Sperm miRNAs as Potential Mediators of the Transgenerational Effects of Stress

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David Alec Dickson

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Advisor: Larry Feig, PhD

Abstract

Recent studies in rodents suggest that epigenetic inheritance of the effect of paternal life experiences is a factor to consider in understanding susceptibility to psychological disorders. Many epigenetic mechanisms involved in this process have been proposed, and the most convincing evidence implicates small non-coding RNA molecules, such as microRNAs, in this process. According to this model, microRNAs are delivered by sperm to zygotes upon fertilization, where they are required for normal mouse embryonic development, and stress-induced increases in sperm miRNAs have been shown to promote altered behaviors in their future offspring, sometimes in a sex-dependent manner. Our studies use a mouse model of Chronic Social Instability (CSI) stress, where mating stressed male mice to control females results in anxiety and defective social interactions specifically in female offspring across at least three generations.

My thesis has implicated specific sperm miRNAs, miR-409-3p, miR-34c, and miR-449a, in the transmission of the effects of stress across generations. miR-409-3p was first implicated because its levels are significantly increased not only in the sperm of CSI stressed F0 males, but also their F1 and F2 male offspring that transmit stress phenotypes to their female offspring. Moreover, manipulations of miR-409-3p levels in early embryos implicate it in establishing baseline anxiety levels in adult females, but not males. In contrast, both miR-34c and miR-449a levels are significantly decreased in the sperm of stressed males and their sons. Strikingly they are also reduced in the sperm of men exposed to high levels of adverse childhood experiences (ACEs) that correlate with elevated risk for future psychiatric disorders. In our mouse model, the decreased

expression of miR-34c and miR-449a is maintained into early embryos from stressed fathers, where knockouts of both miRNAs, but not individually, are known to alter brain development and spermatogenesis in mice. RNA sequencing of early mouse embryos derived from stressed fathers revealed possible mediators of these phenotypes, some of which may be attributed to these implicated miRNAs.

This work is the first to demonstrate altered sperm miRNA profiles in men exposed to early life stress, and has begun to elucidate the mechanisms underlying the inheritance of stress, helping to further understand mental health risk. Considering that women are at an increased risk for many psychiatric disorders, these studies may have begun uncovering a novel sex-specific mechanism leading to the development of mental health disorders in adults.

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List of Abbreviations

ACE – Adverse childhood experiences
Arc – Arucate nucleus of the hypothalamus
BDNF – Brain-derived neurotrophic factor
BNST – Bed nucleus of the stria terminalis
CSI – Chronic Social Instability
CORT - Corticosterone
CVS – Chronic Variable Stress
DES – Diethylstilbestrol
EPM – Elevated plus maze
F0 – The exposed generation
F1 – Direct offspring of F0 exposed subjects
F2 – Direct offspring of F1 subjects
FDR – False discovery rate
LTP - Long-term potentiation
LTD - Long-term depression
miR-FISH – miRNA specific fluorescence *in situ* hybridization
miRNA – MicroRNA
MPO – Medial preoptic area of the hypothalamus
MSUS – Maternal Separation and Unpredictable Stress
MCS - Multiple cloning site
OFT – Open field test
OHS – Offspring of Holocaust survivors
piRNA – Piwi-interacting RNA
PTSD – Post Traumatic Stress Disorder
PVN – Paraventricular nucleus of the hypothalamus
SIJ – Social interaction with a juvenile assay
siRNA – Small interfering RNA
sncRNA – Small non-coding RNA
tRF – Transfer-RNA fragments
WT – Wild type

Chapter 1: Introduction

1.1 A Historical Perspective of Epigenetic Inheritance, with Modern Definitions

Mental illness affects nearly one quarter the world's population, according to estimates from the World Health Organization, yet it still remains underdiagnosed and undertreated. Interestingly, women show not only an increased risk for many disorders, most notably anxiety, depression, and Post Traumatic Stress Disorder (PTSD), but also increased comorbidities and disease burden through mechanisms that remain poorly understood[1-3]. Further complicating this issue is the lack of a consistent framework upon which to model psychiatric disease risk. The so-called "diathesis-stress" model states that an individual's risk for developing a psychiatric disorder is dependent on two main criteria, their 'diatheses', or inherent genetic predisposition for mental illness, and their 'stress' that they accumulate over life from external hardships. However, a growing body of evidence suggests that epigenetic transmission of the lifelong effects of stress on both the mother and father may be an additional factor to consider when assessing mental health risk in individuals.

The concept of the inheritance of acquired traits, one of the early iterations of what we now refer to as epigenetics, was first popularized by Jean-Baptiste Pierre Antoine de Monet chevalier de Lamarck in his 1809 treatise *Philosophie Zoologique*. Published 50 years prior to the emergence of Darwin's *On the Origin of Species* (1859), Lamarck's evolutionary theory was based around two fundamental forces, The Complexifying Force and The Adaptive Force, and two laws that govern the latter[4]. Within his evolutionary framework, all life naturally trends towards increasing complexity, starting as simple organisms and inevitably growing complex

(Complexifying force), and that these organisms will respond to and adapt to their various environments (Adaptive Force) based upon two laws. The first of these laws is his theory of "Use and Disuse", the concept that an individual animal's organs will grow and strengthen in proportion to how often and to the extent that they are used by the animal, and conversely that organs which go unused will continually weaken and eventually disappear entirely[4]. As such, an animal, over the course of its life, will better adapt to their environment based upon what they used to thrive in said environment. It is the aforementioned second of these two laws, the theory of the "Inheritance of Acquired Characters", that pertains to how the biological advantage gained by this animal can be passed on to the next generation in order to further confer an evolutionary benefit to its offspring, stated below.

> "All that nature has caused individuals to gain or lose by the influence of the circumstances to which their race has been exposed for a long time, and, consequently, by the influence of a predominant use or constant disuse of an organ or part, is conserved through generation in the new individuals descending from them, provided that these acquired changes are common to the two sexes or to those which have produced these new individuals[4]"

While the theory of the "inheritance of acquired characteristics" continues to be stubbornly attached to Lamarck's name, so far as dubbing it "Lamarckian evolution", it remains no more than a single aspect of what was truly the first instance of a cohesive, unifying theory of evolution. Also, specific details of this law are often overlooked when referred to today; mainly, the idea that changes could only be passed on if both the mother *and* the father had acquired this trait during their life. Furthermore, Lamarck never claimed to be the originator of this theory, only that he was the first to recognize its importance in the larger framework of evolution[5]. Given the eventual widespread acceptance of Darwinian evolution, it is not surprising that only this aspect of Lamarck's theories persists to this day. However, Lamarck should still be remembered, and celebrated, for providing the world with the first instance of a truly cohesive, unifying, theory of evolution, especially considering it was first introduced half a century before Darwin's work came to light.

It is important to properly distinguish the modern terms used to explain this type of inheritance popularized by Lamarck. While, as mentioned, Lamarck's name is commonly conflated with the term epigenetics, the distinction of popularizing that term is granted to Conrad Hal Waddington, during his work on what he termed "epigenesis" during the 1940s[6]. Although based upon his initial framework, epigenetics as we know it today typically refers to a mechanism underlying changes that occur in response to the surrounding environment that are transmitted from cell to cell after division, or from a whole organism to their offspring, that does not depend on alterations to the DNA sequence. As such, genetically identical cells or individuals can display different phenotypes based upon these epigenetic modifications of the genetic material.

These epigenetic mechanisms are primarily mediated by chromatin remodeling, DNA methylation, and alterations in small RNA profiles[7]. For epigenetics involved in transmission across generations, the subject of my thesis, these changes are studied mainly in the germline (i.e., sperm, oocytes) and during fertilization and early embryogenesis, as these cell types and developmental timepoints show the highest degree of regulation and turnover of these three critical mechanisms[7].

The extent to which these epigenetic marks are perpetuated through future generations further delineates the mechanism of inheritance. Epigenetic changes that are passed on to and affect the first generation of offspring, but do not alter that generation's germ line cells and thus do not affect the second generation, are termed intergenerational[7]. In contrast, changes that continue to alter the subsequent generation's germ line, and thus continually propagate said changes through multiple generations, are termed transgenerational[7].

This important distinction exists in order to identify transmissible traits that are truly epigenetic and are not a result to other confounding variables. One of the most pertinent variables to consider is the maternal effect of an altered *in utero* environment on the development of future offspring. Any change affecting a female either during their life prior to (i.e. stress, anxiety, altered metabolism) or, more significantly, during a pregnancy (direct toxin exposure) could conceivably alter the *in utero* environment during gestation, exposing the developing embryos (and the embryos' own germ cells) to whatever systemic alterations the mother may already be suffering from. This can then affect the related behaviors and/or phenotypes of her first and second generation of offspring. As we will discuss below, this type of inheritance can help explain much evidence of intergenerational epigenetic inheritance in humans, to an even higher degree in the context of toxin exposures. In contrast, similar significant changes affecting the father are insulated from directly affecting their future offspring, considering that males provide only sperm to the developing embryos. If they are also removed from the pregnant female following mating, this presents a much more favorable system to analyze for underlying mechanisms[7].

1.2 Evidence for Epigenetic Inheritance in Humans

Much of Lamarck's work centered around his role as a renowned botanist and biologist, and as such his studies were limited to his observations of plants and small invertebrates. His comments on the possibility of the inheritance of acquired characteristics existing in humans were limited to thought experiments, as he believed their existence to be a self-evident truth[5]. However, many events of the late 20th and 21st century have led to indirect observations of the possibility of such a phenomenon existing in humans. A selection of this evidence is presented below.

First, clear evidence exists for methylation-dependent intergenerational epigenetic inheritance (only to the F1 generation) in the context of genetic imprinting. Imprinting is the expression of only either the maternal or paternal allele of a specific genetic locus in a diploid organism, due to programmed hypermethylation/chromatin modulation leading to silencing of the second allele. Therefore, an identical mutation to one of these otherwise genetically identical loci can produce two distinct phenotypes, dependent upon whether the maternal or paternal copy is lost[7]. For example, loss of a specific region on the maternal proximal long arm of chromosome 15 (15q11-q13) can cause a type of developmental delay characterized by seizures, speech/motor deficits, and an overtly happy demeanor, termed Angelman Syndrome[8]. Loss of this same region, instead on the paternal copy, causes a different type of developmental delay characterized by excessive eating and obsession with food, and decreased muscle tone, termed Prader-Willi syndrome[8]. In both cases, there is loss of the transcriptionally active allele that cannot be compensated for by the remaining imprinted allele that is effectively silenced. While this type of inheritance will only affect the following generation and does not involve modification of this locus via environmental effects or stressors per se, it does demonstrate the existence of a mechanism for variable phenotypes to occur in offspring dependent upon epigenetic changes to either the maternal or paternal genome. However, there is a significant amount of methylation reprogramming during mammalian development that prevents such a mechanism from occurring for most genes, except for a small subset of loci that routinely evade this process[7].

An example of where one's environment influences future generations has been revealed from epidemiological studies on the events surrounding World War II, when a region in the Netherlands was subjected to strict food embargos and subsequent famine from late 1943-1947[9]. Women (F1) who were exposed to these conditions while *in utero* (i.e. their mothers (F0) were pregnant during some part of this famine period) had children (F2) with increased neonatal adiposity and poorer health later in life[9], as well as higher rates of perinatal mortality when they later became parents[10]. These F1 women, but not men, display increased BMI and waist-circumference when 50 years and older[11]. Interestingly, F2 offspring of the F1 men had higher weights and BMIs, a pattern that did not exist for offspring of prenatally malnourished women[12]. In contrast, work on a separate famine cohort exposed during the Siege of Leningrad did not find an association between famine exposure *in utero* and a variety of metabolic outcomes in adult life[13]. A key distinction between these cohorts lies in the fact that the Netherlands was able to return to a normal diet shortly after the end of the embargos, whereas shortages persisted for a significantly longer period of time in the U.S.S.R.[14]. This suggests that the risk for developing these negative outcomes later in life may be increased by the return to a calorie-rich diet following the prolonged periods of famine.

Similar questions were addressed in the analysis of various cohorts in the early 1900s in the Överkalix parish in northern Sweden. The degree of access to food for young men in this region during their slow-growth period (SGP; period before the prepubertal peak growth spurt, ~9-12 years) altered the rates of cardiovascular-related disease and mortality in their grandchildren[15,16]. Additionally, the BMI of the father at the time of conception has been shown to be significantly correlated with the growth of male offspring *in utero*, as well as their cortisol levels in the newborn period[17].

The effects of stress and trauma have been studied in a similar manner, particularly with studies centered around the offspring of Holocaust survivors (OHS). OHS have a higher lifetime risk of PTSD, mood and anxiety disorders, and substance abuse when compared to the regular population[18]. OHS have also been shown to exhibit poorer age-related health, although this was likely mediated by the level of secondary traumatization during their upbringing[19]. Offspring whose survivor parents were diagnosed with PTSD also display low urinary cortisol levels[20] which was further associated with their own risk for PTSD, particularly when their mother had PTSD[18]. Similar findings exist from other studies of war veterans and refugees, demonstrating that the presence of either maternal or paternal psychiatric illness (such as PTSD) significantly correlated with risk for mental health issues later in life[21,22]. Interestingly, in the refugee population, offspring of men with a strong social network actually had a significantly decreased risk for mental health issues later in life, implying the inheritance of a certain level of resiliency[22].

Larger meta-analyses of community-sampled OHS populations, as opposed to the clinical-sampled cohorts referenced in the above studies, have found evidence of increased resilience in not only the offspring of Holocaust survivors, but their own children as well[23]. These dichotomous results have since been replicated in another distinct Israeli cohort, which found that middle-aged OHS reported a significantly higher sense of well-being, while also suffering from significantly more health problems[24]. It is suggested that this protective factor may be a result of the profound level of community and social support amongst the survivors following the atrocities that they lived through[23].

As mentioned previously, there is abundant evidence for the effects of direct toxin exposure to pregnant women. These occurrences offer a much clearer mechanism for transmission (direct effects on the developing F1 generation, and the F1 germ cells that will become the F2 generation). In addition to the well-known maternal exposures to teratogens that affect the F1 generation, such as ethanol (e.g. Fetal Alcohol Syndrome; which may also have transgenerational capacity[25]), one of the clearest examples of this phenomenon is the effect of exposure to the synthetic hormone diethylstilbestrol (DES) during pregnancy. This drug was administered to millions of pregnant women from 1938-1971 in an effort to reduce and/or prevent complications related to their pregnancy[26]. It was later phased out when it was found that firstly, it did not offer any benefit to these women[27], and secondly, that it predisposed female offspring of exposed women to developing a rare vaginal adenocarcinoma[28]. DES has since been linked to increased rates of ADHD in both male and female grandchildren (F2 offspring) of exposed mothers[26], increased risk of male genital tract birth defects in the F2 sons of both men[29] and women[30,31] who were exposed prenatally, and increased rates of menstrual irregularity[32] and ovarian cancer[33] (caveat: low *n*) in the daughters of women exposed *in utero*. In a similar vein, smoking during pregnancy has been shown to increase the risk of autism in the maternal grandchildren[34], and both sons and daughters of chemotherapy-exposed women have significantly fewer children of their own when compared with the general population or cousin-controls[35].

While focused studies on specific populations subjected to extraordinary circumstances, such as those described above, offer valuable insight, it raises the question of whether other more common types of stress and trauma, such as those occurring during adolescence, have similar effects on those individuals' offspring. It is now well established that early life adversity can profoundly influence the health of individuals as adults, a result of the Adverse Childhood Experience (ACE) Study, which is now supported by a preponderance of publications[36]. The original study asked patient participants at Kaiser Permanente's San Diego Health Appraisal Clinic a series of questions pertaining to their life before the age of 18 with the goal of identifying exposures that correlate with the individuals' risk for negative end-health outcomes as adults[37]. This work culminated in the creation of the now widely used ACE Study Questionnaire, where anonymous yes or no answers to just 10 questions involving participant's experiences at home until the age of 18 are quantified. Five are personal questions about physical abuse, verbal abuse, sexual abuse, physical neglect, and emotional neglect. Five relate to other family members: an alcoholic parent, a victim of domestic violence, incarceration, diagnosis with a mental illness, and the disappearance of a parent through divorce, death, or abandonment. A score ≥4 (reported by more than 10% of the population) puts one at serious risk for future mental and physical health problems, such as depression[38], increased suicidal ideation and attempts in adults[39], and increased sensitivity to PTSD in war veterans[40]. Strikingly, mothers with high ACE scores give birth to infants with lower birth weights and decreased gestational ages, the latter at least partially confounded by smoking habits[41]. Increased F0 parental ACE score has also been correlated to an increased risk of deficits in problem-solving, communication, and social/motor skills in their F1 children at the age of 2 years[42]. The mother's ACE score has further been associated with their children's level of developmental risk, although it is likely mediated at least in part by their perception of their own health[43].

As directed experiments of genetically similar human patients are not able to be performed, especially in such a longitudinal manner, much of this data is no more than correlational. As a result, the entire concept of human epigenetic inheritance has been subject to significant debate. Also, many of these results can be attributed to an altered *in utero* environment, particularly relating to the effects of famine, similar to that for direct toxin exposure. However, when viewed as a whole, these observations begin to suggest a strong possibility of epigenetic mechanisms being at play in humans.

1.3 Animal Models of Epigenetic Inheritance

There is now abundant evidence in mouse and other animal models that transgenerational and intergenerational epigenetic inheritance exist and, more importantly, can play a vital role in the development of future offspring. This is likely due in part to the reduced genetic variability of inbred animal strains, as well as the precise control of experimental variables within relatively simple home environments that mouse and animal research affords.

Much early work in the field was performed on simple model systems, including plants and *C. elegans*. Plants do not undergo the high level of methylation reprogramming during embryogenesis that mammalian species do, and thus the inheritance of altered methylation patterns are more likely to be continually transmitted[44]. For example, the degree of methylation at the *cycloidea* gene determines the flowering pattern in a subset of naturally occurring phenotypical mutant *Linaria* flowers that have been continually observed in nature for hundreds of years[45]. Plants also provided some of the earliest evidence for any kind of non-mendelian inheritance, such as the paramutation of the *red1* locus in the 1950s, where a seemingly heterozygous, “recessive” allele containing, plant would only ever produce offspring with the “dominant” coloring[46]. Many other plant genes have demonstrated similar expression patterns, and recent work has implicated RNA-mediated silencing in these phenomena[47]. RNA interference, mediated by small-interfering RNA (siRNA) molecules was first identified in *C. elegans* in the early 1990s[48]. This work eventually led to the discovery of miRNAs[49], and their role in gene regulation, including the ability to alter phenotypes across multiple generations of worms[50]. Following successful cloning of these miRNA genes, investigation into their roles in mammalian systems greatly expanded[51].

Early work on a more complex model system, *D. melanogaster*, led to mechanistic evidence for chromosomal imprinting via chromatin modulation by Heterochromatin Protein 1 (HP1) and its homologues in plant and murine models[52]. This protein was discovered by studying the mechanisms of positive-effect-variegation, where mosaic patterns of gene expression exist between individual cells in the same organism through heterochromatin/euchromatin positioning dynamics. This patterning of expression represented a heritable form of gene repression based upon the extent of spread of heterochromatin into more transcriptionally active, euchromatin-predominant regions.

Following chromatin regulation, additional work appeared regarding epigenetic methylation, pertaining to the agouti viable yellow locus in mice. This locus encodes the agouti gene, which when expressed gives mice a yellow coat color. It was discovered that yellow-colored mothers gave birth to pups that mostly shared their yellow-coat coloration, and that mainly brown-colored mothers gave birth to mainly brown pups (irrespective of the coat color of the father), even though all mice were derived from the same inbred strain and therefore genetically identical[53]. This variability was discovered to arise from the insertion of an endogenous retrotransposon with an alternative promoter site directly upstream of the agouti locus. They discovered that the methylation status of this promoter region in the retrotransposon determines the coat color, either when hypomethylated and active (yellow color) or hypermethylated and silenced (brown color)[53]. Of note, ectopic active transcription of the agouti locus also caused these yellow mice to be obese and much more prone to developing diabetes, likely through agouti paracrine signaling on other tissues[53]. Thus, the methylation status of the mother was at least partially maintained in her offspring, although the mechanism that allows this locus to avoid erasure and reprogramming during embryogenesis is unknown[7]. Follow-up studies later demonstrated that the mother's methylation status can be altered by supplying her with a methyl-rich diet, which readily shifts the coat color of her offspring towards the brown end of the spectrum[54]. This suggests that these epigenetic changes readily respond to, and are altered by, the external environment.

While transgenerational epigenetic inheritance can occur via exposures to both male and female animals, most progress has been made studying the male lineage. The paternal mechanism has been easier to investigate given that transmission occurs while males are present only briefly during mating, drawing specific attention to the environmentally-induced epigenetic changes in sperm. These studies have primarily focused on three different types of environmental effects or exposures: toxin exposures, alterations in the diet, and stress[7]. A large number of studies have demonstrated transgenerational effects of toxin exposure in mice[55], presumably operating under a similar mechanism to those previously discussed in humans. Most relevant, an early study identified that exposure of male mice *in utero* to the antiandrogenic drug vinclozin (used as a fungicide) reduced sperm count, sperm motility, and overall fertility in not only the exposed F0 male mice, but also through three future generations of the male lineage[56]. It has also been shown that nicotine-addicted male mice produce male offspring that, when exposed to nicotine, become metabolically tolerant to other xenobiotic toxins[57].

In the context of diet and metabolism, a variety of changes have been investigated. Paternal preconception fasting results in decreased serum glucose concentrations in both male and female offspring[58]. Offspring of male mice who were fed a low-protein diet exhibited increased expression of a variety of genes involved in lipid/cholesterol synthesis in the liver, and had decreased overall cholesterol levels[59]. A model of caloric undernutrition during late gestation produces low birth weight F1 mice that develop obesity and impaired glucose tolerance as adults[60]. These male F1 mice pass on the low birth weight and impaired glucose tolerance (via altered β-cell function) to their F2 offspring[61]. Male mice fed a chronic high-fat diet produce female offspring with an earlier onset of impaired insulin secretion and glucose intolerance and altered expression of hundreds of genes in their pancreatic islet cells[62]. In a similar paradigm, male mice on a 3-week high-fat diet produced male and female F1 offspring with impaired glucose tolerance and higher percentage of fat mass as adults. Interestingly, this transmission was completely blocked by providing running wheels to the F0 male mice while on the high-fat diet[63].

Most relevant to our work, stress has also been shown to be a potent epigenetic modulator across generations. Various forms of stress, including repeated restraint stress, repeated social defeat, maternal separation and unpredictable stress (MSUS), chronic variable stress (CVS), and chronic social instability (CSI) have all been shown to effect phenotypes in at least the next generation, and in some cases through three generations.

In the model of restraint stress, male C57/BL6 mice were subjected to daily restraint stress for 14 days and then mated to WT naïve females. Both male and female F1 offspring of stressed F0 fathers exhibited elevated blood glucose levels, in both the fed and fasted states, and increased glucose production following a pyruvate challenge, indicating enhanced gluconeogenesis[64]. This was due to enhanced PEPCK protein expression, the rate limiting step for gluconeogenesis, in the livers of the F1 offspring of F0 stressed fathers. Interestingly, these findings in the F1 generation were successfully replicated simply by injecting F0 males with dexamethasone (a synthetic glucocorticoid) daily, for 14 days, and then mating to a WT female[64]. A longer restraint protocol (60 days) has also been shown to significantly reduce anxiety-like behaviors and corticosterone (CORT) expression in both F1 and F2 offspring of F0 stressed male and females[65]. These effects were associated with increased levels of glucocorticoid receptor and BDNF in the brain.

In the model of social defeat stress, male C57/BL6 mice were exposed to an aggressive, larger, CD-1 male for 10 minutes/day, while being housed with the aggressor male separated only by a plexiglass divider that allowed for sensory contact[66]. This paradigm was repeated for 10 days, which induced many anxiety and depression-related behavioral phenotypes in the defeated males[66]. When these defeated males were mated to regular control female mice, both their male and female offspring displayed enhanced anxiety and depressive-like behaviors, however the male offspring also exhibited elevated basal CORT levels and have other stress-related behaviors that were not observed in female offspring[67].

The MSUS model entails removing a dam from her pups randomly for 3 hours every day, from postnatal day 1 through 14, coupled with stressing the mother while she is removed from the litter[68]. This protocol induces both depressive and anxiety-like behaviors in the male mice who were weanling pups during the described separations[68]. Mating these F0 MSUS-exposed males to control females showed that their F1 female offspring also showed increased depressive and anxiety-like behaviors[68]. The F1 males instead displayed elevated levels of social anxiety that were not present in the F0 fathers, and a resilience to acute stressors (social defeat) that was possibly in part mediated by impaired serotonergic signaling[69]. Both male and female F1 offspring also display attenuated metabolic responses in the prefrontal cortex, hippocampus, and striatum in response to acute stressors, further suggesting a degree of resiliency[70]. They also demonstrate significantly reduced long-term potentiation (LTP) and enhanced long-term depression (LTD) in the hippocampus[71].

Interestingly, mating the F1 males of F0 MSUS fathers to normal females produced F2 female offspring with only increased anxiety-like behaviors, while the F2 male offspring showed increased depressive-like behaviors[68]. Recent work from this group further demonstrated that elevated risk-taking behaviors and glucose dysregulation are transmitted through the male lineage through the 3rd generation of offspring[72]. Of note, mating F0 MSUS females to control males transmitted the opposite phenotype to both F1 male and female offspring, which displayed reduced levels of anxiety-like behavior, although to a lesser extent in the females[73].

The CVS model entails exposing male mice to 6 weeks of daily random stressors, including 36 hours of continuous light, 15 minute exposure to a predator scent, 15 min restraint stress, cage composition changes, novel objects in the cage overnight, wet bedding overnight, and a novel 100 dB white noise overnight[74]. Both male and female F1 offspring of F0 male mice subjected to this protocol display a significant reduction in corticosterone responsiveness to acute stress[74]. This pattern of transmission persisted regardless of whether the stress occurred during adolescence, or once the male mice had reached adulthood. This decreased HPA axis responsiveness was associated with gene set expression changes in the PVN and BNST of the affected offspring, including increased expression of a set of glucocorticoid-responsive genes in the PVN[74].

Our lab previously published the effects of CSI stress on future offspring. This paradigm was originally developed by others as a high-throughput mouse model for social stress in humans[75]. They showed that disrupting the social hierarchy of outbred

CD-1 mice during adolescence and adulthood through bi-weekly random cage composition changes for seven weeks induced long-lasting effects in these F0 mice, including increased anxiety and depressive-like behaviors, increased adrenal weight, and other markers of chronic stress exposure[75]. After first replicating the original findings in F0 animals, our lab showed that F0 male mice exposed to CSI stress, when mated with control females, produced F1 female offspring with elevated anxiety and defective social interactions as adults, while the F1 males remained behaviorally unaffected[76]. However, mating these asymptomatic "silent carrier" F1 males with control females revealed that the F2 female offspring again manifest elevated anxiety and defective social interactions, while the F2 males remain asymptomatic[76]. This pattern was further maintained into the F3 generation through the male lineage. This implies that these male mice retain the ability to pass on altered phenotypes to their offspring, while they show no such stress-related phenotypes, further implicating a germ-line, not a behavioral, origin of transmission. In contrast, mating a CSI stressed F0 female to a control male also produced F1 female offspring with elevated anxiety and social deficits, however these changes were only partially maintained into the F2 generation, and not at all passed on to the F3 generation, strongly implicating a maternal altered *in utero* environmental mechanism[76]. The results of increased anxiety-like behaviors in F1 females of CSI stressed parents has since been successfully replicated by a separate group, using a different, inbred, strain of mouse[65].

Not all environmental effects lead to negative, maladaptive behaviors in the offspring, however. For instance, in the MSUS model described above, later work showed that F0 male mice subjected to this early life stress show increased goal-directed behaviors and behavioral flexibility when they become adults[77]. Furthermore, some environments are constructed in order to benefit the exposed animals, such as the concept of enriched environments (EE), where mice are housed in a larger enclosure full of running wheels and novel objects (tunnels, toys). Exposure to an EE has been shown to confer a variety of beneficial effects to the mice exposed to it[78] and has recently been explored for possible benefits on the exposed animals' offspring.

Some of the earliest evidence for intergenerational inheritance in mice, arising in the late 1980s, was centered around work exploring the effects of the maternal pre-pregnancy environment on her offspring. Both male and female F1 offspring of F0 mothers exposed to EE before pregnancy displayed reduced anxiety-like behaviors when compared to offspring of mothers exposed to an impoverished environment prior to pregnancy[79]. It was hypothesized at the time that this could be explained by enhanced maternal care during the postnatal period. A precedence for this proposed mechanism exists, as a high level of maternal care in the postnatal period has been shown to not only influence the stress reactivity of the F1 offspring, but also lead to female offspring who in turn become high-care mothers[80].

Our lab has also studied the effects of EE on the next generation in the context of a genetic knockout mouse line with defective hippocampal LTP, among other learning deficits. Exposing these F0 knockout mice to 2 weeks of EE, starting at postnatal day 15, was sufficient to fully restore LTP back to wild-type (WT) levels, and this rescue persisted until ~ 4 months of age[81]. Interestingly, this did not occur in adult animals, despite the fact that EE has been shown to influence many aspects of the adult brain[82]. A similar enhancement of LTP was observed in WT F0 animals subjected to the same paradigm. Remarkably, F1 offspring of EE exposed knockout animals displayed normal LTP despite never having been exposed to EE themselves, although this enhancement was no longer statistically significant after 3 months of age and was not transmitted to the F2 generation[81]. F1 offspring of EE exposed WT animals also displayed a modest level of LTP enhancement above baseline. Furthermore, this transmission only occurred with EE exposed knockout mothers, not fathers, yet was not affected by cross-fostering experiments, where EE exposed mothers fostering knockout pups from non-exposed knockout mothers did not display any enhancement to LTP[81]. Thus, this transmission was likely independent of postnatal maternal care, in contrast to the earlier studies described above.

This work was eventually expanded upon by a different group more than a decade later, where they discovered that adult male mice, 10 weeks of age, exposed to a much longer EE paradigm (10 weeks) conferred enhanced LTP to their F1 progeny[83]. Similar to the results from our lab, this enhancement of LTP was not passed on to the F2 generation. EE has further been shown to prevent the intergenerational transmission of altered coping behaviors from F0 male mice exposed to MSUS to their F1 male offspring[84]. Of note, exposing mice to an enriched environment leads to a novelty-associated increase in circulating CORT levels[85]. This raises an interesting question as to how different downstream effects on the offspring are mediated in these distinct epigenetic paradigms pertaining to stress.

1.4 Mechanisms of Inheritance

As previously mentioned, much of the mechanistic evidence for paternal epigenetic inheritance centers around DNA methylation, chromatin modulation, and small non-coding RNA (sncRNA) profiles. Available evidence for each of the previously mentioned studies on epigenetic inheritance will be addressed for any mechanistic experiments undertaken.

Many earlier experiments focused on altered methylation as the most likely mechanism. In the study on the effects of vinclozin exposure, the reduced fertility and spermatic defects discovered in the male lineage were associated with altered DNA methylation in the sperm of the male mice[56]. Of note, subsequent work years later implicated both alterations in the sperm sncRNA profile and histone dynamics, together with the previously identified DNA methylation, as being correlated with perpetuating these phenotypes across generations[86]. Interestingly, they discovered a larger number of differential histone retention sites in the sperm of the transgenerational F3 generation when compared to the directly exposed F1 generation and germline-exposed F2 generation[86].

Offspring of mice fed a low-protein diet revealed a variety of modest changes in methylation in the liver centered around the promoter region for the gene *Ppara*, a transcription factor that is a master regulator of lipid metabolism[59]. In the model of maternal caloric undernutrition, F2 offspring showed decreased expression of the β-cell ATP-dependent K^+ channel subunit *Sur1*, however this change was not associated with altered methylation status of the promoter[61]. In contrast, female offspring in the paternal high-fat diet model showed significant hypomethylation of a pancreatic islet cell gene that also showed the highest significant increase in mRNA expression in those cells[62].

In the MSUS paradigm, altered methylation was also considered a probable mechanism. Early work identified that sperm from stressed males showed hypermethylation at the MeCP2 locus, a transcriptional regulator implicated in Rett syndrome and stress modulation, and this hypermethylation was maintained in the cortex of the F1 female offspring of these males[68]. Our lab also explored the possibility of differential methylation in sperm following CSI stress. However, an unpublished in-depth genome wide methylation analysis of sperm derived from F0 CSI stressed males compared to sperm from F0 unstressed males did not identify any significant differences. Of note, a recent genome-wide analysis of heritable methylation states at specific loci (such as the agouti viable peptide allele) found that this type of metastable epiallele that shows evidence for heritable methylation patterns is quite rare, and as such it calls into question the generalizability of methylation-based inheritance patterns[87]. Furthermore, much of the above methylation-based evidence remains no more than correlational.

In contrast, the most convincing mechanistic evidence to date lies in alterations to the sncRNA profile, specifically in the context of microRNAs (miRNAs) and tRNA fragments (tRFs). In the MSUS model, recent work uncovered that in addition to the altered sperm methylation patterns discussed before, the F0 MSUS male mice displayed increased expression of five miRNAs in their sperm when compared to control, unstressed F0 males[88]. Three of these miRNAs showed increased expression in the hippocampus and two of them were decreased in the serum of F1 offspring of these F0 MSUS males. However, these miRNAs were not altered in the sperm of the F1 males, even though this lab previously demonstrated that this paradigm transmits some varied phenotypes through three generations[88]. Most importantly, injection of purified total RNA from F0 MSUS male sperm into normal fertilized zygotes was able to recapitulate the behavioral defects to a similar degree of those observed in direct F1 offspring of F0

MSUS males[88]. Follow-up experiments were necessary to further clarify this result, as injection of pooled total RNA would include all other sncRNAs in addition to the five miRNAs specifically identified. They repeated the microinjection experiments, but this time injected either the small RNA fraction (<200 nt) or long RNA fraction (>200 nt). Mice injected with the small RNA fraction recapitulated the altered body weight and depressive-like behavior, whereas the long RNA fraction recapitulated the altered food intake, insulin tolerance, and decreased anxiety-like behavior[89]. Furthermore, some of the long RNAs altered in the sperm of the stressed males exhibited a similar expression pattern in early embryos derived from the mating of those stressed males, implying they may be exerting their effects shortly after fertilization upon the developing embryo, but some of them showed complete opposite expression as well.

Results from the CVS model have also implicated an altered sperm miRNA profile. Analysis of F0 males exposed to the CVS protocol identified nine miRNAs that were significantly upregulated in the sperm compared to control fathers[74]. Of note, there is overlap of one of the miRNAs, miR-375, with the elevated sperm miRNAs identified in the MSUS model. Injection of a pool of these nine miRNAs, but not a single miRNA, into fertilized zygotes was sufficient to recapitulate the original blunted HPA axis response to restraint stress seen in F1 offspring of F0 CVS stressed fathers[90]. They further demonstrated knockdown of maternal mRNA targets of these miRNAs in early zygotes 24-hours post-injection, but also showed that neither these nine miRNAs nor their top putative targets were altered in the PVN of the injected animals as adults[90]. Instead, RNA sequencing of the PVN of the adult injected animals identified gene expression changes primarily in extracellular matrix associated transcripts[90]. However, these gene expression changes in the injected animals share little, if any, overlap with the transcriptomic changes originally reported for the F1 offspring of F0 CVS stressed males, bringing into question the relevance of those findings[74]. Similarly, there was no attempt to investigate if the maternal mRNA changes in early zygotes seen after injection with the pool of miRNAs were also seen in embryos derived from the mating of stressed fathers. Regardless, this work similarly implies that this altered sperm miRNA profile is influencing early embryo development very shortly after fertilization.

For the model of repeated restraint stress that induced hyperglycemia in the F1 offspring, they discovered that the PEPCK mRNA levels in the livers of the F1 offspring were not affected, even though there was a robust increase in PEPCK protein, implying a post-transcriptional regulation likely through miRNAs. They identified miR-466b-3p, a murine-specific miRNA that is part of a large intronic miRNA cluster (including the miR-466 and miR-467 families), as a putative regulator of PEPCK, and found that its expression was significantly decreased in the livers of the F1 offspring, leading to de-repression of PEPCK protein translation[64]. This was a direct result of hypermethylation of the promoter of the gene inside which this miRNA cluster resides, *sfmbt2*. They further discovered that this locus was originally hypermethylated in the sperm of the F0 stressed males[64]. Furthermore, injection of a glucocorticoid antagonist into F0 males during the stress protocol prevented this hypermethylation from occurring, and also prevented the transmission of the hyperglycemic phenotype to the F1 offspring. In contrast to the above studies, this implies that the F1 phenotypes are due to miRNA changes in an organ in the adult animal, as opposed to being an early developmental defect that manifests later in adulthood.

Pertaining to diet and metabolism, tRFs have been implicated in the transmission of the effects of both low-protein and high-fat diet. As mentioned previously, the low-protein diet was associated with a variety of methylation changes in the F1 offspring, however more recent work on this paradigm identified increased expression of 5' fragments of glycine tRNAs in the sperm of the F0 males on a low protein-diet[91]. Further work demonstrated that the sncRNA payload of sperm changes dramatically throughout the epididymis, and is predominantly comprised of tRFs (with miRNAs and piRNAs second and third in abundance). Most strikingly, the investigators discovered that the sncRNA payload in mature sperm is modulated via direct uptake of exosomes secreted by epididymal cells ("epididymosomes") lining the lumen[91-93]. Single embryo sequencing of embryos derived from IVF using sperm from low-protein diet F0 males identified early embryonic transcriptomic changes attributable to the actions of the previously identified tRFs[91].

A paternal transmission model of high-fat diet was similarly found to alter spermatic tRF expression in the males who transmit altered metabolic phenotypes to their F1 offspring[94]. Injection of the 30-40 nt fraction (which contains the tRFs, but not miRNAs/piRNAs) of RNA purified from sperm of high-fat diet males into fertilized zygotes faithfully recapitulated the metabolic phenotypes seen in F1 offspring of the high-fat diet males[94]. Similar to the low-protein diet paradigm, sequencing of early embryos identified a variety of early transcriptomic changes that could be attributed to the actions of these altered tRFs. Interestingly, injection of a pool of synthesized tRFs (the ones that were increased in the F0 males) was not able to induce any of the metabolic phenotypes in injected animals, and was attributed to enhanced degradation of the synthesized transcripts when compared to the naturally occurring tRFs which possess several post-transcriptional RNA modifications that grant enhanced stability[94].

In the model of chronic social defeat, IVF experiments were carried out to assess for a possible epigenetic mechanism. Interestingly, offspring created with IVF using sperm from the defeated males only retained one of the transmissible phenotypes, increased depressive-like behavior in the forced swim test, suggesting other mechanisms may be necessary for full transmission of the remaining altered phenotypes[67]. The IVF procedure itself involves rather extreme manipulations to both the unfertilized oocyte and sperm, which may also be of an earlier developmental point than the sperm typically available during normal copulation. However, others have demonstrated that using sperm from affected males for artificial insemination, a less-invasive alternative to IVF, successfully transmitted a stress-induced phenotype (decreased postnatal bodyweight) to the next generation[95].

1.5 MicroRNAs: Biogenesis, Function, and Implication in Human Disease

MicroRNAs (miRNAs) are small (20-22nt) noncoding RNA molecules that act as master regulators of gene expression in virtually all cell types. They are transcribed in the nucleus by RNA Pol II as a ~90 nt long primary miRNA (pri-miRNA) hairpin with both a 3’ and 5’ trailing arm[96]. These single-stranded arms and the lower portion of the stem of the hairpin are cleaved off by the Microprocessor complex (containing DROSHA - RNA cleavage, and DGCR8 – RNA binding), resulting in a shorter ~65 nt hairpin, called a pre-miRNA[96]. There are a variety of RNA-binding proteins that have been shown to interact with both the Microprocessor complex and the terminal loop of the pri-miRNA to influence the processing of specific pri-miRNA families[96]. The pre-miRNA is then actively shuttled out of the nucleus in a GTP-dependent manner by exportin 5 (EXPO5).

Once in the cytoplasm, it is further cleaved by the Dicer complex near the terminal loop, resulting in a short double stranded RNA molecule, creating the -3p and -5p arms of the mature miRNA. Similar to the Microprocessor complex, different RNA binding proteins can influence the processing of different miRNA families by interacting with Dicer. The RNA duplex is then loaded onto an Argonaute protein (AGO) to form the immature RNA-induced silencing complex (RISC). Another class of small RNAs, piwi-interacting RNAs (piRNAs; 24-30 nt long) use a unique subclade of AGO proteins (PIWI), and function primarily in germ cells to silence transposable elements[96]. After the duplex is loaded, specific mismatches along the guide strand lead to passive unwinding and removal of the passenger strand, leaving behind the mature RISC. Guide strand selection is determined when the duplex is loaded onto an AGO protein, typically based upon which strand (-3p or -5p) has greater instability of the 5' terminus. The mature RISC then exerts its regulatory effect on specific target mRNAs in the cytoplasm, determined by a 7-nt long section near the 5' end called the "seed sequence" (nt 2-8) that is responsible for base-pairing to the 3' UTR of mRNAs subjected to regulation. Perfect base pairing to a target mRNA's 3' UTR results in AGO2-medated degradation of the transcript, whereas imperfect base pairing results in translational repression[97]. Individual miRNAs have a wide range of effect and can target many different mRNA sequences. There is also a significant amount of overlap between miRNA targets, allowing for functional redundancy for important targets and processes. As such, dysregulation of miRNA expression has been widely implicated in a variety of human disease, including cancer[98], as well as basic biologic processes, such as maintenance of pluripotency and cell fate regulation[99].

There is another type of sncRNA that has gained attention recently, derived from the processing of tRNA molecules, termed tRNA fragments (tRFs)[100]. There are several different types of tRFs, classified by which part of the mature tRNA they arise from, including tRNA halves (31-40 nt), derived from ribonuclease cleavage through the anticodon loop, and smaller tRFs (21-30 nt) derived from cleavage at other sites, such as the D-loop[100]. Certain types of these tRFs interact with AGO proteins (predominantly AGO1) to regulate specific mRNAs (via a "seed sequence") and translational dynamics in a similar manner to miRNAs[100]. They have since been shown to have roles in cell proliferation, viral responses, gene expression, and neurodegeneration[100].

SncRNAs, including miRNAs and tRFs, are vital to many other processes, most importantly embryogenesis and their modulation in germ cells. Soon after sperm were found to contain RNA, contrary to the initial belief that they carried nothing more than the haploid genetic information of the father, it was discovered that they contained a variety of small RNA species, including sncRNAs, piRNAs, and miRNAs, that are delivered to the oocyte upon fertilization[101]. Furthermore, there exists a certain subset of miRNAs that are only expressed in sperm[102], not oocytes, that are vital for early embryo development[103]. Many of these miRNAs, and tRFs, are delivered to the maturing sperm via epididymosomes[92], and the loss of the mRNA fraction specifically leads to poor implantation and eventual failure of early embryos[93].

Additional work on these sperm-borne miRNAs identified one in particular, miR-34c, that was found to be necessary for the first cleavage division in mouse embryos, possibly through regulation of *Bcl2*[102]. The investigators utilized a "sponge" inhibitor, whose specificity is targeted towards the target miRNA's seed sequence, to explore the effects of miR-34c inhibition on these early embryos. This suggested that sperm-borne miRNA were essential for normal early embryo development. However, work from a different group demonstrated that mice with a genetically knocked out miR-34b/c locus were not only viable, but also showed no defects in early embryogenesis[104]. They concluded that the sponge inhibitor used in the original group's experiments was likely producing off-target effects by inhibiting other miRNAs with a similar seed sequence. Interestingly, another miRNA cluster, miR-449a/b/c, is also expressed in sperm, not oocytes, and shares the same seed sequence as the miR-34b/c cluster. Single knockouts of either family caused no undue harm to the mice, however when these two strains were crossed, creating a double knockout mouse lacking both the miR-34 and miR-449 families, there were profound effects on spermatogenesis, brain development, and ciliogenesis in the developing mice[104,105]. This work showed that the miR-34 and miR-449 families were functionally redundant. In addition, it showed that these sperm-borne miRNAs, which are absent from the oocyte before fertilization, do serve a necessary role during development that becomes apparent only after the levels of both families are reduced. This concept becomes important for our analysis of sperm miRNAs we found changed after exposure to either mice or men to early life stress.

Of direct interest to our work is the fact that human sperm miRNAs are also affected by, and respond to, environmental changes. For instance, men who are smokers exhibit a vastly different sperm miRNA profile than age-matched non-smoking control men[106]. This was a small study, however it presented the first evidence of an environmental insult altering the sperm miRNA profile in adult men. A more recent study provided more convincing evidence in regards to obesity. Obese men exhibited a vastly different sperm sncRNA profile, as well as altered sperm DNA methylation patterns, from that of lean, age-matched counterparts[107]. Importantly, when these same obese men underwent gastric bypass surgery and proceeded to lose a significant amount of weight over the next year, their sperm profiles following the weight loss were much more similar to that of their original lean counterparts[107]. This was only performed on a small number of individuals, and was likely influenced by the participants diet before and after surgery. However, this again presented evidence that not only can sperm miRNA content be altered by the external environment, but also that it can presumably change, and reverse, back to a "normal" profile when the environment is corrected.

How these sperm miRNA respond to external stress in F0 subjects, in both mice and in men, as well as how these epigenetic changes may affect the next generation will be addressed in the following chapters.

Chapter 2: Involvement of Early Embryonic miR-409-3p in the Establishment of Anxiety Levels in Female Mice

Dickson, D. A., Mensah, V., Saavedra-Rodriguez, L., Stohn, P., Hernandez, A., Liaw, L., Feig, L. A. Involvement of Early Embryonic miR-409-3p in the Establishment of Anxiety Levels in Female Mice.
To be submitted.

2.1 Introduction

It has long been assumed that sperm's only contribution to the development of zygotes upon fertilization is paternal DNA. However, a growing body of evidences supports the idea that small RNA molecules including, miRNA, piRNAs, siRNAs and tRNA fragments, present in sperm are delivered to zygotes upon conception and also contribute to embryo development[108]. For example, conditional knockout (cKO) mouse that lack the ability to process siRNA and miRNAs in sperm produce zygotes with reduced developmental potential. miRNA content changes observed during the earliest stages of development suggest that they might be key regulatory players during the developmental transition from the fertilized zygote to pluripotent blastocyst[108].

One of the most studied examples of how sperm miRNA delivery to zygotes influences development is how the effects of stress on male mice are transmitted across generations. Male stressors that produce transgenerational effects include social defeat[67], chronic physical restraint[65], multiple variable stressors[74], early maternal separation[68], and chronic social instability[76,109] (CSI). Two sets of reports in mice clearly implicate stress-induced increases in the levels of specific sets of sperm miRNAs in transmitting traits across generations. In one study[88] early maternal separation of males from mothers leads to increased expression of a variety of sperm miRNAs in adult males, and depression and sociability impairments in their future male and female offspring. Injection of total sperm RNA from stressed fathers into zygotes mimics some of these effects when these animals mature. In the other[90], chronic variable stress in adulthood suppresses the HPA axis response in offspring and leads to increased expression of 9 sperm miRNAs. Injection of all of them into zygotes leads to similar phenotypes when they mature. Our recent study on social instability stress implicated reductions in the levels of members of the sperm miRNA 34/449 family, in a model that leads to increased anxiety and reduced sociability specifically in female offspring across multiple generations. A striking aspect of this series of studies, yet to be addressed, is that different stress paradigms produce different effects in offspring through different sperm miRNA changes. They range from enhanced anxiety and depression to blunted HPA axis response, and from female or male specific to no sex specificity in offspring. Although changes in early embryo gene expression patterns have been identified in offspring of stressed fathers, how individual miRNAs contribute to both the modification of stress phenotypes and the sex dependence of the phenomenon remains obscure.

In this study we investigate the role of miR-409-3p in early embryos in the context of stress phenotypes induced across multiple generations by social instability stress. The results imply that baseline levels of miR-409-3p in females, but not male, early embryos contribute to anxiety associated behaviors when they mature to adults. We further identified possible downstream regulators of these phenotypes in embryos derived from stressed fathers. However, elevating levels of this miRNA in early embryos in and of itself may not be sufficient to increase anxiety in adult females.

2.2 Materials and Methods

2.2.1 Animals and care facilities

All mice included in this study are of the CD-1 strain, obtained from Charles River Laboratories. All animals are housed in temperature, humidity, and light-controlled (14 hour on/10 hour off LD cycle) rooms in a fully staffed, dedicated animal core facility led by on-call veterinarians at all hours. Food and water were provided ad libitum. All procedures and protocols involving these mice were conducted in accordance with and approved by the Institutional Animal Care and Use Committee of the Tufts University School of Medicine, Boston MA.

2.2.2 Chronic social instability (CSI) stress

Male “F0” juvenile CD-1 mice arrived at P21 and were given a week to acclimate to our mouse facilities. Starting at P28, the composition of each mouse cage (4 mice per cage) was randomly shuffled twice per week, for seven weeks, such that each mouse was housed with 3 new mice in a fresh, clean cage at each change. This shuffling was randomized to reduce the chance that any mouse would encounter the mouse twice. Control mice were housed four mice per cage with the same cage mates for the duration of the protocol. After seven weeks, mice were housed in pairs with a cage mate from the final cage change and left for two weeks to remove acute effects of the final change. Male mice (both control and stressed) were then “tease” mated with a naive female mouse for 2 days to increase sperm production. Mice were then either sacrificed for sperm collection or mated with control female mice overnight to generate “F1” animals, with successful mating confirmed via presence of copulation plug the following morning. F2 and F3 animals were generated in a similar fashion, using F1 and F2 males, respectively, always through the male lineage starting from F0 stressed fathers. We have previously shown that stressed males can be mated multiple times and still transmit stress phenotypes to their offspring, so male mice were used for mating, embryo collection, or sperm collection as needed.

2.2.3 Mouse sperm collection

Mature, motile mouse sperm was isolated via the swim-up method. Briefly, male mice were anesthetized under isoflurane and sacrificed via rapid decapitation. The caudal epididymis and vas deferens were dissected bilaterally and placed in 1 mL of warm (37˚C) M16 medium (Sigma-Aldrich) in a small petri dish. Under a dissection microscope, sperm were manually expressed from the vas deferens using fine forceps, and the epididymis was cut several times before incubating at 37˚C for 15 minutes to allow mature sperm to swim out, then large pieces of tissue were removed. The remainder of the extraction took place in a 37˚C warm room. The sperm-containing media was centrifuged at 3000 RPM for 8 minutes, supernatant was withdrawn and discarded, and 400 uL of fresh, warm M16 medium was then carefully placed on top of the pellet. The tubes were then allowed to rest at a 45˚ angle for 45 minutes to allow the motile sperm to swim-up out of the pellet into the fresh medium. The supernatant containing the mature sperm was then carefully withdrawn and several tubes worth (n=4-6 mice) were combined (in order to reach sufficient quantities of RNA) and centrifuged again for 8 minutes at 3000 RPM to pellet the motile sperm. Supernatant was withdrawn and discarded, and the pellet was frozen on dry ice for later processing.

2.2.4 RNA extraction and quantitative real-time PCR

Total RNA was extracted from mouse sperm samples using the miRVana miRNA Isolation Kit (Invitrogen) according to the manufacturers protocol. Total RNA was extracted from mouse embryos using the Norgen Single-Cell RNA Isolation kit (Norgen Biotek Corp.) according to manufacturer’s protocol. Concentration of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Genomics) and NanoDrop-1000 (Thermofisher Scientific). Relative miRNA expression for sperm and embryo samples was determined using the Taqman MicroRNA Reverse Transcription Kit and qPCR system (Applied Biosystems) purchased from Thermofisher Scientific. 10 ng of total RNA from sperm, and 1 ng of total RNA from embryos, were used in the initial cDNA synthesis kit. Real-Time PCR was performed for each target and sample in triplicate on a StepOnePlus PCR System (Applied Biosystems). All data was analyzed using the Comparative ΔΔCT method to generate relative expression data using the small noncoding RNA sno202 as the internal control for all samples with this kit.

2.2.5 Zygotic manipulations

To obtain sufficient numbers of embryos, a superovulation protocol was employed. Control adult (8 weeks) female mice were injected with 7.5 U of pregnant mare serum gonadotropin (PMS, National Hormone & Pituitary Program, Harbor-UCLA Medical Center) and 46 h later injected with 7.5 U of human chorionic gonadotropin (HCG, Sigma-Aldrich) and placed into the home cage of the male to mate overnight. In the morning, female mice were checked for copulation plugs and returned to their own cages. If 2-cell and 4-cell embryos were desired, female mice were sacrificed about 1.5 days later, and for 8-cell/morula they were sacrificed about 2 days later. On the day of collection, mice were sacrificed via cervical dislocation. Under a dissection microscope, the uterus was removed and a very high-gauge needle was inserted into the opening of the fallopian tubes and the embryos were flushed out using warm 37 °C EmbryoMax FHM HEPES-buffered medium (1×) w/o Phenol Red (EMD Millipore). The embryos were separated according to cell number, and any unfertilized ova or embryos displaying unusual morphology were discarded. The embryos of interest were pooled by cell stage, and snap frozen on dry ice. Each specific cell-stage sample represents embryos derived from the mating of at least three distinct breeding pairs of mice. Typically, three–four super-ovulated females were mated with three–four males (either F0 control, or F0 stress) for each experiment in order to generate enough embryos needed for a single pool (30–60 embryos total) of a specific cell-stage embryo (2-cell, 4-cell, etc.) to recover sufficient quantities of RNA for downstream analysis. Microinjection experiments were carried out according to standard techniques[110]. Briefly, control zygotes were isolated as described above from female mice the afternoon following mating. 1-2 pL of either miR-409-3p mimic, its associated negative control, or the miR-409-3p inhibitor, or its negative control were injected at 2ng/uL in injection buffer (10 mM Tris-HCL,pH 7.4, 0.25 mM EDTA) into the zygote under a high-powered light microscope. Following injection, embryos were cultured for 48 hours until reaching the 4-cell stage where they were frozen at -80C.

2.2.6 Mouse behavioral assays

All mice were tested at 2 months of age for all behavioral tests. All mice were habituated to the testing room for at least 1 hour prior to beginning testing. All experiments were performed from 1:00PM – 4:00PM, during the light cycle. For the Elevated Plus Maze (EPM), animals were placed into the center neutral square of the apparatus, and then allowed to roam freely for 5 minutes. Speed and position were automatically recorded. For the Social Interaction with a Juvenile (SIJ) assay, mice were placed in a fresh, clean cage for 15 minutes to habituate to the new enclosure. A juvenile, same-sex mouse (age P21-P28) was then placed in the cage for 3 minutes. Videos were then scored, in a blinded fashion, for the amount of time that the experimental mouse spent performing affiliative behaviors on the juvenile mouse (grooming, sniffing, direct contact, etc.). Aggressive behavior was not included in this category. Excessive aggressive behavior was considered sufficient cause to end the behavioral assessment and remove the animals back to their own cages and exclude the animal from analysis.

2.2.7 RNA sequencing analysis

1 ng of total RNA from each embryo sample was used for library prep at the Tufts University Core Facility using the low-input NuGen library preparation kit. All samples (2 per group, 8 groups) were sequenced at the Tufts University Core Facility using an Illumina HiSeq 2500 system with 50bp single-end reads. After initial raw read quality control with FastQC, each sample was processed in parallel on the Tufts High Performance Computing Cluster using cufflinks to align the raw reads to the mouse genome, and HTSeq to count the reads per gene. The aligned read counts for all samples were then analyzed using the R-based DeSeq2 package to obtain an overall DESeq object containing all 16 samples, first filtering out low read counts (count<10 for all samples). Differential expression for the individual groups was performed using the "contrast" argument of the "results" function on the initial DESeq object, using the default $P_{adj} < 0.10$ and $Log2FC > 2$ as cutoffs. Gene set enrichment analyses and pathway analyses were performed using the Ingenuity Pathway Analysis tool (Qiagen) and PANTHER online databases.

2.2.8 Statistical Analysis

For all calculations, a p-value of <0.05 was considered significant. All figures and statistical analyses were performed in Graphpad Prism v7.0 unless otherwise stated. Comparison of miR-409-3p expression in embryos presented at mean +/- technical error for pooled samples of embryos. EPM and SIJ behavioral assays were analyzed by 2-way ANOVA (Main effects: 409-3p manipulation, sex), with posthoc analyses corrected for multiple comparisons using the Sidak method. EPM results from stressed male embryo injection analyzed using 1-way ANOVA. Sequencing statistics discussed in RNA sequencing analysis section.

<u>2.3 Results</u>

2.3.1 Chronic social instability stress elevates levels of sperm miR-409-3p across three generations

We previously published that exposure of adolescent male mice to 7 weeks of chronic social instability (CSI) stress leads to elevated anxiety and defective social interactions in their female, but not male, offspring across at least three generations[76]. We have recently implicated stress induced reductions in sperm miRs-34c and 449a in this process[109]. Because other stress paradigms have implicated sperm miRNAs whose expression rises in this process, we performed a microarray analysis comparing the expression of sperm miRNAs in male mice subjected to the CSI protocol and unstressed control males. We searched for sperm miRNAs whose levels are elevated not only in sperm from stressed males but also in his F1 and F2 male offspring, and confirmed these results via qPCR. Among hundreds of miRNAs analyzed, only miR-409-3p fit this

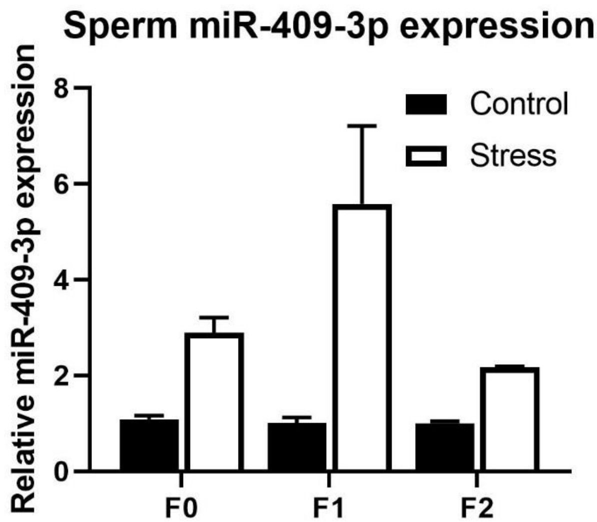

Figure 2.1: Sperm miR-409-3p levels are increased following CSI stress across three generations of the male lineage. qPCR analysis of miR-409-3p expression for 2 groups each of sperm RNA pooled from 4-6 male mice from F0 (original stressed males), F1 (sons of F0 stressed males), and F2 (grandsons of F0 stressed males) generations. Data normalized to expression for control group for each individual generation. Analysis by 2-way ANOVA identified a significant effect due to stress across all three generations (P=0.0039). Data collected by L. Saavedra-Rodriguez.

criteria, where its expression was elevated (~2-6 fold) in sperm across three generations of males derived from CSI stress exposed male mice (**Fig. 2.1**).

2.3.2 Injection of an inhibitor to miR-409-3p into zygotes generates reduced anxiety in female, but not male, offspring

In order to determine whether increased delivery of spermatic miR-409-3p to the early zygote at fertilization is necessary and/or sufficient to enhance anxiety and/or sociability defects in female offspring, we manipulated its levels in early embryos. Proving it is necessary is less stringent, so we began first by injecting zygotes, collected shortly after fertilization from the mating of control animals, with a miR-409-3p inhibitor (or a negative control inhibitor) to see what role early inhibition of miR-409-3p plays in a normal mouse background. Embryos were then re-implanted into foster mothers and stress-associated behaviors were assayed at 2 months of age in the Elevated Plus Maze (EPM) and Social Interaction with a Juvenile (SIJ) assay for anxiety and sociability defects, respectively. A cohort of embryos were collected 2 days after the injection, and showed that miR-409-3p inhibitor injection into early control zygotes induced a large decrease in expression of miR-409-3p at the 4-cell stage (**Fig. 2.2a**), demonstrating efficient and stable knockdown.

Adult F1 females derived from these inhibitor-injected zygotes spent increased time in the open arms of the EPM compared to females derived from control injected zygotes (**Fig. 2.2b**). In contrast, adult males derived from the same set of injected zygotes did not display this anxiolytic phenotype (**Fig. 2.2b**). Moreover, neither male nor female mice displayed altered sociability, as measured by the SIJ **(Fig. 2.2c**). Thus, the phenotype induced by inhibiting baseline miR-409-3p in embryos was, as expected, opposite from what we observed in offspring of stressed males that received elevated sperm miR-409-3p levels at their fertilization. Strikingly, this also reproduced the sex dependence of the anxiety effect we observed in offspring of stressed males. However, it did not influence all behaviors associated with social instability stress, as it had no effect on sociability.

We next attempted to test whether injection of a miR-409-3p inhibitor could reverse the enhanced anxiety observed in offspring of stressed males. In this case we injected the inhibitor or negative control inhibitor into zygotes derived from stressed

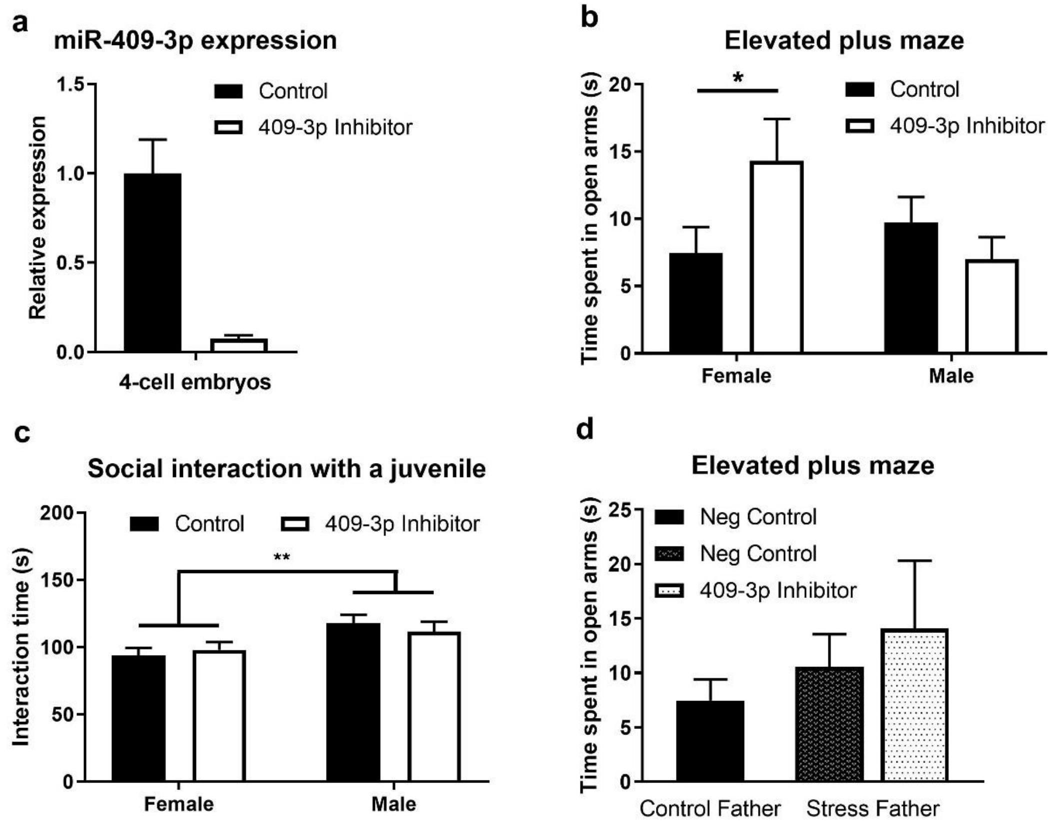

Figure 2.2: Early zygotic inhibition of miR-409-3p levels induce a female-specific anxiolytic phenotype in adult animals. a . qPCR analysis of miR-409-3p expression in pools of 30-50 4-cell embryos injected either with a negative control or a miR-409-3p inhibitor and cultured to the 4-cell stage. **b** Analysis of time spent in the open arms in the elevated plus maze for adult female and male mice injected with either a negative control or miR-409-3p inhibitor as early embryos. Control female, n=41; miR-409-3p inhibitor female, n=38; Control male, n=38; miR-409-3p inhibitor male, n=39. Analysis by 2-way ANOVA identified a significant interaction between 409-3p inhibition and sex (P=0.0307), and posthoc Fisher's LSD analysis of female data identified a significant difference between control and miR-409-3p inhibitor females (P=0.0279). **c** Analysis of time spent performing affiliative interactions with a same-sex juvenile animal on a distinct group of the animals from **b**. Control female, n=21; miR-409-3p inhibitor female, n=17; Control male, n=19; miR-409-3p inhibitor male, n=18. Analysis by 2-way ANOVA identified a significant main effect of sex (P=0.0037). **d** Same analysis as **b**, however this time performed on adult females derived from embryos from stressed fathers, control embryos injected with neg. control included. Control, n=41, Stress father negative control, n=24, Stress father miR-409-3p inhibitor, n=12. No differences identified by 1-way ANOVA. All data presented as mean +/- SEM. Behavior in **b**,**c,d** collected by P. Stohn. Videos for **c** scored by D. Dickson.

males mated to control females. However, when adult females from control inhibitor-injected zygotes were tested in the EPM they did not display the enhanced anxiety normally seen with offspring of stressed males (**Fig. 2.2d**). We surmise that this result could have been due to the requirement of maternal contributions to the transmission process, interfering epigenetic alterations induced by removing, injecting, and re-implanting zygotes, or artifacts associated with the negative-control inhibitor used for the experiments. Thus, while we did produce a phenotype consistent with miR-409-3p contributing to the female specific transmission of anxiety using inhibitor injected zygotes from control matings, we could not test whether this same manipulation could block elevated anxiety in females derived from stressed fathers.

2.3.3 Injection of a miR-409-3p mimic into fertilized zygotes is not sufficient to generate female offspring with elevated anxiety

Next, to investigate whether elevating miR-409-3p levels in early embryos is sufficient to enhance anxiety in female offspring, we injected a miR-409-3p mimic or a scrambled miRNA into early zygotes derived from the mating of control CD-1 males with control CD-1 female mice. After injection, embryos were again re-implanted into foster mothers and assayed behaviorally at 2 months of age. However, this was not sufficient to induce anxiety or sociability changes in the female mice at adulthood (**Figure 2.3**). Thus, it is likely that if elevated levels of this sperm miRNA influence these processes, they are not sufficient to do so, and additional factors must be required.

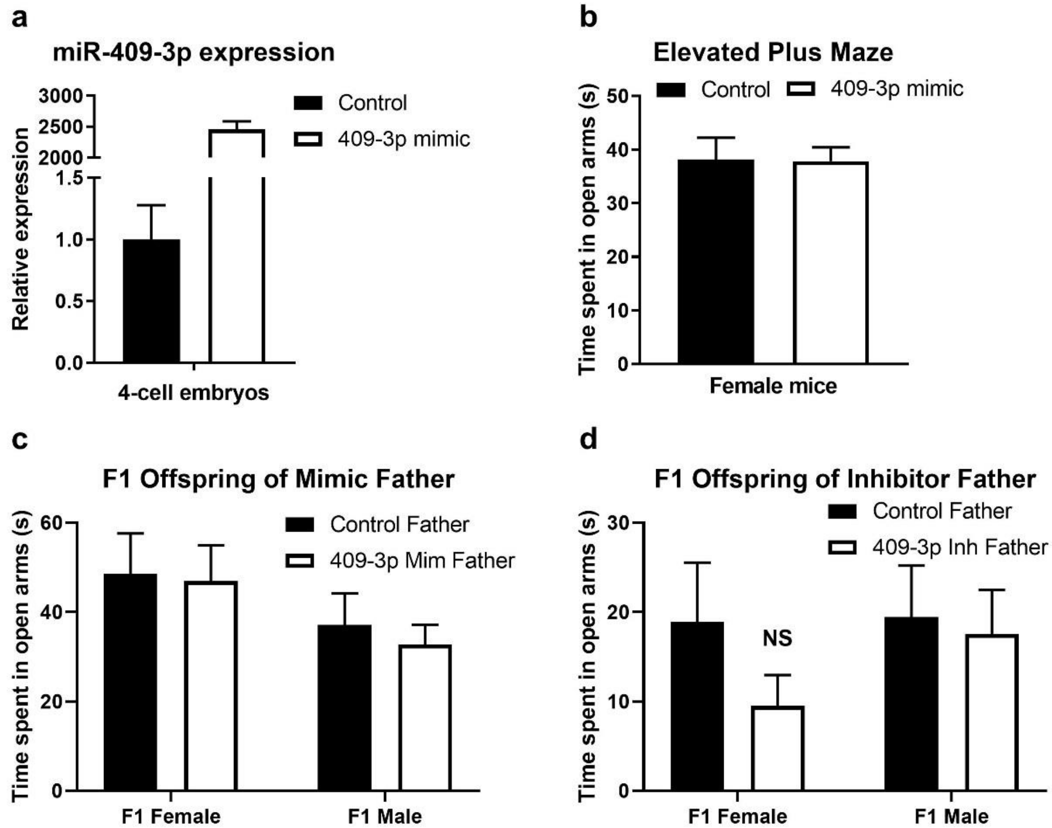

Figure 2.3: Early zygotic injection of a miR-409-3p mimic is not sufficient to alter behavior in female mice, and neither manipulation affects the next generation of offspring. a qPCR analysis of miR-409-3p expression in pools of 30-50 4-cell embryos that were injected either with a negative control or a miR-409-3p mimic as zygotes, and then cultured to the 4-cell stage. **b** Analysis of time spent in the open arms in the elevated plus maze for adult female mice injected with either a negative control or miR-409-3p mimic as early embryos before reimplantation into foster mothers. Control female, n=45; miR-409-3p inhibitor female, n=64. Analysis by unpaired Student's t-test did not identify any significant difference between groups. **c-d** Analysis of time spent in the open arms in the elevated plus maze for adult F1 female and male offspring of F0 fathers injected with either **c,** miR-409-3p mimic or a negative control mimic, or **d,** miR-409-3p inhibitor or a negative control inhibitor, as embryos. Analysis by 2-way ANOVA (Main effects: miR-409-3p manipulation, Sex) did not identify any significant effects in either case. Behavior in **b**,**d** collected by P. Stohn. Behavior in **c** collected by P. Stohn and D. Dickson.

2.3.4 Injection of neither inhibitor nor mimic of miR-409-3p produced phenotypes in the F2 generation

Experiments described above investigated whether a mimic or inhibitor of miR-409-3p injected into zygotes can alter behavior phenotypes in F1 female offspring, as CSI stressed males can upon mating. However, F1 male offspring of CSI stressed fathers are also affected such that they transmit stress phenotypes to F2 offspring while not showing behavioral alterations. Thus, to test whether miR-409-3p may be involved in that process, offspring derived from zygotes injected with miR-409-3p mimic or inhibitor were mated with normal females. When adults, offspring were tested in the EPM. No difference was observed in these females from either set of fathers, indicating that although inhibition of miR-409-3p in zygotes generates an anxiolytic phenotype in female offspring, neither inhibition nor overexpression of miR-409-3p in early embryos is sufficient to prime male offspring to be able to pass on this phenotype to the next generation (**Figure 2.3c,d**).

2.3.5 Paternal stress before mating alters gene expression patterns in 4-cell and 8-cell embryos

We hypothesized that stress-induced alterations in the expression of sperm miRNAs contribute to stress behaviors in F1 female offspring and the ability of F1 males to transmit stress phenotypes to their female offspring by rapidly altering the gene expression profiles of early embryos. This must then lead to changes during development that result in both altered brain function in female offspring, and affect sperm development and/or maturation in male offspring.

To begin to test this hypothesis, we used RNA sequencing to compare the gene expression profiles of both 4-cell and 8-cell embryos derived from the mating of stressed or control males with control females. Each group (4-cell control vs. stress; 8-cell control vs. stress) was then individually analyzed with their own control group to generate lists of differentially expressed genes in each condition. After removing genes with low read counts, and genes expressed in only one sample in each condition, there were a total of 5 genes differentially expressed at the 4-cell stage (*Hopx, Spata22, Dpysl5, Trpv3, Pcdh20*), and 107 genes differentially expressed at the 8-cell stage (**Fig. 2.4a,b**). None of the gene changes seen in 4-cell embryos were maintained into the 8-cell stage. However, it may be the case that specific genes altered by stress may need until the 8-cell stage, where chromatin structure becomes much more consistent, to show significant changes in expression. As such, we identified 5 genes that are significantly changed at the 8-cell stage that show the same direction of change in the 4-cell stage but at a lower level of stringency (*Ndrg4, Nfat5, Srebf1, Slc22a23, Snrpa1*) (**Fig. 2.4c**). Furthermore, the large number of changes in the 8-cell stage that are not seen at the 4-cell stage may be due to alteration of transcription factors or transcription machinery at the 4-cell stage that causes the larger amount of secondary changes seen at the 8-cell stage. Consistent with this hypothesis, the genes significantly altered at the 8-cell stage are significantly enriched for the GO terms "mRNA metabolic process" and "DNA-binding transcription factor activity" (**Fig. 2.4d**).

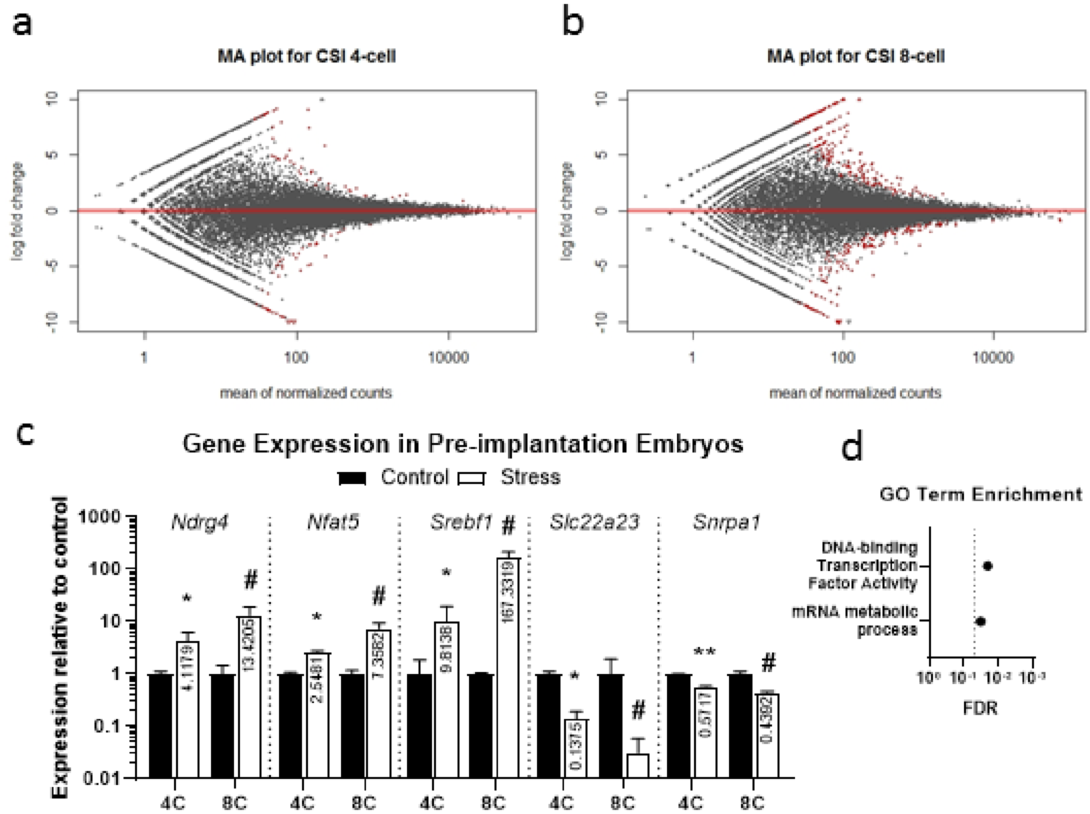

Figure 2.4: Chronic Social Instability alters the gene expression profile of 4-cell and 8-cell embryos. a-b MA plots comparing the fold change in expression between embryos from control fathers and from stressed fathers compared to the average amount of reads for that gene in **a** 4-cell embryos and **b** 8-cell embryos. **c** Gene expression changes for five genes (*ndrg4*, *nfat5*, *srebf1*, *slc22a23*, *snrpa1)* that were regulated in the same direction in both 4-cell and 8-cell embryos. **P*<0.05, ***P*<0.01, #*P*adj<0.10. Fold change listed for each gene, except Slc22a23 in 8-cell embryos (0.0312) which could not fit due to size constraints. **d** GO term enrichment of genes significantly altered at the 8-cell stage. Dotted line corresponds to an FDR cut-off of 0.05. FDR – False Discovery Rate; 4C – 4-cell embryos; 8C – 8-cell embryos.

2.3.6 Injection of a miR-409-3p inhibitor induce changes in 4-cell embryo gene expression consistent with this miRNA playing a role in elevated anxiety levels found in offspring of stressed fathers

Given the findings of female-specific anxiolysis following an early embryonic miR-409-3p inhibitor injection, we performed more RNA sequencing to compare the gene expression profiles of cultured 4-cell embryos derived from the mating of control animals that were injected with a miR-409-3p inhibitor (or the corresponding negative control), and cultured 4-cell embryos derived from the mating of control animals that were injected with a miR-409-3p mimic (or the negative control). Although the mimic injection was not sufficient to induce behavioral changes, it still provides a powerful tool for us to identify putative anxiety-related early embryonic targets of miR-409-3p when used in tandem with the inhibitor injection. Both manipulations induced robust gene expression changes at the 4-cell stage (605 genes for miR-409-3p mimic; 2158 genes for miR-409-3p inhibitor) (**Figure 2.5a,b**).

Analysis of the most significantly altered genes in the inhibitor injected embryos (P_{adj}<0.001) identified several genes with known roles in CNS development or neuronal function, including *cdk5rap2*, *id3*, *pbk*, *sertad1*, and *tdp2*, which represent possible early developmental mediators of the behavioral phenotype seen in inhibitor injected animals (**Figure 2.5c**). Outside of individual targets, on a more global scale GO term and Panther database analyses identified enrichment in a variety of fundamental processes for the sets of genes altered by miR-409-3p inhibitor injection (**Figure 2.5d)**. Taken together, the changes at the 4-cell stage of embryogenesis in miR-409-3p inhibitor injected embryos seems to upregulate genes for modulating signal transduction and binding the cytoskeleton, both critical processes for the developing embryo. More interestingly, there is a profound downregulation of transcription-related processes (initiation, transcription factor binding), and translation-related processes (ribosome synthesis, RNA binding).

To further identify early developmental targets that are altered by paternal stress and may contribute to the development of anxiety in female offspring, we compared the

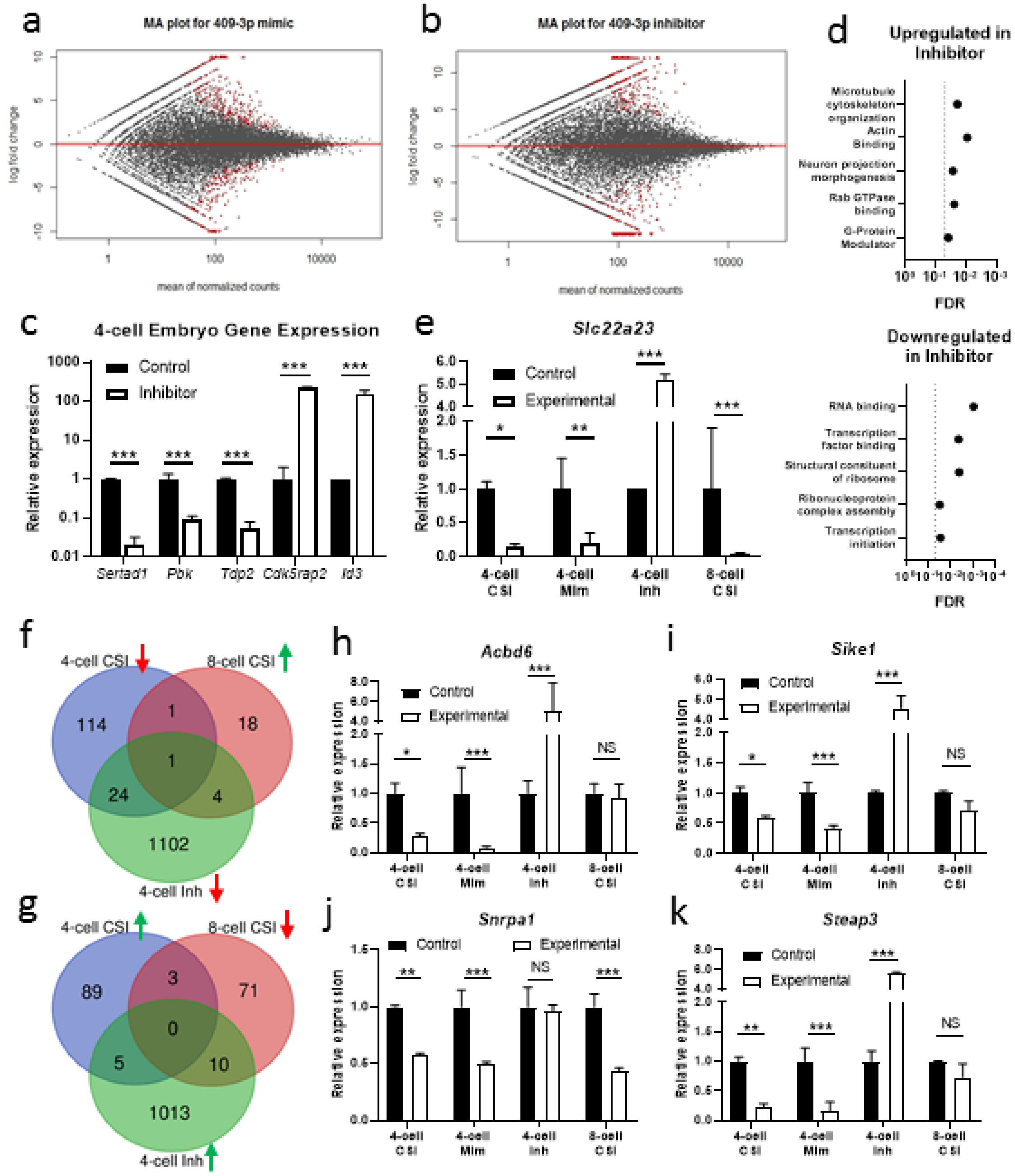

Figure 2.5: Manipulation of early zygotic miR-409-3p levels significantly alters gene expression at the 4-cell stage which may contribute to the development of anxiety in adult animals. a-b MA plots comparing the fold change in expression between embryos injected with either a miR-409-3p mimic (**a**) or inhibitor (**b**) cultured to the 4-cell stage, relative to control injections compared to the average amount of reads for that gene. **c** Gene expression changes for five genes (*sertad1*, *pbk*, *tdp2*, *cdk5rap2*, *id3*) in inhibitor injected embryos. **d** GO term enrichment of genes significantly upregulated (top) or downregulated (bottom) in inhibitor injected embryos. Dotted line corresponds to an FDR cut-off of 0.05. **f-g** Venn diagrams demonstrating the number of genes regulated in the indicated manner that are common between the three groups, 4-cell embryos from CSI

fathers, 8-cell embryos from CSI fathers, and 4-cell embryos injected with the miR-409-3p inhibitor. **e,h-k** Gene expression changes in all sequencing conditions for five identified genes. *P<0.05, **P<0.01, ***Padj<0.10. FDR – False Discovery Rate; NS – not significant.

previous 4-cell and 8-cell sequencing data (again, using the lower stringency for the 4-cell data) to that of the inhibitor-injected embryos, who show a female-specific anxiolysis as adults. We searched for genes whose expression changed in the same direction in 4-cell and 8-cell embryos from stressed fathers, and the opposite direction in inhibitor injected embryos. There are many genes altered in this manner between two of the three groups, and one gene that was common to all three, *slc22a23* (**Figure 2.5e,f,g**).

We then included the sequencing data from the mimic-injected embryos to further identify novel targets. First, we compared the inhibitor and mimic injected datasets to identify genes that are significantly altered in opposite directions in these two data sets, and thus are presumably either direct targets of miR-409-3p, or are secondary changes due to a direct miR-409-3p target. This analysis identified 48 genes that were significantly decreased in mimic injected embryos and significantly increased in inhibitor injected embryos, consistent with the expected direction of miRNA-mediated regulation, as well as an additional 24 genes in the non-canonical direction (up in mimic, down in inhibitor). Ingenuity Pathway Analysis identified HNF4A, TP53, and RICTOR as putative upstream regulators in both sets of data. However, these three genes did not show differential expression in any of our datasets.

By including the mimic-injected data, we further identified several genes that showed similar regulation across our various samples according to the expected biology of miRNAs when compared with the 4-cell embryos from stressed fathers (decreased in embryos from stressed fathers and mimic injected embryos, increased in inhibitor injected embryos). Four genes in particular stood out from this analysis given their consistent pattern of expression across our four groups of samples. *Snrpa1*, mentioned earlier, was also significantly decreased in mimic injected embryos, consistent with the direction of regulation in stressed embryos, as was *slc22a23*, which was also increased by inhibitor injection (**Figure 2.5e**). Furthermore, *steap3*, *sike1*, and *acbd6*, were all found to be decreased in 4-cell embryos derived from stressed fathers, as well as embryos injected with the miR-409-3p mimic, and increased in embryos injected with the miR-409-3p inhibitor (**Figure 2.5h,i,j,k**). Thus, these genes may represent early functional targets of miR-409-3p that are also affected by paternal stress, and may mediate the phenotypes of offspring of stressed fathers.

2.4 Discussion

Results of this study have implicated early embryonic levels of miR-409-3p in establishing normal levels of anxiety specifically in adult female mice. This idea is based on our finding that reducing levels of this miRNA in early embryos leads to below baseline levels of anxiety in female, but not male, adult mice derived from them. This manipulation of early embryo miR-409-3p displayed behavioral specificity because it had no detectable effect on sociability in adult females. The significance of this finding is supported by our other observation that chronic social instability stress leads to the opposite behavior, anxiogenesis, also specifically in female offspring. This transmission is associated with the opposite change in sperm miR-409-3p levels, an increase in expression, in not only the F0 males exposed to it, but also in their F1 and F2 male offspring who transmit elevated anxiety levels to their female offspring.

While these findings imply that normal levels of early embryonic miR-409-3p are necessary for females to express basal levels of anxiety when they mature into adults, we failed to demonstrate that increased miR-409-3p is sufficient to enhance anxiety in them. First, we did not observe an increase in the total levels of miR-409-3p in 4-cell embryos derived from stressed males compared to those from control males (data not shown), suggesting the elevated expression in sperm is not maintained once transcription begins in the developing embryo (around the 2-cell stage). Interestingly, this test has not been demonstrated in other stress models employing injections of sets of miRNA mimics (that rise in stressed males sperm) that induce expected phenotypes in offspring. Regardless, it appears that elevating levels of this miRNA in early embryos, at least by injecting a miR-409-3p mimic into zygotes, is not sufficient to enhance anxiety in them when they become adults. This is not surprising, as others have shown that injection of multiple miRNAs (not including miR-409-3p), but not a single one of these miRNAs, elevated in sperm of stressed males into zygotes can produce stress associated phenotypes[90]. Moreover, in our previous paper we have implicated additional miRNAs, miR-34c and miR-449a, that are reduced in sperm of males exposed to CSI stress and in their F1 male offspring, and they may also be involved in this process given that their decreased expression is maintained through early embryogenesis[109]. However, this conclusion must be tempered by the possibility that this method of elevating miR-409-3p levels in zygotes may have induced secondary artifact effects that blocked its potential activity.

There is large body of evidence supporting the idea that females respond to stress differently than males and have different vulnerabilities to stress related psychiatric disorders. Thus, a striking aspect of both the transgenerational effects of CSI stress exposure to males and the injection of a miR-409-3p inhibitor into early embryos is that both alter the anxiety levels in female, but not male offspring. This raises the possibility that the degree of female sensitivity to stress and/or to stress-associated anxiety disorders could be influenced by one's father's exposure to stress passed on the them epigenetically, or by their mother's experiences that generate in utero exposures that alter early embryo miR-409-3p levels. How changes in sperm or embryonic levels of miR-409-3p specifically alter female behavior in adults remains to be determined. This was a goal of our early embryo sequencing experiments.

Our analysis of early embryos derived from control and stressed fathers identified several genes that were altered in the same direction at both the 4-cell and 8-cell stage in embryos derived from stressed fathers. Thus, paternal life stress prior to conception can significantly alter early embryo gene expression. The finding of few changes at the 4-cell stage, with significantly more at the 8-cell stage is consistent with the timeline of transcriptional and methylation dynamics in early embryos[111,112]. How these early gene expression changes mediate effects into the adult animal is unknown, but some of their known functions offer insight into their possible roles. N-myc down-regulated gene 4 (*ndrg4*) is expressed in neurons throughout the brain, and assists in maintaining the normal levels of brain-derived neurotrophic factor (BDNF) necessary for spatial learning and memory[113]. *Nfat5* is necessary for and highly expressed in the developing fetal brain, and has roles in mediating neuroinflammation and dopaminergic signaling[114]. *Slc22a23*, which was also increased by miR-409-3p inhibition and decreased by miR-409-3p mimic injection, is an organic cation transporter that is expressed abundantly in the developing and mature brain, however its specific role here remains to be elucidated[115]. *Snrpa1*, which was also decreased by miR-409-3p mimic injection, is a key component of spliceosomes where its high expression is necessary for the maintenance of pluripotency in embryonic stem cells[116]. Functioning spliceosomes are also critical for the survival of developing embryonic neurons[117]. Unfortunately, given the limited amount of RNA isolated from small pools of embryos, we did not reach the level of sequencing depth necessary to perform an alternative splicing analysis. Furthermore, without specific manipulations of these targets in early embryos we cannot be sure that they are truly involved in altering phenotypes in F1 offspring.

We initiated this study to identify sperm miRNAs that might mediate the effects of CSI on female offspring. The fact that the level of sperm miR-409-3p is elevated not only in sperm of stressed males, but also in their F1 and F2 male offspring supports this hypothesis, as does the observation that inhibition of miR-409-3p in zygotes leads to the opposite phenotype specifically in females. However, we were unable to generate additional key data to make a more solid case, because we could not test whether inhibiting miR-409-3p in zygotes from stressed males prevents the transmission of stress phenotypes to offspring. As mentioned, it is possible this negative result was the side effect the manipulations needed for these experiments, which are known to affect the epigenetic landscape of early embryos[111]. Alternatively, a maternal contribution to transmission of traits from exposed males has been described in other paradigms[118]. Also, these experiments were performed fewer times than our other studies, and thus may bear repeating in the future on a distinct set of stressed males.

miR-409-3p resides on chromosome 13 and is conserved between mice and humans. Most of what we know about its function relates to its elevated levels in human cancer[119]. Its expression is also altered in the hippocampus in cocaine addiction[120]. However, to date validated gene targets do not give clear hints about how they might affect early embryo development to promote behavior changes in adults. Analysis of the genes altered in inhibitor injected embryos identified several possible targets that could be mediating the anxiolytic phenotype in adult animals. *Sertad1*, a Cdk4 activator, is essential for the apoptosis-mediated neuronal death that occurs throughout early cortical development[121]. *Pbk*, a mitogen-activated protein kinase kinase, plays a vital role in regulating proliferation of neural progenitor cells[122]. *Tdp2* is necessary for expression of many genes in the developing mouse brain and has a role in regulating interneuron density[123]. *Cdk5rap2* loss leads to proliferation and survival defects in neural progenitor cells, and it also regulates brain size[124]. Finally, *Id3* is a known inhibitor of neuronal differentiation, and demonstrates specific spatial expression patterns during the developing brain that likely plays a role in neural precursor proliferation and differentiation[125].

Our combined analysis of both miR-409-3p manipulations identified three putative upstream regulators, HNF4A, RICTOR, and TP53, that could account for a significant portion of the gene changes at the 4-cell stage in these manipulated embryos. Given that these three genes did not show significant changes in expression in any of our data sets, it is possible that, if they are involved, they exerted their effect either before the 4-cell stage, or via a post-transcriptional or post-translational mechanism that does not alter mRNA levels or stability. Of note, RICTOR (aka mTORC2) contains 3 putative 3' UTR target sites for miR-409-3p, and it has also been shown to modulate dopamine-dependent behaviors in the brain of adult animals, through its role in phosphorylating Akt[126].

Both miR-409-3p manipulations induced a much larger change in gene expression than that seen in the embryos of stressed fathers. It is likely that a portion of this altered gene expression profile is a result of the protocol necessary to perform these experiments, as discussed above (zygote extraction, culturing, etc.). It must also be noted that any changes seen in this analysis may only be half the expected magnitude, given that these experiments were performed on total RNA from the entire pool of collected embryos, and thus represents a mix of both male and female embryos. Thus, any changes that may have only occurred in female embryos, or vice versa, will be effectively diluted out by a factor of 2 if that change did not occur in embryos of the other sex. Thus, we may have missed other female-specific mRNA mediators. Future experiments, using single-cell or single-embryo sequencing technologies will be vital to address these issues, and they will simultaneously improve the depth of our sequencing to more than the limited replicates we analyzed here.

Overall, these data implicate miR-409-3p in both the development of anxiety in females, and in our paradigm of transgenerational inheritance of the effects of stress. Furthermore, this paternal stress protocol leads to changes in gene expression in early pre-implantation embryos, and some of these changes may be attributable to the action of miR-409-3p, offering insight into the possible early developmental mechanisms underlying the inheritance of the effects of stress.

Chapter 3: Reduced Levels of miRNAs 449 and 34 in Sperm of Mice and Men Exposed to Early Life Stress[109]

[109] Dickson, D. A., Paulus, J. K., Mensah, V., Lem, J., Saavedra-Rodriguez, L., Gentry, A., Pagidas, K. & Feig, L. A. Reduced levels of miRNAs 449 and 34 in sperm of mice and men exposed to early life stress. Transl Psychiatry 8, 101, (2018).

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3.1 Introduction

Many studies have confirmed that exposure to severe stress during childhood has long-lasting negative health effects. One of the most convincing has been the Adverse Childhood Experience (ACE) Study, which is supported by over 100 publications[36]. It was initiated by collaboration between the Centers for Disease Control and Prevention and Kaiser Permanente's Department of Preventive Medicine. It led to the ACE Study Questionnaire (see http://www.acestudy.org/index.html), where anonymous yes or no answers to 10 questions involving participant's experiences at home until the age of 18 are quantified. Five are personal questions about physical abuse, verbal abuse, sexual abuse, physical neglect, and emotional neglect. Five relate to other family members: an alcoholic parent, a victim of domestic violence, incarceration, diagnosed with a mental illness, and the disappearance of a parent through divorce, death, or abandonment. A score ≥4 puts one at serious risk for future mental and physical health problems, such as a 4.6-fold increased rate of depression[38] and a ~30-fold increased rate of suicidal ideation and attempts in adults[39]. Remarkably, >10% of the population reports scores of ≥4.

There is a growing appreciation that clinicians should be aware of patients' traumatic experiences, particularly when young, because they add to their risk for physical and psychiatric maladies[127,128]. Moreover, sensitivity to PTSD has been shown to correlate with ACE score[40,129,130] implying it can be used as a screening tool to identify people who should take extra precaution to avoid trauma. However, some may not answer the ACE questionnaire accurately due to suppressed memories or because of the sensitive nature of many of the questions, particularly in settings that do not allow anonymity. Thus, discovery of unbiased markers for early trauma could complement ACE surveys in some clinical settings.

Moreover, offspring of those exposed to early life trauma are at elevated risk for psychiatric disorders[131]. This phenomena has also been demonstrated in rodents[132,133]. For example, transmission of the effects of stress across generations has been observed after exposing male mice to a wide variety of psychological stresses, including social defeat[134], chronic physical restraint[135], multiple variable perturbations in adults[136], social instability beginning in adolescence[137] , and early maternal separation[138]. While some evidence in mice points to environmentally induced changes in sperm DNA methylation as a mechanism for transmission of stress phenotypes[138], the best evidence to date supports small RNA species in sperm. Recent studies show that sperm contain various types of cytoplasmic RNAs (e.g., mRNAs, miRNAs, siRNAs, lnc-RNAs, piwi-interacting RNAs, and fragments of tRNAs) that have the potential to contribute to embryo development[139-141]. Two sets of reports on psychological stresses in mice implicate miRNAs. In one[142], early maternal separation of males leads to increased expression of a variety of sperm miRNAs in adult males and depression and social defects in their future offspring. Injection of total sperm RNA from stressed fathers into zygotes mimics some of these effects when these animals matured. In the other[143], chronic variable stress in mice leads to increases in the expression of nine sperm miRNAs, and when these miRNAs are injected into fertilized zygotes, phenotypes similar to those in offspring from stressed fathers are also observed.

Here, we find that severe early life stress is associated with a reduction in sperm of both mice and men of the levels of multiple members of 34/449 miRNA family that all have the same seed sequence and function together to influence brain development and spermatogenesis[144,145]. miR-34 expression has also been implicated in stress regulation in the adult brain[146-148]. In mice, the effect of stress on these sperm miRNAs crosses generations, as reductions in miR-449a and miR-34c are found in both early embryos derived from stressed fathers and in sperm of males derived from these embryos.

3.2 Materials and methods

3.2.1 Animals and care facilities

All mice included in this study are of the CD-1 strain, obtained from Charles River Laboratories. Males used for CSI stress began the protocol at 28 days postnatal age, and control females used for breeding were 8 weeks of age. All animals are housed in temperature, humidity, and light-controlled (14 h on/10 h off LD cycle) rooms in a fully staffed dedicated animal core facility led by on-call veterinarians at all hours. Food and water were provided ad libitum. All procedures and protocols involving these mice were conducted in accordance with and approved by the Institutional Animal Care and Use Committee of the Tufts University School of Medicine, Boston, MA. Sample sizes for the mouse work were based upon our previous experience with these experiments in the lab to yield adequate concentration of RNA for downstream work, and no animals were excluded from use in this study. No randomization procedures were needed to separate mice into groups.

3.2.2 Volunteer recruitment and ACE administration

All research performed on humans and human samples were approved by the University of Louisville School of Medicine Human Subjects Protection Program (IRB

#15.1004). We included only Caucasians to limit confounding by race given possible variation in sperm miRNA and response to the ACE questionnaire, and because Caucasians represented the vast majority of patients in the selected source population. Informed consent to participate in this observational study was obtained from all subjects. Eligible men were administered the adverse child experiences (ACE) survey in a self-reporting format after attaining proper consent. Men then provided a semen sample for their initial workup (n = 28 samples total). Mature motile sperm were isolated using a Percoll gradient to effectively separate mature sperm from immune cells, dead tissue, immature sperm, and other contaminants[149]. An aliquot of purified sperm was saved for expression analysis, the remainder being used for semen analysis and downstream IVF/ICSI procedures. Demographic and health information for all samples was provided by the clinical team in a de-identified, password-protected spreadsheet to the research team, with a password known only to the authors of this study. All men providing samples for this study were compensated with a $50 gift card. This study was funded to recruit eligible participants for a 1-year period. Given typical clinic volumes and anticipated research participation rates, we expected to recruit a sample of 35 subjects over 12 months. A sample size of 35 subjects achieves 80% power to detect a correlation coefficient of 0.45 for the association between miRNA levels and ACE score using a two-sided hypothesis test with a significance level of 0.05. Recruitment was halted at 28 subjects due to the challenge of lower than expected clinic volume.

3.2.3 Microarray analysis

Microarray analysis of ten human sperm samples was performed by LC Sciences Inc. (Austin, TX), using 1 μg of total RNA as starting material. Each sample was run on its own custom µParaflo microfluidic chip developed by LC Sciences. Each chip investigates expression of every experimentally verified human miRNA currently listed in miRbase v21. After hybridization, fluorescence images were collected, analyzed, and quantified by LC Sciences to generate a differential miRNA expression analysis, using the appropriate statistical analysis and correcting for multiple comparisons, between our low ACE group (scores 0–1, n = 5) and high ACE group (scores >4, n = 5).

3.2.4 RNA extraction and quantitative real-time PCR

Total RNA was extracted from both mouse and human sperm samples using the miRVana miRNA Isolation Kit (Invitrogen) according to the manufacturer's protocol. Total RNA was extracted from mouse embryos using the Norgen Single-Cell RNA Isolation kit (Norgen Biotek Corp.) according to the manufacturer’s protocol. Concentration of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Genomics) and NanoDrop-1000 (Thermofisher Scientific). Relative gene expression for all samples was determined using the Taqman Advanced cDNA synthesis and qPCR system (Applied Biosystems) purchased from Thermofisher Scientific. An aliquot of 10 ng of total RNA from sperm and 1 ng of total RNA from embryos was used in the initial cDNA synthesis step. Real-time PCR was performed for each target and sample in triplicate on a StepOnePlus PCR System (Applied Biosystems). All data were analyzed using the Comparative ΔΔCT method to generate relative expression data using miR-192-5p as the internal control for all samples.

3.2.5 Chronic social instability (CSI) stress

Male “F0” juvenile CD-1 mice arrived at P21 and were given a week to acclimate to our mouse facilities. Starting at P28, the composition of each mouse cage (four mice per cage) was randomly shuffled twice per week, for 7 weeks, such that each mouse was housed with three new mice in a fresh, clean cage at each change. This shuffling was randomized to reduce the chance that any mouse would encounter the same mouse twice. Control mice were housed four mice per cage with the same cage mates for the duration of the protocol. After 7 weeks, mice were housed in pairs with a cage mate from the final cage change and left for 2 weeks to remove acute effects of the final change. Male mice (both control and stressed) were then “tease” mated with a naive female mouse for 2 days to increase sperm production. Mice were then either killed for sperm collection or mated with control female mice overnight to generate “F1” animals, with successful mating confirmed via presence of copulation plug the following morning. We have previously shown that stressed males can be mated multiple times and still transmit stress phenotypes to their offspring, so male mice were used for mating, embryo collection, or sperm collection as needed.

3.2.6 Mouse sperm collection

Mature, motile mouse sperm was isolated via the swim-up method. Briefly, male mice were anesthetized under isoflurane and killed via rapid decapitation. The caudal epididymis and vas deferens were dissected bilaterally and placed in 1 mL of warm (37 °C) M16 medium (Sigma-Aldrich) in a small Petri dish. Under a dissection microscope, sperm were manually expressed from the vas deferens using fine forceps, and the epididymis was cut several times before incubating at 37 °C for 15 min to allow mature sperm to swim out, then large pieces of tissue were removed. The remainder of the extraction took place in a 37 °C warm room. The sperm-containing media was centrifuged at 3000 RPM for 8 min, supernatant was withdrawn and discarded, and 400 μL of fresh, warm M16 medium was then carefully placed on top of the pellet. The tubes were then allowed to rest at a 45° angle for 45 min to allow the motile sperm to swim-up out of the pellet into the fresh medium. The supernatant containing the mature sperm was then carefully withdrawn and combined ($n = 4–6$ mice per group pool) in order to reach sufficient quantities of RNA, and centrifuged again for 8 min at 3000 RPM to pellet the motile sperm. Supernatant was withdrawn and discarded, and the pellet was frozen on dry ice for later processing.

3.2.7 Mouse embryo collection

To obtain sufficient numbers of embryos, a superovulation protocol was employed. Control adult (8 weeks) female mice were injected with 7.5 U of pregnant mare serum gonadotropin (PMS, National Hormone & Pituitary Program, Harbor-UCLA Medical Center) and 46 h later injected with 7.5 U of human chorionic gonadotropin (HCG, Sigma-Aldrich) and placed into the home cage of the male to mate overnight. In the morning, female mice were checked for copulation plugs and returned to their own cages. If 2-cell and 4-cell embryos were desired, female mice were sacrificed about 1.5 days later, and for 8-cell/morula they were sacrificed about 2 days later. On the day of collection, mice were sacrificed via cervical dislocation. Under a dissection microscope, the uterus was removed and a very high-gauge needle was inserted into the opening of the fallopian tubes and the embryos were flushed out using warm 37 °C EmbryoMax FHM HEPES-buffered medium (1×) w/o Phenol Red (EMD Millipore). The embryos were separated according to cell number, and any unfertilized ova or embryos displaying unusual morphology were discarded. The embryos of interest were pooled by cell stage, and snap frozen on dry ice. Each specific cell-stage sample represents embryos derived from the mating of at least three distinct breeding pairs of mice. Typically, three–four super-ovulated females were mated with three–four males (either F0 control, or F0 stress) for each experiment in order to generate enough embryos needed for a single pool (30–60 embryos total) of a specific cell-stage embryo (2-cell, 4-cell, etc.) to recover sufficient quantities of RNA for downstream analysis. As the females are sacrificed during the collection process, this entire protocol was repeated with new females for every embryo stage collection to generate one pool each of one specific stage embryo for both F0 control fathers and F0 stressed fathers.

3.2.8 Statistical analysis

Demographic, behavioral, and sperm characteristics were summarized for the study population, stratified by ACE score (low (0–1), medium (2–4), high (≥5)), using the appropriate summary statistic for normally and non-normally distributed data. For all presented data, center values and estimates of variation are stated in the figure legends. For qPCR analysis, miRNA was compared between low (0–1) and high (>4) ACE groups using the Wilcoxon rank-sum test (groups are independent and equivariant). Spearman's correlation coefficients were used to characterize the strength and direction of the linear relationship between continuous variables. Univariate linear regression analysis was performed to estimate slope coefficients, accompanying p values, and 95% confidence intervals. For all analyses described in this section, p values are two-sided and alpha was set to 0.05. All statistical analyses were performed in Graphpad Prism v7.0 unless otherwise stated. The nature of this study did not allow for blinding as only objective measurements are reported for these samples.

3.3 Results

3.3.1 Inverse association between expression of members of sperm miRNA families 449 and 34 with ACE score of Caucasian males

To test whether the extent of childhood trauma influences sperm miRNA content in adulthood, 28 Caucasian volunteers, drawn from men giving sperm samples to the University of Louisville Fertility Clinic, filled out the Adverse Childhood Experience (ACE) study questionnaire. The participants were 32.4 years of age on average, with a mean BMI of 26.7 kg/m2. Among them, ~20% reported scores ≥4, which is higher than the national average (~12%), but close to the average in Kentucky (~16%) (https://www.childtrends.org/wp-content/uploads/2014/07/Brief-adverse-childhood-experiences_FINAL.pdf). The demographic, behavioral, and sperm characteristics of study participants are summarized in **Table 3.1**, stratified by ACE score (low, medium, and high). There were not clinically significant differences noted across the groups defined by ACE score for most parameters. Lower sperm morphology counts were observed in the high ACE group, although a morphology score of 4% is still considered normal according to the most recent World Health Organization guidelines for semen analysis parameters[150]. The other two parameters, sperm count and motility, were also well within normal limits according to these guidelines for all groups (count ≥15 mil/mL; motility ≥40%).

Table 3.1: Demographic, behavioral, and sperm characteristics of the study population, stratified by ACE score

Variable	Low ACE (0–1)	Medium ACE (2–4)	High ACE (≥5)
	n = 14	n = 7	n = 7
Age (years), mean ± SD	30.1 ± 4.1	32.4 ± 6.2	36.7 ± 9.6
BMI (kg/m2), mean ± SD	27.1 ± 5.1[a]	26.7 ± 4.8	26.0 ± 3.9[b]
Ever smoked, n (%)	8 (57%)	4 (57%)	5 (71%)
Current smoker, n (%)	3 (21%)	1 (14%)	2 (29%)
Alcoholic drinks/wk, median (range)	1.5 (0–14)	1 (0–3)	0.25 (0–28)
Illegal drug use, n (%)	3 (21%)	3 (43%)	3 (43%)
Sperm count (mil/mL), mean ± SD	84 ± 57	115 ± 62	97 ± 68
Sperm motility (% motile), median (range)	66 (35–96)	72 (44–83)	59 (1–80)[b]
Sperm morphology (% normal), median (range)	8 (2–21)	11 (2–21)	4 (0–12)[b]

[a]n = 12 for low ACE group
[b]n = 6 for high ACE group

As a first-level screen to find candidate miRNAs whose expression in sperm correlates with ACE score, we randomly chose sperm samples from five men with the highest ACE scores (≥4) and five with the lowest (0–1) and performed miRNA array analysis on each sample. Among hundreds of miRNAs detected, multiple members of the miRNA family miR-34/449, which all code for the same mRNA inhibitory seed sequence, had the most significant differences in expression between the ACE groups. One set includes two of the three paralogs of miR-449, miR-449a, and miR-449b, and the other set, two of the three paralogs of miR-34, miR-34b, and miR-34c. miR-449c and miR-34a were not detected at reliably quantifiable levels in the arrays. qPCR analysis of these miRNAs revealed an approximately four–fivefold reduction in their levels in sperm samples from the high vs. low ACE score groups (**Fig. 3.1a**). To date, no other miRNA tested from this screen has been found to be statistically significantly different between the ACE groups. As examples, we show results for miR-152 and miR-375-3p (**Fig. 3.1a**).

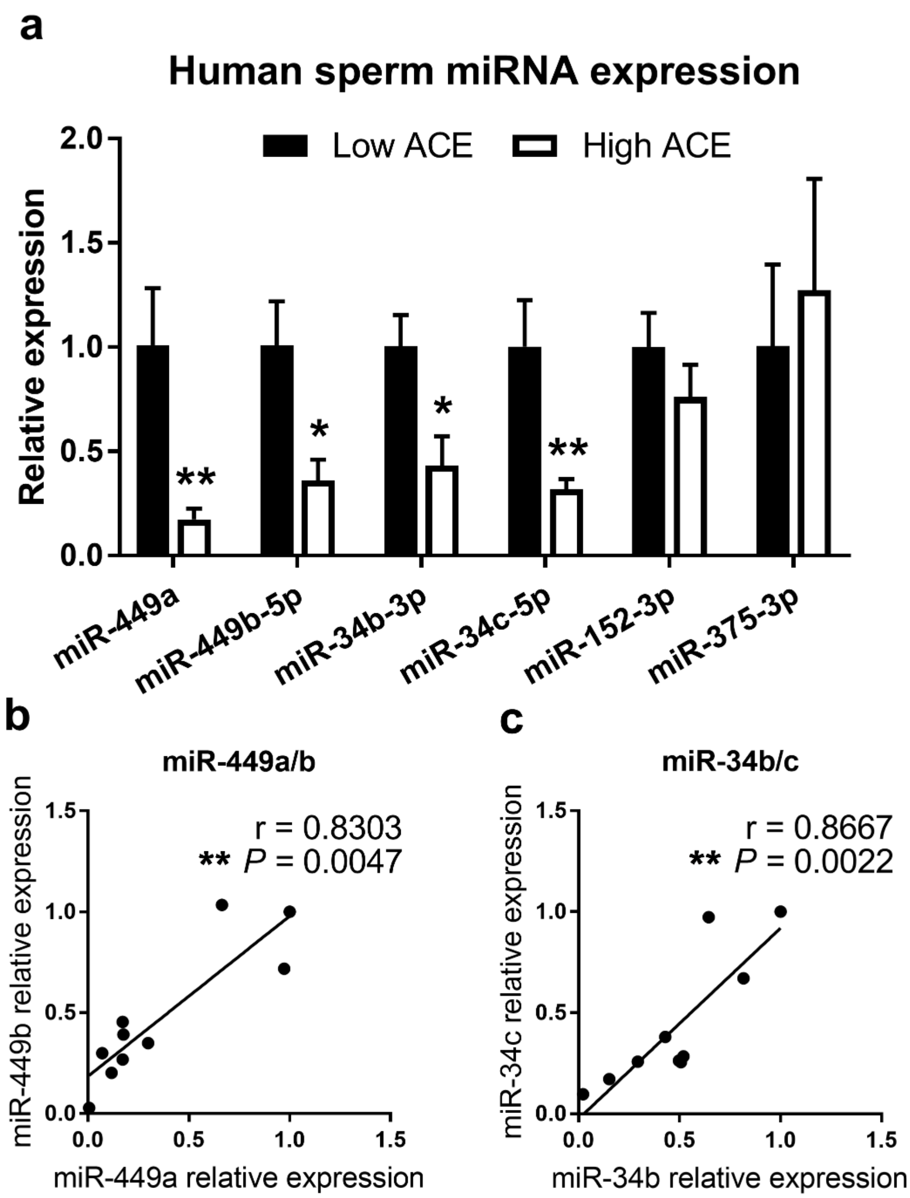

Figure 3.1: Decreased sperm miRNA expression in men with extensive adverse childhood experiences. **a** qPCR analysis of miR-449a, miR-449b-5p, miR-34b-3p, miR-34c-5p, miR-152-3p, and miR-375-3p in sperm RNA from low ACE group (score 0–1, n = 5) vs. high ACE group (score >4, n = 5). Wilcoxon rank-sum test *P < 0.05, **P < 0.01. Data represented as mean ± SEM. **b**–**c** Correlation plot comparing relative expression of miR-449a to miR-449b (b) and miR-34b to miR-34c (c) for individual samples fitted with single-variable linear regression. n = 10, r = Spearman's coefficient with exact p value listed. Each point represents an individual sample.

The former was chosen because array data suggested it might be different, and the latter was chosen because it was identified by two groups as a miRNA elevated in sperm of male mice exposed to two different types of stress[142,143].

We next performed qPCR analysis on these targets in all of the remaining samples. Because the relative levels of miR-449a and miR-449b, as well as miR-34b and miR-34c, were similar to each other in almost every sample (**Fig. 3.1b, c**) miR-449a and miR-34c were used as representatives of each family. We found a statistically significant inverse correlation between ACE score and levels of both miRNAs (miR-449a $r = -0.4357$, $P = 0.0205$; miR-34c-5p $r = -0.397$, $P = 0.0376$), where many of the highest ACE score samples had miR-449a and miR-34c levels as much as ~300-fold lower than many of the low ACE score samples **(Fig. 3.2a, b**). In contrast, no significant correlations were observed for the association between miR-152 and miR-375-3p and ACE score (**Fig. 3.2d, e**). The levels of the miR-449a and miR-34c are coordinately expressed in each sample ($r = 0.913$, $P = 0.001$), implying that stress regulates their levels in sperm by the same mechanism (**Fig. 3.2c**).

Sperm miRNA content has been shown to be influenced by smoking[151,152] and obesity[153,154]; however, in univariate regression analysis neither BMI nor smoking status were significantly associated with expression of sperm miR-449a or miR-34c (Extended Data, **Table 3.2**). Moreover, previous studies found that neither smoking nor obesity influence the levels of sperm miR-449a/b or miR-34b/c[152,154]. miRNA expression was also not associated with behavioral characteristics, such as alcohol and drug use, sperm count, or sperm motility. However, a significant association was observed between sperm morphology score and levels of miR-449a, but not miR-34c. Overall, these data imply

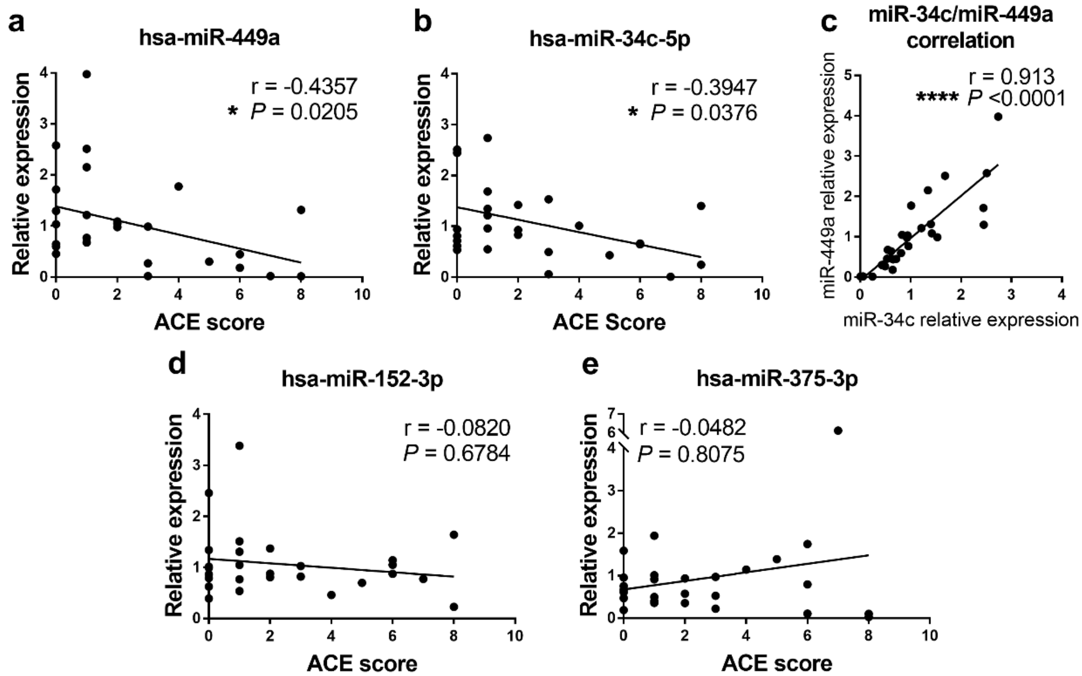

Figure 3.2: ACE score negatively correlates with expression of miR-449a and miR-34c in 28 human sperm samples. **a–b** qPCR analysis of miR-449a, miR-34c-5p, miR-152-3p, and miR-375-3p in all samples, normalized to the overall average expression to generate a relative expression value. Trend lines represent single-variable linear regression. n = 28, r = Spearman's coefficient, with p values listed. Each point represents an individual sample. **c** Correlation plot comparing miR-449a expression to miR-34c expression in individual samples fitted with single-variable linear regression. r = Spearman's coefficient, with exact p value listed. **d–e** qPCR analysis of miR-152-3p and miR-375-3p, data analyzed as in **a, b**

that early life stress in men, as measured by ACE score, is associated with reduced levels of miR-449a and miR-34c.

3.3.2 Levels of miR-449a and miR-34c are decreased in sperm of male mice exposed to chronic social instability stress, early embryos derived from them, and sperm from adult mice derived from these embryos

To determine whether early life stress also regulates sperm miR-449 and miR-34 in mice, we exposed adolescent males to chronic social instability (CSI) stress[155], which induces sociability defects in male mice for at least 1 year after stress ceases. It involves changing the cage composition of mice twice a week for 7 weeks beginning during adolescence. We chose this protocol because like adverse childhood experiences, it involves chronic stress when animals are young (adolescents) that leads to long-term negative effects on behavior, it is performed in outbred CD-1 mice that like humans, but unlike inbred mouse strains, are genetically diverse, and because we have replicated its findings[137]. Both miR-449a and miR-34c are sharply reduced in sperm of these stressed males when they are adults (~fivefold) (**Fig. 3.3a**). In contrast, and consistent with human results described above, no significant difference was found for sperm miR-152 and miR-375-3p, even though the latter was previously shown to increase after exposure of male mice to two different stress paradigms[142,143].

Recently, we[137] and then others[135] showed that CSI stress has transgenerational effects through the male lineage that induces anxiety and sociability defects specifically in F1 female offspring of stressed males. Moreover, even though the F1 males showed no anxiety or sociability defects, we showed that they induce both behavioral defects specifically in their F2 female offspring[137]. A distinguishing feature of miR-449a/b and miR-34b/c is that they are among a small set (14) of miRNAs in mice that are not present in oocytes but delivered to them from sperm upon fertilization[156], suggesting that changes in these miRNAs observed in sperm of stressed males may influence subsequent generations via alterations in early development. Thus, 2 weeks after cessation of CSI stress exposure, stressed male mice and control counterparts were mated and early embryos were collected. Consistent with this hypothesis, we discovered large decreases in the expression of both miR-449a and miR-34c in embryos isolated at the 2-cell, 4-cell,

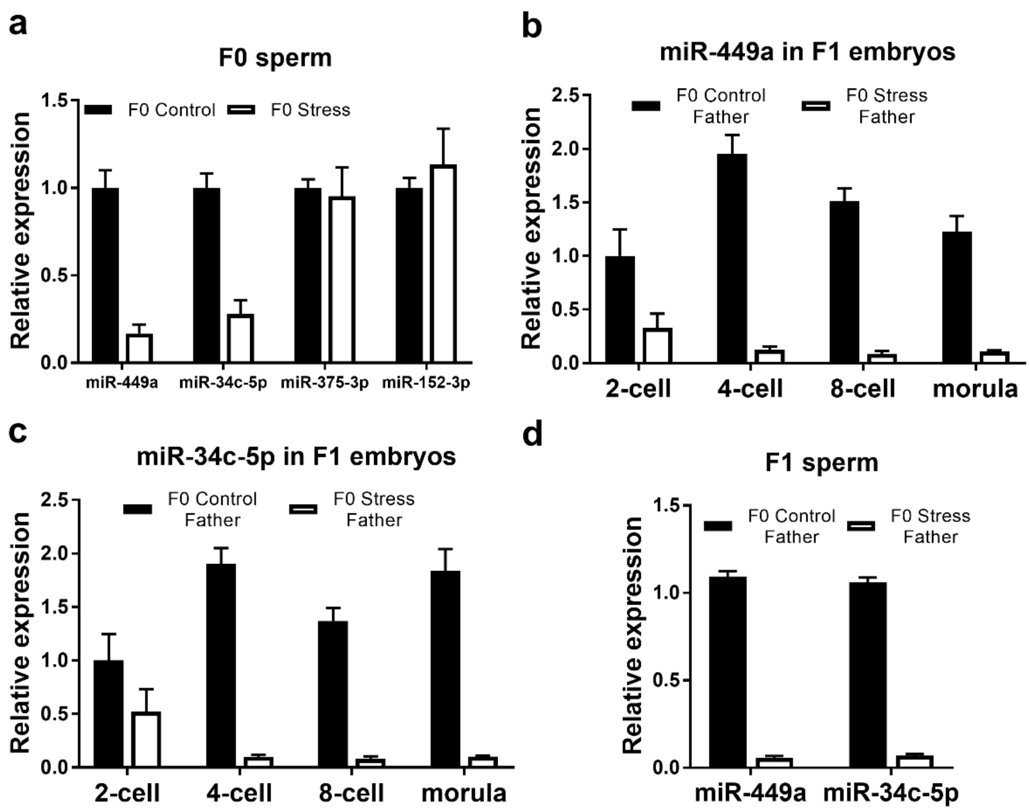

Figure 3.3: miR-449a and miR-34c are downregulated in sperm across generations and in early embryos derived from them in a mouse model of early life stress. **a** qPCR analysis of miR-449a, miR-34c-5p, miR-152-3p, and miR-375-3p in pooled mature motile sperm isolated from stressed or control mice, n = 4–6 males per pool, 1 pool per group. **b–c** qPCR analysis of miR-449a (b), miR-34c-5p (c) in pooled F1 embryos derived from mating control females with stressed or unstressed males. Embryos for each group of fathers and cell stage were pooled prior to analysis (n = 30–60 embryos per pool), 1 pool per group. **d** Same analysis as (a) performed on sperm from offspring of control and stressed fathers. All samples were pooled before triplicate qPCR analysis, presented as fold change (mean ± experimental error) normalized to **a** F0 control, **b** 2-cell control, **c** 2-cell control, **d** F0 control

8-cell, and morula stages (~two–tenfold) from the mating of stressed males to control females, compared to those from the mating of control males **(Fig. 3.3b, c**). When male embryos from stressed fathers reached adulthood, levels of their sperm miR-449a and miR-34c were assayed, and found to also be severely suppressed (~tenfold) compared to those from control males (**Fig. 3.3d**), consistent with our previous finding that these mice transmit stress phenotypes to their F2 offspring[137].

3.4 Discussion

In this study of Caucasian men, an inverse association was found between a measure of exposure to abusive and/or dysfunctional family behavior and sperm levels of multiple members of the miR-449/34 family. Prior studies in humans have documented environmental effects on sperm RNAs. However this study is the first to show that stress affects the levels of human sperm miRNAs, and those found reduced are different from miRNAs previously detected in response to smoking[152] or obesity[154]. In addition, the ACE questionnaire used in this study is designed to capture stressful events occurring ~20 years earlier during one's childhood. Moreover, although a high ACE score (>4) implies a very substantial health risk, only a minority of men (~20–30%) in this category present with significant psychological and physical problems as adults[36]. Despite this, almost all of the men with high ACE scores in our study displayed reduced levels of these miRNAs. If this pattern holds in a larger independent cohort of men, it would indicate that high levels of maltreatment and household dysfunction in childhood are associated with altered sperm miRNA profiles in adulthood regardless of whether men are susceptible or resistant to the psychological burdens associated with high ACE scores.

This is not to imply that current psychiatric state or recent traumatic experiences do not also suppress these sperm miRNAs. In fact, experiments reported here in mice detected severely reduced levels of sperm miRNAs 449a and 34c only 2 weeks after the cessation of social instability stress. Thus, the low levels of sperm miRNAs 449a and 34c observed in some men in this study with low ACE scores could be a consequence of their present psychiatric state or exposure to recent severe stressful experiences. They could also be due to early life traumas that are not surveyed in the ACE questionnaire, such as being bullied. In addition, low-sperm miRNA expression in men reporting low ACE scores could be due to the fact that some people under-report their adverse childhood experiences[157]. This could be because the survey asks sensitive questions such as exposure to sexual abuse that many are uncomfortable admitting occurs, or because some have suppressed these memories. These possibilities support a larger follow-up study, now in the planning stage, that surveys men's present psychiatric state along with their ACE scores and sperm miRNA levels. This may reveal that low levels of sperm miRNAs 449a and 34c are associated with men's present psychiatric problems, in addition to their early exposure to abuse, which could have translational significance.

Interestingly, both sharply reduced levels of sperm miR-449 and miR-34 family members and severe stress have been found to be associated with reduced sperm quality and fertility in men[158,159]. Consistent with these observations, we found low-sperm morphology score associated with low miR-449a expression. Additionally, sperm count and sperm motility showed a trend toward associating with expression of both miRNAs, although these were not significant. Thus, we hypothesize that the connection found previously between stress and sperm quality and fertility may be through, at least in part, stress effects on the levels of sperm miRNAs 449a/b and 34b/c.

This study is the first to identify novel sperm miRNA changes associated with exposure to childhood trauma in men. The relatively small sample size limits our ability to more deeply explore the association between ACE scores and miRNA expression, including additional mediating variables. Nevertheless, these findings will serve to inform a subsequent larger study that can support control for additional confounding factors in a multivariable environment. In addition, this was a cross-sectional design with only a single measurement of miRNA and assessment of exposure to childhood trauma. Longitudinal studies with information on behavioral and psychological factors through adulthood, with repeated measurements of sperm miRNA content, could illuminate how cumulative exposures affect miRNA levels, potential mediating factors and the durability of these effects and the association between these sperm miRNAs levels and fertility in men.

The discovery that low levels of multiple members of the miR-34/449 gene family are associated with men's high ACE scores also has potential implications for the next generation. A growing body of evidence, including phenotypes of knockout mice that cannot process miRNAs[156], supports the idea that paternal miRNAs are delivered to the zygote after fertilization and contribute to early embryo development. Also, other stress paradigms in male mice have been shown to increase expression of specific sperm miRNAs (not miRs 449 and 34), and injection of them into zygotes fertilized by normal males induces a stress phenotype in offspring that is consistent with that obtained by mating stressed fathers[142,143]. Interestingly, among identified sperm miRNAs, only miR-449a/b and miR-34b/c have been shown to be expressed specifically in sperm, not eggs, and are present in zygotes, supporting a mechanism for how the small amounts of these miRNAs in sperm can impact early development. Moreover, in humans, sperm miR-34b/c levels correlate with day-3 embryo quality, implantation rate, and pregnancy after intra-cytoplasmic sperm injection (ICSI) procedures used in fertility treatment, implying this sperm derived miRNA influences early development in humans[159]. Finally, the present study is unique among studies implicating sperm miRNAs in the transgenerational effects of stress by demonstrating that the same stress-induced miRNA expression level changes that occur in sperm of stressed male mice, persist in F1 embryos derived from them, and in sperm of F1 male mice derived from these embryos, which we showed previously[137] pass on stress-associated traits to their F2 offspring.

If a similar phenomenon occurs in humans, we predict that reductions in the levels of sperm miR-449a and miR-34c we detect in men with high ACE scores could be transmitted to their sons. If so, this process could yield another explanation for why some men in our survey display low miR-449a and miR-34c levels despite reporting low ACE scores. They may have inherited these sperm changes from their fathers because of their exposures to abusive and/or dysfunctional family life, a possibility we are planning to test in a follow-up study determining whether fathers of these men have higher than average ACE scores.

miR-449 and miR-34 have the same inhibitory seed sequence and function together in mouse development, such that knockout of either miR-449 or miR-34 paralogs alone does not yield a detectable developmental phenotypes, whereas knockout of both sets of miRNAs mice show defects in brain development and spermatogenesis caused, at least in part, by defective microtubule and associated cilia function[144]. The fact that we detect reduction in expression of paralogs of both miR-34 and miR-449 genes in sperm of men with high ACE scores, as well as in sperm of mice exposed to sociability stress and in embryos derived from them, adds to the potential functional significance of these findings.

The gene targets of these miRNAs that carry out the described developmental functions in both mice and humans have not been clarified, but documented targets in other cell types where these miRNAs are also expressed include p53, CDK6, c-MYC, HDAC1, and BCL-2, all of which could have long-lasting effects on embryo development[160]. Thus, the severe decrease in expression of both miR-449a and miR-34c we detect in early embryos from stressed male mice could alter brain development and the process of spermatogenesis in subtler ways than knockout mice. These developmental changes may contribute to the enhanced anxiety and defective sociability we observed in female offspring, and altered sperm miRNAs we detect in male offspring of male mice exposed to chronic social instability stress[137], a question now being addressed.

Interestingly, miR-34 paralogs have also been implicated in regulating the stress response in adult mice, although the findings are somewhat contradictory[146-148]. However, our evidence to date suggests those effects may be distinct from the transgenerational effect we have observed. First, the effects observed in adults have been detected in male mice, whereas we detect stress-related phenotypes only in adult female offspring of stressed males. Second, complete knockout of all miR-34 paralogs has no effect on basal anxiety, only changes induced by acute stress[144]. In contrast, we detect basal changes in anxiety and sociability in female offspring of stressed males. Third, our phenotype is likely the consequence of suppressing both 34 and 449 members of the miRNA family. Finally, we have observed severe decreases in miR-34c levels in early embryos derived from stressed males. However, to date we have not been able to detect significant changes in its levels in brain regions of adult females derived from these embryos, where these studies have documented the consequences of altering all three paralogs of miR-34.

It is known that children of parents who report high ACE scores are at higher risk for stress-associated behavioral problems, which has been assumed to be due to parental behaviors[161,162]. This study raises the possibility that another component is stress-induced miRNA changes in men's sperm. Thus, just as genetic testing of sperm DNA can assess risk across generations, future studies based on these findings may reveal value in epigenetic testing of sperm miRNA as well.

3.5 Acknowledgements

We thank Emily H. Feig, PhD, for comments on the manuscript. This work was supported by National Institutes of Health; grant number: RO1 MH107536 (to L.A.F.) and the Tufts Center for Neuroscience Research; grant number: P30 NS047243.

3.6 Extended Data

Table 3.2: **Univariate linear regression analysis of behavioral and sperm characteristics association with sperm miRNA levels**

Variable	miR-449a Beta	miR-449a 95% lo	miR-449a 95% hi	miR-449a *p*-value	miR-34c Beta	miR-34c 95% lo	miR-34c 95% hi	miR-34c *p*-value
BMI	-0.0374	-0.1230	0.0483	0.376	-0.0374	-0.1084	0.0336	0.287
Smoking	-0.1587	-0.6180	0.3007	0.484	-0.1697	-0.5281	0.1887	0.340
Drug use	-0.3066	-1.0500	0.4363	0.404	-0.1191	-0.7097	0.4715	0.682
Alcohol use	-0.4969	-1.2840	0.2903	0.206	-0.2419	-0.8735	0.3897	0.438
Sperm count	0.0036	-0.0022	0.0094	0.212	0.0026	-0.0020	0.0072	0.264
Sperm motility	0.0124	-0.0042	0.0290	0.135	0.0098	-0.0030	0.0226	0.127
Sperm morphology	0.0583	0.0020	0.1146	**0.043**	0.0237	-0.0226	0.0701	0.302

Regression analysis performed on all samples with known values of all variables of interest (n=25) using Graphpad Prism v7.0 for all parameters listed. For smoking and Alcohol, use was quantified as an ordinal variable with no smoking/drinking=0, past smoking drinking=1, current smoking/drinking=2, >1 drink/day=3. Significance considered p<0.05. BMI, Body Mass Index (kg/m2).

Chapter 4: Discussion

The overall goal of my thesis was to reveal the mechanisms behind the paternal transmission of the effects of stress across generations via stress-induced changes in sperm miRNA content. A variety of studies using distinct stress paradigms on male mice have shown that these sperm miRNA changes alter the next generation after they are transmitted to zygotes upon fertilization. In mice, I used chronic social instability (CSI) stress, which a former post-doc in the lab, Dr. Lorena Saavedra-Rodriguez showed elevates anxiety and leads to defective sociability specifically in female offspring. While male offspring did not display alterations in these phenotypes, they transmitted them to their female offspring for at least two more generations. Dr. Saavedra also found candidate sperm miRNA mediators miR-409-3p, and miR-467a, which will be discussed shortly, by virtue of the fact that the levels of the former were elevated, and the latter reduced, not only in sperm of F0 stressed males but also in the sperm of their F1 sons and F2 grandsons, who passed on these stress phenotypes to their female offspring.

My contributions, discussed below, added support for a role for miR-409-3p in the sex-dependent influence of paternal stress on anxiety across generations. Moreover, by expanding our studies to men exposed to early life abuse and/or dysfunctional families, I identified additional sperm miRNAs, miR-34b/c and miR-449a/b, that may be involved in this process in both our mouse model, and in human men. In the discussion below I will address the issues that arose from my studies, both regarding their strengths and their limitations, and how they can be interpreted in the context of findings from other groups using different stress paradigms that lead to distinct phenotypes, through distinct sets of sperm miRNAs.

One of the striking features of our mouse work is that only female offspring of stressed fathers display enhanced anxiety and defective sociability. There is a large body of evidence supporting the idea that females respond to stress differently than males and have different vulnerabilities to stress related psychiatric disorders[163]. This raises the possibility that the degree of female sensitivity to stress and/or to stress-associated anxiety disorders could be influenced by: a) one's father's exposure to stress passed on to them epigenetically, as we mentioned with our human study, or b) by their mother's experiences leading to *in utero* exposures that affect early embryo development. However, the step at which this sex specificity arises is of significant debate. It could be the case that in a stressed father, an individual haploid sperm containing an X chromosome could have a different sncRNA profile than that of a sperm containing a Y chromosome. Thus, the payload of RNA delivered to the zygote upon fertilization is different for embryos that will become males than those which will become females. While this could be the case for some miRNAs (the ones we have identified are not X-linked), a thorough analysis of this hypothesis would require a difficult level of single-sperm RNA sequencing that has only recently been met with any level of success[164]. Regardless, our experiments with manipulating miR-409-3p, and similar experiments performed by others, strongly suggest additional factors may be involved and will be discussed in detail below.

4.1 miR-409-3p

We have implicated miR-409-3p in the transmission of stress associated traits across generations by observing that its levels are elevated not only in sperm of stressed fathers but also in their sons and grandsons who can transmit these traits to their offspring. Of note, our study is the first to identify consistent sperm miRNA changes across three generations of the male lineage. Previous studies using other stress paradigms documented that elevated levels of sperm miRNAs, seen only in the original F0 stressed males, have functional consequences because when pools of these miRNAs were injected into normal zygotes they induced stress-associated behaviors in offspring[90]. However, when compared to the injection of a pool, injection of a single miRNA mimic did not demonstrate any observable phenotypes, although only one of the nine miRNAs was tested in this fashion[90].

Nevertheless, we tested miR-409-3p by this approach, via early zygotic microinjection with a miR-409-3p mimic. However, consistent with other studies, we found no behavior defects in female offspring, nor were injected males capable of transmitting phenotypes to their F2 female offspring. This result implied that elevation of levels of this miRNA in zygotes, which presumably mimics what occurs upon fertilization during mating with CSI stressed males, is not sufficient to either alter brain development or spermatogenesis to generate phenotypes in adult offspring. A caveat to this conclusion, however, is that although we attempted to inject a single sperm's worth of RNA into the zygotes (~10 fg[101]), we overexpressed the miR-409-3p mimic to levels in early embryos that were clearly greater than that which likely occurs upon mating of stressed males. This raises the possibility that any potential phenotypes induced might have been masked by an artifact induced by this procedure. It may also be the case that, similar to other groups, manipulations to multiple miRNAs together are necessary, such as the reductions in miR-34b/c and miR-449a/b that we discovered in relation to early life stress in men.

We also tested whether elevated levels of sperm miR-409-3p are necessary, if not sufficient, for the transmission of CSI induced changes across generations. To this end, we attempted to reveal whether injection of a specific miR-409-3p inhibitor into zygotes derived from stressed males could prevent anxiety or sociability defects in female offspring, and/or the ability of male offspring to transmit stress phenotypes to their offspring. However, we could not undertake this rescue experiment because we observed that offspring derived from zygotes of stressed fathers injected with a negative control inhibitor did not display the characteristic enhanced anxiety. It is possible this negative result was a side effect of the zygotic manipulations necessary for these experiments, including flushing them from pregnant mothers, injecting them in culture, and then re-implanting them into foster mothers, or of the negative control inhibitor used. This hypothesis is supported by the fact that these manipulations of zygotes in similar settings have been shown to alter their epigenetic state leading to differential gene expression in both early embryos and in adult animals[111]. Alternatively, maternal contributions to the transmission, a consequence of exposing females to stressed males that is bypassed in this paradigm, could be a necessary component for transmission of anxiety across generations, regardless of other contributing effects. This kind of maternal contribution to the transmission of traits from affected male mates, even when males are not involved in pup rearing, has been described in other paradigms[118].

Nevertheless, we still were able to glean some support for miR-409-3p in producing female offspring of stressed fathers with elevated anxiety, because we found that suppressing miR-409-3p in normal zygotes altered anxiety levels in the predicted direction, making injected animals less anxious than control animals. This manipulation showed a significant level of specificity because it did not alter sociability in the injected animals, and more importantly, just as we find in offspring of stressed fathers, only injected females showed reduced anxiety after inhibitor injection into zygotes. During microinjection of fertilized zygotes, all embryos are injected in an identical fashion, as we cannot select or screen for sex at this stage. Considering that miR-409-3p inhibition resulted in a female specific phenotype in adulthood, leaving males unaffected, this implies that male embryos either responded differently to an identical manipulation to that of their female counterparts, or were simply unaffected by it. Either way, it follows that there is a sex-specific divergent path occurring somewhere downstream of early fertilization. It would not be surprising to find that these phenotypes are influenced by sexual development and differential hormonal changes that occur during adolescence for these animals, although sex-specific gene expression has been demonstrated as early as the 8-cell stage, and much more so at the blastocyst stage of pre-implantation embryos[165]. As briefly mentioned before, none of the miRNAs we have studied (miR-409-3p, miR-34 or miR-449 families) are located on the X chromosome, so an X-linked phenotypic explanation would not explain the sex specificity in this case. This does not rule out such an X-linked miRNA-mediated paradigm from existing, mediating a different aspect of inheritance, or these miRNAs targeting X-linked genes, but it is unlikely in our case for the regulation of these specific miRNAs.

Thus, although not conclusive, this series of experiments suggest that elevating levels of zygote miR-409-3p alone is not sufficient, but may be necessary, for the transmission of anxiety traits to female offspring of CSI stressed fathers. Separate from its role in the transmission of traits across generations, the fact that we generated altered anxiety specifically in females by reducing the levels of early embryo miR-409-3p implies that this miRNA normally plays an early developmental role in brain development required for the normal generation of anxiety. How miR-409-3p in early embryos could alter brain function specifically in females remains to be determined. Unfortunately, its predicted mRNA targets do not give significant clues. Therefore, we attempted to gain insight into how miR-409-3p may influence early embryo mRNA profiles by comparing RNA sequencing data from 4- and 8-cell embryos from mating control female mice to either control, or CSI stressed male mice, and from control zygotes injected with either the miR-409-3p inhibitor or mimic (along with their respective controls) cultured to the 4-cell stage.

We hypothesized that early changes in expression of possibly a methylation regulator or chromatin modulator could induce an altered expression pattern early in development that is maintained into adulthood. Paternal stress altered only a handful of genes at the 4-cell stage, but a much larger amount by the 8-cell stage. This is consistent with the timeline of gene expression and methylation dynamics during early embryogenesis, such that embryos first become transcriptionally active around the 2-cell stage[111], and that a significant proportion of methylation reprogramming has occurred by the 8-cell stage[112].

Several of the genes altered at the 8-cell stage that show the same direction of regulation in 4-cell embryos have known roles in the development of the adult brain that could be plausible mediators of the altered behaviors identified in female offspring of stressed fathers. N-myc down-regulated gene 4 (*ndrg4*) is expressed in neurons throughout the brain and assists in maintaining the normal levels of brain-derived neurotrophic factor (BDNF)[113]. *Nfat5* is necessary for and is highly expressed in the developing fetal brain. It also has roles in mediating neuroinflammation and dopaminergic signaling[114]. *Snrpa1*, which was also decreased by miR-409-3p mimic injection, is a key component of spliceosomes and is necessary for the maintenance of pluripotency in embryonic stem cells[116]. Functioning spliceosomes are also critical for the survival of developing embryonic neurons[117]. Outside of these specific targets, which will need to be directly manipulated in embryos to establish their role in this paradigm, GO term enrichment analyses on all the genes altered at the 8-cell stage identified a significant enrichment in both mRNA metabolic process and DNA-binding transcription factor activity.

To further identify possible early gene targets involved in our paradigm, we turned to our miR-409-3p manipulations. Analysis of the genes altered in inhibitor injected embryos identified several possible targets that could be mediating the anxiolytic phenotype in adult animals. For instance, this includes several genes with known roles in cortical development and regulating neuronal progenitor proliferation, *Sertad1*[121], *Pbk*[122], *Cdk5rap2*[124], and *Id3*[125]. Our combined analysis of both miR-409-3p manipulations further identified three putative upstream regulators, HNF4A, RICTOR, and TP53, that could account for a significant portion of the gene changes at the 4-cell stage seen in both conditions. Given that none of these three genes showed significant changes in expression in any of our data sets, it is possible that if they are involved, they exerted their effect either before the 4-cell stage, or via a post-transcriptional or post-translational mechanism that does not alter mRNA levels or stability. Of note, RICTOR (aka mTORC2) contains 3 putative 3’ UTR target sites for miR-409-3p, and it has also been shown to modulate dopamine-dependent behaviors in the brain of adult animals, through its role in phosphorylating Akt[126]. Of note, the above findings are specific to the miR-409-3p manipulations, which may offer insight on the development of anxiety irrespective of our transgenerational paradigm. However, by combining the data for all of our groups of embryos, we did identify one gene, *Slc22a23*, with an expression pattern consistent with being an early target of miR-409-3p in developing embryos of stressed fathers. It was significantly decreased in both 4-cell and 8-cell embryos from stressed fathers, was decreased in miR-409-3p mimic injected embryos, and was significantly upregulated in miR-409-3p inhibitor injected embryos. *Slc22a23* is an organic cation transporter that is expressed abundantly in the developing and mature brain, however its specific role in neuronal cells or brain development remains to be elucidated[115].

Of note, both manipulations induced a much larger change in gene expression than that seen in the embryos of stressed fathers. It is likely that a portion of this altered gene expression profile is a result of the protocol necessary to perform these experiments, as discussed above (zygote extraction, culturing, etc.). It must also be noted that any changes seen in this analysis may only be half the expected magnitude, given that these experiments were performed on total RNA from the entire pool of collected embryos, and thus represents a mix of both male and female embryos. Furthermore, the conclusions drawn from these analyses are attenuated by the small number of replicate samples for each of our various groups of embryos, and thus can only offer suggestions as to possible mediators in the absence of direct manipulations of said targets.

4.1.1 Unanswered Questions and Future Directions

One of the most pressing unanswered questions for the field is how such small amounts of sperm miRNA delivered to zygotes upon fertilization make such a significant difference in early development, and subsequently adult cellular function. While we were not able to show that elevation of a single miRNA in embryos was capable of inducing phenotypes in offspring, others have by injecting pools of miRNAs. Those results strongly support the idea that delivery of elevated levels of specific sperm miRNAs found in stressed males upon fertilization contributes to the stress phenotypes observed in adult offspring when embryos develop. However, the levels of miRNAs in sperm are much lower than those found in most cells, including early embryos. This raises the question of how changes in their levels could have large impacts on embryos. This is particularly relevant to those very experiments described above that involve miRNAs that are known to already exist in oocytes and early embryos in addition to the small amount provided to the embryo by sperm. In fact, we could not measure increases in miR-409-3p in either F1 or F2 4-cell embryos from stressed fathers (**Figure 4.1**) and none of the other reports that involved injection of specific sperm RNAs have reported on their levels in embryos derived from stressed fathers.

A possible explanation could come from studies on the special regulation miRNAs are subjected to in germ cells and early embryos. The maternal RNA pool is vastly larger than that arising from the sperm, however miRNA function in oocytes is suppressed until the 2-cell stage of embryogenesis, offering a vital time window for paternal contributions to exert their effect[166].

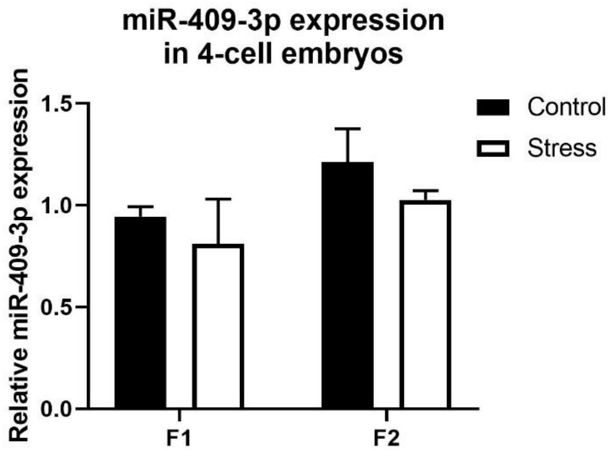

Figure 4.1: miR-409-3p expression in F1 and F2 embryos at the 4-cell stage derived from stressed fathers. qPCR analysis of miR-409-3p expression in 4-cell embryos derived from the mating of F0 stressed fathers (F1, left) or the F1 sons of F0 stressed fathers (F2, right). n=3 pools of 30-50 embryos each. Analysis by Student's t-test for each generation of embryo did not identify any significant differences.

While still only partially understood, post-transcriptional modification to sncRNAs, like 3' tailing of miRNAs, or specific methylation of sperm-borne tRFs[94] seem to confer a level of resistance to degradation for these transcripts. Thus, these modifications may allow for greater stability of the paternal transcripts after fertilization when compared to maternally derived RNA, including miRNAs, which demonstrates significate degradation (~2/3) through the 1-cell and 2-cell stages[166]. This thought is further supported by the finding that oocytes lacking *Dgrc8*, the Dicer associated protein responsible for miRNA processing, can mature in the absence of maternal miRNA function when fertilized by normal sperm[167]. Thus, paternally derived, sperm-delivered sncRNA may function in a privileged environment in the early embryo, allowing for a high level of control of the embryonic transcriptome.

Moving forward, we will need to generate more RNA sequencing data from embryos from CSI stressed and control fathers, as well as directed manipulations of gene targets in embryos, to fully implicate specific mRNAs in mediating these effects. We will pursue these experiments in the context of our other identified miRNAs, miR-34c and miR-449a, as well, to further enhance our understanding of what these miRNAs may be doing during early embryogenesis. We also aim to pursue this using single-cell or single-embryo sequencing, which in recent years has become a much more feasible and viable method to generate sufficient depth of sequencing for our purposes, such as the droplet-based 10X sequencing platform recently acquired by the Tufts Sequencing Core.

4.2 miR-467a

Another miRNA, miR-467a, was implicated in transmitting the effects of CSI stress during our early analysis of stress-associated sperm miRNA. In contrast to miR-409-3p, its expression in sperm from CSI stressed males was severely reduced, yet similar to miR-409-3p, this effect was also maintained through at least 3 generations of the male lineage (**Figure 4.2a**). Moreover, this decrease in expression was maintained through at least the first 4 cell divisions of embryogenesis in both F1 and F2 embryos, similar to miR-34c and miR-449a identified through our human study, supporting the idea that the sperm miRNA change we observed had functional consequences in embryo development that could contribute to phenotypes observed in adult offspring (**Figure 4.2b,c**).

That decreased spermatic levels of miR-467a could have such a strong impact on its embryonic expression may be due to a unique genomic feature of this miRNA's locus. It resides within a large mouse-specific intronic miRNA cluster in the gene *sfmbt2*, and is essential for early placental development[168,169]. Strikingly, this locus is maternally

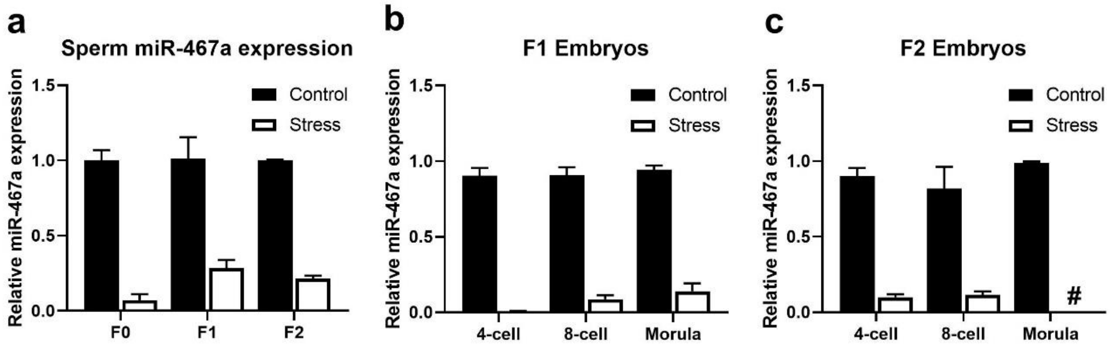

Figure 4.2: miR-467a expression is significantly decreased in the sperm of CSI stressed fathers, the sperm of their F1 sons and F2 grandsons, and in early embryos derived from their mating. a qPCR analysis of miR-467a expression for 2-3 groups each of sperm RNA pooled from 4-6 male mice from F0 (original stressed males), F1 (sons of F0 stressed males), and F2 (grandsons of F0 stressed males) generations. Analysis by 2-way ANOVA identified a significant effect due to stress across all three generations (P=0.0039). **b-c** qPCR analysis of miR-467a expression in 30-50 pooled embryos at 3 different stages; **b** – F1 embryos derived from mating F0 stressed fathers, **c** – F2 embryos derived from mating F1 males of F0 stressed fathers. Data presented as mean +/- SEM (**a**) or Error (**b-c**), and normalized to expression for control group for each individual generation and cell type. **#** - expression approximately 1000-fold decreased compared to control, thus unable to visualize on graph. Data for **a** collected by L. Saavedra. Data for **b,c** collected by L. Saavedra and D. Dickson.

imprinted, such that only the paternal copy of the gene is expressed until after implantation of the embryos, whereupon the maternal copy shows some level of re-activation (~ embryonic day 7.5)[170]. Also, some known mRNA targets for miR-467a are quite provocative in this context, because they include many known modifiers of gene transcription that could have long-lasting impact on embryo development, such as Rex1, Klf2, and Fgf5[171]. In line with this, *fgf5* expression was significantly increased in 8-cell embryos derived from stressed fathers in our RNA sequencing experiments, which is consistent with the severely reduced miR-467a expression we identified at this cell stage.

Interestingly, this miRNA cluster and the associated *sfmbt2* gene were identified by another lab studying a different intergenerational paradigm. They found that male

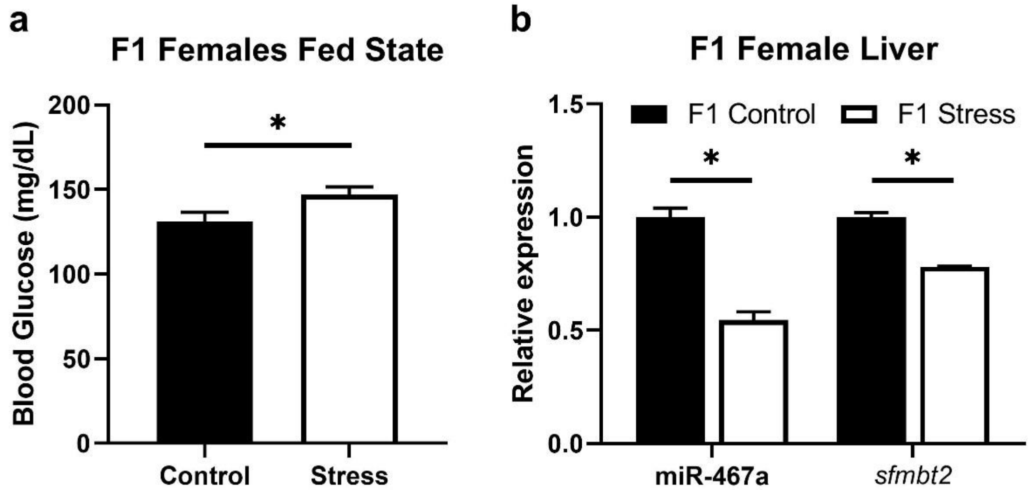

Figure 4.3: F1 Females of CSI stressed fathers display elevated fed blood glucose levels and decreased miR-467a expression in their livers. a Blood glucose measurements for F1 female offspring of control and CSI stressed fathers taken in the fed state during the light cycle, 1200-1400 hours, n=11 mice per group. Unpaired Student's t-test identified a significant difference between groups (*P*=0.0390; t=2.209). **b** qPCR analysis of miR-467a and *sfmbt2* expression in RNA isolated from livers of adult F1 females of control and stressed fathers, n=3 per group. Multiple unpaired Student's t-tests identified a significant decrease in expression for both targets, **P*<0.05.

mice exposed to 2 weeks of restraint stress produced offspring with hyperglycemia, a direct result of decreased miRNA expression from this cluster due to hypermethylation of the *sfmbt2* locus, beginning in the stressed males' sperm, and maintained into his offspring's livers[64]. In fact, we were able to replicate these findings in our F1 female offspring of CSI stressed fathers, who showed significantly higher fed glucose levels, as well as decreased expression of both miR-467a and *sfmbt2* in their livers (**Fig. 4.3a,b**).

While this raised the possibility that the decreased levels of miR-467a we observed in sperm and early embryos was to contribute to altered liver function, it did not rule out the possibility that it could also be involved in altering brain function to induce behavioral changes in female offspring, or sperm miRNA changes in male offspring by a

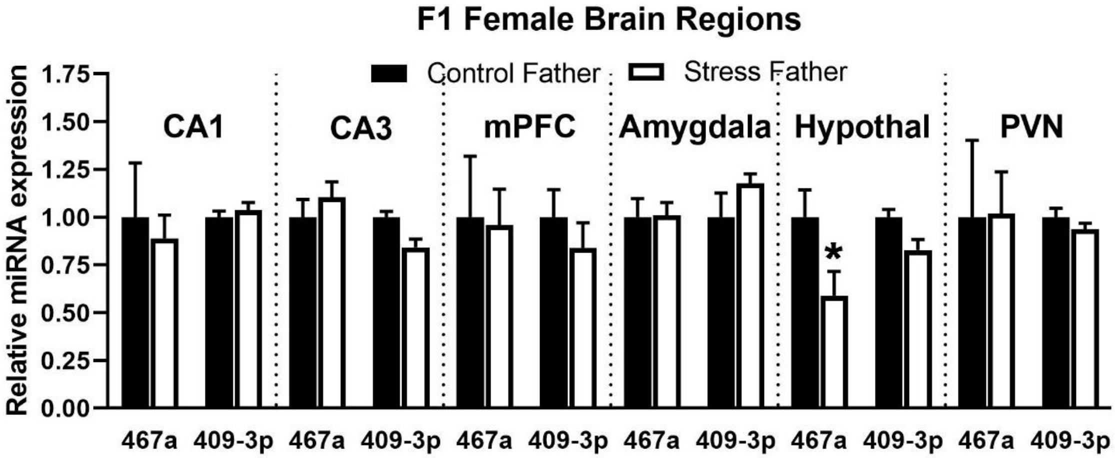

Figure 4.4: qPCR analysis of miR-467a and miR-409-3p expression in the adult brains of F1 female offspring of control and stressed fathers. qPCR analysis of both miRNA targets in a variety of brain regions dissected from the brain of F1 female offspring of control and stressed fathers, n=3 per group. Data analyzed via 2-way ANOVA for each brain region, with posthoc testing via Sidak's method to correct for multiple comparisons. CA1 and CA3 are subregions of the hippocampus; mPFC – medial prefrontal cortex; Hypothal – whole hypothalamus; PVN – Paraventricular nucleus of the hypothalamus. *$P<0.05$.

similar mechanism. We hypothesized that hypermethylation of this locus, if maintained into the brain, could lead to altered miRNA expression in a key region in the adult animals that could explain their altered behaviors. Offspring of mice exposed to the MSUS protocol for instance showed hypermethylation of the *Mecp2* locus in key brain regions[68], and other groups have shown both altered methylation and gene expression of specific loci in livers of offspring of fathers fed a high fat diet[62] or a low-protein diet[59]. However, qPCR analysis revealed no differences in miR-467a expression in a variety of brain regions in F1 female offspring of stressed fathers compared to those of control fathers, except for the hypothalamus (**Figure 4.4**). We also investigated miR-409-3p expression in these regions, which similarly showed no differences in expression in any of those analyzed. While there did seem to be a ~2-fold decrease in the whole hypothalamus for miR-467a, it is an extremely heterogeneous area with a variety of cell types and subregions whose functions range from stress, to social behaviors, to food intake[172]. Given its central role in the HPA axis, we investigated miR-467a expression in the paraventricular nucleus (PVN) of the hypothalamus in an additional cohort of animals, however, there was no difference in expression between F1 female offspring of stressed and control fathers for this subregion either (**Figure 4.4**).

4.2.1 Unanswered Questions and Future Directions

The work conducted on the intronic miRNA cluster residing in *sfmbt2,* especially when taken in the context of much of our data pertaining to miR-467a, raises an important question about this miRNA and the locus as a whole – are these changes solely involved in altering the metabolism of offspring, or could they also serve another function? While miR-467a was shown to have decreased expression in the liver, it was not directly implicated in the mechanism leading to hyperglycemia. That effect was instead attributed to the loss of miR-446b-3p expression, which was directly targeting Pepck, the enzyme for the rate-limiting step of gluconeogenesis[64]. Thus, the loss of miR-467a could be interpreted as just a marker of the hypermethylation of the entire locus. Furthermore, while the authors showed increased methylation in the liver, they never investigated this locus during early embryogenesis. If the methylation that was increased by stress in the father's sperm was maintained into the adult offspring, it was likely hypermethylated during development as well. This is also not to discount the possible role of the gene in which this miRNA resides serving its own function. *Sfmbt2* is a polycomb gene, and thus could have large effects on altering the genetic landscape in early embryos, evidenced by its necessary role in placental development[170].

Furthermore, if we assume that the loss of miR-467a expression could also be contributing the neurologic and behavioral phenotypes we observe in F1 offspring of stressed fathers, could restoring its expression back to normal levels shortly after fertilization rescue any of these phenotypes? We plan to return to this miRNA to test its effects on behavioral changes in offspring using a transgenic inducible mouse paradigm we are developing to test the consequence of reversing the stress-induced miRNA reductions in early embryos described in more detail below.

4.3 miR-34b/c and miR-449a/b

By turning to human samples in our study of Caucasian men, we identified additional sperm miRNAs, the miR-34bc/449ab families, as potential mediators of the transgenerational effects of stress in not only men, but also in mice. In particular, we discovered an inverse association between the degree of men's exposure to abusive and/or dysfunctional family behavior (as measured by the ACE score) until age 18 and sperm expression of multiple members of the miR-34/449 families as adults. Prior studies in humans have documented environmental effects on sperm RNAs, however this work is the first to show that stress alters the levels of human sperm miRNAs. Importantly, the implicated miRNAs are different from miRNAs previously detected in response to smoking[106] or obesity[107].

These results are further bolstered by the fact that the same sperm miRNAs altered in humans were similarly reduced in the sperm of male mice exposed to the CSI protocol, where we first detected changes in sperm miR-409-3p and miR-467a described above. The human study motivated us to reanalyze our mouse miRNA array data and found we had overlooked similar reductions in mouse sperm miR-34b/c and miR-449a/b in CSI stressed fathers. Just as we found for mouse miR-467a, this decrease in sperm miR-34c and miR-449a expression was also maintained through at least the first four cell divisions of embryogenesis in embryos derived from the mating of CSI stressed fathers. This pattern also persisted into the sperm of F1 male offspring of the original stressed fathers who similarly retain the ability to pass on altered behavioral phenotypes to their own female offspring.

In addition to the significance of detecting the same sperm miRNA changes of miR-449 and miR-34 in both mice and men exposed to early life stress, the implications of altering these miRNAs are intriguing because of their known functions. First, they have the same inhibitory seed sequence and function together in mouse development, such that knockout of either miR-449 or miR-34 families alone does not yield a detectable developmental phenotype[104]. However knockout of both sets of miRNAs in mice leads to defects in brain development and spermatogenesis caused, at least in part, by defective microtubule and associated cilia function[105]. Single knockout studies also demonstrated a compensatory increase in expression of the unaffected miRNA family, further suggesting they do in fact share some degree of functional redundancy[104]. While the knockout studies demonstrated profound developmental effects in mice lacking both miRNA families, it may be the case that the decreased expression of miR-449a/34c that we detect in early embryos from stressed male mice could alter brain development and the process of spermatogenesis in subtler ways than knockout mice, leading to more mild phenotypes, considering that there are at least still detectable levels of these miRNAs in the embryos.

The gene targets of these miRNAs that carry out the described developmental functions in both mice and humans have not been clarified, but documented targets in other cell types where these miRNAs are also expressed include p53, CDK6, c-MYC, HDAC1, and BCL-2, all of which could have long-lasting effects on embryo development[173]. These developmental changes may then contribute to the enhanced anxiety and defective sociability we observe in female offspring, and the altered sperm miRNA profiles we detect in male offspring of male mice exposed to chronic social instability stress.

The significant decrease in expression of sperm miR-34c and miR-449a that persists in early embryos derived from stressed fathers raises additional questions as to how stressful experiences induce these changes in sperm. The miRNA profile of sperm is known to change along the length of the epididymis, as is the miRNA profile of the epididymal cells lining the lumen where the sperm mature[174]. It was recently demonstrated that these epididymal cells releases exosomes ("epididymosomes"; containing predominantly tRFs, miRNAs, piRNAs) into the lumen, which can spontaneously fuse with the maturing sperm, thereby altering the sncRNA profile[92]. This process was implicated in an intergenerational inheritance paradigm pertaining to high-fat diet[94] and low-protein diet[91], where the affected animals showed altered tRF expression in the epididymosomes that in turn led to a similarly altered tRF profile in the mature sperm that would eventually fertilize embryos. Of note, this proposed mechanism can be contrasted by that discovered in the context of paternal restraint altering F1 gluconeogenesis, where it was the role of the miRNA in the adult organ (the liver) that mediated the phenotype, regardless if that miRNA was affected in the sperm of the stressed males[64].

Data on the levels of sperm miRNAs as they develop down the length of the epididymis actually supports the epididymosome model for miR-34c. Its levels dramatically increase throughout sperm maturation[174], while sperm are transcriptionally inactive, implying this increase occurs via absorbed miRNAs from epididymal exosomes. Moreover, both miR-34c and miR-449a are two of only 14 miRNAs that are present in sperm, not oocytes, which could help explain how they can have dramatic effects upon delivery to zygotes following fertilization[102]. However, the amount of RNA in individual sperm is much smaller (~10 fg[101]) compared to most cells. In fact, to measure miR-34c and miR-449a we need to pool thousands of sperm, typically from several mice. Nevertheless, we could easily measure them in as little as 30 2 or 4-cell embryos collected from control matings. Since that represents 30 sperms' worth of these miRNAs, the amount of RNA in these embryos delivered by sperm represents only a tiny fraction of the total RNA isolated from pools of embryos. Not only does this minimize the potential role for the sperm-derived miR-34c and miR-449a molecules in early embryo development, it makes it difficult to explain how this decrease in sperm miRNAs found in stressed males can lead to the large decrease in the levels of these miRNAs in early embryos derived from stressed fathers.

An alternative hypothesis is that the majority of miR-34c and miR-449a in embryos is from embryonic transcription of their genes. An in-depth RNA sequencing study of early embryos strongly suggests this is the case for a large number of miRNAs after the first wave of zygotic activation between the 1-cell and 2-cell stage[166]. Moreover, stress decreases the expression of miR-34c and miR-449a in sperm, which could be explained by methylation of these loci. It is possible then that this epigenetic alteration is preserved after fertilization, such that the embryo gene expression remains inhibited. This effect is further magnified by the fact that these miRNAs are not expressed from the maternal genome in oocytes before fertilization.

Alternatively, there may be a feed-forward positive effect of sperm miRNAs on endogenous gene expression of the same miRNA in early embryos that explains this conundrum. Of note, this decreased embryonic expression does not yet seem to be maintained into any adult brain tissue. qPCR analyses on a variety of brain regions, including the hippocampus, amygdala (lateral and central), mPFC, and some hypothalamic subregions, including the medial preoptic area (MPO), arcuate nucleus (Arc), ventromedial hypothalamus (VMH), and PVN, did not identify any consistent changes in expression for either miR-34c or miR-449a in tissues from F1 females of stressed fathers, compared to those from control fathers. This suggests these miRNAs are exerting their effects either earlier on during development, or in a different brain region that we have not yet tested.

Interestingly, both sharply reduced levels of sperm miR-449 and miR-34 family members and severe stress have been found to be associated with reduced sperm quality and fertility in men[175,176]. Consistent with these observations, we found low-sperm morphology scores associated with low miR-449a expression. Additionally, sperm count and sperm motility showed a trend toward associating with expression of both miRNAs, although these were not significant, likely a result of the small size of our study. Thus, we hypothesize that the connection found previously between stress and sperm quality and fertility may be through, at least in part, stress' effects on the levels of men's sperm miRNAs 449a/b and 34b/c.

Our analysis of demographic characteristics did not identify any potential confounding variables in association with ACE score across our three groups (ACE 0-1; ACE 2-4; ACE $\geq$5). While there was a trend for increasing age with higher ACE score, an average age of 36.7 years in the High ACE group when compared to 30.1 years for the Low ACE group does not represent an appreciable clinical difference. A similar paradigm was seen for sperm morphology scores, however all median sperm quality scores, including morphology, motility, and sperm count, were within the normal range according to the most recent guidelines from the World Health Organization[177]. Importantly, neither ACE score, nor miR-34c or miR-449a expression showed a correlation with either BMI or the smoking history of our participants, as both of these factors have been shown by others to alter sperm miRNA content (**Table 3.2**).

The finding that both miRNAs show a consistent correlation with ACE scores raises the possibility that they could serve as biomarkers of early life stress. The ACE survey is a self-reporting measure and has been shown to be influenced by the participants current neurologic state[178]. Furthermore, people may not remember or may have repressed some forms of abuse that occurred during their childhood. Thus, an objectively measured biomarker of early life stress would be of significant use during risk-assessment for clinicians, as it could remove the implicit bias of a self-report measure, and possibly detect other forms of trauma not explicitly discussed in the ACE questionnaire (i.e. those occurring after the age of 18).

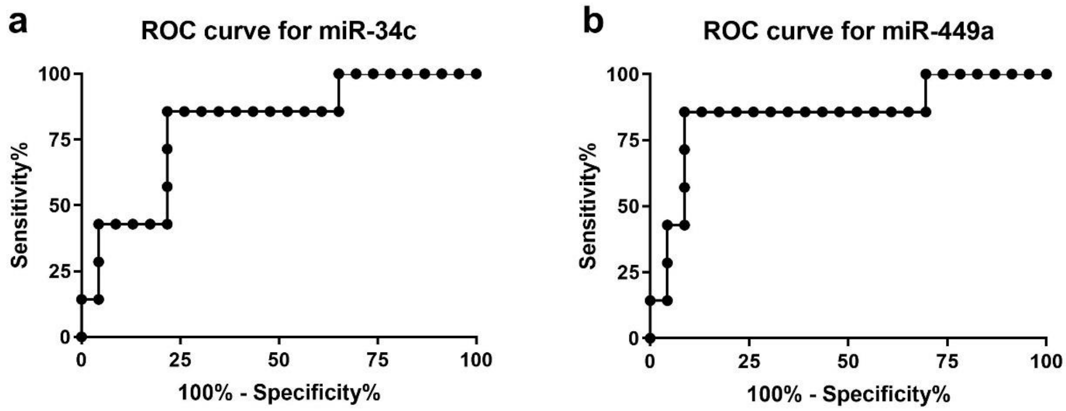

Figure 4.5: Receiver Operator Characteristics Curves for miR-34c and miR-449a show a high degree of sensitivity and specificity in predicting whether men belong to a Low ACE or High ACE group. **a-b** Samples separated into Low ACE group (score: 0-4, n=23) or High ACE group (score: 5-10, n=7) for Receiver Operator Characteristics Curve (ROC) analysis. Total Area Under the ROC Curve (AUC), Std. Error, p-value for both targets are: 0.8012, 0.0943, 0.0174 (**a**: miR-34c), and 0.8509, 0.0952, 0.0056 (**b**: miR-449a). Cutoffs for optimal sensitivity/specificity are 85.71%/78.26% (**a**: miR-34c), and 85.71%/91.30% (b).

Although our study was carried out on a small number of participants, the magnitude of decrease in miR-34c and miR-449a expression in men with highest levels of trauma was close to 300-fold less than that of their low ACE score counterparts. While there were several men with low ACE scores that also had low sperm miRNA expression of miR-34c and miR-449a, only one of the men with a high ACE score began to approach an average amount of these two miRNAs. This suggests that high levels of miR-34c/449a in the sperm could have a remarkable specificity for identifying men without significant levels of childhood trauma. In accordance with these observations, a Receiver Operator Characteristics (ROC) curve based upon our sperm miRNA expression data shows that these two miRNAs are able to quite accurately predict whether patients belong to a Low ACE group (0-4) or High ACE group (≥5) with high levels of sensitivity and specificity (**Figure 4.5**). These cutoffs for these binary groups are based upon the original ACE study, which suggested that patients with ACE scores above 4 were at a significantly increased risk for developing many of the negative health outcomes that were examined. miR-34c expression showed a sensitivity and specificity of 85.71% and 78.26%, respectively, and miR-449a showed 85.71%/91.30%. These correspond to a positive likelihood ratio for miR-34c of 3.943, and for miR-449a of 9.857. This means, for instance, that a man with significantly decreased sperm miR-449a expression (below the threshold used for the test) is ~10 times more likely to have a High ACE score than someone with sperm miR-449a expression above the cutoff. While these statistics are based upon only a small number of patients, limiting their external validity, they at the very least offer a strong suggestion of possible clinical application if they are examined in a much larger and distinct cohort of men in the future.

4.3.1 Unanswered Questions and Future Directions

We next aim to compare the methylation status of the miR-34c and miR-449a genes in sperm and early embryos between control and stressed males. Future experiments will entail a detailed analysis of expression of these targets, their primary transcripts, and their methylation status in sperm and epididymal cells (and their epididymosomes) from the entire length of the epididymis, including the caput, corpus, and cauda. We may find that the decreased miRNA expression exists in sperm even at the earliest stages of epididymal maturation, suggesting a germ-cell alteration that affects sperm miRNA content. Alternatively, similar to the mechanism described for tRF expression in mature sperm, the loss of miR-34b/c and miR-449a/b in mature sperm of CSI stressed males may represent a lack of delivery via epididymosomes of these targets.

This analysis will also help uncover whether these changes are associated with hypermethylation of these loci, either in the epididymal cells providing the exosomes, or in the maturing sperm themselves. As discussed above, alterations in methylation status may help explain the continued lack of expression of these miRNAs seen in embryos derived from CSI stressed fathers.

We will also begin to search for the possibility of transmission of these stress-induced declines in human sperm miR-34c and 449a that we observe in men. If our hypotheses pertaining to the inheritance of these sperm miRNA changes holds true in humans as we have seen it occur in mice, this raises an interesting possibility to explain the large variation in miR-34c and miR-449a expression we saw in men with low ACE scores (see **Figure 3.2**). In the event that these men's fathers had significant early life stress, a man with a low ACE score could theoretically inherit the low sperm miRNA expression from his father. While he did not have a traumatic childhood, his sperm miRNA expression would suggest the opposite, making him an outlier to our paradigm.

Unfortunately, it was not feasible for us to also collect sperm samples from men's fathers considering that sperm quality declines significantly with age[179]. However, it would be possible to administer the ACE survey to these men's father to have an indication of the level of early life trauma their fathers experienced during their life, and then we could attempt to correlate how much the father's experiences influenced the sperm miRNA expression of these "outlier" men. Additionally, while CSI stress results in male offspring with altered sperm miRNA expression, it is only the female offspring that display enhanced anxiety and defective social interactions in our mouse model. Thus, it could be possible that men with low ACE scores and low sperm miR-34/449, whose fathers also had high ACE scores, could have sisters that are at an increased risk for developing anxiety or depression. With this future work, we will aim to survey the sisters of the men providing sperm samples with anxiety and depression scales to see if such a correlation exists. An additional limitation of our study was that we also did not account for current mental health phenotypes, or if the men have taken antidepressants or similar psychiatric medications at any point in their life. These are potential confounders that we will be sure to address in the larger clinical study we are currently planning.

While this study focused mainly on the miR-34 and miR-449 families, our initial microarray screen in these men identified many additional miRNAs, at a lower significance, that also showed a correlation with ACE score (See Appendix, **Figure 5.1**). We did attempt qPCR on some of these that either did not show a correlation across all samples, even though they were changed in our initial cohort (such as miR-152-3p), or were inconsistently detectable via qPCR (such as miR-1273g-3p). However, there are many other miRNAs to test and these will be a subject of future investigations, both in our current and future human samples, and in our mouse model.

While identifying similar sperm miRNA changes in both mice and men exposed to early stress, in the same miRNAs, raises the significance of our findings, the main limitation of this study is that these changes remain no more than correlational at this point. Thus, we will also attempt to prove that the reductions in miR-34c and miR-449a in embryos derived from the mating of stressed fathers contributes to some aspect of the phenotype observed when these offspring mature to adulthood. Our hypotheses are strengthened by the previously discussed knockout mouse models of these two miRNA families showing their involvement in spermatogenesis and brain development, but we have yet to demonstrate mechanistic involvement of these two miRNAs in our model. Again, other labs have shown that injection of either total RNA from sperm of stressed males, or a combination of miRNA mimics, into fertilized zygotes was able to recapitulate most of the phenotypes that offspring of stressed fathers exhibited[88,90]. However, neither group attempted to prevent the transmission of these phenotypes in a rescue experiment. Considering our two miRNAs of interest are regulated in the opposite direction to those implicated in those above studies, we decided to design a transgenic doxycycline-inducible miR-34c expressing mouse in order to answer these questions in a more physiologic manner.

From our mouse work, we know that expression of miR-34/449 is decreased both in the sperm of stressed fathers, and in early embryos derived from mating those fathers. Given that the 34/449 double knockout mice showed altered brain development, we believe these miRNAs are serving a vital role early in embryogenesis that goes mostly unfulfilled in embryos from stressed fathers that do not have the normal expression of both of these miRNA families. Importantly, given the redundant nature of these two families, we also believe that loss of one family can be compensated for by the remaining one. Thus, we hypothesize that by rescuing expression of one of these miRNAs shortly after fertilization (we chose miR-34c, given that its naturally high expression may be more forgiving), we may be able to prevent the development of either the female-specific behavioral phenotypes, or the male-specific altered sperm miRNA expression, or both. Either result would imply that decreased embryonic expression of miR-34c and miR-449a are necessary to transmit these phenotypes to the next generation.

Thanks to recent advances in tetracycline-inducible systems, single plasmid constructs now exist that contain both the tet-responsive transregulatory protein, and the tet-responsive element promoters for a gene of interest. We chose such a commercially available system, the pTetOne construct to accomplish our goals. We first cloned in the miR-34c locus into this plasmid, and then performed some basic induction experiments on HEK293T cells to ensure the plasmid was functioning as expected (See **Appendix Section 5.2 for more details**). Untreated cells transfected with 0.5 ug of plasmid showed only a ~1.5-fold increase in miR-34c expression over un-transfected cells, and doxycycline induced a close to ~200-fold increase in expression over baseline (See **Appendix, Figure 5.3**). As our qPCR kit only detects the mature form of miRNA, this confirmed that our construct design creates functional miR-34c with a robust activation to doxycycline.

We have since created a transgenic mouse line using this plasmid (through the Transgenic core at MMCRI) and have begun characterizing these transgenic animals. Once our colony is established, and consistent miR-34c induction can be demonstrated *in vivo* in these animals, we will employ a unique paradigm in order to rescue miRNA expression shortly after fertilization in a controlled manner. Briefly, we will subject a cohort of transgenic male mice (which we will refer to as tet-34c mice) to the chronic social instability protocol to create CSI stressed male mice. We will then mate these stressed tet-34c males to naïve, wild-type female CD-1 mice who have been maintained on a doxycycline diet. When fertilization occurs, and the paternal genome is exposed to the doxycycline that already exists at steady state in the maternal circulation, the transgene will begin expression of miR-34c, restoring it to normal levels in these embryos. When the pregnant mice are then placed back on normal chow, miR-34c induction will cease, allowing us to have temporal control over the amount of time that we will rescue the miR-34c expression. Furthermore, the concentration of doxycycline in the chow will allow us to titrate the level of induction of miR-34c in this paradigm, which we will give us further control over how much miR-34c is expressed. It may be the case that too much overexpression of miR-34c would lead to deleterious effects, which could actually warrant future investigation into better understanding the role it plays in early development (however, this is outside of the context of these rescue experiments). As the tet-34c male mice will be heterozygous for this transgene, each mating will generate a litter composed of half control, non-transgenic, embryos exposed to dox in the same mother and same *in utero* environment as induced tet-34c embryos, allowing for an excellent level of internal control. Once the mice are raised and behaviorally assayed, they can simply be genotyped after testing to place them into separate groups for analysis.

If we achieve any measure of success in these endeavors, we can use this protocol to harvest early embryos for sequencing to uncover the role miR-34c is playing in early embryogenesis. As mentioned, we can also vary the timeline of miR-34c induction to determine if there is a critical window for rescue to occur. This also offers significant advantages over our previous experimental design by removing the need for the complex embryonic manipulations, including microinjection, culturing, and re-implantation of fertilized embryos, that likely alter the epigenetic landscape. Furthermore, our induction will occur in the same females that mated with the stressed males, allowing for us to include the possible maternal behavioral response mentioned previously, although the males will be removed after successful mating to avoid any component of paternal care.

We will also attempt to follow the expression of these miRNAs (and miR-467a) throughout development to further understand how long we should be rescuing their expression. We have had success using a miRNA-specific fluorescence *in situ* hybridization (miR-FISH) on brain tissue, and will adapt this protocol to study miR-34c, miR-449a, and eventually miR467a expression in developing embryos.

A key component of epigenetic changes is that they imply a level of reversibility, which raises the question - if these miRNA changes do in fact serve a direct role in altering the behavior of the next generation, is there any intervention that could revert their expression back to normal in either the stressed men, or mice, in order to prevent them from passing on these altered phenotypes? Work performed on the MSUS model found that exposure to EE can prevent the transmission of the paternal effects of early trauma onto the next generation[84], and providing mice on a high-fat diet with exercise wheels in the home cage prevents the transmission of metabolic phenotypes to their offspring[63]. We have previously trialed daily fluoxetine injections following stress and found significant behavioral improvement, however the effects on sperm miRNA expression were inconclusive. These experiments bear repeating, and will be a focus in the near future in conjunction with other treatments.

4.4 Different stressors, different phenotypes, different miRNAs

The number of mouse studies pertaining to epigenetic inheritance has been climbing over the last decade. There are a large variety of environmental modulations that have been shown to induce these effects across generations, leading to a large variety of phenotypes in the offspring of the next generation. However, in the context of stress, it is difficult to understand why there is such a significant disparity in offspring outcomes when the final mediator of all stress is, presumably, changes in corticosterone levels, typically increasing after stress. In the MSUS model, stressed male mice transmit altered behavioral phenotypes to both male and female offspring, although male offspring are typically more affected. In the CVS model, stressed male mice transmit a blunted HPA axis response to stress to both male and female offspring. In the chronic social defeat model, stressed male mice transmit altered phenotypes to both their male and female offspring, although more severely to the males. In the repeated paternal restraint stress model, two weeks of restraint led to hyperglycemia in both male and female offspring, and two months of restraint led to decreased levels of anxiety in female offspring. In our model of CSI stress, stressed male mice transmit enhanced anxiety and defective social interactions to only their female offspring.

Furthermore, both the MSUS and CVS models have also implicated altered sperm miRNA expression as a potential mechanism, however there is no overlap in altered miRNAs between our paradigm and either of these, and the only miRNA common to both MSUS and CVS stressed males, miR-375, was not altered by stress in either our human samples or our CSI mouse samples (See **Figure 3.2, 3.3**). This may help explain the distinct phenotypes of the F1 generation, but not how these different sperm miRNAs are altered in these paradigms. In the model of paternal restraint stress leading to hyperglycemia in offspring, daily paternal injection of a synthetic corticosteroid was sufficient to recapitulate the metabolic phenotypes of the next generation. If this is all mediated by CORT levels, where then does the miRNA specificity come into play?

While all of these paradigms employ stress, the timing, length, and severity of the stress vary greatly. Thus, the difference must lie farther upstream, possibly at the level of corticosterone kinetics in the stressed animals. MSUS animals are stressed daily for two weeks, only during the postnatal period, presumably leading to longer, yet regular, daily activations of CORT. It is also the earliest time period for stress in any of these paradigms. The restraint stress model occurs during adulthood, and similar to MSUS likely results in consistent, daily, and robust activations of CORT. The group demonstrating F1 behavioral effects in this model employed an 8-week protocol, the longest of these paradigms. The CVS model (6 weeks long) alters sperm miRNA expression regardless if it begins during early adolescence, like our CSI stress protocol, or later in adulthood. The nature of that stress paradigm likely leads to the least consistent activation of the CORT response of these paradigms, given its random nature and large variety of stressors. Our CSI protocol is the second longest stressor (7 weeks), and likely leads to the longest periods of sustained CORT activation of all the paradigms. Given the social nature of the cage changes, the mice are consistently subjected to a stressful home cage environment, much more so on days with the actual cage changes. Therefore, I hypothesize that the different CORT signaling patterns over different lengths and different developmental periods, must be resulting in distinct downstream signaling pathways, either in sperm germ cell development, or sperm cell maturation along the epididymis, that lead to disparate sncRNA profiles in the mature sperm. Further elucidation of the mechanisms leading to the alteration of sperm sncRNA profiles, either from epididymal cells or another yet undiscovered route, will be vital in elucidating these differences between paradigms.

Of course, this is not to rule out the effects of location and mouse strain on these differential results. We use CD-1 mice, an outbred strain, whereas the MSUS, CVS, and 2-week restraint stress paradigms use inbred C57/BL6 mice. Furthermore, it is impossible to control for the differences in mouse facilities and mouse vendors that could introduce significant changes in how these mice behave, such as through the microbiome. Of note, our CSI protocol is the only transgenerational paradigm to have been replicated by another laboratory, using a distinct strain of mice[65]. What is also unique about our protocol is that it results in decreases, rather than increases, of sperm miRNAs across generations. As described above, there does not appear to be sufficient miRNA expressed in sperm to account for the declines in expression we see in the early embryos of CSI stressed males, suggesting a different mechanism of action.

Thus, we hope to gain significant insight into this problem through a more thorough analysis of the expression dynamics of the miR-34 and miR-449 families, and their methylation status, along the epididymis and sperm maturation process in our stressed animals. Additionally, our work is the only to demonstrate sperm miRNA changes in the F1 generation of males, and as such, parallel experiments in F1 males will be undertaken to identify consistent changes across generations, increasing the potential significance of our findings.

Chapter 5: Appendix

5.1 Human Sperm Microarray Data

Our initial exploratory step to begin investigating the regulation of sperm miRNAs in our human samples was to send off a small cohort of 10 samples for a preliminary analysis to identify putative targets to follow up in all patients individually. We randomly selected 5 samples from men with low ACE scores (ACE 0-1) and 5 samples from men with the highest ACE scores (ACE 5-10). 1 ug of total RNA was sent to LC Sciences Inc. (Austin, TX) for microarray analysis. Each sample was run on its own custom µParaflo microfluidic chip developed by LC Sciences. Each chip investigates expression of every experimentally verified human miRNA currently listed in miRbase v21. After hybridization, fluorescence images were collected, analyzed, and quantified by LC Sciences to generate a list of differentially expressed miRNAs, using the appropriate statistical analysis and correcting for multiple comparisons, between our low ACE group (scores 0–1, n = 5) and high ACE group (scores >4, n = 5). This analysis identified 4 miRNAs differentially expressed at the highest significance ($P<0.01$), an additional 31 miRNAs at $P<0.05$, and another 41 miRNAs at $P<0.10$ (**Figure 5.1**).

5.2 Tet-inducible miR-34c mouse

Using mouse genomic DNA, we synthesized custom primers to clone out the miR-34c gene, including ~100 bp of flanking gDNA on either side of the ~90 nt locus, while synthesizing in an EcoRI and BamHI restriction enzyme site to the 3' and 5' end, respectively. This paradigm has been previously employed with miR-34c for use in bovine embryos[180]. Using these unique restriction enzyme sites, the miR-34c locus was

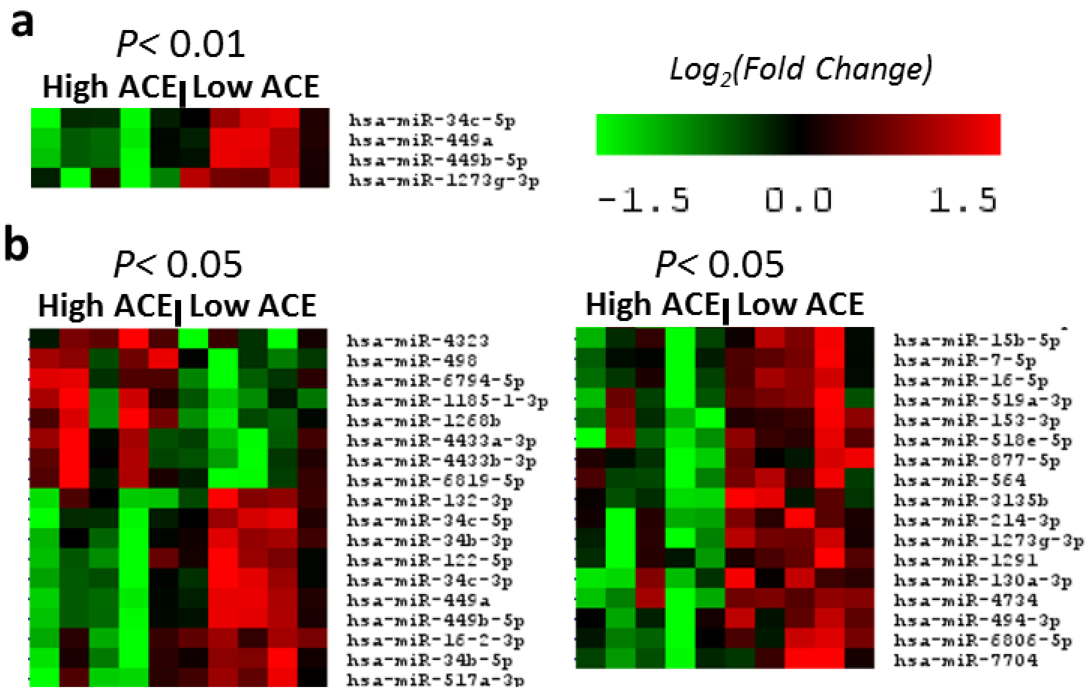

Figure 5.1: Microarray analysis on 10 human sperm samples for differential miRNA expression due to ACE score. a-b Heatmaps displaying results from miRNA microarray differential expression analysis by LC Sciences Inc. Green squares represent a significant decrease in expression compared to the average value for all samples, and red squares represent a significant increase. miRNAs identified in **a** are at a multiple comparison corrected *P*<0.01, and those in **b** are at a multiple comparison corrected *P*<0.05.

ligated into the multiple cloning site (MCS) of the pTetOne plasmid (Takara Bio) (**Figure 5.2**).

Next, we needed to ensure with using this strategy that a) cloning in the entire miR-34c locus, with extra flanking DNA, would produce functional, mature miR-34c, b) that culturing in doxycycline would induce robust overexpression of miR-34c compared to untreated cells, and c) that the baseline levels of miR-34c in 34c-pTetOne transfected, but untreated, cells was not significantly higher than that of un-transfected cells. To investigate these concerns, the 34c-pTetOne plasmid was transfected into HEK293T cells and cultured with and without the presence of doxycycline (1ug/mL) for 48 hours.

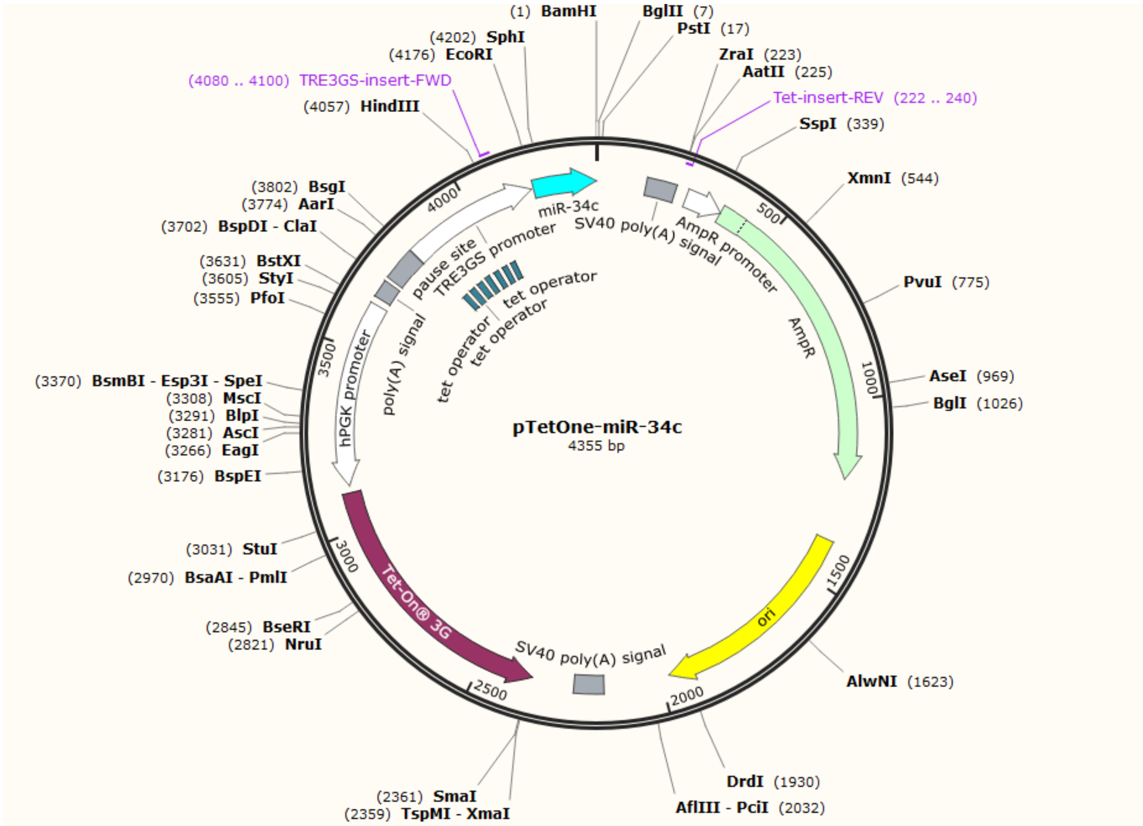

Figure 5.2: Map of the pTetOne plasmid containing the miR-34c locus and flanking genomic DNA. mmu-miR-34c genomic locus and ~100 bp of flanking genomic DNA on either side of the locus were cloned into the pTetOne vector using the BamHI and EcoRI restriction enzyme sites. TRE3GS-insert-FWD and Tet-insert-REV primers used for sequencing to original confirm successful integration of the miR-34c locus into the plasmid, and are also used for genotyping the transgenic animals.

Untreated cells transfected with 0.5 ug of plasmid showed only a 1.5-fold increase in miR-34c expression over un-transfected cells, and doxycycline induced a close to ~200-fold increase in expression over baseline (**Figure 5.3**). As our qPCR kit only detects the mature form of miRNA, we confirmed that this construct design creates functional miR-34c with a robust activation in the presence of doxycycline. A large quantity of this plasmid was grown, carefully purified, and linearized (**Figure 5.3**) using the SspI restriction enzyme site.

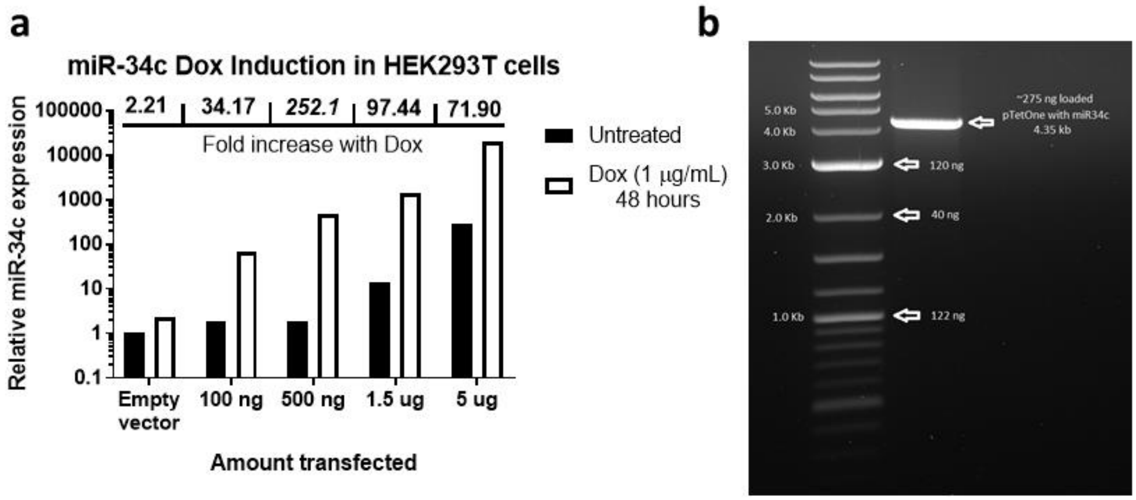

Figure 5.3: Successful doxycycline induction of miR-34c expression from the 34c-pTetOne plasmid transfected into HEK293T cells. **a** qPCR analysis of miR-34c expression in either untreated (black bars) or doxycycline treated (white bars) HEK293T cells transfected with the indicated amount of 34c-pTetOne plasmid. Cells were cultured for 48 hours in tetracycline free-FBS + DMEM at 37C. **b** 275 ng of linearized plasmid were loaded onto a 1.5% agarose gel from the stock of 50 µg of 34c-pTetOne was grown in competent DH5α E. coli cells and purified, and thoroughly cleaned, using the Qiagen Midi Prep Kit, and digested with SspI.

We partnered with Dr. Lucy Liaw's group at the MMCRI mouse transgenic core to inject the linearized version of this plasmid into CD-1 mouse embryos to create our transgenic line. 10 founding animals were created from these injections, one of which displayed aggressive behavior incompatible with mating, however 6 of the remaining 9 successfully transmitted this locus to the next generation of offspring, demonstrating germline integration of the transgene. Early experiments have demonstrated a modest level of miR-34c induction (~60%) via doxycycline in cultured peripheral lymphocytes isolated from tail blood collections on several of the founding male mice, a strategy we employed in order to avoid exposing the entire animal to doxycycline induction, which could have unintended consequences.

Chapter 6: Bibliography

1 McLean, C. P., Asnaani, A., Litz, B. T. & Hofmann, S. G. Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. *J Psychiatr Res* **45**, 1027-1035, doi:10.1016/j.jpsychires.2011.03.006 (2011).

2 Nolen-Hoeksema, S. & Girgus, J. S. The emergence of gender differences in depression during adolescence. *Psychol Bull* **115**, 424-443 (1994).

3 Koenen, K. C. & Widom, C. S. A prospective study of sex differences in the lifetime risk of posttraumatic stress disorder among abused and neglected children grown up. *J Trauma Stress* **22**, 566-574, doi:10.1002/jts.20478 (2009).

4 Lamarck, J. B. P. A. d. M. d. & Elliott, H. S. R. *Zoological philosophy; an exposition with regard to the natural history of animals*. (Macmillan and Co., limited, 1914).

5 Burkhardt, R. W., Jr. Lamarck, evolution, and the inheritance of acquired characters. *Genetics* **194**, 793-805, doi:10.1534/genetics.113.151852 (2013).

6 Bard, J. B. L. Waddington's Legacy to Developmental and Theoretical Biology. *Biological Theory* **3**, 188-197, doi:10.1162/biot.2008.3.3.188 (2008).

7 Boskovic, A. & Rando, O. J. Transgenerational Epigenetic Inheritance. *Annu Rev Genet* **52**, 21-41, doi:10.1146/annurev-genet-120417-031404 (2018).

8 Cassidy, S. B. & Schwartz, S. Prader-Willi and Angelman syndromes. Disorders of genomic imprinting. *Medicine (Baltimore)* **77**, 140-151 (1998).

9 Painter, R. C., Osmond, C., Gluckman, P., Hanson, M., Phillips, D. I. & Roseboom, T. J. Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. *BJOG* **115**, 1243-1249, doi:10.1111/j.1471-0528.2008.01822.x (2008).

10 Lumey, L. H. & Stein, A. D. In utero exposure to famine and subsequent fertility: The Dutch Famine Birth Cohort Study. *Am J Public Health* **87**, 1962-1966 (1997).

11 Ravelli, A. C., van Der Meulen, J. H., Osmond, C., Barker, D. J. & Bleker, O. P. Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* **70**, 811-816, doi:10.1093/ajcn/70.5.811 (1999).

12 Veenendaal, M. V., Painter, R. C., de Rooij, S. R., Bossuyt, P. M., van der Post, J. A., Gluckman, P. D., Hanson, M. A. & Roseboom, T. J. Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. *BJOG* **120**, 548-553, doi:10.1111/1471-0528.12136 (2013).

13 Stanner, S. A. & Yudkin, J. S. Fetal programming and the Leningrad Siege study. *Twin Res* **4**, 287-292, doi:10.1375/1369052012498 (2001).

14 Schulz, L. C. The Dutch Hunger Winter and the developmental origins of health and disease. *Proc Natl Acad Sci U S A* **107**, 16757-16758, doi:10.1073/pnas.1012911107 (2010).

15 Kaati, G., Bygren, L. O. & Edvinsson, S. Cardiovascular and diabetes mortality determined by nutrition during parents' and grandparents' slow growth period. *Eur J Hum Genet* **10**, 682-688, doi:10.1038/sj.ejhg.5200859 (2002).

16 Bygren, L. O., Kaati, G. & Edvinsson, S. Longevity determined by paternal ancestors' nutrition during their slow growth period. *Acta Biotheor* **49**, 53-59 (2001).

17 Chen, Y. P., Xiao, X. M., Li, J., Reichetzeder, C., Wang, Z. N. & Hocher, B. Paternal body mass index (BMI) is associated with offspring intrauterine growth in a gender dependent manner. *PLoS One* **7**, e36329, doi:10.1371/journal.pone.0036329 (2012).
18 Yehuda, R., Bell, A., Bierer, L. M. & Schmeidler, J. Maternal, not paternal, PTSD is related to increased risk for PTSD in offspring of Holocaust survivors. *J Psychiatr Res* **42**, 1104-1111, doi:10.1016/j.jpsychires.2008.01.002 (2008).
19 Shrira, A., Ayalon, L., Bensimon, M., Bodner, E., Rosenbloom, T. & Yadid, G. Parental Post-traumatic Stress Disorder Symptoms Are Related to Successful Aging in Offspring of Holocaust Survivors. *Front Psychol* **8**, 1099, doi:10.3389/fpsyg.2017.01099 (2017).
20 Yehuda, R., Bierer, L. M., Schmeidler, J., Aferiat, D. H., Breslau, I. & Dolan, S. Low cortisol and risk for PTSD in adult offspring of holocaust survivors. *Am J Psychiatry* **157**, 1252-1259, doi:10.1176/appi.ajp.157.8.1252 (2000).
21 Dijanic Plasc, I., Peraica, T., Grubisic-Ilic, M., Rak, D., Jambrosic Sakoman, A. & Kozaric-Kovacic, D. Psychiatric heredity and posttraumatic stress disorder: survey study of war veterans. *Croat Med J* **48**, 146-156 (2007).
22 Vaage, A. B., Thomsen, P. H., Rousseau, C., Wentzel-Larsen, T., Ta, T. V. & Hauff, E. Paternal predictors of the mental health of children of Vietnamese refugees. *Child Adolesc Psychiatry Ment Health* **5**, 2, doi:10.1186/1753-2000-5-2 (2011).
23 Barel, E., Van, I. M. H., Sagi-Schwartz, A. & Bakermans-Kranenburg, M. J. Surviving the Holocaust: a meta-analysis of the long-term sequelae of a genocide. *Psychol Bull* **136**, 677-698, doi:10.1037/a0020339 (2010).
24 Shrira, A., Palgi, Y., Ben-Ezra, M. & Shmotkin, D. Transgenerational effects of trauma in midlife: Evidence for resilience and vulnerability in offspring of Holocaust survivors. *Psychol Trauma* **3**, 394-402, doi:10.1037/a0020608 (2011).
25 Mead, E. A. & Sarkar, D. K. Fetal alcohol spectrum disorders and their transmission through genetic and epigenetic mechanisms. *Front Genet* **5**, 154, doi:10.3389/fgene.2014.00154 (2014).
26 Kioumourtzoglou, M. A., Coull, B. A., O'Reilly, E. J., Ascherio, A. & Weisskopf, M. G. Association of Exposure to Diethylstilbestrol During Pregnancy With Multigenerational Neurodevelopmental Deficits. *JAMA Pediatr* **172**, 670-677, doi:10.1001/jamapediatrics.2018.0727 (2018).
27 Dieckmann, W. J., Davis, M. E., Rynkiewicz, L. M. & Pottinger, R. E. Does the Administration of Diethylstilbestrol during Pregnancy have Therapeutic Value?*†. *American Journal of Obstetrics and Gynecology* **66**, 1062-1081, doi:https://doi.org/10.1016/S0002-9378(16)38617-3 (1953).
28 Herbst, A. L., Ulfelder, H. & Poskanzer, D. C. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med* **284**, 878-881, doi:10.1056/NEJM197104222841604 (1971).
29 Tournaire, M., Devouche, E., Epelboin, S., Cabau, A., Dunbavand, A. & Levadou, A. Birth defects in children of men exposed in utero to diethylstilbestrol (DES). *Therapie* **73**, 399-407, doi:10.1016/j.therap.2018.02.007 (2018).

30 Kalfa, N., Paris, F., Soyer-Gobillard, M. O., Daures, J. P. & Sultan, C. Prevalence of hypospadias in grandsons of women exposed to diethylstilbestrol during pregnancy: a multigenerational national cohort study. *Fertil Steril* **95**, 2574-2577, doi:10.1016/j.fertnstert.2011.02.047 (2011).
31 Brouwers, M. M., Feitz, W. F., Roelofs, L. A., Kiemeney, L. A., de Gier, R. P. & Roeleveld, N. Hypospadias: a transgenerational effect of diethylstilbestrol? *Hum Reprod* **21**, 666-669, doi:10.1093/humrep/dei398 (2006).
32 Titus, L., Hatch, E. E., Drake, K. M., Parker, S. E., Hyer, M., Palmer, J. R., Strohsnitter, W. C., Adam, E., Herbst, A. L., Huo, D., Hoover, R. N. & Troisi, R. Reproductive and hormone-related outcomes in women whose mothers were exposed in utero to diethylstilbestrol (DES): A report from the US National Cancer Institute DES Third Generation Study. *Reprod Toxicol* **84**, 32-38, doi:10.1016/j.reprotox.2018.12.008 (2019).
33 Titus-Ernstoff, L., Troisi, R., Hatch, E. E., Hyer, M., Wise, L. A., Palmer, J. R., Kaufman, R., Adam, E., Noller, K., Herbst, A. L., Strohsnitter, W., Cole, B. F., Hartge, P. & Hoover, R. N. Offspring of women exposed in utero to diethylstilbestrol (DES): a preliminary report of benign and malignant pathology in the third generation. *Epidemiology* **19**, 251-257, doi:10.1097/EDE.0b013e318163152a (2008).
34 Golding, J., Ellis, G., Gregory, S., Birmingham, K., Iles-Caven, Y., Rai, D. & Pembrey, M. Grand-maternal smoking in pregnancy and grandchild's autistic traits and diagnosed autism. *Sci Rep* **7**, 46179, doi:10.1038/srep46179 (2017).
35 Patel, B., Meeks, H., Wan, Y., Johnstone, E. B., Glenn, M., Smith, K. R. & Hotaling, J. M. Transgenerational effects of chemotherapy: Both male and female children born to women exposed to chemotherapy have fewer children. *Cancer Epidemiol* **56**, 1-5, doi:10.1016/j.canep.2018.07.001 (2018).
36 Kalmakis, K. A. & Chandler, G. E. Health consequences of adverse childhood experiences: a systematic review. *J Am Assoc Nurse Pract* **27**, 457-465, doi:10.1002/2327-6924.12215 (2015).
37 Felitti, V. J., Anda, R. F., Nordenberg, D., Williamson, D. F., Spitz, A. M., Edwards, V., Koss, M. P. & Marks, J. S. Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study. *Am J Prev Med* **14**, 245-258 (1998).
38 Chapman, D. P., Whitfield, C. L., Felitti, V. J., Dube, S. R., Edwards, V. J. & Anda, R. F. Adverse childhood experiences and the risk of depressive disorders in adulthood. *J Affect Disord* **82**, 217-225, doi:10.1016/j.jad.2003.12.013 (2004).
39 Dube, S. R., Anda, R. F., Felitti, V. J., Chapman, D. P., Williamson, D. F. & Giles, W. H. Childhood abuse, household dysfunction, and the risk of attempted suicide throughout the life span: findings from the Adverse Childhood Experiences Study. *JAMA* **286**, 3089-3096 (2001).
40 McGuinness, T. M. & Waldrop, J. R. Adverse Childhood Experiences and the Mental Health of Veterans. *J Psychosoc Nurs Ment Health Serv* **53**, 23-26, doi:10.3928/02793695-20150527-55 (2015).

41 Smith, M. V., Gotman, N. & Yonkers, K. A. Early Childhood Adversity and Pregnancy Outcomes. *Matern Child Health J* **20**, 790-798, doi:10.1007/s10995-015-1909-5 (2016).
42 Folger, A. T., Eismann, E. A., Stephenson, N. B., Shapiro, R. A., Macaluso, M., Brownrigg, M. E. & Gillespie, R. J. Parental Adverse Childhood Experiences and Offspring Development at 2 Years of Age. *Pediatrics* **141**, doi:10.1542/peds.2017-2826 (2018).
43 Sun, J., Patel, F., Rose-Jacobs, R., Frank, D. A., Black, M. M. & Chilton, M. Mothers' Adverse Childhood Experiences and Their Young Children's Development. *Am J Prev Med* **53**, 882-891, doi:10.1016/j.amepre.2017.07.015 (2017).
44 Quadrana, L. & Colot, V. Plant Transgenerational Epigenetics. *Annu Rev Genet* **50**, 467-491, doi:10.1146/annurev-genet-120215-035254 (2016).
45 Cubas, P., Vincent, C. & Coen, E. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**, 157-161, doi:10.1038/43657 (1999).
46 Brink, R. A. A Genetic Change Associated with the R Locus in Maize Which Is Directed and Potentially Reversible. *Genetics* **41**, 872-889 (1956).
47 Arteaga-Vazquez, M. A. & Chandler, V. L. Paramutation in maize: RNA mediated trans-generational gene silencing. *Curr Opin Genet Dev* **20**, 156-163, doi:10.1016/j.gde.2010.01.008 (2010).
48 Fire, A., Albertson, D., Harrison, S. W. & Moerman, D. G. Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle. *Development* **113**, 503-514 (1991).
49 Lee, R. C., Feinbaum, R. L. & Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-854 (1993).
50 Billi, A. C., Fischer, S. E. & Kim, J. K. Endogenous RNAi pathways in C. elegans. *WormBook*, 1-49, doi:10.1895/wormbook.1.170.1 (2014).
51 Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297 (2004).
52 Singh, P. B., Miller, J. R., Pearce, J., Kothary, R., Burton, R. D., Paro, R., James, T. C. & Gaunt, S. J. A sequence motif found in a Drosophila heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res* **19**, 789-794 (1991).
53 Morgan, H. D., Sutherland, H. G., Martin, D. I. & Whitelaw, E. Epigenetic inheritance at the agouti locus in the mouse. *Nat Genet* **23**, 314-318, doi:10.1038/15490 (1999).
54 Waterland, R. A. & Jirtle, R. L. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol* **23**, 5293-5300 (2003).
55 Nilsson, E. E., Sadler-Riggleman, I. & Skinner, M. K. Environmentally induced epigenetic transgenerational inheritance of disease. *Environ Epigenet* **4**, dvy016, doi:10.1093/eep/dvy016 (2018).
56 Anway, M. D., Cupp, A. S., Uzumcu, M. & Skinner, M. K. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308**, 1466-1469, doi:10.1126/science.1108190 (2005).

57 Vallaster, M. P., Kukreja, S., Bing, X. Y., Ngolab, J., Zhao-Shea, R., Gardner, P. D., Tapper, A. R. & Rando, O. J. Paternal nicotine exposure alters hepatic xenobiotic metabolism in offspring. *Elife* **6**, doi:10.7554/eLife.24771 (2017).

58 Anderson, L. M., Riffle, L., Wilson, R., Travlos, G. S., Lubomirski, M. S. & Alvord, W. G. Preconceptional fasting of fathers alters serum glucose in offspring of mice. *Nutrition* **22**, 327-331, doi:10.1016/j.nut.2005.09.006 (2006).

59 Carone, B. R., Fauquier, L., Habib, N., Shea, J. M., Hart, C. E., Li, R., Bock, C., Li, C., Gu, H., Zamore, P. D., Meissner, A., Weng, Z., Hofmann, H. A., Friedman, N. & Rando, O. J. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* **143**, 1084-1096, doi:10.1016/j.cell.2010.12.008 (2010).

60 Jimenez-Chillaron, J. C., Hernandez-Valencia, M., Reamer, C., Fisher, S., Joszi, A., Hirshman, M., Oge, A., Walrond, S., Przybyla, R., Boozer, C., Goodyear, L. J. & Patti, M. E. Beta-cell secretory dysfunction in the pathogenesis of low birth weight-associated diabetes: a murine model. *Diabetes* **54**, 702-711 (2005).

61 Jimenez-Chillaron, J. C., Isganaitis, E., Charalambous, M., Gesta, S., Pentinat-Pelegrin, T., Faucette, R. R., Otis, J. P., Chow, A., Diaz, R., Ferguson-Smith, A. & Patti, M. E. Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice. *Diabetes* **58**, 460-468, doi:10.2337/db08-0490 (2009).

62 Ng, S. F., Lin, R. C., Laybutt, D. R., Barres, R., Owens, J. A. & Morris, M. J. Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. *Nature* **467**, 963-966, doi:10.1038/nature09491 (2010).

63 Stanford, K. I., Rasmussen, M., Baer, L. A., Lehnig, A. C., Rowland, L. A., White, J. D., So, K., De Sousa-Coelho, A. L., Hirshman, M. F., Patti, M. E., Rando, O. J. & Goodyear, L. J. Paternal Exercise Improves Glucose Metabolism in Adult Offspring. *Diabetes* **67**, 2530-2540, doi:10.2337/db18-0667 (2018).

64 Wu, L., Lu, Y., Jiao, Y., Liu, B., Li, S., Li, Y., Xing, F., Chen, D., Liu, X., Zhao, J., Xiong, X., Gu, Y., Lu, J., Chen, X. & Li, X. Paternal Psychological Stress Reprograms Hepatic Gluconeogenesis in Offspring. *Cell Metab* **23**, 735-743, doi:10.1016/j.cmet.2016.01.014 (2016).

65 He, N., Kong, Q. Q., Wang, J. Z., Ning, S. F., Miao, Y. L., Yuan, H. J., Gong, S., Cui, X. Z., Li, C. Y. & Tan, J. H. Parental life events cause behavioral difference among offspring: Adult pre-gestational restraint stress reduces anxiety across generations. *Sci Rep* **6**, 39497, doi:10.1038/srep39497 (2016).

66 Krishnan, V., Han, M. H., Graham, D. L., Berton, O., Renthal, W., Russo, S. J., Laplant, Q., Graham, A., Lutter, M., Lagace, D. C., Ghose, S., Reister, R., Tannous, P., Green, T. A., Neve, R. L., Chakravarty, S., Kumar, A., Eisch, A. J., Self, D. W., Lee, F. S., Tamminga, C. A., Cooper, D. C., Gershenfeld, H. K. & Nestler, E. J. Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. *Cell* **131**, 391-404, doi:10.1016/j.cell.2007.09.018 (2007).

67 Dietz, D. M., Laplant, Q., Watts, E. L., Hodes, G. E., Russo, S. J., Feng, J., Oosting, R. S., Vialou, V. & Nestler, E. J. Paternal transmission of stress-induced pathologies. *Biol Psychiatry* **70**, 408-414, doi:10.1016/j.biopsych.2011.05.005 (2011).

68 Franklin, T. B., Russig, H., Weiss, I. C., Graff, J., Linder, N., Michalon, A., Vizi, S. & Mansuy, I. M. Epigenetic transmission of the impact of early stress across generations. *Biol Psychiatry* **68**, 408-415, doi:10.1016/j.biopsych.2010.05.036 (2010).
69 Franklin, T. B., Linder, N., Russig, H., Thony, B. & Mansuy, I. M. Influence of early stress on social abilities and serotonergic functions across generations in mice. *PLoS One* **6**, e21842, doi:10.1371/journal.pone.0021842 (2011).
70 Gapp, K., Corcoba, A., van Steenwyk, G., Mansuy, I. M. & Duarte, J. M. Brain metabolic alterations in mice subjected to postnatal traumatic stress and in their offspring. *J Cereb Blood Flow Metab* **37**, 2423-2432, doi:10.1177/0271678X16667525 (2017).
71 Bohacek, J., Farinelli, M., Mirante, O., Steiner, G., Gapp, K., Coiret, G., Ebeling, M., Duran-Pacheco, G., Iniguez, A. L., Manuella, F., Moreau, J. L. & Mansuy, I. M. Pathological brain plasticity and cognition in the offspring of males subjected to postnatal traumatic stress. *Mol Psychiatry* **20**, 621-631, doi:10.1038/mp.2014.80 (2015).
72 van Steenwyk, G., Roszkowski, M., Manuella, F., Franklin, T. B. & Mansuy, I. M. Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation. *Environ Epigenet* **4**, dvy023, doi:10.1093/eep/dvy023 (2018).
73 Weiss, I. C., Franklin, T. B., Vizi, S. & Mansuy, I. M. Inheritable effect of unpredictable maternal separation on behavioral responses in mice. *Front Behav Neurosci* **5**, 3, doi:10.3389/fnbeh.2011.00003 (2011).
74 Rodgers, A. B., Morgan, C. P., Bronson, S. L., Revello, S. & Bale, T. L. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. *J Neurosci* **33**, 9003-9012, doi:10.1523/JNEUROSCI.0914-13.2013 (2013).
75 Schmidt, M. V., Sterlemann, V. & Muller, M. B. Chronic stress and individual vulnerability. *Ann N Y Acad Sci* **1148**, 174-183, doi:10.1196/annals.1410.017 (2008).
76 Saavedra-Rodriguez, L. & Feig, L. A. Chronic social instability induces anxiety and defective social interactions across generations. *Biol Psychiatry* **73**, 44-53, doi:10.1016/j.biopsych.2012.06.035 (2013).
77 Gapp, K., Soldado-Magraner, S., Alvarez-Sanchez, M., Bohacek, J., Vernaz, G., Shu, H., Franklin, T. B., Wolfer, D. & Mansuy, I. M. Early life stress in fathers improves behavioural flexibility in their offspring. *Nat Commun* **5**, 5466, doi:10.1038/ncomms6466 (2014).
78 Sampedro-Piquero, P. & Begega, A. Environmental Enrichment as a Positive Behavioral Intervention Across the Lifespan. *Curr Neuropharmacol* **15**, 459-470, doi:10.2174/1570159X14666160325115909 (2017).
79 Dell, P. A. & Rose, F. D. Transfer of effects from environmentally enriched and impoverished female rats to future offspring. *Physiol Behav* **39**, 187-190 (1987).
80 Francis, D., Diorio, J., Liu, D. & Meaney, M. J. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science* **286**, 1155-1158 (1999).

81 Arai, J. A., Li, S., Hartley, D. M. & Feig, L. A. Transgenerational rescue of a genetic defect in long-term potentiation and memory formation by juvenile enrichment. *J Neurosci* **29**, 1496-1502, doi:10.1523/JNEUROSCI.5057-08.2009 (2009).

82 van Praag, H., Kempermann, G. & Gage, F. H. Neural consequences of environmental enrichment. *Nat Rev Neurosci* **1**, 191-198, doi:10.1038/35044558 (2000).

83 Benito, E., Kerimoglu, C., Ramachandran, B., Pena-Centeno, T., Jain, G., Stilling, R. M., Islam, M. R., Capece, V., Zhou, Q., Edbauer, D., Dean, C. & Fischer, A. RNA-Dependent Intergenerational Inheritance of Enhanced Synaptic Plasticity after Environmental Enrichment. *Cell Rep* **23**, 546-554, doi:10.1016/j.celrep.2018.03.059 (2018).

84 Gapp, K., Bohacek, J., Grossmann, J., Brunner, A. M., Manuella, F., Nanni, P. & Mansuy, I. M. Potential of Environmental Enrichment to Prevent Transgenerational Effects of Paternal Trauma. *Neuropsychopharmacology* **41**, 2749-2758, doi:10.1038/npp.2016.87 (2016).

85 Baker, E. P., Magnuson, E. C., Dahly, A. M. & Siegel, J. A. The effects of enriched environment on the behavioral and corticosterone response to methamphetamine in adolescent and adult mice. *Dev Psychobiol* **60**, 664-673, doi:10.1002/dev.21633 (2018).

86 Ben Maamar, M., Sadler-Riggleman, I., Beck, D., McBirney, M., Nilsson, E., Klukovich, R., Xie, Y., Tang, C., Yan, W. & Skinner, M. K. Alterations in sperm DNA methylation, non-coding RNA expression, and histone retention mediate vinclozolin-induced epigenetic transgenerational inheritance of disease. *Environ Epigenet* **4**, dvy010, doi:10.1093/eep/dvy010 (2018).

87 Kazachenka, A., Bertozzi, T. M., Sjoberg-Herrera, M. K., Walker, N., Gardner, J., Gunning, R., Pahita, E., Adams, S., Adams, D. & Ferguson-Smith, A. C. Identification, Characterization, and Heritability of Murine Metastable Epialleles: Implications for Non-genetic Inheritance. *Cell* **175**, 1259-1271 e1213, doi:10.1016/j.cell.2018.09.043 (2018).

88 Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E. & Mansuy, I. M. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat Neurosci* **17**, 667-669, doi:10.1038/nn.3695 (2014).

89 Gapp, K., van Steenwyk, G., Germain, P. L., Matsushima, W., Rudolph, K. L. M., Manuella, F., Roszkowski, M., Vernaz, G., Ghosh, T., Pelczar, P., Mansuy, I. M. & Miska, E. A. Alterations in sperm long RNA contribute to the epigenetic inheritance of the effects of postnatal trauma. *Mol Psychiatry*, doi:10.1038/s41380-018-0271-6 (2018).

90 Rodgers, A. B., Morgan, C. P., Leu, N. A. & Bale, T. L. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proc Natl Acad Sci U S A* **112**, 13699-13704, doi:10.1073/pnas.1508347112 (2015).

91 Sharma, U., Conine, C. C., Shea, J. M., Boskovic, A., Derr, A. G., Bing, X. Y., Belleannee, C., Kucukural, A., Serra, R. W., Sun, F., Song, L., Carone, B. R., Ricci, E. P., Li, X. Z., Fauquier, L., Moore, M. J., Sullivan, R., Mello, C. C.,

Garber, M. & Rando, O. J. Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. *Science* **351**, 391-396, doi:10.1126/science.aad6780 (2016).
92 Sharma, U., Sun, F., Conine, C. C., Reichholf, B., Kukreja, S., Herzog, V. A., Ameres, S. L. & Rando, O. J. Small RNAs Are Trafficked from the Epididymis to Developing Mammalian Sperm. *Dev Cell* **46**, 481-494 e486, doi:10.1016/j.devcel.2018.06.023 (2018).
93 Conine, C. C., Sun, F., Song, L., Rivera-Perez, J. A. & Rando, O. J. Small RNAs Gained during Epididymal Transit of Sperm Are Essential for Embryonic Development in Mice. *Dev Cell* **46**, 470-480 e473, doi:10.1016/j.devcel.2018.06.024 (2018).
94 Chen, Q., Yan, M., Cao, Z., Li, X., Zhang, Y., Shi, J., Feng, G. H., Peng, H., Zhang, X., Zhang, Y., Qian, J., Duan, E., Zhai, Q. & Zhou, Q. Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science* **351**, 397-400, doi:10.1126/science.aad7977 (2016).
95 Bohacek, J., von Werdt, S. & Mansuy, I. M. Probing the germline-dependence of epigenetic inheritance using artificial insemination in mice. *Environ Epigenet* **2**, dvv015, doi:10.1093/eep/dvv015 (2016).
96 Ha, M. & Kim, V. N. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* **15**, 509-524, doi:10.1038/nrm3838 (2014).
97 O'Brien, J., Hayder, H., Zayed, Y. & Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol (Lausanne)* **9**, 402, doi:10.3389/fendo.2018.00402 (2018).
98 Paul, P., Chakraborty, A., Sarkar, D., Langthasa, M., Rahman, M., Bari, M., Singha, R. S., Malakar, A. K. & Chakraborty, S. Interplay between miRNAs and human diseases. *J Cell Physiol* **233**, 2007-2018, doi:10.1002/jcp.25854 (2018).
99 Luningschror, P., Hauser, S., Kaltschmidt, B. & Kaltschmidt, C. MicroRNAs in pluripotency, reprogramming and cell fate induction. *Biochim Biophys Acta* **1833**, 1894-1903, doi:10.1016/j.bbamcr.2013.03.025 (2013).
100 Kumar, P., Kuscu, C. & Dutta, A. Biogenesis and Function of Transfer RNA-Related Fragments (tRFs). *Trends Biochem Sci* **41**, 679-689, doi:10.1016/j.tibs.2016.05.004 (2016).
101 Ostermeier, G. C., Miller, D., Huntriss, J. D., Diamond, M. P. & Krawetz, S. A. Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature* **429**, 154, doi:10.1038/429154a (2004).
102 Liu, W. M., Pang, R. T., Chiu, P. C., Wong, B. P., Lao, K., Lee, K. F. & Yeung, W. S. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc Natl Acad Sci U S A* **109**, 490-494, doi:10.1073/pnas.1110368109 (2012).
103 Yuan, S., Schuster, A., Tang, C., Yu, T., Ortogero, N., Bao, J., Zheng, H. & Yan, W. Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development. *Development* **143**, 635-647, doi:10.1242/dev.131755 (2016).
104 Yuan, S., Tang, C., Zhang, Y., Wu, J., Bao, J., Zheng, H., Xu, C. & Yan, W. mir-34b/c and mir-449a/b/c are required for spermatogenesis, but not for the first

cleavage division in mice. *Biol Open* **4**, 212-223, doi:10.1242/bio.201410959 (2015).

105 Wu, J., Bao, J., Kim, M., Yuan, S., Tang, C., Zheng, H., Mastick, G. S., Xu, C. & Yan, W. Two miRNA clusters, miR-34b/c and miR-449, are essential for normal brain development, motile ciliogenesis, and spermatogenesis. *Proc Natl Acad Sci U S A* **111**, E2851-2857, doi:10.1073/pnas.1407777111 (2014).

106 Marczylo, E. L., Amoako, A. A., Konje, J. C., Gant, T. W. & Marczylo, T. H. Smoking induces differential miRNA expression in human spermatozoa: a potential transgenerational epigenetic concern? *Epigenetics* **7**, 432-439, doi:10.4161/epi.19794 (2012).

107 Donkin, I., Versteyhe, S., Ingerslev, L. R., Qian, K., Mechta, M., Nordkap, L., Mortensen, B., Appel, E. V., Jorgensen, N., Kristiansen, V. B., Hansen, T., Workman, C. T., Zierath, J. R. & Barres, R. Obesity and Bariatric Surgery Drive Epigenetic Variation of Spermatozoa in Humans. *Cell Metab* **23**, 369-378, doi:10.1016/j.cmet.2015.11.004 (2016).

108 Reza, A., Choi, Y. J., Han, S. G., Song, H., Park, C., Hong, K. & Kim, J. H. Roles of microRNAs in mammalian reproduction: from the commitment of germ cells to peri-implantation embryos. *Biol Rev Camb Philos Soc* **94**, 415-438, doi:10.1111/brv.12459 (2019).

109 Dickson, D. A., Paulus, J. K., Mensah, V., Lem, J., Saavedra-Rodriguez, L., Gentry, A., Pagidas, K. & Feig, L. A. Reduced levels of miRNAs 449 and 34 in sperm of mice and men exposed to early life stress. *Transl Psychiatry* **8**, 101, doi:10.1038/s41398-018-0146-2 (2018).

110 Nagy, A. *Manipulating the mouse embryo : a laboratory manual*. 3rd edn, (Cold Spring Harbor Laboratory Press, 2003).

111 Kohda, T. & Ishino, F. Embryo manipulation via assisted reproductive technology and epigenetic asymmetry in mammalian early development. *Philos Trans R Soc Lond B Biol Sci* **368**, 20120353, doi:10.1098/rstb.2012.0353 (2013).

112 Okamoto, Y., Yoshida, N., Suzuki, T., Shimozawa, N., Asami, M., Matsuda, T., Kojima, N., Perry, A. C. & Takada, T. DNA methylation dynamics in mouse preimplantation embryos revealed by mass spectrometry. *Sci Rep* **6**, 19134, doi:10.1038/srep19134 (2016).

113 Yamamoto, H., Kokame, K., Okuda, T., Nakajo, Y., Yanamoto, H. & Miyata, T. NDRG4 protein-deficient mice exhibit spatial learning deficits and vulnerabilities to cerebral ischemia. *J Biol Chem* **286**, 26158-26165, doi:10.1074/jbc.M111.256446 (2011).

114 Yang, X. L., Wang, X. & Peng, B. W. NFAT5 Has a Job in the Brain. *Dev Neurosci* **40**, 289-300, doi:10.1159/000493789 (2018).

115 Nigam, S. K. The SLC22 Transporter Family: A Paradigm for the Impact of Drug Transporters on Metabolic Pathways, Signaling, and Disease. *Annu Rev Pharmacol Toxicol* **58**, 663-687, doi:10.1146/annurev-pharmtox-010617-052713 (2018).

116 Kim, Y. D., Lee, J., Kim, H. S., Lee, M. O., Son, M. Y., Yoo, C. H., Choi, J. K., Lee, S. C. & Cho, Y. S. The unique spliceosome signature of human pluripotent stem cells is mediated by SNRPA1, SNRPD1, and PNN. *Stem Cell Res* **22**, 43-53, doi:10.1016/j.scr.2017.05.010 (2017).

117 Sorrells, S., Nik, S., Casey, M., Cameron, R. C., Truong, H., Toruno, C., Gulfo, M., Lowe, A., Jette, C., Stewart, R. A. & Bowman, T. V. Spliceosomal components protect embryonic neurons from R-loop-mediated DNA damage and apoptosis. *Dis Model Mech* **11**, doi:10.1242/dmm.031583 (2018).

118 Mashoodh, R., Franks, B., Curley, J. P. & Champagne, F. A. Paternal social enrichment effects on maternal behavior and offspring growth. *Proc Natl Acad Sci U S A* **109 Suppl 2**, 17232-17238, doi:10.1073/pnas.1121083109 (2012).

119 Josson, S., Gururajan, M., Hu, P., Shao, C., Chu, G. Y., Zhau, H. E., Liu, C., Lao, K., Lu, C. L., Lu, Y. T., Lichterman, J., Nandana, S., Li, Q., Rogatko, A., Berel, D., Posadas, E. M., Fazli, L., Sareen, D. & Chung, L. W. miR-409-3p/-5p promotes tumorigenesis, epithelial-to-mesenchymal transition, and bone metastasis of human prostate cancer. *Clin Cancer Res* **20**, 4636-4646, doi:10.1158/1078-0432.CCR-14-0305 (2014).

120 Chen, C. L., Liu, H. & Guan, X. Changes in microRNA expression profile in hippocampus during the acquisition and extinction of cocaine-induced conditioned place preference in rats. *J Biomed Sci* **20**, 96, doi:10.1186/1423-0127-20-96 (2013).

121 Biswas, S. C., Zhang, Y., Iyirhiaro, G., Willett, R. T., Rodriguez Gonzalez, Y., Cregan, S. P., Slack, R. S., Park, D. S. & Greene, L. A. Sertad1 plays an essential role in developmental and pathological neuron death. *J Neurosci* **30**, 3973-3982, doi:10.1523/JNEUROSCI.6421-09.2010 (2010).

122 Dougherty, J. D., Garcia, A. D., Nakano, I., Livingstone, M., Norris, B., Polakiewicz, R., Wexler, E. M., Sofroniew, M. V., Kornblum, H. I. & Geschwind, D. H. PBK/TOPK, a proliferating neural progenitor-specific mitogen-activated protein kinase kinase. *J Neurosci* **25**, 10773-10785, doi:10.1523/JNEUROSCI.3207-05.2005 (2005).

123 Gomez-Herreros, F., Schuurs-Hoeijmakers, J. H., McCormack, M., Greally, M. T., Rulten, S., Romero-Granados, R., Counihan, T. J., Chaila, E., Conroy, J., Ennis, S., Delanty, N., Cortes-Ledesma, F., de Brouwer, A. P., Cavalleri, G. L., El-Khamisy, S. F., de Vries, B. B. & Caldecott, K. W. TDP2 protects transcription from abortive topoisomerase activity and is required for normal neural function. *Nat Genet* **46**, 516-521, doi:10.1038/ng.2929 (2014).

124 Lizarraga, S. B., Margossian, S. P., Harris, M. H., Campagna, D. R., Han, A. P., Blevins, S., Mudbhary, R., Barker, J. E., Walsh, C. A. & Fleming, M. D. Cdk5rap2 regulates centrosome function and chromosome segregation in neuronal progenitors. *Development* **137**, 1907-1917, doi:10.1242/dev.040410 (2010).

125 Tzeng, S. F. & de Vellis, J. Id1, Id2, and Id3 gene expression in neural cells during development. *Glia* **24**, 372-381 (1998).

126 Dadalko, O. I., Siuta, M., Poe, A., Erreger, K., Matthies, H. J., Niswender, K. & Galli, A. mTORC2/rictor signaling disrupts dopamine-dependent behaviors via defects in striatal dopamine neurotransmission. *J Neurosci* **35**, 8843-8854, doi:10.1523/JNEUROSCI.0887-15.2015 (2015).

127 Waite, R., Gerrity, P. & Arango, R. Assessment for and response to adverse childhood experiences. *J Psychosoc Nurs Ment Health Serv* **48**, 51-61, doi:10.3928/02793695-20100930-03 (2010).

128 Kalmakis, K. A., Chandler, G. E., Roberts, S. J. & Leung, K. Nurse practitioner screening for childhood adversity among adult primary care patients: A mixed-method study. *J Am Assoc Nurse Pract* **29**, 35-45, doi:10.1002/2327-6924.12378 (2017).

129 LeardMann, C. A., Smith, B. & Ryan, M. A. Do adverse childhood experiences increase the risk of postdeployment posttraumatic stress disorder in US Marines? *BMC Public Health* **10**, 437, doi:10.1186/1471-2458-10-437 (2010).

130 Phillips, C. J., Leardmann, C. A., Gumbs, G. R. & Smith, B. Risk factors for posttraumatic stress disorder among deployed US male marines. *BMC Psychiatry* **10**, 52, doi:10.1186/1471-244X-10-52 (2010).

131 Santavirta, T., Santavirta, N. & Gilman, S. E. Association of the World War II Finnish Evacuation of Children With Psychiatric Hospitalization in the Next Generation. *JAMA Psychiatry* **75**, 21-27, doi:10.1001/jamapsychiatry.2017.3511 (2018).

132 Bohacek, J. & Mansuy, I. M. Molecular insights into transgenerational non-genetic inheritance of acquired behaviours. *Nat Rev Genet* **16**, 641-652, doi:10.1038/nrg3964 (2015).

133 Rodgers, A. B. & Bale, T. L. Germ cell origins of posttraumatic stress disorder risk: the transgenerational impact of parental stress experience. *Biol. Psychiatry* **78**, doi:10.1016/j.biopsych.2015.03.018 (2015).

134 Dietz, D. M. Paternal transmission of stress-induced pathologies. *Biol. Psychiatry* **70**, doi:10.1016/j.biopsych.2011.05.005 (2011).

135 He, N. Parental life events cause behavioral difference among offspring: adult pre–gestational restraint stress reduces anxiety across generations. *Sci. Rep.* **6**, doi:10.1038/srep39497 (2016).

136 Rodgers, A. B., Morgan, C. P., Bronson, S. L., Revello, S. & Bale, T. L. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. *J. Neurosci.* **33**, doi:10.1523/JNEUROSCI.0914-13.2013 (2013).

137 Saavedra-Rodriguez, L. & Feig, L. A. Chronic social instability induces anxiety and defective social interactions across generations. *Biol. Psychiatry* **73**, doi:10.1016/j.biopsych.2012.06.035 (2013).

138 Franklin, T. B. Epigenetic transmission of the impact of early stress across generations. *Biol. Psychiatry* **68**, doi:10.1016/j.biopsych.2010.05.036 (2010).

139 Jodar, M., Selvaraju, S., Sendler, E., Diamond, M. P. & Krawetz, S. A. The presence, role and clinical use of spermatozoal RNAs. *Hum. Reprod. Update* **19**, doi:10.1093/humupd/dmt031 (2013).

140 Ostermeier, G. C., Miller, D., Huntriss, J. D., Diamond, M. P. & Krawetz, S. A. Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature* **429**, doi:10.1038/429154a (2004).

141 Sendler, E. Stability, delivery and functions of human sperm RNAs at fertilization. *Nucleic Acids Res.* **41**, doi:10.1093/nar/gkt132 (2013).

142 Gapp, K. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat. Neurosci.* **17**, doi:10.1038/nn.3695 (2014).

143 Rodgers, A. B., Morgan, C. P., Leu, N. A. & Bale, T. L. Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. *Proc. Natl Acad. Sci. USA* **112**, doi:10.1073/pnas.1508347112 (2015).
144 Wu, J. Two miRNA clusters, miR-34b/c and miR-449, are essential for normal brain development, motile ciliogenesis, and spermatogenesis. *Proc. Natl Acad. Sci. USA* **111**, doi:10.1073/pnas.1407777111 (2014).
145 Fededa, J. P. MicroRNA-34/449 controls mitotic spindle orientation during mammalian cortex development. *EMBO J.* **35**, doi:10.15252/embj.201694056 (2016).
146 Andolina, D., Segni, M. & Ventura, R. MiRNA-34 and stress response. *Oncotarget* **8**, doi:10.18632/oncotarget.13923 (2017).
147 Haramati, S. MicroRNA as repressors of stress-induced anxiety: the case of amygdalar miR-34. *J. Neurosci.* **31**, doi:10.1523/JNEUROSCI.1673-11.2011 (2011).
148 Andolina, D. Effects of lack of microRNA-34 on the neural circuitry underlying the stress response and anxiety. *Neuropharmacology* **107**, doi:10.1016/j.neuropharm.2016.03.044 (2016).
149 Kaneko, S. Purification of human sperm by a discontinuous Percoll density gradient with an innercolumn. *Biol. Reprod.* **35**, doi:10.1095/biolreprod35.4.1059 (1986).
150 Check, J. H., Adelson, H. G., Schubert, B. R. & Bollendorf, A. Evaluation of sperm morphology using Kruger's strict criteria. *Arch. Androl.* **28**, doi:10.3109/01485019208987674 (1992).
151 Campbell, J. A., Walker, R. J. & Egede, L. E. Associations between adverse childhood experiences, high-risk behaviors, and morbidity in adulthood. *Am. J. Prev. Med.* **50**, doi:10.1016/j.amepre.2015.07.022 (2016).
152 Marczylo, E. L., Amoako, A. A., Konje, J. C., Gant, T. W. & Marczylo, T. H. Smoking induces differential miRNA expression in human spermatozoa: a potential transgenerational epigenetic concern? *Epigenetics* **7**, doi:10.4161/epi.19794 (2012).
153 Williamson, D. F., Thompson, T. J., Anda, R. F., Dietz, W. H. & Felitti, V. Body weight and obesity in adults and self-reported abuse in childhood. *Int. J. Obes. Relat. Metab. Disord.* **26**, doi:10.1038/sj.ijo.0802038 (2002).
154 Donkin, I. Obesity and bariatric surgery drive epigenetic variation of spermatozoa in humans. *Cell Metab.* **23**, doi:10.1016/j.cmet.2015.11.004 (2016).
155 Schmidt, M. V. Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence. *Psychoneuroendocrinology* **32**, doi:10.1016/j.psyneuen.2007.02.011 (2007).
156 Yuan, S. Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development. *Development* **143**, doi:10.1242/dev.131755 (2016).
157 Reuben, A. Lest we forget: comparing retrospective and prospective assessments of adverse childhood experiences in the prediction of adult health. *J. Child. Psychol. Psychiatry* **57**, doi:10.1111/jcpp.12621 (2016).

158 Abu-Halima, M. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. *Fertil. Steril.* **99**, doi:10.1016/j.fertnstert.2012.11.054 (2013).
159 Cui, L., Fang, L., Shi, B., Qiu, S. & Ye, Y. Spermatozoa micro ribonucleic acid-34c level is correlated with intracytoplasmic sperm injection outcomes. *Fertil. Steril.* **104**, doi:10.1016/j.fertnstert.2015.05.003 (2015).
160 Wang, M. Sperm-borne miR-449b influences cleavage, epigenetic reprogramming and apoptosis of SCNT embryos in bovine. *Sci. Rep.* **7**, doi:10.1038/s41598-017-13899-8 (2017).
161 Merrick, M. T., Leeb, R. T. & Lee, R. D. Examining the role of safe, stable, and nurturing relationships in the intergenerational continuity of child maltreatment--introduction to the special issue. *J. Adolesc. Health* **53**, doi:10.1016/j.jadohealth.2013.06.017 (2013).
162 Holman, D. M. The association between adverse childhood experiences and risk of cancer in adulthood: a systematic review of the literature. *Pediatrics* **138**, doi:10.1542/peds.2015-4268L (2016).
163 Albert, P. R. Why is depression more prevalent in women? *J Psychiatry Neurosci* **40**, 219-221 (2015).
164 Chen, Y., Zheng, Y., Gao, Y., Lin, Z., Yang, S., Wang, T., Wang, Q., Xie, N., Hua, R., Liu, M., Sha, J., Griswold, M. D., Li, J., Tang, F. & Tong, M. H. Single-cell RNA-seq uncovers dynamic processes and critical regulators in mouse spermatogenesis. *Cell Res* **28**, 879-896, doi:10.1038/s41422-018-0074-y (2018).
165 Eakin, G. S. & Hadjantonakis, A. K. Sex-specific gene expression in preimplantation mouse embryos. *Genome Biol* **7**, 205, doi:10.1186/gb-2006-7-2-205 (2006).
166 Yang, Q., Lin, J., Liu, M., Li, R., Tian, B., Zhang, X., Xu, B., Liu, M., Zhang, X., Li, Y., Shi, H. & Wu, L. Highly sensitive sequencing reveals dynamic modifications and activities of small RNAs in mouse oocytes and early embryos. *Sci Adv* **2**, e1501482, doi:10.1126/sciadv.1501482 (2016).
167 Suh, N., Baehner, L., Moltzahn, F., Melton, C., Shenoy, A., Chen, J. & Blelloch, R. MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr Biol* **20**, 271-277, doi:10.1016/j.cub.2009.12.044 (2010).
168 Wang, Q., Chow, J., Hong, J., Smith, A. F., Moreno, C., Seaby, P., Vrana, P., Miri, K., Tak, J., Chung, E. D., Mastromonaco, G., Caniggia, I. & Varmuza, S. Recent acquisition of imprinting at the rodent Sfmbt2 locus correlates with insertion of a large block of miRNAs. *BMC Genomics* **12**, 204, doi:10.1186/1471-2164-12-204 (2011).
169 Inoue, K., Hirose, M., Inoue, H., Hatanaka, Y., Honda, A., Hasegawa, A., Mochida, K. & Ogura, A. The Rodent-Specific MicroRNA Cluster within the Sfmbt2 Gene Is Imprinted and Essential for Placental Development. *Cell Rep* **19**, 949-956, doi:10.1016/j.celrep.2017.04.018 (2017).
170 Miri, K., Latham, K., Panning, B., Zhong, Z., Andersen, A. & Varmuza, S. The imprinted polycomb group gene Sfmbt2 is required for trophoblast maintenance and placenta development. *Development* **140**, 4480-4489, doi:10.1242/dev.096511 (2013).

171 Gu, K. L., Zhang, Q., Yan, Y., Li, T. T., Duan, F. F., Hao, J., Wang, X. W., Shi, M., Wu, D. R., Guo, W. T. & Wang, Y. Pluripotency-associated miR-290/302 family of microRNAs promote the dismantling of naive pluripotency. *Cell Res* **26**, 350-366, doi:10.1038/cr.2016.2 (2016).

172 Gao, Y. & Sun, T. Molecular regulation of hypothalamic development and physiological functions. *Mol Neurobiol* **53**, 4275-4285, doi:10.1007/s12035-015-9367-z (2016).

173 Wang, M., Gao, Y., Qu, P., Qing, S., Qiao, F., Zhang, Y., Mager, J. & Wang, Y. Sperm-borne miR-449b influences cleavage, epigenetic reprogramming and apoptosis of SCNT embryos in bovine. *Sci Rep* **7**, 13403, doi:10.1038/s41598-017-13899-8 (2017).

174 Nixon, B., Stanger, S. J., Mihalas, B. P., Reilly, J. N., Anderson, A. L., Tyagi, S., Holt, J. E. & McLaughlin, E. A. The microRNA signature of mouse spermatozoa is substantially modified during epididymal maturation. *Biol Reprod* **93**, 91, doi:10.1095/biolreprod.115.132209 (2015).

175 Abu-Halima, M., Hammadeh, M., Schmitt, J., Leidinger, P., Keller, A., Meese, E. & Backes, C. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. *Fertil Steril* **99**, 1249-1255 e1216, doi:10.1016/j.fertnstert.2012.11.054 (2013).

176 Cui, L., Fang, L., Shi, B., Qiu, S. & Ye, Y. Spermatozoa micro ribonucleic acid-34c level is correlated with intracytoplasmic sperm injection outcomes. *Fertil Steril* **104**, 312-317 e311, doi:10.1016/j.fertnstert.2015.05.003 (2015).

177 Cooper, T. G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H. W., Behre, H. M., Haugen, T. B., Kruger, T., Wang, C., Mbizvo, M. T. & Vogelsong, K. M. World Health Organization reference values for human semen characteristics. *Hum Reprod Update* **16**, 231-245, doi:10.1093/humupd/dmp048 (2010).

178 Reuben, A., Moffitt, T. E., Caspi, A., Belsky, D. W., Harrington, H., Schroeder, F., Hogan, S., Ramrakha, S., Poulton, R. & Danese, A. Lest we forget: comparing retrospective and prospective assessments of adverse childhood experiences in the prediction of adult health. *J Child Psychol Psychiatry* **57**, 1103-1112, doi:10.1111/jcpp.12621 (2016).

179 Harris, I. D., Fronczak, C., Roth, L. & Meacham, R. B. Fertility and the aging male. *Rev Urol* **13**, e184-190 (2011).

180 Wang, B., Wang, Y., Zhang, M., Du, Y., Zhang, Y., Xing, X., Zhang, L., Su, J., Zhang, Y. & Zheng, Y. MicroRNA-34c expression in donor cells influences the early development of somatic cell nuclear transfer bovine embryos. *Cell Reprogram* **16**, 418-427, doi:10.1089/cell.2014.0016 (2014).