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Expression of polycystic ovary syndrome candidate genes in bovine fetal and adult ovarian somatic cells

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Abstract

Polycystic ovary syndrome (PCOS) is an endocrine metabolic disorder that appears to have a genetic predisposition and a fetal origin. The fetal ovary has two major somatic cell types shown previously to be of different cellular origins and different morphologies and to differentially express 15 genes. In this study, we isolated the somatic gonadal ridge epithelial-like (GREL) cells ($n = 7$) and ovarian fetal fibroblasts ($n = 6$) by clonal expansion. Using qRT-PCR, we compared the gene expression levels of PCOS candidate genes with previous data on the expression levels in whole fetal ovaries across gestation. We also compared these levels with those in bovine adult ovarian cells including fibroblasts ($n = 4$), granulosa cells ($n = 5$) and surface epithelial cells ($n = 5$). Adult cell types exhibited clear differences in the expression of most genes. In fetal ovarian cells, *DENND1A* and *ERBB3* had significantly higher expression in GREL cells. *HMGA2* and *TGFB1I1* tended to have higher expression in fetal fibroblasts than GREL cells. The other 19 genes did not exhibit differences between GREL cells and fetal fibroblasts and *FBN3*, *FSHB*, *LHCGR*, *FSHR* and *ZBTB16* were very lowly expressed in GREL cells and fibroblasts. The culture of fetal fibroblasts in EGF-containing medium resulted in lower expression of *NEIL2* but higher expression of *MAPRE1* compared to culture in the absence of EGF. Thus, the two fetal ovarian somatic cell types mostly lacked differential expression of PCOS candidate genes.

Lay summary

Polycystic ovary syndrome (PCOS) is one of the most common reproductive problems. The cause is not known so there are no specific treatments or prevention strategies. We know it can be linked to issues that occur in the womb and that some people may be more likely to get PCOS due to their genetic makeup. Our recent studies showed that many of the genes linked to PCOS were found to be switched on in the fetal ovary and are likely to be involved in the development of the fetal ovary. In order to improve our understanding of PCOS, we need to identify the type of cells in the fetal ovary where these genes are switched on. In this study, we examined the PCOS genes in two types of cells that mature as the fetal ovary develops and found very little difference between them but bigger differences to their mature adult counterparts.

Keywords: ▶ ovary ▶ fetus ▶ fibroblasts ▶ GREL cells ▶ granulosa cells ▶ surface epithelial cells ▶ polycystic ovary syndrome ▶ gene expression

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine disorders, affecting approximately 10% of women of reproductive age (March *et al.* 2010, Bozdag *et al.* 2016). Women with PCOS are identified with hyperandrogenism and often exhibit male-like body or facial hair (Lizneva *et al.* 2016), menstrual irregularity and polycystic ovaries (Azziz 2018). Furthermore, PCOS is also associated with secondary complications, such as impaired glucose tolerance, type 2 diabetes, sleep disorders, increased anxiety and depression and potentially adverse cardiovascular diseases (Fauser *et al.* 2012, Haoula *et al.* 2012, Cooney *et al.* 2017, Fernandez *et al.* 2018, Teede *et al.* 2018).

The aetiology of PCOS is not completely understood. Studies on twins and families suggested a genetic basis for PCOS. Familial linkage studies and genome-wide association studies (GWAS) have led the genetic understanding of PCOS to a new era by identifying 19 loci associated with risk of PCOS (Jones & Goodarzi 2016, Day *et al.* 2018). The loci contain 25 PCOS candidate genes (*FBN3*, *DENND1A*, *LHCGR*, *THADA*, *C9orf3/AOPOP*, *FSHR*, *HMGA2*, *INSR*, *RAB5B/SUOX*, *SUMO1P1*, *TOX3*, *YAP1*, *ERBB4*, *FSHB*, *GATA4*, *KRR1*, *RAD50*, *PLGRKT*, *ZBTB16*, *MAPRE1*, *FDFT1*, *IRF1*, *ARL14EP*, *ERBB3* and *NEIL2)* (Jones & Goodarzi 2016, Day *et al.* 2018). Moreover, studies on pregnant women with elevated levels of androgens and their offspring indicated that a female fetus exposed to elevated levels of androgens was at an increased risk of developing PCOS in later life, which supported the concept of a fetal or early postnatal origin of PCOS (Sir-Petermann *et al.* 2002, Filippou & Homburg 2017, Tata *et al.* 2018).

In the adult ovary, the follicular thecal cells are responsible for the synthesis of androgens utilising the enzyme cytochrome P450c17 (*CYP17A1*), and in PCOS ovaries, the thecal cells overexpress cytochrome P450c17 resulting in increased androgen production (Chang 2007, Rosenfield & Ehrmann 2016). When mature, the granulosa cells normally convert androgens to oestrogens under the regulation of gonadotrophins (Erickson *et al.* 1992, Kakuta *et al.* 2018). *In vitro* studies on PCOS ovaries found that granulosa cells from women with PCOS had reduced capacity for producing oestradiol and progesterone than normal granulosa cells (Erickson *et al.* 1992). Moreover, granulosa cells in PCOS ovaries appear to be abnormal in their survival and proliferation behaviour, with downregulated expression of proapoptotic factor and upregulated antiapoptotic factor (Das *et al.* 2008, Yuanyuan *et al.* 2019). Ovarian surface epithelium, an essentially single layer of epithelial cells covering the surface of the ovary, is androgen-responsive, and androgens can increase its cell proliferation and decrease cell apoptosis (Edmondson *et al.* 2002). However, elevated levels of androgens are associated with increased risk of ovarian epithelial cancer which can arise from ovarian surface epithelial cells (Thomas 1995, Schildkraut *et al.* 1996, Ose *et al.* 2017). PCOS ovaries tend to be fibrous, which is caused by excessive proliferation of fibroblasts and elevated deposition of extracellular matrix such as collagen type I (Zhou *et al.* 2017).

In terms of the origin of different cell types in the fetal ovary, fibroblasts are derived from the mesonephric stroma which penetrates from the mesonephros into the ovarian primordium and starts proliferating (Hummitzsch *et al.* 2013, Heeren *et al.* 2015, Hartanti *et al.* 2019, Hummitzsch *et al.* 2019). Granulosa cells and ovarian surface epithelial cells are believed to have a common precursor, the gonadal ridge epithelial-like (GREL) cell, which arises from the surface epithelium of the mesonephros (Hummitzsch *et al.* 2013). Later, in gestation, GREL cells differentiate into either pre-granulosa cells or surface epithelium (Hummitzsch *et al.* 2013). GREL cells close to the surface are separated from oogonia/oocytes by the proliferating and migrating stroma and develop into ovarian surface epithelium. GREL cells in the ovigerous cords are in close proximity to oocytes and develop into pregranulosa cells when the ovigerous cords break down and form follicles containing oocytes and GREL cells (Hummitzsch *et al.* 2013).

In our previous studies, we investigated a possible link between the genetic origins and fetal origins of PCOS in both bovine and human fetal ovaries. Initially, we examined the expression of *FBN3* (Hatzirodos *et al.* 2011), which was identified by D19S884 microsatellite in familial linkage studies of PCOS (Stewart *et al.* 2006, Ewens *et al.* 2010), and members of TGFβ signalling pathway (Hatzirodos *et al.* 2011, Azumah *et al.* 2022). The results indicated that this signalling pathway may be involved in the fetal origin of PCOS (Hatzirodos *et al.* 2011). Subsequently, PCOS candidate genes identified by GWAS and additionally *FBN3*, *TGFB1I1* and *AR* were examined in fetal ovaries (Hartanti *et al.* 2020b, Liu *et al.* 2020, Azumah *et al.* 2021). Nearly, all the candidate genes were expressed in the fetal ovary, and the expression pattern of each gene followed one of three patterns: some were expressed early in gestation, some late in gestation and some were expressed throughout gestation (Hartanti *et al.* 2020b, Liu *et al.* 2020). Expression of the early genes was highly correlated

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with each other and so were the late genes (Hartanti *et al.* 2020b, Liu *et al.* 2020). Similarly, in studies on the expression of cell-type-associated genes in the developing ovary (Hatzirodos *et al.* 2019, Hummitzsch *et al.* 2019), hierarchical clustering also exhibited three expression patterns across gestation and higher correlations between genes in the same cluster (Hatzirodos *et al.* 2019). We reasoned that the dynamic expression patterns reflect successive processes like expansion of stroma penetrating from the mesonephros into ovarian primordium and the formation of ovigerous cords early in gestation, and follicle formation, establishment of mature surface epithelium and the tunica albuginea late in gestation (Hummitzsch *et al.* 2013, Hartanti *et al.* 2020a).

In contrast to the fruitful studies of the fetal ovaries, microarray analysis of data from adult human ovaries found that among 25 PCOS candidate genes, only *RAD50* was differentially and significantly higher expressed in PCOS ovaries compared with normal ovaries (Liu *et al.* 2020). So we considered that the PCOS genes expressed in the fetal ovaries maybe vulnerable to disturbance during gestation which possibly leads to a predisposition to PCOS in later life (Hartanti *et al.* 2020b, Liu *et al.* 2020). To obtain more information about the expression of PCOS genes in fetal ovaries, we examined the expression of PCOS candidate genes in cultured fetal GREL cells and fibroblasts isolated from bovine fetuses. These were less than 200 days of gestation to avoid differentiation of GREL cells into granulosa cells and ovarian surface epithelial cells. We also compared these results to the expression of these genes in isolated adult granulosa cells and ovarian surface epithelial cells and cultured adult fibroblasts. Additionally, we also compared these data to previous data on the expression levels in whole fetal ovaries across gestation.

Materials and methods

Animal use statement

The bovine ovaries used in this study were scavenged from animals that were being processed for human consumption and were not owned by the authors or their institutions. As such, the University of Adelaide's Animal Ethics Committee only requires notification of this.

Collection of bovine fetal and adult ovaries

Fetuses from pregnant *Bos taurus* cows and ovaries from non-pregnant *Bos taurus* cows were collected at local abattoirs (Thomas Foods International, Murray Bridge, SA, Australia and Strath Meats, Strathalbyn, SA, Australia) and transported on ice in Hank's balanced-salt solution (HBSS) with calcium and magnesium ($HBSS^{+/+}$) to the laboratory. The gestational ages of the fetuses were estimated from the crown-rump length (CRL) (Russe 1983). Fetuses with a CRL < 8 cm underwent a sex determination as previously reported (Hummitzsch *et al.* 2013). The fetuses for isolation of fetal ovarian cells were at less than 200 days of gestation. Samples of whole fetal ovaries throughout gestation ($n = 27$) were from our previous study (Hartanti *et al.* 2020b) and collected from The Midfield Group, Warrnambool, Victoria, Australia.

Isolation of fetal ovarian GREL cells and fibroblasts

Purified GREL cells and fetal fibroblasts used in this study were isolated and cultured in our previous study (Liu *et al.* 2022). Briefly, fetal ovaries were removed from fetuses and digested in 10 mL of $HBSS^{+/+}$ containing 1 mg/mL collagenase type I (Gibco) and 50 µg/mL DNase I (Sigma–Aldrich) at 37°C. The cell suspensions were seeded in 24-well plates which were coated with collagen type I (Sigma–Aldrich) at 250 cells per well. The cells were cultured in DMEM/F12 medium (Sigma–Aldrich, D8900) containing 5% fetal calf serum (Sigma–Aldrich), 100 IU/mL penicillin, 0.01% streptomycin sulfate (Gibco, 15140122) and 0.1% fungizone (Gibco, 15290-018) and 3 ng/mL human EGF (EGF; Sigma–Aldrich, E9644), and the culture medium was changed every 2 days. Clusters with cells of irregular polygonal shape and with clear visible nuclei as described in our previous study (Liu *et al.* 2022) were observed from day 2 onwards, and the largest purified and healthy clusters were selected on day 3 and day 4. A sterile blunt-ended glass Pasteur pipette was used to scrape and remove other cell types surrounding the selected clusters. Cells were collected from day 10 to day 14. In order to collect pure GREL cell and fetal fibroblast at the end of culture, clusters were scraped with a sterile blunt-ended glass Pasteur pipette and then culture media with the cells was transferred to a sterile Eppendorf tube. After centrifugation at 500 ***g*** for 5 min, the cell pellets were stored at −80°C. Under these culture conditions, GREL cells demonstrated significantly higher gene expression of GREL cell markers (*KRT19* and *PKP2*) and fetal fibroblasts demonstrated higher gene expression of fibroblast markers (*FBN1*, *COL1A1* and *NR2F2*), suggesting that the cells maintained their epithelial and fibroblast characters, respectively (Liu *et al.* 2022).

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Isolation of adult ovarian cells

Samples of adult ovarian fibroblasts, ovarian surface epithelial cells and granulosa cells used in this study were collected in our previous study (Liu *et al.* 2022). Briefly, for fibroblasts, adult individual ovaries were cut into quarters, and tissue below the tunica albuginea (100–200 µm) was dissected out. It was digested in 10 mL of $HBSS^{+/+}$ containing 1 mg/mL of collagenase type I and 50 µg/mL of DNase I at 37°C for 2 h. Cell suspensions were seeded in the same way as fetal ovarian cell suspension. When the culture dishes were 70% covered with cells, the fibroblasts were collected and cell pellets were stored at −80°C. For surface epithelial cells, individual adult ovaries were placed in a 60 mm-culture dish containing culture medium. The surface of the ovary was scraped with the blunt side of a scalpel blade. Culture medium containing ovarian surface epithelial cells from five to seven ovaries was centrifuged at 500 ***g*** for 5 min. Finally, the cell pellets were stored at −80°C until use. Granulosa cells were isolated from healthy antral follicles (9–12 mm). Follicles were cut off from the ovary with scissors and transferred to a dish containing 1 mL of $HBSS^{-/-}$, and they were cut open. Granulosa cells were scraped with a blunt-ended Pasteur pipette. The cell suspension from each follicle was transferred into a sterile eppendorf tube and centrifuged at 500 ***g*** for 5 min. Finally, the cell pellets were stored at −80°C.

RNA isolation, cDNA synthesis and quantitative real-time PCR

RNA from granulosa cells, ovarian surface epithelial cells and adult fibroblasts was extracted using Trizol™ (Thermo Fisher Scientific) according to manufacturer's instructions. RNA from GREL cells and fetal fibroblasts was extracted using RNAqueous micro kit (Thermo Fisher Scientific). RNA of 10 µg of each sample was treated with DNase I (Promega Australia/Life Technologies Australia Pty Ltd) for 20 min at 37°C. DNAse-treated RNA of 200 ng was used for cDNA synthesis as previously described (Hummitzsch *et al.* 2013, Liu *et al.* 2020).

Based on available RNA sequences in NCBI, PCR primers were designed to span introns using Primer3 plus and Net primer (PREMIER Biosoft, Palo Alto, CA, USA), software and primers are listed in Supplementary Table 1 (see section on supplementary materials given at the end of this article). Primer combinations were tested as previously described (Liu *et al.* 2020). The amplification conditions were 95°C for 15 s, then 60°C for 60 s for 40 cycles using Rotor Gene 6000 cycler (Q series, Qiagen GmbH). Ct values were determined using Rotor Gene 6000 software at a threshold of 0.05 normalised fluorescent unit. Gene expression values were determined using $2^{-\Delta Ct}$ method, and ribosomal protein L32 (*RPL32*) and peptidylprolyl isomerase A (*PPIA*) were used as housekeeping genes in the examination of different cell types. We used negative controls of no reverse transcriptase in the cDNA synthesis and of no added RNA.

Treatment of bovine fetal fibroblasts

Fetal fibroblasts (160–198 days, $n = 6$) were cultured and treated with a number of growth factors and hormones as listed in Supplementary Table 2 (see section on supplementary materials given at the end of this article) in a previous experiment (Bastian *et al.* 2016) and the expression of PCOS genes were examined here. Briefly, bovine fetal fibroblasts were seeded at 30,000 cells/well in 24-well plates in DMEM/F12 medium containing 5% (v/v) fetal calf serum, 100 IU/mL penicillin, 0.01% streptomycin sulfate (Gibco, 15140122) and 0.1% fungizone at 38.5°C and 5% CO_2. At 60–70% confluency, cells were treated with 5 or 20 ng/mL of TGFB1 for 18 h in DMEM/F12 medium containing 1% fetal calf serum and then harvested and stored at −80°C for analysis.

Statistical analysis

All statistical analyses were carried out using Microsoft Office Excel 365 (Microsoft Redmond) and IBM SPSS Statistics for Windows, version 25 (IBM Corp.). The $2^{-\Delta Ct}$ data of gene expression in GREL cells and fetal fibroblasts was plotted as scatter plots using GraphPad Prism version 8.00 (GraphPad Software Inc.). To determine significant differences among adult fibroblasts, granulosa cells and ovarian surface epithelial cells, a one-way ANOVA and Tukey's *post hoc* tests were conducted. To compare the difference between the control and the treatments of fetal fibroblasts, one-way ANOVA with Dunnett's *post hoc* tests was conducted. Additionally, Student's *t*-test was conducted to analyse the difference between GREL cells and fetal fibroblasts and the difference between fetal fibroblasts cultured with or without EGF.

Results

Presentation of gene expression data

In Figs. 1, 2, 3 and 4, we present expression data of 25 PCOS candidate genes and 3 other genes (*AR*, *AMH*

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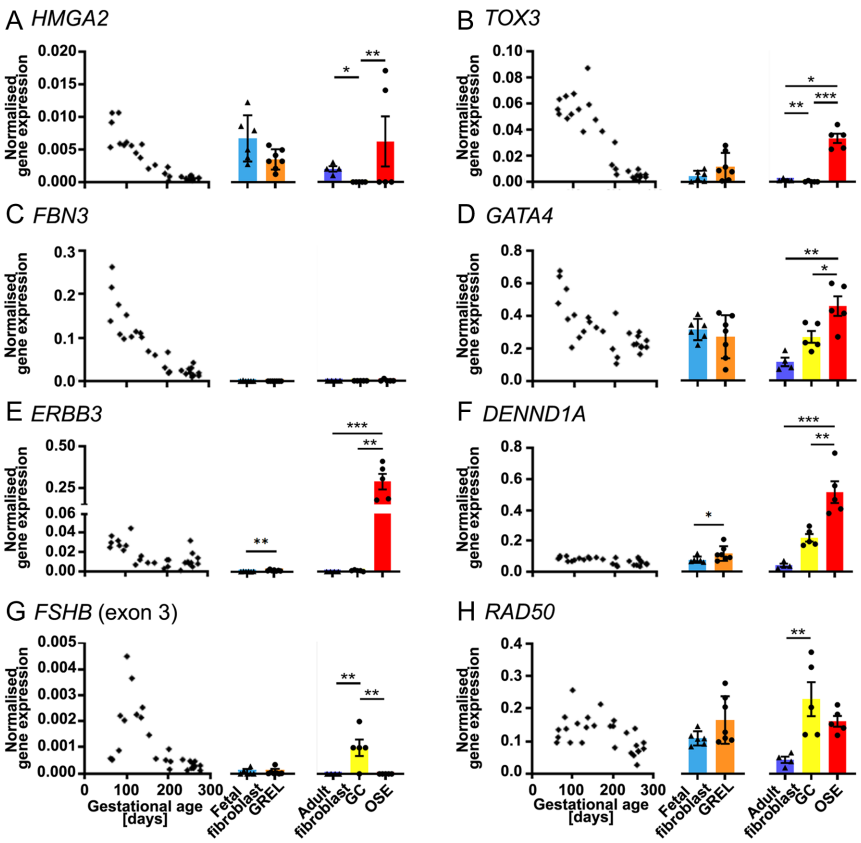

Figure 1 mRNA expression levels of genes expressed during early gestation. Scatter plots on the left in each subfigure show gene expression levels in fetal ovaries ($n = 27$) from our previous publications (Hartanti *et al.* 2020b, Liu *et al.* 2020). Gene expression levels in cultured GREL cells ($n = 7$) and fetal fibroblast ($n = 6$) are presented in the middle graph of each subfigure. Data of gene expression in adult fibroblasts ($n = 4$), granulosa cells (GC; $n = 5$) and ovarian surface epithelial cells (OSE; $n = 5$) are presented as mean ± S.E.M. (normalised to *PPIA* and *RPL32*) in the graph on the right side of each subfigure. Significant differences between the three adult cell types were determined by one-way ANOVA with Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

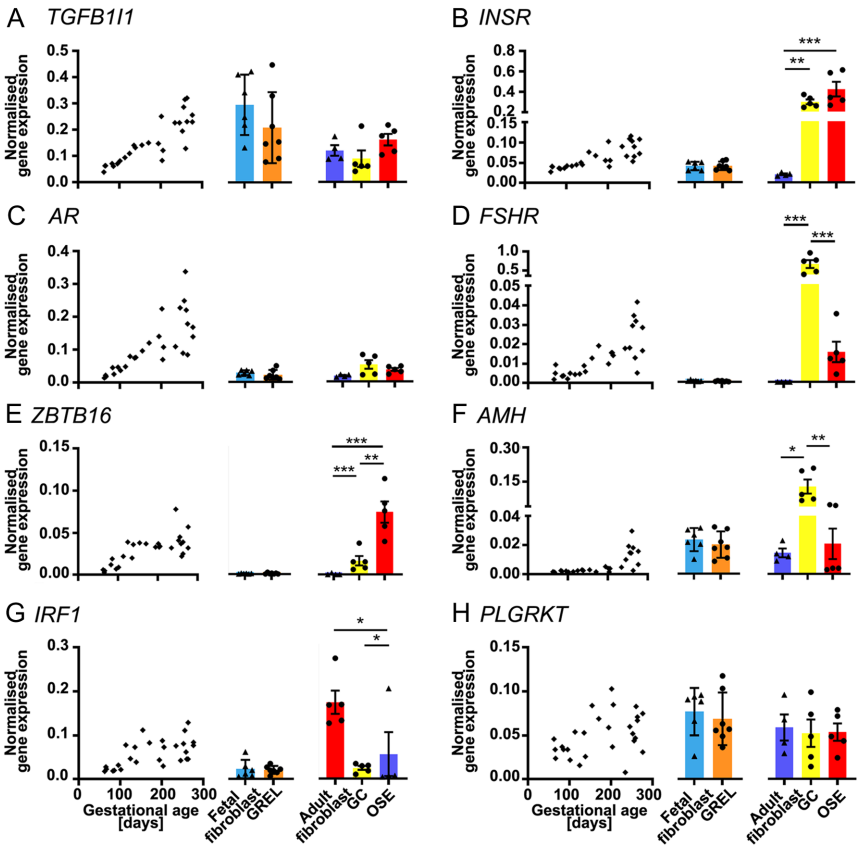

Figure 2 mRNA expression levels of genes expressed during late gestation. Scatter plots on the left in each subfigure show gene expression levels in fetal ovaries ($n = 27$) from our previous publications (Hartanti *et al.* 2020b, Liu *et al.* 2020). Gene expression levels in cultured GREL cells ($n = 7$) and fetal fibroblast ($n = 6$) are presented in the middle graph of each subfigure. Data of gene expression in adult fibroblasts ($n = 4$), granulosa cells (GC; $n = 5$) and ovarian surface epithelial cells (OSE; $n = 5$) are presented as mean ± S.E.M. (normalised to *PPIA* and *RPL32*). Significant differences between the three adult cell types were determined by one-way ANOVA with Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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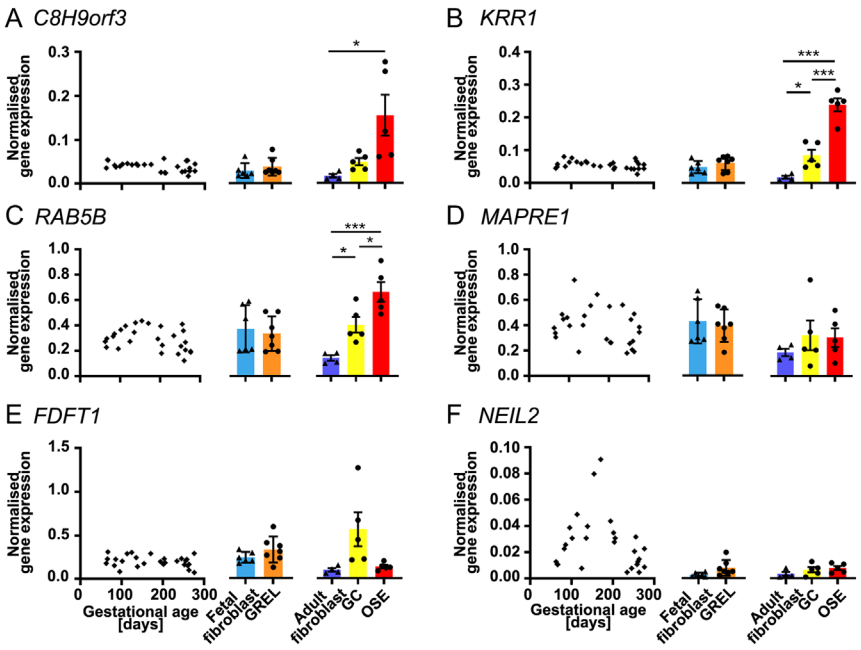

Figure 3 mRNA expression levels of genes expressed throughout gestation. Scatter plots left in each subfigure of gene expression levels in fetal ovaries ($n = 27$) are from our previous publications (Hartanti *et al.* 2020b, Liu *et al.* 2020). Gene expression levels in cultured GREL cells ($n = 7$) and fetal fibroblast ($n = 6$) are presented in the middle graph of each subfigure. Data of gene expression in adult fibroblasts ($n = 4$), granulosa cells (GC; $n = 5$) and ovarian surface epithelial cells (OSE; $n = 5$) are presented as mean ± S.E.M. (normalised to *PPIA* and *RPL32*). Significant differences between the three adult cell types were determined by one-way ANOVA with Tukey's *post hoc* test. $^{*}P < 0.05$, $^{***}P < 0.001$.

and *TGFB1I1*) and group them based on their known expression pattern across gestation in each Figure. For each gene, the expression across gestation is presented, has been published previously (Hartanti *et al.* 2020b, Liu *et al.* 2020) and is acknowledged. We then present the expression in fetal fibroblasts and GREL cells and the statistical comparison between them. We then present the expression in adult fibroblasts, granulosa cells and ovarian surface epithelial cells and the statistical comparison between them. The data for each gene from gestation and from isolated cells in Figs. 1, 2, 3 and 4 are plotted on the same scale for direct comparisons. The mean levels of expression in isolated cells and statistical comparisons of fetal and adult cells are presented in Table 1. The individual data of expression in fetal fibroblasts and GREL cells across gestation are presented in a supplementary figure.

Expression of PCOS candidate genes in fetal ovaries

The gene expression data were from our previous publications (Hartanti *et al.* 2020b, Liu *et al.* 2020). *HMGA2*, *TOX3*, *FBN3*, *GATA4*, *ERBB3*, *DENND1A*, *FSHB*

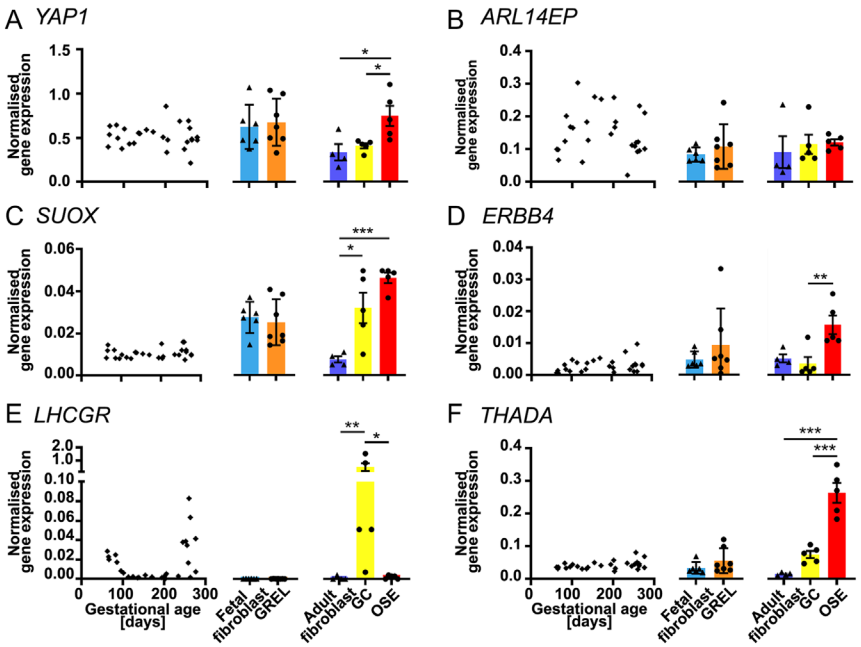

Figure 4 mRNA expression levels of genes expressed throughout gestation (continued). Scatter plots on the left in each subfigure show gene expression levels in fetal ovaries ($n = 27$) from our previous publications (Hartanti *et al.* 2020b, Liu *et al.* 2020). Gene expression levels in cultured GREL cells ($n = 7$) and fetal fibroblasts ($n = 6$) are presented in the middle graph of each subfigure. Data of gene expression in adult fibroblasts ($n = 4$), granulosa cells (GC; $n = 5$) and ovarian surface epithelial cells (OSE; $n = 5$) are presented as mean ± S.E.M. (normalised to *PPIA* and *RPL32*). Significant differences between the three adult cell types were determined by one-way ANOVA with Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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Table 1 Relative gene expression of $2^{-\Delta Ct}$ of genes presented as mean ± S.E.M. for each cell type. Comparisons are between fetal fibroblasts ($n = 6$) with adult fibroblasts ($n = 4$) and then between fetal GREL cells ($n = 7$) with adult ovarian surface epithelial cells ($n = 5$) or granulosa cells ($n = 5$).

Gene	Fetal fibroblasts	Adult fibroblasts	Fetal GREL cells	Adult ovarian surface epithelial cells	Adult granulosa cells
HMGA2	0.007 ± 0.001	0.002 ± 0.000**	0.003 ± 0.001	0.006 ± 0.004	0.000 ± 0.000***
TOX3	0.005 ± 0.002	0.002 ± 0.001	0.010 ± 0.004	0.034 ± 0.004	0.000 ± 0.000***
FBN3	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.001	0.000 ± 0.000
GATA4	0.310 ± 0.026	0.113 ± 0.026***	0.233 ± 0.054	0.452 ± 0.060*	0.265 ± 0.036
ERBB3	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.288 ± 0.047***	0.001 ± 0.000
DENND1A	0.075 ± 0.008	0.040 ± 0.010**	0.101 ± 0.021	0.500 ± 0.068***	0.213 ± 0.024
FSHB (exon 3)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.000**
RAD50	0.109 ± 0.009	0.043 ± 0.010***	0.144 ± 0.031	0.161 ± 0.016	0.228 ± 0.052
TGFB1I1	0.297 ± 0.047	0.118 ± 0.020**	0.183 ± 0.051	0.159 ± 0.022	0.087 ± 0.031*
INSR	0.040 ± 0.004	0.020 ± 0.002**	0.035 ± 0.006	0.421 ± 0.073***	0.295 ± 0.024***
AR	0.028 ± 0.003	0.018 ± 0.002*	0.019 ± 0.006	0.037 ± 0.005	0.052 ± 0.013*
FSHR	0.001 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.016 ± 0.005***	0.631 ± 0.105***
ZBTB16	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.074 ± 0.013**	0.016 ± 0.005*
AMH	0.023 ± 0.003	0.014 ± 0.003	0.017 ± 0.004	0.021 ± 0.011	0.130 ± 0.032**
IRF1	0.022 ± 0.008	0.055 ± 0.049	0.018 ± 0.004	0.172 ± 0.026***	0.024 ± 0.004
PLGRKT	0.076 ± 0.011	0.058 ± 0.015	0.059 ± 0.013	0.053 ± 0.010	0.052 ± 0.015
C8H9orf3	0.029 ± 0.007	0.017 ± 0.004	0.033 ± 0.008	0.156 ± 0.047**	0.049 ± 0.008
KRR1	0.049 ± 0.008	0.018 ± 0.005**	0.054 ± 0.011	0.241 ± 0.020***	0.085 ± 0.017
RAB5B	0.381 ± 0.077	0.144 ± 0.022*	0.300 ± 0.063	0.671 ± 0.079**	0.409 ± 0.062
MAPRE1	0.431 ± 0.072	0.191 ± 0.030*	0.346 ± 0.065	0.310 ± 0.075	0.328 ± 0.120
FDFT1	0.237 ± 0.025	0.097 ± 0.020**	0.285 ± 0.063	0.135 ± 0.019	0.565 ± 0.196
NEIL2	0.003 ± 0.001	0.003 ± 0.002	0.007 ± 0.002	0.008 ± 0.002	0.006 ± 0.002
YAP1	0.639 ± 0.105	0.343 ± 0.096*	0.606 ± 0.125	0.768 ± 0.119	0.420 ± 0.030
ARL14EP	0.082 ± 0.009	0.091 ± 0.049	0.093 ± 0.026	0.122 ± 0.010	0.116 ± 0.030
SUOX	0.028 ± 0.003	0.008 ± 0.001***	0.022 ± 0.005	0.047 ± 0.002	0.032 ± 0.007
ERBB4	0.005 ± 0.001	0.005 ± 0.001	0.008 ± 0.004	0.016 ± 0.003	0.004 ± 0.002
LHCGR	0.000 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.002 ± 0.001	0.494 ± 0.303
THADA	0.033 ± 0.007	0.015 ± 0.002**	0.048 ± 0.014	0.259 ± 0.030***	0.073 ± 0.010

Student's *t*-test was used to analyse the differences between fetal fibroblasts with adult fibroblasts. One-way ANOVA with Tukey's *post hoc* test was used to analyse the differences between GREL cells with adult ovarian surface epithelial cells or granulosa cells.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ report significant differences between the corresponding fetal cells with the adult cells.

and *RAD50* were expressed in the early gestation and then declined (Fig. 1A-H), and the expression of these genes was negatively correlated with gestational age. The gene expression level of *FSHB* was determined with primers which targeted the sequences in exons 3. However, primers to exon 2 and exon 3, which could correctly amplify *FSHB* from anterior pituitary, failed to amplify *FSHB* from ovary samples (Hartanti *et al.* 2020b), suggesting that *FSHB* transcript in fetal ovary cannot encode or produce FSHB. However, we report on the FSHB exon 3 transcript as with GWAS analyses it is not always possible to assign the encoding of a full-length gene to explain the GWAS relationship with the disease. The expression levels of *TGFB1I1*, *INSR*, *AR*, *FSHR*, *ZBTB16*, *AMH*, *IRF1* and *PLGKRT* were low in early gestation and gradually increased until the late stage of gestation (Fig. 2A-H). The other genes *C8H9orf3*, *KRR1*, *RAB5B*, *MAPRE1*, *FDFT1*, *NEIL2*, *YAP1*, *ARL14EP*, *SUOX*, *ERBB4*, *LHCGR* and *THADA* were expressed constantly throughout the gestation (Fig. 3A-F and Fig. 4A-F).

Expression of PCOS candidate genes in fetal ovarian cells

DENND1A and *ERBB3* had significantly higher expression in GREL cells than fetal fibroblasts (Fig. 1E and F; $P < 0.05$ and $P < 0.01$). Even though there were no significant differences between GREL cells and fetal fibroblasts for *HMGA2* and *TGFB1I1* (Fig. 1A and 2A), fetal fibroblasts had the tendency to have higher *HMGA2* and *TGFB1I1* expression than GREL cells of the same gestational age (Supplementary Fig. 1A1 and B1). The other 19 genes did not present significant differences between GREL cells and fetal fibroblasts (Figs. 1-4). Gene expression of *FBN3*, *ERBB3*, *FSHB*, *FSHR*, *ZBTB16* and *LHCGR* was hardly detectable in GREL or fetal fibroblasts (Figs. 1C, 1E, 1G,

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2D, 2E and 4E). The gene expression in GREL cells and fetal fibroblasts was from fetuses younger than 200 days of gestation, which was shorter than from gene expression analyses in fetal ovaries examined throughout gestation.

Expression of PCOS candidate genes in adult ovarian cells

Gene expression levels of *FSHR*, *AMH*, *LHCGR* and a splice variant of *FSHB* exon 3 in adult granulosa cells were significantly higher than in fibroblasts and ovarian surface epithelial cells (Fig. 1G, 2D, 2F and 4E; $P < 0.05$, 0.01 or 0.001). In contrast, *HMGA2* had a significantly lower expression in granulosa cells than adult fibroblasts and ovarian surface epithelial cells (Fig. 1A; $P < 0.05$, 0.01). *TOX3, GATA4*, *ERBB3*, *DENND1A*, *IRF1*, *YAP1* and *THADA* were significantly higher in ovarian surface epithelial cells (Fig 1B, 1D-1F, 2G, 4A and 4F; $P < 0.05$, 0.01 or 0.001) than adult fibroblasts or granulosa cells. *ERBB4* had a similar expression pattern but with no significant difference between adult fibroblasts and ovarian surface epithelial cells (Fig 4D; $P > 0.05$). *INSR*, *ZBTB16*, *KRR1*, *RAB5B* and *SUOX* were expressed significantly higher in both granulosa cells and ovarian surface epithelial cells than in adult fibroblasts (Fig 2B, 2E, 3B, 3C, and 4C; $P < 0.05$, 0.01 or 0.001). Similarly, *RAD50* and *C8H9orf3* had higher expression levels in either granulosa cells or ovarian surface epithelial cells than fibroblasts (Fig. 1H and 3A; $P < 0.05$, 0.01). *FBN3*, *TGFB1I1*, *AR*, *PLGKRT*, *MAPRE1*, *FDFT1*, *NEIL2*, and *ARL14EP* were expressed with no significant differences between the three adult cell types (Fig. 1C, 2A, 2C, 2H, 3D-3F and 4B; $P > 0.05$).

Fetal vs adult cells

HMGA2, *GATA4*, *DENND1A*, *RAD50*, *TGFB1I1*, *INSR*, *AR*, *KRR1*, *RAB5B*, *MAPRE1*, *FDFT1*, *YAP1*, *SUOX* and *THADA* were all more lowly expressed in adult than fetal fibroblasts (Table 1). *GATA4*, *ERBB3*, *DENND1A*, *INSR*, *FSHR*, *ZBTB16*, *IRF1*, *C8H9orf3*, *KRR1*, *RAB5B* and *THADA* were more highly expressed in ovarian surface epithelium than in GREL cells (Table 1). Gene expression of *HMGA2*, *TOX3* and *RAD50* was lower and expression of *DENND1A*, *FSHB* (exon 3), *TGFB1I1*, *INSR*, *AR*, *FSHR*, *ZBTB16* and *AMH* was higher in the granulosa cells than in the GREL cells (Table 1).

Regulation of PCOS candidate genes in fetal fibroblast

We treated bovine fetal fibroblasts ($n = 5$ from 95, 101, 119, 135 and 232 days of gestation) from fetuses in second and third trimesters with different hormones, stimulators and growth factors separately (Supplementary Table 2) and determined their effects on the expression of eight PCOS candidate genes (*ERBB3*, *ZBTB16*, *IRF1*, *PLGRKT*, *MAPRE1*, *FDFT1*, *NEIL2* and *ARL14EP*). The effects of these factors on other PCOS genes have been reported previously (Hartanti *et al.* 2020b) and effects of TGFB1 have been reported elsewhere (Hartanti *et al.* 2020b, Azumah *et al.* 2022). The current results demonstrated that apart from oestradiol (E2) and testosterone (T) of the factors tested (Supplementary Table 2) which increased the expression of *ARL14EP* (Supplementary Fig. 2E5; $P < 0.05$), other treatments had no significant effects on the gene expression ($P > 0.05$). In addition, we compared expression of genes in fetal fibroblasts cultured in EGF-containing medium ($n = 6$ from 51, 56, 73, 110, 127 and 177 days of gestation) with fetal fibroblasts cultured previously (Bastian *et al.* 2016) in medium without EGF and spanning similar times during gestation ($n = 11$ from 95, 101, 119, 135, 160, 165, 168, 170, 180, 198, 232 days of gestation). Fetal fibroblasts cultured in EGF-containing medium had lower expression of *NEIL2* (Fig. 5A; $P < 0.05$) but higher expression of *MAPRE1* (Fig. 5B; $P < 0.05$).

Discussion

This study reports on the expression of PCOS candidate genes in cultured fetal GREL cells and fetal fibroblasts from bovine fetuses younger than 200 days of gestation and compares these data to bovine ovarian fibroblasts, granulosa cells and surface epithelial cells isolated from adult ovaries as well as previously published data from whole fetal ovaries throughout the gestation (Hartanti *et al.* 2020b, Liu *et al.* 2020). Moreover, we determined the effects of different hormones and growth factors on the expression of PCOS candidate genes in order to identify potential regulators of those genes during the development of the fetal ovary.

Although many of the PCOS candidate genes were expressed in bovine fetal ovaries, *FBN3*, *ERBB3*, *FSHB*, *LHCGR*, *FSHR* and *ZBTB16* levels were very low or hardly detectable in GREL cells and fetal fibroblasts isolated and cultured *in vitro*. Fibrillin 3 (FBN3), a structural component of extracellular matrix (Corson *et al.* 2004), previously showed a drastic decline in expression in *in vitro* cultures of bovine and human fetal ovarian fibroblasts (Bastian *et al.* 2016). ERBB3, a receptor in EGF receptor signalling pathways, was mainly expressed early in gestation of human and bovine fetal ovaries (Liu *et al.* 2020). In a study

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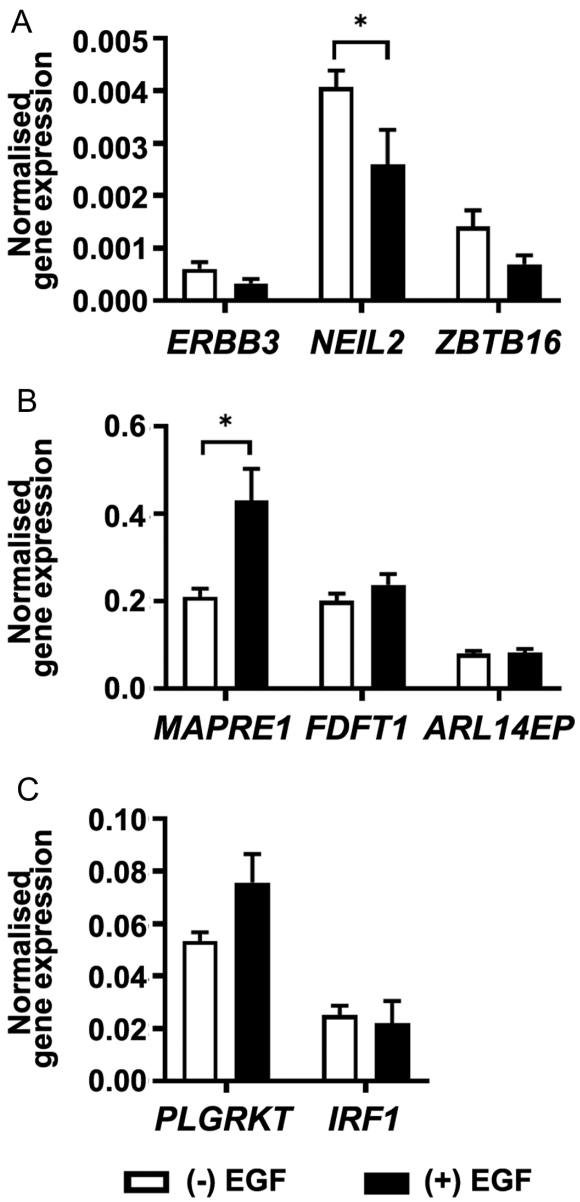

Figure 5 mRNA expression levels of eight newly examined PCOS candidate genes in fetal fibroblasts cultured in the presence or absence of EGF. Columns in white colour and black colours show gene expression in fetal fibroblasts cultured in the absence of EGF ($n = 11$) and in the presence of EGF ($n = 6$), respectively. Data of gene expression are presented as mean ± S.E.M. (normalised to *PPIA* and *RPL32*). Significantly differences between groups were determined by Student's *t*-test. *$P < 0.05$.

on mouse fetal gonads, ERBB3 was detected in primordial germ cells at 12.5 days post coitum, and the expression was downregulated at 14.5 days post coitum when primordial germ cells ceased to grow (Toyoda-Ohno *et al.* 1999). We assume that the decline of *ERBB3* expression in the fetal ovary during gestation (Liu *et al.* 2020) resulted from the differentiation of primordial germ cells into oogonia/oocytes and the expansion of stroma in the fetal ovary (Hartanti *et al.* 2019). FSHB is normally expressed in the anterior pituitary; however, low expression of an alternative splice transcript from exon 3 of FSHB has been detected in the fetal ovary (Hartanti *et al.* 2020b). The absence of *FSHB* expression in GREL cells and fetal fibroblasts indicated that the potential transcriptional activity did not exist in either of the two fetal cell types examined. The receptors for two gonadotrophins, FSH and LH (FSHR and LHCGR), were expressed mostly in late gestation when folliculogenesis begins. This was in line with the observed lack of expression in our cultured fetal GREL cells and fetal fibroblasts which were both isolated from fetal ovaries from early and mid-gestation. *LHCGR* was exclusively expressed in adult granulosa cells and confirms findings of other studies in the same and other species (Cannon *et al.* 2009, Gulappa *et al.* 2017, Convissar *et al.* 2019). *FSHR* appeared to be additionally expressed in ovarian surface epithelial cells but at a lower level than in granulosa cells. FSHR has been identified previously in ovarian surface epithelial cells (Zheng *et al.* 1996, Bose 2008) and FSH has been shown to stimulate growth of ovarian surface epithelial cells (Choi *et al.* 2002). The lack of expression of *FSHR* and *LHCGR* in GREL cells suggests that they are not regulated by FSH or LH, and in any event, these hormones are not secreted until about third to half way through gestation (Lanciotti *et al.* 2018). In mouse granulosa cells, the progesterone receptor binds to an intron of ZBTB16 (Dinh *et al.* 2019) and expression of the progesterone receptor can induce the expression of ZBTB16 (Sriraman *et al.* 2010). Moreover, the progesterone receptor was also found to be expressed in human and non-human primate ovarian surface epithelial cells (Lau *et al.* 1999, Wright *et al.* 2011). Therefore, it could be that the expression of *ZBTB16* in adult granulosa cells and ovarian surface epithelial cells would be relevant for the action of progesterone and the expression of *ZBTB16* in the fetal ovary at the late stage of gestation is likely due to the formation of granulosa cells and ovarian surface epithelial cells.

HMGA2 and *TGFB1I1* were the two genes which had the highest correlations with gestational age in developing bovine fetal ovaries (Hartanti *et al.* 2020b); *HMGA2* was highly expressed in early and *TGFB1I1* late in gestation. High mobility group A (HMGA1 and HMGA2) are small non-histone proteins which can bind DNA and modify the chromatin state. In the human ovary, *HMGA2* was found to be overexpressed in ovarian carcinoma (Tagawa *et al.* 2010) and global gene analysis has revealed HMGA2-mediated tumourigenesis in the ovary (Wu *et al.* 2011). Moreover, the expression of *HMGA2* induced cell proliferation with

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increased expression of cyclin B2 and cyclin E in umbilical cord blood-derived stromal cells and pituitary adenomas (De Martino *et al.* 2009, Yu *et al.* 2013). Likewise, fetal ovarian fibroblasts exhibited greater proliferation and higher gene expression of cyclin B2 and cyclin E than GREL cells (Liu *et al.* 2022), and fetal fibroblasts tended to have potentially higher expression of *HMGA2*. In the bovine fetal ovary, stroma exhibited a higher proliferation index early in gestation than in late gestation (Hartanti *et al.* 2019). Therefore, the high expression of *HMGA2* in early gestation might be responsible for cell proliferation in the fetal ovary, which is critical to early organogenesis (Vignali & Marracci 2020). *TGFB1I1* was expressed higher in late gestation and showed no differences among adult cell types. *TGFB1I1* encodes the coactivator of androgen receptor (AR) and affects cell adhesion, growth and motility which are regulated by androgen (Leach *et al.* 2014). *TGFB1I1* and other genes involved in TGFB1 signalling (*TGFB2* and *3, LTBP2-4, TGFBR2* and *3*) and *AR*, which was highly correlated with *TGFB1I1*, exhibited increased expression levels after 200 days of gestation (Hatzirodos *et al.* 2019, Hartanti *et al.* 2020b). *TGFB1I1* gene expression was detected in adult human cumulus cells and it exhibited higher expression levels in normal cumulus cells than cumulus cells from PCOS ovaries (Haouzi *et al.* 2012). Therefore, TGFB1 signalling potentially is active during the later stages of gestation.

In the adult bovine ovarian cells, *TOX3*, *GATA4*, *DENND1A* and *RAD50* were all predominantly expressed in epithelial cells (granulosa cells and ovarian surface epithelial cells). Combined with the higher expression of *DENND1A* in GREL cells, the declining expression of these four genes in the developing ovary may be due to the increasing proportion of fibroblasts and stroma during gestation (Hartanti *et al.* 2019). On the contrary, *INSR*, *AMH* and *IRF1* which are also expressed predominantly in epithelial cells (granulosa cells and ovarian surface epithelial cells) increased in expression with increasing gestational age (Hartanti *et al.* 2020b). In the human ovary, *AMH* shows only weak expression in granulosa cells of primordial follicles but high expression levels in granulosa cells of secondary, pre-antral and small antral follicles (Weenen *et al.* 2004). *INSR* encodes the insulin receptor, and its expression in granulosa cells is essential for the differentiation of granulosa cells and ovulation (Sekulovski *et al.* 2020). Gene expression of *IRF1* is found in primary cultured human ovarian surface epithelial cells (Zeimet *et al.* 2009), and IRF1 is involved in the regulation of the class II tumour-suppressor *H-REV107-1* in human ovarian surface epithelial cells (Sers *et al.* 2002). Moreover, these three genes were not predominantly expressed in GREL cells. Therefore, we assume that GREL cells do not express *AMH*, *INSR* or *IRF1* until they differentiate into granulosa cells or ovarian surface epithelial cells. Similarly, *C8H9orf3*, *KRR1*, *RAB5B*, *YAP1*, *SUOX*, *ERBB4* and *THADA* demonstrated differences in adult cell types but were constantly expressed throughout the gestation, which indicated those genes did not exhibit differences during gestational stages until neonatal stage or adulthood. On the contrary, the expression of *AR* and *PLGRKT* in the fetal ovary was correlated with gestational age, but the expression of the two genes did not show significant differences among adult cell types. *AR* had a higher expression level in the fetal ovary during the third trimester than adult fibroblasts, ovarian surface epithelial cells and granulosa cells, which was consistent with the earlier comparison of *AR* expression in the fetal ovary and adult ovary (Hartanti *et al.* 2020b). Previous studies demonstrated that high levels of androgen from maternal sources during gestation could lead to a fetal origin of PCOS and result in PCOS in adolescence and adulthood (Sir-Petermann *et al.* 2002, Rosenfield & Ehrmann 2016, Walters 2016, Filippou & Homburg 2017). *PLGRKT* encodes the unique receptor of plasminogen, which is a precursor of plasmin, involved in the degradation of blood plasma clots, and is present in blood. Unlike *AR*, *PLGRKT* demonstrated consistent expression in the fetal ovary indicating that *PLGRKT* was expressed throughout the ovary since the ovarian vascular system was established when the vascularised stroma penetrated into the ovary (Hummitzsch *et al.* 2013). Similarly, *MAPRE1*, *FDFT1*, *NEIL2* and *ARL14EP* were constantly expressed throughout gestation and exhibited no differences in adult cells. Perhaps, they are essential for the normal physiology of ovarian cells from early development of the ovary onwards.

The effects of 18 h of treatments by different hormones, stimulators and growth factors on the expression of PCOS genes in bovine fetal fibroblasts have been reported earlier (Hartanti *et al.* 2020b, Azumah *et al.* 2022), and the current study completes the reporting with a further eight PCOS candidate genes (*ERBB3*, *ZBTB16*, *IRF1*, *PLGRKT*, *MAPRE1*, *FDFT1*, *NEIL2* and *ARL14EP*) examined. Overall, TGFB1 was by far the most important regulator, inhibiting 7 (*INSR*, *C8H9orf3*, *RAD50*, *ERBB3*, *NEIL2*, *IRF1* and *ZBTB16*) of the 25 PCOS candidate genes (Azumah *et al.* 2022). We have also considered that TGFB1 action in the fetal ovary maybe important for the development of PCOS in later life as it also inhibits the expression of *AR* and stimulates its cofactor *TGFB1I1* (Hartanti *et al.* 2020b).

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In the current study, oestradiol and testosterone increased the expression of *ARL14EP*. We also compared the expression of genes in fetal fibroblasts cultured for long term previously (Liu *et al.* 2022) in EGF-containing medium with fetal fibroblasts cultured previously (Bastian *et al.* 2016) in medium without EGF. Fetal fibroblasts cultured in EGF-containing medium had lower expression of *NEIL2* but higher expression of *MAPRE1*. The effects were relatively small but statistically significant and the physiological relevance is not clear at this stage.

Summary and conclusions

The fetal bovine ovary has two major somatic cell types shown previously to be of different cellular origins (Hummitzsch *et al.* 2013) and different morphologies and to differentially express 15 genes (Liu *et al.* 2022). Despite minimal differences between GREL cells and fetal fibroblasts being observed in the current study, there were differences with adult cells. While 14 genes were more lowly expressed in adult fetal fibroblasts than fetal fibroblasts, 11 genes were more highly expressed in ovarian surface epithelium than in GREL cells. Three genes were lower and eight genes higher in granulosa cells than in the GREL cells. One caveat of the current studies is that the cells had to be cultured to achieve purification, and as we showed here and previously, culture (Bastian *et al.* 2016) and the culture media influence the gene expression patterns, at least in ovarian fetal fibroblasts. Clearly, there is a need for a better system of purification of the two somatic cell types to be able to better identify expression patterns of PCOS genes in these cells *in vivo*. This is likely critical for a full understanding of the aetiology of PCOS.

Supplementary material

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-220068.

Declaration of interest

R A A reports consultancy work for Ferring, Merck, IBSA, Roche Diagnostics, NeRRe Therapeutics and Sojournix Inc. The other authors of this manuscript have nothing to declare and no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Ethical approval

The bovine ovaries used in this study were scavenged from animals that were being processed for human consumption and were not owned by the authors or their institutions. As such the University of Adelaide's Animal Ethics Committee only requires notification of this.

Author contribution statement

M L was involved in conceptualisation, formal analysis, investigation, methodology and writing. K H was involved in conceptualisation, formal analysis, funding acquisition, methodology, project administration, resources and editing. R J R was involved in conceptualisation, formal analysis, funding acquisition, methodology, project administration, resources, writing. R A A was involved in funding acquisition, writing, reviewing and editing. N A B and M H D were involved in methodology. H F I-R was involved in project administration and writing.

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