Activation of the aryl hydrocarbon receptor by a component of cigarette smoke reduces germ cell proliferation in the human fetal ovary

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Running title: AhR toxicants reduce human female germ cell proliferation

Key words: Germ cell, smoking, fertility, follicle, oogenesis, ovary, aryl hydrocarbon receptor

Abstract

Fetal life is a critical time for female fertility, when germ cells complete proliferation, initate meiosis and ultimately form the lifetime stock of primordial follicles. Female fertility may be reduced by *in utero* exposure to cigarette smoke, which contains ligands for the aryl hydrocarbon receptor (AhR). The AhR is a critical regulator of ovarian germ cell survival in mice, thus activation of this receptor in the ovaries of fetuses exposed to maternal cigarette smoke *in utero* may provide a mechanism by which female fertility is reduced in later life. We have therefore investigated AhR expression in the human fetal ovary, and examined the effects of an AhR ligand present in cigarette smoke, on germ cells in human fetal ovaries cultured *in vitro*. *AHR* mRNA expression increased 2-fold between first and late second trimester ($p=0.008$). AhR protein was confined to germ cells at all gestations, but varied from expression in most germ cells during the first trimester, to only patchy expression by clusters of germ cells at later gestations. Culture of human fetal ovaries with the AhR ligand 9,10-dimethyl-1,2-benzanthracene-3,4-dihydrodiol (DMBA-DHD; a component of cigarette smoke) did not affect germ cell number *in vitro*, but significantly reduced the proportion of proliferating germ cells by 29% (as assessed by phospho-histone H3 staining ($p=0.04$)). Germ cell apoptosis was not significantly affected. These results reveal that germ cells in the human fetal ovary express AhR from the proliferative stage of development through entry into meiosis and beyond, and demonstrate that AhR ligands found in cigarette smoke have the capacity to impair human fetal ovarian germ cell proliferation.

Introduction

Germ cell development in the human fetal ovary results in the formation of the finite primordial follicle pool that is the ultimate determinant of female fertility and reproductive lifespan (Maheshwari and Fowler, 2008; Tingen *et al.*, 2009). Following migration of primordial germ cells to the gonadal ridge, the key stages are germ cell proliferation, entry into meiosis with subsequent meiotic arrest and association with somatic cells to form primordial follicles (Byskov, 1986; Pepling and Spradling, 2001). The first germ cells enter meiosis in the third month of fetal development with primordial follicles present from approximately 18 weeks gestation (equal to 16 weeks post conception) (Baker, 1963; Kurilo, 1981; Gondos *et al.*, 1986; Sforza *et al.*, 2003; Bendsen *et al.*, 2006). In the human fetal ovary, germ cell proliferation continues long after some cells have entered meiosis, such that during the second trimester of pregnancy a developmental gradient is established across the ovary with less mature and mitotic germ cells present around the periphery of the ovary, with those at increasing stages of maturity towards the centre where the first primordial follicles are formed (Fulton *et al.*, 2005; Stoop *et al.*, 2005; Anderson *et al.*, 2007; Childs *et al.*, 2012).

As entry to meiosis precludes further expansion of the germ cell pool by mitosis, generating an adequate germ cell number prior to meiosis is a key step in establishing female fertility. In addition to intrinsic genetic variability, this process is potentially vulnerable to external influence, and there are increasing data regarding the adverse effects of a range of chemicals on ovarian development in humans as well as other species (Susiarjo *et al.*, 2007; Fowler *et al.*, 2008; Allard and Colaiacovo, 2010; Brieno-Enriquez *et al.*, 2011; Hunt *et al.*, 2012). Cigarette smoking is well recognised

to have a deleterious effect on the fertility of both men and women (Vine *et al.*, 1994; Ramlau-Hansen *et al.*, 2007; Dechanet *et al.*, 2011) and may also affect fetal androgen exposure (Fowler *et al.*, 2011). Smoking advances the age of the menopause (Gold *et al.*, 2013), and *in utero* exposure of human female fetuses to cigarette smoke has been associated with decreased numbers of germ cells and somatic cells in the developing ovary (Lutterodt *et al.*, 2009; Mamsen *et al.*, 2010), and reduced adult female fertility (Jensen *et al.*, 1998; Jensen *et al.*, 2006; Ye *et al.*, 2010).

The chemicals in cigarette smoke include polycyclic aromatic hydrocarbons (PAHs), which are ligands for the aryl hydrocarbon receptor (AhR); a transcription factor which mediates the cellular response to a broad range of xenobiotic molecules with adverse effects on female reproduction (Pocar *et al.*, 2005; Hernandez-Ochoa *et al.*, 2009). We have previously demonstrated that human germ cells in the male express the AhR, and that its activation *in vitro* induces germ cell apoptosis (Coutts *et al.*, 2007). In the fetal mouse ovary, AhR activation results in germ cell apoptosis (Matikainen *et al.*, 2002) and also results in the loss of more mature oocytes in both mouse and human (Matikainen *et al.*, 2001). Consistent with this, $Ahr^{-/-}$ mice have increased numbers of ovarian follicles in the early postnatal period (Benedict *et al.*, 2000; Robles *et al.*, 2000). In the present study we have explored the expression of the AhR in the human fetal ovary and investigated the effect of an AhR ligand on germ cell proliferation and apoptosis, to explore a mechanism whereby cigarette smoke PAHs might impact on female reproductive potential.

Methods

Tissue

Human fetal ovaries were obtained following medical termination of pregnancy during both the first and second trimesters (7 to 20 weeks gestational age). Women gave consent according to national guidelines and the study was approved by the Lothian Research Ethics Committee (REC 08/S1101/1). Termination of pregnancy was induced by treatment with mifepristone (200 mg orally) followed 48 h later by misoprostol (800μg) three hourly per vaginum. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination before termination and confirmed by subsequent direct measurement of foot length. Sex of first trimester specimens was determined by PCR genotyping for the *SRY* gene (primers: Fwd: 5'-ACAGTAAAGGCAACGTCCAG-3', Rev: 5'-ATCTGCGGGAAGCAAACTGC-3' (Friel *et al.*, 2002)**)**. Ovaries were dissected and either snap frozen and stored at -70°C, fixed in Bouin's for 2 hours, followed by processing for immunohistochemistry or immunofluorescence, or cultured *in vitro* as detailed below. Extra-ovarian tissue was dissected from ovaries to be fixed or frozen, but the mesonephros was left attached to samples used in culture experiments.

Quantitative PCR

For quantification of *AHR* and aryl hydrocarbon receptor nuclear translocator (*ARNT)* transcript levels, total RNA was extracted from frozen human fetal ovaries using the RNeasy Mini/Micro Kit (Qiagen, Crawley, UK) with on-column DNaseI digestion, and cDNA synthesised using the Superscript VILO cDNA synthesis kit (Applied Biosystems, Paisley, UK), with duplicate cDNA reactions in which the reverse

transcriptase enzyme was omitted prepared as no-template controls for qPCR. qPCR was performed using an ABI HT7900 real-time PCR instrument (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems). Calculations of mRNA concentrations were made relative to the housekeeping gene *RPL32*, to allow comparisons between cDNAs. Sequences of the oligonucleotide primers used in qPCR are as follows: *AHR* Fwd: 5'-ATACTGAAACAGAGCTGTGC-3', Rev: 5'- AAAGCAGGCGTGCATTAGAC-3' (Ikuta and Kawajiri, 2006); *ARNT* Fwd: 5'-GCTGCTGCCTACCCTAGTCTCA-3', Rev: 5'-GCTGCTCGTGTCTGGAATTGT-3' (Ginis *et al.*, 2004); *RPL32* Fwd: 5'-CATCTCCTTCTCGGCATCA-3', Rev: 5'-AACCCTGTTGTCAATGCCTC-3'.

Immunofluorescence

Paraffin-embedded ovaries were cut into 5µm sections and mounted onto electrostatically charged microscope slides (VWR, Poole, UK), dried overnight, and then dewaxed and rehydrated using conventional methods. Endogenous peroxidases were quenched in 3% hydrogen peroxide in methanol for 30 minutes (min) at room temperature. After a wash in water, slides were transferred into phospho-buffered saline (PBS) (Sigma-Aldrich, Poole, UK) for 5 min and blocked for 30 min in normal serum (Diagnostics Scotland, Carluke, UK) diluted 1:4 in PBS containing 5% Bovine Serum Albumin (BSA). Sections were blocked with avidin (0.01M; 15 min) and then biotin (0.001M; 15min; both from Vector Laboratories, Peterborough, UK) with washes in PBS in between. AHR antibody (Affinity BioReagents/Thermo Fisher Scientific) Cramlington, UK) was diluted 1:150 and applied to sections at 4°C overnight in a humidified chamber. AHR was visualised by tyramide-enhanced fluorescein via an HRP conjugated goat anti-mouse secondary antibody diluted 1:200.

Sections were counterstained with propidium iodide 1:1000. Fluorescent images were captured using a LSM510 confocal microscope. Negative controls incubated with mouse IgG, omitting primary antisera, were included in all runs and showed no positive immunostaining.

Culture of fetal ovaries

Human fetal ovary-mesonephros complexes (8-9 weeks gestational age) were cultured as previously described (Childs *et al.*, 2010) on cell culture inserts (Greiner Bio-One, Stonehouse, UK) in serum free medium (αMEM + GlutaMAX with 1X nonessential amino acids (Applied Biosystems); 2 mM sodium pyruvate and 3 mg/ml BSA Fraction V (both from Sigma-Aldrich); and penicillin/streptomycin/amphotericin B (Cambrex Biosciences, MD, USA)) in the presence of a final concentration of 0.01% dimethyl sulfoxde (DMSO; Sigma-Aldrich) or the AHR ligand 9,10-dimethyl-1,2-benzanthracene-3,4-dihydrodiol (DMBA-DHD (an active metabolite of DMBA); 1μM in DMSO; NCI Chemical Carcinogen Reference Standards Repository, MO, USA) for 7 days in a humidified incubator (37°C, 5% CO_2) to determine effects on PGC number, proliferation and apoptosis. Paired ovaries were used for control and treatment. A complete medium change was performed every 48 hours. After culture, tissues were fixed in Bouin's solution and processed into paraffin for histological assessment.

Immunohistochemical determination of germ cell number, proliferation and apoptosis

Immunohistochemistry was performed to estimate total germ cell number (Activator Protein-2gamma; AP-2γ), germ cell proliferation (phosphorylated histone-H3 (phospho-H3)) and apoptosis (cleaved caspase 3) on adjacent serial sections every 5th

187 section as previously described (Martins da Silva *et al.*, 2004; Childs *et al.*, 2010).
188 Slides were incubated in primary antibody (rabbit polyclonal antibodies to AP-2γ;
189 Santa Cruz Biotechnology, CA, USA; #sc-8977), and cleaved caspase 3 (New
190 England Biolabs, Hitchin, UK; #9601), both diluted 1:100 in Tris Buffered Saline
191 (TBS) supplemented with 20% normal goat serum (NGS) and 5% BSA, at 4^{o}C
192 overnight. Primary antibodies were detected using a biotinylated goat anti-rabbit
193 secondary antibody (Dako, Cambridge, UK), diluted 1:500 in TBS/NGS/BSA and
194 incubated for 1 hour at room temperature. Staining was visualized using streptavidin-
195 horseradish peroxidase (diluted 1:1000 in TBS) followed by 3,3'-diaminobenzidine
196 tetrahydrochloride (DAB; Dako). Immunohistochemical detection of phospho-H3 was
197 performed on an automated Bond Immunostaining Robot using a rabbit polyclonal to
198 phospho-H3 (Upstate Biotechnology, Milton Keynes, UK, #06-570) as the primary
199 antibody, with secondary antibody and detection as above. Images were captured
200 using an Olympus Provis microscope (Olympus, London, UK). PGC counts and
201 determination of areas were determined using a Zeiss Axio Imager A1 microscope
202 (Carl Zeiss) fitted with a camera and automatic stage (Prior Scientific Instruments
203 Ltd., Cambridge, UK) with Image Pro Plus software 4.5.1 with Stereologer Pro 5
204 software (Media Cybernetics, Workingham, UK). PGC numbers were counted using
205 the point-counting tool, and ovarian areas calculated using the freehand draw tool to
206 outline the edge of the tissue section.
207
208 *Statistical analysis*
209 Data are presented as mean±sem. Gene expression across gestation was analysed by
210 ANOVA. Tissue culture experiments were analysed by paired t test or Wilcoxon tests

for data expressed as percentages, as the experimental design involved comparison of treatment effects on paired gonads from each fetal specimen.

Results

AHR gene expression is upregulated during human fetal ovarian development

Expression of *AHR* mRNA was detected in human fetal ovaries at all gestations by qPCR. *AHR* transcript levels increased with gestation, rising 2-fold between the first trimester (8-9 weeks gestation) and late second trimester (17-20 weeks gestation; p=0.008, n=5-6 per group; Figure 1A). Expression of *ARNT*, which encodes the Aryl Hydrocarbon Nuclear Translocator required for AhR transcriptional activity, was unchanged across this period (Figure 1B).

AhR protein is expressed exclusively by germ cells in the human fetal ovary

AhR was detected in human fetal ovaries in all specimens across the gestational range examined. At all stages of development, AhR expression was exclusively confined to germ cells. In the first trimester, AhR was expressed by all germ cells (Figure 2A), whereas in the second trimester AhR was expressed by clusters of germ cells with others not showing expression (Figure 2B and C). AhR-expressing germ cells were predominantly around the periphery of the ovary (i.e. in less mature germ cells) but scattered clusters of immunopositive germ cells were detected throughout the ovary (Figure 2B). Oocytes within primordial follicles (Figure 2D) showed weak/no immunostaining.

The AhR ligand DMBA-DHD reduces germ cell proliferation in the human fetal ovary in vitro

To establish the effect of AhR activation on human fetal germ cell behaviour, first trimester human fetal ovaries were maintained *in vitro* for seven days in the presence of vehicle (0.01% DMSO) or the AhR agonist DMBA-DHD (1μM), before histological assessment of germ cell number, proliferation and apoptosis. First trimester samples were used for this part of the study since i) all germ cells expressed AhR at this stage, ii) germ cells at this stage are less heterogeneous than at later stages of development and iii) first trimester human fetal ovaries can be maintained in culture for at least seven days, which we have previously demonstrated to be a sufficient period to analyse changes in germ cell number, proliferation or apoptosis in response to external stimuli (Childs *et al.*, 2010). Ovarian tissue showed well preserved morphology after 7 days in culture, with ongoing germ cell mitosis detected (as determined by phospho-H3) immunostaining; Figure 3A). Germ cell number (determined by quantifying the number of AP-2γ-positive cells in the ovary (Childs *et al.*, 2010) was not affected by treatment with DMBA-DHD (1.01±0.08 in vehicle controls vs 1.25±0.09 $x10^{-4}/\mu m^2$ in DMBA-DHD treated; Figure 3C), however exposure to DMBA-DHD did reduce the proportion of proliferating (phospho-H3-positive) germ cells by ~30% (8.9±0.8% in controls vs 6.3±1.2% in DMBA-DHD treated; p=0.04, n=4; Figure 3D). Apoptotic (cleaved caspase 3-positive) germ cells were rare (Figure 3B), and the proportion of apoptotic germ cells was not affected by exposure to DMBA-DHD (14.9±5.1% control vs 12.5±2.5% treated, ns; Figure 3E).

Discussion

These data demonstrate that germ cells in the human fetal ovary are a site of expression of the AhR, and that expression of the AhR is developmentally regulated at the gene, protein and cellular level. In the first trimester the great majority of germ

261 cells express the AhR, whereas in the second trimester, after the onset of meiosis,
262 AhR expression was more restricted, with AhR detected in clusters of germ cells,
263 while others showed no expression. There was a modest increase in *AHR* mRNA
264 expression with increasing gestation, interpretation of which is complicated by the
265 changing cellular constituents of the ovary. There was no change in the expression of
266 *ARNT*, which encodes the Aryl Hydrocarbon Translocator, an AhR co-factor.
267 Importantly, we have shown for the first time that functional activation of the AhR by
268 a polycyclic aromatic hydrocarbon (PAH) found in cigarette smoke reduced germ cell
269 proliferation in the first trimester human fetal ovary, but did not affect germ cell
270 apoptosis. Collectively, these data provide a mechanism whereby *in utero* exposure
271 to AhR ligands, as found for example in cigarette smoke and other products of
272 combustion, may influence germ cell proliferation in the ovary and potentially impact
273 on later female reproductive function. This may therefore at least in part contribute to
274 the observed reduced fertility in women exposed to cigarettes prenatally (Jensen *et al.*,
275 1998; Ye *et al.*, 2010).
276
277 The AhR was expressed in germ cells, but not in other cell types in the fetal ovary
278 across the gestational range examined. This pattern of expression is similar to that we
279 previously reported in the fetal testes (Coutts *et al.*, 2007), as is the finding that AhR
280 expression becomes restricted to specific populations of germ cells in the fetal ovary
281 with increasing developmental age. The progressive restriction of AhR expression to
282 a subset of germ cells in the second trimester human fetal ovary implies only a certain
283 stage or stages of germ cell development are associated with AhR expression,
284 following the initiation of meiosis from 11 weeks gestation onwards. The functional
285 significance of this is unclear, but it may be of relevance that the AhR has been

associated with regulation of the cell cycle (Denison and Heath-Pagliuso, 1998). In keeping with this, our functional data indicate that treatment of first trimester fetal ovary with a specific AhR ligand significantly reduced germ cell proliferation, as detected by expression of phosphorylated histone H3. The importance of germ cell proliferation prior to meiotic entry is indicated by the phenotype of mice deficient for Pin1; a regulator of the rate of mitosis, the absence of which results in markedly reduced primordial follicle numbers (Atchison *et al.*, 2003). We were unable to detect a significant reduction in the number of germ cells in the fetal ovary in response to DMBA-DHD. The doubling time of the human fetal ovarian germ cell population has been estimated at approximately 6 days (Bendsen *et al.*, 2006), thus it is likely that the reduction in germ cell proliferation observed here is too small to effect a change in germ cell number of sufficient magnitude to be detected within the short (seven day) period of culture, although this method was able to detect changes in germ cell number associated with increased apoptosis (Childs *et al.*, 2010). Fetuses *in utero* are likely to be exposed chronically to cigarette smoke over a period of weeks or months, which may be long enough for an effect on germ cell proliferation to become manifest as a reduction in germ cell number, and thus reduced adult fertility.

There was no change in the proportion of germ cells undergoing apoptosis, as indicated by detection of cleaved caspase 3. This result therefore differs from our findings in the fetal testes where AhR activation resulted in an increase in germ cell apoptosis (Coutts *et al.*, 2007). Human embryonic stem cells induced to differentiate towards the germ cell lineage also express the AhR, and are sensitive to PAHs (Kee *et al.*, 2010). In that model, DMBA-DHD resulted in reduced expression of primordial germ cell genes, and increased apoptosis, although the suitability of the ES cell

311 system as a model for human ovarian germ cell development *in vivo* remains to be
312 determined. Female mice exposed *in utero* to the AhR ligand benzo(a)pyrene have
313 reduced fertility (MacKenzie and Angevine, 1981), and exposure to dioxin (2,3,7,8-
314 tetrachlorodibenzo-*p*-dioxin, TCDD), also an AhR ligand, has diverse adverse effects
315 on the developing reproductive tract (Wolf *et al.*, 1999; Bruner-Tran and Osteen,
316 2011). *In vitro* studies suggest that PAH exposure resulted in increased germ cell
317 apoptosis in the mouse fetal ovary (Matikainen *et al.*, 2002). This effect on apoptosis
318 therefore differs from the results presented here, possibly reflecting different stages of
319 development of the germ cells exposed to the PAH. Germ cell apoptosis is infrequent
320 in the first trimester human ovary, but is thought to be an important part of germ cell
321 selection at later stages before primordial follicle formation; a hypothesis consistent
322 the marked increase in the number of apoptotic germ cells observed in the late second
323 trimester human fetal ovary (Fulton *et al.*, 2005). The fetal mouse germ cells exposed
324 to PAH by Matikainen *et al* were at embryonic day 13.5, coincident with the onset of
325 meiosis in the fetal mouse ovary. Exposure of meiotic germ cells in second trimester
326 human fetal ovary to an AhR ligand might induce germ cell apoptosis, in contrast to
327 the phenotype of reduced proliferation we see in response to DMBA-DHD treatment
328 of first trimester human fetal ovaries. Interestingly, female mice with targeted
329 disruptions of the *Ahr* gene display increased numbers of primordial follicles in the
330 early postnatal period (Benedict *et al.*, 2000; Robles *et al.*, 2000). This suggests that
331 activation of the AhR by as-yet-unidentified endogenous ligands in the fetal ovary
332 may contribute to the process of widespread germ cell death that occurs during fetal
333 oogenesis under normal physiological conditions.
334

335 The results presented here are consistent with a previous report of reduced numbers of
336 germ cells in the ovaries of fetuses of women who smoked (Mamsen *et al.*, 2010), and
337 indicate that this effect may be mediated by direct effects of PAHs in cigarette smoke
338 on the fetal ovary. Smoking was also associated with a reduced number of somatic
339 cells in the ovary (Mamsen *et al.*, 2010) , and while we found no evidence in the
340 present study that somatic cells expressed the AHR, the close inter-dependency of the
341 two cell types, and extensive bidirectional signalling between them (Robinson *et al.*,
342 2001; Martins da Silva *et al.*, 2004; Coutts *et al.*, 2008; Childs and Anderson, 2009),
343 makes a secondary, germ cell-mediated effect on the development of ovarian somatic
344 cells very plausible.
345
346 In summary, these data provide a functional basis for an adverse effect of *in utero*
347 exposure to AhR ligands including many that are found in cigarette smoke, providing
348 a mechanism for observational studies that have examined the gonads of smoke
349 exposed fetuses (Lutterodt *et al.*, 2009; Mamsen *et al.*, 2010), and epidemiological
350 studies on the subsequent fertility of such individuals (Jensen *et al.*, 1998; Jensen *et*
351 *al.*, 2006; Ye *et al.*, 2010). Together with substantial experimental and
352 epidemiological evidence for an adverse effect of smoking exposure *in utero* on male
353 reproductive function (Jensen *et al.*, 2004; Coutts *et al.*, 2007; Ramlau-Hansen *et al.*,
354 2007) these data highlight the vulnerability of fetal germ cells of both females and
355 males to adverse environmental influences *in utero*.
356
357 **Acknowledgements**

358 This work was funded by the Medical Research Council (G1100357). We are grateful
359 to Anne Saunderson, Joan Creiger and the staff of the Bruntsfield Suite, Royal
360 Infirmary of Edinburgh, for their considerable assistance in patient recruitment.
361

362 **References**

363 Allard P, Colaiacovo MP (2010) Bisphenol A impairs the double-strand break repair
364 machinery in the germline and causes chromosome abnormalities. Proc Natl Acad Sci
365 U S A 107,20405-20410.
366 Anderson RA, Fulton N, Cowan G, Coutts S, Saunders PTK (2007) Conserved and
367 divergent patterns of gene expression in female and male germ cells during
368 development of the human fetal gonad. BMC Dev Biol 7,136-145.
369 Atchison FW, Capel B, Means AR (2003) Pin1 regulates the timing of mammalian
370 primordial germ cell proliferation. Development 130,3579-3586.
371 Baker TG (1963) A quantitative and cytological study of germ cells in human ovaries.
372 Proc Roy Soc Lond B Biol Sci 158,417-433.
373 Bendsen E, Byskov AG, Andersen CY, Westergaard LG (2006) Number of germ cells
374 and somatic cells in human fetal ovaries during the first weeks after sex
375 differentiation. Hum Reprod 21,30-35.
376 Benedict JC, Lin TM, Loeffler IK, Peterson RE, Flaws JA (2000) Physiological role
377 of the aryl hydrocarbon receptor in mouse ovary development. Toxicol Sci 56,382-
378 388.
379 Brieno-Enriquez MA, Reig R, Cabero L, Toran N, Martinez F, Roig I, Garcia Caldes
380 M (2011) Gene expression is altered after Bisphenol A exposure in human fetal
381 oocytes in vitro. Mol Hum Reprod 18,171-183.

382 Bruner-Tran KL, Osteen KG (2011) Developmental exposure to TCDD reduces
383 fertility and negatively affects pregnancy outcomes across multiple generations.
384 Reprod Toxicol 31,344-350.
385 Byskov AG (1986) Differentiation of mammalian embryonic gonad. Physiol Revs
386 66,71-117.
387 Childs AJ, Anderson RA (2009) Activin A selectively represses expression of the
388 membrane-bound isoform of Kit ligand in human fetal ovary. Fertil Steril 92,1416-
389 1419.
390 Childs AJ, Kinnell HL, Collins CS, Hogg K, Bayne RA, Green SJ, McNeilly AS,
391 Anderson RA (2010) BMP signaling in the human fetal ovary is developmentally
392 regulated and promotes primordial germ cell apoptosis. Stem Cells 28,1368-1378.
393 Childs AJ, Kinnell HL, He J, Anderson RA (2012) LIN28 Is Selectively Expressed by
394 Primordial and Pre-Meiotic Germ Cells in the Human Fetal Ovary. Stem Cells Dev
395 21,2343-2349.
396 Coutts SM, Childs AJ, Fulton N, Collins C, Bayne RA, McNeilly AS, Anderson RA
397 (2008) Activin signals via SMAD2/3 between germ and somatic cells in the human
398 fetal ovary and regulates kit ligand expression. Dev Biol 314,189-199.
399 Coutts SM, Fulton N, Anderson RA (2007) Environmental toxicant-induced germ cell
400 apoptosis in the human fetal testis. Hum Reprod 22,2912-2918.
401 Dechanet C, Anahory T, Mathieu Daude JC, Quantin X, Reyftmann L, Hamamah S,
402 Hedon B, Dechaud H (2011) Effects of cigarette smoking on reproduction. Hum
403 Reprod Update 17,76-95.
404 Denison MS, Heath-Pagliuso S (1998) The Ah receptor: a regulator of the
405 biochemical and toxicological actions of structurally diverse chemicals. Bull Environ
406 Contam Toxicol 61,557-568.

407 Fowler PA, Bhattacharya S, Flannigan S, Drake AJ, O'Shaughnessy PJ (2011)
408 Maternal cigarette smoking and effects on androgen action in male offspring:
409 unexpected effects on second-trimester anogenital distance. J Clin Endocrinol Metab
410 96,E1502-1506.
411 Fowler PA, Dora NJ, McFerran H, Amezaga MR, Miller DW, Lea RG, Cash P,
412 McNeilly AS, Evans NP, Cotinot C *et al.* (2008) In utero exposure to low doses of
413 environmental pollutants disrupts fetal ovarian development in sheep. Mol Hum
414 Reprod 14,269-280.
415 Friel A, Houghton JA, Glennon M, Lavery R, Smith T, Nolan A, Maher M (2002) A
416 preliminary report on the implication of RT-PCR detection of DAZ, RBMY1, USP9Y
417 and Protamine-2 mRNA in testicular biopsy samples from azoospermic men. Int J
418 Androl 25,59-64.
419 Fulton N, Martins da Silva SJ, Bayne RAL, Anderson RA (2005) Germ cell
420 proliferation and apoptosis in the developing human ovary. J Clin Endocrinol Metab
421 90,4664-4670.
422 Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A,
423 Carpenter MK, Itskovitz-Eldor J *et al.* (2004) Differences between human and mouse
424 embryonic stem cells. Dev Biol 269,360-380.
425 Gold EB, Crawford SL, Avis NE, Crandall CJ, Matthews KA, Waetjen LE, Lee JS,
426 Thurston R, Vuga M, Harlow SD (2013) Factors Related to Age at Natural
427 Menopause: Longitudinal Analyses From SWAN. Am J Epidemiol 178,70-83.
428 Gondos B, Westergaard L, Byskov AG (1986) Initiation of oogenesis in the human
429 fetal ovary: ultrastructural and squash preparation study. Am J Obstet Gynaecol
430 155,189-195.

431 Hernandez-Ochoa I, Karman BN, Flaws JA (2009) The role of the aryl hydrocarbon
432 receptor in the female reproductive system. Biochem Pharmacol 77,547-559.
433 Hunt PA, Lawson C, Gieske M, Murdoch B, Smith H, Marre A, Hassold T,
434 VandeVoort CA (2012) Bisphenol A alters early oogenesis and follicle formation in
435 the fetal ovary of the rhesus monkey. Proc Natl Acad Sci U S A 109,17525-17530.
436 Ikuta T, Kawajiri K (2006) Zinc finger transcription factor Slug is a novel target gene
437 of aryl hydrocarbon receptor. Exp Cell Res 312,3585-3594.
438 Jensen TK, Henriksen TB, Hjollund NH, Scheike T, Kolstad H, Giwercman A, Ernst
439 E, Bonde JP, Skakkebaek NE, Olsen J (1998) Adult and prenatal exposures to tobacco
440 smoke as risk indicators of fertility among 430 Danish couples. Am J Epidemiol
441 148,992-997.
442 Jensen TK, Joffe M, Scheike T, Skytthe A, Gaist D, Petersen I, Christensen K (2006)
443 Early exposure to smoking and future fecundity among Danish twins. Int J Androl
444 29,603-613.
445 Jensen TK, Jorgensen N, Punab M, Haugen TB, Suominen J, Zilaitiene B, Horte A,
446 Andersen AG, Carlsen E, Magnus O *et al.* (2004) Association of in utero exposure to
447 maternal smoking with reduced semen quality and testis size in adulthood: a cross-
448 sectional study of 1,770 young men from the general population in five European
449 countries. Am J Epidemiol 159,49-58.
450 Kee K, Flores M, Cedars MI, Reijo Pera RA (2010) Human primordial germ cell
451 formation is diminished by exposure to environmental toxicants acting through the
452 AHR signaling pathway. Toxicol Sci 117,218-224.
453 Kurilo LF (1981) Oogenesis in antenatal development in man. Hum Genet 57,86-92.
454 Lutterodt MC, Sorensen KP, Larsen KB, Skouby SO, Andersen CY, Byskov AG
455 (2009) The number of oogonia and somatic cells in the human female embryo and

456 fetus in relation to whether or not exposed to maternal cigarette smoking. Hum
457 Reprod 24,2558-2566.
458 MacKenzie KM, Angevine DM (1981) Infertility in mice exposed in utero to
459 benzo(a)pyrene. Biol Reprod 24,183-191.
460 Maheshwari A, Fowler PA (2008) Primordial follicular assembly in humans--
461 revisited. Zygote 16,285-296.
462 Mamsen LS, Lutterodt MC, Andersen EW, Skouby SO, Sorensen KP, Andersen CY,
463 Byskov AG (2010) Cigarette smoking during early pregnancy reduces the number of
464 embryonic germ and somatic cells. Hum Reprod 25,2755-2761.
465 Martins da Silva SJ, Bayne RAL, Cambray N, Hartley PS, McNeilly AS, Anderson
466 RA (2004) Expression of activin subunits and receptors in the developing human
467 ovary: activin A promotes germ cell survival and proliferation prior to primordial
468 follicle formation. Devel Biol 266,334-345.
469 Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu HY, Laine J, Sakai
470 T, Korsmeyer SJ, Casper RF *et al.* (2001) Aromatic hydrocarbon receptor-driven Bax
471 gene expression is required for premature ovarian failure caused by biohazardous
472 environmental chemicals. Nat Genet 28,355-360.
473 Matikainen TM, Moriyama T, Morita Y, Perez GI, Korsmeyer SJ, Sherr DH, Tilly JL
474 (2002) Ligand activation of the aromatic hydrocarbon receptor transcription factor
475 drives Bax-dependent apoptosis in developing fetal ovarian germ cells. Endocrinology
476 143,615-620.
477 Pepling ME, Spradling AC (2001) Mouse ovarian germ cell cysts undergo
478 programmed breakdown to form primordial follicles. Dev Biol 234,339-351.

479 Pocar P, Fischer B, Klonisch T, Hombach-Klonisch S (2005) Molecular interactions
480 of the aryl hydrocarbon receptor and its biological and toxicological relevance for
481 reproduction. Reproduction 129,379-389.
482 Ramlau-Hansen CH, Thulstrup AM, Storgaard L, Toft G, Olsen J, Bonde JP (2007) Is
483 prenatal exposure to tobacco smoking a cause of poor semen quality? A follow-up
484 study. Am J Epidemiol 165,1372-1379.
485 Robinson LLL, Gaskell TL, Saunders PTK, Anderson RA (2001) Germ cell specific
486 expression of c-kit in the human fetal gonad. Mol Human Reprod 7,845-852.
487 Robles R, Morita Y, Mann KK, Perez GI, Yang S, Matikainen T, Sherr DH, Tilly JL
488 (2000) The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of
489 the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse.
490 Endocrinology 141,450-453.
491 Sforza C, Vizzotto L, Ferrario VF, Forabosco A (2003) Position of follicles in normal
492 human ovary during definitive histogenesis. Early Hum Dev 74,27-35.
493 Stoop H, Honecker F, Cools M, de Krijger R, Bokemeyer C, Looijenga LH (2005)
494 Differentiation and development of human female germ cells during prenatal
495 gonadogenesis: an immunohistochemical study. Hum Reprod 20,1466-1476.
496 Susiarjo M, Hassold TJ, Freeman E, Hunt PA (2007) Bisphenol A exposure in utero
497 disrupts early oogenesis in the mouse. PLoS Genet 3,e5.
498 Tingen C, Kim A, Woodruff TK (2009) The primordial pool of follicles and nest
499 breakdown in mammalian ovaries. Mol Hum Reprod 15,795-803.
500 Vine MF, Margolin BH, Morrison HI, Hulka BS (1994) Cigarette smoking and sperm
501 density: a meta-analysis. Fertil Steril 61,35-43.

502 Wolf CJ, Ostby JS, Gray LE, Jr. (1999) Gestational exposure to 2,3,7,8-
503 tetrachlorodibenzo-p-dioxin (TCDD) severely alters reproductive function of female
504 hamster offspring. Toxicol Sci 51,259-264.
505 Ye X, Skjaerven R, Basso O, Baird DD, Eggesbo M, Uicab LA, Haug K, Longnecker
506 MP (2010) In utero exposure to tobacco smoke and subsequent reduced fertility in
507 females. Hum Reprod 25,2901-2906.
508
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511

512 **Figure legends**

513

514 Figure 1. Expression of *AHR* (A) increases with gestation (P=0.008), but *ARNT* (B)
515 was unchanged (n=5-6 ovaries per group).

516

517 Figure 2. In the first trimester (A, 7 weeks gestation), AhR was expressed by all germ
518 cells (white arrows) with no expression in somatic cells. At later gestations (B, 19
519 weeks and C, 18 weeks), AhR expression remained confined to germ cells in clusters,
520 predominantly but not exclusively localized to the more peripheral regions of the
521 ovary (arrows)). AhR expression was low/absent in primordial follicles (D, 19
522 weeks). All scale bars, 20 μm.

523

524 Figure 3. *In vitro* exposure of human fetal ovaries (8-9 weeks of gestation) to an AhR
525 ligand reduces germ cell proliferation. Representative images of human fetal ovaries
526 cultured for 7 days and immunostained for phosphorylated histone H3 (A and B) and
527 cleaved caspase 3 (C and D) indicating mitotic proliferation and apoptosis
528 respectively (black arrows indicate immunostained cells in A and C). Exposure of
529 first trimester fetal ovaries to the AhR ligand DMBA-DHD (1μM) did not affect germ
530 cell number (E), but significantly reduced human fetal ovarian germ cell proliferation
531 relative to vehicle (DMSO) controls (F; quantified by detection of phospho-H3).
532 Germ cell apoptosis (assessed by caspase 3 immunostaining) was not affected by
533 DMBA-DHD treatment (G). Data are mean±sem of 4 independent experiments.

534

Figure 1

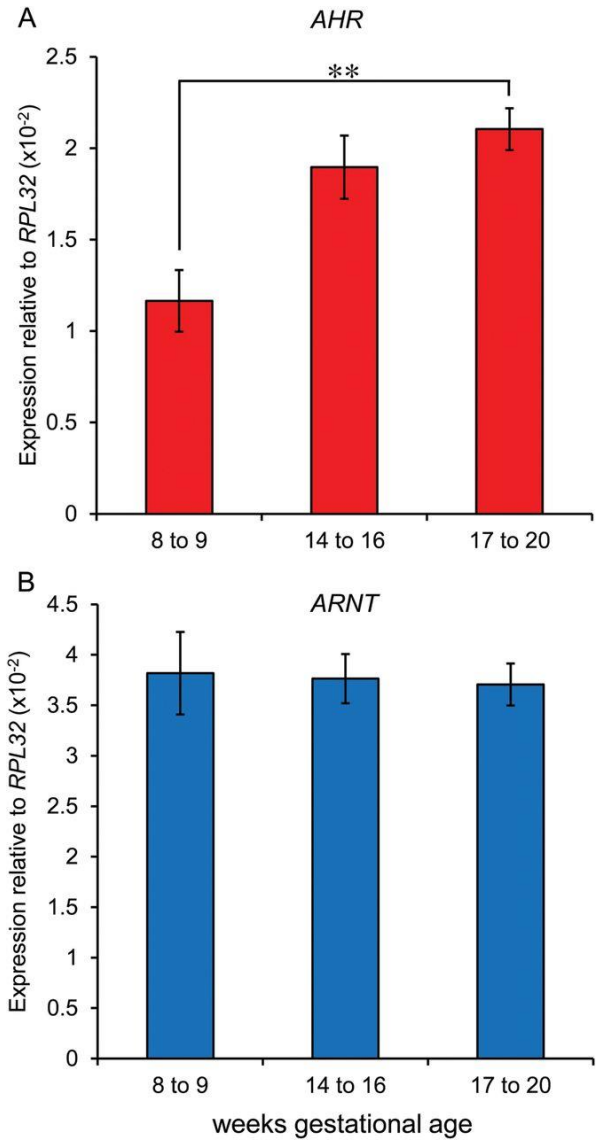

A
AHR
**
Expression relative to RPL32 (x10-2)
2.5
2
1.5
1
0.5
0
8 to 9
14 to 16
17 to 20
B
ARNT
Expression relative to RPL32 (x10-2)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
8 to 9
14 to 16
17 to 20
weeks gestational age

Figure 2

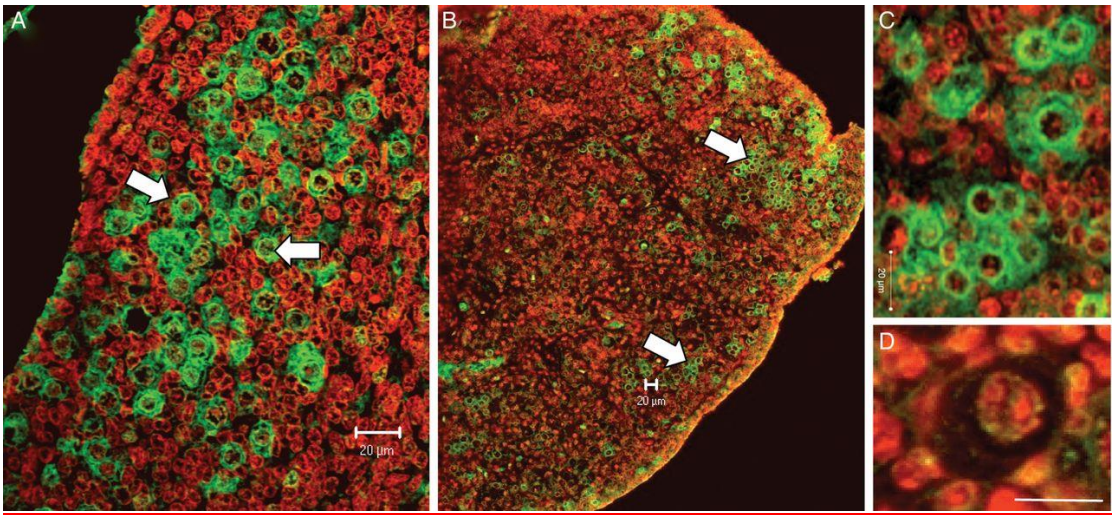
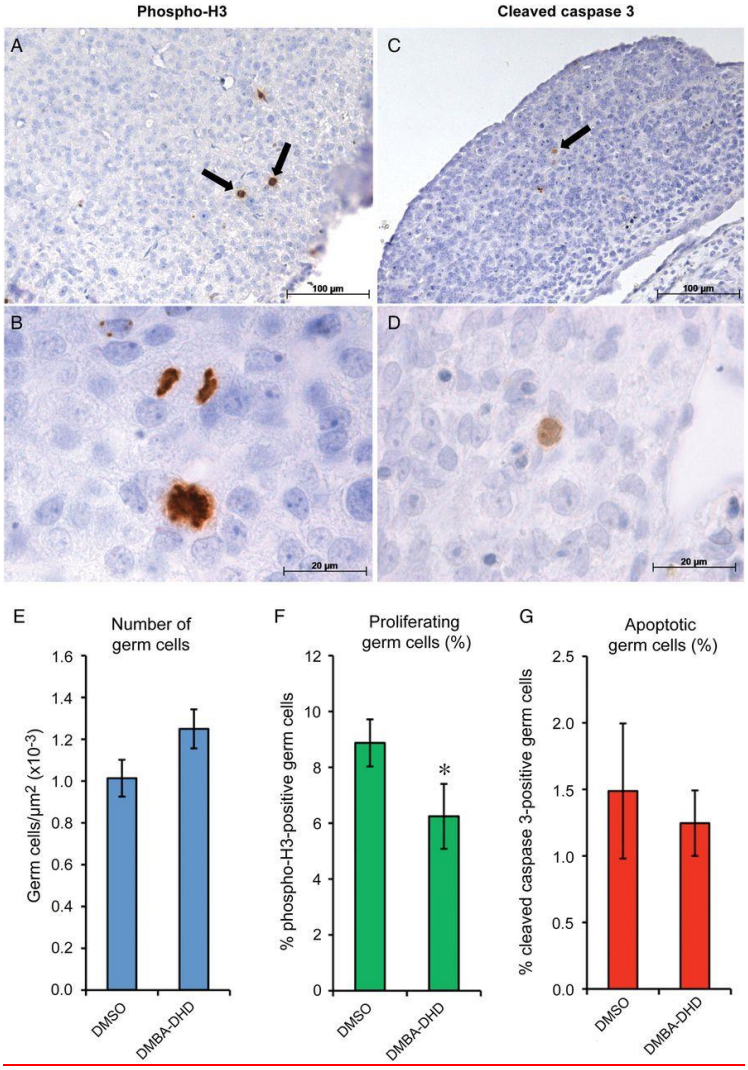


Figure 3


Phospho-H3
Cleaved caspase 3
A
C
100 µm
100 µm
B
D
20 µm
20 µm
E
Number of germ cells
Germ cells/µm² (x10⁻³)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
DMBA-DHD
F
Proliferating germ cells (%)
% phospho-H3-positive germ cells
12
10
8
6
4
2
0
*
DMSO
DMBA-DHD
G
Apoptotic germ cells (%)
% cleaved caspase 3-positive germ cells
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
DMBA-DHD