Aging induces profound changes in sncRNA in rat sperm and these changes are modified by perinatal exposure to environmental flame retardant

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Abstract

Advanced paternal age at fertilization is a risk factor for multiple disorders in offspring and may be linked with age-related epigenetic changes in fathers sperm. Understanding of aging-related epigenetic changes in sperm and environmental factors that modify such changes is needed. Here we characterize changes in sperm sncRNA between young pubertal and mature rats. We also analyze modification of these changes by exposure to environmental xenobiotic 2,2',4,4'-tetrabromodiphenyl ether (BDE-47). SncRNA libraries prepared from epididymal spermatozoa were sequenced and analyzed using DESeq 2. Distribution of small RNA fractions changed with age, with fractions mapping to rRNA and lncRNA decreasing and fractions mapping to tRNA and miRNA increasing. 249 miRNA, 908 piRNA and 227 tRNA-derived RNA were differentially expressed (2-fold change, FDR $p \leq 0.05$) between age groups in control animals. Differentially expressed miRNA and piRNA were enriched for protein-coding targets involved in development and metabolism, piRNA were enriched for LTR targets. BDE-47 accelerated age dependent changes in sncRNA in younger animals, decelerated these changes in older animals and increased the variance in expression of all sncRNA. Our results indicate that the natural aging process has profound effects on sperm sncRNA profiles and this effect may be modified by environmental exposures.

Key words: aging, paternal exposure, sperm, semen, epigenetics, sncRNA, piRNA, miRNA, 2,2′,4,4′-tetrabromodiphenyl ether, PBDE, BDE-47, perinatal, environment.

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1. Introduction

There is a growing trend of delayed parenthood in developed countries as a consequence of increased life expectancy, socioeconomic pressures, and rates of divorce and remarriage. For example, in the USA a 50% increase over the past 30 years has been reported in the number of men fathering children in the age range of 35-44 years (Martin, J. A. et al., 2012) and the mean paternal age has increased over the past 44 years from 27.4 to 30.9 years (Khandwala, Zhang, Lu, & Eisenberg, 2017). Although self-renewal and differentiation of spermatogonial stem cells permits the production of mature sperm throughout the adult life-course, this benefit of continued spermatozoa production may also come at an expense of the accumulation of genetic and epigenetic errors over the life-course, that may in turn, have downstream consequences for the health and development of offspring.

Epidemiologic evidence demonstrates that advanced paternal age is associated with a host of offspring adverse health outcomes. The adverse conditions associated with increased paternal age at conception include stillbirths (Nybo Andersen et al., 2004, Alio et al., 2012), musculoskeletal syndromes (Urhoj, Mortensen & Nybo Andersen, 2015), cleft palate (Bille et al., 2005), acute lymphoblastic leukemia (Sergentanis et al., 2015, Dockerty et al., 2001), retinoblastoma (Moll et al., 1996, Heck et al., 2012), and polygenic neurodevelopmental and psychiatric disorders (Nybo Andersen, Urhoj, 2017), such as schizophrenia (Liebenberg et al., 2016, Malaspina et al., 2001), autism spectrum disorders (Reichenberg et al., 2006, Hultman et al., 2011), bipolar disorder (Frans et al., 2008) and attention deficit/hyperactivity disorder (D'Onofrio et al., 2014); however, the causal link between paternal age and offspring health is not yet well understood.

Spermatozoa have been traditionally deemed vehicles for the transfer of the paternal haploid genome upon fertilization; however, epigenetic marks on sperm (e.g., DNA methylation, histone modifications and small noncoding RNA) can act as a legacy of environmental exposures encountered throughout the life-course that affect early-life development and offspring phenotype (Wu et al., 2015, Marcho, Oluwayiose & Pilsner, 2020). To that end, it has been hypothesized that altered offspring phenotype is associated with age-related accumulation of epigenetic changes in sperm of fathers (Sharma, R. et al., 2015, Perrin, Brown & Malaspina, 2007).

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Increasing evidence indicates that sperm sncRNA is an important vehicle connecting paternal experiences with phenotypes of their offspring (Chen, Yan & Duan, 2016, Marcho, Oluwayiose & Pilsner, 2020, Yan, 2014). The most elaborate research connects paternal diet, composition of sperm sncRNA and offspring development. For example, protein restriction in mice affected sncRNA levels in sperm, including decreased let-7 miRNA and altered composition of tsRNA (Sharma, U. et al., 2016). Specifically, in that study, changes in sperm load of tRF-Gly-GCC were associated with endogenous retroelement MERVL expression in early embryos. In mouse experiments, high-fat diet changed expression of X-linked miRNA (McPherson et al., 2015) and tRNA derived small RNAs (tsRNA) (Chen et al., 2016). Injection of these tsRNAs into normal zygotes altered metabolic pathways in embryo (Chen et al., 2016). Similarly, injection of sperm RNA from mice fed a Western-like diet into naive one-cell embryos produced changes in metabolic phenotype of the resulting progenies normally induced by Western-like diet (Grandjean et al., 2015).

High-fat diet induced obesity in mice was associated with altered expression of around 400 miRNA (Fullston et al., 2013). Interestingly, in this study, paternal phenotype characterized by obesity and insulin resistance was transferred in 2 consecutive generations of both sexes offspring. In another mouse study, high-fat diet altered sperm levels of 15 miRNA, 41 tsRNA and 1092 piRNA (de Castro Barbosa et al., 2015). Contrary, to previous research F1 and F2 female offspring of treated fathers were resistant to high fat diet. Connection between obesity and sperm profile of sncRNA is supported by human research as well. For example, in one study lean and obese men had significantly different levels of 37 piRNAs in their spermatozoa (Donkin et al., 2016).

The ability of chemical exposures to affect composition of sncRNA in sperm only started to emerge. For example, in mice chronic ethanol exposure altered several sncRNAs, including tsRNAs, mitochondrial small RNA, and miRNA (Rompala et al., 2018). The same study reports also that tsRNAs were similarly altered in sperm and epididymosomes, supporting the hypothesis that altered tsRNAs are loaded to spermatozoa in epididymis (Vojtech et al., 2014). To our knowledge, effects of paternal exposures to environmental xenobiotics on the composition of sperm sncRNA were not yet addressed by existing research. Similarly, there is a knowledge gap in the understanding of the role of paternal age for the sperm load of sncRNA.

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Given the general trend of delayed parenthood in developed countries, it is important to clarify the effect of paternal aging on the sperm epigenome, as well as the impact of environmental exposures on age-related changes in the sperm epigenome. In the current study, we first examine the effect of aging on the sperm small RNA by comparing sncRNA profiles in control rats on PND65 and PND120, corresponding approximately to young pubertal and mature men, respectively (Robb, Amann & Killian, 1978, Zanato et al., 1994). Furthermore, we analyze how observed age-dependent changes in sncRNA are modified by perinatal exposure to an ubiquitous environmental flame retardant 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in relevant doses to background human exposures. Our results indicate that the natural aging process has profound effects on sperm profiles of sncRNA and these effects may be modified by environmental exposures.

2. Materials and Methods

2.1. Animals and Treatment

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at University of Massachusetts, Amherst (protocol #2013-0069). Seven-week-old Wistar rats were purchased from Charles River Laboratories (Kingston, NY, USA) on the sixth day of pregnancy, housed in a temperature- and humidity-controlled room with a 12-h light cycle and maintained at 23±2 °C. All rats were fed ad libitum with a rodent chow (Prolab Isopro RMH 3000, Cat. # 5P75, LabDiet, St. Louis, MO). Between pregnancy day 8 and postnatal day 21 (PND21) dams were fed daily from the tip of a pipette 0.2 µl/g body weight of vehicle (tocopherol stripped corn oil, MP Biomedicals, Solon, OH) or same volume of 1 mg/ml solution of BDE-47 (AccuStandard, Inc., New Haven, CT; 100% purity) daily (*n=6* per exposure group). In the latter group, it resulted in exposure level of 0.2 mg/kg body weight BDE-47 per day. This method of exposure was developed to substitute oral gavage, which induces significant stress response and may interfere with analyzed health outcomes (Vandenberg et al., 2014). The litters were not culled after delivery to avoid catch-up growth that may be associated with significant increase in nutrient availability following culling (Suvorov, Vandenberg, 2016). Pups were weaned on PND21. On PND65 and PND120 one male pup was randomly selected from each litter

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fasted for 2 hours and euthanized using cervical dislocation. Other pups were used in a different study. All euthanasia was done during morning hours, between 9 and 10 am. At each euthanasia both distal cauda epididymis were collected, incised longitudinally and incubated at 37°C for 30 min in 1 ml of sperm wash buffer (Cat. # ART1006, Origio, Denmark) to collect motile spermatozoa via the swim-up procedure. All animal experiments were performed at the University of Massachusetts, Amherst.

2.2. Extraction of sperm RNA

For sperm sncRNA analysis, we used sperm collected from 6 animals per time-point per exposure group. All animals were randomly selected from individual litters, one animal per each litter. To remove somatic cells contamination spermatozoa samples were loaded on top of density gradient (40% Isolate, Irvine Scientific, USA) and centrifuged for 25 min at 500 g. Pelleted spermatozoa were used to extract RNA using a protocol described elsewhere (Goodrich, Anton & Krawetz, 2013) with modifications. In short, pelleted spermatozoa were homogenized for 5 min using max speed setting of Disruptor Genie (Scientific Industries) and glass sand in 0.5 ml of RLT buffer with 7.5 µl β-mercaptoethanol. Homogenate was mixed with 0.5 ml QIAzol (Cat. # 79306, Qiagen) and subjected to 2 more minutes of homogenization with the same settings. Homogenate was than mixed with 0.22 ml of chloroform, incubated for 3 min at room temperature and centrifugated at 12000 rpm, 4°C, 15 min. Aqueous phase was collected and mixed with 1.5x volume of 96% ethanol and RNA was purified using RNeasy Mini Kit (Cat. # 74104, Qiagen) protocol with DNase digestion step (RNase-Free DNase Set (50), Cat. # 79254, Qiagen). Purified RNA was eluted in 30 µl of nuclease-free water and 1 µl of DTT and 0.5 µl of RNase Block (Cat. # 12091021, Stratagene) were added immediately after purification.

2.3. Preparation of sncRNA libraries and sequencing

Libraries of small RNA were constructed using NEBNext Multiplex Small RNA Library Prep Set for Illumina (Cat. # E7330, New England BioLabs) as per the manufacture's guidelines, with size-selection of 147-160 nucleotides fragments using high-resolution gel containing 0.8% agarose, 0.4% polygalactomannan and 1.6% γ-polygalactomannan in TAE buffer. Libraries were sequenced on NextSeq 500 (Illumina). RNA extraction,

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library preparation and sequencing were performed in the Laboratory of Evolutionary Genomics, A.N. Belozersky Research Institute of Physico-Chemical Biology, Moscow State University.

2.4. Identification of differentially expressed small RNA

Reads quality was checked using FastQC (Andrews, 2010). Adapters trimming was done using cutadapt (Martin, M., 2011). Reads without adapters were first mapped to UniVec (Rigden, Fernandez-Suarez & Galperin, 2016) contaminants database to filter out non-rat sequences. At the next step, ribosomal RNAs were filtered out by aligning the remaining readings to the SILVA v132 rat ribosomal RNA database (Quast et al., 2013). Remaining reads were aligned in the following order: miRNA > piRNA > tRNA. After each step, only unmapped reads from previous step were used in analysis. Reads were mapped to rat miRBase v22 for miRNA (Kozomara, Birgaoanu & Griffiths-Jones, 2019), piRNAdb v1.7.5 for piRNA (www.pirnadb.org) and GtRNAdb v18.1 for tRNA (Chan, Lowe, 2016) using bowtie aligner (Langmead et al., 2009), samtools (Li et al., 2009) for SAM/BAM data manipulation and bedtools (Quinlan, Hall, 2010) for mapped reads counting. If a read was mapped to several small RNAs, it was assigned randomly to one of sequences. 3'CCA sequences were not removed prior to tRNA mapping. Raw read counts for MicroRNAs , tRNAs and for piRNAs were loaded into DESeq2 (Love, Huber & Anders, 2014) R package, processed by variance-stabilizing transformation (VST) (Anders, Huber, 2010) and values of differential expression were identified using default independent filtering and Cook's cut-off parameters of DESeq2 package (Bourgon, Gentleman & Huber, 2010). VST counts were used for samples relationship visualization using PCA and heatmap.

2.5. Statistical analysis

Differences in distribution of sncRNA subtypes were evaluated using Chi square test. Variance in expression values in each group was analyzed using T. Test. Pearson's correlation and T. Test were used to compare expression values and fold change values of significant age dependent sncRNA between exposure groups. Fisher Exact Test was used to analyze enrichment of piRNA targeting different genomic elements. Statistical analysis listed in this paragraph was done using Microsoft Excel 2016 (16.0.5056.1000).

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2.6. Functional analyses

For the enrichment analysis based on the differential expression of miRNA, the list of significantly differentially expressed miRNAs (fold change ≥ 2 or ≤ -2 and FDR adjusted $p \leq 0.05$) was first uploaded into miRDB, an online database for miRNA target prediction (Liu, Wang, 2019, Wong, Wang, 2015), to identify gene-targets of the miRNAs. Targets with prediction score ≥ 80 were selected for enrichment analysis as recommended by miRDB developers. To identify molecular pathways enriched with predicted targets Metascape (Tripathi et al., 2015) was used with default settings.

To analyze functional significance of changes in piRNA expression, we first analyzed enrichment of piRNA targeting different transposable elements (LINE, SINE, LTR and satellite) and protein coding genes using Fisher Exact test. Data on piRNA targets were downloaded from piRBase, which maintains a manually curated annotation of more than 4 million rat piRNA (Wang et al., 2019).

3. Results

We found no significant relationship between litter size and exposure group, with the number of pups varying from 10 to 15 per litter. Litter size was 12.33 ± 0.51 in control group and 12.71 ± 0.56 in BDE-47 exposed group (all data are for mean ± SE). No weight differences were observed between the control and exposed dams or pups throughout the experiment.

3.1. Changes in the profile of small RNA expression

Sequencing was completed with an average 16.5 million reads per sample with a range 6.5 – 23.3 million reads and 80% average alignment to the reference genome. Sequenced fragments were distributed in a range between 16 and 46 nucleotides. Distribution of different types of non-coding RNA as analyzed using RNAcentral shows high consistency across samples within age groups (Fig. 1A). Distribution of sncRNA subtypes was different between age groups (Chi-square p = 8.91E-13 for controls and 3.70E-05 for BDE-47 exposed groups). Age-related changes were similar for both, exposed and control groups Fig. 1A. Percent reads mapping to rRNA and lncRNA decreased with age from 46.2±2.5% to 37.1±2.3% for rRNA and from 24.4±1.3% to 16.3±2.0% for lncRNA (mean±SE). Percent reads mapping to tRNA, precursor miRNA and miRNA increased with age from 9.8±1.5%

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to 16.5±1.5% for tRNA, from 2.4±0.4% to 9.4±1.8% for precursor miRNA and from 0.6±0.1% to 3.2±0.7% for miRNA. Percent reads of piRNA did not change significantly with age (16.6±1.6% on PND65 and 17.7±1.5% on PND120). Distribution of reads mapping to different RNA types was not significantly different between exposure groups in each age (Chi square p = 0.68 for PND65 and 0.69 for PND120).

Principle component analysis of all 24 biological samples based on miRNA expression shows clear grouping of control animals by age (Fig. 1B). Samples collected from exposed animals do not show clear grouping. Similarly, control samples cluster by age in a heatmap of the top 100 miRNAs with the highest standard deviations, while exposed samples do not form clear age-related clusters (Fig. 1C)

Values of differential expression were identified for 1,038 individual miRNAs and precursor miRNAs, 37,459 piRNA and 444 tRNA (Supplemental Table 1) using default independent filtering and Coock's cut-off parameters of DESeq2 package (Bourgon, Gentleman & Huber, 2010). Given that the distribution of RNA sizes in our study does not cover precursor miRNA and tRNA, identification of these molecules is likely due to the presence of their fragments in our samples or due to multiple mapping of shorter RNA, such as mapping of miRNA to precursor miRNA. Numbers of individual sncRNA significantly differentially expressed (fold change ≥ 2 or ≤ -2 and FDR adjusted p ≤ 0.05) in age and exposure groups are shown in Table 1. Age has the highest effect on non-coding RNA expression (1.384 differential expressed sncRNA in control group) while exposure has only minor effect (1 and 24 differential expressed sncRNA on PND65 and 120 respectively). Interestingly, exposure seemingly attenuates effect of age on RNA expression, (1.384 in control group vs 165 in exposed group).

Table 1. Number of individual miRNA, piRNA and tRNA significantly (FDR adjusted p ≤ 0.05) 2-fold differentially expressed in rat sperm in relation to age and exposure to BDE-47.

	Effect of age		Effect of exposure	
	Control	BDE-47 exposed	PND65	PND120
miRNA*	249	68	1	18
piRNA	908	44	0	0
tRNA	227	53	0	6

Footnote: * miRNA and precursor miRNA

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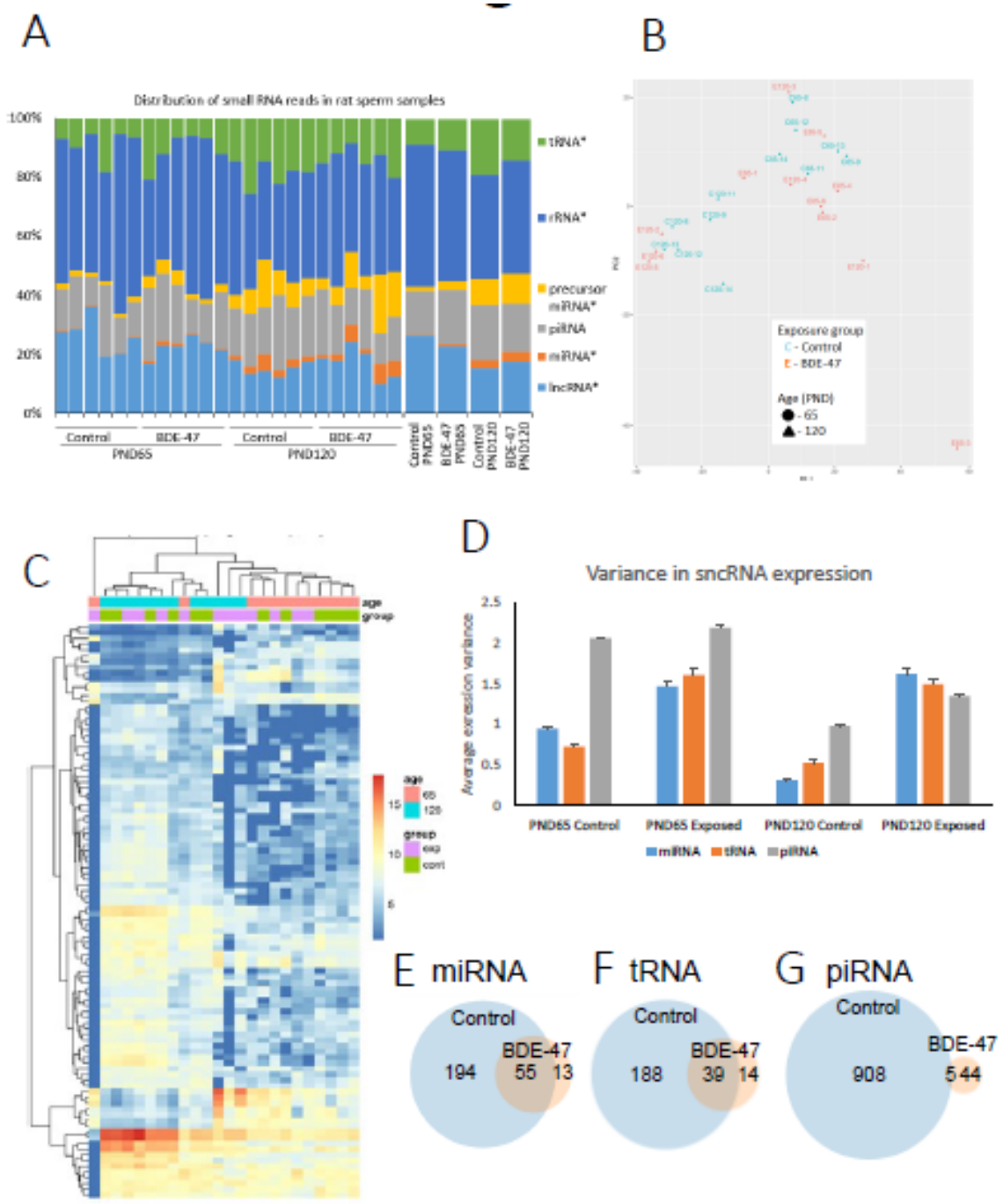

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306 Figure 1. Age and BDE-47 dependent changes in profiles of sncRNA expression; A –
307 distribution of different types of RNA fractions across biological samples, B – PCA plot of
308 all biological samples based on miRNA expression, C – expression heatmap and
309 hierarchical clustering of biological samples based on expression of 100 miRNA with top
310 highest standard deviations, D – variance of sncRNA expression in age and exposure
311 groups, E-G – overlap of differentially expressed miRNA (E), tRNA (F) and piRNA (G) in
312 control and BDE-47 exposed animals.

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We hypothesized that the later observation may be at least partly due to the fact that exposure increases variance in expression levels across biological replicates; and therefore, smaller number of individual RNAs passes our threshold of FDR adjusted p-value. To test this hypothesis, we compared variance in expression values across age and exposure groups (Fig 1D). This comparison shows that indeed exposure increases variance of non-coding RNA in both age groups. It is difficult to infer if increased variance is the only factor responsible for the decreased number of significantly differentially expressed age-dependent RNA. To test if additionally exposure attenuates effect of age we compared across all groups expression values of age-dependent sncRNAs significantly altered between two age groups in control animals (Supplemental Figures 1-3).

According to the distribution of expression values, exposure to BDE-47 produces changes concordant with accelerated aging in younger animals and attenuates the effect of age in older animals. Specifically, expression of miRNA and piRNA undergoing age-dependent suppression in control rats is lower in exposed animals on PND65 than in controls of the same age (Supplemental Figures 1C and 2C). Likewise, piRNA undergoing age-dependent increase in expression have higher levels of expression in young exposed animals then in young control animals (Supplemental Figure 2A). Thus, exposure induced changes in expression of these groups of sncRNA in young rats resemble age-dependent changes in control animals. Similarly, exposure attenuates age-dependent changes in some groups of sncRNA. In particular, miRNA and piRNA undergoing age-dependent increase in expression have lower levels of expression in older exposed animals as compared with control rats (Supplemental Figures 1B and 2B). Same trend is seen for tRNAs (Supplemental figure 3).

Among significant age-dependent sncRNA, 55 miRNA, 39 tRNA and 5 piRNA overlapped between control and exposed groups (Fig 1E-G). Out of the 55 overlapping miRNA, 52 underwent age-dependent changes in the same direction in both exposure groups and for 3 miRNA, the direction of change was opposite in control and exposed groups. All overlapping tRNA and piRNA had the same direction of age-dependent change in control and exposed groups. Pearson's correlation of Log2 fold change values in exposed and control animals was 0.61 for the list of overlapping miRNA, and 0.96 for overlapping tRNA or piRNA (Supplemental Figure 4B,D,F). To check if in both exposed and control animals age-dependent changes occur in the same direction, we also calculated Pearson's

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correlation of Log2 fold change values for merged lists of sncRNA that were significantly differentially expressed in either exposure group or both. Pearson's correlation was 0.60 for the merged list of 262 miRNA, 0.78 for the merged list of 241 tRNA, and 0.52 for the merged list of 947 piRNA (Supplemental Figure 4A,C,E).

Exposure to BDE-47 altered expression of only 1 miRNA on PND65 (Supplemental Table 2), which was upregulated 18-fold in exposed animals. On PND120, 18 miRNA were significantly upregulated in response to exposure (Supplemental Table 2). Changes in expression of these RNAs on PND65 and 120 were not consistent (Supplemental Table 2). Six tRNA were significantly differentially expressed in response to BDE-47 on PND120. However, no tRNA passed a threshold of significance on PND65, although expression of 4 tRNA out of 6 differentially expressed on PND120 were changed in the same direction (Supplemental Table 2). No piRNA passed the threshold of significance on either time-point.

Interestingly, the majority of sncRNA altered in response to exposure (1 out of 1 miRNA changed on PND65, 16 out of 18 miRNA changed on PND120 and 6 out of 6 tRNA changed on PND120) were in the merged list of significant age-dependent RNA, i.e they were differentially expressed between PND65 and 120 in control or exposed groups or both.

3.2. Functional analysis of age-dependent changes in miRNA expression

We first uploaded to miRDP database (Liu, Wang, 2019, Wong, Wang, 2015) the list of 249 age-dependent miRNAs differentially expressed in control animals, and identified 4908 genes – targets of miRNA with prediction score ≥ 80. Many identified genes were targets of several differentially expressed miRNA (Supplemental Table 3). Given that Metascape can analyze only datasets not exceeding 3000 genes, we uploaded to this tool a list of 2654 genes, targets of at least 2 miRNA. This list was highly enriched with high statistical significance (-log 10(P) ranged 14-23) for a broad range of developmental categories (Fig. 2A). To add detail to this analysis, we further restricted it to genes-targets of ≥ 5 miRNA undergoing age-dependent increase or decrease in expression.

Targets of upregulated miRNA were enriched for different categories related to embryonic and other development (ex.: axon development, appendage development, sensory organ development, odontogenesis), apoptosis and cell cycle (ex.: cell death signaling via

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NRAGE, NRIF and NADE, positive regulation of cell cycle, leucocyte apoptotoic process), lipid metabolism (ex.:NR1H2 and NR1H3-mediated signaling, regulation of lipid metabolic process) and oxidative stress (response to redox state) (Supplemental figure 5A). Targets of downregulated miRNA were also enriched for developmental categories (ex.: embryonic organ development, response to growth factor, reelin pathway), oxidative stress (AGE-RAGE signaling pathway in diabetic complications), and cell cycle (positive regulation of mitotic cell cycle), as well as for metabolism of carbohydrates (ex.: glucose transmembrane transport, response to carbohydrate) and other metabolism regulation (ex.: positive regulation of cold-induced thermogenesis, fat cell differentiation) and transcriptional and posttranscriptional regulation (ex.: poly(A)+ mRNA export from nucleus, transcriptional regulation of pluripotent stem cells, production of miRNA involved in gene silencing in miRNA) (Supplemental figure 5B).

The majority of differentially expressed miRNA between PND65 and PND120 in exposed animals overlapped with age-dependent miRNA in control animals (Fig. 1E). Therefore, in the next step, we focused on 55 significantly differentially expressed miRNA between PND65 and PND120 in both control and BDE-47 exposed animals. Using miRDP database, we identified 2261 genes-targets of these 55 miRNA with prediction score ≥ 80. The use of the entire list of these genes-targets for Metascape analysis showed high enrichment with high statistical significance (-log 10(P) ranged 12-27) of mostly the same developmental categories as these affected by age in control animals (Fig 2B, C).

3.3. BDE-47 exposure-dependent changes

Neurotransmitter receptor transfer to plasma membrane was the top biological category enriched for 106 targets of the one miRNA differentially expressed on PND65 in response to BDE-47 exposure (Supplemental figure 5C). We then identified 688 genes-targets of 18 differentially expressed miRNA on PND120 in response to BDE-47 exposure. The top biological category enriched by these genes was modulation of chemical synaptic transmission (Fig. 2D). Additionally enriched biological categories include pathways of growth and development (ex.: regulation of growth, developmental growth, regulation of bone resorption, cell part morphogenesis), regulation of carbohydrate metabolism (ex.: regulation of glucose transmembrane transport, phosphatidylinositol signaling system, insulin resistance) and others. However it should be noted that significance of BDE-47 exposure-dependent changes are smaller (-log 10(P) ranged 2-6) than for age-dependent changes.

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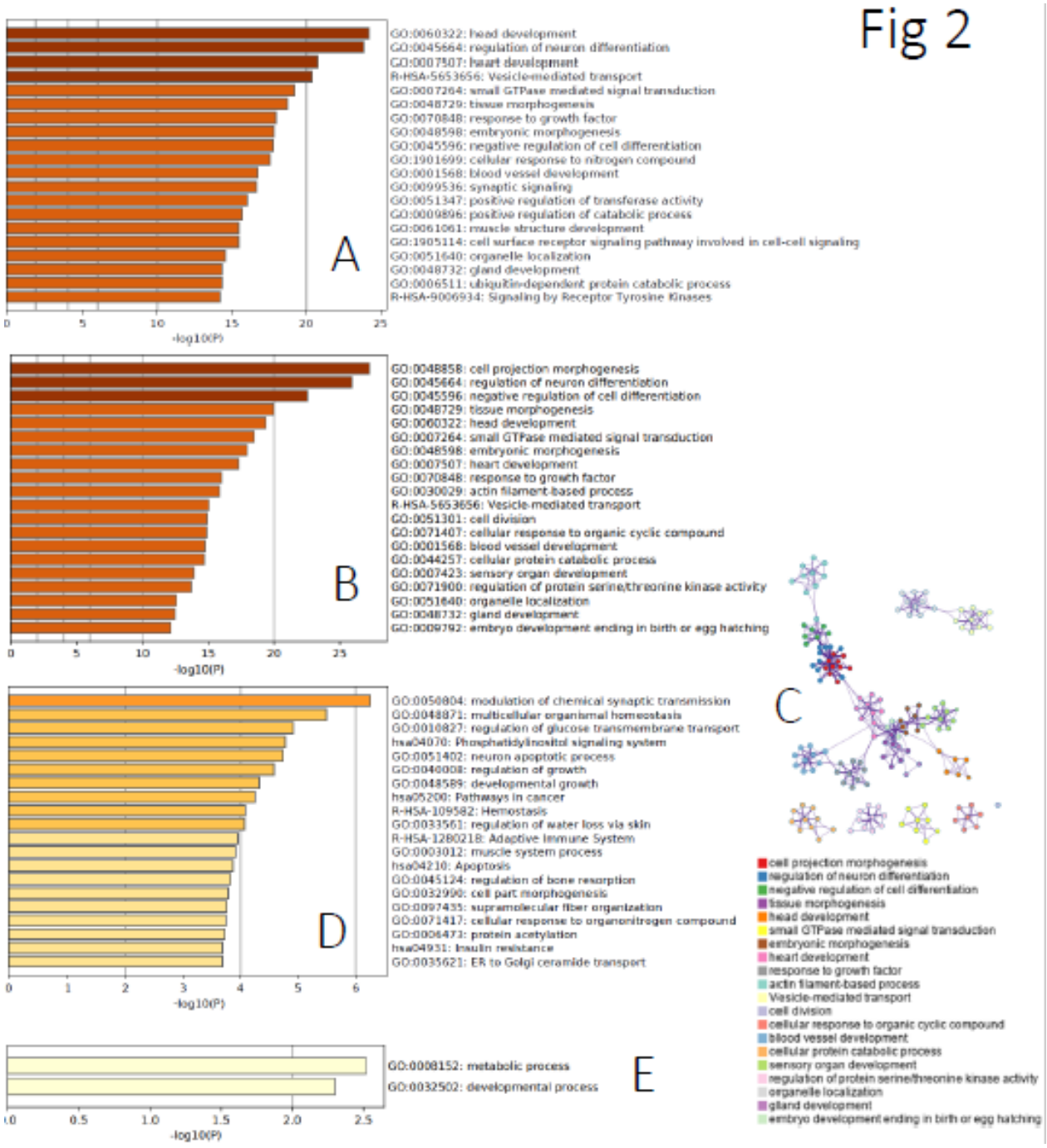

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412 Figure 2. Functional enrichment of differentially expressed sncRNA: A – enrichment of
413 biological categories with 2654 genes-targets of ≥2 age-dependent miRNA differentially
414 expressed in control animals, B,C - enrichment of biological categories (B) and network
415 of enriched terms (C) for 2261 targets of age-dependent miRNA differentially expressed
416 in both control and BDE-47-exposed animals, D - enrichment of biological categories
417 with 688 genes-targets of miRNA differentially expressed in BDE-47 animals on
418 PND120, E - enrichment of biological categories with 42 genes-targets of piRNA
419 differentially expressed between two age groups of control animals.

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3.4. Functional analysis of age-dependent changes in piRNA expression

Using data on rat piRNA functional annotation from piRBase (Wang et al., 2019) we analyzed if age-dependent differentially expressed piRNAs in sperm are enriched with piRNA targeting different genomic elements. Our null hypothesis assumed that age and expression in sperm of piRNA targeting specific genomic elements are independent variables. Compared with numbers predicted from the null hypothesis we have found that in sperm of control animals piRNA targeting transposable elements LINE (long interspersed elements) and SINE (short interspersed elements) as well as satellite DNA sequences were not significantly over- or under-represented among age-dependent piRNA (Table 2).

piRNA targeting LTR (long terminal repeats) and protein coding genes were significantly ($p < 0.00001$) overrepresented in sperm of control animals among age-dependent piRNA with 5.3 and 11.8 fold enrichment, respectively (Table 2). In exposed animals, enrichment of age-dependent piRNA for different genomic elements was significant only for LTR (p = 0.026, enrichment fold change = 3.5).

Table 2. Functional enrichment of significant age-dependent piRNA

Targets	Identified non-age-dependent piRNA	Identified age-dependent piRNA	Enrichment, fold change	p-value	Direction of change of age dependent genes
All	36,551	980	--	--	139 down 841 up
LINE	1,557	33	-1.2	0.40	4 down 19 up
SINE	1,648	47	1.1	0.33	6 down 41 up
LTR	521	80	5.3	<0.00001	13 down 67 up
Satellite	52	1	-1.28	1	1 up
Protein coding	353	142	11.8	<0.00001	24 down 118 up

Lastly, in spermatozoa of control animals, we identified 142 age-dependent piRNA targeting protein coding genes. These piRNAs were involved in 158 piRNA-gene pairs (some piRNA targeting more than one gene) with 42 genes (some genes targeted by more

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than one piRNA). Among these genes, Ppil1 was targeted by 88 piRNA, Add2 by 14, Stox1 by 9, Loc500567 by 5, Brinp2 by 3 and Loc360933 and Cep830s by 2 piRNA. All other genes were targets of one piRNA. Metascape analysis of the list of 42 genes – targets of age-dependent piRNA showed enrichment of metabolic and developmental process (Fig. 2E). In exposed animals, enrichment of age-dependent piRNA for different genomic elements was significant only for LTR ($p = 0.026$, enrichment fold change = 3.5) (Supplemental Table 4).

4. Discussion

In this study, we report for the first time that the composition of sncRNAs in rat sperm undergoes age-dependent changes, where fractions of rRNA-derived sncRNA and lncRNA decreased with age, while fractions of tRNA-derived sncRNA and miRNA increased with age and fractions of piRNA did not changing with age. These findings may have fundamental importance for the understanding of the mechanisms involved in age-dependent changes in epigenetic information transferred by spermatozoa, as well as age-dependent changes in sperm quality and fertility. In our study, more than 1000 sncRNAs, including miRNA, tRNA and piRNA underwent significant age-dependent change in expression in rat spermatozoa, which were enriched in the regulation of genes involved in developmental and metabolic processes. Finally, perinatal exposure of rats to low dose of environmental flame retardant modifies these age-dependent changes in sncRNA by accelerating age-dependent changes in younger animals and attenuating them in older animals.

4.1. SncRNA in sperm

The role of sncRNA in spermatogenesis was reviewed in a recent paper (Natt, Ost, 2020). Composition of different sncRNAs changes dynamically throughout spermatogenesis cycle (74 days in humans, 56 days in rat (Creasy, 1997)) (Sharma, U. et al., 2016, Chen, Yan & Duan, 2016). piRNA are mostly germline-specific sncRNA, which have two waves of expression in spermatogenesis. One population of piRNA is upregulated in primordial germ cells and another in pachytene spermatocytes (Reik, Dean & Walter, 2001, Fu, Q., Wang, 2014). The major recognized role of piRNA consists in suppression of transposable elements during epigenetic reprograming events (Aravin,

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Hannon & Brennecke, 2007). Concordant with this role, piRNAs of the first wave mostly target transposable elements, while piRNAs of the second wave are enriched in protein coding mRNA targets (Gou et al., 2014). In mature human spermatozoa, piRNA expression decreases and miRNA and small RNA derived from tRNA and rRNA are present in much higher quantities. In fact, the two later types of RNA constitute about 75% of all sncRNA in humans (Donkin et al., 2016, Hua et al., 2019).

After leaving the testis, mature spermatozoa receive additional loads of miRNA and fragments of tRNA and rRNA delivered by epididimosomes (Trigg, Eamens & Nixon, 2019) and prostasomes (Zijlstra, Stoorvogel, 2016). Mouse models suggest that the load of these extracellular vesicles have significant functional roles and may affect sperm competition, fertilization, embryo development and intergenerational inheritance (Sharma, U. et al., 2016, Sharma, U. et al., 2018, Conine et al., 2018). The described dynamic and dramatic changes of sncRNA content during spermatogenesis result in highly selective sncRNA loads of mature spermatozoa. Although emerging evidence indicates that sncRNA in spermatozoa may serve as an important channel of epigenetic information transfer to the next generation, our knowledge of factors that affect sperm sncRNA profiles only is starting to emerge. For examples, existing research identified that high-fat diets and low-protein diets can alter expression of miRNA, piRNA, and tRNA-derived RNA in spermatozoa (reviewed in (Klastrup, Bak & Nielsen, 2019)). Diet induced changes in the sncRNA profile were associated with phenotypes displaying insulin resistance, altered body weight, and glucose intolerance. To our knowledge, there still exists a knowledge gap in the understanding of age-dependent changes in sperm sncRNA. Additionally, the ability of environmental factors to affect age-dependent changes in sperm sncRNA was not previously reported.

4.2. Justification of the model

We analyzed age-dependent changes in sperm sncRNA by comparing sperm of young pubertal (PND65) and mature rats (PND120). Male reproductive system undergoes significant change between these time-points. Sexual maturation in rats occurs between 41 and 54 days of age when growth hormone pulse amplitudes increase twofold (Gabriel, Roncancio & Ruiz, 1992). According to Robb et al. (Robb, Amann & Killian, 1978), spermatozoa are first registered in testis by PND45 and in the epididymis tail by

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PND50 in Wistar rats. Sperm production increases till PND75 and testis weight increases till PND100. Blood testosterone starts to increase on PND40-45, reaches its maximum by PND76 (Zanato et al., 1994), and then decreases gradually till reaching its adult level by PND97. PND55 male rats are also less successful in insemination of female rats than 90-95-day old male rats as indicated by number of pregnancies (Zemunik et al., 2001). Thus, the two age groups in our study (PND65 and PND120) represent distinct stages of reproductive maturation in rats, which corresponds to young pubertal and mid-life periods in humans.

To analyze the effect of environmental exposure on age-dependent changes in sperm sncRNA, we tried to simulate exposure of the general population to brominated flame retardant BDE-47, the most prevalent congener of PBDE in human tissues. Median PBDE concentration in adipose tissue from New York urban population is 399 ng/g lipids (Johnson-Restrepo et al., 2005). In our previous study, exposure of pregnant rats to 0.2 mg/kg body weight of BDE-47 (the same dose used in the current study) resulted in accumulation of 234.3 ng BDE-47/g lipid in adipose tissue of dams (Suvorov et al., 2009). Additionally, our dosing paradigm was designed to simulate human exposure dynamics over different life stages. In the general population, exposure to lipophilic brominated flame retardants is highest during the perinatal period of development. Human studies and animal experiments suggest that these compounds, accumulate in maternal adipose tissue, mobilize during pregnancy and lactation, and are delivered *via* cord blood and breast milk to the developing organism (Antignac et al., 2008, Schecter et al., 2006, Shi et al., 2013). BDE-47 easily crosses the placenta (Zhao et al., 2013), and is found in the majority of fetal samples in North America (Herbstman et al., 2007, Doucet et al., 2009). Toddlers are exposed to higher doses of brominated flame retardants than adults because of higher rates of dust ingestion (Wilford et al., 2005) and higher rates of food intake (Schecter et al., 2006). To mimic this exposure scenario, we exposed males perinatally only, thus all exposures occurred via cord blood and breast milk. This period covers prospermatogonia development recognized as a sensitive window of epigenetic reprograming (Wu et al., 2015, Smallwood, Kelsey, 2012). During this period, primordial germ cells experience comprehensive loss of methylation (around E13.5) and establishment of de novo methylation (around E16). It also includes drastic changes in histone modification (Wu et al., 2015, Gkountela et al., 2013).

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4.3. Age-dependent changes in sncRNA fraction

We observed age-dependent change in composition of different sncRNA types in rat sperm. The fact that many sperm parameters are changing with age is well recognized (Paoli et al., 2019). Additionally, a plethora of offspring health effects is associated with paternal age indicating that age is an important factor that determines what information can be transferred to the next generation via spermatozoa. The mechanisms involved in age-dependent changes of spermatozoa physiology as well as changes in information transferred to the next generation are not yet well understood. Our study demonstrates that composition of sncRNA may be one potential candidate molecular mechanism involved in these changes. This hypothesis is supported, for example, by findings that smaller content of RNA derived from rRNA is associated with low-quality embryos after in vitro fertilization (Hua et al., 2019, Natt et al., 2019).

Additionally, to changes in sncRNA fractions, we analyzed functional enrichment of differentially expressed miRNA and piRNA between the two age groups, corresponding approximately to young pubertal and mature men. Targets of differentially expressed miRNA were highly enriched with developmental, (including neurodevelopment) and metabolic categories. Similarly, protein-coding genes, targets of differentially expressed piRNA, were also enriched for metabolic and developmental processes. Differentially expressed piRNA were also highly enriched for LTR targets. Interestingly, the rat genome contains approximately 556,000 copies of LTR elements, representing 9% of the whole genome (Garcia-Etxebarria, Jugo, 2016). LTRs, also referred to as endogenous retroviruses, are traditionally viewed as threat to genomic stability (Doolittle, Sapienza, 1980, Yoder, Walsh & Bestor, 1997). To avoid exponential amplification of transposable elements (TE), mammalian organism have developed mechanisms of TE silencing, with the piRNA pathway being the core mechanism of genome protection from TE in a germline. In spermatogenesis, piRNA ensure genome integrity and male fertility (Chuma, Nakano, 2013). Emerging evidence demonstrates, however, that over a course of symbiotic evolution these initial parasitic sequences have developed important functions such as increasing fitness of the host genomes (Fu, B., Ma & Liu, 2019, Evsikov, Marin de Evsikova, 2016). Specifically, TEs play critical role during early stages of embryo development. Cleavage stage embryos undergo global epigenetic reprograming and thus provide an environment suitable for the transcription of LTRs (Burton, Torres-Padilla, 2014). Activation of LTRs during early embryonic development is

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critical for zygotic genome activation (Fu, B., Ma & Liu, 2019) – a critical event in preimplantation embryo development, when 2-cell stage embryos switch from maternal control to zygotic genome control. Many genes activated at this step contain promoters derived from LTR (Schoorlemmer et al., 2014, Peaston et al., 2004). For example, expression of murine LTR MERVL peak during 2-cell embryo (Fu, B., Ma & Liu, 2019), and downregulation of MERVL through RNA interference result in developmental arrest at that stage (Huang et al., 2017).

Thus, our results present more than one line of evidence indicating that age-dependent changes in sperm sncRNA specifically target molecular mechanisms involved in basic developmental process. Such results are in line with epidemiological and clinical assisted reproduction outcomes indicating that increase paternal age is associated with a host of early-life developmental indicators including longer time to pregnancy (Hassan, Killick, 2003), reduced embryo quality (Frattarelli et al., 2008), and lower rates of fertilization (Aboulghar et al., 2007) and live birth (Frattarelli et al., 2008).

4.4. BDE-47 modifies effect of aging

Compared to age-dependent changes in sncRNA expression, we found only small effects of BDE-47 exposure on sncRNA expression. In fact only one miRNA was differentially expressed on PND65 in response to exposure, while on PND120, six tRNA and 18 miRNA were differentially expressed. Interestingly, almost all of these sncRNA changes in response to exposure were also altered in age-dependent manner in control animals, suggesting that exposure modifies normal dynamics of age-dependent changes in sncRNA expression in sperm. This conclusion is also supported by the fact that unsupervised clustering of sncRNA profiles identifies distinctive age-groups of control animals but not of exposed animals (Fig. 1B,C). Much smaller number of sncRNA differentially expressed between the two age groups in exposed animals than in controls is likely a result of the following effects of exposure. First, sncRNA profiles of exposed animals undergo changes that may be interpreted as acceleration of age-dependent changes in younger animals and their deceleration in older animals (Supplemental Figures 1-3). Second, our data indicate that exposure increases variance in expression of sncRNA between biological replicates, suggesting that at least partly, increased variance may

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explain why some age-dependent changes in exposed animals did not reach significance threshold.

4.5. Limitations and future directions

Emerging evidence indicate that tRNA-derived small RNAs, including tRNA-derived fragments (tRFs) and tRNA halves (tiRNAs) and rRNA derived small RNA (rsRNA), may have significant regulatory functions in spermatozoa (Natt et al., 2019). New bioinformatic methods are being developed in recent years to analyze functional role of these fragments (Xie et al., 2020). These types of small RNA are not yet well characterized and annotated in rat, making it difficult to produce meaningful functional analysis of observed changes in abundance of these scnRNA. We hope that this analysis will become possible as new databases of rat small RNA will be developed.

Additionally in our study, the first time-point when sperm was collected was PND65. Although first spermatozoa appear in the caudal epididymis 2 weeks earlier, it is possible, but unlikely, that part of spermatozoa collected on PND65 represent the first wave of spermatogenesis. Hypothetically, the first wave of spermatogenesis may have the process of epigenetic maturation not yet well established, suggesting that part of differences reported in our study may be explained by the comparison of spermatozoa resulting from different developmental phases of spermatogenesis maturation, rather than just from different age mature animals. Future research is needed to analyze changes in sncRNA over the whole reproductive lifespan.

5. Conclusions

Our study compared sncRNA in sperm of young pubertal and mature rats. Aging was associated with changes in the composition of sncRNAs including age-dependent decrease of rRNA and lncRNA and increases in tRNA and miRNA. Changes in expression of individual miRNA and piRNA were enriched for targets associated with developmental and metabolic process. Differentially expressed piRNA were highly enriched for LTR targets. Perinatal exposure to environmentally relevant doses of BDE-47 accelerates age dependent changes in sncRNA in younger animals, decelerates these changes in older animals and increases variance in expression of all sncRNA. Future research is needed to identify the mechanisms involved in age-dependent

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changes in sperm sncRNA profiles as well as the downstream effects on the health and development of future progeny.

Competing interests

All authors declare no conflict of interest.

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Author Contributions

Conceptualization, Alexander Suvorov, J.Richard Pilsner and Oleg Sergeyev; Data curation, Alexander Suvorov, Vladimir Naumov, Victoria Shtratnikova and Oleg Sergeyev; Formal analysis, Alexander Suvorov, Vladimir Naumov, Victoria Shtratnikova, Anna Zheludkevich, Evgeny Gerasimov and Maria Logacheva; Funding acquisition, Alexander Suvorov and Oleg Sergeyev; Investigation, Alexander Suvorov, Vladimir Naumov, Victoria Shtratnikova, Evgeny Gerasimov and Oleg Sergeyev; Methodology, Alexander Suvorov, Vladimir Naumov, Victoria Shtratnikova, Anna Zheludkevich, Evgeny Gerasimov, Maria Logacheva and Oleg Sergeyev; Project administration, Alexander Suvorov and Oleg Sergeyev; Resources, Alexander Suvorov and Oleg Sergeyev; Supervision, Alexander Suvorov, J.Richard Pilsner and Oleg Sergeyev; Validation, Alexander Suvorov, Victoria Shtratnikova and Anna Zheludkevich; Visualization, Alexander Suvorov and Vladimir Naumov; Writing – original draft, Alexander Suvorov; Writing – review & editing, Alexander Suvorov, J.Richard Pilsner and Oleg Sergeyev.

Additional files

Supplemental Figure 1. Comparison of miRNA VST-transformed expression values (VST) between control rats and rats perinatally exposed to BDE-47. Only miRNA undergoing significant age-dependent change in control animals are shown, and are ranked in accordance with their VST values in control animals. A - expression on PND65

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of miRNA undergoing age-dependent up-regulation in control animals; B - expression on PND120 of miRNA undergoing age-dependent up-regulation in control animals; C - expression on PND65 of miRNA undergoing age-dependent down-regulation in control animals; D - expression on PND120 of miRNA undergoing age-dependent down-regulation in control animals; E – comparison of average expression values for miRNA across ages, exposure groups and direction of age-dependent regulation of expression (mean ± SE, *p*-value is for T.Test).

Supplemental Figure 2. Comparison of piRNA VST-transformed expression values (VST) between control rats and rats perinatally exposed to BDE-47. Only piRNA undergoing significant age-dependent change in control animals are shown, and are ranked in accordance with their VST values in control animals. A - expression on PND65 of piRNA undergoing age-dependent up-regulation in control animals; B - expression on PND120 of piRNA undergoing age-dependent up-regulation in control animals; C - expression on PND65 of piRNA undergoing age-dependent down-regulation in control animals; D - expression on PND120 of piRNA undergoing age-dependent down-regulation in control animals; E – comparison of average expression values for piRNA across ages, exposure groups and direction of age-dependent regulation of expression (mean ± SE, *p*-value is for T.Test).

Supplemental Figure 3. Comparison of tRNA VST-transformed expression values (VST) between control rats and rats perinatally exposed to BDE-47. Only tRNA undergoing significant age-dependent change in control animals are shown, and are ranked in accordance with their VST values in control animals. A - expression on PND65 of tRNA undergoing age-dependent up-regulation in control animals; B - expression on PND120 of tRNA undergoing age-dependent up-regulation in control animals; C - expression on PND65 of tRNA undergoing age-dependent down-regulation in control animals; D - expression on PND120 of tRNA undergoing age-dependent down-regulation in control animals; E – comparison of average expression values for tRNA across ages, exposure groups and direction of age-dependent regulation of expression (mean ± SE, *p*-value is for T.Test).

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Supplemental Figure 4. Coherence in age-dependent changes in expression of miRNA (A,B), tRNA (C,D) and piRNA (E,F) in sperm of control and BDE-47 exposed animals. Values of differential expression are shown for merged (A, C, E) and overlapping (B, D, F) lists of differentially expressed RNA in control and exposed animals.

Supplemental Figure 5. Metascape enrichment of genes-targets of differentially expressed miRNA. A – enrichment of 222 genes-targets of 5 and more miRNA upregulated in older unexposed rats; B – enrichment of 106 genes-targets of 5 and more miRNA downregulated in older unexposed rats; C – enrichment of 106 genes-targets of the single miRNA differentially expressed in animals exposed to BDE-47 on PND65.

Supplemental Table 1. Expression of sncRNA in rat sperm samples.

Supplemental Table 2. sncRNA significantly differentially expressed in rat sperm in response to perinatal exposure to BDE-47 and their log2 fold changes on PND65 and 120. sncRNA highlighted in bold are age-dependent). Footnote: *-significant change, FDR adjusted $p<0.05$

Supplemental Table 3: Genes – targets of 249 age-dependent miRNAs differentially expressed in control animals with prediction score ≥ 80.

Supplemental Table 4. Functional enrichment of significant age-dependent piRNA in exposed animals.

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