

Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress

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Epigenetic signatures in germ cells, capable of both responding to the parental environment and shaping offspring neurodevelopment, are uniquely positioned to mediate transgenerational outcomes. However, molecular mechanisms by which these marks may communicate experience-dependent information across generations are currently unknown. In our model of chronic paternal stress, we previously identified nine microRNAs (miRs) that were increased in the sperm of stressed sires and associated with reduced hypothalamic–pituitary–adrenal (HPA) stress axis reactivity in offspring. In the current study, we rigorously examine the hypothesis that these sperm miRs function postfertilization to alter offspring stress responsivity and, using zygote microinjection of the nine specific miRs, demonstrated a remarkable recapitulation of the offspring stress dysregulation phenotype. Further, we associated long-term reprogramming of the hypothalamic transcriptome with HPA axis dysfunction, noting a marked decrease in the expression of extracellular matrix and collagen gene sets that may reflect an underlying change in blood–brain barrier permeability. We conclude by investigating the developmental impact of sperm miRs in early zygotes with single-cell amplification technology, identifying the targeted degradation of stored maternal mRNA transcripts including sirtuin 1 and ubiquitin protein ligase E3a, two genes with established function in chromatin remodeling, and this potent regulatory function of miRs postfertilization likely initiates a cascade of molecular events that eventually alters stress reactivity. Overall, these findings demonstrate a clear mechanistic role for sperm miRs in the transgenerational transmission of paternal lifetime experiences.

transgenerational | epigenetic | stress | microRNA | paternal

Evidence that offspring behavior and physiology can be shaped by parental life experiences has stimulated new consideration of the factors that underlie disease risk and resilience. Notably, perturbations such as parental stress, malnutrition, infection, or advanced age have been associated with an increased incidence of neurodevelopmental disease in offspring (1), and alterations in their hypothalamic–pituitary–adrenal (HPA) stress axis response may be central to increased disease predisposition (2, 3). Studies in diverse animal models have demonstrated similar outcomes, particularly that of offspring HPA axis dysregulation, following either maternal or paternal stress exposure (4–7); yet mechanisms by which parental lifetime stress experience modify offspring development and adult phenotypes remain unclear. In particular, transgenerational transmission via the maternal lineage likely relies on the complex maternal–fetal/neonatal interaction, whereas transmission through the paternal lineage suggests germline reprogramming (8).

Germ cell epigenetic marks, vulnerable to environmental stimuli and capable of directing profound developmental change, may mediate the effects of parental lifetime environmental exposures on offspring behavior and physiology (9, 10). Rodent models examining paternal transmission have identified epigenetic signatures in mature sperm as possible substrates of transgenerational programming, namely patterns of retained histone modifications, DNA methylation, and/or populations of small noncoding RNAs (11–21). RNA populations are of primary interest, as they may be altered through intercellular communication via epididymosomes even in transcriptionally

inert mature sperm, where DNA condensation impedes other epigenetic change (22–24). Studies in which manipulation of total RNA content in postfertilization zygotes reproduced aspects of a paternally transmitted phenotype highlight the critical importance of RNA as a germ cell epigenetic mark and support the potential role of small RNAs, including microRNAs (miRs), in trait transmission (20, 25). However, neither the identity of specific sperm miRs responsive to environmental challenge, nor mechanisms by which they may function to impact offspring development, have been determined.

We previously established that the adult offspring of male mice exposed to chronic stress before breeding exhibit a significantly blunted HPA stress axis response and reprogramming of relevant gene sets within the paraventricular nucleus (PVN) of the hypothalamus. In this model, we identified nine specific miRs in the sires' sperm as the potential germ cell mark sensitive to paternal stress experience (11). Here, to confirm sperm miR content as a mechanism of epigenetic transmission, we microinjected the nine miRs into single-cell zygotes (multi-miR injection). Zygotes were then implanted into surrogate females, reared normally, and examined for adult HPA stress axis sensitivity and long-term reprogramming of PVN gene expression as a recapitulation of the paternal stress phenotype (Fig. 1A). Further, to elucidate the currently unknown function of sperm miRs following fertilization, we evaluated the potential targeting of stored maternal RNA in early zygotes.

Results

Recapitulation of Blunted HPA Axis Response. To determine if the multi-miR injection elicited changes in HPA stress axis responsivity similar to that previously observed in our paternal stress model, we examined plasma corticosterone levels following a brief restraint

Significance

Studies examining paternal exposure to diverse environmental stimuli propose that epigenetic marks in germ cells, including small noncoding RNAs such as microRNA (miR), transmit experience-dependent information from parent to offspring. However, these nongenetic mechanisms of transgenerational inheritance are poorly understood, specifically how these germ-cell marks may act postfertilization to enact long-term changes in offspring behavior or physiology. In this study, through zygote microinjection of nine specific sperm miRs previously identified in our paternal stress mouse model, we demonstrate that sperm miRs function to reduce maternal mRNA stores in early zygotes, ultimately reprogramming gene expression in the offspring hypothalamus and recapitulating the offspring stress dysregulation phenotype.

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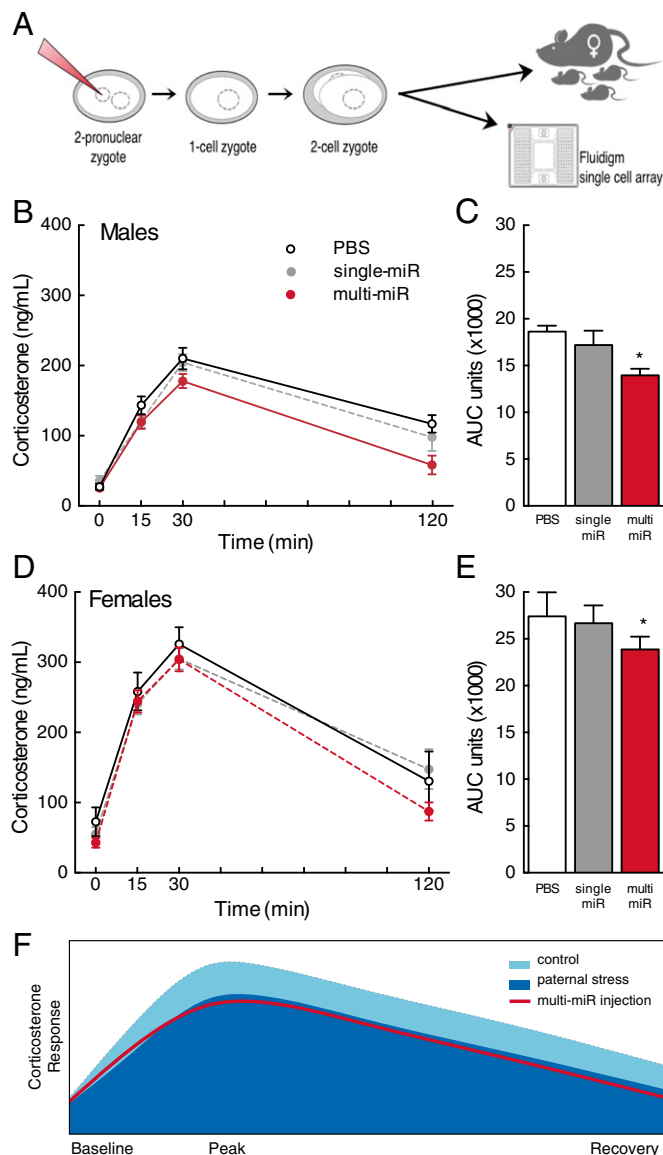


Fig. 1. Zygote microinjection of nine sperm miRs recapitulated the HPA axis phenotype. (A) Schematic of experimental design in which single-cell mouse zygotes were injected with nine miRs (multi-miR; 1 ng/ μ L final concentration of miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, and miR-698) or with one of two control conditions: an injection control (PBS only) or a control for the total amount of injected miRs (single miR; 1 ng/ μ L final concentration miR-193-5p alone) before implantation into surrogate females or preparation for later analyses. (B and D) Corticosterone levels in male and female adult mice were significantly reduced in the multi-miR group relative to PBS controls in response to a 15-min restraint from $t = 0$ –15 min. (C and E) Total AUC of corticosterone production over 120 min was significantly decreased in the multi-miR group compared with PBS controls, and expected sex differences were observed. Data are presented as mean \pm SEM $n = 6$ –10 mice per group. * $P < 0.05$ multi-miR vs. PBS. (F) Model demonstrating the similarity in the shape of the male corticosterone response curve in the multi-miR-injected group with the outcome previously reported in offspring following paternal stress (11).

stressor in adult male and female mice derived from injected zygotes. To ensure that observed outcomes resulted from the action of the nine miRs (multi-miR injection: miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, and miR-698) independent of experimental techniques and the potential effect of concentration, control conditions included both a PBS injection and a single-miR injection using miR-193-5p

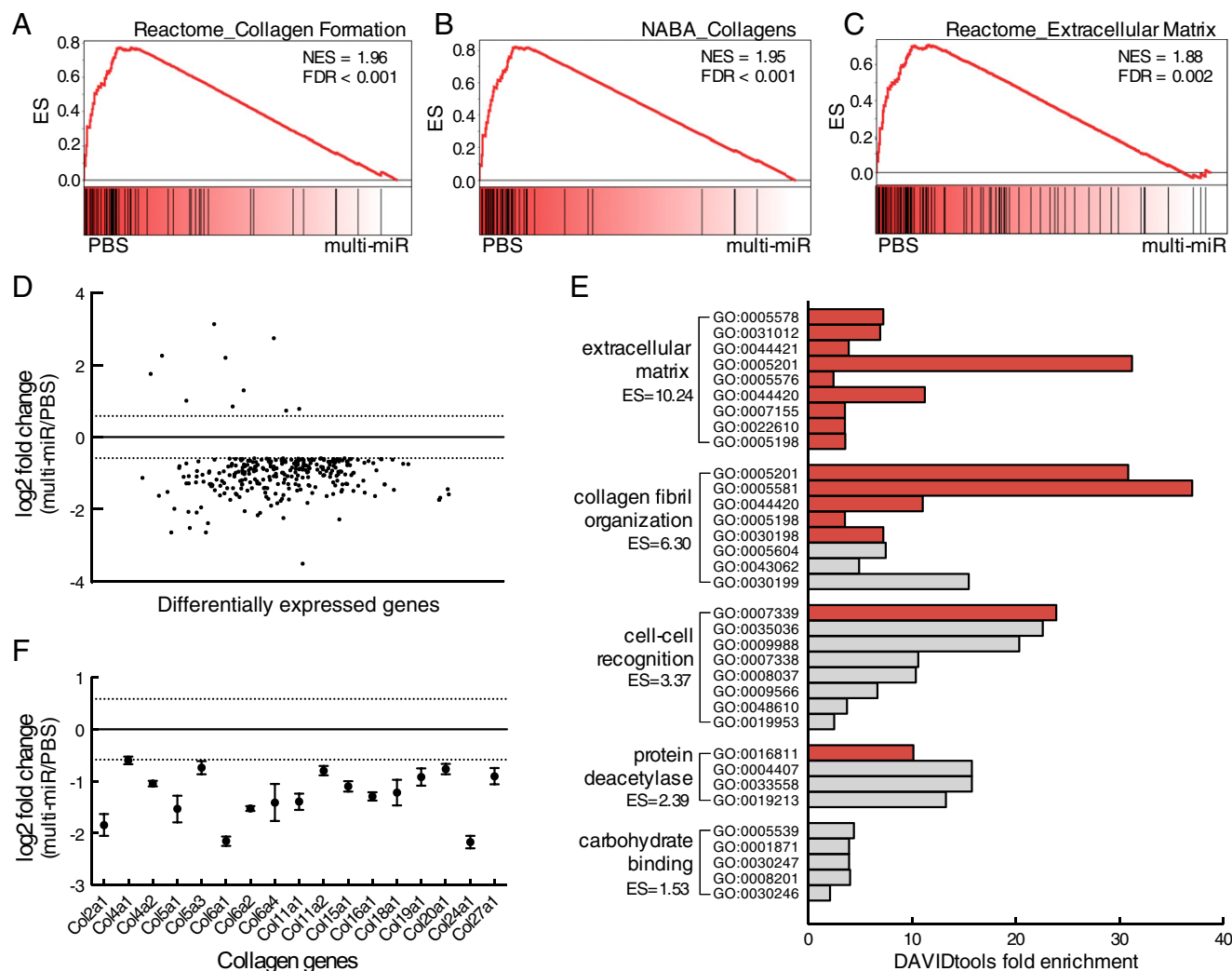
(randomly selected from the nine used in the composite) at the same total miR concentration. Corticosterone response was significantly reduced by miR injection [treatment ($F_{2,43} = 3.32$, $P = 0.046$)] and this effect did not vary across time (no interaction, Wilks' lambda = 0.83, $F_{6,82} = 1.31$, $P = 0.26$), (Fig. 1 B and D). Expected changes in corticosterone levels across time were observed ($F_{3,41} = 32.03$, $P < 0.0001$), as well as expected basal sex differences in corticosterone ($F_{1,43} = 63.82$, $P < 0.0001$). The main effect of sex significantly interacted with time ($F_{3,41} = 33.51$, $P < 0.0001$), but not treatment condition (no interaction, $F_{2,43} = 0.005$, $P = 0.99$). There was no interaction of time, sex, and treatment (Wilks' lambda = 0.90, $F_{6,82} = 0.73$, $P = 0.62$). Assessment of total corticosterone production in 120 min, measured as area under the curve (AUC), confirmed the significant and noninteracting effects of zygote injection and sex [treatment ($F_{2,37} = 3.44$, $P = 0.043$); sex ($F_{1,37} = 45.02$, $P < 0.0001$); and interaction ($F_{2,37} = 0.33$, $P = 0.72$)]. Total corticosterone was reduced in the multi-miR-injected group compared with PBS ($P < 0.05$) (Fig. 1 C and E). Animals were otherwise normal in weight and appearance (Table S1).

Programmatic Changes in PVN Transcriptome in Response to Zygote miR Injection. Gene expression patterns in the adult PVN were

miR Injection. Gene expression patterns in the adult PVN were analyzed using RNA sequencing (RNA-seq) to investigate long-term reprogramming of the HPA axis. Gene set enrichment analysis (GSEA) of the PVN transcriptome in PBS vs. multi-miR groups revealed the robust enrichment of curated gene sets for collagen formation and extracellular matrix organization in the PBS group (26, 27), indicating that the expression of member genes was decreased in the multi-miR-injected group (Fig. 2A–C). No gene sets were significantly increased in the multi-miR group (Table S2). Differential gene expression analysis using stringent threshold and significance criteria of $P < 0.05$, false discovery rate (FDR) < 0.05 , and \log_2 fold change $\geq |0.585|$ identified 298 genes with altered expression in the PVN following correction for multiple comparisons (Table S3), and the majority of these genes (288 of 298) exhibited decreased expression (Fig. 2D). Gene ontology (GO) analysis of the 298 dysregulated PVN genes using DAVID tools functional annotation clustering similarly identified an enrichment of collagen and extracellular matrix-related terms (28, 29), driven in part by the decreased expression of 17 collagen genes (Fig. 2E and F). Notably, expression of the nine injected miRs was unchanged in the adult PVN of the multi-miR group (Fig. S1).

Further, to ensure zygote miR injection did not impact overall development of the HPA axis, gene expression in the PVN, pituitary gland, and adrenal gland was examined. Markers for primary and dominant PVN cell populations were unchanged in the multi-miR injected group [arginine vasopressin (AVP), $t(10) = 0.19, P = 0.85$; oxytocin (OT), $t(10) = 0.00027, P = 0.99$]; corticotropin releasing factor (CRF), $t(10) = 0.48, P = 0.64$]; integrin alpha M (CD11b) $t(10) = 0.49, P = 0.64$]; glial fibrillary acidic protein (GFAP), $t(10) = 0.50, P = 0.62$]] (Fig. 3.4). Stress-regulatory genes in peripheral sites of the HPA axis were also unchanged in the multi-miR-injected group [pituitary corticotropin releasing factor receptor 1 (Crfr1), treatment ($F_{1,24} = 0.0006, P = 0.98$) by sex ($F_{1,24} = 4.57, P = 0.043$) with no interaction ($F_{1,24} = 0.13, P = 0.72$); pituitary proopiomelanocortin (POMC), treatment ($F_{1,26} = 0.0017, P = 0.97$) by sex ($F_{1,26} = 2.73, P = 0.11$) with no interaction ($F_{1,26} = 0.060, P = 0.81$); adrenal melanocortin receptor 2 (Mc2r), treatment ($F_{1,26} = 0.098, P = 0.76$) by sex ($F_{1,26} = 33.53, P < 0.0001$) with interaction ($F_{1,26} = 5.43, P = 0.023$) driven by an effect of treatment in males [$t(14) = 2.46, P = 0.028$], but not in females [$t(13) = 1.47, P = 0.16$]; adrenal corticosteroid 11 β -dehydrogenase (11 β HSD-1), treatment ($F_{1,27} = 0.013, P = 0.91$) by sex ($F_{1,27} = 3.69, P = 0.065$) with no interaction ($F_{1,27} = 0.78, P = 0.38$)] (Fig. 3 B–E).

Reduction of Stored Maternal mRNA by Sperm miRs. To investigate the potential function of sperm miRs postfertilization, we directly assessed sperm miR targeting of stored maternal mRNAs, using single-cell amplification technology to examine gene expression levels in miR-injected zygotes 24 h postinjection (Fig. 1A).



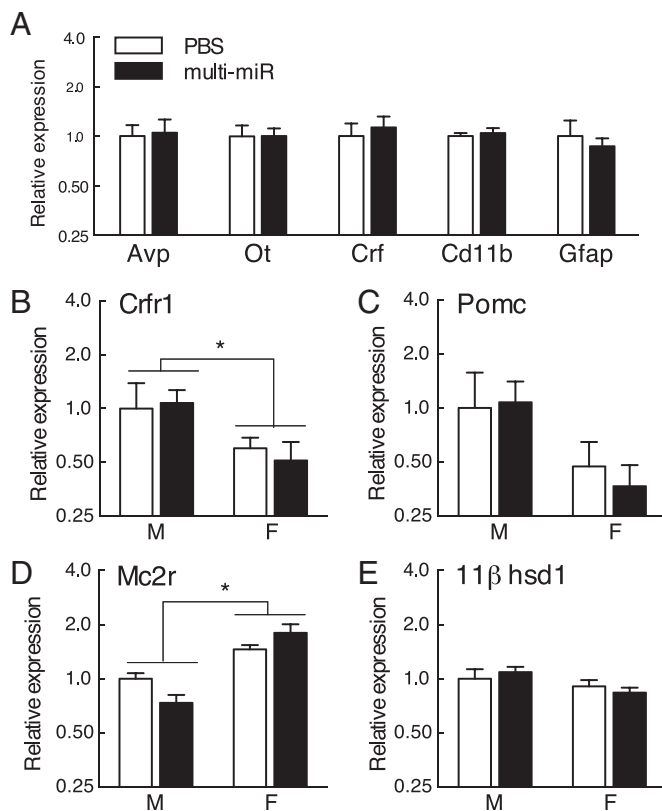


Fig. 3. No differences in adult expression of stress-regulatory genes in the HPA axis. (A) Markers of major neuronal and nonneuronal cell populations in the PVN were assessed by qRT-PCR and were unchanged in the multi-miR-injected group: Avp, arginine vasopressin; Ot, oxytocin; Crf, corticotropin releasing factor; Cd11b, integrin alpha M; Gfap, glial fibrillary acidic protein. $n = 6$ mice per group. (B–E) Stress-axis related gene expression in the pituitary and adrenal glands was not significantly different between PBS and multi-miR-injected animals. No effect was observed for pituitary Crfr1, pituitary Pomc, adrenal Mc2r, or adrenal 11 β hsd-1. Data presented as mean \pm SEM, $n = 6$ –9 mice per group. * $P < 0.05$.

(RISC) (33). Therefore, control injection conditions included a PBS injection to control for the zygote injection process itself, and a single-miR injection (randomly selected from one of the nine used in the composite) at the same overall concentration to control for miR concentration effects.

Remarkably, and important for our understanding of paternal RNA involvement in postfertilization development, the multi-miR injection produced a phenotype nearly identical to that of our paternal stress model, where both male and female mice from the multi-miR injections mounted a significantly blunted corticosterone response to an acute restraint stress as adults. A striking overlap was observed in the magnitude of effect on corticosterone production and its rate of rise and of recovery between multi-miR-injected animals and our previously reported paternal stress offspring (as shown in the schematic in Fig. 1F). The corticosterone response curve elicited by an acute stressor is multifaceted, with aspects of the curve (e.g., baseline, maximal rise, and rates of rise and recovery) regulated by specific brain regions, including the PVN, thalamus, and hippocampus (34). Thus, parallels in the shape of the corticosterone curves between this study and our paternal stress model emphasize similarities in programming mechanisms elicited by paternal sperm miRs. The single-miR injection did not affect the HPA axis corticosterone levels, suggesting that the specific and combinatorial activity of these sperm miRs alters stress axis responsivity. Certainly, as we did not examine each miR independently or in all possible combinations to determine the minimal complement necessary to produce the effect, future studies will need to examine the contribution of each of the nine

miRs. Additionally, although expected sex differences in corticosterone levels were observed, sex did not interact to impact miR programming of stress responsivity. Further, similar to what we reported following paternal stress exposure, the expression of off-spring stress-regulatory genes in the pituitary (CRF receptor-1 and proopiomelanocortin) and adrenal glands (melanocortin receptor-2 and 11 β HSD-1) were unaffected in the zygote multi-miR-injected mice as adults. However, the interaction of zygote miR injection with sex on adrenal Mc2r expression suggests that miR reprogramming of the HPA axis may involve a convergent sex difference, whereas the endpoint effect on the HPA is ultimately the same (35).

In examination of the long-term changes in neurodevelopmental programming in the multi-miR-injected mice, we completed RNA-seq analysis on micropunches from the adult PVN, the hypothalamic regulator of the HPA stress axis. GSEA of the PVN transcriptome revealed a robust enrichment of extracellular matrix and collagen formation gene sets in control animals, reflecting a decreased expression of these genes in the PVN of the multi-miR-injected group. Functional annotation clustering of differentially expressed genes using DAVID tools confirmed this outcome, where affected genes primarily associated with GO terms for the extracellular matrix and collagen organization. In fact, 17 unique collagen genes were down-regulated in the multi-miR-injected group PVN compared with controls. These findings may reflect deficits in cerebral circulation and/or blood-brain barrier permeability in the PVN, effects that would have significant impact on neuroendocrine function (36, 37). Furthermore, the PVN is one of the most highly vascularized regions in the brain, suggesting that it may be particularly susceptible to developmental changes in circulation or permeability (38). This heightened plasticity of stress homeostatic mechanisms to transgenerational reprogramming could confer a selective advantage or disadvantage depending on environmental conditions.

Additionally, in examination of RNA-seq results, we noted that the majority of differentially expressed genes (288 of 298) in the PVN exhibited a dramatic decrease in expression, similar to what we reported previously in our paternal stress model and supporting the idea that an increase of these specific nine miRs in the zygote in either model (delivered through sperm in response to stress or experimentally by microinjection) can elicit long-term genetic reprogramming (11). As the overall focus of these repressed genes is in the extracellular matrix and collagen organization, this mouse model suggests that hypothalamic reprogramming governs neuroendocrine function in control of the HPA axis. Gross PVN development was not impacted by the zygote injection, as markers of the primary and dominant cell populations within the PVN were examined and did not differ between control and multi-miR-injected groups. Instead, this robust suppression of the transcriptome may suggest regulation by an upstream epigenetic mechanism, such as a histone repressive mark. The programming of such a change at the level of the brain is likely a complex and multifaceted process, whereby miRs initiate a cascade of molecular events at fertilization that, through many steps, impact regulatory mechanisms such as chromatin modifications or DNA methylation to eventually alter neurodevelopment and related behaviors or physiology (9). As we would predict, we found that the expression of the nine specific sperm miRs was unchanged in the adult PVN. Ultimately, stepwise investigation of the complex cascade by which a germ cell mark, such as sperm miRs, can impart changes in the developing offspring is critical to our understanding of the transgenerational transmission of paternal experiences, and must begin with an investigation of its activity immediately following fertilization in the single-cell zygote.

During normal embryogenesis, stores of maternal mRNA initially present in the zygote are selectively degraded post-fertilization, and miR function within the zygote RISC complex is essential to this developmental process (39, 40). As this regulatory role has previously only been attributed to maternally derived miRs present in the oocyte, and as a postfertilization

poly-A enrichment (RS-122-2101) according to the manufacturer's protocol. Six biological replicates per group (12 total cDNA libraries) were multiplexed and sequenced on two identical HiSeq2000 lanes (Illumina).

Single-Cell Amplification. Criteria for selection of candidate genes for analysis in individual zygotes were as follows: present in late-stage MII mouse oocytes^{3a}, in single-cell mouse zygotes^{3b}, and/or homologously in human and mouse mature oocytes⁴ (two or more of three lists) (30, 31) and predicted target of one or more of nine miRNAs in both miRWalk and miRanda algorithms (32). qRT-PCR was conducted with the two-step single-cell protocol with EvaGreen Supermix on the BioMark HD System using DELTAgene assays (Table S4) according to the manufacturer (Fluidigm).

Statistics. Corticosterone levels were analyzed by two-way ANOVA with time as a repeated measure. Corticosterone AUC, body weight and length, and adrenal and pituitary gene expression were analyzed by two-way ANOVAs. Litter statistics were analyzed by one-way ANOVA and PVN gene expression was analyzed by two-tailed *t* tests. Significance was set at $P < 0.05$. When appropriate, a post hoc Fisher's least significant difference or Student's *t* test was used to

explore main effects. Differential gene expression in single-cell analysis was calculated with the Fluidigm Singular toolset 2.0 using $P < 0.05$. The expression pattern of all candidate genes was compared using a χ^2 test. GSEA (v2.2.0, Broad Institute) of c2_CP gene sets from the Molecular Signature Database (MsigDB v5.0, Broad Institute) was run using 1,000 gene_set permutations (26, 27). Differential gene expression analysis of RNA-seq data was conducted by a modified Fisher's exact test with EdgeR (44). Statistical criteria for significance were an adjusted $P < 0.05$, FDR < 0.05 , and log2 fold change $> |0.585|$ (1.5 linear fold change). Functional annotation clustering of all GO annotations was performed with DAVID v6.7, with cluster enrichment score of 1.3, equal to an α of 0.05, considered significant enrichment (28, 29). FDR correction for multiple comparisons was applied to GO annotation enrichment *P* values.

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