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RESEARCH ARTICLE

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# Glucocorticoid exposure modifies the miRNA profile of sperm in the guinea pig: Implications for intergenerational transmission

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## Abstract

Approximately 1%–3% of the adult population are treated with synthetic glucocorticoids (sGCs) for a variety of conditions. Studies have demonstrated that adversities experienced by males prior to conception may lead to abnormal neuroendocrine function and behaviors in offspring and that epigenetic factors including microRNA (miRNA) within sperm may be responsible for driving these effects. However, it remains unclear where in the epididymis sperm miRNA changes are occurring. Here, we hypothesized that sGC exposure will alter the miRNA profile of sperm in the epididymis in a region-specific manner. Adult male guinea pigs were exposed to regular drinking water (Ctrl) or water with the sGC dexamethasone (Dex; 3mg/kg) ($n$ = 6/group) every other day for 48 days. Sperms were collected from epididymal seminal fluid in the caput and cauda regions of the epididymis and total RNA was extracted. miRNAs were assessed by miRNA 4.0 microarray; data were processed by TAC 4.0.1 and R. miRNA analysis revealed one miRNA in the caput that was significantly decreased by Dex in sperm. In the cauda, 31 miRNAs were reduced in sperm following Dex-exposure. The findings of this study demonstrate that Dex-exposure influences miRNA profile of sperm in the cauda but not the caput of the epididymis. This suggests that glucocorticoids target the epididymis to modify sperm miRNA and do not modify the miRNA content during spermiation in the testes.

**KEYWORDS**

epididymis, epigenomics, glucocorticoids (dexamethasone), miRNA, spermatozoa

**Abbreviations:** AR, androgen receptor; circRNA, circular RNA; Dex, dexamethasone; FDR, false discovery rate; GR, glucocorticoid receptor; HBSS, Hank's balanced salt solution; miRNA, microRNA; piRNA, piwi-interacting RNA; sGC, synthetic glucocorticoid; TAC, Transcriptome Analysis Console; TCAG, The Centre for Applied Genomics; tRNA, transfer RNAs.

## 1 | INTRODUCTION

At the end of spermiation, sperms are released into the caput of the epididymis moving through the corpus into the cauda.[1] When sperms reach the distal end of the cauda, they are stored and primed for fertilization.[1] This process is tightly regulated and influenced by endocrine factors, such as glucocorticoids. Synthetic glucocorticoids (sGCs) are commonly used to treat a variety of conditions ranging from autoimmune disorders to cancer.[2–6] It has been estimated that ~1%–3% of the global adult population are being prescribed sGCs at any given time.[2] A Danish study has estimated that the annual prevalence of glucocorticoid use was roughly 3%.[7] In this population, 46% are males and 54% are between the ages of 20–59.[7] These data suggest that there is a large population of males that are prescribed glucocorticoids during their reproductive years. Furthermore, sGCs such as dexamethasone, have recently been administered to patients hospitalized with Coronavirus 2019 (Covid-19).[8] Patients receiving sGCs experience acute relief; however, chronic exposure has been shown to increase susceptibility to diabetes, hypertension, adrenal insufficiency, and various neuropsychiatric diseases.[3,5,6] Furthermore, long-term exposure to sGCs has been shown to negatively impact male fertility by impairing sperm quality,[9,10] inducing germ cell apoptosis,[11] and altering androgen production and spermatogenesis,[12] which may have implications for intergenerational transmission.

Recent evidence suggests that environmental adversities experienced by male rodents prior to breeding impacts embryo development and alters offspring behavior.[13,14] Male mice that experienced chronic stress sired offspring with a dysregulated stress response.[14] Other studies using various paradigms of environmental challenges have highlighted the importance of the paternal preconception period for offspring health.[15–20] Sperm DNA is critical for zygote formation; however, sperm also carry non-DNA epigenetic factors that are able to influence early embryo development.[21,22] Long non-coding RNAs, transfer RNAs (tRNAs), and miRNAs are present within sperm. miRNAs are small non-coding RNAs that regulate gene expression by binding to the 3' UTR of target mRNAs for degradation or inhibition of translation.[23] Studies have demonstrated that miRNA within sperm are altered after paternal adversity and that these changes are associated with abnormal offspring outcomes.[24–26] Rodgers et al.[24] evaluated the miRNA profile of mice sperm after chronic stress exposure and found nine significantly enriched miRNA. Injection of these miRNA into zygotes recapitulated the phenotypic changes in HPA function previously observed in offspring from the chronic stress experiments,[14] highlighting that miRNAs may be a key factor in facilitating intergenerational transmission. However, the location within the reproductive tract wherein these sperm miRNA changes are occurring has not yet been fully elucidated.

It has been suggested that epididymosomes released from epididymal epithelial cells facilitate miRNA alterations, as they are crucial in shaping the molecular landscape of sperm.[25] Epididymosomes (50-250 nm) bind to the sperm head, a process important for sperm maturation[25,26] and offspring health.[27] It has been hypothesized that the miRNA cargo within epididymosomes becomes altered after environmental adversity, and once delivered into sperm can influence gene expression in the embryo.[21,22] However, it is currently unclear whether epididymosomes originating from the caput or cauda, or both are responsible for influencing sperm miRNA after adversity. Thus, elucidating the epididymal region where differences in miRNA levels emerge after glucocorticoid exposure would allow for better understanding of the mechanisms involved in paternal intergenerational transmission. We hypothesized that sGC exposure will alter the miRNA profile of sperm in the epididymis, and that these effects will be dependent on the region of the epididymis where the sperm were derived.

## 2 | METHODS

### 2.1 | Animals and treatments

Twelve-week-old male Dunkin-Hartley guinea pigs (Charles River; St. Constant, QC, Canada) were singly housed in temperature (23°C) and humidity controlled rooms on a 12-h light–dark cycle (lights on 0700 h, off 1900 h). Food and water were available ad libitum. All protocols were approved by the Animal Care Committee at the University of Toronto in accordance with the Canadian Council on Animal Care. The guinea pig model has been widely utilized in reproductive studies and for investigating developmental origins of health and disease.[28] Furthermore, like the human, the endogenous glucocorticoid of guinea pigs is cortisol[29] and androgen production in the testes and adrenal cortex is similar to humans.[29] Therefore, in the context of this study, the epididymal physiology of guinea pigs may better resemble humans than other rodent models. Food and drinking water were measured every day and pilot data demonstrated that adult male guinea pigs drink ~125–150 mL of water/day. Guinea pigs were exposed to the sGC dexamethasone 21-phospate disodium salt in the drinking water (Dex; 3 mg/kg; Sigma Aldrich, St. Louis, MO) every other day for 48 days ($n = 6$). This duration of exposure encompasses the entire spermatogenic process of the guinea pig[30] so that sperm were exposed to sGC at multiple stages of

spermatogenesis as well as maturation during epididymal transit. Controls received untreated water ($n = 6$). Water intake and weight measurements were used to calculate Dex concentrations to equate ~3 mg/kg in the drinking water. Baseline measurements were taken for 5 days prior to treatment. Salivary samples were collected four times a week by providing the animals with a cotton swab to chew for 20 s. Saliva was extracted with centrifugation (5 min, 10 000×*g*, 25°C) and stored at −20°C for future analysis of cortisol by ELISA. Guinea pigs were euthanized following treatment using isoflurane anesthetic followed by decapitation.

## 2.2 | Hormone measurement

Saliva samples for analysis of free cortisol levels were collected at 12:00 pm for two consecutive days followed by two days of rest for a total of four times a week throughout treatment. Salivary cortisol levels were assayed using a Cortisol ELISA Kit (Abcam, Cambridge, MA) following the manufacturer's protocol. Intra- and interassay coefficients of variance were 6.5% and 12.5% respectively. Plasma samples for analysis of testosterone levels were collected at time of euthanasia ($n = 6$/group). Plasma testosterone levels were assayed using a Testosterone ELISA Kit (Abcam, Cambridge, MA) following the manufacture's protocol. All samples were analyzed in the same assay.

## 2.3 | Analysis of sperm

The epididymis was isolated and divided into caput and cauda sections to isolate sperm. Sperm were isolated using a modified protocol from Moohan and Lindsay[31] and Sharma et al.[16] Briefly, epididymal fluid from each section was extruded by mechanical compression of the epididymis into warm PBS (3 mL; 37°C) and incubated for 15 min. Aliquots (100 µL) were used to visually assess sperm parameters such as sperm motility, morphology, vitality, and count using a modified protocol from the World Health Organization (WHO-2010)[32] and performed by the same technician. Investigators were blinded to source location (caput vs cauda) and group (control vs. Dex) at time of assessment. *Sperm motility*: Sperm motility was evaluated immediately after isolation. Epididymal fluid was placed on a warmed slide (37°C) and examined on a microscope (200x magnification). Five random fields of view were evaluated, and results were expressed as a percent of sperm that were progressive motile, non-progressive motile, or immotile. *Sperm morphology and vitality*: An eosin nigrosine stain was used to evaluate sperm morphology and vitality immediately following collection. Epididymal fluid (50 µL) was added to the eosin nigrosine stain (50 µL). The mix (10 µL) was placed onto a microscope slide and examined at 200× magnification on a light microscope. A total of 250 sperm were evaluated for both caput and cauda in each animal and assessed for morphology (normal or abnormal) and vitality (alive or dead). Morphological abnormalities included irregular, duplicated, detached, coiled, or elongated head and tail defects. Dead sperm exhibited dark pink heads while alive sperm exhibited white/light pink heads under the eosin nigrosine stain. Morphology and vitality were expressed as a percent. *Sperm count* was evaluated by adding epididymal fluid to warmed PBS (37°C). The solution (10 µL) was placed in a hemocytometer and was counted at 200× magnification. Sperm count was expressed in millions of sperm per mL. Epididymal fluid that was not used for sperm parameter analysis was used for RNA analysis. Epididymal fluid was centrifuged (5 min, 800×*g*, 4°C) and the resultant pellet was carefully layered over 40% Percoll (Sigma Aldrich, St. Louis, MO)/60% 10× Hank's balanced salt solution (HBSS; Invitrogen Carlsbad, CA), followed by further centrifugation (15 min, 800×*g*, 4°C). The final resultant pellet was re-suspended in PBS and centrifuged (800×*g*, 5 min, 4°C) prior to being stored at −80°C until RNA extraction.

## 2.4 | RNA extraction

Total RNA was extracted from sperm using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations with some modifications. Briefly, samples were homogenized with Trizol using the Tissuelyser II (Qiagen, 25 Hz, 5 min), and incubated with chloroform and centrifuged (15 min, 12 000×*g*). The aqueous phase was isolated, and isopropanol added. Following further centrifugation, EtOH (75%) was added to the pellet, centrifuged then air dried. RNA was eluted with RNase-free water (31 µL) and heated (60°C, 15 min). RNA quality and quantity was evaluated with the Synergy™ HTX Multi-Mode Microplate Reader (BioTek Canada) and Agilent Bioanalyzer (Agilent technologies); performed by The Centre for Applied Genomics (TCAG), The Hospital for Sick Children, Toronto, Canada.

## 2.5 | miRNA microarray

Sperm miRNA profiling was evaluated with Applied Biosystems™ GeneChip miRNA 4.0 Array (Affymetrix) performed at TCAG. Total RNA from caput and cauda ($n = 6$/group) were submitted for analysis. Each array contained miRNA probes for 203 organisms derived from the Sanger miRBase miRNA database v20.[33]

## 2.6 | qPCR

miRNA microarray data were validated with real-time PCR quantification. Briefly, total RNA from sperm was reverse transcribed using the miScript II Reverse Transcription Kit (Qiagen, Toronto, ON). Real-time quantitative PCR (qPCR) was performed using the SensiFAST Probe Hi-ROX Kit (Bioline USA Inc.) and quantified by the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Canada Inc.) (RRID:SCR_018064) according to the manufacturer's instructions. miRNA primers used in the present study were designed with miRprimer2 (RRID:SCR_000480)[34] and are listed in Table 1. Levels of target miRNAs relative to the reference gene *RNU6* were assessed using the $2-\Delta\Delta ct$ method.

## 2.7 | Statistical analysis

miRNA analysis for caput and cauda microarrays were analyzed by importing CEL files with the Affymetrix Transcriptome Analysis Console 4.0.1(TAC) software (available at www.thermofisher.com) (RRID:SCR_016519), using RMA + DMG (all organisms) and removing batch effects. Differential analysis was conducted between control and Dex groups for both caput and cauda microarrays and calculated using one-way ANOVA with Benjamini-Hochberg Step-UP FDR correction. miRNA probes were determined to be significantly altered at a fold change $\geq |1.5|$ and a false discovery rate (FDR) $p < .05$. All analyses were conducted using the TAC software. miRNA set enrichment analysis was performed using DIANA-miRPath v.3 (RRID:SCR_017345).[35] KEGG pathways were predicted from DIANA-TarBase v7.0 and significant (FDR-corrected $p < .05$) targeted pathways were identified. All other analyses were conducted using Prism and SPSS. Data were assessed for normal distribution using a Shapiro–Wilk test, and outliers were detected and excluded using a ROUT test. A one-tailed Students *t*-test was used to analyze PCR data. A two-tailed Students *t*-test test was used to analyze testosterone data. Two-way Mixed ANOVA was used to analyze weight, food intake, salivary cortisol (treatment × times), and sperm parameters (treatment × epididymal location) and Tukey HSD post-hoc tests were used where applicable.

# 3 | RESULTS

## 3.1 | Physical and endocrine effects of Dex-treatment

Control and Dex-treated animals gained weight throughout treatment. There was a significant interaction effect of Dex treatment on weight gain over time $F(26,260) = 3.242$, $p < .0005$ (Figure 1A), but post-hoc analysis revealed no significant differences between groups at any time point (Figure 1A). There was no significant effect of Dex treatment on food intake throughout treatment $F(52,364) = 0.765$, $p = .881$ (Figure 1B).

Dex treatment did not significantly impact caput or cauda sperm morphology or vitality. There was a significant main effect of location where motility was dependent on caput or cauda sections of the epididymis ($p < .0001$). As expected, in the caput, sperm exhibited less progressively motile sperm and more immotile or non-progressively motile sperm compared to the cauda (Table 2). However, Dex-treatment did not influence sperm motility in either the caput or cauda. Similarly, sperm count was not impacted by Dex-treatment in either the caput or cauda sections of the epididymis (Table 2). There was a main effect of location such that there were fewer sperm in the caput compared to the cauda ($p = .01$).

There was a significant interaction between Dex treatment and time on salivary cortisol concentrations, $F(13,130) = 1.813$, $p = .047$ (Figure 1C). Post-hoc analysis revealed that salivary cortisol was significantly reduced at day 37 ($p = .005$) day 49 ($p = .019$), with a trend ($p = .08$) on day 46 (Figure 1C). Dex treatment had no significant effect on plasma testosterone levels (Control $0.59 \pm 0.21$ ng/mL, Dex $0.54 \pm 0.14$ ng/mL, $p > .05$).

## 3.2 | miRNA in caput and cauda sperm

Analysis of miRNA levels in sperm revealed one modified miRNA in the caput and 31 miRNAs decreased in the cauda from Dex-treated animals (Figure 2A,B) (Supporting Information Tables S1 and S2). qPCR was performed on selected miRNAs shown to be significantly affected by Dex in cauda sperm by miRNA microarray. miR-199, miR-449a and miR-125 were selected for targeted qPCR analysis because these have been implicated in embryo development,[20,36,37]

**TABLE 1** List of miRNAs used for qPCR.

| Target | Forward | Reverse |
|---|---|---|
| miR-125b-5p | GCAGTCCCTGAGACCCT | CCAGTTTTTTTTTTTTTTTCACAAGT |
| miR-199-3p | GCAGTACAGTAGTCTGCAC | TCCAGTTTTTTTTTTTTTTTAACCAATG |
| miR-449a-5p | GCAGTGGCAGTGTATTGTTAG | GTCCAGTTTTTTTTTTTTTTTACCAG |
| RNU6-1 | AGGTTAGCACTCCCCTTGAC | GCAATGCCTTAACCGTATGC |

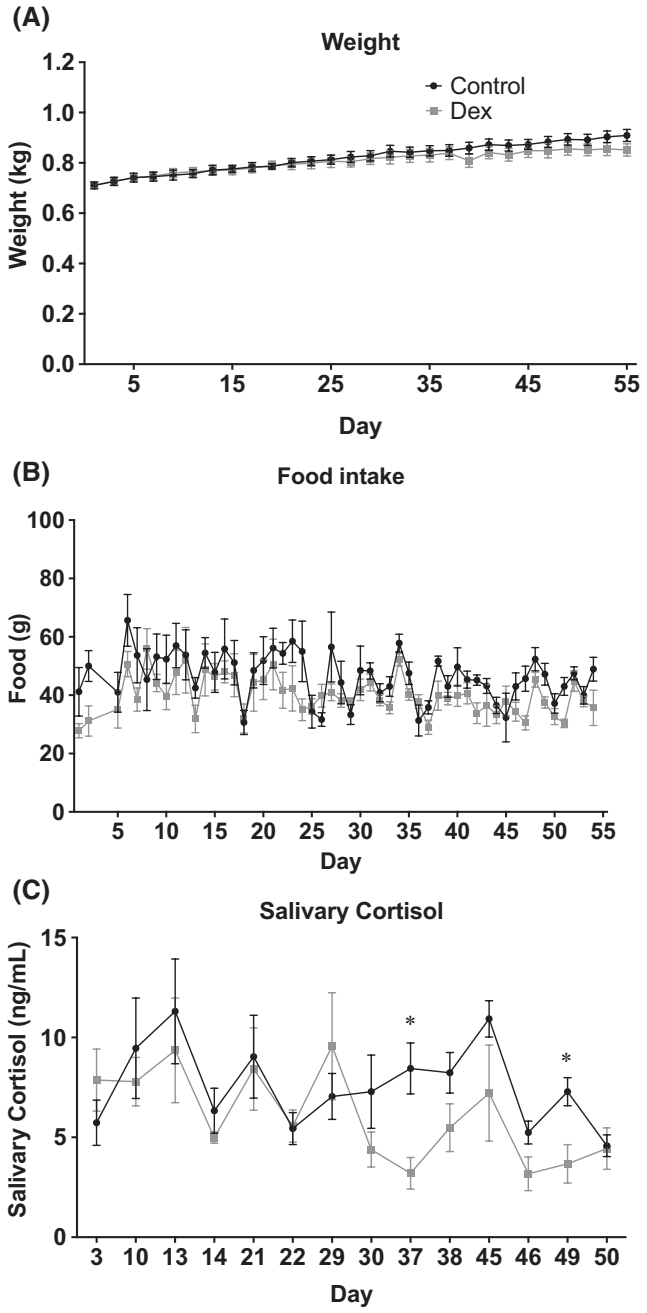


**FIGURE 1** Weight, food, and salivary cortisol measurements during Dex exposure ($n = 6$/group). Baseline measurements were taken for the first 5 days of the experiment prior to Dex treatment. (A) Guinea pig weight was measured every other day throughout the experiment. (B) Food intake (g) was measured daily. (C) Salivary cortisol (ng/mL). Dex-treated animals (squares), Control animals (circles); Values are presented as mean ± Standard Error Mean (SEM); *$p < .05$.

and in investigations of the mechanisms of paternal intergenerational transmission.[14] miR-199 and miR-449a exhibited significantly reduced levels in cauda sperm ($p < .05$). While the direction of change for miR-125 was similar to the microarray, the effect did not achieve statistical significance by qPCR ($p = .15$) (Figure 3).

To better understand the potential role of the altered miRNA after Dex-expsoure, miRNA set enrichment analysis was performed on the significant cauda sperm miRNAs. 33 KEGG terms were identified in the pathway analysis for sperm. Interestingly, terms related to steroidogenesis, signaling pathways, and metabolism were identified (Figure 4, Supporting Information Table S3).

## 4 | DISCUSSION

In the present study, we have shown that exposure of adult male guinea pigs to Dex modifies the miRNA profile in epididymal sperm. These effects on sperm miRNA are much greater in the cauda than the caput of the epididymis. Together, these findings suggest that the epididymis represents an important target for the actions of glucocorticoids, and we speculate that these effects likely involve modification of epididymosome miRNA cargo and/or release.

Studies have demonstrated that as sperm transit from the caput to cauda, they acquire specific miRNA signatures.[38,39] Our finding that most miRNA alterations occurred at the level of the cauda following Dex exposure are likely due to region-specific effects within the epididymis. Indeed, one previous study demonstrated that treatment with Dex in adrenalectomized rats led to modified glucocorticoid receptor (GR) and androgen receptor (AR) levels in the epididymis.[40] Interestingly, the effects of glucocorticoid exposure on AR levels occurred in the cauda, and not in the caput. Another recent study demonstrated that exposure to glucocorticoids leads to altered GR expression in an epididymal epithelial cell line, in vitro, as well as alterations in the miRNA content of the epididymosomes that they produce.[41] The AR has also been implicated in miRNA production in epididymal epithelial cells.[42] Thus, the GR and AR may be involved in the region-specific actions of glucocorticoids in the epididymis.

The miRNA processing enzymes, dicer and drosha, are key regulators of miRNA production.[43,44] Interestingly, sGCs have been shown to decrease the expression of dicer and drosha leading to significant downregulation of miRNAs in lymphocytes.[43] Furthermore, Yuan et al.[21] generated germ cell-specific dicer and drosha cKO mice and showed that these mice exhibited an altered sperm miRNA profile. Embryos derived from these sperm showed reduced preimplantation developmental potential. Interestingly, this effect was rescued when small RNAs from wildtype mice were introduced to the oocytes fertilized with the dicer/drosha cKO sperm. These findings highlight the importance of dicer and drosha for miRNA production and normal embryo development. Thus, the alterations in miRNA in cauda sperm identified

**TABLE 2** Caput and cauda sperm visually assessed for morphology, vitality, motility and expressed as a mean percentage ± SEM.

| | Caput $N = 6$ | | Cauda $N = 6$ | |
|---|---|---|---|---|
| **Parameter** | **Control** | **Dex** | **Control** | **Dex** |
| %Progressive motility (±SEM) | 16.4 ± 3.5 | 12.5 ± 3.3 | 64.6 ± 1.9 | 42.1 ± 12.1 |
| %Non-progressive motility (±SEM) | 47.3 ± 2.0 | 54.0 ± 4.4 | 15.9 ± 2.3 | 21.2 ± 5.3 |
| %Immotile (±SEM) | 36.2 ± 2.4 | 33.4 ± 3.2 | 19.3 ± 1.3 | 26.5 ± 8.5 |
| %Normal morphology (±SEM) | 57.0 ± 6.7 | 54.0 ± 4.9 | 59.9 ± 4.7 | 50.8 ± 7.9 |
| % Vitality (±SEM) | 59.2 ± 4.9 | 59.6 ± 3.8 | 62.4 ± 5.0 | 53.3 ± 6.6 |
| Count (millions/mL ± SEM) | 155.5 ± 17.4 | 233.9 ± 19.8 | 292.8 ± 16.7 | 402.2 ± 79.9 |

*Note*: Sperm visually counted and expressed as millions of sperm/mL. Adult male guinea pigs were chronically exposed to Dex or normal drinking water (Control) $n = 6$/group. There was no effect of Dex on any sperm parameters evaluated in the caput and cauda.

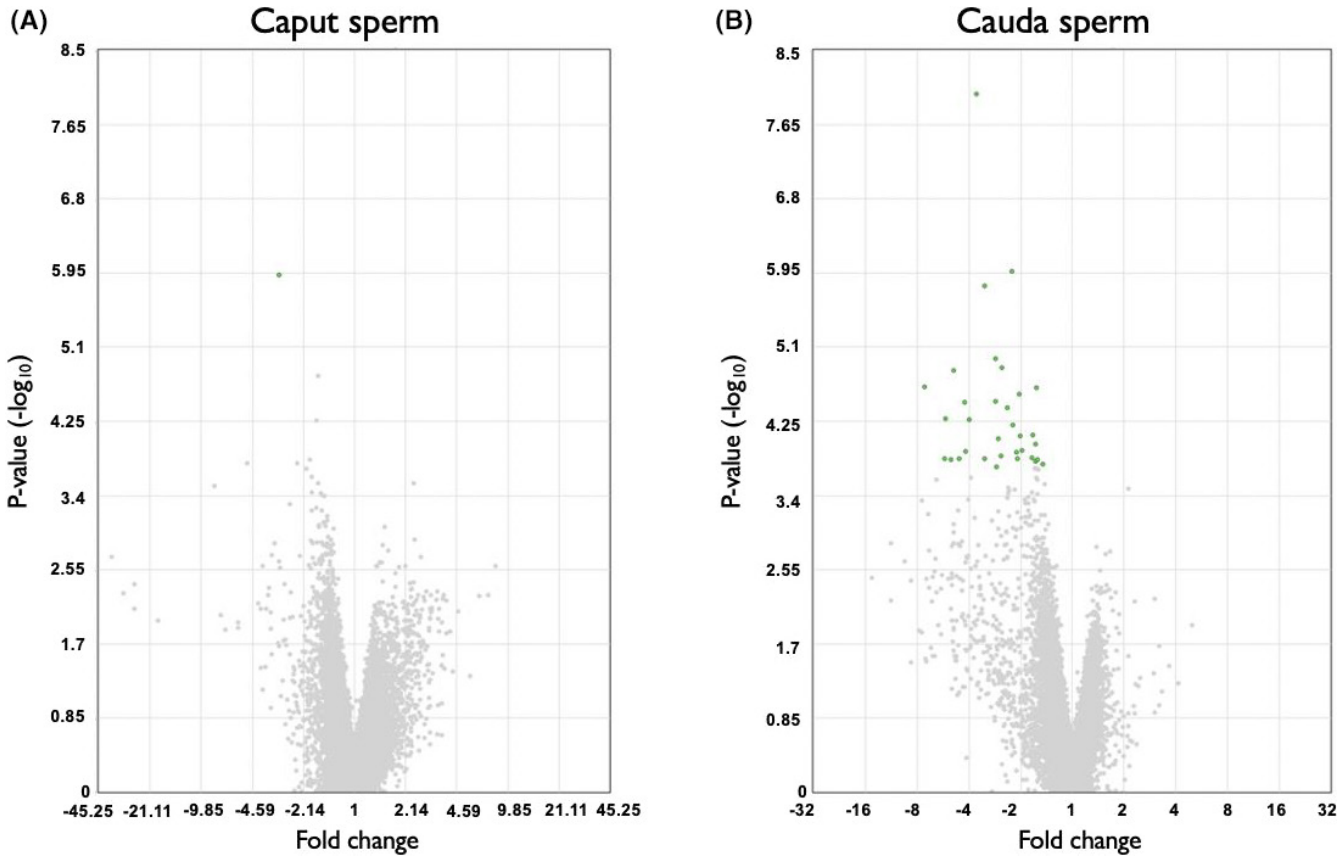


**FIGURE 2** Volcano plot comparing the miRNA levels in caput (A) and cauda (B) sperm following Dex exposure ($n = 6$/group). Significantly reduced levels of miRNA are represented by green dots. Fold change ≥ |1.5| and a false discovery rate (FDR) $p < .05$ was considered significant.

in the present study may be driven by altered expression of dicer and drosha in the cauda of epididymal epithelial cells following sGC treatment. Future studies should be undertaken to investigate the impact of sGC exposure on dicer and drosha in cauda epididymal epithelial cells.

miRNAs are transported to sperm from epididymosomes and can influence maternal mRNA stability and gene expression in the developing zygote and embryo, respectively.[20,21] An elegant study by Chan et al.[27] treated a mouse caput epididymal epithelial cell line with corticosterone. The resultant epididymosomes were then incubated with naïve sperm, which were then injected into mouse oocytes to generate offspring. The offspring exhibited altered brain gene expression and a dysregulated stress response, highlighting the significance of these extracellular vesicles and their cargo. In the current study, it is likely that the altered miRNA levels in cauda sperm result from glucocorticoids acting on the epididymal epithelial cells.

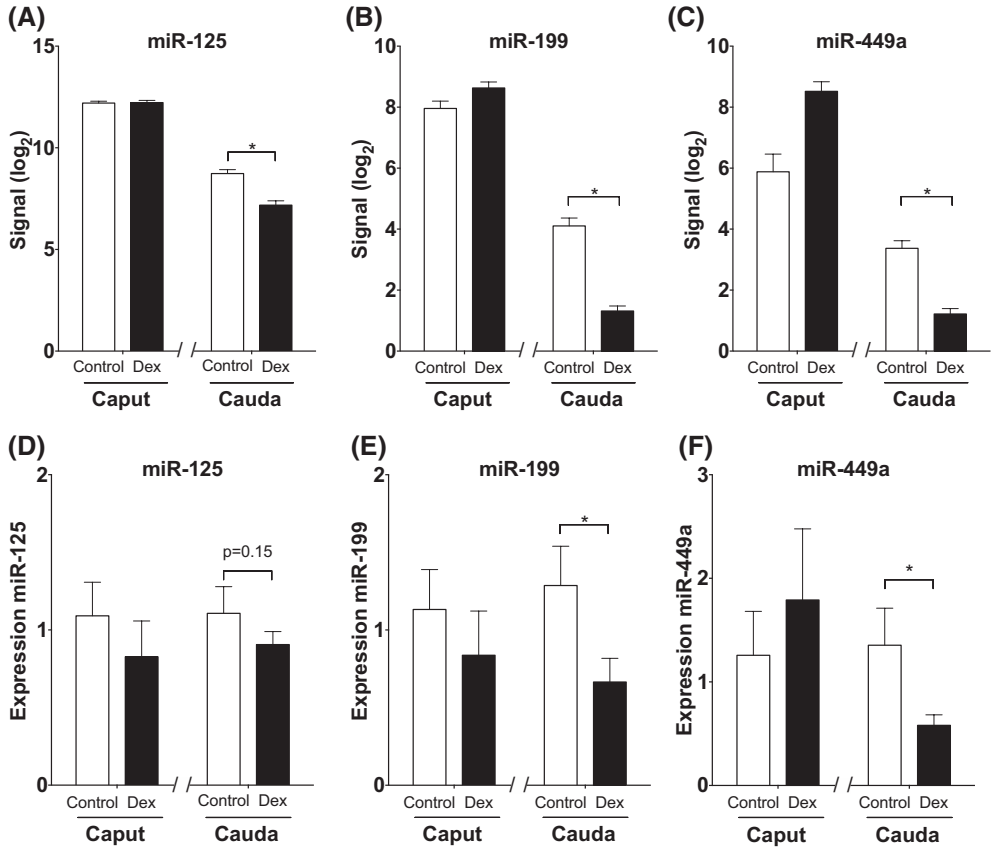


**FIGURE 3** miRNA levels following Dex exposure in epididymal sperm by miRNA microarray in caput and cauda sperm (A–C) miRNA levels were quantified by RT-qPCR in caput and cauda sperm (D–F) RT-qPCR samples were run in triplicate ($n = 6$/group, miR-449a caput RT-qPCR $n = 5$/group). RNU6 was used as a reference gene to normalize the levels of miRNA for RT-qPCR. Data are presented as mean ± SEM; *$p < .05$.

Previous studies have shown that Dex exposure modifies exosome release and exosome miRNA levels from macrophages.[45] Exosomes derived from macrophages exhibited a reduction in production and decreased levels of miR-155 following Dex exposure.[45] Further studies designed to determine the release and composition of the epididymosomes in the cauda following sGC exposure are required to better understand the function of these extracellular vesicles in fertility and intergenerational transmission.

Previous studies have demonstrated that glucocorticoids can impact sperm miRNA.[19,46] However, the miRNA profile of sperm that we report in the present is quite different from that reported previously.[19] This likely results from species differences, the duration and dose of treatment and the area of the epididymis where sperm were collected. Short et al.[19] exposed adult male mice to corticosterone (25 μg/mL) every day for 4 weeks in their drinking water, but did not undertake analysis in the different regions of the epididymis. In contrast Gapp et al.[46] utilized a single intraperitoneal injection of an extremely high concentration of Dex (2 mg/kg) for mice. In the present study, we utilized a chronic exposure to Dex in the drinking water, which reduces the risk associated with single or repeated injection.

The miRNA changes that we identified in sperm may have implications for both fertility and embryo development. Specifically, miR-199, miR-449a and miR-125 were significantly decreased in cauda sperm (by microarray analysis). Previous studies have demonstrated that miR-199 is important for regulating spermiogenesis by binding to the 3'-UTR of one of its target gene, Tekt1.[47] Tekt1 has been demonstrated to influence the production and maturation of sperm.[47,48] Other studies have demonstrated that reduced miR-199 levels in IVF blastocysts decreases their viability and developmental potential.[49] miR-449 has been demonstrated to be significantly reduced in sperm of mice exposed to chronic stress and these changes persist to the early embryo.[20] Further, miR-449 levels have been demonstrated to be inversely correlated with Adverse Childhood Experience (ACE) scores in human sperm.[20] These data highlight miR-449 as a potential biomarker in sperm for stress-induced miRNA changes that may influence the next generation. miR-125 may also impact offspring development as this miRNA has been shown to be important in driving HPA phenotypes after paternal trauma in mice.[18] Male mice that were exposed to traumatic stress exhibited an altered sperm miRNA profile, leading to

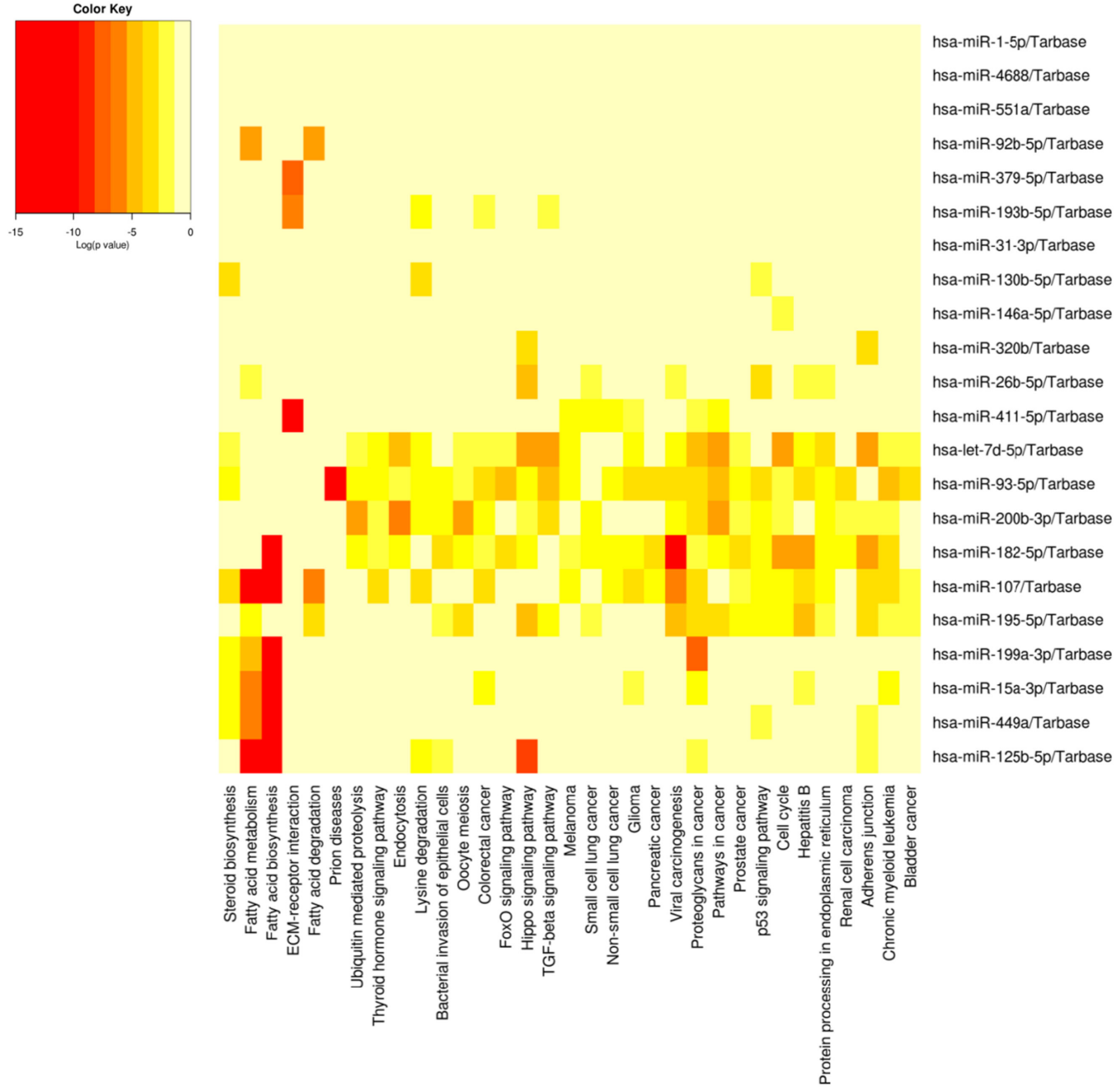


**FIGURE 4** Heatmap of miRNA-set enrichment analysis of significant miRNAs identified in cauda sperm following Dex-exposure using DIANA-miRPath v.3. Each row depicts a miRNA, and each column represents a significant predicated pathway. Color of the bar represents $-\log_{10}$ adjusted $p$ of the pathway predicted to be a target of the miRNA. Red represents highly significant, yellow represent less significant.

behavioral alterations in offspring.[18] When these miRNAs were injected into wildtype zygotes, the offspring recapitulated the same outcomes as those from the paternal stress experiments. Future mechanistic studies are required to determine the pathways as well as molecular mechanisms by which miRNA are altered in the sperm following sGC exposure, and the region-specific nature of these mechanisms.

The miRNAs identified to be affected by sGC treatment in cauda sperm have been shown to impact steroid biosynthesis, metabolism, and other signaling pathways. Steroid biosynthesis is essential for the maintenance of epididymal function.[50] The finding of reduced miRNA levels in cauda sperm following Dex exposure may be due to reduced levels of these miRNAs in epididymal epithelial cells and epididymosomes. Interestingly, other studies have demonstrated that miRNA alterations following early life stress are associated with steroidogenesis.[51] Adult male mice that underwent early life stress exhibited modifications of miRNA in cauda epididymosomes,

these changes were associated with steroid biosynthesis.[51] Further, mice exhibited reduced levels of androgens in the caput epididymis.[51] The miRNAs identified in the present study are also associated with signaling pathways such as FOXO and p53. These signaling pathways are important for early embryo development.[52,53] These data indicate the potential importance of sperm miRNA for embryo development and regulation of fertility.

In the present study sGC-exposure did not influence sperm motility, morphology, vitality or count in either the caput or cauda sections of the epididymis and did not influence plasma testosterone concentrations. This is in contrast to previous studies that demonstrate significant reductions to sperm parameters in other species.[9,12] For instance, a study in rams demonstrated that two intramuscular Dex (0.25 mg/kg) injections resulted in decreases in sperm motility, sperm density, semen volume, and increased abnormal sperm rate.[9] Similarly, a study in mice found that seven day intraperitoneal injection of Dex (7 mg/kg/day) significantly decreased sperm motility, the number of spermatocytes, spermatogenesis index and serum testosterone levels.[12] The main difference between the current study and previous finding is the route of exposure and dose. Previous studies have used acute high doses of Dex that were administered through injection. Injections, particularly intraperitoneal injections, can result in stress-activation of the HPA axis, increasing endogenous glucocorticoid concentrations.

It is clear that stress[14,54] and corticosterone exposure[19] in male rodents before conception can impact offspring behavior and physiology. miRNAs are key in facilitating these intergenerational effects. There was not complete overlap in the miRNAs identified in this study and the study investigating preconception stress.[14] However, other studies investigating the impact of preconception stress on miRNA in sperm have demonstrated significant reductions in miR-449a in sperm,[20] and the current study observed a similar profile in sperm exposed to sGC. In the context of stress, endogenous glucocorticoids bind to both GR and MR, whereas sGCs primarily target the GR.[2] Future studies will be required to investigate the specific roles of MR and GR in the modulation of changes in miRNA following stress and glucocorticoid exposure. However, it is important to note that other factors including tRNA, piwi-interacting RNA (piRNA) and circular RNA (circRNA) may also be altered in sperm and signal to the oocyte, zygote, and embryo.[38,46,55] Changes in these other sncRNA in sperm may also result from changes in the epididymal epithelial cells and subsequent epididymosomes cargo. For instance, tRNAs in sperm have been demonstrated to be acquired through epididymosomes as they transit from the caput to cauda.[39] These tRNAs have also been demonstrated to be transferred to 2-cell embryos from sperm.[46] These data highlight another potential epigenetic mediator of transmitting paternal experiences to offspring.

In conclusion, we have demonstrated that chronic sGC exposure influences the miRNA profile of sperm in the cauda but not the caput of adult male guinea pigs. This suggests that the corpus and cauda of the epididymis are an important site of sGC action in modifying sperm miRNA. These findings are highly clinically relevant considering 1%–3% of the global adult population are using sGCs at any given time,[2] especially with the added use in treatment for Covid-19.[8] Future studies will determine whether an acute treatment (<10 days) of sGCs would also influence the sperm miRNA profile. These studies also have ramifications for animal populations and in animal production since males sire many offspring from multiple females, and because of the increasing use of IVF in animal breeding.[56] A better understanding of the molecular mechanisms responsible for the transmission of paternal experiences could help in the development of new approaches to break the cycle of intergenerational transmission, and identify novel predictive biomarkers of embryo, fetal and postnatal health.

## AUTHOR CONTRIBUTIONS

**Christopher Casciaro:** Designed research, performed research, analyzed data, writing - reviewing and editing. **Hirotaka Hamada:** Performed research, analyzed data. **Alisa Kostaki:** Designed research, performed research. **Stephen G. Matthews:** Designed research, analyzed data, writing, reviewing and editing, supervision, project administration, and funding acquisition.

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## DISCLOSURES

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The microarray data were deposited in NCBI's Gene Expression Omnibus Database (https://www.ncbi.nlm.nih.gov/geo/) and are available through GSE series accession number GSE226168. All other data that support the findings from the study are available from the corresponding author upon request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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