A paternal environmental legacy: Evidence for epigenetic inheritance through the male germ line

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Literature on maternal exposures and the risk of epigenetic changes or diseases in the offspring is growing. Paternal contributions are often not considered. However, some animal and epidemiologic studies on various contaminants, nutrition, and lifestyle-related conditions suggest a paternal influence on the offspring's future health. The phenotypic outcomes may have been attributed to DNA damage or mutations, but increasing evidence shows that the inheritance of environmentally induced functional changes of the genome, and related disorders, are (also) driven by epigenetic components. In this essay we suggest the existence of epigenetic windows of susceptibility to environmental insults during sperm development. Changes in DNA methylation, histone modification, and non-coding RNAs are viable mechanistic candidates for a non-genetic transfer of paternal environmental information, from maturing germ cell to zygote. Inclusion of paternal factors in future research will ultimately improve the understanding of transgenerational epigenetic plasticity and health-related effects in future generations.

Keywords:
developmental origins of health and disease (DOHaD); environment; epigenetics; imprinted genes; offspring; paternal exposures; spermatogenesis; transgenerational effects

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Abbreviations:
A^{vy}, agouti viable yellow; **DMR,** differentially methylated region; **DNMT,** DNA (cytosine-5)-methyltransferase; **DOHaD,** developmental origins of health and disease; **IGF2,** insulin-like growth factor 2; **PAHs,** polycyclic aromatic hydrocarbons; **PCP,** pentachlorophenol; **ROS,** reactive oxygen species; **SGP,** slow growth period.

Introduction

A number of animal and human studies demonstrate that periconceptional and in utero developmental maternal exposures to a variety of environmental factors affect the risk for disease development in subsequent generations. Early observations of intrauterine exposure to maternal malnutrition as a determinant for type two diabetes in the offspring led to Barker's "thrifty phenotype hypothesis" [1]. This theory has now been extended to reflect a wider scope of exposures, and is called the "Developmental Origins of Health and Disease" (DOHaD) hypothesis. DOHaD concepts include exposures to environmental chemicals and toxins, use of medicines, infections, nutritional status, and other stressors in pre-pregnancy, during in utero development, and during the first years of life (reviewed by [2]). Poor health outcomes in children associated with harmful maternal exposures include congenital abnormalities [3], obesity, and insulin resistance [4], cardiovascular diseases [5], behavioral disorders [6], and potentially even cancer [7]. Following a landmark study by Waterland and Jirtle [8] using the agouti viable yellow (A^{vy}) mouse model, the biological mechanisms underlying these associations in humans are now proposed to involve alterations in the epigenome, including DNA methylation, histone modifications, and transcription of non-coding RNAs. During the development of gametes, DNA methylation is

uniquely regulated. Primordial germ cells undergo a nearly complete epigenetic erasure, followed by reprogramming of DNA methylation patterns in a sex-specific manner, such as at imprinted genes [9–11]. Imprinted genes are characterized by parent-of-origin dependent monoallelic expression; their functional haploid state being controlled by differentially methylated regions (DMRs) [12, 13]. The establishment of inherited imprint methylation marks at these DMR sites during gametogenesis is essential [9, 14, 15], and their aberrant methylation is associated with infertility and several chronic disorders [16–20]. Hence, paternal influences on the formation of epigenetic marks during spermatogenesis, and their impact on the health of the offspring are important biological endpoints to investigate. Human epidemiologic studies covering two or more generations are difficult to conduct, and only a few have provided evidence for the inheritance of epigenetic information through the male germ line [21, 22]. Nevertheless, a significant number of epidemiologic studies report unexplained father-child effects from various occupational or other environmental exposures. Although DNA damage or mutations are often suggested or assumed as the biological background for these harmful outcomes, the literature does not always provide sufficient evidence for an exposure-related mutagenic effect. An increasing number of animal experiments confirm that the offspring's epigenetic profile and health status is influenced by paternal preconceptional insults, such as exposures to endocrine disruptors or toxins [23], ionizing radiation [24], and nutritional status [25, 26]. Hence, an additional (or sole) epigenetic component responding to the environmental insult cannot be excluded. In this essay, we explore paternal exposures to various pollutants and lifestyle-related conditions, and their potential effect on the health status of future generations. We discuss the accumulating evidence that epigenetic mechanisms are important in the transfer of information from one generation to the next through the male germ line.

Do paternal exposures to environmental toxins promote transgenerational epigenetic changes?

Fathers occupationally exposed to high levels of carcinogenic substances may not only endanger themselves, but also their children. The realization that paternal occupational exposure to chemical substances can affect the integrity of spermatogenesis, and potentially result in the transmission of carcinogenic defects to the children, was initially reported by Fabia and

Box 1

Animal models: Evidence for transgenerational epigenetic effects from paternal exposures to environmental toxins.

Animal models indicate that male exposure to pesticides or other harmful chemicals causes defects in the gametes and abnormal development of the offspring. Insecticides, such as chloropyrifos, affect sperm quality, and pregnancy outcomes in mice [52]. The fungicide vinclozolin induces fertility problems and abnormalities in rats for at least four subsequent generations. Interestingly, this effect was most pronounced in males and was correlated with altered DNA methylation patterns in the germ line [23, 53]. Reproductive consequences of vinclozolin appear at relatively low doses; lower than the dose defined by the US Environmental Protection Agency (EPA) as "no observed adverse effect level" (NOAEL) [54]. Chronic exposures to low doses of vinclozolin not only affect male fertility, but also affect the levels of mRNA in mice testes [54]. Similarly, very low doses of a herbicide used worldwide, Simazine, administered during pregnancy did not elucidate measurable toxicity in the mother, but adversely affected normal development and reproductive activity of male offspring, accompanied with changes in expression of several genes in the testes [55]. These results on low-dose exposures are concerning, especially given the fact that toxicological classifications are generally based on mutagenic or other non-epigenetic tests. Another report on the commercially available pesticide Roundup reveals that low dose exposure during prepuberty in rats negatively affects fertility and causes overproduction of ROS in the testis [56]. Increased levels of ROS potentially cause cellular damage. Unbalanced ROS have been linked with impaired spermatogenesis, DNA damage and epigenetic alterations that ultimately increase risk of development of diseases [57, 58]. Pollutants such as plasticizers (e.g. phthalates) and heavy metals (e.g. lead) also stimulate testicular ROS generation, resulting in (at least) impaired spermatogenesis [59–61]. Plastic-derived endocrine compounds bisphenol-A (BPA), bis(2-ethyl-hexyl)phthalate (DEHP) and dibutyl phthalate (DBP) and other toxins such as the hydrocarbon mixture JP-8 (or jet fuel) cause permanent changes to DNA methylation in F3 generation animals, testis or pubertal abnormalities, and several adult onset pathologies [62, 63]. Another study in mice confirmed the concept that environmental toxins induce transgenerational epigenetic changes in sperm DNA. Adult males treated with methoxychlor, an insecticide, showed a decrease in methylation at the paternally imprinted *Dlk1/Meg3* (Delta-like, drosophila homolog1/maternally expressed gene3) gene, and an increase in methylation at the maternally imprinted genes *Peg1/Mest* (paternally expressed gene1/mesoderm specific transcript), Snrpn (small nuclear ribonucleoprotein polypeptide N), and Peg3 (paternally expressed gene3) in sperm. Interestingly, administration of methoxychlor in pregnant mice seemed to encompass the erasure of the methylation marks and the beginning of the methylation resetting within imprinted genes in sperm of offspring over two generations [64]. Although the mechanisms have not yet been elucidated, these observations suggest that environmentally induced epigenetic defects can survive transgenerational reprogramming.

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Thuy [27]. Subsequently, a number of other studies have also reported that paternal occupational exposures to chemicals are associated with harmful health outcomes in the offspring. Wilkins and Sinks [28] showed that children born to painters were six times more likely to develop Wilms' tumor than children from fathers with other occupations. Other case-control studies or cohorts confirm associations between paternal exposures to paints, thinner, turpentine, dyes, or pigments and childhood cancer, such as leukemia, neuroblastoma, and brain cancer; however, the magnitude of the correlations often differs by region and time when the study was performed [29–31]. Since paint composition has evolved over the years, including a change to lead-free paint since the late 1970s, comparisons of results of studies spanning decades need to take these changes into account. Lead is a reproductive toxicant [32]; and early-life environmental lead exposure has been linked with defects in brain development and Alzheimer's disease; epigenetic mechanisms have been suggested as the underlying cause [33]. Although a direct epigenetic effect on a male's gametes and his offspring has not yet been reported in humans, this question should be addressed in epidemiological studies in which lead exposures are evaluated.

Paternal exposures to other hydrocarbons, such as the industrial solvent trichloroethylene, mineral oils, and polycyclic aromatic hydrocarbons (PAHs) present in cigarette smoke are strongly related to childhood leukemia [34]. Recently, epidemiological studies show significant associations between PAH exposures and poor human sperm quality and increased levels of bulky DNA adducts [35, 36].

Pesticides belong to another category of substances widely discussed in literature as affecting sperm quality and chromatin integrity [37, 38]; but, potentially also affecting the offspring through preconceptional exposure of the father. A recent meta-analysis provides evidence that paternal occupational exposure to herbicides, such as pentachlorophenol (PCP) used in wood-related industries, increases the risk of lymphoma and leukemia in the exposed individual and in their children [39]. Agricultural work is also associated with an increased risk of congenital malformations [40] and cancer in the offspring [29, 41]. However, it is not always clear to what extent the potential environmental effect is related to the father only, especially in a domestic or agricultural context. If the outcome varies by gender of the parent it is easier to separate paternal from maternal influences. In a case-control study of families residing in industrially or agriculturally polluted regions of the Yangtze River, an association was reported between high concentrations of PCP in the father's urine and unexplained spontaneous abortions; while similar significant associations were not found for urinal PCP in mothers [42]. A recent meta-analysis, focusing on case-control and cohort studies where information on preconceptional exposures of both parents was available, provided evidence for increased risk of childhood brain cancer if the father was exposed to pesticides through occupational activity or the use of household or garden pesticides; maternal exposures were also linked to the incidence of childhood cancers, but cancer sites were different [43]. Not all studies show an association between paternal exposure to pesticides and health outcomes in the offspring; and many miss the assessment of exposure-risk gradients. Additionally, the evaluation of broad classes of pesticides may dilute the potential effect(s) [44]. Hence, there is a need for studies that better define and quantify the exposures to the different categories of biocides used at home or through occupation. Organophosphates and organochlorides (or dioxins) are likewise health burdens on a large scale, but they are still used as pesticides and as flame retardants. High concentrations of some of these organophosphate chemicals in house dust, such as tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP), are associated with decreased semen quality [45]. Whether flame retardants present in furniture and clothing also affect the offspring remains unanswered. Exposure to other contaminants, such as bisphenol A (BPA), phthalates, heavy metals, and other toxic compounds through maternal exposures are known to affect pregnancy outcomes and the offspring's health potentially by altering the epigenome; however, the consequences of *paternal* exposures to the male germ line and offspring are unknown.

The striking consequences of involuntarily human exposures to high concentrations of chemicals, such as during war, confirm that paternal chemical exposures before conception can affect the offspring's health. "Agent orange", a mixture of chemicals including herbicides and dioxin, used by the US Army in the Vietnam war, not only caused devastating disabilities and birth defects in the Vietnamese population, it also affected the offspring of exposed US soldiers. A meta-analysis conducted by Ngo et al. [46] describes a strong association between congenital malformations in offspring, and the exposure of veterans and Vietnamese; with an overall estimated relative risk of 1.95 (95%CI: 1.6–2.4) and 3.0 (95%CI: 2.2–4.1), respectively. In a subsequent meta-analysis, a twofold increased risk of spina bifida was reported in children from agent orange-exposed Vietnam veterans [47].

Both genomic and epigenetic pathways have been suggested to explain the transmissible effects of environmental contaminants, including sperm DNA mutations, genomic instability, suppression of germ-cell apoptosis, and imprinting errors [48]. However, most epidemiologic studies do not include evidence for these mechanisms, and many assume that the (only) mechanistic underlying cause is a genetically inherited mark. Since epidemiologists, environmental toxicologists and molecular biologists have just begun to explore these questions through interdisciplinary research, yet undiscovered epigenetic effects from occupational or environmental exposures through the paternal germ line will undoubtedly be revealed in the future. An interesting study performed in people who migrated from agricultural areas to urban settings in India showed that having a malformed or aborted child is associated with high DNA damage and high reactive oxygen species (ROS) levels in semen [49]. In the same population, high seminal ROS was also found in men who fathered children with retinoblastoma (personal communication with Rima Dada). We know from animal models that high ROS in testes is related to epigenetic changes in sperm. Hence, it is possible that paternal occupational

exposure to pesticides may have affected some genetic and epigenetic characteristics of the sperm through altered ROS, and ultimately increased the risk for disorders in the offspring. Further investigation in this and other populations is necessary to confirm our hypothesis. Noteworthy is the study of Warmlander et al. [50] on the skeletal phenotypes of Ancient Californian Indians and their use of bitumen more than 2,000 years ago. Bitumen (tar) is a mixture of PAHs that was used in Indian manufacturing techniques, from the making of leak-free water baskets to the sealing of fishermen's canoes. Skeletal analyses revealed an association between the increase in use of bitumen over centuries and a decrease in population stature, reflecting a decline in health conditions. Although caution is warranted when drawing conclusions from these ancient data, since the exact exposure levels are unknown, a gender-related decline in cranial volume was observed over multiple generations; the effect appears to be stronger in males [50]. If the current evolving technology makes it possible ultimately to determine PAH levels in these archeological specimens, and if next-generation sequencing technologies are included to perform (epi) genome-wide analyses, we may be able to decipher the effects of environmental changes in the past on human adaptation. Recent research on ancient bison bones indicates that DNA methylation patterns are faithfully retained along with nuclear DNA over evolutionary timescales [51]; making these ancient samples ideal tools to explore the role of environmentally induced epigenetic modifications and their effects on evolution. Research on animal models shows that toxin-induced epigenetic changes are measurable in the germ line and can survive several generations. Epigenetic effects from different harmful chemicals in animals are summarized in Box 1.

Do paternal exposures to low dose ionizing radiation promote transgenerational epigenetic changes?

Ionizing radiation induces germ line genomic instability and may have adverse effects on the offspring [48]. Men who received radiation treatment for childhood cancers have an elevated sperm DNA fragmentation index, and are at increased risk of having fertility problems when compared to controls [65]. Transgenerational effects from paternal exposure to radiation through occupation, airport scans, medical treatment and diagnosis, and other man-made sources of radiation presently remain mostly unknown. An unsolved epidemiologic finding relates to the Sellafield case. Public concerns in the 1980s prompted the UK government to investigate the excess of malignant

Box 2

Animal models: Evidence for transgenerational epigenetic effects from paternal exposures to ionizing radiation.

High doses of ionizing radiation in mice, administrated before mating, cause an accumulation of DNA double strand breaks in somatic cells of the offspring, which is accompanied by global hypomethylation, changes in the levels of methyltransferases, and altered microRNA expression [24, 87]. An acute gamma-irradiation of male mice destabilizes the sperm genome and F1 brain genome, indicating a transgenerational instability triggered by a certain threshold dose of acute paternal irradiation [88]. Koturbash et al. [87] speculate that sperm cells damaged by radiation may interfere with the epigenetic programming of the fertilized egg, causing genomic instability and potential carcinogenesis in the progeny of the exposed parent. Additionally, an epigenetic bystander effect occurs in the cells of unirradiated organs of rats after cranial radiation with high doses. These changes included loss of global DNA methylation, altered levels of miRNAs, and downregulation of DNA methyltransferases and methyl CpG binding protein 2 (Mecp2) [89, 90]. Since these epigenetic effects occur in organs that neighbor the exposed tissues, it is possible that germ cells may also be affected through a bystander process, thereby causing heritable defects in the offspring. The same research group investigated the effects of chronic low dose radiation exposure on somatic cells in an in vivo murine model, and found that it induced epigenetic changes, such as genome-wide hypomethylation; while acute low dose administration showed no direct measurable effects [91].

Bernal et al. [92] recently showed, with the use of the agouti viable yellow (A^{vy}) mouse model, that maternal exposure to doses of X-rays used in diagnostic CT-scans (0.7–7.6 cGy) alters the epigenome in the offspring. The offspring were irradiated at implantation stage, while an effect of paternal irradiation on the epigenome of the offspring has not yet been determined. The results of this study demonstrate that low doses of X-rays induce dose- and sex-dependent increases in DNA methylation at the A^{vy} locus, causing a significant shift in the coat color distribution of the offspring from yellow to brown [92]. Dietary antioxidants taken during pregnancy negate the radiation-induced increase in DNA methylation, indicating that low doses of ionizing radiation increase DNA methylation at the A^{vy} locus in part through the generation of ROS. Persistent induction of ROS as a response to radiation exposure has been suggested earlier [93], and free radical injury can profoundly alter DNA methylation levels [58, 94]. Thus, events such as exposure to X-rays during early pregnancy may alter the cellular redox state in pluripotent stem cells, determining the ultimate methylation status at the A^{vy} locus at birth. Once the uteroplacental circulation is established, dietary antioxidants may reduce the abundance of highly reactive ROS, and reduce the epigenetic consequences. Although this is speculative, this intriguing possibility needs to be investigated.

diseases in children living in the vicinity of the Sellafield nuclear plant. A population-based analysis confirmed the high incidence of leukemia and lymphoma in the young residents of Seascale, the village near the Sellafield plant, when compared to those in national registries and surrounding areas [66]. In a cohort study of children attending school at Seascale, an increased rate of leukemia and other cancers was observed among children born in Seascale, but not in children who moved to the village after birth [67]. A case-control study indicated that children of fathers working at the nuclear plant at the time of conception had a three times higher risk of developing leukemia or non-Hodgkin's lymphoma before the age of 25. Interestingly, the same study suggested a preconceptional dose–response relationship [68]. An independent case-control analysis confirmed the association between excess risk of childhood leukemia and lymphoma in the area when paternal radiation exposure occurred at the time of conception, but not when radiation exposure occurred three to six months before conception [69]. These studies ultimately led to "Gardner's Hypothesis", which proposes a causal relationship between paternal exposure to ionizing radiation and cancer risk in the offspring [68, 70]. Gardner's hypothesis has been widely criticized, and was ultimately rejected [71–73]; in part, because after a comparison with other studies, such as those on the atomic bomb survivors in Japan, no evidence was found for increased cancer incidence in children from exposed fathers [74–76]. Some attributed the increased risk of childhood leukemia near the nuclear plant to population mixing and a yet unidentified infectious agent [69, 77, 78]. Furthermore, additional studies on populations near nuclear plants in other countries did not show significant effects on the young population living in the vicinity of nuclear plants, with some exceptions [79]. Other clusters of childhood cancers were reported near the Krümmel nuclear power plant in Germany [80], the Dounreay nuclear reactor in Scotland [77], and the nuclear fuel reprocessing plant near La Hague, France [81]. Nevertheless, no plausible (genetic) explanations have been proposed to date for these clusters, and because of a lack of knowledge regarding the biological mechanism behind these observations, it was concluded that there were "no indications" for an increased risk of childhood cancer [79]. But, in his critical report [82], Nussbaum rightfully reminded epidemiologists that they should not ignore a fundamental rule earlier espoused by Altman and Bland [83], "The absence of evidence of an effect does not constitute evidence of absence of that effect."

Although the possibility that paternal exposure to ionizing radiation increases the susceptibility of the offspring to cancer remains controversial, it cannot be excluded that epigenetic effects may play a role in the unexplained excess of cancer incidence observed in children from fathers working in the nuclear industry. Notably, studies in human and animal populations exposed to radiation from the Chernobyl nuclear power plant accident in 1986 showed DNA damage in sperm and an overall increase in generation of reactive oxygen metabolites [84, 85]. Further analyses in the offspring of fathers exposed to low doses of radiation during cleanup of the nuclear plant showed an elevated frequency of chromosome aberrations, which may lead to increased morbidity over their lifetimes [86]. To our knowledge, no studies on Chernobyl victims were performed to verify potential transgenerational epigenetic effects through the paternal germ line. Analyses on epigenetic markers and long-term follow-up studies are needed to help resolve this important question. Animal data provides evidence for transgenerational epigenetic changes from exposure to high and low dose ionizing radiation (see Box 2).

Does paternal lifestyle, diet, or obesity promote transgenerational epigenetic changes?

Maternal smoking before and during pregnancy is a well-recognized risk factor for adverse health outcomes in the child; however, paternal preconceptional exposure to PAHs from cigarette smoking is likewise associated with childhood cancer [34]. The Avon Longitudinal Study of Parents and Children (ALSPAC) indicates that the earlier the father starts smoking in life, the higher is his son's BMI [95]. Paternal cigarette smoking at the time of conception is also linked with DNA damage in cord blood of the offspring, while maternal passive smoke exposure is not a predictor for DNA damage [96]. This indicates that cigarette smoke metabolites may induce transgenerational epigenetic inheritance of vulnerability for DNA integrity defects through the paternal germ line. Interestingly, cigarette smoke induces changes in miRNA profiles of human spermatozoa. The altered miRNAs were suggested to target epigenetic compounds important in DNA methylation and histone modification [97].

Another lifestyle-related factor is obesity. The obesity burden and concomitant health problems are global issues. More alarming is that obese parents tend to give birth to children who will also become obese [98]. Furthermore, these children not only have a higher BMI than children from non-obese parents, there is also a higher risk of congenital abnormalities at birth [3], behavioral problems [6], cardiometabolic dysfunction, and other chronic disorders in later life [5]. As with other parent-child associations, these harmful health conditions are generally attributed to the mother's lifestyle or diet. However, Figueroa-Colon et al. [99] emphasized for the first time in humans that the father's body composition can also affect the offspring. They reported a significant association between paternal body fat and long-term changes in the percentage of body fat in their prepubertal children. In contrast, a separate correlation analysis on maternal anthropometric characteristics did not show a significant influence of BMI on the offspring's body composition.

Long-term cohort studies, such as the Framingham Heart Study, show associations between early-onset paternal (but not maternal) obesity and aberrant levels of circulating alanine transaminase (ALT) in the offspring. Elevated ALT levels are associated with liver dysfunction and obesity, providing evidence for an as yet unknown underlying transgenerational influence on metabolic processes affecting the offspring of obese fathers [100].

Box 3

Animal models: Evidence for transgenerational epigenetic effects from paternal lifestyle and nutrition-related exposures.

It is widely accepted that the intrauterine environment, including maternal nutrition, is important in determining an offspring's birth weight and risk for chronic disorders in childhood and adult life (reviewed by [2]). Research with the A^{vy} mouse model also demonstrated that maternal nutrition during pregnancy can alter the phenotype of the offspring by changing the epigenome [8]. Experiments in rats also show that environmental factors, such as a parental high-fat diet, already affect the offspring if the exposure takes place before in utero development. A high-fat diet of the parents before and during mating results in offspring with increased body-fat accumulation, increased weight gain, and altered expression of lipoprotein lipase and leptin in adipose tissues [108]. Maternal obesity in rat disturbs postnatal steroid levels and development of male germ cells [109]. Paternal food deprivation in male mice before conception leads to offspring with impaired glucose metabolism [25]. Carone et al. [110] demonstrated that male mice consuming a low-protein diet from weaning to sexual maturity had different RNA content and chromatin packaging of sperm as compared to controls; they also fathered offspring with altered DNA methylation at specific liver CpG islands, including a potential enhancer for the key lipid regulator PPARα; and, expression of hepatic genes involved in lipid and cholesterol biosynthesis were elevated. Ng et al. reported that a paternal high-fat diet results in lower DNA methylation at a putative regulatory region of the *interleukin 13 receptor alpha 2* gene, coupled with impaired glucose tolerance and increased body weight in rat offspring [26]. These data strongly indicate that a suboptimal diet during male gametogenesis can influence the metabolic status of the offspring and affect phenotypic outcomes. Although a diet-induced epigenetic effect during spermatogenesis was suggested, a "sperm signature" from paternal diet was only recently reported by the group of Michelle Lane. High-fat paternal diet may increase histone acetylation [111], ROS and sperm DNA damage [112–114]. In addition, male mice consuming a high-fat diet showed altered global methylation and microRNA content in mature sperm, and altered transcriptional profiles in the testes; interestingly, these alterations were linked to metabolic disturbances in the next generations [115].

Genistein is a dietary compound also known to affect DNA methylation patterns. It is a phytoestrogen present in soy or soy-derived products. While its effects on the epigenome have been widely discussed in offspring after relatively short perinatal or in utero exposures [116, 117], and changes in DNA methylation have been demonstrated in organs of exposed animals, including mouse prostate [118], Eustache et al. explored genistein exposures from conception to adulthood in male mice and found deleterious effects on male reproductive development, adult reproductive organs, and fertility. Another important finding of this lifelong genistein exposure was a change in the testis transcriptome, with a general repressive effect on gene expression [54]. Importantly, a major effect was seen at low doses as compared to high doses, and variable results were detected if mixtures with vinclozolin, an anti-androgenic food contaminant, were used. These results underscore the complex interplay of synergistic or antagonistic actions of food-born nutrients and/or contaminants, resulting in different molecular or phenotypic outcomes.

Other examples of nutritional compounds or supplements influencing the epigenome are vitamins. Singh et al. [119] suggested that nutritional deficiencies of vitamins or micronutrients, are potential triggers for disturbances in chromatin packaging and DNA integrity during spermatogenesis, and hence the maintenance of the male reproductive health. Folate is a naturally occurring water-soluble vitamin and a key source of the one-carbon group necessary to methylate DNA. Folate, or its synthetic form folic acid, is crucial for normal embryonic development; hence, most studies focus on effects of folate deficiencies during pregnancy (reviewed by [120]). An interesting approach by Mejos et al. [121] showed that both maternal and paternal folate deficiency can influence global DNA methylation in rat offspring. Further studies on animal models are needed to confirm a direct epigenetic effect of folate on sperm DNA and to explore the mechanisms involved to transmit these defects permanently through fertilization and embryonic growth.

Literature showing a direct effect of other lifestyle-related exposures on sperm epigenetics is scarce. To date, two studies demonstrate a correlation between alcohol use and demethylation at gene regulatory sites of *IGF2* and *H19* in humans [122], and in mice [123].

A Swedish study on historical data of three generations indicates a transgenerational response to variable food availability during the slow growth period (SGP), before the prepubertal growth peak. Longevity of males was reduced if the grandfather [101] or the father [102] was exposed to an excess of food during the SGP. Similar results were found in females if the paternal grandmothers were exposed to an excess of food supply [102]. Furthermore, the risk of death from diabetes in the descendants was four times increased if the paternal grandfather was exposed to a plentiful food supply in his SGP [21]. These remarkable gender-specific associations suggest that during the SGP epigenetic changes in the germ line may underlie these transgenerational effects, but this has not yet been determined.

The first epidemiologic evidence of epigenetic changes in the offspring being triggered by paternal obesity came from analyses of DNA methylation in the Newborn Epigenetics STudy

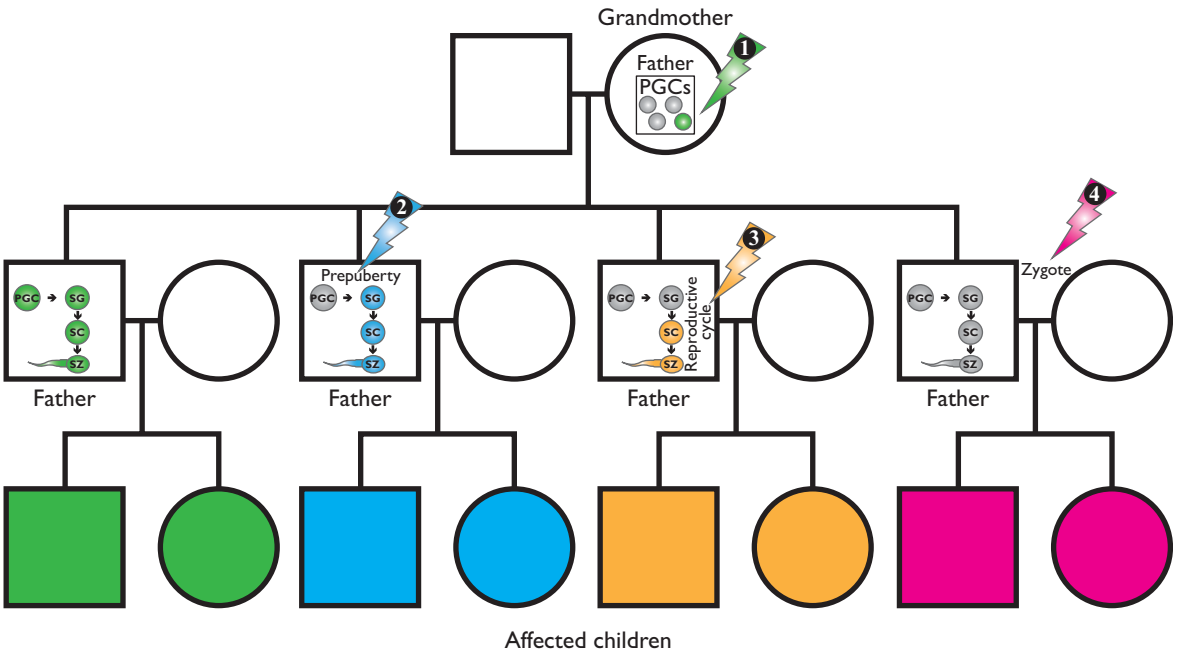

Figure 1. Susceptibility windows for environmentally induced epigenetic changes through the paternal germ line. Hypothetical pedigree chart of children with altered epigenetic profiles that may increase risk for disease. Changes in epigenetic profiles may have different causes that vary by timing (lightning bolts) and type of exposure, including – but not limited to – environmental toxins, pollutants, endocrine disruptors, ionizing radiation, smoking, nutrition, etc. Windows of heritable epigenetic damage include: 1. during migration of primordial germ cells (PGCs) to the genital ridge (before week 6 of development of the future father in the grandmother), when genome-wide epigenetic erasure occurs; 2. before puberty, from PGC (or gonocyte) to spermatogonia, during which methylation profiles are largely established; 3. during each reproductive cycle, from spermatogonium (SG) to spermatocyte (SC) and finally the spermatozoon (SZ), when DNA methylation should be fully established; and 4. in the zygote, when the acquired methylation marks need to withstand post-zygotic epigenetic reprogramming at specific regions (e.g. imprinted genes).

(NEST) birth cohort. The father's BMI was shown to be inversely related to the level of DMR methylation at the imprinted insulin-like growth factor 2 (*IGF2*) locus [22]; similar results were seen at DMRs of other imprinted genes involved in early growth regulation [103]. Interestingly, these results were independent of maternal obesity. As stated before, the inherited methylation marks for DMRs involved in regulating imprinted gene expression are established during gametogenesis [15], and their deregulation is associated with several chronic or metabolic diseases in the offspring [16–18]. A follow-up analysis of NEST children is needed in order to correlate the obesity-induced findings with metabolic outcomes at later age.

Two study cohorts on maternal nutrition highlight the importance of the timing of exposure to nutritional insults. Periconceptional exposure to food deprivation in the Dutch famine cohort or seasonal dietary circumstances in a Gambian study cohort showed strong associations with poor health outcomes and altered DNA methylation in the offspring [104, 105]; however, neither study addressed the importance of the fathers' diet. Since the fathers were likely to be exposed to the same famine or nutritional conditions as the mothers, a paternal effect cannot be excluded in these cohorts. As suggested by Lecomte et al. [106], large epidemiological studies are needed where stratified analyses by maternal and paternal influences are carried out, and attempts to dissociate parental obesity from nutritional status should help us understand which phenotype is related to which nutrient deficiency or abundance. Finally, an extensive nutritional study on multiple populations was published in 1939 by Weston Price, an American nutritionist and dentist who investigated multiple tribal diets around the world [107]. Price reported that in several primitive tribes there was a consciousness that the food eaten by both parents before conception has significant influences on birth characteristics, ultimate overall health, and character of the child. He noticed a higher frequency of facial deformities when these "primitive" tribes adopted the Western diet. Furthermore, he commented specifically about the effect of the father's diet on the offspring's dental and facial phenotypes. Nevertheless, since Price did not report his original measurements or statistical analyses, these observations need to be interpreted with some caution. Studies on animal males consuming different diets provide evidence for epigenetic effects in the male reproductive system and in the offspring (see Box 3).

Epigenetic mechanisms: How and when is the paternal environmental information transmitted to the next generation?

The increasing number of reports on associations between paternal environmental exposures and fertility or risk of disease in the next generation evokes the compelling question of how and when the effects of environmental

exposures are transferred to the male gametes, and how these effects are sustained through developmental processes. Besides the potential for genetic damage or DNA mutations in sperm cells, animal studies, and epidemiological data indicate that transfer of information through generations may also occur via epigenetic mechanisms. There are a number of potential windows of susceptibility during the lifespan of the father where environmental effects can impact the epigenetic profile of his gametes. We summarized four windows of susceptibility during development of the paternal germ line and zygote in Fig. 1, and discuss the potential roles of DNA methylation, histone modifications, and/or presence of non-coding RNAs during these early developmental processes, in the following paragraphs.

Paternal embryonic development: A first window of susceptibility

During embryonic development, primordial germ cells undergo genome-wide epigenetic erasure as they migrate to the genital ridge. Animal models indicate that this process may appear in waves of active and passive DNA demethylation mechanisms, affecting the bulk of the genome and imprinted genes at different times. However, some portions of the genome have been reported to remain resistant to DNA methylation erasure [124, 125]. These protected genomic regions, currently limited to IAPs, LTR-ERV1 elements, and a few single-copy sequences, open the potential for transgenerational inheritance of DNA methylation profiles over multiple generations. Defects in complete erasure, or in maintenance of the protected regions, could be the first potential effect from internal or external factors during early development (green lightning bolt, Fig. 1).

Paternal prepuberty and spermatogenesis: Second and third windows of susceptibility

Following the epigenetic erasure, DNA methylation is gradually re-established throughout spermatogenesis [9, 10]. The literature suggests that de novo methylation at imprinted gene loci occurs mainly during differentiation from primordial germ cells to spermatogonia, i.e. before puberty in human. Hence, this period of life represents a second, and presumably important, window of susceptibility (blue lightning bolt, Fig. 1). Given that methylation patterns seem to be established by the time germ cells are differentiated to mature spermatocytes [14, 15], the early phase of each reproductive cycle (i.e. development from spermatogonium to spermatocytes) is a third potential vulnerability window (orange lightning bolt, Fig. 1).

De novo methylation and its maintenance are established through DNA (cytosine-5)-methyltransferase (DNMT) enzymes, including DNMT3A and DNMT3B, and DNMT1, respectively. DNMTs are expressed throughout spermatogenesis [15]. Further, the DNA methyltransferase-like protein DNMT3L possesses no DNA methyltransferase activity, but is crucial to the establishment of DNA methylation patterns during spermatogenesis [126, 127]; it interacts and stimulates de novo methylation activity of DNMT3A and DNMT3B [128, 129]. It has been suggested that maintenance of paternal imprints during all stages of spermatogenesis is a dynamic process that might result in a fluctuation in methylation at some CpG dinucleotide sites [15]. This normal fluctuation may be vulnerable to skewing by exposure to environmental factors that influence the transcriptional activity of DNMTs. The autocrine human growth hormone (hGH) has been described to influence DNA methylation through activation of signaling pathways that lead to transcriptional upregulation of the de novo DNA methyltransferase enzymes [130]. Environmental toxins, such as endocrine disruptors, or circulating obesity-related hormones (e.g. estrogen, leptin, and insulin) can accumulate in scrotal fat, potentially affecting sperm DNA methylation through their influence on DNMT activity; thereby contributing to infertility and pregnancy failures [131]. Pathak et al. reported how the estrogen pathway could be involved in genomic imprinting. In rat spermatozoa, the estrogen-estrogen receptor β complex interacts with Dnmt1 and binds an estrogen response element at the *H19* DMR, catalyzing methylation of the *H19* CpG island. DNA methylation at this site may be counteracted by administration of an estrogen receptor modulator, Tamoxifen [132]. These reports strengthen the idea that environmental estrogens may interfere with a normal crosstalk between estrogen signaling and imprinting during spermatogenesis.

Nutritional compounds, such as dietary fatty acids, can directly stimulate transcription of specific genes or transcription factors (such as PPARα) [133, 134], potentially also affecting the establishment of epigenetic mechanisms during spermatogenesis; but this is still an unexplored area. Supplementation of methyl donors including folic acid and vitamin B12 are able to increase the flux through a DNA methylation pathway at specific loci, resulting in DNA hypermethylation. This has been studied mainly in maternal or pregnancy models [8, 135], while a recent animal study on males suggests that also the paternal germ line is susceptible to DNA methylation changes through dietary folate intake [121]. Besides a potential environmental epigenetic effect through interaction with hormonal signaling pathways during spermatogenesis, other downstream effects of the surrounding environment on sperm and surrounding cells, e.g. Sertoli cells and leucocytes, include altered ROS concentrations. Various factors such as long-term exposures to chemicals or pesticides [56, 59], heavy metals [61], low dose ionizing radiation [93], chronic conditions such as diabetes [136, 137] and obesity [113, 114], or increasing levels of fatty acids [112] can promote ROS generation. Changes in ROS may modulate sperm DNA methylation and chromatin structure, ultimately influencing regulation of imprinted genes important in growth and development; or other genes, such as those responsible for maintenance of genome stability, altering DNA damage responses and repair mechanisms. Consequently at birth, cord blood or samples of other tissues may reveal DNA strand breaks and/or DNA methylation abnormalities, sustained throughout life and increasing risk for disease.

Periconception and zygote stage: Fourth window of susceptibility

In order to persist throughout embryogenesis, the acquired epigenetic signature needs to withstand reprogramming that occurs after fertilization. Until recently, the role of histones in conveying epigenetic information in mature spermatozoa was doubted because it was believed that all histone proteins are replaced by protamines (i.e. protamines 1 and 2) during late spermatogenesis. This facilitates a highly condensed state of the chromosomes, represses transcriptional activity, and prevents DNA damage [19]. After fertilization, the protamines are removed and replaced with maternal histone proteins, which then undergo the epigenetic modifications required for cellular differentiation. It is now clear, however, that there is selective retention of up to ~10% of the histone proteins in condensed chromatin of the sperm DNA (reviewed by [138]). The modifications present on histone proteins in sperm provide one mechanism by which epigenetic information is carried to the next generation. This retention of histones may relate to the selective establishment of specific DNA methylation patterns, as observed for developmental genes critical to early embryogenesis, including imprinted genes, microRNAs, and homeobox genes [139]. For these genes, histone retention provides a regulatory mechanism in which paternal DNA is poised for immediate activation after fertilization [20]. Lane et al. [124] demonstrated in mouse that intracisternal A-particles (IAPs) are largely resistant to DNA demethylation during preimplantation. Consequently, acquired epigenetic states of IAPs can also lead to persistent heritable changes in transcription of neighboring genes. Histone retention at yet undefined genes or gene promoters cannot be excluded as a potential mechanism for inheritance of environmentally induced epigenetic marks through this window. Importantly, nucleosomal patterns are disrupted in men with infertility [140]. Although the processes described here may explain in part how early environmental messages can be transmitted to the embryo, the complex mechanisms of selective removal, replacement, and retention of epigenetic factors (such as histones or methylation marks) in the fertilized oocyte makes this period of development vulnerable to environmental damage; hence, we define the zygote as a fourth developmental stage where paternal periconceptional influences may play an indirect role (pink lightning bolt, Fig. 1).

Non-coding RNAs as potential messengers of epigenetic information

Besides DNA methylation and histone modifications, the presence of RNA molecules in spermatozoa suggests another type of regulatory mechanism that is implicated in conveying epigenetic information; thereby potentially leading to phenotypic changes in the next generation [141]. An extensive review on the "hidden features" of RNAs in spermatozoa has been authored by Kumar et al. [49]. The types of RNA molecules present include mRNAs and non-coding RNAs. Their retention in spermatozoa begins to occur during early stages of spermatogenesis. Among the non-coding RNAs, several small RNAs have been identified in the sperm, which raises the possibility that these small molecules can carry hereditary information from one generation to the next [142, 143]. RNAs present in the mature spermatozoa are delivered to the oocyte, and from RNA-Seq analysis, many seem to have essential functions during early embryogenesis [144]. The genetic origins of RNAs identified in sperm are highly correlated with regions of the genome that are hypomethylated and enriched in histone proteins, especially those with H3K4me3 (but not H4K27me3) modifications. Spermatozoal RNAs seem to possess the capacity to direct histone modifications and DNA methylation, for instance in response to paternal smoking [97], while the chromatin structure and DNA modifications in turn may affect transcription of RNAs. This "epigenetic crosstalk", suggested by Rando, may be influenced by the paternal environment [145]. The longevity of such effects is presently unclear, but enzymes such as DNMT2 may protect these small RNA molecules from stress conditions during early embryogenesis [146].

Conclusions and outlook

Environmental variation may lead to transgenerational epigenetic changes resulting in differences in gene expression and ultimately different phenotypes or diseases in the following generations. Besides the generally assumed inherited genomic defects from environmental insults, epigenetic changes may accumulate (i.e. from chronic exposures) or persist and result in heritable modifications of the epigenome. This may influence male fertility or if the epigenetic modification is subtle it may be transmitted to the zygote through yet unidentified carriers, ultimately affecting health status in the offspring. Aside from the need to better understand the mechanisms by which environmental factors can alter epigenetic reprogramming in sperm, there are a number of unanswered questions. Is there a threshold of tolerance for epigenetic skewing beyond which there is certainty of an effect on the next generation? Environmental insults to epigenetic programming in male gametes may not cause equivalent defects in every cell stage of sperm maturation. Indeed, analysis of individual cloned alleles shows some variability in methylation at sporadic CpG dinucleotide sites during normal spermatogenesis [15]. Paternal age is also likely to be a major contributory factor to the epigenetic integrity of the sperm. Advanced paternal age is a well-established risk factor for child morbidities, and it is plausible that the ability to reprogram the epigenome declines with advancing years, as do other cellular processes [147]. We hypothesize that the male gametes are at higher risk for epigenetic damage during the epigenetic reprogramming periods, and that environmental factors can alter the fidelity of this process. Such an "environmental message" may be carried to the next generation through epigenetic modifications in the form of incomplete or unstable methylation at certain regions of the genome, changes in the levels of DNMTs, histone modifications, and defects in the transmission of non-coding RNAs during the process of

fertilization. Research on human sperm, and long-term epidemiologic investigations of multiple generations are necessary in order to obtain better insights into the epigenetic mechanisms underlying transgenerational environmental effects through the paternal lineage. This will lead to a better understanding of the etiology of certain (childhood) disorders, and may ultimately have implications for public health recommendations. We conclude that a healthy occupational environment and lifestyle for future fathers may be more important than ever realized before.

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Conflict of interest

The authors declare that they have no competing interests.

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