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Germline epigenetic inheritance: Challenges and opportunities for linking human paternal experience with offspring biology and health

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Abstract

Recently, novel experimental approaches and molecular techniques have demonstrated that a male's experiences can be transmitted through his germline via epigenetic processes. These findings suggest that paternal exposures influence phenotypic variation in unexposed progeny–a proposal that runs counter to canonical ideas about inheritance developed during the 20th century. Nevertheless, support for paternal germline epigenetic inheritance (GEI) in nonhuman mammals continues to grow and the mechanisms underlying this phenomenon are becoming clearer. To what extent do similar processes operate in humans, and if so, what are their implications for understanding human phenotypic variation, health, and evolution? Here, we review evidence for GEI in human and nonhuman mammals and evaluate these findings in relation to historical conceptions of heredity. Drawing on epidemiological data, reproductive biology, and molecular embryology, we outline developments and opportunities for the study of GEI in human populations, emphasizing the challenges that researchers in this area still face.

KEYWORDS

adaptation, evolution, germline, heritability, sperm

1 | INTRODUCTION

Understanding how traits are inherited, and what contributes to phenotypic continuity across generations, underpins the study of human variation, evolution, and disease.[1–3] Although the genetic component of heritability estimates have generally been assumed to reflect the inheritance of DNA alone (Figure 1A), research starting in the latter part of the 20th century has expanded the range of biological mechanisms known to contribute to the transmission of phenotypes across generations.[1,4] This view, which is part of a broader "extended evolutionary synthesis,"[5] has led to a growing appreciation for the importance of a wider array of non-DNA sequence based processes capable of contributing to biological inheritance.[1,6] Of particular interest are *epigenetic processes* (Box 1), which comprise a diverse set of molecular systems involved in gene regulation and cellular identity but that do not involve permanent changes to the DNA sequence itself.

While DNA sequence is for the most part identical in all bodily cells and stable across an individual's lifetime,[7,8] epigenetic processes—which include *DNA methylation*, *histone modifications*, and *noncoding RNAs* (*ncRNAs*; Box 1)—are generally more dynamic and environmentally responsive. Epigenetic changes have been related to a host of environmental and health-related behaviors, from socioeconomic status to smoking,[9–11] and appear to be involved in the development and progression of a long list of health disorders and diseases.[12,13] Environmental effects on an individual's epigenome are increasingly being traced back to prenatal life, potentially as far back as early embryonic development.[14–16] What is more surprising, however, are recent suggestions that some of the epigenome may be inherited, and that environmentally induced epigenetic changes

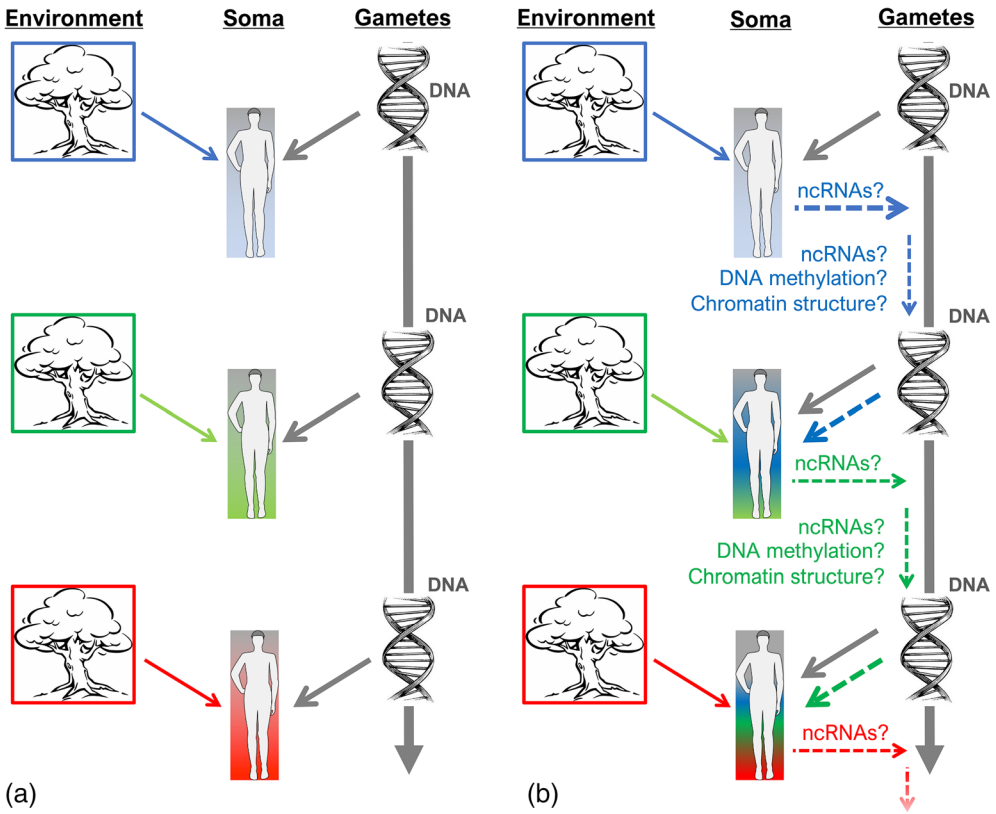

FIGURE 1 Contrasting models of inheritance. (a) Classical inheritance after Weismann. DNA is inherited through gametes (solid gray line) and interacts with the environment (solid colored lines) during development to produce phenotypes (soma). The environment of previous generations has no impact on the phenotype; (b) Inheritance under germline epigenetic inheritance (GEI). Environments affect the current generation but may also trigger epigenetic processes (dotted lines) that are transmitted back to gametes and passed onto subsequent generations. Acquired phenotypes may be inherited, in some cases across multiple offspring generations [Color figure can be viewed at wileyonlinelibrary.com]

experienced by one generation might be transmitted to subsequent generations (Figure 1B). The possibility of germline epigenetic inheritance (GEI) resurrects an idea that was common in the past but has lain dormant for much of the 20th century: the idea that parents'—or grandparents'—environmental exposures live on in the phenotype of their unexposed progeny.[17–19] The new literature examining this question in human and nonhuman mammals forms the subject of this review.

While the transmission of epigenetic information between generations applies equally in theory to both male and female lineages, emerging research on GEI has concentrated almost exclusively on the *male* germline. Studying paternal transmission as a model for GEI helps researchers minimize the potential influence of other pathways of nongenetic inheritance common to female mammals but that are not present in males, including gestation and lactation. Focusing on males, researchers can eliminate all contact between the exposed male and the mother of his offspring (and consequently, contact with his offspring), while constituents of sperm or male seminal fluid may be manipulated experimentally. This male-centered approach—when carried out in tightly-controlled laboratory conditions using isogenic populations—has proven effective at minimizing genetic (DNA) and environmental confounds. It has also yielded some of the most compelling mechanistic evidence to date that environmentally-induced epigenetic information is packaged in the germline and inherited by offspring, impacting phenotypes in future generations.[20–25] As we will discuss, findings from this rapidly growing literature are challenging the universality of several canonical ideas in genetics and evolutionary biology, including Mendel's particulate theory of inheritance[26] and Weismann's germ-plasm theory of heredity,[27,28] while also blurring the classic distinction between genotype and phenotype.[29]

In this paper, we review the evidence for GEI in mammals—with a focus on the more extensive literature examining male transmission—and critically evaluate the potential significance of these postulated pathways to questions in human development, health, and evolution.

We open with a brief review of prevailing notions of biological inheritance—several of which are being challenged by research on GEI—and situate them within the historical context of late 19th and early 20th century thinkers, including Darwin and Weismann. We discuss how Weismann's theoretical conceptions of heredity were derived from, and came to be interpreted in the context of, advances in cell and molecular biology. We also discuss how axioms of 20th century genetics are being reconsidered in light of new discoveries in these same fields. Following this historical overview, we review current animal model research that implicates nongenetic pathways in the direct transmission of environmentally-induced molecular information through the germ line (Figure 1B). We summarize current understandings of the exposures, phenotypic outcomes, and candidate mechanisms linking experiences in one generation with phenotypes in subsequent generations. We then review the present state of evidence that similar effects might operate in humans. We conclude by discussing the possible implications of these findings for understanding human health and evolution and some of the major issues that pose challenges to the study of GEI in humans.

BOX 1 Defining epigenetic processes

Epigenetic processes: A collection of chemical processes and modifications that are associated with cellular differentiation and mitotically- or meiotically heritable gene expression states, in the absence of the original perturbation, and not arising as a direct result of the underlying genetic (DNA) sequence.[198] There is considerable ambiguity and confusion over the term "epigenetic" owing to separate uses of the term through time and a divorce of the modern uses from its historical roots.[199–201] The term was coined by Conrad Waddington to describe the "causal mechanisms" connecting genotype to phenotype, in particular through development and cellular differentiation.[202] The term was later popularized by Nanney,[203] who described it as a form of "cellular memory," a homeostatic state based on "self-regulating metabolic patterns." It is Nanney's definition that is closest to contemporary uses of the term, although the term "epigenetic" is often used even in the absence of any evidence for mitotic/meiotic heritability or an effect of cell fate.[204]

DNA methylation (DNAm): The chemical attachment of a methyl group to a nucleotide side chain, most commonly that of cytosine, and usually in the context of a cytosine-guanine (CpG) dyad.[198] Methylation can occur de novo through enzymatic reactions or can be "copied" to the daughter strand following cell division based on the hemimethylated state of the template strand.[6] Demethylation, such as that occurring during epigenetic reprogramming, can arise passively through cell division/DNA replication in the absence of remethylation of daughter strands, or actively through TET DNA demethylase.[6] Hydroxymethylation of cytosine, and methylation of adenine, also occur in mammals, but their role in cellular differentiation and the regulation of gene expression are only beginning to be understood.[198]

Histone modifications: A collection of chemical modifications to histones, the proteins that make up nucleosomes.[198] Nucleosomes are the structural units around which DNA is wrapped as part of chromatin packaging and the regulation of gene expression, with tightly coiled regions forming transcriptionally-quiescent heterochromatin. Histone modifications are associated with changes in the biochemical interactions between histones and DNA, and between the histones themselves, which ultimately affects DNA packaging density and chromatin structure.[205,206] Histone states and chromatin structure across the genome change with cell transcriptional activity and during tissue differentiation.

Non-coding RNAs (ncRNAs): A subclass of RNA that does not code for proteins, but can affect gene expression through transcriptional and post-transcriptional processes.[207] Transcriptional repression by ncRNAs involves the recruitment of proteins and other epigenetic machinery that induce local repressive DNA methylation or histone modifications.[198] Post-transcriptional silencing by ncRNAs can occur by degradation of the targeted RNA or blocking protein translation. Well-known examples of ncRNAs include the germline silencing of transposable elements by PIWI-interacting RNAs (piRNAs) and X-chromosome inactivation by long intergenic RNAs (lincRNAs).[208]

Epigenetic reprogramming: Process in which epigenetic information, especially DNAm, is globally erased and reestablished. There are three major reprogramming events in males. The first (postfertilization reprogramming) occurs immediately following fertilization, when the differentiation states involved in generating "sperm" and "egg" cells are cleared.[6,52] Following post-fertilization reprogramming, a second reprogramming event occurs during the formation of primordial germ cells (PGCs), when PGC lineage-specific epigenetic marks are cleared and replaced with sex-specific DNAm in the developing germline. A third "reprogramming" stage occurs during spermatogenesis, and involves global changes in DNAm.[183] A dramatic instance of histone reprogramming also occurs during spermatogenesis, when numerous histone modifications occur and roughly 90% of human histones are replaced with protamines.[61] The "histone-to-protamine transition" is necessary for sperm DNA to be sufficiently compact, transcriptionally-silenced, and protected from environmental insults from the late stages of spermiogenesis and fertilization of the ovum in the female reproductive tract.[55] See Figure 2 for a visual representation of these reprogramming events.

piRNAs: PIWI-interacting RNAs; small, non-coding RNAs involved in germline epigenetic silencing of retrotransposons and transposable elements.[209,210] By limiting germline transposable element activity and mutagenic potential, piRNAs maintain genome integrity and are described as a "genomic immune system".[209] Surprisingly, some piRNAs in the human male testis also appear to target protein coding genes.[191]

microRNAs (miRNAs): A class of small (~22 nucleotides) non-coding RNAs involved in the post-transcriptional regulation of protein expression. Repression through miRNAs occurs through translational repression, mRNA cleavage, and deadenylation, and a single miRNA can target anywhere from one to 100 s of target mRNAs. There are an estimated 2,300 miRNAs in the human genome.[211]

Inter- and transgenerational epigenetic inheritance: Definitions of epigenetic inheritance can be based on the proximity of the original exposure to the individual/generation of interest. To affect the phenotype of subsequent generations, intergenerational epigenetic alterations must affect germ cells, pass through spermatogonial epigenetic reprogramming (males only), and bypass at least early stages of post-fertilization reprogramming (both males and females). In transgenerational epigenetic inheritance, epigenetic alterations must also persist through the reprogramming of primordial germ cells (PGCs). While transgenerational GEI is subject to an additional reprogramming barrier relative to intergenerational GEI, any nonmutagenic environmental exposure capable of passing through either of the major reprogramming events is relevant to questions about heritability and phenotypic variation and classified here as GEI. See Figure 2 for more detail.

2 | PREVAILING CONCEPTIONS OF BIOLOGICAL INHERITANCE IN HISTORICAL CONTEXT

A cornerstone of modern genetics and evolutionary biology is the assumption that DNA is the primary hereditary molecule and that any biological or phenotypic effects of experience or the environment on DNA sequence is both random and irreversible.[7,30] Traits acquired through mutations arising from an animal's interactions with the environment are widely-accepted to be either harmful and nonspecific responses to the original exposure, or simply not transmitted to offspring. These assumptions are in fact quite novel and trace primarily to developments in the late 19th and early to mid-20th century.[27,31,32] Early thinkers as far back as Hippocrates had theories of phenotypic variation that included the influence of the parental environment,[33,34] and Darwin himself was a proponent of ideas about heredity that are now often more commonly associated with Lamarck.[35] As Darwin developed his ideas, he needed to explain not only the similarities between parents and offspring, but also the source of new variation upon which natural selection could act.

With the discovery of the role of DNA in heredity nearly a century away, Darwin proposed a model of inheritance that he called *pangenesis* (Box 2).[36,37] Darwin proposed that cells from peripheral organs and tissues shed minute hereditary particles called *gemmules* that then travel to the reproductive organs, where they are later transferred to offspring (Box 3 in Reference 38) In the developing embryo, gemmules aggregate to recreate the same structures from which they were derived in the parent. Darwin proposed that gemmules could lie dormant across the lifecycle and across multiple generations and that their quantity and composition determined the qualities of the trait that they generated. To Darwin, when use or disuse modifies a structure, the number of gemmules or their nature also changes. If this changed pattern of use persisted for multiple generations, the new, changed gemmules would eventually outnumber gemmules generated by the older conditions, leading to the emergence of the newly-acquired phenotype. Gemmules therefore comprised the primary

BOX 2 Key terms

Pangenesis: Darwin's theory to explain the origins and transmission of heritable phenotypic variation.[36] All body organs and systems were viewed as shedding small hereditary particles (gemmules) that traveled to the reproductive organs to be passed on to offspring, thus shaping phenotypes in the next generation.[38,39] Novel variants were thought to result from changed experiences or use/disuse of existing structures, which modified the gemmules conveyed across generations.[36]

Germinal selection: Theory of heredity proposed by August Weismann.[27] Weismann proposed a physical separation between the *germplasm* (i.e., gametes) and *soma* (i.e., all other bodily cells), often referred to as *Weismann's barrier*. The germplasm remained sequestered and continuous through each generation, while the cells of the soma arose from the germplasm and changed during development. Development involved the selection and differential partitioning of *determinants* composed of *biophores*. Determinants were proposed to compete for nutrition, growing, or shrinking according to their share of nutrition.[42] In this way, quantitative changes in determinants could produce qualitative phenotypic traits that could be shaped by natural selection.

Spermatogonial stem cells (SSCs): Pluripotent germ cells vital to spermatogenesis and fertility. SSCs are self-renewing and give rise to spermatogonia. Spermatogonia differentiate into primary spermatocytes, which undergo meiosis to become spermatids.[212] Spermatids pass through a series of epigenetic reprogramming events, including global demethylation and the histone-protamine transfer, and are transported into the epididymis where they are stored and mature into functional sperm. Transit time in the human epididymis is 2–4 days.[184] See Figure 2 for more detail.

Blood-testis barrier: Physical barrier dividing the seminiferous epithelium into apical and basal components, restricting the flow of water, ions, hormones, and—importantly—creating an immunological barrier to protect developing haploid spermatids from the immune system.[45] The blood-testis barrier is broadly consistent with Weismann's barrier. See Figure 2 for more detail.

Protamines: Arginine-rich proteins abundant in sperm and involved in the condensation, protection, and packaging of nuclear DNA.[213] Protamines bind between 85 and 95% of DNA in sperm, and are thought to prime the paternal epigenome and transcriptional landscape for postfertilization epigenetic reprogramming.[60]

Imprinted genes: Genes that are monoallelically expressed or silenced depending on the sex of the parent of origin.[214] Imprinting/expression can vary with developmental stage and tissue type.[66] Imprinted genes are often organized into clusters with related functions and regulated by imprinting control regions.

Paramutation: A process in which DNA sequence variation in one allele (the paramutagen) affects the expression of itself as well as of the other, wild-type allele (the paramutable allele). While paramutation initially involves a change in DNA sequence, the expression profile of the paramutagenic allele is transferred to the unaffected locus in *trans* via a process referred to as "chromosome kissing".[215] This effect is heritable and persists across several generations even in the absence of the original paramutagenic allele.[67] A well-known example of paramutation in mice occurs at the *Kit* locus.[68]

hereditary unit and their proportions in germ cells, altered by parental exposures, generated variation.

Despite the widespread acceptance of similar notions of inherited acquired traits during Darwin's time, Darwin lacked any physical evidence for the existence of gemmules, let alone any change in their proportion through differential use and disuse or their accumulation in the germline.[39] Even though evolution by natural selection as a concept was not dependent upon the specific sources of variation that Darwin proposed, the lack of a mechanism that could address these critiques meant that many biologists of the day were reluctant to accept natural selection as the engine that generated adaptive phenotypes.[40] At the time, the model of pangenesis was also directly tested—and challenged—on experimental grounds (but see Reference 39 for numerous examples conducted during the 20th century that refute these early experiments). The concept of pangenesis as a source of phenotypic novelty eventually faded from biological debate.

The modern conception of heredity, in which the processes that generate heritable phenotypes are viewed as distinct from the environments that exert selection on them, was instead heavily influenced by the ideas of the German evolutionary biologist August Weismann. Cytogeneticists in the late 19th and early 20th centuries had discovered the separation of cells into nucleoplasm and cytoplasm. They were also revealing that both cells and chromosomes undergo mitosis during development and meiosis during the production of gametes. Weismann recognized the importance of these discoveries to development, heritability, and evolution. He saw how sexual reproduction and the resulting recombination of chromosomal components could provide a source of variation that is transmitted to offspring but that did not rely on inherited acquired characteristics to generate novel phenotypes.[41] Evoking the principles of division and compartmentalization developing among cytogeneticists, Weismann proposed his own model of inheritance and development in which the full complement of hereditary material is present in the zygote ("germinal selection," Box 2). This hereditary material, which he called *determinants*, was progressively divvied up as daughter cells branched into cell lines with specialized functions.[42] This solution to the problem of development in turn required that a full complement of hereditary material—which he called the *germplasm*—be stowed away and sequestered from the cells that would generate the body, collectively called the *soma*.[27] This ensured that a complete, pristine copy of the hereditary material would be available to pass on to the next generation.

While some argue that Weismann's sequestered germline primarily allowed him to reconcile cellular differentiation with the problem of heredity,[43,44] more recent understandings of developmental biology have rendered this model of development unnecessary (see below). Regardless of his motivations, the idea of a germline that is separated from the somatic tissues was surprisingly prescient and has had lasting impacts on modern biology. We now know that the traffic of proteins and other molecules into the germline from surrounding tissues is tightly regulated at the blood-testis barrier in males[45] and that similar processes may insulate the female germline.[46] Over time, documentation of this physical barrier reinforced Weismann's theoretical barrier, which provided what many viewed as the final nail in the coffin of the concept of inherited acquired characteristics. Since Weismann, it has been widely assumed that the modifications made to the body (the soma) as a result of experience die along with the body and that information flow is always one way, from the genome to the developing body, and never in reverse (Figure 1A).

2.1 | Updating the firewalls: Epigenetic reprogramming and germline-body barriers

Despite Weismann's enduring impacts on mainstream biology, it is well appreciated that the diversity of cell types that make up metazoans do not vary by virtue of a mosaic model in which the DNA sequence present in each cell varies.[47,48] Rather, cellular lineages arise as a function of transcriptional and proteomic profiles. Apart from a few exceptions (e.g., red blood cells and germ cells), all cells contain the genome in its entirety, and cellular differentiation is achieved through selective silencing of certain genes and the expression of others. In vivo, such stable gene regulation is in part inherited with cell lineage, as well as through communication with nearby cells.[49] Because cellular differentiation is based in processes of epigenetic silencing and expression of individual genes, insulating offspring phenotypes from the effects of parental experience requires not only retaining DNA sequences in their unmodified form, but also maintaining an epigenetic state capable of being transmitted to offspring to become any cell type (totipotency). Totipotency must in turn be reestablished in the gametes of each generation. In mammals, development from fertilization to gametogenesis does in fact involve several major epigenetic "reprogramming" events that reset the epigenetic state in germ cells (Top panel, Figure 2).[50]

For DNA methylation there are two distinct *epigenetic reprogramming* events (Top panel, Figure 2; Box 1). The first occurs immediately following fertilization, when the cellular differentiation states involved in programming sperm and egg cells are passively (through division without remethylation) and actively (through maternally-derived TET DNA demethylase) cleared.[51] This reprogramming step is necessary for germ cells, which are highly differentiated cells, to "de-differentiate" into a totipotent zygote capable of producing all of the cell and tissue types in offspring, as well as the trophoblast.[51] Cell-line specific epigenetic and transcriptional profiles are then reconstituted during development, resulting in epigenomic signatures and corresponding patterns of gene expression that drive cellular and tissue differentiation.[52]

Following postfertilization reprogramming, a second reprogramming event occurs during the formation of primordial germ cells (PGCs), when PGC lineage-specific epigenetic marks are cleared and replaced with sex-specific DNA methylation (DNAm) in the developing germline (top panel, Figure 2).[50] In essence, the undifferentiated PGCs that will eventually give rise to the germline must be reprogrammed to match the unique expression profiles of spermatogonial or oogonial stem cells, depending on the biological sex of the fetus.[53,54] These two epigenetic reprogramming events form the barriers of *inter- and transgenerational epigenetic inheritance* (Box 1),

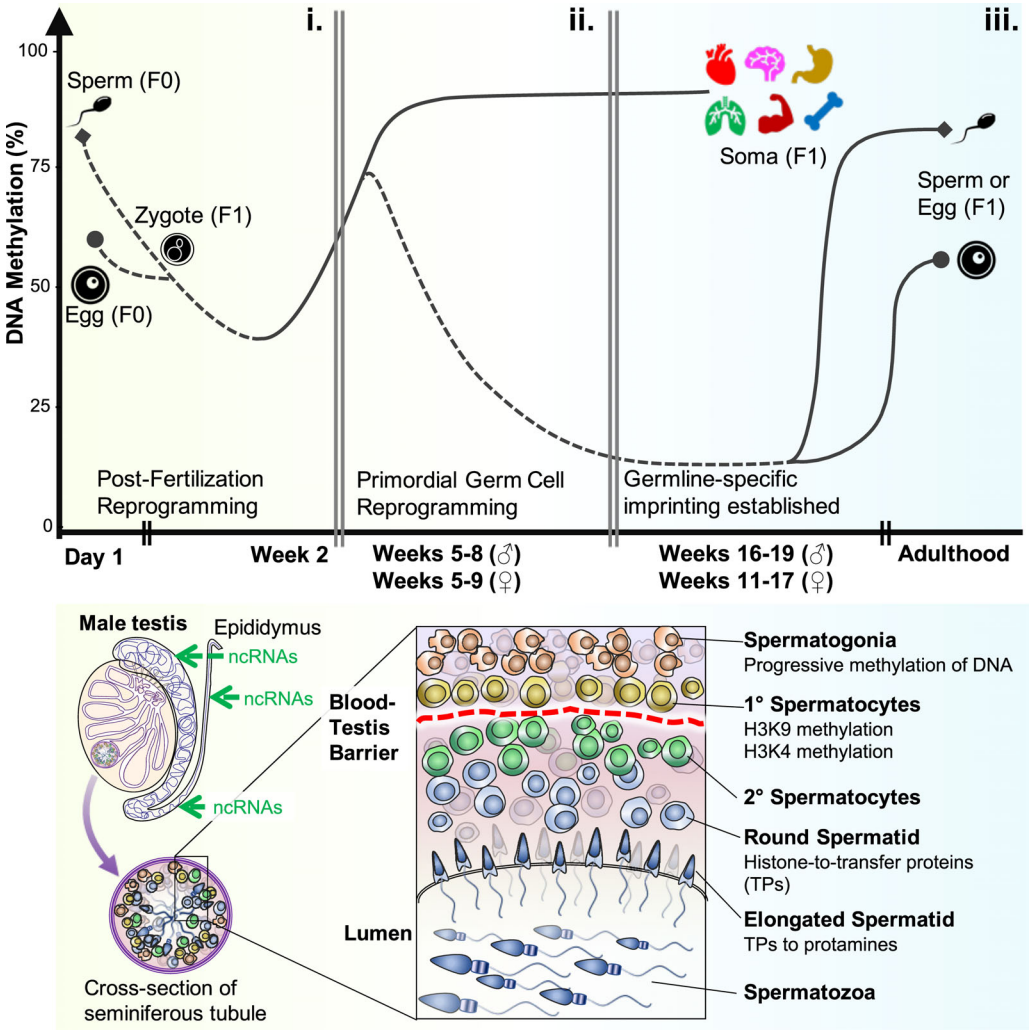

FIGURE 2 Epigenetic reprogramming in embryonic development and male gametogenesis (top panel) reprogramming of the fetal methylome. The germline (sperm and egg) specific methylome is cleared in the early zygote during the formation of the blastocyst. DNA methylation (DNAm) levels increase with cell specification during the differentiation of somatic tissues and primordial germ cells (PGCs). After migration to the genital ridge, the PGC methylome is extensively cleared and sex-specific imprinting is established. An epigenetic alteration from an exposure to the F0 generation that passes through post-fertilization reprogramming (i.) is inter-generational in the F1 offspring. Epigenetic changes in the F0 that persist through both reprogramming stages (i. and ii.) affecting the F2 are transgenerational. An exposure to the F1 while in utero that affects the F2 can be considered intergenerational if it occurs after PGC reprogramming and establishment of imprinted genes (iii.), but transgenerational if it occurs before (i. or ii.). Other epigenetic processes (histone modifications and chromatin structure) show analogous reprogramming processes, though are less well-understood. Non-coding RNAs may bypass reprogramming, providing a source of non-genetic paternal inheritance. (bottom panel) anatomy of the testis and epigenetic reprogramming of the adult male gamete. The testis is made up of thousands of coiled seminiferous tubules. Spermatogonia mature into spermatocytes in the walls of the seminiferous tubules as they move from the outer wall toward the lumen. Maturing spermatogonia are surrounded by Sertoli cells, which nourish maturing sperm cells and create tight-junctions between primary and secondary spermatocytes, referred to as the blood-epididymis or blood-testis barrier. Male gametes undergo numerous epigenetic alterations as they mature, including widespread methylation of the genome and the transfer of histones to compact protamine toroids via transfer proteins (TPs) during the late stages of spermatogenesis. Spermatozoa mature in the epididymis, where non-coding RNAs transferred through epididymosomes may provide a source of non-genetic paternal inheritance [Color figure can be viewed at wileyonlinelibrary.com]

subclasses of epigenetic inheritance which can be defined by the timing of the exposure relative to the stages of epigenetic reprogramming. For example, intergenerational epigenetic inheritance occurs when at least half of an individual's chromosomes—in the form of their parent's gametes—are exposed sometime after PGC reprogramming. Under these conditions, any epigenetic changes pass through postfertilization reprogramming but not PGC reprogramming. In contrast, transgenerational epigenetic inheritance occurs if epigenetic changes have crossed through both postfertilization reprogramming and the reprogramming that occurs in PGCs (Top panel, Figure 2; Box 1).

To complicate matters, *spermatogonial stem cells* (SSCs—Box 2) undergo a third epigenomic reprogramming event as they pass through the *blood-testis barrier*[55,56] (bottom panel, Figure 2; Box 2). This highly regulated barrier between the body and dividing

spermatocytes is formed by tight junctions between Sertoli cells.[57–59] These junctions correspond well with Weismann's theoretical barrier between the soma and the germline, reinforcing the idea that there is little or no information transfer between the soma and developing sperm. In fact, the blood-testis barrier does appear largely impervious to the immune system and the passage of DNA.[58] Moreover, during maturation, sperm undergoes massive, genome-wide reconfigurations in both DNAm and chromatin structure, above and beyond the postfertilization and primordial germ cell reprogramming that occurs in utero with each generation (bottom panel, Figure 2; Box 2). During spermatocytogenesis, spermatogonia undergo wide-spread passive demethylation, while during spermiogenesis up to 90% of histones are replaced by *protamines* (Box 2).[60,61] These sperm-specific shifts in DNAm and the transfer of sperm DNA from histones to more compact but transcriptionally repressive protamines provide the protection and compactness necessary for sperm delivery through the immunologically and chemically hostile female reproductive tract.[56] (the female reproductive tract itself has evolved immunological responses and microbial responses to infection that select for these responses in sperm.) In summary, the epigenetic remodeling needed for successful sperm delivery and fertilization creates an additional information bottleneck that further limits the potential for epigenetic information transfer through the male germline (Figure 1B). Although exceeding the scope of the present literature and this review, the female equivalent of a germline barrier—the blood-follicle barrier—is similarly believed to limit opportunities for nongenetic germline inheritance.[46]

During the 20th century, the blood-epididymis/follicle barriers, two epigenetic reprogramming events, and the third epigenetic reprogramming that occurs in sperm, came to be viewed as insulating each generation from any epigenetic influence arising from the lived experiences of ancestors.[28] This finding provided an updated version of Weismann's hypothesis that environments do not contribute to the variation upon which natural selection acts. As the century came to a close, what remained was a canonical model in which DNA was accepted as the only hereditary material of importance and largely impervious to parental experience.[7] Evolution came to be defined as arising solely from selection, mutation, gene flow, and drift. Inheritance followed Mendelian laws and even the epigenome was thought to be genetically programmed and reset every generation.

2.2 | How recent work is revealing pathways that work around the firewalls

While the model theorized by Weismann was supported through much of the 20th century, the firewalls described above may be turning out to be more porous—or at least more complex—than previously thought. First, although epigenetic reprogramming immediately after fertilization is extensive, it is not absolute. In genomic imprinting, genes are selectively transcribed or silenced depending on the sex of the parent.[62] The epigenetic processes that facilitate genomic imprinting pass through postfertilization reprogramming (but not germline reprogramming) intact and their phenotypic effects in offspring are widely-documented.[63,64] In males, differential methylation in imprinted regions also persists through spermatogenesis, including the process of chromatin repackaging. Thus, the epigenetic state of *imprinted genes* (Box 2) in sperm is retained to varying degrees in individual offspring tissues throughout their lives,[65,66] and provide an example of intergenerational epigenetic inheritance (Box 1). The extent to which imprinted regions are modified by the environment, and later transmitted to subsequent generations (i.e., transgenerationally by passing through primordial germ cell reprogramming; Box 1) is less well documented. Nevertheless, paramutation provides a well-documented example in which a gene's expression state is inherited through epigenetic processes for multiple generations (Box 2).[67] Unlike imprinted genes, which are reestablished with each generation and so do not technically pass through reprogramming of the primordial germline, the transcriptional state of paramutated sites is retained faithfully through both postfertilization and germline reprogramming.[68,69] While imprinted genes and paramutation may well be exceptions rather than the rule when considering epigenetic inheritance,[70] they provide "proof-of-concept" illustrations that the theoretical and physical firewalls laid down throughout the 20th century are far from impenetrable—and that Weismann's theory might be only part of the story.

The blood-testis barrier—although tightly controlled—is also now understood to be permeable to a range of biologically active molecules,[71,72] including numerous proteins and a rich assemblage of coding and noncoding RNAs[73,74] (Bottom panel, Figure 2; Box 1). Both coding and noncoding RNAs circulate ubiquitously in the body in "exosomes," tiny lipid vesicles that are secreted from most if not all cell and tissue types. These RNA-containing exosomes are abundant in the blood, lymph, cerebrospinal fluid, breast milk, and semen,[75] and are able to pass through the blood-testis barrier. Challenging Weismann's barrier, RNA-containing exosomes—especially a subclass secreted from epididymal epithelial cells during the final stages of sperm maturation (i.e., epididymosomes)—are considered a likely conduit for information transfer between the soma and the germline.[76] Preliminary evidence even suggests that epididymal epithelial cell secretions and their RNA payloads may be regulated by circulating hormones, including both androgens and glucocorticoids.[20,77] Noncoding RNAs also provide an alternative information pathway to DNAm and chromatin conformation, which are both extensively remodeled during spermatogenesis. Several subclasses of noncoding sperm RNAs, including *microRNAs* (Box 1) and tRNA fragments, are delivered intact during fertilization, appear in the zygote, and are essential for normal embryonic development.[78] Thus, noncoding RNAs provide a means of by-passing the germline/somatic and epigenetic reprogramming firewalls thought to insulate each generation from the biological effects of ancestral experience. This work lies at the heart of current interest in GEI, which we turn to now.

3 | EVIDENCE FOR GEI: NONHUMAN MAMMALS

3.1 | Evolutionarily novel exposures in nonhuman mammals: Chemicals and drugs

One of the earliest examples suggestive of GEI is the work of Spergel and colleagues in the 1970's,[79] which demonstrated that low dose paternal (and maternal) exposure to a diabetogenic compound (alloxan) induced glucose intolerance and diabetes in subsequent generations. Similar effects have been attributed to other diabetogenic compounds, anesthetics, and chemical insults.[80,81] It was not until 2005 that the heritability of these kinds of inter/transgenerational effects was proposed to involve epigenetic processes. In their seminal paper, Anway et al.[82] found that the endocrine disruptors vinclozolin and methoxychlor were tied to decreased sperm capacity and infertility in the male offspring of exposed pregnant dams (F1). These effects persisted through several generations (followed out to F4) and were tied to altered DNA methylation in the male germline.[82] Demonstration of transgenerational phenotypic effects (accompanied by germline alterations in DNA methylation) from other chemical insults and endocrine disruptors followed. Since then, a long list of paternal chemical exposures have been linked to offspring phenotype, presumably through GEI. Paternal exposure to cocaine,[83,84] nicotine,[85,86] betel nut,[87] and alcohol[88,89] have all been linked to inter- or transgenerational alterations in germline epigenetic processes, including DNA methylation, histone modification, and noncoding RNAs (Table S1).

3.2 | Evolutionarily ancient exposures in nonhuman mammals: Fear, famine, and learning

While these findings point to potentially intriguing evidence for GEI, many of the examples remain confounded by the possibility of predominantly mutagenic—rather than strictly epigenetic—effects.[81] This stems from the fact that many of the toxins and chemicals mentioned are known mutagens and because genetic variation affects epigenetic variation—a major obstacle to studies of GEI (discussed in more detail below). In recent years, work on GEI has expanded beyond toxicants, endocrine disruptors and related chemical exposures to explore the effects of evolutionarily less novel stimuli like psychosocial stress, diet, learning, and aging.

3.2.1 | Psychosocial stress

The impact of psychosocial stress across generations has been an active area of investigation in GEI. One of the earliest demonstrations of such effects came from the work of Drake and colleagues,[90] who reported that prenatal exposure to a synthetic glucocorticoid (dexamethosone) in male rats was associated with decreased birth weight and increased hepatic PEPCK activity in their offspring.[91] More recent work has shown that experimentally inducing stress through various protocols (Table S1) can lead to increased risk taking behavior,[92] poorer performance in a forced swim task,[93,94] and a blunted hypothalamic–pituitary–adrenal (cortisol/stress) response in offspring.[95,96] These effects have been linked to changes in DNAm or gene expression in the offspring brain,[92,93,95] liver,[97] or circulation.[94] Several studies have revealed changes in the sperm epigenome that point to mechanisms of epigenetic inheritance. These include DNA methylation,[93,94,96–98] but as noted above, sperm RNAs in particular have emerged as prime candidates for inter- and transgenerational transmission of nongenetic information through the germline.

In 2010, a postnatal maternal separation protocol in one generation (P0) was found to induce depressive-like symptoms among the subsequent generation (F1). These exposures were tied to changes in DNAm in sperm of exposed F1 males, as well as in the brains of unexposed offspring.[93] Postnatal maternal separation—as well as corticosterone injections—were later linked to changes in sperm RNAs. Importantly, injections of microRNA harvested from the sperm of exposed animals recapitulated the stress-induced phenotype among offspring of unexposed fathers.[22,94] More recent studies examining the effect of injecting small and long ncRNAs from stress-exposed males found that different classes of RNA each recapitulate a different subset of metabolic and behavioral phenotypes.[22] Similar findings have been reported by other groups, including the finding that paternal exposure to chronic stress led to blunted stress reactivity among unexposed offspring.[96] These changes coincided with gene transcription enrichment patterns in the brains of offspring, as well as changes in the composition of nine sperm miRNAs[96] (Box 1). Later work showed that microinjection of these nine miRNAs into an unexposed zygote led to a reduction in certain maternal mRNAs and recapitulated the phenotype of offspring of exposed fathers.[95] More recently, this group has shown that exposure of epididymal endothelial cells to glucocorticoids causes widespread changes to the histone code as well as the miRNA content of secreted epididymosomes.[20] In a rare example that explored possible interventions, Gapp et al.[99] found that the negative transgenerational effects of early life paternal stress on offspring phenotype were mitigated by paternal social (i.e., cage-mates) and environmental (i.e., mazes, wheels) enrichment. The protective effect on offspring behavior coincided with changes in DNAm in both the exposed father's sperm and in the hippocampi of offspring.[99] Interestingly, a follow-up study found that transgenic mice with reduced epididymal cell glucocorticoid receptor expression did not exhibit the intergenerational transmission of the stress-induced phenotype, suggesting that the glucocorticoid receptor plays an important role in mediating the GEI-based transmission of paternal stress.[20]

In one of the most highly publicized—but also critically received—studies, researchers exposed male mice to an odor (acetophenone or propanol) along with a mild electric shock.[98] They observed changes in the olfactory bulbs, as well as odor-potentiated startle response, of F1 offspring. These same changes were observed through cross-fostered, as well as in IVF fertilized F1 offspring, and changes in paternal sperm DNAm at a gene associated with olfactory bulb development point to GEI as a pathway for such effects. These findings suggest a

fascinating example of GEI in which fear conditioning is itself passed down through the male germline. Nevertheless, as with other studies in nonhuman mammals, researchers have questioned these findings on design and statistical grounds (e.g., lack of clear, a priori, testable hypotheses; multiple comparisons; reporting differences in significance, i.e., one group is significant, and one is not—and not significant differences—between groups[100–103]) and the field awaits independent replication of this finding.

3.2.2 | Nutritional stress

Nutritional stress—typically a high-fat or high-sugar diet—has also been extensively studied with respect to GEI. As with the psychosocial stress experiments described above, there has been much variation in study design with respect to the timing of nutritional exposure (i.e., prenatal, juvenile, and adult), as well as the offspring phenotypic and epigenetic processes examined. Despite this variation, evidence is converging on the conclusion that paternal diet can impact epigenetic processes in the germline, as well as the epigenotype and phenotypes in subsequent generations. A high-fat, low-protein paternal diet has been linked to offspring body weight or composition,[104–110] liver function and gene expression,[104,111–114] insulin levels,[21,104,106,114,115] and glucose intolerance.[38,104–108,110,114] The role of epigenetic processes in the heritability of these traits has been supported by observing changes in DNA methylation,[110,112,113,115] chromatin structure,[111,113] and noncoding RNAs,[23,104–106,111,112] in the sperm of exposed males or their unexposed progeny (but see Reference 116). In several cases, epigenetic changes similar to those observed in sperm are detectable in the tissues of progeny (e.g., the liver), supporting a causal link between epigenetic alterations in the paternal germline and the offspring phenotype (e.g., References 106, 113, 115).

As with psychosocial stress experiments, sperm RNAs have become prime candidates for the inter- and transgenerational transmission of paternal nutritional stress. In an early study in this area, Rando and colleagues reported that a high-fat paternal diet caused wide-spread changes in offspring liver gene expression, but found no evidence that these were mediated by changes in sperm DNAm.[111,116] Nevertheless, they showed that IVF fertilization of eggs using sperm from fathers fed a high-fat diet caused the same changes in hepatic gene expression among offspring, indicating that some form of germline inheritance was in effect.[111,116] Later studies confirmed the role of ncRNAs in such effects. Convergent findings by two separate groups simultaneously attributed inheritance of metabolic phenotypes to a subclass of noncoding tRNAs.[21,23] Microinjection of tRNA fragments, particularly those between 30 and 40 nucleotides in length and a fragment derived from a glycine tRNA (tRF-Gly-GCC), induced changes in gene expression and developing offspring phenotype, beginning at the blastocyst stage. These changes appear to arise through the effect of the tRNAs on the expression of the endogenous retroelement MERVL, which itself regulates the expression of numerous genes involved in cell fate and totipotency (but see also Reference 70).

In addition to the studies of psychosocial or nutritional stress described above, several other studies are worth mentioning. The first examined the effect of experimental liver damage on hepatic fibrogenesis (scarring) in offspring and grandoffspring of male rats.[117] Researchers found that offspring and grandoffspring of treatment animals had less hepatic fibrosis when exposed to the same hepatotoxin as their fathers (or grandfathers) and exhibited altered liver DNAm and mRNA in several genes associated with hepatic scar formation. Treatment was linked to changes in F0 sperm chromatin organization in genes involved in the scarring process.[117] Astonishingly, the effect on fibrosis was observed only in damaged liver tissue and not in experimentally damaged renal tissue, indicating tissue specificity of the treatment effect. Furthermore, some of the transgenerational effects on gene expression and scarring were recapitulated through injections of serum from fibrotic rats to unexposed animals.[117] Notably, several of the key genes involved in the transgenerational effect of liver damage were also found to differ in the livers of humans with mild compared to severe liver fibrosis.

In another study, researchers examined the effect of paternal social (i.e., cage-mates) and environmental (i.e., mazes and wheels) enrichment on offspring long-term potentiation (a measure of synaptic plasticity) and measures of cognitive performance in mice.[118] Offspring of mice exposed to enriched environments had greater synaptic plasticity and cognitive performance than control mice, and these phenotypic effects were replicated through injection of sperm RNAs from treatment males.[118] Phenotypic effects were linked specifically to two miRNAs (miR202/132) in treatment male sperm that have been found elsewhere to be involved in learning in mice. Notably, miR202/132 were elevated in the hippocampi of treatment mice within weeks of environmental enrichment, but were only detected in sperm after 10 weeks of treatment,[118] supporting a delay in the transfer of environmental stimuli to epigenetic alterations in sperm.

The role of epigenetic processes in aging has become a topic of great interest,[119,120] and work by Xie et al.[121] suggests that the rate of biological aging may be affected by paternal age at conception. They showed that older mice sire offspring with shorter lifespans and accelerated histopathological and molecular measures of aging. The effect of paternal age at conception was associated with differences in DNAm, miRNAs, and chromatin composition in paternal sperm and these differences were more likely to occur in genes involved in aging, senescence, and longevity.[121] In addition to these changes to F0 sperm epigenetics, F1 offspring exhibited altered DNAm and mRNA levels in the hippocampus. These differentially methylated and expressed genes in the brain were also tied to aging-associated pathways (e.g., mTor signaling) and were disproportionally found in the same genes and pathways that were altered in the sperm of older fathers. Importantly, researchers were able to narrow the candidate pathways to mTOR1 and ameliorated age-associated transcriptional changes and a subset of age-related phenotypes in mice of older fathers using the mTOR inhibitor rapamycin.[121] These findings suggest a set of pathways by which a father's age can impact the biology of aging in offspring, operating through GEI-mediated epigenetic pathways (for related work on telomeres see Reference 122).

4 | EVIDENCE FOR GEI: HUMANS

Despite evidence for GEI in nonhuman mammals like mice and rats, and even stronger support for similar processes in fruit flies (*Drosophila melanogaster*) and roundworms (*Caenorhabditis elegans*; reviewed in Reference 34) evidence in humans remains patchy. A number of studies have claimed support for GEI in humans in response to evolutionarily novel (e.g., chemicals and drugs) and not-so novel (nutritional deprivation and trauma) exposures. Research on GEI in humans generally falls into one of two categories: epidemiological studies supporting inter- and transgenerational impacts but lacking evidence for a biological mechanism, and studies supporting a mechanism through alterations to sperm epigenetic profiles, but that lack data to support inter- or transgenerational phenotypic effects. There are a number of challenges inherent in studying GEI in humans and the skepticism should not be pushed aside.[123] However, before considering the major challenges to studying GEI in humans, we first describe the most noteworthy human studies that have explored GEI. As with the nonhuman animal literature, we focus on those thought to be transmitted through the male germline.

4.1 | Evolutionarily novel exposures in humans: Chemicals and drugs

Building on the finding that the ingestion of Betel nut (*Areca catechu*) in mice has diabetogenic effects on unexposed F1 and F2 offspring,[87] Chen et al[124] found an increased risk of metabolic syndrome among a large (n = 5,037) sample of children of fathers who consumed Betel nut in Taiwan, with a dose–response relationship linking the duration and quantity of Betel quid consumed to offspring metabolic health.[124] Similarly, using the large Avon Longitudinal Study of Parents and Children (ALSPAC), Northstone and colleagues found that the sons of fathers who started smoking regularly prior to age 11 had significantly higher BMI, waist circumference, and total fat mass.[125] Supporting these epidemiological findings, a number of other studies have found differences in the sperm epigenome (miRNAs, histone-protamine ratio, and LINE-1 methylation) of healthy smokers and nonsmokers, providing a potential mechanistic link between paternal smoking and offspring phenotype,Table 1 in References 61, 126 although so far no single study has brought these two lines of evidence together.

4.2 | Evolutionarily ancient exposures in humans: Famine, fear, and learning

Perhaps the most widely discussed evidence for transgenerational inheritance in humans comes from a study of nutritional stress using historical records from the rural Swedish municipality of Överkalix (e.g., Reference 127, reviewed in Reference 128). Here, researchers linked records of harvest quality during certain periods of childhood growth with the mortality of grandoffspring. Evidence for an exposure-mortality relationship was only found to be transmitted through the father and to vary depending on the sex of the grandoffspring: mortality risk was elevated for females only when the paternal grandmother was exposed to good nutrition between ~8 and 10 years of age, while it was elevated for males only when the paternal grandfather was exposed to good nutrition between 9 and 12 years of age.[129] Although less often discussed, the authors also found evidence that mortality at Överkalix was *reduced* if the same-sex paternal grandparent experienced favorable nutrition around the time of gestation and early infancy. These findings have been questioned on a range of study design and statistical grounds, and pathways of inheritance not involving germline epigenetic transmission (discussed in more detail below) must be considered as alternative explanations for these findings (e.g., Reference 130). However, a recent study using separate Swedish historical records set out to replicate their findings with larger sample sizes.[131] While their findings were still somewhat mixed, these researchers were able to replicate one of the main (but still counter-intuitive) findings of Överkalix: proxies of good nutrition just prior to puberty among the paternal grandfather was associated with higher mortality in grandsons.[131]

4.3 | Molecular studies connecting environmental exposures to sperm epigenetics

While epidemiological findings provide one line of support, other research has focused on the putative mechanistic underpinnings of GEI in paternal exposure to metabolic or nutritional changes. An early study of newborn umbilical cord blood DNA found a relationship between paternal obesity and differences in DNAm at the *IGF2* imprinted locus.[132] This finding is consistent with more recent work of Donkin and colleagues[133] on the effect of obesity and bariatric (gastric-bypass) surgery-induced weight loss on men's sperm. This work shows that obese men have distinct patterns of DNAm and small ncRNA in sperm when compared to men of healthy weight. Importantly, they showed that obese men displayed dramatic remodeling of the sperm epigenome in response to surgery.[133] Roughly 1,500 changes in methylation were observed 1 week after surgery, many of which were still present 1 year later. Importantly, extensive changes in sperm methylome observed following surgery corresponded closely to sperm methylation differences observed between lean and obese men who had not been subjected to the surgery.[133] The same research group recently demonstrated that experimental endurance exercise training led to wide-spread remodeling of the sperm epigenome (DNAm and RNAs),[134] confirming changes in the sperm methylome among healthy young men experimentally assigned to exercise training previously reported elsewhere.[135]

A number of studies focusing on molecular processes thus suggest that alterations to the sperm arising from metabolic and nutritional change can have both rapid and persistent effects on the sperm epigenome.[133] Whether or not these changes are transmitted to offspring is at present unclear. However, a large (n > 8,000) Swiss study reported that individuals exhibiting active brown-adipose tissue (BAT) were more likely to have been conceived during colder

TABLE 1 Major challenges facing researchers interested in studying epigenetic inheritance through the paternal germline (GEI) in humans, and potential solutions, opportunities, and examples to address them

Major challenges to studying GEI in humans…		Solutions and opportunities	Examples
Humans are long-lived—Many exposures over lifetime	→	Critical developmental and molecular windows narrow down timing of exposures	5–9 weeks gestation, 11–19 weeks gestation, 11–14 years of age Spermatogenesis (74 days), epididymal transit (2–4 days)
Exposures are often correlated (e.g., SES and nutrition)	→	Some exogenous exposures are less correlated than others	Natural disasters, epidemics
Exposures are rarely random, but biased toward certain genotypes/phenotypes (e.g., racism and structural inequality)	→	Some exposures are less systematic than others with respect to genotype/phenotype (although this should be considered carefully)	Natural disasters, epidemics
Exposure can affect offspring indirectly by affecting parental phenotype (e.g., affect offspring via parental behavior, not epigenetic processes)	→	Some short-lived/transient exposures may have minimal effect on parental phenotype	In utero nutritional deprivation/stress during natural disaster or similar short-lived emergency
Exposure can affect rearing environment independently of parent (e.g., post-disaster environment differs)	→	Some short-lived/transient exposures may have minimal impact on long-term rearing environment	In utero nutritional deprivation/stress during natural disaster or similar short-lived emergency
Pathways are many (e.g., genetic, physiological, social)—GEI is rarely the most parsimonious	→ →	Large datasets with genetic, epigenetic, environmental and social information across multiple generations are becoming available Innovative study designs (e.g., involving siblings before/after exposures) can help control for other pathways of inheritance	Histone-retention sites (e.g., ACOX3, PGGT1B, TSEN15, ZFP281), escapees (e.g., SVA and L1PA retroelements, SRRM2, CSNK1D, TACC2)

Note: See the text for more detail.

months (October and February) than during the warmer parts of the year.[24] This finding was replicated using cold exposure in mice, whose offspring also exhibited alterations consistent with an adaptive response to cold-exposure.[24] A substantial subset of the genes showing differential expression in mouse offspring were also detected in mouse paternal sperm, pointing to potential mechanistic links between paternal cold-exposure and the cold-resistant phenotype.[24] Despite these promising mechanistic findings in mice, it seems puzzling that a short-term temperature experience by a human father would affect the BAT activity over his child's lifetime, and a number of other explanations for the season of birth/BAT effect in humans must first be ruled out; we return to some of these issues in later sections.

Psychosocial stress and trauma have also been the subject of several studies of GEI in humans. In a highly publicized study by Yehuda et al.,[136] the children of Holocaust survivors exhibited differences in DNAm at CpG sites proximal to the *FKBP5* gene when compared to the offspring of individuals living outside Europe during WWII.[136] Limitations in this study's design and statistical approach have been noted[137] and alternative pathways of inheritance (discussed below), including exposure to the parents' own post-traumatic stress disorder symptoms, are potentially more parsimonious explanations. Nevertheless, a more recent study of the progeny of American Civil War POWs using historical medical records found that the sons of men who had been imprisoned when camp conditions were harshest had a higher mortality rate than those with fathers who had not been imprisoned, or imprisoned under less challenging conditions.[138] In this study, a number of factors were statistically controlled for, including socioeconomic effects, family cohesion and structure, and maternal and paternal health. However, no mechanistic or biological pathways were evaluated in this study, leaving the contribution of GEI to these effects unclear.

Studies that combine human research with experimental data from mice or other model organisms may be a particularly fruitful approach to studying GEI. In one example, Dickson et al.[139] reported an inverse relationship between men's adverse childhood experience (ACE) scores and the quantity of miR-449 and miR-34 miRNA in their sperm. Importantly, they were able to induce these same changes in sperm miRNA in male mice using a chronic social instability stressor and showed that not only were these changes present in early embryonic development, but that they could also be detected in the sperm of unexposed F1 offspring.[139] Such studies, which combine human historical or health records with experimental animal work, provide some of the most compelling evidence to date for GEI in humans (see also Reference 24).

5 | DISCUSSION

While much remains to be determined about the extent of GEI in humans and—if it exists, its mechanistic bases—a number of political and policy related issues hinge on understanding this

phenomenon.[140,141] Epigenetic processes on their own have already shifted our conceptions of what constitutes our "innate," inherited biology and are blurring the boundaries between genes and the world that we inhabit.[142,143] GEI potentially expands the reach of the environment to include multiple generations and longer timescales. Studies linking obesity and exercise with rapid changes in sperm epigenetic variation hint that intergenerational effects of paternal behavior might be present in humans. If paternal experiences and behaviors like stress, smoking, and dietary fat intake do impact subsequent generations, the intergenerational stakes for men's health behavior will be elevated, but new targets for behavioral and policy intervention may also be revealed.[141] Nevertheless, demonstrating that inter- or transgenerational relationships trace to GEI as a causal pathway is not trivial. All of the obstacles common to work aimed at identifying the long-term biological consequences of early life experiences are compounded by others that are unique to the biology of this unusual set of pathways. GEI is but one of many explanations for the transmission of environmental information between generations, and of these, rarely the most parsimonious. Here we will consider the major alternative pathways and processes that can confound studies of GEI in humans and that need to be ruled out before concluding GEI is the best possible explanation.

6 | ALTERNATIVE PATHWAYS OF INHERITANCE TO GEI IN HUMANS

6.1 | Genetic inheritance

Any study of inheritance must consider the potential confounding effects of genotype and genetic relatedness.[1] After tissue-specific variation in epigenetic profiles (e.g., different cell lineages possess markedly different epigenomes), underlying genetic sequence is thought to be the predominant driver of inter-individual differences in epigenetic profiles.[144,145] Genetic sequence explains most inter-individual (including intergenerational) similarity in DNAm among family members and an estimated 20% of the variation in DNAm in populations as a whole.[145,146] For example, a mutation to a cytosine in a CpG dyad will remove the possibility of methylation at that site, while a single SNP in other cases can predict methylation at as many as 200 CpG sites across the genome.[145] Although certain CpG loci show evidence of "epigenetic supersimilarity," possibly due to stochastic epigenetic changes prior to embryonic cleavage during twinning,[147] and some researchers suggest that heritability estimates for DNAm may be exaggerated,[148] it is still important to consider genetic similarity between individuals when studying GEI in humans or other outbred species.

Genetic variation can also affect studies of GEI through less direct processes, such as through differential genetic selection. If the different genotypes of a man's spermatozoa differ in their ability to fertilize the ova or survive gestation under certain conditions (i.e., nutritional stress), the observed living cohort will not represent the conception cohort and estimates of the impact of paternal environment on offspring phenotypes will be correspondingly skewed.[149] It is possible to circumvent this issue by examining the genetic distribution of offspring across exposure groups, but this remains challenging in large, living human populations. Another way that genetic variation can affect estimates of GEI in humans is through assortative mating, in which individuals preferentially mate based on genetic, physical, behavioral, or other phenotypic similarities or dissimilarities.[150] An exposed male with certain phenotypes who mates with a female with similar phenotypes increases the odds that his offspring will share those traits, whether they initially arose through shared exposures or shared genetic backgrounds. Other factors, like maternally-inherited mitochondrial DNA, can also confound estimates of heritability through indirect genetic effects.[151] For example, maternal nutritional status can alter mitochondrial DNA copy number in her offspring, affecting offspring cellular energy production and metabolism.[151] For these and other reasons, researchers should, as feasible, design studies that take into account the role of genetics when attempting to test hypotheses about GEI. Analyzing methylation quantitative trait loci (mQTLs) to check for genetic correlations with candidate sites and/or incorporating exposed/unexposed sibling comparisons to reduce genetic confounds are starting points toward realizing this goal.

6.2 | Social/physical environment

In human societies, the continuity of social and environmental conditions across generations are major pathways of inheritance and should be considered when studying epigenetic inheritance.[1,5] Although the relevance of inherited environments is well-recognized, its confounding role in intergenerational phenotypic effects can take unanticipated forms. As one hypothetical example, a parent who experiences political violence prior to the birth of the child may harbor residual concerns about their physical safety and so alter the home environment, the opportunities available to their children, or the emotional valence around certain people, places, or things. These changes in the environment in turn can shape a child's development and epigenome.[152] The inheritance of cultural practices and norms is particularly pronounced in humans[4] and is likely to be a major contributor to many would-be examples of phenotypic and epigenetic inheritance.[44] High-risk behaviors, such as alcoholism, drug abuse, gambling, and promiscuity—all linked to experiences of psychosocial or physical trauma[153] —might also indirectly affect the home environment and social norms experienced by a child. More benign cultural factors downstream of early or ancestral experiences, such as education, socioeconomic status, health behavior, political orientation, or religious practices, can also affect the child's rearing environment, impacting their phenotype and epigenotype.[9,10,154]

6.3 | G × E and rGE

The tendency for offspring to have similar genes and environments to their parents means that genotype–environment (G × E) interactions and correlations must also be considered. As an example, carriers of

genotypes thought to be more "reactive" to social stressors, such as certain variants of *5-HTTLP* or *DRD4*, may show varying epigenetic and phenotypic susceptibility to some exposures.[155] By constraining the range or threshold of epigenetic and phenotypic effects, underlying genetic sequence can affect the lability of response to exposures to social and physical environments.[14] As a result, two generations of genetically related individuals may exhibit similar epigenetic, metabolic, or psychological profiles due to similar genetically-based environmental sensitivities and similar environments—the epigenetic processes themselves may not be inherited.

Another way that underlying genetic sequence can contribute to epigenetic inheritance is through G × E correlations (rGE), in which an individual's genotype may not alter their response to an environment or exposure (as with G × E interactions), but rather the probability that they will be exposed in the first place.[156] rGE can take three major forms: passive, evocative, and active. Passive rGE occurs because children share genes with their parents but are also often raised by them. A parent who smokes, for example, may pass on both the genetic predisposition to smoke and an early childhood environment characterized by smoke exposure. Evocative rGE occurs when individuals indirectly shape their environment or elicit certain social outcomes—a more outgoing or physically attractive child can evoke certain responses from their peers or caregivers that may not be available to individuals characterized by less socially- or culturally-valued traits.[157] Finally, active rGE occurs when individuals actively seek out some environments over others, altering the probability of exposure. Extroversion and openness to new experiences, for example, can cause individuals to seek out social experiences and environments outside the repertoire of less gregarious and adventuresome individuals.[158] Insofar as genotype biases the environments individuals choose for themselves or are exposed to by others, rGE can confound studies of GEI.

6.4 | Maternal effects

The shared physiological environment of the mother and offspring during pregnancy and breastfeeding is an enormously important nonGEI pathway for the transmission of metabolic, immunological, or toxicological cues that impact developing phenotypes.[159–161] Pathways can include such diverse effects as maternal hormones priming endocrine regulation,[162–164] effects of energetic and nutrient constraints on organ development or body growth,[165–167] or teratogenic effects of toxic exposures.[168] Similar effects may be conveyed after birth via signals, such as growth factors, immune components, and other signals transferred in breast milk.[169] In addition, recent work is highlighting the inheritance of the maternal microbiome[170,171] and noncoding RNAs[172,173] as emerging forms of nongenetic, nongermline biological information that connects maternal phenotype and experience with offspring biology. As noted above, the complex influences of maternal gestational and lactational physiology and metabolism on offspring phenotype help explain the predominant focus on paternal transmission of environmentally-induced epigenetic information in experimental animal model studies, in which the potential confounding role of such effects are minimized. In research conducted in human populations, maternal effects generally may not be eliminated in the same way, which presents an important challenge to efforts to isolate GEI as a source of phenotypic variation in offspring.

7 | THE FUTURE OF STUDYING GEI IN HUMANS

While it is rarely possible to account for all possible confounders, understanding the various factors that can affect phenotypic outcomes provides opportunities to minimize their effects by paying careful attention to study design and statistical analyses. The major criticisms of studies purporting to document GEI in humans have centered on correlated exposures between generations, poorly defined biological pathways for transmission, and a lack of a priori predictions between exposures and phenotypic outcomes. A more general critique of GEI stems from the overwhelming focus of nearly all of this work on documenting the negative effects of traumas or stressors. In theory a much wider array of exposures, including beneficial or enriching experiences, should have important epigenetic and phenotypic effects (for an expanded discussion see[140,142]). The focus on pathology likely stems in part from the priority of funding agencies, which fund work aimed at clarifying the pathways that cause disease. However, focusing solely on negative effects of stress or trauma runs the risk of stigmatizing disadvantaged groups who are most impacted by them.[140,142] Despite these pitfalls, stressors are often easier to reconstruct retrospectively from historical records and can facilitate resolution of the timing of past exposures. We next consider principles of study design that would help limit the potential for confounding by alternative pathways and move the field closer to causal inference, even if these studies are likely to primarily illuminate the negative impacts of GEI triggered by stress or trauma.

In studies of GEI, an ideal exposure would be acute and vary randomly with respect to genotype and phenotype, thus approximating a natural experiment (Table 1). The exposure would also not have long-term impacts on parental phenotypes or the rearing environment. In practice, these criteria will rarely be entirely met, but an acute exposure that approximates a quasi-experiment, that is experienced during a critical developmental period (discussed in more detail below), and that has minimal impact on the parental epigenotype, phenotype, and rearing environment, is a workable goal (Table 1). Examples of such exposures might include short-term economic or sociopolitical instability or disease outbreaks, ideally experienced during epigenetic reprogramming events or critical windows of spermatogenesis.[168,174] Other factors, such as famine, war, or natural disasters may meet some of these requirements,[154,175] but are more likely to have long-term indirect effects through alternative pathways like altered maternal physiology or childhood rearing environment. Family studies with multiple siblings provide opportunities to minimize the impact of some

classes of confounding factors, for instance by minimizing variation in certain domains of the parental or rearing environment. Comparing individuals to siblings born before and after an exposure will be useful for GEI research, whereas twins may be less useful given that they share both genetic similarities in addition to paternal exposure. The phenotypes studied in offspring may be informed from past animal model research, or alternatively could be detected through functional analysis of regions that circumvent the histone-to-protamine transfer (e.g., *ACOX3*, *PGGT1B*, *TSEN15*, *ZFP281*, etc.)[176] or demethylation during spermatogenesis and fertilization (Bottom panel, Figure 2),[177,178] including the so called "escapees" of primordial germline epigenetic reprogramming (e.g., SVA and L1PA retroelements, *SRRM2*, *CSNK1D*, *TACC2*, etc.)[54](top panel, Figure 2; Table 1).

7.1 | Temporal windows: Insights from empirical and epidemiological studies

Designing a study with appropriate timing of exposure is particularly challenging when evaluating GEI. The critical period effects on metabolic and physiologic outcomes documented by the DOHaD field has greatly facilitated testing of these effects in humans by allowing researchers to focus on conditions, like stress or famine, experienced during particular trimesters of pregnancy.[179,180] An outstanding question in the study of paternal GEI is whether there are similar critical periods during which environmental exposures can impact germline epigenetic processes or other developmental windows when alterations in GEI do not occur. With respect to male GEI, there does not appear to be evidence for clear critical windows in rats and mice as pup, juvenile, and adult exposures have all been associated with germline epigenetic modifications and/or trans- or intergenerational effects. Furthermore, the fusion of RNA-containing exosomes with maturing sperm in the epididymis—currently the best-supported mechanism for GEI in mammals—points to ongoing incorporation of signals from environmental exposures across adulthood (bottom panel, Figure 2; but see Reference 118).

However, as discussed above, several studies[129,131,139] have been interpreted as evidence that childhood may represent a period of heightened epigenetic sensitivity in human males (Table 1). Biological precedent for epigenomic sensitivity at this age is lacking and the absence of any mechanistic rationale for restricting effects to late childhood—and the claim that these differ by sex—has prompted critique of this work.[127] Although some have suggested that clonal expansion of spermatogonia prior to puberty (~12–14 years of age) in males could constitute a window of epigenetic sensitivity,[61] most available mechanistic data in humans do not support this concept. As described above, studies of humans have shown that adult experiences like cold-exposure, weight loss, or adopting an exercise regime can lead to changes in the sperm epigenome in a short time frame,[24,133,135] arguing for an epigenetic sensitivity to experience that persists across adulthood.

7.2 | Temporal windows: Insights from molecular and developmental biology

Although work on ncRNA has failed to identify critical periods outside of which environments do not have effects, other modes of epigenetic inheritance have characteristics more consistent with this concept. In particular, several features of genomic imprinting allow the framing of a priori hypotheses that could be tested in humans: (a) there are known windows of epigenetic erasure and remethylation in both the male and female germline; (b) there is some evidence that imprinting is itself sensitive to early embryonic experiences, including nutrition in utero[132]; (c) these changes in epigenetic state persist through the lifetime of the individual until the time of fertilization; (d) they pass through the initial reprogramming that occurs immediately after fertilization; and (e) imprinted genes, once inherited by offspring, have well-described effects on growth, development, cognition, and behavior.[63,181,182]

Sex-specific imprinting is established in the gonadal primordial germ cells after the second global demethylation event (top panel, Figure 2). Several aspects of this process are worth highlighting. First, in human primordial germ cells demethylation of imprinted genes occurs between 5 and 9 weeks of gestation in males while remethylation occurs at 11–19 weeks[54] (Table 1). These dynamic periods of epigenetic remodeling may be particularly vulnerable (or amenable) to environmentally-induced epigenetic change (Table 1). Second, the windows for remethylation of the germline differ for males and females, with female germline imprinting established slightly earlier than that of the male germline (top panel, Figure 2).[50] This provides an a priori starting point for assessing the effects of parent-specific timing of exposure on epigenetic inheritance. Third, in addition to a number of evolutionarily young and active retrotransposons, Tang et al.[54] found evidence for thousands of non-repetitive genomic regions that bypass germline reprogramming in human PGCs. Outside of repetitive elements, these regions are disproportionately located in enhancers, CpG-islands, promoters, and gene bodies, pointing to their possible regulatory roles.[54] Even more surprising is the fact that the regions found to escape reprogramming were functionally enriched in processes tied to neural and brain development and many of these genes have been associated with traits and diseases showing complex inheritance patterns, including obesity, schizophrenia, and multiple sclerosis (e.g., *SRRM2*, *CSNK1D*, *TACC2*, etc.)[54] (Table 1). Thus, imprinted genes and the regions that escape reprogramming described above may be especially sensitive during relatively narrow developmental windows, with the timing of critical windows varying according to sex of the exposed embryo. These effects could then be evaluated by linking information on these parent-of-origin exposures (including sex-specific embryonic critical periods) to offspring phenotypes. For example, if opposite sex dizygotic twins (F1) experience brief exposure to malnutrition or shock in utero, the offspring (F2) of one sex but not the other would be expected to exhibit intergenerational effects due to the non-overlapping windows of sensitivity of PGC reprogramming (Top panel, Figure 2).

The epigenetic reprogramming that occurs during spermatogenesis and sperm maturation also point to candidate windows of epigenetic sensitivity during adulthood. In addition to the clonal expansion of spermatogonia prior to puberty, several major epigenetic transitions occur during the ~74-day window required for spermatogenesis (Bottom panel, Figure 2; Table 1). During the early stages of *spermatocytogenesis* (Box 2), cell division of primary spermatogonia is accompanied by passive demethylation, which is then followed by numerous histone modifications and the histone-protamine transition.[183] Spermiogenesis, when sperm mature and become active through exposure to a cocktail of proteins, mRNAS, and ncRNAs in the epididymis, is also a critical period of epigenetic activity.[61] Indeed, sufficient maturational time in the epididymis is critical for sperm viability and the epididymis has become the focus of nonhuman research in GEI. To the extent that epididymosomes are important sources of sperm-born epigenetic signaling in humans, as has been increasingly demonstrated in other species, the 2–4 days transit time of sperm in the human epididymis[184] may define a relatively narrow period of information transfer that can be incorporated into study designs (Table 1).

If de- and re-methylation (reprogramming) are vulnerable periods in germline development (i.e., when the F1 male offspring is in utero), these temporal windows could also provide useful opportunities to study epigenetic inheritance in humans. Specifically, exposures of the mother (F0) during these periods could affect the offspring's germline (F2) more significantly than the F1 offspring themselves (top panel, Figure 2). This leads to the hypothesis that maternal exposure will result in phenotypic effects that "skip" a generation, providing some control over the indirect effects of exposure to the mother or developing male fetus. Epigenetic and phenotypic changes that are observed in the F2 but not in the F1, and only when the F0 mother is exposed during critical periods of germline epigenetic reprogramming (5–8 weeks and 16–19 weeks postconception in males),[53,54] would support germline epigenetic inheritance (Table 1). Researchers concentrating on the aforementioned temporal windows and candidate genomic regions, and accompanying these findings with experimental work in mice, rats, or nonhuman primates, may be particularly well positioned to help to resolve our understanding of GEI in humans and its role in human health and evolution.

8 | IS GEI ADAPTIVE?

Despite the challenges of isolating such effects in human population studies, it has become incontrovertible that sperm RNAs and other epigenetic processes are essential for normal embryonic development.[78] As reviewed above, there is also increasing evidence that chemical, nutritional, and psychological stressors experienced by the father can affect these processes in mammals—ncRNAs in particular. These findings raise new questions about the adaptive role of these putative inter- and transgenerational environmental effects. Indeed, some have pointed out the similarities between GEI-related processes like exosomes, which convey experiential information between generations, and Darwin's proposal of gemmules.[39,185] Although the evolutionary importance of GEI is beginning to receive more attention,[186] there is currently no consensus about the evolutionary origins or adaptive implications of GEI in humans or other mammals. If eventually replicated, studies suggesting inter- or transgenerational effects of cold-exposure,[24] fear conditioning,[98] learning,[118] or wound healing[117] in rodents certainly seem consistent with an adaptive process. However, there are no studies demonstrating any effect of GEI on fitness and numerous examples are clearly *not* adaptive. Evidence that endocrine disrupting compounds can lead to traits like reduced sperm count and impaired fertility across at least four generations[82] demonstrates that epigenetic dysregulation can be maintained and replicated across multiple generations, even if clearly harmful to Darwinian fitness.

To serve an adaptive role, GEI should—at a minimum—demonstrate the following characteristics. First, an informative signal about the internal (e.g., physiology, metabolism) or external environment must be induced in nongermline tissues and then be transmitted to the germline. Exosomes, epididymosomes, and the highly-targeted nature of ncRNAs show that this is indeed very likely for some classes of stimuli or experience.[78,187] Second, the signal must survive through the physical and biochemical changes that are required for spermatogenesis and sperm maturation, as well as the epigenetic reprogramming that occurs immediately after fertilization. As discussed above, there is growing evidence consistent with such effects in mice.[38] (For *trans*generational effects, the signal would also have to survive primordial germline reprogramming and the epigenetic reprogramming that occurs in sperm—there is less evidence for this in mammals). Finally, the captured signal must predictably influence offspring development in a manner that produces phenotypic changes commensurate with the original exposure and do so in such a way as to improve behavioral or physiological adaptation—and ultimately Darwinian fitness—in response to similar exposures experienced by offspring. This last step is probably the most controversial and least documented in the literature, and the most difficult to demonstrate in humans.

In support of an adaptive function for some GEI-mediated responses, many traits impacted in offspring involve the same systems or traits that interface with or help the body cope with the inducing stimuli experienced by the ancestor.[24,103,117,118] For instance, psychosocial stressors influence offspring stress response or physiology, while offspring metabolism has been shown to be altered by the diet consumed by fathers. This correspondence between inducing stimulus and functional effect need not be the case. Nevertheless, few studies in mammals have shown what could be interpreted as a *positive* outcome of paternal exposure and GEI (i.e., one that would lead to greater offspring number or survival). It is hard to imagine how metabolic dysregulation, poor performance on behavioral tests, or cocaine-seeking behavior would be adaptive intergenerational responses. Moreover, more general epigenetic dysregulation and developmental abnormalities unrelated to the original exposure could easily be missed by researchers focused only on the systems and traits they believe should be affected. It is thus possible that inducing stimuli lead

to less targeted, widespread responses in offspring, but that this is overlooked as a result of study designs (i.e., the so-called streetlight effect). Evidence of extensive, nonspecific effects of specific inducing stimuli would run contrary to a targeted adaptive (i.e., a neo-Lamarckian) role of these responses.

From an evolutionary perspective, the value of the signal should be tied to predictability of the environment.[188,189] In this regard, the short life cycles for examaple of rodents increases the potential for adaptive GEI in these species. Following this logic, environmental predictability—and hence the potential for adaptive intergenerational signals—should be poorer for long-lived species like humans, at least for some stimuli, such as nutrition or season of birth in regions with annual fluctuations in temperature or precipitation. Poor predictability in long-lived species is evidence against an adaptive function to some of the findings in humans. For instance, why should children conceived in October produce more heat via brown fat *across their entire lives*, when compared to peers conceived in July?[24] Similarly, of what use is a grandparents' nutritional status during late childhood to grandchildren half a century later (one's same sex grandchildren alone, no less!)?[129] Evidence that epigenetic changes in sperm can arise within weeks of a change in diet[133] or exercise[135] present similar problems—of what adaptive value is a signal of nutrition or exercise if the signal can change before the fetus reaches the end of its first trimester of gestation? An adaptive role for GEI in humans could be supported if evidence for more predictable stimuli, such as an effect of paternal exposure to altitude or latitude,[190] were reported.

If a particular example of GEI is not a driver of developmental adaptation in humans, why might such effects exist? The first possibility, mentioned above, is that some components of GEI are simply manifestations of epigenetic dysregulation and thus serve no adaptive purpose whatsoever. Another is that GEI is present in humans because we inherited these systems from short-lived, phylogenetically more distant evolutionary ancestors in which they served an adaptive role. GEI-mediated changes that were once adaptive, owing to the relative ease of predicting future experiences in short-lived mammals, could persist in humans if their continued presence is largely selectively neutral, or if such processes are pleiotropically linked to other functions that are phylogenetically constrained due to strong stabilizing selection. Sperm-based ncRNAs (e.g., *piRNAs*, Box 1) that act as a genomic "immune system" against rogue retroviral activity in the germline might be an example of a function that could convey environmental information as a by-product of other more functionally relevant effect of maintaining genomic stability.[191,192] While adding another layer of complexity, yet another possibility is that GEI represents an example of intergenerational and parental conflict.[193] Both males and females may incorporate environmental information to fine tune resource extraction and allocation in an attempt to maximize their own fitness, even if it comes at the expense of the fitness of their offspring. Such a proposal would be consistent with findings reporting environmental perturbations on imprinted loci,[132] the ability of imprinted regions to "escape" at least postfertilization reprogramming,[65,66] and their known effects on offspring phenotypes.[63,181,182]

9 | CONCLUSIONS

DNA provides a fundamental substrate for heritable variation, but is accompanied by a complex supporting cast of dynamic and malleable molecular regulatory processes.[194,195] During the 20th century, biologists described a series of firewalls that were viewed as insulating the phenotype from ancestral environments, including the soma-germline barrier, epigenetic reprogramming, and sperm reprogramming. New research in GEI is showing that these barriers may be more porous than previously appreciated and thus may be circumvented to varying degrees. Although research into environmentally-induced GEI is currently proceeding rapidly in animal systems, relatively little is known about the extent to which such findings apply to primates, including humans. The efforts that have been made are commendable, but convincing demonstration of GEI in humans and nonhuman primates awaits future studies. We believe that human biology and biological anthropology are well positioned to make fundamental contributions to our understanding of GEI and its importance in humans. In addition, long-running primate studies with detailed information on environmental exposures across multiple generations, along with well-defined pedigrees, provide unusually rich opportunities to explore these pathways in a wider array of environments and life history contexts (e.g., The Amboseli Baboon Research Project,[196] The Karisoke Research Center).[197]

Designing more definitive tests of GEI in humans is a major challenge given unanswered questions about when, where, and how to look for relevant exposures, outcomes, and epigenetic pathways. Furthermore, our distinctively long lives, genetic diversity (relative to isogenic model species), environmental heterogeneity, and ability to construct and transmit social information and physical environments across generations, all compound the challenges of exploring this biological question. There are no easy ways to control the exposures, partition confounding pathways, and detect what might be subtle phenotypic outcomes. Nevertheless, the growing scale and resolution of public databases, declining costs of high-throughput genomic, methylomic, and transcriptomic methods, and increased understanding of the molecular and epigenetic processes involved, hold the promise to bring us closer to realizing this goal.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

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