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Intergenerational Mechanisms Of Paternal Stress Transmission

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Abstract
Evidence that the intergenerational transmission of parental experiences can influence offspring outcomes prompts new consideration for the molecular mechanisms underlying disease risk and resilience. The role of the paternal preconception environment has been of particular interest, stimulating characterization of germ cell epigenetic marks that can respond dynamically to environmental insults and transmit this information at fertilization. Given such exciting potential for sperm epigenetic marks, how these marks are changed by the environment and subsequently impact offspring development are key questions that require investigation. In this dissertation, we address these questions using our established mouse model of paternal stress, where specific sperm microRNA altered by paternal chronic stress exposure causally reprogram offspring hypothalamic-pituitary-adrenal (HPA) stress axis reactivity and the hypothalamic transcriptome. First, we examined the role of glucocorticoids, a major component of the HPA stress response, as a signal for sperm microRNA changes. To ensure similar levels of glucocorticoids are produced in response to stress and thus are available for paternal cellular signaling, we developed an approach to confirm the stress sensitivity and reactivity of experimental mice. We next demonstrated that glucocorticoids are involved in communicating stress to the caput epididymis, a somatic tissue that secretes extracellular vesicles (EVs) to deliver microRNA from epididymal epithelial cells to maturing sperm. Using an in vitro model where we administered glucocorticoids to caput epididymal epithelial cells, we showed altered EV microRNA content and within epithelial cells, changes to histone post-translational modifications and increased glucocorticoid receptor levels, mimicking aspects of our in vivo paternal stress model. Further, we demonstrated the crucial role of caput epididymal glucocorticoid receptors in paternal stress transmission by transgenic knockdown, preventing offspring HPA axis and hypothalamic programming. In our final study, we provided evidence for the specificity of paternal stress sperm microRNA effects on embryonic brain and placental transcriptomes, indicating a tightly regulated process by which sperm microRNA are coordinated and function to influence offspring development. Together, the research presented in this dissertation provides insight into the mechanisms contributing to paternal transmission and support the paternal preconception environment as an influential factor in offspring disease risk and resilience.

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ABSTRACT

INTERGENERATIONAL MECHANISMS OF PATERNAL STRESS TRANSMISSION

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Tracy L. Bale

Evidence that the intergenerational transmission of parental experiences can influence offspring outcomes prompts new consideration for the molecular mechanisms underlying disease risk and resilience. The role of the paternal preconception environment has been of particular interest, stimulating characterization of germ cell epigenetic marks that can respond dynamically to environmental insults and transmit this information at fertilization. Given such exciting potential for sperm epigenetic marks, how these marks are changed by the environment and subsequently impact offspring development are key questions that require investigation. In this dissertation, we address these questions using our established mouse model of paternal stress, where specific sperm microRNA altered by paternal chronic stress exposure causally reprogram offspring hypothalamic-pituitary-adrenal (HPA) stress axis reactivity and the hypothalamic transcriptome. First, we examined the role of glucocorticoids, a major component of the HPA stress response, as a signal for sperm microRNA changes. To ensure similar levels of glucocorticoids are produced in response to stress and thus are available for paternal cellular signaling, we developed an approach to confirm the stress sensitivity and reactivity of experimental mice. We next demonstrated that glucocorticoids are involved in communicating stress to the caput epididymis, a somatic tissue that secretes extracellular vesicles (EVs) to deliver

microRNA from epididymal epithelial cells to maturing sperm. Using an *in vitro* model where we administered glucocorticoids to caput epididymal epithelial cells, we showed altered EV microRNA content and within epithelial cells, changes to histone post-translational modifications and increased glucocorticoid receptor levels, mimicking aspects of our *in vivo* paternal stress model. Further, we demonstrated the crucial role of caput epididymal glucocorticoid receptors in paternal stress transmission by transgenic knockdown, preventing offspring HPA axis and hypothalamic programming. In our final study, we provided evidence for the specificity of paternal stress sperm microRNA effects on embryonic brain and placental transcriptomes, indicating a tightly regulated process by which sperm microRNA are coordinated and function to influence offspring development. Together, the research presented in this dissertation provides insight into the mechanisms contributing to paternal transmission and support the paternal preconception environment as an influential factor in offspring disease risk and resilience.

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CHAPTER 1

GENERAL INTRODUCTION

Much of the content of this chapter was originally published in Biological Psychiatry, 2018, May 15, Vol. 83(10): 886-894, PMID: 29198470, Biological Psychiatry *(under review)*, or adapted from work originally published in Hormones, Brain and Behavior 3rd edition, 2016 November 26, Vol. 5: 117-132.

I. Stress axis programming and neuropsychiatric disease

Stress is pervasive in the environment, and the individual variation in the response to stress is a considerable factor for neuropsychiatric disease risk. First, what is considered stress? Stress has been defined as "…a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioral responses. In biomedicine, stress often refers to situations in which adrenal glucocorticoids and catecholamines are elevated because of an experience" (McEwen, 2000). Stressful experiences can be chronic or acute, deriving from life events (e.g. death of a loved one, divorce), life trauma (e.g. war, domestic violence), or daily agitations (e.g. financial struggles, relationship problems, work-related stressors). The severity and duration of stress, as well as the individual's coping ability and genetics, often influence the long-term physiological and psychological impacts of a stressor. For example, overcoming acute, mild adversity can stimulate learning, resilience and social bonding, while exposure to chronic, severe stress can be maladaptive and promote social instability and ill health (McEwen et al., 2010). In addition, the developmental window during which stress is experienced can greatly impact the outcome (Heim et al., 2012). For instance, a healthy adult may experience stress for several months, but with proper

social support may not experience life-altering, long-term consequences. In contrast, during specific neurodevelopmental critical periods, such as early life and pubertal periods when the brain is undergoing dramatic maturation, several months of stress can have severe and long-lasting impacts on an individual's health and behavior (Eiland et al., 2013).

Stress responsivity and homeostasis is controlled by the Hypothalamic-Pituitary-Adrenal (HPA) stress axis (Brown et al., 1985; Vale et al., 1981). The HPA stress axis is a self-regulating system designed to maintain homeostasis in response to challenges, i.e., stress. The primary neuronal contributors are corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus (PVN) of the hypothalamus ("H" in HPA). These neurons release CRF, a neuropeptide, to activate corticotrope cells in the anterior pituitary ("P" in HPA). The pituitary releases adrenocorticotropic hormone (ACTH) into circulation where it reaches the adrenal gland ("A" in HPA). ACTH activates melanocortin-2 receptors in the adrenal gland cortex to then synthesize and release glucocorticoids, including cortisol (in humans) and corticosterone (in rodents) that bind to their cognate low and high affinity receptors, glucocorticoid (GR) and mineralocorticoid (MR) receptors, respectively (de Kloet et al., 2005). GR and MR largely act as transcriptional regulators or participate in rapid, non-genomic signaling to alter cellular function (Evans, 1988; Makara et al., 2001; Tasker et al., 2006). In general, glucocorticoid-mediated activation of GR and MR initiate catabolic processes throughout the body, essential in stress signaling and maintenance of organismal homeostasis (Hasselgren, 1999). Importantly, subsequent glucocorticoid action in the brain, including

at ventral hippocampal neurons and CRF neurons in the PVN, impedes CRF secretion and thus, completes a negative feedback loop (de Kloet, 2005).

Critical to understanding disease mechanisms, HPA stress axis dysregulation is a common endophenotype across neuropsychiatric disorders. Aberrant responses to stress in the environment can often precipitate or worsen disease progression, depending "on the degree to which an individual has control over a given stressor" (McEwen, 2010). Both hyper- and hypo-reactivity of the HPA axis have been implicated in patient populations as underlying features of disease risk (de Kloet, 2005). For example, hypo-reactivity of the HPA axis has been observed in subsets of patients with major depressive disorder or post-traumatic stress disorder (Meewisse et al., 2007; Sherin et al., 2011). Importantly, stress dysregulation is suggested to precede, and therefore increase the risk for, disease onset rather than emerging as a result of disease (Yehuda, 2009). Thus, considerable research, including this dissertation, focus on understanding the factors that contribute to HPA axis programming as a readout of neuropsychiatric disease risk.

Key programming of HPA axis function and responsivity occurs during early development, largely preceding birth. In both human studies and animal models, parental susceptibility to stress and/or exposures to stress prior to parturition can influence HPA axis development in offspring (Bale, 2015; Ostiguy et al., 2011; Yehuda et al., 2007). To identify the specific contributors of HPA axis programming, animal models are used to control the types and amount of stress in the environment (Nestler et al., 2010). For example, stress can be imparted in preclinical rodent studies using psychological and/or physical challenges, including immobilization, social defeat, and isolation (Campos et al.,

2013). During pregnancy, maternal stress alters the maternal milieu that can directly or indirectly impact fetal development *in utero* (Bale, 2016; Weinstock, 2005). Because the impact of stress is transmitted from parent to offspring, the term 'intergenerational transmission' has been applied (Klengel et al., 2016). However, preconception stress in either parent can impact germ cells, thus influencing development in one or more generations, resulting in transgenerational effects (Lane et al., 2014; Rodgers & Bale, 2015). Importantly, the specificity of intergenerational and transgenerational effects can depend on the parent-of-origin, lying downstream of sex-dependent genetic factors (e.g. sex chromosomes or genomic imprinting) or reproductive tissues (e.g. placenta, testes, epididymis) (Bale, 2015; Gabory et al., 2009). While efforts to understand parental stress inheritance have largely focused on maternal gestational stress, recent research using mouse models support the contribution of paternal lifetime stress in influencing offspring outcomes. Moreover, modeling parental stress in mice necessitates the careful characterization of parental mouse strains and their associated baseline stress vulnerability, which can impact offspring HPA axis development outside of environmental triggers (Bogaert et al., 2006; Mozhui et al., 2010). This dissertation explores parental influences on HPA axis programming with a specific focus on the paternal lineage, downstream of both baseline strain-dependent stress phenotypes and chronic stress in the environment, investigating mechanisms whereby paternal tissues and germ cells influence offspring neurodevelopment.

II. Parental transmission of stress phenotypes

In both human studies and animal models, intergenerational transmission of stress exposures through both the maternal and paternal lineages have been associated with endophenotypes of stress-related neuropsychiatric disease in adult offspring, including disruption of the HPA stress axis (Bale, 2015; Bowers et al., 2016; Lehrner et al., 2014; Yehuda et al., 2014). Understanding the mechanisms by which parental stress exposure is ultimately communicated to the developing fetal brain is critical for elucidating the complex etiology of mental health disorders. Epigenetic control of gene expression, including DNA methylation, histone post-translational modifications (PTMs), and non-coding RNAs, evolved to regulate and establish cell- and tissue-specific gene expression programs and to control normal cellular functions (Gibney et al., 2010; Jaenisch et al., 2003). Stress experienced during critical developmental windows when these epigenetic patterns are generated can result in reprogramming of cellular epigenomes, leading to long-term changes in patterns of gene expression and cellular function. More specifically, stress exposure can lead to such epigenetic alterations in brain, peripheral tissues, and sperm and oocytes, resulting in transmission of altered marks to the pluripotent zygote (Bale, 2011; Nugent et al., 2015; Rodgers, 2015). Following conception, stress exposure can also directly alter epigenetic programming of the fetus by disrupting the function of extra-embryonic tissues, including the placenta, to promote alterations in key developmental signals throughout gestation. These epigenetic signals are mechanisms by which transient stress experienced during critical periods in offspring brain development produce long-term HPA stress axis dysregulation. Thus, parental stress exposures during

the preconception and prenatal windows can have lasting consequences on offspring development and, subsequently, adult outcomes (Figure 1.1).

Human evidence of intergenerational stress transmission

Epidemiological studies provide abundant evidence linking parental stress to offspring health outcomes. Historically, efforts to understand parental stress inheritance have largely focused on maternal gestational exposures where prenatal stress has been associated with an increased risk for autism spectrum disorders, schizophrenia, affective disorders, and attention deficit hyperactivity disorder in offspring, related to the specific stage of pregnancy in which stress experience occurred (Weinstock, 2005). For example, maternal psychological stressors, such as those associated with war and other traumatic life events, experienced during the first and second trimester of pregnancy have been associated with an increased risk of schizophrenia in male, but not female children (Khashan et al., 2008; van Os et al., 1998). In contrast, late gestation may be a sensitive period wherein stress exposure can lead to long-term alterations in cognitive function and risk for ADHD, particularly in females (LeWinn et al., 2009; Li et al., 2013; Ronald et al., 2010). Maternal preconception stress effects on offspring disease risk have been less explored. However, there have been significant associations between maternal childhood abuse and poor psychological outcomes in future children (Dubowitz et al., 2001; Miranda et al., 2011). Other studies in human populations have linked maternal grief within the year prior to conception with increased risk of offspring neurodevelopmental and affective disorders and infant mortality (Class et al., 2013; Rieder et al., 2013;

Yehuda et al., 2008b; Yehuda, 2014). Additionally, offspring born to mothers who were Holocaust survivors had greater GC sensitivity and decreased methylation in the GR promoter of blood samples, suggesting preconception maternal stress has intergenerational effects (Lehrner, 2014; Yehuda, 2014).

More recently, research efforts have focused on understanding the contribution of paternal lifetime exposures on offspring development. For example, well-kept records from the town of Overkalix, Sweden documented the births and deaths of its citizens as well as periods of nutrient abundance and scarcity. Using these records, researchers linked paternal and grand-paternal food supply during the slow-growth period, a window of adolescence, to mortality and cardiovascular risks in subsequent generations (Bronson et al., 2017; Clifton, 2010; Gabory et al., 2012; Howerton et al., 2013). In other retrospective epidemiological studies, adult offspring whose fathers were Holocaust survivors presented with increased rates of major depressive disorders and anxiety, reduced GR sensitivity, and increased methylation at the GR promoter in blood samples (Lehrner, 2014; Yehuda et al., 2001; Yehuda, 2014; Yehuda et al., 2008). Other paternal exposures including smoking (Deng et al., 2013; Ji et al., 1997; Pembrey et al., 2006a), alcohol abuse (Abel, 2004; Day et al., 2016) and advanced age (Malaspina et al., 2001; Reichenberg et al., 2006) have also been associated with changes to offspring health, supporting that the paternal preconception environment has intergenerational effects as well.

How specifically can parental stress modulate offspring development? Parental experiences were predominately thought to impact offspring health by shaping parental

behavior and care. For example, experiencing trauma prior to conception and presenting with symptoms of post-traumatic stress disorder during childrearing is a strong predictor of offspring neuropsychiatric disease risk (Yehuda et al., 2001). However, as discussed, parental stress transmission may also lie downstream of cellular programming events, implicating maternal and paternal germ cells (i.e. oocyte and eggs) or the trophoblast cells of the placenta. Efforts to determine specific cellular mechanisms of intergenerational stress transmission have recently focused on rodent models of paternal, rather than maternal, preconception stress for two major reasons: 1) the mechanisms whereby maternal stress imparts life-long neurodevelopmental dysfunction during both prenatal and preconception windows are complex, with stress affecting the oocyte, intrauterine environment, placenta, fetus and maternal care simultaneously, and 2) in most rodent models, males do not participate in offspring rearing, allowing researchers to isolate the specific contribution of epigenetic changes in paternal germ cells on offspring development. Thus, the animal studies discussed in this dissertation focus on mechanistic insights from models of paternal stress transmission.

Animal models of paternal stress

Corroborating findings in humans, male mice exposed to periods of chronic or defeat stress sire offspring that have behavioral, physiological or metabolic dysfunction characteristic of stress-sensitive neuropsychiatric disorders (Carone et al., 2010; Dietz et al., 2011; Franklin et al., 2010; Lambrot et al., 2013; Rodgers et al., 2013). As the males are often immediately removed from the female's cage following copulation to limit any

influence on maternal behavior/care (Curley et al., 2011), these studies implicate epigenetic mechanisms within germ cells as mediators of intergenerational transmission. Indeed, rodent studies have demonstrated germ cell susceptibility to stressful environments across the paternal lifespan. For instance, male mice exposed to maternal separation stress during the perinatal period sired offspring with depressive-like behaviors (Franklin, 2010). Our lab has shown that male mice exposed to stress *in utero* present with altered stress coping behaviors and a heightened HPA stress response and transmit this phenotype only to their male, but not female, offspring in the next generation (Morgan et al., 2011). These were two of the first rodent studies demonstrating that male germ cells can be reprogrammed by stress experience during early development. Sperm has distinct periods of differentiation, development, and maturation, and therefore the timing of stress exposure likely impacts distinct mechanisms (Rodgers, 2015). During the prenatal and perinatal periods, development and epigenetic patterning of germ cell precursors and the surrounding reproductive tissues is dynamic; therefore, stress exposure during these critical windows may disrupt the organization of important processes unique to this period (Ly et al., 2015).

Other studies examining paternal transmission have demonstrated that stress exposure of adolescent and adult animals alters germ cell programming. For example, male mice exposed to chronic variable stress sire male and female offspring that exhibit a significantly blunted HPA stress response (Rodgers, 2013). Interestingly, this paternal effect occurred whether the sires were exposed to stress over the pubertal window or solely during adulthood, suggesting that stress exposures post-puberty (i.e. following

maturation of the male reproductive system) evoke similar mechanisms. In contrast, retrospective studies from Swedish famine cohorts associated nutritional challenge during preadolescence with changes in grandson longevity, while such challenges later in life produced no transgenerational effects (Bygren et al., 2001). This disparity in the timing of germ cell vulnerability between our findings in stress-exposed rodents and the findings from the Swedish cohorts may be dependent on species, timing of exposure, or type of perturbation (e.g. psychosocial vs. nutritional). Therefore, further studies are needed in order to identify the windows of germ cell vulnerability in humans. Other paternal exposures on offspring phenotypes have been described using rodent models, including chronic intake of alcohol or drugs of abuse (Finegersh et al., 2014; Vallaster et al., 2017; Vassoler, et al., 2012), nutritional challenges (Carone, 2010; Chen et al., 2015; Lambrot, 2013; Ng et al., 2010), advanced age (García-Palomares et al., 2009; Smith et al., 2009), and environmental toxicants (Guerrero-Bosagna et al., 2014; Skinner et al., 2013), substantiating paternal germ cells as versatile vectors of environmental information to developing offspring.

Strain-dependent transmission of paternal stress

Rodent studies offer strong supporting evidence for observations of paternal stress transmission in human populations, enabling examination of the specific effects of germ cell programming on offspring outcomes. In studies using inbred mouse lines, for example, one fundamental advantage that is impossible in human cohorts is the ability to control the genetic background of the individuals experiencing stress that governs the

physiological and/or behavioral response to stress. Critical to the use of mouse models and the extent of paternal stress effects is selection of the mouse genetic background where different inbred mouse strains have distinctive characteristics, such as variations in physiological responses, cognitive performance, or stress susceptibility (Anisman et al., 2001; Contet et al., 2001; Holmes et al., 2002; Mozhui, 2010; Shanks et al., 1990; Tannenbaum et al., 2003). While paternal strain selection can clearly influence offspring outcomes through inheritance of the genetics characteristic of that strain (Jacobson et al., 2007), it can also control the paternal response to the environment that may alter the non-genetic (i.e. epigenetic) germ cell components delivered at fertilization. For example, the degree of stress susceptibility in mice can determine the extent of the HPA axis response, or vice versa, influencing levels of glucocorticoids available for cellular programming (Ebner et al., 2017; Nasca et al., 2015; Reichardt et al., 2000) and, subsequently, offspring outcomes. In our lab's mouse model of paternal stress, the use of a paternal mouse background (C57BL/6:129 mixed F1 background) known to elicit ample levels of glucocorticoids results in downstream programming of the offspring HPA axis (Rodgers, 2013; Võikar et al., 2001). In comparison, the same paternal stress protocol used in a stress-resistant mouse strain (C57BL6/J) did not recapitulate offspring effects (Rompala, 2018), potentially owing to the known disparities in stress sensitivity between these mouse backgrounds (Võikar, 2001). Moreover, the effects of paternal strain on offspring can be modulated by maternal strain, where strain differences in maternal care (Champagne et al., 2007; Chourbaji et al., 2011) or parent-of-origin genomic imprinting (Barlow et al., 2014; Chaillet, 1994) can influence offspring development, making the

assignment of parental mouse strains an important factor in the intergenerational transmission of stress signals. Thus, baseline stress susceptibility and reactivity as a result of genetic background, especially when compounded by additional environmental perturbations, is an essential consideration for modeling paternal stress in mice. Methods that evaluate paternal stress susceptibility and the extent of HPA axis activation are needed in order to ensure similar levels of offspring programming and for investigation into the cellular mechanisms involved in paternal stress transmission.

III. Paternal mechanisms of intergenerational stress transmission

Stress Programming of Epigenetic Marks in Sperm

The observation that stress exposures across the male lifespan can lead to transmission of offspring phenotypes has brought mounting attention to examination of epigenetic marks in male germ cells (Jirtle et al., 2007). Epigenetic marks have been described in mature sperm in both humans and rodents, including DNA methylation, histone PTMs, and small noncoding RNAs, and have been implicated in transmitting environmental information to the next generation (Bohacek et al., 2015; Rodgers, 2015). The male germ cell undergoes unique and continuous waves of development and proliferation, called spermatogenesis, where patterns of DNA methylation, histone distribution, and small RNA populations are dynamically regulated (Belleannée, 2015; Ly, 2015; McLay et al., 2003). Each step of this process may be vulnerable to environmental stimuli during a male's lifetime. The majority of epigenetic patterning of the male germ cell occurs prenatally by programming primordial germ cells with non-

somatic patterns of DNA methylation (Hajkova et al., 2002). Then, in the postnatal testes, immature sperm cells born from a self-renewing stem cell population migrate through the seminiferous tubules where they rely on Sertoli and Leydig cells to provide immune, nutritional, hormonal and structural support (Cheng et al., 2010). At this stage, sperm histones are actively replaced by protamines, highly charged proteins that allow condensation of sperm chromatin to one-tenth that of somatic cells (Miller et al., 2010). As a result, mature sperm become transcriptionally inert, and are considered resistant to external influences. Following spermatogenesis, the immotile spermatozoa are discharged into the head of the epididymis (the caput) for post-testicular maturation. It is here in the caput that spermatozoa acquire the abilities to swim and fertilize before the fully mature sperm travel to the caudal region of the epididymis where they are stored (Cornwall, 2009). However, recent studies have turned this dogma upside down, demonstrating that mature sperm are responsive to homeostatic challenges, including dietary disruption, stress or trauma, and exposure to drugs of abuse, during spermatogenesis or the maturation stage that occurs in the epididymis (Chen, 2015; Lambrot, 2013; Rodgers, 2013; Sharma et al., 2016; Siklenka et al., 2015; Vassoler, 2012).

DNA Methylation in Sperm

Sperm DNA methylation patterns are well described in normal germ cell development, and specific changes to these patterns have been reported in response to paternal stress exposure, such as maternal separation stress and odor-paired fear conditioning (Dias et al., 2014; Franklin, 2010). During embryogenesis, the developing

germ cell undergoes global erasure of DNA methylation marks. Following this process, *de novo* DNA methyltransferases specify germ cell methylation patterns that are distinct from those in somatic cells (Ly, 2015). An additional wave of active DNA demethylation of the paternal gamete occurs immediately post-fertilization in the zygote (Wu et al., 2010). Importantly, some genomic loci are resistant to demethylation, a process of genomic imprinting critical for normal development, as mistakes at imprinted loci can result in neurodevelopmental disorders, including Angelmans and Prader-Willi syndromes (Hackett et al., 2013; Lawson et al., 2013). Changes to sperm DNA methylation have been reported in rodent models of chronic stress experience (Dias, 2014; Franklin, 2010; Wu et al., 2016). For example, males that experienced odor-paired fear conditioning as adults had decreased DNA methylation at the specific genomic locus of the corresponding odor receptor in their sperm, suggesting a mechanism by which stress experience may produce offspring with specific behavioral changes (Dias, 2014). Intriguingly, in the same study, these sperm DNA methylation changes corresponded to increased offspring behavioral sensitivity to the associated odor. However, DNA methylation changes at this odor receptor were not present in the brains of these offspring, suggesting sperm DNA methylation changes may influence other epigenetic mechanisms, such as histone PTMs, to program the offspring brain. In another study, males exposed postnatally to maternal separation stress sired offspring with depressive-like behaviors (Franklin, 2010). These altered behaviors were also associated with changes in DNA methylation patterns at loci related to stress regulatory genes and epigenetic pathways in both the paternal germ cell and in the offspring brain. However,

how stress induces such site-specific sperm methylation changes and how these changes influence the programming of adult offspring tissues to produce behavioral phenotypes, are not known.

Histone Post-Translational Marks (PTMs) in Sperm

Histone PTMs are also potential epigenetic signals in sperm. Roughly 1% of histones in mice and 10% of histones in humans are retained in sperm chromatin following the active exchange of histones with protamines during late spermatogenesis (Brykczynska et al., 2010; Miller, 2010). As a result, any information written into the sperm histone code regarding paternal exposures was assumed to be lost. However, retained histones have been mapped to regions of important developmental genes, suggesting a designation for those that are critical for post-fertilization function in the zygote (Hammoud et al., 2009). Histone PTMs associated with transcriptional activation in sperm may increase the dosage of important developmental genes and/or allow for paternal-driven gene expression in the zygote. As evidence to this point, disruption of the specific histone mark, H3K4me2, in sperm altered gene expression in the two-cell zygote and severely impaired offspring development (Siklenka, 2015). In addition, sperm from male rats that were administered chronic cocaine showed increased H3 acetylation specifically at the *Bdnf* promoter in both paternal sperm and in the offspring brain, supporting the hypothesis that retained histone PTMs may denote genes important to offspring development (Vassoler, 2012; Wimmer et al., 2017). In addition to histone PTMs, protamine biochemical modifications have also been reported, supporting a

potential protamine code in sperm that imparts transcriptional effects on embryo development (Brunner et al., 2014). However, as protamines are rapidly replaced with maternal histones post-fertilization (McLay, 2003), how such protamine modifications could influence embryogenesis requires further investigation.

Small Non-coding RNAs in Sperm

While the central dogma describes mature sperm as transcriptionally inert, their content is now understood to change through the maturational stage in the epididymis. Indeed, populations of small noncoding RNAs (~22-34 bp) have been well described in the mature sperm of humans and animals, including microRNA (miRs), PIWI-associating RNAs (piRNAs), and transfer RNA-derived fragments (tRFs) (Kawano et al., 2012; Krawetz et al., 2011; Peng et al., 2012; Sendler et al., 2013). Small non-coding RNAs have the capacity to respond rapidly to environmental cues and regulate gene expression, making them primary candidates for transmission of paternal experience. In particular, studies suggest that miRs are critical for proper embryogenesis (Bernstein et al., 2003; Liu et al., 2012; Lykke-Andersen et al., 2008). miRs are ~22 bp non-coding RNAs that post-transcriptionally regulate gene expression. In the nucleus, the enzyme Dicer preprocesses miR precursors and loads them into the RNA-induced Silencing Complex (RISC). The RISC employs miRs as guides to target complementary mRNAs for degradation or to inhibit translation. The capacity for each miR to regulate hundreds of genes suggests that miRs can have extensive programmatic effects. In the zygote, loss of Dicer or Argonaut-2, the catalytic component of the RISC, results in embryonic lethality

(Bernstein, 2003; Lykke-Andersen, 2008). Moreover, inhibition of miR-34c, a known sperm-derived miR, arrests the zygote before the two-cell stage, again supporting the important role of sperm miRs (Liu, 2012).

Stress-dependent changes to sperm small RNAs have been reported in rodent models of chronic stress, dietary challenges, and substances of abuse (Chen, 2015; de Castro Barbosa et al., 2016; Fullston et al., 2013; Gapp et al., 2014; Rodgers, 2013; Rompala et al., 2018; Sharma, 2016; Short et al., 2016). In our lab, male mice administered a chronic variable stress paradigm sired offspring with stress dysregulation as adults, with increased levels of specific sperm miRs as potential molecular links (Rodgers, 2013). Additionally, changes to sperm tRF levels have been identified in response to both low protein and high fat diets in male rodents (Chen, 2015; Sharma, 2016). In order to test the specific contribution of sperm RNA on transmitting paternal experiences to offspring development, microinjection techniques can be used to directly inject experience-altered RNAs found in sperm into fertilized zygotes. These zygotes can then be examined for the direct effects of sperm RNA or implanted into foster females to be reared and tested as adults. Such manipulations enable researchers to separate the effects of sperm RNAs from confounding factors, present in both human studies and animal models, that can also influence offspring outcomes, such as changes to paternal or maternal behavior (Curley, 2011). Indeed, zygote microinjection of total sperm RNA, specific miRs or specific tRFs phenocopied transmission of paternal experiences (Chen, 2015; Gapp, 2014; Grandjean et al., 2015; Rassoulzadegan et al., 2006; Rodgers et al., 2015; Sharma, 2016). For example, we previously demonstrated that animals resulting

from zygote microinjection of the sperm miRs altered by paternal chronic stress recapitulated the offspring stress phenotype (Rodgers, 2015). These studies demonstrate that sperm small RNA populations, including miRs, are sensitive to a variety of psychological and physiological stressors, and are causal mediators of offspring brain programming. Recently, RNA modifications in sperm have also been implicated in paternal transmission of high-fat diet (Chen, 2015; Chen et al., 2016); however, more studies are needed to generalize their role to other paternal perturbations and to humans.

Sperm RNA Programming: Soma-to-Germline Communication by Extracellular Vesicles

The plethora of evidence supporting germ cell epigenetic modifications in response to environmental perturbations prompts consideration for the mechanisms by which the male reproductive tract can alter germ cell content. Historically, contrary to this line of thinking was August Weismann's popular theory published in 1893, known as the 'Weismann barrier', describing a one-way information transfer from germ cells to somatic cells (Weismann, 1893). This theory implied that environmental changes to somatic cells could not be inherited through the germline and, in effect, Lamarck's theory of inheritance of acquired characteristics was impossible (Eaton et al., 2015). In an attempt to mechanistically explain Lamarck's observations, Darwin proposed his pangenesis theory. The pangenesis theory suggested that all somatic cells shed minute particles termed 'gemmules' that could accumulate in the gonads and integrate with the germline, conferring inheritance of acquired parental characteristics to the next

generation (Liu, 2008). This theory was largely rejected during Darwin's lifetime; however, recent studies shed new light on soma-to-germline communication.

Accumulating evidence support soma-derived factors as causal mediators of germ cell changes. For example, in a rat model of paternal liver fibrosis, chronic treatment with the hepatotoxin CCl_4 resulted in suppressed hepatic wound healing across multiple generations. In the exposed male rats, chronic treatment induced chromatin remodeling in sperm. Subsequently, serum transfer from CCl_4-treated rats to naïve rats recapitulated sperm chromatin remodeling, suggesting blood-borne soluble factors can induce germ cell epigenetic changes (Zeybel et al., 2012). While the serum soluble factor responsible for these effects were not identified, hormones have been suggested as potential somatic signals of paternal experience (Sharma, 2013), though this has not been well-examined.

Mechanistically, how molecular substrates in blood can regulate sperm content is not well understood. In the male reproductive tract, very real biological manifestations of Weismann's theoretical barrier can be identified. For example, sperm develop and mature in the lumen of the immune-privileged testes and epididymis, whereby tight junctions of the blood-testis and blood-epididymis barriers formed by Sertoli cells or epididymal epithelial cells, respectively, physically restrict many blood-borne molecules from interacting with sperm in order to generate a microenvironment distinct from the surround interstitium (Mital et al., 2011; Sullivan et al., 2013). Transporters lining the apical and basolateral membranes of these barriers additionally regulate the permeability of these tissues (Mital, 2011), prompting consideration for how circulating molecules can penetrate these barriers to alter the luminal microenvironment regulating sperm content.

Of particular interest to this question is the observation that, despite losing much of its cytoplasmic volume and transcriptional activity upon entering the stage of post-testicular maturation (Cooper, 2005; Neto et al., 2016), sperm continue to gain additional functions through changes in its lipid, protein, and RNA content throughout the epididymis (Machtinger et al., 2016; Sullivan, 2013). Here, extracellular vesicles (EVs), that normally deliver cargo from epididymal epithelial cells to maturing sperm, have been proposed as Darwin's 'gemmules', acting as dynamic intermediaries between paternal environmental exposures, somatic responses, and sperm changes (Liu, 2008). We will discuss the role of epididymal EVs in specifically altering sperm small RNAs, as multiple studies now demonstrate their causal and functional role in transmitting paternal lifetime exposures.

Extracellular Vesicles – bypassing the Weismann Barrier

Extracellular vesicles (EVs) are small membrane bound particles produced by most, if not all, eukaryotic cells (Tetta et al., 2013). EVs have been classified primarily by their subcellular origin and the tissue that produces them. Some EVs, often referred to as microvesicles (50-1000 nm in diameter), bud directly from the cell membrane. Others are generated inside multivesicular bodies and released upon fusion of these compartments with the plasma membrane (Raposo et al., 2013; Théry et al., 2006). These are generally termed as exosomes (40-100 nm in diameter), though it is also common to see this name altered to reflect their tissue of origin – for instance, exosomes produced by epithelial cells in the epididymis are often called epididymosomes, while prostasomes

originate from the prostate (Tkach et al., 2016). EVs play a recently appreciated role in intercellular communication and have advantages over other mechanisms in that rather than a given signal consisting of a single molecule, they can deliver complex payloads of communicating factors, including proteins, lipids, and nucleic acids (Raposo, 2013; Tetta, 2013). Once they reach their targets, EVs can transmit their signal by presenting a membrane-bound ligand to a cellular receptor, by inducing their internalization via endocytosis, or by fusing directly to the plasma membrane, passing on membrane bound constituents and/or releasing an internal cargo to act inside a targeted cell (Tkach, 2016). EVs are produced at high levels by the tissues of the male reproductive tract, such as the epididymis, and play a critical role in the intercellular signaling of these tissues with sperm (Belleannée, 2015; da Silveira et al., 2018).

Within the epididymis, regulation of EV content is regionally distinct in the three main segments of the epididymis: the caput, the corpus, and the cauda (Belleannée et al., 2013). Although most of the initial work characterizing the role of epididymal EVs in shaping post-testicular sperm development focused on changes in lipid and protein profiles, recent studies have emphasized EV-mediated delivery of small RNAs to transcriptionally silent sperm. For example, a recent study in mice found that more than 80% of epididymal EV miRs were shared by sperm isolated from the same epididymal region (Reilly et al., 2016). Interestingly, this was a significantly greater degree of overlap in miR content than existed between the epididymal EVs and the epithelial tissue that produced them. An independent group made the same observation in the bovine epididymis, suggesting that EV miRs are actively tailored for export, rather than simply

reflecting the miR profile of the originating tissue (Belleannée, 2013). Additionally, epididymal EVs can package tRFs that contribute approximately 80% of the small RNA content of sperm in the cauda epididymis (Sharma, 2016). This was not the case for sperm isolated directly from the testes, suggesting that sperm gained the tRFs as they matured in the epididymis.

Given their origin from a somatic tissue capable of receiving physiological signals, epididymal EVs and their content may be influenced by paternal exposures. For example, the epididymis is a hormone-responsive tissue containing receptors for androgens and glucocorticoids (O'Hara et al., 2011; Silva et al., 2010; You et al., 1998), suggesting a potential mechanism whereby chronic pharmacological treatment with glucocorticoids alone was sufficient to produce sperm changes (Petropoulos et al., 2014; Short, 2016; Wu, 2016). Moreover, in a mouse model of paternal low protein diet, caput epididymal EVs had altered tRF profiles, resulting in changes in the sperm tRF content delivered to the oocyte (Sharma, 2016). Thus, epididymal EVs may act as key players in soma-to-germline communication, where paternal perturbations can be communicated to transmit heritable information to offspring

Targets and Functions of Sperm miRs

Though many studies have now related paternal stress experiences with changes to sperm small RNA content, how sperm small RNAs subsequently act at fertilization to alter the trajectory of offspring development remains unclear. To understand the direct effect of sperm RNAs, the majority of studies have focused on changes to the zygote and

early embryo (Chen, 2015; Rodgers, 2015; Sharma, 2016). Interestingly, two distinct cell lineages derive from the early embryo to form embryonic and extra-embryonic membranes, generating the fetal tissues and placenta, respectively. Aberrant development of the fetal tissues can result in improper tissue functions in adulthood, increasing the risk for disease later in life (Calkins et al., 2011). Proper placental function is also a crucial contributor to offspring viability and health, acting as the interface between maternal and fetal circulation (Cross, 2006). Thus, sperm RNA action in the zygote that impairs downstream development and functions of these tissues may mechanistically contribute to offspring reprogramming.

Oocyte/Zygote

As the relative abundance of RNA delivered by one sperm cell (~10 fg) (Boerke et al., 2007; Krawetz, 2005) is so little compared to the amount of RNA in a single oocyte (0.5-1.5 ng), the role of sperm RNA has been considered negligible for embryogenesis (Olszanska et al., 1990). This view was substantiated by the generation of parthenogenic mice – offspring produced from only maternal germ cells (Kono et al., 2004), suggesting successful reproduction does not require the paternal gamete, let alone paternal RNAs. However, the survival rate of parthenogenic mice was low, with only 0.6% (2/371) of parthenogenic embryos transferred to recipient females living to adulthood (Kono, 2004). Therefore, the paternal contribution at fertilization likely includes elements, perhaps RNAs amongst others, critical for facilitating proper development. The argument for an important role for sperm RNAs is substantiated by a study where idiopathic infertility in

men was correlated with a lack of sperm RNAs (Jodar et al., 2015). Further, a study in mice demonstrated that sperm treated with RNases, resulting in a 90% decrease in RNA levels, led to reduced morula-blastocyst transitions and live birth rates (Guo et al., 2017). However, these effects of RNase-treated sperm were partially rescued by supplementation with wildtype RNA (Guo, 2017), supporting a functional role for sperm RNAs in embryogenesis.

Considering the important presence of sperm RNAs, what then is their contribution to development? Here, we focus on the recent evidence for functional roles of sperm small RNAs during embryogenesis. In particular, sperm miRs have been implicated in fertilization and pre-implantation development. Sperm miR-34c, for example, when inhibited in the zygote, suppressed DNA synthesis and zygotic cleavage (Liu, 2012), suggesting this sperm miR plays a critical role in fertilization, despite its reported functional redundancy (Wu et al., 2014). Following fertilization, another critical stage for embryogenesis is the maternal-to-zygotic transition, wherein maternal mRNAs are degraded before zygotic transcription occurs (Tadros et al., 2009). Given the canonical function of miRs to degrade mRNAs, sperm miRs transferred and present in the zygote may facilitate this process. For example, germ cell-specific knockout of Dicer1 or Drosha, two enzymes critical for processing miR precursors into their mature forms, resulted in aberrant miR profiles in sperm (Yuan et al., 2016). Zygotes resulting from these knockout sperm had impaired maternal mRNA turnover and development (Yuan, 2016), suggesting that sperm miRs promote embryogenesis by facilitating the maternal-to-zygotic transition, as suggested for other miRs present during this

developmental stage (Giraldez et al., 2006). Importantly, when miRs important for maternal mRNA degradation are absent, maternal mRNA clearance is delayed (Giraldez, 2006), suggesting developmental delay may occur depending on the miRs present during this sensitive window, thus influencing offspring outcomes.

As environmental perturbations, such as stress, during the paternal lifetime can alter sperm miR populations, miR regulation of mRNA in the zygote may be a mechanism whereby paternal exposures influence offspring development. To test this hypothesis, we used our paternal chronic stress model where specific sperm miRs reprogrammed stress axis reactivity and hypothalamic transcription in offspring (Rodgers, 2013; Rodgers, 2015). Following zygote microinjection of the stress-altered sperm miRs, we examined the expression levels of maternal mRNAs that were predicted targets of these specific miRs in the two-cell zygote. As expected, the majority of these predicted mRNAs were repressed (Rodgers, 2015). Interestingly, the two most downregulated transcripts were *Sirt1* and *Ube3a*, which play important roles during mammalian development and have been implicated in neurodevelopmental and metabolic disorders in humans (Greer et al., 2010; Herskovits et al., 2014). In our paternal stress model, neither the expression of *Sirt1* or *Ube3a* were altered in the adult offspring hypothalamus, suggesting repression of these genes by sperm miRs may act during the early sensitive window in the zygote to influence subsequent developmental events, ultimately reprogramming adult offspring tissue (Rodgers, 2015).

Other small noncoding RNAs in sperm, such as tRFs, may have similar roles during offspring development. Derived from the 5’ or 3’ ends of tRNAs, tRFs can silence

viral transcripts with complementary sequences and inhibit translation (Raina et al., 2014). When delivered by sperm, tRFs in the zygote repress genes associated with endogenous retroelements active in pre-implantation embryos (Sharma, 2016). Further, microinjection of sperm tRFs altered by high fat diets resulted in distinct transcriptomic changes at the 8-cell and blastocyst stages, with few overlapping differentially expressed genes between these stages (Chen, 2015). These studies suggest that sperm small RNAs can directly impact gene expression in the zygote, thus initiating a cascade of transcriptional events that alters the development of later embryonic stages, ultimately guiding towards a phenotype reflective of the paternal environment.

Placenta

Until recently, the placenta has been often overlooked as a factor in the developmental origins of disease (Cross, 2006). Lying between the maternal milieu and fetal circulation, the placenta plays a critical role by providing the fetus with essential nutrients and gases and blocking maternal immune signals (Nugent, 2015). Importantly, shifts in placental function or regulation can disrupt brain development, resulting in changes to adult behaviors, metabolism, and HPA stress axis reactivity (Bronson, 2017; Howerton et al., 2014; Nelissen et al., 2011; Wu et al., 2017). As the developing placenta derives from the embryo, it is likely sensitive to post-fertilization events, such as sperm small RNA actions in the zygote, and their downstream consequences. In fact, specification of the trophectoderm that forms the placenta begins at the 8-cell stage, when blastomeres separate into embryonic and extra-embryonic cell lineages (Red-Horse et al.,

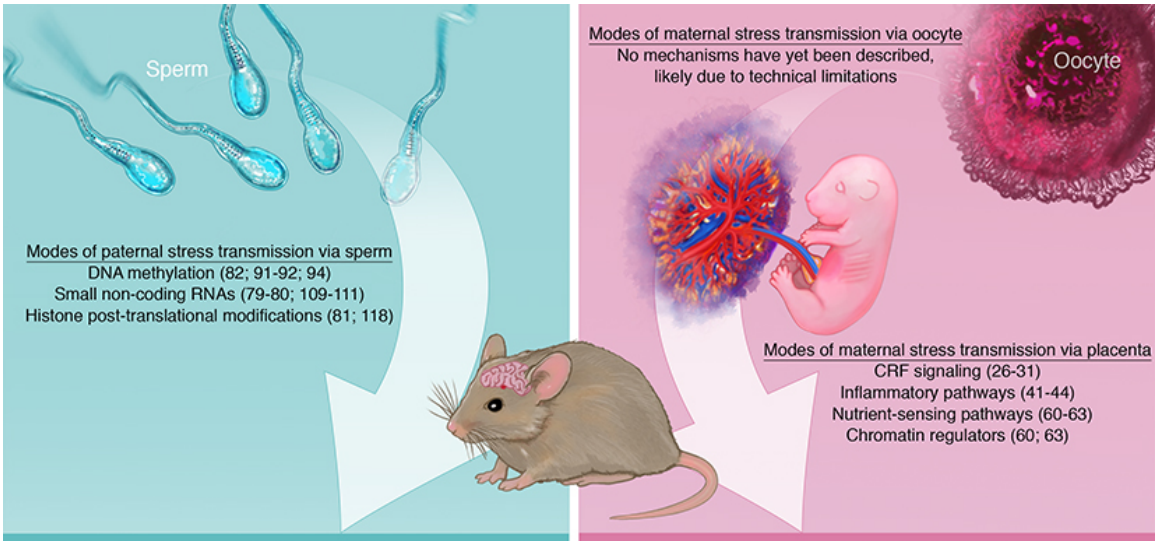
2004). Such specification of these cell lineages is a careful coordination of both transcriptional and epigenetic regulation present during earlier development (Maccani et al., 2009; Red-Horse, 2004). Therefore, paternal delivery of functional RNAs, such as miRs, may have the capacity to alter the regulatory transcriptional events upstream of trophectoderm specification and subsequently, placental regulation of offspring neurodevelopment.

While evidence for the role of sperm small RNAs on placental development and function is in its infancy, two studies to date have associated paternal exposures with placental alterations. In one study, a paternal low folate diet altered the DNA methylation profile in sperm and resulted in transcriptional dysregulation in the placenta (Lambrot, 2013). Importantly, these changes in sperm and placenta were further associated with negative offspring outcomes, including craniofacial and musculoskeletal deformations (Lambrot, 2013). In another study, a paternal high-fat diet impaired placental growth and gene expression, and associated these changes with reduced fetal growth and viability (Binder et al., 2015). Therefore, paternal exposures may contribute to offspring reprogramming through changes in the placenta. Further studies should examine the effects of sperm RNA, such as miRs, on placental development and function.

IV. Overview of Dissertation

Considering the number of studies establishing experience-dependent changes in sperm small RNAs, including miRs, as conveyors of the paternal preconception environment, the main goal of this dissertation was twofold: 1) to identify the upstream

cellular mechanism whereby paternal stress exposure is communicated from somatic tissues to the germ cells, and 2) to examine the actions of sperm miRs during embryogenesis that can influence adult offspring outcomes. In Chapter 2, we describe a strategy to characterize and confirm paternal stress susceptibility and reactivity in mouse models that require strain selection, with a focus on the use of transgenic mice for probing mechanisms involved in stress signals. In Chapter 3, we use our established paternal stress mouse model to determine a cellular mechanism within the epididymis that programs sperm miRs and the downstream offspring stress dysregulation phenotype. In Chapter 4, we build on the evidence that sperm miRs can influence regulation of offspring neurodevelopment by disrupting the transcriptome of the embryonic brain and placenta. Finally, we conclude this dissertation in Chapter 5 with a general discussion of this work, its potential implications, and future directions.



Exposure	Species	Offspring outcomes	Ref
Swedish famine	H	Increased longevity and decreased risk for cardiovascular disease in grandsons	83-85; 98
Preconception trauma	H	Reduced cortisol levels, reduced GR sensitivity increased risk of depression and PTSD	74; 86
Chronic variable stress	M	Reduced HPA stress axis responsivity in male and female offspring	79
Social defeat	M	Increased depressive-like and anxiety-like behaviors, increased glucocorticoid levels	93
Odor-paired fear conditioning	M	Increased odor behavioral sensitivity in males	91
Maternal separation	M	Increased depressive-like behaviors; altered exploratory behavior	94
Dietary challenge	M	Metabolic dysfunction, developmental defects	78; 80; 82

Exposure	Species	Offspring outcomes	Ref
Psychological stress in pregnancy	H	Increased risk of ASD and schizophrenia in males; increased risk for affective disorders in females	13-19
Infection during pregnancy	H	Increased risk of ASD and schizophrenia in males	34-40
Childhood abuse	H	Increased disruptive behavior, externalizing behavioral problems	71-72; 75
Preconception trauma	H	Poor perceived emotional health, increased depressive and anxiety symptoms, increased PTSD risk, greater glucocorticoid sensitivity	73-74; 86
Prenatal chronic variable stress	M	Increased HPA stress axis sensitivity, cognitive dysfunction, metabolic dysfunction in males	21-23; 60
Prenatal infection	M	Social deficits; altered exploratory behavior; increased repetitive behaviors	42-43
Prenatal glucocorticoids	R	Impaired coping and learning; reduced growth	32-33

Figure 1.1. Intergenerational transmission of maternal and paternal stress can impact offspring neurodevelopment. Paternal stress exposures influence offspring outcomes (left table), potentially through changes in sperm epigenetic marks. Maternal stress during pregnancy alters placental signaling to reprogram offspring neurodevelopment (right table). Few studies to date have examined maternal preconception stress effects on the oocyte, likely due to current technical barriers. References (Ref) correspond with the bibliography where this figure was originally published in Biological Psychiatry, 2018, May 15, Vol. 83(10): 886-894, PMID: 29198470. H, human; M, mouse; R, rat. Illustration by Jay LeVasseur / www.appliedartstudio.com.

CHAPTER 2

STRAINED IN PLANNING YOUR MOUSE BACKGROUND? USING THE HPA STRESS AXIS AS A BIOLOGICAL READOUT FOR BACKCROSSING STRATEGIES

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Main Text

The use of transgenic mouse lines over the last several decades has been indispensible for modeling human conditions and probing mechanisms underlying disease. Importantly, selecting the correct mouse background strain can be difficult and is, no doubt, critical to experimental outcomes, interpretation of results, and reproducibility. In the fields of stress and neuropsychiatric disease research, strain-dependent differences in treatment sensitivity, neuroanatomical development, stress and physiological responses, and performance on behavioral tests have directed laboratories toward using preferred mouse strains for reliable, robust results.

In the last 25 years, transgenic mice have paved the way toward a greater understanding of genes involved in disease pathology. However, when examining the Jackson Laboratories mouse inventory, the chances of finding a transgenic mouse on your preferred background strain are unlikely. In fact, the standard transgenic mouse-production pipeline of injecting embryonic stem cells (ES) with the incorporated transgenic modification into blastocysts typically uses 129-derived ES cells and C57BL/6 blastocysts (Ledermann, 2000). The resulting successful chimeras are then backcrossed on a C57BL/6 background to determine transgene transmission. As agouti coat color is dominant over black, this cross allows penetrance determination. For convenience and to save time, labs then often continue breeding the mice to a C57BL/6 background, thus resulting in the extensive usage of this strain of mice for neurobehavioral outcomes. Numerous studies have corroborated the usefulness of the C57BL/6 mouse in neuroscience research as this strain excels in learning-dependent tasks (Holmes, 2002),

shows clear preferences for sucrose and alcohol (Pothion et al., 2004; Yoneyama et al., 2008), gains weight in diet-induced obesity models (West et al., 1992), exhibits high levels of immobility in the forced swim test (Lucki et al., 2001), has high sensitivity to pain (Mogil et al., 1999), and responds well to antidepressant treatment compared to other inbred strains (Lucki, 2001). In comparison, substrains of the 129 background display significant difficulty with hippocampal-dependent learning tasks and fear extinction (Camp et al., 2012; Hefner et al., 2008), and another popular inbred strain, FVB/N, also has problems with learning tasks and prominent visual impairments (Brown et al., 2007). Moreover, the C57BL/6 mouse was the first rodent to have its genome completely sequenced, again adding to its popularity amongst researchers and the disproportionately available literature and resources for this strain (Consortium et al., 2002).

For many labs, however, the C57BL/6 mouse is not advantageous. In 2005, Ducottet and Belzung reported that among 8 inbred mouse strains tested, C57BL/6 mice were relatively resistant to mild chronic variable stress, rendering this strain less relevant for studies examining the role of stress experiences in the etiology of neuropsychiatric disorders (Ducottet et al., 2005). Strain differences in baseline stress responsiveness and anxiety-like behaviors are likely critical components of strain disparities in stress susceptibility (Homanics et al., 1999; Mozhui, 2010) In contrast to C57BL/6 mice, BALB/c and 129 mouse strains produce significantly greater levels of corticosterone in response to an acute stress (e.g. restraint). These 2 strains also exhibit more anxiety-like behaviors in the elevated plus maze, light-dark box, and open field tests, and show

greater susceptibility to chronic stress (Anisman et al., 2005; Homanics, 1999). Therefore, the 129 and BALB/c strains are more likely to provide face validity in studies modeling susceptibility to stress experiences as an increased risk for neuropsychiatric-related disorders than C57BL/6 mice (Anisman, 2005; Ducottet, 2005). Importantly, the use of specific inbred mouse strains based on behavioral and physiological characteristics to model disease does not parallel the relatively heterogeneous characteristics of the human population. However, for some disorders in which there is phenotypic vulnerability, including increased stress sensitivity as a predisposition for affective disorders, it may be effective to develop rodent models by strategically selecting mouse strains based on their known characteristics (Bale, 2006).

More recently, to apply the mutual advantages of these known strain-specific attributes, many labs are using mixed-strain background mice (Curley et al., 2012; Mueller et al., 2008; Ridder et al., 2005). Researchers selectively breed two strains that have desired characteristics with the resulting offspring demonstrating a hybrid vigor (Birchler et al., 2006). For instance, in order to produce a mouse strain that is susceptible to chronic stress and still produces robust phenotypes on behavioral tasks including cognitive performance, our lab uses C57BL/6:129 F1 hybrids for many of our studies. This method allows our lab to use a new transgenic line arriving on a C57BL/6 background for experimental testing after only one generation by crossing them with 129S1/SvImJ mice to generate the F1 hybrid. However, in the case of conditional knockouts and more complex crosses in which two or more transgenic lines are on a C57BL/6 background, one or more lines need to be backcrossed onto a 129 strain before

we can produce F1 hybrids for testing. With each mouse breeding requiring substantial time – each generation taking at least three months - we wanted to develop a biological assay as a readout to determine how many generations a C57BL/6 mouse needed to be backcrossed before producing experimental animals that would more closely resemble traits of the 129 strain.

To resolve this question, we used the robust strain differences in the hypothalamus-pituitary-adrenal (HPA) stress axis between the low stress-responding C57BL/6 and the high stress-responding 129 mouse strains as a physiological readout for inheritance of background genetics. We utilized the HPA stress axis due to its known strain differences, and as it is a robust, quantitative, and relatively non-invasive procedure that provides predictive validity for chronic stress susceptibility. There are certainly other stress measures that may also be relevant (e.g. prefrontal cortex and/or amygdala reactivity) that could be explored in specific mouse strains as well (Kumar et al., 2014; Williams et al., 2015). Following each backcross, we examined the HPA stress response of the resulting offspring. The generation in which the strain differences in HPA reactivity disappeared designated the minimal number of backcrosses necessary for a mouse of C57BL/6 origin to inherit the genes underlying our desired characteristic - in this case, a heightened HPA stress axis responsiveness.

In addition, as labs interested in developmental outcomes may be backcrossing male and/or female mice of either strain, we also considered the important potential contribution of maternal vs. paternal genotype for inheritance of background genetics utilizing the HPA stress axis phenotype as our outcome measure. **Figure 2.1 A&B**

suggest that for inheritance in both male and female F1 offspring, penetrance of stress-responsive genes was greater with a maternal 129 x paternal C57BL/6 cross, suggesting an effect of maternal/paternal imprinted genes or maternal care in the intergenerational transmission of stress phenotypes. As F1 hybrids resulting from a maternal C57BL/6 x paternal 129 cross had a less reactive HPA stress response, we continued to backcross the male F1 hybrid offspring with 129 females. We exploited the known sex differences in the magnitude of the HPA stress response (i.e. females having a more robust response compared to males), and focused on the female offspring from subsequent generations to produce the greatest strain differences in the HPA response curve. Surprisingly, only three backcrosses were required for the hybrid offspring to show a stress response identical to 129 mice (**Figure 2.1 C&D**), suggesting F3 mice can be used to generate experimental animals for stress exposure studies.

Clearly, research requires great care in selecting the appropriate mouse strain to ensure reproducibility, face validity, and measurable outcomes across research laboratories and models. Mathematically, 10 generations are needed to ensure a strain has achieved 99.9% genetic similarity to the designated pure strain it has been crossed with (Eisener-Dorman et al., 2009). However, timing and mouse care expenses render this expectation extraordinarily expensive, both in terms of cage costs and research time to produce the experimental animals (i.e., nearly 3 years and over 12K in housing costs to generate experimental animals). We propose here that for researchers interested in stress-related research and who are confronted with backcrossing mice to a preferred strain, the HPA stress axis can be used as a robust outcome measure to designate when a mouse

strain has been sufficiently backcrossed to pass on genes sufficient for stress responsivity. In our studies, 3 generations of backcrosses with 129 females were adequate for C57BL/6 mice to produce a 129-like HPA stress response, effectively reducing the number of necessary backcrosses to produce experimental animals by years. Following generation of experimental animals, researchers can continue backcrossing their mice with new breeders to achieve more genetic similarity with the designated pure strain, and to avoid genetic drift that may occur within their mouse colony (Casellas, 2010).

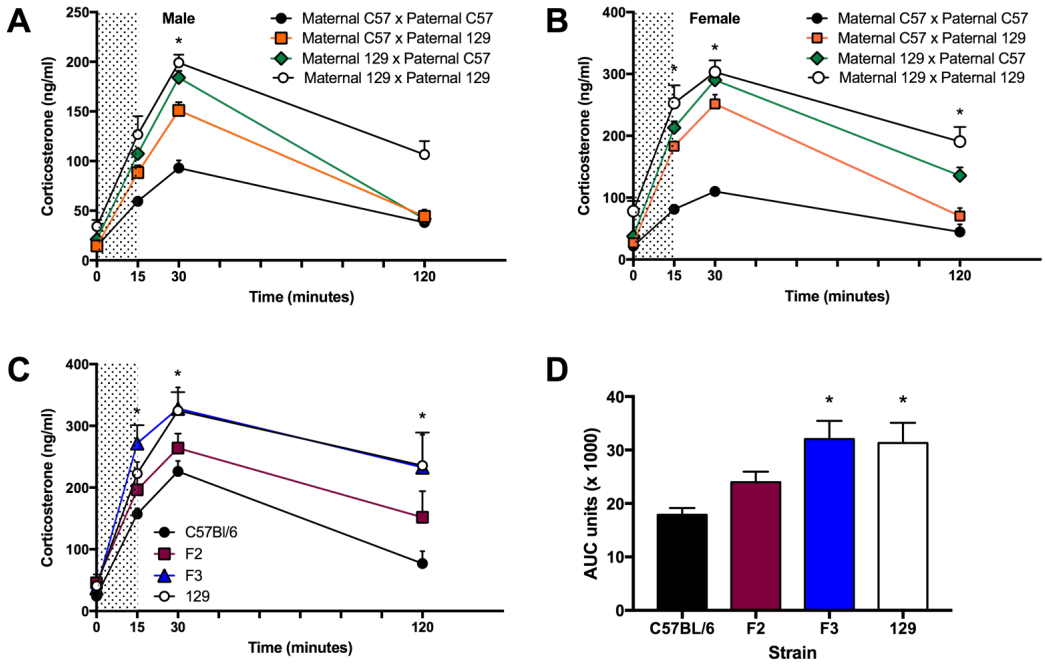

Figure 2.1. Strain-dependent differences in HPA stress axis responsiveness can be used to identify a backcrossing strategy. (**A**) Male and (**B**) female F1 hybrid offspring resulting from the Maternal 129 x Paternal C57 breeding scheme had greater stress reactivity than offspring from a Maternal C57 x Paternal 129 scheme, indicated by greater levels of corticosterone at the 30-minute time point in males: $F_{3,32}=32.61$, $p<.0001$; and a main effect on the corticosterone response curve in females: parental strain: $F_{3,34}=41.25$, $p<.0001$; time: $F_{3,102}=172.1$, $p<.0001$; interaction: $F_{9,102}=6.82$, $p<.0001$. *$p<.05$ Maternal 129 x Paternal C57 vs. Maternal C57 x Paternal 129 and Maternal 129 x Paternal 129 vs. Maternal C57 x Paternal C57. N=6-11/group/sex. (**C**) Female mice of 129 and F3 strains had significantly different corticosterone responses from C57BL/6 female mice over time (strain: $F_{3,25}=6.302$, $p=.0025$; time: $F_{3,75}=55.94$, $p<.0001$; interaction: $F_{9,75}=1.28$, $p=.26$. N=6-8/group. *$p<.05$ compared to C57BL/6). (**D**) Total corticosterone levels by area under the curve (AUC) measurements of 129 and F3 strains were significantly greater than C57BL/6 levels ($F_{3,25}=5.005$, $p=.0074$. N=6-8/group. *$p<.05$ compared C57BL/6 by post-hoc analysis). Data are mean ± SEM.

CHAPTER 3

EPIDIDYMAL GLUCOCORTICOID RECEPTORS PROMOTE INTERGENERATIONAL TRANSMISSION OF PATERNAL STRESS

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Abstract

Paternal preconception exposures and insults, including stress, dietary challenge and drugs of abuse, can shape offspring health and disease risk outcomes, as evidenced from retrospective human studies and more recent animal models (Carone, 2010; Chen, 2015; Dias, 2014; Dietz, 2011; Donkin et al., 2016; Franklin, 2010; Kaati et al., 2007; Lambrot, 2013; Lehrner, 2014; Ng, 2010; Pembrey, 2006; Rodgers, 2013; Vallaster, 2017; Vassoler, 2012; Wu, 2016; Yehuda, 2014). Mechanistic examination has implicated small noncoding RNA populations in sperm, including microRNA (miRs), as carriers of paternal environmental information that consequently influence offspring development (Chen, 2015; Gapp, 2014; Grandjean, 2015; Rassoulzadegan, 2006; Rodgers, 2015; Sharma, 2016). However, the cellular mechanisms by which these paternal signals are relayed to sperm and how they may persist remain unknown. Here, using our previously established paternal stress mouse model we identify caput epididymal epithelial glucocorticoid receptors as crucial upstream mediators of long-lasting germ cell programming. We show that glucocorticoid treatment of caput epididymal epithelial cells results in increased glucocorticoid receptor levels and enduring changes to the miR content of secreted extracellular vesicles (EVs), or epididymosomes, known to interact with sperm and alter their RNA content (Belleannée, 2015; Reilly, 2016). Further, significant changes were detected in the caput epididymal histone code long after stress ended, both in vitro and in vivo, as a potential mechanism whereby stress programmed enduring changes to EV miRs. Genetic targeting to reduce caput epididymal epithelial-specific glucocorticoid receptors reversed stress-induced chromatin remodeling and

promoted cellular resilience to paternal stress, ultimately rescuing transmission of a stress dysregulated offspring phenotype. Taken together, these studies identify glucocorticoid receptor regulation of EV miRs in the caput epididymis as a key contributor in the intergenerational transmission of paternal environmental stress experiences.

Main Text

The contribution of preconception insults in the etiology of disease has garnered great interest in recent years, yet the crucial molecular mechanisms whereby an environmental insult is transmitted from somatic to germ cells and is able to persist long after the insult had ended, are not known. To address this, we utilized our established paternal stress mouse model in which we have previously demonstrated that stress-altered sperm miRs causally promote offspring brain reprogramming and stress dysregulation (Rodgers, 2013), an endophenotype common to many neuropsychiatric disorders (Bale, 2006). We focused on the contribution of glucocorticoids as an essential and necessary component of stress signaling that when elevated, bind and activate the low-affinity glucocorticoid receptor, a ubiquitously expressed molecule critical for the orchestration of cellular responses and chromatin remodeling (de Kloet, 2005; Deroo et al., 2001). Further, extracellular vesicles (EVs) from caput epididymal epithelial cells deliver important proteins, lipids, and RNAs, including miRs, to maturing sperm, altering sperm content (Belleannée, 2015, 2013; Reilly, 2016; Sharma, 2016; Sullivan et al., 2007). Therefore, we hypothesized that in response to stress, caput epididymal epithelial glucocorticoid receptors are poised to contribute both to changes in the composition of the secreted EV miRs that interact with and shape maturing sperm, and also to coordinate local somatic epigenetic remodeling, as paternal-experienced stress produces effects that endure long after stress end (Hunter, 2012).

Therefore, to identify the molecular and epigenetic marks involved in the persistence of sperm miR alterations by stress, we administered chronic stress to male

mice and collected epididymal sperm 1- or 12-weeks post-stress end (**Fig. 3.1a,** top) to compare the acute and enduring (allowing approximately two cycles of sperm turnover following stress exposure (Oakberg, 1956)) effects. We performed small RNA sequencing and identified two distinct populations of differentially expressed sperm miRs (adjusted $P < 0.05$), with no similarly stress-altered miRs shared between these populations (**Fig. 3.1b**), suggesting that a unique mechanism emerges following the acute response to stress to induce enduring changes in sperm miR populations.

As we previously established that intergenerational transmission of paternal stress can continue for months after stress has ended, we investigated the epigenetic mechanism whereby epididymal EV miR changes, that are likely to impact sperm content during maturation, are maintained. To examine the specific population of caput epididymal EVs, we treated cultured DC2 mouse caput epididymal epithelial cells with corticosterone *in vitro*. Using DC2 cells allowed us to isolate EVs secreted into the media produced from a specific cell population. As all mammalian tissues secrete EVs into circulation, such select isolation *in vivo* is not possible (Tetta, 2013). Further, this allowed for the controlled administration of corticosterone, the primary glucocorticoid in rodents produced by activation of the hypothalamus-pituitary-adrenal (HPA) stress axis and known to activate the low affinity glucocorticoid receptors (de Kloet, 2005). We confirmed the purity of EVs isolated from DC2 cells using validated EV markers (Théry, 2006) (**Supplementary Fig. 3.1**). To develop an accurate modeling of the timing of events of this 'stress in a dish' model compared to our *in vivo* paternal model, we first examined three distinct concentrations of corticosterone that included the range of the

mouse physiological baseline (low) and stress response (medium), as well as a supraphysiological (high) concentrations, at three time points post-treatment to examine the acute, intermediate, and enduring effects of treatment in DC2 cells (**Fig. 3.1a,** bottom). We then used rank-rank hypergeometric overlap (RRHO) analyses to evaluate the extent of overlap between stress- and corticosterone-altered miRs *in vivo* and *in vitro*, respectively. We compared control v. stress enduring differential expression profiles in sperm miRs and the vehicle v. corticosterone differential expression profiles in DC2 EV miRs at each collection point post-treatment allowing for threshold-free identification followed by quantification of statistically significant overlap between datasets (Plaisier et al., 2010). Using this approach, we compared the populations of significantly overlapping EV miRs following small RNA sequencing and found distinct groups of altered EV miRs that were dependent on the time post-treatment, where the degree of overlap increased dramatically at 8-days compared to 1-day post-treatment at all corticosterone concentrations (**Fig. 3.1c** and **Supplementary Fig. 3.2a**), supporting enduring effects present in our *in vitro* model. Following quantification of total overlapping EV miRs at all corticosterone concentrations and times, we confirmed that 8-days following treatment with the stress-relevant concentration of corticosterone most-closely matched the *in vivo* enduring paternal stress sperm (**Fig. 3.1d** and **Supplementary Fig. 3.2b**), where the total proportion of significantly overlapping miRs rose to 31.4% (116/369). No doubt, the complexity of sperm miR composition reflects additional interactions from along the reproductive tract and therefore will not completely mirror the DC2 EVs, as has been described (Belleannée, 2013; Jerczynski et al., 2016).

To ensure corticosterone treatment did not disrupt the endogenous properties and tissue selectivity of DC2 EVs *in vivo*, we quantified and characterized the size distribution of DC2 EVs using Nanosight particle tracking analysis. Interestingly, corticosterone treatment reduced EV mean size, but not mode, consistent with possible changes to lipid or protein composition that may affect EV performance at select tissues (Record et al., 2014) (**Fig. 3.1e**). We labeled and isolated vehicle- and corticosterone-treated DC2 EVs with a near-infrared, lipophilic DiR dye, and injected 50 million EVs intravenously into naïve male mice (**Fig. 3.1f**, top schematic). 24-hours post-injection, we imaged the tissues to evaluate the bio-distribution of caput epididymal EV targeting. As expected, there was substantial accumulation of EVs in the liver and spleen, as previously described for EVs from most other cellular sources (Wiklander et al., 2015). However, specific to EVs from these epididymal epithelial DC2 cells, there was substantial accumulation along the reproductive tract, including the caput epididymis and testes, and a surprising localization to the brain (**Fig. 3.1f**). Importantly, corticosterone treatment of DC2 cells did not alter EV tissue targeting (**Fig. 3.1g** and **Supplementary Fig. 3.3**). These results suggest that stress at the level of the caput epididymis impacts EV and sperm miR content without disruption to endogenous EV tissue selectivity. The local effects of EV miRs on paternal tissues such as the brain remain to be evaluated.

To assess the role of glucocorticoid receptors in the prolonged timing effects of EV miRs, we performed immunoblotting on DC2 cells 1- and 8-days following corticosterone treatment. While there were no significant changes in glucocorticoid receptor levels immediately following corticosterone treatment end (**Fig. 3.2a,** left),

glucocorticoid receptor levels were increased 8-days post-treatment at all corticosterone concentrations (**Fig. 3.2a,** right). We hypothesized that increased nuclear glucocorticoid receptors may coordinate chromatin remodeling to promote enduring changes to EV miR content. As changes to histone composition and post-translational modifications (PTM) are a likely candidate for upstream broad transcriptional control of miR genes, we performed unbiased quantitative histone mass spectrometry in DC2 cells 8-days post-corticosterone treatment. We applied Random Forests classification analysis to our dataset to identify the histones and PTMs altered by corticosterone. Random Forests is an ensemble-learning algorithm that identifies groups of features (i.e. histone PTMs) altered together, and ranks these features according to their importance to the model's accuracy (Breiman, 2001). Using this approach, we identified the top thirteen histone PTMs, as determined by ten-fold cross-validation of the model (**Fig. 3.2b,** inset), that most accurately discriminate vehicle v. corticosterone-treated DC2 cells (**Fig. 3.2b**). To confirm these Random Forests results, we performed Mann-Whitney U tests (**Fig. 3.2c**), demonstrating long-term remodeling of the histone code that corresponded with post-treatment glucocorticoid receptor increases.

We then compared these enduring *in vitro* DC2 epigenetic changes to our *in vivo* paternal stress model. We again performed histone PTM mass spectrometry on whole caput epididymal tissue from control and stress males 12-weeks post-stress end, and used Random Forests analyses. We identified ten histone PTMs that most accurately classified our model (**Fig. 3.2d**), and that were substantiated by Mann-Whitney U tests (**Fig. 3.2e**). We deconvoluted these histone PTMs identified by Random Forests in our *in vitro* and *in*

vivo models and identified five overlapping histone PTMs (**Fig. 3.2f**), approximately 45% (5/11) of total treatment-discriminating *in vivo* histone PTMs, supporting that our paternal 'stress in a dish' model, where glucocorticoids were administered, extensively mimics features of endogenous paternal stress programming. These five common histone PTMs include modifications to two H1 variants (H12 and H15), H2A1 K5 acetylation, H3 K18 monomethylation, and H3 K14 acetylation. Interestingly, H3 K14ac has been implicated in driving stress effects at the chromatin level in other stress models (Covington et al., 2009; Johnsson et al., 2009; Li et al., 2003). While the literature is scarce regarding the remaining histone PTMs, these data suggest that post-stress glucocorticoid receptor increases may mediate chromatin remodeling at specific loci, including those of EV miRs, to alter their expression, consistent with previous reports (John et al., 2008; Jubb et al., 2017; Paakinaho et al., 2010). Moreover, these data support that stress in the environment is able to promote lasting modifications to cellular transcriptional machinery within reproductive tissues, functionally modifying germ cell content.

To then examine a causal role of epididymal epithelial glucocorticoid receptors in the intergenerational transmission of paternal stress *in vivo*, we genetically targeted glucocorticoid receptors in male mice to reduce expression (GR^{Het}) specifically in caput epididymal epithelial cells using the lipocalin-5 (Lcn5) promoter (Xie et al., 2013) crossed with GR^{flox} mice (Brewer et al., 2003) (**Fig. 3.3a**). We additionally incorporated the transgenic RiboTag line, allowing isolation of mRNA specifically from the HA-tagged ribosomal subunit, Rpl22, in caput epididymal epithelial cells for RNA

sequencing (Sanz et al., 2009). In these mice, we verified transgenic reduction as well as the inhibition of stress-mediated increases in glucocorticoid receptors 12-weeks post-stress in GR^{Het} mice (**Fig. 3.3b**). We hypothesized that inhibiting post-stress glucocorticoid receptor increases here would prevent the enduring intergenerational transmission of the paternal stress phenotype. To test this, we bred control and stressed GR^{WT} and GR^{Het} males to wildtype females, and examined the offspring HPA stress axis response. Remarkably, there was a significant paternal treatment x paternal genotype interaction in the offspring response to an acute restraint whereby paternal epididymal GR^{Het} prevented the blunted offspring stress response to control levels (**Fig. 3.3c**). We extended this finding by examining the effect in response to an additional type of HPA activation, an acute predator odor, as a more ethologically relevant challenge in mice. Again, paternal stress GR^{WT} offspring presented with a dysregulated HPA axis response compared to paternal control offspring, and this heightened response was again rescued in paternal stress GR^{Het} offspring (**Fig. 3.3d**). Importantly, neither treatment nor genotype affected paternal reproductive function or litter characteristics (**Supplementary Table 3.1**). The paraventricular nucleus (PVN) of the hypothalamus is key to HPA stress regulation and we have previously demonstrated transcriptional dysregulation of the PVN in paternal stress offspring (Rodgers, 2013). Therefore, we next examined the ability of the GR^{Het} paternal genotype to rescue these gene expression changes in the offspring PVN. As expected, hierarchical clustering of all genes altered by paternal stress exposure demonstrated that the greatest difference in PVN gene expression was between control and paternal stress GR^{WT} offspring, supporting a strong programming effect of paternal

stress, while paternal GR^{Het} mitigated the extent of PVN changes by paternal stress (**Fig. 3.3e**), demonstrating that caput epididymal glucocorticoid receptors govern paternal stress transmission of the offspring brain and stress response.

To then determine the mechanism within the caput epididymis that can promote or prevent enduring transmission, we performed differential expression analyses on the actively translating genes isolated from HA-tagged Rpl22 subunits (RiboTag) in Lcn5+ cells. Remarkably, comparing within genotype for the effects of stress, there were 1826 differentially expressed genes (adjusted $P < 0.05$) affected by stress 12-weeks following stress-end between GR^{Het} mice, but very few (65 genes) between GR^{WT} mice (**Fig. 3.4a**), suggesting that reducing caput glucocorticoid receptors results in a robust, compensatory response to stress within these epididymal cells. Importantly, there were 176 differentially expressed genes between control mice and 810 genes between stress mice; therefore, the robust response of GR^{Het} mice to stress was not attributed to glucocorticoid receptor reduction alone. In comparison, using the same pipeline, there was a modest caput epididymal response acutely post-stress, where the total number of genes altered (adjusted $P < 0.05$) in all comparisons totaled 62 (**Fig. 3.4b**). Comparing the number of acute v. enduring stress-altered differentially expressed genes, there was a 3-fold induction in GR^{WT} males and a 200-fold induction in GR^{Het} males (**Fig. 3.4c**), supporting the time post-stress as a crucial window whereby stress is processed to promote long-term transmission. Lastly, to determine the functional pathways broadly affected by the interaction of treatment and genotype in the caput epididymis that remain altered long-term, we performed cluster analyses and identified three clearly distinct groups of co-

regulated genes (**Fig. 3.4a**, heatmap side). For each cluster, we used ClueGO for functional annotation analysis and gene ontology (GO) terms for biological processes to inform us as to pathways that may be driven by stress, by genotype, or by both (Bindea et al., 2009). Genes from cluster 1 were clearly changed specifically in control GR^{Het} mice, an effect driven by a reduction in glucocorticoid receptors alone, and enriched for GO terms including cell-cell signaling and vesicle-mediated transport (data not shown), suggesting caput glucocorticoid receptors normally regulate epithelial cell communication with other cell types. Related to our hypothesis, cluster 2 genes were upregulated in GR^{WT} mice by prior stress experience, and intriguingly, were reversed in GR^{Het} stressed mice, supporting that this cluster includes genes involved in enduring programming of intergenerational transmission. Genes from cluster 2 were most significantly enriched for GO terms representing chromatin-modifying processes and intracellular transport (**Fig. 3.4d**, left), again corroborating that stress reprograms the histone code long-term. Cluster 3 genes were upregulated specifically in GR^{Het} mice exposed to prior stress, and were enriched for ribosomal biogenesis, mitochondrial transport and metabolic processes (**Fig. 3.4d**, right), suggesting increased oxidative phosphorylation capacity by mito-ribosome biogenesis may be a counteractive response to stress (Silva et al., 2015). Altogether, these data indicate that paternal stress transmission is glucocorticoid receptor-dependent, where caput epididymal glucocorticoid receptor reduction reverses stress-induced chromatin remodeling and enhances mitochondrial function, promoting cellular resilience to environmental challenges (Du et al., 2014) and preventing transmission of an offspring phenotype.

In summary, these studies identify a cellular mechanism whereby paternal stress experience produces lasting consequences for future offspring neurodevelopment. Our findings implicate caput epididymal epithelial glucocorticoid receptors as important orchestrator of environmental stress contributing to enduring epigenetic reprogramming and epididymal epithelial EV and sperm miR changes. We show that caput epididymal glucocorticoid receptor reduction coordinates a compensatory response to stress, including reversal of chromatin modifications that ultimately rescues paternal stress transmission of the offspring phenotype. These studies establish the paternal caput epididymis as a key determinant in the intergenerational transmission of environmental experience and programming of offspring disease risk.

Materials and Methods

Animals. Male C57BL/6J and female 129S1/SvImJ mice were obtained from Jackson Laboratories and were used to produce C57BL/6:129 F1 hybrids. F1 hybrids were used for all paternal stress studies. For the caput epididymal epithelial cell-specific reduction of GR and RiboTag breedings, GR^{flox} (B6.129S6-*Nr3c1*$^{tm2.1Ljm}$/J) and RiboTag (B6N.129-*Rpl22*$^{tm1.1Psam}$/J) mice were crossed with 129S1/SvImJ females for minimally 3 generations (Chan et al., 2017). Lcn5-Cre male mice on a C57Bl/6J background were purchased from the Model Animal Research Center of Nanjing University and were bred to double heterozygous GR^{flox}; RiboTag 129 females to generate experimental animals. All mice were housed in a 12:12 light:dark cycle with temperature 22°C and relative humidity 42%. Food (Purina Rodent Chow; 28.1% protein, 59.8% carbohydrate, 12.1% fat) and water were provided ad libitum. All studies were performed according to experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee, and all procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Chronic Variable Stress. Administration of chronic variable stress was performed as previously described (Rodgers, 2013). At PN28, males were weaned, pair-housed with a same-sex littermate, and randomly assigned to a control or stress group. Chronic variable stress occurred over 28 days (PN28-56). One stressor was administered each day and the order of stressors was randomized each week. Stressors include the following: 36 h constant light, 1 h exposure to predator odor (1:5000 2,4,5-trimethylthiazole (Acros

Organics) or 1:2000 phenethylamine (Sigma)), 15 min restraint, novel object (marbles or glass vials) overnight, multiple cage changes, 100 dB white noise overnight, and saturated bedding overnight.

Breeding. Following completion of chronic variable stress (PN56), males were all left undisturbed for at least 1 week to remove the acute effects of stress. Males were then housed with virgin, stress-naïve F1 hybrid females at either 1- or 12-weeks following the end of stress exposure for a maximum of 3 nights. To minimize male-female interactions that may impact maternal investment or care (Curley, 2011), observation of a copulation plug within 1 h after lights on signaled the immediate removal of the female to her own cage containing a nestlet.

Adult tissue collection. Sires were rapidly decapitated under isoflurane anesthesia 24 h following copulation. The testes, caput and corpus epididymis were removed and flash frozen in liquid nitrogen. Sperm were obtained by mincing the caudal epididymis into 1% BSA and subsequently isolated at 37°C through a double swim-up assay. The supernatant containing motile sperm was centrifuged for 5 min at 4000 rpm and the sperm pellets were stored at -80°C. Adult offspring were dissected at ~20 weeks of age. Whole brains were removed, frozen on dry ice, and stored at -80°C.

HPA axis assessment. Plasma corticosterone was measured in response to an acute 15 min restraint stress in a 50mL conical tube. Testing occurred 2-5 h after lights on. Tail

blood was collected at onset and completion of restraint (0 and 15 min) and 15 and 115 min after the end of restraint (30 and 120 min). Samples were immediately mixed with 50 mM EDTA and centrifuged 10 min at 5000 rpm. 3ul of plasma was collected at stored at -80°C until analysis. Corticosterone levels were determined by ^{125}I-corticosterone radioimmunoassay (MP Biomedical) according to manufacturer's protocol. For HPA axis responsivity to fox odor exposure, 1:5000 2,4,5-trimethylthiazole (Acros Organics) was administered on a Q-tip cotton swab in a separate testing room for 15 min to minimize odor exposure during recovery. For each experiment, no more than two littermates were included in each group.

Cell culture and corticosterone treatment. Immortalized mouse distal caput epididymal epithelial (DC2) cells were purchased from Applied Biological Materials and cultured as previously described (Araki et al., 2002). Briefly, cells were seeded in 75cm^2 Nunc EasYFlasks (Thermo Fisher) coated in collagen type 1, rat tail (Millipore). Cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% exosome-free fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). At monolayer confluency, the media was replaced and cells were either treated with 1:1000 vehicle (ethanol; resulting in 0.1% ethanol) or 1:1000 corticosterone in ethanol (Sigma; low concentration 144μM, medium concentration 1.4mM, high concentration 14.4mM - resulting in about 50, 500, or 5000 ng/ml of corticosterone, respectively). Cells were treated every 24 h for 3 days for a total of three treatments. The media was replaced 24 and 96 h following the last treatment. Media and cells were collected at 24, 96, or 192 h

following the last treatment. For cell collection, cells were trypsinized in 0.25% trypsin-EDTA (Gibco), centrifuged at 1500 rpm for 3 min, and frozen at -80°C until further analysis.

Extracellular vesicle (EV) isolation. EVs were isolated from exosome-free media (Gibco) using differential centrifugation (Théry, 2006). Briefly, cellular debris was removed from the media by centrifugation at 200g for 10 min, 2000g for 10 min, and 10,000g for 30 min. EVs were pelleted by ultracentrifugation at 100,000g for 1 h using the Optima L-90K Ultracentrifuge and SW 32 Ti swinging bucket rotor (Beckman Coulter). The EV pellet was resuspended in PBS or TriZol reagent and frozen at -80°C until further analysis.

Nanoparticle tracking analysis. All samples were run on a NanoSight NS500 to determine the size distribution of EV particles at the Center for Nanotechnology in Drug Delivery at the University of North Carolina. All samples were diluted to a concentration between 1EE08-5EE08 particles/mL in filtered PBS. Five 40 sec videos were taken of each sample to capture particles moving by way of Brownian motion. The nanosight software tracked the particles individually and using the Stokes-Einstein equation, calculated the hydrodynamic diameters.

IVIS Spectrum Imaging of labeled EVs. EVs isolated 8 days following 3 day treatment were labeled with XenoLight DiR Fluorescent Dye (PerkinElmer) per manufacturer's

instruction. Briefly, EV pellets were resuspended in 600 µl cold PBS and incubated with 20 µl 10mM DiR dye for 5 min at RT. As a non-EV control, 600 µl PBS alone was processed in parallel. The total volume was brought up to 38 ml with PBS and ultracentrifuged at 100,000g for 1 h. The dyed EV pellet was resuspended in PBS and 5e7 particles were injected intravenously via the tail vein into naïve adult F1 hybrid male mice. 24 h following injection, the mice were sacrificed and their tissues were collected for imaging using an IVIS Spectrum (PerkinElmer). The excitation filter was set at 745 and the emission filter was set at 800. For quantification, total radiant efficiency was calculated using Living Image software, with the minimum set at 1e7 and the maximum set at 1.45e7. Total radiant efficiencies for each tissue were normalized to total radiant efficiency of 0.1 g liver to control for success of the injection.

Protein extraction and western immunoblotting. Cell pellets were processed for immunoblotting using established protocols. For nuclear extractions, samples were homogenized with a pestle in cold sterile PBS, homogenates were centrifuged at 1200 g for 10 min at 4°C, pellets were washed with PBS, and resuspended in Buffer A (10mM Hepes pH 7.8, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Sigma)). Following a 15 min incubation on ice, 0.05% NP-40 was added, samples were vortexed, and nuclear extracts pelleted at 14,000 x g for 30 sec. Nuclear pellets were resuspended in Buffer B (50 mM Hepes pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, protease inhibitor cocktail, phosphatase inhibitor cocktail). For whole cell extracts and EV protein extraction,

samples were homogenized and resuspended in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (Sigma), rotated for 2 h at 4°C, and pelleted at 5000 x g for 10 min. Protein quantification was done using Bradford assay (BioRad). For immunoblotting, twenty μg of protein was loaded per lane for gel electrophoresis onto a NuPAGE 4-12% Bis-Tris gel (Life Technologies). After running, gels were cut and the same molecular weight sections for all samples were transferred together to enable multiple probing and to control for transfer conditions. After transfer of proteins to a nitrocellulose membrane (Life Technologies), membranes were blocked with Odyssey blocking buffer (Li-Cor) and probed with rabbit anti-GR (1:10000; Abcam ab109022), mouse anti-beta actin (1:30000; Sigma A5441), rabbit anti-CD63 (1:1000; Systems Biosciences EXOAB-CD63A-1), rabbit anti-Lamp1 (1:1000; Abcam ab22595), and/or rabbit anti-Calnexin (1:1000; Abcam ab24170), followed by incubation in IRDye800-conjugated donkey anti-rabbit secondary (1:20,000; Li-Cor) and/or IRDye680-conjugated goat anti-mouse secondary (1:20,000; Li-Cor).

Histone extraction, bottom-up nanoLC MS/MS and data analysis. Samples were processed as previously described (Sidoli et al., 2016). Briefly, whole caput epididymides or DC2 cell pellets were homogenized in nuclei isolation buffer (15mM Tris-HCl pH 7.5, 60 mM KCl, 15mM NaCl, 5mM MgCl2, 1 mM CaCl2, 250 mM sucrose) with 1 mM DTT, 1% phosphatase inhibitor (Sigma), 1 pellet protease inhibitor (Roche), 10mM sodium butyrate (Sigma), and 10% NP-40. Histones were acid extracted from nuclei by rotating overnight in 0.4N H2SO4 at 4°C and precipitated with 100% trichloroacetic acid overnight at 4°C. Extracted histones were washed with acetone and quantified by

Bradford reagent according to manufacturer's protocol (Sigma). ~20ug histones were derivatized using propionic anhydride (Sigma) and digested with 1:10 trypsin (Promega). Samples were subsequently desalted by binding to C18 material from a solid phase extraction disk (Empore), washed with 0.5% acetic acid, and eluted in 75% acetonitrile and 5% acetic acid. Peptides were separated in EASY-nLC nanoHPLC (Thermo Scientific, Odense, Denmark) through a 75 μm ID x 17 cm Reprosil-Pur C_{18}-AQ column (3 μm; Dr. Maisch GmbH, Germany) using a gradient of 0-35% solvent B (A = 0.1% formic acid; B = 95% acetonitrile, 0.1% formic acid) over 40 min and from 34% to 100% solvent B in 7 minutes at a flow-rate of 250 nL/min. LC was coupled with an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) with a spray voltage of 2.3 kV and capillary temperature of 275 °C. Full scan MS spectrum (m/z 300−1200) was acquired in the Orbitrap with a resolution of 60,000 (at 200 *m/z*) with an AGC target of 5x10e5. At Top Speed MS/MS option of 2 sec, the most intense ions above a threshold of 2000 counts were selected for fragmentation with higher-energy collisional dissociation (HCD) with normalized collision energy of 29, an AGC target of 1x10e4 and a maximum injection time of 200 msec. MS/MS data were collected in centroid mode in the ion trap mass analyzer (normal scan rate). Only charge states 2-4 were included. The dynamic exclusion was set at 30 sec. Where data-dependent acquisition (Sidoli et al., 2015) was used to analyze the peptides, full scan MS (*m/z* 300−1100) was performed also in the Orbitrap with a higher resolution of 120,000 (at 200 m/z), AGC target set at the same 5x10e5. The difference is in the MS/MS though also performed in the ion trap, was with sequential isolation windows of 50 *m/z* with an

AGC target of 3x10e4, a CID collision energy of 35 and a maximum injection time of 50 msec. MS/MS data were collected in centroid mode. For both acquisition methods, peak area was extracted from raw files by using our in-house software EpiProfile (Yuan et al., 2015). The relative abundance of a given PTM was calculated by dividing its intensity by the sum of all modified and unmodified peptides sharing the same sequence. For isobaric peptides, the relative ratio of two isobaric forms was estimated by averaging the ratio for each fragment ion with different mass between the two species.

RNA isolation. Total RNA extraction from epididymal sperm and EV pellets were done using the TRIzol reagent (Thermo Fisher) according to manufacturer's protocol. For RNA extraction of PVN punches, the RNeasy Micro Kit was used according to manufacturer's protocol (Qiagen).

RiboTag mRNA immunoprecipitation. To obtain actively translating mRNA, RiboTag mice were used as previously described (Sanz, 2009). Briefly, whole caput epididymal tissue were dounce homogenized in 1 ml supplemented homogenization buffer (50 mM Tris pH 7.5, 100 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1mM DTT, 200U/mL RNasin (Promega), 1mg/mL heparin, 100 μg/mL cyclohexamide, protease inhibitor cocktail (Sigma)). Following centrifugation at 10,000g for 10 min, 800μl of the supernatant was incubated with 5μl of anti-HA.11 clone 16B12 antibody (Biolegend) for 4 h at 4°C. 400μl of Dynabeads Protein G (Life Technologies) were washed with supplemented homogenization buffer and incubated with the supernatant-antibody complex overnight at

4°C. The next morning, bead-antibody-protein complexes were washed 3 times for 10 min with high salt buffer (50 mM Tris pH 7.5, 300 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 100 μg/mL cyclohexamide). Immediately following washes, Qiagen Buffer RLT with beta-mercaptoethanol was added and the RNeasy protocol was followed according to manufacturer's protocol to isolate RNA from the complexes.

mRNA sequencing and analysis. Total RNA from caput epididymal RiboTag and PVN punches were quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific). Libraries for RNA-seq were made using a TruSeq Stranded mRNA Sample Preparation Kit (Illumina) with 250ng RNA according to manufacturer's protocol. All library sizes and concentrations were confirmed on a TapeStation 4200 (Agilent) and Qubit 3.0 Fluorometer (Thermo Fisher). Individually barcoded libraries were pooled and sequenced on an Illumina NextSeq 500 (75-bp single-end). Fastq files containing an average of 50 million reads were processed for pseudoalignment and abundance quantification using Kallisto (version 0.43.1) (Bray et al., 2016). The transcriptome was aligned to the EnsemblDB Mus musculus package (version 79).

Small RNA sequencing and analysis. Small RNA libraries were constructed using the NEBNext Small RNA Library Prep Set for Illumina (NEB) on 200ng total RNA according to manufacturer's protocol. All library sizes and concentrations were confirmed on a TapeStation 4200 (Agilent) and Qubit 3.0 Fluorometer (Thermo Fisher). Individually barcoded libraries were pooled and sequenced on an Illumina NextSeq 500

(75-bp single-end). Fastq files containing an average of 10 million reads per sample were aligned and quantified using miRDeep2 (version 2.0.0.8) (Friedländer et al., 2012).

Bioinformatics analyses: All analyses were performed using R version 3.3.3 and Bioconductor version 3.4.

Random Forests. The R package randomForest (Breiman, 2001) was used to analyze histone mass spectrometry ratio data with the parameters ntree=1000 and mtry=$\sqrt{p}$ for classification analysis, based on calculation of p where p=total number of histone modifications identified. This approach ranks each histone modification by the percent decrease (MDA) to the model's accuracy that occurs if the histone mark is removed, allowing for the identification of a histone code that discriminates between treatment groups. To estimate the minimal number of histone modifications required for prediction, ten-fold cross-validation using the 'rfcv' command was implemented through the randomForest package.

Rank-rank hypergeometric overlap (RRHO). The R package RRHO was used to evaluate the degree and significance of overlap in threshold-free differential expression data between *in vivo* sperm and *in vitro* EV miR datasets (Plaisier, 2010). For each comparison, one-sided enrichment tests were used on $-\log_{10}$(nominal p-values) with the default step size, and corrected Benjamini-Yekutieli p-values were calculated. Each pixel

represents one miR comparison between the two datasets, with the degree of significance color-coded.

Differential expression analysis. The R package DESeq was used to perform pairwise differential expression analyses on RNA-seq datasets using the negative binomial distribution (Anders et al., 2010). For PVN and RiboTag mRNA-seq, count data were filtered for at least 10 counts per gene across all groups, normalized, and dispersions were estimated per condition with a maximum sharing mode. Small RNA-seq data were filtered for greater than 2 counts in at least 3 samples across all groups, normalized, and dispersions were estimated per-condition using empirical values. Significance for all differential expression was set at an adjusted P-value<0.05. Heatmaps were generated using the R package gplots heatmap.2 function. All heatmaps are plotted as average Z scores per treatment group and arranged through hierarchical clustering of groups. Clusters of co-regulated differentially expressed genes were determined with the R package Stats using hierarchical clustering of genes (complete method) followed by 'cutree', k=3.

ClueGO. Functional annotation analysis was performed on co-regulated differentially expressed gene clusters with the Cytoscape plug-in ClueGO (Bindea, 2009). ClueGO identifies enriched pathways using Gene Ontology (GO) terms and can reduce redundancy of GO terms that share similar genes by sorting into parent categories. For each cluster of differentially expressed genes, ClueGo was used to determine the enriched

GO biological processes. Redundant terms were allowed to fuse with related terms that had similar associated genes. Networks of GO terms visualized using Cytoscape were linked using kappa statistics, with connecting nodes sized according to *P*-values corrected by Bonferroni step down. For each group of related GO terms, the leading group was determined by highest degree of significance. Top enriched groups of GO terms for each cluster were determined by the corrected group *P*-value.

Statistics. Corticosterone levels were analyzed by two-way ANOVA with time as a repeated measure. Corticosterone AUC, litter characteristics, testis weights, and gene expression data were analyzed by two-way ANOVAs. Outliers for HPA axis assessment were excluded at all time points and determined by data greater than two standard deviations away from the group mean or corticosterone levels greater than 150 ng/mL at the 120 min time point, indicating no stress recovery. Immunoblotting data were analyzed using one-way ANOVAs or two-tailed *t*-tests. Nanosight, and IVIS radiant efficiency were analyzed using two-tailed *t*-tests. Histone mass spectrometry ratio data were analyzed Mann-Whitney U tests. When appropriate, Bonferroni's multiple comparisons or Student's *t*-tests were used to explore main effects. Proportions of reproductive success were analyzed using chi-square tests. Significance was set at $P<0.05$.

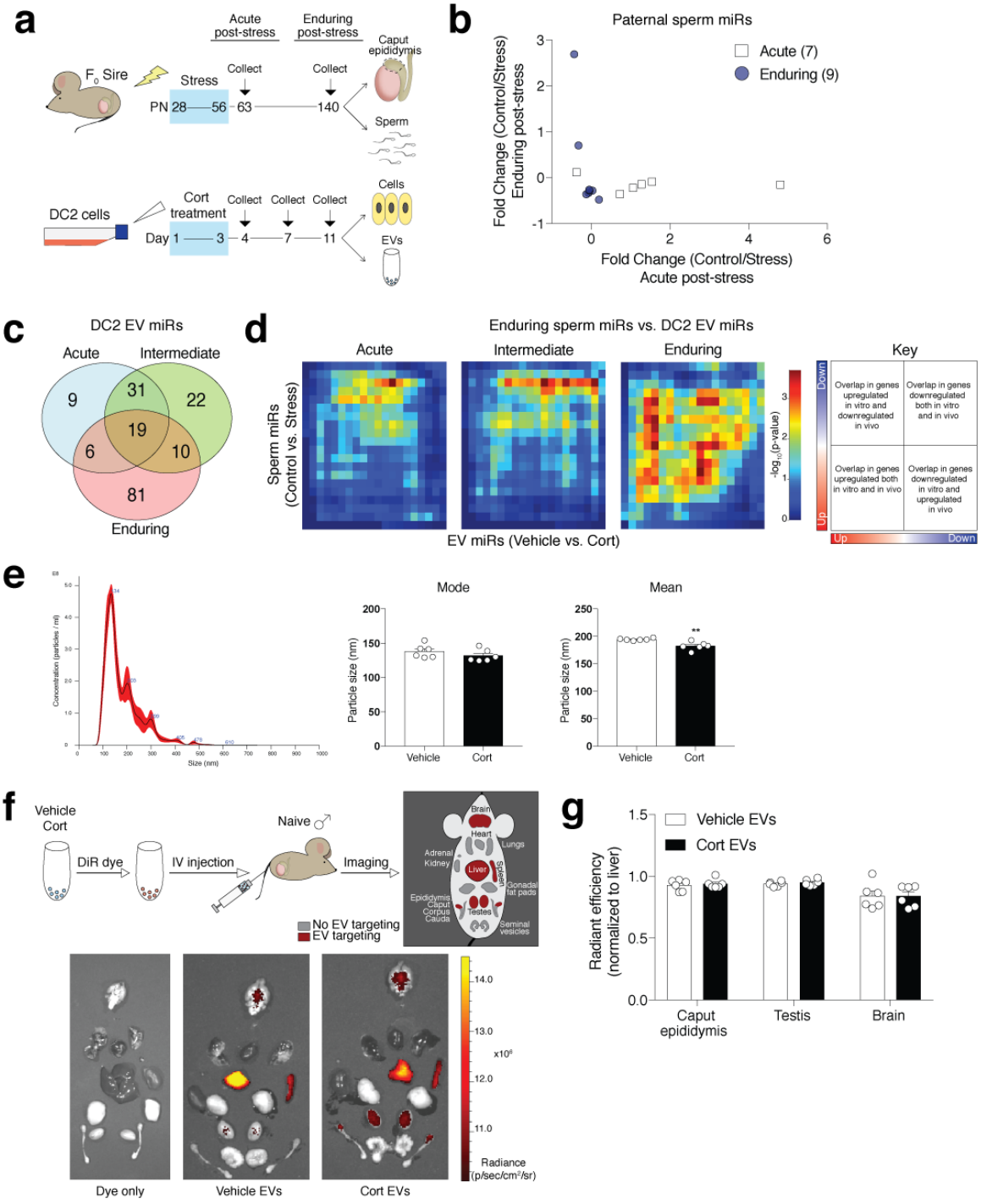


Figure 3.1. Glucocorticoid-treated DC2 mouse caput epididymal epithelial cell EVs *in vitro* mimic paternal stress programming of enduring sperm miRs *in vivo*. (**a**) Male mice were exposed to stress from postnatal days (PN) 28-56. Sperm and caput epididymal tissue were collected at 1-week (acute) and 12-weeks (enduring) post-stress. To mimic chronic stress, DC2

cells were administered three concentrations of corticosterone (cort) (50ng/ml (low), 500 ng/ml (medium), or 5 µg/ml (high)) for 3-days. Epididymal cells and secreted EVs isolated at 1 (acute), 4 (intermediate), and 8-days (enduring) post-treatment were examined for similar changes as those from paternal stress tissue. (**b**) Differential expression analysis of paternal sperm miRs identify distinct populations altered at 1- and 12-weeks post-stress, with each point representing one miR, suggesting unique mechanisms for acute and enduring sperm miRs post-stress. N = 6-8; adjusted $P < 0.05$. (**c**) Rank-rank hypergeometric overlap (RRHO) analysis was used between enduring *in vivo* paternal sperm miRs and *in vitro* DC2 EV miRs post-treatment to determine the best-matched period of miR regulation in DC2 cells. Venn diagram of significantly overlapping EV miRs from cells treated with the medium (physiologically relevant) corticosterone concentration following small RNA-sequencing, demonstrating the greatest overlapping number of miRs at 8-days post-treatment. N = 3-4; max $-\log_{10}(P\text{-value})$= 3. These data are represented visually using (**d**) RRHO heatmaps where each pixel represents one miR comparison color-coded for degree of significance, with the most upregulated miRs at the bottom left corner and downregulated miRs at the top right corner (as described in the schematic, right). (**e**) Representative particle tracking plot (left) using Nanosight confirm DC2 EV size distribution. Corticosterone treatment did not affect the mode (Student's t-test, $t(10) = 1.165$, $P = 0.2712$), but reduced the mean (Student's t-test, $t(10) = 3.865$, $P = 0.0031$) of EV particle size 8-days post-treatment, suggesting altered lipid composition/function. N = 6. Student's t-test, $^{**}P < 0.01$. (**f**) Representative image of tissue-specific selectivity of 5E7 near-infrared DiR dye-labeled DC2 EVs treated with vehicle or corticosterone. (**g**) There were no differences between treatment for each tissue in total radiant efficiency of caput epididymis (Student's t-test, $t(10) = 0.4757$, $P = 0.6445$), testes (Student's t-test, $t(10) = 0.8337$, $P = 0.4239$), and brain (Student's t-test, $t(10) = 0.00912$, $P = 0.9929$) between EVs injected, suggesting corticosterone-treated EVs retain endogenous tissue selectivity. N = 6. Data are mean ± SEM, with individual data points overlaid.

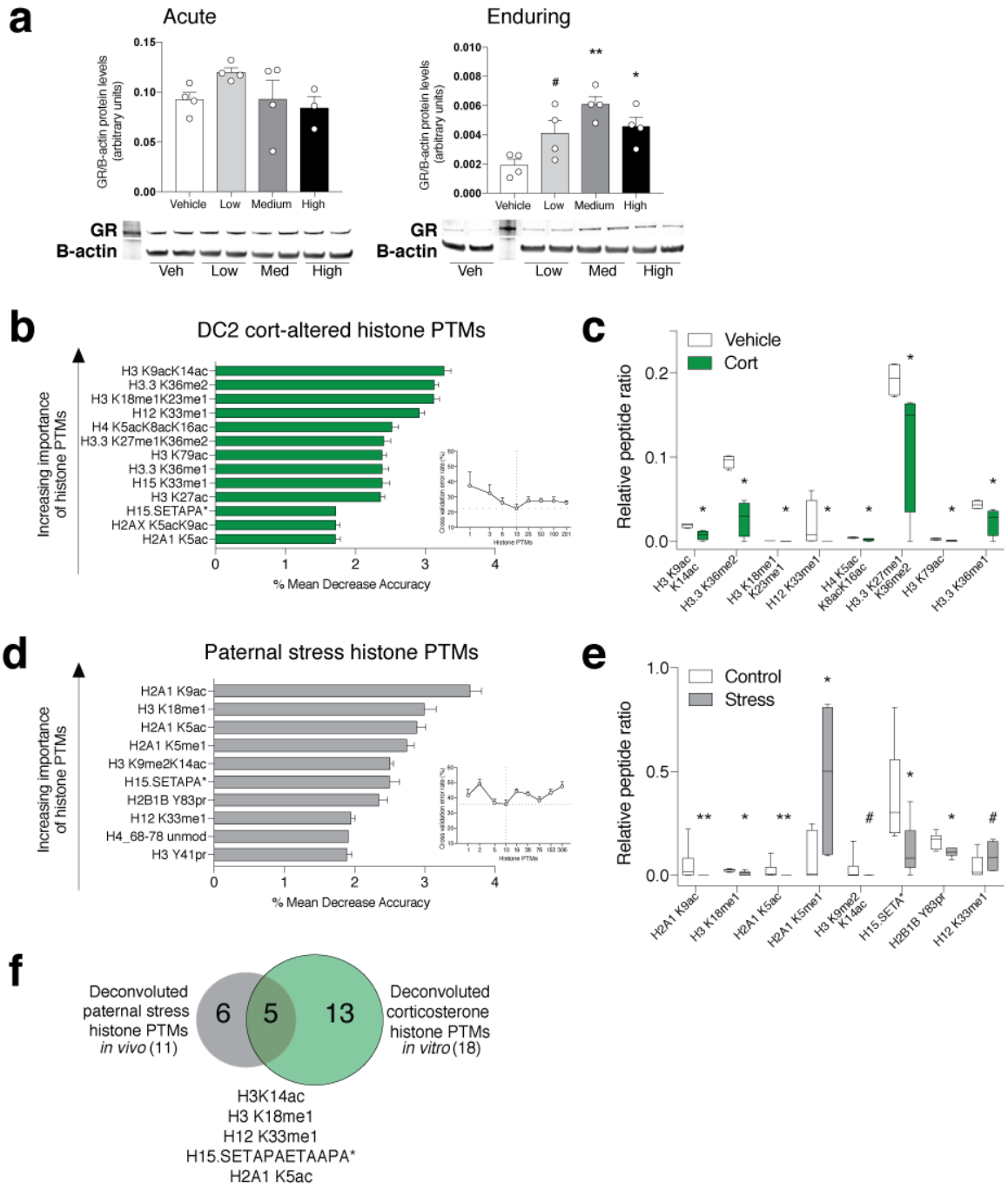


Figure 3.2. Glucocorticoid receptors are increased post-stress and correspond with enduring reprogramming of the caput epididymal histone code. (**a**) Immunoblotting of glucocorticoid receptor levels 1- (acute time point, left) and 8-days (enduring time point, right) post-treatment. No effect of corticosterone treatment at the acute time point (one-way ANOVA, $F(3, 11) = 1.644$, $P = 0.2360$). There were significant treatment effects at the enduring time point (one-way ANOVA, $F(3,12) = 7.306$, $P = 0.0048$. Bonferroni's post-hoc analysis showed

significant differences between vehicle (Veh) v. medium (Med) concentration ($t(12) = 4.625$, adjusted $P = 0.0018$); vehicle v. high concentration ($t(12) = 2.93$, adjusted $P = 0.0378$), and a nonsignificant difference between vehicle v. low concentration ($t(12) = 2.416$, adjusted $P = 0.0977$), suggesting glucocorticoid receptors (GR) are involved in enduring EV miR alterations. N=3-4; one-way ANOVA with Bonferroni's correction, $^{**}P < 0.01$, $^{*}P < 0.05$, $\#P < 0.1$. Data are mean ± SEM, with individual data points overlaid. (**b** and **d**) Random Forests analysis of quantitative histone post-translational modifications (PTM) mass spectrometry identified the top histone PTMs, ranked by importance, that most accurately discriminate at the enduring time point between (**b**) vehicle v. corticosterone treatment of DC2 cells *in vitro,* and (**d**) control v. stress caput epididymis *in vivo*. Mean decrease accuracy indicates the percent decrease in model accuracy if the histone PTM is removed. $N = 4$-6. Error bars are ± SD. (**c** and **e**) Relative abundance of the top eight histone PTMs determined by Random Forests were confirmed by Mann-Whitney U tests between treatment for each individual histone PTM in (**c**) DC2 cells and (**e**) paternal stress caput epididymis. Data are median ± interquartile range. $^{**}P < 0.01$, $^{*}P < 0.05$, $\#P < 0.1$. (**f**) Venn diagram of total deconvoluted histone PTMs discriminating treatment groups, as determined by Random Forests analysis, between *in vivo* caput epididymis (gray) and *in vitro* DC2 cells (green), and their overlap (histone PTMs listed below).

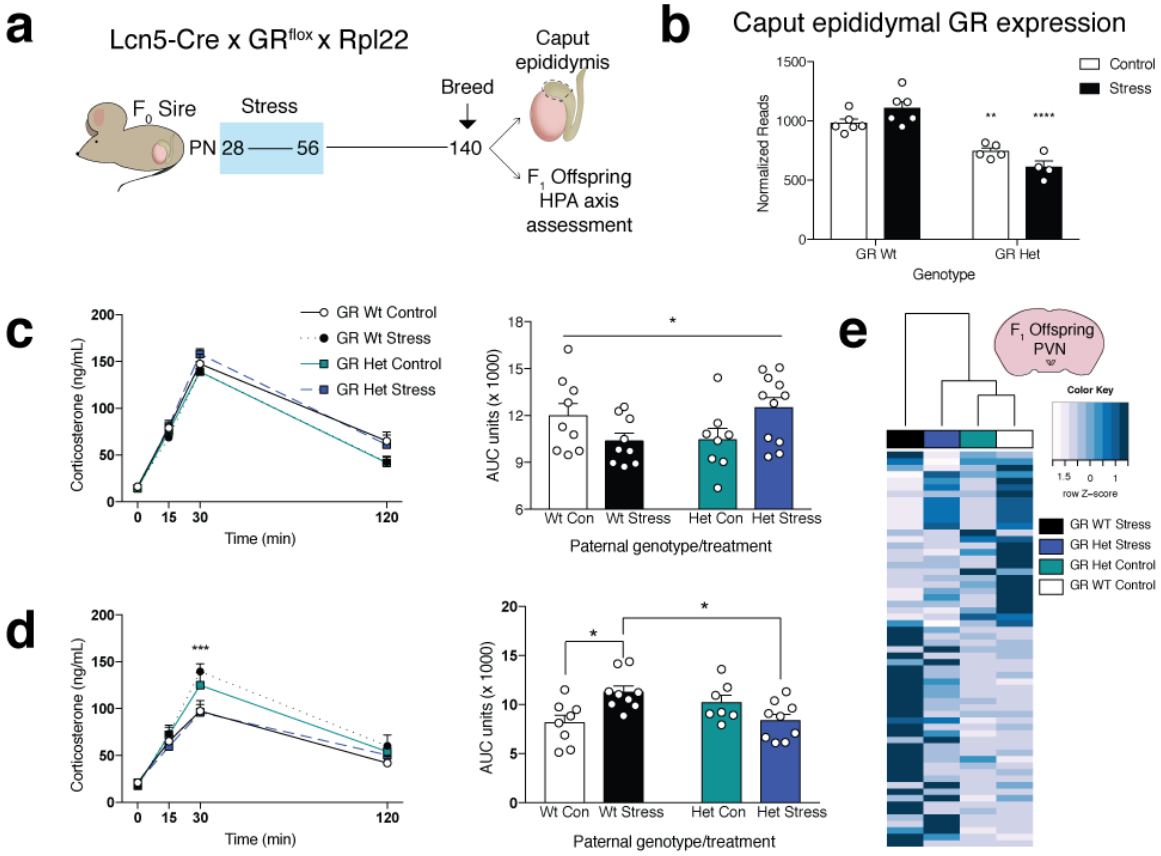


Figure 3.3. Genetic reduction of caput epididymal epithelial glucocorticoid receptors *in vivo* rescues paternal stress programming of offspring stress dysregulation. (**a**) Caput epididymal epithelial cell-specific Lcn5-Cre x GR^{flox} x Ribotag (Rpl22) male mice were exposed to stress, as above, and were bred 12-weeks post-stress. Adult offspring were assessed for HPA stress axis responsivity. (**b**) To ensure transgenic glucocorticoid receptor (GR) reduction and inhibition of post-stress glucocorticoid receptor increases, glucocorticoid receptor mRNA expression from paternal caput epididymal epithelial cells was examined using Ribotag technology (two-way ANOVA, main effect of genotype ($F(1, 17) = 68.71$, $P < 0.0001$), interaction of genotype x treatment ($F(1,17) = 8.652$, $P = 0.0091$). Tukey's post-hoc analysis showed significant differences between Control GR^{WT} and Control GR^{Het} ($t(17) = 5.527$, adjusted $P = 0.0056$) and between Stress GR^{WT} and Stress GR^{Het} ($t(17) = 10.89$, adjusted $P < 0.0001$)). $N = 4$-6; Tukey's post-hoc test, $^{**}P < 0.01$, $^{****}P < 0.0001$. (**c**) There was a significant interaction for paternal treatment x genotype for the offspring HPA area under the curve (AUC), where the reduced response to an acute restraint in wildtype (Wt) paternal stress offspring was normalized by paternal glucocorticoid receptor reduction (two-way ANOVA, interaction of paternal genotype x paternal treatment, $F(1, 34) = 4.902$, $P = 0.0336$). $N = 8$-11; two-way ANOVA, $^{*}P < 0.05$. (**d**) Similarly, there was a significant interaction in the AUC (two-way ANOVA, interaction of paternal genotype x paternal treatment, $F(1, 29) = 12.65$, $P = 0.0013$. Tukey's post-hoc analysis showed significant differences between Control GR^{WT} offspring v. Stress GR^{WT} offspring ($t(29) = 4.554$, adjusted $P = 0.0158$) and between Stress GR^{WT} offspring v. Stress GR^{Het} offspring ($t(29) = 4.369$, adjusted $P = 0.0216$)) and a main effect on the curve (two-way ANOVA with time as a

repeated measure, main effect of treatment ($F(3, 29) = 3.325$, $P = 0.0333$) and main effect of time ($F(3, 87) = 97.71$, $P < 0.0001$). Tukey's post-hoc analysis showed significant differences at the 30-minute time point between Control GR^{WT} offspring v. Stress GR^{WT} offspring ($t(116) = 5.183$, adjusted $P = 0.0021$) and between Stress GR^{WT} offspring v. Stress GR^{Het} offspring ($t(116) = 5.125$, adjusted $P = 0.0009$)), whereby paternal GR^{Het} prevented the paternal stress-altered HPA response to an acute predator odor exposure in offspring. $N = 7\text{-}9$; Tukey's post-hoc test on the curve, $^{***}P < 0.001$; Tukey's post-hoc test on the AUC, $^{*}P < 0.05$. Data are mean ± SEM, with individual data points overlaid. (**e**) Hierarchical clustering and heatmap of all differentially expressed genes between paternal stress and control groups from RNA-sequencing of the paraventricular nucleus (PVN) from naïve adult offspring, showing caput epididymal GR^{Het} mitigation of paternal stress programming. $N = 5\text{-}6$; adjusted $P < 0.05$.

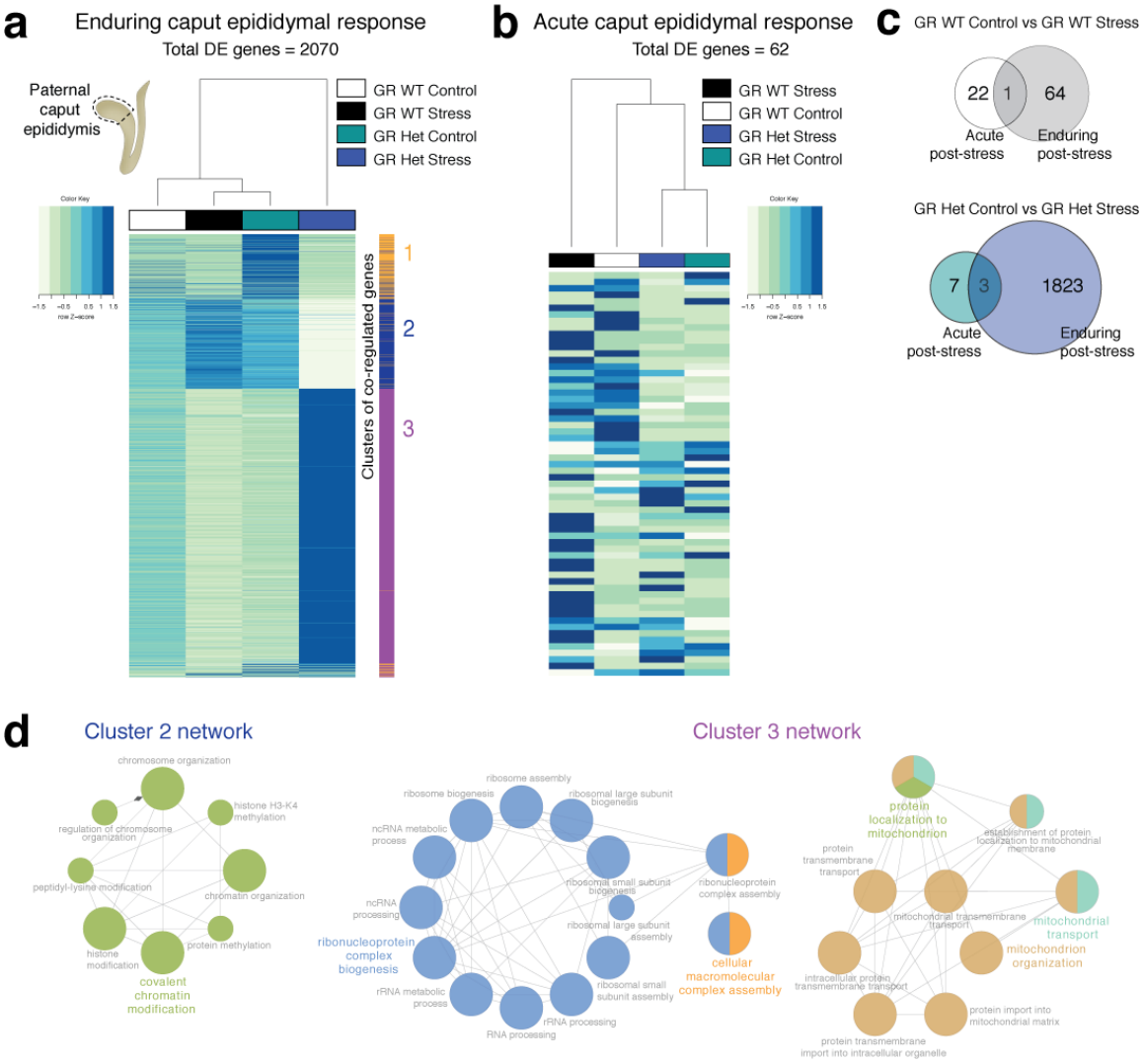
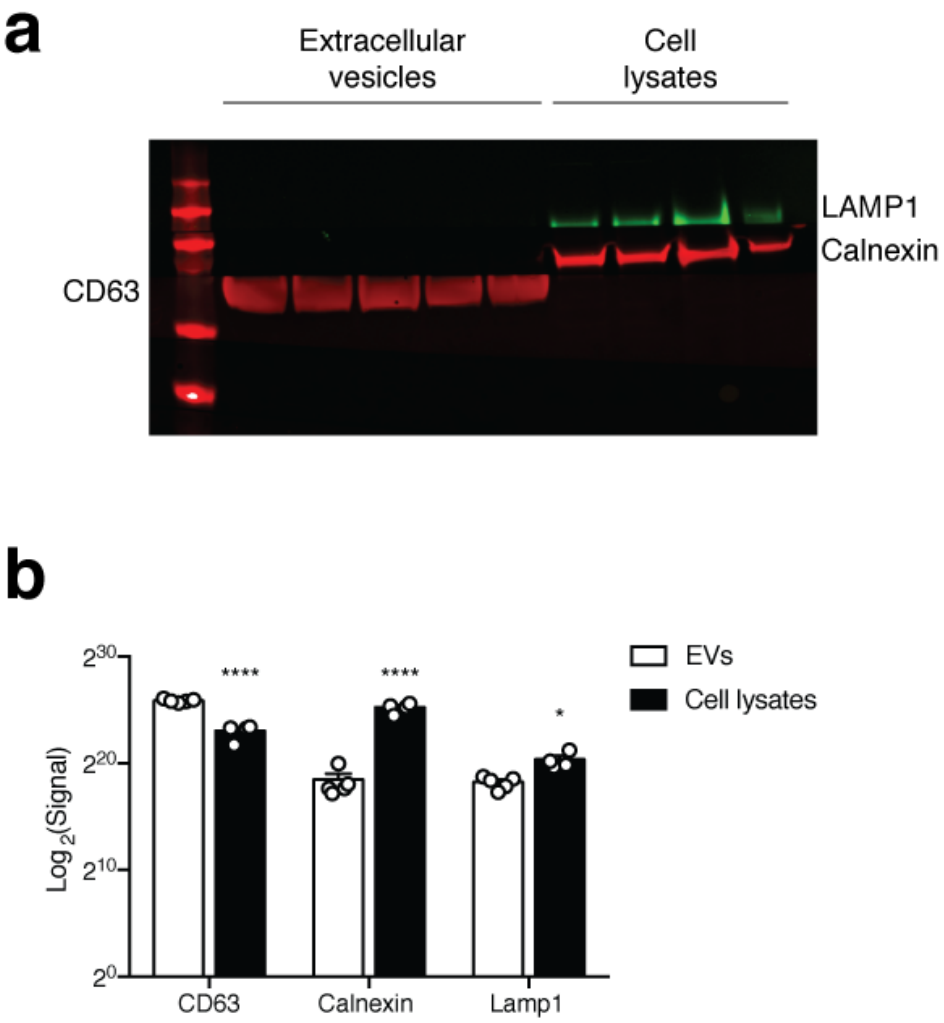
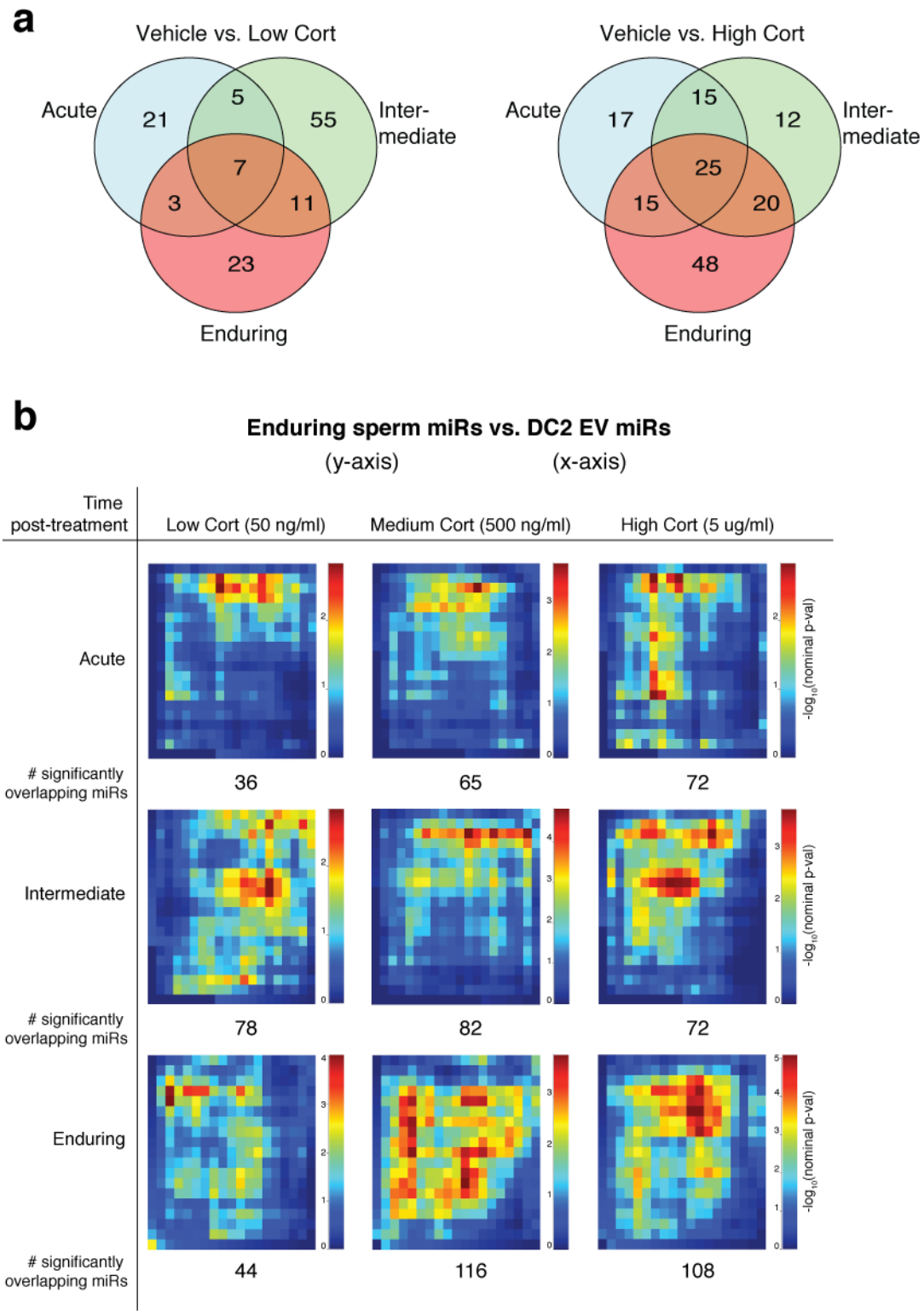


Figure 3.4. Reduction of caput epididymal glucocorticoid receptors reverses stress-induced epigenetic programming and promotes ribosomal and mitochondrial processes. (**a, b**) Heatmap of all differentially expressed (DE) genes from RNA-sequencing of paternal caput epididymal epithelial cells isolated using Ribotag technology at (**a**) 12- and (**b**) 1-week post-stress. $N = 4$-6; adjusted $P < 0.05$. Hierarchical clustering of co-regulated genes for (**a**) is depicted by color blocking on right of heatmap for functional annotation analysis. (**c**) Venn diagrams of the acute v. enduring caput epididymal epithelial response to prior stress exposure between GR^{WT} males (**top**) and GR^{Het} males (**bottom**), substantiating a post-stress mechanism that mediates enduring changes. (**d**) Functional annotation analysis of the enduring caput epididymal response 12-weeks post-stress using gene ontology terms (biological processes) for cluster 2 (genes increased in Stress GR^{WT} and decreased in Stress GR^{Het}, **left**) and cluster 3 (genes increased only in Stress GR^{Het}, **right**) reveal pertinent enriched pathways, determined by ClueGo and depicted as a network. Edges indicate degree of connectivity between terms. Node size indicates statistical

significance, with the leading term (large, colored descriptor) determined by greatest degree of significance. Node colors indicate number of groups associated with the gene ontology term.



Supplemental Figure 3.1. Validation of extracellular vesicles (EV) isolated from culture media of DC2 caput epididymal epithelial cells via differential centrifugation. (**a**) Representative western blot and (**b**) quantification of CD63, a known EV tetraspanin, Calnexin, an endoplasmic reticulum-associated protein, and Lamp1, a lysosome-associated protein. CD63 (Student's t-test, $t(7) = 13.96$, $P < 0.0001$) is typically found on EV membranes, while Calnexin (Student's t-test, $t(7) = 7.678$, $P = 0.0001$) and Lamp1 (Student's t-test, $t(7) = 3.138$, $P = 0.0164$) are typically found from cell lysates, suggesting minimal cellular contamination in isolated EV populations. $N = 4$-5. Data are mean ± SEM, with individual data points overlaid. Student's t-test, $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$.


a
Vehicle vs. Low Cort
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20
48
b
Enduring sperm miRs vs. DC2 EV miRs
(y-axis)
(x-axis)
Time
post-treatment
Low Cort (50 ng/ml)
Medium Cort (500 ng/ml)
High Cort (5 ug/ml)
Acute
significantly
overlapping miRs
36
65
72
Intermediate
significantly
overlapping miRs
78
82
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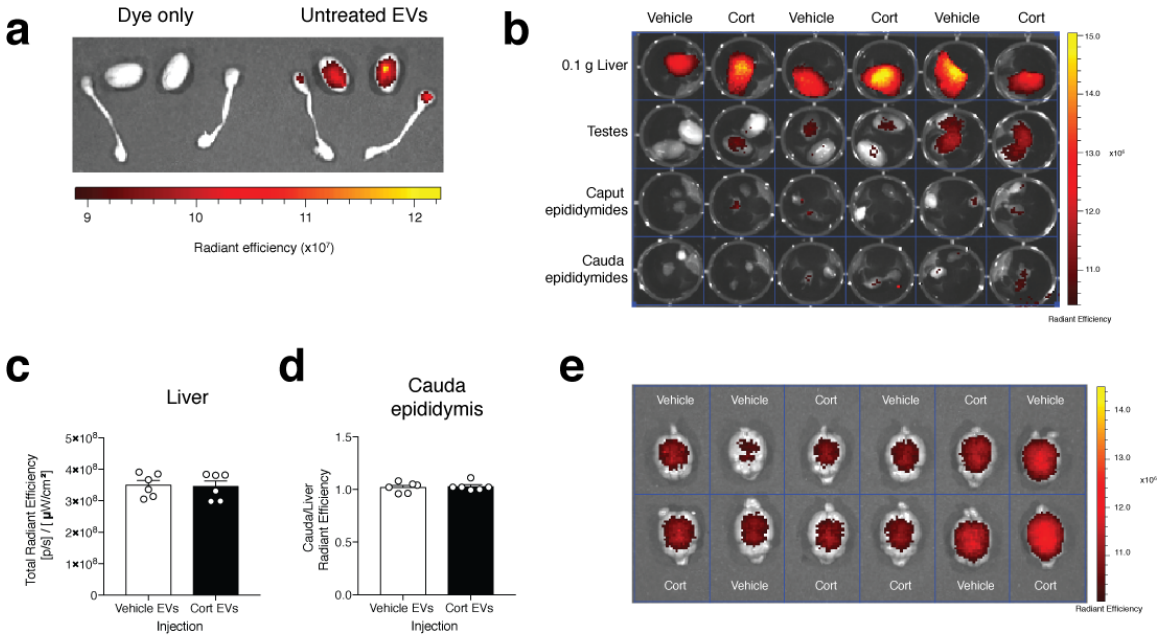
Enduring
significantly
overlapping miRs
44
116
108
-log10(nominal p-val)

Supplemental Figure 3.2. Determining timing and concentration of corticosterone to optimally recapitulate paternal stress programming *in vitro.* (**a**) Venn diagrams of significantly overlapping EV miRs by RRHO (max $-\log_{10}$(*P*-value) = 5) from DC2 caput epididymal epithelial cells treated with (left) low (50 ng/ml) or (right) high (5 μg/ml) concentrations of corticosterone, demonstrating distinct groups of EV miRs changed at each time point post-treatment, with the number of overlapping EV miRs increased at 8- compared to 1-day post-treatment. *N*=3-4. (**b**) Rank-rank hypergeometric overlap (RRHO) analysis to determine corticosterone concentration and timing post-treatment of *in vitro* DC2 mouse caput epididymal epithelial cell-derived EV miRs that most closely match enduring sperm miRs altered 12-weeks post-stress *in vivo*. Overlap data are plotted as sperm miRs (increasing down the y-axis) or EV miRs (increasing left along the x-axis). Each pixel represents one miR, with the color coded according to degree of significant overlap. Quantification for total number of significantly overlapping miRs are presented below each plot, showing that miR changes by the medium concentration of corticosterone 8 days post-treatment (enduring time point post-treatment, bottom middle plot) has the greatest degree of overlap with enduring sperm miRs altered post-stress.



Supplemental Figure 3.3. Quantification and imaging of tissues from male mice injected i.v. with DiR-labeled extracellular vesicles (EVs) secreted from DC2 caput epididymal epithelial cells. (**a**) Testes and epididymal tissue from DiR-only PBS control and DiR-labeled untreated EV injections, showing that caput epididymal EVs specifically target testes and caput epididymal tissues, regardless of treatment. (**b**) Liver, testes, caput and cauda epididymal tissue from mice injected with vehicle or corticosterone treated DC2 secreted EVs. (**c**) Quantification of total radiant efficiency of liver (Student's t-test, t(10) = 0.1691, $P = 0.8691$) and (**d**) cauda epididymis (Student's t-test, t(10) = 0.3298, $P = 0.7483$) normalized to radiant efficiency of 0.1 g liver to control for injection success. (**e**) Imaging of brains from mice injected with vehicle or corticosterone-treated DC2 secreted EVs. Quantification of caput epididymis, testes, and brain target are presented in the main text. N=6. Data are mean ± SEM.

Supplementary Table 3.1. Assessment of Reproductive Function

	Control GR^{wt}	**CVS GR^{wt}**	**Control GR^{het}**	**CVS GR^{het}**
% Plugged	77.78	66.67	75.0	88.89
% Pregnant	55.55	66.67	75.0	72.22
% Testis weight	0.36 ± 0.04	0.38 ± 0.05	0.38 ± 0.04	0.36 ± 0.02
Litter characteristics				
Average size	9.67 ± 3.27	7.6 ± 3.44	9 ± 2.19	8.56 ± 1.81
% Male	54.95 ± 26.34	62.09 ± 16.31	56.21 ± 12.93	65.24 ± 8.92

% Plugged indicates ratio of females found with a copulation plug within 3 nights of breeding.
% Testis weight is (g left testis weight/g body weight) * 100.

CHAPTER 4

PATERNAL STRESS SPERM MICRORNA IMPACTS FETAL BRAIN AND PLACENTAL DEVELOPMENT

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Abstract

Germ cell epigenetic marks responding to a variety of environmental exposures, including stress, dietary challenges and substances of abuse, have been implicated to shape offspring development. In particular, sperm small RNA populations such as microRNA (miRs) transmit paternal environmental information sufficient to shift adult offspring outcomes. Furthermore, sperm miRs delivered at fertilization function to repress gene expression in the zygote. Yet, how sperm miRs at fertilization promote lasting consequences during later development and adulthood is not well understood. Here, we utilize our established paternal stress model where specific sperm miRs responsive to chronic stress reprogram the adult offspring hypothalamus and stress axis reactivity. Using zygote microinjection, we tested the specific impact of our paternal stress sperm miRs against a composite of randomly selected sperm miRs on the developing brain and placenta, as these tissues are known to contribute to hypothalamic development. We show that microinjection of stress miRs produced robust, transcriptional dysregulation of the embryonic brain compared to few differences by random miRs. In contrast, the placental transcriptome was sensitive to both miR groups, suggesting sperm miR effects depend on the miR population and tissue target. Lastly, changes in histone post-translational modifications in the embryonic and adult brain suggest a potential mechanism by which sperm miRs promote long-term transcriptional dysregulation. These studies demonstrate the importance of sperm miRs in producing developmental antecedents to adult offspring outcomes, emphasizing the paternal environment as a potential factor underlying disease risk.

Introduction

Mounting evidence that germ cell epigenetic marks can carry information regarding the preconception environment to influence offspring development has brought new attention to the players involved at fertilization. Germ cell epigenetic marks, including DNA methylation, histone post-translational modifications and small RNA populations, have been described to respond to a variety of environmental stimuli and perturbations, including chronic stress/trauma, dietary challenges, or substances of abuse (Carone, 2010; Chen, 2015; Dias, 2014; Dietz, 2011; Franklin, 2010; Lambrot, 2013; Rodgers, 2013; Siklenka, 2015; Vallaster, 2017; Vassoler, 2012). Notably, sperm small RNAs, such as microRNA (miRs) and tRNA-derived fragments, causally link the paternal preconception environment to changes in offspring outcomes (Benito et al., 2018; Chen, 2016; Gapp, 2014; Rodgers, 2015; Sharma, 2016). We previously established a paternal chronic stress mouse model where exposed males sired offspring with dysregulated hypothalamic-pituitary-adrenal (HPA) stress axis reactivity and an altered transcriptome in the paraventricular nucleus (PVN) of the hypothalamus in adulthood (Rodgers, 2013). As a potential mode of paternal stress transmission, we identified specific miRs increased in sperm following stress exposure. Indeed, zygote microinjection of these altered sperm miRs recapitulated the dysregulated stress phenotype in adult offspring (Rodgers, 2015). Further, we demonstrated that these sperm miRs functioned to alter gene expression in the pluripotent zygote (Rodgers, 2015), supporting sperm small RNAs as influential regulators of embryogenesis. However, the downstream effects of sperm miRs introduced at fertilization in modulating development

of offspring tissues, such as the brain, to promote phenotypic changes in adulthood is not well understood.

The canonical function of miRs to post-transcriptionally repress gene expression by binding complementary sequences on mRNA positions sperm miRs as influential regulators of early transcriptional events (Zhao et al., 2007). In the zygote, development relies on the presence of stored maternal mRNAs from the oocyte, and zygotic transcription is stalled until the maternal mRNAs are cleared (Li et al., 2014). The capacity for miRs present during this window to deplete maternal transcripts in the zygote has been shown (Bushati et al., 2008; Giraldez, 2006; Lund et al., 2009; Tang et al., 2007), where one miR may regulate up to thousands of transcripts and a single mRNA can be targeted by multiple miRs (Sevignani et al., 2006). Such regulatory control by miRs has suggested their role in 'fine-tuning' crucial transcriptional events, such as during early embryonic stages where rapid regulation of transcription factor or morphogen levels is important for determining cell fate decisions (Zhao, 2007). This highly efficient system is further regulated by the specific composition of miRs present during these critical windows, where groups of co-regulated miRs can coordinately modulate entire cellular pathways or compound repression of individual targets (Ivanovska et al., 2008). Given the sensitivity of transcriptome regulation in the pluripotent zygote, the delivery of functional miRs by sperm may have complex consequences on the trajectory of offspring development.

During early embryogenesis, the first cell fate specification event begins at the 8-cell stage and becomes apparent in the blastocyst, where the outer layer forming the

trophectoderm is segregated from the inner cell mass (Red-Horse, 2004). The inner cell mass has unlimited developmental potential, eventually giving rise to all fetal tissues through a complex, organized cascade of molecular and transcriptional events (Wobus et al., 2005). Additionally, the trophectoderm gives rise to the trophoblast cells that form the placenta, a crucial tissue that provides nutrients, gases, and growth factors to the developing fetus and simultaneously protects it from maternal immune signals (Nugent, 2015). Importantly, shifts in placental regulation can disrupt fetal neurodevelopment, promoting cognitive, behavioral and physiological phenotypes in adulthood, including disruption of the HPA stress axis (Bronson et al., 2014; Bronson, 2017; Howerton, 2014; Hsiao et al., 2012). Thus, changes in offspring outcomes and tissue development may derive from reprogramming of key regulatory transcriptional events either through the embryonic and/or extra-embryonic lineages. However, whether sperm miRs shift these events to reprogram offspring outcomes by a direct regulatory cascade of molecular events from the zygote to differentiated tissues, such as the brain, and/or through changes in placental signaling has not been examined.

Therefore, in this study we utilized our paternal stress mouse model to test the hypothesis that coordinated changes in specific sperm miRs in response to chronic stress exposure alter the development of offspring tissues. Nine miRs previously identified in sperm to transmit paternal stress effects were selected for our 'Stress miRs' treatment group. To examine the specificity of miRs altered by paternal stress in influencing offspring development, we incorporated another treatment group that includes nine miRs present in sperm but not altered by stress exposure, termed 'Random miRs'. We

microinjected these two miR groups (Stress or Random) into single-cell zygotes. Following cleavage, two-cell zygotes were transferred into surrogate foster females, where embryos from one treatment group (X) were transferred into the left uterine horn and embryos from a second treatment group were transferred into the right uterine horn (Y), thereby allowing control of the maternal intrauterine environment on embryo development (**Figure 4.1a**). As our lab has previously demonstrated transcriptomic changes in the embryonic brain and placenta as developmental antecedents of HPA axis reprogramming (Bronson, 2017; Howerton, 2013; Mueller, 2008), we collected embryos at embryonic day E12.5 to examine these tissues. We next performed quantitative histone mass spectrometry in E12.5 brains following zygote microinjection as a potential mechanism whereby sperm miRs promote downstream transcriptional consequences. Lastly, we examined the histone PTM profiles of adult PVN from paternal stress offspring to compare the relevance and persistence of histone PTMs altered by Stress miRs in the E12.5 brain. Together, this approach provides an opportunity to compare the specificity and developmental effects of experience-dependent sperm miRs, and to evaluate mechanisms whereby sperm miRs introduced transiently at fertilization can produce outcomes during development and adulthood.

Materials and Methods

Animals. C57BL/6J females (Jackson Laboratories) were superovulated with 5 IU pregnant mare serum gonadotropin (Sigma) and 5 IU human chorionic gonadotropin (Sigma). Following, they were mated with 129S6/SvEvTac males (Taconic) and F1

hybrid fertilized zygotes were collected 14-16 hours after human chorionic gonadotropin injection. CD-1 females (Charles River) used for zygote transfer were mated with vasectomized males. On the day of zygote transfer, recipient CD-1 females were identified by observation of a copulation plug. All mice were housed in a 12:12 light:dark cycle with temperature 22°C and relative humidity 42%. Food (Purina Rodent Chow; 28.1% protein, 59.8% carbohydrate, 12.1% fat) and water were provided ad libitum. All studies were performed according to experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee, and all procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Microinjection and miR selection. Zygote microinjection was performed as previously described (Rodgers, 2015). Briefly, C57/BL6:129S6/SvEvTac hybrid mouse zygotes were randomly assigned for microinjection of either nine previously identified paternal stress miRs (miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-36, miR-698), nine random miRs that were selected based on low expression levels in mature sperm (average Ct > 25 for Rodent Taqman microRNA array or average normalized reads < 50 for small RNA sequencing), that were not responsive to stress in two in-lab datasets (miR-132-3p, miR-149-5p, miR-15a-5p, miR-223-5p, miR-292a-3p, miR-301a-5p, miR-326-3p, miR-466d-3p, miR-709), or 1x DPBS. The miR treatments groups had a final concentration of 1 ng/µl, with each miRIDIAN mimic diluted to 0.11 ng/µl in DPBS. Microinjected zygotes were cultured overnight in KSOM media

(Millipore) and transferred into recipient CD-1 foster females (Charles River). To control for the intrauterine environment, 7-10 zygotes from one treatment group were transferred into the left uterine horn, and 7-10 zygotes from a separate treatment group were transferred into the right uterine horn. Following injection, CD-1 dams were singly housed until embryo collection.

Paternal stress and offspring brain collection. Administration of chronic variable stress to sires was performed as previous (Rodgers, 2013). Briefly, stressed males received one of seven different stressors across 28 days: 36 h constant light, 15 min exposure to fox odor (1:5000 2,4,5-trimethylthiazole; Acros Organics, Geel, Belgium), novel object (marbles) overnight, 15 min restraint in a 50 mL conical tube, multiple cage changes, novel 100 dB white noise (Sleep Machine; Brookstone, Merrimack, NH) overnight, and saturated bedding overnight. Following chronic variable stress, males were bred with naïve females for a minimum of 3 nights, where observation of a copulation plug signaled the removal of the female to her own cage. Resulting offspring were weaned at postnatal day 28 and left undisturbed until brain collection at ~15 weeks.

PVN micropunching. Whole brains were cryosectioned at -20°C. Brain regions were micropunched using a hollow needle (Ted Pella Inc.) according to the Paxinos and Franklin atlas (Paxinos, 2013) with the following coordinates: 1.00 mm punch along the midline from two successive 300μm slices -0.50 to -0.80 and -0.80 to -1.10 relative to bregma, atlas figs. 36-40.

Analysis of oocye/zygote gene targets. The targets of the Stress or Random miRs groups were predicted by the miRWalk database, which identifies putative miR target sequences in the 3' UTR of mRNA transcripts (Dweep et al., 2011). Target mRNAs were considered if they were predicted by miRWalk, miRDB.org, miRanda, and TargetScan algorithms. Putative gene targets determined by all four algorithms were cross-referenced with gene lists previously established from: late stage MII mouse oocytes or single-cell zygotes (Potireddy et al., 2006) and/or homologously shared between both human and mouse mature oocytes (Stanton et al., 2001) to identify sperm miR targets most likely present post-fertilization. The resultant gene lists were used for functional annotation clustering using David (Huang et al., 2009).

Embryonic tissue collection. Pregnant CD-1 dams were deeply anesthetized with isoflurane on E12.5, and each uterine horn was removed where conceptuses were harvested. Fetal brains, placentas, and tails were flash frozen in liquid nitrogen and stored at -80°C until processing. All dissections were completed between 11:00 and 15:00. Tails were used for determination of sex by Jarid genotyping, as we have previously described (Bronson, 2014).

RNA isolation. Total RNA extraction from whole embryonic heads and placentas were done using the TRIzol reagent (Thermo Fisher) according to manufacturer's protocol.

mRNA sequencing and analysis. Total RNA from brains and placentas were quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific). Libraries for RNA-seq were made using a TruSeq Stranded mRNA Sample Preparation Kit (Illumina) with 250ng RNA according to manufacturer's protocol. All library sizes and concentrations were confirmed on a TapeStation 4200 (Agilent) and Qubit 3.0 Fluorometer (Thermo Fisher). Individually barcoded libraries were pooled and sequenced on an Illumina NextSeq 500 (75-bp single-end). Fastq files containing an average of 50 million reads were processed for alignment and abundance quantification using Rsubread (version 1.20.2) (Liao et al., 2013). The transcriptome was aligned and assembled to the Ensembl Mus musculus reference genome GRCm38.p5.

Histone extraction, bottom-up nanoLC MS/MS and data analysis. Samples were processed as previously described (Sidoli et al., 2016). Briefly, PVN micropunches were pooled 4 per sample across litters and within sex and treatment, with a final N = 4-5, with 16-20 total brains used. Pooled PVN samples were homogenized in nuclei isolation buffer (15mM Tris-HCl pH 7.5, 60 mM KCl, 15mM NaCl, 5mM MgCl2, 1 mM CaCl2, 250 mM sucrose) with 1 mM DTT, 1% phosphatase inhibitor (Sigma), 1 pellet protease inhibitor (Roche), 10mM sodium butyrate (Sigma), and 10% NP-40. Histones were acid extracted from nuclei by rotating overnight in 0.4N H2SO4 at 4°C and precipitated with 100% trichloroacetic acid overnight at 4°C. Extracted histones were washed with acetone and quantified by Bradford reagent according to manufacturer's protocol (Sigma). ~20ug histones were derivatized using propionic anhydride (Sigma) and digested with 1:10

trypsin (Promega). Samples were subsequently desalted by binding to C18 material from a solid phase extraction disk (Empore), washed with 0.5% acetic acid, and eluted in 75% acetonitrile and 5% acetic acid. Peptides were separated in EASY-nLC nanoHPLC (Thermo Scientific, Odense, Denmark) through a 75 μm ID x 17 cm Reprosil-Pur C18-AQ column (3 μm; Dr. Maisch GmbH, Germany) using a gradient of 0-35% solvent B (A = 0.1% formic acid; B = 95% acetonitrile, 0.1% formic acid) over 40 min and from 34% to 100% solvent B in 7 minutes at a flow-rate of 250 nL/min. LC was coupled with an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) with a spray voltage of 2.3 kV and capillary temperature of 275 °C. Full scan MS spectrum (m/z 300−1200) was acquired in the Orbitrap with a resolution of 60,000 (at 200 m/z) with an AGC target of 5x10e5. At Top Speed MS/MS option of 2 sec, the most intense ions above a threshold of 2000 counts were selected for fragmentation with higher-energy collisional dissociation (HCD) with normalized collision energy of 29, an AGC target of 1x10e4 and a maximum injection time of 200 msec. MS/MS data were collected in centroid mode in the ion trap mass analyzer (normal scan rate). Only charge states 2-4 were included. The dynamic exclusion was set at 30 sec. Where data-dependent acquisition (Sidoli et al., 2015) was used to analyze the peptides, full scan MS (m/z 300−1100) was performed also in the Orbitrap with a higher resolution of 120,000 (at 200 m/z), AGC target set at the same 5x10e5. The difference is in the MS/MS though also performed in the ion trap, was with sequential isolation windows of 50 m/z with an AGC target of 3x10e4, a CID collision energy of 35 and a maximum injection time of 50 msec. MS/MS data were collected in centroid mode. For both acquisition methods, peak

area was extracted from raw files by using our in-house software EpiProfile (Yuan et al., 2015). The relative abundance of a given PTM was calculated by dividing its intensity by the sum of all modified and unmodified peptides sharing the same sequence. For isobaric peptides, the relative ratio of two isobaric forms was estimated by averaging the ratio for each fragment ion with different mass between the two species.

Rank-rank hypergeometric overlap (RRHO). The R package RRHO was used to evaluate the degree and significance of overlap in threshold-free differential expression data between in vivo sperm and in vitro EV miR datasets (Plaisier, 2010). For each comparison, one-sided enrichment tests were used on –log10(nominal p-values) with the default step size, and corrected Benjamini-Yekutieli p-values were calculated. Each pixel represents one miR comparison between the two datasets, with the degree of significance color-coded.

Differential expression analysis. The R package DESeq was used to perform pairwise differential expression analyses on RNA-seq datasets using the negative binomial distribution (Anders et al., 2010). Count data were filtered for at least 10 counts per gene across all groups, normalized, and dispersions were estimated per condition with a maximum sharing mode. Significance for all differential expression was set at an adjusted P-value < 0.05.

Statistics. Histone mass spectrometry data were analyzed by nonparametric Mann-Whitney U tests, with significance set at $P < 0.05$.. Functional annotation clustering was performed with DAVID version 6.8, for all gene ontology terms for biological processes, with a cutoff of 1.3 for cluster enrichment score and Benjamini-corrected $P < 0.05$ (Huang, 2009).

Results

Selection of Random miRs and functional annotation of predicted targets

Nine miRs for the Stress group were select from previous examination of paternal stress sperm. For the Random miRs treatment group, miRs were selected based on three criteria applied to two large data sets examining sperm miR expression: the miR must be 1) detectable, 2) lowly expressed, and 3) be unaffected by stress exposure (**Table 4.1**). From this filtered list, nine miRs were randomly chosen for the Random miR treatment group. To examine the specificity of miR targeting on cellular pathways, we used miRWalk 2.0 to computationally predict the targets of each miR group based on the 3'UTR seed sequence, and compared the results with three other algorithms and databases (Dweep, 2011). Using genes predicted by all four programs as putative targets of our miR groups, we next filtered these for genes reported previously in the mouse oocyte or single-cell zygote (Potireddy, 2006; Stanton, 2001) in order to examine those gene sets directly relevant to sperm function post-fertilization. Next, we performed functional annotation analysis on these relevant gene sets (**Supplementary Table 4.1**) using DAVID tools for enrichment of Gene Ontology (GO) terms for biological

processes, with a cutoff of Benjamini-corrected *P*-value < 0.05 and Enrichment Score > 1.3. Remarkably, the putative Stress miR targets significantly enriched for seven clusters of GO terms, including cellular component organization, cellular response to stress, and neuron development (**Table 4.2**). In contrast, there were no clusters of GO terms significantly enriched for targets of the Random miR group (**Table 4.3**), suggesting specific composites of sperm miRs, and not any random population of miRs, are conserved to function post-fertilization.

Specificity of sperm miRs in changes to embryonic brain transcriptome

To determine the specificity and downstream impact of sperm miRs on neurodevelopment, we performed RNA-sequencing on E12.5 brains from male offspring and compared the Stress and Random miR treatment groups to the PBS group. Differential expression analysis identified 702 genes (adjusted $P < 0.05$) altered by microinjection of Stress miRs compared to PBS, whereas Random miR microinjection resulted in 4 differentially expressed genes that were shared with the Stress miR group (**Fig. 4.1b, c**). The differences produced by the Stress and Random miRs suggest that the population of sperm miRs delivered at fertilization is carefully coordinated to modulate brain development. We corroborated this hypothesis by using Rank-rank hypergeometric overlap (RRHO) analysis, allowing the comparison of differential expression profiles between Stress v. Random miRs in a threshold-free manner (Plaisier, 2010). This approach subsequently allows for the quantification of statistically significant genes overlapping between these two comparisons, and the directionality of gene changes. As

expected, there were very few (35/10784 = 0.32%) statistically significant overlapping genes (max $-\log_{10}$(*P*-value) = 15), where all 35 overlapping genes were increased in both miR microinjection groups compared to PBS (**Fig. 4.1d**), suggesting these genes may be affected by the increased concentration and/or number of miRs delivered to the zygote. There were no differences in the characteristics of each microinjected litter, including number of zygotes implanted per uterine horn or sex ratio (**Supplementary Table 4.2**).

To determine the functional pathways broadly affected by Stress sperm miRs in the embryonic brain, we used DAVID functional annotation clustering tools on the 702 differentially expressed genes for enrichment of GO terms for all biological processes, with a cutoff of Benjamini-corrected *P*-value < 0.05 and Enrichment Score > 1.3. Notably, plotting one representative statistically significant GO term for the top eight enriched GO clusters indicated that processes important to neurodevelopment were dysregulated by zygote microinjection of Stress miRs (**Fig. 4.1e**). We did not perform functional annotation analysis on the genes altered by Random miRs as this is below the recommended number for robust analysis.

Placental transcriptome is sensitive to sperm miRs from both treatment groups

As fetal development can be influenced by changes to placental signaling, we next examined whether sperm miRs at fertilization dysregulated placental development by performing mRNA sequencing on E12.5 male placentas following zygote microinjection. Differential expression analysis identified 82 significantly altered genes (adjusted *P*-value < 0.05) by Stress miRs and 460 genes by Random miRs (**Fig. 4.2a, b**).

Strikingly, the majority of differentially expressed genes in both groups was downregulated, where genes altered by Stress miRs were largely shared with the Random miRs group. These results were confirmed using RRHO analysis, with the greatest degree of overlap (max $-\log_{10}(P\text{-value}) = 605$) in the top right corner (**Fig. 4.2c**), suggesting these genes were broadly influenced by the microinjection of miRs in the zygote and were not specific to one group of sperm miRs. We performed functional annotation analysis for GO terms of biological processes and identified several clusters shared between these two groups (**Figure 4.2d**). The most enriched processes altered by Stress miRs included transport and localization of macromolecules and lipids, inflammatory responses, and blood regulation. These pathways were also significantly enriched by Random miRs, but to a lesser extent than organization of the extracellular matrix and response to external stimuli. While the placental transcriptome was disrupted by both miR microinjection groups, there were more genes altered by Random miRs, supporting that placental changes depend on the miRs present in the zygote. This suggests the placenta is sensitive to the presence of specific miRs during early development, but is not the main contributor of brain programming by sperm miRs.

Sperm miRs influence chromatin remodeling in the brain

To examine the mechanism by which sperm miRs promote long-term alterations in the brain transcriptome, we investigated the potential for zygote-microinjected miRs to epigenetically reprogram the brain. We performed quantitative histone mass spectrometry on whole E12.5 brains to compare the complete profile of histone post-translational

modifications (PTMs) between the PBS and Stress miR groups. We identified four histone PTMs significantly altered by Stress miRs (**Figure 4.3a**), suggesting sperm miRs can crosstalk with chromatin modifiers during development. Next, to assess whether chromatin remodeling continued in the adult hypothalamus and also pertained to our paternal stress model, we performed the same pipeline on pooled PVN from the hypothalamus in adult paternal stress offspring. We identified changes in the abundance of five histone PTMs (**Figure 4.3b**), suggesting an upstream mechanism whereby sperm miRs at fertilization can produce lasting transcriptional dysregulation in the developing and adult offspring brain. Finally, to test the persistence of developmentally altered histone PTMs, we examined the overlap between altered histone PTMs in the E12.5 brain vs. adult PVN. We observed no similarly changed histone marks between these time points, suggesting changes to the brain histone code by sperm miRs likely depends on a stepwise modulation of chromatin regulators and their expression levels across development.

Discussion

Germ cell epigenetic marks have been implicated in mediating paternal stress transmission following exposures to stress, nutritional challenge, and substances of abuse (Carone, 2010; Dias, 2014; Dietz, 2011; Finegersh, 2014; Franklin, 2010; Lambrot, 2013; Rodgers, 2013; Vallaster, 2017; Vassoler, 2012). In particular, sperm miRs have been causally linked with offspring programming through zygote microinjection of experience-dependent miRs and examination of the resulting offspring in adulthood. In

our model of paternal stress, specific changes in sperm miRs transmitted a dysregulated HPA stress axis response and transcriptional reprogramming in the PVN of the hypothalamus in offspring (Rodgers, 2013; Rodgers, 2015). Moreover, microinjection of these sperm miRs repressed maternal mRNA stores in the two-cell zygote. In the current study, we pursued the downstream effects of sperm miR action in developing offspring with a focus on the embryonic brain and placenta, two tissues known to contribute to programming of the HPA stress axis. Moreover, we compared the effects of nine miRs altered by paternal stress experience to the effects of nine randomly selected miRs to assess the specificity of sperm miR action post-fertilization.

To evaluate the specific composition of sperm miRs co-regulated by environmental stress, we used bioinformatic methods to identify the putative targets of these miRs and whether these targets were enriched for biological processes relevant to the zygote. Unsurprisingly, there were no significantly enriched pathways for the predicted targets of the Random miRs group, whereas there were interesting, relevant developmental pathways predicted as targets of Stress miRs in the post-fertilization zygote. These data suggest changes in the paternal environment trigger a coordinated response, leading to a specific population of miRs altered in sperm. Such an organized mechanism is likely conserved to most efficiently and precisely regulate developmental processes, e.g. miRs that together tune the expression of various members of a developmental cellular pathway compared to miRs that have no relevant targets or that may compete with each other for targets (Sevignani, 2006; Zhao, 2007), resulting in less effective regulation, as has been suggested (Nyayanit et al., 2015). Related to our paternal

stress model, one significantly enriched cluster of GO terms for predicted Stress miR targets included processes relevant to neurodevelopment. Notably, one miR included in the Random group, miR-132, has been identified in another mouse model where paternal environmental enrichment transmitted cognitive benefits to offspring (Benito, 2018). In that model, increases in miR-132 were responsible for transmitting this phenotype, suggesting this miR in the proper context (i.e. composite of other miRs) may have influential, developmental effects. Moreover, a previous study in our lab showed that microinjection of one miR increased by paternal stress (miR-193-5p), at the same concentration as microinjection of nine Stress miRs combined, was ineffective in regulating mRNA in the zygote or reprogramming the adult hypothalamic transcriptome (Rodgers, 2015), supporting that paternal transmission occurs as result of a specific population of miRs delivered by sperm.

We further demonstrated the accuracy, as well as the specificity, of the pathways predicted as targets of Stress miRs in our examination of the embryonic brain transcriptome. Remarkably, we identified robust differences in the number of differentially expressed genes in embryos resulting from zygote microinjection of Stress miRs, compared to very few changes in those resulting from Random miRs. As these differentially expressed genes enriched for processes related to neurogenesis, neuron differentiation and regulation of synaptic transmission, stress miRs may delay or promote the rate of neurodevelopment, though this directionality is difficult to conclude, as the genes enriching for these processes were both up- and down-regulated. Importantly, the limited impact of Random miRs on neurodevelopment reinforces that it is the

composition of miRs delivered at fertilization, rather than the concentration or number of miRs, that results in developmental programming.

As miRs are unlikely to be maintained past numerous rounds of cell division and lineage specification (Gantier et al., 2011; Zhang et al., 2012), how then can sperm miRs influence embryonic brain development twelve days post-fertilization? We previously demonstrated that the Stress miRs repress maternal mRNA in the zygote, where the two most down-regulated genes were *Sirt1* and *Ube3a* (Rodgers, 2015). Given the known developmental roles these two genes play in post-transcriptional regulation and neurodevelopment (Calvanese et al., 2010; Greer, 2010; Herskovits, 2014), they may initiate a cascade of molecular events upstream of brain transcriptome reprogramming. Indeed, neither *Sirt1* nor *Ube3a* was altered in the whole E12.5 brain or adult paraventricular nucleus of the hypothalamus following zygote microinjection (Rodgers, 2015), suggesting they act during a sensitive window of development to catalyze long-term changes.

Gene expression differences in important developmental regulators in the pluripotent zygote suggest that sperm miRs likely influence development of multiple offspring tissues. As we were interested in identifying developmental antecedents to our adult stress dysregulation phenotype, we additionally focused on the placenta. Interestingly, we showed broad repression in the placental transcriptome by zygote microinjection of both miR groups, suggesting the placenta is sensitive to the early presence of miRs, regardless of the miR population. However, specific miRs within the Random miR group may be responsible for the robust downregulation of genes. For

example, miR-223, miR-149 and miR-301a have been linked with preeclampsia and preterm birth in humans (Choi et al., 2013; Gray et al., 2017; Vashukova et al., 2016; Yang et al., 2015), suggesting these miRs may be specifically important for placental development. As the fetally-derived trophoblasts of the placenta are one of the first cell lineages to differentiate (Red-Horse, 2004), there are fewer steps between direct sperm miR action in the zygote and placental development than for other embryonic tissues, suggesting the placenta is more vulnerable to miR functions in the zygote. Indeed, our data suggest some tissue specificity in sperm miR effects, as Random miRs result in broad transcriptional reprogramming in the placenta, but not in the embryonic brain. Moreover, these data suggest that, for this model, the placenta is an unlikely contributor to brain reprogramming, and that paternal stress sperm miRs impact the developing brain via changes to the embryonic, and not extra-embryonic, lineage that lies upstream of neural differentiation.

In order to understand how sperm miRs introduced transiently at fertilization results in persistent transcriptional reprogramming in the brain, we examined histone PTMs as changes to the histone code can govern transcriptional regulation and neurodevelopment (Dulac, 2010; Fagiolini et al., 2009; Yoo et al., 2009). Moreover, following zygote microinjection of Stress miRs, the histone deacetylase *Sirt1* was repressed in the zygote, and the expression of numerous known histone modifiers (e.g. the H3K36 methyltransferase *Setd2,* and the H3K27 demethylase *UTY*) was significantly altered in the E12.5 brain, suggesting sperm miRs may crosstalk with other epigenetic modifiers to produce gene expression changes. Indeed, there were significant alterations

in histone PTMs including H3K27 and H3K36, consistent with our RNA sequencing data that modifiers of those histone peptide sites were altered at this time point. Interestingly, the regulation of these two marks is thought to be tightly intertwined, such that methylation on one site antagonizes the methylation status of the other (Yuan et al., 2011), suggesting a careful balance of transcriptional repression and activation. Moreover, genetic ablation of readers and writers of these marks are associated with neurodevelopmental disorders, including Wolf-Hirschhorn and Weaver syndromes (Parkel et al., 2013), supporting the role of these marks in guiding neuroplasticity that ultimate reprograms adult brain function.

We next extended these histone PTM findings in the adult paternal stress offspring PVN as a means of relating our observations from microinjected embryos with animals from our paternal stress model. We again detected significant differences in histone PTMs in the PVN that may lie upstream of the transcriptional repression we previously observed. As the control center of the HPA stress axis response, chromatin regulation of these changes in the PVN transcriptome may produce the stress dysregulation phenotype in our model. Indeed, methylation of H3K9 and acetylation of H4 lysines have been implicated as crucial regulators of both the acute and chronic effects of stress in other brain regions (Ferland et al., 2014; Hunter et al., 2009; Kenworthy et al., 2014; Levine et al., 2012; Pathak et al., 2017; Sun et al., 2013). Interestingly, in a separate model of paternal stress transmission, H4K5ac was implicated in promoting the behavioral phenotypes in offspring (Gapp et al., 2016), suggesting the influential role of these histone marks in their convergent response to acute, chronic, and

paternal stress. As the PVN encompasses a small region within the hypothalamus, technical limitations currently restrict our ability to test this relationship in our model.

Next, we assessed whether the same histone PTMs governed transcriptional dysregulation during embryonic development vs. adulthood. Comparing the significantly altered histone PTMs by either zygote microinjection of Stress miRs or by paternal stress transmission, we observed no shared histone marks altered between these time points. Unsurprisingly, the composition of histone marks and variants mature across development and adulthood (Maze et al., 2015). Thus, the functional importance of histone PTMs in our model may also be time-specific, resulting from a cascade of epigenetic modifiers changing over time, consistent with our data, or the interaction of Stress miR-induced remodeling with age. However, the comparison of an entire embryonic brain with the specific region of the PVN, and/or the differences in transmission of paternal stress with sperm miR microinjection may also contribute to this observation.

Together, our findings demonstrate that sperm miRs altered by paternal stress can influence offspring neurodevelopment through stepwise changes in chromatin and transcriptional regulation. Disruption of the embryonic brain transcriptome suggests developmental antecedents in the paternal transmission of offspring endophenotypes. Moreover, specificity in the sperm miR population at fertilization suggests a conserved and coordinated process by which paternal tissues convey information capable of offspring programming. These studies confer the importance of germ cell epigenetic

marks in influencing offspring outcomes, and further emphasize the paternal preconception environment as a potential factor in the etiology of disease.

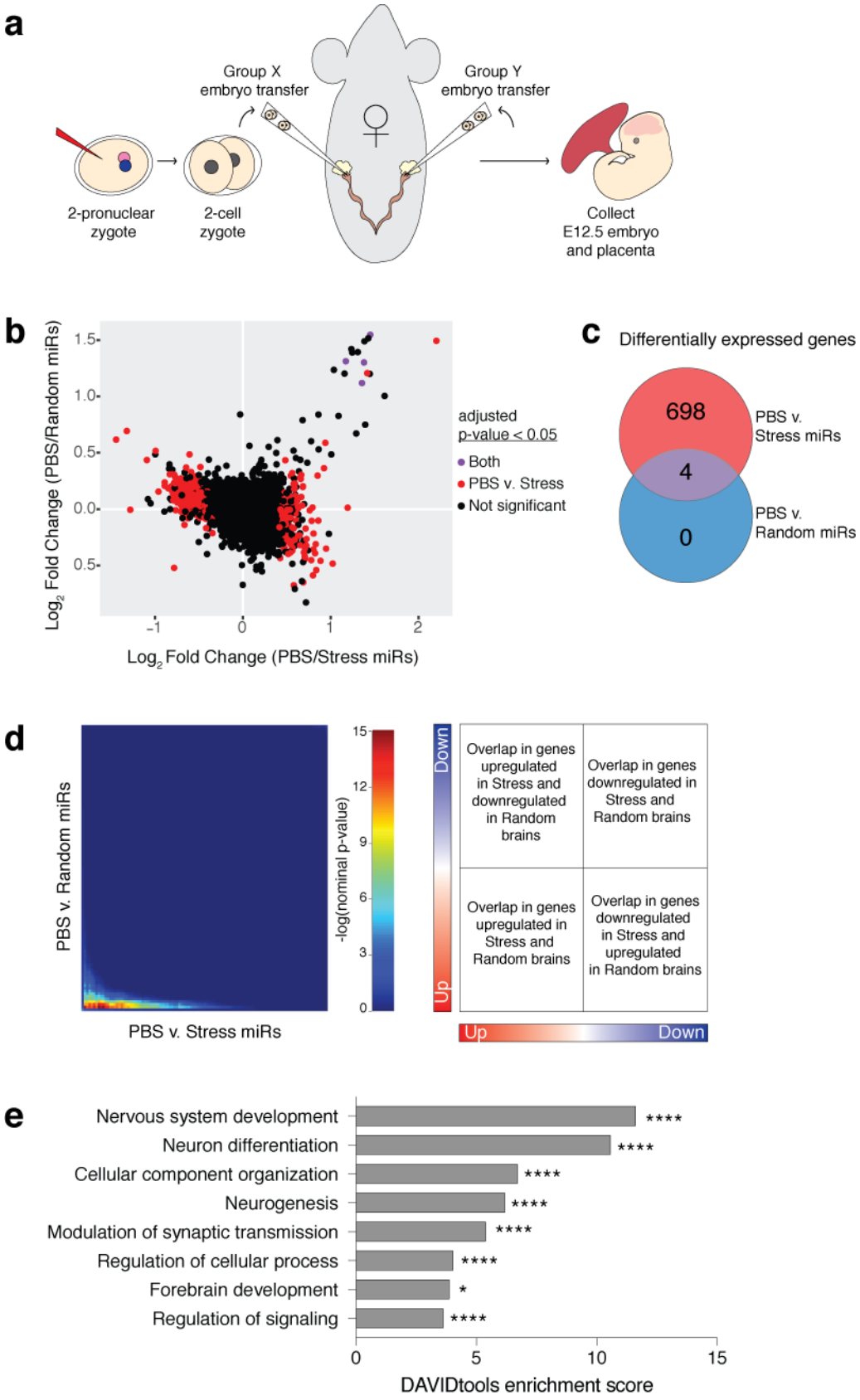
a
Group X
embryo transfer
Group Y
embryo transfer
2-pronuclear
zygote
2-cell
zygote
Collect
E12.5 embryo
and placenta
b
Log2 Fold Change (PBS/Random miRs)
Log2 Fold Change (PBS/Stress miRs)
adjusted
p-value < 0.05
Both
PBS v. Stress
Not significant
c
Differentially expressed genes
698
PBS v.
Stress miRs
4
0
PBS v.
Random miRs
d
PBS v. Random miRs
PBS v. Stress miRs
-log(nominal p-value)
Overlap in genes upregulated in Stress and downregulated in Random brains
Overlap in genes downregulated in Stress and Random brains
Overlap in genes upregulated in Stress and Random brains
Overlap in genes downregulated in Stress and upregulated in Random brains
Down
Up
e
Nervous system development
Neuron differentiation
Cellular component organization
Neurogenesis
Modulation of synaptic transmission
Regulation of cellular process
Forebrain development
Regulation of signaling
DAVIDtools enrichment score

Figure 4.1. Paternal stress sperm miRs specifically program the embryonic brain transcriptome. (**a**) Schematic of experimental design: single-cell zygotes were microinjected with either nine miRs previously identified in transmitting paternal stress effects (Stress miRs), a composite of nine miRs that are lowly expressed in sperm and unresponsive to stress exposure (Random miRs), or a PBS control. Resultant 2-cell zygotes were transferred into surrogate fosters where one treatment group (X) was transferred to the left uterine horn and another group (Y) was transferred to the right uterine horn. As embryos were collected at E12.5, this allowed for harvesting of each individual uterus and the control of the maternal intrauterine environment. (**b**) Differential expression analyses of E12.5 male brains showing the robust and specific effects of Stress miRs are presented as the $\log_2$ fold change of each miR group compared with PBS, where each dot represents one gene color-coded for significance. Red: adjusted *P*-value < 0.05, PBS v. Stress. Purple: adjusted *P*-value < 0.05, PBS v. Stress and PBS v. Random. Black: not significant by either comparison. N = 6 embryos/group. (**c**) Venn diagrams of significant differentially expressed genes for each miR microinjection comparison with PBS, and their overlap. (**d**) Rank-rank hypergeometric overlap (RRHO) was used between the differential expression profiles of PBS v. Stress miRs and PBS v. Random miRs, showing the degree of significant overlap in a threshold-free manner (max $-\log_{10}$(*P*-value) = 15. Few overlapping genes clustered in the bottom left corner, indicating upregulation by both miR microinjection groups (see key, right). (**e**) Functional annotation cluster analysis of all differentially expressed genes by Stress miR microinjection using gene ontology (GO) terms for biological processes, with a cutoff of Benjamini-corrected *P*-value < 0.05 and Davidtools Enrichment Score > 1.3. For each significantly enriched cluster, one GO term is plotted.

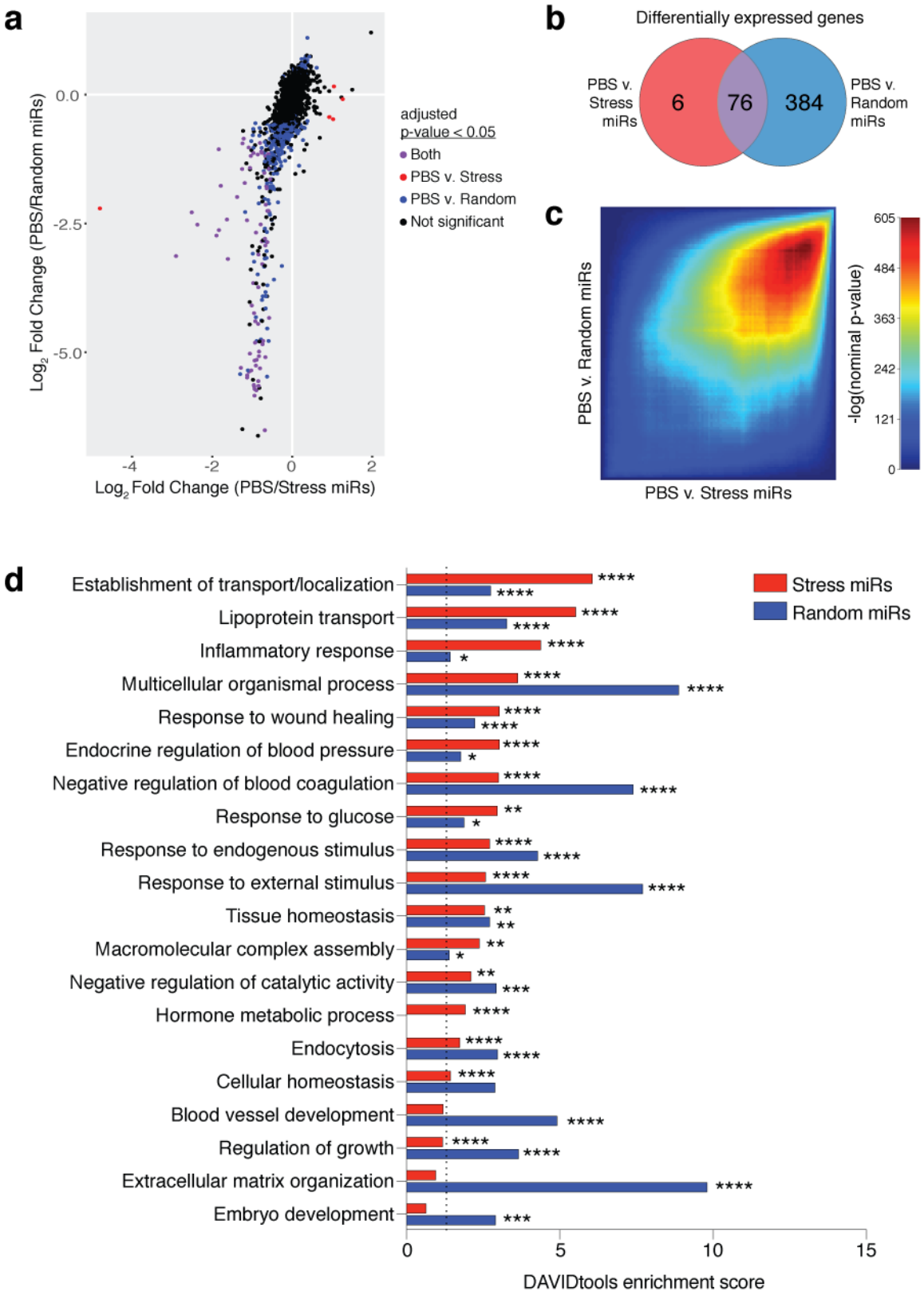
a
0.0
-2.5
-5.0
-4
-2
0
2
Log2 Fold Change (PBS/Random miRs)
Log2 Fold Change (PBS/Stress miRs)
adjusted
p-value < 0.05
Both
PBS v. Stress
PBS v. Random
Not significant
b
Differentially expressed genes
PBS v. Stress miRs
6
76
384
PBS v. Random miRs
c
PBS v. Random miRs
PBS v. Stress miRs
-log(nominal p-value)
605
484
363
242
121
0
d
Stress miRs
Random miRs
Establishment of transport/localization
Lipoprotein transport
Inflammatory response
Multicellular organismal process
Response to wound healing
Endocrine regulation of blood pressure
Negative regulation of blood coagulation
Response to glucose
Response to endogenous stimulus
Response to external stimulus
Tissue homeostasis
Macromolecular complex assembly
Negative regulation of catalytic activity
Hormone metabolic process
Endocytosis
Cellular homeostasis
Blood vessel development
Regulation of growth
Extracellular matrix organization
Embryo development
0
5
10
15
DAVIDtools enrichment score

Figure 4.2. Sperm miRs at fertilization influence the placental transcriptome. (**a**) Differential expression analyses of E12.5 male placentas showing transcriptional downregulation as a result of both miR microinjection groups are presented as the $\log_2$ fold change of each miR group compared with PBS, where each dot represents one gene color-coded for significance. Red: adjusted *P*-value < 0.05, PBS v. Stress. Blue, adjusted *P*-value < 0.05. Purple: adjusted *P*-value < 0.05, PBS v. Stress and PBS v. Random. Black: not significant by either comparison. N = 5-6 samples/group. (**b**) Venn diagrams of significant differentially expressed genes for each miR microinjection comparison with PBS, and their overlap. (**c**) Rank-rank hypergeometric overlap (RRHO) was used between the differential expression profiles of PBS v. Stress miRs and PBS v. Random miRs, showing the degree of significant overlap in a threshold-free manner (max - $\log_{10}$(*P*-value) = 605. There was strong overlap in the genes in the top right corner, indicating extensive repression in placental gene expression by both miR microinjection groups (see key, Fig. 1d, right). (**d**) Functional annotation cluster analysis of all differentially expressed genes by Stress miRs (red bars) and Random miRs (blue bars) using GO terms for biological processes, with a cutoff of Benjamini-corrected *P*-value < 0.05 and Davidtools Enrichment Score > 1.3. One representative GO term was plotted for the top significantly enriched clusters for each miR group analysis, showing the majority of enriched placental processes affected by both Stress and Random miRs.

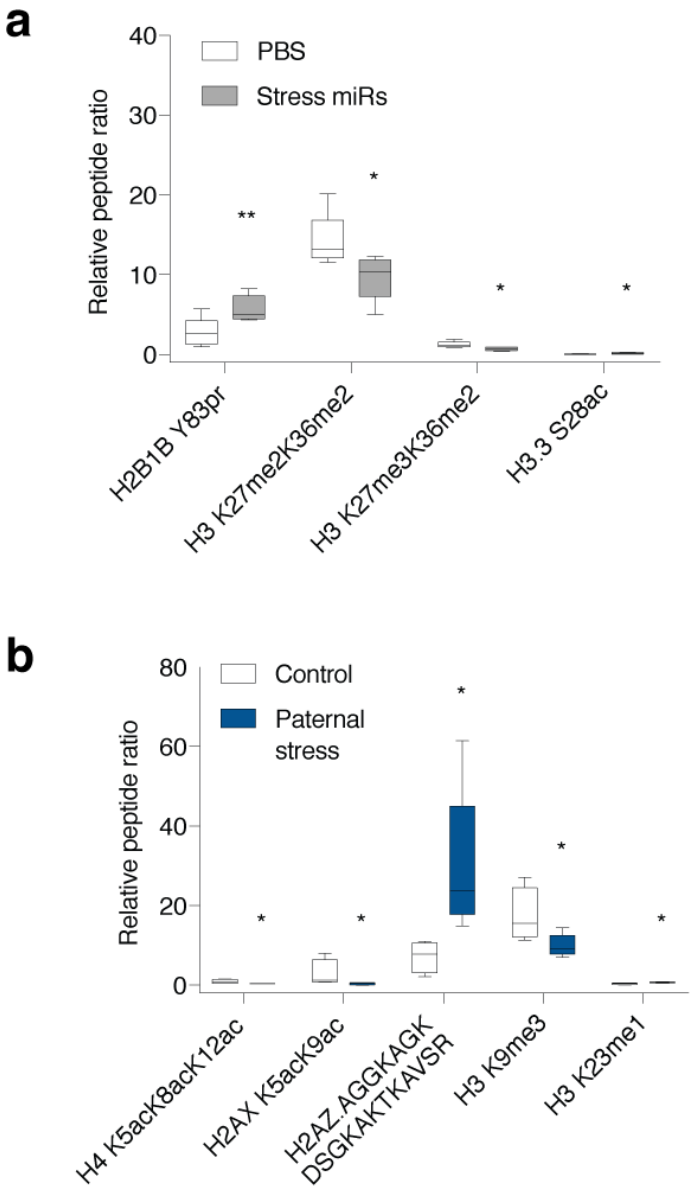

Figure 4.3. Paternal stress sperm miRs influence the histone code in developing and adult brains. (**a**) To examine the how sperm miRs at fertilization can promote long-term transcriptional changes, we performed quantitative histone mass spectrometry on E12.5 whole brains following zygote microinjection. We identified four histone PTMs significantly altered by Stress miRs. N = 4-5 embryos/group; Mann-Whitney U, $^{**}P < 0.01$, $^{*}P < 0.05$. (**b**) Histone mass spectrometry of adult paternal stress offspring pooled paraventricular nuclei samples was conducted to examine continued chromatin remodeling in adulthood as related to our paternal stress mouse model. We similarly identified five histone PTMs significantly altered by paternal stress exposure. N = 4-5; Mann-Whitney U, $^{**}P < 0.01$, $^{*}P < 0.05$. Box plots indicate median ± interquartile range (edges of box) and range (bars).

Table 4.1. Composition of microinjection treatment groups

PBS	Stress miRs	Random miRs
-	mmu-miR-29c	mmu-miR-132-3p
-	mmu-miR-30a	mmu-miR-149-5p
-	mmu-miR-30c	mmu-miR-15a-5p
-	mmu-miR-32	mmu-miR-223-5p
-	mmu-miR-193-5p	mmu-miR-292a-3p
-	mmu-miR-204	mmu-miR-301a-5p
-	mmu-miR-375	mmu-miR-326-3p
-	mmu-miR-532-3p	mmu-miR-466d-3p
-	mmu-miR-698	mmu-miR-709

Table 4.2. Functional annotation clustering of putative Stress miR targets in the zygote

Annotation Cluster 1	Enrichment Score: 3.61	
Term	Fold Enrichment	Benjamini
GO:0016043~cellular component organization	1.62	0.03
GO:0071840~cellular component organization or biogenesis	1.58	0.01
GO:0044085~cellular component biogenesis	1.82	0.06
GO:0006996~organelle organization	1.46	0.25

Annotation Cluster 2	Enrichment Score: 2.73	
Term	Fold Enrichment	Benjamini
GO:0033554~cellular response to stress	2.49	0.02
GO:0006950~response to stress	1.60	0.12
GO:0051716~cellular response to stimulus	1.16	0.58

Annotation Cluster 3	Enrichment Score: 2.64	
Term	Fold Enrichment	Benjamini
GO:0051179~localization	1.55	0.03
GO:0071702~organic substance transport	1.95	0.05
GO:0061024~membrane organization	2.90	0.05
GO:1902580~single-organism cellular localization	2.76	0.05
GO:0006810~transport	1.62	0.05
GO:0051640~organelle localization	3.96	0.05
GO:0034613~cellular protein localization	2.29	0.05
GO:0070727~cellular macromolecule localization	2.27	0.05
GO:0046907~intracellular transport	2.39	0.05
GO:0044802~single-organism membrane organization	3.01	0.06
GO:0045184~establishment of protein localization	2.06	0.06
GO:0051234~establishment of localization	1.57	0.06
GO:0015031~protein transport	2.07	0.07
GO:0033036~macromolecule localization	1.79	0.07
GO:0010256~endomembrane system organization	3.42	0.07
GO:0008104~protein localization	1.84	0.08
GO:0051656~establishment of organelle localization	3.97	0.09
GO:0051641~cellular localization	1.82	0.10
GO:0051649~establishment of localization in cell	1.99	0.11
GO:1902582~single-organism intracellular transport	2.93	0.13
GO:0006886~intracellular protein transport	2.26	0.22
GO:0033365~protein localization to organelle	2.36	0.22
GO:0016192~vesicle-mediated transport	2.00	0.25
GO:1902578~single-organism localization	1.45	0.31
GO:0044765~single-organism transport	1.45	0.36
GO:0006605~protein targeting	2.33	0.37
GO:0072594~establishment of protein localization to organelle	2.31	0.44
GO:0051049~regulation of transport	1.36	0.71

Annotation Cluster 4	Enrichment Score: 2.21	
Term	Fold Enrichment	Benjamini
GO:0048666~neuron development	3.00	0.02
GO:0031175~neuron projection development	3.22	0.01
GO:0030182~neuron differentiation	2.60	0.02
GO:0048699~generation of neurons	2.45	0.02
GO:0030154~cell differentiation	1.69	0.04
GO:0022008~neurogenesis	2.28	0.05
GO:0030030~cell projection organization	2.35	0.05
GO:0007399~nervous system development	1.93	0.06
GO:0044707~single-multicellular organism process	1.44	0.06

GO:0048869~cellular developmental process	1.57	0.06
GO:0048468~cell development	1.92	0.06
GO:0048812~neuron projection morphogenesis	3.28	0.06
GO:0048731~system development	1.52	0.09
GO:0032502~developmental process	1.41	0.10
GO:0007275~multicellular organism development	1.44	0.12
GO:0000904~cell morphogenesis involved in differentiation	2.52	0.14
GO:0044767~single-organism developmental process	1.38	0.15
GO:0048856~anatomical structure development	1.37	0.17
GO:0048667~cell morphogenesis involved in neuron differentiation	2.95	0.17
GO:0032989~cellular component morphogenesis	1.92	0.24
GO:0000902~cell morphogenesis	1.94	0.25
GO:0048858~cell projection morphogenesis	2.25	0.25
GO:0007409~axonogenesis	2.96	0.26
GO:0048513~animal organ development	1.46	0.27
GO:0032990~cell part morphogenesis	2.17	0.29
GO:0061564~axon development	2.74	0.31
GO:0009653~anatomical structure morphogenesis	1.48	0.37
GO:0006928~movement of cell or subcellular component	1.63	0.40
GO:0007411~axon guidance	3.68	0.40
GO:0097485~neuron projection guidance	3.66	0.41
GO:0040011~locomotion	1.63	0.46
GO:0045664~regulation of neuron differentiation	2.06	0.48
GO:0050767~regulation of neurogenesis	1.89	0.50
GO:0048870~cell motility	1.62	0.52
GO:0051674~localization of cell	1.62	0.52
GO:0051960~regulation of nervous system development	1.69	0.62
GO:0032501~multicellular organismal process	1.12	0.68
GO:0006935~chemotaxis	1.45	0.92
GO:0042330~taxis	1.45	0.92

Annotation Cluster 5	Enrichment Score: 2.18	
Term	Fold Enrichment	Benjamini
GO:0048522~positive regulation of cellular process	1.65	0.02
GO:0051254~positive regulation of RNA metabolic process	2.45	0.04
GO:0048518~positive regulation of biological process	1.56	0.05
GO:0006357~regulation of transcription from RNA polymerase II promoter	2.20	0.05
GO:0006366~transcription from RNA polymerase II promoter	2.20	0.05
GO:0051173~positive regulation of nitrogen compound metabolic process	2.15	0.05
GO:0045935~positive regulation of nucleobase-containing compound metabolic process	2.19	0.05
GO:0045893~positive regulation of transcription, DNA-templated	2.33	0.06
GO:1903508~positive regulation of nucleic acid-templated transcription	2.33	0.06
GO:1902680~positive regulation of RNA biosynthetic process	2.32	0.06
GO:0006351~transcription, DNA-templated	1.74	0.06
GO:0010557~positive regulation of macromolecule biosynthetic process	2.17	0.06
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	2.53	0.06
GO:0048523~negative regulation of cellular process	1.55	0.07
GO:0019219~regulation of nucleobase-containing compound metabolic process	1.59	0.09
GO:0031325~positive regulation of cellular metabolic process	1.75	0.09
GO:0044260~cellular macromolecule metabolic process	1.32	0.09
GO:0051252~regulation of RNA metabolic process	1.61	0.10
GO:0010604~positive regulation of macromolecule metabolic process	1.73	0.10
GO:0031328~positive regulation of cellular biosynthetic process	2.01	0.10
GO:2000112~regulation of cellular macromolecule biosynthetic process	1.57	0.10
GO:0009891~positive regulation of biosynthetic process	1.97	0.11
GO:0048519~negative regulation of biological process	1.47	0.11

GO:0051171~regulation of nitrogen compound metabolic process	1.51	0.12
GO:0010556~regulation of macromolecule biosynthetic process	1.53	0.12
GO:0034645~cellular macromolecule biosynthetic process	1.46	0.13
GO:0006355~regulation of transcription, DNA-templated	1.58	0.13
GO:1903506~regulation of nucleic acid-templated transcription	1.58	0.13
GO:2001141~regulation of RNA biosynthetic process	1.58	0.13
GO:0018130~heterocycle biosynthetic process	1.52	0.14
GO:0019438~aromatic compound biosynthetic process	1.51	0.14
GO:0010628~positive regulation of gene expression	1.92	0.15
GO:0009059~macromolecule biosynthetic process	1.42	0.17
GO:0009893~positive regulation of metabolic process	1.61	0.17
GO:0097659~nucleic acid-templated transcription	1.54	0.17
GO:0090304~nucleic acid metabolic process	1.43	0.17
GO:0031323~regulation of cellular metabolic process	1.37	0.17
GO:0032774~RNA biosynthetic process	1.53	0.17
GO:0043170~macromolecule metabolic process	1.25	0.17
GO:1901362~organic cyclic compound biosynthetic process	1.47	0.18
GO:0031326~regulation of cellular biosynthetic process	1.47	0.18
GO:0009889~regulation of biosynthetic process	1.45	0.21
GO:0034654~nucleobase-containing compound biosynthetic process	1.46	0.22
GO:0016070~RNA metabolic process	1.40	0.25
GO:0044237~cellular metabolic process	1.19	0.25
GO:0046483~heterocycle metabolic process	1.33	0.26
GO:0006725~cellular aromatic compound metabolic process	1.32	0.29
GO:0010468~regulation of gene expression	1.39	0.29
GO:1901360~organic cyclic compound metabolic process	1.31	0.30
GO:0044249~cellular biosynthetic process	1.31	0.30
GO:0044271~cellular nitrogen compound biosynthetic process	1.35	0.30
GO:0009058~biosynthetic process	1.29	0.31
GO:0080090~regulation of primary metabolic process	1.30	0.31
GO:0019222~regulation of metabolic process	1.28	0.33
GO:1901576~organic substance biosynthetic process	1.28	0.34
GO:0006139~nucleobase-containing compound metabolic process	1.30	0.34
GO:0060255~regulation of macromolecule metabolic process	1.28	0.37
GO:0034641~cellular nitrogen compound metabolic process	1.26	0.38
GO:0006807~nitrogen compound metabolic process	1.24	0.39
GO:0010467~gene expression	1.25	0.48
GO:0044238~primary metabolic process	1.14	0.48
GO:0008152~metabolic process	1.12	0.50
GO:0071704~organic substance metabolic process	1.12	0.52
GO:0065007~biological regulation	1.10	0.53
GO:0051716~cellular response to stimulus	1.16	0.58
GO:0050794~regulation of cellular process	1.08	0.73
GO:0050789~regulation of biological process	1.07	0.74
GO:0007165~signal transduction	1.08	0.87

Annotation Cluster 6	Enrichment Score: 1.80	
Term	Fold Enrichment	Benjamini
GO:0046847~filopodium assembly	16.24	0.05
GO:0051491~positive regulation of filopodium assembly	20.11	0.06
GO:0051489~regulation of filopodium assembly	12.99	0.12
GO:0031346~positive regulation of cell projection organization	3.19	0.22
GO:0031344~regulation of cell projection organization	2.35	0.31
GO:0044087~regulation of cellular component biogenesis	1.97	0.47
GO:0060491~regulation of cell projection assembly	3.90	0.51
GO:0030031~cell projection assembly	2.23	0.61
GO:0044089~positive regulation of cellular component biogenesis	1.76	0.84

Annotation Cluster 7	Enrichment Score: 1.71	
Term	Fold Enrichment	Benjamini
GO:0006357~regulation of transcription from RNA polymerase II promoter	2.20	0.05
GO:0006366~transcription from RNA polymerase II promoter	2.20	0.05
GO:0051252~regulation of RNA metabolic process	1.61	0.10
GO:0045934~negative regulation of nucleobase-containing compound metabolic process	1.98	0.18
GO:0051172~negative regulation of nitrogen compound metabolic process	1.85	0.22
GO:0031324~negative regulation of cellular metabolic process	1.64	0.24
GO:0051253~negative regulation of RNA metabolic process	1.94	0.25
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	2.29	0.29
GO:0009892~negative regulation of metabolic process	1.51	0.36
GO:2000113~negative regulation of cellular macromolecule biosynthetic process	1.73	0.38
GO:0010629~negative regulation of gene expression	1.63	0.43
GO:0010558~negative regulation of macromolecule biosynthetic process	1.66	0.44
GO:0031327~negative regulation of cellular biosynthetic process	1.59	0.48
GO:0010605~negative regulation of macromolecule metabolic process	1.45	0.48
GO:0009890~negative regulation of biosynthetic process	1.56	0.50
GO:0045892~negative regulation of transcription, DNA-templated	1.68	0.51
GO:1903507~negative regulation of nucleic acid-templated transcription	1.66	0.52
GO:1902679~negative regulation of RNA biosynthetic process	1.64	0.54

Annotation Cluster 8	Enrichment Score: 1.70	
Term	Fold Enrichment	Benjamini
GO:0061024~membrane organization	2.90	0.05
GO:1902580~single-organism cellular localization	2.76	0.05
GO:0044802~single-organism membrane organization	3.01	0.06
GO:0010256~endomembrane system organization	3.42	0.07
GO:1903729~regulation of plasma membrane organization	9.50	0.09
GO:1903076~regulation of protein localization to plasma membrane	8.66	0.22
GO:1904375~regulation of protein localization to cell periphery	8.54	0.22
GO:0072657~protein localization to membrane	3.09	0.24
GO:1904377~positive regulation of protein localization to cell periphery	11.41	0.32
GO:1903078~positive regulation of protein localization to plasma membrane	11.41	0.32
GO:0007009~plasma membrane organization	3.35	0.35
GO:0090003~regulation of establishment of protein localization to plasma membrane	9.17	0.39
GO:0072659~protein localization to plasma membrane	3.75	0.40
GO:0060341~regulation of cellular localization	1.98	0.41
GO:1990778~protein localization to cell periphery	3.42	0.44
GO:1903827~regulation of cellular protein localization	2.25	0.46
GO:0090150~establishment of protein localization to membrane	2.93	0.52
GO:0032880~regulation of protein localization	1.71	0.55
GO:0090002~establishment of protein localization to plasma membrane	3.34	0.75
GO:1903829~positive regulation of cellular protein localization	1.77	0.89
GO:1904951~positive regulation of establishment of protein localization	1.49	0.91

Annotation Cluster 9	Enrichment Score: 1.68	
Term	Fold Enrichment	Benjamini
GO:0010941~regulation of cell death	2.02	0.11
GO:0012501~programmed cell death	1.92	0.12
GO:0008219~cell death	1.87	0.12
GO:0006915~apoptotic process	1.93	0.12
GO:0042981~regulation of apoptotic process	2.00	0.16
GO:0043067~regulation of programmed cell death	1.98	0.17
GO:0097190~apoptotic signaling pathway	2.68	0.22
GO:0010942~positive regulation of cell death	2.51	0.26
GO:2001237~negative regulation of extrinsic apoptotic signaling pathway	6.43	0.30

GO:0043065~positive regulation of apoptotic process	2.43	0.34
GO:0043068~positive regulation of programmed cell death	2.41	0.35
GO:0043066~negative regulation of apoptotic process	2.00	0.37
GO:0043069~negative regulation of programmed cell death	1.97	0.38
GO:2001234~negative regulation of apoptotic signaling pathway	3.66	0.41
GO:0097191~extrinsic apoptotic signaling pathway	3.33	0.46
GO:0060548~negative regulation of cell death	1.80	0.47
GO:2001236~regulation of extrinsic apoptotic signaling pathway	3.87	0.51
GO:2001233~regulation of apoptotic signaling pathway	2.45	0.54
GO:0001655~urogenital system development	2.14	0.73

Annotation Cluster 10	Enrichment Score: 1.56	
Term	Fold Enrichment	Benjamini
GO:0080135~regulation of cellular response to stress	4.00	0.02
GO:0043412~macromolecule modification	1.72	0.05
GO:0036211~protein modification process	1.71	0.06
GO:0006464~cellular protein modification process	1.71	0.06
GO:0031325~positive regulation of cellular metabolic process	1.75	0.09
GO:0044260~cellular macromolecule metabolic process	1.32	0.09
GO:0010604~positive regulation of macromolecule metabolic process	1.73	0.10
GO:0065009~regulation of molecular function	1.84	0.11
GO:0010648~negative regulation of cell communication	2.28	0.11
GO:0044093~positive regulation of molecular function	2.21	0.11
GO:0023057~negative regulation of signaling	2.28	0.11
GO:0080134~regulation of response to stress	2.22	0.12
GO:0044267~cellular protein metabolic process	1.45	0.15
GO:0042327~positive regulation of phosphorylation	2.34	0.17
GO:0031399~regulation of protein modification process	1.93	0.17
GO:0009893~positive regulation of metabolic process	1.61	0.17
GO:0043170~macromolecule metabolic process	1.25	0.17
GO:0046330~positive regulation of JNK cascade	6.29	0.18
GO:0048583~regulation of response to stimulus	1.55	0.18
GO:0031401~positive regulation of protein modification process	2.16	0.19
GO:0019538~protein metabolic process	1.38	0.21
GO:0001934~positive regulation of protein phosphorylation	2.27	0.22
GO:0006796~phosphate-containing compound metabolic process	1.58	0.22
GO:0006793~phosphorus metabolic process	1.57	0.22
GO:0009968~negative regulation of signal transduction	2.13	0.23
GO:0032874~positive regulation of stress-activated MAPK cascade	5.38	0.23
GO:0070304~positive regulation of stress-activated protein kinase signaling cascade	5.34	0.24
GO:0010646~regulation of cell communication	1.57	0.24
GO:0019220~regulation of phosphate metabolic process	1.81	0.24
GO:0051174~regulation of phosphorus metabolic process	1.81	0.24
GO:0023051~regulation of signaling	1.55	0.25
GO:0045937~positive regulation of phosphate metabolic process	2.07	0.25
GO:0010562~positive regulation of phosphorus metabolic process	2.07	0.25
GO:0042325~regulation of phosphorylation	1.88	0.25
GO:0001932~regulation of protein phosphorylation	1.91	0.26
GO:0046328~regulation of JNK cascade	4.56	0.30
GO:0006468~protein phosphorylation	1.71	0.31
GO:0007254~JNK cascade	4.40	0.31
GO:0032268~regulation of cellular protein metabolic process	1.56	0.35
GO:0016310~phosphorylation	1.59	0.37
GO:0009966~regulation of signal transduction	1.52	0.37
GO:0051246~regulation of protein metabolic process	1.51	0.38
GO:0048584~positive regulation of response to stimulus	1.61	0.39

GO:0032872~regulation of stress-activated MAPK cascade	3.75	0.40
GO:0035556~intracellular signal transduction	1.51	0.40
GO:0070302~regulation of stress-activated protein kinase signaling cascade	3.73	0.40
GO:0048585~negative regulation of response to stimulus	1.73	0.41
GO:0010647~positive regulation of cell communication	1.67	0.42
GO:0023056~positive regulation of signaling	1.67	0.43
GO:0051403~stress-activated MAPK cascade	3.42	0.44
GO:0050790~regulation of catalytic activity	1.59	0.46
GO:0031098~stress-activated protein kinase signaling cascade	3.22	0.48
GO:0051247~positive regulation of protein metabolic process	1.64	0.48
GO:0043410~positive regulation of MAPK cascade	2.34	0.50
GO:0007154~cell communication	1.20	0.52
GO:0023052~signaling	1.19	0.57
GO:0051716~cellular response to stimulus	1.16	0.58
GO:0007166~cell surface receptor signaling pathway	1.37	0.61
GO:0009967~positive regulation of signal transduction	1.53	0.63
GO:0043408~regulation of MAPK cascade	1.85	0.63
GO:0044700~single organism signaling	1.17	0.63
GO:0000165~MAPK cascade	1.80	0.65
GO:0023014~signal transduction by protein phosphorylation	1.79	0.66
GO:0043085~positive regulation of catalytic activity	1.62	0.67
GO:1902531~regulation of intracellular signal transduction	1.41	0.71
GO:1902533~positive regulation of intracellular signal transduction	1.55	0.75
GO:0007165~signal transduction	1.08	0.87
GO:0051338~regulation of transferase activity	1.42	0.88
GO:0051347~positive regulation of transferase activity	1.58	0.89
GO:0043549~regulation of kinase activity	1.39	0.91
GO:0045860~positive regulation of protein kinase activity	1.61	0.93
GO:0071900~regulation of protein serine/threonine kinase activity	1.54	0.94
GO:0071902~positive regulation of protein serine/threonine kinase activity	1.81	0.94
GO:0033674~positive regulation of kinase activity	1.47	0.95
GO:0045859~regulation of protein kinase activity	1.27	0.96
GO:0044710~single-organism metabolic process	0.86	1.00

Annotation Cluster 11	Enrichment Score: 1.40	
Term	Fold Enrichment	Benjamini
GO:1902110~positive regulation of mitochondrial membrane permeability in apoptotic process	18.71	0.22
GO:1902686~mitochondrial outer membrane permeabilization in programmed cell death	18.71	0.22
GO:0035794~positive regulation of mitochondrial membrane permeability	16.70	0.23
GO:1902108~regulation of mitochondrial membrane permeability involved in apoptotic process	14.61	0.26
GO:0046902~regulation of mitochondrial membrane permeability	9.95	0.37
GO:0090559~regulation of membrane permeability	8.82	0.40
GO:0006839~mitochondrial transport	3.54	0.43
GO:0007006~mitochondrial membrane organization	5.50	0.55
GO:0008637~apoptotic mitochondrial changes	4.33	0.65
GO:0007005~mitochondrion organization	1.61	0.79

Annotation Cluster 12	Enrichment Score: 1.38	
Term	Fold Enrichment	Benjamini
GO:0030154~cell differentiation	1.69	0.04
GO:0048869~cellular developmental process	1.57	0.06
GO:0042221~response to chemical	1.45	0.22
GO:0070887~cellular response to chemical stimulus	1.55	0.29
GO:0071310~cellular response to organic substance	1.56	0.39
GO:0010033~response to organic substance	1.36	0.51
GO:1901700~response to oxygen-containing compound	1.40	0.69

GO:0014070~response to organic cyclic compound	1.55	0.71
GO:0071495~cellular response to endogenous stimulus	1.44	0.75
GO:1901701~cellular response to oxygen-containing compound	1.47	0.76
GO:0009719~response to endogenous stimulus	1.26	0.85
GO:0009725~response to hormone	1.33	0.91

Annotation Cluster 13	Enrichment Score: 1.34	
Term	Fold Enrichment	Benjamini
GO:0007420~brain development	2.51	0.18
GO:0007417~central nervous system development	2.25	0.18
GO:0060322~head development	2.36	0.22
GO:0021543~pallium development	4.35	0.32
GO:0021537~telencephalon development	2.92	0.53
GO:0030900~forebrain development	2.26	0.60
GO:0021987~cerebral cortex development	3.93	0.69
GO:0021953~central nervous system neuron differentiation	2.24	0.89

Annotation Cluster 14	Enrichment Score: 1.31	
Term	Fold Enrichment	Benjamini
GO:0007416~synapse assembly	6.39	0.18
GO:0031344~regulation of cell projection organization	2.35	0.31
GO:0050808~synapse organization	3.27	0.47
GO:0050803~regulation of synapse structure or activity	2.78	0.55
GO:0050807~regulation of synapse organization	3.65	0.72

Table 4.3. Functional annotation clustering of putative Random miR targets in the zygote

Annotation Cluster 1	Enrichment Score: 1.80	
Term	Fold Enrichment	Benjamini
GO:0030163~protein catabolic process	3.49	0.68
GO:0044257~cellular protein catabolic process	3.76	0.58
GO:0019941~modification-dependent protein catabolic process	4.20	0.49
GO:0043632~modification-dependent macromolecule catabolic process	4.14	0.34
GO:0044265~cellular macromolecule catabolic process	3.25	0.35
GO:0009057~macromolecule catabolic process	2.90	0.31
GO:0051603~proteolysis involved in cellular protein catabolic process	3.55	0.36
GO:0006511~ubiquitin-dependent protein catabolic process	3.80	0.40
GO:0044248~cellular catabolic process	2.29	0.45
GO:1901575~organic substance catabolic process	2.09	0.51
GO:0009056~catabolic process	1.98	0.53
GO:0051865~protein autoubiquitination	13.58	0.53
GO:0006508~proteolysis	1.88	0.60
GO:0070647~protein modification by small protein conjugation or removal	2.02	0.75
GO:0042787~protein ubiquitination involved in ubiquitin-dependent protein catabolic process	4.70	0.75
GO:0043161~proteasome-mediated ubiquitin-dependent protein catabolic process	2.97	0.77
GO:0000209~protein polyubiquitination	4.29	0.78
GO:0032446~protein modification by small protein conjugation	2.04	0.80
GO:0010498~proteasomal protein catabolic process	2.72	0.81
GO:0016567~protein ubiquitination	1.83	0.90

Annotation Cluster 2	Enrichment Score: 1.46	
Term	Fold Enrichment	Benjamini
GO:1902680~positive regulation of RNA biosynthetic process	2.73	0.85
GO:0051254~positive regulation of RNA metabolic process	2.62	0.42
GO:1903508~positive regulation of nucleic acid-templated transcription	2.55	0.31
GO:0045893~positive regulation of transcription, DNA-templated	2.55	0.31
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	2.80	0.35
GO:0010557~positive regulation of macromolecule biosynthetic process	2.32	0.38
GO:0006357~regulation of transcription from RNA polymerase II promoter	2.20	0.40
GO:0045935~positive regulation of nucleobase-containing compound metabolic process	2.24	0.43
GO:0010628~positive regulation of gene expression	2.15	0.48
GO:0031328~positive regulation of cellular biosynthetic process	2.14	0.46
GO:0051173~positive regulation of nitrogen compound metabolic process	2.10	0.46
GO:0009891~positive regulation of biosynthetic process	2.10	0.44
GO:0090304~nucleic acid metabolic process	1.53	0.44
GO:0006351~transcription, DNA-templated	1.72	0.45
GO:0044260~cellular macromolecule metabolic process	1.31	0.56
GO:0006366~transcription from RNA polymerase II promoter	2.01	0.55
GO:0043170~macromolecule metabolic process	1.26	0.54
GO:0006139~nucleobase-containing compound metabolic process	1.41	0.57
GO:0010604~positive regulation of macromolecule metabolic process	1.66	0.58
GO:0006355~regulation of transcription, DNA-templated	1.56	0.59
GO:1903506~regulation of nucleic acid-templated transcription	1.56	0.59
GO:2001141~regulation of RNA biosynthetic process	1.55	0.59
GO:0048522~positive regulation of cellular process	1.42	0.60
GO:0019219~regulation of nucleobase-containing compound metabolic process	1.49	0.59
GO:0034645~cellular macromolecule biosynthetic process	1.42	0.59
GO:0046483~heterocycle metabolic process	1.38	0.59
GO:1901360~organic cyclic compound metabolic process	1.36	0.59
GO:0034641~cellular nitrogen compound metabolic process	1.34	0.58
GO:0097659~nucleic acid-templated transcription	1.52	0.60
GO:0006725~cellular aromatic compound metabolic process	1.36	0.59

GO:0032774~RNA biosynthetic process	1.51	0.59
GO:0051171~regulation of nitrogen compound metabolic process	1.45	0.59
GO:0051252~regulation of RNA metabolic process	1.50	0.59
GO:0006807~nitrogen compound metabolic process	1.31	0.59
GO:0009059~macromolecule biosynthetic process	1.39	0.60
GO:0080090~regulation of primary metabolic process	1.35	0.60
GO:0031325~positive regulation of cellular metabolic process	1.59	0.60
GO:0044237~cellular metabolic process	1.20	0.61
GO:0009893~positive regulation of metabolic process	1.55	0.62
GO:0009058~biosynthetic process	1.32	0.62
GO:1901362~organic cyclic compound biosynthetic process	1.42	0.62
GO:0044249~cellular biosynthetic process	1.32	0.62
GO:0031399~regulation of protein modification process	1.81	0.63
GO:0034654~nucleobase-containing compound biosynthetic process	1.42	0.63
GO:0048518~positive regulation of biological process	1.33	0.63
GO:1901576~organic substance biosynthetic process	1.30	0.64
GO:0018130~heterocycle biosynthetic process	1.40	0.64
GO:0019438~aromatic compound biosynthetic process	1.40	0.65
GO:0031323~regulation of cellular metabolic process	1.29	0.67
GO:2000112~regulation of cellular macromolecule biosynthetic process	1.39	0.69
GO:0044238~primary metabolic process	1.17	0.69
GO:0071704~organic substance metabolic process	1.15	0.71
GO:0010556~regulation of macromolecule biosynthetic process	1.36	0.71
GO:0009889~regulation of biosynthetic process	1.34	0.71
GO:0019222~regulation of metabolic process	1.24	0.73
GO:0044271~cellular nitrogen compound biosynthetic process	1.29	0.74
GO:0016070~RNA metabolic process	1.30	0.76
GO:0008152~metabolic process	1.12	0.75
GO:0060255~regulation of macromolecule metabolic process	1.23	0.76
GO:0031326~regulation of cellular biosynthetic process	1.30	0.77
GO:0006468~protein phosphorylation	1.55	0.79
GO:0010468~regulation of gene expression	1.26	0.81
GO:0010467~gene expression	1.22	0.82
GO:0009719~response to endogenous stimulus	1.37	0.92
GO:0042127~regulation of cell proliferation	1.34	0.93

Annotation Cluster 3	Enrichment Score: 1.46	
Term	Fold Enrichment	Benjamini
GO:0033365~protein localization to organelle	3.39	0.37
GO:0017038~protein import	5.20	0.35
GO:1902578~single-organism localization	1.84	0.33
GO:0044765~single-organism transport	1.87	0.31
GO:0042990~regulation of transcription factor import into nucleus	11.11	0.42
GO:0042991~transcription factor import into nucleus	10.98	0.42
GO:0051234~establishment of localization	1.59	0.46
GO:0051179~localization	1.48	0.47
GO:0042992~negative regulation of transcription factor import into nucleus	22.21	0.45
GO:0071702~organic substance transport	1.88	0.46
GO:0006810~transport	1.58	0.45
GO:0046822~regulation of nucleocytoplasmic transport	5.50	0.50
GO:1902580~single-organism cellular localization	2.55	0.54
GO:0034613~cellular protein localization	2.12	0.57
GO:0051051~negative regulation of transport	3.35	0.55
GO:0070727~cellular macromolecule localization	2.10	0.56
GO:0072594~establishment of protein localization to organelle	3.16	0.54
GO:0042308~negative regulation of protein import into nucleus	12.42	0.56

GO:1904590~negative regulation of protein import	12.42	0.56
GO:0006913~nucleocytoplasmic transport	3.61	0.56
GO:0008104~protein localization	1.75	0.57
GO:0051169~nuclear transport	3.58	0.57
GO:0006606~protein import into nucleus	4.38	0.58
GO:1902593~single-organism nuclear import	4.38	0.58
GO:0044744~protein targeting to nucleus	4.38	0.58
GO:0051170~nuclear import	4.35	0.57
GO:1902582~single-organism intracellular transport	2.92	0.59
GO:0046823~negative regulation of nucleocytoplasmic transport	10.62	0.59
GO:1900181~negative regulation of protein localization to nucleus	10.32	0.59
GO:0006605~protein targeting	2.85	0.58
GO:0042306~regulation of protein import into nucleus	5.55	0.58
GO:1904589~regulation of protein import	5.43	0.59
GO:0051223~regulation of protein transport	2.49	0.60
GO:0045184~establishment of protein localization	1.81	0.59
GO:0033036~macromolecule localization	1.63	0.59
GO:0051641~cellular localization	1.69	0.59
GO:0015031~protein transport	1.83	0.60
GO:0032880~regulation of protein localization	2.20	0.61
GO:0090317~negative regulation of intracellular protein transport	8.33	0.61
GO:0070201~regulation of establishment of protein localization	2.33	0.62
GO:0034504~protein localization to nucleus	3.33	0.63
GO:1900180~regulation of protein localization to nucleus	4.38	0.63
GO:0051224~negative regulation of protein transport	4.32	0.63
GO:0006886~intracellular protein transport	2.18	0.64
GO:0032387~negative regulation of intracellular transport	6.79	0.64
GO:1904950~negative regulation of establishment of protein localization	4.12	0.64
GO:0046907~intracellular transport	1.87	0.67
GO:0032879~regulation of localization	1.55	0.67
GO:1903828~negative regulation of cellular protein localization	5.27	0.72
GO:1902532~negative regulation of intracellular signal transduction	2.66	0.73
GO:1903533~regulation of protein targeting	3.27	0.73
GO:0032386~regulation of intracellular transport	2.55	0.75
GO:0051649~establishment of localization in cell	1.56	0.78
GO:1903827~regulation of cellular protein localization	2.20	0.82
GO:0033157~regulation of intracellular protein transport	2.62	0.82
GO:0051049~regulation of transport	1.47	0.83
GO:0060341~regulation of cellular localization	1.70	0.89
GO:0042981~regulation of apoptotic process	1.15	0.99
GO:0043067~regulation of programmed cell death	1.14	0.99
GO:0097190~apoptotic signaling pathway	1.26	1.00

Annotation Cluster 4	Enrichment Score: 1.30	
Term	Fold Enrichment	Benjamini
GO:0015849~organic acid transport	10.98	0.42
GO:1905039~carboxylic acid transmembrane transport	19.29	0.45
GO:1903825~organic acid transmembrane transport	19.29	0.45
GO:0006820~anion transport	3.47	0.58
GO:0015711~organic anion transport	4.13	0.59
GO:0098656~anion transmembrane transport	7.64	0.62
GO:0006865~amino acid transport	7.12	0.63
GO:0046942~carboxylic acid transport	3.91	0.67
GO:0006811~ion transport	1.62	0.81
GO:0034220~ion transmembrane transport	1.78	0.91
GO:0071705~nitrogen compound transport	1.40	0.98

Supplementary Table 4.1. Putative sperm miR target genes in the oocyte and/or zygote

Stress miRs	Random miRs
Dnmt3a	Pex13
Ubn1	Epha4
Arf4	Iqgap2
Pnn	Ralbp1
Ptpn21	Ptpn7
Bnip3l	Cnn3
Vapa	Ptp4a2
Ptpn13	Cd81
Eed	Svil
Sfrs7	Mkln1
Rap1b	Hivep1
Slc30a4	Slc7a2
Becn1	Ube2a
Cdh2	Yes1
Ilf2	Rcn2
Tomm70a	Ier3
Trip12	Rb1
Atf2	Slc6a6
Gtf2h1	Pik3r1
Jak1	Gtf2h1
Mest	Ube3a
Snx4	Irf1
Nasp	Usp1
Ppm1d	Ptprr
Wasl	Ppm1b
Fmr1	Gtf2b
Atrx	Ubqln2
Btg2	Tagln2
Evi5	Timm10
Ptpro	Api5
Map2k4	Golph3
Itga6	Nrf1
Ptpre	Elf2
Prkar1a	Fdft1
Slc16a2	Mcm5
Pik3r1	Pcyox1
Cdk9	Med28
Traf4	Stx17
Nfe2l1	Nln

Large	Cadps2
Papolg	Abcc1
Gpt2	Gnb4
Capza1	Slc7a11
Rab38	Tnfaip8
Xpr1	Mpp5
Ttc13	Rbm9
Ndel1	Tjp2
Lrrfip2	Arhgef11
Usp47	Ubap2
Pitpnm2	Qtrtd1
Ube2g1	Ctdsp1
Fbxo42	Wdr32
Dlgap4	Scamp5
Tnfaip8	Rybp
Ripk1	Mllt3
Rnf122	Topbp1
Mkrn1	Mpzl1
Dicer1	Sfrs1
Ap1s3	Jag1
Mrps25	Tnpo1
Dyrk1a	Lamc1
Farp1	Tollip
Dpp10	Dffb
Mllt3	Slc4a7
Dnajc14	2810407c02rik
Dusp19	Il1rap
Fbxl19	C1galt1c1
Spop	Hectd2
Ss18l1	1110067d22rik
Nr6a1	D12ertd551e
Elf2	Anp32e
Osbp	Dclre1a
Abcb6	Slc30a5
Wdr32	Ccnt2
Spna2	Pkd2
Rnf12	Depdc1a
Pigc	Mlstd2
Per3	-
Srpr	-
Myo1b	-
Dock5	-
Stx17	-
Rbpms2	-
Tob1	-
Plekhm1	-

Yap1	-
Hipk2	-
Slc6a7	-
Myo5b	-
Dnajb1	-
Zfp687	-
Zswim4	-
Srf	-
Mbtps1	-
B4galt2	-
Slc4a7	-
Atad2	-
Rev3l	-
Stag2	-
Ptgfrn	-
Slc41a2	-
Ccnt2	-
Ddx19b	-
Pdcl	-
Unc5c	-
Acvr1	-
Slc12a6	-
Col9a3	-
Drctnnb1a	-
Ssr3	-
Tmem2	-
Epha7	-
Elovl5	-
Rhot1	-
2810485i05rik	-
Kctd5	-
Il1rap	-
Cdca4	-
Dbt	-
Lamc1	-
Kif5b	-
Cog3	-
Rsbn1	-
Unc84a	-
Slc12a5	-
Gpm6a	-

Supplementary Table 4.2. Summary of embryo and litter statistics

Injected litters	PBS	Stress miRs	Random miRs	ANOVA	*P*-value
litter *N*	5	5	5	-	-
zygotes per uterine horn	5.2 ± 2.9	4.6 ± 2.9	5.4 ± 2.5	$F_{2, 12} = 0.11$	0.89
implantation (%)	53.3 ± 17.0	50.1 ± 20.5	59.8 ± 31.2	$F_{2, 12} = 0.23$	0.79
male (%)	41.0 ± 10.2	47.2 ± 15.0	47.6 ± 33.0	$F_{2, 12} = 0.13$	0.87

CHAPTER FIVE

GENERAL DISCUSSION

Stress, encompassing various environmental challenges that disrupt homeostasis, is a well-established risk factor for neuropsychiatric disorders across the lifespan (de Kloet, 2005; McEwen, 2000). With recent studies, such as those in this dissertation, we now understand that stress occurring during parental preconception windows can also influence disease risk in subsequent generations (Lane, 2014). Parental germ cells (spermatozoa and oocytes) that receive environmental inputs across the lifetime can respond to these cues, resulting in changes in epigenetic marks that are disseminated at fertilization. In particular, evidence from numerous rodent models now demonstrate that paternal lifetime exposures to a variety of stressors are signaled to sperm to influence offspring behavior, physiology, and disease risk. In our established mouse model, paternal stress exposure alters sperm miR content to disrupt offspring HPA stress axis responsivity (Rodgers, 2013), an underlying feature of many neuropsychiatric disorders such as major depression, post-traumatic stress disorder, schizophrenia, and autism (Arborelius et al., 1999; Corbett et al., 2014; Nestler et al., 2002; Walker et al., 2008; Yehuda, 2009). While HPA axis dysregulation alone is not indicative of disease, cumulative aberrant responses to lifetime stressors can precipitate disease onset or exacerbate existing symptoms, contributing to the extensive multifactorial etiology of neuropsychiatric disorders (Lupien et al., 2009; Russo et al., 2012). Thus, expanding on the ongoing examination of gene x environment interactions that underlie disease

vulnerability, mechanisms by which paternal stress experiences are transmitted to influence offspring outcomes should be considered. Currently, there are two major gaps in understanding intergenerational transmission of paternal stress: 1) the soma-to-germline mechanism whereby the reproductive tract senses perturbations in the paternal environment to alter sperm content, and 2) how sperm epigenetic marks subsequently shift the trajectory of offspring development to reflect the paternal environment.

In order to answer these questions, we used our established paternal stress mouse model, exploiting the known relationship between a paternal exposure, resultant offspring phenotype, and specific sperm miRs that can be manipulated in the zygote (Rodgers, 2013; Rodgers, 2015). First, we hypothesized that glucocorticoids, a major component of stress signaling following activation of the HPA axis, were involved in communicating with the male reproductive tract to alter sperm miRs following chronic stress exposure. However, critical to testing this hypothesis, not all mouse lines used in rodent research produce the same levels of glucocorticoids in response to stress (Shanks, 1990), making some mouse strains more susceptible to the effects of glucocorticoids in experimental stress paradigms and therefore, the choice of mouse strain a critical factor in experimental reproducibility (Anisman, 2005; Benedetti et al., 2012). In Chapter 2 of this dissertation, we developed an approach to test the stress susceptibility and reactivity of several mouse strains. We quantified and compared the extent of HPA axis activation in response to stress between mouse strains in order to standardize the level of glucocorticoids released into circulation, as this indicates the amount available for cellular programming in our model. Though the use of mouse strains based on stress susceptibility is not a novel

concept, our goal was to identify a simple and quantifiable method to ensure all experimental mice in our model shared similar stress reactivity, glucocorticoid levels, and, thus, germ cell and offspring programming. However, the utility of this approach is more generalizable in the field of stress research, especially when incorporating new transgenic mouse lines that arrive on stress resistant backgrounds. Using the HPA stress axis as a readout, these new lines can be backcrossed until exhibiting appropriate stress reactivity for experimental conditions.

Having this method in our arsenal, we next investigated the role of glucocorticoids in programming somatic cells of the reproductive tract, presented in Chapter 3 of this dissertation. We examined the role of the epididymis, where the involvement of extracellular vesicles (EVs) produced from caput epididymal epithelial cells (termed 'epididymosomes') was recently suggested to deliver miRs to maturing sperm (Belleannée, 2013; Reilly, 2016). Using both our mouse model and cultured DC2 caput epididymal epithelial cells, we showed that chronic stress *in vivo* and glucocorticoid administration *in vitro* resulted in long-term increases in glucocorticoid receptor (GR) levels and reprogramming of the epididymal histone code, consistent with previous research regarding the capacity for glucocorticoids to remodel chromatin via GR (John, 2008). Further, we demonstrated that glucocorticoid treatment alone could produce similar changes in the miR content of EVs secreted from DC2 cells as we saw in sperm following chronic stress. This study provided a central finding as, despite the recent surge in EV research as a novel mode of intercellular communication within numerous bodily systems (Raposo, 2013; Tetta, 2013), scarce studies to date have examined the impact of

glucocorticoids or even chronic stress on EV content/function. However, the most striking finding of this dissertation lie in the rescue of paternal stress transmission of offspring HPA axis dysregulation using a combination of three transgenic mouse lines to block stress-induced GR increases specifically within the paternal caput epididymal epithelial cells, showing a molecule within a somatic cell that signaled germ cell reprogramming in response to the paternal environment. Moreover, each data point had different effects depending on time collected post-stress (acute vs. enduring), suggesting that it is not only the type and length of paternal exposure, but also processing time post-treatment that impacts germ cell programming. These data demonstrated a cellular pathway whereby stress resulted in long-term increases in caput epididymal GR expression, remodeling the epididymal histone code to produce enduring changes in EV miR content that fuse with sperm, integrating what is known within the fields of stress, EV, epigenetic, and andrology research to produce a unique mechanism of paternal transmission.

Understanding how paternal stress is communicated from the caput epididymis to alter sperm miRs, we next questioned how these changes in sperm miRs influenced offspring development. We hypothesized that sperm miRs repress maternal mRNAs in the zygote immediately post-fertilization, initiating a cascade of transcriptional and molecular events that guide neurodevelopment towards a paternal stress phenotype (Rodgers, 2015). With the ability to microinject specific sperm miRs altered by paternal stress into a naïve, fertilized zygote, we examined the downstream effects of these miRs on neurodevelopment. Multiple research groups interested in paternal transmission have

implicated different populations of sperm miRs (Fullston et al., 2016; Gapp, 2014; Rompala, 2018), prompting our examination of the specificity of sperm miRs altered by paternal stress. Previous unpublished work in the lab examined the expression of our nine paternal stress miRs in intergenerational transmission models of chronic cocaine administration (Vassoler, 2012) and odor-paired fear conditioning (Dias, 2014) where they were not altered in sperm, suggesting that the paternal exposure induces expression of a distinct population of sperm miRs. In Chapter 4, we expanded on these results with the addition of a Random miR group, including nine sperm miRs that were present but unchanged by stress exposure. We examined the effects of the Stress vs. Random miR groups on the embryonic brain and placenta, two tissues previously described with the capabilities to disrupt development of the HPA stress axis (Bronson et al., 2016; Kapoor et al., 2006). In this study, we found that these two populations of miRs had distinct targets during development, where our paternal stress miRs resulted in broad transcriptional changes in the developing brain, compared to practically no differences in the Random miRs group. In comparison, both groups disrupted the placental transcriptome, showing both the specificity of sperm miR composites and their tissue targeting. Moreover, both embryonic whole brains and paternal stress PVN showed chromatin remodeling, demonstrating that sperm miRs influence epigenetic regulators to promote lasting downstream effects. The changes to these histone PTMs may lie upstream of the transcriptional dysregulation that promotes a stress dysregulation phenotype; however, technical limitations regarding the size of the PVN restricts our ability to make these associations for now. This study was important in adding

developmental time points to our model, confirming our previous conclusions that paternal stress sperm miRs impact neurodevelopment by functioning during a sensitive window to initiate a cascade of events, rather than being maintained in expression until adulthood.

Together, these studies on the mechanisms of paternal stress transmission received an R37 merit award from the National Institute for Mental Health, providing ten years of funding and showing the substantial interest in advancing this field of research. This interest spans multiple disciplines, as researchers in the fields of metabolism, drug abuse, epigenetics, and fertility have built considerable evidence supporting intergenerational transmission through the paternal lineage. Thus, the following discussion will address the implications of this work, including future directions and potential translational approaches.

Does paternal stress transmission equate to disease transmission?

Neuropsychiatric disorders are multifactorial in etiology, where some factors can produce disease in a subset of individuals and not others. For example, while most individuals experience chronic psychological stress across the lifetime, the incidence of disorders such as schizophrenia and autism spectrum disorders remains at 30% of the general population (Blaxill, 2004; Chen et al., 2007; Elsabbagh et al., 2012; Simonoff et al., 2008; Weintraub, 2011). Additionally, hyporeactivity of the HPA stress axis is observed in many, but not all, patients with post-traumatic stress disorder (Meewisse, 2007; Sherin, 2011). In a similar manner, paternal preconception stress may transmit

endophenotypes of risk (i.e. HPA axis dysregulation) that alone do not equate to disease, but can prime an individual if accumulated with other risk factors. This idea is known as the two- or multiple-hit hypothesis, originally proposed in the context of mutations contributing to cancer (Knudson, 1971), but now applied more broadly to neuropathology (Gershon et al., 2011; Giovanoli et al., 2013; Maynard et al., 2001). This hypothesis generally considers the interaction of gene x environment, where a genetic vulnerability compounded by trauma may precipitate disease. We can apply our results from Chapters 2 and 3 of this dissertation, where a mouse genetic background underlying a robust physiological response to stress (C57BL/6:129 mixed F1) was required in order to produce paternal stress effects. As previously noted, the use of a pure stress-resistant C57BL/6J strain for this model was insufficient to produce offspring HPA axis reprogramming (Rompala, 2018), suggesting our model actually incorporates three sequential hits - requiring a 1) genetic vulnerability for 2) paternal stress effects to transmit offspring 3) HPA axis dysregulation.

With this hypothesis in mind, what might happen to naïve paternal stress offspring that show no other cognitive or behavioral impairments but are downstream of three "hits", if administered stress during the lifetime? We expect that additional stress may precipitate other disease endophenotypes, such as depression-like behaviors or social impairments on behavioral tests, though these experiments have not yet been conducted. Interestingly and relevant to other studies in our lab, stress during *in utero* development may also compound paternal stress effects. In other words, can maternal gestational stress act as an additional hit? As disruption of placental development and signaling is a major

component of maternal stress effects, our data from Chapter 4 of this dissertation, where zygote microinjection of sperm miRs disrupted the placental transcriptome, suggest this is an intriguing possibility. Indeed, human studies suggest that the combined impact of paternal and maternal trauma increases offspring risk of anxiety, depressive, and post-traumatic stress disorders (Lehrner, 2014; Yehuda, 2008a, 2014). However, there have been limited studies examining the interaction of paternal and maternal stress effects on offspring risk.

Despite our discussion thus far that paternal preconception stress can promote disease risk, in reality this is an oversimplified interpretation of the role of paternal transmission. Evolutionarily, germ cell programming may reflect the inheritance of phenotypes that, in fact, are advantageous. In our model, transmission of altered HPA axis reactivity may better prepare offspring for a stressful environment based on paternal experiences. Furthermore, unpublished data from our lab suggest that paternal stress offspring exposed to caloric restriction in adulthood lose less weight than control offspring, suggesting they may be more metabolically equipped for the potential of famine in their lifetime. Certainly, these conclusions must be made with caution, though other paternal transmission studies have similar findings (Benito et al., 2018; Gapp, et al., 2014). For example, in a study of paternal chronic cocaine exposure, cocaine-sired offspring self-administered less cocaine than control offspring, suggesting inheritance of a resistance phenotype (Vassoler, 2012). Therefore, paternal preconception exposures may transmit risk or resilience to disease dependent on the offspring environment.

Are miR delivery mechanisms a broad mode of stress programming?

MiRs are an enticing regulatory mechanism given their essential role in the development and function of all tissues, responsiveness to external triggers, and ability to repress numerous transcripts (Cai et al., 2009; Leung et al., 2010; Morgan et al., 2012). Many miRs acting on genes post-transcriptionally in a cell are themselves transcribed in the nucleus and processed into the cytoplasm. Recent descriptions of exogenous or extracellular miRs being delivered to and functioning within cells provide an exciting mechanism for intercellular and inter-tissue communication (da Silveira, 2018; Tetta, 2013). As we have described, EVs delivering a payload of miRs, as well as proteins, lipids and other RNA populations, from one cell to another to regulate various processes, including neural and glial communication (Lafourcade et al., 2016; Morel et al., 2013), immune function (Valadi et al., 2007), and sperm maturation (Reilly, 2016; Sullivan, 2007). Subsequently, we now know that sperm can deliver miRs to the oocyte (Ostermeier et al., 2004), where they act to impact offspring development. Given the recent literature and the studies included in this dissertation, the intercellular transport of miRs provide an intriguing mechanism whereby tissues responsive to stress signals can communicate with each other or relay those signals to less responsive or unexposed tissues.

The influential role of miRs delivered to other cells is vast, where one sperm carrying <10 fg of miRs to a stress-naïve oocyte containing about 1 ng RNA (a ratio of $1{:}10^5$), can shift maternal mRNA control of zygote development to incorporate paternal signals in offspring phenotypes (Boerke, 2007; Krawetz, 2005). However, skeptics of

sperm miR-mediated transmission question the raw numbers of miRs actually carried in by sperm, and whether microinjection experiments reflect these numbers. To test this question, the combination of small RNA standards must be incorporated with single cell sequencing of one sperm cell, which has not yet been accomplished. Moreover, as we now understand that epididymal epithelial cells produce EV populations containing miR profiles reflective of sperm content, some of these epididymal EVs do not fuse with sperm but are instead incorporated into the seminal fluid along with EVs from the seminal vesicle glands and prostate (Aalberts et al., 2013; Belleannée, 2015; Machtinger, 2016; Sullivan, 2013). When ejaculated into the female reproductive tract, these populations of EVs can interact with female cells or further with spermatozoa (Aalberts, 2013; Robertson et al., 2013, 2016). Thus, it is possible the epididymal EVs can fuse further with or get "stuck" on sperm cells following ejaculation, bringing further RNA cargo to the oocyte. Indeed, sperm that are stringently washed of seminal factors prior to methods of in vitro fertilization (IVF), such as intracytoplasmic sperm injection (ICSI), for use in fertility clinics or transgenic mouse cores often produce lower birth rates or changes in offspring outcomes (Anthony, 2002; Cox et al., 2002; Ecker et al., 2004; Fernandez-Gonzalez et al., 2004; Giritharan et al., 2007; Rybouchkin et al., 1995; Yoshida et al., 2007). This has been previously attributed to many potential factors (Odom et al., 2010; Rinaudo et al., 2004; Rinaudo et al., 2006; Stouder et al., 2009), including the protein content of seminal fluid factors, such as prostasomes through their modulation of female immune cells and capacitation of sperm (Aalberts, 2013; Bromfield et al., 2014; Robertson, 2016). However, seminal fluid EV miRs, amongst other EV

components such as lipids or tRFs, may be poised to act on female reproductive and immune cells and sperm post-coitus as well, as has been described in the vagina (Madison et al., 2015; Vojtech et al., 2014). Ongoing studies examining this possibility for seminal fluid EV miRs to influence the pre-implantation microenvironment post-ejaculation should be conducted as an additional mode of paternal to offspring communication of stress signals.

Given the capacity for EV miRs to relay stress signals between somatic and germ cells within one reproductive system or between paternal and maternal cells in the female genital tract, might EV miRs also communicate between somatic tissues within one individual? Recent evidence suggests this inter-system regulatory possibility, where EV miRs can communicate axon injury between sensory neurons and macrophages (Simeoli et al., 2017) and glucose regulation between adipose tissue and the liver (Thomou et al., 2017). Our data presented in Chapter 3 (**Fig. 3.1f,g**) and collected from ongoing studies add to this growing, complex network of intercellular conversation. Injection followed by imaging of dye-labeled EVs collected from a pure population of cultured caput epididymal epithelial cells into the bloodstream of male mice revealed selective EV accumulation in the caput epididymis, testes, and brain. Given the physical connection between the caput epididymis and testes via the efferent ducts (Cornwall, 2009), such communication within the male reproductive tract is unsurprising, though the function of epididymal EVs traveling upstream to the testes has not been tested. Remarkably, that epididymal EVs may localize to the brain suggest an exciting mechanism whereby reproductive tissues can convey information centrally. Moreover, as we know that

glucocorticoid treatment alters epididymal EV miR content, and likely other components as well, can these EV miRs regulate gene expression in the brain and potentially influence behavior or physiology? These questions are of course not limited to the epididymis, as all cells in the body are implicated to secrete EVs (Raposo, 2013; Tetta, 2013) and comprise GR (Oakley et al., 2013). Thus, the role of cellular EVs in delivering stress signals, perhaps in the form of miRs, to other cells in the body is an exciting potential mode of stress programming of both the individual exposed to stress and subsequent offspring.

What is the translational potential of paternal transmission research?

The majority of studies supporting paternal transmission and sperm miRs thus far are derived primarily from mouse models. As sperm is an easily obtainable biological material in humans, the potential use of sperm miRs as biomarkers of prior stress exposure or disease risk is an exciting prospect. However, evidence in human populations that sperm miRs reflect paternal exposures and contribute to offspring outcomes is limited. The principal epidemiological studies underlying paternal transmission were retrospective examinations, restricting those researchers from collecting tissue. More recently, prospective studies have enabled recruitment of men and collection of their semen for RNA analysis. For example, a study comparing smokers to non-smokers observed altered miR profiles in sperm (Marczylo et al., 2012), suggesting a potential link for previous retrospective observations that paternal smoking influenced offspring health (Deng, 2013; Ji, 1997; Pembrey, et al., 2006). The ability to classify specific miR

profiles in sperm as indicative of paternal exposures or offspring endophenotypes would be immensely useful in the development of predictive disease biomarkers. The potential to identify at-risk individuals may then inform clinical decisions, including altering prenatal care and earlier interventions for children. However, there are still many questions that require investigation in human cohorts.

While mouse models of paternal transmission have identified several populations of sperm miRs that, through proof of concept experiments, are involved in programming offspring outcomes, whether these miRs have similar functions in humans is not known. For example, one of our paternal stress sperm miRs, miR-204-5p, is transcribed from chromosome 19 in mice but chromosome 9 in humans despite sharing sequence homology, as determined by miRBase (Griffiths-Jones et al., 2008), suggesting distinct mechanisms of miR regulation between humans and mice. Further, whether stress-responsive sperm miRs across species converge to act on the same biological pathways in the zygote has not been examined. Therefore, the identity of sperm miRs that respond to paternal exposures in humans needs to be specifically surveyed. Adding to the intricacy of sperm miR regulation in humans is timing from the initial exposure. Our mouse data suggests that the timing of post-stress processing, appended to the effects of stress duration, contributes to sperm miR programming. Given these dynamic changes in a controlled laboratory setting, it is likely that the profile of sperm miRs responsive to the environment also fluctuates over time in humans. Studies that repeatedly sample sperm at multiple time points following stress exposure could address and outline this dynamic regulation of sperm miRs in men. Another complication regarding the timing of sperm

regulation is the continuously changing environment across the human lifespan, as the impact of cumulative life experiences on sperm miR profiles is not known. It is conceivable that sequential life events can ablate or override the expression of previously responsive miRs, or perhaps accumulate to create a unique population. This can be examined in prospective human cohorts, where at the time of semen collection, questionnaires accounting for stress, diet and other life events can be administered to associate specific sperm miR populations with one or a combination of exposures, and to assess how these miR levels change over time and/or in response to additional triggers.

Concluding remarks

Mechanisms by which the paternal lineage can influence offspring development were historically reduced to the delivery of genomic material by sperm. In this dissertation, we challenged these previous notions by describing three levels by which the paternal germ cell contribution is altered by stress to impact offspring outcomes: 1) at the level of sperm, by delivering stress-sensitive miRs capable of disrupting offspring neurodevelopment, 2) at the level of the paternal epididymis, which can detect stress in the environment and alter sperm content, and 3) at the level of the paternal physiological response to stress, which governs the degree of reproductive tissue programming. Importantly, we have built on the growing intergenerational transmission literature by establishing a soma-to-germline mechanism whereby an environmental trigger is communicated to sperm, demonstrating a fundamental biological process for the regulation and transmission of non-genetic signals at fertilization. Altogether, these

studies provide insight into the factors and processes that shape disease risk and resilience, prompting new consideration for the role of the paternal environment as a key determinant of offspring development.

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