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Profiling nonhuman primate germline RNA to understand the legacy of early life stress

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Abstract

Exposure to stress is a risk factor for perturbed mental health, including impoverished regulation of emotional and physiological responses that accompany anxiety and mood disorders, substance abuse and behavioral disorders. Such disruptions to well-being could be triggered by discrete environmental events or pervasive early life stress (ELS) resulting for example from adverse caregiving. Recent data mostly collected from rodents exposed to anthropogenic stressors suggest that one way via which the detrimental effects of such stress extend beyond the exposed population to future offspring is via stress-induced alterations of RNA found in the paternal germline. In contrast, less attention has been paid to how naturally occurring stress in males might influence offspring biology and behavior. In this study, we used a translational nonhuman primate model of ELS caused by naturally occurring adverse caregiving of infant macaques to (1) profile total RNA in the adolescent male germline, and (2) identify how those RNA profiles are affected by exposure to ELS. Our findings that the top 100 transcripts identified correspond to transcripts related to germline biology and reproduction demonstrate the validity and feasibility of profiling RNA in the germline of rhesus macaques. While our small sample sizes precluded definitive assessment of stress-induced alterations of RNA in the male germline of rhesus macaques that experienced ELS, our study sets the foundation for future investigations of how early adversity might alter the male germline, across species and in experimental protocols that involve anthropogenic vs natural stressors.

KEYWORDS
adolescence, early life stress, infant maltreatment, nonhuman primate, RNA, sperm

1 | INTRODUCTION

Stress profoundly impacts mental health in the generation directly exposed. Among the many potential exposures to stress, early life stress (ELS) is the most insidious in setting up exposed children for increased risk of poor mental and physical health (Felitti et al., 1998). ELS, including childhood maltreatment (MALT), is a major risk factor for the emergence of psychopathology such as poor emotional and stress regulation seen in anxiety and mood disorders, substance abuse and behavioral disorders (Cicchetti & Toth, 2005; Douglas

et al., 2010; Sinha, 2008). If such influences of ELS on the exposed individuals were not grave enough, accumulating literature provides evidence that detrimental effects of ELS often extend beyond the generation directly exposed (Cao-Lei et al., 2014; Costa et al., 2018), and offspring of parents that have experienced stress are at a higher risk for mental health disorders (Brand et al., 2006; Halligan et al., 2007; Klarić et al., 2008; Stein et al., 2009; Toth, 2015; Yehuda et al., 2005). For example, children born to mothers who were abused present with higher depressive- and anxiety-related metrics even if they were conceived after the time of the maternal abuse (Jovanovic et al., 2011). Research in the context of the Holocaust has provided evidence for non-trauma-exposed offspring of parents with PTSD being more likely to display PTSD-like symptoms, compared to offspring of parents without PTSD (Rosenheck, 1986; Yehuda & Bierer, 2008; Yehuda et al., 2001, 2007). While such intergenerational influences of stress are now being increasingly appreciated, the mechanisms underlying such increased risk for psychopathology in offspring as a consequence of parental exposure to stress are not well understood. Filling this gap in knowledge is a first step toward not only identifying individuals who might be at risk to bear the burden of intergenerational legacies of stress, but also eventually developing interventions to break the perpetuation of the stress effects across generations.

Abusive parental care, stress experienced while in utero, and parental stress experienced before conceiving that impacts the germline are three possible mechanisms that could underlie intergenerational legacies of stress. A wealth of literature has illuminated how abusive parental care and the maternal–fetal placental interface during pregnancy program offspring neurobiology and consequently derail the offspring's mental health (Bos, 2017; O'Donnell et al., 2014; Bronson & Bale, 2016; Christian, 2012; Kaffman & Meaney, 2007). In contrast, much less is known about how alterations that might occur in the germline before conceiving offspring may perpetuate intergenerational legacies of stress after fertilization. Studies in rhesus macaques have shown that infants of nursery-reared fathers, not mothers, showed greater emotional reactivity and higher plasma cortisol levels, despite being raised in the absence of parent-offspring social contact (Kinnally & Capitanio, 2015). Further, descendants of nursery-reared macaque fathers have shown behavioral and immune differences across 2–3 generations raised in a semi-naturalistic environment (Kinnally et al., 2018), suggesting biological influences of paternal experiences that do not have a social component. By focusing on paternal stress and using approaches like in vitro fertilization using sperm from stressed males, work in rodents has been able to study intergenerational legacies of stress independently of any influence from socially transferred information of stress exposure (Aoued et al., 2019; Babb et al., 2014; Dias & Ressler, 2014; Gapp et al., 2014, 2020; Rodgers et al., 2013, 2015). RNA contained in sperm have emerged as an important mechanism that passes along the baton of paternal experiences to offspring. Via intra-zygotic injections of RNA contained in sperm of male rodents exposed to different experiences, RNA in paternal sperm have been shown to mediate the impact of paternal dietary manipulations, stress and salient chemical exposure on metabolism, physiology, and behavior of offspring (Aoued et al., 2019; Chen et al., 2016; Gapp et al., 2014, 2020; Rodgers et al., 2015; Sharma et al., 2016). While these findings suggest that paternal germ cells respond and are vulnerable to environmental exposures and may set up the next generation to bear the legacy of paternal stress, most of these studies have been rodent-centric and carried out in overly controlled settings in which stress has been experimentally imposed. While extremely important, these approaches leave a contextual vacuum in our understanding of the etiology of intergenerational influences of stress that occur de novo and without design in our daily lives.

To begin to fill this void in our understanding of how the germline may be affected in males directly exposed to ELS, we turned to a highly translational nonhuman primate (NHP) model that leverages naturally occurring early adverse caregiving (infant maltreatment) in rhesus macaques living in large social groups in semi-naturalistic settings (Drury et al., 2017; Howell et al., 2013, 2017; McCormack et al., 2006, 2009; Morin et al., 2019; Morin et al., 2020). We examined total RNA signatures in sperm of adolescent macaques and compared the total RNA profiles of males that experienced competent maternal care versus ELS (maltreatment) during infancy.

2 | METHODS

2.1 | Subjects and housing

Seven adolescent male rhesus macaques (*Macaca mulatta*) ages 5.5–6 years old were included in this study. These animals were part of a larger longitudinal study of developmental outcomes of ELS in 42 animals and were well-characterized throughout infancy and the juvenile pre-pubertal period (Drury et al., 2017; Howell et al., 2013, 2017; Morin et al., 2020). They were born and lived with their mothers and families in complex social groups at the Yerkes National Primate Research Center (YNPRC) Field Station breeding colony. Groups consisted of 75–150 adult females, their sub-adult and juvenile offspring, and 2–3 adult males. These groups were housed in outdoor compounds, with access to climate-controlled indoor areas. Standard high fiber, low fat monkey chow (Purina Mills Int., Lab Diets) and seasonal fruits and vegetables were provided twice daily, in addition to enrichment items. Water was available ad libitum.

Three of the subjects experienced ELS in the form of spontaneous maternal maltreatment (MALT), and the other four received competent maternal care (Control). In this model, infant maltreatment is defined by co-morbid experience of maternal physical abuse and rejection of the infant during the first three months of life, never exhibited by Control, competent mothers, which causes pain, emotional distress, and elevations in stress hormones (Drury et al., 2017; Howell et al., 2013, 2017; Maestripieri & Carroll, 1998; Maestripieri et al., 2000; McCormack et al., 2006, 2009, 2015). Each infant was randomly assigned at birth to be cross-fostered to a Control or MALT foster mother in an effort to disentangle and control for effects of prenatal and heritable factors that may confound the effects of ELS in the larger developmental study,

where groups were also counterbalanced by social dominance status and infants were assigned from different matrilines and paternities to provide genetic and social diversity (Drury et al., 2017; Howell et al., 2017; Morin et al., 2020). All seven animals included in this study were cross-fostered to a foster mother of the same biological group (Control or MALT); therefore, the prenatal and postnatal environments were congruent. Behavioral measures of maternal care were collected during the first 3 postnatal months to characterize competent care received by Control infants, and to quantify rates of maternal physical abuse and rejection rates received by MALT infants (see detailed description of the infant rhesus maltreatment model and behavioral methods in Drury et al., 2017; Howell et al., 2017). Interestingly, similar rates of abuse and rejection are exhibited by maltreating foster mothers towards foster infants than towards previous biological infants. Control mothers did not exhibit physical abuse or rejection towards cross-fostered infants from biological Control or MALT mothers in the larger developmental study. This suggests that maltreatment is a maternal trait, and not triggered by the infant.

At approximately 4 years of age, the seven adolescents were transferred to the YNPRC Main Station. Upon arrival, animals were pair-housed in home cages and fed Purina monkey chow (Ralston Purina), supplemented with fruit and vegetables daily, and water was available ad libitum. Environmental enrichment was provided on a regular basis. The colony is maintained at an ambient temperature of 22 ± 2°C at 25%–50% humidity, and the lights set to a 12-h light/dark cycle (lights on at 7 h; lights off at 19 h). Following several months of acclimation to the move and new housing environment, the animals underwent behavioral tasks, neuroendocrine assessments and MRI scans, before semen collection.

All procedures and animal care were in accordance with the Animal Welfare Act and the U.S. Department of Health and Human Services "Guide for the Care and Use of Laboratory Animals" and approved by the Emory Institutional Animal Care and Use Committee (IACUC).

2.2 | Semen collection

Sperm was collected during mid-adolescence (5.79 ± 0.09 years old). Subjects were previously trained, using positive reinforcement, to enter a vertical primate chair from their home cage. Semen samples were collected between 7:30–8:30 am under light sedation with ketamine (0.42–2.5 mg/kg BW IM) following previously published methods (Chan & Yang, 2009), and were kept at 37°C for ~20 min before sperm purification. Animals were then returned to their home cage and monitored until recovery from the effects of ketamine.

2.3 | Sperm purification

Semen was purified within 60 min of collection using the PureSperm® 40/80 density gradient technique (Nidacon International AB, Sweden). Establishing this gradient maintains the sperm at a pH of 7.4–7.8 and facilitates the separation of motile sperm from seminal plasma, bacteria, cell debris, and epithelial cells. Additionally, removal of immature sperm and lymphocytes decrease damage to DNA/RNA caused by reactive oxygen species. Purified sperm was stored at −80°C until sperm was collected from all males and RNA could be extracted across all samples simultaneously.

2.4 | RNA extraction and sequencing

RNA was extracted using a standard guanidinium thiocyanate-phenol-chloroform, or TRIzol® (Thermo Fischer Scientific) extraction protocol, which has been shown to be a robust method for total RNA extraction, especially isolation of microRNAs (Mraz et al., 2009). Extracted RNA was stored at −80°C before sequencing. Total RNA sequencing, including stranded library prep and treatment with Ribogone™ (Takara Bio USA Inc.) and alignment of RNA reads to the MacaM genome (Zimin et al., 2014) using STAR software (Dobin et al., 2013) was performed by the YNPRC Genomics Core.

2.5 | Bioinformatics analysis

Following alignment, the open source SAMtools utility package was used to efficiently retrieve and count all reads aligning to loci in the MacaM gene annotation file using featureCounts (Li et al., 2009). To determine read coverage, reads were first separated based on alignment to the forward or reverse strand, using SAMtools. The coverage of each exon in the MacaM annotation by these reads was then calculated, using the bedtools coverage function on each stranded. bam file. Sense read coverage was calculated from forward strand reads that aligned to genes read on the + strand, or reverse strand reads that aligned to genes read on the – strand. Antisense read coverage was calculated from the inverse, forward strand reads that aligned to genes read on the – strand, or reverse strand reads that aligned to genes read on the + strand.

Gene Ontology (GO) Enrichment Analyses were performed with biological processes gene sets within the Macaca mulatta reference list to identify broad cellular and molecular pathways where RNA in sperm may play a role. The top 100 genes by expression among Control animals were included in the analysis, with sense and antisense reads analyzed separately, to examine normative biology of sperm.

A differential expression analysis was performed using Limma-Voom (Law et al., 2014) to examine group differences. A powerful approach was taken, only analyzing genes with > = 80% coverage across groups (determined separately for sense and antisense reads), to reduce multiple comparison burden. Based

on this threshold, 463 and 64 genes were analyzed in the sense and antisense datasets respectively.

3 | RESULTS

3.1 | Mapping

Alignment to the MacaM genome was successful, and the majority of reads were mapped to either one or multiple loci (Figure 1a). Across the seven animals, there were 53,340,938.71 ± 6,230,299.43 total reads, 45,765,685.29 ± 5,520,314.97 uniquely mapped reads, and 6,764,395.57 ± 857,255.73 reads mapped to multiple loci (reported as mean ± standard error of the mean). Sperm-specific transcripts (PRM1, PRM2, TNP2) were 100% covered by sequencing in both control and maltreated animals (Figure 1b). Coverage of coding genes by sequencing (including both sense and antisense) were 81.00 ± 1.63% for control and 85.24 ± 7.78% for maltreated animals (mean ± *SEM*) (Figure 1c). Coverage and expression levels were found to be positively correlated (sense: control [$r = .39$, $p < .0001$], maltreated [$r = .38$, $p < .0001$]; antisense: control [$r = .44$, $p < .0001$], maltreated [$r = .44$, $p < .0001$]), suggesting that high expressing genes were more likely to have high coverage (Figure 1d).

3.2 | GO analysis

The top 100 expressed genes of sense and antisense reads in control animals include sperm-specific transcripts PRM1 and PRM2, which are more highly expressed compared to other genes among sense reads (Tables S1 and S2). Pathways that are enriched by the top 100 expressing genes of sense reads include many expected

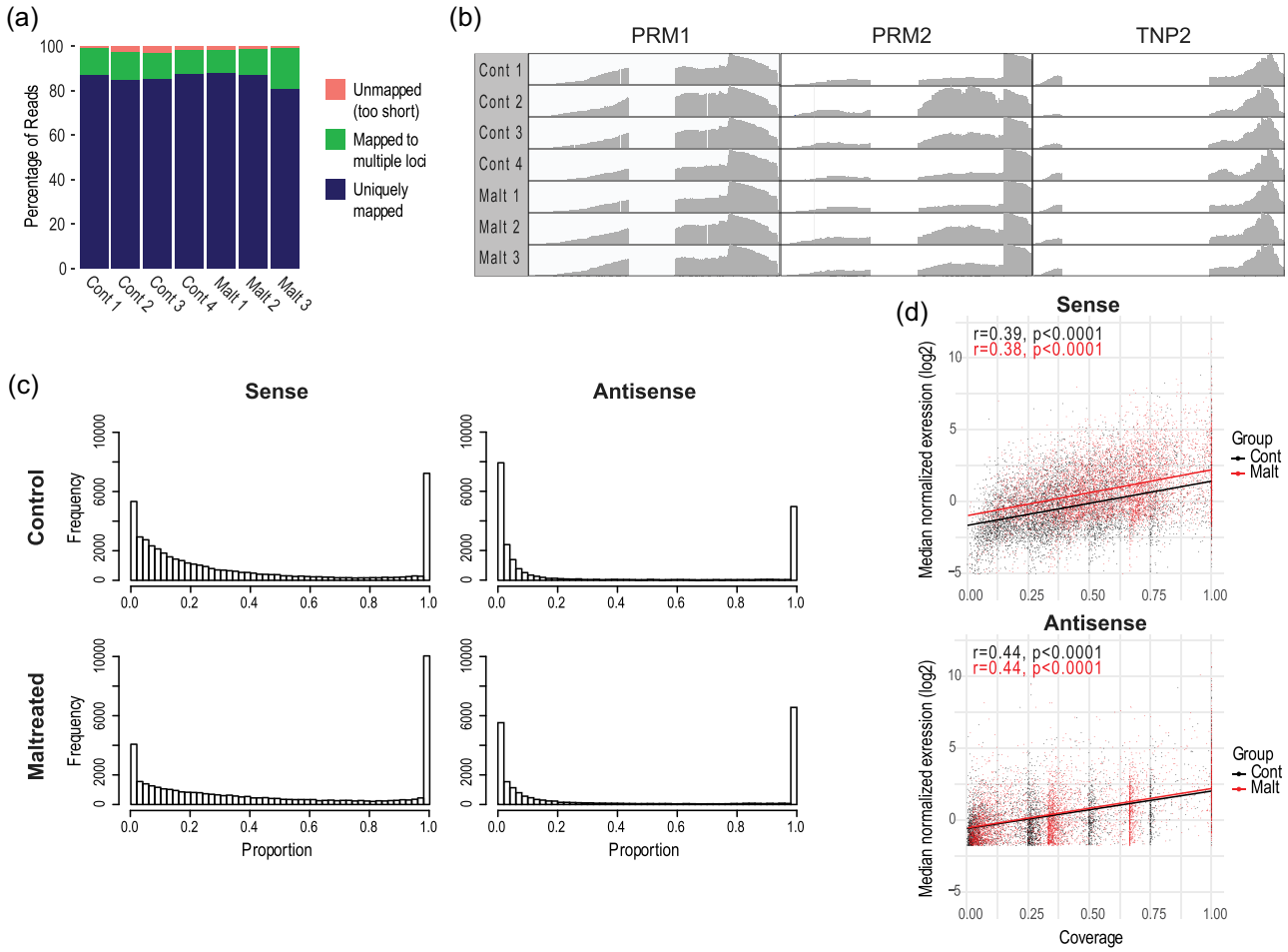

FIGURE 1 Quality control analysis of sequencing illustrates successful mapping and greater gene coverage by reads among highly expressed genes. (a) Mapping of reads to MacaM genome by STAR alignment with >97% mapped reads, of which >80% are uniquely mapped, across all animals. (b) Sperm-specific transcripts in the MacaM genome have complete coverage by aligned reads across all animals. Genome locations visualized (chromosome: start, stop sites) are as follows: PRM1—chr16:11290482-11290981; PRM2—chr16:11284765-11285608; TNP2—chr16:11276903-11278394. (c) The distributions of coverage (proportion of base pairs) across genes, stratified by sense and antisense reads and group, and summed across individuals, reveals a large population of genes with high to full coverage. (d) Coverage and expression levels were found to be moderately positively correlated, suggesting that high expressing genes were more likely to have high coverage

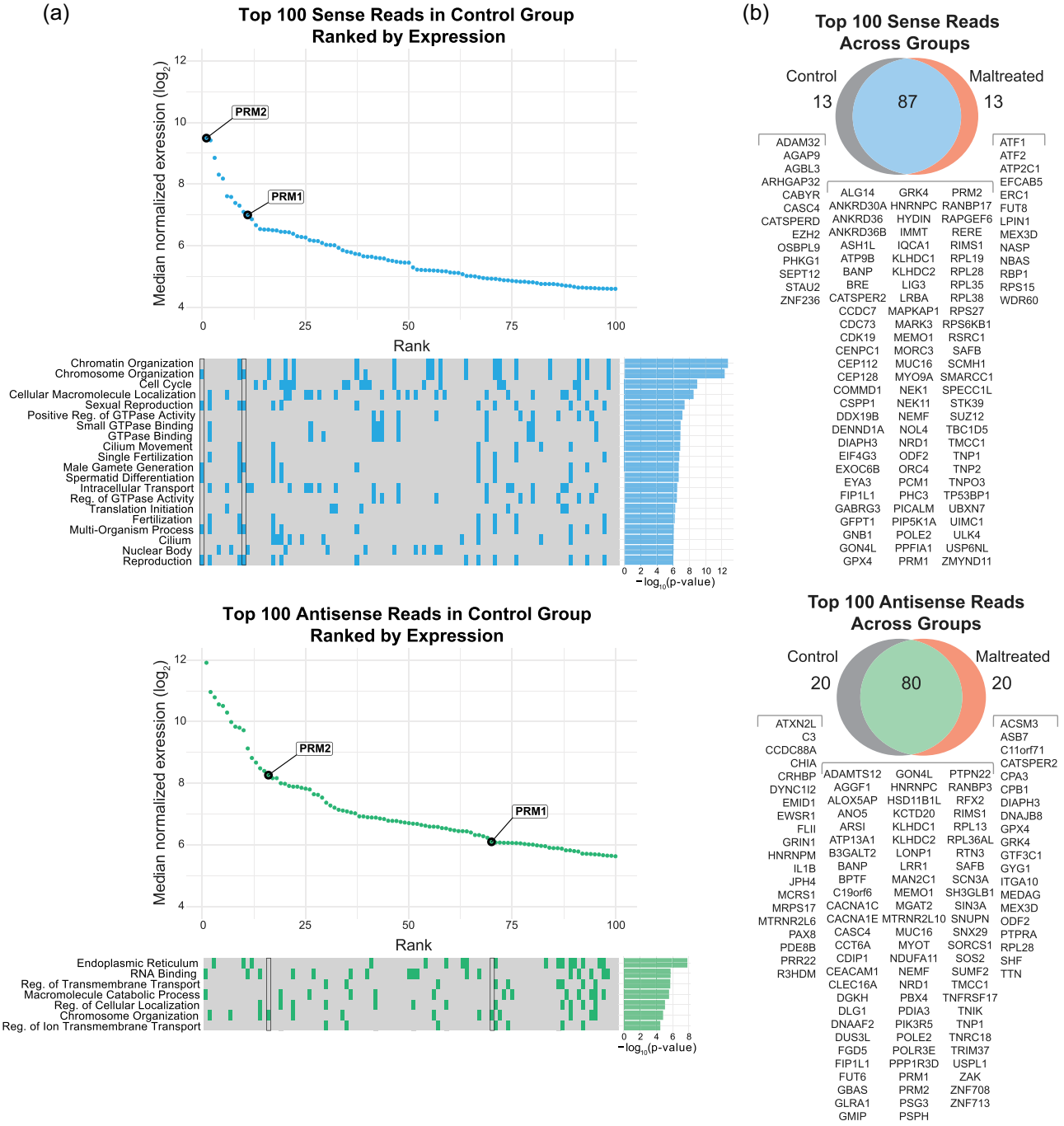

FIGURE 2 Biological analysis of reads examining enriched gene sets among high expressing genes. (a) The top 100 expressing genes in the control group, which include sperm-specific transcripts PRM1 and PRM2, are plotted by rank, with sense and antisense reads visualized separately. Directly below each gene, the enriched gene sets identified by GO analysis for which this gene is included, are highlighted. Many significantly enriched gene sets among sense reads are relevant for fertilization, reproduction, sperm generation. (b) Venn diagrams show common and unique genes between the top 100 expressed genes in each group, stratified by sense/antisense reads

germline-relevant mechanisms, such as sexual reproduction, single fertilization, male gamete generation, spermatid differentiation, fertilization, and reproduction (Figure 2a). Of the top 100 highest expressing genes, there is significant overlap between groups (87/100 for sense reads, and 80/100 for antisense reads) (Figure 2b).

3.3 | Differential expression analysis

There were no genes found to be differentially expressed over and above what would be expected by random chance among either sense or antisense reads (Tables S3 and S4).

4 | DISCUSSION

Here we used a translational NHP model of ELS caused by naturally-occurring infant maltreatment in macaques to (1) profile total RNA in the adolescent male germline for the first time and (2) to identify how those RNA profiles are affected by exposure to ELS. We found that the top 100 genes for which RNA sequences aligned to the sense strand were relevant to sperm-related biology and reproduction. While these data might seem unsurprising, they demonstrate the validity and feasibility of profiling RNA in macaque sperm and provide us with confidence that the nature and analyses of our RNA sequencing is not spurious. We posit that such quality control of our data is a needed component for future analyses that may seek to profile RNA in NHP sperm. Most studies of RNA in male sperm in rodents and humans have focused on small noncoding RNA that we did not profile. To do so, we would have to specifically enrich for short RNA and then generate libraries for sequencing. Therefore, it is challenging to compare and contrast our total RNA sequencing data from the macaque with small noncoding RNA profiles found in rodents and humans. Future analyses across species would provide information about RNA signatures and biological processes that could influence normative development in an evolutionarily conserved manner.

In our analyses, we found sequences that aligned to the antisense strand of genes. While we cannot definitively say what the functional roles of these antisense transcripts are in sperm, antisense transcripts play an important role in regulation of gene expression via various molecular mechanisms (Khorkova et al., 2014; Modarresi et al., 2012; Nishizawa et al., 2015; Pelechano & Steinmetz, 2013; Villegas & Zaphiropoulos, 2015) and it is very plausible that these antisense transcripts serve to orchestrate gene expression in the zygote soon after fertilization. In the differential expression analysis based on expressed genes with good gene coverage, we did not identify any differentially expressed genes between groups, so we did not pursue any gene-set or pathway analyses. While our small sample sizes may have precluded definitive assessment of stress-induced alterations of RNA in sperm of macaques that experienced ELS, our study sets the foundation for future studies that examine how early adverse experiences might alter the male germline, across species and in experimental protocols that involve anthropogenic vs natural stressors.

Of all the molecular entities contained in sperm that include but are not limited to histone modifications, DNA methylation, and RNA, we sought to profile RNA because of the accumulating literature from rodent studies that demonstrate sperm-located RNA to be robust intergenerational transducers of acquired parental experiences (Aoued et al., 2019; Chen et al., 2016; Gapp et al., 2014; Rodgers et al., 2015; Sharma et al., 2016). Most of these studies have focused on microRNA and tRNA fragments as playing important roles in passing on intergenerational influences of paternal stress. However, given that a recent case has been made for RNA in sperm that are >200 bp being important for behavioral consequences of paternal stress in offspring (Gapp et al., 2020) and the lack of a specific reference library for macaque microRNA, we decided to focus on sequencing total RNA rather than specifically focusing on short noncoding RNA. We intended for this study to inform us of how RNA in the male germline in a NHP species responds to ELS. Our focus on total RNA and the lack of significant group differences leaves open the possibility that RNA may not the main molecular entity in sperm that may register exposure to ELS and suggests the need for future sequencing to query not only short noncoding RNA, but also DNA methylation and histone modifications in sperm. Missing from our analyses is a profile of RNA in eggs of female rhesus macaques that had experienced maltreatment as infants. With literature demonstrating the transmission of behavioral and physiological legacies of infant maltreatment along maternal lineages of rhesus macaques (Drury et al., 2017; Klengel et al., 2019), understanding how the female germline responds to ELS will undoubtedly provide new clues as to how intergenerational legacies of stress perpetuate across generations.

Alternate explanations for not finding differences between the groups may be that the time between exposure to ELS in infancy and the maturation of the adolescent male germline may be long enough for alterations of RNA in the immature germline to be transient, short-lived and labile and therefore no longer present in adolescence. Also, all animals were raised at the Yerkes National Primate Research Center Field station and subsequently moved to the Main Center at about 4 years of age, resulting in relocation stress. The fact that both groups were exposed to this stress may have neutralized some group differences that would otherwise have been observed in its absence.

Our study was motivated by the intent to understand how the male germline responds to ELS in a translational model of infant maltreatment that occurs spontaneously in rhesus macaques (Drury et al., 2017; Howell et al., 2013, 2017; McCormack et al., 2006, 2009; Morin et al., 2019; Morin et al., 2020). Our lack of significant group differences might question the utility of trying to publish these data. Two points are worth noting to address this criticism. First, this was a pilot, exploratory, study with a very small sample size. Second, publication bias and only publishing positive results are concerns that undermine confidence in science (Bespalov et al., 2019; Button et al., 2013; Joober et al., 2012; Turner, 2013), and the need to reject such publication bias motivate our impetus to publish our work. Third, no studies to our knowledge have attempted to profile the germline of rhesus macaques, and the rodent-centric studies that have done so used experimentally-induced stress. Therefore, our profiling of RNA in sperm of rhesus macaques informs future analyses that could be conducted with the intent of comparing and contrasting how the paternal germline responds to stress in NHP, across species and in experimental protocols that involve anthropogenic vs natural stressors.

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CONFLICT OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Elyse L. Morin, Kristie M. Garza, Brian G. Dias, and Mar M. Sanchez conceived and planned the experiments. Elyse L. Morin, Erin R. Siebert and Hadj Aoued collected and purified samples. Hadj Aoued, Soma Sannigrahi, and Kristie M. Garza optimized the RNA extraction methods, and Kristie M. Garza performed the RNA extraction. Mar M. Sanchez designed the larger longitudinal developmental study that these animals were a part of and generated the animals with Brittany R. Howell. Elyse L. Morin performed the analysis and interpreted results with Hasse Walum and Brian G. Dias. Elyse L. Morin and Brian G. Dias wrote the manuscript in consultation with Hasse Walum. All authors commented on the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data sets generated and code used in the analysis for this study can be found at GEO Accession No. GSE168978 and https://github.com/elmorin/Monkey_Sperm_RNAseq.

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