Epigenetics in male reproduction: effect of paternal diet on sperm quality and offspring health

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Abstract | Epigenetic inheritance and its underlying molecular mechanisms are among the most intriguing areas of current biological and medical research. To date, studies have shown that both female and male germline development follow distinct paths of epigenetic events and both oocyte and sperm possess their own unique epigenomes. Fertilizing male and female germ cells deliver not only their haploid genomes but also their epigenomes, which contain the code for preimplantation and postimplantation reprogramming and embryonal development. For example, in spermatozoa, DNA methylation profile, DNA-associated proteins, protamine 1:protamine 2 ratio, nucleosome distribution pattern, histone modifications and other properties make up a unique epigenetic landscape. However, epigenetic factors and mechanisms possess certain plasticity and are affected by environmental conditions. Paternal and maternal lifestyle, including physical activity, nutrition and exposure to hazardous substances, can alter the epigenome and, moreover, can affect the health of their children. In male reproductive health, data are emerging on epigenetically mediated effects of a man's diet on sperm quality, for example through phytochemicals, minerals and vitamins, and nutritional support for subfertile men is already being used. In addition, studies in animal models and human epidemiological data point toward a transgenerational effect of the paternally contributed sperm epigenome on offspring health.

Epigenetics
The study of mechanisms that regulate gene expression without changing the underlying DNA sequence, for example, gene silencing through addition of methyl groups to DNA and/or to histones.

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doi:10.1038/nrurol.2016.157
Published online 31 Aug 2016

Until around 5 years ago, sperm cells were generally presumed to deliver only their genome to the oocyte at fertilization. However, in subfertile couples, only 15–30% of male partners with idiopathic infertility carry genetic alterations, such as chromosomal aberrations and Y chromosome microdeletions[1]. In addition, if the DNA sequence was the only information transferred at fertilization, the existence of various but specific transcription programmes in the 30–40 trillion cells (including different populations of germ cells) that make up the human body and share exactly the same genes remains unexplained. Since the importance of epigenetics was recognized at the turn of the millennium, the influential role of the cell-specific epigenetic landscape in the coordination and fine-tuning of gene expression has become clear. This mechanism enables the transformation of the same genome into hundreds of different transcriptomes and might explain the diversity of transcriptomes of human cells. Moreover, this new research impressively demonstrates the plasticity of the epigenetic landscape, which makes genes responsive to changing environmental conditions. Inheritance of epigenetic signatures guarantees the maintenance of every single cell-specific epigenome and corresponding transcriptome throughout the generations.

Many existing review articles describe negative effects of the environment (for example, radiation, heavy metals or endocrine disrupters) and lifestyle factors (for example, alcohol, caffeine or nicotine) on general health and risk of cancer, but some also discuss effects on reproductive health[2–10]. In this Review, we summarize current knowledge of sperm-specific epigenetic signatures and discuss epigenetically mediated effects of a man's diet on sperm quality, focusing on DNA methylation and histone modification processes. We also review the transgenerational effects of the paternally contributed sperm epigenome on offspring health.

Sperm-specific epigenetic signatures

Sperm cells show many characteristic epigenetic features[10–12] (FIG. 1). An important phenomenon in haploid spermatids is the replacement of the majority of DNA-binding histones by protamines[13]. This process can be considered a sperm-specific epigenetic mechanism, as

Key points

- Spermatozoa have a unique epigenetic signature, consisting of their DNA methylation profile, DNA-associated proteins, protamine 1:protamine 2 ratio, nucleosome distribution pattern, post-translational histone modifications, stored RNA and nonhistone and nonprotamine proteins
- Dietary compounds, especially phytochemicals, minerals and vitamins, can effect changes in epigenetic signatures of somatic as well as germ cells by influencing enzymes and other proteins responsible for epigenetic modifications
- Modifications of the epigenetic landscape by dietary compounds can affect overall health but also the reproductive health of both sexes
- Studies in animal models and human epidemiological data point toward a transgenerational effect of parental nutrition on offspring health
- Male germ cell development can be divided into distinct stages, each representing a time window of susceptibility to epigenetic alterations, resulting in specific epigenetic changes in descendants and their phenotypes

protamine-induced high-order chromatin packaging results in a global stop of transcription. Thus, sperm relies on epigenetic regulation more than any other cell.

In 2014, the existence of a protamine code in addition to the well-known histone code in sperm cells has been suggested[14]. A meta-analysis published in 2016 demonstrated that abnormal histone–protamine exchange followed by protamine deficiency is related to both increased sperm DNA damage and male subfertility[15]. Analysis of data from 12 studies found a significant association between protamine deficiency and DNA damage ($n = 845$; $P < 0.001$). The relationship between protamine ratio and male fertility was analysed using data from nine studies, demonstrating a significantly higher protamine ratio in subfertile men compared with healthy volunteers ($n = 633$ versus 453, respectively; $P < 0.00001$)[15]. The replacement of histones by protamines might also affect the epigenetic landscape in sperm, as aberrant protamine levels in oligozoospermic men have been correlated with an altered DNA methylation pattern at seven imprinted genes (*KCNQ1OT1*, *MEST*, *SNRPN*, *PLAGL1*, *PEG3*, *H19*, *IGF2*)[16].

Interestingly, around 5–10% of the haploid paternal genome are excluded from the protamine replacement and remain arranged in nucleosomes[17,18]. Retained sperm histones exhibit a variety of epigenetic signatures that can be paternally inherited by the offspring[19,20] and affect DNA access of transcription factors in the early embryo[21]. Whole-genome analyses revealed that the remaining nucleosomes are not randomly distributed throughout the genome. Early studies found enrichment of sperm nucleosomes at loci of developmental importance and imprinted genes[17,22–24], but two studies published in 2014 have shown that sperm nucleosomes are primarily localized in gene-poor regions and are associated with retrotransposable long and short interspersed nuclear elements (LINEs and SINEs, particularly LINE1)[18,25]. The discrepant findings between previous and current studies might be explained by different protocols for nuclease treatment of the isolated sperm genome. Sperm nucleosomes retained at developmental important loci might be more stable to nuclease digestion, possibly indicating the existence of specific histone variants and modifications at these locations. However, consensus exists in so far that epigenetic signals on sperm histones are thought to be transferred to the oocyte and to be involved in the regulation of gene expression in the early embryo[26,27].

Another feature of germ cells is the epigenetic tagging of imprinted genes, resulting in the expression of only one allele in a parent-of-origin-dependent manner. Parent-specific imprinting signatures must be established in the germline, maintained throughout life and, after fertilization, erased and re-established in the germline of the next generation[28]. In male germ cells, four imprinting genes have been studied in detail in the mouse, namely *Igf2* and *H19* (REFS 29,30), *Rasgrf1* (REF. 31) and *Gtl2* (REF. 31). As has been demonstrated for paternally expressed *Igf2* domain and maternally expressed *Kcnq1*, the silent allele has hypermethylated promoters associated with histone H3 trimethylated at Lys9 (H3K9me3) and histone H4 trimethylated at Lys20 (H4K20me3), whereas the active allele has hypomethylated promoters associated with acetylated histones H3 and H4, as well as histone H3 dimethylated at Lys4 (H3K4me2) and histone H3 trimethylated at Lys4 (H3K4me3)[32].

Unlike somatic cells, germ cells have hypomethylated DNA. Using restriction landmark genomic scanning, 2,600 gene loci distributed randomly throughout the genome have been analysed in the mouse[33]. The DNA methylation profile in testicular tissue showed eightfold more hypomethylated gene loci relative to that of somatic tissue. Further analyses demonstrated that hypomethylated regions are generally located within nonrepetitive sequences outside gene promoters and are correlated with GC-rich DNA sequences[34]. Subsequent studies in chimp and human sperm revealed that centromere regions, but also LINE1s, are hypomethylated and associated with preserved nucleosomes[18,35].

Hypermethylation in germ cells has been shown to affect human male fertility. For example, hypermethylation of the *CREM* promoter in spermatids results in an abnormal protamine 1:protamine 2 ratio and is associated with incomplete sperm chromatin compaction, reduced sperm motility and male subfertility[36]. Moreover, in a study in 63 men whose female partners took part in an *in vitro* fertilization (IVF) programme, genome-wide hypermethylation of sperm DNA was associated with pregnancy failure ($P < 0.05$)[37].

In addition to histone modification and DNA methylation, sperm RNA can act as an epigenetic regulator. Although spermatozoa are transcriptionally inactive cells[38], they contain both mRNAs[39–41] and noncoding RNAs (ncRNAs), including microRNAs (miRNAs)[41], small interfering RNAs (siRNAs)[42,43] and piwi-interacting RNAs (piRNAs)[44,45]. Although sperm RNA is transmitted to the oocyte[46] and inhibition of sperm-delivered miRNAs in mice results in developmental delay in the zygote[47], to what extent sperm RNA contributes to the regulation of gene expression in the early embryo is still being debated. Two studies reported that injection of sperm RNA from obese mice into normal one-cell embryos generated altered gene expression in the early embryos, which was followed by metabolic

Protamines
In haploid male germ cells, histones are replaced by arginine-rich proteins termed protamines, resulting in high-order nuclear chromatin compaction.

Imprinted genes
Genes that are expressed in a parent-of-origin-dependent manner depending on genomic imprinting. For example, if the paternally inherited allele is imprinted (for example, silenced due to methylated cytosines within the gene promoter) only the maternal allele is expressed.

Nucleosomes
A 147 bp DNA sequence wound around a histone octamere that consists of two molecules each of histones H2A, H2B, H3 and H4.

LINES and SINEs
In repetitive DNA, short interspersed nuclear elements (SINEs, containing 100–500 bp) and long interspersed nuclear elements (LINEs, containing 6–8 kbp) make up ~52% of all known repeat elements, which are mainly localized in heterochromatin.

Epigenetic tagging
Addition of methyl or acetyl groups to DNA and/or histones by specialized enzymes results in specific epigenetic signatures that can act as a 'cellular memory' when inherited by offspring.

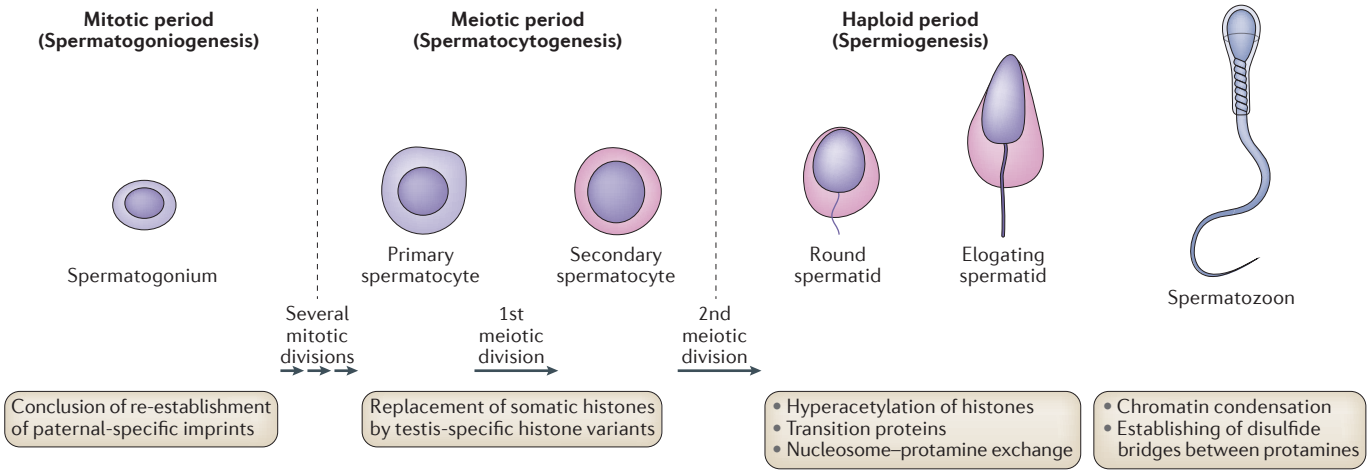

Figure 1 | **Epigenetic processes during male germ cell development.** Spermatogenesis can be divided into a mitotic, a meiotic and a haploid period. Re-establishment of paternal-specific imprints mainly takes place in primordial germ cells and concludes in spermatogonia. During meiosis, most of the somatic-type histones are exchanged for testis-specific histone variants. During spermiogenesis, complete reorganization and extensive condensation of the nuclear chromatin occur, involving the replacement of most nucleosomes by protamines.

disorders in adult animals[48,49]. These findings suggest an important role of sperm-derived RNAs in the epigenetic regulation of gene expression in the early embryo.

Diet and male reproductive epigenetics

Studying effects of diet on epigenetic signatures. When interpreting possible effects of dietary factors on epigenetic signatures a number of confounding factors have to be considered in general. For example, the magnitude of change produced by a specific food ingredient is in itself small, but cumulative. As a consequence, results of short-term nutritional studies (~3 months) might differ from those of long-term nutritional studies (~6 months). In addition, differences between effects in various cell types and tissues might exist, owing to cell-specific epigenetic landscapes. Furthermore, when using whole food products instead of single food ingredients in a study, other ingredients within the same product might interact with the factor to be investigated. Exercise can have a considerable effect on DNA methylation profiles and might, therefore, influence results in individuals adhering to a healthy diet, as these individuals are also likely to take care of adequate exercise[50]. In addition, epigenetic signatures can also be affected by gut bacteria, as these microorganisms produce bioactive substances that are involved in epigenetic regulation, such as acetyl coenzyme A (acetyl-CoA)[51], biotin[51], butyrate[52] and folate[53]. Furthermore, our gut microbiota contributes to the absorption of minerals, such as selenium and zinc, which are essential cofactors of enzymes that participate in epigenetic processes[54].

Dietary effects on epigenetics of germ cells. Regarding reproductive health, studies in mice have shown that dietary factors can change the epigenetic landscape in germ cells, and sperm can pass on altered epigenetic signatures to the offspring[20,55,56]. However, a direct effect of dietary factors on the sperm epigenome is unlikely, as sperm cells carry protamine-packaged high-order nuclear chromatin, are transcriptionally inactive and fully differentiated without any further cell division. Thus, the primary target cells of epigenetic modification are not the mature spermatozoa within the epididymis but the developing germ cells within the testis. In men, the development of a spermatogonial stem cell into mature spermatozoa takes 64 days[57], followed by a 2-week epididymal maturation period; hence, any effect of a change in dietary habits on sperm quality will not be perceptible for 3 months after the change.

In addition, in the era of assisted reproductive technology (ART), the considerable differences in the embryo's environment between IVF and natural conception have to be considered. The environmental exposure might alter epigenetic signatures and negatively affect the health of the embryo in adult life. In mice conceived via IVF, an elevated blood pressure[58], increased fasting glucose, impaired glucose tolerance and aberrant insulin signalling[59], as well as altered gene expression in liver, pancreas, muscles and adipose tissues[60] have been reported. Elevated blood pressure and fasting glucose levels have also been observed in IVF-conceived children[61]. Of note, different sperm cells within an ejaculate exhibit variable epigenetic landscapes, which is relevant in case of intracytoplasmic sperm injection (ICSI), during which a single sperm cell is transferred into the oocyte. Applying CpG island microarray analysis, numerous DNA-methylation-variable positions have been identified in human spermatozoa, with the highest degree of variation occurring in promoter CpG islands and repetitive elements[62].

Epigenetic regulators and dietary compounds. Dietary factors do not directly change epigenetic signatures but act on enzymes that add or remove epigenetic tags to or from DNA and histones (FIG. 2). DNA methyltransferases (DNMTs) add methyl groups to cytosine residues within CpG islands of gene promoters. Specifically,

Restriction landmark genomic scanning
A method to visualize differences in DNA methylation levels across the genome, consisting of DNA digestion by restriction enzymes followed by radioactive labelling and 2D electrophoresis.

Microbiota
The sum of all microorganisms hosted by an individual in an environmental niche.

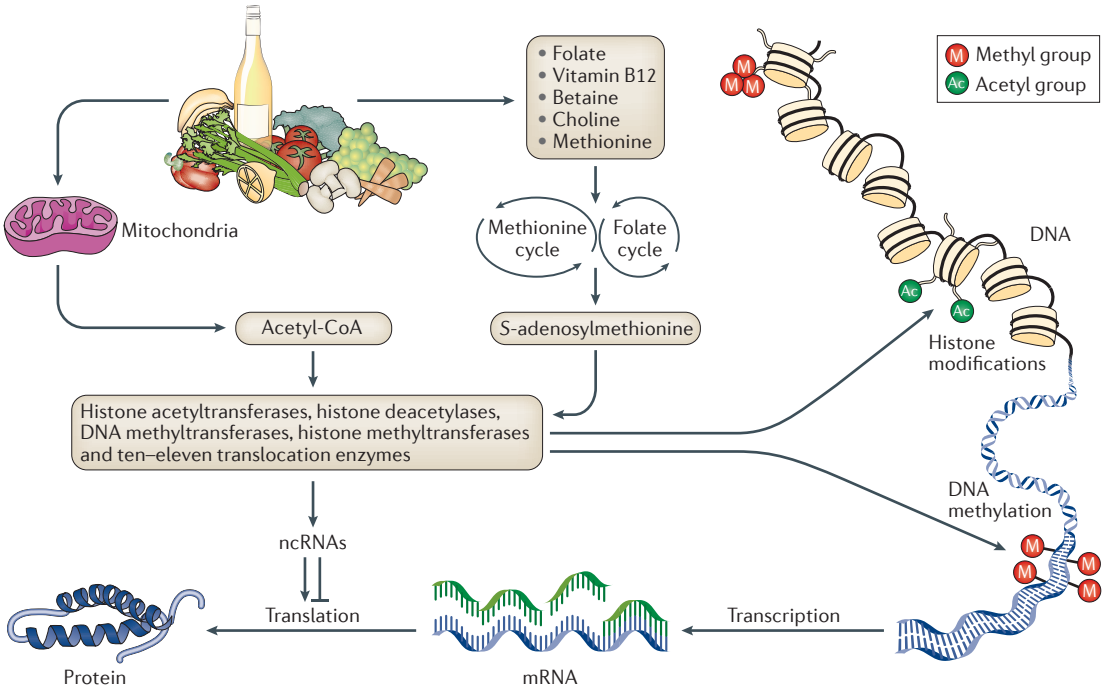

Figure 2 | **Effects of diet on the main epigenetic processes.** Epigenetic regulation of gene expression can occur at the transcriptional level via DNA methylation and/or histone modifications and the translational level via noncoding RNAs (ncRNAs). Methyl and acetyl groups required for these processes are derived from components of the daily diet. Some dietary factors, such as folate, vitamin B_{12}, betaine, choline and methionine, are methyl donors that transfer a methyl group to S-adenosylmethionine in the folate and methionine cycles (one-carbon metabolism). Acetyl groups are generated in the mitochondria (for example via oxidative decarboxylation of pyruvate and β-oxidation of fatty acids) and transferred to acetyl-CoA. Methyl and acetyl groups are transferred to DNA and/or histones by DNA methyltransferases, histone methyltransferases and histone acetyltransferases.

DNMT1 maintains existing methylation profiles after replication by adding methyl groups to hemimethylated CpG sequences[63], DNMT3a and DNMT3b perform *de novo* methylation of unmethylated CpG sequences[64]. Methylation of CpG sequences either prevents binding of transcription factors within the gene promoter[65] or promotes binding of proteins that form repressor complexes with histone deacetylases, resulting in chromatin compaction[66]. Methyl groups are provided via the folate and methionine cycles, also known as the one-carbon metabolism, resulting in the production of *S*-adenosylmethionine. Demethylation of DNA occurs either passively via cell division without *de novo* methylation or actively by ten–eleven translocation (TET) enzymes catalysing the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine. DNA hypermethylation is associated with transcriptional repression, whereas DNA hypomethylation results in transcriptional activation[67]. DNA methylation seems to be precisely regulated even when dietary methyl group donors are restricted. Mice fed a methionine-choline-deficient diet for 1 week showed reduced *S*-adenosylmethionine levels in the liver but stable DNA methylation patterns, as well as upregulated expression of *Dnmt1*, *Tet2* and *Tet3* mRNA[68]. One hypothesis is that methyl deficiency causes oxidative stress and activates the DNA repair pathway involving active DNA demethylation. As methylation levels within a cell are strictly regulated, decreasing DNA methylation results in *Dnmt1* upregulation; however, this mechanism fails if the methyl-deficient diet lasts for >9 weeks[69].

Histone acetyltransferases add acetyl groups provided by acetyl-CoA to lysine residues within the N-terminal end of histones, resulting in local chromatin expansion and a transcriptionally active gene region[70,71]. By contrast, removal of acetyl groups by histone deacetylases leads to chromatin condensation and transcriptionally inactive gene regions[72]. Histone methyltransferases add methyl groups provided by the one-carbon metabolism to lysine residues (1–3 methyl groups) and arginine residues (1–2 methyl groups), and histone demethylases remove methyl groups[70,73]. In contrast to DNA methylation, histone methylation is associated with either repression or activation of gene expression depending on the position and the number of methylated amino acid residues. Methylation of histone H3 at Lys4 (H3K4), Lys36 (H3K36) and Lys79 (H3K79) has an activating effect on gene expression, whereas methylation of histone H3 at Lys9 (H3K9) and Lys27 (H3K27) is associated with gene silencing[73–75].

The influence of several epigenetic processes and regulators on epigenetic signatures during spermatogenesis and possible effects on male fertility have been investigated. For example, analyses of testicular biopsy samples from subfertile men showed a precocious hyperacetylation of histone H4 in spermatocytes

One-carbon metabolism
Folate and methionine cycles constitute a one-carbon metabolism, as only one carbon group is transferred, for example, a methyl group via *S*-adenosylmethionine.

instead of spermatids[76]. In mice, application of the histone deacetylase inhibitor trichostatin A was associated with male infertility, which could be reversed by trichostatin A withdrawal[77]. Reduced expression of the histone deacetylase NAD-dependent protein deacetylase sirtuin 6, encoded by *Sirt6*, owing to chronic high-fat diet, resulted in an increase of histone acetylation in elongating spermatids in mice[78]. An increased expression of the lysine-specific histone demethylase 1A during spermatogenesis specifically reduced methylation of H3K4me2 in mouse sperm and resulted in reduced survivability of the offspring, suggesting that histone modifications alone, without involvement of DNA methylation, can act as mediators of transgenerational epigenetic inheritance[20]. TET enzymes also have a role in epigenetic remodelling during human spermatogenesis, suggested by the observation that 5-hydroxymethylcytosine levels decrease while 5-methylcytosine levels remain constant[79]. Furthermore, a study published in 2016 showed that TET enzymes are successively expressed during human spermatogenesis, starting with TET2 in late pachytene spermatocytes and followed by TET1 and TET3 in step 1 and step 3 round spermatids, respectively[80]. Oligozoospermic and asthenozoospermic men had reduced TET1–3 in sperm in comparison with healthy donors. TET2 levels, but not TET1 and TET3 levels, in sperm were significantly associated with pregnancy rates in couples undergoing IVF treatment ($P = 0.006$).

Potential effects of dietary compounds on epigenetics of male germ cells. Evidence is accumulating that certain micronutrients, such as minerals, vitamins and phytochemicals, have a substantial effect on overall health. In reproductive health, the primary target cells of epigenetic modification are the testicular germ cells. Dietary compounds act on histone-modifying enzymes that are present in all cell types; hence, the mechanisms affecting epigenetic signatures in differentiating germ cells are likely to be very similar to those involved in developing cancer cells, in which numerous studies reported an important role of phytochemicals in the epigenetic regulation of gene expression[81–86] (FIG. 3).

In various cancer cell lines, epigallocatechin gallate, the main polyphenol present in green tea, has been demonstrated to affect DNA methylation by inhibiting DNMT1 (REFS 87,88) or dihydrofolate reductase[89], histone modification by inhibiting histone acetyltransferases[90], histone deacetylases[91] or Polycomb group protein complexes[92], as well as miRNA expression[93].

Resveratrol, a polyphenol found in blueberries, cranberries, peanuts, grapes and red wine, has a weak inhibitory effect on DNA methyltransferase activity[94] and a specific inhibitory effect on class III histone deacetylases (sirtuins)[95] but also modifies expression patterns of miRNAs[96].

Curcumin, a polyphenol extracted from turmeric, has been reported to inhibit DNMT1 activity by blocking the catalytic thiol group of the Cys1226 binding site[97,98]. Furthermore, curcumin can modulate both histone acetyltransferase and histone deacetylase enzyme activity[99] and can suppress acetylation of nonhistone proteins[81].

Although their exact function needs further clarification, a modulating potential on epigenetic regulation of gene expression in cancer cells has also been reported for other phytochemicals: sulforaphane, phenyl isothiocyanate and indole-3-carbinol, which are present in cruciferous vegetables, such as broccoli, cauliflower and cabbage; lycopene, a terpenoid in tomatoes; organosulfur compounds (especially diallyl sulfides) present in garlic; and genistein, an isoflavone in soy beans and soy products[81–86]. In rats, chronic low-dose (equivalent to that in human diet) exposure to genistein was associated with male infertility, resulting in decreased sperm counts, reduced sperm motility and an aberrant transcriptome in testicular germ cells[100].

Apart from phytochemicals, minerals and vitamins can also affect epigenetic signatures. Several studies reported an improvement of human sperm quality through dietary supplementation with folate[101,102], vitamin C[101–103], vitamin D[104,105], vitamin E[102], β-carotene[103], lycopene[101,103] and zinc[102]. Functional studies showed that vitamin D also interacts with the epigenome, as critical genes in the vitamin D signalling pathway (for example, *VDR* encoding the vitamin D_3 receptor) have large CpG islands in their promoter regions that can be silenced by DNA methylation[106]. However, the primary epigenetic effect of vitamin D seems to be linked to histone acetylation, as the vitamin D_3 receptor interacts with cofactors that are in contact with histone acetyltransferases involved in transcriptional activation[107]. Finally, a number of genes encoding chromatin modifiers are primary targets of the vitamin D_3 receptor[108,109].

Folate is a key source of the one-carbon metabolism involved in DNA methylation[110]. In rats, both paternal and maternal folate deficiency 4 weeks before mating was followed by aberrant DNA methylation profiles, reduced folate and increased plasma homocysteine levels in livers of their offspring[111]. However, the studied animal groups were small (total $n = 28$) and pubs were observed for 3 weeks only. In one study in mice, maternal folate deficiency during pregnancy and lactation resulted in aberrant DNA methylation patterns in offspring's sperm[55]. Chronic paternal folate deficiency resulted in delayed onset of spermatogenesis and was associated with decreased pregnancy rates.

Stressed sperm and paternal obesity

In high-quality sperm, protamines protect the DNA from harmful environmental factors and safeguard the paternal genome until fertilization. Subfertile men have an incorrect histone to protamine exchange and an aberrant protamine 1:protamine 2 ratio[112,113], which results in reduced condensation of nuclear chromatin that is prone to oxidative stress and represents the predominant cause of DNA fragmentation[114–119]. Sperm DNA fragmentation, however, can result in *de novo* mutations in the embryo[120]. Thus, increasing numbers of IVF clinics offer their patients oral antioxidant therapy to improve sperm quality by reducing oxidative stress[119,121]. In one study, a 4-month daily nutritional support of a combination of B vitamins, vitamin E and zinc improved fertility of male partners in couples with at least two failed

Polycomb group protein complexes
Cluster of proteins belonging to one family that are involved in chromatin remodelling to facilitate epigenetic gene silencing.

DNA fragmentation
A hallmark of apoptosis during which endonucleases cleave chromatin into nucleosomal units representing multiples of ~180 bp.

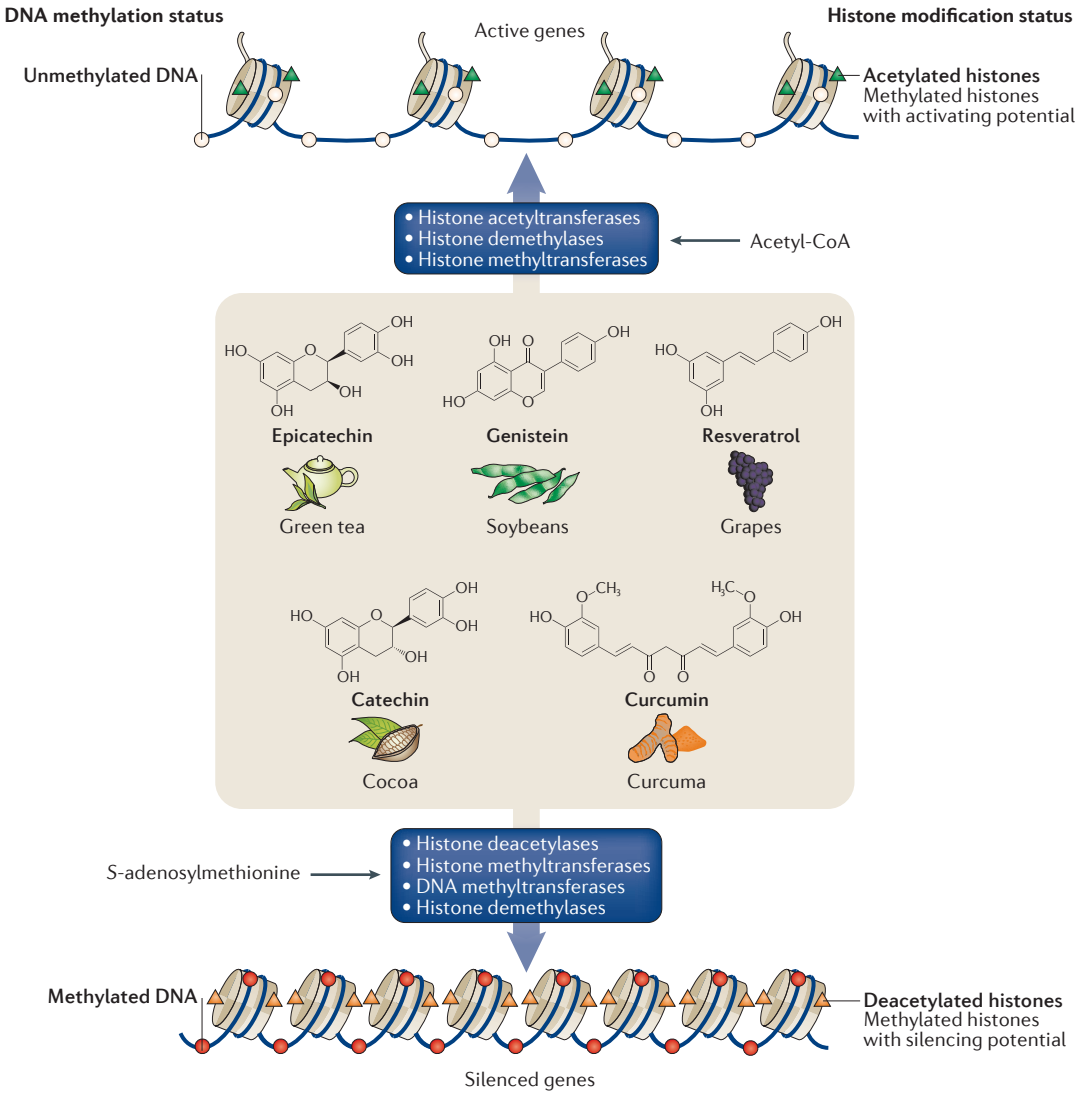

Figure 3 | **Bioactive substances that can affect enzymes involved in epigenetic processes.** Genes located in areas containing unmethylated DNA, acetylated histones and/or methylated histones with activating potential (for example, H3K4, H3K36 and H3K79) are thought of as being transcriptionally active. By contrast, genes in areas of methylated DNA, deacetylated histones and methylated histones with silencing potential (for example, H3K9 and H3K27) have decreased levels of transcription or are transcriptionally silent. Acetyl groups are provided by acetyl-CoA and added to histones by histone acetyltransferases. Methyl groups are provided by *S*-adenosylmethionine and added to DNA or histones by DNA methyltransferases or histone methyltransferases, respectively. Removal of acetyl groups or methyl groups from histones is catalysed by histone deacetylases or histone demethylases, respectively. Bioactive substances contained in phytochemicals that are part of the daily diet have the potential to inhibit one or more of these enzymes and can, therefore, influence gene expression at multiple levels.

ART attempts ($n = 84$) and, after 12 months, resulted in spontaneous pregnancies and live births in 18 couples and third ART attempts that lead to 22 pregnancies and 15 live births[122]. A Cochrane review published in 2014 analysed 48 randomized controlled trials including a total of 4,179 subfertile men aged 20–52 years and reported that antioxidant supplementation can improve both clinical pregnancy rates and live birth rates but demanded further placebo-controlled studies[123].

Paternal obesity, which in Western nations approaches an estimated 70% of the adult male population[124], is associated with reduced sperm quality[125], owing to an increased level of reactive oxygen species, resulting in increased sperm DNA fragmentation[126–130], reduced pregnancy rates[131–133] and increased miscarriage risk[133]. A meta-analysis published in 2015 of 30 articles involving 115,158 participants reported a significant correlation between male obesity and reduced reproductive potential (OR 1.66, 95% CI 1.53–1.79), as well as decreased live birth rates (OR 0.65, 95% CI 0.44–0.97)[134]. Following weight loss, an improvement of sperm count, morphology, motility and DNA integrity has been observed in both men[135] and mice[136].

In 2000, paternal obesity in humans was reported to affect body fat composition in daughters[137]. In animals, two studies found a direct effect of paternal

Reactive oxygen species
Highly reactive chemical species (radicals) formed as a natural byproduct of oxygen metabolism. During oxidative stress, levels of reactive oxygen species can increase and effect cell damage.

obesity on the metabolic health in offspring of rats[138] and mice[139], and one study demonstrated that paternal insulin resistance can be passed down to the following two generations[140]. Male obese mice exhibit an altered transcriptional profile in their testes, as well as aberrant DNA methylation and miRNA expression patterns in their sperm[140]. Thus, obesity-induced male subfertility might also be caused by changes in sperm epigenetic signatures, resulting in altered accessibility of parentally derived genes during early embryogenesis[120]. Aberrant DNA methylation profiles in human[141,142] and mouse[139] sperm occur predominantly at imprinting genes and repeat elements and are linked to reduced pregnancy rates[21]. In addition to DNA methylation, male mice on high-fat diets have a decreased expression of the histone deacetylase sirtuin 6 in elongating spermatids, resulting in increased DNA fragmentation and increased histone acetylation[78].

Transgenerational epigenetics

Data from human epidemiological studies. Our knowledge of transgenerational epigenetics in humans is mainly based on epidemiological studies, most using data from the Dutch famine birth cohort (3,307 children born between 1944 and 1947) and the Överkalix cohort (1,818 children and grandchildren from parents born between 1890 and 1920). Data from the Dutch famine birth cohort, also known as the Hungerwinter cohort, showed for the first time that maternal undernutrition during gestation increases the susceptibility for diseases in the later life of their children[143,144]. Interestingly, the offspring's risk for specific diseases varied, depending on which trimester the fetus was exposed to the calorie restriction. Famine in the first trimester was associated with cardiovascular diseases and reduced cognitive function, exposure during the second trimester resulted in impaired kidney and lung function and during the third trimester was followed by impaired glucose tolerance[145]. In addition, data from this cohort provided evidence for the involvement of DNA methylation in transgenerational inheritance: periconceptional exposure to famine was associated with significantly decreased methylation of the imprinted *IGF2* promoter compared with unexposed same-sex siblings ($P = 5.9 \times 10^{-5}$)[146]. To date, all studies analysing the Dutch famine birth cohort discuss their observations in the context of maternal effects only and exclude any paternal effects on offspring.

By contrast, paternal effects have been involved in studies based on the Överkalix cohort. Sons and grandsons had decreased longevity and an increased risk of mortality from heart disease or diabetes when either their father or their paternal grandfather was exposed to an excess of food at age 9–12 years[147–150]. Sons, but not daughters, had an increased BMI and an increased waist circumference and fat mass in their later lives if their fathers had been smoking before the age of 11 years[148].

Animal models of transgenerational epigenetic effects. Concomitant with the expansion of research into epigenetics, interest in animal models that enable environmental epigenomic studies on developmental origins of disease increased. Probably the most prominent model is the agouti-viable-yellow (A^{vy}) mouse, which carries a metastable agouti gene (*Asip*), owing to a retroviral intracisternal A particle (IAP) upstream of the agouti transcription start site[151,152]. The degree of IAP methylation correlates with the coat colour of the animals, ranging from brown (methylated IAP) to yellow (unmethylated IAP). Mice with any yellow fur due to agouti gene expression will become obese in later life and are prone to develop diabetes and/or cancer[152,153]. Feeding pseudoagouti pregnant mice with a normal diet results in unhealthy offspring[154]. However, feeding a diet supplemented with folate, vitamin B_{12}, methionine, choline, betaine (a building block of the chief methyl-donor *S*-adenosylmethionine[155]) and zinc (a cofactor for DNMT 1 (REF. 156)) alters the epigenetic regulation of the agouti gene in the offspring and results in a healthy, pseudoagouti phenotype.

A^{vy} mice represent excellent biosensors for functional environmental epigenomic studies, but for appropriate interpretation of the results careful attention must be given to the experimental conditions employed[152,157]. An absolute requirement is the assessment of not only the coat colour according to the five-categories classification (yellow, slightly mottled, mottled, heavily mottled, pseudoagouti)[158] but also the degree of DNA methylation[152,158] and histone modification[159] within the IAP promoter region. For transgenerational studies, knowing that the A^{vy} allele is passed through the paternal lineage is important, as the epigenotype is reset with paternal, but not with maternal transmission of the A^{vy} locus. Thus, male A^{vy}/a mice of varying coat colour must be generally mated with female virgin a/a mice[160].

These data suggest that nutritional factors affect addition or removal of chemical tags to or from DNA or histones, and that these epigenetic signatures are heritable and able to switch genes on or off in the offspring. Thus, our health in adulthood is not only determined by our nutrition but also influenced by prenatal factors and by the nutrition of our parents. The majority of existing studies has a clear focus on the mother and the *in utero* development of the embryo, but interest in epigenetic signatures in sperm and the paternal factors is increasing.

Effects of paternal diet on offspring health

In humans, the first evidence for paternal-diet-induced epigenetic effects in offspring was based on data from the Newborn Epigenetics Study (NEST), which showed significantly decreased DNA methylation within the differentially methylated region (DMR) of the imprinted gene *IGF2* in children from obese fathers ($P = 0.003$)[161]. A follow-up study found abnormal DNA methylation in the DMRs of six out of 12 imprinted genes: hypomethylation at *MEG3* ($P = 0.02$), *NDN* ($P = 0.02$), *SNRPN* ($P = 0.02$) and *SGCE/PEG10* ($P = 0.01$); and hypermethylation at *MEG3-IG* ($P = 0.04$) and *H19* ($P = 0.03$)[162].

In male mice, low-protein diet produced offspring with a decrease of histone H3 trimethylated at Lys27 (H3K27me3) and an increase of DNA promoter methylation at *Ppara* in the liver[163]. In male rats, high-fat diet resulted in aberrant glucose and insulin signalling, as

Transgenerational epigenetics
Transmission of parental epigenetic signatures and their effects further than the first generation of children (F1 generation; classified as intergenerational epigenetics) to grandchildren and subsequent offspring (F2 and following generations).

Agouti-viable-yellow (A^{vy}) mouse
In this model, expression of the metastable A^{vy} allele depends on the methylation status of an intracisternal A particle located upstream of the *Asip* transcription start site. Low methylation levels of CpG sites result in high agouti-signalling protein expression from *Asip* and the agouti phenotype (yellow coat colour), whereas methylated CpG sites result in low expression levels and the pseudoagouti phenotype (brown coat colour).

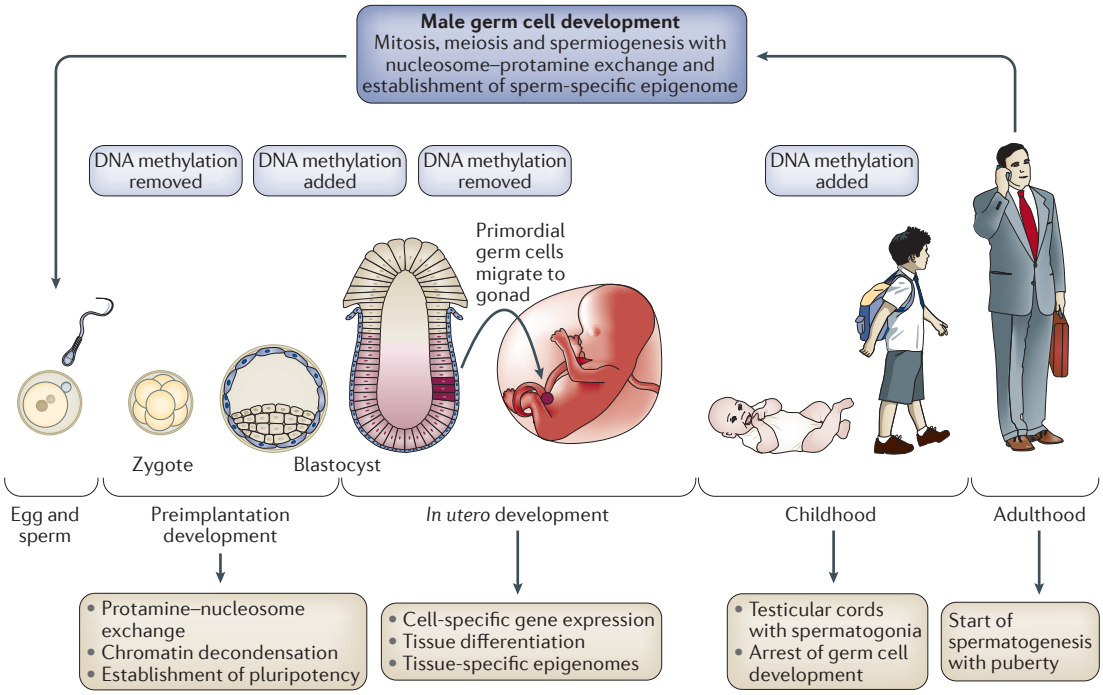

Figure 4 | **Key epigenetic events during human development.** To enable gene expression in the early embryo, protamines are replaced by nucleosomes resulting in chromatin decondensation. Directly after fertilization, preimplantation reprogramming establishes totipotency, enabling cell-lineage-specific somatic cell differentiation in the developing embryo. Germline reprogramming that establishes sex-specific inherited epigenetic profiles starts in migrating primordial germ cells during *in utero* development and concludes in spermatogonia during childhood. In adulthood, the sperm epigenome is established through hypermethylation of the condensing DNA and reorganization of the epigenetic signatures associated with retained histones. The sperm epigenome, together with the sperm genome, are transferred to the oocyte at fertilization.

well as decreased DNA promoter methylation at *Il13ra2* and aberrant expression of 642 pancreatic islet genes in the female offspring[138]. In male mice, a folate-deficient diet was followed by decreased DNA methylation in the liver of the offspring[111], whereas, in female mice, a folate-deficient diet during pregnancy and the lactation period resulted in an altered DNA methylation profile in offspring's sperm[55]. Also in mice, paternal life-long folate deficiency was associated with a delayed onset of meiosis and delayed expression of histone H3 methylated at Lys4 (H3K4me1), as well as decreased pregnancy rates (50% versus 85% in controls) and increased birth defects (27% versus 3% in controls) in the offspring, including craniofacial and musculoskeletal malformations[55]. A genome-wide DNA methylation analysis in sperm identified 57 DMRs in genes implicated in development, chronic diseases, such as cancer and diabetes, and neurological diseases. In the placenta of offspring, >300 genes were differentially expressed, of which two genes (*Cav1* and *Txndc16*) correspond to genes with differential methylation in sperm[55]. Aberrant sperm DNA methylation patterns in offspring were also observed when female mice were undernourished during pregnancy[56]. In contrast to another study[164], differential methylation was not maintained in somatic tissue of the F2 generation[56]. In addition, high-fat diet in male mice resulted in offspring producing sperm with an aberrant DNA methylation[139,165] and miRNA[139] profile, and increased acetylation of histones H3 and H4 was present in testicular spermatids[139], possibly due to a decreased expression of the histone deacetylase sirtuin 6 (REF. 78).

Male germ cell development can be divided into distinct stages (FIG. 1); each stage represents a potential time window of establishing a susceptibility to specific diseases in the later life of the offspring[7,10] (FIG. 4). Information on the postnatal, prepubertal period is sparse but more evidence exists for the period between fertilization and implantation (preimplantation and early embryogenesis). The one-cell embryo contains a mixture of paternal-specific and maternal-specific epigenetic signatures and has to undergo two rounds of epigenetic reprogramming: preimplantation reprogramming to gain totipotency and germline reprogramming to establish the sex-specific epigenetic signatures.

Preimplantation reprogramming. Erasure of the DNA methylation profile involves both passive and active processes. Passive demethylation is caused by the absence of the Dnmt1 cofactor E3 ubiquitin-protein ligase UHRF1 (REF. 166), whereas active demethylation of DMRs is mediated by TET enzymes[167]. In addition, an erasure of imprinted marks takes place via histone remodelling, particularly depletion of histone H3 trimethylated at Lys27 (H3K27me3) and removal of the histone variant H2A.Z[168]. Only epigenetic signatures involved in the regulation of imprinted genes escape

this reprogramming process and are maintained into adulthood[169,170]. This limitation is important to protect parent-specific epigenetic information transmitted by the gametes. Sperm DNA methylation differs from that of somatic cells, as it occurs predominantly outside of promoter regions[171], and from that of oocytes, revealing >1,600 CpG islands that are differentially methylated in addition to the known imprinting control regions[172]. The sperm genome had nearly complete coverage of methylation, except in the CpG-rich regions, whereas oocytes exhibit global hypomethylation. Sperm contributes DMRs that are largely intergenic and become hypermethylated after the blastocyst stage[170].

Parallel to preimplantation reprogramming, protamines within the paternal pronucleus have to be replaced by oocyte-derived histones[173]. By contrast, sperm nucleosomes and sperm-derived RNAs have the potential to affect both gene expression in the early embryo and transgenerational inheritance[174].

Germline reprogramming. Primordial germ cells (PGCs) originate from the somatic yolk sac at embryonic day 6.5 in the mouse[175] and during gestational week 2 in humans[176]. Germline reprogramming starts when PGCs migrate along the hindgut to enter the genital ridge at embryonic day 11.0 (mouse) or during gestational week 5 (human). After entering the gonads, PGCs, now called gonocytes, start to establish sex-specific epigenetic profiles (for example, genomic imprinting), which in mice involves decreasing DNA methylation levels from 71% to 14%[177], as well as demethylation of H3K9me2 and enrichment of H3K27me3 (REF. 178). Reprogramming of the paternally imprinted genes *Rasgrf1*, *Meg3* and *H19* starts between embryonic days 12.5 and 17.5 but will be completed in later life[31]. DNA demethylation in spermatogonia occurs predominantly in interspersed repeat sequences[34]. *De novo* DNA methylation starts in prospermatogonia at imprinted loci and repetitive elements, but will be completed with the onset of puberty in pachytene spermatocytes that are enriched in non-repeat sequences located within flanking gene bodies and paternal DMRs[34]. Histone modifications might be involved in redirecting DNA remethylation to the appropriate sites. In somatic cells, the methylated allele is associated with H4K20me3 and H3K9me3, whereas the unmethylated allele is enriched for H3K4me2 and H3ac, as is the case in spermatogonia[179]. Currently unclear is the regulatory function of the coexistence of both repressive H3K27me3 and active H3K4me3 within the same gene promoter[180–182]. In fetal germ cells of the mouse, 513 genes in male cells and 727 genes in female cells have been reported to be bivalently enriched for these two histone modifications with none of the associated genes being shared between the sexes[181].

Conclusions

Existing studies provide evidence that the influence of environmental factors on offspring's health is not restricted to the mother but is shared by the father, as sperm-specific epigenetic signatures are transferred to the oocyte and can, therefore, affect embryo development and offspring's health in their later life. However, our knowledge of transgenerational epigenetics is still sparse and, in humans, mainly based on epidemiological studies focusing on undernutrition and obesity. To improve our understanding of the underlying molecular mechanisms, animal models are used to analyse possible effects of environmental exposures to chemical toxins (for example, endocrine disrupters and smoking) and dietary factors (for example, phytochemicals and obesity). To date, we do not know why epigenetic mutations caused by environmental effects are not corrected during the two comprehensive waves of epigenetic reprogramming that occur between fertilization and implantation. Future studies have to clarify whether interventions can prevent or modify specific detrimental epigenetic signatures to optimize metabolic and reproductive health in offspring's later life.

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Acknowledgements
The authors wish to acknowledge grant support from the German Research Foundation (DFG), Clinical Research Unit KFO181 and the University Medical Center Giessen and Marburg (UKGM; 29/2015GI).

Author contributions
All authors researched data for the article, made a substantial contribution to discussion of content, wrote and reviewed and/or edited the article before submission.

Competing interests statement
The authors declare no competing interests.

Review criteria
The PubMed database was searched for articles with the terms “male germ cells”, “sperm”, “epigenetics”, “DNA”, “RNA”, “protamine”, “histone”, “methylation”, “acetylation”, “diet”, “nutrition”, “early embryo development” and “animal model”. If possible, original full-text articles published in English were retrieved. The reference lists of identified articles were searched for further papers within the Review’s scope. Although no limits were set regarding the year of publication, this Review focuses on articles published within the past 5 years and includes landmark studies published earlier.