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Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications

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Abstract | Once deemed heretical, emerging evidence now supports the notion that the inheritance of acquired characteristics can occur through ancestral exposures or experiences and that certain paternally acquired traits can be 'memorized' in the sperm as epigenetic information. The search for epigenetic factors in mammalian sperm that transmit acquired phenotypes has recently focused on RNAs and, more recently, RNA modifications. Here, we review insights that have been gained from studying sperm RNAs and RNA modifications, and their roles in influencing offspring phenotypes. We discuss the possible mechanisms by which sperm become acquisitive following environmental–somatic–germline interactions, and how they transmit paternally acquired phenotypes by shaping early embryonic development.

The idea of the inheritance of acquired traits, which suggests that ancestral life experiences acquired in the environment can be inherited by offspring, was once under heated debate[1]. Recently, great interest in this topic has been rekindled, owing to accumulating evidence from well-designed experiments in different animal species[2–8]. These studies demonstrated that certain paternal traits that are acquired in response to ancestral exposures, such as toxicant contact, mental stresses and diet changes, can be inherited by the offspring, suggesting that epigenetic inheritance can occur through the sperm. This conclusion was further supported by recent data obtained using *in vitro* fertilization (IVF) or the injection of sperm heads to fertilize oocytes[9–11], which essentially ruled out germline-independent paternal factors (such as seminal fluid and male–female contacts[12]), thus indicating that sperm could be the carrier of ancestral epigenetic memory. The resurrected idea of germline inheritance of acquired traits has the potential to further our understanding of the aetiology of many modern human diseases that may have originated from environmentally induced transgenerational effects and thus may have widespread medical and social implications.

One of the major reasons for the previous dismissal of the 'inheritance of acquired traits' hypothesis was the knowledge that epigenetic reprogramming in the zygote and primordial germ cells resets the epigenome to a naive state by removing most of the epigenetic marks (for example the 5-methylcytosine (5 mC) mark produced by DNA methylation) that result from environmental exposures[13]. However, changes in DNA methylation in sperm have been detected after exposure to environmental stresses[14], presumably having escaped from epigenetic reprogramming, suggesting that these DNA modifications represent a form of molecular carrier for acquired epigenetic memory[15]. Nevertheless, recent studies based on comprehensive DNA methylation analyses cast doubts on the role of sperm DNA methylation as a molecular mark of epigenetic inheritance that responds to environmental exposure; stochastic variations in sperm DNA methylation were found to make a greater contribution to the sperm methylome than diet[16]. In other studies, in which sperm DNA methylation patterns showed alterations after environmental exposure, the changed patterns of DNA methylation were not maintained in subsequent generations[17–19]. These data suggest that other molecular information carriers in sperm are required for the intergenerational transfer and maintenance of acquired traits; such information carriers could include histone marks that can be maintained across generations[20–22] and non-coding RNAs (ncRNAs), which can act in *trans* to escape the reprogramming process[23]. Recently, proof of a direct causal role of sperm RNAs in transferring acquired traits across generations in mammals has emerged; that is, the injection of total sperm RNAs or a subset of sperm RNAs (for example, microRNAs (miRNAs) or tRNA-derived small RNAs (tsRNAs; also known as tRNA-derived RNA fragments (tRFs)) into normal zygotes can generate offspring that recapitulate paternal phenotypes, including mental stress and

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Box 1 | **Historical view of sperm RNAs**

The mission of a sperm was once believed solely to be the delivery of paternal DNA to the oocyte. Reports of the identification of RNAs in sperm were initially sporadic and controversial. However, the emergence of high-throughput strategies, such as microarray and RNA sequencing (RNA-seq) analyses, has begun to end this controversy by providing solid evidence of the existence of RNA in sperm[127]. The demonstration of the delivery of sperm RNAs into oocytes unequivocally ended the controversy[128] and gave rise to new questions. What are the roles of sperm RNAs? Are sperm RNAs simply negligible remnants of spermatogenesis, or could they represent something more?

Whether sperm RNAs are necessary for early embryonic development remains controversial[129–131], particularly given the recent generation of parthenogenetic mice with appreciable survival rates[132,133]; nonetheless, their potential synergistic action with maternal RNAs have been discussed with interest[134]. Thus far, the most significant biological functions identified for sperm RNAs are their involvement in non-Mendelian inheritance in mammals, such as the paramutation phenomenon in mice (BOX 2), and their contribution to the intergenerational inheritance of paternally acquired traits, including mental and nutritional stresses[9,24–26].

metabolic changes[9,24–26]. Furthermore, sperm RNA modifications and RNA-editing events have been implicated as active participants in the intergenerational transfer of epigenetic information[9,27,28], adding layers of complexity to the mechanisms that underlie this process and pointing to a new direction for future studies.

In this Review, we outline the current understanding of acquired epigenetic inheritance through sperm, mainly in mammalian species, focusing on the roles of sperm RNAs and RNA modifications. We discuss the potential mechanisms by which sperm RNAs become acquisitive and the flow of information from the environment to somatic cells and then to sperm. We also discuss known and potential mechanisms by which sperm RNAs reshape embryonic development and offspring phenotypes, and the roles of sperm RNA modifications in this process. We avoid extensive discussion of other candidate epigenetic carriers in sperm (for example, DNA methylation marks and histone modifications, which have been extensively reviewed elsewhere[15,29]), unless they are highly relevant to the functions and mechanisms of sperm RNAs.

Acquired inheritance through sperm

The first evidence of sperm-mediated epigenetic inheritance of phenotypic traits in mammals was derived from nuclear transplantation experiments in mice[30]. Such embryo manipulations induced abnormalities in the offspring — including growth defects and aberrant gene expression — which were documented to be inherited by the next generation of offspring through the paternal germ line[30], probably owing to altered epigenetic features. A more representative case of environmentally induced epigenetic inheritance through sperm was based on a chemical exposure experiment[7]: when pregnant female rats were exposed to vinclozolin (a pesticide), the male offspring of the exposed mothers showed a wide range of disease phenotypes, which were inherited by most of the males of the subsequent generations examined that were not directly exposed to vinclozolin. In these early studies, genetic and epigenetic mechanisms cannot be fully distinguished, because nuclear transplantation and chemical exposure can cause gene mutation. Nevertheless, these studies drew great interest in the potential for epigenetic inheritance and triggered further studies to explore whether phenotypes induced by other forms of environmental exposure could also be inherited through the male germ line. Now, data from independent laboratories have demonstrated that paternal characteristics acquired during environmental exposures (including a high-fat diet (HFD)[5,9,10,26,31], a low-protein diet[5,11], mental stresses[8,25,32], odour sensitivity to specific chemicals[6] and susceptibility to irradiation[33]) can indeed be inherited by the offspring through the paternal germ line in a non-Mendelian fashion, thus supporting the idea that certain life experiences and environmental cues can be 'memorized' in sperm as epigenetic information.

Is DNA methylation the cause?

At the molecular level, a plausible explanation for this paternal germline inheritance could be environmentally induced aberrant DNA methylation patterns that are maintained in the sperm and escape at least two waves of epigenetic reprogramming[13], resulting in the persistence of these patterns in the offspring. Evidence supporting the escape of DNA methylation marks from epigenetic reprogramming comes from well-established mouse studies on intracisternal A particle (IAP) retrotransposon-controlled agouti viable yellow (A^{vy})[34] or axin fused ($Axin^{Fu}$)[35] gene activity, in which the methylation state of the IAP was incompletely erased during reprogramming of the male germ line, providing an explanation for the epigenetic inheritance of coat colour or kinky-tail phenotype, respectively. However, such clearly identified inheritance of DNA methylation state in mammals is rare, and consistent changes in gene expression in subsequent offspring resulting from environmentally induced aberrant sperm DNA methylation have not been demonstrated thus far. Furthermore, there is controversy regarding the influence of environmental stresses on DNA methylation[14,16], and recent data appear to contradict the role of methylated DNA as a persistent mark that maintains phenotypes in subsequent generations[17,36]. More comprehensive DNA methylomic analyses with improved coverage and depth[37] are required to draw a final conclusion. Uncertainties surrounding the role of DNA methylation have inspired the search for other epigenetic information carriers in sperm that could potentially transfer environmentally acquired traits, which has led to a focus on sperm RNAs.

Sperm RNAs mediate transmission of acquired phenotypes. The existence and functions of sperm RNAs were once a subject of debate (BOX 1). High-throughput technologies, such as microarray analysis and RNA sequencing (RNA-seq), have led to the recent discovery and characterization of various types of sperm RNAs[23] (FIG. 1). Sperm RNAs were first implicated in mediating offspring phenotypes in early studies on the transvection[38–40] and paramutation[41–44] phenomena in mice (BOX 2), and these studies led to the discovery of sperm RNAs as the trans-acting molecules that mediate

Parthenogenetic
A type of asexual reproduction that occurs when a female gamete develops a new individual without being fertilized by a male gamete

RNA-editing events
Molecular processes by which specific nucleotide sequences in an RNA molecule are changed, such as C-to-U and A-to-I editing.

Kinky-tail phenotype
A mouse phenotype characterized by a kinked tail containing a sharp bend at an angle to the main tail axis.

Transvection
An epigenetic phenomenon that involves the interaction between two homologous chromosomes, resulting in either gene activation or repression at an allele.

Paramutation
Interaction between two alleles at a single locus during meiosis, resulting in epigenetic transfer of information from one allele to another that is heritable for generations.

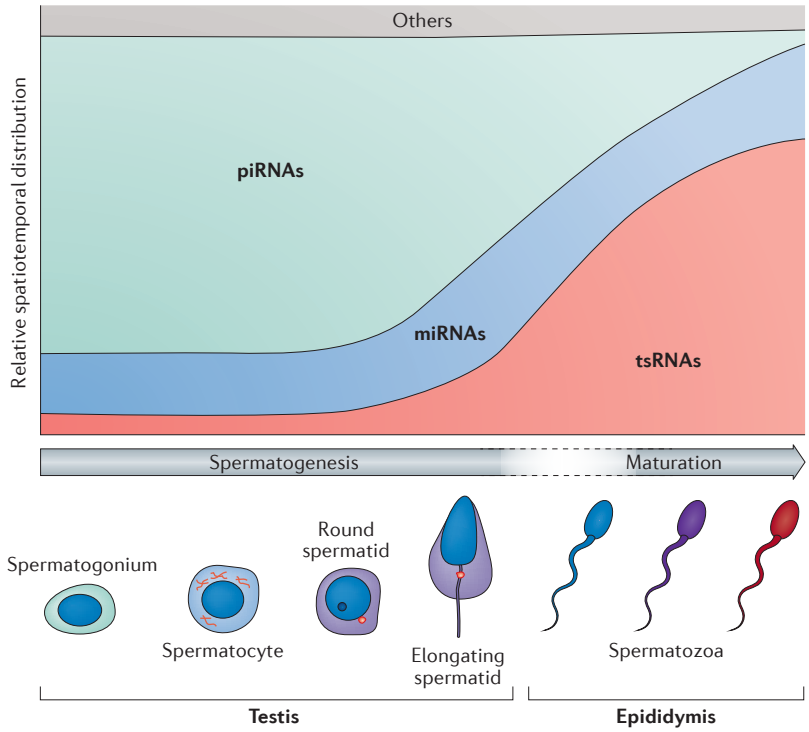


Figure 1 | **Small non-coding RNAs in mouse male germ cells.** Spatiotemporal distribution of major types of small non-coding RNAs (for example, PIWI-interacting RNAs (piRNAs), microRNAs (miRNAs) and tRNA-derived small RNAs (tsRNAs)) in sperm precursors (for example, spermatogonia, spermatocyte, round spermatid and elongated spermatid) during spermatogenesis in the testis, and in spermatozoa during sperm maturation in the epididymis (which occurs during the transition from the caput epididymis to the cauda epididymis).

these phenomena[41–43]. More recent studies have further demonstrated that sperm RNAs can function as molecular carriers that transmit paternally acquired characteristics to the offspring[9,24–26]. The first experiment to directly demonstrate the causal relationship between sperm RNAs and inherited characteristics involved the injection of total sperm RNAs from mentally stressed male mice into normal zygotes, which generated offspring that reproduced the behavioural and metabolic alterations observed in the father[24]. Importantly, it was noted that the stress phenotypes in the offspring were passed onto the subsequent generation, suggesting that transgenerational inheritance is due to the induction of stable, heritable epigenetic marks[24]. Similarly, two independent groups showed that the injection of total sperm RNAs from males that were fed a chronic HFD[9] or a high-fat–high-sugar diet[26] into normal zygotes generated offspring that either partially[9] or fully[26] recapitulated the paternal metabolic disorders. Because mouse sperm contain various types of mRNAs and ncRNAs (for example, miRNAs, PIWI-interacting RNAs (piRNAs), tsRNAs, mitochondrial genome-encoded small RNAs (mitosRNAs) and long ncRNAs (lncRNAs)), identification of the subpopulations of sperm RNAs that are responsible for the transmission of paternal phenotypes is of great interest, and recent emerging evidence has put sperm miRNAs and tsRNAs in the spotlight.

Epididymal maturation
Spermatozoa from testis undergo a maturation process during transit from the proximal to the distal end of the epididymis, acquiring motility and fertility.

Sperm miRNAs. Several studies have reported alterations in sperm miRNA profiles after various types of paternal exposure[8,31,33,45], but the functions of sperm miRNAs in transmitting paternally acquired phenotypes were reported only recently[25,26]. In one mouse study, following the zygotic injection of a combination of nine miRNAs that were altered in sperm after chronic paternal stress, the offspring developed stress-dysregulation phenotypes that recapitulated those of the father[25]. In another study, zygotic injection of miR-19b (the expression of which is upregulated in the sperm of mice consuming a high-fat–high-sugar diet) induced obesity and glucose intolerance in the offspring, recapitulating paternal phenotypes[26]. Moreover, the glucose-intolerance phenotype induced in male offspring persisted in subsequent generations despite incomplete penetrance after mating with healthy females[26], suggesting that the epigenetic changes induced by miR-19b injection were maintained in the germ line, although concerns were discussed by the authors regarding overdosage of a single miRNA injection, which might not faithfully represent physiological conditions[26].

Sperm tsRNAs. The enrichment of tsRNAs in mature sperm was first discovered through sperm RNA-seq analysis in mice[46]. Sperm tsRNAs are mainly derived from the 5′ end of tRNAs, range in size from 29 nt to 34 nt and are more abundant than miRNAs, constituting the majority of small ncRNAs in sperm[9,11,46]. Although details of their biogenesis remain unknown, sperm tsRNAs show altered profiles after a HFD[9] or a low-protein diet[10] in mice, after a HFD[45] or an environmental-compound exposure in rats[47], and in obese humans[48], suggesting that sperm tsRNAs can function as sensitive markers of environmental exposure. Further functional evidence obtained by injecting tsRNA-enriched RNA fragments from the sperm of mice fed a HFD into zygotes indicated that tsRNAs could induce metabolic disorders in offspring, which is similar to the effect of injecting total sperm RNAs[9]. This effect may be exerted in a synergistic manner along with other sperm or oocyte RNAs or proteins. Moreover, zygotic injection of a combination of tsRNAs[9] or a single tsRNA ($tsRNA^{Gly(GCC)}$)[11] could change early embryonic gene expression, indicating that sperm tsRNAs play an active part in the transmission of paternally acquired traits by reshaping early embryonic development.

A recent study revealed that sperm tsRNAs show a dramatic increase in abundance during late spermatogenesis and epididymal maturation[46] (FIG. 1). In addition to the possibility that tsRNA biogenesis occurs through specific cleavage of tRNAs during these stages, recent evidence suggests that sperm may obtain tsRNAs from extracellular vesicles (EVs) that are produced by epididymal epithelial cells[11]. Moreover, sperm tsRNAs were found to harbour numerous RNA modifications that contribute to the stability of tsRNAs[9], and levels of 5-methylcytidine (m^5C) and N^2-methylguanosine (m^2G) in sperm tsRNAs were significantly increased after paternal HFD consumption[9]. These discoveries raised exciting new possibilities regarding the mechanisms

through which sperm gain information from the environment and the potential roles of RNA modifications in mediating epigenetic memory. We discuss these topics in detail below.

Acquisitive sperm — information flow

The production of functional sperm begins with spermatogenesis in the testis, which is followed by maturation in the epididymis; each stage involves complex regulatory processes. How can developing sperm sense environmental changes, and how do they become acquisitive and enable the storage of epigenetic memory? Understanding the flow of information (physiological, cellular and genetic) from the environment to somatic cells and then to germ cells is key to answering these questions. Interestingly, heated debate over the flow of hereditary information between the soma and the germ line had already started more than 100 years ago (BOX 3). The once widely accepted 'Weismann barrier' theory is now increasingly being challenged[49], whereas the once disregarded Darwin's 'pangenesis' theory is receiving increasing attention[50], owing, in particular, to advances in research on EVs that can deliver proteins[51] and RNAs[11] to sperm, and to the discovery of various extracellular mobile RNAs that can be found outside EVs[52].

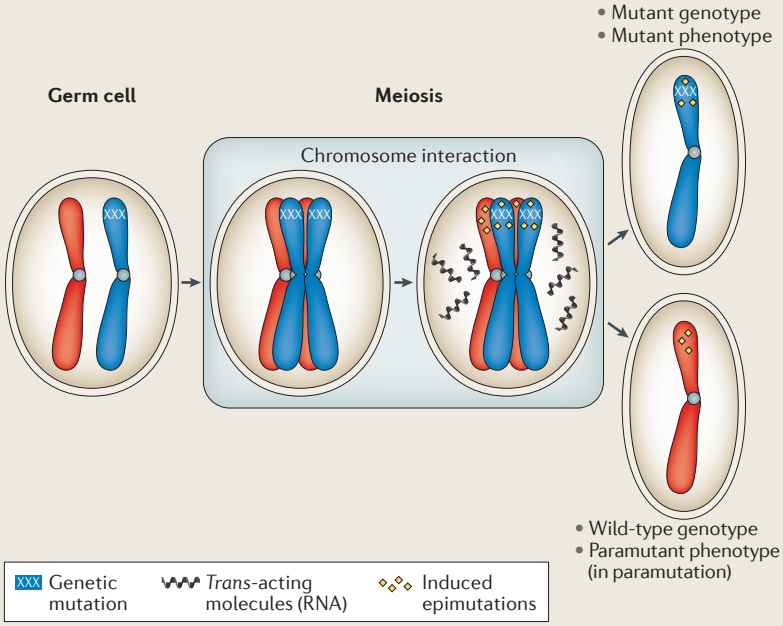
Transfer of RNAs from soma to sperm

Extracellular vesicles. EVs are membrane-bound microvesicles that are ubiquitously found in biofluids. They display sensitive responses to bodily conditions[53] and are frequently found in mammalian reproductive tracts, particularly in the epididymis, semen and uterine fluids[54]. Cellular communication through EVs represents a new frontier in cell biology, bringing novel approaches for biomarker diagnostics[55] and drug delivery[56], and raising the possibility of transferring RNA cargoes from somatic cells to germ cells as a way to deliver environmentally obtained information (either harmful or adaptive). Recent data have shown that EVs in the epididymis (called epididymosomes) contain abundant tsRNAs, similar to mature sperm[11], and *in vitro* experiments have demonstrated that epididymosomes can fuse with and transfer tsRNAs into sperm[11]. This scenario suggests a transfer of tsRNAs between the epididymis and sperm, and is highly intriguing, as a similar transfer might occur in the seminal fluid, where EVs are enriched with miRNAs and tsRNAs[57]. Moreover, an enrichment of miRNAs and tsRNAs is also found in serum[58,59] and neuronal synaptic vesicles[60], both of which exhibit sensitive physiological changes to bodily conditions, such as inflammation, ageing and calorie restriction[58,59,61]. These findings implicate potential connections through EVs at the organismal level and suggest the possibility that EVs can be transported from the soma to the germ line, along with their RNA cargoes. Indeed, a recent study reports soma-to-sperm transmission of RNAs in mice with xenografts of human tumour cells that expressed tumour-specific enhanced green fluorescent protein (*EGFP*) RNAs, which were subsequently found in the bloodstream and sperm of these mice[62], although the acquisition of phenotypic features were not examined in their offspring. In another study, soluble serum factors (of unknown identity, but were possibly EVs or RNAs) from rats with liver damage induced chromatin changes at specific loci in the sperm, which then transmitted the liver-specific wound-healing capacity to subsequent generations[63]. In addition to the evidence from mammals, long-distance transport of RNAs from soma to germ line has been reported in *Caenorhabditis elegans* and was shown to generate transgenerational gene silencing[64–68].

Box 2 | **Transvection and paramutation in mice**

Transvection occurs during chromosome pairing such as during the chromosome synapsis stage of male meiosis in mice, in which an epigenetic modification in one allele is transferred to the other. The transvection phenomenon in mice was first noticed during transgene manipulation[38–40]. In these cases, induced DNA methylation on one allele (triggered by transgene manipulations at this allele) was transferred to the other allele, the genotype of which remained wild type. The ectopically methylated region of the wild-type allele could then be maintained in subsequent generations in the absence of the initial triggering factors[38,39], although no associated phenotype was reported. These phenomena suggest that the manipulation of one allele triggers certain mobile molecules in the cell that could act in *trans*, thus affecting both the manipulated and the intact alleles, and may also contribute to the maintenance of the effect in the wild-type allele. These *trans*-acting molecules could be RNAs.

The paramutation phenomenon is conceptually the same as transvection regarding the molecular interactions between two pairing alleles, yet, it is represented by a prominent phenotype. Paramutation was first documented in plants[135]. In the first paramutation phenomenon reported in mice, a 'white tail' phenotype, representing disruption of the *Kit* gene at one allele, was surprisingly observed in the genotypically wild-type offspring in which the wild-type allele had been exposed to the disrupted *Kit* allele during meiosis[43]. It was then demonstrated that abnormally generated RNAs from the *Kit* loci that were ectopically maintained in wild-type sperm caused the white tail phenotype[43], and this finding was repeated by an independent group[136]. Additional paramutation phenomena in mice have since been reported in other studies[28,36,41,42,44]. These phenomena implicate diffuse molecules, such as RNAs, that are triggered by one mutated allele acting in *trans* to induce a heritable change in the other genotypically intact allele, leading to a paramutant phenotype in offspring with a wild-type genotype. The figure depicts the common principle underlying transvection and paramutation phenomena that have been reported in mice. Note that both paramutation and transvection produced changes at the epigenetic level, whereas only paramutation produced a phenotype at the animal level.

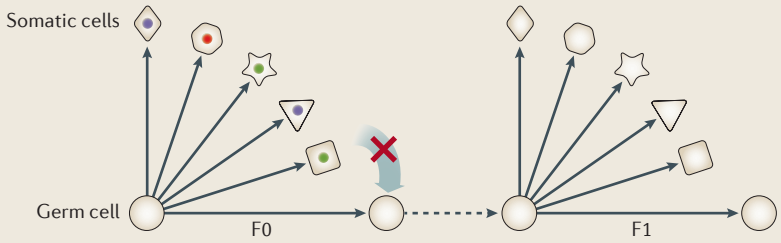


Box 3 | **Somatic–germline barrier: unbreachable?**

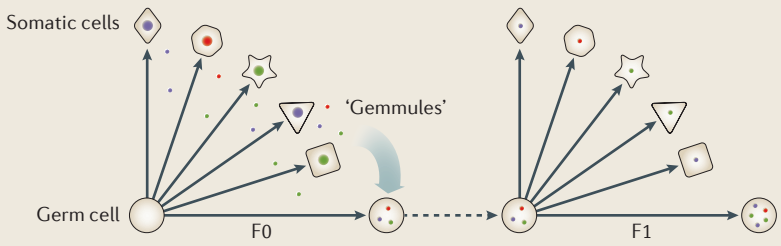
The Weismann barrier is a theoretical barrier proposed by August Weismann in 1893; the central idea was that the flow of information can only move from germ cells to somatic cells and not in the reverse direction (see the figure). Based on this theory, traits acquired during environmental exposure cannot be inherited through the germ line.

Darwin's pangenesis is a theory developed by Charles Darwin in 1868 as a possible mechanism to explain Jean-Baptiste Lamarck's theory of evolution (proposed in 1801) regarding the inheritance of acquired characteristics. In the pangenesis theory, hypothetical particles called 'gemmules' can transfer information between somatic cells and between somatic and germ cells (see the figure), enabling the possible inheritance of acquired traits during ancestral exposure. Interestingly, in a series of debates on pangenesis in 1871 (REF. 137), it was foreseen that "The supposed gemmules must be much more minute than the smallest particles that can be seen by the highest magnifying powers used in these days," and that "…the gemmules are actual particles suspended and not dissolved in the fluid". These descriptions were visionary, as the properties of gemmules proposed approximately 150 years ago appear to match well with those of extracellular vesicles (EVs) (for example, exosomes), which are the topic of intense research today. EVs carry a range of active molecules, including various regulatory RNAs (for example, microRNAs (miRNAs) and tRNA-derived small RNAs (tsRNAs)), and indeed show the ability to transfer information between somatic cells[53] and from somatic cells to germ cells[62].

a **Weismann barrier**



b **Darwin's pangenesis**



In addition, recent reports have shown that semen samples harbour a range of microbiota[69], which can release outer membrane vesicles that are similar to EVs[70] and thus may also deliver unexpected RNA cargoes to sperm. It would be of great interest to explore whether sperm could also gain exogenous RNAs from EVs or bacterial outer membrane vesicles during or after ejaculation. Notably, even after ejaculation into the female reproductive tract, sperm remain immersed in an EV-enriched environment that includes EVs from not only the male, but also the female partner or even from another male under polyandrous conditions in which the sperm could experience cryptic female choice, sperm cooperation and competition (BOX 4). It would be interesting to investigate a role under such circumstances for RNA-containing EVs as communication units mediating sperm–sperm and/or female–sperm communication. Studies such as these have the potential to develop new theories in evolution, particularly regarding environmentally driven, rapid or adaptive evolution.

Specificity and regulation of EV transport. Does the ubiquitous existence of EVs in bodily fluids suggest that cells (including sperm) constantly exchange information via EVs in a non-selective manner? This appears to be counterintuitive. Indeed, recent advances in cancer biology have revealed that EVs are active players in organ-specific metastasis, using distinct membrane integrins as a mechanism for tissue-specific cargo docking[71]. In addition, pathological conditions have been shown to facilitate the transport of RNA-containing EVs to unanticipated locations. For example, an inflammatory response substantially increased the transport of EVs through the blood–brain barrier, resulting in the direct delivery of functional RNAs from blood cells to neurons, which is rarely observed in healthy mice[72]. In this regard, it is notable that both obesity[73] and mental stress[74] are well established to be inflammatory in nature, and both HFD-induced obesity[75] and psychological stress[76] have been reported to disrupt the blood–testis barrier, which might facilitate the transport of environmentally induced somatic EVs to the developing spermatogenic cells and sperm. The exploration of targeting specificities of RNA-containing EVs under physiological and pathological conditions is still in its infancy, but is of great importance as it should help to unravel the mystery of which types of acquired traits are transmitted to offspring through the germ line and the circumstances under which this is likely to occur. Furthermore, although IVF and sperm head injection protocols have demonstrated that the sperm itself carries sufficient information to transmit paternally acquired phenotypes[9–11], it is also possible that non-sperm factors, such as EVs in the semen, contribute to the final phenotype of offspring, especially given the recent experimental evidence for telegony[77,78] — a mysterious phenomenon in which offspring can inherit the traits of a male that mated previously with their mother.

Mobile RNAs. Small RNAs are carried as cargo in EVs, but can also be found in bodily fluids outside EVs[58,79]. Maintenance of their stability outside EVs requires binding proteins, specific secondary structures or RNA modifications[58,79], which might also allow their transport into and out of cells. In *C. elegans*, specific RNA import by cells involves selective transmembrane RNA-gated channels, such as systematic RNA-interference defective protein-1 (SID-1) and SID-2 (REFS 80,81), which allow the transport of RNAs into germ cells, leading to transgenerational gene silencing[64]. The existence and functions of the mammalian homologues of SID family proteins remain to be determined. A recently defined intriguing aspect of small RNA secondary structure is that certain tsRNAs can adopt an RNA G-quadruplex conformation that contributes to neuroprotective responses[82]. Most interestingly, tsRNAs with a G-quadruplex structure can be spontaneously taken up by neuronal cells, suggesting that this specific structure

Polyandrous
The mating behaviour of animals in which the females mate with more than one male in a single breeding cycle.

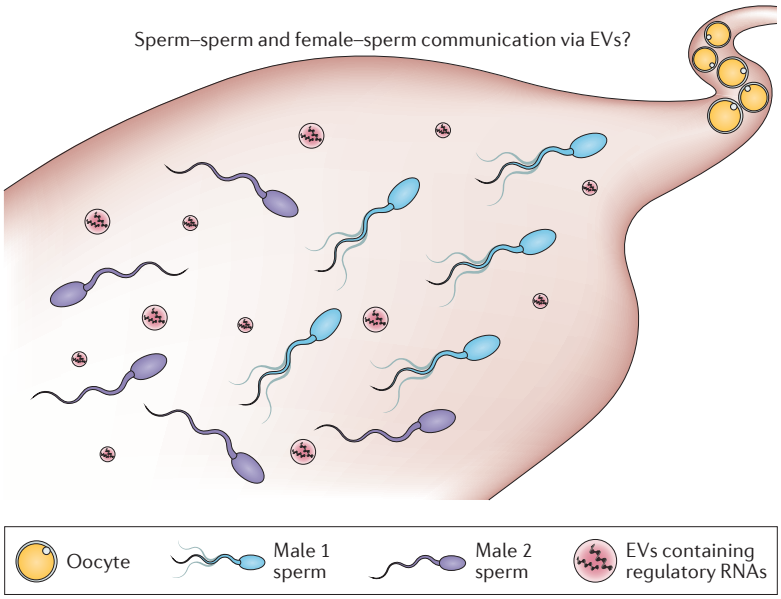
G-Quadruplex conformation
Guanine-rich oligonucleotides that assembled into intra- or inter-molecular guanine tetrad structures, which can be formed by both DNA and RNA.

Box 4 | **Post-copulatory female cryptic choice and sperm behaviour**

In polyandrous species, the female can influence the events that occur after copulation leading to biased sperm selection, resulting in the successful fertilization of the oocyte by the sperm of one male over that of another[138]. This process, termed post-copulatory female cryptic choice, is among the most mysterious biological events ever known; yet, it is important because it biases paternity and influences the trajectory of evolution.

Sperm can also be influenced by each other (see the figure). After ejaculation into the female reproductive tract, individual sperm can group together to form certain networks for mutual advantage in a phenomenon called sperm cooperation that has been well documented in certain species[139]. By contrast, sperm competition describes the process in polyandrous species in which the sperm of different males compete in the female reproductive tract to fertilize a given set of oocytes, representing an important series of post-copulatory events that drives evolution[140].

Despite the evolutionary importance of these well-documented phenomena, the molecular basis of female–sperm and sperm–sperm communication remains enigmatic. Could RNA-containing microvesicles, which are enriched in the semen and uterine fluid, be involved in the mechanism of information exchange during these post-copulatory phenomena?



could facilitate the transmembrane movement of mobile RNAs. This discovery may be far-reaching because most serum tsRNAs are outside EVs[58] and might enter sperm cells by adopting specific RNA structures such as the G-quadruplex. Moreover, evaluating the potential structure of mobile RNAs might also provide fresh insights into the much-debated issue of cross-kingdom transport of small RNAs (for example, the ingestion of plant RNAs by the human body[83–87]), which is probably affected by RNA modifications because they have been shown to protect miRNAs[88] and tsRNAs[9] from degradation.

Alternative ways to alter RNA profiles in sperm

An alternative way of changing sperm RNA profiles in response to environmental stimuli is to alter gene expression during spermatogenesis. Indeed, reports have shown that a HFD can change the testicular transcriptome in mice[31], which may lead to altered sperm RNA contents that mirror the environmental exposure. Moreover, injection of total RNAs extracted from the testes of HFD-fed males into normal zygotes induced metabolic disorders in the offspring, similarly to the injection of total RNAs from mature sperm[26], suggesting that the HFD led to changes in testis RNAs that were inherited by the sperm and dictated the phenotype of the offspring. The implications of these findings are twofold: the testis may gain somatic RNAs through EVs or mobile RNAs, or testicular transcription may be modulated to generate novel RNA profiles that mirror the HFD conditions, or both. However, it is difficult to imagine how dietary changes can be precisely mirrored by changing the testicular transcriptome (and probably the proteome as well), and how such alterations are transformed into sperm-borne information without loss of the original specificities (that is, metabolic disorders). Nevertheless, such a scenario is possible because recent evidence supports the precise transfer of intergenerational information, ranging from the inheritance of a specific olfactory memory[6] to a tissue-specific wound-healing response[63]. A highly integrated interplay among spermatogenic transcription, sperm RNA biogenesis and storage, DNA modifications and chromatin remodelling might underlie such a possibility. Moreover, recent data also show that mutations in or aberrant expression of certain genes (including RNA-editing enzymes[28], chromatin modulators[36,44] and RNA methyltransferases[27]) are responsible for the transgenerational non-Mendelian inheritance of phenotypes and that toxicant-induced epimutations can promote genetic mutations in sperm[89]. It is likely that some of these genes are modulated during certain types of environmental exposure and contribute to the inheritance of acquired traits. Similar gene-regulation feedback mechanisms have recently been shown to control the duration of transgenerational small RNA inheritance in *C. elegans*[90].

Mechanisms of action

The mechanism by which sperm RNAs reshape early embryonic development to recapitulate a paternally acquired phenotype in offspring remains unclear. In addition, how the initial changes caused by sperm RNAs are converted into a stable form of information to allow transgenerational inheritance remains a major puzzle. Recently, emerging evidence has shed new light on these intriguing questions.

Reshaping embryonic development: the transcriptional cascade. The injection of RNAs into mammalian zygotes has been reported to alter offspring phenotypes[41–43] and to mimic paternally acquired traits[9,24–26]. These studies provide proof-of-principle evidence that perturbations in RNA profiles have a profound impact on early embryonic development, although the amount of RNA injected in these studies was larger than a single sperm could deliver. Under physiological conditions, the amount of RNAs delivered by a single sperm is infinitesimal compared with the RNA reservoir of oocytes. However, data have indeed shown that the incoming sperm can provide information to the host oocyte cytoplasm to functionally influence the order of cell division and spatial patterning in mouse embryos[91].

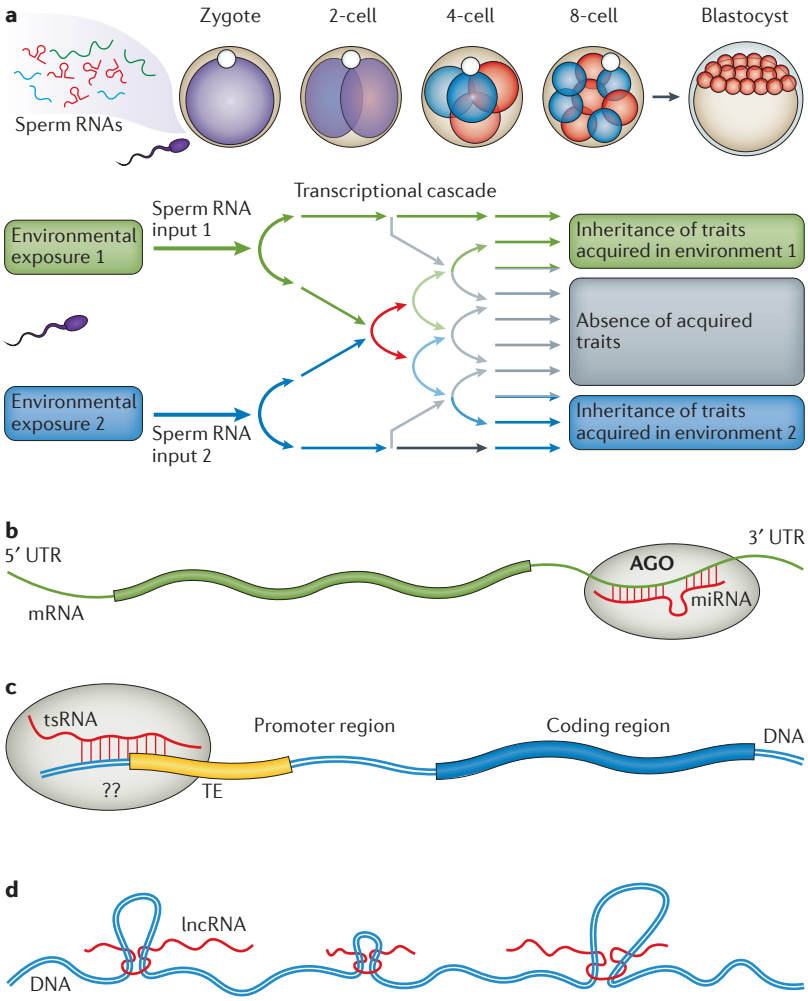


Figure 2 | **Potential mechanisms involving sperm RNAs in early embryos. a** | Sperm RNA input following certain environmental exposures may trigger a transcriptional cascade in the early embryo that affects the symmetry-breaking process, and may generate phenotypes in the offspring that mirror the paternally acquired phenotypes. **b** | Sperm microRNAs (miRNAs) could mediate mRNA stability and/or degradation by binding to the 3′ untranslated region (UTR), which is facilitated by Argonaute (AGO), to regulate mRNA metabolism. **c** | Sperm tRNA-derived small RNAs (tsRNAs) showed sequence matches to gene promoters that are associated with transposable elements (TEs), with unidentified mechanisms or related binding proteins. **d** | Sperm long non-coding RNAs (lncRNAs) may reshape chromatin 3D structure.

A recent single-blastomere RNA-seq analysis of mammalian early embryos suggested that small variations in the transcriptome will inevitably emerge at the two-cell embryo stage, owing to imperfect cleavage division[92]. These initial biases will subsequently be transformed into a more defined asymmetrical transcriptional pattern upon progression of zygotic transcriptional activation, a dynamic symmetry-breaking process that defines different lineage fates[92]. Recent evidence showing biased distributions of essential molecules that can bias cell fate in early embryonic stages are supportive of this model[93–96]. In this regard, even the smallest biases in RNA distribution (for example, sperm miRNAs that can regulate mRNAs) at early embryonic stages (FIG. 2a,b) have the potential to influence the trajectory of development through a butterfly effect. In theory, the altered sperm RNA contents could convey epigenetic information that is specific for paternally acquired traits if the embryonic transcriptional cascade is regulated with analogue and/or digital precision[97] (FIG. 2a). Indeed, RNA-seq analyses of early embryos after the injection of sperm tsRNAs from a HFD-fed father revealed a down-regulation of metabolic regulation-related genes in both early embryos and pancreatic islets of offspring, supporting the idea that regulation of the transcriptional cascade continues throughout development[9], although the detailed regulatory network remains unknown.

Regulating transposable elements. Transcriptional regulation might explain the phenotypes observed in the immediate offspring following paternal environmental exposure, but how might the phenotypes induced by sperm RNAs be transferred to further generations? RNA-guided DNA methylation has been well established in plants[98], and it has been observed in mammals that piRNAs can mediate site-specific DNA methylation[99] and retrotransposon silencing[15]. Therefore, RNA-guided DNA methylation could be one possible mechanism that underlies transgenerational inheritance of acquired phenotypes. Intriguingly, sperm tsRNAs exhibit sequence matches at various gene promoters[9], many of which are associated with transposable elements (TEs)[100], suggesting that transcriptional regulation of these genes occurs through TEs. Indeed, injection of a single tsRNA ($tsRNA^{Gly(GCC)}$) into a mouse zygote can repress the genes associated with murine endogenous retrovirus with leucine tRNA (MERVL), a type of TE[11]. Whether the interactions between tsRNAs and TEs in the early embryo can establish a stable mark (for example, altered DNA methylation or histone modifications) and thus change the expression of TE-associated genes in a heritable manner remains an interesting possibility (FIG. 2c). Testing this hypothesis requires comprehensive, high-coverage, genome-wide DNA methylation analysis, such as whole-genome shotgun bisulfite sequencing (WGSBS), in both early embryos and the sperm of offspring. Notably, although recent WGSBS data have suggested that DNA methylation is not the initial molecular carrier for diet-induced epigenetic inheritance in the sperm of exposed males[16], whether DNA methylation could act as the secondary effector that relays the initial effects of sperm RNAs remains unresolved. This possibility should be examined carefully with sufficient sequencing coverage and depth[37], particularly for TEs in proximity to genes and promoters. In addition to the well-studied 5 mC DNA methylation discussed above, the recent discovery of N^6-methyladenine (m^6A) DNA methylation in mammalian cells and its association with TEs[101] also raise the intriguing possibility of m^6A as a potential heritable mark for epigenetic inheritance in mammals, as has been suggested in *C. elegans*[102].

Remodelling chromatin structure. Mature sperm contain numerous mRNAs, small RNAs and lncRNAs[23]. Both small RNAs and lncRNAs have been shown to

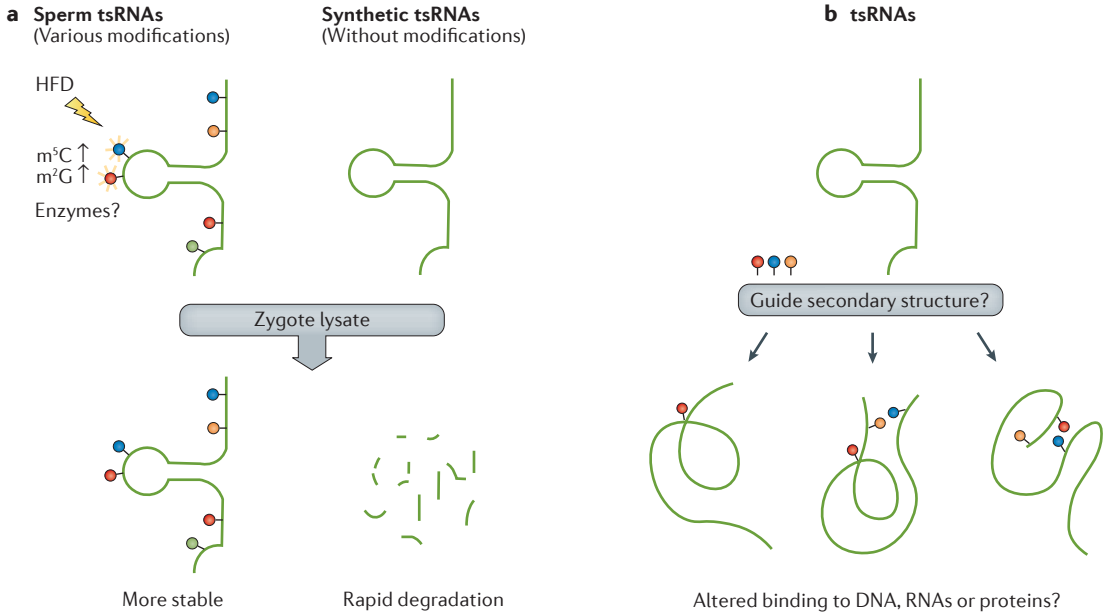


Figure 3 | **Current knowledge of sperm RNA modifications. a** | Sperm tRNA-derived small RNAs (tsRNAs) contain various RNA modifications (coloured dots) that contribute to their stability in zygote lysate; a high-fat diet (HFD) in male mice increases 5-methylcytidine (m^5C) and N^2-methylguanosine (m^2G) levels in sperm tsRNAs, by unknown enzymes and mechanisms. **b** | RNA modifications may alter the secondary structure of tsRNAs, which could affect their stability and targeting specificity.

regulate chromatin structure[98,103]. In particular, lncRNAs mediate long-range chromatin interactions, generating unexpected 3D organization of the chromosomes[104–107] with extensive topological domains, as revealed by whole-genome chromosome conformation capture (Hi-C) analysis[108] (FIG. 2d). Recently, using a transgenic mouse line overexpressing lysine-specific histone demethylase 1 (LSD1; encoded by *Kdm1a*) it was observed that LSD1 overexpression in one generation impaired offspring development, owing to multiple defects that persisted in subsequent generations in the absence of the initial transgene expression. These transgenerational defects were not transmitted by altered DNA methylation in CpG-rich regions but were associated with abnormal RNA expression in wild-type sperm from the offspring of the transgenic mice[36]. In this case, the abnormal sperm RNA profiles appeared to be the underlying cause of the defects in offspring, and the sperm of the offspring maintained the aberrant RNA expression. Could this be due to a feedback loop in which abnormal sperm RNAs induce changes in chromatin structure that, in turn, induce the same abnormalities in RNA expression? These are remote but tantalizing possibilities, and a similar RNA-based feedback loop mechanism has been suggested in *C. elegans*[68,109].

Sperm RNA modifications

In addition to RNA sequences, RNA modifications have recently emerged as a new layer in the transcriptional and epigenetic regulation of various biological processes, bringing about a new era of RNA epigenetics[110,111]. Recently, a study using liquid chromatography–tandem mass spectrometry (LC–MS/MS) to simultaneously identify and quantify multiple RNA modifications in mice[112] revealed that sperm small RNAs harbour various RNA modifications. The tsRNA fraction is particularly sensitive in the response to a paternal HFD, showing a significant increase in m^5C and m^2G (REF. 9) (FIG. 3a). The significance of such alterations remains unclear, yet the RNA modifications present in tsRNAs seem to increase RNA stability, as chemically synthesized tsRNAs without RNA modifications showed more rapid degradation in mouse zygote lysates[9] and serum[58] (FIG. 3a). This increased RNA stability might prolong the half-life of sperm RNA actions after fertilization and thus preserve their function in transferring paternally acquired traits to offspring[9]. In addition, RNA modifications could change the structure of RNAs *in vivo* and alter the specificity of their interactions with other RNAs, DNA or proteins (FIG. 3b), which may represent a new layer of regulation[111]. By far, the greatest challenge is to reveal the full map of RNA modifications in sperm tsRNAs and other types of RNAs, because the LC–MS/MS approach cannot pinpoint the exact location of each RNA modification. Future combinational use of LC–MS/MS and other methods — for example, antibody-based pull-down and chemical approaches that are already successfully used for genome-wide profiling of RNA modifications (such as, m^5C, m^6A, m^1A and pseudouridine[110,111]) — as well as the existing databases of RNA modifications[113,114], will help to reveal the full map of RNA modifications in sperm RNAs. In particular, RNA-modification maps for tsRNAs and miRNAs will be of great interest, given their probable roles in the inheritance of acquired traits.

RNA modification enzymes in epigenetic inheritance. The specific enzymes responsible for regulating each sperm RNA modification remain largely unknown, yet several studies have demonstrated the great potential of such enzymes to mediate epigenetic inheritance. One report showed that RNA-mediated epigenetic heredity requires the DNA (cytosine-5)-methyltransferase-like protein 2 (DNMT2)[27], which is known to be responsible for adding m^5C to several types of tRNAs[115,116]. Deletion of *Dnmt2* also causes increased production of tsRNAs, particularly under stress conditions[115,117]. Another report showed that the deletion of DEAD box polypeptide 1 (*Ddx1*), which encodes an RNA helicase that has broad functions in regulating RNA structure and metabolism (including tRNA splicing[118] and promoting miRNA maturation[119]), resulted in transgenerational inheritance of wild-type lethality in wild-type mice derived from heterozygote intercrossing[120], which might involve an interplay between abnormal RNAs and a perturbed chromatin structure. Moreover, deletion of apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1 (*Apobec1*), an RNA-editing enzyme with cytidine deaminase activity that converts C to U, resulted in the transgenerational inheritance of susceptibility to testicular germ-cell tumours in the first to the third generations of wild-type male offspring of heterozygous female mice[28], suggestive of RNA-mediated paramutation effects. All of these transgenerational effects are closely related to abnormal post-transcriptional RNA modifications, including nucleotide modifications, RNA editing, RNA ligation and/or unwinding. This evidence strongly suggests that RNA modifications have a central role in the modulation of epigenetic memory, a hypothesis that warrants future investigations.

Sequencing bias due to RNA modifications. At the technical level, it is important to note that certain RNA modifications can block reverse transcriptase during RNA-seq library preparation, resulting in a failure to detect some modified RNAs and thus generating biased readouts[121,122]. This is particularly true for tRNAs and tsRNAs, which contain extensive modifications[9,113,114,123]. Recently, the use of *Escherichia coli* α-ketoglutarate-dependent dioxygenase (AlkB) in a treatment protocol, which can demethylate reverse-transcriptase-blocking RNA methylations before RNA library constructions, has led to the discovery of abundant, previously undetected tRNAs and tsRNAs[121,122]. These findings suggest that previously published data sets of small RNAs, particularly those including heavily methylated RNAs (for example, sperm tsRNAs), will require a comprehensive re-analysis using new approaches. Until then, caution should be exercised when interpreting inherited phenotypes based solely on observed changes in one or a few sperm small RNAs, as such observations could be misleading.

Perspectives and conclusion

Evidence for RNA-mediated inheritance of ancestrally acquired traits is now expanding, and solid data have become available from studies on plants, nematodes, fruitflies and mammals. These and other studies that support the inheritance of acquired traits hypothesis have set the stage for a re-evaluation of long-held paradigms that describe the physiological and molecular barriers of hereditary information, as well as the flow of information at both the molecular and physiological levels. The versatility of sperm regulatory RNAs, along with the even more complex forms of RNA modifications and the potential interactions of sperm RNAs with other epigenetic factors, raise important questions regarding the epigenetic inheritance of acquired traits. For example, how many items of information can be stored in the form of sperm RNAs, and how specific can a heritable acquired trait be? Could sensitivity to a specific odour, like or dislike of a specific food, fear of a specific animal or scenario, or adaptation to a lifestyle be inherited? Indeed, recent reports have demonstrated that inheritance of acquired traits could be as precise as the epigenetic memory of a specific odour[6], giving rise to wide-ranging possibilities for the inheritance of other life experiences. It would be remarkable if such heritability were to extend beyond passive exposures to environmental factors (for example, chemicals, nutrition or stress) to learned or intentional behaviours or characteristics (such as kindness and charisma).

However, demonstrating the contributions of sperm RNAs to the transfer of acquired traits is only the first step. A fundamental question concerns the nature of the 'coding mechanism' through which specific acquired traits become encoded in sperm; could a trait be encoded in the form of a single RNA or a combination of RNAs, or is additional interplay between sperm RNA and other epigenetic factors required? The mechanisms that 'encode' this information in the sperm and that 'decode' it in the offspring, and the potential transformation of RNA-borne 'codes' into other forms of hereditary information (for example, DNA methylation and chromatin structure), are key issues that require investigation. Furthermore, in addition to the flow of information from the environment to sperm RNAs and RNA modifications, and the means by which these factors reshape development, it is important to understand the reversibility of acquired traits over generations, which may have widespread medical and social implications.

Answering these questions will require elegantly designed experiments at both the animal and molecular levels, as well as the improvement of technologies for 'single-cell omics' (for example, transcriptomics, DNA methylomics, ChIP–seq (chromatin immunoprecipitation followed by sequencing) and Hi-C)[124] for use in sperm and early embryo investigations, which will ideally involve parallel double-[125], triple-[126] or even panomic approaches. Such advances will probably require at least another decade's worth of efforts, but may revolutionize our understanding of hereditary information flow, stimulate further interest in old theories (including Lamarckism and Darwin's pangenesis (BOX 3)) and inspire new waves of research in the fields of genetics and epigenetics.

Lamarckism
The idea that an organism can pass on characteristics that are acquired during its lifetime to its offspring.

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Competing interests statement
The authors declare no competing interests.

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