Published in final edited form as:

Curr Opin Behav Sci. 2017 April ; 14: 140–147. doi:10.1016/j.cobeha.2017.02.007.

Transgenerational paternal transmission of acquired traits: Stress-induced modification of the sperm regulatory transcriptome and offspring phenotypes

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Abstract

In recent years, it has become evident that pre-conceptual exposure of males to various environmental factors induces epigenetic changes in sperm, which can mediate the transmission of acquired traits in their offspring. The most thoroughly examined paternal exposures involve stress and elevated corticosterone, which have been shown to modulate offspring phenotypes in a manner that is relevant to predisposition to brain disorders, and psychiatric illness in particular. Recent seminal studies have demonstrated that key epigenetic information transmitted via the paternal germline involves small non-coding (snc) RNA transcripts such as microRNAs. Following fertilisation, these sncRNAs appear to regulate development so as to modify the phenotype of the offspring. Understanding the mechanisms involved in such transgenerational effects may facilitate future screening of human sperm for 'epigenetic health' and the tailoring of therapeutic interventions according to genetic and epigenetic contributions to illness.

Introduction

Environmental influences during critical periods of development contribute to an individual's vulnerability or resilience to stress-induced psychopathological conditions [1–

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Conflict of interest

The authors have no conflicts of interest to declare.

4]. For example, maternal stress experienced during the first trimester of pregnancy is associated with an increased risk of schizophrenia for the offspring in adulthood [5]. There is also a strong link between postnatal depression in care-giving mothers and the onset of anxiety-related disorders in their adolescent offspring [6]. On the other hand, exercise, positive mother–infant interactions, and enriched lifestyles are associated with later-life resilience to stress-linked psychopathologies [7]. In recent years, the transgenerational effects of the paternal environment have received much scrutiny. One major challenge is to identify the biological mechanisms underlying the transgenerational influence of parental environmental factors, including their specificity with respect to particular sorts of environmental signals.

It is firstly essential to clarify the mechanistic differences between inter- and transgenerational inheritance. Intergenerational inheritance implies that the parent (F0) and offspring (F1) are subject to direct exposure to the environmental factor (e.g. stress during pregnancy impacting on *in utero* development of the foetus). In contrast, transgenerational inheritance occurs when the environmental exposure is prior to conception of the F1 generation with phenotypic changes also manifesting in the F2 generation [see 8 for further reading]. Epigenetics refers to the molecular mechanisms that regulate heritable changes in gene expression which do not depend on variations in the underlying genetic code [see 9 for recent review on key epigenetic mechanisms]. These include chromatin remodelling through DNA methylation, histone protein modifications and changes to the expression levels of non-coding RNAs. Traditionally, this epigenetic variability was thought to be primarily associated with establishing and maintaining cellular diversity and/or cell fate decisions across the lifespan. Growing evidence indicates that perturbation of transgenerational epigenetic inheritance processes results in negative health trajectories of subsequent generations. For example, abnormal genomic imprinting (the inheritance of DNA methylation patterns on specific genes) results in a range of syndromes [10]. A range of parental non-genetic/environmental factors (e.g. smoking, obesity) are now known to impart subtle alterations to the offspring epigenome, in a manner such that the offspring are at greater risk for cardiovascular diseases, obesity and Type II diabetes in offspring [11–14]. Importantly, this conferred risk is transmitted to the offspring without any direct exposure of the offspring to the initial environmental influences which impacted on the parents. Interestingly, these same epigenetic processes also play a role in the brain, and one major neural function is in mediating vulnerability to development of stress-related disorders [15,16]. For example, early-life stress has been linked to sustained changes in the expression patterns of key regulators of the physiological stress response, which have been shown to be mediated by epigenetic mechanisms, including DNA methylation [4,17–19].

In recent years, the notion that the paternal environment exerts a broad range of transgenerational influences on behavioral phenotypes has been more thoroughly investigated. This is now supported by a growing body of preclinical evidence that paternal factors such as stress, diet and exposure to drugs of abuse have the capacity to modify offspring health outcomes [reviewed in 20]. Further understanding of this pattern of non-Mendelian inheritance has been enabled by the additional discovery of the dynamic nature of the epigenetic content of male germ cells. There is now compelling evidence that male germ cells are responsive to certain environmental signals and effectively encode exposure

to these cues in the form of epigenetic alterations such as DNA methylation, histone modifications, and non-coding RNA expression [21–24]. These impact the offspring by steering them on a developmental trajectory towards either vulnerability or resilience for certain health conditions across their lifetime.

However, there is still controversy over how this process occurs and there are several reasons why the relevant mechanisms have not been fully elucidated. Firstly, there is limited evidence and poor understanding of the contribution of the paternally-derived epigenome to embryology, which is required in order to explain the transgenerational inheritance of acquired traits. Secondly, these early years of preclinical research have revealed inconsistent behavioral and molecular outcomes of the paternal environmental modifiers. Thirdly, the technology required to probe these mechanisms at the molecular level, including low-input transcriptome-wide sequencing and methods for the direct quantification of rare RNA species, has only recently become widely available. Here we highlight the recent discoveries in this domain, focussing specifically on the transgenerational effects of paternal stress.

Paternal stress influences male offspring anxiety and depression-related behaviors by modification of sperm microRNAs

Dietz and colleagues demonstrated a robust transgenerational impact of paternal stress using a chronic social defeat mouse model [25]. The male offspring of defeated sires, in the absence of any behavioral or pharmacological triggers, manifested core symptoms of major depressive disorder – social withdrawal, anhedonia and increased anxiety. However, animals generated through IVF were limited to a subtle pro-depressive response in the forced-swim test leading the authors to speculate that a transgenerational effect of paternal stress is not borne out through sperm or that the extent of the epigenetic influence is somewhat limited. They addressed several caveats of the IVF process including epigenetic erasure and reprogramming. However, in light of more recent evidence that IVF alters DNA methylation [26,27] and the expression of microRNAs in the fetal brain that regulate neuronal migration, differentiation and development [28], one should be cautious in interpreting IVF experiments with respect to epigenetic mechanisms mediating transgenerationally transmitted effects of paternal stress [see 22 for further discussion of this issue].

The role of sncRNAs, specifically microRNAs, in transgenerational transmission has emerged from the enlightening work of the Mansuy and Bale laboratories [29–31]. Their studies revealed the crucial role that sperm-borne microRNAs play in influencing offspring behavior and stress response. Gapp and colleagues used a modified protocol combining maternal separation with unpredictable maternal stress (MSUS) which yielded male offspring manifesting reduced anxiety levels but greater depressive behavior [29]. The involvement of small non-coding RNAs was firmly established when they microinjected whole populations of small RNAs isolated from MSUS-exposed sperm into fertilised oocytes and recapitulated the spectrum of anxiety and depression-related behaviors in offspring. A limited cluster of 43 microRNAs up-regulated in the sperm of MSUS-effected mice was identified, amongst which miR-375 was subsequently confirmed to be up-

regulated in the hippocampus following *in vivo* injection of the rodent stress hormone, corticosterone, implying modification of the physiological stress response.

Rodgers and colleagues further elucidated the role of microRNAs in transgenerational transmission by identifying nine microRNAs that were consistently up-regulated in sperm of two separate groups of mice exposed to chronic variable stress either during adolescence or adulthood [30]. While the paternal interventions had no impact on offspring behavior, offspring had significantly reduced serum corticosterone levels following restraint stress suggesting a dysregulation of the hypothalamic-pituitary-adrenal axis and, more broadly, altered vulnerability or resilience to stress-induced psychopathology. The over-expression of these nine microRNAs was confirmed to alter the physiological stress response of offspring through zygote microinjection (post-fertilization) resulting in reprogramming of the hypothalamic transcriptome [31]. Interestingly, four microRNAs - miR-30c, miR-204, miR-375, miR532 – had also been identified by Gapp et al. (2014) and it would be interesting to determine if MSUS is also a transgenerational modifier of offspring stress response.

Our group took a reductive approach in an attempt to dissociate the behavioral and physiological aspects of stress impacting the father. We demonstrated that dysregulation of paternal HPA axis activity modifies male offspring anxiety, simply by supplementing sires with a low dose of corticosterone over the course of one spermatogenic cycle [32]. The elected dose and dosing period was insufficient to alter anxiety and depression-related measures of the sires themselves, and therefore the discovery that corticosterone-treatment resulted in an up-regulation of >100 microRNAs (a number far exceeding that reported by Gapp et al. and Rogers et al. using paternal stress paradigms) was impressive [32]. There could be several reasons for this discrepancy including the discovery platforms employed (see Table 1) or differences in the type and the timing of stressor. Alternatively, daily corticosterone-supplementation could have prevented the homeostatic adaptation of the HPA axis to the daily surges in serum corticosterone levels resulting in an exaggeration of the down-stream impact on microRNA levels. Regardless of the interpretation, these collective findings provide the opportunity for cross-data comparisons which could inform us of microRNAs with distinct roles in regulating offspring behavior and the physiological stress response. However, while there are between-study microRNA matches (see Table 1), no single microRNA was found to be consistently altered across all studies.

There are a range of possible variables, discussed above, that could explain the different findings regarding the effects of physical stressors and corticosterone administration on sperm microRNA profiles (Table 1). Importantly, it should be noted that the different stress paradigms used also induced variable behavioral effects on the offspring [29–32]. In addition to the obvious differences in paternal stress paradigms, versus paternal corticosterone administration, subtle genetic differences in substrains of mice used, housing conditions (of both the experimental animals and their ancestors) and behavioral assays, among other factors, could contribute to the exact nature of paternally mediated behavioural modulation of offspring phenotypes.

While there remains much to be discovered about the biological role of microRNAs, evidence supports an expansion of the previously assumed function of sperm microRNAs in fertilisation and preimplantation embryonic development [33]. MicroRNAs must now be considered as long-term regulators of offspring behavior and stress response. These findings are in addition to evidence of the involvement of microRNAs in paternal diet-linked modification of offspring metabolism and obesity [34–36]. Of course, we cannot exclude the involvement of other subspecies of sncRNAs. For example, a recent report highlights transfer RNAs (tRNAs) and 5' fragments of tRNAs in sperm that may also exert a transgenerational influence, although currently limited to offspring metabolism [37,38]. Interestingly, delving into the field of reproduction biology may provide many clues to guide future research.

Location and mechanism for stress-induced modification of sperm microRNA: where and what?

It remains unknown how exposures to behavioral or physiological stress result in changes to sperm microRNAs and other subclasses of sncRNAs. The challenge lies in first identifying the specific cell-types which respond to stress and have the additional capacity to directly impact on the sncRNA content of sperm. Secondly, these cells must have the inherent means to directly signal to developing and/or mature sperm. There is strong evidence that epithelial cells of the epididymis secrete exosomes containing microRNAs, and these exosomes are capable of delivering their cargo to the developing sperm.

Exosomes are endosomal microvesicles ranging 30–100nm in diameter which contain a variety of proteins involved in membrane transport and fusion, as well as microRNAs [39]. They are secreted into the intraluminal space where they can be involved in direct cell-to-cell signalling by fusion with the acceptor cell, or facilitate organ-to-organ signalling via body fluids such as blood, saliva and milk [40]. It was first established in plants that exosome-mediated transfer of microRNAs could function as a key regulatory process for cell differentiation and growth [41,42]. In mammals, exosomal delivery of microRNAs is implicated in T-cell maturation and the priming of an immune response [43,44]. Lung epithelial cells respond to environmental challenges such as hyperoxia-induced oxidative stress by releasing microRNA-rich exosomes to initiate a macrophage-regulated inflammatory response [45]. As an example of organ-to-organ signalling, exosomal microRNAs secreted by endometrial epithelial cells have been proposed to comprise a major signalling process for endometrium-embryo crosstalk [46]. Under certain conditions, serum exosomes may also bear distinct microRNA profiles, and there is on-going research to exploit this as a means of early detection of glioblastoma [47] and pancreatic cancer [48,49].

Recent profiling studies of spermatozoa in mice have revealed that the microRNA profile of sperm is concordant with the microRNA profile of cauda epididymal epithelial cells [50]. That led to a subsequent characterisation of >350 microRNAs that epithelial cell-derived exosomes, termed 'epididymosomes', deliver to sperm [51]. Importantly, these epithelial cells express the glucocorticoid receptor (GR, nr3c1) - the key nuclear receptor regulating the physiological stress response. While GR is highly expressed throughout the brain and is

implicated in many psychiatric conditions, it is also highly expressed in the male reproductive tract including the cauda epididymis [52]. In rats, manipulating corticosterone levels either by adrenalectomy or dexamethasone (a GR antagonist) significantly altered epididymal epithelial cell GR mRNA and protein levels [52]. Thus, chronic variable stress, MSUS and corticosterone-supplementation (each established to down-regulate GR expression in the brain) could exert their common transgenerational influence by modifying glucocorticoid receptor signalling of epididymal epithelial cells. Based on the collective evidence, we propose that the transgenerational mechanism of paternal stress involves a simple but tri-layered stress-to-cell-to-sperm signalling pathway (see Figure 1 for proposed mechanism and explanation). Additional studies need to be conducted to determine, 1) if stress alters epididymal GR expression, 2) if the severity of stress correlates with the extent of that alteration, and 3) if the various stress paradigms differentially impact GR signalling across the epididymis.

Another key issue that needs to be addressed is the intracellular impact of stress on microRNA biogenesis. A clinical study of post-traumatic stress disorder with co-morbid depression discovered that blood DICER1 expression was reduced, correlating with increased amygdala activation by a fearful stimulus [53]. DICER1 is an RNaseIII endonuclease involved in the canonical microRNA biogenesis pathway [54]. It is essential for spermatogenesis and male fertility [55]. Deletion of DICER1 is embryonic lethal and tissue-specific studies have confirmed its vital role in the development of the adrenal cortex [56]. Interestingly, a previous study of the molecular pathways associated with behavioral resilience of mice to chronic social defeat stress uncovered evidence that DICER1 serves an essential modulatory role down-stream to β -catenin/Wnt signalling [57], i.e. knocking down DICER1 abolished behavioral resilience to stress. Chronic stress up-regulates cellular oxidative stress, which is known to inhibit microRNA biogenesis by modulating DICER1 activity [58] and altered intracellular reactive oxygen species production has been proposed to be a cause of idiopathic male infertility [59]. Future studies are required to clarify the mechanisms involved.

Using sperm epigenetics to inform preventative and therapeutic approaches

Whilst most of the experimental data on paternal transgenerational epigenetics has been generated in laboratory mice and rats, various studies suggest that parallel processes occur in humans [1–4,60]. Therefore, future studies will likely endeavour to characterize the human sperm epigenome and its modulation by environmental factors. Longitudinal cohort studies are necessary to firmly establish that paternal transgenerational transmission of acquired traits, and predisposition to illness, exists in humans. However, it will admittedly be far more challenging to uncover definitive evidence of human transgenerational effects due, in part, to the significant degree of genetic heterogeneity of the human population. The direct interaction between parents and offspring is a major hindrance for clear causal interpretations; although adoption studies might be a possibility to account for this. There will also undoubtedly be non-genetic co-variables to consider, such as race, socio-economic status and education. Despite the current lack of any definitive mechanistic evidence that

sperm epigenetic modifications mediate transgenerational processes in humans, it is nonetheless intriguing to speculate on the possibilities. One implication is that such data could be used to screen sperm samples for epigenetic health to determine a health risk probability of children conceived at that time, akin to current genetic testing for disease-related gene mutations. Such information could eventually be used to tailor personalised medicine approaches, based on relative genetic and epigenetic contributions to disease predisposition. For example, we know that diet directly impacts on sperm quality and influences the reproductive health of offspring [61]. This kind of ‘sperm epi-genotype’ data could not only inform public health messages targeted at males planning parenthood, but also help identify the potential causes (and thus therapeutic options) of medical conditions which occur in later generations.

Conclusion

The past few years have witnessed a revolution in our understanding of the impact of the environment on the sperm epigenome and the subsequent impacts on paternal inheritance of acquired traits. It is a sobering scientific lesson that Jean-Baptiste Lamarck’s evolutionary ideas should not have been dismissed out of hand, and that the hypotheses he generated should have been experimentally tested as soon as the appropriate tools became available. In fact, Charles Darwin was influenced by some of Lamarck’s ideas and presciently proposed the existence of ‘gemmules’ which he theorised were minute particles which could transfer information between somatic and germ cells via ‘pangenesis’ [for recent discussion see 62].

Whilst the present article has focused on paternal stress, other studies have explored paternal diet [63,64], and additional paternal exposures such as environmental enrichment [65] have recently been shown to have transgenerational effects. Furthermore, a positive paternal intervention (environmental enrichment) can prevent the transgenerational transmission of the negative effects of paternal stress [66]. There is still much to be done with respect to defining aspects of the paternal ‘envirome’ which induce transgenerational modulatory effects via epigenetic modifications of sperm. The findings described above raise many fascinating questions regarding the mechanisms whereby such transgenerational information can be transformed from paternal environmental exposures into the sperm epigenome, including regulatory RNAs as well as DNA methylation and other possible epigenetic changes. Major additional challenges are posed regarding our understanding of how such paternally derived epigenetic information can act post-conceptually to specifically modify embryogenesis, brain development and adult function. Furthermore, as the evidence that such transgenerational epigenetic inheritance of acquired traits occurs in humans, it raises major public health issues, including the spectre of future epidemics which could be seeded through paternal (and maternal) shifts in population-wide environmental exposures, or ‘enviromes’ [67]. Therefore, this should be a priority area in public health and medical research, as we try to establish how parental enviromes modulate germ cell epigenetics to change offspring phenotypes, with respect to biological traits and associated predispositions of offspring to a wide range of disorders. Finally, these recent seminal discoveries of epigenetic inheritance have major implications outside of human biology and disease. Understanding the transgenerational inheritance of acquired traits in other animal species

will have significant impacts on fields as diverse as agriculture, ecology, conservation biology and behavioral science.

Acknowledgments

The authors' research described in this article was supported by an NHMRC Project Grant to AJH, TWB and TYP. AJH is an NHMRC Principal Research Fellow. We thank Katie Fennell, Shlomo Yeshurun and other members of the Hannan Laboratory for discussions and experimental work supporting the ideas presented in this article.

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HIGHLIGHT

- Recent evidence supports the paternal transgenerational transmission of acquired traits
- Paternal stress had impacts on offspring behaviours and neuroendocrine function
- Evidence supports specific epigenetic mechanisms of transgenerational transmission
- Small non-coding RNAs, including microRNAs, in sperm appear to be key epigenetic mediators

Figure 1. Stress-associated transgenerational effects involve a change in sperm microRNA profile
 The hypothalamic-pituitary-adrenal axis exists as a negative feedback loop involving glucocorticoids (cortisol in humans, corticosterone in rodents) to regulate stress response. The glucocorticoid receptor, GR, is also highly expressed in the testes and epididymis. Epididymal epithelial cells exocytose small-RNA containing exosomes ‘epididymosomes’ into the epididymal lumen which fuse with maturing sperm. Chronic stress alters levels of circulating stress hormones resulting in abnormal GR-signalling and we propose that this causes altered microRNA biogenesis in epithelial cells. As a result, sperm can acquire a different stress-specific microRNA profile. However, it is unknown if the epithelial cells mount a uniform response, or if there is variability which results in sub-populations of sperm with different exosome cargo (as illustrated).

MicroRNAs potentially mediating the transgenerational transmission of paternal stress identified by independent studies.

Table 1

	microRNA discovery platform	miRs reported	let7f	let7g	let7i	miR21a	miR26b	miR30a	miR30b	miR30c	miR32	miR103	miR194	miR204	miR375	miR449a	miR532
Roger s <i>et al</i> 2013	Applied Biosystems Taqman Array microRNA A+B cards	9	n/a	n/a	n/a	n/a	n/a	+	n/a	+	+	n/a	n/a	+	+	n/a	+
Gapp <i>et al</i> 2014	Illumina HiSeq2000	43	+	+	+	+	+	n/a	+	+	n/a	+	+	+	+	+	+
Short <i>et al</i> 2016	Illumina HiSeq200 0	101	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-
Expression pattern of selected microRNAs across epididymal segments																	
Nixon <i>et al</i> 2015	Illumina HiSeq2000	Directionality of expression as indicated	Corpus > cauda Caput < cauda	◇	Caput > corpus Corpus > cauda	#	◇	Caput > corpus Corpus < cauda	Corpus > cauda Caput > cauda	Caput > corpus Corpus < cauda	ND	Caput > corpus Corpus < cauda	Caput > corpus Corpus < cauda	Caput > corpus Corpus > cauda	Caput > corpus Corpus < cauda	Corpus < cauda	Caput > corpus Corpus < cauda

Cross referencing of data sets reveals common sperm microRNAs identified by independent studies that are altered by behavioral (Rogers *et al.*, 2013; Gapp *et al.*, 2014) and pharmacological (Short *et al.*, 2016) paradigms of paternal stress. +denotes significantly up-regulated, - denotes unchanged, n/a denotes not reported. Expression patterns of the 15 microRNAs were obtained from Nixon *et al.*, 2015 and the relative expression levels across the epididymis is indicated. # No annotation of *miR21a* by Nixon *et al.* ◇> Expression of *miR26b* and *miR352-3p* not significantly altered across the epididymis. ND denotes microRNA not annotated by Nixon *et al.*