Aging-induced changes in sperm DNA methylation are modified by low dose of perinatal flame retardants

J. Richard Pilsner[1], Alex Shershebnev[1], Haotian Wu[1], Chelsea Marcho[1], Olga Dribnokhodova[2], Victoria Shtratnikova[3,4], Oleg Sergeyev[3,5], Alexander Suvorov[1,3]

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J. Richard Pilsner - rpilsner@umass.edu

Alex Shershebnev - ashershebnev@umass.edu

Haotian Wu - haotianw@umass.edu

Olga Dribnokhodova - dribnokhodova@cmd.su

Victoria Shtratnikova - vtosha@yandex.ru

Chelsea Marcho - cmarcho@mcb.umass.edu

Oleg Sergeyev - olegsergeyev1@yandex.ru

Alexander Suvorov - asuvorov@umass.edu

[1] Department of Environmental Health Sciences, School of Public Health and Health Sciences, University of Massachusetts, 686 North Pleasant Street Amherst, MA 01003

[2] Federal Budget Institution of Science "Central Research Institute of Epidemiology" of The Federal Service on Customers' Rights Protection and Human Well-being Surveillance, Novogireevskaya-3a, 111123 Moscow, Russia

[3] Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Leninskie Gory 1/40, 119992 Moscow, Russia

[4] Center for Data-Intensive Biomedicine and Biotechnology, Skolkovo Institute of Science and Technology, 143028, Moscow, Russia

[5] Chapaevsk Medical Association, Meditsinskaya str. 3a, 446100 Chapaevsk, Samara Region, Russia

Corresponding author: Alexander Suvorov

Work telephone number: 413-545-3487

Work fax number: 413-545-6536

Abstract

Advanced paternal age at fertilization has been suggested to be a risk factor for neurodevelopmental, psychiatric and other disorders in offspring. One emerging hypothesis suggests that altered offspring phenotype is linked with age-related accumulation of epigenetic changes in the sperm of fathers. Given that paternal age is increasing in the developed world, understanding aging-related epigenetic changes in sperm is needed as well as environmental factors that modify such changes. In this study, we characterize age-dependent changes in sperm DNA methylation profiles between young pubertal (postnatal day (PNDs) 65) and mature (PND120) Wistar rats. We also analyze these changes in rats exposed perinatally to 0.2 mg/kg of ubiquitous environmental xenobiotic 2,2',4,4'-tetrabromodiphenyl ether (BDE-47). Reduced representation bisulfite sequencing (RRBS) libraries were prepared from caudal epididymal sperm DNA and differentially methylated regions (DMRs; ≥ 10x coverage depth, ≥ 3 CpGs per cluster, ≥ 5% methylation change, $q < 0.05$) were identified via MethPipe package. In control animals, 5,319 age-dependent DMRs were identified, with 99.3% DMRs hypermethylated in mature animals compared to young pubertal rats. These age-related DMRs were enriched for functional categories essential for embryonic development, such as pattern specification, forebrain and sensory organ development, Hippo and Wnt pathways. Age-related changes in sncRNA, reported in different study, target similar list of genes and biological categories.In BDE-47 exposed rats, sperm DNA methylation was higher in young pubertal and lower in mature animals when compared to controls, which resulted in a significant attenuation in the number of age-dependent DMRs (N = 189) identified in the exposed group. In conclusion, our results indicate that the natural aging process has profound effects on sperm methylation levels and this effect may be modified by environmental exposures. Moreover, our results further support the role of epigenetic mechanisms as a likely link between paternal age and offspring health and development.

Key words: aging, paternal exposure, sperm, advanced paternal age, epigenetics, DNA methylation, RRBS, 2,2′,4,4′-tetrabromodiphenyl ether, PBDE, BDE-47, perinatal, environment.

1. Background

Developed countries experience growing trends for delayed parenthood. Over the past 30 years, the number of men fathering children at their 35-44 increased 1.5 fold in the USA (Martin et al., 2012) and up to 40% in the UK (Bray, Gunnell & Davey Smith, 2006). Mean age on achieving fatherhood is 30.9 years in the USA (Khandwala et al., 2017) and 35 years in Italy (Paoli et al., 2019). While age-related declines of reproductive potential in females are linked to ovarian reserves that are fixed at birth, the self-renewal and differentiation of spermatogonial stem cells permits the production of mature sperm throughout the adult life-course. However, this benefit of continuous spermatogenesis until older age may be outweighed by life-long accumulation of genetic mutations and epimutations in spermatozoa.

Compelling epidemiologic data links advanced paternal age with many adverse health conditions in the offspring. These include stillbirths (Nybo Andersen et al., 2004, Alio et al., 2012), leukemia (Sergentanis et al., 2015, Dockerty et al., 2001), retinoblastoma (Moll et al., 1996, Heck et al., 2012), cleft palate (Bille et al., 2005), autism spectrum disorders (Reichenberg et al., 2006, Hultman et al., 2011), attention deficit/hyperactivity disorder (D'Onofrio et al., 2014), and a range psychiatric conditions (Nybo Andersen, Urhoj, 2017, Liebenberg et al., 2016, Malaspina et al., 2001, Frans et al., 2008). Biological mechanisms responsible for the transfer of offspring adverse phenotype via the germ cell of older fathers are not well understood.

Recent studies elucidate the role of epigenetic changes in spermatozoa as a potential mechanism that connect paternal legacy of lifestyle and exposures to environmental stressors with early-life development and offspring (Wu, Hauser et al., 2015). An association between age-dependent changes in fathers' sperm epigenome (e.g., DNA methylation, histone modifications and small noncoding RNA profiles) and altered offspring phenotype has been suggested (Sharma et al., 2015, Perrin, Brown & Malaspina, 2007).

Recent population research showed that age-related changes in sperm DNA methylation are enriched for regions associated with neurobehavior (Jenkins et al., 2014), such that among men providing two semen samples ranging from 9-19 years apart, most (94%) of the significant intra-individual differences in sperm DNA methylation were hypomethylated with aging, including methylation changes in genes associated with schizophrenia and bipolar disorder. Similarly, an age-dependent loss of sperm DNA methylation in regions association with transcriptional regulation was observed in 129SvEv/Tac mice and offspring of older fathers displayed transcriptional dysregulation of developmental genes implicated in neurobehavior (Milekic et al., 2015). Most recently, sperm methylation between young and old C57BL/6J mice revealed widespread sperm methylation changes with 62% of regions hypomethylated and enriched in pathways associated with senescence, aging and mTOR signaling (Xie et al., 2018).

With existing trend for delayed parenthood and ever expanding preconception period of paternal exposure to environmental stressors it is important to understand effects of age and environmental factors on the sperm epigenome. Therefore, we utilized existing data from our previous study that examined the effects of perinatal exposure to 2,2',4,4'-tetrabromodiphenylether (BDE-47), the most ubiquitous congener of polybrominated diphenyl ethers (PBDE), on the sperm methylome in adult rat offspring (Suvorov et al., 2018). In this study, perinatal BDE-47 exposure resulted in 21 and 9 differentially methylated regions (DMRs) in sperm collected on postnatal day (PND) 65 and PND120, respectively (Suvorov et al., 2018). In the current study, we first examine the effect of aging on the sperm methylome by comparing DNA methylation 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 DNA methylation are modified by BDE-47 exposure. We also compare our findings with another study from our group which analyzed age- and BDE-4-dependent changes of sncRNA in the same rat model (Suvorov et al., 2020). Our results indicate that the natural aging process has profound effect on sperm epigenome and this effect 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 (Figure 1A). 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/gram 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 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.

2.2.   Sperm DNA extraction

For sperm DNA-methylation analysis we used sperm collected from 6 animals per time-point per exposure group. To remove somatic cells contamination 1.5 ml sperm samples were loaded on

top of 1 ml density gradient (40% Isolate, Irvine Scientific, USA) in 15 ml conical tubes and centrifuge for 25 min at 500 g. Pelleted spermatozoa were used to extract DNA using the rapid method developed in Dr. Pilsner's laboratory (Wu, de Gannes et al., 2015).

2.3.   Reduced representation bisulfite sequencing (RRBS)

Bisulfite converted libraries were prepared from 100 ng of sperm DNA using Ovation RRBS Methyl-Seq System (Cat. # 0353, NuGEN) and EpiTect Fast DNA Bisulfite Kit (Cat. # 59824, Qiagen) following manufacturers' protocols. Sequencing of libraries was done on HiSeq 2500 (Illumina) in Deep Sequencing Core Facility of the University of Massachusetts School of Medicine (Shrewsbury, MA). DNA methylation by RRBS were validated by pyrosequencing (Supplemetal Table 1). DNA was bisulfite converted using EZ DNA Methylation Gold Kit (Zymo Research) following manufacturer's instructions. Following PCR amplification all sequences were sequenced using a PyroMark Q24 (Qiagen). PCR and sequencing primers were designed using PyroMark® Assay Design SW 2.0 (Qiagen).

2.4.   Identification of differentially methylated regions

Differentially methylated regions were analyzed using a pipeline described in our previous study (Suvorov et al., 2018). In short, raw reads were processed in accordance with recommended protocol for libraries prepared with Ovation RRBS Methyl-Seq System (NuGEN) and then mapped to rn6 Rattus norvegicus reference genome using Bismark (version 0.16.1) (Krueger, Andrews, 2011) and bowtie-2 (version 2.2.9) (Langmead, Salzberg, 2012). PCR-duplicates were removed using nudup.py (version 2.2). Subsequent analyses were restricted to CpGs with ≥ 10x coverage to comply with ENCODE recommendations (ENCODE, 2011) and recommendations of MethPipe developers (Song et al., 2013). Differentially methylated regions (DMRs) were identified using MethPipe (Song et al., 2013). All DMR spatial annotations with regard to various genome structures were produced using closest-features (v. 2.4.25) tool from bedOps package (Neph et al., 2012). Gene coordinates were obtained from refGene using UCSC Table Browser (Karolchik et al., 2004). For promoters 2kbp regions around gene transcription start site (-1500bp

upstream…500bp downstream) were used. Unsupervised hierarchical clustering of DMRs was performed using Qlucore Omics Explorer v 3.3 (Qlucore AB, Lund, Sweden). Final DMRs used for subsequent functional analysis were limited to windows less than 1 kb total. DMRs had to be within 1,500bp upstream of the transcription start site or overlapping an annotated gene to be included in the ontology analysis.

2.5. Functional analyses

To explore enriched functional terms, Metascape (Tripathi et al., 2015) was used with default settings. All analyses were performed for the lists of age-dependent hyper- and hypomethylated genes (q < 0.05, ≥ 10x coverage depth, ≥ 3 CpGs, and window length < 1kb). To analyze if different epigenetic mechanisms have synergistic effects on age-dependent regulation of genes in sperm, we used Fisher Exact test to compare genes associated with age-dependent DMRs with lists of genes – predicted targets of age-dependent sncRNA, identified in another study using the same rat model (Suvorov et al., 2020).

3.0. Results

There were no significant differences in litter size between exposure group. Litter size was 12.3 ± 0.5 in control group and 12.7 ± 0.6 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. Spermatozoa of untreated rats undergo age-related changes in methylation

To evaluate the effect of natural aging on sperm DNA methylation, we conducted RRBS of epididymal sperm collected from young pubertal and mature control rats and rats perinatally exposed to 0.2 mg/kg body weight BDE-47 (Figure 1A). RRBS was completed with an average 18 million unique reads per sample with an average 70.0% (66.2% – 72.4%) reads aligned to the reference genome (see Additional File 1 at (Suvorov et al., 2018) for details). Total average CpG coverage was 2.1 million CpG per sample with, which represents approximately 10% of all CpGs

in the rat genome. Among all identified CpGs, 58% had 10x or higher coverage. Samples

obtained from animals of different age and exposure group did not differ in the mean of total

unique reads, percent alignment, total number of covered CpGs and number of 10x covered

CpGs. To validate RRBS results we conducted pyrosequencing for 5 regions each including 5-12

CpGs. RRBS and pyrosequencing provided similar assessment of DNA methylation levels for
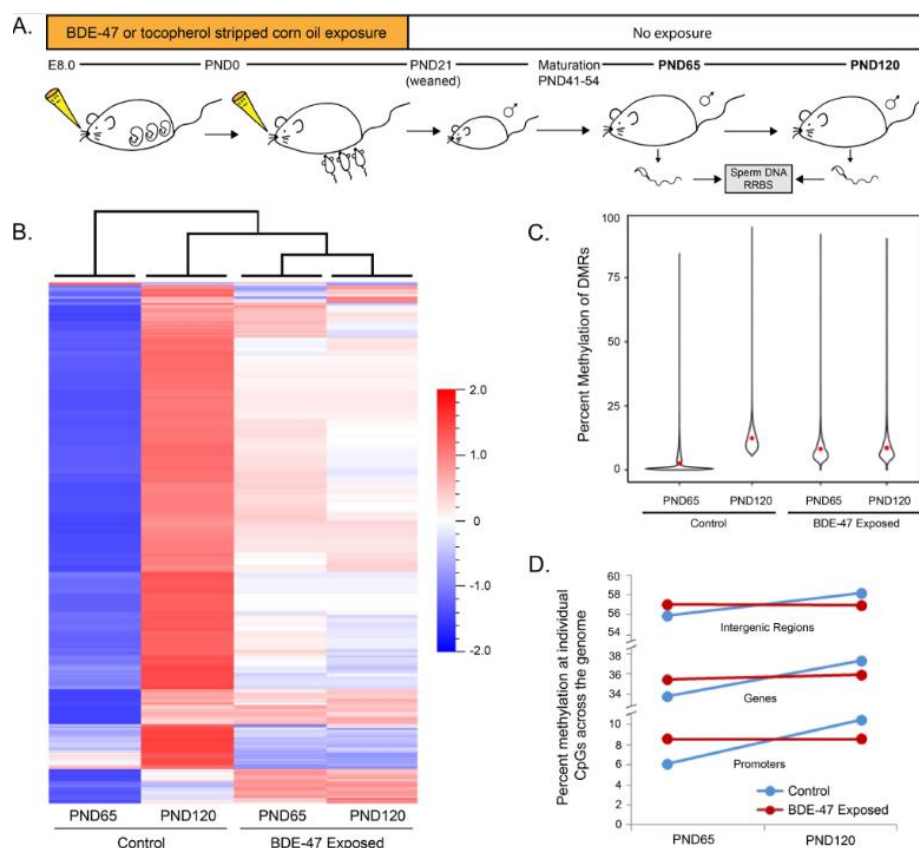
these selected sites.

Figure 1. Age-induced changes in sperm DNA methylation is altered in BDE-47 exposed rats. A).

Summary of the experimental design. Pregnant rats were administered either BDE-47 or

tocopherol stripped corn oil (vehicle) starting at E8.0 and continuing through PND21. Weaned

male mice were allowed to grow to maturity without additional exposure. Sperm was collected

from PND65 and PND120 males for reduced representation bisulfite sequencing (RRBS). B).

Heatmap and hierarchical clustering based on all 5,319 significant age-dependent DMRs (≥ 10x

coverage depth, ≥ 3 CpGs per cluster, ≥ 5% methylation change, q < 0.05) in rat sperm identified

in control group or exposed group or both as a result of comparison PND65 and PND120

animals. The heatmap color scale is based on the standard deviations (SD) from the mean of all samples ranging from +2 SD (red) to -2 SD (blue). C). Violin plot of methylation percent of significant DMRs with a total window length < 1kb (3,866). Circle represents the mean of all DMRs within condition. D). Age-dependent changes in the average methylation across all individual CpGs of epididymal sperm within intergenic regions, genes and promoters among animals exposed to perinatally to vehicle or 0.2 mg/kg body weight BDE-47.

To better understand DNA methylation changes during aging, we first examined age-dependent changes in sperm DNA methylation in young and mature control rats. When restricting DMRs with ≥ 10x coverage depth, ≥ 5% methylation change and ≥ 3 CpGs per cluster, we identified 5,319 age-dependent significant (q < 0.05) sperm DMRs (Figure 1B, Supplemental Table 2). For control animals, 5,283 (99.3%) out of the 5,319 sperm DMRs displayed age-dependent increases in DNA methylation, while only 36 (0.7%) DMRs had lower methylation in mature animals compared to young. Of the 5,319 age-dependent DMRs, 3,866 DMRs had a total window length less than 1kb. The distribution of percent methylation at the 3,866 DMRs highlights the shift in methylation from PND65 to PND120 (Figure 1C). Majority of DMRs undergoing age-dependent hypermethylation in control animals are found in regions with initially low level of methylation. Consistent with age-dependent methylation increases in DMRs, average methylation of individual CpGs also increased in an age-dependent manner across genomic features: gene bodies (+3.5%), promoters (+2.5%) and intergenic regions (+1.1%) (Figure 1D).

In order to understand the biological relevance of the age-related DMRs, we conducted gene ontology enrichment analysis on all 3,866 DMRs (≥ 10x coverage depth, ≥ 5% methylation change, ≥ 3 CpGs per cluster, q < 0.05, window length < 1kb). These DMRs were associated with 3,066 individual genes. Interestingly, ontology results from genes associated with age-dependent sperm DMRs identified in control animals revealed an overall enrichment of terms

related to embryonic and early-life development, including neuron and brain development (Supplemental Table 3). For example, genes undergoing age-dependent changes in methylation, were enriched for the following GO terms: embryo development, sensory organ development, pattern specification, cell differentiation and forebrain development (Figure 2 and Table 1). Specifically, the top enriched molecular functions, biological processes, and cellular components indicate enrichment of genes participating in DNA binding, transcription regulation, and chromatin organization (Table 1). The top enriched domain was homeobox, which is found within genes encoding transcription factors (including Hox transcription factors) involved in the regulation of patterns of embryonic development. Top enriched pathways include pathways of embryonic development (Figure 2A, B), such as Hippo and Wnt (Figure 3), and pathways regulating pluripotency of stem cells (Table 1).
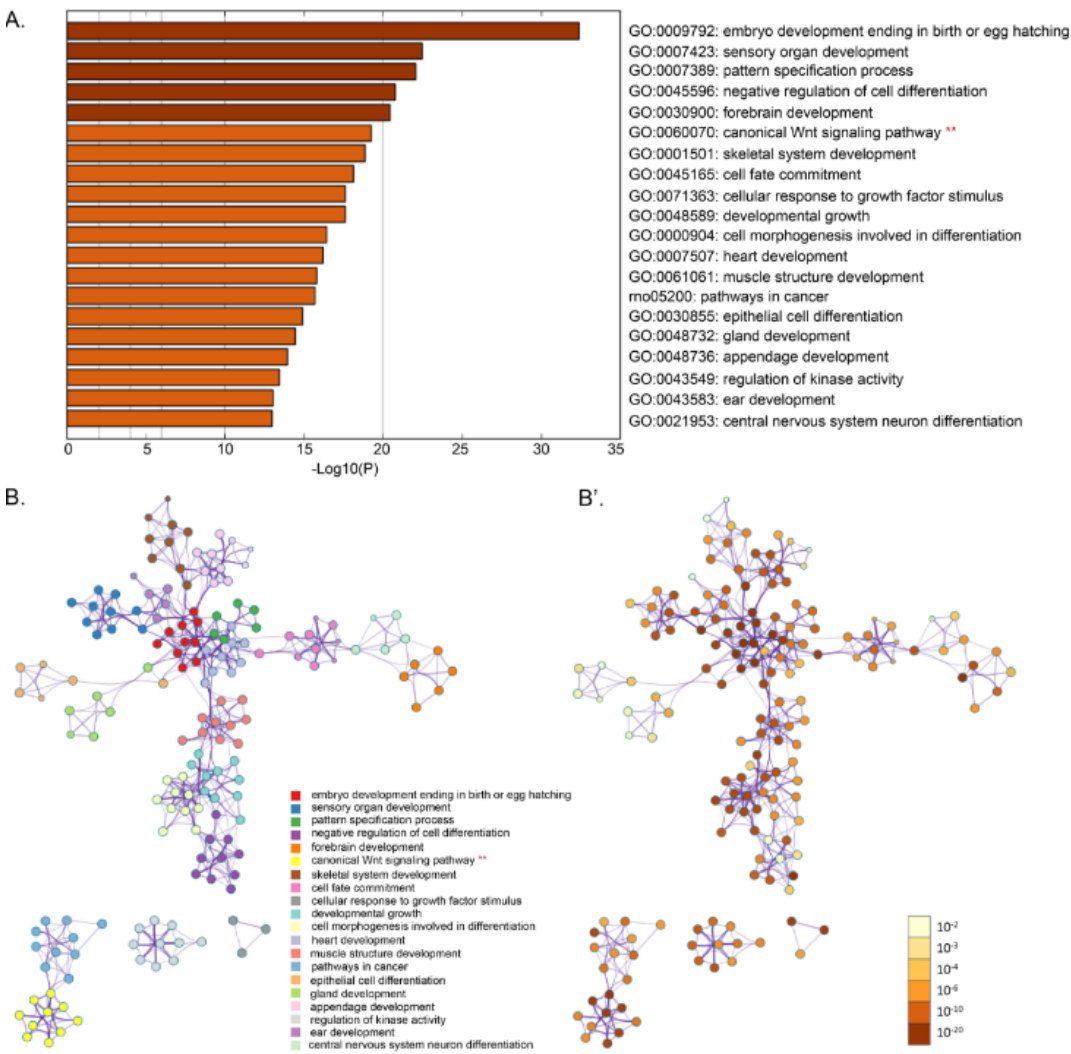
Figure 2. Gene ontology enrichment analysis of age-related DMRs. A). Most significantly enriched biological categories based on Metascape analysis of the list of genes associated with 5% altered DMRs (q < 0.05) with ≥ 10x coverage depth, ≥ 3 CpGs, and window length < 1kb (3,866) undergoing age-dependent hypomethylation and hypermethylation in sperm. B). Network of ontology terms enriched in the list of significant DMRs with window length < 1kb based on Metascape analysis. Network colored by cluster ID. B'). Network colored by p-value.
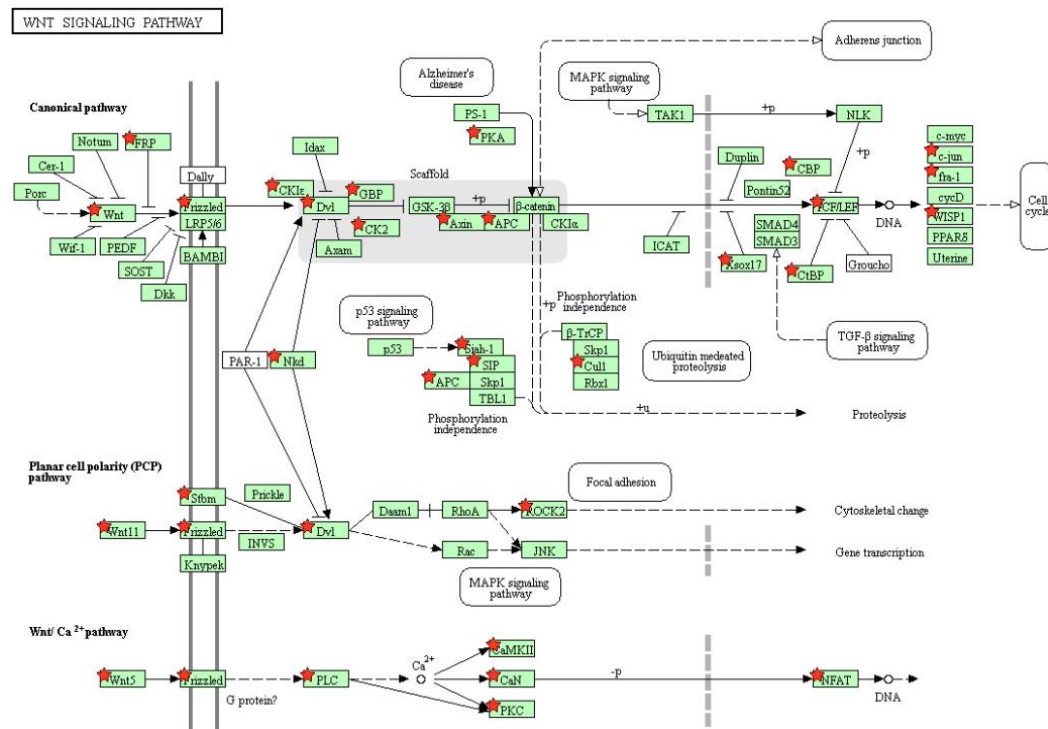
Figure 3. KEGG map of the Wnt signaling pathway (Kanehisa, Goto, 2000) as an example of developmental pathway enriched with genes (red starts) associated with 5% altered DMRs (q < 0.05, ≥ 10x coverage depth, ≥ 3 CpGs, window length < 1kb (3,866)) undergoing age-dependent hypermethylation or hypomethylation in rat sperm.

Table 1. Top biological categories enriched with genes associated with DMRs undergoing age-dependent hypermethylation in sperm. Based on ToppGene analysis.

Source: ID	Name	q-value FDR B&Y	Hit Count in Query List	Hit Count in Genome
Molecular Function				
GO:0043565	sequence-specific DNA binding	1.19E-37	323	1096
GO:0000981	DNA-binding TF activity, RNA polymerase II-specific	9.10E-34	224	675
GO:0001067	regulatory region nucleic acid binding	1.04E-33	265	866
GO:0044212	transcription regulatory region DNA binding	4.72E-33	262	860
GO:1990837	sequence-specific double-stranded DNA binding	8.61E-30	228	735
Biological Process				
GO:0006357	regulation of transcription by RNA polymerase II	1.87E-51	523	1916
GO:0009790	embryo development	6.87E-43	345	1135
GO:0051254	positive regulation of RNA metabolic process	3.36E-39	428	1593
GO:1903508	positive regulation of nucleic acid-templated transcription	3.92E-38	412	1528
GO:0045893	positive regulation of transcription, DNA-templated	3.92E-38	412	1528
Cellular Component				

GO:0005667	transcription factor complex	5.81E-11	107	368
GO:0045202	synapse	5.56E-09	195	870
GO:0043005	neuron projection	1.70E-07	244	1194
GO:0044456	synapse part	3.62E-07	158	708
GO:0060076	excitatory synapse	3.62E-07	72	252
Domain				
Pfam: PF00046	Homeobox	1.08E-11	81	234
PROSITE: PS00027	HOMEOBOX_1	1.08E-11	81	236
InterPro: IPR017970	Homeobox_CS	1.08E-11	69	186
SMART: SM00389	HOX	1.08E-11	81	237
InterPro: IPR009057	Homeodomain-like	1.08E-11	102	332
Pathway				
KEGG: 83105	Pathways in cancer	2.12E-11	115	395
KEGG: 1026136	Signaling pathways regulating pluripotency of stem cells	1.58E-06	48	139
KEGG: 1435207	Breast cancer	1.58E-06	49	144
KEGG: 749777	Hippo signaling pathway	1.62E-06	51	154
KEGG: 83061	Wnt signaling pathway	7.19E-06	47	143

3.2.   Exposure leads to altered age-related shift in DNA methylation levels

Additionally, we were interested in examining age-dependent changes in sperm DNA methylation among animals that were perinatally exposed to BDE-47. Unexpectedly, we identified a significantly lower amount of age-dependent sperm DNA methylation changes in exposed animals (Figure 1B-D, Supplemental Table 2); whereby we identified only 189 sperm DMRs (52 DMRs (29%) hypomethylated; 137 DMRs (71%) hypermethylated) in BDE-47 exposed animals at PND120 compared to PND65. The effect of age on the average methylation across all individual CpGs genome-wide was minimal in exposed animals: gene bodies (+0.4%), promoters (0%) and intergenic regions (-0.1%) (Figure 1D). Upon further investigation into the low number of sperm DMRs in exposed animals, we found it was largely attributable to higher methylation at DMRs of young exposed animals compared to young controls (Figure 1B,C). Similarly, average methylation at individual CpGs at genes, promoters, and intergenic regions for the two time-points show that sperm methylation levels were higher in young and lower in mature BDE-47 exposed animals compared to controls (Figure 1D).

Of the 189 age-dependent DMRs in the BDE-47 exposed animals, 178 (94%) overlapped with age-dependent DMRs in control animals, and out of these overlapping DMRs, 154 (86%) had the same direction (e.g., hypo- or hypermethylated) and similar magnitudes of methylation change (correlation coefficient = 0.8174, Figure 4A, Supplemental Table 4). Gene ontology enrichment analysis revealed that both hyper- and hypomethylated DMRs are enriched for genes involved in regulation of development (Figure 4B,C, respectively). Specifically, hypermethlyated DMRs were associated with signaling pathways involved in pluripotency, which is important for embryonic development, as well as histone modifications and endosome organization (Figure 4B); whereas, hypomethylated DMRs were associated with regulation of developmental growth, regulation of vesicle-mediated transport, and regulation of anatomical structure size (Figure 4C).
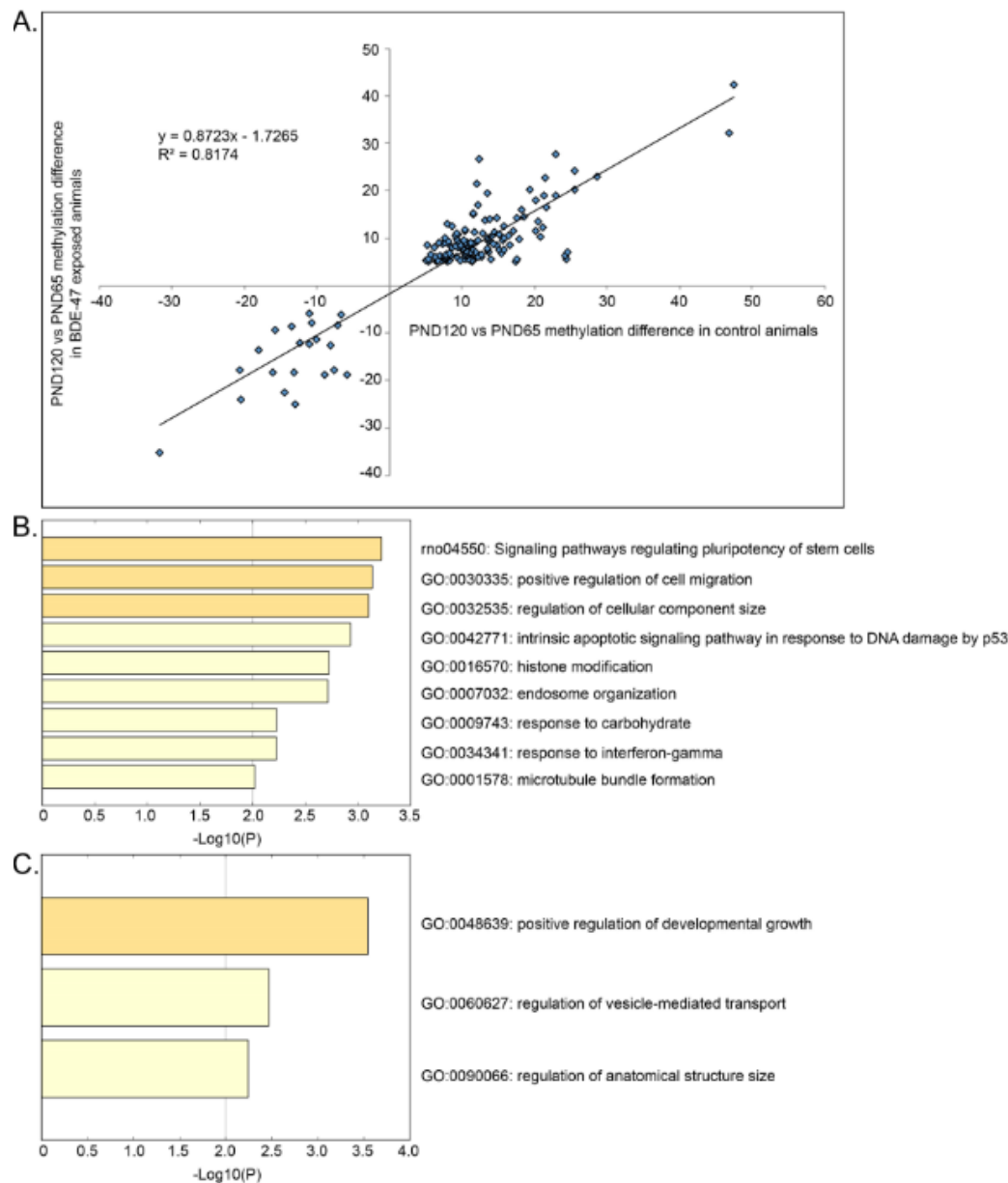
Figure 4. Analysis of overlapping age-related differentially methylation regions significantly altered in the same direction between control and BDE-47 exposed rats. A). Methylation differences in age-dependent DMRs in control and BDE-47 exposed rats. B,C). Gene ontology enrichment analysis of 154 overlapping age-related DMRs. Most significantly enriched biological categories based on Metascape analysis of the list of genes associated with 5% altered DMRs (q < 0.05) with ≥ 10x coverage depth, ≥ 3 CpGs, and window length < 1kb undergoing age-dependent (B) hypermethylation and (C) hypomethylation in sperm.

3.3.   Synergy of age-dependent epigenetic mechanisms

The rat model analyzed in the current study was previously used to dissect age-dependent changes in sncRNA in sperm (Suvorov et al., 2020). This study identified sncRNA differentially expressed in sperm of unexposed animals between PND65 and 120. Furthermore, it identified 4908 protein-coding genes – targets of differentially expressed miRNA and 42 genes – targets of differentially expressed piRNA. We compared the list of 3,066 genes associated with age-dependent DMRs (≥ 10x coverage depth, ≥ 5% methylation change, ≥ 3 CpGs per cluster, q < 0.05, window length < 1kb) with lists of gene-targets of differentially expressed age-dependent sncRNA. We found 1052 genes overlapping between DMR-associated genes and gene-targets of miRNA (Supplemental Table 5), while 9 genes were common between DMR-associated genes and gene-targets of piRNA. Using Fisher Exact test, we identified that DMR-associated genes were significantly enriched with gene-targets of age-dependent miRNA (p < 0.00001) and piRNA (p = 0.035). Metascape analysis of the 1052 overlapping genes from miRNA targets and DMR-associated genes showed high enrichment for developmental categories (embryonic morphogenesis, brain development, heart development, gland development and other) with high statistical significance (-log 10(P) ranged 14-22) (Supplemental Figure 1).

4.0.   Discussion

In this study, we identified >5,000 age-dependent DMRs in rat spermatozoa in which 99% of DMRs underwent an increase in DNA methylation in older animals. Furthermore, we found that DMRs were associated with genes significantly enriched for biological categories related to embryonic development, morphogenesis and brain development. Finally, perinatal exposure of rats to low dose of environmental flame retardant resulted in substantial decrease in age-dependent sperm DMRs. This was largely due to higher genome-wide methylation levels in younger rats (PND65) that were exposed to BDE-47, whereby sperm from younger exposed animals more closely resembled sperm from mature (PND120) controls. Such results suggest

that perinatal exposure resulted in sperm from young animals to harbor an advanced epigenetic aging phenotype.

Enriched biological categories identified in our ontology analyses are largely related to embryonic morphogenesis, development, and cell differentiation. Among enriched molecular functions the most significant categories were related to DNA and chromatin binding. These enrichments were due to the altered methylation in large number of transcription factors and histone and chromatin remodeling genes. Among transcription factors, significant number of genes contain a homeobox domain, the most conserved regulators of embryonic development (Holland et al., 2017). Among the most enriched molecular pathway identified by our enrichment analysis were Hippo and Wnt pathways. Wnt pathway is conserved mechanism that acts on multiple tissues and developmental stages to specify cell fate and patterning in animal embryos (Sokol, 2015). Hippo is also highly conserved mechanism, which controls organ size in animals through the regulation of cell proliferation and apoptosis (Pan, 2010). Additionally, we identified 154 age-depended sperm DMRs in which the direction and magnitude of methylation change was similar in exposed and control animals. These age-dependent DMRs that are resistant to environmental exposures may represent the most conserved group of DMRs responsive to aging. Remarkably, these DMRs are enriched in biological pathways related to development and cell differentiation.

Interestingly, another study from our group using the same rat model has recently shown recently that age-dependent changes in sncRNA in rat sperm target similar sets of developmental genes (Suvorov et al., 2020) as we show here for age-dependent changes in DNA-methylation. Thus, different age-dependent changes in the sperm epigenome (DNA-methylation, expression of miRNA and piRNA) may have synergistic effects on the regulation of the expression of genes essential for early embryo development. These results may be relevant for reports of reduced embryo quality in association with increased paternal age (Frattarelli et al., 2008). Other potentially relevant health outcomes include lower rates of fertilization (Aboulghar et

al., 2007), implantation, pregnancy (Ferreira et al., 2010) and live birth (Frattarelli et al., 2008) associated with advanced paternal age.

We analyzed age-dependent changes in DNA methylation by comparing sperm of young pubertal (PND65) and mature rats (PND120). Between postnatal days 41 and 54 growth hormone pulse amplitudes increase twofold (Gabriel, Roncancio & Ruiz, 1992). In Wistar rats, spermatozoa are first registered in the epididymis tail by PND50 (Robb, Amann & Killian, 1978). After that, reproductive system continues to undergo maturation until approximately PND100. It fact, testis weight increases until PND100, sperm production increases until PND75, and blood testosterone reaches its maximum by PND76 (Zanato et al., 1994), and then decreases gradually till reaching its adult level by PND97. PND55 male rats are less successful in insemination of female rats than 90-95-day old male rats (Zemunik et al., 2001). Thus, two age groups in our study represent distinct stages of reproductive maturation in rats, which corresponds to young pubertal and mid-life periods in humans.

In our study, 99% of age-dependent DMRs in control sperm underwent age-dependent increases in methylation. Not surprisingly, different genomic elements, such as intergenic regions, genes bodies and promoters also demonstrate age-dependent increases in methylation, indicating that such changes are not restricted to specific genomic features. Age-dependent increases in methylation in our study are in contrast to a loss of methylation previously reported in older mice (12-14 months old) compared to younger mice (3 months old) in regions associated with transcriptional regulation identified using Methyl-MAPS approach (Milekic et al., 2015). In another study of total sperm DNA methylation, only slight increases in methylation levels in long interspersed nuclear elements were found in sperm collected from 17-months old compared to 18-week-old mice (Kobayashi et al., 2016). Comparison of global methylation levels in sperm of 6- and 21-24-months old Brown Norway rats did not show any effect of age, while ribosomal DNA was shown to undergo age-dependent hypermethylation (Oakes et al., 2003). Comparison of these studies indicate that age-related changes in sperm DNA methylation may be species-

and even strain-specific or may have more complex dynamics than just linear increase or decrease in methylation. Other possibility is that different methods of DNA methylation sequencing and analysis enrich for different genomic elements resulting in discrepancy across studies. Detailed studies following standardized protocols are needed to further characterize dynamics of sperm epigenetic aging across the lifespan in humans and laboratory rodents.

Changes in the dynamics of DNA methylation induced by BDE-47 were achieved using a dose that approximates environmental exposures in the general population. Exposure of pregnant rats to the same dose of BDE-47 in our previous study (Suvorov et al., 2009) resulted in accumulation of 234.3 ng BDE-47/g lipid in adipose tissue of dams. This concentration is comparable to PBDE concentrations in adipose tissue from American urban populations (New York - 399 ng/g lipids, (Johnson-Restrepo et al., 2005)).

Additionally, we designed our exposure protocol to simulate exposures in the general population. In human populations exposures to bioaccumulative flame retardants peak at early steps of ontogenesis. PBDEs accumulate in maternal adipose tissue and during pregnancy and lactation the developing organisms is exposed *via* cord blood and breast milk (Antignac et al., 2008, Schecter et al., 2006, Shi et al., 2013). These findings are supported by the fact that PBDEs are found in the majority of fetal samples in North America (Herbstman et al., 2007, Doucet et al., 2009). Additionally, during early postnatal life exposure levels of kids is higher than of their parents due to higher rates of dust ingestion (Wilford et al., 2005) and higher rates of food intake (Schecter et al., 2006).

Both animal experiments and human studies show male reproductive toxicity of BDE-47 and other PBDE congeners. Developmental (Kuriyama et al., 2005, Khalil et al., 2017) and adult exposures (Zhang et al., 2013, van der Ven et al., 2008) to PBDE in rats were associated with altered weight of testis, epididymis and seminal vesicles, abnormalities in sperm morphology, decreased sperm count and percent of apoptotic sperm cells. In our recent published study,

using same dosing paradigm as in the current study, significant changes in testes transcriptome were observed in adult male rats, including suppression of genes, essential for spermatogenesis. In particular, exposed animals had 4-fold decreased expression of protamine and transition protein genes in testis, suggesting dysregulation of histone-protamine exchange in the course of spermiogenesis (Khalil et al., 2017). In humans, blood BDE-47, BDE-100 and ΣBDEs were negatively associated with sperm mobility (Abdelouahab, Ainmelk & Takser, 2011). In another study men recruited in Michigan and Texas from 2005 to 2009 demonstrated decreased semen quality, specifically increased percentages of abnormal morphology in relation to blood PBDEs concentrations (Mumford et al., 2015).

Our results demonstrate that exposure to BDE-47 during perinatal period of development significantly alters DNA methylation levels, such that we observed 5,319 age-dependent DMRs in controls compared to 189 age-dependent DMRs in exposed rats. In our previous study, BDE-47 exposure, compared to controls, resulted in the identification of only 21 and 9 sperm DMRs in young pubertal and mature animals, respectively (Suvorov et al., 2018). It must be noted that unsupervised hierarchical clustering of age-dependent DMRs (Figure 1B) revealed that sperm methylations of young and mature BDE-47 exposed rats are more similar to sperm of mature controls. Such results indicate that the methylation profiles of sperm from perinatal BDE-47 exposure resembles those of more "aged" PND120 control sperm. This notion is further supported by our analyses showing that total CpG methylation within genomic regions (intergenic, genes, promoters) is higher in young exposed rats compared to young controls (Figure 1D). Moreover, between PND65 and PND120, sperm methylation increases across all genomic regions in controls, whereas in exposed rats the slope between the two time points is relatively flat, indicating little to no age-dependent changes in DNA methylation. Taken together, our results suggest that perinatal exposure to BDE-47 alters age-dependent dynamics of DNA methylation. In particular, perinatal BDE-47 exposure appears to accelerate sperm epigenetic aging in younger animals (<PND65) and impedes the normal aging process in older animals (between PND65 and PND120). Based on our recent findings, BDE-47 exposure had very

similar effect on age-dependent sncRNA in spermatozoa (Suvorov et al., 2020). 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. This effect of exposure makes sncRNA profiles of younger and older animals look much more alike and seemingly decreases age-dependent changes. For example, in control animals 249 miRNA, 908 piRNA and 227 tRNA were significantly differentially expressed between PND65 and 120, while in exposed animals the same numbers were 68, 44 and 53, respectively (Suvorov et al., 2020). Additionally research is needed to identify the effects of environmental exposures on the sperm epigenome as well as to understand interaction between exposures and sperm epigenetic changes that occur during the natural aging process.

Our study compares sperm DNA methylation in young pubertal and mature rats. Although first spermatozoa are registered in testis around 3 weeks before PND65 (Robb, Amann & Killian, 1978), there is a small but unlikely chance that some fraction of PND65 spermatozoa represent the first wave of spermatogenesis, suggesting that some differences reported in our study may be due to the comparison of spermatozoa resulting from different developmental phases of spermatogenesis maturation. Thus, future research is needed to analyze changes in DNA methylation over the whole reproductive lifespan.

5.0.   Conclusions

In our rat model, paternal aging is associated with increase in methylation of over 5,000 DNA regions in sperm, enriched with genes essential for embryonic development. Similar set of genes and biological functions was associated with age-dependent changes in expression of miRNA and piRNA in our previous study (Suvorov et al., 2020), suggesting synergy between age-dependent changes in different epigenetic mechanisms in sperm. Exposure to BDE-47 increases sperm DNA methylation in young animals, making their sperm methylation profiles similar to those of older unexposed animals. Similarly, exposure to BDE-47 increased "epigenetic age" of

sncRNA profile in sperm of young animals (Suvorov et al., 2020), suggesting, that response to exposure is coordinated between the two epigenetic mechanisms. More research is needed to understand inter- and transgenerational phenotypes associated with these changes and to identify specific mechanisms and developmental windows responsible for epigenetic profiles reprograming by chemical exposures.

6.0.   Summary points

- Advanced paternal age at fertilization is a risk factor for many disorders in offspring, however effect of age and environmental exposures on sperm epigenome are poorly understood.

- Comparison of sperm DNA methylation between young pubertal and mature rats identified 5,319 DMRs, with 99.3% DMRs hypermethylated in mature animals.

- Age-related DMRs were enriched for functional categories essential for embryonic development.

- Comparison of age-dependent DMRs with age-dependent changes in sncRNA from different study indicate that both target similar list of genes and biological categories.

- In BDE-47 exposed rats, sperm DNA methylation was higher in young pubertal and lower in mature animals when compared to controls, which resulted in significantly smaller number of age-dependent DMRs (N = 189).

- Similarly, BDE-47 decreases the difference in sperm expression of age-dependent sncRNA betwen young and mature animals, suggesting common response of different age-dependent epigenetic changes to environmental stressor.

- Our results indicate that the natural aging process has profound effects on sperm methylation levels and this effect may be modified by environmental exposures.

- Our results support the role of epigenetic mechanisms as a likely link between paternal age and offspring health and development.

7.0.   Disclosure

Competing interests

All authors declare that they have no any competing interests.

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Ethical approval

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.

Acknowledgements

Author contributions

ASu conceived the study and participated in its design, coordination, data analysis and drafting of the manuscript. JRP led sperm DNA methylation analysis and participated in drafting of the manuscript. ASh and CM conducted bioinformatic analysis of RRBS data. VS and OD conducted validation of RRBS data. HW carried out DNA purification and library preparation for RRBS. OS

participated in the study design, coordination and data validation. All authors read critically

revised and approved the final manuscript.

Data availability

Original data used in this manuscript are available upon request.

Supplemental material

Supplemental Table 1: Pyrosequencing primers and method validation for six control samples,

PND120.

Supplemental Table 2. All significant DMRs (q < 0.05) with ≥ 10x coverage depth and ≥ 3 CpGs

identified between PND65 and PND120 among control and/or BDE-47 exposed animals.

Supplemental Table 3. Gene ontology enrichment for significant DMRs (q < 0.05) with ≥ 10x

coverage depth, ≥ 3 CpGs, and window length < 1kb (3,866) identified between PND65 and

PND120 among control and/or BDE-47 exposed animals.

Supplemental Table 4: Overlapping age-dependent DMRs significantly altered in the same

direction (e.g., hypo- or hypermethylated) in both control and BDE-47 exposed animals between

PND65 and PND120.

Supplemental Table 5: Overlap between genes associated with significant age-dependent DMRs

in control animals and gene-targets of miRNA and piRNA significantly differentially expressed

between two age groups in control animals.

Supplemental Figure 1: Metascape enrichment of biological categories with 1052 genes

overlapping between genes associated with significant age-dependent DMRs in control animals

and gene-targets of miRNA significantly differentially expressed between two age groups in

control animals.

References

Abdelouahab N, Ainmelk Y, Takser L. (2011). Polybrominated diphenyl ethers and sperm quality. Reprod Toxicol 31:546-550.

Aboulghar M, Mansour R, Al-Inany H, Abou-Setta AM, Aboulghar M, Mourad L, Serour G. (2007). Paternal age and outcome of intracytoplasmic sperm injection. Reprod Biomed Online 14:588-592.

Alio AP, Salihu HM, McIntosh C, August EM, Weldeselasse H, Sanchez E, Mbah AK. (2012). The effect of paternal age on fetal birth outcomes. Am J Mens Health 6:427-435.

Antignac JP, Cariou R, Maume D, Marchand P, Monteau F, Zalko D, Berrebi A, Cravedi JP, Andre F, Le Bizec B. (2008). Exposure assessment of fetus and newborn to brominated flame retardants in France: preliminary data. Mol Nutr Food Res 52:258-265.

Bille C, Skytthe A, Vach W, Knudsen LB, Andersen AM, Murray JC, Christensen K. (2005). Parent's age and the risk of oral clefts. Epidemiology 16:311-316.

Bray I, Gunnell D, Davey Smith G. (2006). Advanced paternal age: how old is too old?. J Epidemiol Community Health 60:851-853.

Dockerty JD, Draper G, Vincent T, Rowan SD, Bunch KJ. (2001). Case-control study of parental age, parity and socioeconomic level in relation to childhood cancers. Int J Epidemiol 30:1428-1437.

D'Onofrio BM, Rickert ME, Frans E, Kuja-Halkola R, Almqvist C, Sjolander A, Larsson H, Lichtenstein P. (2014). Paternal age at childbearing and offspring psychiatric and academic morbidity. JAMA Psychiatry 71:432-438.

Doucet J, Tague B, Arnold DL, Cooke GM, Hayward S, Goodyer CG. (2009). Persistent organic pollutant residues in human fetal liver and placenta from Greater Montreal, Quebec: a longitudinal study from 1998 through 2006. Environ Health Perspect 117:605-10.

ENCODE. (2011). ENCODE and modENCODE Guidelines For Experiments Generating ChIP, DNase, FAIRE, and DNA Methylation Genome Wide Location Data 2016.

Ferreira RC, Braga DP, Bonetti TC, Pasqualotto FF, Iaconelli A,Jr, Borges E,Jr. (2010). Negative influence of paternal age on clinical intracytoplasmic sperm injection cycle outcomes in oligozoospermic patients. Fertil Steril 93:1870-1874.

Frans EM, Sandin S, Reichenberg A, Lichtenstein P, Langstrom N, Hultman CM. (2008). Advancing paternal age and bipolar disorder. Arch Gen Psychiatry 65:1034-1040.

Frattarelli JL, Miller KA, Miller BT, Elkind-Hirsch K, Scott RT,Jr. (2008). Male age negatively impacts embryo development and reproductive outcome in donor oocyte assisted reproductive technology cycles. Fertil Steril 90:97-103.

Gabriel SM, Roncancio JR, Ruiz NS. (1992). Growth hormone pulsatility and the endocrine milieu during sexual maturation in male and female rats. Neuroendocrinology 56:619-625.

Heck JE, Lombardi CA, Meyers TJ, Cockburn M, Wilhelm M, Ritz B. (2012). Perinatal characteristics and retinoblastoma. Cancer Causes Control 23:1567-1575.

Herbstman JB, Sjodin A, Apelberg BJ, Witter FR, Patterson DG, Halden RU, Jones RS, Park A, Zhang Y, Heidler J, Needham LL, Goldman LR. (2007). Determinants of prenatal exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) in an urban population. Environ Health Perspect 115:1794-800.

Holland PW, Marletaz F, Maeso I, Dunwell TL, Paps J. (2017). New genes from old: asymmetric divergence of gene duplicates and the evolution of development. Philos Trans R Soc Lond B Biol Sci 372:10.1098/rstb.2015.0480.

Hultman CM, Sandin S, Levine SZ, Lichtenstein P, Reichenberg A. (2011). Advancing paternal age and risk of autism: new evidence from a population-based study and a meta-analysis of epidemiological studies. Mol Psychiatry 16:1203-1212.

Jenkins TG, Aston KI, Pflueger C, Cairns BR, Carrell DT. (2014). Age-associated sperm DNA methylation alterations: possible implications in offspring disease susceptibility. PLoS Genet 10:e1004458.

Johnson-Restrepo B, Kannan K, Rapaport DP, Rodan BD. (2005). Polybrominated diphenyl ethers and polychlorinated biphenyls in human adipose tissue from New York. Environ Sci Technol 39:5177-5182.

Kanehisa M, Goto S. (2000). KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28:27-30.

Karolchik D, Hinrichs AS, Furey TS, Roskin KM, Sugnet CW, Haussler D, Kent WJ. (2004). The UCSC Table Browser data retrieval tool. Nucleic Acids Res 32:D493-6.

Khalil A, Parker M, Brown SE, Cevikae SE, Guo LW, Jensen J, Olmsted A, Portman D, Wu H, Suvorov A. (2017). Perinatal exposure to 2,2′,4′4′ −tetrabromodiphenyl ether induces testicular toxicity in adult rats. Toxicology.

Khandwala YS, Zhang CA, Lu Y, Eisenberg ML. (2017). The age of fathers in the USA is rising: an analysis of 168 867 480 births from 1972 to 2015. Hum Reprod 32:2110-2116.

Kobayashi N, Okae H, Hiura H, Chiba H, Shirakata Y, Hara K, Tanemura K, Arima T. (2016). Genome-Scale Assessment of Age-Related DNA Methylation Changes in Mouse Spermatozoa. PLoS One 11:e0167127.

Krueger F, Andrews SR. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics 27:1571-1572.

Kuriyama SN, Talsness CE, Grote K, Chahoud I. (2005). Developmental exposure to low dose PBDE 99: effects on male fertility and neurobehavior in rat offspring. Environ Health Perspect 113:149-154.

Langmead B, Salzberg SL. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357-359.

Liebenberg R, van Heerden B, Ehlers R, Du Plessis AM, Roos JL. (2016). Advancing paternal age at birth is associated with poorer social functioning earlier and later in life of schizophrenia patients in a founder population. Psychiatry Res 243:185-190.

Malaspina D, Harlap S, Fennig S, Heiman D, Nahon D, Feldman D, Susser ES. (2001). Advancing paternal age and the risk of schizophrenia. Arch Gen Psychiatry 58:361-367.

Martin JA, Hamilton BE, Ventura SJ, Osterman MJ, Wilson EC, Mathews TJ. (2012). Births: final data for 2010. Natl Vital Stat Rep 61:1-72.

Milekic MH, Xin Y, O'Donnell A, Kumar KK, Bradley-Moore M, Malaspina D, Moore H, Brunner D, Ge Y, Edwards J, Paul S, Haghighi FG, Gingrich JA. (2015). Age-related sperm DNA methylation changes are transmitted to offspring and associated with abnormal behavior and dysregulated gene expression. Mol Psychiatry 20:995-1001.

Moll AC, Imhof SM, Kuik DJ, Bouter LM, Den Otter W, Bezemer PD, Koten JW, Tan KE. (1996). High parental age is associated with sporadic hereditary retinoblastoma: the Dutch retinoblastoma register 1862-1994. Hum Genet 98:109-112.

Mumford SL, Kim S, Chen Z, Gore-Langton RE, Boyd Barr D, Buck Louis GM. (2015). Persistent organic pollutants and semen quality: The LIFE Study. Chemosphere 135:427-435.

Neph S, Kuehn MS, Reynolds AP, Haugen E, Thurman RE, Johnson AK, Rynes E, Maurano MT, Vierstra J, Thomas S, Sandstrom R, Humbert R, Stamatoyannopoulos JA. (2012). BEDOPS: high-performance genomic feature operations. Bioinformatics 28:1919-1920.

Nybo Andersen AM, Urhoj SK. (2017). Is advanced paternal age a health risk for the offspring?. Fertil Steril 107:312-318.

Nybo Andersen AM, Hansen KD, Andersen PK, Davey Smith G. (2004). Advanced paternal age and risk of fetal death: a cohort study. Am J Epidemiol 160:1214-1222.

Oakes CC, Smiraglia DJ, Plass C, Trasler JM, Robaire B. (2003). Aging results in hypermethylation of ribosomal DNA in sperm and liver of male rats. Proc Natl Acad Sci U S A 100:1775-1780.

Pan D. (2010). The hippo signaling pathway in development and cancer. Dev Cell 19:491-505.

Paoli D, Pecora G, Pallotti F, Faja F, Pelloni M, Lenzi A, Lombardo F. (2019). Cytological and molecular aspects of the ageing sperm. Hum Reprod 34:218-227.

Perrin MC, Brown AS, Malaspina D. (2007). Aberrant epigenetic regulation could explain the relationship of paternal age to schizophrenia. Schizophr Bull 33:1270-1273.

Reichenberg A, Gross R, Weiser M, Bresnahan M, Silverman J, Harlap S, Rabinowitz J, Shulman C, Malaspina D, Lubin G, Knobler HY, Davidson M, Susser E. (2006). Advancing paternal age and autism. Arch Gen Psychiatry 63:1026-1032.

Robb GW, Amann RP, Killian GJ. (1978). Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil 54:103-107.

Schecter A, Papke O, Harris TR, Tung KC, Musumba A, Olson J, Birnbaum L. (2006). Polybrominated diphenyl ether (PBDE) levels in an expanded market basket survey of U.S. food and estimated PBDE dietary intake by age and sex. Environ Health Perspect 114:1515-1520.

Sergentanis TN, Thomopoulos TP, Gialamas SP, Karalexi MA, Biniaris-Georgallis SI, Kontogeorgi E, Papathoma P, Tsilimidos G, Skalkidou A, Iliadou AN, Petridou ET. (2015). Risk for childhood leukemia associated with maternal and paternal age. Eur J Epidemiol 30:1229-1261.

Sharma R, Agarwal A, Rohra VK, Assidi M, Abu-Elmagd M, Turki RF. (2015). Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 13:35-015-0028-x.

Shi Z, Jiao Y, Hu Y, Sun Z, Zhou X, Feng J, Li J, Wu Y. (2013). Levels of tetrabromobisphenol A, hexabromocyclododecanes and polybrominated diphenyl ethers in human milk from the general population in Beijing, China. Sci Total Environ 452-453:10-8.

Sokol SY. (2015). Spatial and temporal aspects of Wnt signaling and planar cell polarity during vertebrate embryonic development. Semin Cell Dev Biol 42:78-85.

Song Q, Decato B, Hong EE, Zhou M, Fang F, Qu J, Garvin T, Kessler M, Zhou J, Smith AD. (2013). A reference methylome database and analysis pipeline to facilitate integrative and comparative epigenomics. PLoS One 8:e81148.

Suvorov A, Shershebnev A, Wu H, Medvedeva Y, Sergeyev O, Pilsner J. (2018). Perinatal Exposure to Low Dose 2,2′,4,4′-Tetrabromodiphenyl ether (BDE-47) Alters Sperm DNA Methylation in Adult Rats . Reproductive Toxicology 75:136-143.

Suvorov A, Naumov V, Shtratnikova V, Pilsner J, Zheludkevich A, Gerasimov E, Logacheva M, Sergeyev O. (2020). Aging Induces Profound Changes in sncRNA in Rat Sperm and these Changes are Modified by Perinatal Exposure to Environmental Flame Retardant. Preprints.

Suvorov A, Vandenberg LN. (2016). To Cull or not to Cull? Considerations for studies of endocrine disrupting chemicals. Endocrinology:en20161145.

Suvorov A, Girard S, Lachapelle S, Abdelouahab N, Sebire G, Takser L. (2009). Perinatal exposure to low-dose BDE-47, an emergent environmental contaminant, causes hyperactivity in rat offspring. Neonatology 95:203-209.

Tripathi S, Pohl MO, Zhou Y, Rodriguez-Frandsen A, Wang G, Stein DA, Moulton HM, DeJesus P, Che J, Mulder LC, Yanguez E, Andenmatten D, Pache L, Manicassamy B, Albrecht RA, Gonzalez MG, Nguyen Q, Brass A, Elledge S, White M, Shapira S, Hacohen N, Karlas A, Meyer TF, Shales M, Gatorano A, Johnson JR, Jang G, Johnson T, Verschueren E, Sanders D, Krogan N, Shaw M, Konig R, Stertz S, Garcia-Sastre A, Chanda SK. (2015). Meta- and Orthogonal Integration of Influenza "OMICs" Data Defines a Role for UBR4 in Virus Budding. Cell Host Microbe 18:723-735.

van der Ven LT, van de Kuil T, Verhoef A, Leonards PE, Slob W, Canton RF, Germer S, Hamers T, Visser TJ, Litens S, Hakansson H, Fery Y, Schrenk D, van den Berg M, Piersma AH, Vos JG. (2008). A 28-day oral dose toxicity study enhanced to detect endocrine effects of a purified technical pentabromodiphenyl ether (pentaBDE) mixture in Wistar rats. Toxicology 245:109-122.

Vandenberg LN, Welshons WV, Vom Saal FS, Toutain PL, Myers JP. (2014). Should oral gavage be abandoned in toxicity testing of endocrine disruptors?. Environ Health 13:46-069X-13-46.

Wilford BH, Shoeib M, Harner T, Zhu J, Jones KC. (2005). Polybrominated diphenyl ethers in indoor dust in Ottawa, Canada: implications for sources and exposure. Environ Sci Technol 39:7027-7035.

Wu H, Hauser R, Krawetz SA, Pilsner JR. (2015). Environmental Susceptibility of the Sperm Epigenome During Windows of Male Germ Cell Development. Curr Environ Health Rep.

Wu H, de Gannes MK, Luchetti G, Pilsner JR. (2015). Rapid method for the isolation of mammalian sperm DNA. BioTechniques 58:293-300.

Xie K, Ryan DP, Pearson BL, Henzel KS, Neff F, Vidal RO, Hennion M, Lehmann I, Schleif M, Schroder S, Adler T, Rathkolb B, Rozman J, Schutz AL, Prehn C, Mickael ME, Weiergraber M, Adamski J, Busch DH, Ehninger G, Matynia A, Jackson WS, Wolf E, Fuchs H, Gailus-Durner V, Bonn S, Hrabe de Angelis M, Ehninger D. (2018). Epigenetic alterations in longevity regulators, reduced life span, and exacerbated aging-related pathology in old father offspring mice. Proc Natl Acad Sci U S A 115:E2348-E2357.

Zanato VF, Martins MP, Anselmo-Franci JA, Petenusci SO, Lamano-Carvalho TL. (1994). Sexual development of male Wistar rats. Braz J Med Biol Res 27:1273-1280.

Zemunik T, Peruzovic M, Capkun V, Zekan LJ, Milkovic K. (2001). Pregnancy in adolescent rats, growth and neurodevelopment in their offspring. Arch Physiol Biochem 109:450-456.

Zhang Z, Zhang X, Sun Z, Dong H, Qiu L, Gu J, Zhou J, Wang X, Wang SL. (2013). Cytochrome P450 3A1 mediates 2,2',4,4'-tetrabromodiphenyl ether-induced reduction of spermatogenesis in adult rats. PLoS One 8:e66301.