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Transgenerational Epigenetic Inheritance: An open discussion

By: Corina Nagy[1] and Gustavo Turecki[1,2]

[1]McGill Group for Suicide Studies, Douglas Hospital University Institute, 6875 Lasalle boul, Montreal,QC, Canada
[2]Department of Psychiatry, McGill University, Montreal, QC, Canada
*Author for correspondence: gustavo.turecki@mcgill.ca

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Abstract

Much controversy surrounds the idea of transgenerational epigenetics. Recent papers argue that epigenetic marks acquired through experience are passed to offspring, but as in much of the field of epigenetics, there is lack of precision in the definitions and perhaps too much eagerness to translate animal research to humans. Here we review operational definitions of transgenerational inheritance and the processes of epigenetic programming during early development. Subsequently, based on this background, we critically examine some recent findings of studies investigating transgenerational inheritance. Finally, we discuss possible mechanisms that may explain transgenerational inheritance, including transmission of an epigenetic blueprint, which may predispose offspring to specific epigenetic patterning. Taken together, we conclude that presently, the evidence suggesting that acquired epigenetic marks are passed to the subsequent generation remains limited.

Introduction

Conrad Waddington first used the term epigenetics over half a century ago and defined it as ''the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being". A more modern definition of epigenetics is; heritable chemical modifications to DNA capable of influencing transcriptional activity with no direct alteration to the DNA sequence itself. It should be noted that heritable in this definition refers to "mitotic stability" [1] or, in other words, to the fact that epigenetic information is passed to, and maintained, in the daughter cell upon mitotic division. Certainly, this type of epigenetic heritability is of relevance to cancer research [2]. However, the concept of epigenetic heritability has broadened over the years. Studies have progressively suggested that not only might epigenetic marks be inherited through mitotic processes, but also through meiosis, a concept akin to genetic inheritance. That is, similar to the way we inherited genetic traits from our parents through germline transmission; epigenetic marks might also be propagated forward through the germline to offspring. This notion was fuelled by both research studies in plants and longitudinal epidemiological studies in humans, which have suggested that the effects of environmental influences were observed in subsequent generations. Studies in the plant *Arabidopsis thaliana* showed that alterations in DNA methylation can be transmitted through several generations [3]. Famously in humans, epidemiological studies examining the Dutch famine found that those individuals who were exposed to the famine while *in utero,* had increased susceptibility to diseases in adulthood such as, diabetes, cardiovascular disease and obesity, to name a few [4]. These findings begged the question of lastingness, expressly, whether these predisposing factors, which seemingly stemmed from in utero exposure to famine, could be transmitted to the next generation. Though there is no direct molecular link to these major health concerns, second-generation offspring was found to demonstrate the same lower birth weights, as seen in first-generation affected individuals [4].

In contrast to the mitotic stability seen in somatic cells, transgenerational epigenetics requires germline transmission of acquired epigenetic information

across generations. In cases of male exposure, the second generation may be sufficient to assess transgenerational effects. This is because exposure will affect the F0 male as well as his germline (F1), therefore only starting with the F2 generation can the effects of transgenerational epigenetic inheritance can be considered. In females, however, studies require a third generation to test whether any of these DNA modifications are truly transmitted from one generation to the next. This is because one needs to rule out in utero exposure of the second generation to an environmental effect associated with epigenetic changes. As an illustration, exposing a gestating female, her fetus and thus the fetal germline to a given environment, produces an F1 generation with an associated epigenetic change reflecting direct environmental effects through in utero exposure (Figure 1). Prior to the F2 generation in males and the F3 generation in female, the inheritance detected in the offspring can only be considered intergenerational i.e. parental effect due to direct exposure. This is important information to consider when reviewing some of the recent publications in this field.

Early developmental mechanisms: Epigenetic reset

Early epigenetic reprogramming has been extensively studied in rodents. Though the same processes of early developmental epigenetic erasure occur in humans, the definitive characteristics and timeline of these events have still to be established [5]. Work in mice has demonstrated that maternal and paternal cells not only undergo a global erasure of DNA methylation but the chromatin also undergoes active remodelling. Global erasure occurs at two points. First, as the primordial germ cells (PGC) or gamete precursors develop and become part of the embryo, the epigenetic landscape is cleared to allow for cellular totipotency and the development of future generations through PGCs. This stage is followed by gametogenesis when the genomes undergo *de novo* methylation, a process that occurs later in the maternal genome. The second wave of demethylation occurs after fertilization as the gametes fuse to form the zygote (figure 2). Here, however, imprinted genes escape the second round of demethylation (figure 2, dotted lines), to carry forward parent-specific monoallelic expression in somatic cells. If epigenetic marks were to be

maintained across generations, it is presumably at this point that they could be carried forward. In fact, some loci, including repeated elements such as retrotransposons, do not undergo reprogramming during this phase [6, 7]. Interestingly, Molaro and collegues [8] found a striking difference in methylation of retrotransposon subfamilies when examining the sperm of humans and chimps. Further, when comparing these sperm methylomes to embryonic stem cells, which they consider a mature germ cell having the product of the two reprograming events in mammal, they found distinct characteristic, suggesting that sperm and somatic cells have different features, which determine DNA methylation patterns in each cell type. Furthermore, approximately 100 non-imprinted, non-repetitive genes have been identified as maintaining promoter methylation levels throughout a range of developmental stages, from mature gametes to blastocysts [9]. Non-mammalian organisms on the other hand, appear to not undergo whole genome demethylation upon fertilization [10]. In a comprehensive study using zebrafish, Jiang and colleagues [10] were able to demonstrate that no global erasure occurs at fertilization and by the midblastula stage the embryo's methylome is almost identical to the of sperm, while the oocyte's methylome contribution lessens by the 16-cell state. This suggests that the early reprogramming mechanisms are different between mammalian and non-mammalian organisms.

As mentioned above, both the mammalian maternal and paternal genomes undergo demethylation and they also experience active chromatin remodelling. This is particularly evident for the paternal genome. The process of spermatogenesis in humans requires that anywhere between 85-96% of histones be replaced by protamines, resulting in a 10-fold compaction of the DNA [11, 12]. It is believed that this process protects the paternal genome from physical and chemical damage. Protamines, like histones, carry posttranslational modifications such as phosphorylation. The role of these modifications remains unknown, although they are believed to act similarly to histone modifications altering the availability of the DNA sequencing to the cellular transcriptional machinery [11]. Thus, the majority of paternally derived histone marks are lost during this process while the histones

not replaced by protamines are thought to belong to genes expressed early in development [12]. Interestingly, the repressive mark, histone H3 lysine 27 trimethylation, is retained on some genes in the human and mouse spermatozoa. This is arguably another possible avenue for inheritance; conversely, it could be a methodological artifact, and simply be the result of a histone mark being re-established so quickly that its period of absence is not detected. Gradually, studies are demonstrating the impressive dynamics of chromatin, for example the protein involved in heterochromatin formation HP1 binds for just a few minutes [13] and the wrapping and unwrapping of nucleosomes, a process which erases histone modifications, is so rapid that the turnover is faster than a complete cell cycle [14]. Thus, the highly dynamic nature of histone marks makes them less likely to act as a mechanism of transgenerational epigenetics. Equally, canonical forms of histones in the maternal genome, such as H2A, are replaced with H2A.Z during early embryonic development [15]. It is thought that H2A.Z is responsible for establishing heterchromatin in early development [16], furthermore, its absence in the early embryonic development results in death shortly after implantation [15]. The maternal genome has also been shown to transfer components of a repressive complex, PRC1, to the paternal genome after fertilization [17], resulting in direct silencing in the zygote. At gamete fusion, the maternal genome takes the form of nucleosome, whereas following the histone to protamine exchange during spermiogenesis, the paternal genome incorporates histones from the maternal genome [17]. Importantly, epigenetic processes in early mammalian development result in non-canonical forms of histones in both the maternal and paternal genomes.

Finally, there is interesting epigenetic asymmetry between parental genomes, which remains poorly understood. For example, de novo methylation in gametes, and demethylation after fertilization occurs more rapidly in paternal genome, denoted respectively by the blue and pink line graph in figure 2. In males, during protamine removal, the paternal genome is repackaged into new histones, as well as into maternally derived histones, whereas maternal chromatin maintains histone

methylation throughout early cleavage [18]. The timing of methylation could be directly related to the presence or absence of histone marks like H3K9me2, which are intimately related to DNA methylation, the absence of this mark may promote DNA demethylation [5]. For instance, PGCs show sex differences in the imprinted *Igf2r* gene, in both the timing of demethylation in males and de novo methylation in females [6]. This asymmetry is likely an explanation of why some traits are only propagated through one parental line, examples of which are discussed in later sections.

Escaping Intergenerational reprogramming

In light of early developmental reprogramming, the question researchers must address is whether certain epigenomic transcriptional drivers, in the form of DNA methylation or histone modifications, escape this prototypical intergenerational reprogramming.

The classic experiment in the agouti viable yellow mouse (A^{vy}) was the first study to report intergenerational inheritance in mammals. If a repeat element called an intracisternal A particle (IAP), of which there are thousand of copies throughout the mouse genome, inserts itself upstream of the agouti locus, phenotypic variations can arise including altered coat color and obesity [19]. DNA methylation at the promoter region of this IAP, which in turn regulates the agouti gene, is inversely related to its transcriptional activity [19]. As indicated above, repeat elements may escape reprograming, which appears to be the case for this epiallele that is passed along the maternal but not paternal germline [19]. Interesting as this may be, this is not the result of a past environmental factor being propagated forward via epigenetic inheritance. Though the insertion of the IAP at the agouti locus may not be entirely random [20], it does not result from the effects of environmental factors. However, once the IAP is inserted and susceptible to methylation changes, environment can play a role. This has been demonstrated by altering the food source of pregnant dams. Diets high in methyl groups such as folic acid or betaine can effectively "turn off" the A^{vy} locus by methylating the promoter region of the IAP

[21, 22]. Though it is tempting to look at this as transgenerational inheritance, it is rather the result of direct environmental exposure on the germline, and is thus the result of parental influence, more appropriately termed, intergenerational inheritance.

Early life environment is also known to alter methylation states as classically demonstrated through rat maternal behaviour paradigms [23]. Caution must be taken when conducting transgenerational experiments to control for both *in utero* and early life experiences to avoid confounding early-life environmental effects with transgenerational inheritance. To avoid these potential confounding effects, both in utero and in early life, studies in rodent models have focused on paternal lineage as males are not involved in the *in utero* environment and are less likely to be involved in the early-life postnatal environment. An interesting example of this was found using outbred rats exposed to the fungicide, vinclozolin *in utero*. As a result, males exhibited diminished fertility over three to four generations of offspring, which was found to be transmitted through the male germline [24]. Interestingly, these effects were not observed with another strain of rats. It is possible that this discrepancy results from methodological differences between studies but it also raises the possibility that interaction with genetic variation might be responsible for the observed effect [25]. If this is the case, it represents an example of secondary epimutations, whereby DNA sequence variants have an effect on epigenetic marks (in *cis* or *trans)* [26]. Indeed a number of studies have demonstrated that genetic-based alterations can be responsible for the inheritance of an epigenetic state [27-31]. In other words transmission of epigenetic marks is secondary to inherited DNA sequence mutations.

Epigenetic Inheritance: Acquiring Knowledge

A number of recent studies have implied that epigenetic alteration induced by environmental factors affect the behaviour of subsequent generations. Vassoler and colleagues [32] found decreased cocaine self-administration in the first generation offspring of cocaine exposed male rats. The behavioural outcome suggests an almost

protective effect of fathers consuming cocaine, which is not consistent with the clinical literature [33, 34]. Molecularly, this associated with increased *Bdnf* levels as well as increased H3 acetylation, an epigenetic mechanism potentially responsible for this change. The authors of this intriguing study correctly suggested that more generations are required to definitively conclude that cocaine exposure results in transgenerational epigenetic alterations [35]. Certainly, cocaine itself can alter chromatin states [36] but it is possible that cocaine directly influenced the gametes of the parental generation, and thus the F1 progeny. In a similar vein, a recent study in C. elegans showed paternal transmission to the embryo of the repressive mark H3K27me3, which is under the control of the Polycomb repression complex 2 [37]. Interestingly, histones containing this mark are not replaced by protamines during spermatogenesis in mammals [12]. This very interesting finding demonstrates mechanistic properties for the propagation of epigenetic marks through generations, but as pointed out previously, there is a need to demonstrate transgenerational inheritance beyond the first generation of progeny.

A study examining early stress in mice addressed the question of whether transgenerational inheritance could be explained by the effect of parental environment on the gametes or through the transmission of acquired epigenetic marks to the offspring, by observing the transmission of depressive-like behaviours up to the third generation [38, 39]. The behavioural traits co-segregated with altered DNA methylation in the male germ line. The same genes were tested for methylation levels in both the F2 sperm and the F2 female brains. The methylation patterns in these cell types were similar, though certainly not identical. However, of note was a discrepancy in behavioural phenotype, where the F1 and F3 males showed depressive-like behaviours but not the F2 males. The depressive-like phenotype was only seen in the F2 females and the molecular changes reported in this study were correlational, as there was no experiment in this study showing a causal link between methylation changes and phenotype. Furthermore the exposure of females to stressed males, even for a short period, could have had subtle effects on the female behaviours and hormonal expression, which in turn may have affected

maternal care. *In vitro* fertilization (IVF) of the sperm directly into control females has been used to avoid these potential confounding effects from fathers. When IVF was used in social defeat paradigms, which is an animal model of depression, the depressive-like phenotype that seemed to be propagated forward under normal mating conditions was largely absent [39]. Together, these results suggest that broad behavioural conditioning can be inherited. Although there is a possibility that these may co-segregate with specific epigenetic profiles, additional work is necessary to definitively make such conclusions.

Another study using the same early stress paradigm in mice showed depressive-like behaviours associated with an upregulation of several micro RNAs (miRNA) in sperm which appeared to affect serum and hippocampal miRNAs levels in the subsequent generation [40]. Because small RNAs are highly present in sperm, they have become candidate vectors for conveying transgenerational inheritance [41]. However, in this study, although the behavioural phenotype was detected in the F3 generation, the miRNA levels of 5 candidate miRNAs in F2 sperm were unchanged, and the alterations seen in these candidate miRNAs from F2 were no longer detectable in F3. The authors suggest that the initial change in miRNA levels resulting from early stress may have been transferred to other epigenetic marks, but give no evidence of this effect. Another study from the same group set out to demonstrate a causal mechanism for the inherited stress responses in the offspring of stressed sires [42]. In this study the authors were able to pharmacologically induce the same behavioural traits seen in the stressed offspring, with corresponding alteration to gene expression levels resulting from histone methyl- and acetyl- transferase inhibitors, suggesting a causal role for mineralcorticoid receptors in the altered stress response of paternally stressed offspring. Here however, the epigenetic changes seen in the male germline differed from what was found in the adult hippocampus of the offspring, specifically methylation changes where seen in the sperm whereas altered histone modifications were seen in the brains. Collectively, it appears that whatever environmentally induced epigenetic alteration is taking place in one generation may be propagated forward, but as

noted above, although possible, it is still premature to conclusively suggest a clear association between one acquired epigenetic mark and transgenerational behavioral phenotypes. An enticing possibility is that epigenetic changes transmitted across generations may act in a probabilistic manner. In other words, these changes are more or less likely to occur in broad genomic regions, resulting in altered behaviour without producing a consistent molecular phenotype. A recent paper demonstrating that paternal sugar consumption in fruit flies influences the metabolic properties of the F1 generation, leading to an obesity phenotype, supports this concept. Here, the authors demonstrate that a network of genes is required for proper intergenerational metabolic reprograming involving a number of changes to chromatin structure. No specific sites where identified but rather a pattern or signature of gene dysfunction was identified as conferring susceptibility to obesity [43].

Epigenetic Inheritance: From Rodents to Humans

The vast majority of studies examining transgenerational inheritance have been conducted in animals other than humans. Though mice and rats provide convenient models for human disorders and behavioral traits, there are different physiological and biological processes between humans and rodents. For instance, a recent paper exploring the methylation landscape of early human embryos showed features distinctive to humans, specifically levels of methylation and timing of genome-wide demethylation [44], while another study suggests the symmetry of epigenetic reprogramming cycles may differ between species [6]. Moreover, many transcriptionally relevant epigenetic marks show considerable sequence divergence between mice and humans [45]. Furthermore, information about epigenetic erasure during early development comes primarily from mouse models, and it is possible to be different in humans, but remains, as yet, untested. Transgenerational effects have been observed in humans through longitudinal studies [46-48] whereas gene-environment interplay is suspected as a mediating factor in health outcome. Intuitively, most people can recognize that their environment plays a role in health and behaviour, but we still lack conclusive evidence to indicate that such

transgenerational effects are explained by acquired epigenetic mechanisms inherited from one generation to the next.

Conclusions

The studies reviewed above suggest that there is some evidence that certain epigenetic marks escape erasure in early development. There is also evidence that certain acquired behavioral phenotypes are transmitted through subsequent generations. Although promising, the evidence suggesting that acquired epigenetic marks co-segregate with acquired behavioral phenotypes remains inconclusive and open to a number of potential alternative interpretations, including methodological explanations.

One should be particularly cautious when interpreting the results of studies testing transgenerational epigenetic inheritance in animals to the lay media or general public. Though likely not the intention, as pointed out previously [49] animal studies have been vulgarized and commonly anthropomorphized into a public message that overemphasizes the molecular impact of the environment, including parents and grandparents onto their children.

The field of epigenetics is fascinating and holds great potential in medicine, both to uncover disease biomarkers and therapeutic interventions. The results suggesting that acquired epigenetic factors may be transmitted through generations and explain acquired phenotypic traits are promising and intriguing, but nevertheless, studies published thus far have limitations and prevent us from making definitive conclusions.

Future Perspectives

An important missing link in the study of transgenerational epigenetic inheritance is a mechanism by which gene-regulatory information is transferred from somatic cells to germs cells. Efforts to uncover these mechanisms have been made in non-mammalian animals. For example, a number of studies in *Caenorhabditis elegans* (C.

elegans) have implicated small RNAs in the process of inherited epigenetic marks [50-52]. It was recently demonstrated in that dsRNA act as mobile elements that mediating intertissue transfer of regulatory information by entering the cytosol via a dsRNA-selective importer [53]. Equally, it has been speculated, notably by a paper discussed here [40] and others [54, 55], that small non-coding RNAs, like microRNA mediate soma to germline transfer of regulatory information in mammals, however, experimental evidence in mammals is lacking. Tackling this question in mammals and particularly in humans is an important next step in the research of transgenerational epigenetics.

Moving forward in the field of transgenerational epigenetics requires more precision in experimental design. First, studies should investigate both acquired phenotypic traits and acquired epigenetic marks, and their co-segregation, through three generations at least. In addition to correlational evidence, studies should also investigate causative links between the molecular changes investigated and the phenotypes. Finally, although more difficult to conduct, studies are also necessary in humans.

Executive Summary

Early developmental mechanisms: Epigenetic reset

- Epigenetic reprogramming occurs at two points during early development, after fertilization and in primordial germ cells.
- Some genomic features, such as retrotransposons, as well as certain histone marks can carrier forward epigenetic marks in spite of these erasure mechanisms, supporting the possibility that epigenetic marks can be maintained across generations.

Escaping Intergenerational reprogramming

- Mice and rat models have been used to show that certain epigenetic marks can escape intergenerational reprogramming, affecting the phenotype of future generations.
- Effects resulting from parental influences result in intergeneration inheritance, which is not to be confused with transgenerational inheritance (effects that survive across generations in the absence of direct exposure).
- Secondary epimutations represent another factor that can influence possible transgenerational inheritance. In these cases, epigenetic marks are secondary to inherited DNA sequence mutations.

Epigenetic Inheritance: Acquiring Knowledge

- A number of studies including recent article providing mechanistic insight show that epigenetic marks can be inherited intergenerational.
- Data for transgenerational inheritance on the other hand show certain inconsistences, which may be suggestive of probabilistic epigenetic changes capable of influencing phenotypic outcome in subsequent generations.

Epigenetic Inheritance: From Rodents to Humans

- It is difficult to provide concrete evidence that transgenerational epigenetics occurs in humans owing to the lack of studies in humans and the physiological and biological differences between humans and rodents.

Conclusion

- There is evidence that epigenetic inheritance occurs intergenerations by escaping erasure in early development.
- However, evidence is lacking for the co-segragation of acquired epigenetic marks with acquired behavioural traits transgenerationally.

- The field is very promising but more studies are required in order to provide definitive conclusions on the topic of transgenerational inheritance, particularly in humans.

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Figure Legends:

Figure 1: Schematic diagram demonstrating the difference between intergenerational and transgenerational epigenetic inheritance in females and males. F0 represents maternal or paternal exposure to environmental factors, which directly impacts the fetus (F1) and its already formed germline (F2) in females or the germline in males (F1). This demonstrates the importance of studying the F2 generation in males and the F3 generation in females to avoid parental effects i.e. intergenerational epigenetics. Studying the F2 (in males) and F3 (in females) and/or their subsequent generations could provide information on transgenerational epigenetics as they would be free of direct environmental exposure and parental effects.

Figure 2: Very early embryonic development corresponds to epigenetic programing (from [56, 57]). Primordial germ cells (PGC) in the embryo undergo global DNA methylation erasure, or "reprograming" from their epiblast state (red arrow). This first wave of demethylation is also denoted in the methylation cycle graph depicting both the male and female genomes devoid of DNA methylation, including imprinted genes. Gametes are then de novo methylated at different rates, with maternal methylation marks being established later (graph pink line) than paternal marks. A second round of 'reporgramming' occurs upon fusion of the gametes (sperm and oocyte) producing totipotent or pluripotent cell states. At this point, demethylation occurs more rapidly in the paternal genome (graph blue line), moreover, imprinted genes escape erasure (graph dotted lines) maintaining their methylation marks. Genome-wide remethylation occurs in both parental genomes at implantation (green arrow). The timeline denoted in this schematic refer to event in the mouse life cycle. The timeline in humans is not yet full defined, though the events are considered to occur in a similar manner.

Figure 1:

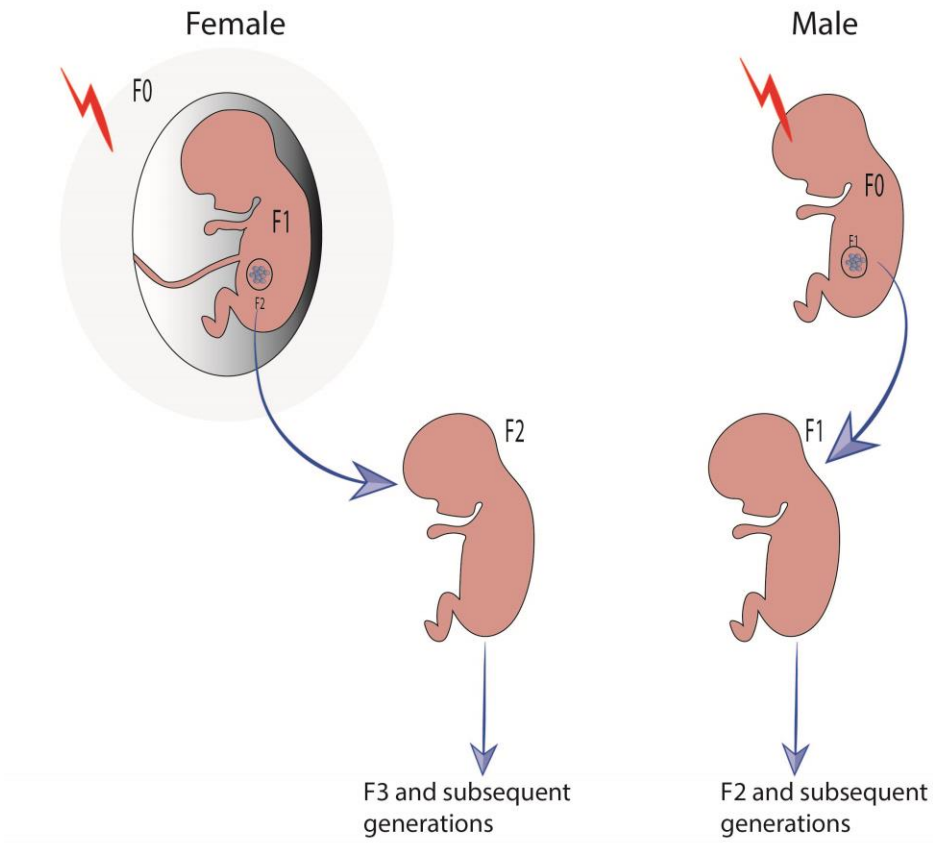
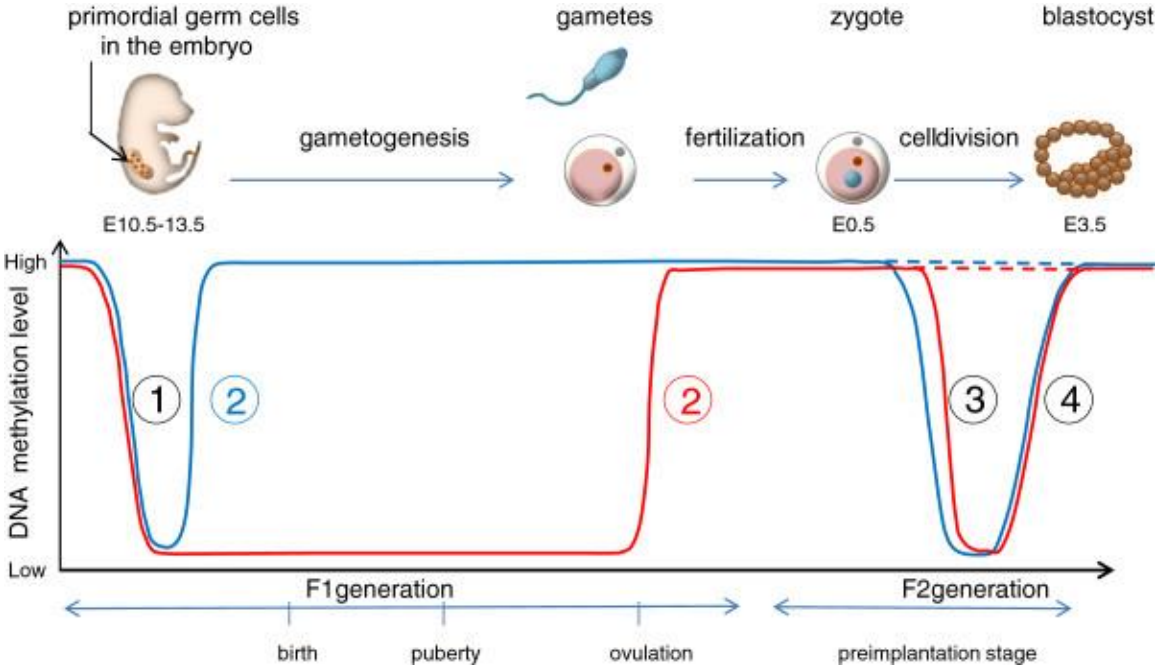

Female
F0
F1
F2
F3 and subsequent generations
Male
F0
F1
F1
F2 and subsequent generations

Figure 2


primordial germ cells
in the embryo
gametes
zygote
blastocyst
gametogenesis
fertilization
celldivision
E10.5-13.5
E0.5
E3.5
High
DNA methylation level
Low
1
2
2
3
4
F1generation
F2generation
birth
puberty
ovulation
preimplantation stage