Reproductive Toxicology

BPA disrupts meiosis I in oogonia by acting on pathways including cell cycle regulation, meiosis initiation and spindle assembly.

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Corresponding Author:	Béatrice Mandon-Pépin, Ph.D. INRAE Centre Île-de-France Jouy-en-Josas Antony: Institut National de Recherche pour l'Agriculture l'Alimentation et l'Environnement Centre Ile-de-France Jouy-en-Josas Antony Jouy-en-Josas, Île-de-France FRANCE
First Author:	Benoit Loup, Ph.D.
Order of Authors:	Benoit Loup, Ph.D.
	Elodie Poumerol
	Luc Jouneau
	Paul A Fowler
	Corinne Cotinot
	Béatrice Mandon-Pépin, Ph.D.
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Suggested Reviewers:	Severine Mazaud-Guittot, Dr Researcher, Rennes 1 University: Universite de Rennes 1 severine.mazaud@univ-rennes1.fr Works on endocrine disruptors and fetal gonads
	Richard Lea University of Nottingham School of Veterinary Medicine and Science richard.lea@nottingham.ac.uk Works on endocrine disruptors on ruminants
	Virginie Rouiller-Fabre, Professor Institut de biologie François Jacob: Commissariat a l'energie atomique et aux energies

	alternatives Institut de biologie Francois Jacob virginie.rouiller-fabre@cea.fr Works on endocrine disruptors and mouse gonads
	Pauliina Damdimopoulou, Dr PI, KI: Karolinska Institutet pauliina.damdimopoulou@ki.se Works on effects of chemicals on reproductive health in women
	Catherine Viguié, Dr, DMV Research Director, INRAE Occitanie-Toulouse: Institut National de Recherche pour l'Agriculture l'Alimentation et l'Environnement Centre Occitanie-Toulouse catherine.viguie@inrae.fr Works on understanding the toxicokinetic mechanisms leading to fetal exposure to endocrine disruptors.
	Gabriel Livera, Dr Pr, CEA Jacob: Commissariat a l'energie atomique et aux energies alternatives Institut de biologie Francois Jacob gabriel.livera@cea.fr Works on the different stages of fetal meiosis - effects of endocrine disruptors
Response to Reviewers:	

Highlights

- Development of a meiosis targeting culture model of the fetal ovary in sheep.
- BPA reduced the expression of meiotic marker genes in exposed explants.
- BPA altered expression of genes involved in cell cycle transition, meiosis, and spindle formation.
- Long-term effects could result in a reduction of the ovarian reserve and an increased frequency of aneuploidy.

ABSTRACT

The negative *in utero* effects of BPA on female reproduction are of concern since the ovarian reserve of primordial follicles is constituted during the fetal period. This time-window is difficult to access, particularly in humans. Animal models and explant culture systems are, therefore, vital tools for investigating EDC impacts on PGCs. Here, we investigated the effects of BPA on prophase I meiosis in the fetal sheep ovary. We established an *in vitro* model of early gametogenesis through retinoic acid-induced differentiation of sheep PGCs that progressed through meiosis. Using this system, we demonstrated that BPA ($3x10^{-7}$M, $3x10^{-5}$M) exposure for 20 days disrupted meiotic initiation and completion in sheep oogonia and induced transcriptomic modifications of exposed explants. After exposure to the lowest concentrations of BPA ($3x10^{-7}$M), only 2 probes were significantly up-regulated corresponding to NR2F1 and TMEM167A transcripts. In contrast, after exposure to $3x10^{-5}$ M BPA, 446 probes were deregulated, 225 were down- and 221 were up-regulated following microarray analysis. Gene Ontology (GO) annotations of differentially expressed genes revealed that pathways mainly affected were involved in cell-cycle phase transition, meiosis and spindle assembly. Differences in key gene expression within each pathway were validated by qRT-PCR.

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BPA disrupts meiosis I in oogonia by acting on pathways including cell cycle regulation, meiosis initiation and spindle assembly

Benoit Loup[a], Elodie Poumerol[a], Luc Jouneau[a], Paul A Fowler[b], Corinne Cotinot[a], Béatrice Mandon-Pépin[a*]

[a] Université Paris-Saclay, UVSQ, ENVA, INRAE, BREED, 78350, Jouy-en-Josas, France; loup_benoit@hotmail.com (BL); elodie.poumerol@inrae.fr (EP); luc.jouneau@inrae.fr (LJ); p.a.fowler@abdn.ac.uk (PAF); corinne.cotinot@inrae.fr (CC).

[b] Institute of Medical Sciences, School of Medicine, Medical Sciences & Nutrition, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK.

[*] **Corresponding author**: beatrice.mandon-pepin@inrae.fr (BMP); phone: +0033-134652567

Full postal address: INRAE, UMR BREED, Batiment 230/231, Domaine du Vilvert, 78350 Jouy en Josas, France

ABSTRACT

The negative *in utero* effects of bisphenol A (BPA) on female reproduction are of concern since the ovarian reserve of primordial follicles is constituted during the fetal period. This time-window is difficult to access, particularly in humans. Animal models and explant culture systems are, therefore, vital tools for investigating EDC impacts on primordial germ cells (PGCs). Here, we investigated the effects of BPA on prophase I meiosis in the fetal sheep ovary. We established an *in vitro* model of early gametogenesis through retinoic acid (RA)-induced differentiation of sheep PGCs that progressed through meiosis. Using this system, we demonstrated that BPA ($3x10^{-7}$ M & $3x10^{-5}$ M) exposure for 20 days disrupted meiotic initiation and completion in sheep oogonia and induced transcriptomic modifications of exposed explants. After exposure to the lowest concentrations of BPA ($3x10^{-7}$ M), only 2 probes were significantly up-regulated corresponding to NR2F1 and TMEM167A transcripts. In contrast, after exposure to $3x10^{-5}$ M BPA, 446 probes were deregulated, 225 were down- and 221 were up-regulated following microarray analysis. Gene Ontology (GO) annotations of differentially expressed genes revealed that pathways mainly affected were involved in cell-cycle phase transition, meiosis and spindle assembly. Differences in key gene expression within each pathway were validated by qRT-PCR.

This study provides a novel model for direct examination of the molecular pathways of environmental toxicants on early female gametogenesis and novel insights into the mechanisms by which BPA affects meiosis I. BPA exposure could thereby disrupt ovarian reserve formation by inhibiting meiotic progression of oocytes I and consequently by increasing atresia of primordial follicles containing defective oocytes.

Keywords: fetal ovary, organ culture, meiosis, oogonia, BPA, sheep

Abbreviations:

RA, Retinoic acid; BPA, Bisphenol A; AM580, retinoic acid receptor agonist; STRA8, Stimulated by Retinoic acid gene 8; PGC, primordial germ cell; DPC, days post coitum; SAC, spindle assembly checkpoint; COC, cumulus–oocyte complex;

Highlights

- Development of a meiosis targeting culture model of the fetal ovary in sheep.
- BPA reduced the expression of meiotic marker genes in exposed explants.
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1. Introduction

Following initiation of the female cascade by the WNT4/RSPO1/β-catenin pathway, granulosa cell fate is enforced by expression of FOXL2 in XX fetal gonads[1–5]. This somatic sex determination occurs from around 8 weeks of gestation in human ovaries [6], 34-36 days *post coitum* (d*pc)* in the sheep and goat, [7,8] and 12.5 d*pc* in mouse [9]. Following sex determination commitment, a species-dependent period of ovarian somatic and germ cell proliferation occurs [10], influenced by estrogens in non-rodent mammals [11]. Next, the primordial germ cells (PGC) switch from mitosis to meiosis. The onset of meiosis is, at least in part, triggered by retinoic acid [12,13], which stimulates the up-regulation of the pre-meiosis marker STRA8 and, subsequently, the meiotic signalling cascade that includes SYCP1, SPO11 and DMC1, [13–15]. In sheep, this occurs around 55 d*pc* [7,16]. Once germ cells are blocked in diplotene at the end of prophase I and enclosed in ovigerous nests, primordial follicle formation then commences, at around 75 d*pc* in sheep [17]. Like other female mammals, the sheep oocytes within the primordial follicles remain arrested in the first meiotic division until the re-activation of follicles in the postnatal ovary. These constitute the ovarian reserve that is established for the entire reproductive life of females.

There are notable differences regarding the differentiation processes of the mammalian fetal ovary [18]. While in sheep and cattle, similar to humans, meiosis and follicle development progresses from the inner to the outer regions of the cortex, in rodents these events progress in an anterior to posterior direction. This may signal species differences either in the site of retinoic acid production (mesonephros vs ovary) or in the timing and organization of mesonephric cell penetration of the cortex [18]. Collectively, these studies indicate that, the production and/or delivery of factors and signalling mechanisms involved in the initiation of meiosis is the subject of species

differences between rodents and larger mammals [18]. In species with delayed meiosis, such as sheep and humans, there is an extended period between gonadal sexual differentiation and the onset of meiosis. An intense germ cell proliferation phase occurs during this period. In the developing sheep ovary, germ cell numbers increase from approximately 50,000 to 805,000 [19].This proliferative period coincides with a peak in aromatase expression and oestrogen production. While the ovary does not produce steroids during early fetal life in mice [20], in many other mammalian species, such as ruminants and humans, the developing ovary is steroidogenically active [21–25]. Therefore, these species may be particularly sensitive to estrogen-mimetic compounds, unlike rodents.

Bisphenol A [BPA; 2, 2-bis (4-hydroxyphenyl) propane; CAS#80-05-7] is one of the most widely produced chemicals globally and is an endocrine disrupting compound (EDC) that can affect ovarian development as it can bind to estrogen receptors [26-27]. Adult mouse oocytes exposed to BPA exhibit improperly aligned chromosomes at the spindle equator during metaphase II and unbalanced chromosome sets while pups derived from exposed oocytes have higher abortion rates than controls [28–32].
However, the cellular and molecular processes are very different between fetal meiosis (initiation from oogonia in mitosis, homologous recombination then arrest in prophase I at the diplotene stage) and adult meiosis (activation of small groups of oocytes I regulated FSH and LH signaling, reductional meiosis which will give two cells of different sizes. Then after ovulation, the oocyte II engages in its second division of meiosis but will be blocked in metaphase II. The extrapolation of the effects observed on oocytes II of adult mice to human fetal oogonia is questionable. This is why we proposed a culture model of fetal gonads in a species presenting characteristics of

ovarian differentiation close to human to elucidate mechanisms involved in adverse impacts of EDCs on fetal ovary differentiation. Very few studies have been published in humans for ethical reasons and the scarcity of biological material. They show that human fetal BPA-exposed oocytes exhibit delayed meiotic progression, characterised by a fall in the percentage of oocytes reaching pachytene and a reduced oocyte viability in culture [33].

The sheep was selected as a relevant generic model for the extrapolation of BPA fetal kinetics to the human fetus because it is an acknowledged model for characterizing BPA disposition during the prenatal period [34]. Furthermore, BPA glucuronidation in humans and sheep is very similar [35,36].

We developed an *ex vivo* culture model on sheep fetal ovary that recapitulates the key stages of prophase I meiosis in oogonia. Using this ovarian explant culture, we investigated the effects of BPA on early female gametogenesis, identifying possible underlying mechanisms. Ranges of total BPA concentrations in human pregnancy are: maternal plasma 1.5–80 ng/mL [37,38], amniotic fluid 0.3-10 ng/mL [39–42], and cord blood 0.1-50 ng/mL [43–45].

It is noteworthy that the results obtained in near-term fetuses are not necessarily representative of BPA fetal exposure at earlier stages of pregnancy. As shown by Corbel et al. 2015 [46], the ovine fetus in the first third of pregnancy (around 50 d*pc*), expresses limited hepatic BPA glucuronidation activity, with an intrinsic clearance rate about 30-fold lower than in the last third of pregnancy.

In addition, the conjugation/deconjugation balance is clearly in favour of BPA-G deconjugation in ovine fetal gonads (ovaries and testes) with an activity of BPA-G hydrolysis 10-fold higher than the activity of BPA conjugation [46]. The possible

reactivation of BPA-G into BPA could contribute to an increased exposure of fetal sensitive tissues to bioactive BPA *in situ*.

In order to model the exposure conditions of the human fetus, we selected two concentrations to expose the ovine fetal ovaries in a culture model: $3x10^{-7}$ M (68.5 ng/mL) and $3x10^{-5}$ M (6.85 µg/mL). The lowest concentration corresponds to that measured in the biological fluids of human fetuses in the last third of pregnancy [44-45] and the higher concentration accounts for the low conjugation capacity of the fetal liver, the trapping of BPA-G at the fetal compartment and the capacity of the fetal gonads to deconjugate BPA-G.

We showed that initiation and progression of *ex-vivo* prophase I meiosis in sheep explants needed retinoic acid and that BPA is a potent meiotic toxicant altering expression of numerous genes involved in cell cycle transition, prophase I meiosis, and spindle formation. Therefore, exposure to BPA could affect the formation and differentiation of primordial follicles that would contain oocytes unable to complete prophase I.

2. Materials and methods

The experimental procedure is summarised in Fig. 1.

2.1. Collection of fetal sheep gonads

Procedures for handling sheep were conducted in compliance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching (authorization from local ethical committee CEEA n°45). All sheep fetuses were obtained from pregnant Pré-Alpes females, following hormonal treatment as previously described [7]. Sheep fetuses were collected at our local slaughterhouse (INRAE, Jouy en Josas, France) at 50, 60, 70 d*pc*. The genetic sex of all fetuses was determined by PCR amplification of the *SRY* and *ZFY/ZFX* genes using liver genomic DNA as previously described [7].

2.2. Organ culture

Each fetal ovary was cut into 2 pieces. Ovarian pieces were processed and cultured in Waymouth medium MB 752/1 supplemented with 25 mg/L pyruvic acid, sodium salt (Sigma, France), 1 ml/100ml of media of ITS+ (insulin, transferrin, and selenous acid; Becton Dickinson, France), 50 UI/ml penicillin, 50 µg/ml streptomycin (P433-Sigma-Aldrich). Ovarian pieces were rinsed in the culture medium and then transferred onto 30-mm-diameter uncoated culture plate inserts (Millicell Cell Culture Insert, 0.4 µm pore size; Millipore SAS, France) in individual wells of a 6-well culture dish containing 1.1mL of media. The cultures were incubated at 38.5 C in an incubator gassed with

5% CO2 and 95% air. Cultures were equilibrated for 24 hours and then considered as day 0. On day 0, representative pieces of fetal ovaries were fixed in Bouin's liquid or flash frozen in liquid nitrogen and then stored at -80° C, to serve as non-cultured controls. Ovarian explants were then cultured for 10 or 20 days under various exposure conditions. The medium in each well was replaced daily. Retinoic acid (RA) 10^{-6}M (R2625, Sigma-Aldrich) or AM580 10^{-6} M (product number sc-203505, Tebu-bio, France) were added to cultures for 20 days or 7 days, respectively. The compound 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtamido) benzoic acid (AM580) is a synthetic stable analogue of retinoic acid (RA) that acts as a selective RARα agonist and is resistant to CYP26 metabolism. For this last reason, AM580 was added only the first 7 days. In contrast, RA needs to be added continuously during all the culture period because it was rapidly degraded by endogenous CYP26 enzymes. Stock solutions of the various retinoids were prepared in dimethyl sulfoxide (DMSO, D2650-Sigma-Aldrich) under dimmed light and stored at -80 C and protected from light until use.

2.3. Chemicals

Bisphenol A (BPA, 4,4'-dihydroxy-2,2'-diphénylpropane) (N°CAS 80-05-7, Interchim) was dissolved in 0.01% dimethyl sulfoxide (DMSO, D2650-Sigma-Aldrich) and was added to culture medium for obtaining a final concentration of $3x10^{-5}$ M (6.85µg/mL, BPA1) or $3x10^{-7}$M (68.48ng/mL, BPA2). Explants at 50 d*pc* were first cultured in control culture medium for 24 h (D0). Then the culture was pursued for 10 and 20 days, corresponding to 60 and 70 d*pc* with half of the wells added with BPA and the other half in basal medium (+DMSO only) to serve as controls.

2.4. Histology

For histological studies, fetal ovaries were fixed in Bouin's Solution. Fixed tissues were dehydrated, embedded in paraffin by standard procedures with a Citadel automat (Thermo Fisher Scientific - Shandon Citadel 1000) and cut into 5-µm sections. Bouin-fixed sections were stained with haematoxylin and eosin (HES). Images were captured using a digital slide scanner (Hamamatsu Photonics, Massy, France) and images were analysed with NDP view software (Hamamatsu). As previously described [47], oogonia were identified as small cells with high nucleocytoplasmic ratio and the presence of prominent nucleoli.

2.5. RNA extraction

Total RNA from freshly explanted fetal ovaries or from cultured ovarian explant pieces were isolated using Trizol Reagent (Life Technologies, Paisley, UK), DNAse-treated and purified with RNeasy Mini kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. Total RNAs were quantified by Nanodrop ND-1000 UV measurement or by Qubit® Fluorometric Quantitation (Thermo Fisher Scientific, Illkirch, France) and the RNA integrity was verified using an Agilent 2100 Bioanalyser (Matriks, Norway). Only samples with RNA Integrity Number greater than 9 were used for quantitative PCR or microarray hybridizations.

2.6. Real-time quantitative PCR

In order to measure gene expression after the different condition cultures or exposures, reverse transcription was performed with the Thermo Scientific Kit Maxima Fist Strand

231 cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Illkirch, France) on each
232 extracted total RNA. Quantitative RT-PCR was performed using the Step One system
233 with Fast SYBR® Green Master Mix (Applied Biosystems, Courtaboeuf, France).
234 YWHAZ, HPRT1 and H2AFZ were used as reference genes for the comparative CT
235 method for relative quantitation of gene expression. Results were analysed using
236 qBase software (Biogazelle NV, Ghent, Belgium). The sequences of primers used for
237 qRT-PCR are presented in Table 1.
238

Table 1: Primer sequences used for qPCR.

Gene	**Primers forward 5'-3'**	**Primers reverse 5'-3'**
AURKA	GTGGAAGACGGACTCAGAGC	CACACAGGACTGGGAAGGTT
BUB1	CTCAGTGGCTTTCGGACTGT	TGCGCTAAATCTGCTACACC
CDKN1	ATATGGGTCTGGGAGCTGTG	AGGATGCTACAGGAGCTGGA
CITED2	ACCGTTCTGGATCAGGAAAA	CCACTGACGACATTCCACAC
DMC1	TTGCGAAAAGGAAGAGGAGA	CCCAATTCCTCCAGCAGTTA
ESR1	CAGGTGCCCTATTACCTGGA	GCCACCTTGACGTCGATTAT
ESR2	GCCTCCATGATGATGTCCTT	CACTTGGTCGTACAGGCTGA
GPR30	AGGTGTTCAACCTGGACGAG	GAGGAAGAAGACGCTGCTGT
H2AFZ	GCGTATTACCCCTCGTCACTTG	CAGCAATTGTAGCCTTGATGAGA
HPRT1	GAACGGCTGGCTCGAGATGT	TCCAACAGGTCGGCAAAGAA
KIF18A	TTCCTTTTGTTTTGTGCTTTTG	CCACCACACTGACTCAGGAA
NR2F1	TTCTTCGTCCGTTTGGTAGG	CCAAGGTCTAGGAGCACTGG
REC8	TGGTGGAGACTGACCTACCC	TCCACAGACATCATCCCAAA

SPO11	TACCGAGGAGGAGTCTGCAT	GTTCACCTTGGTGCCATCTT
STRA8	CACCCCTGAGGAGATCCTTT	AGCACGGAACTGAGGCTAGA
SYCP1	CCCATGCTTGAACAGACTGA	GTCTGCTCATTGGCTCTGAA
TMEM167	TGCAGTGTGCTGTATCGTGA	GGCAGATCAGTCCCTTTTTG
TPX2	CTCCTGCCCGAGTGACTAAG	GTGCAAGGGGAACGTAGGTA
YWHAZ	GGAGCCCGTAGGTCATCTTG	CTCGAGCCATCTGCTGTTTTT

For each experiment, median values were plotted with GraphPad Prism, and statistical analyses were performed with KrusKal-Wallis test in R software (Rcmdr package (p-value<0.05) (p-value between 0.01 and 0.05 = **, p-value less than 0.01 = ***).

2.7. Customized ovine microarray hybridization

Transcriptome analysis was conducted on a custom 15K Agilent oligo sheep microarray previously described in [48]. Labelling and hybridization were performed at the “Plate-forme Biopuces et Sequençage” (http://genomeast.igbmc.fr/). RNA integrity and quantity was checked, respectively, by Bioanalyzer (Agilent) analysis and Nanodrop ND-1000 UV measurement. Labelling and hybridisation were carried out using the Quick Amp Labelling kit for one-color labeling (Agilent, 5190-0442) and the One-Color RNA Spike-in Kit (Agilent, 5188–5282) according to the manufacturer’s instructions with 200 ng of total RNAs starting quantities. Arrays were scanned with the Agilent DNA Microarray Scanner Model G2565B and image analysis performed with Agilent Feature Extraction software v9.5.3.1. In order to verify the quality and hybridization reproducibility of the array, hybridisation tests with RNA from control sheep ovaries were performed using one-color labelling and standard hybridization:

70–80% of spots were significantly hybridised and replicate experiments showed good reproducibility. For gene array, data processing and analysis were conducted using Bioconductor packages suite (http://www.bioconductor.org/index.html) and LIMMA package50 with the R statistical program. Raw median signal from Feature Extraction array files was used as non-processed signal and log2 transformed. Background was then subtracted locally, and intra-array normalisation performed by subtracting the array median signal from each spot signal on the same array. Multiple testing corrections were applied and differentially expressed probes were considered under a False Discovery Rate (FDR) of 5% [49].

2.8. Gene ontology enrichment

Differentially expressed probes were analysed with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway membership with Database performed using the DAVID Bioinformatic Database 6.8 (https://david.ncifcrf.gov/) [50,51]. These analyses and pathways were considered significant for a Benjamini corrected enrichment p-value of less than 0.05.

3. Results

3.1. Initiation and progression of prophase I meiosis

Newly explanted 50 d*pc* ovaries contained mitotic germ cells, some at pre-leptotene stages, many with prominent nucleoli (Fig. 2A-A). Germ cells at zygotene were observed at 60 *dpc* with the first diplotene stage germ cells seen at 70 d*pc* (Fig. 2A-B, 2A-C). In order to better characterise the timing of meiosis initiation and progression, we analysed the expression of key meiotic markers: STRA8, SYCP1, DMC1 and SPO11 in freshly collected sheep fetal ovaries ranging from 50 to 70 d*pc* (Fig. 2B, C, D, E). Strikingly a 10-fold increase in *STRA8* mRNA (between 50 and 55 d*pc*) and a 4-fold increase in *SYCP1* mRNA (between 50 and 60 d*pc*) were observed in the sheep fetal ovary coincident with the onset of meiosis. In contrast to mouse, a lack of synchronicity in the expression of the various meiotic markers was observed, with *DMC1*, and *SCYP1* being expressed initially while *SPO11* was up-regulated from 70 d*pc*.

Sheep fetal ovaries freshly isolated at 50 d*pc* were cultured for 10 or 20 days in culture medium with or without retinoic acid (RA 10^{-6}M) or AM580 (10^{-6}M). When the fetal ovaries were cultured without retinoic supplementation (control), no features of meiosis were observed 20 days later and the germ cells remained in a proliferative state (Fig. 3A). The addition of RA or AM580 enabled oogonia to enter meiosis (Fig. 3A). At 10 and 20 days of culture RA- or AM580-supplemented fetal ovaries demonstrated a histologically normal appearance, containing germ cells and somatic cells similar to that in tissue from uncultured age-matched controls (Figs. 2&3). After 20 days of

culture with RA or AM580 germ cells in pachytene/diplotene and diplotene stages had appeared (Fig 3A). RA and AM580 up-regulated mRNA levels of meiotic markers, particularly, SYCP1, and SPO11 (Fig. 3B). However, the addition of 10^{-6}M Am580 during the first 7 days of culture yielded histological and transcriptional characteristics closest to those observed ex-*vivo* at 70 d*pc* and appeared more effective than RA itself (Figs 2, 3B).

3.2. Effects of BPA on 20 day-exposed fetal explants

BPA down-deregulates meiotic marker gene expression

In order to determine whether prophase I meiosis is altered by BPA exposure in sheep fetal ovary explants, and which mechanisms were involved, we analysed mRNA expression of several candidate meiotic genes using qRT-PCR from gonads cultured in control (BPA-free+AM580+DMSO) conditions and after exposure to BPA concentrations. Following *in vitro* exposure to $3x10^{-7}$ M and $3x10^{-5}$ M BPA for 20 days, changes in mRNA levels of *STRA8, DMC1, REC8* and *SYCP1* occurred (Fig. 4). Meiotic gene expression levels were reduced at both BPA concentrations, except for REC8 where only the highest BPA concentration led to a marked reduction in expression.

Control and BPA-exposed ovaries after 20 days of culture were then analysed using a custom sheep microarray containing 15K probes. After exposure to the lowest concentrations of BPA ($3x10^{-7}$ M), only 2 probes were significantly up-regulated (FDR 5% or adj-pval<0.05) corresponding to Nuclear Receptor Subfamily 2 Group F Member 1 (NR2F1) and transmembrane protein 167A (TMEM167A) transcripts (Table S1,

sheet #1). The protein encoded by NR2F1, is a nuclear hormone receptor and transcriptional regulator whereas TMEM167A is involved in constitutive secretory pathway. Both were also deregulated in the same manner with the highest concentration of BPA. Validation of these expression changes was performed by qRT-PCR. The results confirmed microarray data, especially at the highest concentration (10^{-5} M BPA). At the lowest concentration, only NR2F1 presented an up-regulation by RT-qPCR. (Fig. 6 A, B).

In contrast, after exposure to $3x10^{-5}$ M BPA, 446 probes were deregulated, 225 were down- and 221 were up-regulated with FDR 5% and threshold of ±0.7 on the log2 transformed fold change (Log2FC) and 677 probes were deregulated (259 down- and 418 up-regulated) with FDR 5% and Log2FC<±0.2 (Table S1, sheet #2, Fig.5A). Among the down-regulated probes, many corresponded to transcripts expressed in pre-meiotic or meiotic female germ cells, such as BOULE (BOLL), DAZL and its targets TEX11, SMC1B and also TRIP13, RAD18, RAD51,MAD2L1 [52]. The expression of the meiosis gatekeeper STRA8 was also decreased. Two members of the doublesex and mab-3-related transcription factor family (DMRT) present in the fetal ovaries at the time of meiotic initiation, DMRTC2 (DMRT7) and DMRT1 are transcriptionally reduced. MAEL (Maelstrom), an evolutionarily conserved protein that interacts with other piRNA components to silencing of transposable elements was down-regulated after BPA exposure. In the same way, Topaz1 (Testis and Ovary-specific PAZ domain gene 1) a germ cell specific gene potentially involved in piRNA pathway was also reduced [16], as well as, MEIOB, which is specifically implicated in meiotic homologous recombination [53].

BPA alters cell cycle regulation and spindle assembly pathways

To further understand biological functions and pathways altered by BPA, the 677 sheep differentially (adj pval<0.05 and Log2FC threshold ±0.2) expressed probes (control *versus* $3x10^{-5}$ M BPA conditions) were functionally annotated based on Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway through DAVID ontology database. Uncharacterised putative genes and redundant probes were removed before analyses. Overall, 516 official gene symbols were subjected to DAVID analyses. To increase the depth of genes with GO annotations, we used the *Homo Sapiens* genome annotation as background (recommended in [54], and obtained statistically enriched biological processes and molecular functions in which the proteins are involved. The transcripts were classified in the ontological categories: Biological process (BP), Cellular component (CC), and Molecular function (MF) (Table S2, Fig.5). It is important to note that an individual transcript could be represented in several categories.

GO enrichment analysis showed that the Top 10 of biological processes significantly enriched from differentially expressed genes following BPA exposure were: cell cycle (GO:0007049 & GO:0022402), organelle fission (GO:0048285), nuclear division (GO:0000280), mitotic nuclear division (GO:0007067), chromosome segregation (GO:0098813 & GO:0007059), mitotic cell cycle (GO:0000278 & GO:1903047), sister chromatid segregation (GO:0000819) and meiotic cell cycle (GO:0051321) (Fig. 5B). The associated Cellular Component GO terms were focused on chromosomes (condensed chromosome GO:0000793, centromeric region GO:0000775), kinetochore (GO:0000776) and spindle (GO:0005819). The most significant affected molecular functions were: macromolecular complex binding (GO:004487), chromatin binding

(GO:0003682) enzyme binding (GO:0019899) and identical protein binding (GO:0042802), (Fig. 5C and Table S2,). In accordance with GO analysis, the most significant terms of KEGG pathway analysis included Cell cycle (pvalue: 8.88E-04) and Oocyte meiosis (pValue: 0.04) (Fig.5D). In conclusion, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses revealed that the majority of differentially expressed (BPA vs Control) were involved in specific biological process associated to gametogenesis, and specifically germ cell meiosis. (Fig. 5 and Table S2).

In order to confirm the deregulation of these pathways, selected genes were analysed by qRT-PCR. Among them, CDKN1A and CITED2, involved into the cell-cycle regulation, were both up-regulated in microarray analysis at the highest BPA concentration. qRT-PCR analysis confirmed response following exposure to 10^{-5} M BPA only (Fig.6 C, D). KIF18A and BUB1B, two key factors of spindle assembly, were down-regulated in microarray and qRT-PCR analysis at 10^{-5} M BPA (Fig.6 E, F). The same was observed for AURKA and TPX2: decreased expression in BPA exposed explants analysed by microarray and qRT-PCR (Fig.6 G, H).

4. Discussion

We have demonstrated, in a fetal ovine ovarian explant system recapitulating meiosis initiation and progression, that BPA disrupted fetal ovarian meiosis (prophase I). We showed that BPA caused at human-relevant doses, inhibition of meiosis I by affecting not only meiotic marker gene expression but also key factors involved in cell cycle transition and with spindle assembly, microtubule organizing centre and chromosome alignment and segregation. However, certain expression deregulations were only demonstrated at the highest dose of BPA ($3x10^{-5}$M). Validation by qRT-PCR revealed that the expression of several transcripts was significantly affected at the lowest dose ($3x10^{-7}$M), such STRA8, SYCP1, DMC1, SPO11, NR2F1, BUB1 and TPX2. This could be due to the lower sensitivity of the microarray technique that requires greater gene expression differentials than qRT-PCR. Moreover, BPA effects on meiosis could occur at different concentrations according to the mode of action, such as methylation changes of key regulator genes, disturbance of estrogen or retinoic acid signaling pathways. It is possible that each molecular process be sensitive to different BPA concentrations and that the highest dose recapitulates all the effects.

4.1. Comparison between *in vivo* and *in vitro* ovary culture

New approaches for *in vitro* prophase I meiotic toxicity testing are required in order to increase the number of molecules tested per animal, thus reducing animal numbers. Critical barriers to overcome included: (1) the maintenance of a coherent 3D structure in the explants, and (2) the development of *in vitro* culture conditions essential for entrance into meiosis and sustained progression of prophase I meiosis in species where these phenomena extend over several weeks, such as ruminants or humans.

To our knowledge, we showed here the first characterisation of a sheep fetal ovary explant model that allows the initiation and progression of prophase I meiosis *in vitro* in human developmentally-relevant animal model.

Meiotic commitment is a two-step process: (1) the acquisition of intrinsic factors enabling germ cells to initiate meiosis, notably through the expression of DAZL [55]; (2) the reception of a meiosis-inducing signal that triggers STRA8 expression, ultimately leading to meiotic entry [13,14, 56-58]. Retinoic acid (RA) induces germ cells to express both STRA8, a gene required for meiotic initiation [59-65], and REC8, a gene required for meiotic progression [64]. In sheep, DAZL is expressed from 49 d*pc* and STRA8 expression in fetal ovaries is detected as early as 50 d*pc* with a peak at 55 d*pc* followed by rising expression of meiotic genes like DMC1 and SYCP1, MSH4 and MSH5 [7]. This time course of expression was confirmed here by qRT-PCR analysis of freshly explanted ovaries at 50, 55, 60 and 70 d*pc*.

In order to trigger prophase I meiosis, retinoids was added at 50 d*pc* when the oogonia become competent to receive the meiosis inducing factor following expression of DAZL. In cultures from 50 d*pc* supplemented with RA or its analogue AM580, expression profiles of STRA8, DMC1, SYCP1 and SPO11 were analogous to the corresponding *in vivo* developmental stages. The *in vitro* model of ovary explant culture described here, preserved tissue integrity and morphology and supported the survival of 50 d*pc* -ovary explants for over 3 weeks with appropriate development of the female germline. Moreover, 50 d*pc* -explants after 10 and 20 days of cultures were characterised by similar prophase I meiosis stages to those of freshly explanted ovaries at 60 and 70 d*pc* (*i.e*. pachytene and diplotene stages). The time course,

444 initiation and progression of meiosis were comparable to those of *in vivo* ovaries [7].
445 Since the AM580 RA analogue induced results similar to those obtained with RA, the
446 action of RA is likely mainly mediated by RARα.
447
448 As in the human fetal ovary [12,13], no initiation and progression of meiosis was
449 observed in sheep ovary explants cultured without RA, confirming that meiosis is not
450 spontaneous in the sheep fetal ovary. In contrast, meiotic cells spontaneously appear
451 in both mouse and rat fetal ovaries cultured for a few days in a defined culture medium
452 (i.e. with no serum or retinoids for 3 or 4 days) [65].
453
454 **4.2 Effects of BPA exposure on oocyte prophase I meiosis progression**
455
456 We focused on the effects of BPA on prophase I meiosis and the underlying molecular
457 mechanisms. BPA decreased expression of STRA8, REC8, DMC1 and SYCP1 in
458 exposed ovary explants after 20 days of culture (Fig. 4). This down-regulation of
459 meiotic markers will necessarily have consequences for meiotic progression, either by
460 non-initiation of prophase I, or by a process of delay or acceleration of the various
461 stages. Further studies at intermediate culture durations (5, 10, 15 days) will be needed
462 to decide between these possibilities. Several *in vitro/ex-vivo* systems have been
463 developed to culture human fetal ovaries [33,66–68]. In humans, independently of the
464 BPA concentration used (1, 5, 10, 20 and 30 µM), BPA affects meiotic progression by
465 increasing the proportion of oocytes at leptonema and reducing the proportion of
466 oocytes that reach pachynema *in vitro* [33].
467

We showed by microarray analysis that $3x10^{-7}$ M BPA exposure affected expression of two genes, *TMEM167A* (transmembrane protein 167A) and *NR2F1* (nuclear receptor subfamily 2, group F, member 1, also called COUP-TF1 chicken ovalbumin upstream promoter transcription factor-1), which were up-regulated. TMEM167A is involved in the regulation of vesicular trafficking and contributes to aggressiveness of gliomas by deregulation of vesicle transport system [69,70], The presence of *TMEM167A* mRNA in fetal ovary is however confirmed by RNAseq data from fetal mouse ovary [71] (https://rgv.genouest.org/) [72] (Fig. S1A) and is also observed in human developing fetal ovaries from 6 to 17 PCW [73]. These data are presented in Figure S1B. Further investigations will be needed to determine its role in fetal ovary.

COUP-TFs are orphan receptors of the nuclear receptor superfamily. In human fetal ovaries, COUP-TF1 (NR2F1) was located to the cytoplasm of some oocytes and to the nuclei of scattered somatic cells in the second trimester (15 weeks) [74]. *COUP-TF1* transcript expression was significantly increased in human fetal ovary following endocrine disruption due to maternal smoking [74]. COUP-TF1 interacts with ER and ER ligand influences COUP-TF-ERE half-site binding [75]. It is therefore possible that the binding of BPA with ER could modify the interactions with COUP-TF1 and deregulate the COUP-TF-responsible target genes.

Several studies have suggested that COUP-TF could be part of retinoid signalling pathways both *in vivo* and in cell culture systems [76–79]. Moreover, the trend towards increased NR2F1 may also disturb retinoic acid signalling [80], with potential disruption of meiosis entry. This agrees closely with our observations that increased *NR2F1* could be associated with deregulation of *STRA8* and consequently of numerous other meiosis-related genes following BPA exposure. COUP-TF1 appears as a master

493 regulator in several signalling pathways such as ER, AHR and RA signalling and its
494 deregulation could broadly impact the fetal ovarian gene expression program.
495
496 **4.3 Biological Processes Impacted by BPA**
497
498 At the higher BPA ($3x10^{-5}$M) concentration, exposure dysregulated expression of
499 numerous genes (677 probes) with 418 up- and 259 down-regulated probes. BPA-
500 induced STRA8 deregulation via disturbance in RA signalling is likely contributory to
501 many of these changes in gene expression. This is supported by recent studies in mice
502 showing that STRA8 acts as a transcription factor and regulates the expression of
503 thousands of genes including meiotic prophase genes, factors mediating DNA
504 replication and the G1-S cell-cycle transition, and genes that promote the lengthy
505 prophase unique to meiosis I [81]. Strikingly, these pathways involved in cell-cycle
506 regulation were affected in our BPA-exposed ovary explant GO-term analysis.
507 *CDKN1A* and *CITED2,* known to be associated with ovarian dysfunction and affecting
508 both germ cells and somatic cells [90, 91], were amongst the up-regulated genes. Both
509 presented a similar profile, increasing at the higher BPA concentration, first evidence
510 that these are BPA target genes.
511 CDK inhibitor p21 (CDKN1A or p21) inhibits cell cycle progression by interacting with
512 cyclin–CDK complexes and the expression of Cdkn1a (p21) normally decreases during
513 the transition from mitosis to meiosis, as shown by single-cell RNA sequencing of early
514 mouse female germ cells [82]. The observed up-regulation of CDKN1A/p21 in our
515 ovarian explants in response to BPA $3x10^{-5}$ M could therefore result into a blockage of
516 mitosis/meiosis transition.

CITED2 (CBP/p300 interacting transactivator 2 with GLU/ASP-rich C terminal domain 2), involved in oocyte development, is markedly increased at the initiation of oocyte growth in mouse primordial follicles [83]. Cited2 is also expressed in mouse and human cumulus cells [84,85] and high levels in these cells are associated with polycystic ovary syndrome [86] and diminished ovarian reserve in humans [84]. The observed increase in our BPA-exposed ovarian cultures could lead to similar phenotypes in adult female offspring.

Previous studies on BPA or BPS exposure during *in vitro* meiotic maturation (IVM) has shown an impairment of the progression to metaphase-II (MII) as well as disrupted meiotic spindle assembly and organization in mouse [28,29], porcine [87], bovine [88] and human [89] oocytes. Recently, Yang et al shown that a brief (4h) exposure of mouse ovulated oocytes to increasing concentrations (5, 25, 50 µg/ml) of BPA or BPF disrupted spindle organization in a dose-dependent manner. They identify a link between these microtubule defects and altered distribution of key spindle associated factors, as well as Aurora Kinase A activity. [90]. Our microarray transcriptomic analysis also revealed a decreased expression of multiple spindle-associated factors such as BUB1, Kinesin Family Member 18 and 23 (KIF18A & KIF23), TPX2 and also AURKA. Recently, Blengini et al. demonstrated that female oocyte-specific AURKA knockout mice are sterile, and their oocytes fail to complete meiosis I [91]. The microtubule-associated protein TPX2 is also a key mitotic regulator that contributes through distinct pathways to spindle assembly. TPX2 functions in the activation, stabilisation and spindle localisation of the Aurora-A kinase [92] while TPX2 expression in somatic cells exposed to BPA is also disrupted [93]. This is in agreement with our

data showing altered expression of factors essential for the assembly of the spindle after BPA exposure.

BUB1B encodes BUBR1, a crucial spindle assembly checkpoint (SAC) component involved in cell division [94-96]. The SAC is a safeguard mechanism to avoid premature chromosome segregation before correct kinetochore binding to the spindle. A strong reduction of BubR1 has been observed in oocytes of women approaching menopause and in ovaries of aged mice, which led to the hypothesis that a gradual decline of BubR1 contributes to age-related aneuploidization [97,98]. Exposure to 20µg/mL BPA of cumulus cells led to significantly decreased expression of BUB1B [99]. All these data reinforce our results showing BPA exposure induced a reduction of BUB1B expression that can lead to abnormal spindle assembly and chromosome mis-segregation.

Finally, Kif18a, a member of the kinesin-8 family, was found to be expressed in mouse oocytes, being closely associated with microtubules [100] and Kif18a knock down caused the failure of first polar body extrusion, resulting in severe chromosome misalignment. [100]. The decrease of kif18A in our 10^{-5} M BPA-exposed cultures could also contribute to spindle disorganisation.

It is remarkable that several gene deregulations observed following BPA exposure of adult follicles containing oocytes II were also found in our study. This implies that some molecular mechanisms impacted in adult and fetal models are shared. However, the disruption of the early stages of meiosis can generate more drastic effects because the alteration of the ovarian reserve can lead to premature ovarian failure and infertility. Indeed, this reserve built up during fetal life is not renewed during the reproductive life of female mammals.

5. Conclusion

We have developed a culture model enabling initiation and maintenance of prophase I meiosis *in vitro* in sheep fetal ovary explants. Cultured oogonia in these explants followed the same pattern of gene expression changes as seen in non-cultured fetal oogonia of matching ages. This is important since mono-ovulatory sheep represents a physiologically human-relevant model for testing ovary toxicity of endocrine disruptor exposures during fetal life. Our results show that BPA exposure affects oogenesis in fetal sheep ovary, disrupting the meiotic process via three different pathways. Some of these deregulations are also found in the process of late meiosis that takes place in oocytes II in adults (spindle assembly). The other two altered processes (mitosis/meiosis transition and prophase I) are specific to fetal stages.

Importantly, such meiotic changes induced during these fetal stages would decrease the ovarian reserve and increase the frequency of chromosomally abnormal oocytes (aneuploidy) both leading to sub or infertility in the adult female. There are multiple underlying mechanisms and BPA could act either by interfering with the RA pathway and disturbing STRA8 action and/or via the ER pathway by controlling cell-cycle progression. Overall, our findings provide novel insight regarding the multiple effects of BPA exposure on fetal oogonia that go beyond deregulation of factors directly involved in meiotic recombination by also disrupting meiotic spindle organization as well as the mitosis / meiosis transition.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure Legends

Figure 1: Schematic description of the experimental procedure.

Fetal ovaries were collected at 50 days *post coitum* (d*pc*), cut widthways into ~2 mm pieces (2 pieces/ovary) and one piece treated for histological analysis while the other was cultured. Three pieces from different fetuses were placed in each well. Cultures were incubated at 38.5C for up to 7 or 20 days with retinoids (RA or AM580) under a humidified atmosphere of 95% air and 5% CO2. The medium was replaced with fresh media after the first 24 h (D0) and then every 24 h. Explants were exposed to treatments on D0 by adding either vehicle at a final concentration of 0.1% v/v (dimethyl sulfoxide; DMSO) or BPA (0.3 or 30 μM). At the end of cultures, the explants were snap-frozen on dry ice and stored at −80C. Subsequently RNA extraction was performed, and RNA samples were analysed either by qRT-PCR or microarray.

Figure 2: Prophase I meiosis in sheep fetal ovary occurs between 55 d*pc* and 70d*pc*.

A timeline of ovine ovarian development is shown at the top of the figure. The key stages of prophase I of meiosis are illustrated at the bottom of the figure.

A-A) At culture onset, the 50 d*pc* fetal ovaries contained numerous mitotically active oogonia and some pre-leptotene and leptotene (L) oocytes had appeared. A-B) In 60 d*pc* ovary explants, numerous oocytes had reached the zygotene stage (Z). A-C) 70 d*pc*-ovary explants contained meiotic germ cells at different stages of prophase I, diplotene oocytes had appeared but pachytene (P) and zygotene are still present.

Transcript expression of *STRA8* (B), *DMC1* (C), *SYCP1* (D) and *SPO11* (E) was determined using qRT-PCR from in vivo fetal ovaries collected at 50, 55, 60 and 70 d*pc*. Each stage corresponded to 5-6 ovary fragments, except day 55 that contained

only 2 fragments of uncultured ovaries. Gene expression values were normalized relative to *YWHAZ, HPRT1* and *H2AFZ* reference genes. Statistical analyses were performed with KrusKal-Wallis test in R software (Rcmdr package) (** = $p < 0.05$ and *** $p < 0.01$).

Figure 3: Retinoids (RA & AM580) initiate entry and progression of meiosis prophase I in fetal ovary explant cultures.

A- Histological observations of explants of sheep fetal ovaries from 50 d*pc* cultured with 10^{-6} M RA or AM580 or without (CTR) for 20 days. Black arrows indicate oogonia and oocytes in hematoxylin/eosin stained sections. In controls, germ cells were mainly in meiotic proliferation (black arrows show preleptotene stages). In contrast when explants were cultured with retinoids (AM580 or RA), oocytes initiated and progressed into prophase I meiotic stages, arrows indicated oocytes in pachytene or diplotene.

B- mRNA expression of meiosis regulators in explants cultured with 10^{-6} M RA or AM580 or without (CTR) for 20 days. RNA was extracted, reverse transcribed and analysed by qRT-PCR. Transcript expression of *STRA8* (A), *DMC1* (B), *SYCP1* (C) and *SPO11* (D) was determined from 2-6 ovary explants according to presence of RA (circled in dotted lines, n=6), or AM580 (circled in full line, n=2). Cultures in control conditions contained 3 explants. Gene expression values were normalized relative to *YWHAZ, HPRT1* and *H2AFZ* reference genes. Statistical analyses were performed with KrusKal-Wallis test in R software (Rcmdr package) (** = pval < 0.05 and *** pval < 0.01).

Figure 4: Downregulation of meiosis marker expression in explant cultures exposed to BPA

995 Explants of sheep fetal ovaries at 50 d*pc* were cultured with 10^{-6} M AM580 (CTR 20d)
996 and exposed at 2 concentrations of BPA (0.3µM and 30µM) for 20 days. RNA was
997 extracted, reverse transcribed and analysed by qRT-PCR. Transcript expression of
998 *STRA8* (A), *DMC1* (B), *SYCP1* (C) *SPO11* (D) and *REC8* (E) was determined from 6
999 control and 2 exposed ovary explants. Gene expression values were normalized
1000 relative to *YWHAZ, HPRT1* and *H2AFZ* reference genes.
1001
1002 Figure 5: Deregulation of gene expression and alteration of cell cycle processes by
1003 30 µM BPA exposure
1004 (A) Number of differential probes according to their fold change. Only probes that met
1005 an FDR of 5% (or adj-pval<0.05) and a threshold of ±0.2 on the log2-transformed fold
1006 change (LogFC) are displayed and represented according to their positive (shades of
1007 yellow) or negative (shades of blue) fold change. 677 differentially expressed probes
1008 were identified after 20 days of exposure to 30µM BPA *versus* control medium
1009 (AM580+DMSO).

1010 (B-D) Among these 677 probes, 516 are known and unique official gene symbols were
1011 obtained and submitted to DAVID analyses. Their biological significance was explored
1012 by GO term enrichment analysis (DAVID) including biological process (B), molecular
1013 function (C) and cellular component (D). Gene ontology (GO) analysis revealed that
1014 cell cycle, cell proliferation, cell death processes and reproduction were the most
1015 enriched biological processes (B); nucleotide and small molecule binding pathways,
1016 the most enriched molecular functions (C) and nucleoplasm, microtubules cytoskeleton
1017 and chromosomes, the most affected cellular components (D).
1018

Figure 6: Validation by qRT-PCR of expression deregulation of 8 genes following BPA exposure

After 20 days of culture, explants exposed to BPA (0.3 or 30 µM) or not (CTR 20d) were analysed by qRT-PCR. Transcript expression of *NR2F1*(A), *TMEM167* (B), *CDKN1* (C), *CITED2* (D), *Kif18A* (E), *BUB1* (F), *AURKA* (G) and *TPX2* (H) was determined from 3 control and 2 exposed ovary explants at both BPA concentrations. Gene expression values were normalized relative to *YWHAZ, HPRT1* and *H2AFZ* reference genes.

Supplementary material

Figure S1 TREM167 expression in mouse and human fetal ovary

(A) TPM-normalized average expression levels from mouse fetal ovary (pink) or testis (light blue) (from 10.5 to 13.5 d*pc*, days postcoïtum) according to the data of Zhao et al. [65].

(B) FPKM-normalized average expression levels from human fetal ovary (red) or testis (blue) (from 6 to 17 PCW, postconceptional weeks; e7: early 7 PCW; l7: late 7 PCW) according to the data of Lecluze et al. [67].

Table S1:

sheet 1_ Fetal ovarian genes altered by 0.3µM BPA in *in vitro* culture

sheet2_ Fetal ovarian genes altered by 30µM BPA in *in vitro* culture

Table S2: Functional annotation of differentially expressed probes

Figure 1

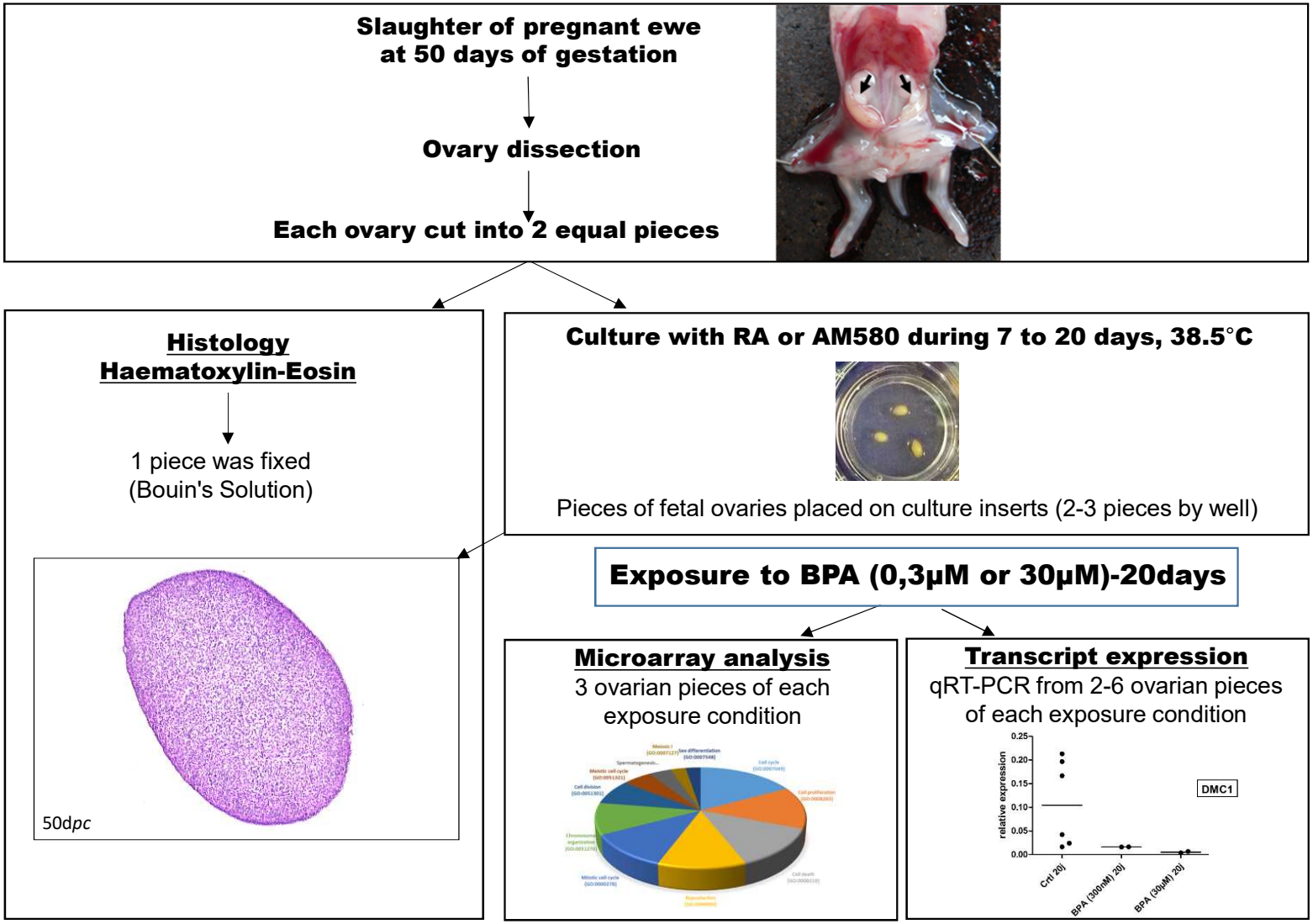
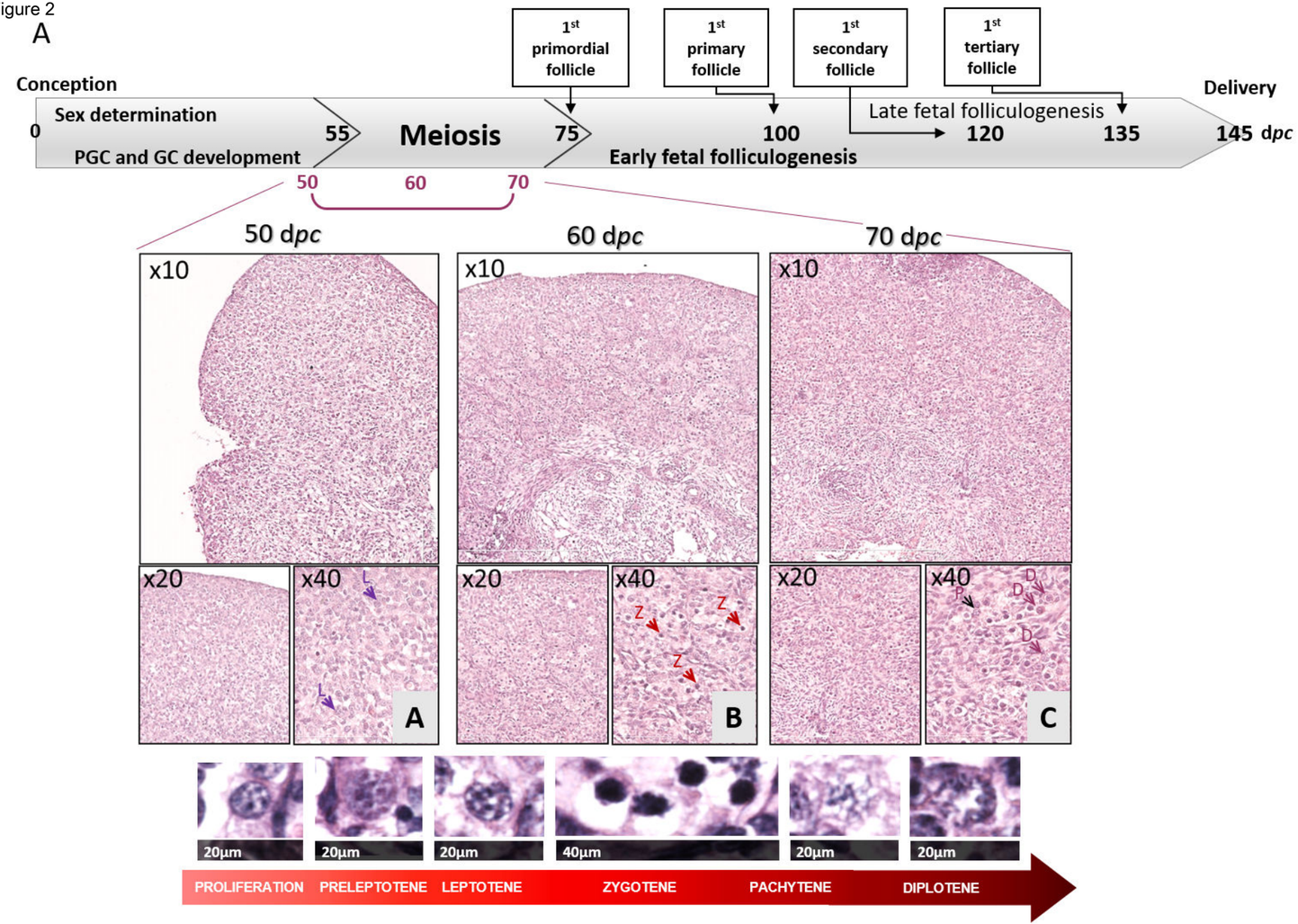
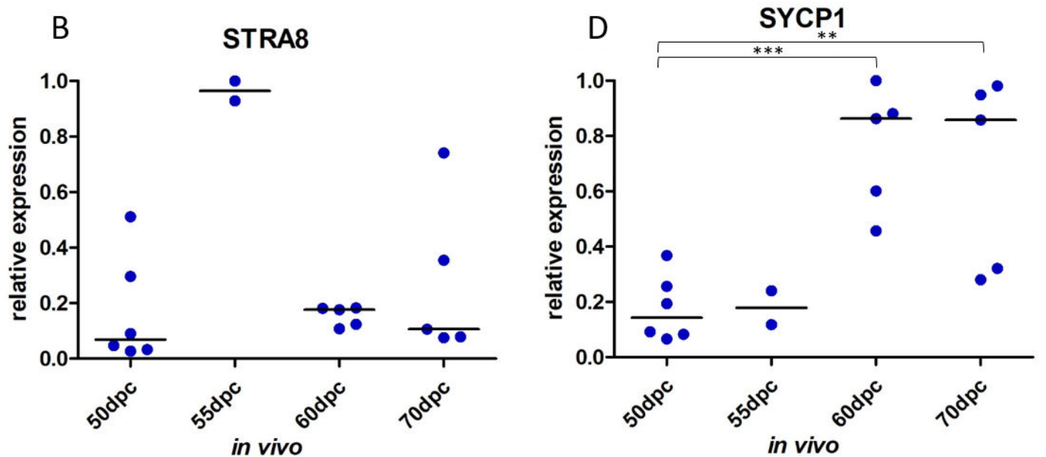


Figure 2





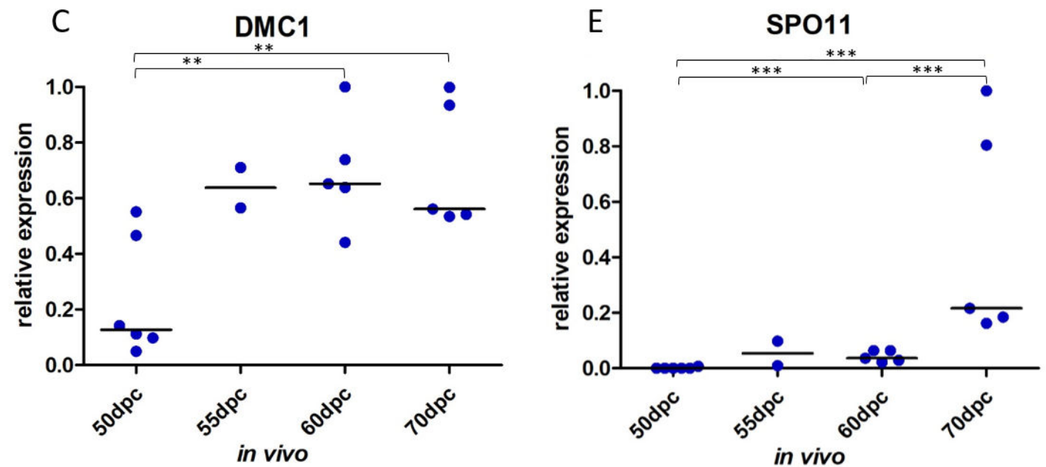


Figure 3

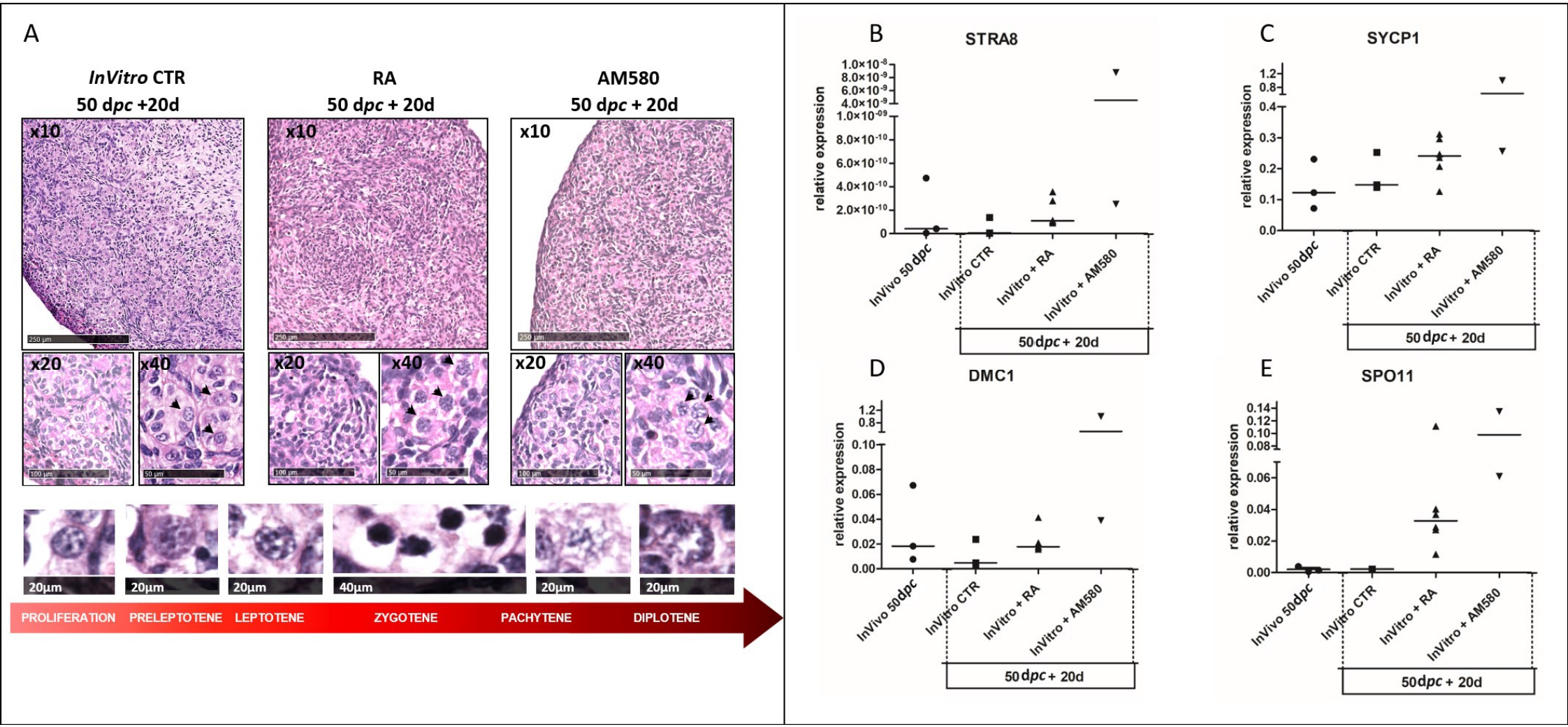
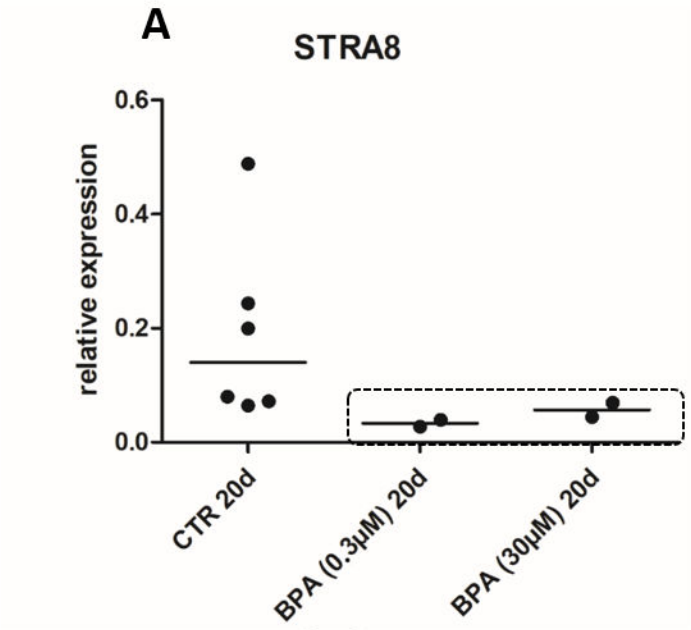
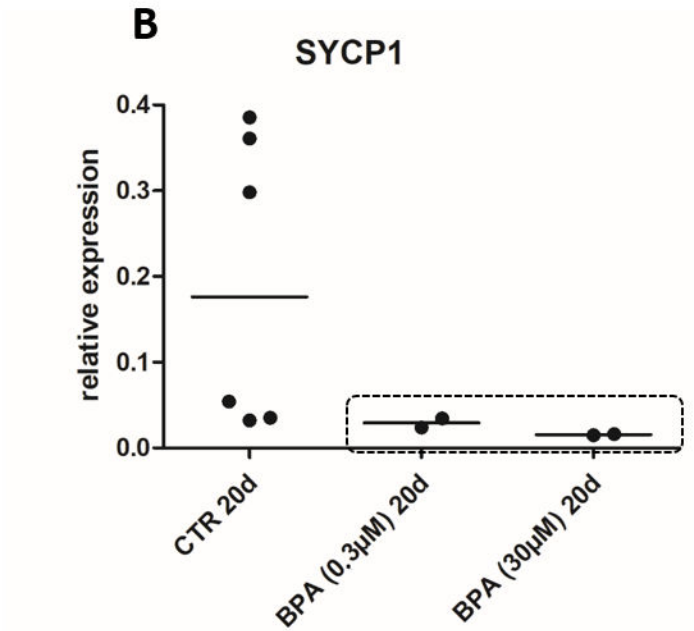
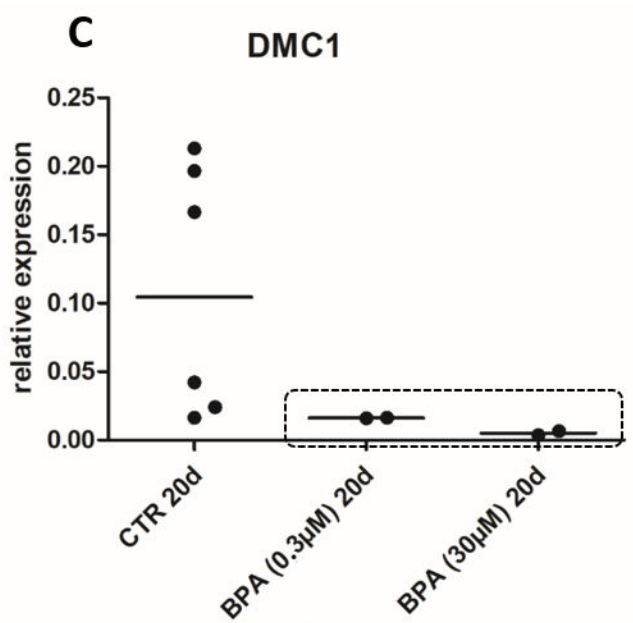
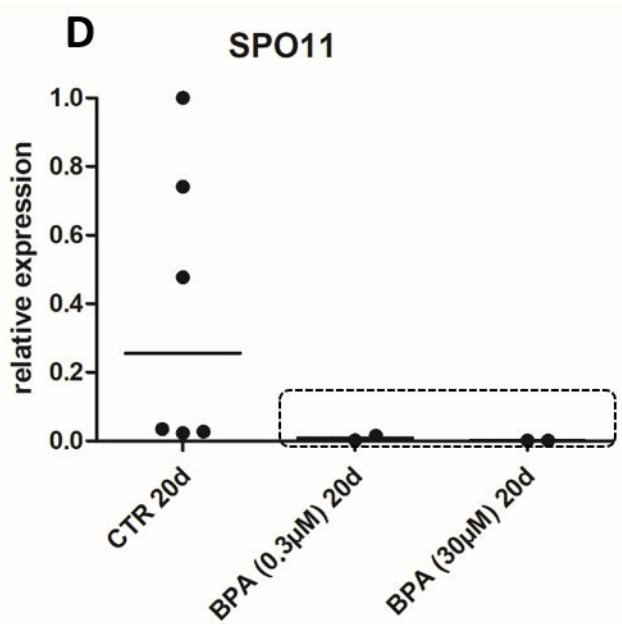


Figure 4









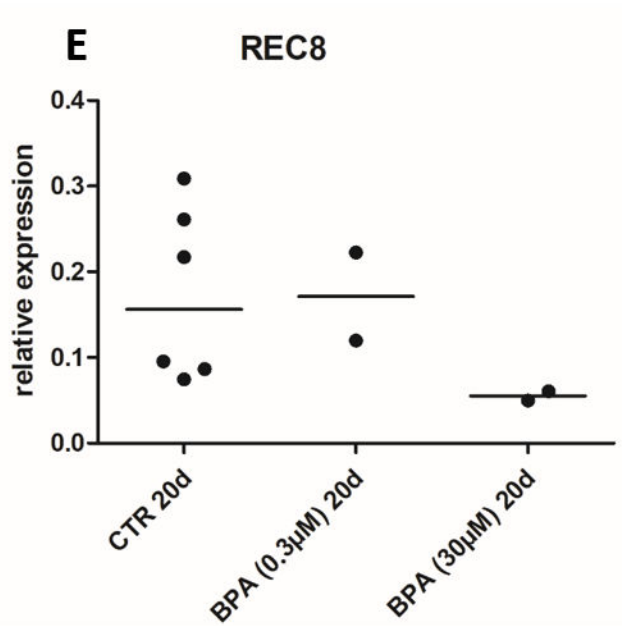


Figure 5

Click here to access/download;Figure;Figure 5-revised.pdf

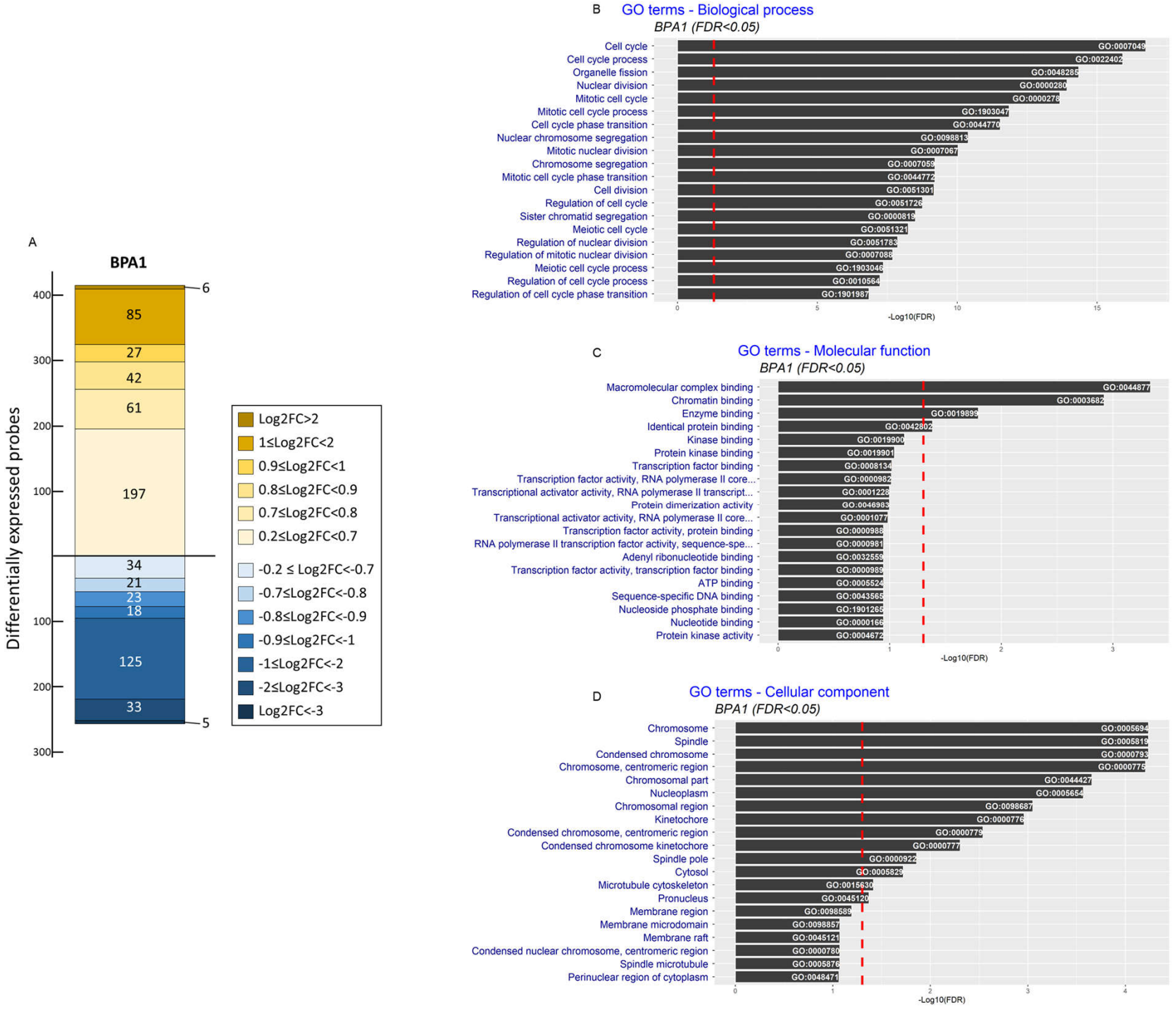
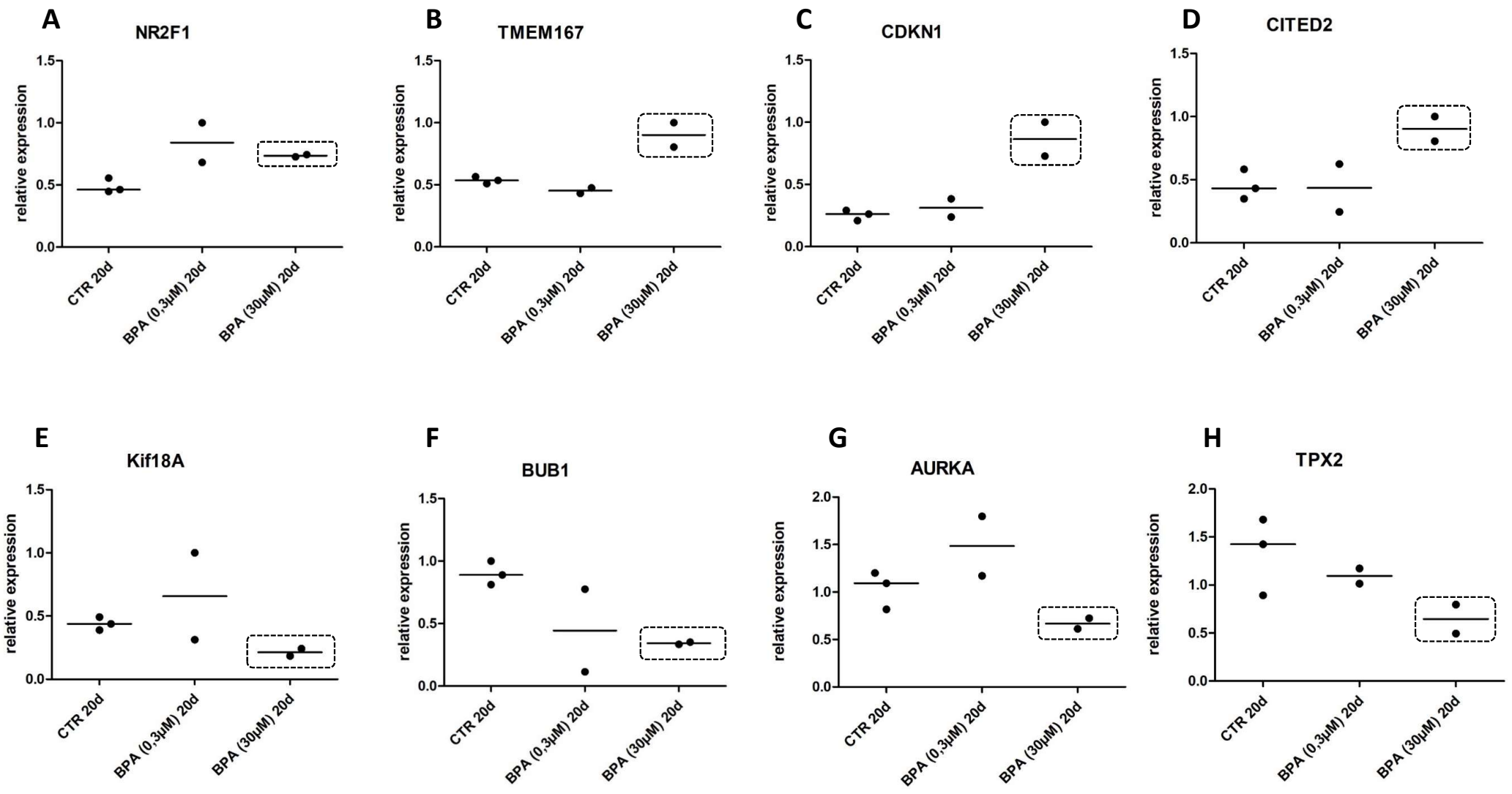


Figure 6



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Benoit Loup
Elodie Poumerol
Luc Jouneau
Paul A. Fowler
Corinne Cotinot
Béatrice Mandon-Pépin

Table S1

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Supplementary Material

Table S1_final.xlsx

Table S2

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Supplementary Material

Table S2_BMP.xlsx

Figure S1