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# Sperm RNA-mediated epigenetic inheritance in mammals: challenges and opportunities

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## Abstract

Emerging evidence now shows that in addition to delivering a haploid DNA, the mammalian sperm also carry various types of RNAs that respond to the paternal environment, which can mediate the intergenerational transmission of certain phenotypes to the offspring relating to the paternal environmental exposures (e.g. diet, mental stress). Improved analytical tools are beginning to decipher the complexity of sperm RNAs, RNA modifications and their spatial compartmentalisation, which support the concept of 'sperm RNA code' in programming specific offspring phenotypes during embryonic development. In this commentary article, I discuss the challenges and opportunities in solidifying the field of mammalian sperm RNA-mediated epigenetic inheritance, including the identification of the key sperm RNAs that are responsible for the paternal phenotype transmission, and the cellular and molecular events that are triggered by sperm RNAs during embryo development. I also discuss the translational application potential by harnessing the knowledge of sperm RNA code to improve farm animal production and human health.

### Keywords

epigenetic inheritance; lncRNA; miRNA; PANDORA-seq; precision medicine; reductionism; RNA modification; rsRNA; systems biology; tsRNA

## Introduction

Solid observations have been reported repeatedly in mammalian models that a range of paternal (F0) environmental exposures (e.g. diet, mental stress, inflammation, toxins, endocrine disruptor and temperature) can lead to phenotypical changes in the immediate F1 offspring (and sometimes to the following generations) relating to the paternal exposure (Perez and Lehner 2019; Fitz-James and Cavalli 2022). Many of these observations are thought to be independent of directly altering the DNA sequence in the sperm, thus suggesting the involvement of epigenetic mechanisms beyond the DNA sequence-based information, including DNA methylation, histone modifications, Chromatin 3D structure

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and RNA-based mechanisms, probably in a synergistic manner. These potential 'epigenetic information carriers' are supposed to encode paternal environmental experiences in the sperm, and drive offspring phenotypes by regulating embryo development. While different epigenetic mechanisms (Perez and Lehner 2019; Fitz-James and Cavalli 2022) and general principles (Zhang and Chen 2019) have been extensively discussed in recent reviews, the present commentary will focus on the revelation of sperm RNAs as a causal factor in intergenerational transmission of phenotypes (Chen *et al*. 2016*a*) in mammals and discuss the challenges and opportunities extending from the emerging concept of 'sperm RNA code' (Zhang *et al*. 2019).

## The 'sperm RNA code' in programming offspring phenotypes

One of the major advances in studying mammalian epigenetic inheritance over the last decade has been the demonstration that sperm RNAs, along with their RNA modifications, can act as a causal factor to induce a range of offspring phenotypes (Chen *et al*. 2016*a*; Zhang *et al*. 2019), where the injection of either total sperm RNAs or a subset of sperm RNAs (e.g. 30–40 nt fraction) from exposed males into healthy zygotes can robustly induce offspring phenotypes relating to paternal exposures, including paternal unhealthy diet (Grandjean *et al*. 2016; Chen *et al*. 2016*b*; Sarker *et al*. 2019; Raad *et al*. 2021), mental stress (Gapp *et al*. 2014; Rodgers *et al*. 2015; Gapp *et al*. 2020; Wang *et al*. 2021), inflammation (Zhang *et al*. 2021*a*), ageing (Guo *et al*. 2021) and drug administration (Gapp *et al*. 2021).

The sperm RNA profile is now increasingly recognised to be highly complex, mixed with a diversity of messenger RNAs (mRNAs), long non-coding RNAs (lncRNAs) and small non-coding RNAs (sncRNAs), with the sncRNAs being categorised into major subtypes including microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), tRNA-derived small RNAs (tsRNAs) and rRNA-derived small RNAs (rsRNAs), most can sensitively respond to paternal environmental changes (Chen *et al*. 2016*a*; Zhang *et al*. 2019). The sperm RNAs also harbors various types of RNA modifications as detected by high-throughput approaches such as liquid chromatography–tandem mass spectrometry (LC–MS/MS) from different sperm RNA fractions, certain types of RNA modifications (e.g. $m^5C$, $m^2G$) are shown to be sensitive to environmental changes (e.g. high-fat diet) in the tsRNA/rsRNA-enriched sperm RNA fraction (30–40 nt), and are involved in the transmission of phenotype to the offspring (Chen *et al*. 2016*b*; Zhang *et al*. 2018). Importantly, synthesised tsRNAs without RNA modifications are degraded faster in mouse zygote lysates (Chen *et al*. 2016*b*) or serum (Zhang *et al*. 2014) compared to the tsRNAs extracted from *in vivo*, and that certain modifications can change the secondary structures and functions of sncRNAs from *in vitro* experiments (Zhang *et al*. 2018), all suggesting that RNA modifications are an integral part of enabling proper sncRNA functionality (Zhang *et al*. 2016).

The information capacity enabled by diverse types of sperm RNAs and various RNA modifications, coupled with the observation that the injection of sperm RNAs can induce specific phenotypes relating to paternal exposures (such as sperm RNAs from diet-exposed father induce metabolic disorders, or sperm RNAs from mental stress-exposed father induce behaviour changes) has led to the proposition that each specific environment may encode a

specific signature of 'sperm RNA code' (Zhang *et al.* 2019) to confer offspring phenotype under different contexts of paternal epigenetic inheritance.

While the 'sperm RNA code' represents an attractive concept in explaining the reported data regarding sperm RNA-mediated epigenetic inheritance in mammals, it remains largely unknown which (group of) sperm RNAs are the most important casual factors under each specific paternal exposure, and how these modified RNAs exert their effects that penetrate the embryo development process. Addressing these key questions requires high resolution in analysing the sperm RNA code, which involves accurate identification and profiling of a diverse range of sperm RNAs and the associated RNA modifications that are integral to their function, as well as the dissection of possible cellular and molecular mechanisms in the context of embryo development. I'll discuss these issues with my thoughts below.

## Updated landscape of sperm RNAs with increasing resolution

Like the research history of the RNAs, our understanding of the sperm RNA population is constantly changing, mostly due to the ever-evolving technologies available, and also to our revelation in critically assessing our previous views when technical revolution arrives – every time we realise that we have seen only part of the larger picture (Shi *et al.* 2022).

### Improved methods in profiling sperm RNAs and RNA modifications

Identification of RNAs in sperm has been reported since the 1970s (Betlach and Erickson 1973), and were sporadic and controversial at first; in the 2000s the emergence of high-throughput methods such as microarray began to provide more evidence on the existence of RNA in sperm, initially focused on mRNAs (Ostermeier *et al.* 2002). The early reports that sperm RNAs can alter offspring phenotypes (Rassoulzadegan *et al.* 2006; Wagner *et al.* 2008; Grandjean *et al.* 2009) further triggered interests to study sperm RNAs in more depth. The application of RNA sequencing (RNA-seq) and improved bioinformatic analyses have further identified various types of sncRNAs in mature sperm (Shi *et al.* 2018), for example, the mature mouse sperm contain a dominant set of tsRNAs and rsRNAs, in addition to a less abundant level of miRNAs and piRNAs (Peng *et al.* 2012; Chen *et al.* 2016*b*, 2018; Sharma *et al.* 2016; Chu *et al.* 2017; Sharma *et al.* 2018; Zhang *et al.* 2018). The sperm sncRNA composition are distinct from the testicular spermatogenetic cells and seems to be species-specific (Peng *et al.* 2012; Donkin *et al.* 2016; Schuster *et al.* 2016; Chen *et al.* 2020; Sellem *et al.* 2020, 2021). Sperm sncRNAs are now known to be dynamically regulated by a range of environmental exposures (Zhang *et al.* 2019), genetic factors (Zhang *et al.* 2018; Zhang *et al.* 2021*a*) and exercise training (Ingerslev *et al.* 2018; Stanford *et al.* 2018); and can also be used to separate sperm with low versus high fertility potential in clinical settings (Hua *et al.* 2019; Chen *et al.* 2021*a*).

In addition to the sequence diversity of sperm RNAs, various RNA modifications have been identified in sperm RNAs using LC–MS/MS, which can be regulated by environmental factors and specific enzymes (Chen *et al.* 2016*b*; Zhang *et al.* 2018; He *et al.* 2021). Notably, a combined RNA modification signature (e.g. $m^1G$, $m^5C$, $m^2G$ and $m^1A$) in sperm RNAs have been shown recently to be correlated with sperm motility, providing the potential for diagnostic value in IVF clinics (Guo *et al.* 2022); other tissue- or blood- based RNA

modification signatures are also implicated as biomarker under different disease conditions (Zhang *et al.* 2020, 2022).

Importantly, several types of RNA modifications in sncRNAs (e.g. $m^1A$, $m^1G$ and $m^3C$) can block the reverse transcription process during the conversion of sncRNA into cDNAs, and some modified sncRNA termini cannot be efficiently ligated to adaptor sequences during the cDNA library preparation (Chen *et al.* 2021*b*; Shi *et al.* 2022). These RNA modifications have generated substantial biased results when using the traditional sncRNA sequencing method, as the sncRNAs harbouring these terminal or internal modifications cannot be efficiently included in the cDNA library, thus cannot be detected by traditional RNA-sequencing methods (Shi *et al.* 2022). To conquer these problems, recently improved methods such as PANDORA-seq (Shi *et al.* 2021), can resolve these RNA modifications by step-wise enzymatic treatments, leading to the discovery of more abundant modified sncRNAs (mostly tsRNAs and rsRNAs) in sperm that were previously undetectable (Shi *et al.* 2021). This would also suggest that many previously reported sncRNA alternations under different paternal exposure deserve to be sequenced again using updated methodology, potentially leading to updated insights.

Moreover, sperm also harbour unique sets of large RNAs (e.g. mRNAs, lncRNAs and circular RNAs) with the potential to transmit certain types of stress related phenotypes (Gao *et al.* 2020; Gapp *et al.* 2020, 2021). Although a majority of sperm large RNAs are supposed to be fragmented as exemplified by the fragmented rRNAs, a recent study using PacBio-based third-generation long-read sequencing has shown that a substantial portion of sperm mRNAs remain intact, many of these intact mRNAs are enriched for translation related proteins such as small ribosomal subunits (RPSs) and large ribosomal subunits (RPLs) (Sun *et al.* 2021). These translation related mRNAs add to the diversity of the ‘sperm RNA code’, which might be directly used during post-fertilisation and contribute to the translational regulation program in the early embryo (Zhang *et al.* 2019).

## Developmental origins and compartmentalisation of sperm RNAs

Mature sperm has a unique structure comparing to other cell types, with minimal cytoplasmic contents, a condensed nucleus, and a long tail contains mitochondria. The composition of sperm RNAs are developmentally regulated, where the RNA signature in epididymal sperm is distinct from the testicular spermatogenic cells due to several layers of regulation (Zhang *et al.* 2019). First, the mature sperm get rid of most of the cytoplasmic contents and RNAs, and selectively enrich RNA species that remain in the sperm head and tails (Zhang *et al.* 2019). Second, *de novo* fragmentation of longer RNAs (e.g. cleavage of tRNAs/rRNAs into tsRNAs/rsRNAs) can happen during epididymal transition thus modify the repertoire of sperm RNA population (Zhang *et al.* 2018; Zhang *et al.* 2021*a*). Third, sperm can potentially gain somatic RNAs during epididymal transition via exosome dependent or independent pathways (Sharma *et al.* 2018; Chan *et al.* 2020; van Steenwyk *et al.* 2020; Trigg *et al.* 2021), which resonate with Darwin’s pangenesis theory raised in 1868 (Darwin 1868) which in turn supports Lamarck’s idea regarding inheritance of acquired characteristics and is now gaining renewed interests (Liu and Chen 2018).

RNAs in the sperm are also spatially compartmentalised, for example, by comparing the sncRNA profile in sperm heads (with nude nucleus) and whole sperm, it was found that the miRNA/tsRNA/rsRNA signature in the sperm heads is distinct from that of the whole sperm, with more tsRNAs enriched in the sperm heads (Shi *et al.* 2021), suggesting their unique function in the nuclei – a hypothesis awaits to be systematically analysed in the context of sperm RNA-induced epigenetic inheritance. In addition, a transitional structure called the cytoplasmic droplet, which is involved in osmoadaptation and energy metabolism (Chen *et al.* 2011; Yuan *et al.* 2013), may also carry a unique reservoir of RNAs that may represent the cytoplasmic content of testicular spermatogenic cells. The compartmentalised sperm RNA signature could bear significant biological significance, for example, RNAs in the sperm head (nuclei) might be more directly engaged in the epigenetic reprogramming progress after fertilisation, whereas the RNAs contained the tail and cytoplasm would be quickly diluted in the oocyte cytoplasm after fertilisation (some RNAs may have a longer half-life due to certain RNA modifications or function synergistically with RNAs/proteins deposited in the oocyte/zygote). Also, sncRNAs compartmentalised in different sperm region may respond differentially to the same environmental stimuli, a recent study showed that mitochondrial tsRNAs and rsRNAs are more sensitive to a short-term high-sugar diet in human (Nätt *et al.* 2019), possibly due to the cleavage of tRNA/rRNA in the sperm mitochondria where they are exposed to a higher level of oxidative stress upon acute high-sugar diet (Zhang and Chen 2020).

## Challenges and opportunities in solidifying sperm RNA-mediated phenotype transmission

While evidence from RNA zygotic injection has shown that sperm RNAs can act as causal factors to induce offspring phenotypes, which (group of) sperm RNAs are the most important casual factors under each specific condition, and how could these modified RNAs exert their effect that penetrates the embryo development process remain largely unknown. These represent the two main concerns during future exploration.

### Identifying key regulatory sperm RNAs: reductionism vs systems biology

In the search of key regulatory sperm RNAs under each paternal exposure condition, an important direction is the application of state-of-the-art methods to comprehensively capture and analyse various RNA species in the sperm along with their RNA modification status. Explorations on this direction have in fact, triggered a series of methodological advances in the past few years regarding the panoramic detection of sperm sncRNAs, large RNAs and RNA modifications that were previously unavailable (Shi *et al.* 2021; Sun *et al.* 2021). These advanced tools in turn call for re-evaluation of the sperm RNA changes under different paternal exposures. This direction may look ‘descriptive’ but would be absolutely necessary before further functional/mechanistic dissection, because the accurate discovery of key sperm RNA changes would depend on the right methods being used.

Also, in the search for key sperm RNAs that may have causal function, one of my concerns is that there is an increasing trend in publications in which the researchers tend to focus on only one or a very few altered sperm RNAs (e.g. miRNAs, tsRNAs) and use bulk injection

of the selected sncRNAs (or their antagonist) into zygote followed by offspring phenotype tracing, and attribute the observed offspring phenotype to one or few sncRNAs. This type of study, in my opinion, tends to generate biased conclusions as there are many caveats involved, including the choosing of the few 'right' RNA candidates (usually out of many altered RNAs), the property (e.g. modification status) and quantity of the selected RNAs being injected, and the interpretation of the phenotypes (focusing on the paternal exposure-related phenotypes while ignoring other potential phenotypical changes). These biases may represent an excessive use of 'reductionism' while lacking the thinking from a 'systems biology' angle that different RNAs act in a synergistic manner, and many phenotypes do not stand alone.

Perhaps focusing more on a comprehensive descriptive discovery (including the sperm RNA alterations and the phenotypes changes) without rushing into conclusions such as claiming that a key RNA is fully responsible for a specific phenotype, would make the field more resilient and leave room for more in-depth explorations – which usually happens more slowly but may stand the test of time.

## Mechanisms of sperm RNAs during development, one step a time

For the mechanism by which mammalian sperm RNAs impact embryo development that influence offspring phenotype, although it has been shown that injection of sperm RNAs can robustly change the early embryonic transcriptome that relate to the offspring phenotypes (Chen *et al*. 2016*b*; Gapp *et al*. 2021; Wang *et al*. 2021), there is, by far, no further satisfactory molecular mechanisms provided, especially how the effect can penetrate the whole development.

Unlike in other model animals where the effect sncRNAs can be amplified via RNA-dependent RNA polymerase (RdRP) or positive feedback between small RNAs and histone marks (Zhang *et al*. 2019), the RdRP system has not been found in mammals. Thus, this has put to the question how sperm RNAs can make its effect in the zygote and influence the embryo development. There are at least several directions of hypotheses to explore, including the RNA-histone/DNA mark interactions, the nuclear function of tsRNA/rsRNA that result in long-lasting effects such as by interacting with nuclear RNPs, generating ribosome heterogeneity, and affecting embryonic lineages as previously discussed (Chen *et al*. 2018, Chen *et al*. 2021*b*; Zhang *et al*. 2019).

Important considerations when exploring these possible mechanisms would include the knowledge based on the compartmentalisation of sperm RNAs (e.g. head vs tail), the modifications of the RNAs, and the resulting half-life and interaction potential in the zygote/embryo. Addressing these fundamental questions would need explorations one step at a time, sometimes requiring *in vitro* cell models first before moving to actual mammalian embryos due to the scarcity of the material.

Finally, genetic mouse models remain a powerful means of understating key genes/pathways that contribute to the phenotype transmission, such as in the example of deleting Dnmt2 (Kiani *et al*. 2013; Zhang *et al*. 2018; Yu *et al*. 2021) or Angiogenin (Zhang *et al*. 2021*a*), which prevent the transmission of sperm RNA-mediated phenotype to the offspring, and is

in part due to the alterations of sncRNA biogenesis and RNA modifications. Meanwhile, we should keep in mind that genetic deletion models in mice may tell us how to disrupt a sperm RNA code to prevent the transmission of a phenotype, but not about how a functional sperm RNA code is built – a case of necessity versus sufficiency. Nonetheless, these models provided unique opportunities to understand the nature of sperm RNA signatures under each condition and possibly lead to the manipulation of sperm RNA code with precision.

## Translational applications of sperm RNA code in humans and farm animals

Even without fully understanding the RNA-mediated molecular mechanisms, we may still make good use of the existing knowledge of sperm RNA code for translational applications to benefit human health and farm animal production. Importantly, since human sperm RNA profiles are known to be efficiently altered by improved body conditions such as trained exercise or surgical assisted reduction of body weight (Donkin *et al.* 2016; Ingerslev *et al.* 2018), the sperm RNA signature could be monitored as a clinical biomarker before a planned pregnancy, and clinical guidance (such as improvements to lifestyle) can be introduced to reshape the sperm RNA code to intergenerationally prevent disease susceptibilities (Zhang *et al.* 2019). Due to ethical and safety issues, synthetic modified RNAs will apparently not be allowed to be used in human embryos in a predicted future to counteract disease-associated sperm RNA signatures, but it could be tested in mouse as a pilot study and might be used in large farm animals to enhance certain traits in the offspring. Indeed, recent studies have shown the potential that sperm sncRNAs (e.g. miRNAs, tsRNAs) can regulate early embryo function in farm animals (Chen *et al.* 2020; Wu *et al.* 2020).

For the study of sperm RNA functionality in large farm animals such as bulls, it might be essential to first generate a comprehensive database for sperm RNA signature from different breeds (Sellem *et al.* 2020) with updated methods such as PANDORA-seq, and to establish the correlation between different sperm RNA codes and specific traits of breeds. This would represent an emerging direction that is worth exploring in the near future. In addition, important agricultural economic species such as sheep may also be studied as a novel model for environmental exposure experiments and the knowledge obtained could be informative for humans. Finally, the potential limitation of small RNA annotation in large animals might be encountered due to relatively less comprehensive full-genome annotation, and this could be a direction to be explored by the interested groups.

## Outlook

In addition to the main topics discussed above, there are other thought-provoking aspects regarding sperm RNA-mediated phenotype transmission. For example, whether the sperm RNA code can encode traits with great precision such as the sensitivity to a specific paternal olfactory experience that are controlled by specific olfactory sensory neurons (Dias and Ressler 2014), or perhaps the sperm RNA code can only program offspring phenotype with less precision such as generate an overall altered metabolism due to the cascade effect starting from early embryo development, resulting in abnormal fetus–placenta development; or perhaps a grey area in between. Also, what is the deciding factor that controls the penetrance of RNA-mediated phenotypes in the offspring (which do not fit to Mendelian

pattern); and what is the cause of the sex-specific phenotypes that are usually observed in the phenotypical tracing of the offspring (Sandovici *et al.* 2022); and finally, whether and to what extent the sperm RNA-mediated phenotypes in F1 offspring can be transmitted to F2 or further generations, and whether this could be under adaptive selection.

Technology-wise, new methods will undoubtedly keep emerging to bring new insights into the complexity and chemical nature of the sperm RNAs. For example, recently developed MLC-seq, a mass spectrometry-based direct sequencing method, can simultaneously unravel the sequences and quantitatively map multiple RNA modifications of tRNAs/tsRNAs (Zhang *et al.* 2021*b*), which could be used to study the RNA modifications of sperm sncRNAs (e.g. tsRNAs) with unprecedented precision. Emerging third-generation sequencing such as those based on Nanopore and PacBio technologies also hold great promise in panoramically revealing the sequence and modification profiles of sperm RNAs (Shi *et al.* 2022). We may need to stay open-minded with the emerging new knowledge ahead and be ready to constantly introspect and to forge new ideas.

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## Data availability.

Data sharing is not applicable as no new data were generated or analysed during this study.

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