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REVIEWS: CURRENT TOPICS

DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging☆

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Abstract

Experimental studies demonstrated that maternal exposure to certain environmental and dietary factors during early embryonic development can influence the phenotype of offspring as well as the risk of disease development at the later life. DNA methylation, an epigenetic phenomenon, has been suggested as a mechanism by which maternal nutrients affect the phenotype of their offspring in both honeybee and agouti mouse models. Phenotypic changes through DNA methylation can be linked to folate metabolism by the knowledge that folate, a coenzyme of one-carbon metabolism, is directly involved in methyl group transfer for DNA methylation. During the fetal period, organ-specific DNA methylation patterns are established through epigenetic reprogramming. However, established DNA methylation patterns are not immutable and can be modified during our lifetime by the environment. Aberrant changes in DNA methylation with diet may lead to the development of age-associated diseases including cancer. It is also known that the aging process by itself is accompanied by alterations in DNA methylation. Diminished activity of DNA methyltransferases (Dnmts) can be a potential mechanism for the decreased genomic DNA methylation during aging, along with reduced folate intake and altered folate metabolism. Progressive hypermethylation in promoter regions of certain genes is observed throughout aging, and repression of tumor suppressors induced by this epigenetic mechanism appears to be associated with cancer development. In this review, we address the effect of folate on early development and aging through an epigenetic mechanism, DNA methylation.
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1. Introduction

Epidemiologic studies indicate that impaired intrauterine growth and development are associated with higher risk of cardiovascular disease, diabetes mellitus, obesity and osteoporosis in the adult life of offspring [1–6]. Among many candidate mechanisms by which *in utero* and early-life conditions affect adult health and diseases, epigenetic machinery including DNA methylation has been highlighted [7]. Epigenetics is a phenomenon that affects gene expression without altering the genomic sequence. DNA methylation is a major epigenetic phenomenon that modifies DNA by methylating cytosine bases at the carbon-5′ position in CpG dinucleotide residues and regulates gene expression and integrity. Appropriate DNA methylation is essential for embryogenesis, and early fetal development [8] as inadequate establishment of DNA methylation patterns by insufficient maternal diet such as low folate diet may induce pediatric developmental diseases and even affect health in later life [9,10].

Established epigenetic patterns during the fetal period can be changed in adult life by environmental factors including nutrition [8]. For example, identical twins possess the same genotype and no distinguishable epigenetic differences in their early life, but they show remarkable differences in

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genomic DNA methylation and histone acetylation patterns in their later life, so that these epigenetic differences may result in different gene expression and disease susceptibility [11]. This observation emphasizes the concept that the exposure to a particular environment is important during the whole life span. This review summarizes the accumulated body of knowledge for a possible mechanistic explanation on how folate affects both embryonic development and aging.

2. Folate, DNA methylation and early development

2.1. Embryonic development and DNA methylation

During embryonic development, each cell, tissue and organ acquires different prototypes of gene expression, which are thought to be mediated by epigenetic modifications such as DNA methylation. In fact, the mammalian genome undergoes profound reprogramming of DNA methylation patterns in the germ cells and the early preimplantation embryos [12]. Upon fertilization, gamete methylation patterns from parents are erased by a genome-wide demethylation event, and during the implantation, methylation patterns are newly established through *de novo* methylation [13]. These embryonic marks are important for early embryonic development and establishment of totipotency or pluripotency [14]. Interestingly, embryonic and fetal exposure to nutrients, which are mainly maternally derived, can affect this dramatic epigenetic phenomenon, thereby affecting fetal development and even later life health status [15,16].

DNA methylation plays a crucial role in genomic imprinting as a result of repression of one allele inherited from either parent by DNA methylation. Abnormal derepression of imprinted allele can cause pediatric developmental diseases such as Prader–Willi syndrome and cancer disease in later life [17–19]. Even though the parental methylation in imprinted genes escapes from the process of demethylation and *de novo* methylation and a number of imprinted genes remain imprinted throughout the life, it is noteworthy that many genes are imprinted in a tissue-specific manner or during a limited period, indicating that genomic imprinting by DNA methylation is reversible [20]. Most recently, a study using a DNA methyltransferase 1 (*Dnmt1*) conditional knockout mouse model demonstrated that a lack of maternal and zygotic Dnmt1 proteins induces demethylation of imprinted *loci* in blastocysts, indicating that Dnmt1 proteins are a prerequisite for the maintenance of methylation imprints in preimplantation embryo [21]. Thus, alterations in genomic imprinting by maternal diets that are known to affect DNA methylation may also result in pediatric developmental diseases as well as diseases in their life.

2.2. Maternal diet for the development of honeybee

An excellent model demonstrating the effect of maternal diet on the offspring's phenotype is the honeybee model. Female larvae fed different diets develop to either worker or queen bees, even though they have identical genomic sequences [22]; a royal jelly fed to larvae by young nurse bees let female larvae develop as queens and other larvae fed usual bee bread develop as worker bees. Adult queens are quite different from workers in morphology, reproductive capability, behavioral repertoires and life span. This developmental difference seems to depend on the different expression of an entire collection of genes involved in larval fate [23–26], as microarray analysis demonstrated that 240 genes were differentially expressed between queens and workers [24]. In workers, developmental genes were more up-regulated, while in queens, physiometabolic genes were more up-regulated, including genes for metabolic enzymes, mass-transforming processes and the general growth of the organism [24]. This difference in gene activation can be explained by an epigenetic mechanism, DNA methylation. Recently, Kucharski et al. [27] suggested that DNA methylation determined by nutritional input plays a critical role for different developmental fates between fertile queens and sterile workers. Silencing the expression of DNA methyltransferase 3 (*Dnmt3*) by injection of small interfering RNA (siRNA) in pooled larvae induced the development of large ovary like virgin queens raised on royal jelly. Interestingly, DNA methylation in CpG islands of *dynactin p62* gene, which is known to be methylated during development in social insects [28], is significantly decreased in both queen larvae and siRNA-treated larvae compared with worker larvae [27]. This study is helpful for the understanding of the potential nutritional effects on reprogramming in early development in humans because honeybees also have three functional DNA methyltransferases (Dnmts) and with similar *in vivo* properties to human Dnmts [28,29].

2.3. Methyl donor nutrients for mouse phenotypes

The viable yellow agouti (A^{vy}) mouse is a good animal model to determine the effect of methyl donor nutrients such as folate, methionine and choline on the phenotype of offspring. This model is in fact easy to control, and the phenotypes determined by DNA methylation status at the metastable epiallele A^{vy} are clearly definable (e.g., coat color and obesity) [30]. Metastable epialleles are expressed variably in genetically identical individuals by epigenetic modifications during development [16,31], and the A^{vy} allele is the first metastable epiallele known to be influenced by nutrition [32,33]. The origin of metastable epiallele is not yet known, but its characteristic has been regarded as similar to that of transposable elements (transposons) [10], which are regarded as remnants of ancestral infection and mostly silenced by CpG methylation.

In the agouti mouse model, Cooney et al. [34] demonstrated that maternal methyl supplements increase DNA methylation in the A^{vy} allele and determine the change of coat color, which then represents an indicative pattern of

future health and particularly of future development of obesity and insulin resistance. The percentage of phenotypes with more agouti coat (combined black and yellow pigment in the hair) are higher as increasing levels of methyl supplement are added to the diet. This shift in the distribution of mouse coat colors was correlated with DNA methylation of the A^{vy} allele [34]. Waterland and Jirtle [35] further demonstrated that the dietary methyl supplementation of *a/a* dams with extra folic acid, vitamin B12, choline and betaine alters the phenotype of their A^{vy}/*a* offspring via increasing CpG methylation at each of seven A^{vy} pseudoexon 1A (PS1A) CpG sites. Dolinoy et al. [36] also demonstrated that maternal exposure to bisphenol A (BPA), an endocrine-active compound that is known to cause adverse effects on the reproduction and the development of animals [37], shifted the coat color distribution of A^{vy} mice offspring toward yellow by decreasing CpG methylation in the intracisternal A particle (IAP) of the *agouti* gene. Intracisternal A particle sequences are known as endogenous retrovirus-like mobile elements, present at 1000 copies in the mouse genome [38]. Maternal nutritional supplementation such as folic acid or the phytoestrogen genistein counteracts BPA-induced DNA hypomethylation and shifts coat color distribution to black [39].

Another murine metastable epiallele, axin fused ($Axin^{Fu}$), was also examined by Waterland et al. [40]. Intron 6 of the $Axin^{Fu}$ gene has a spontaneous IAP, and the severity of the tail kinking is negatively associated with the degree of DNA methylation at the IAP in $Axin^{Fu}$ [41]. Methyl donor supplementation of female mice during gestation period increased DNA methylation at $Axin^{Fu}$, silenced the expression from the cryptic promoter and decreased the incidence of tail kinking in $Axin^{Fu}$/+ offspring [40]. This observation indicates that maternal diets may affect epigenetically more than one locus and change the various phenotypes of offspring. More interestingly, maternal methyl donor supplementation during midgestation also prevents the tail-specific loss of $Axin^{Fu}$ methylation, indicating that nutritional influences on epigenetic regulation of metastable epialleles are not limited to early embryonic development.

There is still some debate over whether maternal diet-induced epigenetic change can be inherited to the next generation. Cropley at al. [42,43] demonstrated that methyl donor supplementation of the pregnant dam shifts A^{vy} phenotypes not only in exposed fetuses but also in the fetus's offspring, implicating that maternal diet may influence succeeding generations, independently of their later changes in diet. However, a recent study reported that diet-induced hypermethylation at A^{vy} is not inherited in the female germ line [44].

2.4. Maternal folate status, DNA methylation and later life outcomes

Folate metabolism is closely associated with fetal development and growth including neural tube defects [45,46]. It has been hypothesized that inhibition of methyl transfer or reduced folate intake could increase the risk of human neural tube defects by reducing DNA methylation, based on animal studies that demonstrated the importance of adequate methyl availability during cranial neural tube closure [9,47]. During pregnancy, folate status is positively associated with DNA methylation [48]. In a recent study using a hyperhomocysteinemia rat model, Kim et al. [49] demonstrated that folate-supplemented diet increased and folate-depleted diet decreased placental DNA methylation. Placenta DNA methylation was also positively correlated with hepatic folate and hepatic *S*-adenosylmethionine, the unique methyl donor for DNA methylation [49].

The protein-restricted diet model also indicates the association among maternal folate status, DNA methylation and offspring's health. Rats fed a protein-restricted diet during pregnancy, which can induce high blood pressure and alter cardiovascular events in offspring [50,51], showed decreased promoter methylation and increased expression of the glucocorticoid receptor gene (*GR*) and peroxisome proliferator-activated receptor gene (*PPARa*) in the liver of the adult offspring along with reduced *Dnmt1* expression. However, folic acid supplementation reversed DNA hypomethylation and reduced the expression of those genes [52,53]. Sinclair et al. [54] demonstrated that methyl-deficient diet during early development in female mature sheep results in alterations of promoter DNA methylation and leads offspring to obesity, altered immune responses, insulin resistance and elevated blood pressure; these effects were most obvious in male offspring.

Two possible mechanisms to explain the epigenetic effect of maternal nutrients on phenotypes in their offspring were proposed by Waterland and Michels [55]: (1) reduced methyl availability may alter the establishment of DNA methylation at metastable epialleles by affecting either one-carbon metabolism or the activity of Dnmt1 and (2) repression of critical genes may occur during *de novo* DNA methylation in early fetal development. Moreover, this early nutritional effect can result in a permanent defect of epigenetic regulation through DNA methylation, suggesting a possible mechanism for the development of later life diseases [56].

3. Folate, DNA methylation and aging

3.1. DNA methylation and aging

Studies for the relationship between aging and DNA methylation have consistently demonstrated that aging is associated with genomic DNA hypomethylation and gene-specific promoter DNA hypermethylation in a tissue-specific manner [57–61]. Total methylcytosine contents are prone to decrease by aging, leading to genomic hypomethylation in most vertebrate tissues [62,63], whereas promoter regions tend to undergo hypermethylation in many genes (Table 1). Recently, Bjornsson et al. [64] demonstrated that global DNA methylation changes occurred over time in two

Table 1
Age-associated promoter DNA methylation

Gene	Tissues, species	Description	References
ER α	Colon, human	Hypermethylation is associated with gene inactivation	[65]
	Heart/vessels, human	Hypermethylation with aging	[66,67]
	Breast, rat	Hypomethylation with aging and tumor	[68]
	Prostate, human	Methylation increases with aging	[69,70]
IGFII	Colon, human	Methylation increases with aging	[71]
	Brain, rat	Altered methylation in aged brain DNA	[72]
	Fibroblast, human	Hypermethylation in SV40-infected aged fibroblast cell	[73]
p16INK4a	Colon, mouse	Hypermethylation with aging	[74]
	Liver, human	Multiple genes are hypermethylated in aged liver	[75]
	Stomach, human	Multiple genes are progressively methylated with aging	[76]
Msh2	Multiple organ, mouse	Hypermethylation in old breeder mice	[77]
hTERT	Peripheral, blood, human	Hypermethylated in Alzheimer disease	[78]
N-cadherin	Kidney, rat	Highly methylation in kidney but not liver	[79]
E-cadherin	Bladder, human	Hypermethylation with aging and tumor	[80]
	Stomach, human	Hypermethylation in promoter of five genes with aging	[81]
COL1A1	Periodontal ligament	Methylation decreases the gene expression	[82,83]
C-fos	Liver, human	Methylation increases with aging	[84]
RASSF1A	Kidney, human	Age is associated with methylation in kidney	[85]
	Breast, human	Methylation increases linearly between ages 32 and 55	[86]
	Lung, human	Methylation is associated with age in lung cancer	[87]

different cohorts, and this alteration may be under genetic control. This study demonstrated that 8% to 10% of individuals in both populations showed greater than 20% methylation changes over 11 to 16 years and further methylation changes of familial clustering.

Estrogen receptor (*ER*) gene was first studied to show an association between age and promoter DNA methylation [62]. Issa et al. [65] demonstrated that the *ER* gene is repressed by promoter hypermethylation in the aged human colonic mucosa, which is similar to the earliest epigenetic event that predispose to colonic tumorigenesis. However, methylation status in this gene is tissue specific; hypermethylated in aged heart, arteries, vessels [66,67] and prostate [69,70] and hypomethylated in aged breast tissue [68]. Another gene studied by the same group is the insulin-like growth factor II gene (*IGF2*) [71]. During aging, this promoter methylation also becomes more extensive and involves the originally unmethylated allele in human colon. Similar methylation patterns are reported in other tissues such as rat brain and human fibroblast [72,73].

Other genes for tumor suppression, cell cycle, apoptosis, detoxification, lipid metabolism and housekeeping [88] are also associated with altered promoter DNA methylation in aging. In a young and old mice study, aged mice colon demonstrated *p16* promoter hypermethylation compared with young mice, and the *p16* promoter methylation increase paralleled with the increased dietary folate levels [74]. Recently, multiple tumor suppressor genes including lysyl oxidase (*LOX*), runt-related transcription factor 3 (*RUNX3*) and tazarotene-induced gene 1 (*TIG1*) showed aberrant methylation in their promoter regions in normal aged liver [75] and gastric epithelium [76]. Many other genes have been studied in a variety of tissues to demonstrate associations between aging and DNA methylation (Table 1). However, longevity-associated genes such as *SIRT3*, *SMARCA5* and *CDH1* in peripheral blood did not show any significant differences in promoter methylation between young and elderly groups [78]. Evidence also indicates that the aging process induces gene silencing by other epigenetic phenomena such as histone modifications and chromatin remodeling in combination with DNA methylation [88,89].

3.2. Mechanisms for altered DNA methylation in aging

Several potential mechanisms are suggested for age-associated changes in genomic DNA methylation [62]. (1) The progressive decrement of genomic DNA methylation during aging results mainly from passive demethylation by the progressively diminished activity of Dnmt1 that maintains the hypermethylated status of heterochromatic DNA [90]. Expression of *Dnmt1* declines significantly from birth to age, and decreased Dnmt1 activity may lead to reduced replication of methylation patterns during mitosis [73]. (2) Aging *per se* increases homocysteine, a sulfur-containing amino acid formed in folate-mediated one-carbon metabolism [91]. A major cause of hyperhomocysteinemia is the disturbance of methyl transfer in one-carbon metabolism, which is known to decrease genomic DNA methylation status by increasing the cellular *S*-adenosylhomocysteine that inhibits DNA methyltransferases [92]. (3) Friso et al. demonstrated that estrogen replacement therapy in menopause women reduces total plasma homocysteine concentration and increases genomic DNA methylation of peripheral mononuclear cells [93]. This study indicates that the decline of sex hormone during aging may also reduce genomic DNA methylation. (4) Nutritional factors associated with aging are also involved in DNA hypomethylation [62]. Folate status

declines with aging due to both decreased folate intake and altered folate availability [94], and folate depletion results in DNA hypomethylation in elderly women [95,96]. Both a folate-deficient diet and a methyl donor-deficient diet are known to decrease methyl-CpG-binding protein and Dnmts, resulting in genomic DNA hypomethylation in the rat liver [97,98]. Deficiency of trace elements such as zinc and selenium can also cause genomic DNA hypomethylation by altering one-carbon metabolism in the elderly [99].

The mechanism for aging-associated promoter hypermethylation is not yet clear. In contrast to reduced genome-wide methylation in aged cells by reduced Dnmt1 activity, gene-specific promoter hypermethylation observed during aging may be linked to increased *de novo* methylation by other Dnmts such as Dnmt3b, which showed increased activity in aged and immortalized cells [100]. It is also suggested that heterochromatin, which is highly methylated and has the ability to propagate and influence gene expression in a region-specific and sequence-independent manner, may spread to euchromatin, which is less methylated and easily transcribed, over the boundary elements by aging. This encroachment of heterochromatin may progressively methylate the promoter regions of adjacent genes by aging [101].

3.3. DNA methylation, folate and life span

Lin et al. [102] demonstrated that overexpression of *Drosophila* DNA methyltransferase 2 (*dDnmt2*) could extend the life span of *Drosophila melanogaster*. The upstream-activating sequences-*dDnmt2* transgene (*UAS-dDnmt2*)-inserted fly, which overexpresses *dDnmt2*, shows a greater mean life span compared with controls. Although the functional role of the Dnmt2 proteins is still unclear and Dnmt2 is more known to mediate RNA methylation [103], this experiment implies that DNA methylation might affect aging or longevity of eukaryotes. Interestingly, mice with disruption of proliferation-associated SNF-2-like gene (*PASG*), which displayed a loss of 33% to 43% of total methylcytosine, demonstrated cellular senescence and an age-associated phenotype, indicating that altered DNA methylation can result in cell senescence and reduced longevity [104,105].

Telomere shortening is associated with reduced human life span and has been proposed as a mechanism of age-associated disorders such as cancer [106]. Increasing amounts of evidence suggest that telomere length is regulated by neighboring histone modifications [107–109] and DNA methylation of the subtelomeric region adjacent to the telomere, which is composed of repeated $(TTAGGG)_n$ sequences lacking CpG dinucleotides necessary for Dnmts [110]. During aging, subtelomeric regions become hypomethylated due to decreased Dnmt activity, resulting in telomere shortening [110].

Homocysteine is known to accelerate the onset of endothelial progenitor cell senescence by the suppression of telomerase activity, which results in shortening of telomere [111]. A recent population-based cohort study demonstrated that plasma homocysteine concentrations are negatively correlated with leukocytic telomere length [112]. Based on these observations, it can be hypothesized that a low folate diet or methyl-deficient diet, both of which can induce hyperhomocysteinemia, might also be involved in the shortening of telomere length. However, it is not known whether the effects of hyperhomocysteinemia on telomerase activity or the length of telomere are conveyed through genomic DNA hypomethylation or promoter DNA hypermethylation [113].

Low dietary folate can alter, genetically, the sequence and length of telomere [114]. Under low folate condition, thymidine-rich telomere sequences are prone to DNA strand breaks, which may lead to shortening of telomere length. It is known that folate deficiency increases uracil misincorporation into DNA by reducing the synthesis of thymidylate, thereby producing DNA strand breaks by simultaneously increased activity of uracil glycosylases and reduced DNA repair [113].

3.4. DNA methylation, an epigenetic transition between aging and cancer

A possible epigenetic link between aging and cancer has been suggested because genomic DNA hypomethylation, promoter DNA hypermethylation and altered *Dnmt* expression can occur in both aging and cancer [115]. Toyota et al. [116] demonstrated that a similar pattern of promoter methylation in colon cancer clones can occur in normal colonic cells during the aging process. Since epigenetic silencing of tumor suppressor genes by promoter DNA hypermethylation can initiate tumorigenesis [117,118], it appears that progressively accumulated promoter hypermethylation by aging could induce cellular transformation to malignancy [90].

Genomic DNA hypomethylation, which frequently occurs during aging, is also observed in many malignant tissues, but the role of aging-associated genomic hypomethylation in carcinogenesis is not yet fully understood [115]. Decreased DNA methylation is thought to promote chromosomal instability, eventually leading to carcinogenesis [119,120]. Genomewide DNA hypomethylation also affects transcription through loss of imprinting [121], ectopic oncofetal gene expression and up-regulation of silent genes, all of which might induce tumor development [8,122].

3.5. Interactions between folate and aging in cancer

As a coenzyme in one-carbon metabolism, folate participates in both nucleotide synthesis (genetic pathway) and DNA methylation (epigenetic pathway) [94]. Fig. 1 shows a complex mechanism through which folate and aging interact genetically and epigenetically, thereby promoting cancer development.

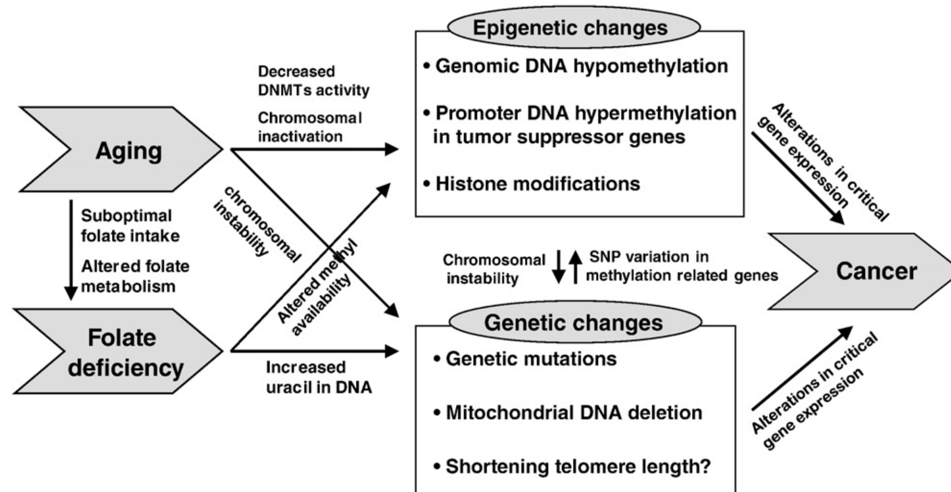

Fig. 1. Genetic and epigenetic interactions between aging and folate deficiency in carcinogenesis.

Inadequate folate intake reduces the synthesis of thymidylate from deoxyuridylate, resulting in excessive uracil misincorporation into DNA, consequently leading to mutagenesis [123,124]. Choi et al. [125] demonstrated that elder rats increased uracil content in colonic DNA compared with young rats, and dietary folate depletion further increased uracil misincorporation. Aging and folate also affect the integrity of mitochondrial DNA, the instability of which is thought to play an important role in tumorigenesis [126–128].

In one-carbon metabolism, folate depletion decreases *S*-adenosylmethionine, the universal methyl donor, and increases *S*-adenosylhomocysteine, an inhibitor of methyltransferases. Aberrant DNA methylation mediated by folate depletion has been regarded as one candidate mechanism that explains the association between folate and cancer [94]. Since aging by itself affects DNA methylation, a synergistic effect between folate status and aging on DNA methylation has been investigated. In the elder rat liver, folate supplementation increases genomic DNA methylation in a dose-dependent manner [129]. Keyes et al. [74] demonstrated that genomic DNA methylation and promoter methylation of *p16* increased in parallel with an increment of dietary folate levels in old mice colon but not in the young. Interestingly, DNA methylation may interact with genetic mechanisms because (1) genomic DNA hypomethylation is associated with instability of chromosome and DNA [130,131] and (2) specific allelic sequence variants such as single-nucleotide polymorphisms (SNPs) may act in *cis* or *trans* to influence DNA methylation [132]. Genetic variations in genes that regulate epigenetic phenomena such as *Dnmt* genes or polymorphisms of genes involved in one-carbon metabolism such as 5,10-methylenetetrahydrofolate reductase gene (*MTHFR*) can affect DNA methylation [133–136]. In another example, a G>C SNP in the *LRP1B* promoter creates an additional CpG site and increases the probability that this allele becomes methylated [137].

4. Conclusions

As seen in the experimental models, maternal diet can affect the offspring's phenotype as well as the risk of disease development at the later life. The amount of methyl donors in the maternal diet is especially critical for embryonic development by affecting DNA methylation in animal models. In this regard, folate, which ultimately transfers methyl groups for DNA methylation, is expected to significantly influence the extent of DNA methylation reprogramming during the embryonic development period that is particularly vulnerable to the low methyl availability.

During aging, genomic and gene-specific DNA methylation can be altered in a tissue-specific manner. Folate status further modifies DNA methylation in the elderly. It appears that aging and folate deficiency synergistically provide an epigenetic milieu toward cancer development.

For healthy embryonic growth and aging, as well as for the prevention of aging-associated chronic diseases, proper folate status is needed to secure optimal DNA methylation status. Further studies to investigate the effect of folate on fetal epigenetic programming and aging focused on the role of DNA methylation and other major epigenetic phenomena will provide us with a better understanding of maintaining health and preventing chronic diseases.

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