Estradiol supports *in vitro* development of bovine early antral follicles

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Abstract

Antrum formation and estradiol (E₂) secretion are specific features of oocyte and granulosa cell complexes (OGCs). This study investigates the effect of E₂ on the *in vitro* development of bovine OGCs derived from early antral follicles as well as on the expression of genes in granulosa cells (GCs). The supplementation of culture medium with either E₂ or androstenedione (A₄) improved the *in vitro* development of OGCs and the nuclear maturation of enclosed oocytes. When OGCs were cultured in medium containing A₄, developmentally competent OGCs secreted more E₂ than OGCs that were not competent. In addition, fulvestrant inhibited the effect of both E₂ and A₄ on OGCs development. Comprehensive gene expression analysis using next-generation sequence technology was conducted for the following three types of GCs: i) GCs of OGCs cultured for 4 days with E₂ (1 µg/ml; E₂(+)), ii) GCs of OGCs cultured for 4 days without E₂ (E₂(-)) or iii) OGCs that formed clear antrum after 8 days of *in vitro* culture in medium containing E₂ (1 µg/ml; AF group). GCs of the E₂(+) group had a similar gene expression profile to the profile reported previously for the *in vivo* development of large follicles. This genetic profile included factors implicated in the up-regulation of E₂ biosynthesis and down-regulation of cytoskeleton and extracellular matrices. In addition, a novel gene expression profile was found in the AF group. In conclusion, E₂ impacts the gene expression profile of GCs to support the *in vitro* development of OGCs.

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Introduction

Granulosa cells (GCs) that are derived from early antral follicles (EAFs) secrete estradiol (E₂) and are involved in antral formation. When oocyte and granulosa cell complexes (OGCs) are collected from bovine EAFs and cultured *in vitro*, granulosa cells (GCs) form antral-like cavities secrete E₂ into the culture medium. After 12–16 days in culture, oocytes grown *in vitro* acquire full developmental competence (Mingoti *et al.* 2002, Hirao *et al.* 2004). The relationship between E₂ in follicular fluid (FF) or in culture medium and oocyte competence has been reported (Xu *et al.* 2006, West-Farrell *et al.* 2009) in mice. However, studies on aromatase KO mice suggest that the significance of E₂ on *in vivo* oocyte development is limited (Huynh *et al.* 2004). The aromatase KO mouse has an ovulation defect, but the oocytes were shown to have full developmental competence *in vitro*. Estrogen receptor β KO mice also have defects in ovulation, differentiation of the GCs and follicle maturation (Couse *et al.* 2005, Emmen *et al.*

2005, Drummond 2006). To culture OGCs derived from EAFs *in vitro*, culture conditions have to maintain and/or develop functions of the GCs; however, the effect of E₂ on the *in vitro* development of OGCs and the functions of the granulosa cells, as well as the molecular basis underlying the beneficial effect of E₂, are not clearly understood.

The aim of this study is to examine the role of E₂ during the *in vitro* development of OGCs by answering the following questions: i) does E₂ directly improve the *in vitro* development of OGCs derived from EAFs; ii) does the ability of OGCs to secrete E₂ relate to the developmental competence of the OGCs and 3) what is the molecular basis associated with the beneficial effect of E₂ on the growth of OGCs?

Results

In Experiment 1, the ratio of antral cavity formation was very low at day 4 of the culture period. Subsequently, formation rapidly increased until day 8 (Table 1). When

Table 1 Effect of estradiol in culture medium on development of OGCs.

E_2 ($\mu\text{g/ml}$)	No. of trials	No. of OGCs	Rate (%) of OGCs with antrum, mean $\pm$ S.E.M.				Oocyte diameter		Maturation ratio M2/total
			4 Days	8 Days	12 Days	16 Days	<i>n</i>	Mean $\pm$ S.E.M. (μm)	
0	10	100	0.0*	5.0 $\pm$ 2.2*	6.0 $\pm$ 2.7*	7.0 $\pm$ 3.3*	6	115.4 $\pm$ 1.5	0/50 (0)*
0.1	10	100	1.0 $\pm$ 1.0*,†	34.8 $\pm$ 7.1†	37.7 $\pm$ 4.8†	42.7 $\pm$ 5.3†	17	118.6 $\pm$ 1.1	6/50 (12.0)†
1	10	100	5.0 $\pm$ 2.2*,†	38.0 $\pm$ 7.3†	41.0 $\pm$ 5.7†	36.0 $\pm$ 6.7†	22	117.3 $\pm$ 1.7	13/50 (26.0)*,†
10	10	100	8.0 $\pm$ 2.5†	49.0 $\pm$ 6.6†	55.0 $\pm$ 4.3†	56.0 $\pm$ 5.8†	31	122.2 $\pm$ 1.2	17/50 (34.0)†

*,† P <0.05.

OGCs were cultured in medium without E_2 supplementation, the rate of antral cavity formation was 7.0% and no oocytes reached the M2 stage. Supplementation of the culture medium with E_2 improved the cavity formation, and the highest ratio of antrum cavity formation was obtained with the medium containing 10 $\mu\text{g/ml}$ E_2 (56.0%). The average diameter of the *in vitro* grown oocytes was 122.2 μm . When the oocytes grown *in vitro* were cultured further for 23 h, ~30.0% of the oocytes reached M2 stage. Supplementation of the culture medium with 1 and 10 $\mu\text{g/ml}$ A_4 improved antrum formation (26.7%), and 7.8 and 6.7% of OGCs reached the M2 stage respectively (Table 2). When OGCs were cultured in medium containing 1 $\mu\text{g/ml}$ A_4 , the E_2 concentration in the medium of competent OGCs was 5.5 ± 0.9 ng/ml, which was significantly greater than that in un-competent OGCs (3.1 ± 0.5 ng/ml, P <0.05; Table 3). When the A_4 concentration was increased to 10 $\mu\text{g/ml}$ from 1 $\mu\text{g/ml}$, the E_2 concentration of the competent OGCs was significantly increased to 11.3 ng/ml, which was significantly greater than that of un-competent OGCs (4.5 ng/ml, P <0.05). There was a significant interaction observed between A_4 concentrations in the culture medium and E_2 concentrations secreted from OGCs.

Fulvestrant (1 $\mu\text{g/ml}$) completely inhibited OGC antrum formation of OGCs that were cultured with either 1 $\mu\text{g/ml}$ A_4 or 0.1 $\mu\text{g/ml}$ E_2 . The inhibitory effect of fulvestrant was ameliorated by increasing the concentration of E_2 from 0.1 to 1 $\mu\text{g/ml}$ (Table 4).

Table 5 lists genes that have been previously reported as markers of follicle cells for the largest follicle in cows (Hayashi *et al.* 2010). With a few exceptions, many of the genes that are reported to be highly expressed in the largest follicle tend to have their expression enhanced by E_2 treatment. The *FOS*, *SCD* and *FST* had slightly but significant low expression ratio in E_2 (+) group to those

seen in E_2 (-) group (-1.22, -1.04 and -1.04 respectively, P <0.01). When OGCs were cultured for 8 days, expression of *FOS* further decreased, while expression of *SCD* and *FST* increased in the AF group compared with the level observed in the E_2 (+) group (P <0.01). Conversely, the expressions of many genes that are reported to be highly expressed in the second largest follicle were depressed by E_2 treatment. Continued *in vitro* culture showed that the expression of *CRABP2* and *SERPINE1* increase in the AF group compared with the expression observed in the E_2 (+) group. Table 6 shows the expression of genes related to steroid biosynthesis, WNT/CTNNTB1 signalling and members of the *THBS*, *VEGF*, *JUN* and *FOS* families. *HSD17B1* were significantly up-regulated by E_2 treatment and antrum formation, whereas *CYP11A* and *HSD3B1* decreased. *CYP19A1* increased in E_2 (+) and AF groups (1.35- and 1.78-fold respectively) but the increasing did reach significant due to low RPKM (reads per kilobase of exon per million reads of mapped reads) value. The expression of genes related to WNT2/CTNNB1 was altered in the E_2 (+) and AF groups (Table 6 and Fig. 1). The relative expression levels in E_2 (+) and E_2 (-) are shown in Table 6 and were -1.43, -2.55 and 1.31 for *WNT2B*, *SFRP4* and *NR5A1* respectively (P <0.05). The expression of *FZD1* and *FZD9*, the receptor of WNT2 and the co-receptor LRP6 were not affected by E_2 treatment, although the expression of LPR5 slightly but significantly increased. During continued *in vitro* culture, the expression of *FOXO1* significantly decreased (P <0.01) and the genes contained in destruction complex (*APC*, *AXINs*, *GSK3B*, and *CSNK1E*) tend to decrease (Table 6), and many differentially expressed genes in the AF group were linked to low expression of *FOS* (Fig. 1). The expression of members of the *FOS* and *JUN* families was also significantly low in the E_2 (+) group compared with the

Table 2 Effect of supplementation of culture medium with androstenedione on development of OGCs.

A_4 ($\mu\text{g/ml}$)	No. of trials	No. of OGCs	Rate (%) of OGCs with antrum, mean $\pm$ S.E.M.				No. (%) of oocytes at M2
			Day 4	Day 8	Day 12	Day 16	
0	9	90	0	5.0 $\pm$ 3.6*	2.5 $\pm$ 1.5*	0*	0/90 (0)*
1	9	90	0	22.2 $\pm$ 5.7†	26.7 $\pm$ 6.0†	26.7 $\pm$ 6.7†	7/90 (7.8)†
10	9	90	0	28.9 $\pm$ 6.8†	31.1 $\pm$ 7.5†	26.7 $\pm$ 6.7†	6/90 (6.7)†

*,† P <0.05.

Table 3 Relationship between estradiol secretion and antrum cavity formation of OGCs.

Antrum cavity formation	A ₄ (µg/ml)			
	1		10	
	No. of samples examined	E ₂ concentration (ng/ml), mean ± S.E.M.	No. of samples examined	E ₂ concentration (ng/ml), mean ± S.E.M.
+	24	5.5 ± 0.9* [‡]	20	11.3 ± 1.0* [‡]
–	53	3.1 ± 0.5 [†]	20	4.5 ± 1.2 [†]

*[†],[‡],[§] $P < 0.05$.

E₂(–) group (Table 6). A network of genes differentially expressed between the E₂(+) and E₂(–) conditions are shown in Fig. 2. The expression of *THBS1* and *THBS2* significantly decreased with E₂ treatment (–3.15 and –1.96), whereas *VEGFB* increased ($P < 0.05$). This gene expression is linked to low expression of *IGFBP3* and 5, *COL1A*, *FN1*, and *AMH*. However, the expression of *THBS1* was significantly increased in the AF condition compared with the E₂(+) group (Table 6). Genes implicated in cardiovascular system development and function were among the genes that most frequently had differential expression in the E₂(+) and E₂(–) conditions (Supplementary Table 1, see section on supplementary data given at the end of this article). Of the TGFβ family members, *TGFβ1* were down-regulated in the E₂(+) group. Expression of *BMP1* slightly but significantly decreased in E₂(+) group, the receptor for BMPs (*BMPRII*) much decreased in the AF group compared with the expression observed in the E₂(+) group (Table 7). The expression of most BMP antagonists decreased in the E₂(+) group compared with the E₂(–) group, but in AF medium, *FST* and *FSTL3* increased to over 1.5-fold the level observed in the E₂(+) medium ($P < 0.01$; Table 7). As observed in Figs 3 and 4, E₂ treatment of OGCs tended to decrease the expression of genes related to the cytoskeleton and extracellular matrix (ECM), representing significant low expression of *ACTA2* (–2.18), *ACTG2* (–2.15), *KRT8* (–1.90), *COL1A1* (–2.95), *COL4A1* (–1.55) and *LAMA5* (–1.46) (Supplementary Table 2, see section on supplementary data given at the end of this article); genes related to each function were selected using Kegg (<http://www.genome.jp/kegg/brite.html>). In addition, the

changes in gene expression were very robust in the AF conditions. The functions of the genes that are differentially expressed in the AF and E₂(+) groups include roles in cancer development, cellular movement, cellular growth and proliferation, apoptosis and cellular differentiation (Supplementary Table 1).

Discussion

This study shows that endogenous and exogenous E₂ directly supports the *in vitro* development of OGCs and, to the best of our knowledge, it is the first to show gene profiles either in GCs of OGCs treated with E₂ or in those of OGCs that formed antrum *in vitro*.

This study provides evidence for the following: i) both E₂ and A₄ improve the *in vitro* growth of OGCs, ii) competent OGCs have a greater ability to secrete E₂ into the culture medium than un-competent OGCs and iii) inhibition of E₂ prevents both A₄ and E₂ from supporting the *in vitro* development of OGCs. In addition, increasing the E₂ concentration from 1 to 10 µg/ml ameliorated the inhibitory effect of the inhibitor on OGC development. Taken together, we conclude that endogenous and exogenous E₂ maintains or helps to develop the functions of GCs of OGCs cultured *in vitro*. This finding sheds light on the long disputed question regarding the significance of E₂ on *in vitro* follicle development (Walters *et al.* 2008, Okutsu *et al.* 2010, Romero & Smits 2010, Taketsuru *et al.* 2011). It is worth noting that the E₂ concentration used in this study is much greater than that is in blood (Mann & Lamming 2001). OGCs are enclosed in FF, which contains a high concentration of E₂ compared with that in blood. We measured the concentration of E₂ in bovine FF previously and found the highest E₂ concentration to be about 0.1–0.2 µg/ml. In addition, the E₂ concentration in bovine antral follicles reportedly ranges from 0 to 0.3 µg/ml (Ouellette *et al.* 2005, Monniaux *et al.* 2008, Nishimoto *et al.* 2009, Green *et al.* 2011). Furthermore, Bridges *et al.* (2005) reported that when bovine follicle cells were cultured *in vitro*, the concentration of E₂ secreted was 0.2 µg/ml. The concentration of E₂ used in this study is 1 µg/ml, which is still higher than that found in antral FF. Because the concentration is measured using well-mixed FFs, it is plausible that the concentration of E₂ proximal to the GC

Table 4 Effect of fulvestrant on the effects of E₂ and A₄ on development of OGCs.

	No. of trials	No. of OGCs	Rate (%) of OGCs with antrum, mean ± S.E.M.			
			Day 4	Day 8	Day 12	Day 16
1 µg/ml A ₄	10	100	0.0 ± 0.0	20.0 ± 4.4*	28.0 ± 6.1*	24.0 ± 4.7*
1 µg/ml A ₄ + fulvestrant	10	100	0.0 ± 0.0	0.0 ± 0.0 [†]	0.0 ± 0.0 [†]	0.0 ± 0.0 [†]
0.1 µg/ml E ₂	10	100	0.0 ± 0.0	17.0 ± 6.0	21.0 ± 5.7*	18.0 ± 6.1*
0.1 µg/ml E ₂ + fulvestrant	10	100	0.0 ± 0.0	10.0 ± 4.2	4.0 ± 3.1 [†]	0.0 ± 0.0 [†]
1 µg/ml E ₂	10	100	3.0 ± 1.5	42.0 ± 4.4	46.0 ± 4.8	34.0 ± 4.0
1 µg/ml E ₂ + fulvestrant	10	100	1.0 ± 1.0	29.0 ± 5.7	35.0 ± 6.2	28.0 ± 6.1

*[†] $P < 0.05$.

Table 5 Expression of marker genes in granulosa cells.

Marker genes, Hayashi <i>et al.</i> (2010)	RPKM value			Ratio ^a					
	E ₂ (+)	AF	E ₂ (-)	E ₂ (+)/E ₂ (-)	P value*	AF/E ₂ (+)	P value	AF/E ₂ (-)	P value
Largest									
<i>FOS</i>	33.85	17.73	41.13	-1.22	0.001	-1.91	0.026	-2.32	0.000
<i>RGN</i>	7.28	3.88	8.33	-1.14	0.178	-1.87	0.381	-2.15	0.030
<i>SCD</i>	1010.82	1335.50	1052.84	-1.04	0.000	1.32	0.000	1.27	0.000
<i>FST</i>	775.87	1359.46	805.32	-1.04	0.000	1.75	0.000	1.69	0.014
<i>TNFAIP8</i>	1.50	2.10	1.51	-1.01	0.618	1.41	1.000	1.39	0.613
<i>SRGN</i>	5735.20	9677.05	5717.45	1.00	0.000	1.69	0.000	1.69	0.000
<i>PIGF</i>	15.69	17.61	14.75	1.06	0.136	1.12	0.865	1.19	0.203
<i>GUC1A1A</i>	4.24	18.61	3.61	1.18	0.465	4.39	0.003	5.16	0.123
<i>AMH</i>	85.01	106.06	71.91	1.18	0.007	1.25	0.219	1.47	0.115
<i>SERPINF2</i>	2.37	3.17	1.98	1.20	0.618	1.34	1.000	1.60	1.000
<i>GCLC</i>	365.31	411.73	298.41	1.22	0.000	1.13	0.223	1.38	0.000
<i>SLC39A14</i>	44.02	55.03	35.50	1.24	0.079	1.25	0.367	1.55	0.324
<i>PLA2G1B</i>	3.86	4.21	3.04	1.27	0.704	1.09	1.000	1.39	0.700
<i>INHBA</i>	77.57	212.50	59.67	1.30	0.050	2.74	0.000	3.56	0.000
<i>INHA</i>	875.33	1917.79	655.32	1.34	0.000	2.19	0.000	2.93	0.000
<i>HSD17B1</i>	348.75	554.18	267.63	1.30	0.000	1.59	0.000	2.07	0.227
<i>CITED1</i>	16.01	17.76	11.88	1.35	0.432	1.11	0.865	1.49	0.567
<i>CYP19A1</i>	12.26	21.84	9.08	1.35	0.498	1.78	0.123	2.41	0.576
<i>FSHR</i>	41.57	48.34	30.01	1.39	0.269	1.16	0.674	1.61	0.477
<i>TMEM20</i>	15.01	15.43	9.14	1.64	0.833	1.03	1.000	1.69	0.831
<i>IGFBP2</i>	71.45	28.27	38.28	1.87	1.000	-2.53	0.000	-1.35	0.000
<i>GPX3</i>	38.78	20.32	16.52	2.35	0.487	-1.91	0.013	1.23	0.167
Second largest									
<i>ADAMTS1</i>	9.43	2.90	19.20	-2.04	0.001	-3.25	0.088	-6.62	0.000
<i>CCDC80</i>	28.57	30.52	60.16	-2.11	0.000	1.07	0.898	-1.97	0.000
<i>CRABP2</i>	756.06	1100.79	1121.66	-1.48	0.000	1.46	0.000	-1.02	0.000
<i>GADD45A</i>	24.36	13.35	24.33	1.00	0.048	-1.83	0.070	-1.82	0.000
<i>IGFBP5</i>	77.42	8.36	134.50	-1.74	0.000	-9.27	0.000	-16.10	0.000
<i>KRT8</i>	89.70	60.65	170.78	-1.90	0.000	-1.48	0.012	-2.82	0.000
<i>LOXL4</i>	6.87	2.64	29.38	-4.28	0.000	-2.60	0.220	-11.13	0.000
<i>OLR1</i>	6.16	2.34	21.21	-3.44	0.000	-2.63	0.173	-9.06	0.000
<i>OXT</i>	57.23	3.52	91.62	-1.60	0.000	-16.27	0.000	-26.05	0.000
<i>PDK4</i>	6.46	2.65	6.52	-1.01	0.244	-2.44	0.337	-2.46	0.039
<i>PLAUR</i>	5.82	1.53	15.88	-2.73	0.000	-3.80	0.173	-10.39	0.000
<i>SERPINE1</i>	59.93	164.47	179.08	-2.99	0.000	2.74	0.000	-1.09	0.000
<i>SFRP4</i>	6.71	9.77	17.10	-2.55	0.000	1.45	0.630	-1.75	0.004
<i>SLC1A5</i>	11.73	10.73	11.65	1.01	0.140	-1.09	0.837	-1.09	0.083
<i>THBS2</i>	54.13	9.15	106.00	-1.96	0.000	-5.92	0.000	-11.59	0.000
<i>TIMP1</i>	181.46	14.30	251.89	-1.39	0.000	-12.69	0.000	-17.62	0.000

*P values are calculated by Fisher's exact test.

^aWhen the ratio is <1, it is converted to its negative inverse.

is higher than that of mixed FF. Considering these findings and this possibility, I concluded that 0.1 or 1 µg/ml of E₂ is relevant to yield full-grown oocytes *in vitro*. However, in the current culture system, it remains unclear why the low concentration of E₂ does not support antrum formation. Thus far, there have been reports showing the relationship of the robust developmental ability of mouse OGCs with either high concentrations of E₂ secreted from GCs or high *Cyp19a1* expression in GCs (Xu *et al.* 2006, West-Farrell *et al.* 2009). In addition, studies on KO mice suggest that E₂ mainly affects follicle maturation and ovulation including GC function (Couse *et al.* 2005, Drummond 2006). In this context, we examined comprehensive gene expression of E₂(-), E₂(+) and AF GCs.

Our first question was whether the OGCs cultured in the *in vitro* medium containing E₂ develop normally? In

this study, we compared the expression of genes in GCs of OGCs developed *in vitro* with the expression of previously reported marker genes related to GCs from *in vivo* developed, healthy, large follicles in cows (Hayashi *et al.* 2010). In the report, the expression of several marker genes was compared between GCs of the largest follicle and the second largest follicles. Upon comparing our data with the reported results, many results are consistent, genes highly expressed in the largest follicle are highly expressed in E₂(+) compared with those observed in E₂(-). With continued *in vitro* culture, the expression of many parts of these genes is enhanced in the AF group. In addition, *ITGA6*, *THBS1*, *THBS2*, *VEGF*, *AMH*, *FST* and *INHBA* were reported to express high in growing follicles (Le Bellego *et al.* 2002, Nilsson *et al.* 2003, Greenaway *et al.* 2005, Tesfaye *et al.* 2009, Rosales-Torres *et al.* 2010, Bonnet *et al.* 2011) and

Table 6 Expression of genes related to steroidogenesis, WNT/CTNNB1 signalling, THBSs, VEGFs, JUNs and FOSs.

Gene symbol	RPKM value			Ratio ^a					
	E ₂ (+)	AF	E ₂ (-)	E ₂ (+)/E ₂ (-)	P value*	AF/E ₂ (+)	P value	AF/E ₂ (-)	P value
<i>CYP11A1</i>	53.62	24.17	68.62	-1.28	0.000	-2.22	0.000	-2.84	0.000
<i>CYP19A1</i>	12.26	21.84	9.08	1.35	0.498	1.78	0.123	2.41	0.576
<i>HSD3B1</i>	25.24	12.58	37.01	-1.47	0.000	-2.01	0.051	-2.94	0.000
<i>HSD17B1</i>	348.75	554.18	267.63	1.30	0.000	1.59	0.000	2.07	0.227
<i>WNT2B</i>	38.91	63.54	55.46	-1.43	0.000	1.63	0.023	1.15	0.009
<i>SFRP4</i>	6.71	9.77	17.10	-2.55	0.000	1.45	0.630	-1.75	0.004
<i>FZD1</i>	9.26	6.97	10.02	-1.08	0.149	-1.33	0.624	-1.44	0.043
<i>FZD3</i>	16.17	16.56	15.41	1.05	0.136	1.02	1.000	1.07	0.192
<i>FZD9</i>	8.08	7.69	6.47	1.25	0.583	-1.05	1.000	1.19	0.578
<i>LRP5</i>	51.30	30.26	47.47	1.08	0.011	-1.70	0.014	-1.57	0.000
<i>LRP6</i>	13.35	10.05	12.20	1.09	0.211	-1.33	0.536	-1.21	0.070
<i>APC</i>	12.07	9.13	11.86	1.02	0.140	-1.30	0.518	-1.30	0.038
<i>AXIN1</i>	18.03	17.03	17.82	1.01	0.081	-1.06	0.866	-1.05	0.049
<i>AXIN2</i>	5.78	3.43	7.85	-1.36	0.099	-1.69	0.337	-2.29	0.021
<i>CSNK1E</i>	8.15	5.13	9.33	-1.14	0.136	-1.59	0.417	-1.82	0.025
<i>GSK3B</i>	21.04	18.42	19.78	1.06	0.101	-1.14	0.632	-1.07	0.026
<i>CTNNB1</i>	375.27	503.82	382.68	-1.02	0.000	1.34	0.000	1.32	0.000
<i>FOXO1</i>	54.28	31.08	52.43	1.04	0.004	-1.75	0.009	-1.69	0.000
<i>NR5A1</i>	91.98	99.01	70.27	1.31	0.040	1.08	0.828	1.41	0.075
<i>THBS1</i>	22.00	59.76	69.34	-3.15	0.000	2.72	0.000	-1.16	0.000
<i>THBS2</i>	54.13	9.15	106.00	-1.96	0.000	-5.92	0.000	-11.59	0.000
<i>THBS3</i>	6.14	5.12	7.06	-1.15	0.244	-1.20	0.771	-1.38	0.125
<i>VEGFA</i>	93.18	337.51	63.38	1.47	0.209	3.62	0.000	5.32	0.000
<i>VEGFB</i>	38.54	39.20	36.03	1.07	0.029	1.02	0.910	1.09	0.020
<i>JUN</i>	37.93	28.18	52.07	-1.37	0.000	-1.35	0.178	-1.85	0.000
<i>JUNB</i>	23.96	10.35	32.39	-1.35	0.001	-2.32	0.015	-3.13	0.000
<i>JUND</i>	63.25	83.02	73.53	-1.16	0.000	1.31	0.159	1.13	0.001
<i>FOS</i>	33.85	17.73	41.13	-1.22	0.001	-1.91	0.026	-2.32	0.000
<i>FOSL1</i>	4.25	6.78	7.88	-1.85	0.033	1.59	0.549	-1.16	0.173
<i>FOSL2</i>	41.43	8.31	43.62	-1.05	0.002	-4.99	0.000	-5.25	0.000
<i>PPARG</i>	63.62	101.22	62.66	1.02	0.001	1.59	0.008	1.62	0.325
<i>AHR</i>	3.32	3.91	5.59	-1.68	0.076	1.18	1.000	-1.43	0.105

*P values are calculated by Fisher's exact test.

^aWhen the ratio is <1, it is converted to its negative inverse.

the expression of these genes express high in E₂(+) compared with those observed in E₂(-). These results suggest that E₂ treatment induces OGCs to develop *in vitro* and that OGCs having antrum after 8 days of *in vitro* culture are on the way to normal follicle development. However, there were several genes that were exceptions to this trend, which indicates that OGCs may be slightly diverted from normal development; thus, we should look at these genes carefully. These genes were *CTGF*, *CRABP2*, *FOS*, *GPX3*, *GREM1*, *IGFBP2*, *NOG* and *SERPINE1*, etc.

West-Farrell *et al.* (2009) cultured mouse OGCs derived from EAFs *in vitro* using optimal and suboptimal culture conditions, OGCs cultured in optimal condition secrete more E₂ and had specific gene expression pattern that included high *Cyp19a1* expression and low *Cyp11a* and *Hsd3b* expression. In agreement with this report, *CYP19A1* and *HSD17B1* were up-regulated by E₂ treatment and antrum formation, whereas *CYP11A1* and *HSD3B1* were down-regulated. In addition, competent OGCs secreted more E₂ than un-competent OGCs. These results suggest that E₂ secretion is closely related to OGCs developmental competence and that E₂ itself induces OGCs to secrete E₂.

WNT/CTNNB1 is one of the three signalling WNT factors, and it reportedly regulates *CYP19A1* expression (Parakh *et al.* 2006, Boyer *et al.* 2010). The expression of *Wnt2* is high in healthy mouse follicles compared with that of atretic follicles, and both *WNT2* and *CTNNB1* induce the DNA synthesis of the GCs and prevent these cells from undergoing apoptosis (Wang *et al.* 2010). Figure 5 summarises the genes related to WNT2/CTNNB1 signalling and *CYP19A1* expression (details were presented in Boyer *et al.* (2010)). *SFRP4* binds to *WNT2/4*, preventing the WNTs from binding to the receptor *FZDs*. E₂ treatment decreased the expression of *SFRP4* to a relative level of 39% of the E₂(-) group ($P < 0.01$; Table 6). *FOXO1A* binds to *CTNNB1* to inhibit it, and the level of *FOXO1* expression in the AF group is 57% of the E₂(+) ($P < 0.01$). *APC*, *AXIN*, *CSNK1E* and *GSK3B* are major components of the β -catenin destruction complex (Kimelman & Xu 2006), and expression of these factors tended to decrease in the E₂(+) and AF conditions. *NR5A1* cooperates with *CTNNB1* to regulate *CYP19A1* expression, and expression of the *NR5A1* was significantly high ($P < 0.05$, 1.31; Table 6) in the E₂(+) group compared with that observed in the E₂(-) group. In addition, expression of *CTNNB1* itself increased in the

Figure 1 The network of genes showing different levels of expression between the AF and $E_2(+)$ groups. The functions of the molecules in the network are largely those related to the skeletal and muscular system development and function. The red-coloured symbols represent genes that are up-regulated in the AF group, and the green-coloured symbols represent those that were down-regulated. The number under each molecule indicates the ratio of the expression level in the AF group to that in the $E_2(+)$ group. PP, protein-protein binding; PD, protein-DNA binding; RE, reaction; MB, group/complex membership; A, activation; E, expression (includes metabolism/synthesis for chemicals); T, transcription; TR, translocation.

AF group ($P < 0.01$). These results suggest that WNT/CTNNB1 signalling has a role in the E_2 biosynthesis from OGCs cultured in medium containing E_2 . In addition, we used gene cards (<http://www.genecards.org/>) to select transcriptional factors (AHR, NFKB1, PPARG, E2F1, E2F2, E2F3, E2F4, FOS, JUN and ATF2) that bind to the

promoter region of WNT2B and CYP19A1. E_2 treatment significantly reduced the expression of member of JUN and FOS, and AHR, but increased PPARG, expression of PPARG also increased in AF group, these changes may be involved in the kinetics of WNT2B and CYP19A1. In addition, CTNNB1 colocalised with CDH1 (Wang

Figure 2 The network of genes with different expression patterns in the $E_2(+)$ and $E_2(-)$ groups. The functions of the molecules in the network are largely those related to cancer, gastrointestinal disease and cardiovascular system development and function. The red-coloured symbols represent genes up-regulated in the $E_2(+)$ group, and the green-coloured symbols represent those that were down-regulated. The number under each molecule indicates the ratio of the expression level in the $E_2(+)$ group to that in the $E_2(-)$ group. PP, protein-protein binding; PD, protein-DNA binding; RE, reaction; MB, group/complex membership; A, activation; E, expression (includes metabolism/synthesis for chemicals); T, transcription; TR, translocation.

Table 7 Expression of genes related to TGF β family and BMPs and BNP antagonists.

Gene symbol	RPKM value			Ratio ^a					
	E ₂ (+)	AF	E ₂ (-)	E ₂ (+)/E ₂ (-)	P value*	AF/E ₂ (+)	P value	AF/E ₂ (-)	P value
ACVR1	4.23	2.80	5.42	-1.28	0.295	-1.51	0.722	-1.93	0.135
ACVR1B	7.66	6.96	7.33	1.04	0.420	-1.10	0.801	-1.05	0.264
ACVR2A	3.45	2.43	3.30	1.05	0.433	-1.42	0.683	-1.36	0.349
ACVR2B	5.50	3.90	5.15	1.07	0.535	-1.41	0.543	-1.32	0.291
TGFB1	9.91	2.06	17.44	-1.76	0.004	-4.82	0.021	-8.48	0.000
TGFB11	3.76	2.74	4.92	-1.31	0.295	-1.37	0.722	-1.80	0.135
TGFB1	21.09	19.94	19.74	1.07	0.101	-1.06	0.876	1.01	0.047
TGFB2	2.01	1.04	3.75	-1.87	0.194	-1.94	0.619	-3.62	0.052
TGFB3	6.88	5.59	5.35	1.29	0.764	-1.23	0.788	1.04	0.530
TGFBRA1	7.48	6.45	7.72	-1.03	0.178	-1.16	0.788	-1.20	0.093
BMP1	78.34	74.74	83.13	-1.06	0.000	-1.05	0.687	-1.11	0.000
BMP6	7.31	9.14	7.60	-1.04	0.178	1.25	0.804	1.20	0.312
BMP1A	12.78	11.27	11.52	1.11	0.211	-1.13	0.686	-1.02	0.083
BMP1B	33.87	12.12	32.42	1.04	0.029	-2.80	0.001	-2.68	0.000
BMP2	11.20	9.39	10.67	1.05	0.181	-1.19	0.659	-1.14	0.063
AMH	85.01	106.06	71.91	1.18	0.007	1.25	0.219	1.47	0.115
INHBA	77.57	212.50	59.67	1.30	0.050	2.74	0.000	3.56	0.000
INHA	875.33	1917.79	655.32	1.34	0.000	2.19	0.000	2.93	0.000
TWSG1	46.86	62.89	40.21	1.17	0.043	1.34	0.183	1.56	0.408
HTRA1	138.71	120.91	148.27	-1.07	0.000	-1.15	0.173	-1.23	0.000
NBL1	9.75	21.83	11.09	-1.14	0.113	2.24	0.051	1.97	1.000
FST	775.87	1359.46	805.32	-1.04	0.000	1.75	0.000	1.69	0.138
FSTL3	46.15	74.13	65.33	-1.42	0.000	1.61	0.018	1.13	0.003
CTGF	16.32	1.99	33.69	-2.06	0.000	-8.21	0.001	-16.94	0.000
CHRD	3.51	6.63	4.62	-1.32	0.295	1.89	0.549	1.43	0.763
NOG	7.65	2.84	9.78	-1.28	0.086	-2.70	0.140	-3.45	0.002
GREM1	123.67	73.32	133.89	-1.08	0.000	-1.69	0.000	-1.83	0.000

*P values are calculated by Fisher's exact test.

^aWhen the ratio is <1, it is converted to its negative inverse.

et al. 2009), and the expression of both *CTNNB1* and *CDH1* tend to increase in the AF group compared with the E₂(+) group (1.31 and 1.39 respectively, [Supplementary Table 2](#)). The presence of other factors regulating *CYP19A1* expression in the E₂(+) group and the other functions of *CTNNB1* need to be explored.

Of specific networks of genes differentially expressed between the E₂(+) and E₂(-) groups, one of the most prominent functions affected by E₂ treatment-induced expression modifications is angiogenesis ([Supplementary Table 1](#) and [Fig. 2](#)). In small antral follicles, where expression of VEGF is low, THBS1 and THBS2 prevent GCs from performing angiogenesis ([Garside et al. 2010](#)). As the follicle develops, THBS1 and THBS2 decrease and VEGF increases ([Greenaway et al. 2005](#)). Our results agree with these previous observations. However, the expression of *THBS1* increased in the AF group, which also indicates that an abnormal event may occur within follicles that are cultured for long period *in vitro*.

Oocyte and follicle growth is regulated by well-orchestrated interactions among oocytes, GCs and thecal cells. The TGF β superfamily, KIT and KITL are major players in these interactions ([Knight & Glistler 2006](#)). The TGF β superfamily contains BMPs, AMH, activins, inhibins and GDF9, and BMP15 and GDF9 are well-studied oocyte secretion factors. In this study, the expression of *BMP1* was high throughout *in vitro* culture period and was lightly but significantly affected by E₂

treatment. The functions of various BMPs depend on many BMP antagonists, which are expressed in GCs ([Fenwick et al. 2011](#)). We detected expression of *TWSG1*, *HTRA1*, *NBL1*, *FST*, *FSTL3*, *CTGF*, *CHRD*, *NOG* and *GREM1*. The expression of these genes is reduced in the E₂(+) group compared with the E₂(-) group, indicating that E₂ treatment impacts GC–oocyte interactions. Once OGCs formed antrum, some of these antagonists increased or decreased over twofold. Our results show that in order to form antrum, each antagonist has a unique contribution and relies on complex mutual interactions. The expression of *CTGF* is depressed by E₂ treatment and antrum formation to the extent that the relative expression level in the AF group to that of the E₂(-) group is 6% ($P < 0.01$). Conversely ([Harlow et al. 2007](#)), it has been reported that the expression of *CTGF* and *CYP19A1* increases as follicles develop in rats. Studies with *Ctgf* conditional KO mice have reported that expression of *Ctgf* is also necessary for follicle development ([Nagashima et al. 2011](#)). These mice also show a decline in the expression of *Myc*, *Adams1* and an increase in the expression of *Hsd3b1*, *Hsd17b7*, *Sfrp4*, and *Kitl*. In this study, while expression of *CTGF* was decreased following E₂ treatment and antrum formation, *ADAMTS1* also significantly decreased ([Table 5](#)), which agrees with the report. However, *KITL* and *HSD3B1* in the AF group tended to decrease to 40 and 50% of the levels observed in E₂(+)

Figure 3 The network of genes with differential expression between the $E_2(+)$ and $E_2(-)$ groups. The functions of the molecules in the network are largely those related to cell-to-cell signalling and interaction, cellular assembly and organisation and cellular movement. PP, protein-protein binding; PD, protein-DNA binding; RE, reaction; MB, group/complex membership; A, activation; E, expression (includes metabolism/synthesis for chemicals); T, transcription; TR, translocation.

($P=0.052$ and $P=0.072$ respectively), indicating that there may be some disorder in the regulation of the TGF β superfamily in the OGCs cultured *in vitro*.

Follicle development is accompanied by remodelling of the ECM, cytoskeleton and cell-to-cell junctions. Overall, E_2 treatment induced a reduction in the expression of actins, keratins and ECMs including types 1 and 4 collagen, laminin and integrin (Figs 3 and 4,

Supplementary Table 2). N-cadherin, JAM-A, afadin and cingulin are the main components of adherence junctions in mouse GCs and KRT8, characteristic of epithelial cell, in GCs decreases as follicles develop (Mora *et al.* 2012). We also detected these factors and the level of *KRT8* expressed in GCs decreases with follicle development. Zalewski *et al.* (2012) reported that collagen type 4 was highly expressed in the follicle of

Figure 4 A schematic diagram of the expression of genes related to the cytoskeleton, cell-to-cell junctions and ECMs (A) in $E_2(+)$ compared with $E_2(-)$ and (B) in AF compared with $E_2(+)$. Green, decrease; red, increase. T.J, tight junction; G.J, gap junction; A.J, adherence junction.

Figure 5 A schematic diagram of genes relate to WNT/CTNNB1 signalling in granulosa cells. The figure is created based on the previous reports (Boyer *et al.* 2010) and the Kegg pathway (<http://www.genome.jp/kegg/pathway.html>). SFRP4 prevents WNT molecule signalling by binding to WNT, WNT binds to the FZDs/LRP co-receptor, CTNNB1 is freed from the APC/CSNK1E/AXIN/GSK3B complex and translocates to the nucleus, where CTNNB1 binds to the transcription factor, NR5A1, which regulates CYP19A expression. FOXO1a inhibits CTNNB1 by binding to it.

estrogen receptor β KO mice, indicating a negative relationship between E_2 and these ECMs. In addition, bovine expression of COL4A1, 2, 3, 4 and 5 has been reported to decrease as follicles increase in size (Rodgers *et al.* 1998). Furthermore, LAMA1, LAMB2 and LAMC1 are expressed in bovine follicular basal lamina of healthy antral follicles but have reduced expression in the atretic antral follicle (van Wezel *et al.* 1998). Rodgers *et al.* (2003), suggesting that decreased type 4 collagen and increased laminin are markers for healthy follicle development. Bovine GCs cultured on type 1 collagen gel exhibit a round shape and enhanced E_2 secretion (Huet *et al.* 2001). In this study, when OGCs formed antrum following 8 days of culture with E_2 , the expression of COL4A1, COL4A2 and LAMB1 decreased. Taken together, regarding collagens and the cytoskeleton, OGCs cultured with E_2 develop normally, but the opposing kinetics of laminin induce part of the OGCs to transition to atresia.

Oocytes in EAFs have great potential to yield embryos, but the ongoing *in vitro* culture system is not currently optimised to produce embryos with high efficiency. For example, in our preliminary experiment, the developmental ratio to the blastocyst stage was only 6.9% (12/174 OGCs) for oocytes grown *in vitro* whereas it was 30% for oocytes collected from medium antral follicles (3–6 mm in diameter). This result suggests that there were some abnormal events in current culture system for OGCs derived from EAFs. In addition, several genes

deviated from normal gene expression profiles during long-time *in vitro* culture and these genes might be an important candidate to improve the current culture condition. This study shows that E_2 is a powerful supplement for *in vitro* development of OGCs, but other side effects should be considered because many parts of E_2 functions are categorised as cancer related (Supplementary Table 1).

In conclusion, this study demonstrates the significance of E_2 in the *in vitro* development of OGCs derived from EAFs. Developing OGCs have a specific gene expression profile that includes E_2 biosynthesis, and treatment of OGCs with E_2 impacts several genes to support the establishment of these gene expression profiles.

Materials and Methods

Drugs and media

Unless otherwise stated, all drugs were purchased from Sigma–Aldrich. TCM 199 medium was used for *in vitro* culture of OGCs (Gibco BRL) and was supplemented with 5.56 mM glucose (final concentration, 11.2 mM), 0.1 mM pyruvic acid, various concentrations of E_2 or androstenedione (A_4), 0.02 mAU/ml FSH (Kawasaki Mitaka, Tokyo, Japan), 4% polyvinylpyrrolidone (PVP) (360K), 4 mM hypoxanthine and 10% FCS (5703H, ICN; Costa Mesa, CA, USA). Stock solution of E_2 and A_4 was prepared in ethanol at 1000-fold the required concentrations and the maximum ethanol concentration was never above 1 μ l/l. The TCM 199 medium used for the *in vitro* maturation (IVM) of oocytes contained 5% FCS.

Collection of ovaries and oocytes from EAFs

Bovine ovaries were collected from a slaughterhouse and transported in PBS containing antibiotics, 10 mM sucrose, 10 mM glucose and 1 mM pyruvic acid at 30 °C within 4 h. Ovaries were wiped with 70% ethanol, the ovarian surface was sliced and EAFs (400–700 μ m in diameter) were collected under a stereomicroscope using a scalpel in TCM 199 medium containing 3 mg/ml BSA. In our preliminary experiment, average diameter of oocytes in EAFs was 102.7 ± 1.0 μ m (no. 92).

Culture and IVM of OGCs

OGCs were washed in culture medium, individually transferred to 200 μ l medium in a well (96-well plate, Becton Dickinson, Franklin Lakes, NJ, USA) and cultured for 16 days. Half of the medium was replaced with fresh medium and antrum formation was examined every 4 days (4, 8, 12 and 16 days in culture). After the culture period, OGCs having antra were subjected to IVM for 23 h. After maturation, oocytes were denuded from surrounding cumulus cells and were fixed, permeabilised and mounted with antifade that contained 4'-diamidino-2-phenylindole (DAPI) (Pro-long gold antifade reagent with DAPI; Invitrogen) on glass slides. The nuclear maturation was examined using a fluorescence digital microscope (BZ-8000; Keyence, Tokyo, Japan).

Figure 6 OGCs were cultured in 96-well plates with a single complex in each well. The medium was collected at day 4 before the OGCs formed antrum, and these OGCs were subsequently cultured for 12 days. At the end of culture period, OGCs were classified as OGCs having clear antrum (+) or OGCs having no antrum. The E₂ concentration in the medium was compared between competent (+) and un-competent (–) OGCs.

Estradiol concentration

As described in the experimental design, the culture medium was sampled at day 4 of the *in vitro* culture period. The medium was stored at –80 °C. The E₂ concentration was measured using the E₂ immunoassay kit (DELFLIA estradiol reagents, PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol. All measurements were conducted in one plate, and the coefficient of variation value was 1.36.

Gene expression analysis

For the analysis of gene expression, total RNA was extracted from GCs of OGCs that had been exposed to no or 1 µg/ml E₂ for 4 days or OGCs that formed antrum at 8 days, using the RNAqueous Kit (Life Technologies Corp.) according to the manufacturer's protocol. RNA quality and quantity were assessed on a 2100 Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA, USA). Libraries were prepared from the samples using the TruSeq RNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA), and the prepared samples were used to generate clusters on cBot (Illumina, Inc.). Two lanes per group were sequenced on a HiSeq 2000 (Illumina, Inc.) as 50 bp reads (single-read). The image analysis and basecalling were performed with CASAVA ver. 1.8.2 (Illumina, Inc.) according to the manufacturer's instructions. High-quality sequences (those passing the default quality-filtering parameters in the Illumina pipeline GERALD stage) were retained and aligned to the bovine genome sequence (bosTau6) and the exon–exon splice junction database downloaded from the UCSC sequence and annotation database (<http://hgdownload.cse.ucsc.edu/>

[downloads.html#cow](#)). Both the raw and normalised (RPKM; Mortazavi *et al.* 2008) read counts were obtained using CASAVA and Genome Studio ver. 2011.1 (Illumina, Inc.). For the genes in which the normalised gene counts were >1, the fold-changes of the normalised counts between the groups were calculated. Statistical significance was assigned to each pairwise group comparison (E₂(+):E₂(–), AF:E₂(+) and AF:E₂(–) groups) by setting up a Fisher's exact test, comparing the number of weighted, mapped reads for each gene to the total number of mapped reads for that group. Genes that were differentially expressed were interpreted in the context of their biological processes and functions and by their networks and pathways by Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Inc., Redwood City, CA, USA). A detailed description of the method for performing IPA can be found at www.ingenuity.com. Fisher's exact test was used in the analysis of gene set enrichment in the functional categories.

Experimental design

Experiment 1

The effect of E₂ on the *in vitro* development of OGCs derived from EAFs was examined. OGCs were cultured in medium containing 0, 0.1, 1 and 10 µg/ml E₂ for 16 days, and antrum formation was examined. Experiments were repeated ten times. In the first five trials, oocytes of OGCs having antrum were used to measure the diameter. In the last five trials, OGCs having antrum were subjected to IVM and nuclear maturation of the oocytes was examined.

Experiment 2

The effect of A₄ on OGCs development, as well as the relationship between the E₂ concentration and the development of OGCs, was examined. As shown in Fig. 6, OGCs were

Figure 7 OGCs were collected from a donor cow and divided into two groups. OGCs were cultured in a 96-well plate individually. OGCs were cultured in medium containing no or 1 µg/ml E₂ for 4 days, and granulosa cells were collected (E₂(+) and E₂(–) group). AR group; OGCs were cultured for an additional 8 days in medium containing 1 µg/ml E₂ and granulosa cells were collected from OGCs forming antrum.

cultured in medium containing 0, 1 and 10 µg/ml A₄. At day 4 of the culture period, half of the culture medium was sampled and stored for later E₂ concentration measurement. At that time, no OGCs formed antrum. The OGCs were subsequently cultured for an additional 12 days. At the end of the culture period, OGCs were categorised as OGCs having antrum (competent OGCs) or OGCs having no antrum (un-competent OGCs). OGCs having antrum were further cultured in IVM medium, and the ratio of nuclear maturation was subsequently examined. Ten OGCs were used as replicates, and the experiment was repeated nine times. The E₂ concentration was measured from randomly selected medium samples, and the concentrations were compared between the competent and un-competent OGCs.

Experiment 3

The ability of fulvestrant to inhibit the effects of E₂ and A₄ on OGC development was examined. OGCs were cultured in medium containing either fulvestrant and E₂, or fulvestrant and A₄ for 16 days, and antrum formation was examined. The combinations of fulvestrant and hormones were as follows: i) A₄, 1 µg/ml and A₄, 1 µg/ml+fulvestrant, 1 µg/ml; ii) E₂, 0.1 µg/ml and E₂, 0.1 µg/ml+fulvestrant, 1 µg/ml and iii) E₂, 1 µg/ml and E₂, 1 µg/ml+fulvestrant, 1 µg/ml. This experiment was repeated ten times, and the ratio of antrum formation was compared between the hormone group and the hormone plus inhibitor group.

Experiment 4

The initial three experiments suggested that E₂ improves OGC development *in vitro*. In Experiment 4, we aimed to identify the molecular basis associated with the beneficial effect of E₂ on OGC development. As shown in Fig. 7, we collected 20 OGCs from a donor, and ten OGCs were cultured in medium containing 0 or 1 µg/ml E₂. At day 4, three OGCs were randomly selected from the ten OGCs and GCs were collected from these three OGCs, which were defined as the E₂(-) and E₂(+) groups respectively. Other OGCs were cultured in medium for an additional 4 days, and the antrum formation was examined. Antrum formation was observed in medium with E₂ supplementation but no OGCs formed in the medium without E₂ supplementation. The GCs collected from the OGCs forming antra in medium containing E₂ were defined as AF groups. A total of 25 cows were used for these experiments, and GCs of the E₂(-), E₂(+) and AF groups were collected from 75, 75 and 50 OGCs respectively. These GCs were subjected to comprehensive gene expression analysis.

Statistical analysis

The ratio of antrum formation and oocyte diameter was analysed by a one-way ANOVA followed by Tukey's *post hoc* test. The ratio of antrum formation was arcsin transformed before analysis. The nuclear maturations were compared using χ^2 test. And the Bonferroni's method was used to adjust the *P* values for multiple comparisons. E₂ concentrations were compared by a two-way ANOVA. *P* values <0.05 were considered significant.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0319>.

Declaration of interest

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

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