RESEARCH

Daily exposure to phthalates and alkylphenols alters miR biogenesis and expression in mice ovaries

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Abstract

Reproductive hormone imbalance in infertile women is correlated to high levels of phthalates and alkylphenols, which are among endocrine-disrupting chemicals (EDCs). Previous studies have shown that they interfere with gene expression by deregulating levels of microRNAs (miRs), small non-coding RNAs targeting mRNAs encoding enzymes in the hormone biosynthesis pathway. However, this effect depends on the target organ, dose and whether or not they are alone or in mixtures. Our goal was to study whether the biosynthesis, and a specific group of miRs targeting mRNAs encoding enzymes in steroid hormone biosynthesis, are deregulated in the ovaries of female mice chronically exposed to a mixture of three phthalates (DEHP+DBP+BBP) and two alkylphenols (NP+OP) at a human environmentally relevant dose. We performed qPCR and Western blot assays along with a bioinformatics approach and found that this mixture modified the biogenesis machinery of miRs, inducing an increase in the mRNA levels of *Drosha* and *Dicer1* and DROSHA protein levels. In addition, we found changes in the precursor and mature forms of miR-96-5p, miR-200b-3p, miR-365-3p, miR-378a-3p and miR-503-5p which target steroidogenic pathway enzymes. Finally, using primary granulosa cell culture, we confirmed that miR-200b-3p targets *Cyp19a1*, transcript encoding CYP19A1, the enzyme that produces estradiol (E₂). These results indicate that chronic exposure to phthalates and alkylphenols mixture alters the biogenesis of ovary miRs and increases the expression of miRs implicated in the control of steroidal hormone synthesis in female mice, thus contributing to reproductive pathologies.

Key Words

- ▶ endocrine disruptors
- ▶ ovary
- ▶ miRs
- ▶ gene regulation

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Introduction

Humans are exposed to ~85,000 different molecules throughout life (Fenner-Crisp *et al.* 2000), some of which have the property to alter hormone homeostasis and are known as endocrine-disrupting chemicals (EDCs). Phthalates such as bis (2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and benzyl butyl phthalate (BBP) are used primarily to soften PVC, and to increase

transparency, durability, flexibility and longevity. Alkylphenols such as 4-nonylphenol (NP) and 4-tert-octylphenol (OP) are building blocks in the production of detergents, personal care products, cosmetics, and additives for lubricants, fuels and polymers (Guart *et al.* 2011, Dodson *et al.* 2012, Hwang & Choi 2015, Mitro *et al.* 2016, Broe *et al.* 2017). Several evidences indicate that these

compounds migrate from their containers and enter the body acting as EDCs (Chang *et al.* 2014, Sathyanarayana *et al.* 2017). However, most of the toxicological studies on animal models have focused on single compound exposure during a specific developmental window, with high and not environmentally relevant doses; also, in most of them, the method of administration (e.g. intraperitoneal injection) is not related to physiological exposure (Luu *et al.* 2017, Liu *et al.* 2018).

In women, these compounds are present in many types of tissues and biological fluids, like the serum, milk, and follicular fluid (Du *et al.* 2016, 2019, Parikh *et al.* 2018; <https://clinicaltrials.gov/show/NCT01385605>), and they are correlated to fertility problems (Tranfo *et al.* 2012, Du *et al.* 2016, Messerlian *et al.* 2016, Parikh *et al.* 2018, Martinez *et al.* 2019; <https://clinicaltrials.gov/show/NCT01385605>). In addition, *in vitro* and *in vivo* murine studies have shown that single and/or mixed exposure to phthalates or alkylphenols influences the development of ovarian follicles and changes gene expression associated with the regulation and synthesis of steroid hormones as well as steroid hormone levels, mainly aromatase (CYP19A1)/E₂ in ovary and testes (Willoughby *et al.* 2005, Hannon & Flaws 2015, Bunay *et al.* 2017, 2018, Patino-Garcia *et al.* 2018). However, the molecular mechanism by which phthalates and alkylphenols deregulate the expression of steroidogenic pathway enzyme genes has not yet been revealed.

Epigenetic regulation by small non-coding RNAs such as microRNAs (miRs) is a conserved mechanism of post-transcriptional gene expression (Uzumcu *et al.* 2012, Bunkar *et al.* 2016). The biogenesis of miRs involves multiple elements and alternative pathways, but four enzymes are considered key factors in the canonical miR pathway. DROSHA and DICER1 are involved in the processing of miRs from pri-miRs (primary miRs), pre-miRs (miR-precursors) to mature miRs and EXPORTIN-5 (Xpo5), which is implicated in the export of pre-miRs from the nucleus to the cytoplasm, and AGO2 participates in the RNA-induced silencing complex (RISC) that finally binds to target mRNA (Gavin & Akbarian 2012, Garcia-Lopez *et al.* 2013, Bhaskaran & Mohan 2014). The evidence in mammals shows that miRs regulate follicle development and steroidogenesis in the ovaries (Sang *et al.* 2013, Li *et al.* 2015, Toms *et al.* 2017). Furthermore, gene ablation of *Drosha* and *Dicer1* alters ovary physiology and decreases fecundity (Lykke-Andersen *et al.* 2008, Nagaraja *et al.* 2008, Otsuka *et al.* 2008, Lei *et al.* 2010, Kuang *et al.* 2014, Kim *et al.* 2016, Guo *et al.* 2017). In women, urinary concentrations of phthalate metabolites

are associated with the expression of extracellular vesicle miRs in follicular fluid (Martinez *et al.* 2019). The exposure to DEHP altered the ovarian miR expression profile of newborn mice (Zhang *et al.* 2019). In a previous work, we found that female mice chronically exposed to a mixture of phthalates and alkylphenols show a decrease in the expression of ovarian steroidogenesis genes, including CYP19A1, along with a significant decrease in E₂ plasmatic levels (Patino-Garcia *et al.* 2018). We hypothesized that this may be due to alterations in post-transcriptional regulation of genes encoding the steroidogenesis pathway. For this reason, in the present work, our general aim was to study changes in the genes responsible for encoding proteins involved in the biogenesis of miRs as well as in the expression pattern of miRs which act as post-transcriptional regulators of steroidogenic gene expression in mice ovaries exposed to a mixture of phthalates and alkylphenols from conception until adult life.

Materials and methods

Chemicals

DEHP, DBP, BBP, NP, OP, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Ethanol was acquired from Winkler (Santiago, Chile). A bulk stock solution containing three phthalates (DEHP, DBP, and BBP) diluted in DMSO and two alkylphenols (NP and OP) diluted in ethanol was used to prepare the final mixture. For vehicle and treatments, the final DMSO and ethanol doses were 2.5 g/kg-bw/day and 0.06 g/kg-bw/day. In mice, orally tolerable doses were established at 7.9 g/kg for DMSO and 2.5 g/kg for ethanol (Gad *et al.* 2016). The mixture dose assayed in this study was 1 mg/kg-bw/day, which was chosen based on the human non-occupational exposure to these compounds (NTP-CERHR 2003a,b, Jonsson 2006, Shelby 2006, Ademollo *et al.* 2008).

Animals and *in vivo* dosing regimen

Adult female C57BL/6J mice aged 60 days were obtained from the Animal Facility of PUC and maintained in bisphenolA-free H-TEMP polysulfone cages (TECNIPLAST). The mice were housed in an environmentally controlled room under a 12 h light:12 h darkness cycle that was maintained at 22 ± 1°C. Phytoestrogen-free food and water were provided for *ad libitum* consumption. The experimental protocol was approved by the Ethical Scientific Committee for the Care of Animals and the Environment at the PUC No. 141222004. All animal

protocols were endorsed by the Chilean National Fund of Science and Technology (FONDECYT).

The mixture used in the present study represents the most abundant phthalates and alkylphenols in everyday products (Snedeker & Hay 2014, Mitro *et al.* 2016). Doses were chosen based on non-occupational exposure to these compounds (0.3 mg/kg-bw/day for each phthalate and 0.05 mg/kg-bw/day for each alkylphenol) (FDA 2001, Kavlock *et al.* 2002, Jonsson 2006, Kavlock *et al.* 2006, Hines *et al.* 2011, Mitro *et al.* 2016, Somasundaram *et al.* 2017). In this way, we generated the dose of this mixture resembling non-occupational (1 mg/kg-bw/day) human exposure. It should be noted that these doses are, in relation to NOAEL and LOAEL of DEHP and NP, ~1000 times lower for endocrine-reproductive adverse effects in humans, rats and mice (Chapin *et al.* 1999, ATSDR 2002, Osimitz *et al.* 2015) which made human-relevant doses.

The final dose was calculated according to the body weight and volume of water ingested by the mice recorded in a pilot study and in agreement with data in the literature referring to these parameters (Bachmanov *et al.* 2002, Spangenberg *et al.* 2014). Water intake was controlled each day. The global water intake was not affected by exposure to the phthalates and alkylphenols mixture or vehicle. The mixture of phthalates and alkylphenols was freshly made according to the changes in animal body weight (bw) and administered in the drinking water and throughout the experimental period. The bottle was covered with foil to prevent photolysis of the compounds.

To emulate chronic human exposure to an environmental mixture of phthalates and alkylphenols, we administered the mixture (1 mg/kg/day; containing 0.3 mg/kg-bw/day of each phthalate (DEHP, DBP and BBP) and 0.05 mg/kg-bw/day of each alkylphenol (NP and OP)) or vehicle (control: DMSO and ethanol) to pregnant mice from day 0.5 post-coitus (biological *n*) during pregnancy and lactation as described above (Table 1). At weaning

(day 21), only females were selected and maintained in a group of three to four individuals per cage. We continued the administration of the mixture of phthalates and alkylphenols or control until adulthood. At the end of the treatment, females (60 days old) in estrous were killed, and both ovaries were aseptically removed and; cleaned of interstitial tissue.

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from the ovary samples using TRIzol reagent (Invitrogen). The quantity and integrity of total RNA were determined by a Thermo Scientific Nanodrop 2000 spectrophotometer. Gene and primary-precursor (pri-pre-miR) cDNA was synthesized from 1 µg RNA by RT Master Mix (Promega) and random primers. The cDNA of miRs was synthesized using a previously described protocol (Cirera & Busk 2014). Briefly, 100 ng of total RNA was poly(A)-tailed and reverse-transcribed in a single reaction tube containing 0.1 mM of ATP, 0.1 mM of dNTPs, 100 units of MMLV reverse transcriptase (New England Biolabs), 1 µL of 10x poly(A) polymerase buffer, 1 unit of poly(A) polymerase (New England Biolabs) and 1 µM of Oligo (dT) Adaptor (Applied Biological Materials, Richmond, Canada) in a final volume of 10 µL. The qPCR was performed using 1 µM of primers and 2XSyBR Green Fast qPCR MasterMix (Biotool, TX, USA). Expression data were normalized using the $2^{-\Delta\Delta C_t}$ method with *Gapdh* and *Actb* as endogenous reference genes for mRNAs and U6 snRNA for miRs. Primers for miR-200b-3p (MPM00977) and miR-503-5p (MPM01369) were obtained from Applied Biological Materials. The miRprimer (Busk 2014) software was used to design functional forward primers for the miR-96-5p, miR-365-3p and miR-378a-3p. Both mRNAs and specific miR forward primers (Table 2), and the 3' universal primer (Applied Biological Materials) were used for endogenous miR analysis.

Table 1 Body weight and water consumption by the mice dam.

Variables	Gestational and lactation stages			
	Beginning of pregnancy	Mid pregnancy	End of pregnancy	Lactation
Body weight (g)				
Vehicle	18.5 ± 0.43	25.6 ± 1.46	35.0 ± 1.00	26.1 ± 0.40
EDCs	18.6 ± 0.37	25.4 ± 1.42	33.5 ± 1.19	24.6 ± 0.56
Water intake (mL)				
Vehicle	4.6 ± 0.06	6.4 ± 0.23	8.8 ± 0.30	23.1 ± 2.33
EDCs	4.7 ± 0.09	6.6 ± 0.13	8.4 ± 0.34	22.1 ± 1.78

Values represent the mean ± s.e.m., *n* = 4. No differences were observed to compare vehicle and EDCs. EDCs (mixture of phthalates and alkylphenols).

Protein extraction and Western blotting

Protein extraction and Western blotting were performed as previously published (Patino-Garcia *et al.* 2019). Briefly, the homogenization of ovaries was performed in a buffer containing 1 M NaCl, 1 mM EDTA, 10 mg/mL PMSF, 1% Triton X-100, and 20 mM Tris-HCl pH 7.4, plus a general metalloprotease inhibitor, BB-94 10 μ M, and protease inhibitor cocktail (Sigma), and then centrifuged for 10 min at 10,000 *g* at 4°C. Then, samples of 20 μ g of protein were separated by electrophoresis on a 10% polyacrylamide gel (SDS-PAGE) under denaturing and reducing conditions before being transferred to a nitrocellulose membrane (Thermo Scientific) at 350 mA for 2 h. Next, membranes were incubated with sodium citrate solution 0.01 M, pH 6.0, for 10 min at 95°C to expose the antigens and blocked with a solution of 3% (w/v) BSA 0.1% (v/v) Tween in Tris-buffered saline, pH 7.4 and incubated overnight with the respective primary antibodies for DROSHA (1:1000) (Santa Cruz Biotechnology) and ACTB (1:5000)

(Abbeva, Cambridge, UK) as a loading control. Finally, the second incubation took place with their respective secondary antibodies conjugated with horseradish peroxidase (KPL, Gaithersburg, MD, USA) diluted 1:5000 in blocking solution for 1 h at room temperature. Peroxidase activity was detected using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer).

Bioinformatics analyses

Predicted miR targets for steroidogenic mRNAs were obtained using the searches of several databases that including miRWalk, miRecords and MicroCosm. To filter the large number of obtained miRs, presence in the ovary was determined using miRNA. Also, to verify whether miR levels were affected by some of the compounds evaluated in this work, we used ChemiRs. Finally, validated mRNA targets for certain miRs were obtained using the miRWalk database and DIANA-TarBase v7.0. Validated miR-mRNA target interactions were performed using the

Table 2 Sequence of qPCR primers.

Name	Primer	Sequence (5'-3')
<i>Drosha</i>	Forward primer	GGACCATCACGAAGGACACTT
	Reverse primer	ATGCCAGTTCCTCTGCTACCT
<i>Xpo5</i>	Forward primer	GAAGTAGCCCCCTCGTCTGT
	Reverse primer	AGCAGTGCTGTGCAGACATC
<i>Dicer1</i>	Forward primer	GGATGCGATGTGCTATCTGGA
	Reverse primer	GCACTGCTCCGTGTGCAA
<i>Ago2</i>	Forward primer	GCCGTCCTTCCCACTACCAC
	Reverse primer	GGTATTGACACAGAGCGTGTGC
Pri-pre-let-7f	Forward primer	GATTGTATAGTTGTGGGGTAGTGA
	Reverse primer	TCAGGGAAGGCAATAGATTGTA
Pri-pre-miR-96	Forward primer	CCGATTTTGGCACTAGCAC
	Reverse primer	CATATTGGCATGCACATGA
Pri-pre-miR-200b	Forward primer	CCGTGGCCATCTTACTGG
	Reverse primer	TCCGCCGTCATCATTACC
Pri-pre-miR-203	Forward primer	GCTGGGTCCAGTGTTCTTA
	Reverse primer	GCCGGGTCTAGTGGTCCTA
Pri-pre-miR-221	Forward primer	CACCATCCAGGTCTGGGG
	Reverse primer	TTCCAGGTAGCCTGAAACCC
Pri-pre-miR-365	Forward primer	TCACCTCGGCTCATCTGG
	Reverse primer	TGACCTTCCTTGGGCACT
Pri-pre-miR-378a	Forward primer	AGGGCTCCTGACTCCAGG
	Reverse primer	AGGCCTTCTGACTCCAAGTC
Pri-pre-miR-503	Forward primer	GCCAGATCTTTGCCCTATTCTTGCC
	Reverse primer	GCCGAATTCTCTGATTGGGAAGGATTTT
<i>Gapdh</i>	Forward primer	GCTGATGCTCCCATGTTCTGTAT
	Reverse primer	GTGGTGCAAGAGGCATTGCTGAC
<i>Actb</i>	Forward primer	GCTTCTTTGCGCTCCTTCGT
	Reverse primer	CCAGCGCAGCGATATCG
miR-96-5p	Forward primer	TTTGGCACTAGCACATTTTGTCT
miR-365-3p	Forward primer	TAATGCCCCATAAATCCTTAT
miR-378a-3p	Forward primer	ACTGGACTTGGAGTCAGAAGG
<i>U6</i>	Forward primer	CGCTTCGGCAGCACATATAC
	Reverse primer	AAAATATGGAACGCTTCACGA

Cytoscape software. Functional analysis for potential mRNA targets in biological processes of gene ontology (GO) domains was performed using a Cytoscape plugging ClueGO.

Statistical analysis

All data represent the mean $\pm$ S.E.M. We analyzed the differences in the averages through the unpaired *t*-test and the Mann-Whitney *U*-test or one-way ANOVA along with Tukey's post hoc test using GraphPad Prism version 5.0 (GraphPad Software); *P* values < 0.05 were considered statistically significant. Heatmap was performed using QCanvas 1.2.1. GO enrichment analysis was carried out using a hypergeometric test with a *P* value less than 0.05 and Kappa score of 1.

Results

Phthalate and alkylphenol mixtures changed the expression levels of genes involved in the biogenesis of miRs

Since exposure to phthalates and alkylphenols affects the biogenesis machinery of miRs in the testes (Bunay *et al.* 2017, 2019), we wondered if this also occurred in the ovaries of females under the same experimental conditions. To answer this question, we quantified the mRNA expression levels of genes encoding for *Drosha*, *Dicer1*, *Ago2* and *Xpo5*, along with DROSHA protein levels. We found significantly increased levels of *Drosha* and *Dicer1* mRNA and DROSHA protein in the ovaries of exposed mice (Fig. 1). However, mRNA levels of *Xpo5* and *Ago2* were similar to those of control mice (Fig. 1A). Thus, some elements of the machinery for biogenesis are upregulated by exposure to phthalates and alkylphenols mixture in ovaries, which might explain the change in the miR global profile.

The exposure to phthalates and alkylphenols mixture alters pri-pre-miRs

The next step was to determine whether exposure to the phthalates and alkylphenols mixture impaired primary-precursor (pri-pre-miR) levels to mature miRs targeting mRNAs encoding enzymes in steroid hormone biosynthesis. First, we aimed to identify predicted miRs that target *Star* and *Cyp19a1*, given that this mixture

Figure 1

Exposure to the mixture of phthalates and alkylphenols induces changes in the miR biogenesis machinery. (A) RT-qPCR analysis of mRNA levels of genes that encode proteins involved in miR biogenesis and miR nuclear export and editing. (B) DROSHA protein levels. Values represent the mean $\pm$ S.E.M., Mann-Whitney *U*-test, *n* = 4 (each data point represents one animal), **P* < 0.05, ***P* < 0.01. EDCs (mixture of phthalates and alkylphenols), and AU (arbitrary units). A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0149>.

of compounds decreased the mRNA levels of those transcripts in our previous study (Patino-Garcia *et al.* 2018). During *in silico* analysis, we detected that nine mature miRs would potentially target *Star* (mmu-miR-96-5p, mmu-miR-365-3p and mmu-miR-378a-5p), and *Cyp19a1* (mmu-let-7f-1-3p, mmu-miR-200b-3p, mmu-miR-203-3p, mmu-miR-221-5p, mmu-miR-365-3p, mmu-miR-378a-3p and mmu-miR-503-5p). The evaluation of pri-pre-miR levels in the ovaries of exposed females showed a decrease in the levels of pri-pre-miRs for mmu-let-7f-1-3p, mmu-miR-203-3p and mmu-miR-221-5p. On the other hand, we found an increase in the levels of pri-pre-miRs of mmu-miR-96-5p, mmu-miR-200b-3p, mmu-miR-365-3p, mmu-miR-378a-3p, mmu-miR-378a-5p, and mmu-miR-503-5p (Fig. 2A). Next, we validated the increase in these pri-pre-miRs by quantifying their mature miRs. The results showed that levels of the mature forms of all five upregulated pri-pre-miRs were also increased compared to control-treated mice (Fig. 2B).

Figure 2

Exposure to the mixture of phthalates and alkylphenols alters precursor and mature miRs. RT-qPCR analysis of (A) pri-pre-miRs and (B) miRs. Heat map of the 8 pri-pre-miRs in female mice ovaries following EDCs treatment. The differential expression of pri-pre-miRs can be easily followed by a color-coded approach, indicating low levels of pri-pre-miR content with green, and high levels with red. The data for pri-pre-miRs are presented as fold change with respect to control. Values represent the mean $\pm$ S.E.M., Mann-Whitney *U*-test, $n = 4$ (each data point represents one animal), $*P < 0.05$. EDCs (mixture of phthalates and alkylphenols) and AU (arbitrary units). A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0149>.

The exposure to phthalates and alkylphenols mixture alters the miR levels in ovary of adult females

Given that the upregulation of miRs is involved in the post-transcriptional control (mostly repression) of target mRNAs, we developed an integrative *in silico* analysis of validated miR-mRNA target interactions. This analysis revealed that three out of five upregulated miRs (miR-200b-3p, miR-378a-3p and miR-503-5p) are involved in the down-regulation of a set of genes related to steroidogenic pathways (Fig. 3A), affecting reproductive processes such as the regulation of follicle-stimulating hormone secretion, follicle and uterus development, estrogen receptor signaling and response to gonadotropin (Fig. 3B).

Of the three miRs targeting the *Cyp19a1* mRNA (miR-378a-3p, miR-503-5p and miR-200b-3p), we chose to study miR-200b-3p because it is increased in women with PCOS (Xue *et al.* 2018, He *et al.* 2019) and the other two have

Figure 3

Gene ontology (GO) enrichment analysis of genes targeted by upregulated miRs. The miRs that are upregulated in mice ovaries after exposure to mixture of EDCs are shown in red rhombuses and its validated target genes are shown in green ovals (A). Enrichment analysis of downregulated genes (B). GO enrichment analysis was carried using a hypergeometric test with a *P*-value less than 0.05 and Kappa score of 1. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0149>.

already been experimentally validated to target *Cyp19a1* mRNA (Lei *et al.* 2010, Xu *et al.* 2011, Pan *et al.* 2015). First, we reconfirmed the predicted results for *Cyp19a1* as a target of miR-200b-3p using the online tool, FindTar3 (Ye *et al.* 2008), which offers a qualitative assessment for miR-mRNA binding and was rated 'excellent' for the mouse and 'good' for the human gene (Fig. 4A). In order to experimentally validate the *in silico* results, we used a primary cell culture from mouse granulosa cells, which are the somatic cells surrounding the oocyte and produce and secrete E_2 . The results showed a significant decrease in E_2 secretion as well as mRNA and protein levels of CYP19A1, when cells were transfected with agomiR-200b-3p, which mimics the action of endogenous miR-200b-3p (Fig. 4B, C and D). On the other hand, only protein levels of CYP19A1 increased when granulosa cells were transfected with the antagomiR-200b-3p (Fig. 4D), which antagonize the action

A

miRNA	mRNA	Position	Structure	Loop Score	ΔG	Recommendation
mmu-miR-200b-3p	NM_007810	716-737	3' AGUAGUAUUG-UCCG- UCAAUU 5' ***** : 5' AAAACA-TGCTAAGT AGTATTA 3'	25.00	-18.10	excellent
hsa-miR-200b-3p	NM_000103	738-763	3' AGUAGUAUUG-UCCG- UCAAUU 5' ***** : 5' TAGTAATAGCTCGGTT AGTATTA 3'	15.00	-16.40	good

B

C

D

E

Figure 4

The miR-200b-3p suppresses E₂ production and CYP19A1 protein levels in mice granulosa cells. Online prediction software predicted relationship between miR-200b-3p and *Cyp19a1* in mice (top) and human (bottom) (A). Levels of E₂ in culture medium collected from granulosa cells transfected with miR-200b-3p. RT-qPCR of *Cyp19a1* in granulosa cells transfected with miR-200b-3p (C). CYP19A1 protein levels after miR-200b-3p transfection (D), and miR-200b-3p transfection control (E). Values represent the mean $\pm$ S.E.M., Mann-Whitney *U*-test, *n* = 3 with two technical replicates each, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. EDCs (mixture of phthalates and alkylphenols), AU (arbitrary units), and E₂ (estradiol). A full color version of this figure is available at <https://doi.org/10.1530/IME-20-0149>.

of endogenous miR-200b-3p (Fig. 4E). Further data confirm the increase or decrease miR-200b-3p levels after treatment with agomiR or antagomiR, respectively (Fig. 4E). Therefore, these in vitro results strongly suggest that miR-200b-3p targets *Cyp19a1* mRNA in mouse granulosa cells.

Overall, these data suggest that the dysregulation of the biosynthetic machinery of miRs and levels of miRs targeting mRNAs encoding steroidogenic enzymes, in the ovary of females treated with a mixture of phthalates and alkylphenols, is a mechanism that underlies their endocrine-disrupting action.

Discussion

Recent evidence shows that changes in reproductive tissue function due to exposure to environmental EDCs, such as bisphenol A (Lin *et al.* 2017) or DBP (Gaido *et al.* 2007), could be explained by the disruption in the post-transcriptional regulatory machinery, where a pivotal step is the mRNA stability regulated by miRs. Here, we show that chronic exposure to a mixture of three phthalates and two alkylphenols, from conception to adulthood, induces the upregulation of *Drosha* and *Dicer1* in the ovary of exposed female mice, suggesting a possible disruption mechanism in the processing of miRs that may explain the changes at the endocrine and reproductive level (Patino-Garcia *et al.* 2018).

Selective inactivation of *Dicer1* in prophase I oocytes of the fetal ovary led to compromised folliculogenesis, premature ovarian failure and infertility in adult mice

(Yuan *et al.* 2014). Therefore, the analysis of *Drosha* and *Dicer1* in infertile women would also provide further clues about the genetic etiology underlying premature ovarian failure and infertility during EDCs exposure, such as phthalates and alkylphenols.

Previous studies have shown that some elements of the canonical biogenesis machinery, like *Drosha* and *Dicer1* are dysregulated by single EDCs, xenobiotics and/or environmental chemicals in vitro or in vivo. For example, the expression of *Drosha* mRNA was decreased by BPA (Tait *et al.* 2015, Lin *et al.* 2017, Mahemuti *et al.* 2018); and it was increased by dioxin/ethinyl (Boverhof *et al.* 2008), flutamide (Laifenfeld *et al.* 2014), titanium dioxide (Jennen *et al.* 2010) and dicrotophos (an organophosphate insecticide) (Hseu *et al.* 2017). In the same way, *Dicer1* mRNA expression was decreased by benzo(a)pyrene (Jennen *et al.* 2010, Magkoufopoulou *et al.* 2011), BPA (Ali *et al.* 2014), ochratoxin A (a mycotoxin) (Qi *et al.* 2014) and DBP (Gaido *et al.* 2007). Interestingly, our previous study in testes from mice exposed to the same mixture, and under the same conditions as those described in this work, show an increase in *Drosha* but no change in the levels of *Dicer1*, *Ago2* or *Exportin-5*, suggesting some similarities and differences between the sexes (Bunay *et al.* 2017). In this way, the regulation of miR expression by the biogenesis enzymes such as DROSHA, DICER1 and others (e.g. TUT4-7 and LIN28B) would be more than a general mechanism, a selective mechanism which also involves the decay and degradation of other miRs (Kim *et al.* 2015, Bunay *et al.* 2017).

Drosha and *Dicer1* are transcriptionally regulated by different pathways. One of the most relevant is c-Myc, a transcription factor that directly transactivates *Drosha* mRNA (Wang *et al.* 2013) and possibly also *Dicer1* mRNA through E2F/c-Myc loop (Fernandez *et al.* 2003, Leung *et al.* 2008, Blandino *et al.* 2012). In this way, it has been reported that DBP and NP, two molecules of our mixture, increase c-Myc mRNA levels (Kostka *et al.* 2010, Lü *et al.* 2010). On the other hand, the transcriptional regulation of *Dicer1* is complex, due to many factor cell-, tissue- or stage-specific factors (Kurzynska-Kokorniak *et al.* 2015), and where ADAR1 and TUT4, two *Dicer1*-positive regulators, are increased by phthalates and alkylphenols mixture in male mice (Bunay *et al.* 2017).

Some works show that altered ovary phenotypes after a single exposure to phthalates (DEHP and DBP) or alkylphenols as BPA correlate with global changes in the transcriptome (Lawson *et al.* 2011, Li *et al.* 2016, Liu *et al.* 2018). New research has proposed a link between these changes in mRNA expression and the loss of the post-transcriptional regulation by miRs or other sncRNAs (Sandhu *et al.* 2012) affecting female fertility (Ahmed *et al.* 2017, Lite *et al.* 2019). Studying the miRome (miR expression) profile, Zhang *et al.* showed that only some miRs were deregulated and the progesterone-mediated oocyte maturation signaling pathway was one of the most altered in newborn mice ovaries after exposure to DBP (Zhang *et al.* 2019). Interestingly, mmu-miR-200a-5p was reported to be upregulated. This miR is part of the miR-200 family, which includes miR-200b (Senfter *et al.* 2016), and was implicated in ovarian development, folliculogenesis and female fertility (Ahn *et al.* 2010, Zou *et al.* 2020). Here, we report an increase in both the precursors and mature miRs (miR-96-5p, miR-200b-3p, miR-365-3p, miR-378a-3p and miR-503-5p) giving an account of the relationship of miR expression between pre- and mature forms (Donadeu *et al.* 2017). In addition, predicted miRs that target *Star* and *Cyp19a1* increased while the target transcripts decreased (Patino-Garcia *et al.* 2018). Interestingly, *in silico* analysis revealed that three upregulated miRs (miR-200b-3p, miR-378a-3p and miR-503-5p) target a set of mRNAs encoding proteins related to the steroidogenic pathway and ovarian follicle development. Among their targets are *Lhcgr*, *Fshr*, *Ar*, *Esr1*, *Esr2*, *Igf1*, *Tgfb1*, *Cyp19a1*, *Inha* and *Acvr2* which are critical for steroid biosynthesis, the steroid signaling pathway, progression from pre-antral to antral follicles and pre-ovulatory follicle development (Thomas *et al.* 2003, Drummond 2006, Young & McNeilly 2010, Gleicher *et al.* 2011, Sanchez & Smitz 2012, Wood & Rajkovic 2013, Nayak *et al.* 2016).

Here, we use *in silico* analysis to show that three miRs (miR-200b-3p, miR-378a-3p and miR-503-5p) expressed in mice and human ovaries target the mRNA of *Cyp19a1*, the enzyme that produces E₂ from testosterone (Liang *et al.* 2007, Ahn *et al.* 2010). In addition, miR-503-5p is expressed at low levels during ovarian folliculogenesis (McBride *et al.* 2012) in the cytoplasm of granulosa cells (Lei *et al.* 2010). By contrast, miR-378a-3p presents higher expression levels in granulosa but no theca cells from atretic follicles (Donadeu *et al.* 2017). Recent studies have shown upregulated levels of miR-200b-3p in both the blood and follicular fluid of anovulatory women (Huang *et al.* 2016, Eisenberg *et al.* 2017, Xue *et al.* 2018). Here, we experimentally validate that *in vitro* miR-200b-3p targets *Cyp19a1* mRNA, suggesting that this could be a mechanism *in vivo* by which exposure to phthalate and alkylphenol mixtures could downregulated the levels of E₂ and contribute to the observed effects in follicle progression disruption and imbalances in hormone levels. Likewise, the downregulation of miR-145a-5p in neonatal mouse ovary culture increases the expression of *TGFBR2*, inducing activation of the *TGFB* signaling pathway, which is important for primordial to primary follicle transition (Yang *et al.* 2013). Thus, the overexpression of miR-200b-3p, miR-378a-3p and miR-503-5p could alter the pre-antral to antral follicle progression through the repression or degradation of steroidogenic transcripts such as *Cyp19a1* by phthalate and alkylphenol exposure, compromising female fertility.

In conclusion, these findings add evidence to the emerging role of miRs in the effect of exposure to phthalates and alkylphenols in a non-occupational manner. We identify a putative upregulation mechanism of certain miRs involved in phthalates and alkylphenols mixture-induced hormone disruption (mainly estradiol) and ovarian dysregulation. Validates analysis suggested dysregulation of the steroidogenesis signaling pathway and reproductive processes. Overall, these data laid the foundation for further investigations into the use of miRs as markers of ovarian insult by phthalates and alkylphenols mixtures exposure.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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