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Review

Can our understanding of epigenetics assist with primary prevention of congenital defects?

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Abstract

Having identified teratogenic factors, primary prevention of congenital defects is possible by the implementation of specific measures in pregnant women or those planning pregnancy. Our current understanding of the epigenetic processes acting during reproductive events raises new possibilities to prevent both heritable and sporadic congenital anomalies. Cell differentiation during embryonic-foetal development involves different epigenetic processes which, if altered, may affect either somatic or germ cells. Epigenetic alterations can occur in somatic cells at different stages of life, from fecundation to adulthood, and when germ cells are affected, such changes can even be passed on to future generations.

This review summarizes the main epigenetic processes that influence gene expression and cell specification at different stages of development. The experimental and epidemiological evidence of environmental agents that cause epigenetic alterations is evaluated, as well as their effects in males and females. As a result, new avenues for primary prevention are proposed.

Words: 149

Introduction

It is clear that identifying the environmental factors that disrupt human embryonic-foetal development (teratogens) will facilitate the implementation of primary prevention strategies to avoid their adverse effects. Indeed, many epidemiological studies have analysed environmental risk factors that affect embryonic development, leading to the recommendation of a series of preventive measures for women prior to and during pregnancies [1-3]. At present, primary prevention of heritable conditions is only considered to be possible through assisted reproduction techniques (ART) and pre-implantation diagnosis. However, interventions such as prenatal diagnosis followed by termination of the affected pregnancies do not avoid the embryonic-foetal alterations and as such, they are considered by some as secondary prevention [1-3].

The spectacular amount of biological information generated by experimental and epidemiological studies in humans now permits that this can be evaluated in a more integrated manner [4]. Therefore, our understanding of epigenetics and embryonic development is summarized here, to assess their implications for primary prevention of birth defects. The first part of this review focuses on the epigenetic processes that influence gene expression and cell specification, while the second sets out our current understanding of the initial stages of development from fecundation to gamete formation and maturation. Finally, we will summarize some of the evidence that environmental agents alter epigenetic processes during development in mammals and humans.

Overview of epigenetic mechanisms

The term *epigenetics* refers to the heritable changes in gene expression and cell phenotype in the absence of alterations to the DNA sequence. Hence, while the genome generally remains uniform in all the different cells of a complex organism, the *epigenome* controls the differential gene expression in most cell types, silencing or activating genes, and defining when and where they are expressed. In molecular terms, these events commonly involve chromatin modifications and cytosine methylation at CpG dinucleotides. Chromatin is principally made up of histones (H2A, H2B, H3 and H4), proteins that are predominantly comprised of positively charged amino acids that bind to the negatively charged DNA. These histones coil DNA into nucleosomes, consisting of a core octamer of histones around which the DNA is wrapped. The amino-terminal tail of each core histone extends out from the nucleosome and it contains residues that can be epigenetically modified. Thus, the DNA/histone association is regulated by epigenetic mechanisms that inactivate gene transcription by condensing chromatin, activating it when the chromatin opens [5]. For

example, the acetylation of histones neutralizes their positive charge and relaxes the chromatin structure, permitting gene transcription [6].

Several epigenetic mechanisms are known to regulate gene expression, such as: DNA methylation; chromatin remodelling; and complex histone modifications including acetylation for gene expression, phosphorylation for gene activation and repression (mainly through different families of methyl transferases, acetyl transferases and deacetylases) [6-9]. In addition, there are other less well understood protein modifications (e.g. ubiquitination, sumoylation), as well as other rapidly emerging epigenetic processes involving small non-coding RNAs, called microRNAs, that regulate gene function and expression during prenatal development and during germ-line formation in both sexes [10-13]. In addition, genomic *imprinting* is a type of epigenetic modification that leads to the selective expression of only one allele of autosomal genes or differential methylated regions (DMR), depending on their parental origin. That is, instead of the maternal and paternal alleles/DMR being expressed equally, their expression/repression depends on whether they are inherited from the mother or the father (**Fig. 1**). Hence, expression/repression of the same gene/DMR differs in the male and female gametes across generations [14]. In mammals, most of the known imprinted genes lie in clusters, or they are found in close proximity to one another, and the mechanisms responsible for the coordinated expression of these imprinted genes are now beginning to be understood [15-16].

Due to its inherent stability, DNA methylation is one of the most generalized epigenetic mechanisms in mammals and it represents a means to silence gene expression [17]. Unlike histone acetylation -which is a relatively dynamic modification- the methylation of CpG dinucleotide results in a stable yet potentially reversible in gene expression. Methylation occurs in the one-carbon cycle, where a methyl group from the S-adenosyl-methionine (SAM) is covalently added to the carbon 5' of the DNA cytosine ring (CpG dinucleotide). This process is highly dependent on the levels of folate and it is catalysed by several DNA methyltransferases (DNMTs) [17-18], particularly: DNMT1, which maintains methylation patterns throughout DNA replication in the mouse and human genome; DNMT2, which appears to be involved in embryonic stem cells; DNMT3A and DNMT3B that participate in *de novo* methylation at CpG sites. In addition, DNMT3A appears to participate in germ cell imprinting through an interaction with another DNMT3 (DNMT3L), which is essential for genomic imprinting [19-21]. The protein groups that are involved in epigenetic modifications are closely coordinated and they form multiprotein complexes [22]. There are three types of DNA methylation/demethylation: *de novo* methylation, and passive or active demethylation. While passive demethylation is due to the failure to maintain methylation during DNA replication, such that the newly replicated strand is not methylated [23-24], the mechanisms underlying active demethylation are poorly understood in mammals. However, recent studies [25-26] suggested that this DNA demethylation is initiated by the same

enzymes that establish the methylation marks in parental imprinting (DNMT3A and DNMT3B), although other possibilities have also been evaluated [27].

The methylation of the genome is not random in mammals and since it is tissue-specific, it is also responsible for different types of cells differing in their epigenetic stability. It was considered that DNA methylation patterns established during prenatal development may remain stable throughout life. However, we now know that far from being permanent, epigenetic modifications undergo important changes at particular stages of development. Moreover, it has been postulated that DNA-demethylase helps maintain the DNA methylation equilibrium in post-mitotic cells and that the DNA methylation machinery remains active throughout life [28]. Indeed, there is even evidence suggesting that at least some types of DNA methylation might change later in postnatal life [29-30].

Since some epigenetic processes active during embryonic and foetal development are conserved between mice and humans [28], our understanding of these processes in human development is growing dramatically.

Epigenetic processes during fecundation and during embryonic and foetal development, or gamete formation

At fecundation, the two parental gametes and genomes differ in their morphological asymmetry and in several other aspects (**Fig. 2**):

a) The paternal gamete is already haploid, while the maternal one is arrested at metaphase II containing a single set of chromosomes with double the DNA content.

b) The paternal genome is highly condensed by substituting the histones with protamines (which fold the DNA without forming nucleosomes). By contrast, the maternal chromatin is formed by nucleosomes.

c) Whereas the paternal gamete is mainly restricted to its DNA content, the maternal one has duplicated most of its cytoplasm structures. It includes an important amount of maternally inherited transcription factors that are acquired during oocyte maturation (about 14,000 proteins from different families) [31]. These factors are fundamental to regulate the transition of the paternal and maternal pronuclei to the zygote. Among these factors are those that are necessary for histone modification and reprogramming of the paternal genome, as well as those needed to acquire pluripotency and for DNA synthesis during the first zygote divisions [32].

d) When the parental genomes come together in the cytoplasm of the oocyte, both are methylated including the maternal and paternal imprinted areas. Consequently, the zygotic genome needs to restore totipotency by undergoing epigenetic reprogramming.

e) The reprogramming of the highly condensed paternal genome replaces the protamines with histones, and rapidly initiates extensive genomewide demethylation. By contrast, the maternal genome needs to remain methylated until it finishes the second (asymmetric) meiotic division, forming the pronucleus and the second polar body (PB-II) that

mainly contains DNA. Subsequently, the maternal genome will undergo passive DNA demethylation during the first zygote cleavages.

The importance of epigenetic mechanisms in critical processes during the initial stages of mammalian development is becoming ever more evident [33-46]. Moreover, two major phases of transcriptional activity have been identified in the transition from maternal zygotic control: a) from fecundation to the late 2-cell embryo; and b) the subsequent phases up to the blastocyst stage [33-35]. From studies in mice we know that the PB-II forms at the moment of fecundation (**Fig. 3A**) and that it remains tethered to the egg at the site where the 2nd meiotic division occurred, considered to be the animal pole (lying opposite to the vegetal pole) [36-37]. The position of PB-II also determines the zygote's plane of polarity and the animal-vegetal axis that persists in the zygote [38-40], along which the first cleavage occurs by a meridian division to generate the first two blastomeres (about 30 hours after human fecundation: **Fig. 3B,1,2**). This meridian division is favoured by the molecules anchored in the cortical cytoplasm near the PB-II, which form an organizing centre that captures microtubules that extend from the poles of the zygote's first mitotic division spindle (**Fig. 3B,1**) [36-37]. This first cleavage breaks the embryo's symmetry, generating blastomeres with different developmental characteristics that predict the blastocyst axis (**Fig. 3B,2,3**) [38-40].

Although there are different opinions regarding how the two cell populations in the blastocyst are formed [36,38-43], a consensus exists that some blastomeres develop into the embryonic part of the blastocyst, while others form the mural trophectoderm and the superficial part of the ICM or polar trophectoderm [40-41]. Indeed, epigenetic mechanisms are implicated in the cell fate decisions made from the earliest embryonic stages, and cell position and epigenetic information both influence cell fate determination [43]. Through experimental methylation of histone H3, the methylation state of the first two cell lineages was shown to depend on the orientation of the first blastomeres with respect to the animal-vegetal axis, which is meridian at the first division (2-cell stage) and equatorial at the second. In addition, blastomeres with methylated histone H3 almost exclusively contribute to the inner cell mass (ICM) rather than to the trophectoderm (which is highly demethylated) [43]. These observations further support the notion that epigenetic mechanisms are implicated in cell fate decisions from the earliest embryonic stages, and that they distinguish the first two cells of the embryo (**Fig. 3B**), influencing their fate [43].

Interestingly, at the 8-cell morula stage genetic and epigenetic modifications occur that appear to influence the subsequent allocation of the cells to the blastocyst. Indeed, the DNA methyl-transferase-1 α (Dnmt1 α) protein, synthesized during the maturation of the mouse oocyte, is translocated to the nucleus at the 8-cell morula stage, while it remains in the cytoplasm at all the other pre-implantation stages [44-45]. Thus, Dnmt1 α is only observed in the nucleus of the 8-cell morula, where it works together with Dnmt1 β , another Dnmt1 protein expressed in the nucleus of early embryos, to maintain methylation and to ensure the

accurate inheritance of DNA methylation imprinting in the mouse embryo [44-45]. In addition, the 8-cell morula embryo undergoes the process of compaction, whereby each blastomere acquires an apical-basal polarity, through epigenetic modifications. The molecular basis for polarization in the mouse embryo resides in the positioning of some transcription factors, such as mRNA from the *Cdx2* gene (Caudal type homeobox transcription factor 2) [46-47]. In the polarized blastomeres of the 8-cell morula, most of the *Cdx2* transcripts are at the apical poles and very few are in the basal poles.

These characteristics of the 8-cell morula appear to influence the subsequent allocation of the cells in the blastocyst, determining the position of the ICM during cavitation [40, 48] (**Fig. 3C,1**). At the 16-32 cell stage, the cells of the morula undergo symmetric or asymmetric divisions depending on their previously established fates (**Fig. 3C,2**) [41, 48]. Accordingly, symmetric divisions generate two similar outer cells while asymmetric divisions generate an outer and an inner cell that differ in their polarization status. Indeed, when these cells undergo symmetric division, the mRNA from *Cdx2* is equally distributed between the two new cells. Conversely, when they divide asymmetrically, most of the *Cdx2* mRNA is located in the outer daughter cell and with a lesser contribution in the inner cells, evidence of a true asymmetric division. As a result, cell polarity affects the spatial distribution of *Cdx2* transcripts and in turn, *Cdx2* expression reciprocally reinforces cell polarity. Consequently, cells with more *Cdx2* transcripts in the 8-cell morula and that divide symmetrically will essentially contribute to the trophectoderm more than the cells with less *Cdx2* transcripts, which will give rise to the ICM [46-47]. Cavitation commences at about the 32 cell stage, just before implantation, in the vicinity of the cells that have undergone more symmetric than asymmetric divisions [37, 41] (**Fig. 3C,3**). It is believed that cavitation is driven by these cell divisions, since the outer cells from these divisions adhere more strongly to their inner sisters than the two outer cells produced by symmetric division [37, 41].

During gastrulation the primitive ectoderm cells from the ICM form the epiblast cells, which then give rise to two different cell lineages: the embryonic cells, and the primordial germ cells (PGCs) that represent the sole source for the next generation. As in mice, the formation of mature human germ cells (both sexes) commences very early in embryonic development, at the end of the 2nd to 3rd week after fecundation in the caudal wall of the yolk sac, prior to their migration towards the primitive sex glands (developing gonad). Once the PGCs arrive at the primitive gonads (between the 4th and 5th weeks of human gestation), extensive epigenetic reprogramming commences by erasing the specific parental imprinting, since the new imprinting must be established in the gametes according to the sex of the embryo [49- 53] (**Fig. 1**). At this stage the PGCs are bipotential since they may develop into either testis or ovaries, irrespective of their chromosomal constitution. However, it was recently demonstrated that at the time the PGCs arrive at the primitive gonads in the male mouse, the *Sry* gene (which is essential for initiating Sertoli cell differentiation in mammals) acts during a narrow time window (only 6 hours). In this period it initiates and maintains the

expression of the autosomal *Sox9* gene, which is required for testis formation [54]. Without the appropriate activity of *Sry*, the PGCs could develop female gonads even if the chromosomal sex is male. Afterwards, the PGCs will start to differentiate into the mature gametes, although this process occurs at different moments in the female and male.

In a human female embryo, the PGCs will transform into oogonias through successive mitotic divisions that occur throughout the 2nd month of gestation in humans. During the 3rd month, genetic and epigenetic processes will help them acquire their germ cell characteristics. At this time, the oogonia begin to differentiate into primary oocytes by initiating a meiotic prophase. After the diplotene stage of the prophase, once cross-over has occurred, the process is arrested and a long resting period is initiated that lasts into postnatal life. At puberty, the growth and maturation of the oocytes involves the progress of meiosis, the methylation of maternal imprints and *de novo* methylation to acquire the characteristics of the primary oocyte. This requires hypertrophy since the PB-I only includes a small portion of the cytoplasm and consequently, the intracellular organelles in the oocyte are doubled (lysosomes, endoplasmic reticulum, Golgi complex, mitochondria and others). Additionally, all the transcription factors and other substances necessary for the zygote to function until the end of the pre-implantation period must be synthesised. Thus, the hallmarks of having successfully completed oogenesis are those needed to acquire the specific post-fecundation activity [55-57].

In human male embryos, unlike females, the differentiation of PGCs into spermatogonia does not commence during development. By the 2nd month of gestation the PGC enter into mitotic arrest (prospermatogonia) and they remain unchanged in the developing gonads throughout foetal development. In postnatal life, at puberty, the male PGCs initiate a continuous process of mitotic multiplication that will persist throughout adult life, generating spermatogonia that undergo meiosis to form gametes. As soon as meiosis starts, genome-wide DNA methylation occurs prior to the pachytene phase of meiosis and paternal imprints are progressively acquired. The meiotic process terminates in the formation of four spermatids that will give rise to the mature spermatozooids [57-58] (**Fig.2**).

Conversely, somatic cells generally maintain their gene expression/repression throughout embryonic-foetal development because they are mitotically heritable [17]. However, we know that the patterns of DNA methylation are highly dynamic in post-mitotic tissues, and not only in cancer cells. This dynamism suggests that the methylation/demethylation equilibrium reflects the gene/environment interactions. Therefore, as the epigenome is responsible for multiple cell signalling events, it may be vulnerable to deregulation by environmental factors that produce teratogenic and adverse epigenetic effects, which could even be transmitted to subsequent generations (when they affect PGCs and gametes). In addition, such deregulation events may produce stages of susceptibility to develop different diseases during postnatal life [59-67], referred to as “*the foetal basis for adult disease*” [68]. This concept explains how environmental factors acting during prenatal

development can affect cell plasticity and adult susceptibility to develop different types of diseases under certain conditions, such as type II diabetes, cancer and others [66-67].

Evidence that epigenetic deregulation may be caused by environmental agents

There has been a dramatic increase in the evidence that environmental agents interfere with epigenetic processes during prenatal development, as well as during gamete formation and maturation, provoking different effects. These processes can have implications for primary prevention.

Lessons from diethylstilbestrol (DES). DES is a synthetic oestrogen that was widely prescribed to pregnant women in the mid-1900s. This drug was then demonstrated to affect all the aforementioned cell types [69-73], increasing the risk of genital tract malformations in infants [70], clear cell adenocarcinoma in females at puberty [71] and of breast cancer after the age of 40 [72]. Moreover, some of the effects of DES were also observed in the granddaughters and grandsons (second generation) of exposed mothers [70, 73]. Currently we know that DES may induce such effects by altering normal hormonal signalling through different gene targets involved in cancer, and through the response of others genes to oestrogen exposure [69]. Indeed, DES affects HOX gene expression in prenatally exposed mice, as well as in human uterine and cervical tissues in culture [74]. The HOX genes are transcription factors that regulate cell differentiation during embryonic implantation, development, and in the reproductive tract [75-76]. HOXA10 and HOXA11 are necessary for uterine endometrial cell receptivity, and for implantation in mice and humans [75]. Indeed, an oestrogen responsible element (ERE) in human HOXA10 mediates differential ligand-specific oestrogen-responsive transcription and hence, the ligand specificity of the HOXA10 ERE represents a molecular epigenetic mechanism by which DES can influence development [76].

Other endocrine disruptors (ED). The effects of DES stimulated further studies into ED and environmental chemicals. Studies in pregnant rats exposed to the endocrine disruptor vinclozolin (an anti-androgenic pesticide) and methoxychlor (a fungicide with mixed estrogenic and anti-androgenic activity), demonstrated epigenetic alterations and effects over at least four generations [77-78]. ED are also regarded as the cause of embryonic testicular alterations and increased apoptosis of sperm cells in adult testes. Similarly, there is evidence in humans of the effects on germ cells of chemicals such as pesticides, polycyclic aromatic hydrocarbons, organophosphates, metals and life-style (alcohol, tobacco) [79-81].

Inhibitors of histone deacetylases (HDACs). Epigenetic processes are also altered by factors that inhibit HDACs. Indeed, conventional HDAC inhibitors produce defects quite similar to those observed after prenatal exposure to Valproic acid (VPA) [82], a potent inhibitor of HDACs [83]. Studies in mice demonstrated that VPA and Trichostatin drugs induce hyperacetylation in embryos at the level of the caudal neural tube and somites. Therefore, the effects of these drugs are likely to be mediated through the inhibition of

HDACs, which may represent the molecular starting point for their teratogenic effects [81-83]. Moreover, VPA induces demethylation at CpG sites in endogenous genes, indicating that VPA induces both hyperacetylation and demethylation [84]. Recently, Topiramate (TPM), another anticonvulsant drug, was also shown to inhibit HDACs and to possibly produce teratogenic effects in humans [85-86]. Hence, it is tempting to speculate that these drugs (and probably other substances) silence/activate some genes in a similar way as mutations in the same gene, mimicking their phenotypes and producing “phenocopies”.

Chemical agents that affect the epigenome as a whole. General alterations of genomic methylation (either hypomethylation or hypermethylation) have been observed in adult mice both *in vivo* and *in vitro*. For example, the cytotoxic agent 5-Aza-2'-deoxycytidine (5-Aza-CdR) is a strong inhibitor of DNA methylation and it stimulates the expression of some genes [87]. In humans, global hypomethylation in T cells occurs in systemic lupus erythematosus (SLE) [86], and some DNA binding proteins implicated in demethylation are more strongly expressed in T cells from patients with SLE [88]. Other drugs such as procainamide (anti-arrhythmic) and hydralazine (anti-hypertensive) produce global hypomethylation in T cells by inhibiting DNA methylation, producing lupus like symptoms and providing evidence of how an environmental agent can induce a late onset disease [89]. Epigenetic modifications acquired during embryonic-foetal development also appear to increase the susceptibility to develop certain diseases later in life, such as cancer and type II diabetes [90]. In addition, DNA methylation in monozygotic twins also emerge later in life [91].

Nutritional deficiencies. These are among the most important environmental agents affecting the epigenome as a whole, as evident through the effects of starvation and severe nutritional alteration during pregnancy. Such effects were observed in individuals born to starving mothers during the Dutch famine [92-93]. Indeed, the problems identified in this population affected fertility and fecundity, obesity and the risk of disease in later life, and certain changes were transmitted to subsequent generations [93]. Additionally, epidemiological studies on women with other types of nutritional imbalances during gestation demonstrated an increased risk for birth defects and other reproductive problems [94].

Assisted reproduction technologies (ART). All the procedures used in ART have been implicated in congenital defects, including altered imprinting. ART have been related with an increased risk of infants having Beckwith-Wiedemann (BWS), Prader-Willi/Angelman and Silver-Russell syndromes, among other defects [95-97]. In a cohort study of discordant MZT for BWS, an imprinting defect was observed in KCNQ10T1 of a twin with BWS that was not present in the healthy co-twin [98]. Indeed, the failure to maintain the methylation imprinting centre 2 (DMR2), due to the abnormal expression of DNMT1, was postulated as an underlying cause of BWS [99]. Moreover, inactivation of Dnmt1s was shown to reduce the methylation in regions where methylation must be maintained after fecundation [97].

Comments and conclusions

The equilibrium between environmental alterations and genome function is the result of a network of DNA activities that show a high degree of plasticity to produce a wide range of specific responses to different types of environmental modifications. However, at the same time, DNA may be vulnerable to deregulation by environmental agents [59]. Taking all the aforementioned observations into consideration, it has become clear that epigenetic alterations can affect gene expression, producing different types of defects and diseases that may be transmitted to descendants, even over several generations.

These findings have important implications for the primary prevention of birth defects and for some long-term diseases, which cannot be ignored. First, because the biological processes and the effects of their alterations, have already been documented in some cases. Second, because the interventions to efficiently prevent such effects are easy, safe and cheap to implement. Indeed, we only need to spread the information that current preventive measures to prevent birth defects should be followed by both parents from three months before attempting to become pregnant, and they should be followed until the beginning of the second month of gestation by the father, and during the entire pregnancy by the mother. In this sense, to increase their efficacy it would be important to spread this information to the pre-pubescent and childbearing populations, irrespective of gender.

To conclude, our current understanding of epigenetic processes opens the door to promote more efficient primary prevention for a wide spectrum of birth defects and other heritable and sporadic diseases.

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Figure 1. Paternal *imprinting*. Only the maternal allele is expressed.

Figure 2. Male and female gamete formation. Differences according to gender and certain epigenetic processes.

Figure 3: Modified from: Plusa et al. (2002) [38] and Zernicka-Goetz (2006)[41]

A. (1) Upon fecundation the maternal oocyte completes meiosis II. The whole paternal genome undergoes DNA demethylation, except for the paternally imprinted genes, centromeres, heterochromatin and some repetitive elements. (2) Once the two pronuclei are formed, they approximate to form the zygote. (3) The zygote anchors the second polar body (PB-II) to mark the animal pole, while the vegetal pole is established at the diametrically opposite side.

B. (1) The animal pole determines the plane of the first mitotic cleavage division in the zygote. (a) indicates the microtubules extending from the spindle poles anchored near the PB-II. (2) The first two blastomeres result from a meridian division. (3) Afterwards, at the blastocyst stage the embryonic-abembryonic axis will be established from these first two blastomeres (the colours are related (see text)).

C. Detailed representation of development from the (1) 8-cell morula onwards. (2) At the 16-32 cell stage, two types of divisions occur: asymmetric and symmetric, and subsequently (3) cavitation takes place to generate (4) the blastocyst. Black lines indicate symmetric division, giving two external sister cells. Grey lines indicate symmetric division of internal cells. White lines indicate asymmetric division giving one external and another internal sister cell. ZP= zone pellucida. TF=trophectoderm. TEP=trophectoderm polar. TEM= trophoctoderm mural. The coloured cells are related.

Figure 1. Paternal *imprinting*. Only the maternal allele is expressed.

During early embryo development, precursors of PGCs need to suppress the somatic gene expression patterns. This occurs in PGCs upon arrive at primitive gonads by extensive demethylation including erasures of parental imprints.

Figure 2. Gametes formation. Differences by sex, and occurrence of some epigenetic processes

Figure 3