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 decreased 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 sir1 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.

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 germ-line 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 epididylosomes 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 micro-RNAs (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 significant 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 sensitivity similar to that previously observed in our paternal stress model, we examined plasma corticosterone levels following a brief restraint
2.46, P = 0.49, (10) 0.83, P = 0.13, 0.99); corticotropin releasing factor (CRF), treatment (F_{1,26} = 3.44, P = 0.043; sex (F_{1,27} = 45.02, P < 0.0001) and interaction (F_{1,27} = 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 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. 2 A–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 log2 fold change ≥ 0.585 identified 298 genes with altered expression in the PVN following correction for multiple comparisons (Table S3), and the majority of these genes (259/298) were down-regulated (28, 29), driven in part by the decreased expression of 17 collagen genes (Fig. 2 E 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; giall fibrillary acidic protein (GFAP), t (10) = 0.50, P = 0.62] (Fig. 3). Stress-regulatory genes in peripheral sites of the HPA axis were also unchanged in the multi-miR–injected group [putty corticotropin releasing factor receptor 1 (Cfr1r), treatment (F_{1,24} = 0.0006, P = 0.95) by sex (F_{1,24} = 4.57, P = 0.043) with no interaction (F_{1,24} = 0.13, P = 0.72); putty proopiomela- nocortin (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} = 0.06, P = 0.81); adrenal melancortin 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 11β-dehydrogenase (11βHSD-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. L4).

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_{0.42} = 1.31, P = 0.26)] (Fig. 1 B and D). Expected changes in corticosterone levels across time were observed (F_{1,41} = 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_{3,43} = 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_{1,27} = 45.02, P < 0.0001); and interaction (F_{2,27} = 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).

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/μL 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) with the outcome previously observed in 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 ± 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).
Candidate genes included in the analysis (n = 75) were selected as predicted targets of one or more of the nine miRs cross-referenced against maternal mRNAs reported in the mouse late-stage oocyte and/or single-cell zygote (30–32), (Table S4). We found that the multi-miR injection reduced the expression of many more stored maternal mRNA than the single miR injection (χ² = 11.53, P < 0.005), (Fig. 4A). Further, we detected a significant two- to fourfold decrease in expression of eight genes: Sirt1 (F2,31 = 8.54, P = 0.0011), Ubc3a (F2,31 = 4.00, P = 0.029), Srsf2 (F2,31 = 12.39, P < 0.0001), Iilost (F2,31 = 3.80, P = 0.033), Ncl (F2,31 = 6.37, P = 0.0048), Aars (F2,31 = 4.52, P = 0.019), Aggl (F2,31 = 3.47, P = 0.044), and Rablp1 (F2,31 = 3.39, P = 0.047) in the multi-miR-injected group (Fig. 4B–E). The down-regulation of these eight genes was not maintained into the mature brain, as their expression in the adult PVN was unaffected (Fig. S1).

**Discussion**

Posttranslational histone modifications, DNA methylation patterns, and populations of small noncoding RNAs in sperm have been implicated in the transgenerational transmission of paternal experience, with changes in these epigenetic marks observed following male exposure to such diverse stimuli as stress, malnutrition, and drugs of abuse (11–21). In particular, the role of sperm RNA as a mechanistic link between paternal experience and its consequences on offspring behavior and physiology has been emphasized by recent studies that characterize offspring phenotypes following in vitro fertilization and/or the experimental manipulation of total sperm RNA content (12, 20). In our model of paternal stress, a reduced HPA axis response in offspring was associated with the increased expression of nine miRs (miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532p, and miR-698) in paternal sperm following chronic stress exposure (11). In the current study, to confirm causality of these miR changes in offspring neurodevelopmental programming, the nine miRs (multi-miR injection) were micro-injected into single-cell zygotes. Two components were critical to control for these experiments to ensure outcomes were clearly interpreted: (i) the injection process itself, and (ii) the increased overall miR concentration, which could overwhelm and inhibit the endogenous activity of the zygote RNA-induced silencing complex.

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**Fig. 2.** Altered transcriptome profile in adult PVN from multi-miR-injected zygotes supports a lasting epigenetic modification. (A–C) GSEA of RNA-seq data identified three significantly enriched C2 gene sets with normalized enrichment score (NES) > 1.8 and FDR < 0.05, illustrating a decrease in collagen formation and extracellular matrix genes in the multi-miR–injected group. (D) Differential expression analysis identified 298 (10 up, 288 down) genes significantly changed by at least 1.5 fold (log2 fold change ≥ 0.585, dotted lines). Genes are presented in ranked order of expression from low to high. (E) Functional annotation clustering of dysregulated genes in the multi-miR group according to GO annotation (DAVID tools, ES = 1.3 equivalent to α = 0.05, gray bar indicates P < 0.05, and FDR < 0.05 for unique GO terms). (F) Significantly decreased expression of 17 collagen genes found in top extracellular matrix–enriched cluster. P < 0.05, FDR < 0.05. Data are shown relative to PBS-injected zygotes and presented as mean ± SEM, n = 6 biological replicates per group. ES, cluster enrichment score.
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 Crf1, pituitary Pomc, adrenal Mc2r, or adrenal 11β-hsd-1. Data presented as mean ± SEM, n = 6–9 mice per group. *P < 0.05.

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-injected groups exhibited 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 recovery between multi-miR-injected and our previously reported PBS-injected group PVN-exposed mice with controls. These findings suggest that the specific and combinatorial activity of these sperm miRs is essential to this developmental process (35). 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.

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

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Sirt1, Ube3a, Srsf2, IL6st, Ncl, Aars, Agfg1, and Ralbp1 genes in zygotes was reduced by multi-miR injection than by the single-miR injection. *P < 0.05 multi-miR vs. single-miR. Figure 4. Single-cell amplification studies confirmed sperm miR targeting of stored maternal mRNA. (A) Expression of 8 of the 75 candidate genes in zygotes was reduced by multi-miR injection than by the single-miR injection. P < 0.005. (B–I) Expression of 8 of the 75 candidate genes—Sirt1, Ube3a, Srsf2, IL6st, Ncl, Aars, Agfg1, and Ralbp1—was significantly reduced by the multi-miR injection, with expression levels measuring log2 fold change > 1 (dotted lines) relative to PBS controls. Expression of these genes was unaffected in single-miR injection controls. Data are shown relative to PBS-injected zygotes and presented as mean ± SEM, n = 10–13 zygotes per group. *P < 0.05 multi-miR vs. PBS, P > 0.05 multi-miR vs. single miR.

function of paternally derived miRs has not been established, we examined the ability of these nine paternally derived sperm miRs to impact stores of maternal mRNA. To do so, injected zygotes were cultured to the one- to two-cell stage, and the mRNA within each individual zygote was then amplified to examine differences in expression. The specific mRNAs examined were selected as being both present in published lists of the transcriptome in mouse late-stage oocytes and/or single-cell zygotes and predicted targets of one or more of the nine sperm miRs in the miRWalk database (30–32). Impressively, the pattern of candidate maternal mRNAs expression in single-cell zygotes was overall reduced in the multi-miR-injected group, supporting our hypothesized function that sperm miRs selectively target maternal mRNA leading to the posttranscriptional silencing of expression. Further, eight of the candidate mRNA—Sirt1, Ube3a, Srsf2, IL6st, Ncl, Aars, Agfg1, and Ralbp1—were dramatically affected by the multi-miR zygote injection, with expression levels reduced to between a half and a quarter of control levels. Importantly, the known developmental functions of the targeted genes, including the notable involvement of Ube3a and Sirt1 in chromatin remodeling and neurodevelopmental disorders (41, 42), support the hypothesis that sperm miRs may initiate lasting, programmatic changes that ultimately impact neurodevelopment and stress reactivity of the developing embryo. Again, as the single-miR injection did not affect the expression of stored maternal mRNA, the reduction in mRNA levels appears related to the specific composite activity of the nine miRs and not simply to changes in overall miR concentration. Future studies will need to determine how the targeted reduction in stored maternal mRNAs by paternal sperm miRs can induce reprogramming of the hypothalamus, but these findings certainly suggest an important influence of the paternal epigenome on early zygote development, a process previously considered maternally driven.

Together, our findings demonstrate a definitive involvement of the sperm miRs previously identified in our paternal stress model in the recapitulation of offspring HPA stress axis phenotype and hypothalamic reprogramming (11). Identification of a novel postfertilization function of these miRs in targeting stored maternal mRNAs offers exciting insight into the role of paternal RNA transmission and epigenetic marks. These studies emphasize a stepwise mechanistic approach toward a greater understanding of the transgenerational transmission of paternal lifetime experiences and offer valuable insight into the novel factors influencing offspring disease risk and resilience.

Materials and Methods

Microinjection. C57Bl/6j:129S6/SvEvTac hybrid mouse zygotes were randomly assigned for microinjection of nine miRs together (1 ng/μL final concentration of 0.11 ng/μL each miRIDIAN mimic miR-29c, miR-30a, miR-30c, miR-32, miR-193-5p, miR-204, miR-375, miR-532-3p, miR-698 in 1× DPBS) (Table S5) or to one of two control conditions: 1× DPBS only, to control for the injection process itself, or a single miR (1 ng/μL final concentration of miR-193-5p in 1× DPBS) to control for the total increased concentration of miRs within the zygote while minimizing nonspecific targeting effects that would be produced by alternate groups of miRs. Injected zygotes were transferred into recipient CD-1 females (Charles River) or cultured overnight in potassium-supplemented simplex optimised medium (KSMOS, EMD Millipore) and frozen at –80°C. Experimental protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (43).

HPA Axis Assessment. Naïve 13–14 wk male and female mice were restrained in flat bottom animal restrainers (Braintree Scientific) for 15 min beginning at time 0 min and were immediately returned to their home cage at the conclusion of restraint. As previously described, a single tail snap was made at time 0 min, removing <1 mm tissue from the distal tip. A total of 10 μL tail blood was collected by micropipette at 0, 15, 30, and 120 min into EDTA-treated tubes, first blotting the blood clot and then gently milking the tail to stimulate blood flow (11). Corticosterone levels were determined by radioimmunoassay (MP Biomedicals) in 3 μL plasma. All testing was completed 2–5 h after lights on.

RNA-Seq. Illumina single-end cDNA libraries of adult male PVN mRNA were prepared from 250 ng total RNA using the TruSeq Stranded mRNA Kit with
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 MI mouse oocytes, in single-cell mouse zygotes, and/or homologously in human and mouse mature oocytes (two or more of three lists) (30, 31) and predicted target of one or more of nine mirt 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 PWN 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 χ² 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 adjusted by 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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