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Potential roles of noncoding RNAs in environmental epigenetic transgenerational inheritance

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Abstract

"Epigenetic Transgenerational Inheritance" (ETI) has been defined as germline (sperm or egg) transmission of epigenetic information between generations in the absence of direct exposures or genetic manipulations. Among reported cases of ETI in mammals, the majority are induced by environmental factors, including environmental toxicants [e.g. agricultural fungicide vinclozolin, plastic additive bisphenol A, pesticide methoxychlor, dioxin, di-(2-ethylhexyl) phthalate, dichlorodiphenyltrichloroethane, and hydrocarbons] and poor nutritional conditions. Although the ETI phenomenon is well established, the underlying mechanism remains elusive. Putative epimutations, including changes in DNA methylation and histone modification patterns, have been reported, but it remains unclear how these epimutations are formed in the first place, and how they are memorized in the germline and then get transmitted to subsequent generations. Based on recent advances in our understanding of regulatory noncoding RNAs (ncRNAs), I propose that ncRNAs are involved in ETI, during both the initial epimutation formation and the subsequent germline transmission of epimutations. ncRNAs can function at epigenetic levels by affecting DNA methylation and histone modifications, thereby changing gene transcriptional activities, which can lead to an altered mRNA transcriptome associated with a disease phenotype. Alternatively, novel or altered ncRNA expression can cause dysregulated post-transcriptional regulation, thus directly affecting the mRNA transcriptome and inducing a disease phenotype. Sperm-borne ncRNAs are potential mediators for epigenetic memory across generations, but they alone may not be sufficient for stable transmission of epimutations across generations. Overall, research on ncRNAs in the context of ETI is urgently needed to shed light on the underlying mechanism of ETI.

Keywords

Gamete; Sperm; Oocyte; Epigenetics; Genetics; Environment; Inheritance; Disease etiology

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1. Introduction

A recent definition refers to “epigenetics” as “the molecular factors/processes around the DNA that regulate genome activity independent of DNA sequence, and these processes are mitotically stable” (Skinner, 2014). Although epigenetic regulation does not involve changes in DNA sequences, it can significantly influence genome activities and gene expression levels, leading to phenotypic changes (Feinberg, 2007). Therefore, it has been widely accepted that epigenomic changes represent aspects of the physiological, or pathophysiological, response to changes in the environment in which an organism lives (Bonasio et al., 2010; Feinberg, 2007). Adverse epigenetic changes can lead to disease conditions, whereas beneficial epigenetic modifications may enhance the physiological fitness. Epigenetic changes in the somatic cells are not passed on to future generations. For organisms of sexual reproduction, these changes must be reflected in the germline in order to transmit to subsequent generations. However, even if epigenetic changes arise in the germline (sperm or oocyte), it has long been believed that the altered epigenetic information would not be passed on to subsequent generations because of the two waves of global epigenetic reprogramming that occur right after fertilization, and during primordial germ cell development in the fetal gonads (Fig. 1) (Bao and Yan, 2012; McCarrey, 2014). In mice, shortly after fertilization, the paternal and maternal genomes undergo active and passive demethylation, respectively; by the time of implantation, the genome of the embryo is largely demethylated. However, soon after implantation, the embryonic genome is rapidly remethylated followed by further methylation changes over the course of organogenesis. The physiological implication of this first wave of reprogramming lies in the erasure of gamete epigenetic marks, including some of those essential for gamete identity, and those potentially gained during parental gametogenesis, so that the new embryonic epigenome can be established to support early development (Seisenberger et al., 2013). When the embryonic primordial germ cells (PGCs) arise and start migrating towards the genital ridges, the PGC genome undergoes global demethylation. Upon arrival in the genital ridges, the PCG genome is largely demethylated; Soon after sexual differentiation in the primitive gonads, both the prospermatogonial, and the oocyte, genomes gain their germlinespecific methylation patterns, which are further modified in a relatively subtle manner during gametogenesis in adulthood. The second wave of reprogramming in PGCs is critical for germ cell fate commitment (Seisenberger et al., 2013). It was previously believed that the two waves of reprogramming events remove the vast majority, if not all, of the methylation marks gained from the parental germline (Seisenberger et al., 2013). However, subsequent studies reveal that neither of the two waves of DNA demethylation is complete as many of the imprinted loci and numerous repetitive sequences [e.g., IAPs (*Intracisternal A-particle*)] remain methylated after the first wave of reprogramming in the somatic cell lineages, or even after the second wave of reprogramming in the germline (Arney et al., 2001; Mochizuki and Matsui, 2010). Although several DNA-binding factors (e.g. MBD3, ZFP57, ARID4A and ARID4B) have been found essential for the maintenance of several specific genomic imprints (Bartolomei, 2009), the underlying molecular mechanisms remain largely unknown.

Because of these reprogramming events, it has been widely accepted that epigenetic changes acquired during the lifetime of one individual would be completely erased and reset in the germline, and thus, there would be no chance for those epigenetic changes to be transmitted to next generations (McCarrey, 2014). It was furthermore believed that even if some of the acquired epigenetic information were to be transmitted to the immediate next generation, the reprogramming events would eventually erase those epigenetic marks in subsequent generations (McCarrey, 2014). Therefore, the concept of transgenerational epigenetic inheritance (ETI) has been controversial (McCarrey, 2014; Skinner, 2011). However, increasing lines of evidence suggest that certain induced pheno-types indeed can be passed on to subsequent generations through the germline, and that this non-Mendelian transmission of pheno-type is most likely mediated by epigenetic mechanisms.

2. Environmental epigenetic transgenerational inheritance (EETI)

Environmental epigenetic transgenerational inheritance (EETI) refers to the phenomenon in which exposure of the gestating mother (F0), along with the developing fetus (F1), to environmental insults, such as endocrine disruptors, toxicants, poor nutrition, etc., causes epimutations that are transmitted to the F3 generation, and beyond, through the germline, in the absence of continued exposure (Cuzin et al., 2008; Gluckman et al., 2007; Skinner, 2011; Skinner and Guerrero-Bosagna, 2009; Whitelaw and Whitelaw, 2008). The Skinner lab first reported, in 2005, that exposure of gestating female rats to vinclozolin, an environmental endocrine disruptor, during the time window (E8.5–E14.5) of global demethylation in the PGCs in the fetal rat gonads (F1) causes a phenotype characterized by increased incidence of adult onset diseases, including disrupted spermatogenesis, prostate or kidney diseases, immune abnormalities, and tumorigenesis, in not only F1 and F2 generations, but also the F3 generation that is never directly exposed to vinclozolin (Anway et al., 2005) (Fig. 2). More interestingly, the phenotype can only be transmitted through the paternal germline, i.e., sperm. This non-Mendelian inheritance and lack of segregation suggest that epigenetic mechanisms are responsible for the transgenerational propagation of the initial effects (Anway et al., 2005; Skinner, 2008). Subsequent studies demonstrate that this transgenerational effect is associated with epimutations manifested as altered DNA methylation patterns, termed differential DNA methylation regions (DMRs), which are propagated specifically through the male germline (Anway et al., 2008; Clement et al., 2010; Guerrero-Bosagna et al., 2010; Skinner et al., 2008). These studies imply that the epimutations caused by the initial vinclozolin exposure may possess features similar to those of imprinted genes, thus allowing them to avoid reprogramming and to achieve transgenerational propagation. Moreover, recent data from the Skinner lab demonstrate that sperm epimutations subsequently alter the epigenomes and transcriptomes of developing Sertoli cells or granulosa cells, which may be responsible for the adult onset spermatogenic and ovarian disruptions leading to fertility defects (Nilsson et al., 2012; Skinner et al., 2012).

Since the report of vinclozolin-induced EETI in rats (Anway et al., 2005) (Fig. 2), similar phenomena have been reported in other species, including plants (Schmitz et al., 2011), worms (Greer et al., 2011), insects (Ruden and Lu, 2008), mice (Anway et al., 2005; Doyle et al., 2013) and humans (Pembrey, 2010), when exposed to various environmental factors, e.g., poor nutrition (Burdge et al., 2011; Hoile et al., 2011), cell culture conditions

(Mahsoudi et al., 2007), vinclozolin (Anway et al., 2005), bisphenol A (Salian et al., 2009), dioxin (Bruner-Tran and Osteen, 2011), di-(2-ethylhexyl) phthalate (DEHP) (Doyle et al., 2013; Manikkam et al., 2013), dithiothreitol (DTT) (Kabasenche and Skinner, 2014; Skinner et al., 2013) and alcohol (Ramsay, 2010). Abnormalities manifested in the F1 (developing fetus) and F2 (progeny of F1) generations are not surprising because the F1s are directly exposed to the environmental factors, and F2s are derived from the sperm or eggs of F1s, which are also directly exposed during the fetal development (Fig. 2). However, observation of the same phenotype in the F3 generation, and beyond, is unexpected because these subsequent generations were never directly exposed (Daxinger and Whitelaw, 2012; Skinner, 2011; Whitelaw and Whitelaw, 2008). As discussed above; the two waves of reprogramming during post-fertilization and PGC development (Fig. 1) would erase the environmentally induced epimutations, if they were not protected through some means.

These data clearly demonstrate connections between DMRs, the altered mRNA transcriptome, and the adult onset disease pheno-type, which may provide some mechanistic insights into the EETI phenomenon. However, many questions remain unanswered, e.g.: How are specific genomic regions recognized and targeted for methylation to form DMRs? How do limited initial epigenetic changes induce alterations in expression profiles of many genes located outside those DMRs? How do those epimutations avoid correction by the global epigenetic reprogramming during post-fertilization and PGC development? How are those epimutations memorized by the germ cells and then transmitted to subsequent generations? Increasing lines of evidence suggest that the answers may lie in noncoding RNAs (ncRNAs).

3. Involvement of noncoding RNAs in the initial formation of epimutations

The majority of the noncoding regions of the mammalian genome are transcribed and processed into small and large noncoding RNAs (ncRNAs) (Cech and Steitz, 2014). ncRNAs can be divided into two main groups based on size; those with a size of >200 nt are called large noncoding RNAs (lncRNAs), while ncRNAs <200 nt represent small noncoding RNAs (sncRNAs). Accumulating data have shown that ncRNAs, especially large intergenic noncoding RNAs (lincRNAs), can function as epigenetic regulators by interacting directly with epigenetic factors involved in chromatin remodeling, DNA methylation, histone modifications or epigenetic memory (e.g. genomic imprinting and paramutations), thus achieving regulation of complex gene networks (Orom and Shiekhattar, 2013). Alternatively, lincRNAs can indirectly regulate epigenetic states by affecting transcriptional or translational activity, or by affecting the stability of mRNAs encoding epigenetic factors (Aravin and Hannon, 2008; Clark and Mattick, 2011; Costa, 2008; Esteller, 2011; Kaikkonen et al., 2011; Mattick, 2007, 2011; Morris, 2009). Therefore, ncRNAs, in theory, should have multiple molecular means to participate in EETI.

First, some of the long noncoding RNAs (lncRNAs), especially lincRNAs, are transcribed from sites within, or close to, the genomic regions that are destined to be silenced (Esteller, 2011; Gupta et al., 2010; Khalil et al., 2009; Tsai et al., 2010). These lincRNAs can serve as “sequence guides”, attracting chromatin remodeling complexes, or other epigenetic machineries, to achieve regional silencing through DNA methylation, or repressive histone

modifications (Gupta et al., 2010; Khalil et al., 2009; Tsai et al., 2010). For example, the methylation of *H19*, a paternally imprinted gene, requires another lincRNA that is transcribed from the *H19* locus (Gabory et al., 2010; Kwong et al., 2006). This lincRNA is essential for maintaining the hypermethylation status of the paternal copy of the *H19* gene. Recently, it has also been demonstrated that the differential expression patterns in the *HOXC* gene cluster depend upon the expression of a lincRNA, called *HOTAIR* (Gupta et al., 2010; Rinn et al., 2007; Tsai et al., 2010). *HOTAIR* is transcribed in a region close to the *HOXC* gene cluster, and it enables the tethering of two distinct repressive complexes to chromatin for coupled H3K27 methylation and H3K4 demethylation (Gupta et al., 2010; Tsai et al., 2010). In this way, the *HOXC* gene cluster displays a wide variety of expression profiles in multiple organs of the body during development. Additionally, lincRNA-induced epigenetic silencing through chromatin remodeling may not have to occur in a sequence-specific manner, e.g., lncRNA *XIST*-mediated X chromosome inactivation (XCI) (Ng et al., 2007). *XIST* is expressed on the targeted X chromosome; it attracts and binds polycomb group protein complexes (PcGs), which, in turn, trimethylate H3K27 in *cis*, thus initiating the panchromosomal epigenetic silencing (Ponting et al., 2009). The fact that ~20% of lincRNAs expressed in human cells are bound by PcGs suggests that a similar mechanism may be utilized by other genes or gene clusters (Khalil et al., 2009). Therefore, it is conceivable that some lincRNAs may be involved in the initial formation of epimutations, caused by environmental exposures, possibly during the global epigenetic reprogramming that occurs in PGCs, or during gametogenesis.

Second, a subclass of sncRNAs that are associated with MIWI2 are exclusively expressed in PGCs, and thus, called MIWI2-piRNAs (or pre-pachytene piRNAs) (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). MIWI2-piRNAs are mainly derived from repetitive sequences (e.g. transposons) (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). These piRNAs are required for remethylation of transposons, like LINE1 elements, during the latter half (E14.5– E18.5) of reprogramming in male germ cells (Aravin et al., 2008; O'Donnell and Boeke, 2007), and for the maintenance of the hypermethylation status of at least one of several known imprinted genes, *Rasgrf1* (Watanabe et al., 2011). In the MIWI2 KO mice, the activity of LINE1 transposons is drastically elevated, and the methylation pattern of *Rasgrf1* is massively altered (Aravin et al., 2008; Bao et al., 2014; Kuramochi-Miyagawa et al., 2008; Zheng et al., 2010). Therefore, it is also possible that the initial epimutations induced by the environmental factors may involve some of the MIWI2-piRNAs.

Supporting these hypotheses, studies have shown that expression of lincRNAs and piRNAs occurs prior to heterochromatin formation, hypermethylation of DNA or repressive histone modifications (Kaikkonen et al., 2011; Morris, 2009). More importantly, these ncRNAs are expressed either from, or close to, the regions or loci that are targeted for silencing (Kaikkonen et al., 2011; Morris, 2009). These facts suggest that ncRNAs may serve as "sequence guides" that direct chromatin modifying protein complexes to the targeted regions/loci for epigenetic modifications. Therefore, it would be interesting to examine whether an environmental exposures induce the expression of unique lncRNAs and piRNAs

from regions within, or proximal to, the exposure-specific DMRs in both somatic and germ cells.

Third, the DMRs identified by the Skinner lab (Skinner et al., 2012) contain numerous miRNAs, endo-siRNAs and pachytene/MIWI2-piRNAs, all of which mainly function by regulating mRNA stability and translational efficiency (Kaikkonen et al., 2011). Another group of ncRNAs, including promoter-associated RNAs (PARs) and enhancer RNAs (eRNAs), have been determined to bind the promoter regions of mRNA genes, and function as transcriptional activators by interacting with the transcriptional machinery (e.g. transcription factors, RNA Pol II, RNA-binding proteins, etc.) (Kaikkonen et al., 2011). If a DMR contains ncRNAs that regulate gene expression at transcriptional (PARs and eRNAs) or post-transcriptional (miRNAs, endo-siRNAs, and pachytene piRNAs) levels, then the implicated ncRNAs could have effects on the expression of numerous target genes, which could be encoded by genes distributed throughout the genome.

Alternatively, ncRNAs can also be involved in the distal effects of DMRs on the expression of multiple genes constituting an epi-genetic control region (ECR), through the following two potential mechanisms: First, environmental factors (e.g. vinclozolin) cause primary DNA methylation changes (DMRs) which, in turn, affect the expression of ncRNAs that are adjacent to the DMRs (Fig. 3, upper panel). Altered ncRNA expression then causes disrupted expression of many of its target genes, even those located distally. Second, environmental factors (e.g. vinclozolin) can directly affect the production of ncRNAs, especially large intergenic noncoding RNAs (lincRNAs), which are essential for sequence-specific DNA methylation and chromatin remodeling. Aberrant ncRNA production leads to altered DNA methylation patterns generally manifesting in DMRs, which, in turn, affect the expression of multiple mRNA genes located throughout the genome (Fig. 3, lower panel). Therefore, mapping ncRNAs located within, or proximal to, the DMRs would allow for the dissection of the relationship between changes in ncRNAs and alterations in the mRNA transcriptome.

4. Sperm-borne ncRNAs as the epigenetic memory

The paternal transmission of epimutations, resulting from the *in utero* exposure of an F0 mother to vinclozolin, through the mother's descendants, suggests that the epimutations are ingrained into the “epigenetic memory” of the male germline, and that this information can be transmitted *via* sperm. The sperm epigenome includes the entire methylome, which is largely established during fetal germ cell development, and further completed during spermatogenesis (Carrell and Hammoud, 2010; Jenkins and Carrell, 2011; Talbert and Henikoff, 2010). Sperm chromatin is protamine-dominant, and only a small amount of histones are retained. Recent data suggest that sperm retained histones exist in various forms of modifications, and are associated with specific genomic regions that encode early developmental genes or sncRNAs (Anway et al., 2005; Brykczynska et al., 2010; Hammoud et al., 2009; Ihara et al., 2014; Molaro et al., 2011). In addition, sperm-borne RNAs, including RNAs that are associated with the sperm plasma membrane and sperm chromatin, also represent an integral part of the sperm epigenome because these RNA molecules can be delivered into the oocyte during fertilization (Ostermeier et al., 2002, 2004). The fact that

the use of “membrane-free” sperm heads yields similar fertilization and birth rates through intracytoplasmic sperm injection (ICSI), as compared to unaltered sperm, suggests that the RNAs located in parts other than the head are not critically required for fertilization and subsequent embryonic development (Yan et al., 2008; Yanagimachi, 2005).

Soon after fertilization, the paternal genome undergoes reprogramming, which involves genome-wide demethylation, chromatin decondensation and histone restoration (Reik et al., 2001; Wilkins, 2005). Epigenetic reprogramming events in the preimplantation embryo, along with the subsequent germline-specific reprogramming events, would be expected to correct all acquired epimutations, unless those epimutations were to adopt an imprinting-like mechanism, thus persisting in the germline. Alternatively, sperm-borne RNAs, especially ncRNAs, may function as templates or sequence guides, to induce similar epimutations once released into oocytes during fertilization (Fig. 4). Sperm-borne RNAs, both large (mRNAs and lncRNAs) and small (sncRNAs), have been confirmed in many mammalian species (Krawetz et al., 2011; Lalancette et al., 2008; Martins and Krawetz, 2005; Miller et al., 2005; Ostermeier et al., 2002, 2004; Peng et al., 2012; Yan et al., 2008). These RNA molecules are delivered to the oocyte during fertilization, but their physiological roles remain elusive (Ostermeier et al., 2004). Previous studies suggest that sperm-borne RNAs may contribute to paramutational effects (i.e., one allele affects the expression of the other allele of a gene) (Rassoulzadegan et al., 2006). In plants, sperm-borne mRNAs and ncRNAs can serve as templates for modifying DNA sequences (Alleman et al., 2006; Lolle et al., 2005; Storici et al., 2007). However, this dramatic effect has not been observed in animal kingdom. Nevertheless, given that the epimutations induced by vinclozolin are selectively transmitted through the male germline, it is plausible to hypothesize that the RNA contents associated with sperm chromatin may contribute to this transgenerational inheritance (Fig. 4). Therefore, it would be interesting to determine changes in sperm-borne ncRNA profiles, in exposed sperm, across three or more generations, and to explore the effects of altered sperm ncRNA contents on the formation of specific DMRs and/or the disruption of mRNA expression in preimplantation embryos and adults. Similar work should also be conducted on oocytes because oocyte-mediated transgenerational epigenetic inheritance has been documented (Skinner, 2014; Skinner et al., 2013; Waterland et al., 2007), and similar mechanisms may function in the female germline to promote transgenerational epigenetic inheritance.

In summary, ncRNAs might be the missing link between DNA sequences and their ultimate epigenetic states. ncRNAs can either act directly at epigenetic levels by affecting epigenetic modifications of the genome, or function to control the expression of epigenetic modulators and thus, indirectly induce epigenetic changes. ncRNAs exist and function in both somatic and germ cells; ncRNA changes in somatic cells lead to altered mRNA transcriptomes, and could potentiate disease phenotypes, whereas germline ncRNAs may mediate the transmission of epigenetic memory. These hypotheses may, or may not, be proven true, but are worth investigation, as elucidation of the underlying mechanisms of ETI would have a profound impact on our understanding of disease etiology.

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Highlights

- ncRNAs may be involved in epigenetic transgenerational inheritance.
- ncRNAs can function at epigenetic levels by affecting DNA methylation and histone modifications.
- Novel or altered ncRNA expression can cause dysregulated post-transcriptional regulation.
- Sperm-borne ncRNAs are potential mediators for epigenetic memory.

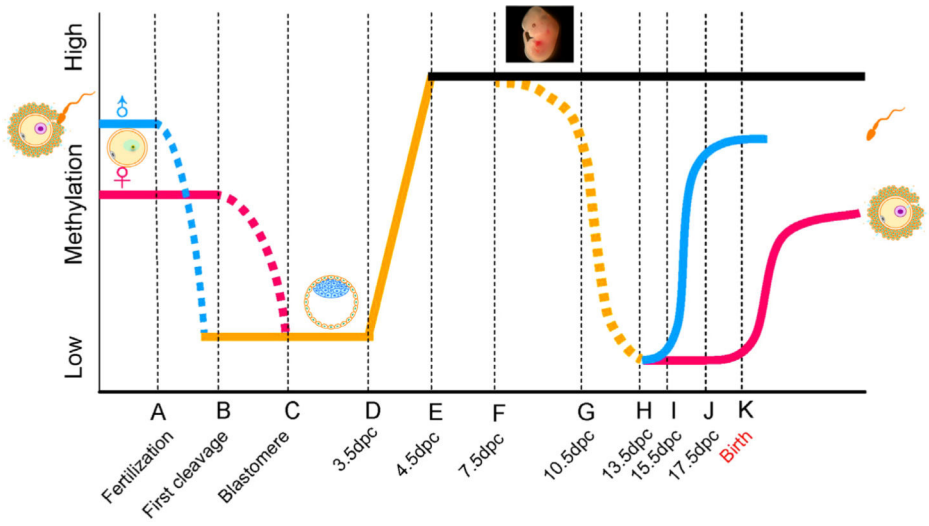

Fig. 1.

Schematic illustration showing the two global reprogramming events during murine development. Relative genomic methylation levels are represented on the vertical axis, whereas key stages of preimplantation and fetal germ cell development are shown on the horizontal axis. Blue and red lines denote the paternal and the maternal genomes, respectively. Dash lines represent the progression of demethylation. “dpc” stands for “days post-cotium”. This figure is adapted from (Bao and Yan, 2012).

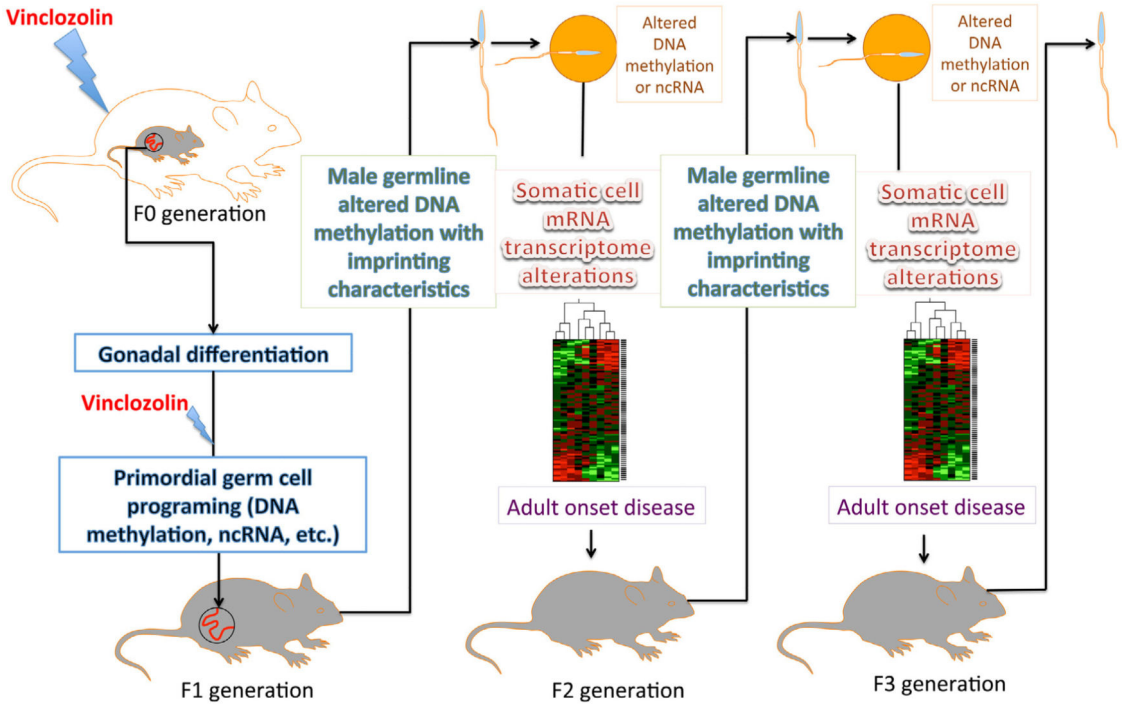

Fig. 2.

Schematic presentation of vinclozolin-induced transgenerational epigenetic inheritance of the adult onset disease phenotype. When a gestating rat (F0) is exposed to vinclozolin in the time window of primordial germ cell (PGC) reprogramming (E10.5–E13.5), epimutations [e.g. altered DNA methylation leading to the formation of differential methylation regions (DMRs)] arise and persist throughout spermatogenesis in F1 and also the post-fertilization global reprogramming in F2 embryos. These epimutations can directly cause aberrant expression of many mRNA genes, or indirectly affect mRNA expression by altering ncRNA expression. Dysregulated mRNA transcriptome results in different adult onset diseases in various organs. Epimutations and the disease phenotype can then be stably transmitted through the male germline, to not only the exposed generations (F1 and F2), but also subsequent generations that have never been exposed to vinclozolin (F3, F4, and beyond).

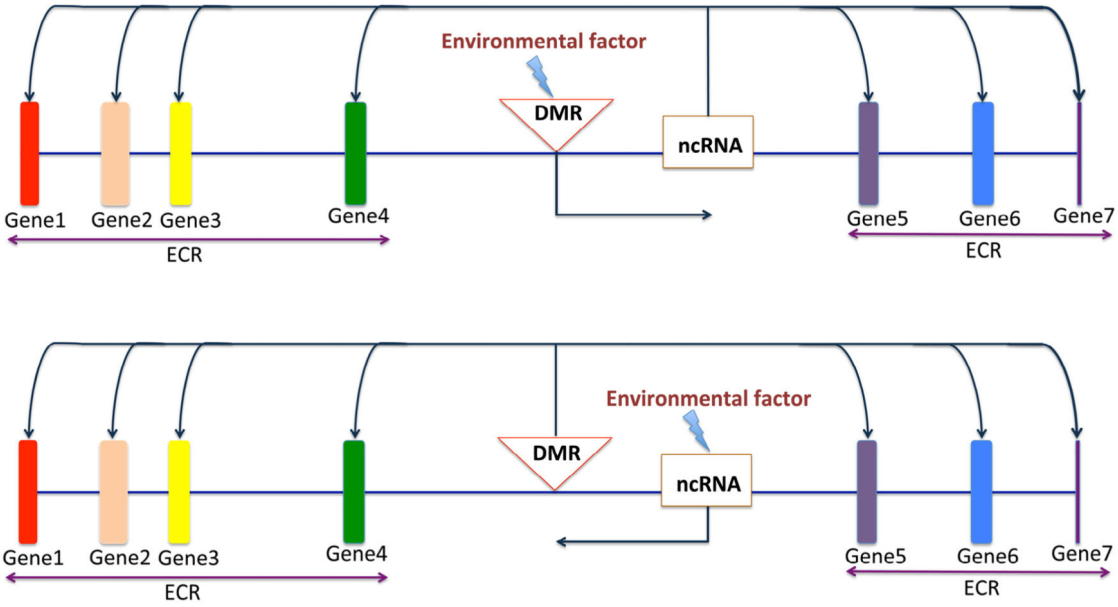

Fig. 3.

Two potential mechanisms by which ncRNAs are involved in distal effects of differential DNA methylation regions (DMRs) on expression of multiple genes constituting an epigenetic control region (ECR). *Upper panel:* Environmental factors (e.g. vinclozolin) cause primary DNA methylation changes (DMRs), which, in turn, affect the expression of ncRNAs that are adjacent to the DMRs. Altered ncRNA expression then affects the expression of their target genes located distally. *Lower panel:* Alternatively, environmental factors (e.g. vinclozolin) can directly affect the production of ncRNAs, especially those large intergenic noncoding RNAs (lincRNAs), which are essential for sequence-specific DNA methylation (e.g. H19 lincRNA for the imprinting of *H19* locus) and chromatin remodeling (e.g. HOTAIR for the epigenetic control of the *Hox* gene cluster). Aberrant ncRNA production leads to altered DNA methylation patterns manifested as DMRs which, in turn, affect expression of multiple mRNA genes located throughout the genome.

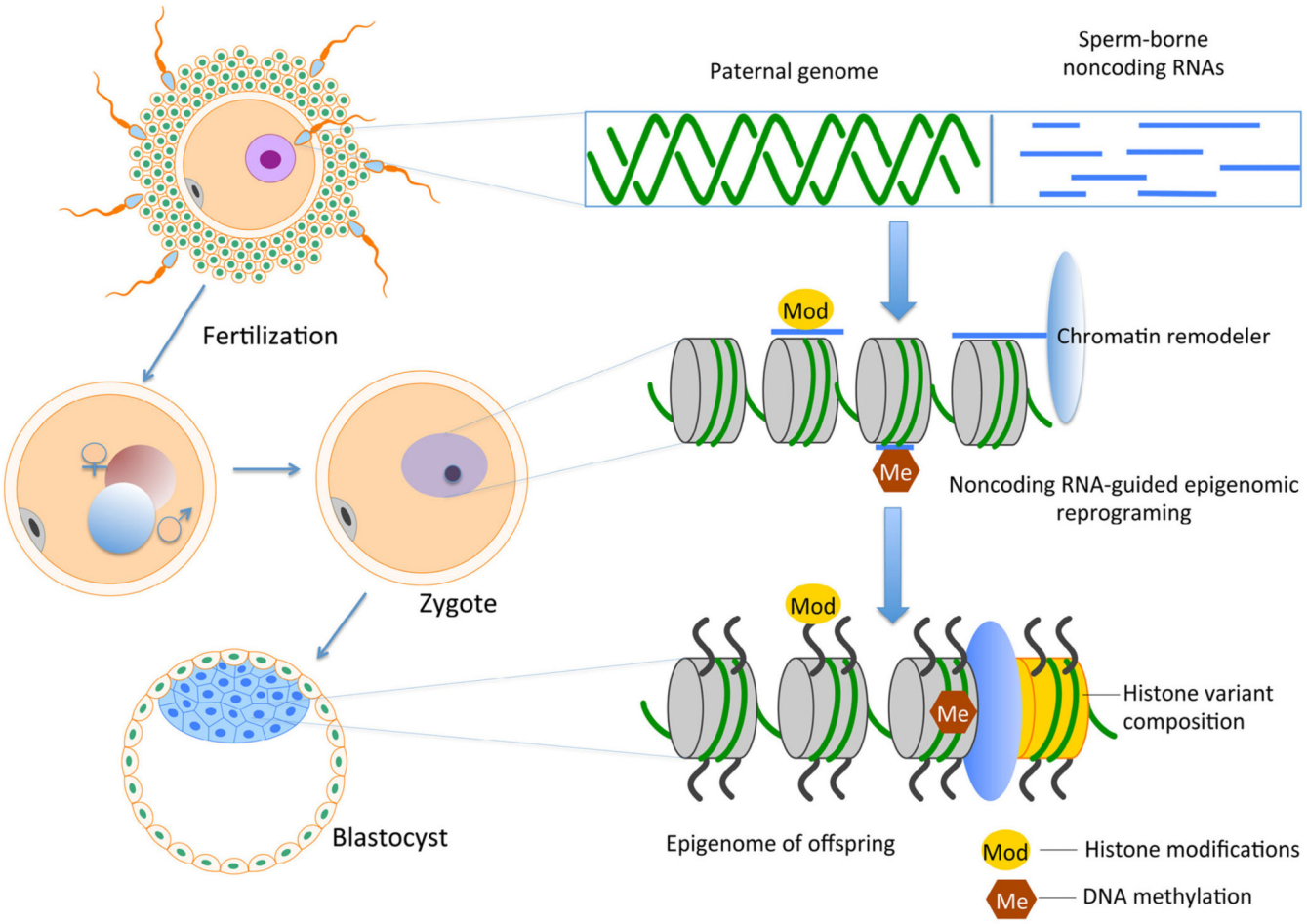

Fig. 4.

Schematic illustration of a potential mechanism by which sperm-borne ncRNAs mediate transgenerational epigenetic inheritance. In this model, sperm-borne ncRNAs are released during paternal chromatin decondensation. In the subsequent reprogramming of the preimplantation embryos, these paternal ncRNAs may function as the “sequence guide” to direct epigenetic machineries for DNA methylation, histone modification and chromatin remodeling to set up the epigenome of the early embryo. Some, if not all, of these ncRNA-mediated epigenetic marks may possess features of those imprinted loci, and thus, can avoid erasure during the 2nd round of reprogramming in the male germline, leading to transgenerational inheritance. The key in this model is that the ncRNAs are required for reprogramming.