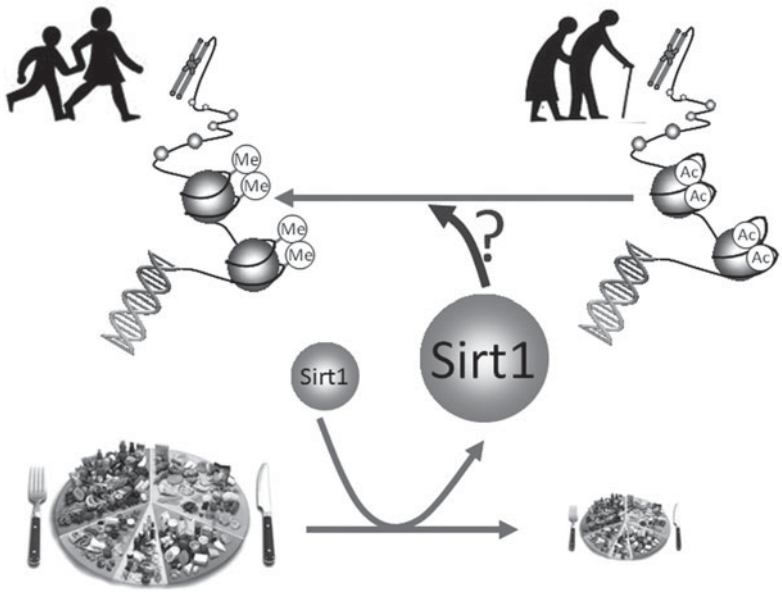

Fig. 2. Schematic representation of a hypothesis of sirtuin 1 (Sirt1)-mediated maintenance of epigenetic status in response to dietary restriction (DR). We propose that changes in DNA methylation (and other epigenetic markings, such as histone acetylation) that accompany and may be causal in the ageing process are reversed or prevented through epigenetic actions of the histone deacetylase Sirt1, which is up-regulated in response to DR.

SIRT1 overexpression and knockdown, which we are using to confirm Sirt1-dependent changes in methylation and expression of genes highlighted through this *in silico* analysis and also to analyse the transcriptomic response to Sirt1 manipulation, to investigate if genes/pathways affected by DR and/or ageing-dependent changes in methylation status and/or with links to processes relevant to ageing are affected.

In related research, we set out to examine effects of resveratrol on histone modifications in Caco-2 and MCF-7 cells and observed, rather than changes in histone acetylation, a substantial reduction in histone protein expression in both cell lines that was abrogated by an ER antagonist, indicating an ER-mediated response[17]. We are currently investigating the extent to which the response of the transcriptome to resveratrol includes genes associated with the histone protein fraction that is affected by resveratrol treatment and if these genes or pathways have relevance to ageing or ageing-related phenomena, to gain insight into whether or not this effect on histone protein expression is a major mechanism through which resveratrol may have an impact on healthy ageing.

Summary, conclusions and future priorities

Bodies of evidence that (i) the epigenetic architecture, and in particular DNA methylation, changes as individuals age, (ii) diet has an impact on ageing and (iii) DNA methylation is affected by diet coalesce to indicate that diet may affect the ageing process through epigenetic effects. The practice of DR in particular is robust with respect to extending lifespan and/or promoting 'healthier ageing' and likely epigenetic actions of a central mediator of the effects of DR, Sirt1, suggest a hypothesis that some of the effects of DR that delay or reverse some of the physiological changes associated with ageing are through Sirt1-mediated effects on epigenetic status. Our preliminary, unpublished results lend support to this hypothesis. We hope that the work will reveal new gene targets or pathways to investigate as important mediators of the ageing process that are labile to dietary influences. The hypothesis itself and relevant data remain somewhat crude, in that there is a need in the future to consider the extent to which such interactions may be tissue-specific. Evidence for tissue-specific DNA methylation profiles, including methylation marks that are labile with respect to ageing and environmental influences, including diet, is emerging[9,86]. There will be a need to identify also which specific epigenetic 'signatures' are relevant to healthy ageing and the extent to which dietary measures can have a positive effect on these profiles, perhaps guiding efforts to identify dietary or pharmaceutical mimetics of the effects of DR. The increasing availability in the public domain of high volume, high-density data on DNA methylation and how it is affected by ageing and/or environmental exposures is an opportunity for hypothesis-driven research to understand better how we may use dietary practices to slow this aspect of the ageing process, perhaps then guiding intervention studies. These ideas are based on an assumption that epigenetic changes are causal in the ageing process, and not simply a parallel phenomenon. There is thus an urgent need to better unravel links between cause and effect with respect to changes in epigenetic status with ageing. An informative approach may

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be to mimic experimentally in 'non-aged' models the changes in epigenetic marking that are observed with ageing and then test for effects on readouts of ageing. These suggested priorities highlight the technical and conceptual challenges still to meet in this area. Pharmacological tools at our disposal allow manipulation of epigenetic markings, but effects are very much more global than the changes observed in ageing. Moreover, use of such tools is, to a large extent, limited to cell line models, where identifying an appropriate readout of ageing is itself a challenge. Transgenic mouse models offer the opportunity to manipulate (tissue specific) expression of enzymes involved in modulating the epigenetic architecture, but identification of the models to best mimic features of the ageing process requires a better understanding of the mechanisms underlying ageing-associated changes in epigenetic markings. This area of research is still very much at an early stage, but offers much promise for future exploitation to guide nutritional strategies to promote health and longevity.

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