Does The Apple Fall Far From The Tree?

In utero exposure to persistent organic pollutants alters sperm miRNA expression across multiple, unexposed generations.

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Introduction

Numerous reports indicate that the prenatal environment can affect the parental germline and influence future generations. Our laboratory has used persistent organic pollutants (POPs) to show toxicant-induced disorders are transmitted from father to offspring over multiple generations coincident with alternations in sperm methylation, suggesting paternally-mediated epigenetic inheritance. However, the role of other epigenetic marks and the effect on his early embryo offspring have not yet been investigated.

Hypotheses

A. Prenatal paternal exposure to POPs alters sperm miRNA expression in sperm and the sperm of his offspring

B. POPs-induced epigenetic dysregulation of miRNA expression is reduced or prevented by nutritional folic acid (FA).

Project design

Composition Arctic POPs mixture

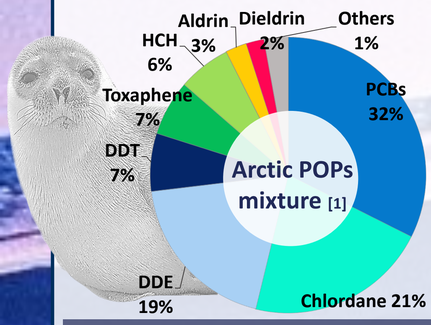
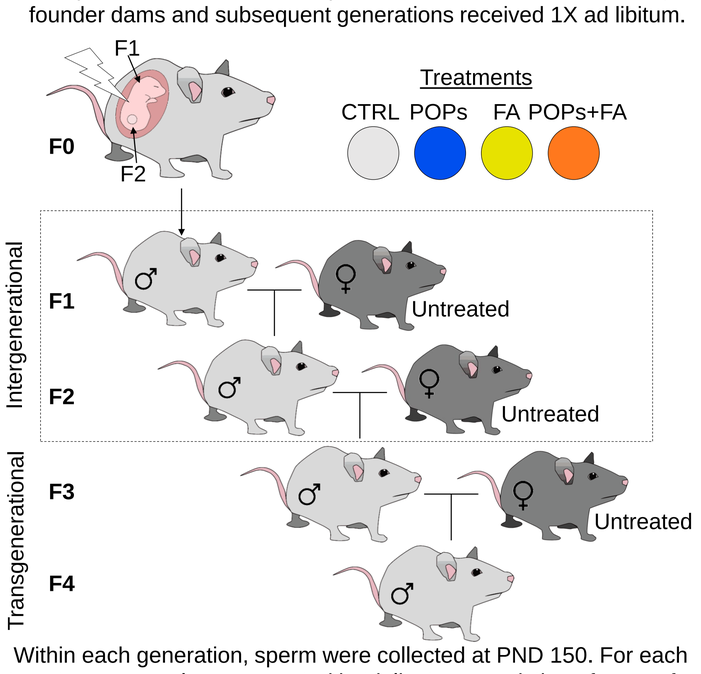


***Figure 1**. The Arctic POPs mixture represents the composition found in Ringed Seal blubber of Northern Quebec. The POPs mixture and dosing protocol are based on previous work, and are confirmed to generate plasma POP concentrations resembling to those observed in populations experiencing high exposure to POPs through the food chain (Anas et al, 2005). POPs were dissolved in corn oil.*

[1] Muir, D. *et al.* 1999.

Experimental set-up

F0 founder dams were exposed to POPs, 3x a week, 5 weeks before gestation and until parturition. FA diets were provided ad libitum. After 9 weeks, all F0 founder dams and subsequent generations received 1X ad libitum.



Within each generation, sperm were collected at PND 150. For each treatment, sperm pools were created in triplicate – consisting of sperm from 4 different males with each a different F0 mother (if possible)

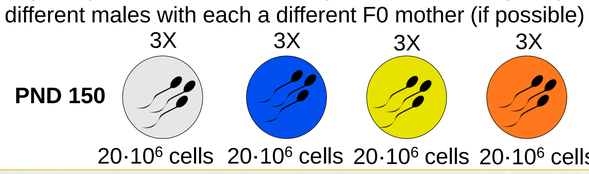


***Figure 2**. Folic acid supplementation was given in the form of supplemented food pellets. 2 mg/kg diet (1X) recommended daily allowance (RDA) of 0.2 mg dietary folate found in fortified foods. 6 mg/kg diet (3X) equals the additional dietary supplementation guidance amount for pregnant women.*

Methods

- Total miRNA extraction using mirVana™ miRNA Isolation Kit
- Sequencing Illumina MiSeq ~13 million reads/sample
- Gene Ontology Ingenuity Pathway Analysis and Metascape
- Validation several miRNAs using qPCR

Results

POPs alter sperm miRNA expression

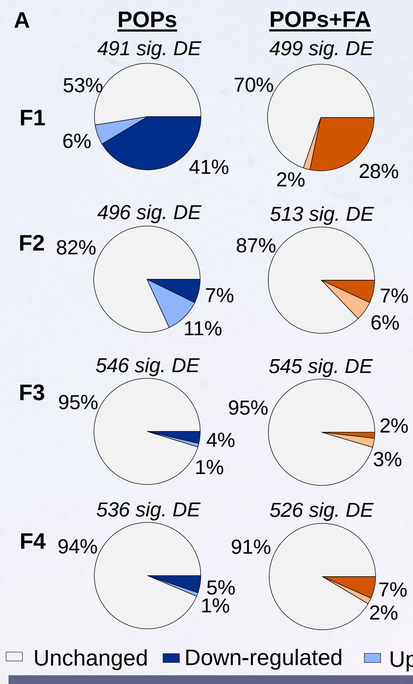


***Figure 3. In utero* exposure to POPs and POPs+FA display altered miRNA expression profiles in F1-F4.** Pie charts illustrating proportions of the differentially expressed (≥1.5 fold change ≤-1.5) and unchanged (<1.5 fold change >-1.5) miRNAs in POPs (blue) or POPs+FA (orange) compared to CTRL sperm. A total of 747 miRNAs were identified in the sperm of rats from the CTRL, POPs, FA and POPs+FA lineages in F1-F4 generations. Among the 747 identified miRNAs, ~ 65%-73% of miRNAs were expressed > 10 counts in F1-F4. For the F1 generation, *in utero* exposure to POPs dysregulated the expression of 47% of the miRNAs at least 1.5-fold compared to CTRL. POPs+FA supplementation caused fewer, 30%, miRNAs to be dysregulated in F1, indicating that maternal consumption of 3X FA diets protected her offspring's sperm epigenome from toxicant-induced perturbation.

GO-term of altered miRNAs by POPs & POPs+FA

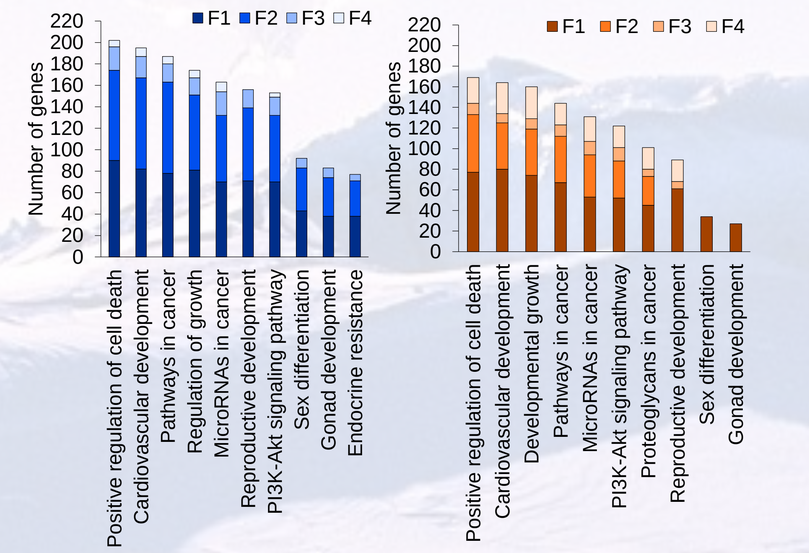


***Figure 4.* Gene ontology and pathway analysis based on validated miRNA-targeted genes for POPs (blue) and POPs+FA (orange).** Top 10 significant (P < 0.05) GOs and KEGG enriched pathways predicted by dysregulated miRNAs in F1-F4 are presented. Compared to POPs, POPs+FA affected miRNAs alter less genes involved in sex differentiation and gonad development F1 and none in F2-F4.

POPs+FA counteracts POPs effect on sperm miRNA

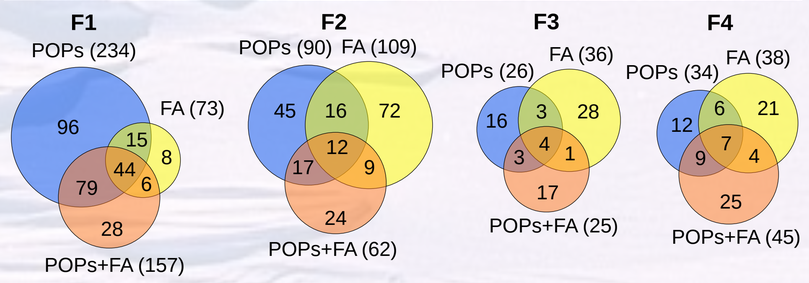


***Figure 5.* Combining FA with POPs counteracts the effect of POPs on sperm miRNA expression.** Venn diagrams comparing the number of differentially expressed (≥1.5 fold change ≤-1.5, >10 reads) between treatments with respect to controls in generation F1-F4.

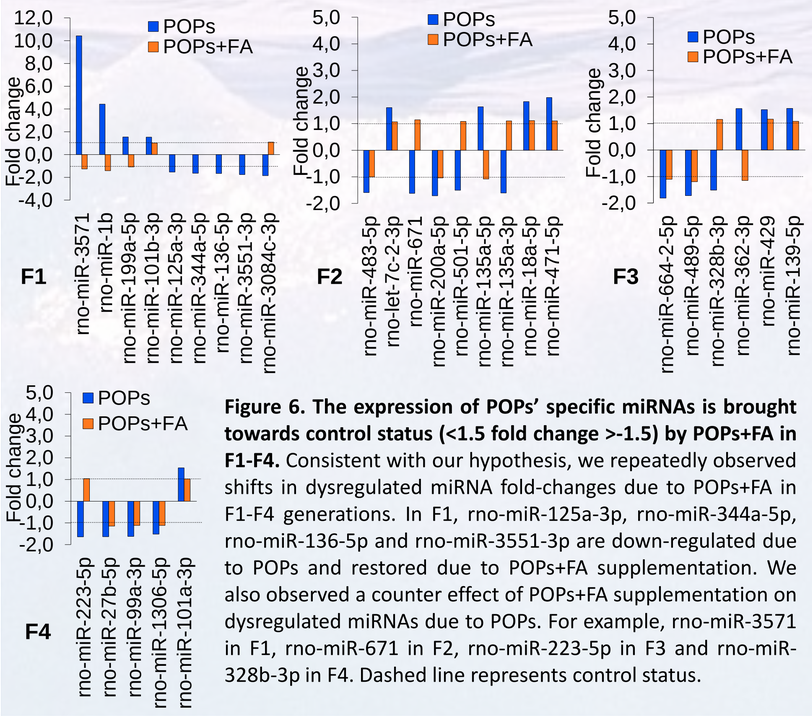
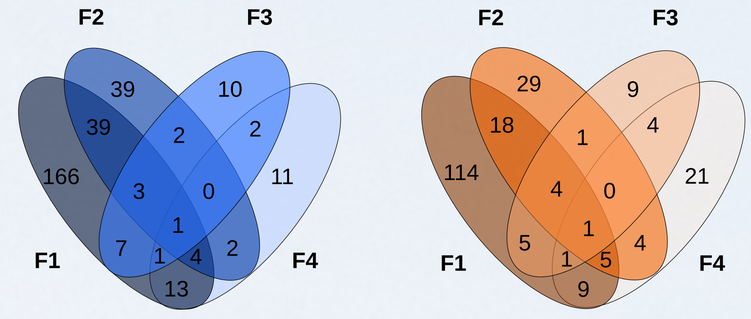


Figure 6. The expression of POPs' specific miRNAs is brought towards control status (<1.5 fold change >-1.5) by POPs+FA in F1-F4. Consistent with our hypothesis, we repeatedly observed shifts in dysregulated miRNA fold-changes due to POPs+FA in F1-F4 generations. In F1, rno-miR-125a-3p, rno-miR-344a-5p, rno-miR-136-5p and rno-miR-3551-3p are down-regulated due to POPs and restored due to POPs+FA supplementation. We also observed a counter effect of POPs+FA supplementation on dysregulated miRNAs due to POPs. For example, rno-miR-3571 in F1, rno-miR-671 in F2, rno-miR-223-5p in F3 and rno-miR-328b-3p in F4. Dashed line represents control status.

Results

POPs & POPs+FA cause transgenerational effect



		Fold Change			
miRNA	**Experimentally validated mRNA targets**	**F1**	**F2**	**F3**	**F4**
Rno-mir-32-5p	BCL2L11, BMPR2, CCNE2, CDKN1A, CDKN1C, ENPP6, FBXW7, IKZF1, ITGA5, ITGB3, MAP2K4, MAPRE1, MYLIP, PTEN, ZEB2	1.56	4.00	1.61	
Rno-mir-678		-1.96	-1.59	-1.77	
Rno-mir-3586-5p	BCL6, GRM3	-2.89	-1.60	-1.88	-1.50
Rno-mir-99a-3p		-2.91	-1.69	-1.62	

		Fold Change			
miRNA	**Experimentally validated mRNA targets**	**F1**	**F2**	**F3**	**F4**
Rno-mir-32-5p	BCL2L11, BMPR2, CCNE2, CDKN1A, CDKN1C, ENPP6, FBXW7, IKZF1, ITGA5, ITGB3, MAP2K4, MAPRE1, MYLIP, PTEN, ZEB2	1.70	1.64	1.57	
Rno-mir-129-5p	AGO3, BMPR2, SOX4, TNPO1, TP53INP1	-1.99	-1.91	-2.01	1.52
Rno-mir-3586-5p	BCL6, GRM3	-2.38	-1.50	-1.76	
Rno-mir-329-5p		-1.65	-1.57	-1.56	
Rno-mir-451-5p	ABCB1, MIF	1.99	-1.52	1.53	

***Figure 7. In utero* exposure to both POPs and POPs+FA affects sperm miRNA expression transgenerationally (F1-F4).** (**a**) Venn diagram depicting the overlap of differentially expressed miRNAs (≥1.5 fold change ≤-1.5, >10 reads) due to POPs between F1-F4. (**b**) Venn diagram depicting the overlap of differentially expressed miRNAs (≥1.5 fold change ≤-1.5, >10 reads) due to POPs+FA between F1-F4. (**c**) Transgenerational miRNAs and correlating fold change from F1-F4 due to POPs. (**d**) Transgenerational miRNAs and correlating fold change from F1-F4 due to POPs+FA.

POPs+FA counteracts POPs effect on sperm miRNA

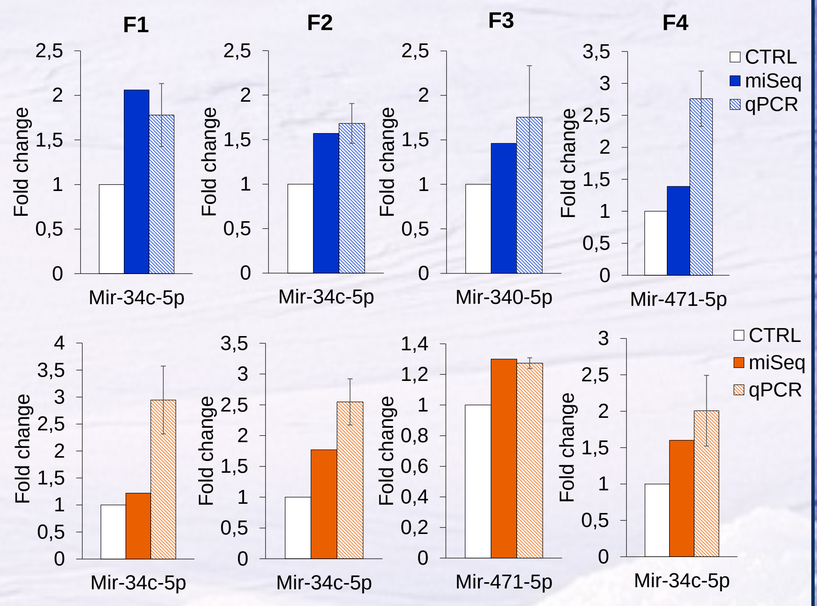


Figure 4. Validation of miRNA sequencing data using real-time PCR. Total RNA was extracted from CTRL, POPs and POPs+FA sperm. The expression of miRNAs relative to endogenous control RNA was determined by real-time PCR. The results are expressed as a fold change of POPs or POPs+FA to CTRL. Data are presented as means ± S.D. from 3-5 rats, each assay performed in triplicate.

Conclusion

Sperm miRNA expression is altered inter- and transgenerationally due to prenatal POPs & POPs+FA exposure. Data indicate a possible protective effect of dietary FA supplementation against POPs.

To obtain a complete overview of multigenerational epigenetic changes, we will investigate the impact of *in utero* exposure to POPs and POPs+FA on the two-cell transcriptome in generation F2 to F4. In addition, we investigate sperm DNA methylation marks and histone marks in generation F1 to F4 – to have a complete overview of the sperm epigenome.