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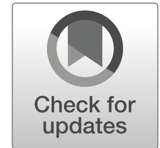

Transcription profile of the insulin-like growth factor signaling pathway during human ovarian follicular development

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Abstract

Purpose The IGF signaling cascade exerts important regulatory functions in human ovarian folliculogenesis. The scope of this study was to evaluate the transcription profile of insulin-like growth factor (IGF) genes during human ovarian follicle development and to analyze follicle fluid levels of key IGF proteins.

Methods Gene expression profiling was performed with microarray gene analysis. The analysis was assessed from ovarian follicles and granulosa cells (GCs) obtained from isolated stage-specific human ovarian follicles, including preantral follicles, small antral follicles, and preovulatory follicles. Numerous genes involved in the IGF signaling pathway was evaluated and key genes were validated by qPCR from GCs. Protein levels of various IGF components of human follicular fluid (FF) were measured by ELISA and time-resolved immunofluorometric assays (TRIFMA).

Results The gene expression levels of *PAPPA*, *IGF2*, *IGF receptors* and intracellular IGF-activated genes increased with increasing follicle size. This was especially prominent in the late preovulatory stage where *IGF2* expression peaked. Protein levels of intact IGF binding protein-4 decreased significantly in FF from large preovulatory follicles compared with small antral follicles concomitant with higher protein levels of PAPP-A. The IGF modulators IGF-2 receptor, IGFBPs, stanniocalcins, and IGF-2 mRNA binding proteins were all observed to be expressed in the different follicle stages.

Conclusions This study confirms and highlights the importance of PAPP-A regulating bioactive IGF levels throughout folliculogenesis and especially for the high rate of granulosa cell proliferation and expression of key ovarian hormones important in the last part of the follicular phase of the menstrual cycle.

Keywords IGF system · IGFBPs · PAPP-A · Stanniocalcins · Human ovarian follicles

Introduction

Ligands of the insulin-like growth factor (IGF) system entails two small peptides IGF-1 and IGF-2 structurally related to proinsulin. IGFs are intimately involved in cell growth, differentiation, and metabolism in numerous cell types and tissues [1–3] and initiate signaling through the IGF receptor type 1 (IGF1R) [4]. The IGFs also interact with the insulin receptor (INSR) although with lower affinity than that of insulin [5, 6]. In the circulation, the total level of IGFs is approximately 1000 times higher than insulin, which makes masking of IGF bioactivity extremely important. Regulation of the biological activity of the IGFs is very complex and involves six different IGF-binding proteins (IGFBP-1 to 6) that bind the IGFs with different affinities. Hereby, the IGFBPs prolong the IGFs' half-lives or block the signaling to a varying extent [6, 7].

Specifically, limited proteolytic cleavage of IGFBPs is believed to be a major mechanism by which bioactive IGF is released from IGFBP/IGF complexes. One key IGFBP protease is pregnancy-associated plasma protein-A (PAPP-A),

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which cleaves IGFBP-2, IGFBP-4, and IGFBP-5, hereby releasing bioactive IGFs [8, 9]. Cleavage of IGFBP-4 may be limited to PAPP-A only, while other proteinases are capable of cleaving IGFBP-2 and IGFBP-5 [9, 10]. Cleavage of IGFBP-5 is also accomplished by PAPP-A2 (the only global homolog of PAPP-A), HTRA serine peptidase 1 (HTRA1), and the metalloproteinase ADAM9 [11–13]. Cleavage of IGFBP-5 appears to be crucial for proteolysis of IGFBP-4/IGF complexes by PAPP-A since IGFBP-5 sequesters IGFs from IGFBP-4 [14].

When the IGFs bind and activate their corresponding receptors, intracellular signaling proteins termed insulin receptor substrate 1 and 2 (IRS1 and IRS2) become phosphorylated and subsequently activate additional signaling proteins such as AKT that activates intracellular pathways important for cell growth and metabolism [1, 15].

IGF activity is also modulated by the IGF-2 receptor (IGF2R), which is homologous to the cation-independent mannose-6-phosphate receptor. IGF2R internalizes the IGFs upon binding and transports the ligands to lysosomes, thus, downregulating IGF bioavailability [16]. Additionally, the IGF2R exists as a soluble form found in the circulation, where it reduces the biological activity of IGF-2 [17, 18].

Several studies have shown that the IGF system impact on human ovarian follicular development [19–23]. The IGFs' signal via the IGF1R that is expressed on both granulosa cells (GCs) and theca cells, and has an effect on follicular growth, steroidogenesis, and development important for a successful pregnancy [23–26]. In cultured bovine and human theca cells, androgen production is enhanced when stimulated with IGF-2 in synergy with LH, while androgen production is blocked by adding an IGF1R antibody [27, 28]. Previously, IGF-2 have shown to stimulate estradiol production and follicle diameter in human preantral follicles in culture [29]. This effect was inhibited by IGFBP-4. Further, FSH has been shown to enhance IGF-2 expression in cultured human cumulus cells, which synergistically with FSH increased cell proliferation and expression of *CYP19a1* [30]. A recent study in our laboratory supports these earlier studies and proposed that PAPP-A plays an important role in regulating IGF activity [31]. In addition, PAPP-A antigen was found to shift from the theca cells in the small antral follicles to the GCs layer in larger antral follicles with highest staining in GCs from preovulatory follicles, possibly reflecting importance of an augmented IGF activity mediating a high proliferation rate of GCs in growing antral follicles.

Women who become pregnant after undergoing ovarian stimulation, which results in high levels of estradiol, show significantly lower levels of PAPP-A as compared to women who become pregnant naturally [32, 33]. This has been measured at the end of the first trimester in connection with prenatal screening, and the reference interval for normal PAPP-A levels is reduced if the woman underwent ovarian stimulation. In fact, the higher the estradiol concentration during ovarian stimulation, the lower the levels of PAPP-A at prenatal screening [32], which suggests that aberrant IGF signaling is an underlying cause. Fetal growth and development may also be influenced by this abnormal PAPP-A/IGF environment since studies have shown that low levels of PAPP-A associate with high risk of having a child with low birth weight [34, 35].

Previously, two potent inhibitors of PAPP-A, stanniocalcin 1 and 2 (STC1 and STC2), have been characterized [36, 37]. These proteins are known to be expressed and form complexes with PAPP-A in the human ovaries [38]. Additionally, the proteolytic function of PAPP-A is inhibited by the proform of eosinophil major basic protein (proMBP), which forms a heterotetrameric disulfide-bound 2:2 complex with PAPP-A [39, 40].

Post-transcriptional regulation of IGF-2 is an extra layer of control, which occurs via IGF-2 mRNA binding proteins (IMPs). Previous studies have shown antigen levels of IMPs in the adult human ovary [41].

Thus, numerous reports suggest that the IGF system is intimately involved in ovarian follicle development and pregnancy and involves a highly complex regulation of bioactive IGFs.

The aim of the present study was to analyze the transcription profile of IGF genes in isolated stage-specific human follicles or granulosa cells spanning the entire human folliculogenesis and to evaluate follicular fluid (FF) levels of IGF proteins.

Materials and methods

Human ovarian tissue

Granulosa cells and FF from preantral and antral follicles (prior to the preovulatory stage) for mRNA microarray analysis, qPCR, ELISA, and TRIFMA was isolated from patients undergoing fertility preservation by having one ovary excised. This procedure is offered to women diagnosed with cancer or other diseases, where gonadotoxic treatments leave the patients with a high risk of infertility. Only the cortex tissue is cryopreserved, whereas the medulla tissue is normally discarded or used for research purposes [42, 43]. The patients included in this study were diagnosed with mainly breast cancer, Hodgkin's lymphoma, cervical cancer, and others. None of the patients ($N = 48$, aged 24–34 years (median = 30)) had any endocrinological and/or ovarian disease. Preantral follicles were isolated as previous described [44] with a diameter ranging from approximately 45 to 200 μm. Isolated preantral follicles from the same patient were pooled and snap-frozen in liquid nitrogen or lysed in RNA lysis buffer and stored at − 80 °C until RNA purification. FF from small antral follicles exposed on the surface of the ovary was collected with a small

syringe, and the GCs were isolated from the FF by centrifugation [45]. The small antral follicles ranged in size from 4 to 6 mm in diameter. GCs and FF were snap-frozen in liquid nitrogen and stored at − 80 °C until RNA purification and measurements.

GCs and FF from preovulatory follicles were obtained from women (N = 24, aged 27.9 ± 3.4 years) undergoing IVF treatment. One sample was obtained prior to ovulation induction (pre-OI) and one sample from the same woman was obtained at oocyte aspiration 36 h after ovulation induction (post-OI) as previously described [46, 47]. None of the women had any endocrine abnormalities (e.g., PCOS, endometriosis). Additionally, mural and cumulus cells from preovulatory follicles post-OI were collected from IVF patients (N = 20, aged 24–33 years) as previously described [48]. These patients were referred for IVF due to male factor and/or tubal disease, unexplained infertility, and mild endometriosis.

The use of surplus ovarian tissue, FF, and GCs was approved by the Ethical Committee of the Capital Region (nos. H-4-2011-102 and H-2-2011-044). The use of GCs from follicles pre- and post-OI was approved by the Danish Scientific Ethical committee (SJ-156) and conducted in accordance with Helsinki Declaration II. The use of paired mural and cumulus cells from preovulatory follicles were approved by the Danish Ethical Committee (VN2004/61).

Microarray

Microarray data from four previously published studies were analyzed (Table 1 shows references, number of follicles included in each stage, and the section describing statistical methods shows how normalization was performed) [46–49]. Despite being published in separate studies, all data were generated on the same platform and by the same laboratory: all on the Affymetrix Human Gene ST v1.0 GeneChip array platform (Affymetrix, Santa Clara, California, USA). The amplification and labeling were performed using the Pico amplification kit version 2 from Nugen (Nugen, San Carlos, CA, USA) following the manufacturer's guidelines. All arrays were stained with phycoerytrin conjugated streptavidin (SAPE) using the Affymetrix Fluidics Station®450 and scanned in the Affymetrix GeneArray® 2500 scanner to obtain fluorescent images as described previously [49]. RNA quality was evaluated with an Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Pico LabChip (Agilent Technologies, Waldbronn, Germany) and all samples showed a high RIN value. These previous studies have not examined the expression of genes related to the IGF system in detail; only a minor analysis has been included in earlier studies showing expression of IGF genes in the preantral stages and *IGF2* expression in the preovulatory stage [47, 49]. Microarrays included in this study covered the entire human folliculogenesis from the preantral stage to after induction of ovulation.

Validation of microarray results by quantitative reverse transcriptase (RT)-PCR

For the RT-PCR analysis, GCs were isolated from FF from small antral follicles with a diameter of approximately 6 mm during the cryopreservation procedure. In addition, RT-PCR analysis was also performed on paired mural and cumulus cells from preovulatory follicles post-OI aspirated from women undergoing IVF treatment.

RNA isolation for quantitative RT-PCR

RNA for quantitative RT-PCR measures was isolated from the GCs using Trizol reagents (cat. no. 15596026, Ambion; Life Technologies) and 1-bromo-3-chloropropane (cat. no. B9673-200ml; Sigma) and subsequently with an Rneasy minikit 250 (cat. no. 74106; Qiagen) following the manufacturer's protocol.

Quantitative RT-PCR analysis

For each sample, first-strand cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit from Invitrogen (Invitrogen, 2012; Life Technologies Corporation) following the manufacturer's protocol. First-strand cDNA was stored at − 80 °C until quantitative RT-PCR analysis.

Quantitative RT-PCR analysis was performed using TaqMan technology (Applied Biosystems), applying TaqMan Gene Expression Master Mix from Invitrogen (Invitrogen, 2012; Life Technologies Corporation) and predesigned Taq-Man Gene Expression Assays for the following genes: *IGFBP5* and *PAPP-A* (ID = Hs00181213_m1 and Hs01032307_m1) (Invitrogen, 2012; Life Technologies Corporation). The cDNA samples were amplified in duplicates using the LightCycler 480 quantitative PCR instrument

Table 1 Overview of the included ovarian tissue samples for microarray analysis

Follicle stage	Reference	Number of follicles
Preantral follicles (from < 60 μm to > 150 μm in diameter)	[46, 49]	125
Antral follicles 4–6 mm in diameter	[46]	5
Preovulatory follicles (pre- and post-OI) > 13 mm in diameter	[47]	18
Preovulatory follicles (post-OI)	[48]	35

(Roche). The expression of *IGFBP5* and *PAPP-A* was normalized to *GAPDH*, and relative quantification according to the comparative cycle threshold method (LightCycler480 Software, Roche) was used to quantify gene expression.

ELISA measurements

Quantification of total and intact IGFBP-4 protein together with total IGFBP-5 protein and detectable free IGF-2 (no pretreatment was performed to separate IGF-2 from its binding proteins) in FF from small antral follicles and in FF from preovulatory follicles pre- and post-OI were performed with ELISA (AL-126, AL-127, AL-128, and AL-131; Ansh Labs, Texas, USA). This was completed according to the manufacturer's instructions and calibrator A (0 ng/ml antigen) supplied with the ELISA kits was used for FF dilution. FF were diluted 1:2 for IGFBP-4 measurements, 1:5 for IGFBP-5, and 1:3 for IGF-2 measurements.

This study also combined ELISA data from previous studies of PAPP-A protein level in FF from small antral follicles and in FF from preovulatory follicles pre- and post-OI [31, 50].

Time-resolved immunofluorometric assay

Quantification of STC2 protein was measured in FF from small human antral follicles and in FF from preovulatory follicles pre- and post-OI using a time-resolved immunofluorimetric assay (TRIFMA) based on a previously published assay [36]. Samples were diluted 1:10 in 1% (wt/vol) BSA, TBS-Tween 0.05% (pH 7.4), and incubated in monoclonal STC2-coated wells (2 μg/ml, [36]) at 37 °C for 1 h. After washing, 2 μg/ml biotinylated STC2 antibody [36] was diluted in 1% BSA and 0.05% TBS-Tween and incubated for 2 h. After washing, Eu^{3+}-labeled streptavidin was diluted 1:1000 in TBS-T supplemented with 25 μM EDTA and incubated at RT for 1 h. Subsequently, wells were washed and enhancement buffer (Perkin Elmer) was added. The resulting fluorescent was measured using an Enspire Multimode Plate Reader (Perkin Elmer) with excitation 340 nm/emission 615 nm. Blank values were subtracted, and data were analyzed using GraphPad Prism 7.

Statistical analyses

The microarray data analysis was accomplished with R 3.5.1 with the limma and frma packages from Bioconductor (version 3.8). Background subtraction, quantile normalization of probe expression, and summarizing of probe intensities into gene expression were performed using the frozen Robust Multichip Average (fRMA) preprocessing algorithm established by McCall and colleagues [51, 52] and used in several previous studies [46, 53, 54]. The fRMA algorithm allows one to examine microarrays separately and afterwards combine the data for analysis. Information on estimates of probe-specific effects and variances were available from large microarray databases, and these were used on the new arrays to normalize and summarize data. This is important to remove variation from the different arrays and making them comparable [51, 52]. In order to test for statistically significant difference between the expression levels of the selected genes in the different follicular stages, a moderated *t* test after linear model fit was used.

Analysis of the qPCR, ELISA, and TRIFMA results from the group of small antral follicles and the groups of preovulatory follicles obtained from women undergoing IVF treatment was performed using a *t* test, since the GCs and FF samples from women undergoing IVF was paired. When analyzing intact IGFBP-4 levels, the Wilcoxon rank sum and Wilcoxon signed-rank test were used since these values did not follow a normal distribution. A p value below 0.05 was considered statistically significant.

Results

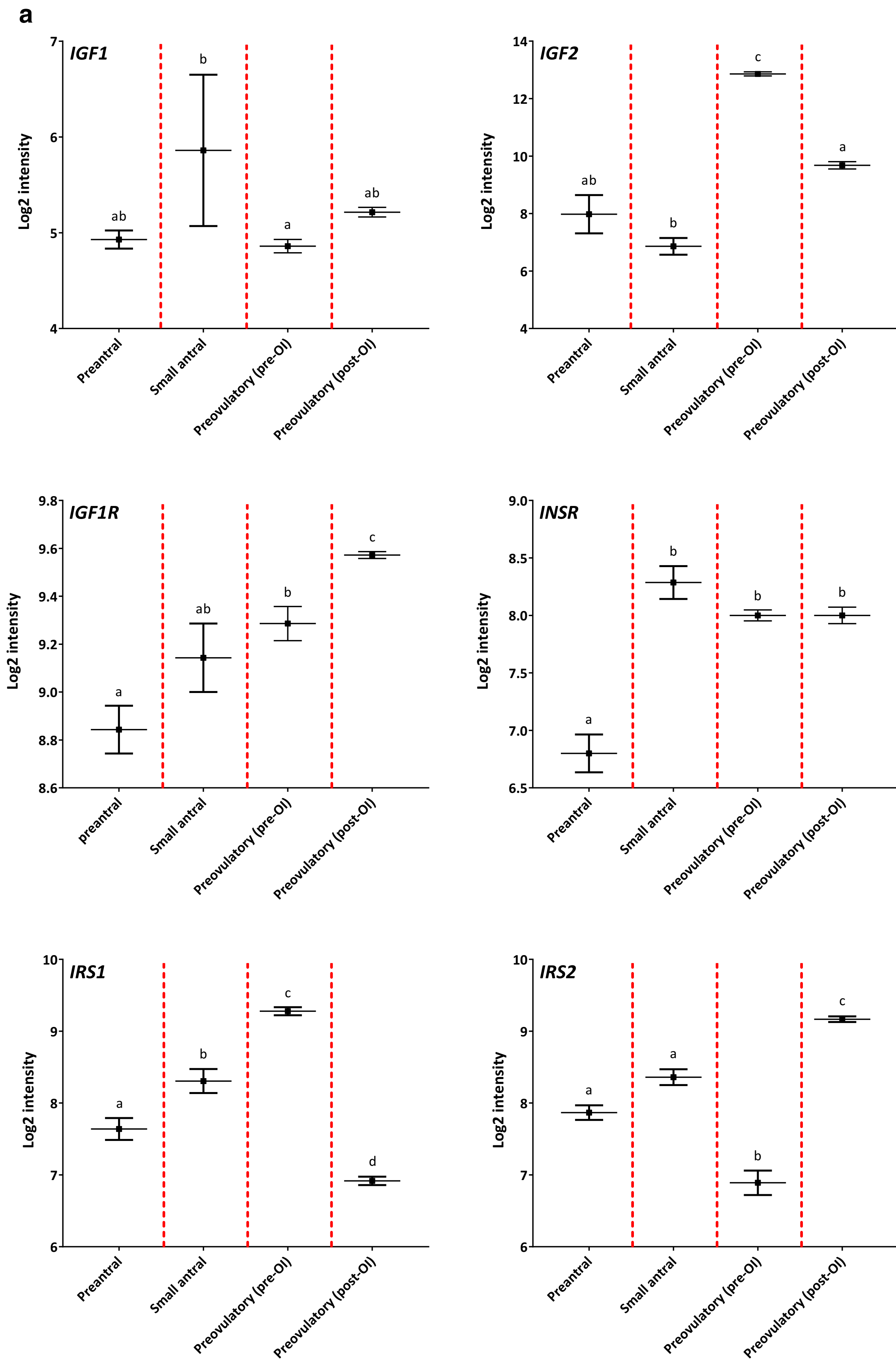
Microarray

A total of 85 individual microarrays from individual GC or follicle samples were included in this study (Table 1). In this study, we describe log2 intensity values ≤ 6 as low gene expression, log2 intensity values between 6 and 10 as moderate gene expression, and log2 intensity values ≥ 10 as high gene expression (Fig. 1).

Insulin-like growth factor 1 and 2 (IGF1 and IGF2)

The expression level of *IGF1* was overall low in human GCs irrespective of follicular developmental stage (Fig. 1a). In contrast, *IGF2* was highly expressed and showed pronounced fluctuations throughout follicular development (Fig. 1a). The expression of *IGF2* was moderate in preantral follicles and in small antral follicles, but from this nadir *IGF2* expression increased approximately 64-fold in GCs from preovulatory follicles collected pre-OI ($p < 0.001$) (Fig. 2c). In GCs collected

Fig. 1 Gene expression profiles of IGF genes during human folliculogenesis. The log2-transformed intensity from the microarray gene expression dataset covering the human folliculogenesis is shown on the *y*-axis. The following four follicle/granulosa cell (GC) groups are shown on the *x*-axis: preantral follicles from < 60 μm to > 150 μm in diameter (Preantral), GCs from 4 to 6 mm small antral follicles (Small antral), GCs isolated prior to ovulation induction (Preovulatory (pre-OI)), and GCs isolated after ovulation induction (Preovulatory (post-OI)). The gene name is shown in the upper left corner for each expression profile. Panel (**a**) shows the profile for *IGF1*, *IGF2*, *IGF1R*, *INSR*, *IRS1*, and *IRS2*. Panel (**b**) shows the profile for *IGF2R* and *IMP3*, *IGFBP2*, *IGFBP3*, *IGFBP4*, and *IGFBP5*. Panel (**c**) shows the profile for *PAPP-A*, *ProMBP*, *STC1*, and *STC2*. Different letters between follicle/GC groups indicate statistical significance. Error bars indicate SEM values


a
IGF1
Log2 intensity
7
6
5
4
ab
b
a
ab
Preantral
Small antral
Preovulatory (pre-OI)
Preovulatory (post-OI)
IGF2
Log2 intensity
14
12
10
8
6
4
ab
b
c
a
Preantral
Small antral
Preovulatory (pre-OI)
Preovulatory (post-OI)
IGF1R
Log2 intensity
9.8
9.6
9.4
9.2
9.0
8.8
8.6
a
ab
b
c
preantral
Small antral
Preovulatory (pre-OI)
Preovulatory (post-OI)
INSR
Log2 intensity
9.0
8.5
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7.5
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Preantral
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Log2 intensity
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Preovulatory (post-OI)
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Log2 intensity
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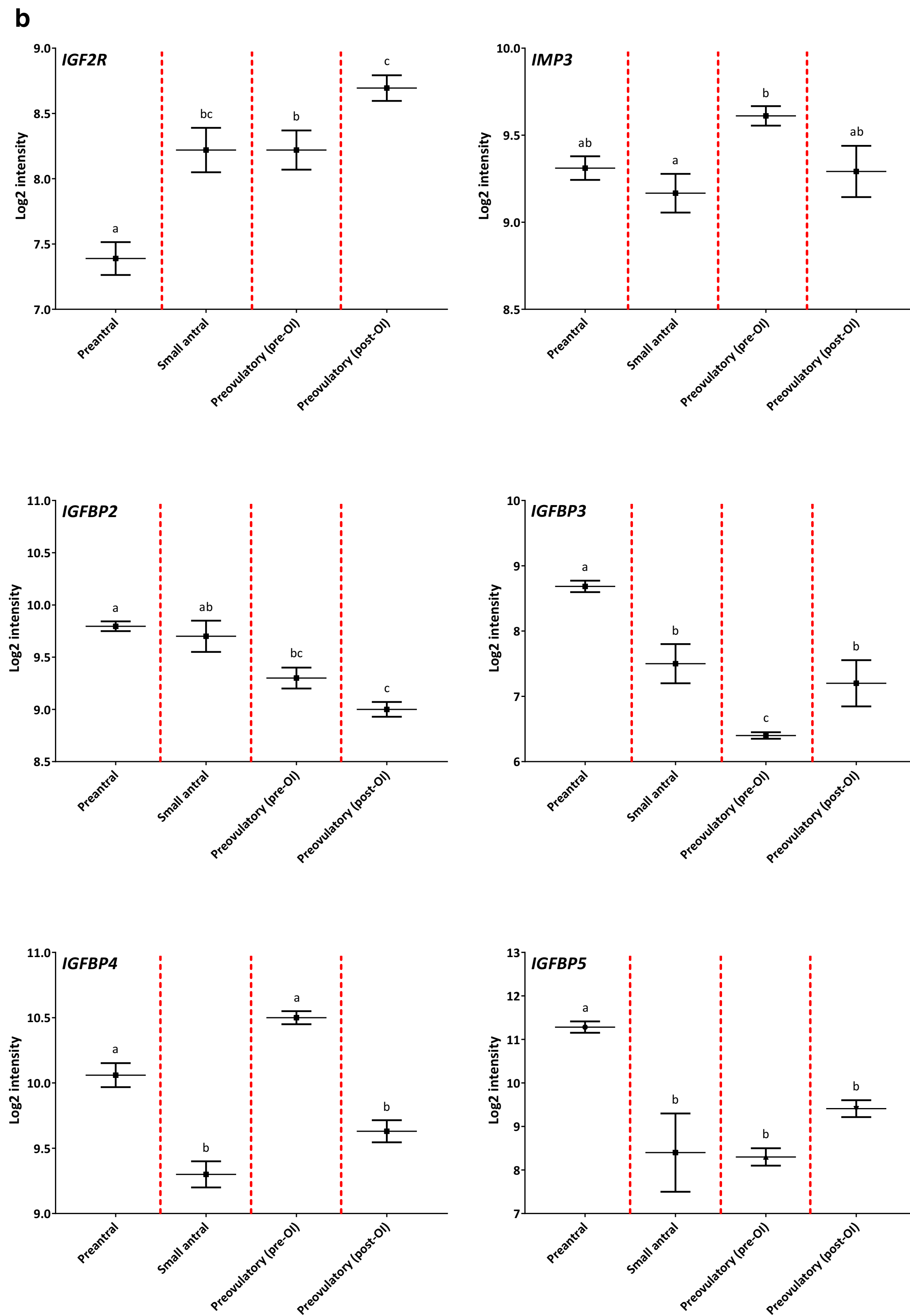

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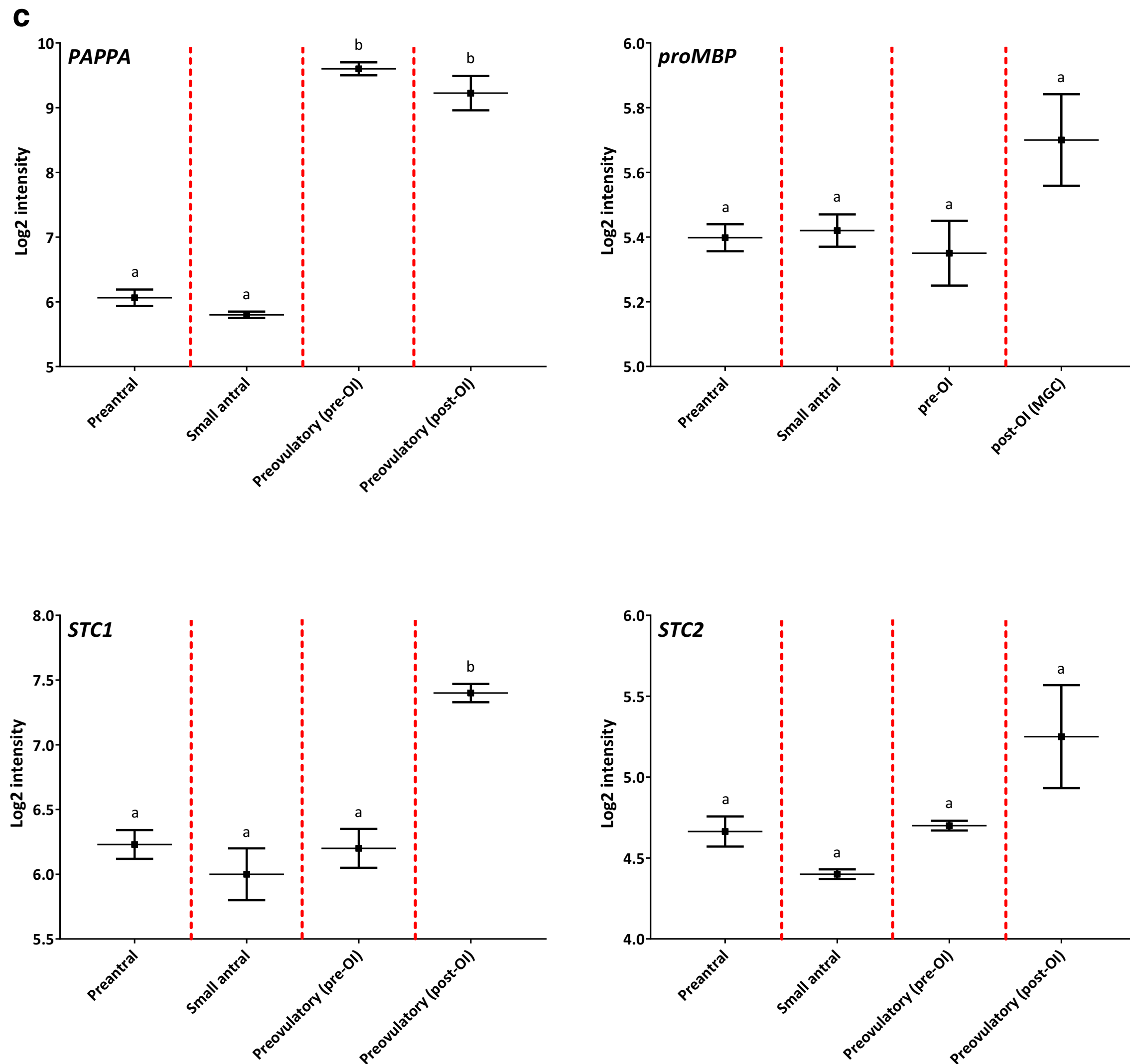


Fig. 1 (continued)

from preovulatory follicles post-OI, the *IGF2* expression was reduced 8-fold compared to the peak at pre-OI, but the expression level was significantly higher compared to the level observed in GCs from small antral follicles ($p < 0.001$).

Insulin-like growth factor receptor type 1 (IGF1R) and insulin receptor (INSR)

The expression level of *IGF1R* and *INSR* remained moderate throughout follicular development with an increase as follicle diameter increases (Figs. 1a and 2).

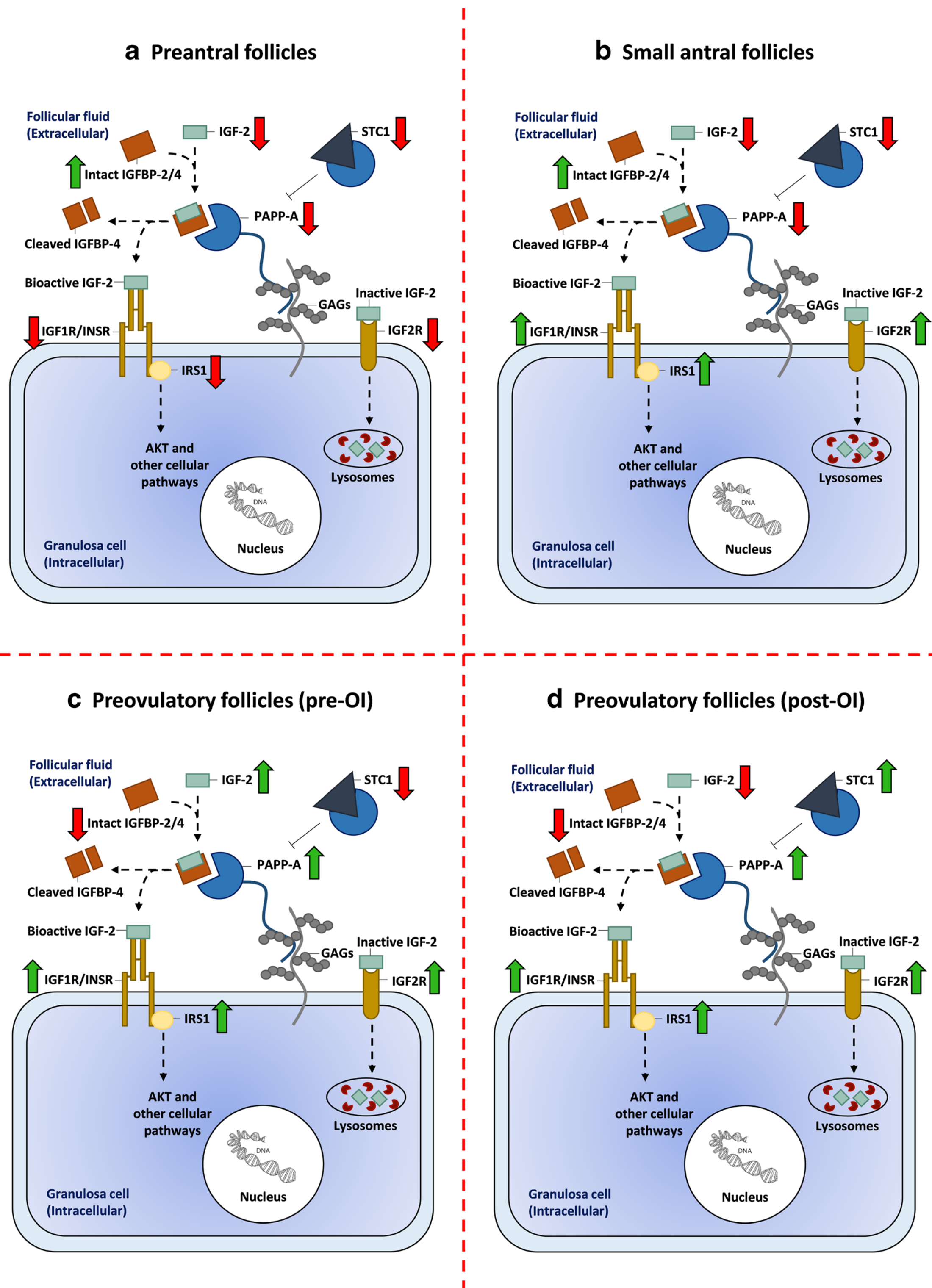
Intracellular signaling proteins (IRS1 and IRS2)

The expression level of *IRS1* was moderately expressed in human follicles (Fig. 1a). An increase was observed from preantral follicles to small antral follicles (Figs. 1a and 2). The expression of *IRS1* increased significantly in GCs from preovulatory follicles pre-OI compared with small antral follicles ($p < 0.001$), while the expression level was significantly lower in GCs from preovulatory follicles post-OI compared to earlier stages ($p < 0.001$).

The expression level of *IRS2* was also moderately expressed throughout folliculogenesis (Fig. 1a). The expression level of *IRS2* was significantly lower in GCs from preovulatory follicles pre-OI compared to the other follicle groups ($p < 0.001$); however, the expression level of *IRS2* in GCs from preovulatory follicles post-OI was significantly higher compared to the earlier follicle stages ($p < 0.001$).

Insulin-like growth factor receptor type 2 (IGF2R) and insulin-like growth factor-2 binding protein-3 (IMP3)

The expression level of *IGF2R* was moderately expressed in human follicles and increased with increasing follicle size (Figs. 1b and 2). The expression level of *IMP3* was also moderately expressed in human follicles (Fig. 1b).



Insulin-like growth factor binding proteins (IGFBPs)

The expression level of *IGFBP2* was high in the different follicle stages, but a decline was observed in preovulatory follicles (Figs. 1b and 2). A decline in *IGFBP3* expression levels was observed in small antral follicles compared with preantral follicles and a further reduction was observed in preovulatory follicles pre-OI compared with small antral follicles ($p < 0.001$). The expression of *IGFBP4* was high in human follicles (Fig. 1b). A significant drop in *IGFBP4* expression was observed in small antral follicles compared with preantral follicles. Preovulatory follicles pre-OI expressed

◀ **Fig. 2 Regulation of IGF-2 bioactivity at the surface of an ovarian granulosa cell.** Regulation of IGF bioactivity includes multiple levels of regulatory proteins. This figure depicts some of the most relevant IGF proteins expressed in human ovarian follicles and how they are up- or downregulated in granulosa cells (GCs) from four stages of folliculogenesis: **a** preantral follicles (< 60 μm to > 150 μm in diameter), **b** small antral follicles (4–6 mm in diameter), **c** preovulatory follicles prior to ovulation induction (pre-OI), and **d** preovulatory follicles after ovulation induction (post-OI). Arrows indicate upregulation (green) or downregulation (red) when comparing the four follicular stages. The following functional statements are based on the observed up- and downregulation of IGF genes in the four follicular stages evaluated in this study: **a** at the preantral follicle stage, GCs express less IGF-2, IGF1R/INSR, IRS1, PAPP-A, and more IGFBPs that may reflect a limited IGF-2 signaling; **b** in small antral follicles, GCs continue to express high levels of IGFBPs and less IGF-2 and PAPP-A. In addition, the IGF2R is upregulated in GCs from small antral follicles, which reduces IGF-2 bioactivity. However, small antral follicles also begin to upregulate the IGF1R/INSR, and IRS1 genes important for transducing the signal, indicating a shift to a more active signaling system; **c** at the preovulatory stage pre-OI, expression of IGF-2, PAPP-A, IGF1R/INSR, and IRS1 increases significantly, whereas IGFBP-2 expression and intact IGFBP-4 protein decreases. This implies an upregulation of IGF bioactivity important for the high rate of GC proliferation and aromatase expression; **d** at the preovulatory stage post-OI, the GCs downregulate IGF-2 expression and upregulate STC1 that inactivates and modulates PAPP-A activity, which may imply a strict regulation of PAPP-A-mediated IGF signaling after ovulation induction

higher levels of *IGFBP4* compared to small antral follicles ($p < 0.001$), while the level of *IGFBP4* was lower in preovulatory follicles post-OI compared to follicles pre-OI ($p < 0.001$). The expression level of *IGFBP5* was high in preantral follicles but declined significantly in small antral follicles and larger preovulatory follicles ($p < 0.001$) (Fig. 1b).

Supplementary Fig. 5 displays transcription profiles of *IGFBP1* and *IGFBP6*.

Pregnancy-associated plasma protein-A (PAPPA)

The expression of *PAPPA* was low to moderate in preantral follicles and in small antral follicles (Fig. 1c). The expression of *PAPPA* increased markedly in GCs from preovulatory follicles ($p < 0.001$) (Fig. 2c).

PAPP-A inhibitors: ProMBP, STC1, and STC2

The expression level of *proMBP* was overall low throughout the different follicle stages (Fig. 1c).

The expression level of *STC1* was low to moderate in preantral follicles, small antral follicles, and in GCs from preovulatory follicles pre-OI (Fig. 1c). An increase in the expression level of *STC1* was observed in preovulatory follicles post-OI ($p < 0.001$) (Figs. 1c and 2d). The expression levels of *STC2* remained low throughout the different follicle stages (Fig. 1c).

Quantitative RT-PCR of IGFBP5 and PAPPA

To validate the microarray data, mRNA levels of *IGFBP5* and *PAPPA* were measured with qPCR in GCs from small antral follicles together with mural and cumulus cells from preovulatory follicles post-OI (Fig. 3). The gene expression level of *IGFBP5* was non-significantly higher in small antral follicles compared to the mural and cumulus cells from preovulatory follicles post-OI. Cumulus cells showed a significantly higher expression of *IGFBP5* than mural cells ($p = 0.01$).

The expression level of *PAPPA* was significantly higher in mural and cumulus cells from preovulatory follicles post-OI compared to GCs from small antral follicles ($p = 0.006$ and $p < 0.001$, respectively). This confirms the microarray data results.

Protein levels of total IGFBP-4, intact IGFBP-4, total IGFBP-5, PAPP-A, STC2, and detectable free IGF-2 in FF from small human antral follicles and preovulatory follicles

The level of total IGFBP-4 protein showed no significant variation between small antral follicles and preovulatory follicles pre-OI and post-OI (Fig. 4). In contrast, intact levels of IGFBP-4 were significantly lower in FF from preovulatory follicles ($p < 0.001$) compared to FF from small antral follicles (Figs. 2c and 4). The protein levels of total IGFBP-5 were significantly higher in FF from preovulatory follicles compared to small antral follicles ($p < 0.001$).

This study also combined ELISA data from previous studies to highlight that PAPP-A protein level increased significantly in FF from preovulatory follicles compared to small antral follicles ($p < 0.001$) [31, 50].

The protein levels of STC2 showed no significant variation in the FF from the three groups of follicles.

Intrafollicular levels of detectable free IGF-2 were significantly higher in small antral follicles compared to preovulatory follicles pre-OI and post-OI ($p < 0.001$).

Figure 2 is an attempt to provide a schematic display of IGF signaling at four stages of human follicular development.

Discussion

The exceptional complex regulation of the IGF system may reflect its action in many different organs of the body that requires local regulatory mechanisms for controlling bioactivity. Indeed, the human ovarian follicle is no exception, and various regulatory steps interact to control the access of the IGFs to their receptors on the cell surface. Once the receptor is activated, other mechanisms affect and control both intracellular signaling pathways and IGF synthesis.

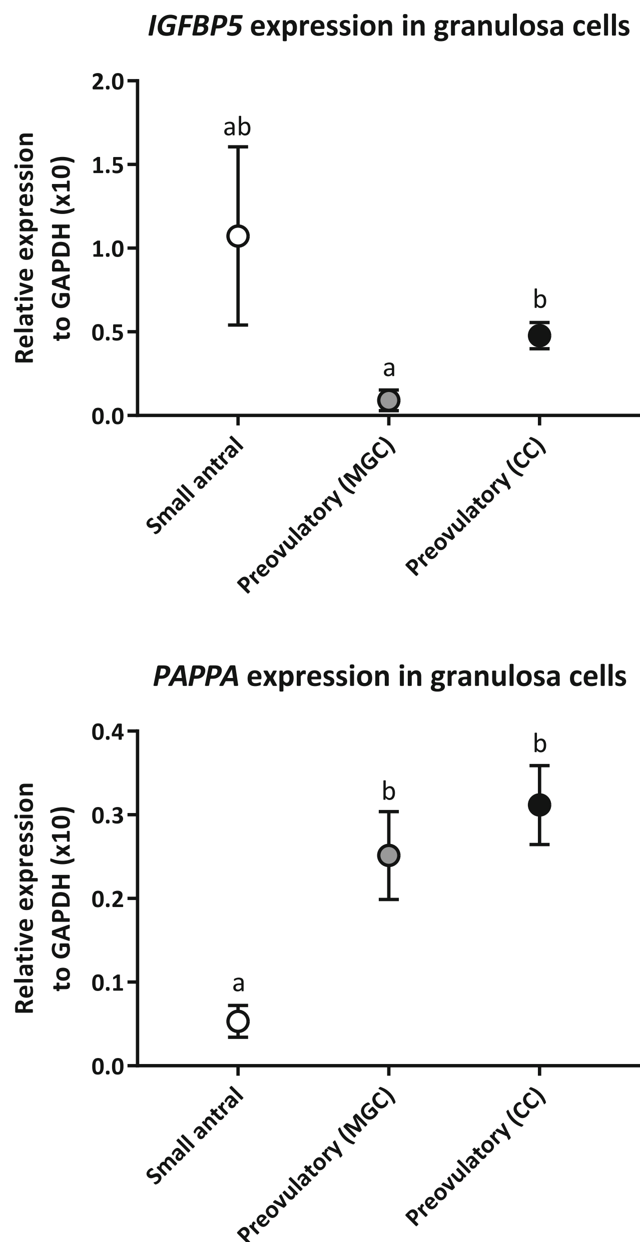


Fig. 3 RT-qPCR validation of *IGFBP5* and *PAPPA* expression in GCs from small antral follicles and in mural and cumulus GCs from IVF patients isolated after ovulation induction. *IGFBP5* and *PAPPA* gene expression were normalized to *GAPDH* expression and the relative expression is displayed. Data are presented as mean values of GC samples measured in duplicates from small antral follicles (Small antral), mural GCs isolated after ovulation induction (Preovulatory (MGC)), and cumulus cells isolated after ovulation induction (Preovulatory (CC)). Error bars indicate SEM values. Different letters between follicle groups indicate statistical significance. Statistical significance for *IGFBP5* expression: mural GCs vs. cumulus GCs: $p = 0.01$. Statistical significance for *PAPPA* expression: small antral follicles vs. mural GCs: $p = 0.006$, small antral follicles vs. cumulus GCs: $p < 0.001$

This study is to our knowledge the first to evaluate the majority of components in the IGF signaling cascade during human folliculogenesis. Despite the complexity, the present study suggests that the IGF system is especially active in the preovulatory phase of follicular development (Fig. 2c). The expression of *IGF2* is upregulated approximately 64-fold in the preovulatory stage prior to the midcycle surge of gonadotropins compared to small antral follicles (Fig. 1a), where *PAPP-A* expression also showed to peak (Fig. 1c). On a functional level, this is backed by the observation showing that PAPP-A protein levels significantly increased while intact IGFBP-4 protein levels significantly decreased in preovulatory follicles compared to small antral follicles (Fig. 4). Since PAPP-A is believed to be the only protease cleaving IGFBP-4 to release IGFs, the reduction of intact IGFBP-4 confirms increased PAPP-A activity and IGF signaling.

The expression of the *IGF receptors* together with increase of the intracellular signaling molecules, IRS1 and IRS2, with increasing follicle size (Fig. 1a) further supports the hypothesis of a highly active IGF system in late folliculogenesis.

This study confirmed expression of all six IGFBPs as well as the inhibitors of PAPP-A, STC1, and STC2 (Fig. 1b, c). The IMP proteins interfering with translation of IGF-2 were also expressed. The expression levels of most of these genes were relatively constant during follicular development, which suggests that their role in regulating the IGF system is constant and not related to any stage of folliculogenesis.

The present study therefore suggests that the IGF system on a physiological level impacts on the key events taking place in the preovulatory follicle, which is characterized by a very intense proliferation of GCs simultaneous with a substantial synthesis of estradiol and progesterone. Further, the present study enforces that the membrane anchored PAPP-A probably secure IGF signaling by releasing IGF-2 close to the IGF1R present on the cell surface, which is reflected by the significantly reduced levels of intact IGFBP-4.

These data are corroborated by earlier findings in FF, which also suggest a strong involvement of the IGF system in determining steroid output and GC mitosis activity [19, 22, 31, 55–57]. Following the midcycle surge of gonadotropins, the high GC proliferation is terminated, and the steroid output is reorganized to reflect the luteal phase. The expression profiles of the IGF system also reflect this transition with reduced IGF signaling (Fig. 2d). Collectively, the present results suggest that the IGF system is intimately involved in the regulation of follicular growth and development especially in the preovulatory phase of the menstrual cycle.

The importance of the IGF system for fertility has recently been highlighted in a mouse model, where a conditional knockdown of the IGF1R in GCs caused sterility and the ovaries were smaller than controls. Antral follicles did not develop nor were ovulated oocytes observed after ovarian stimulation [24].

Previous studies have suggested that IGF signaling possibly via PAPP-A regulation is important for steroid production in human follicle development [31, 55–58]. In addition, earlier studies showed strong immunoexpression of PAPP-A in GCs

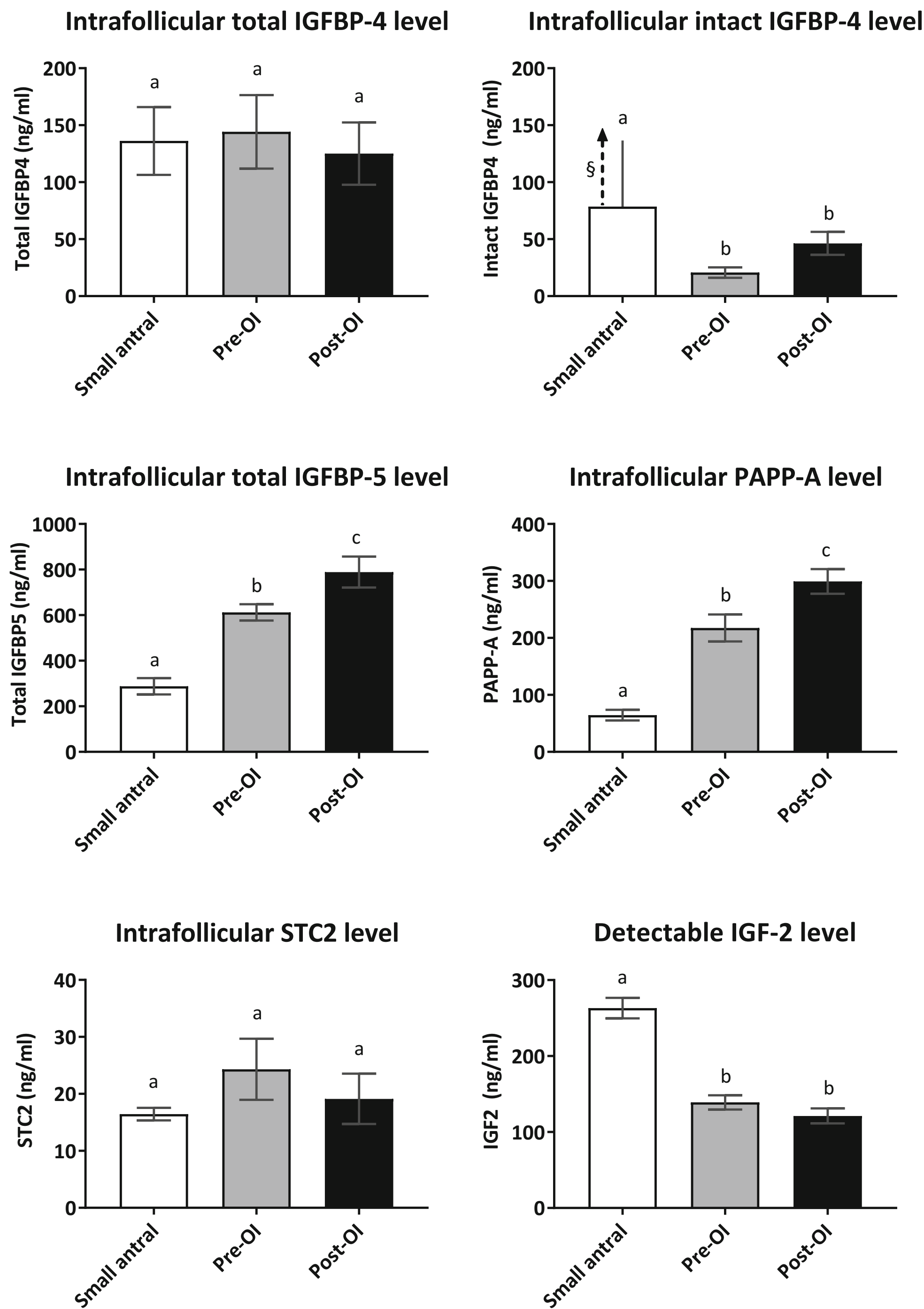


Fig. 4 Antigen levels of total IGFBP-4, intact IGFBP-4, total IGFBP-5, PAPP-A, STC2, and detectable free IGF-2 in FF from small human antral follicles and preovulatory follicles. Data are mean (± SEM) of the intrafollicular levels of total IGFBP-4, intact IGFBP-4, total IGFBP-5, PAPP-A, STC2, and detectable free IGF-2 in FF of small antral follicles (Small antral), preovulatory follicles prior to ovulation induction (Pre-OI), and preovulatory follicles after ovulation induction (Post-OI). §The arrow shows that the mean intact IGFBP-4 protein level in FF from small antral follicles was higher than the upper calibrator of 78 ng/ml supplied with the ELISA kit; thus, an exact mean is not shown. Different letters between follicle groups indicate statistical significance. Statistical significance for FF levels of intact IGFBP-4 protein: small antral follicles vs. follicles pre-OI: $p < 0.001$, and small antral follicles vs. follicles post-OI: $p < 0.001$. Statistical significance for FF levels of total IGFBP-5: small antral follicles vs. follicles pre-OI: $p < 0.001$, small antral follicles vs. follicles post-OI: $p < 0.001$, and follicles pre-OI vs. follicles post-OI: $p = 0.02$. Statistical significance for FF levels of PAPP-A: small antral follicles vs. follicles pre-OI: $p < 0.001$, small antral follicles vs. follicles post-OI: $p < 0.001$, and follicles pre-OI vs. follicles post-OI: $p = 0.02$. Statistical significance for FF levels of detectable free IGF-2: small antral follicles vs. follicles pre-OI: $p < 0.001$ and small antral follicles vs. follicles post-OI: $p < 0.001$

from preovulatory follicles together with a strong positive correlation between FF levels of PAPP-A and follicle size [31, 59]. Thus, the presence of increased expression of *PAPP-A* in large preovulatory follicles confirms and expands on previous observations.

Genes encoding differentiation markers like *CYP19a1*, *LH receptor*, and *StAR* are known to be upregulated by FSH and studies of cultured human cumulus cells have shown that this upregulation is highly dependent on IGF1R activity [60]. This fits with our results showing upregulation of *IGF2* expression together with increasing PAPP-A levels at the preovulatory stage underscoring the importance of IGF signaling for differentiation of GCs and the estradiol output, especially when the follicle is approaching ovulation.

Expression of all six IGFBPs, which inhibit the action of IGFs or prolong their half-life, was observed throughout follicle development (Fig. 1b), however with low expression of *IGFBP1* and *IGFBP6* (Supplementary Fig. 5). Previously, IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 have been observed in human GCs and FF [61–63], which is confirmed and extended by the present results. The present study for the first time demonstrated that the intrafollicular protein level of IGFBP-5 in FF from human preovulatory follicles was significantly higher compared with small antral follicles. As the affinity constant between IGF-2 and IGFBP-5 is around ten times higher than that for IGFBP-4 [14], it is likely that the majority of IGF-2 will be bound by IGFBP-5 rather than IGFBP-4. As PAPP-A cleaves both binding proteins and the levels of intact IGFBP-4 becomes significantly reduced, it is hypothesized that this may reflect a higher conversion of IGFBP-4 by PAPP-A, which only cleaves IGFBP-4 when occupied by IGF-2 thereby leading to higher IGF signaling.

The observed significant rise in *IGF2* expression in large preovulatory follicles collected pre-OI in the present study is in line with an earlier study, showing a markedly higher *IGF2* expression level in GCs of a 14-mm large follicle compared to smaller follicles [64]. The significant higher expression of *IGF2* in GCs from follicles pre-OI compared to follicles post-OI was validated with qPCR by Wissing and colleagues [47]; however, we have not been able to validate the difference in *IGF2* expression between GCs isolated from small antral follicles and follicles pre-OI due to lack of material. Previous studies have found significantly higher FF levels of total IGF-2 in human E_2-dominant follicles compared to androgen-dominant follicles [19, 55]. The present study found significantly reduced levels of detectable free IGF-2 protein in FF from preovulatory follicles compared to FF from small antral follicles (Fig. 4), which may appear contradictory. However, the assay used to measure detectable free IGF-2 may only partly reflect the true free levels of IGF-2 since this will depend on the affinity constant of the employed antibody, the concentration, cleavage state of the binding proteins, and the soluble IGF2R, which also show high binding affinities toward IGF-2. Thus, it may be difficult to detect the true free biological active IGF-2 concentration in FF, which furthermore is likely to be modulated close to the cell surface. Thus, the biological activity of IGF-2 affecting the receptor is not clarified by the present study, which, however, suggests that at least the cellular synthesis of IGF-2 is highly increased in GCs of preovulatory follicles. Thus, the available assays add another level of complexity analyzing IGF signaling. Assays detecting bioactive IGF-2 in biological fluids are to our knowledge currently not available, and therefore it becomes crucial to address total and intact binding proteins, proteases, and inhibitors of the proteases that may reflect the potential for activation.

Giudice and colleagues showed that FSH increased the expression of *IGF2* in human preantral follicles in vitro [29]. In the present study, GCs collected from preovulatory follicles before the midcycle surge are obtained from women who underwent ovarian stimulation with exogenous FSH administration resulting in supra-physiological levels of FSH, while GCs from small antral follicles on the contrary were collected from women in their natural menstrual cycle. Therefore, it cannot be excluded that the 64-fold difference in *IGF2* expression is less pronounced when compared to women in their natural menstrual cycle.

The expression levels of the intracellular signaling protein IRS1 was observed to increase significantly in follicles pre-OI compared to small human antral follicles (Figs. 1a and 2c). Previous studies have shown an increase in immunoexpression of IRS1 in human GCs with increasing follicle size, which our findings confirm and extend [65]. The IRS1 pathway has also been shown to be essential for follicle growth and a low expression of IRS1 has been associated with a reduced ovarian activity in mice [66]. The expression level of *IRS2* peaked in GCs from preovulatory follicles post-OI, which point toward a possible shift in IGF signaling in the periovulatory events and in the luteal transition being mediated to a higher degree through IRS2 (Fig. 2d).

Another local level of regulation has recently been suggested by the findings of co-localization and formation of inhibitory complexes between STCs and PAPP-A during follicle development [38]. This previous study also found a positive correlation between the total PAPP-A level and the activity in FF from preovulatory follicles pre-OI with markedly lower PAPP-A activity post-OI. This decrease in proteolytic activity was suggested to be modulated by the STCs. In the present study, the expression level of the *STC1* was observed to increase in preovulatory follicles post-OI (Figs. 1c and 2d) supporting the previous findings, which suggests a stage-dependent upregulation of *STC1* expression important for modulation of PAPP-A mediated IGF signaling after ovulation induction. This may be important for the decline in GC proliferation at the midcycle surge. No significant differences were observed between STC2 protein levels in FF from small antral follicles and preovulatory follicles (Fig. 4).

Previous studies observed a significant increase in STC1 mRNA levels in mice ovaries after treatment with hCG [67].

This might explain the upregulation of STC1 in preovulatory follicles after ovulation induction as shown in this study. However, only little is known on the specific regulation of STCs synthesis in the human ovary, and this needs to be studied in more detail in coming studies.

The present study is mainly based on gene expression data and may not reflect actual functional protein levels. In this context, it is also important to notice that the present study found expression of the IMPs that regulates translation of IGF-2. Our data confirm previous studies [41] and highlight that gene expression does not necessary reflect translation especially in connection with IGF-system. In our study, however, gene expression levels of *PAPPA*, *IGFBP4*, *IGFBP5*, and *STC2* were confirmed by qPCR and protein measures and are in line with previous studies from our laboratory [31, 59].

The patients from whom one ovary was excised for fertility preservation had different diagnosis, which could affect the results in this study since different types of cancers have been shown to affect levels of proteases like PAPP-A and levels of IGFs [68, 69]. However, all patients were early-stage disease patients, and further, this study included samples from patients with different types of diagnoses, thus, not reflecting one specific disease. None of the patients had any ovarian disease, and the change in expression associated with different cancer types may be tissue specific and affects organs with tumor cells.

Conclusion

In conclusion, the expression of PAPP-A with its specific proteolytic activity toward IGFBP-4/IGF-2 complexes increased significantly in preovulatory follicles coinciding with a reduction of intact levels of IGFBP-4, which potentially reflects an increased IGF signaling (Fig. 2c). The increased IGF-2 is likely to augment steroid production and rapid GC proliferation as seen in final stages of follicle development, prior to the midcycle surge. Transcription levels of intracellular signaling proteins increase with increasing follicle size, supporting the hypothesis that IGF signaling is essential in late folliculogenesis. The moderate expression of IGF components in the early stages of follicular development implies a role for IGF activity regarding GC differentiation and growth in preantral follicles; however, the high expression of IGFBPs and IMP3 may also be important to maintain the follicles in their arrested state. An increase in *STC1* expression and FF-levels of IGFBP-5 protein seems to be important to sustain a strict regulation of the IGF signaling in the periovulatory phase.

These data show how the expression of IGF components fluctuate during human ovarian folliculogenesis, and a better understanding of essential pathways like this may provide markers of follicular health or targets for induction of follicle activation and hereby help in improving treatments at the IVF clinics.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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