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ORIGINAL ARTICLE ***Reproductive biology***

In utero exposure to cigarette smoke dysregulates human fetal ovarian developmental signalling

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STUDY QUESTION: How does maternal cigarette smoking disturb development of the human fetal ovary?

SUMMARY ANSWER: Maternal smoking increases fetal estrogen titres and dysregulates several developmental processes in the fetal ovary.

WHAT IS KNOWN ALREADY: Exposure to maternal cigarette smoking during gestation reduces human fetal ovarian cell numbers, germ cell proliferation and subsequent adult fecundity.

STUDY DESIGN, SIZE, DURATION: The effects of maternal cigarette smoking on the second trimester human fetal ovary, fetal endocrine signalling and fetal chemical burden were studied. A total of 105 fetuses were studied, 56 from mothers who smoked during pregnancy and 49 from those who did not.

PARTICIPANTS/MATERIALS, SETTING METHODS: Ovary, liver and plasma samples were collected from electively terminated, normally progressing, second trimester human fetuses. Circulating fetal hormones, levels of 73 fetal ovarian transcripts, protein localization, density of oocytes/primordial follicles and levels of 16 polycyclic aromatic hydrocarbons (PAHs) in the fetal liver were determined.

MAIN RESULTS AND THE ROLE OF CHANCE: Circulating fetal estrogen levels were very high and were increased by maternal smoking (ANOVA, $P = 0.055–0.004$ versus control). Smoke exposure also dysregulated (two-way ANOVA, smoking versus gestation weeks interaction, $P = 0.046–0.023$) four fetal ovarian genes (cytochrome P450 scc [*CYP11A1*], NOBOX oogenesis homeobox [*NOBOX*], activator of apoptosis harakiri [*HRK*], nuclear receptor subfamily 2, group E, member 1 [*NR2E1*]), shifted the ovarian Inhibin βA/inhibin α ratio (*NHBA/INHA*) transcript ratio in favour of activin (ANOVA, $P = 0.049$ versus control) and reduced the proportion of dominant-negative estrogen receptor 2 (ERβ: *ESR2*) isoforms in half the exposed fetuses. PAHs, ligands for the aryl hydrocarbon receptor (AHR), were increased nearly 6-fold by maternal smoking (ANOVA, $P = 0.011$ versus control). A fifth transcript, COUP transcription factor 1 (nuclear receptor subfamily 2, group F, member 1: *NR2F1*, which contains multiple AHR-binding sites), was both significantly increased (ANOVA, $P = 0.026$ versus control) and dysregulated by (two-way ANOVA, smoking versus gestation weeks interaction, $P = 0.021$) maternal smoking. NR2F1 is associated with repression of *FSHR* expression and smoke-exposed ovaries failed to show the normal increase in *FSHR* expression during the second trimester. There was a significantly higher number of DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*DDX4*) VASA-positive (ANOVA, $P = 0.016$ versus control), but not POU domain,

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class 1, transcription factor 1 (*POU5F1*) OCT3/4-positive, oocytes in smoke-exposed fetuses and this matched with a significantly higher number of primordial follicles (ANOVA, $P = 0.024$ versus control).

LIMITATIONS, REASONS FOR CAUTION: The effects of maternal smoking on establishment of the maximum fetal primordial follicle pool cannot be reliably studied in our population since the process is not completed until 28 weeks of gestation and normal fetuses older than 21 weeks of gestation are not available for study. Our data suggest that some fetal ovaries are affected by smoke exposure while others are not, indicating that additional studies, with larger numbers, may show more significant effects.

WIDER IMPLICATIONS OF THE FINDINGS: Fetal exposure to chemicals in cigarette smoke is known to lead to reduced fecundity in women. Our study suggests, for the first time, that this occurs via mechanisms involving activation of AHR, disruption of inhibin/activin and estrogen signalling, increased exposure to estrogen and dysregulation of multiple molecular pathways in the exposed human fetal ovary. Our data also suggest that alterations in the *ESR2* positive and dominant negative isoforms may be associated with reduced sensitivity of some fetuses to increased estrogens and maternal smoking.

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Key words: ovary / fetal human / cigarette / polycyclic aromatic hydrocarbons / AhR

Introduction

Maternal cigarette smoking has wide-ranging harmful effects on fetal development and the health of subsequent offspring. These effects include reduced growth (Harvey *et al.*, 2007), reduced academic achievement (Birney *et al.*, 2006), increased likelihood of metabolic syndrome (Chen *et al.*, 2006) and reproductive abnormalities and reduced fertility (Weinberg *et al.*, 1989; Jensen *et al.*, 1998, 2006, 2007; Ramlau-Hansen *et al.*, 2007; Werler, 2007; Ye *et al.*, 2010). The incidence of smoking has generally fallen over the last 20 years but significant numbers of women continue to smoke before and during pregnancy (Tong *et al.*, 2009). In Scotland, 31% of women are smokers when first pregnant (Prabhu *et al.*, 2010) and fewer than 4% of these will stop smoking (Tappin *et al.*, 2010). Smoke exposure *in utero* remains, therefore, a major cause of adult ill-health and it is essential that we understand the mechanisms leading to altered fetal and post-natal development.

Primordial follicle formation is the single most important event in establishing potential adult fecundity and the timing of menopause in women (Laissue *et al.*, 2008). In mice, primordial follicle numbers are reduced by exposure to cigarette smoke (Tuttle *et al.*, 2009), an effect probably mediated by various chemicals, especially polycyclic aromatic hydrocarbons (PAHs), in the smoke (Jurisicova *et al.*, 2007). PAHs are activating ligands for the aryl hydrocarbon receptor (AHR) and are amongst the active compounds known to affect female reproductive development in rodents (Matikainen *et al.*, 2001). We have also shown that the fetal human ovary expresses the AHR, its nuclear translocator (*ARNT*) and that activating the AHR in the human fetal ovary *in vitro* significantly reduces germ cell proliferation (Anderson *et al.*, 2014). This highlights a potential mechanism by which maternal smoking may affect female reproductive development but which requires further study. Many women delay childbirth to 35–40 years of age, at which point they will be left with only ~5% of their original reserve of non-growing follicles (Wallace and Kelsey, 2010). It is likely, therefore, that reduced fertility associated with *in utero* smoke exposure, when combined with delayed childbirth, will result in a significant decline in conception rate.

Given the prevalence of maternal smoking and the exposure of pregnant women to a wide range of environmental chemicals, it is critical that we understand the effects of *in utero* exposure to chemicals in cigarette smoke on the formation of human primordial follicles (from ~18 weeks of gestation onwards (Fowler *et al.*, 2009a)). We report here that maternal smoking increases the exposure of the female fetus to PAHs, increases fetal circulating estrogens and dysregulates ovarian development and paracrine/endocrine signalling. The evidence suggests that these effects may be mediated through induction of AHR pathways and AHR-estrogen receptor (ESR) crosstalk.

Materials and Methods

Sample collection and study design

The collection of fetal material (Fowler *et al.*, 2008) was approved by the National Health Service Grampian Research Ethics Committees (REC 04/S0802/21). Women seeking elective, medical terminations of pregnancy were recruited with written, informed consent. Only normally progressing pregnancies from women over 16 years of age and between 11 and 21 weeks of gestation were collected. Fetal ovaries were: (i) snap-frozen and stored at −85°C, (ii) processed for histology or (iii) processed for *in vitro* culture. The fetal livers were snap-frozen and stored at −85°C. In total, 105 female fetuses were included in this study, 56 from mothers who smoked during pregnancy and 49 from those who did not. Circulating hormones were assayed in a subset of 45 fetuses, with sufficient cardiac blood, and, in a further subset of 22 fetuses, the ovarian expression levels of key developmental genes were determined and liver PAH content measured. In 10 fetuses around 19 weeks of gestation circulating estrogens were measured while 3 further fetuses, from non-smoking mothers, were used for ovary culture to determine ovarian estrogen production (Tables I, II and III, Figs 1 and 2). The remaining 25 fetuses contributed to the overall assessment of smoke exposure effects on growth.

Hormone and cotinine assays

LH, FSH, prolactin (PRL) and intact hCG were measured using DELFIA kits (Fowler *et al.*, 2008, 2009a,b), with detection limits and inter- and intra-assay

Table I **Maternal cigarette smoking during pregnancy does not significantly affect second trimester growth or pituitary hormone profile in the female human fetus (mean ± SEM).**

Measure	All fetuses included		Endocrine group[a]		qPCR/PAH group[a]		E1/E2 group[a,b]		Ovary
	Control *n* = 49	Smoke-exposed *n* = 56	Control *n* = 19	Smoke-exposed *n* = 26	Control *n* = 10	Smoke-exposed *n* = 12	Control *n* = 5	Smoke-exposed *n* = 5	Culture group *n* = 3
Maternal characteristics									
Age (years)	24.3 ± 0.9	24.0 ± 0.8	23 ± 1	24 ± 1	27 ± 2	24 ± 2	27.2 ± 3.9	21.2 ± 1.5	21.7 ± 2.2
BMI (kg/m^3)	24.2 ± 0.6	24.7 ± 0.7	24 ± 1	24 ± 1	26 ± 2	25 ± 2	24.8 ± 2.1	27.3 ± 3.5	25.7 ± 1.9
Cigarettes per day	0	12 ± 1	0	13 ± 1	0	11 ± 1	0	9 ± 1	0
Fetal characteristics									
Age (weeks)	14.9 ± 0.3	14.9 ± 0.3	16.1 ± 0.6	15.6 ± 0.5	15.4 ± 0.8	14.9 ± 0.8	18.6 ± 0.2	19.2 ± 0.4	17.0 ± 0.6
Weight (g)	87.3 ± 11.0	92.4 ± 11.5	125 ± 9	118 ± 8	118 ± 30	82 ± 27	235 ± 32	229 ± 20	147.2 ± 9.1
Crown-rump length (mm)	101.0 ± 4.3	101.9 ± 3.9	116 ± 2	114 ± 2	113 ± 10	100 ± 9	156 ± 5	145 ± 6	129.4 ± 6.0
Ovary weight (mg)	14.4 ± 1.8	14.4 ± 2.2	23 ± 3	19 ± 2	18 ± 5	16 ± 4	34.6 ± 3.6	35.8 ± 7.6	30.6 ± 2.5
Intact hCG (U/l)			106 ± 33	133 ± 29	148 ± 82	169 ± 71			
LH (U/l)			48 ± 5	51 ± 4	44 ± 10	51 ± 9			
FSH (U/l)			8 ± 3	13 ± 3	3 ± 4	7 ± 4			
PRL (mU/l)			119 ± 29	107 ± 24	136 ± 42	116 ± 33			
Cotinine (ng/ml)[c]	4 ± 2	41 ± 2	2 ± 2	47 ± 1	2 ± 1	43 ± 3	1 ± 1	42 ± 9	3 ± 3

[a]The quantitative PCR (qPCR)/polycyclic aromatic hydrocarbons (PAH) group includes some of the Endocrine group.
[b]To enable 100 μl of plasma for analysis, only well characterized 18- and 19-week-old fetuses were used but not all had other endocrine data.
[c]Fetal plasma cotinine significantly ($P < 0.001$) higher in smoke-exposed fetuses.
BM, body mass index; LH, lutenising hormone; FSH, follicle stimulating hormone; PRL, prolactin; E1/E2, estrone/estradiol.

Table II Effects of maternal cigarette smoking on the mRNA transcript levels of 57 key genes in the human female fetal ovary (mean ± SEM, based on the qPCR/ PAH group shown in Table I).

Gene symbol	Gene product	Control[a]	Smoke-exposed[a]	Two-way ANOVA	
				Smoking	Weeks–smoke interaction
		n = 10	*n* = 12	P[b]	P[b]
Endocrine signalling: receptors					
1. *PGR*	Progesterone receptor	11.8 ± 5.38	7.97 ± 3.65	0.701	0.308
2. *ESR1*	Estrogen receptor α (ERα)	20.3 ± 5.3	20.4 ± 5.6	0.534	0.427
3. *ESR2*	ERβ (all isoforms)	18.0 ± 6.6	17.6 ± 5.3	0.337	0.152
	ERα/ERβ ratio	1.44 ± 0.17	1.28 ± 0.12	0.354	0.154
4. *ESR2*	Estrogen receptor β dominant negative isoforms b and c	0.95 ± 0.28	0.60 ± 0.16	0.227	0.198
	ESR2 a,d/b,c ratio (active/dominant negative ratio)	16.2 ± 1.48	29.2 ± 6.68	0.069	0.709
5. *GPER*[c]	G-protein-coupled estrogen receptor 1	6.87 ± 3.42	4.37 ± 2.97	0.534	0.518
6. *AR*	Androgen receptor	6.66 ± 1.90	5.02 ± 1.57	0.984	0.665
7. *LHCGR*	LH/hCG receptor	0.07 ± 0.04	0.02 ± 0.02	0.279	0.169
8. *FSHR*	FSH receptor	0.71 ± 0.34	0.28 ± 0.08	0.157	0.500
9. *PRLR*	PRL receptor	0.93 ± 0.21	0.66 ± 0.19	0.719	0.715
10. *ACVR1B*	Activin receptor type 1B	23.3 ± 9.6	16.5 ± 4.9	0.877	0.674
11. *ACVR2A*	Activin receptor type 2A	21.8 ± 8.6	15.3 ± 5.1	0.756	0.124
Endocrine signalling: hormones					
12. *INHA*	Inhibin α	2.14 ± 0.47	1.61 ± 0.40	0.391	0.494
13. *INHBA*	Inhibin βA	4.50 ± 1.15	5.22 ± 1.17	0.343	0.198
	Inhibin βA/inhibin α ratio	**2.23 ± 0.40**	**3.78 ± 0.52**	**0.049**	0.453
14. *INHBB*	Inhibin βB	0.35 ± 0.15	0.43 ± 0.12	0.451	0.834
	Inhibin βB/inhibin α ratio	0.14 ± 0.04	0.33 ± 0.09	0.294	0.679
15. *FST*	Follistatin	8.3 ± 2.4	7.1 ± 2.1	0.640	0.857
16. *PROK2*	Prokineticin 2	2.12 ± 1.42	1.54 ± 0.95	0.639	0.847
17. *AMH*	Anti-Mullerian Hormone	0.03 ± 0.01	0.07 ± 0.05	0.617	0.900
Xenochemical signalling					
18. *AHR*	Arylhydrocarbon receptor	34.2 ± 13.2	26.9 ± 7.0	0.209	0.376
19. *ARNT*	Arylhydrocarbon receptor nuclear translocator	32.0 ± 9.02	25.5 ± 6.73	0.606	0.374
	AHR/ARNT ratio	0.90 ± 0.12	1.07 ± 0.21	0.123	0.057
Steroidogenesis					
20. ***CYP11A1***	**Cytochrome P450 scc**	**6.16 ± 2.02**	**4.13 ± 1.14**	0.912	**0.035**
21. *CYP17A1*	Cytochrome P450-C17	1.85 ± 0.99	0.95 ± 0.36	0.509	0.573
22. *CYP19A1*	Cytochrome P450 aromatase	16.0 ± 4.5	10.3 ± 2.3	0.383	0.270
23. *STAR*	Steroid acute regulatory protein	4.25 ± 0.96	3.55 ± 0.62	0.862	0.116
24. *HSD17B1*	Hydroxysteroid (17-β) dehydrogenase 1	1.24 ± 0.27	1.05 ± 0.27	0.582	0.296
25. *HSD17B3*	Hydroxysteroid (17-β) dehydrogenase 3	0.10 ± 0.04	0.06 ± 0.02	0.193	0.335
26. *HSD3B2*	3 β-hydroxysteroid dehydrogenase/Δ5→4-isomerase type 2	0.67 ± 0.25	0.31 ± 0.10	0.340	0.103
27. *STS*	Steroid sulfatase (microsomal), isozyme S	12.4 ± 4.1	10.1 ± 3.5	0.463	0.857
Wnt pathway signalling					
28. *WNT4*	Wnt-4	1.81 ± 0.33	2.23 ± 0.48	0.542	0.325
29. *WNT5A*	Wnt-5a	2.51 ± 0.57	1.74 ± 0.35	0.324	0.212
30. *WNT6*	Wnt-6	4.35 ± 1.47	3.11 ± 0.99	0.476	0.722
31. *WNT9A*	Wnt-9a	0.26 ± 0.13	0.16 ± 0.09	0.905	0.905
32. *FZD8*	Frizzled-8	40.3 ± 9.4	47.0 ± 14.8	0.616	0.582

Continued

Table II *Continued*

Gene symbol	Gene product	Control[a]	Smoke-exposed[a]	Two-way ANOVA	
				Smoking	Weeks–smoke interaction
		n = 10	*n* = 12	P[b]	P[b]
Transcription factor/regulation					
33. *FIGLA*	Factor in the germ-line α	9.13 ± 5.27	6.46 ± 3.84	0.175	0.108
34. *FOXR1*	Forkhead box protein R1	53.3 ± 35.2	28.7 ± 12.3	0.972	0.649
35. *FOXL2*	Forkhead box protein L2	144 ± 60.5	99.0 ± 36.7	0.232	0.786
36. *SOHLH1*	Spermatogenesis and oogenesis specific basic helix-loop-helix-containing protein 1	4.66 ± 3.34	2.34 ± 1.08	0.297	0.282
37. *LHX8*	LIM/homeobox protein Lhx8	9.27 ± 5.96	5.38 ± 3.78	0.949	0.307
38. ***NR2E1***	**Nuclear receptor subfamily 2 group E member 1**	**2.00 ± 1.14**	**1.71 ± 0.89**	**0.007**	**0.046**
39. ***NOBOX***	**Homeobox protein NOBOX**	**14.5 ± 8.1**	**11.4 ± 6.8**	0.125	**0.023**
40. *POU5F1*	POU domain, class 1, transcription factor 1 (OCT4)	272 ± 65	473 ± 139	0.123	0.462
41. *SMAD2*	Mothers against decapentaplegic homolog 2	19.7 ± 5.4	16.3 ± 4.5	0.774	0.196
Developmental gene					
42. *ZAR1*	Zygote arrest protein 1	6.65 ± 4.28	3.28 ± 2.38	0.783	0.342
43. *DDX4*	DEAD box protein 4 (VASA)	94.6 ± 38.4	88.7 ± 31.5	0.447	0.257
44. *MBNL1*	Muscleblind-like, isoform CRA_f	66.4 ± 20.5	57.8 ± 15.6	0.778	0.161
45. *CAPN3*	Calpain 3	7.26 ± 2.42	5.57 ± 2.08	0.535	0.688
46. *KIT*	Mast/stem cell growth factor receptor Kit (c-kit)	15.5 ± 5.7	11.0 ± 2.9	0.315	0.199
47. *KITLG*	Kit ligand (SCF)	70.2 ± 17.9	65.6 ± 14.4	0.779	0.304
	KIT/KITLG ratio	0.18 ± 0.03	0.15 ± 0.02	0.542	0.406
Protein/receptor binding/folding					
48. *HSPD1*	Heat shock 60 kDa protein	95.0 ± 27.9	91.7 ± 27.8	0.297	0.105
49. *NALP5*	NACHT, LRR and PYD domains-containing protein 5	28.4 ± 19.9	20.3 ± 15.4	0.098	0.089
50. *ZP2*	Zona pellucida sperm-binding protein 2	8.94 ± 6.68	2.09 ± 1.47	0.637	0.325
51. *ZPBP2*	Zona pellucida-binding protein 2	30.8 ± 20.4	17.1 ± 7.53	0.825	0.938
52. *NXPH1*	Neuroexophillin-1	9.04 ± 1.96	11.0 ± 3.13	0.866	0.924
Apoptosis/proliferation/tumour suppression					
53. ***HRK***	**Activator of apoptosis harakiri**	**1.90 ± 0.56**	**1.91 ± 0.40**	0.099	**0.027**
54. *BAX*	Apoptosis regulator Bax	15.9 ± 3.7	14.0 ± 4.3	0.366	0.816
55. *BCL2*	Apoptosis regulator Bcl2	1.92 ± 0.52	1.48 ± 0.40	0.751	0.163
	BAX:BCL2 ratio	10.6 ± 1.9	14.8 ± 6.3	0.560	0.360
56. *PCNA*[c]	Proliferating cell nuclear antigen	23.9 ± 14.4	26.1 ± 11.7	0.877	0.674
57. *PTEN*	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase	172 ± 83.6	112 ± 47.1	0.945	0.966
Vault complex, nucleo-cytoplasmic transport, multidrug resistance					
58. *MVP*	Major vault protein (LRP, VAULT1)	22.8 ± 6.0	19.5 ± 4.0	0.627	0.306

[a]qPCR is expressed as mRNA relative to luciferase.
[b]Statistically significant *P*-values are shown in bold. Where relevant, multi-function genes are categorized according to their function most appropriate to ovarian development.
[c]Transcript expression below limit of detection in ≥50% of ovaries.

coefficients of variation of 0.05 U LH/l, 2.4% and 4.2%, 0.5 U FSH/l, 2.8% and 2.0%, 0.04 mU PRL/l, 2.0% and 3.0% and 0.5 U hCG/l, 4.1% and 4.6%, respectively. Cotinine, a marker of smoking, was measured using a commercial kit (Cozart Plc, Abingdon, Kent, UK), with values of 0–12 ng cotinine/ml being considered negative (Fowler *et al.*, 2008). Fetal plasma and ovary-conditioned culture medium samples were assayed for estrogens by gas chromatography linked to mass spectrometry (GC-MS/MS). The plasma preparation procedure (extraction and purification) used to isolate the target steroids has been previously described (Courant *et al.*, 2007, 2010). Briefly, an enzymatic hydrolysis was followed by purification and partitioning to separate androgens and estrogens. An Agilent 7890A gas chromatograph coupled to a 7000 GC triple-quadrupole device (Agilent, San José, CA, USA) was used for analyte detection and quantification in the selected reaction monitoring acquisition mode.

Table III Concentrations of PAHs in the human female fetal liver (μg/kg tissue weight, mean ± SEM, based on the qPCR/PAH group shown in Table I).

PAH name	PAH	Control	Smoke-exposed	Two-way ANOVA[c]	
		n = 8[a]	*n* = 8[a]	Smoke	Weeks-smoke
Acenaphthene	AC	0.16 ± 0.08	5.67 ± 4.54	0.809	0.165
Benzo[ghl]perylene	BghlP	0.46 ± 0.30	0.67 ± 0.67	0.620	0.732
Fluoranthene	FA	1.73 ± 1.73	7.29 ± 7.08	0.659	0.295
Fluorene	FL	6.48 ± 2.83	72.8 ± 38.8	**0.021**	0.128
Phenanthren	PHE	13.7 ± 4.18	89.1 ± 47.5	**0.025**	0.112
Naphthalene	NA	7.44 ± 1.13	16.9 ± 10.4	0.807	0.097
Bezo[b]fluoranthene	BbF	0.83 ± 0.83	1.07 ± 1.02	0.947	0.168
Anthracene	AN	1.32 ± 0.92	19.0 ± 6.71	0.999	0.277
Pyrene	PY	<LOD[b]	11.37 ± 6.77	***0.044***	0.099
Benzo[k]fluoranthene	BkF	<LOD[b]	1.00 ± 0.86	0.175	0.744
Benzo[a]pyrene	BaP	<LOD[b]	1.37 ± 1.37	0.388	0.518
Benzo[a]anthracene	BaA	1.42 ± 0.59	1.43 ± 0.62	0.966	0.129
Chrysene	CHR	3.56 ± 3.21	0.01 ± 0.01	0.352	0.670
Acenaphthalene	ACL	0.45 ± 0.44	<LOD[b]	0.809	0.165
Total		37.6 ± 4.3	216.3 ± 123.5	***0.011***	***0.018***

[a]Some livers were pooled to ensure sufficient material for analysis, *n* above refers to either separate or pooled livers used for each group from the same fetuses used for qPCR.
[b]PAHs < limits of detection (<LOD) in all livers, excluded from analysis.
[c]Statistically significant *P*-values are shown in bold, italicized for ANOVA results using LOD values for <LOD PAHs. Weeks-smoke = two-way ANOVA interaction between weeks of gestation and smoke exposure.

Phenotypic investigation of smoking effects on the fetal ovary and immunohistochemistry

Ovarian sections (5 μm) were either stained with hematoxylin and eosin (H&E) or using a Leica BOND-MAX™ automated immunostainer (Leica Microsystems, Newcastle Upon Tyne, UK). The primary antibodies, all with pH 6 antigen retrieval, were: (i) nuclear receptor subfamily 2 group E member 1 (NR2E1): 2 μg/ml: rabbit polyclonal, Abcam (Cambridge, UK), ab30942, (ii) activator of apoptosis harakiri (HRK): 1:50: goat polyclonal, Santa Cruz Biotechnology (Santa Cruz, CA, USA), sc-6972, (iii) major vault protein (MVP): 1:200: mouse monoclonal, Abcam, ab14562, (iv) POU domain, class 1, transcription factor 1 (*POU5F1*, OCT4): 1:300, goat polyclonal, Santa Cruz, sc-8628, (v) DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*DDX4*, VASA): 1:200: rabbit polyclonal, Abcam, ab13840, (vi) COUP transcription factor 1 (nuclear receptor subfamily 2, group F, member 1: NR2F1): 1:2000: rabbit polyclonal, Abcam, ab96846, (vii) COUP transcription factor 2 (nuclear receptor subfamily 2, group F, member 2: NR2F2): 1:1000: mouse monoclonal, R&D Systems (R&D Systems Europe Ltd, Abingdon, UK), PP-H7147–00. Sections were visualized with Bond 'Refine' 3,3′-diaminobenzidine (DAB), counter-stained with haematoxylin. IgG-negative sections, exposed to non-immune mouse and rabbit or goat serum in the absence of primary antibody, were included in all runs and showed no positive immunostaining. Human fetal ovarian localization for cytochrome P450 scc (CYP11A1) and homeobox protein NOBOX (NOBOX) have previously been reported (e.g. (Huntriss *et al.*, 2006; Fowler *et al.*, 2011)).

Ovaries from ten 17- to 19-week-old fetuses (five control, five smoke-exposed, for details see Supplementary data, Table SI) were scanned using a Zeiss LSM700 and Zen Blue 2012 software and images captured with a Zeiss Mrm camera and visually assessed by a single observer blinded to group identity. The number of (i) primordial follicles and (ii) DDX4, POU5F1 and MVP positive germ cells were determined in single sections (2–4 sections in the case of MVP) and normalized relative to the total area of the relevant sections using ImageJ software.

Western blot

Proteins were extracted from fetal ovaries with Qiagen AllPrep DNA/RNA/Protein mini kits (Qiagen Ltd, Crawley, UK). Tissue was homogenized in the presence of protease inhibitors (Protease Inhibitor Cocktail, Sigma-Aldrich Company Ltd, Gillingham, UK). Proteins (30 μg/lane) and Odyssey Two-Color molecular weight markers (LI-COR Biosciences UK Ltd, Cambridge, UK) were electrophoresed on a single dimension, 4–12% Bis-Tris gels (Invitrogen Ltd, Paisley, UK) under reducing conditions and transferred to immobilon-FL membranes (Millipore (UK) Ltd, Watford, UK). Membranes were blocked with Odyssey Blocking Buffer (927–4000: LICor) and incubated with primary antibodies: (i) steroid sulfatase (microsomal), isozyme S (STS): 1:500: rabbit polyclonal, Sigma-Aldrich Company Ltd, Poole, UK, HPA002904, (ii) G protein-coupled estrogen receptor 1 (GPER): 1:1000: rabbit polyclonal, Sigma-Aldrich Company Ltd, HPA027052, (iii) MVP: 2 μg/ml: mouse monoclonal, Abcam, ab14562, (vi) NR2F1: 1:500: rabbit polyclonal, Abcam, ab96846. Protein bands were visualized using an Odyssey infrared fluorescence imager (LI-COR) and analysed using TotalLab TL120 software (v2008.1; Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) (see (O'Shaughnessy *et al.*, 2011)).

Ovarian estrogen production

Three human fetal ovaries from control fetuses (Table I) were dissected free of other tissues, and then cut longitudinally and transversely. Tissue pieces were placed on cell culture inserts (PICM ORG 50, Millipore UK Ltd), each well receiving half an ovary. The cells contained 1 ml Waymouth media (Fisher Scientific UK Ltd, Loughborough, UK) supplemented with ITS+ (BD Biosciences), penicillin/streptomycin (Sigma-Aldrich), pyruvic acid (Merck) and follistatin (0.4 μg/ml, R&D Systems Europe Ltd, Abingdon, UK). Fetal ovary pieces were cultured at 37°C in 5% $C0_2$ and the media was replaced every 48 h. Culture medium representing 1–48 and 49–72 h culture periods were used for estrogen assay.

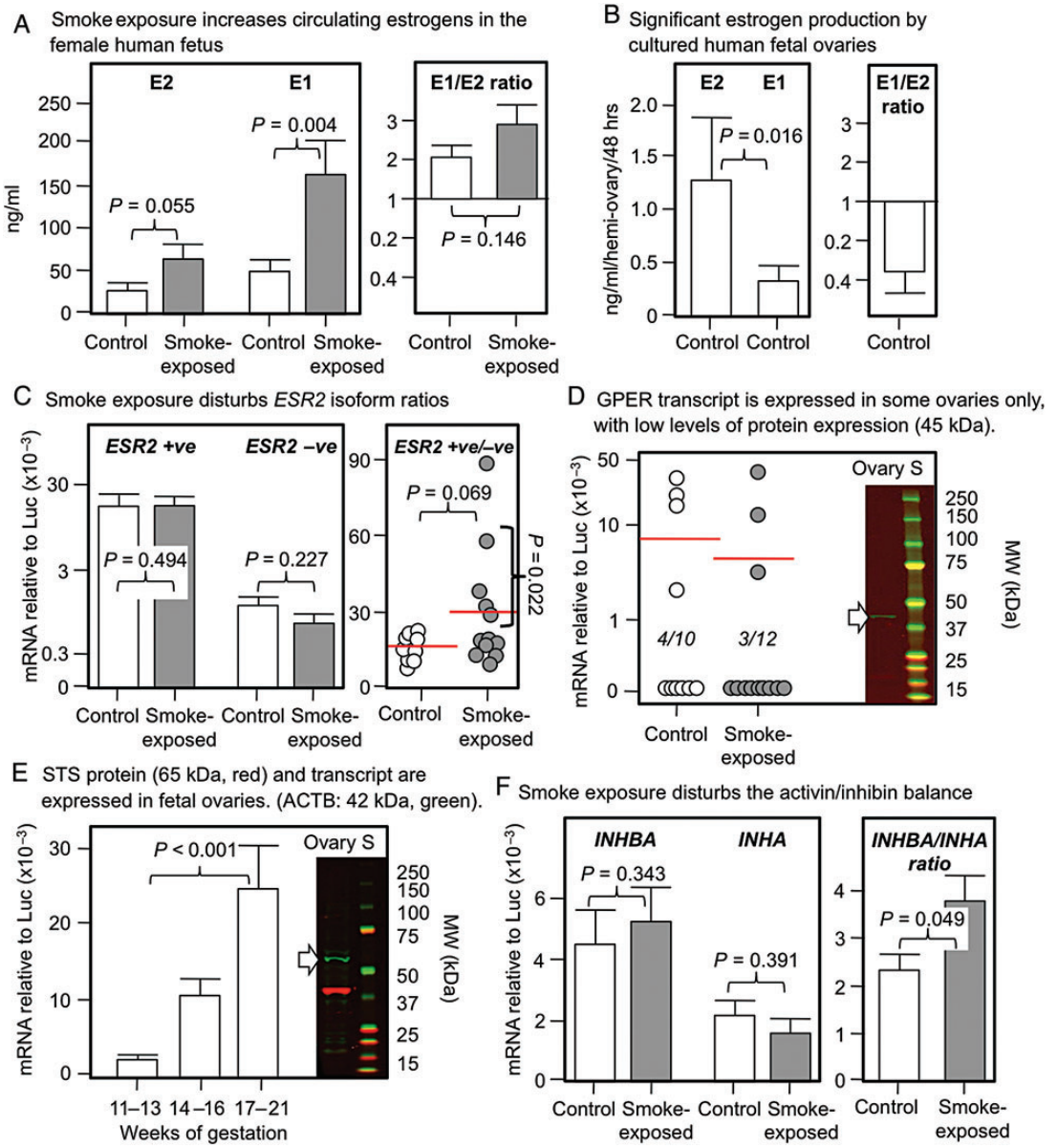

Figure 1 The estrogen and inhibin/activin signalling systems in human fetus and ovary are affected by maternal smoking. (**A**) *In utero* smoke exposure increases circulating fetal estrogen during the late second trimester (estrone [E1] predominates) although the E1/estradiol [E2] ratio is not significantly different between groups. (**B**) The fetal human ovary (from control fetuses) itself produces significant quantities of E2 and E1 *in vitro* (E2 predominates, $P = 0.016$). Values for the first 48 h of culture are shown, but were very similar during the second 48 h of culture. (**C**) The human fetal ovary expresses higher levels of estrogen receptor β (*ESR2*) isoforms a and d (*ESR2* +ve: values were calculated as isoforms b and c subtracted from total *ESR2*) than the dominant-negative isoforms b and c (*ESR2* −ve). Although these are not significantly affected by maternal smoking, the ratio between the two shows a significantly different variance (Levene's test $P = 0.022$) with 5/12 smoke-exposed fetuses deviating from the control range. (**D**) Transcripts for the putative non-nuclear estrogen receptor G-protein-coupled estrogen receptor 1 (*GPER*) are expressed in fewer than half the ovaries examined and only very low levels of protein could be detected. (**E**) The fetal human ovary expresses increasing *STS* transcript across the second trimester and also expresses the mature protein. (**F**) Smoke exposure skews the Inhibin βA/inhibin α (*INHBA:INHA*) transcript expression ratio in favour of activin A.

PAH determinations

The fetal liver concentrations of 16 PAHs known to be in cigarette smoke were determined as previously described (Fowler *et al.*, 2008). Briefly, seven internal standards (0.05 μg) were added to freeze-dried livers and PAHs extracted and quantified using GC/MS operated in the single ion-monitoring mode (Thermo Electron Trace MS (Hemel Hempstead, UK) linked to a Trace 2000 GC fitted with an AS2000 auto-sampler).

Real-time quantitative PCR (qPCR, Supplementary data, Table SII)

For quantification of specific mRNA species in fetal ovaries (see (Fowler *et al.*, 2009a,b, 2011)), qPCR was used after reverse transcription of isolated RNA (see (O'Shaughnessy *et al.*, 2007)). To allow specific mRNA levels to be expressed per ovary, 5 ng external standard (luciferase mRNA: Promega UK, Southampton, UK) was added to each ovary at the start of the RNA

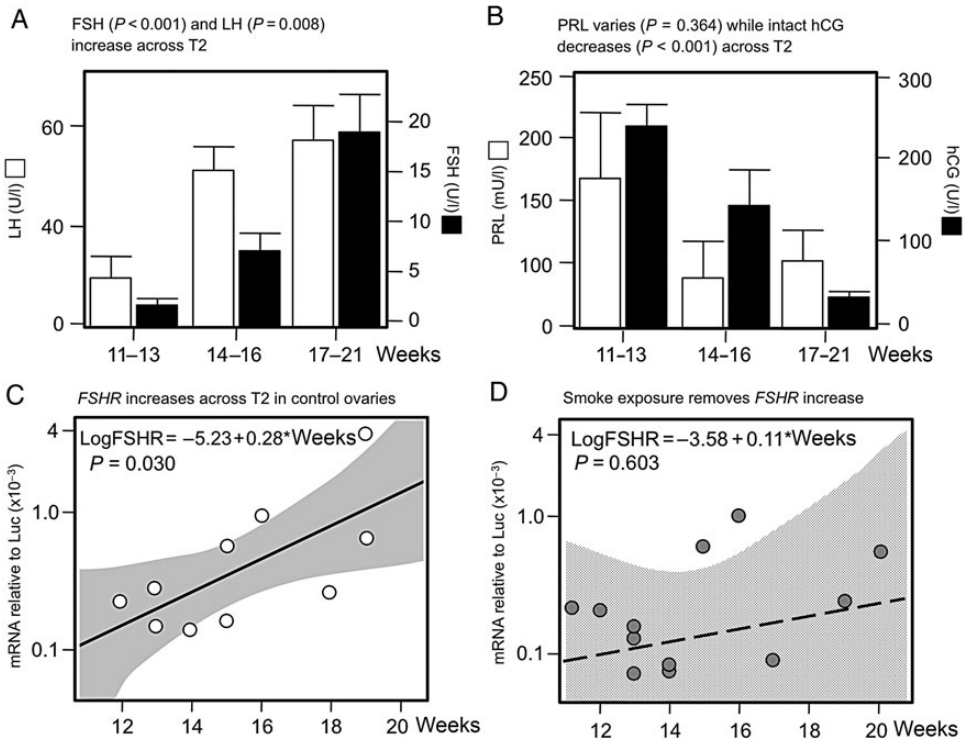

Figure 2 Circulating gonadotrophins in the human fetus are not affected by maternal smoking although fetal ovarian FSH receptor expression is disturbed. (**A**) Circulating fetal LH and FSH increase significantly across the second trimester. (**B**) Circulating fetal PRL is variable, showing no significant pattern while intact hCG in the fetal circulation declines sharply between 11 and 21 weeks of gestation. However expression of the FSHR is affected by maternal smoking with the significant increase in FSH receptor (*FSHR*) transcript expression across the second trimester in control fetal ovaries (**C**) abolished by maternal smoking (**D**). Data are shown as mean ± SEM and *P*-values are derived from one-way ANOVA for differences in means. In (A) and (B), the data are derived from the 45 fetuses of the Endocrine group shown in Table I. In (C) and (D), linear regression fits (based on the 22 fetuses of the qPCR/PAH group shown in Table I) are shown by solid (control) or dashed (smoke-exposed) lines and 95% confidence limits by solid (control) and stippled (smoke-exposed) shading.

extraction (Baker and O'Shaughnessy, 2001). Ovarian RNA was extracted using TRIzol (Life Technologies, Paisley, UK). The real-time PCR approach utilized the SYBR green method using a Stratagene MX3000 cycler. The quantity of each measured cDNA from the real-time PCR was expressed relative to the standard luciferase cDNA in the same sample. Transcripts were selected from our own gene array study of primordial follicle formation (Fowler *et al.*, 2009a) and from the literature.

Data analysis

Statistical analyses of data were performed using JMP 9.0.2 software (Thomas Learning, London, UK). Normality of data distribution was tested with the Shapiro–Wilk test and non-normally distributed data were log-transformed and re-checked for normality prior to analysis by analysis of variance (ANOVA) and Tukey-Kramer honest significant difference test. Care was taken to ensure that groups were balanced in terms of fetal age. Because most of the parameters investigated showed developmental changes in expression across the second trimester, two-way ANOVA was used to test the combined effects of gestational age (weeks) and maternal smoking (yes/no) on morphological and biochemical data and gene expression levels. A statistically significant increase in variation in the smoke-exposed group and a reduced correlation between transcript levels and weeks of gestation (two-way ANOVA: interaction between smoking and weeks of gestation, $P < 0.05$) was observed for four transcripts. This statistically significant disturbance of transcript expression patterns across the second trimester is described as dysregulation. Levene's test was used to probe the significance of differences in variances between control and smoke-exposed groups.

Bioinformatics

Following on from our previous study (Anderson *et al.*, 2014) showing AHR expression in the late first and second trimester human fetal ovary and AHR-induced reduction in late first trimester human fetal ovary, we utilized a combined literature search and IPA pathway analysis strategy to look for further AHR target genes. Data mining and bioinformatics were carried out using the University of California and Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/index.html). Analysis was carried out using the Human Mar. 2006 (NCBI36/hg18) assembly. Highlighted binding sites have been conserved between humans and rodents (75 million years of divergent evolution) and calculated using the Transfac Matrix Database (v7.0) set at its most stringent setting (*Z* score cut-off = 2.33).

Results

Maternal cigarette smoking does not significantly affect gross female fetal morphology

There were no significant morphological differences between control and smoke-exposed groups and no statistically significant interaction

between weeks of gestation and maternal cigarette smoking (Table I). Neither fetal cotinine, nor cigarettes smoked per day (reported by the mother), correlated significantly with fetal weight ($r = 0.106$, $P = 0.301$; $r = -0.063$, $P = 0.563$, respectively) or crown-rump length ($r = 0.160$, $P = 0.191$; $r = -0.042$, $P = 0.70$, respectively) or ovary weight ($r = -0.001$, $P = 0.991$; $r = -0.040$, $P = 0.711$, respectively).

Maternal cigarette smoking disturbs female fetal endocrine signalling

Both estrone (E1) and estradiol (E2) concentrations were extremely high in the fetal circulation and were further increased (2- to 3-fold) by maternal smoking (Fig. 1A, Table I). In cultured ovaries (Fig. 1B) of fetuses from non-smoking women, E1 release was significantly less than E2 release, with an E1:E2 ratio (0.35 ± 0.11) much lower than that in the fetal circulation, where E1 was dominant (E1:E2 ratio = 2.0 in control and 2.9 in smoke-exposed fetuses). Mean levels of transcripts encoding the estrogen receptor 2 (*ESR2*) were not affected by maternal smoking (Table II) but separate analysis of active and dominant-negative *ESR2* isoform transcripts (Table II, Fig. 1C) showed that maternal smoking altered the ratio of active/dominant negative transcripts so that variance was significantly altered (Levene's test, $P = 0.022$). Within the smoke-exposed group, five fetuses showed a marked shift in balance between the two types of isoforms, favouring expression of transcripts encoding the active receptor isoform. Putative estrogen detection may also occur through GPER, although most ovaries did not have detectable levels of transcripts (Fig. 1D, Table II). Expression of *STS* (Fig. 1E) in the fetal ovary increased across the second trimester showing that the ovary can probably de-conjugate estrogens although there was no effect of maternal smoking (Table II). Maternal smoking was, however, associated with a significant 1.7-fold increase in the intra-ovarian Inhibin βA/inhibin α ratio (*INHBA/INHA*) transcript ratio (Fig. 1F), which would tend to shift the balance from inhibin towards activin signalling.

Circulating LH and FSH (Fig. 2A) significantly increased across the second trimester but PRL (Fig. 2B) showed no trend and intact hCG decreased (Fig. 2B). Maternal smoking did not significantly affect fetal LH, FSH, PRL or hCG (Table I) or endocrine receptor transcripts (Tables I and II). The statistically significant increase in FSH receptor (*FSHR*) across the second trimester seen in control ovaries (Fig. 2C) was, however, not observed in smoke-exposed ovaries (Fig. 2D).

Maternal cigarette smoking disturbs oocyte/follicle progression in the second trimester human fetal ovary

In ovaries from fetuses averaging 18 weeks of gestation (see Supplementary data, Table SII) there was an increase in primordial follicle number in smoke-exposed fetuses (Fig. 3A). In the same group there was also an increase in DDX4+ve oocyte number although there was no smoke exposure effect on the numbers of POU5F1+ve oocytes between the two groups (Fig. 3C). MVP in the human fetal ovary is oocyte-specific and both MVP transcript and protein increased markedly across the second trimester (13.4-fold from 11–3 to 17–21 weeks of gestation: Fig. 3E and F). However, transcript levels and the number of MVP+ve oocytes were not significantly affected by maternal smoking (Table II, Fig. 3G).

Maternal cigarette smoking dysregulates key fetal ovarian transcripts

Most of the 58 ovarian transcripts investigated (Table II) showed no statistically significant effect of maternal smoking (e.g. follistatin [*FST*]). Considerable inter-fetus variation precluded statistical significance for some transcripts with large differences between means (e.g. *POU5F1*). Four fetal ovarian transcripts (cytochrome P450 scc [*CYP11A1*], homeobox protein NOBOX [*NOBOX*], *HRK*, *NR2E1*), however, showed significant dysregulation with smoke exposure (Fig. 4). The dysregulation manifested as a statistically significant increase in variation in the smoke-exposed group and a reduced correlation between transcript levels and weeks of gestation (2-way ANOVA: interaction between smoking and weeks of gestation, $P < 0.05$). No statistically significant dysregulation was observed for other ovarian transcripts or any other measures from either the whole population or subpopulations detailed in Table I (e.g. body weight, hormone profiles). This indicates that the smoke exposure-associated disturbance of these four transcripts is not due to sample selection or effects on fetal growth rates. *NR2E1* ovarian transcript levels (Fig. 4E) were also significantly higher in smoke-exposed fetuses <16 weeks old. NR2E1 protein was localized to somatic cell nuclei and was absent from most germ cells. Most pre-granulosa cells in primordial follicles showed strong NR2E1 staining, which was also seen in many somatic cells around nests. Mesenchymal cell streams were mostly NR2E1 negative (Fig. 4F). HRK protein was localized to scattered germ cells, especially those in nests while those enclosed in primordial follicles were mostly HRK-negative (Fig. 4D).

Maternal cigarette smoking increases female fetal liver PAH content

Overall liver PAHs were significantly higher in smoke-exposed livers than control livers (5.8-fold, $P = 0.011$, Table III). Pyrene, benzo[k]fluoranthene and benzo[a]pyrene were only detectable in smoke-exposed fetuses while acenaphthalene was detectable only in controls. Benzo[a]anthracene concentrations showed no change as a result of maternal smoking, while chrysene was 323-fold higher in control livers although extremely variable between fetuses. Of the 10 PAHs that were detectable in both groups, 8 were between 1.3-fold and 35.8-fold higher in smoke-exposed fetuses, although only fluorene (11.2-fold, $P = 0.021$) and phenanthrene (6.5-fold, $P = 0.025$) reached statistical significance.

NR2F1, an AHR target gene, is a candidate mediator the effects of maternal smoking on the fetal ovary

Fifteen AHR target genes were further investigated, based on known or possible roles in ovarian development and xenochemical detection or metabolism (Table IV). *NR2F1* was significantly dysregulated ($P = 0.021$) and increased ($P = 0.026$) in smoke-exposed fetuses (Fig. 5A, Table IV). The closely related *NR2F2* was not affected by maternal smoking (Fig. 5B). Expression of NR2F2 was nuclear in ovarian somatic cells and testis Leydig and peritubular myoid cells (labelled inset, Fig. 5C and D), but the protein was also located in the cytoplasm of some oocytes earlier in gestation and then also in the nuclei of some oocytes at later stages. The major band recognized by the NR2F1

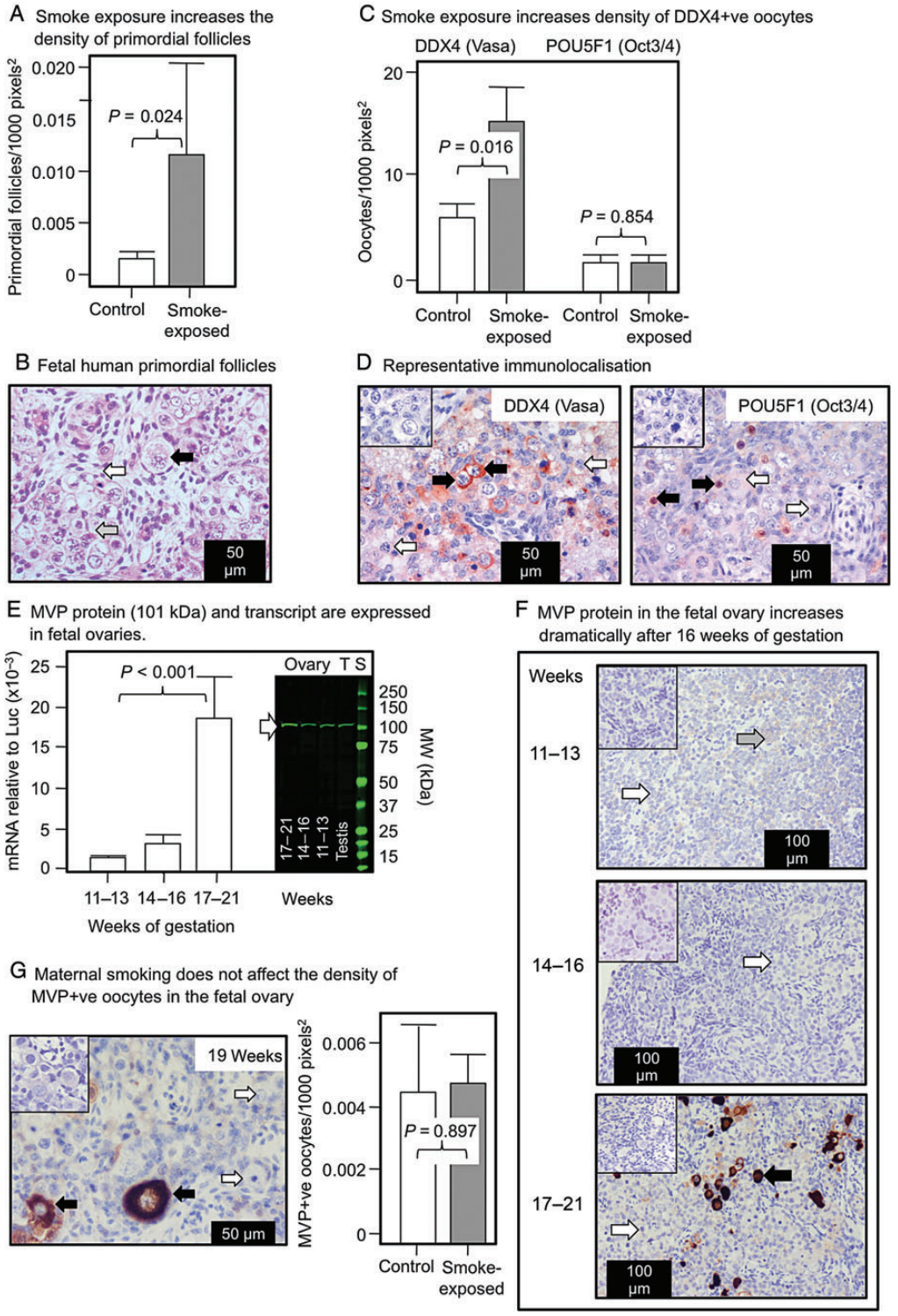

Figure 3 Maternal cigarette smoking affects fetal human ovarian germ cell development at 17–21 weeks of gestation. Smoke-exposed fetal ovaries have a higher density of primordial follicles (**A**, **B**) as well as an increased density of DEAD box protein 4 (DDX4)+ve oocytes (**C**). In contrast, neither POU domain, class 1, transcription factor 1 (POU5F1)+ve (C) nor major vault protein (MVP)+ve (**G**) oocyte densities were affected by maternal smoking. MVP expression increases sharply across the second trimester (**E**, **F**). MVP was localized to oocyte cytoplasm (F, G) although expression prior to 17–21 weeks was very infrequent and faint. Even at 19 weeks and later there were many MVP-ve oocytes visible. In (**D**), (F) and (G), the stain is DAB (brown) and counterstain is haematoxylin, visualized by brightfield view. The inset panels show the absence of staining in IgG-negative (non-immune serum) controls. In (B) the black arrow highlights a primordial follicle, the white arrow a mesenchymal cell stream and the grey arrow an oocyte nest while in (D), (F) and (G), black arrows denote +ve staining, white arrows −ve stain.

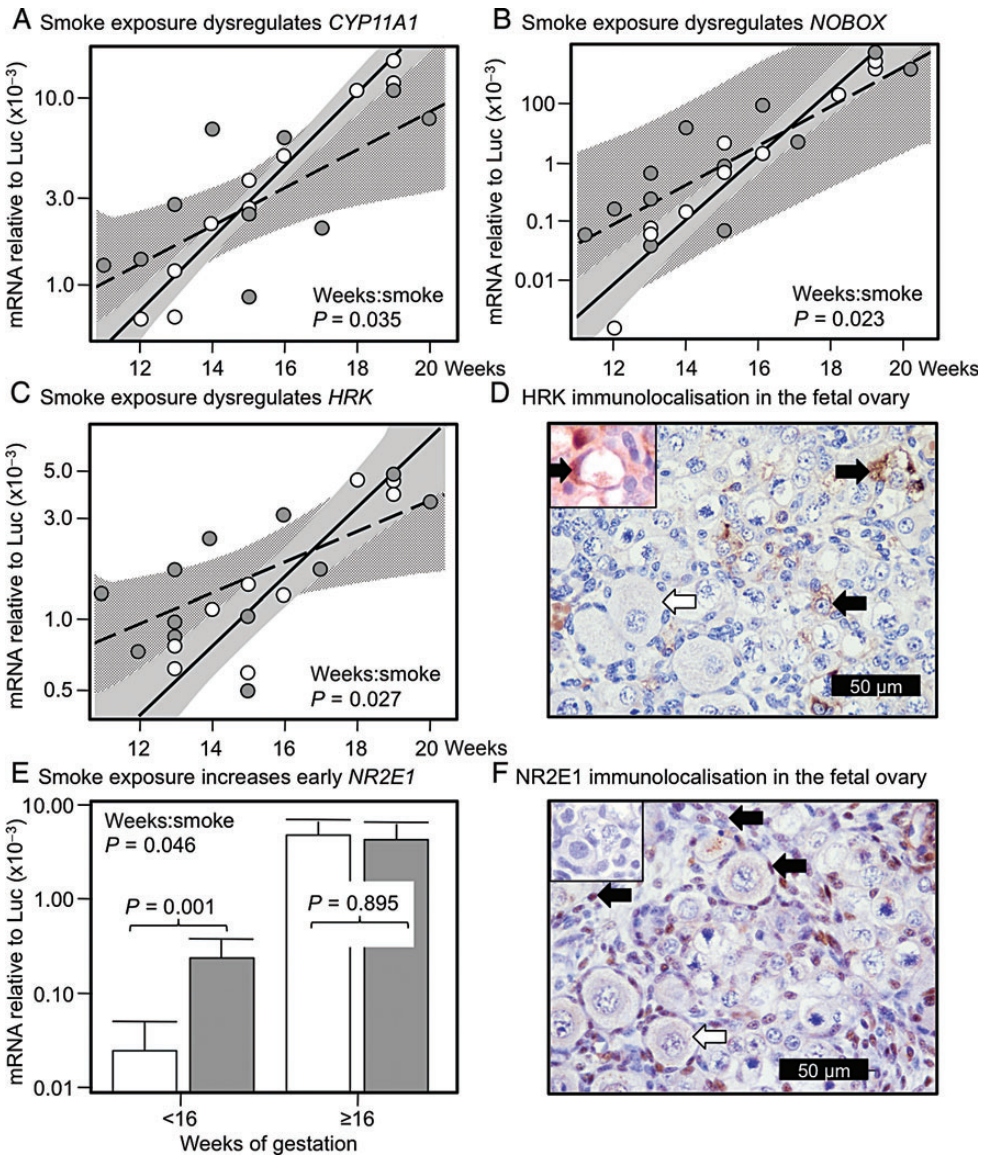

Figure 4 *In utero* smoke exposure dysregulates expression of key ovarian transcripts. Dysregulation is defined as a statistically significant increase in variation in the smoke-exposed group and a reduced correlation between transcript levels and weeks of gestation (assessed by two-way ANOVA: interaction between smoking and weeks of gestation, $P < 0.05$): (**A**) cytochrome P450 scc [*CYP11A1*] (controls: log *CYP11A1* $= -6.03 + 0.47$*weeks, $P < 0.001$; smoke-exposed: log *CYP11A1* $= -2.65 + 0.25$*weeks, $P = 0.013$), (**B**) homeobox protein NOBOX [*NOBOX*] (controls: log *NOBOX* $= -20.28$ - + 1.30*weeks, $P < 0.001$; smoke-exposed: log *NOBOX* $= -11.49 + 0.78$*weeks, $P = 0.013$), (**C**) activator of apoptosis harakiri [*HRK*] (controls: log *HRK* $= -5.71 + 0.38$*weeks, $P < 0.001$; smoke-exposed: log *HRK* $= -2.13 + 0.17$*weeks, $P = 0.021$), (**E**) nuclear receptor subfamily 2 group E member 1 [*NR2E1*] dysregulation (controls: log *NR2E1* $= 2.05 + 0.18$*weeks, $P = 0.008$; smoke-exposed: log *NR2E1* $= 2.28 + 0.16$*weeks, $P = 0.022$) is very similar to that shown for *HRK* and therefore not shown. However, younger fetuses demonstrate clear smoke-induction of *NR2E1*, with histograms and error bars denoting mean ± SEM. In (A)–(C) linear regression fits are shown by solid (control) or dashed (smoke-exposed) lines and 95% confidence limits by solid (control) and stippled (smoke-exposed) shading. Controls are shown by white circles/bars, smoke-exposed by grey circles/bars. (**D**) HRK was localized to scattered germ cells while most germ cells in primordial follicles were HRK-negative. HRK was also localized in some somatic cells, especially pre-granulosa cells associated with primordial follicles (inset). (**F**) NR2E1 was localized to somatic cell nuclei and absent from germ cells. Most pre-granulosa cells in primordial follicles showed strong NR2E1 staining as did many somatic cells around nests. In (D) and (F) the stain is DAB (brown) and counterstain is haematoxylin, visualized by brightfield view. The inset panels show the absence of staining in IgG-negative (non-immune serum) controls. Black arrows denote +ve staining, white arrows −ve stain.

antibody on a western blot (Fig. 5D) was at 46 kDa as expected. Of the other two major xenosensors, nuclear receptor subfamily 1 group I member 2 (*NR1I2* [*PXR*]) transcript was detected in most ovaries and unaffected by maternal smoking while nuclear receptor subfamily 1 group I member 3 (*NR1I3* [*CAR*] was detected in a high proportion of control ovaries (Fig. 5E, Table IV).

Table IV Effects of maternal cigarette smoking on the mRNA transcript levels of selected AHR responsive/target genes and/or matching binding sites on genes identified in Table II and Fig. 4 as dysregulated by maternal smoking (cytochrome P450 scc [CYP11A1], NOBOX oogenesis homeobox [NOBOX], activator of apoptosis harakiri [HRK], nuclear receptor subfamily 2, group E, member 1 [NR2E1]) in the human female fetal ovary (mean ± SEM, based on the qPCR/ PAH group shown in Tables I and II).

Gene symbol	Gene product	Control[a]	Smoke-exposed[a]	Two-way ANOVA	
				Smoking	Weeks–smoke interaction
		n = 10	*n* = 12	*P*[b]	*P*[b]
1. *UGT1A*	UDP-glucuronosyltransferase 1-1	0.032 ± 0.013	0.033 ± 0.015	0.975	0.970
2. *NQO1*	NAD(*P*)H dehydrogenase [quinone] 1	1.772 ± 0.442	1.650 ± 0.476	0.384	0.191
3. *ESRRG*	Estrogen-related receptor gamma	1.920 ± 0.462	1.903 ± 0.523	0.923	0.399
4. *CTNNB1*	Catenin beta-1	166.9 ± 53.74	121.6 ± 34.00	0.667	0.222
5. *CDKN1B*	Cyclin-dependent kinase inhibitor 1B (p27Kip1)	22.56 ± 8.702	16.73 ± 5.942	0.365	0.423
6. *SMAD4*	Mothers against decapentaplegic homolog 4	64.50 ± 19.70	59.69 ± 18.67	0.936	0.351
7. *POR*	NADPH–cytochrome P450 reductase	9.768 ± 2.723	8.800 ± 2.266	0.825	0.281
8. *CYP1A1*	Cytochrome P450 1A1	0.115 ± 0.045	0.076 ± 0.022	0.743	0.656
9. ***CAR***[c]	Nuclear receptor subfamily 1 group I member 3 *(NR1I3)*	0.075 ± 0.041	0.005 ± 0.005	**0.031**[c]	NA
10. *PXR*	Nuclear receptor subfamily 1 group I member 2 *(NR1I2)*	0.269 ± 0.084	0.257 ± 0.087	0.650	0.799
11. ***NR2F1***	**COUP transcription factor 1**	**7.375 ± 2.544**	**8.467 ± 1.843**	**0.026**	**0.021**
12. *NR2F2*	COUP transcription factor 2	153.1 ± 30.06	131.1 ± 26.54	0.613	0.789
13. *REST*	RE1-silencing transcription factor *(NRSF)*	76.69 ± 20.87	65.52 ± 19.97	0.802	0.344
14. *FOXP3*	Forkhead box protein P3	2.263 ± 0.917	1.235 ± 0.448	0.406	0.444
15. *MYC*	Myc proto-oncogene protein	22.75 ± 6.007	19.46 ± 3.975	0.872	0.307

[a]qPCR is expressed as mRNA relative to luciferase.
[b]Statistically significant *P*-values are shown in bold. Where relevant, multi-function genes are categorized according to their function most appropriate to ovarian development.
[c]Transcript expression below limit of detection in ≥50% of ovaries.

We also interrogated the UCSC Genome Bioinformatics Website to trawl for AHR-related transcription factor binding sites (Table V) and found that of the maternal-smoking dysregulated genes *CYP11A1* had a conserved AHR/ARNT binding site, as did *FSHR* and *NR2F1*. Furthermore, *NR2E1*, *CYP11A1* and *FSHR* all had NR2F1 binding sites.

Discussion

This extensive study shows that exposure to the complex mixture of chemicals in cigarette smoke disturbs human fetal ovarian development *in utero* and increases the fetal burden of AHR-activating chemicals. The significance of this is highlighted by our demonstration that *in vitro* activation of AHR reduces human fetal ovarian germ cell proliferation. We also show that the female human fetus is normally exposed to very high levels of endogenous estrogens, which markedly increase if the mother smokes. Overall, our findings suggest, for the first time, that prenatal maternal cigarette smoking induces AHR activation, dysregulates genes important in ovarian development (including inhibin/activin and FSH signalling), reduces germ cell proliferation and elevates estrogen exposure in the human. We propose that these changes probably contribute significantly to the observed reduced fecundity in women exposed to cigarettes prenatally (Ye *et al.*, 2010).

Our data on maternal smoking effects on primordial follicle number and number of DDX but not POU5F1 positive oocytes clearly support the conclusion that primordial follicle formation is dysregulated in smoke-exposed fetuses. Nevertheless, the relatively small sample size of five ovaries/group and low number of primordial follicles in the population available means that this finding needs to be confirmed in a larger study, especially one using full stereological techniques to count oocytes and follicles. However, it must be noted that the data are internally consistent with increased primordial follicle number associated with increased DDX4 positive oocytes, which would be expected (Anderson *et al.*, 2007). Previous studies suggest that cigarette smoke PAHs, which are AHR ligands, disturb ovarian development (Jurisicova *et al.*, 2007; Tuttle *et al.*, 2009) and here we report significantly increased PAH concentrations in livers of female fetuses exposed to maternal cigarettes. Numerous reports exist of adverse effects of AHR ligands on reproductive development and function (e.g. (Coutts *et al.*, 2007; Sobinoff *et al.*, 2011)) and the trend for dysregulated *AHR:ARNT* transcript ratios in the smoke-exposed fetal ovaries (Table II, $P = 0.057$) suggests alteration in AHR signalling. Further studies are required, however, to relate agonist bioactivity to levels in human fetuses. In the female human fetus, cigarette exposure is linked to decreased germ cell and somatic cell numbers (Lutterodt *et al.*, 2009; Mamsen *et al.*, 2010). Furthermore, the reduced germ cell proliferation we previously reported in fetal ovaries

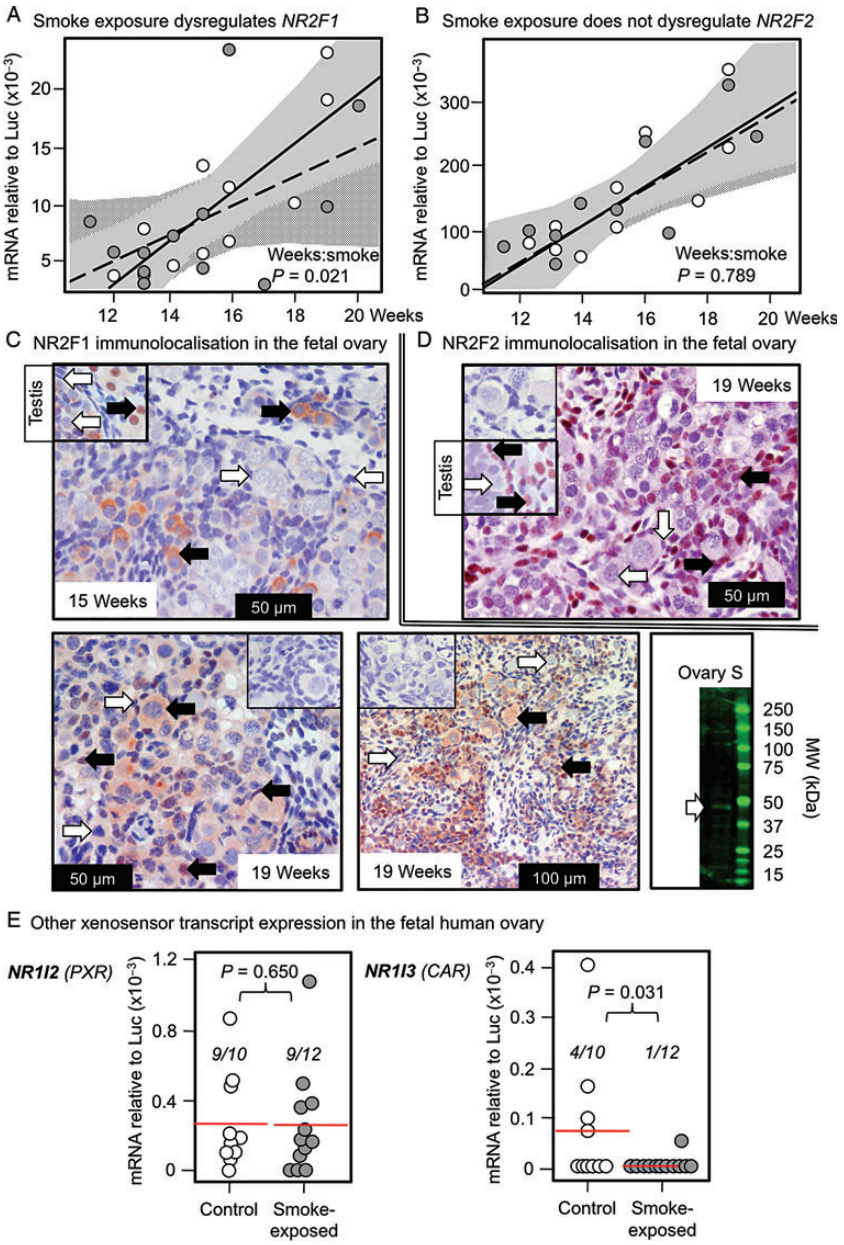

Figure 5 Maternal smoking dysregulates COUP transcription factor I (*NR2F1* [*COUP-TF1*]) but not COUP transcription factor 2 (*NR2F2* [*COUP-TF2*]) expression in the fetal ovary. Dysregulation is defined as a statistically significant increase in variation in the smoke-exposed group and a reduced correlation between transcript levels and weeks of gestation (assessed by two-way ANOVA: interaction between smoking and weeks of gestation, $P < 0.05$): (**A**) *NR2F1* (controls: log *NR2F1* $= -5.35 + 0.43$*weeks, $P = 0.006$; smoke-exposed: log *NR2F1* $= 0.33 + 0.11$*weeks, $P = 0.147$), (**B**) *NR2F2* (controls: log *NR2F2* $= 2.05 + 0.18$*weeks, $P = 0.008$; smoke-exposed: log *NR2F2* $= 2.28 + 0.16$*weeks, $P = 0.022$). In (A) and (B) linear regression fits are shown by solid (control) or dashed (smoke-exposed) lines and 95% confidence limits by solid (control) and stippled (smoke-exposed) shading. Controls are shown by white circles/bars, smoke-exposed by grey circles/bars. NR2F1 was localized (**C**) to the cytoplasm of some oocytes and the nuclei of scattered somatic cells earlier in the second trimester but later was also expressed in the nuclei of some oocytes and in some somatic cells, including some pre-granulosa cells. Many mesenchymal cell streams were NR2F1 −ve. In the testis NR2F1 was localized predominantly to Leydig cell nuclei. NR2F2 (**D**) was localized to the nuclei of many somatic cells, including mesenchymal cell stream nuclei, and also to smaller oocytes. NR2F2 was also localized in some pre-granulosa cell nuclei and predominantly in Leydig and peritubular myoid cell nuclei in the testis. In (C) and (D) the stain is DAB (brown) and counterstain is haematoxylin, visualized by brightfield view. The inset panels show the absence of staining in IgG-negative (non-immune serum) controls or, if labelled 'Testis', show matching expression in the second trimester human fetal testis. Black arrows denote +ve staining, white arrows −ve stain. The xenosensor nuclear receptor subfamily 1 group I member 2 (*NR1I2* [*PXR*]) (E) was detectable in most ovaries examined but was not affected by maternal smoking. In contrast the xenosensor nuclear receptor subfamily 1 group I member 3 (*NR1I3* [*CAR*]) (E) was detectable in few ovaries and was significantly reduced by maternal smoking.

Table V Coordinates, DNA methylation status, polymorphic status and location relative to coding sequence of predicted highly conserved (vertebrates) transcription factor binding sites from UCSC browser (NCB136/hg18).

Gene and coordinates	Conserved TF binding sites	Coordinates	CpG	Methyl	SNP?	Position 5′, 3′ coding or intronic
NR2E1 chr6:108593955-108616706	NR2F1 (COUP-TF1)	chr6:108592946-108592959	No	No	No	1009 bp 5′ of NR2E1 TSS (may also lie within Intron 5 of flanking OSTM1 gene). Nested within PPARG site below.
	PPARG	chr6:108592942-108592962	No	No	No	Overlaps COUP-1 above.
		chr6:108593899-108593921	Yes	ND	rs2233486	At transcriptional start site of NR2E1 gene.
CYP11A1 chr15:72424166-72447134	AHR ARNT.	chr15:72419098-7419113	Yes	Yes	No	Exon 4 of CYP11A1 gene. 12 base pairs from TCF11 binding site.
	NR2F1 (COUP-TF1)	chr15:72412478-72412491	No	No	No	5068 bp of CYP11A1 gene or within intron 16 of flanking CCDC33 gene.
NOBOX chr7:143726972-143738253	PPARG	chr7:143721185-143721207	No	No	No	5742 3′ of NOBOX gene
HRK chr12:115783410-115803615	TAL1A and TAL1B,	chr12:115801860-115801875	Yes	No	No	Highly conserved non-coding region in HRK intron 1
	NRSF	chr12:115803362-115803382	Yes	Yes	No	HRK Exon 1
	PAX6 and	chr12:115805441-115805461	Yes	Yes	No	Highly conserved region 5′ of HRK TSS
	TCF3 (E47)	chr12:115803257-115803,71	Yes	No		Within Exon 1 of HRK gene
FSHR chr2:49043779-49236657	NR2F1 (COUP-TF1)	chr2:48974670-48974683	No	No	No	Overlaps PPARG and lies 69 kb from FSHR 3′ end.
	ARNT	chr2:49235172-49235191	No	No	No	Within highly conserved region 30 bp from FSHR TSS
NR2F1 chr5:92944799-92955542	AHR/ARNT	chr5:92955089-92955107	Yes	Yes	No	Within Last Exon of NR2F1 gene.
	NR2F1 (COUP-TF1)	chr5:92944637-92944650	No	No	No	149 bp from NR2F1 TSS within PPARG site.

TF, transcription factor; CpG, site of DNA methylation in TF binding site; Methyl, CpG is methylated in cortical neurones derived from 57-year-old male; SNP, TF site contains a single nucleotide polymorphism; TSS, transcriptional start site; UTR, untranslated region; ND, no data.

cultured with an AHR-activating ligand (Anderson *et al.*, 2014) would be a key component in disturbing the germ/somatic cell balance that underpins appropriate primordial follicle formation in the developing ovary.

Levels of fetal circulating estrogens measured in the present study were very high and markedly greater than those reported previously (Shutt *et al.*, 1974; Smith *et al.*, 1975). The reason for this discrepancy is not clear but the early studies used an indirect competitive protein binding assay method that does not give information on the analyte identity. In their detailed review of more recent studies Kuijper *et al.* (2013) assembled data from a number of studies reporting similarly high female fetal estrone and estradiol levels, supporting our findings reported here. The fetal estrogen levels reported here are also markedly higher than those in the maternal circulation (Holl *et al.*, 2009). During pregnancy the placenta is the main source of estrogens and differences between fetal and maternal estrogen levels are probably due to concentration effects of placental estrogens passing into the much smaller fetal blood volume. In pregnant women estrogen levels are decreased and androgen levels are increased by smoking (Bernstein *et al.*, 1989; Toriola *et al.*, 2011). The different response to smoking in the fetus is probably related to altered steroid metabolism by the mother, placenta and fetus. Increased fetal estrogen is likely to be due to increased placental production, conjugation and protein binding in the fetus. In addition, human fetal ovarian and hepatic cytochrome P450 aromatase (CYP19A1) (Fowler *et al.*, 2009a, 2011; O'Shaughnessy *et al.*, 2013), which converts androgen to estrogen, will probably contribute to elevated estrogens in smoke-exposed fetuses. Our data shown here, combined with the steroid machinery reported in Fowler *et al.* (2009a, 2011), strongly suggest that the fetal ovary itself may be a major contributor to intra-ovarian estrogen titres.

The increased *INHBA*:*INHA* sub-unit ratio in smoke-exposed ovaries would be expected to increase activin A, which is expressed in germ cells immediately prior to follicle formation (Martins da Silva *et al.*, 2004). Indeed, administration of activin to neonatal mice during the period of nest breakdown and primordial follicle formation significantly increases the number of primordial follicles formed (Bristol-Gould *et al.*, 2006). Activin A suppresses Kit ligand (KITLG) expression in adjacent somatic cells, part of the system regulating the timing of follicle formation (Coutts *et al.*, 2008; Childs and Anderson, 2009), thus providing a mechanism for an effect on subsequent female fertility. The absence of changes in circulating gonadotrophins in female fetuses exposed to maternal smoking agrees with male data (Fowler *et al.*, 2008, 2009b), although LH and FSH were higher in female than male fetuses (up to 4.8-fold, $P < 0.001$). The latter sex difference is likely to be due to negative feedback by testosterone in the male fetuses.

In many species E2 reduces primordial follicle formation but, in the human, the fetal ovary can produce high levels of E2 (present study) and primordial follicle formation occurs in the presence of high fetal and maternal estrogen levels. In some model species, FSH is required

for primordial follicle formation (e.g. hamsters (Roy and Albee, 2000; Wang and Roy, 2004)) and the high levels of FSH in the female fetal circulation reported here would be consistent with this mechanism in the human. While circulating fetal FSH is unaffected by maternal smoking, the pronounced trend for reduced *FSHR* expression (a similar effect of smoking was reported in a study published in Polish for granulosa cell *FSHR* in women (Josiak *et al.*, 2006)) and lack of increasing *FSHR* expression with gestational age (Table II, Fig. 2C and D) suggests that adverse effects of smoking on primordial follicle formation may occur via altered FSH signalling. In hamsters, FSH-regulated, ovary-produced E2 is critical for germ and somatic cell survival and differentiation, thereby underpinning primordial follicle formation, but this effect is lost when E2 levels rise beyond a threshold (Wang and Roy, 2007). Since *in utero* exposure of women to diethylstilbestrol (DES) is associated with an increased risk of infertility and early menopause (Hoover *et al.*, 2011), high circulating and intra-ovarian estrogens, together with a reduced proportion of ovarian dominant-negative *ESR2* isoforms (in nearly half of smoke-exposed fetuses reported here), support the hypothesis that excessive estrogenic signalling will impair human fetal folliculogenesis. The fetal baboon, like the human (Fowler *et al.*, 2011), also expresses *ESR2* in fetal oocytes (Bocca *et al.*, 2008) and, using experimental approaches impossible in the human, multiple roles of estrogen in the fetal baboon ovary have been identified. These include the regulation of α-inhibin (Billiar *et al.*, 2003), folliculogenesis (Zachos *et al.*, 2002; Billiar *et al.*, 2003; Pepe *et al.*, 2006), *FSHR* expression and oocyte proliferation (Zachos *et al.*, 2003; Burch *et al.*, 2009). Furthermore, estrogen deprivation does not alter levels of mothers against decapentaplegic homolog (SMAD)-transducing activin signalling, suggesting that estrogen regulates fetal baboon folliculogenesis by modulating the intra-ovarian activin:inhibin ratio (Billiar *et al.*, 2004). This is identical to one of the effects reported here for maternal smoking: a significant alteration in activin:inhibin transcript ratio. Such an alteration might be expected to exert negative effects on human follicle formation (Coutts *et al.*, 2008; Childs and Anderson, 2009). We had previously observed a decrease in MVP protein in fetal sheep exposed to a complex cocktail of environmental chemicals (Bellingham *et al.*, 2013) and therefore investigated MVP in the human fetal ovary. However, transcript levels and the number of MVP+ve oocytes were not significantly affected by maternal smoking, suggesting differences in the affected ovarian pathways between the human and the sheep model.

Smoke exposure dysregulated expression of four fetal ovarian transcripts (*CYP11A1, HRK, NR2E1, NOBOX*), each of which may play a role in normal follicle formation and development. Dysregulation of *CYP11A1* (which is localized in oocytes (Fowler *et al.*, 2011)), for example, suggests smoke exposure will affect ovarian steroidogenesis. Similarly, PAH exposure negatively affects the primordial follicle pool in mice by inducing HRK in the presence of apoptosis regulator Bax (BAX) (Matikainen *et al.*, 2001; Jurisicova *et al.*, 2007) and HRK is mostly expressed in the oocytes and pre-granulosa cells associated with primordial follicles in the fetal human ovary. Dysregulation and increased expression of the transcriptional repressor *NR2E1* in <16-week-old human fetal ovaries exposed to cigarettes (present study) may be related to the induction of *POU5F1* expression (transcript levels nearly doubled by maternal smoking, Table II) through activation of AKT signalling (Chavali *et al.*, 2011). This is important since the AHR ligand 7,12-Dimethylbenz(a)anthracene (DMBA) causes ovotoxicity in mice by activating PI3K/AKT and mTOR signalling pathways (Sobinoff *et al.*, 2011) and maintenance of POU5F1 in fetal germ cells would disrupt primordial follicle formation (Anderson *et al.*, 2007). Deficits in the oocyte-specific transcript NOBOX leads to impaired somatic-germ cell communication and somatic cell invasion during ovarian development (Lechowska *et al.*, 2011). Dysregulated *NOBOX* would be expected, therefore, to contribute to impaired germ cell nest breakdown and thereby alter primordial follicle formation.

Dysregulation of the AHR-responsive transcription factor *NR2F1* and the presence of highly conserved AHR/ARNT and/or NR2F1 binding sites on the genes for *NR2E1, CYP11A1* and *FSHR* are highly suggestive of adverse consequences of AHR activation on the human fetal ovary through several pathways. Firstly, *NR2F1* (and *NR2F2)* is a repressor of *FSHR* in gonadal cells (Xing *et al.*, 2002). The cytoplasmic as well as nuclear expression of NR2F1 has previously been reported (Tang *et al.*, 2005). In contrast, the related transcription factor *NR2F2*, which has been proposed as part of the mechanism leading to masculinization disorders (van den Driesche *et al.*, 2012), was unaffected in the human fetal ovary. Dysregulated *NR2F1* expression and AHR activation, as a result of maternal smoking, may therefore contribute to the disconnection between gestational stage and *FSHR* expression. Secondly, the trend towards increased *NR2F1* may also disturb retinoic acid signalling (Zhuang and Gudas, 2008), with potential disruption of the coordination of meiosis entry, which is important in the fetal ovary (Childs *et al.*, 2011). Lastly there is also evidence linking NR2E1, NR2F1, FSH and the developmental/transcription regulator B-cell CLL/lymphoma 11A (zinc finger protein) (BCL11A), with the pathways considered important in oocyte-granulosa cell interactions (Perlman *et al.*, 2006; Chan *et al.*, 2013).

Based on a synthesis of our data and the supporting literature we propose a model (Fig. 6) in which disturbed coordination of a complex sequence of events from nest breakdown to primordial follicle formation, via several pathways, will impair ovarian development of some, but not all, fetuses. Increased estrogen and activin signalling and AHR-activating ligands (e.g. PAHs) and reduced FSH signalling would be expected to alter the coordination of pathways leading to primordial follicle formation. The disturbed balance between proliferation and apoptosis is likely to contribute to reduced numbers of germ and somatic cells and impact negatively on primordial follicle formation. Dysregulation of CYP11A1 would be expected to affect the pattern of steroidogenesis within forming primordial follicles. Altered expression of the somatic cell orphan receptor NR2E1 may increase transcription repression and activate AKT signalling prior to the start of primordial follicle formation. At the same time, dysregulated expression of NOBOX and a shift in the intra-ovarian inhibin/activin signalling balance in favour of activin A would probably disturb germ cell nest breakdown and the synchronization of primordial follicle formation. Early inhibition of AMH by elevated E2 might also disrupt the timing of primordial follicle formation (Grynberg *et al.*, 2012). The observation that maternal smoking alters specific transcript levels in some individuals and not others is likely to contribute to the changed incidence of reduced fertility: i.e. some individuals are affected but not all. Finally, activation of the AHR might modulate estrogen actions, potentially increasing anti-estrogenic effects of AHR ligands in cigarette smoke through ESR2 mediation of ARNT co-activation (Swedenborg and Pongratz, 2010). Overall, it is also likely that altered *NR2F1* plays an important role in these processes. These hypotheses are based on a relatively large number of samples but given the large individual variation in response to maternal

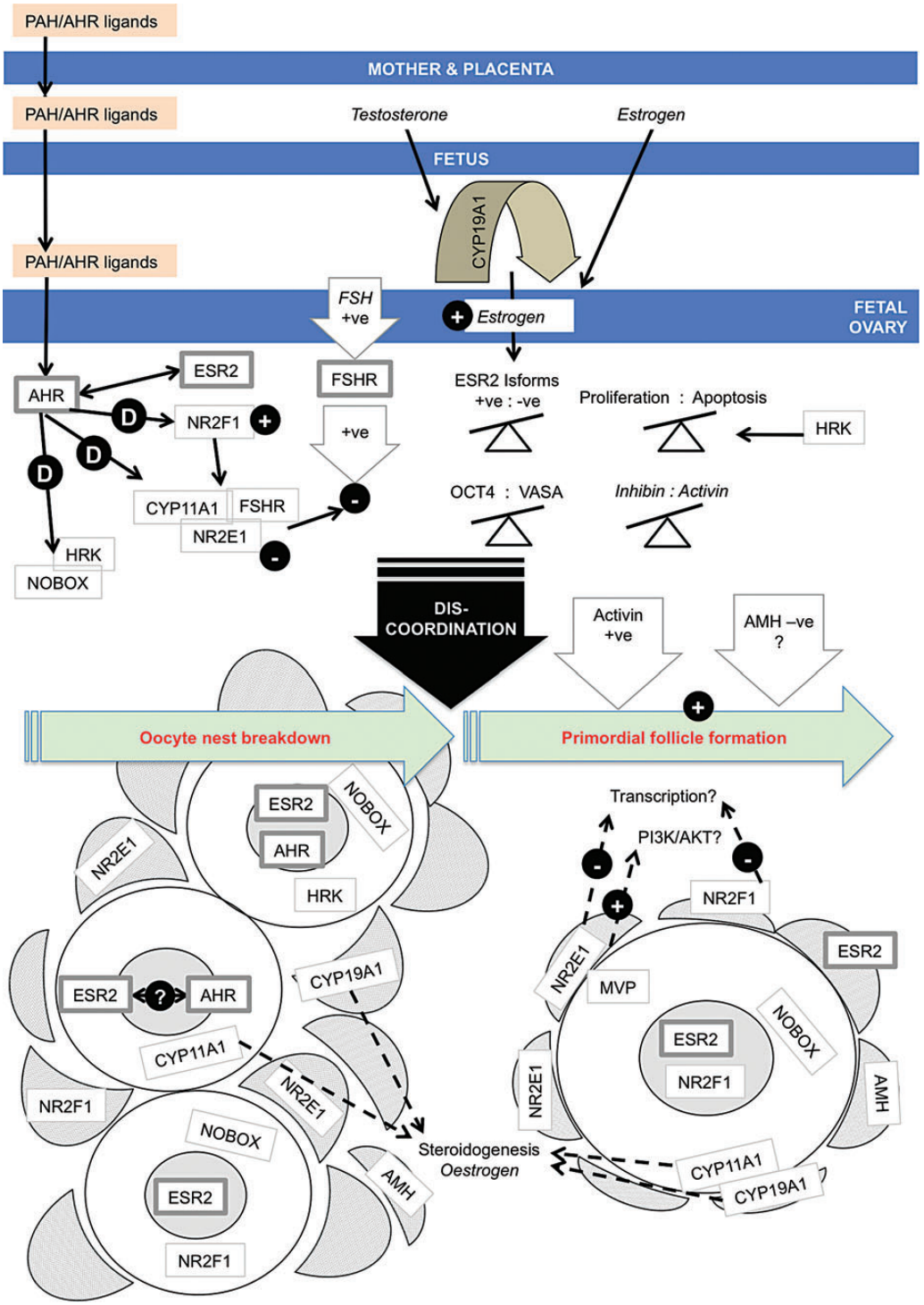

Figure 6 Diagram summarizing dysregulation of fetal ovarian development and primordial follicle formation by maternal cigarette smoking. Increased circulating estrogen in the presence of high intra-ovarian estradiol (E2) and aryl hydrocarbon receptor (AHR) activation would lead to reduced proliferation and dysregulation of activator of apoptosis harakiri (*HRK*)-mediated apoptosis. Disturbed nuclear receptor subfamily 2 group E member 1 (*NR2E1*) and COUP transcription factor 1 (*NR2F1*) may be expected to increase transcription repression and PI3K/ACT and mTOR signalling, especially prior to 16 weeks gestation. Dysregulated homeobox protein NOBOX (*NOBOX*) and FSH receptor (*FSHR*) is likely to affect germ cell nest breakdown while dysregulated germ cell *CYP11A1* may affect steroidogenesis. Increased activation of estrogen receptor β (ESR2) (partly by reduced relative dominant-negative *ESR2* isoforms in some smoke-exposed ovaries) may lower intra-ovarian anti-Mullerian hormone (AMH), which, together with a shift in intra-ovarian inhibin/activin signalling towards activin, would desynchronize primordial follicle formation.

smoking, they will need to be followed up by more detailed mechanistic studies with greater power.

In summary, these data provide a possible mechanistic explanation of how maternal cigarette smoking may negatively impact the human female fetus, explaining the link between maternal smoking and reduced fecundity in daughters. Maternal cigarette smoking during pregnancy causes significant dysregulation of key fetal ovarian genes and endocrine signalling during the second trimester, partly driven by an increase in the liver burden of AHR-activating ligands, PAHs.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

P.A.F.: conception, design, analysis, interpretation, drafting, revision and approval. A.J.C.: interpretation, drafting, revision and approval. F.C.: acquisition, analysis, revision and approval. A.Ma.: analysis, interpretation, drafting and approval. S.M.R.: design, acquisition, analysis, writing, revision and approval. J.-P.A.: acquisition, analysis, revision and approval. B.L.B.: acquisition, analysis, revision and approval. P.F.: acquisition, analysis, revision and approval. F.E.: acquisition, analysis, revision and approval. S.F.: acquisition, analysis, revision and approval. Ab.M.: conception, design, interpretation, drafting, revision and approval. S.B.: conception, design, drafting, revision and approval. An.M.: acquisition, analysis, revision and approval. R.A.A.: interpretation, drafting, revision and approval. P.J.O.S.: conception, design, analysis, interpretation, drafting, revision and approval.

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Conflict of interest

The authors declare they have no competing interests, be it financial, personal or professional.

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