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# Utilising FGF2, IGF2 and FSH in serum-free protocol for long-term *in vitro* cultivation of primary human granulosa cells

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ABSTRACT

Human granulosa cells acquired as leftover from IVF treatment can be used to study infertility problems and are a valuable tool in the research of follicle maturation and ovulation. There is a need for more defined and long-term culture protocols for studying the response of granulosa cells upon treatment with selected hormones/chemicals. In the current study, we tested the effect of adding FGF2, IGF2 and FSH into defined basal medium in order to find culture conditions that would support proliferation of cumulus and mural granulosa cells along with the expression of common granulosa cell type markers such as *FSHR*, *AMHR2*, *LHR* and *CYP19A1*. We found that FGF2, IGF2 together with FSH helped to retain granulosa cell marker expression while supporting cell survival at least for two weeks of culture. The defined serum-free culture conditions for long-term culturing will be valuable in providing new standards in the research of human granulosa cells.

## 1. Introduction

### 1.1. The function of granulosa cells in folliculogenesis

Follicle maturation and ovulation are complex biological processes that primarily determine the fertility of women. During maturation, the oocyte heavily depends on external support from granulosa cells (GCs) and theca cells (TCs), which provide the oocyte with metabolites and regulatory molecules (Edson et al., 2009). Granulosa cells are divided into two types: cumulus GCs (CGCs) that surround the oocyte, and mural GCs (MGCs) that are positioned peripherally in the follicle. During the follicular cycle TCs synthesize androstenedione as a response to luteinizing hormone (LH) released from the pituitary gland, while GCs express aromatase (CYP19A1) as a response to follicle stimulating hormone (FSH). CYP19A1 converts androstenedione into estradiol, which is necessary to achieve ovulation (Hillier, 1994; Lasley et al., 1975; Ryan and Petro, 1966; Sasano et al., 1989). Therefore, disturbances in GC function can lead to reduced fertility or even infertility.

### 1.2. Molecular markers of GCs

Several different markers can be used to characterise GC phenotype and differentiation status. The FSH receptor (FSHR), anti-Müllerian hormone (AMH) and aromatase are often used to characterise these cells. GCs start expressing FSHR during the primary follicular phase (Oktay et al., 1997) and still express it during preantral and Graafian stages (Yamoto et al., 1992), while AMH and its receptor (AMHR2) are expressed during primary, secondary and small antral phases of the follicle (Xu et al., 2016) and expression decreases notably at later stages (Cheon et al., 2018; Weenen et al., 2004). The LH receptor (LHR) expression is detectable in early antral follicles and later stages of maturation (Yung et al., 2014). Aromatase is already detectable in small follicles, but its expression increases as the follicle matures (Sasano et al., 1989). In conjunction with the traditional markers, one can use additional targets to characterise GCs differentiation status. Cytochrome P450 side-chain cleavage enzyme (CYP11A1, also known as P450scc), required for the first stages of steroidogenesis, is usually considered to be abundant in TCs, but it is also detectable in GCs, especially in luteinized GCs (Sasano and Sasano, 1989). Gonadotropin-releasing hormone receptor (GnRHR) is present only in preovulatory

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**Abbreviations**

| | |
|---|---|
| GCs | granulosa cells |
| CGCs | cumulus granulosa cells |
| MGCs | mural granulosa cells |
| TCs | theca cells |

and luteinized GCs (Choi et al., 2006).

### 1.3. In vitro cultivation of GCs

Over the years, several GC lines have been developed, that are relatively easy to work with (Bayasula et al., 2012; Hosokawa et al., 1998; Ishiwata et al., 1984; Nishi et al., 2001; Nitta et al., 2001; Rainey et al., 1994; Tajima et al., 2002; van den Berg-Bakker et al., 1993). However, these cell lines may differ from primary GCs by hormone synthesis and responsiveness, aromatase activity and even the number of chromosomes (Havelock et al., 2004). Thus, there is a need for a reliable cell culture protocol that would allow to cultivate primary human GCs while maintaining GC identity, including responsiveness to relevant hormones. Several protocols have been published (Sarah C Baumgarten et al., 2015; Brůcková et al., 2008; Chang et al., 2016; Chatterjee et al., 2014; Figenschau et al., 1997; Grossman et al., 2008; Karamouti et al., 2008; Kossowska-Tomaszczuk et al., 2010; Kranc et al., 2017; Ophir et al., 2014; Prapa et al., 2015; Schipper et al., 1993; Taieb et al., 2011; Tureck and Strauss, 1982; Wickham et al., 2013). However, most of them are either short-term cultures (up to 7 days) or use undefined serum in growth medium. Inclusion of serum can make interpretation of the results more difficult and may not be consistent over longer periods of time, since serum batches can differ from each other and that can influence the results of the experiment.

Several of these protocols use growth factors to promote cell proliferation and steroidogenesis. Insulin-like growth factors (IGFs) are important players in regulation of ovarian steroidogenesis in humans (Giudice, 1992). Actions of IGFs can be mediated via insulin receptors (INSR), type I IGF receptors (IGF1R) and type II IGF receptors (IGF2R) (Rechler and Nissley, 1986). Growth factors IGF1 and IGF2 mainly modulate the steroidogenic response via IGFR1 and enhance GC steroid production *in vitro* (Willis et al., 1998). It has been found that human GCs from preovulatory follicles selectively express IGF2, and IGF1 expression is not detectable (el-Roeiy et al., 1993; Geisthovel et al., 1989). The expression of IGF2 by human GCs is enhanced by FSH and together these molecules stimulate CYP19A1 expression in CGCs and regulate their proliferation and differentiation (Sarah C Baumgarten et al., 2015). While an early study analysing the proliferative effect of IFG2 has found that human GCs respond positively to the IGF2 treatment (Di Blasio et al., 1994), a more recent study states that IGF2 alone does not enhance GC proliferation, but the proliferative effect is detected only together with FSH (Sarah C Baumgarten et al., 2015).

Fibroblast growth factors (FGFs) are potent regulators of growth and differentiation in reproductive tissues, where FGF2 (also known as basic fibroblast growth factor - bFGF) is the most studied and has been shown to promote GC proliferation while decreasing steroidogenesis of these cells (Gospodarowicz and Bialecki, 1979; McAllister, 1995; Price, 2016). The expression of FGF2 and its receptors in human GCs has been demonstrated (Di Blasio et al., 1993; Watson et al., 1992). Out of the main types of FGF receptors, FGFR2 and FGFR3 are present in GCs of primordial, primary and secondary follicles and FGFR3 can be detected also in antral follicles, whereas FGFR4 is missing from primordial follicles, but is present in primary and secondary follicles (Ben-Haroush et al., 2005). The expression of FGF2 protein has been found in GCs from primary and secondary follicles and similarly to the expression FGF receptors, it is only detectable in adult women (Ben-Haroush et al., 2005). Interestingly, transcripts of FGF2 together with all the four receptors were detected by qPCR from primary to antral follicles (Ben-Haroush et al., 2005). The latter is in concordance with a more recent study where the analysis of transcriptional profiles of antral granulosa cells showed evidence about the presence of all the four main FGF receptor types (Kõks et al., 2009). It has also been noted that the mRNA expression of *FGF2* decreases during early follicle development from non-growing to small secondary follicles (Quennell et al., 2004).

### 1.4. The aim of the study

The aim of this study was to develop a serum-free primary human GC cultivation protocol that would support proliferation of the FSH responsive (expressing *FSHR*) cells for *in vitro* cellular experiments, providing a reliable tool to study the biology of human GCs.

## 2. Materials and methods

### 2.1. Granulosa cells retrieval and purification

The study was approved by the Research Ethics Committee of the University of Tartu (289/M-8). GCs were derived in the Women's Clinic of Tartu University Hospital during intracytoplasmic sperm injection (ICSI) procedure from women undergoing *in vitro* fertilization (IVF) treatment. The samples were collected anonymously and thus no patient information was made available for the researchers. Ovarian hormonal stimulation was conducted according to the GnRH antagonist (Cetrotide, Merck Serono, Geneva, Switzerland) protocol with the administration of recombinant FSH (Gonal-F, Merck Serono, Darmstadt, Germany) to all patients. All patients underwent ovum pick-up (OPU) after 36h of human chorionic gonadotropin (hCG) administration (Ovitrelle, Merck Serono, Germany) with the puncture of follicles $\geq$15 mm in size. Cumulus and mural GCs were collected separately.

CGCs were collected 4h after OPU during oocyte denudation for ICSI, lasting up to 5 min with type IV-hyaluronidase extracted from bovine testes (Sigma-Aldrich, Saint Louis, MO, USA) and diluted in Sperm preparation Medium (MediCult, Jyllinge, Denmark). The cells were pooled together and centrifuged at 450g for 10 min, and the supernatant was removed. The cells were separated on a 50% density gradient of PureSperm 100 (Nidacon, Mölndal, Sweden) in Universal IVF Medium (MediCult), and washed three times in Universal IVF Medium at 37 °C.

For MGCs, the follicular fluid of all patients was pooled, centrifuged at 450g for 10 min, and the supernatant was removed. The cells were separated on a 50% density gradient of PureSperm 100 (Nidacon) in Universal IVF Medium (MediCult), and washed three times in Universal IVF Medium at 37 °C.

### 2.2. Cell culture

The cells were frozen, thawed, pooled together (pools of 10–30 patients, depending on the size of the experiment). Live cells were counted manually with hemocytometer and trypan blue staining. 500 000 live cells were plated onto Matrigel-covered (Corning, New York, NY, USA) 6-well plates in DMEM/F12 growth medium (Lonza AG, Basel, Switzerland) with 20% KnockOut Serum Replacement (KSR) (Thermo Scientific, Waltham, MA, USA), 1% penicillin/streptomycin (Naxo, Tartu, Estonia) and Primocin (100 μg/ml) (InvivoGen, San Diego, CA, USA). IGF2 (50 ng/ml) (Bio-Techne, Minneapolis, MN, USA), FGF2 (8 ng/ml) (Enantis, Brno-Bohunice, Czech Republic), both, or none were added to the growth medium. The effect of FSH (1 U/ml) (Gonal-F, Merck Serono) was tested in the IGF2 and FGF2-containing medium starting from day 4, because it has been shown that GCs are not responsive to hormones right after collection (Ophir et al., 2014). Growth medium was changed daily. Cells were passaged using TrypLE (Thermo Scientific), half of the cells were replated and half were lysed using QIAzol lysis reagent (Qiagen, Hilden, Germany). Cell

proliferation was analysed on the days of passaging by counting the live cells in a hemocytometer. Cell morphology was analysed by visual observation. All cell culture experiments were repeated three times. In preliminary experiments the effect of freezing and thawing cells, as well as the effect of passaging was studied and no significant effects on the expression levels of the selected target genes were observed.

### 2.3. RNA purification, cDNA synthesis and qPCR

For RNA purification miRNeasy Micro Kit (Qiagen) was used according to manufacturer's guidelines. All RNA samples were treated with DNase from RNase-Free DNase Set (Qiagen). For cDNA synthesis RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used. Based on literature *FSHR, AMHR2, LHR, CYP19A1*, *GnRHR*, *AMH and CYP11A1* were selected as GC markers to be analysed in the current study. Additionally, *IGF1, IGF2, IGF1R, IGF2R, INSR, FGF2, FGFR1, FGFR2, FGFR3,* and *FGFR4* were selected for qPCR analysis. Primers were designed using Primer-BLAST online software (Ye et al., 2012). All used primer sequences are available in supplementary table ST1. qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific) in Roche LightCycler 480 and the results were analysed using the manufacturer's software.

### 2.4. Statistical analyses

During subsequent analyses the expression levels of all targets were normalized to succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) and were shown relative to the starting point of frozen and thawed cells using the ΔΔCt method. Statistical analyses were done using R Project software (version 3.5.2). For pairwise comparisons Student's t-test was used and the differences between groups were considered statistically significant when $p < 0.05$ after Bonferroni correction for multiple testing.

## 3. Results

### 3.1. Granulosa cell marker genes FSHR, AMHR2, LHR, CYP19A1, GnRHR and AMH have similar expression level in cumulus and mural cell types after cell retrieval

First, we characterized and compared the expression of our selected genes of interest in freshly thawed CGCs and MGCs. In addition to common GC-associated genes, we analysed the expression levels of *IGF1/2, FGF2* and their main receptors in these cells. Growth factors IGF1/2 and FGF2 have been used before to support granulosa cell growth (Di Blasio et al., 1994), but comprehensive analysis of GC marker gene expression in long-term cell culture has not been studied under serum-free conditions. We did not detect any significant differences in the expression of most of the selected granulosa marker genes such as *FSHR, AMHR2, LHR, CYP19A1*, *GnRHR* and *AMH* between these two GC types (Fig. 1). However subtle differences in the expression of *CYP11A1, IGF2* and *IGF1R* between CGCs and MGCs were statistically different, while the difference in the expression of *IGF1* was not due to larger differences between biological replicates. The expression of FGFR2 in both cell types together with FGFR3 in MGCs was barely detectable and the expression of IGF1 and FGFR3 in CGCs was detected at very low level compared with the other targets.

### 3.2. Combining FGF2 with IGF2 in GC growth medium supports CGC cell proliferation while retaining the expression of FSHR longer during 24 days in culture

Here our goal was to find serum-free culture conditions for primary GCs which could support cell growth and maintain the expression of main GC marker genes. We selected a more defined serum alternative, KnockOut™ Serum Replacement and a DMEM/F12 basal medium together with the usage of Matrigel coated cultureware to grow GCs. Based on literature we added FGF2 to support GC proliferation and IGF2 as a crucial regulator of GC proliferation and differentiation. To study the effect of growth factors IGF2 and FGF2 on the expression levels of our selected target genes, we cultivated CGC cells for 24 days in four different growth media: regular (no growth factors added), with IGF2, with FGF2, and with IGF2 + FGF2. Time points were taken on days 0, 4, 13 and 24.

The CGCs that were cultivated without growth factors or with IGF2 only, had similarly low survival rate (Fig. 2b). Out of 500 000 cells plated, only around 150 000 survived until day 24. The CGCs cultivated in FGF2 medium had the highest proliferation speed, as cell count increased 2.58 times in 24 days. Cells in IGF2 + FGF2 medium multiplied too, but not as fast as in the FGF2 medium. By day 24 the cell count had increased 1.74 times, suggesting that FGF2 increases and IGF2 decreases cell proliferation speed.

The morphology of CGCs slowly changed over time from small and round to long and narrow, more similar to fibroblasts. The culture mediums supporting the cell proliferation seemed to be more prone to induce this phenotype. Photos of CGCs in different growth media are shown in supplementary figure SF1.

The results of qPCR (supplementary table ST2) showed that from GC markers only *FSHR* expression increased by day 4 in all media but quickly decreased after that (Fig. 2a). The expression of other markers that were tested in this experiment – *AMHR2, LHR, CYP19A1* and *GnRHR* – either started decreasing immediately or stayed around the same level by day 4 and decreased after that. The expression levels of *IGF2* and all IGF receptors all decreased rapidly after the start of the experiment and stayed low until the end of the experiment at day 24. The expression of *IGF1* started out below the detection level and increased slightly above it by the end of the experiment. The expression of *FGF2* and its main receptor *FGFR1* decreased by day 4 but started rapidly increasing after that. The other FGF receptors had low expression levels for the whole duration of the experiment.

As our aim was to select culture conditions that support cell proliferation together with *FSHR* expression, we found FGF2 and IGF2 synergistically support this phenotype the most during the 24 days in cell culture compared to the other tested culture conditions (Fig. 2). Culture medium with FGF2 supported proliferation, but the expression of *FSHR* decreased faster during the 24 days than in the FGF2-IGF2 combined medium. The regular medium without any additional growth factors along with the medium only with IGF2 supported the expression of FSHR, but the proliferation of cells was compromised.

### 3.3. FSH induces expression of GC marker genes FSHR, AMHR2, LHR, CYP19A1, GnRHR, CYP11A1, but not AMH in CGCs under FGF2 and IGF2 supplemented growth medium and prolongs their expression in long-term cell culture

We hypothesized that adding FSH to growth medium containing IGF2 and FGF2 might help to retain the GC markers' expression and

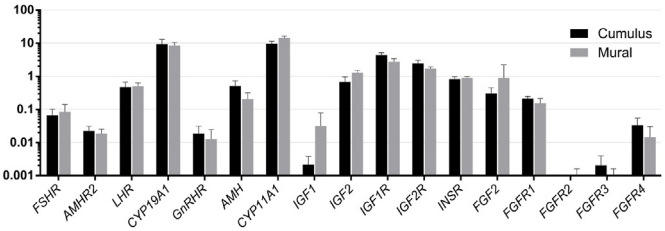



**Fig. 1.** Gene expression of shown genes in CGCs and MGCs after freezing, thawing and pooling. Expression levels are shown as mean ± SD. For CGCs n = 9, except for *AMH* and *CYP11A1* n = 6; for MGCs n = 6, except for *AMH* and *CYP11A1* n = 3. *, $p < 0.05$; Student's t-test after Bonferroni correction for multiple testing. The "n" marks number of biological replicates.

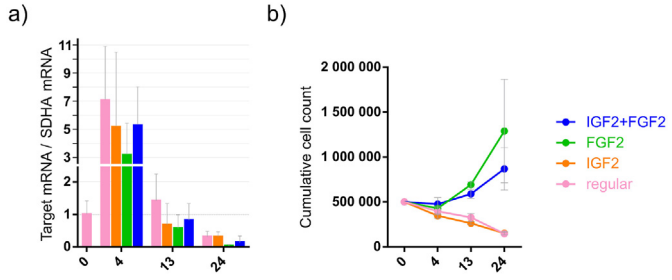



**Fig. 2.** *FSHR* expression level change relative to day 0 in different mediums (a). Cumulative cell count of CGCs cultivated in different growth media on days 0, 4, 7, 13 and 24 (b). Expression levels and cell counts are shown as mean ± SD; n = 3.

maintain the original GC morphology, because IGF2 has been shown to be important factor together with FSH in regulating CYP19A1 expression and FGF2 supported proliferation of these cells.

To study the effect of FSH to CGCs, the cells were cultivated in IGF2 + FGF2 growth medium and FSH (1 U/ml) was added to half of the cells starting from day 4 of culture. Time points were taken on days 0, 4, 7, 13 and 24 of the experiment. The CGCs cultivated in the growth medium with FSH did not proliferate as fast as the cells cultivated without FSH (Fig. 3a). By day 24, cell count of the CGCs that were grown without FSH, had increased 3.32 times, and with FSH in the medium, 1.82 times. The morphology of CGCs still changed from small and round to long and narrow, however that change was much slower in the medium where FSH was added (Fig. 3b).

The results of qPCR showed that adding FSH to the growth medium helped to upregulate most of the GC markers that we studied (Fig. 4a, supplementary table ST3). The expression levels of *FSHR, AMHR2, LHR* and *CYP19A1* followed a similar pattern – in the FSH medium they stayed well above the starting level on days 7 and 13 but dropped to barely detectable levels by day 24. Without adding FSH to the medium the expression levels dropped below the original level much faster. The expression of *GnRHR* followed a similar pattern, except by day 24, when the expression levels were comparable to the original expression level. The expression of *CYP11A1* acted differently, dropping below detection level on day 7 but rapidly increasing by day 13, with added FSH, followed by a decrease by day 24. In the medium without FSH the decrease was steadier but ended up at the same level. The expression of *AMH* however, dropped right after the start of the experiment and stayed very low until the end of cultivation in both growth media.

The differences between the two growth media were not as big when comparing the expression levels of growth factors and their receptors (Fig. 4b). The expression levels of *IGF1*, *FGF2* and *FGFR1* increased in both growth media by the end of the experiment while remaining lower in the FSH medium. The expression levels of *IGF2, IGF1R, IGF2R* and *INSR* generally decreased but were higher in the FSH medium. The expression of *FGFR1* increased in both growth media but more so in the medium without FSH. The expression of *FGFR2* was undetectable in the beginning of the experiment and increased above the detection limit in both growth media by the end of the experiment. The expression levels of *FGFR3* and *FGFR4* dropped in both media, except *FGFR3* expression that increased by day 24 in both growth media.

### *3.4. FSH induces expression of GC marker genes FSHR, AMHR2, LHR, CYP19A1, CYP11A1, but not AMH and GnRHR in MGCs under FGF2 and IGF2 supplemented growth medium and prolongs their expression in long-term cell culture*

To study the effect of FSH to MGCs, the same protocol as used for CGCs was utilized. Cells were cultivated in IGF2 + FGF2 growth medium and FSH (1 U/ml) was added to half of the cells starting from day 4 of culture. Time points were taken on days 0, 4, 7, 13 and 24 of the experiment.

The MGCs cultivated with or without FSH had similar proliferation

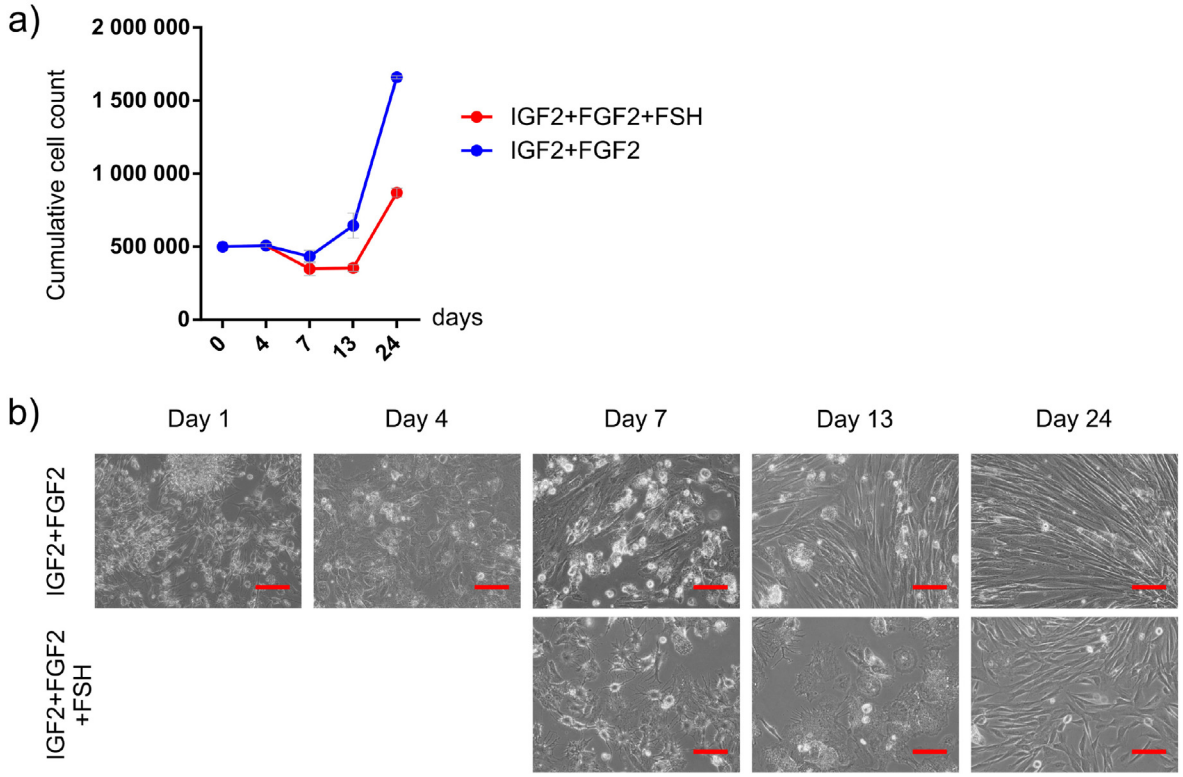



**Fig. 3.** Cumulative cell count of CGCs cultivated in different growth media on days 0, 4, 7, 13 and 24 (a). CGC morphology cultivated in different growth media on days 1, 4, 7, 13 and 24 before passaging. The scale bar corresponds to 100 μm (b). Cell counts are shown as mean ± SD; n = 3.

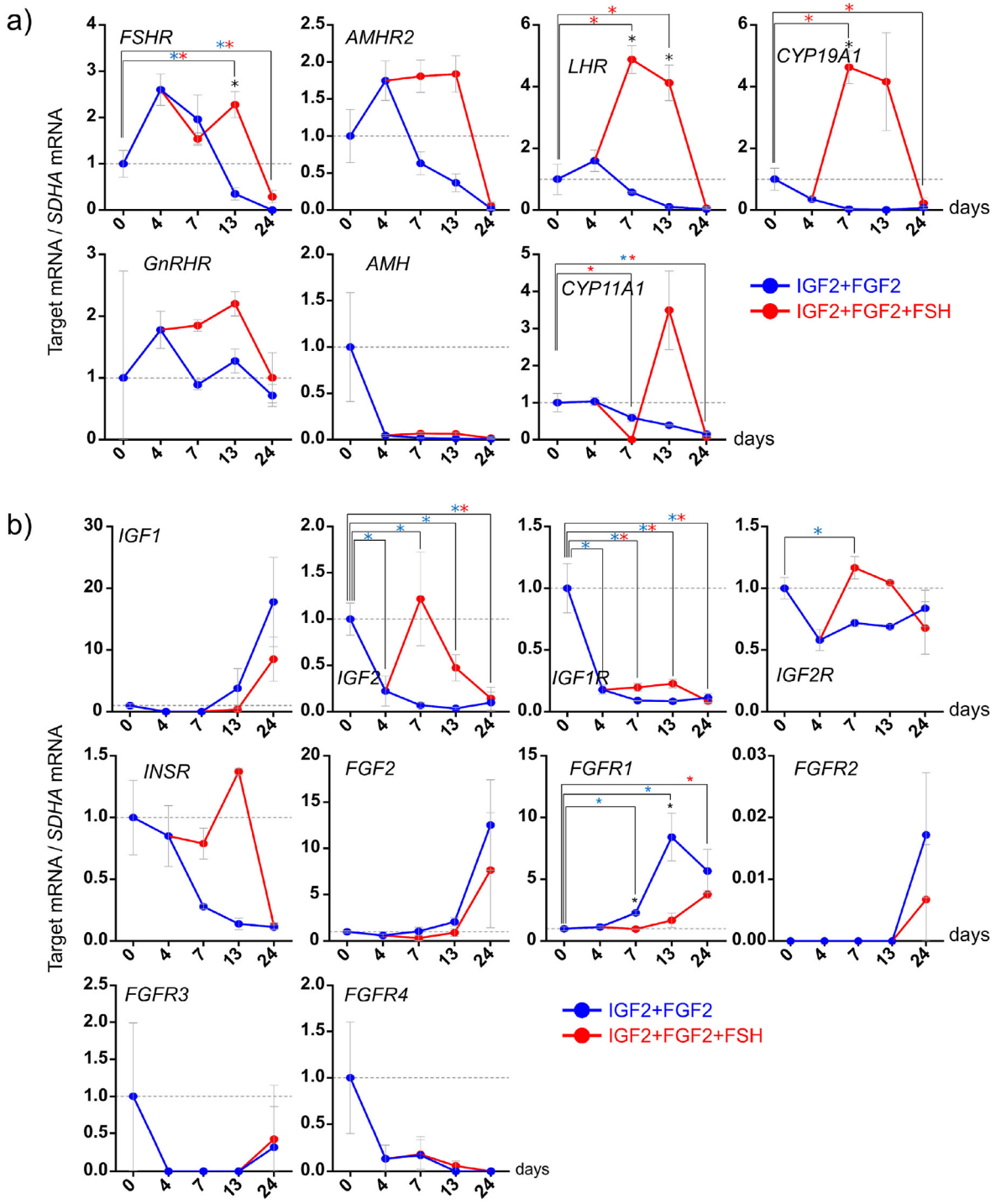



**Fig. 4.** Gene expression of shown genes in CGCs that were cultured in serum-free growth medium with IGF2 and FGF2. FSH (1 U/ml) was added to half of the cells starting from day 4. Growth medium was exchanged daily and time points were taken on days 0, 4, 7, 13 and 24. qPCR results of GC markers (a) and other targets (b) were analysed using ΔΔCt method, where *SDHA* was used as a reference gene and the results are shown in fold changes relative to day 0, except for *FGFR2*, in which case the gene expression levels are normalized to *SDHA*. Results are expressed as mean ± SD; n = 3. * (blue asterisk), $p < 0.05$ gene expression in IGF2 + FGF2 medium between day 0 and the corresponding time point; * (red asterisk), $p < 0.05$ gene expression in IGF2 + FGF2 + FSH medium between day 0 and the corresponding time point; * (black asterisk), $p < 0.05$ gene expression in the corresponding time point between IGF2 + FGF2 medium and IGF2 + FGF2 + FSH medium; Student's t-test after Bonferroni correction for multiple testing. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

speeds (Fig. 5a). The biggest difference was seen on day 7, where the cell count in the medium without FSH was about 480 000 and in the FSH medium about 312 000. On day 13 the cell counts were close to 500 000; and on day 24 the cell counts were around 2 million.

The morphology of MGCs changed again from small and round to long and narrow (Fig. 5b). This change was slower in the FSH medium, but the difference wasn't as big as for CGCs.

The results of qPCR showed that, in general, the expression of GC markers in MGCs (Fig. 6, supplementary table ST4) changed quite similarly to CGCs, however, they were mostly down-regulated faster

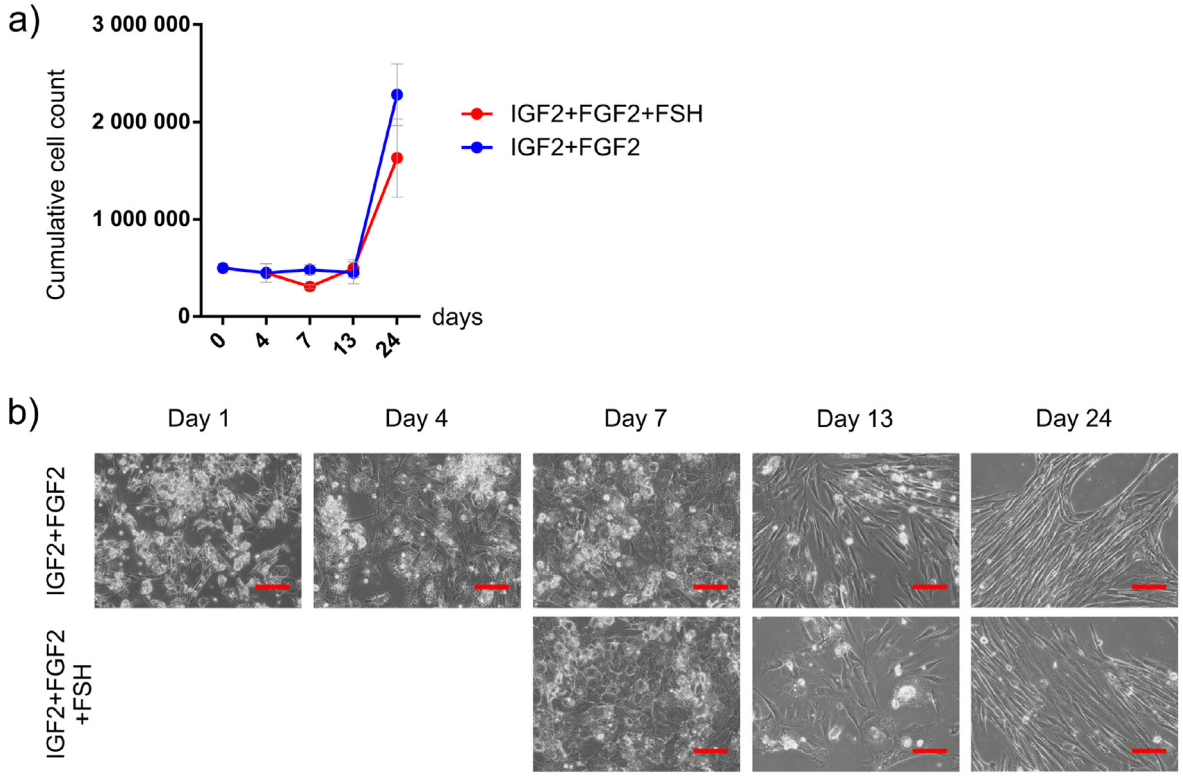



**Fig. 5.** Cumulative cell count of MGCs cultivated in different growth media on days 0, 4, 7, 13 and 24 (a). MGC morphology cultivated in different growth media on days 1, 4, 7, 13 and 24 before passaging. The scale bar corresponds to 100 μm (b). Expression levels and cell counts are shown as mean ± SD; n = 3.

(Fig. 6a) and there were markers which expression was regulated differentially on day 13 between CGCs and MGCs when FSH was added to the medium (supplementary table ST5). The expression levels of *FSHR*, *AMHR2*, *LHR*, *CYP19A1* and *CYP11A1* all increased in either FSH medium or both media, but then decreased to barely detectable levels by the end of the experiment. In contrast to CGCs, *GnRHR* expression increased in both media by day 7 and then dropped back to the original level by days 13 and 24. The expression of *AMH* dropped in both media already by day 4 and stayed low until the end of the experiment, just like in CGCs.

In general, the expression levels of our chosen growth factors and their receptors followed similar patterns in MGCs and in CGCs, although the average differences between the two media were smaller in MGCs (Fig. 6b). The subtle differences we noted between the two granulosa cell types were associated with the FSH response on day 13 (supplementary table ST5).

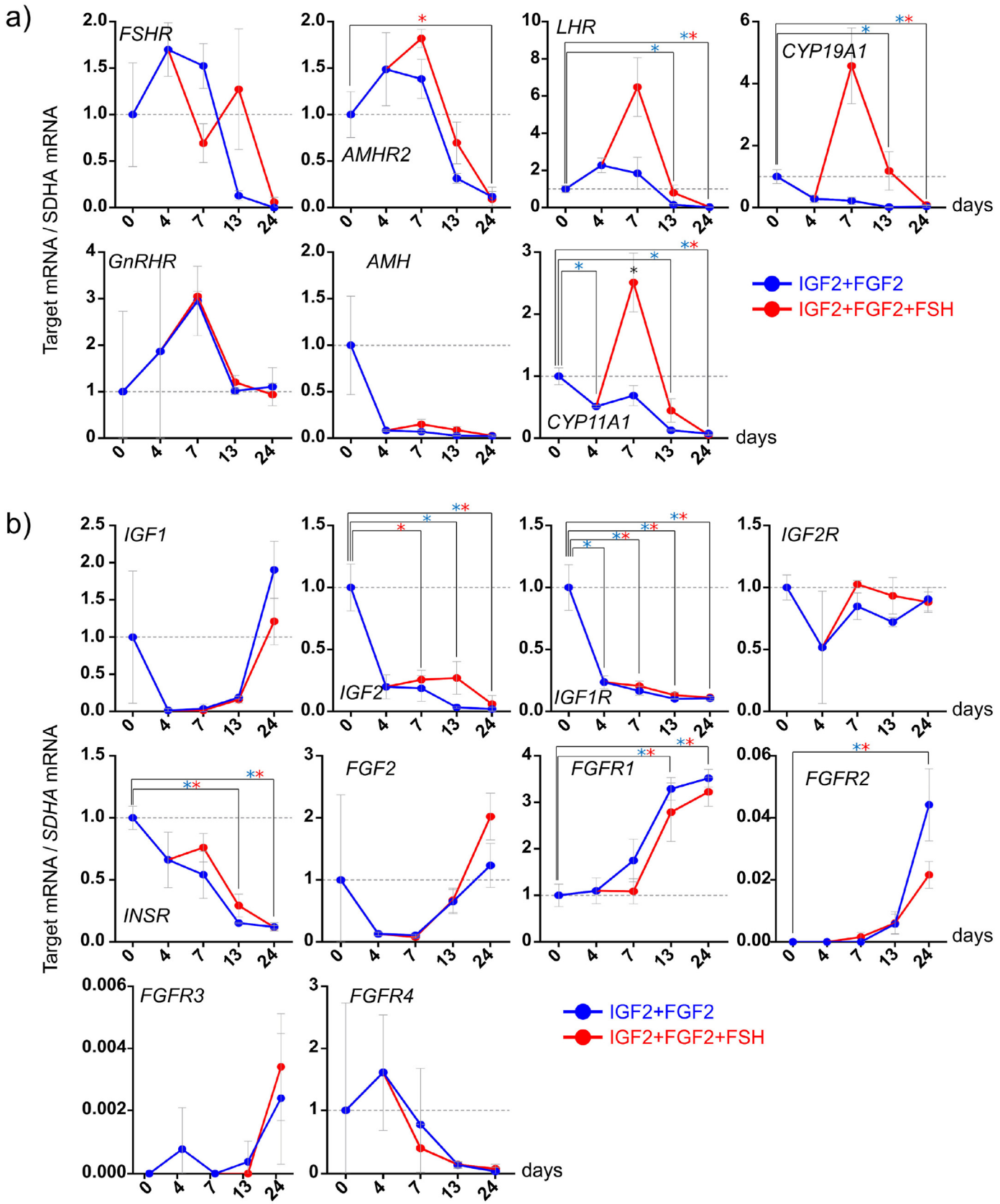
## 4. Discussion

Cultivation of primary human GCs has provided important knowledge on follicular biology that is crucial for understanding impaired folliculogenesis in female infertility. However, these studies have been challenging for a long time, because the primary cells: 1) are limited in availability; 2) proliferate very slowly in culture; and 3) tend to lose their characteristic marker gene expression in cell culture. Additionally, GCs are usually obtained during IVF procedure, meaning that they are in the luteinized state and are unresponsive to gonadotropin stimulations immediately after derivation (Ophir et al., 2014).

Most of the current GC culture protocols are not suitable for culturing cells more than a week or they complement growth medium with FBS, the composition (including various growth factors) of which can vary and therefore cause unpredicted differences in the results of multiple experiments. In the current study, we established a serum-free protocol for *in vitro* cultivation of primary human GCs, by further developing the protocols published by Ophir et al. (2014) and Baumgarten et al. (2015), and making them more suitable for long-term cell culture, so that the expression of GC marker genes is maintained for at least two weeks.

Although our initial focus was on finding optimal culture conditions for long-term culturing of primary granulosa cells, the serum-free culture protocol allowed us to monitor the response to FSH in CGCs and MGCs for up to 20 days, highlighting distinctive response patterns in these two granulosa cell types. With the exception for *GnRHR* and *AMH* and by looking only the response trends, both cell types responded to FSH with the eventual upregulation of common granulosa markers such as *FSHR*, *AMHR2*, *LHR*, *CYP19A1* and *CYP11A1*, however, there were differences when and for how long the upregulated expression was maintained. While the *FSHR* expression patterns were similar in both cell types, the expression of *AMHR2* and *LHR* proved to be statistically different between CGCs and MGCs on day 13. The expression levels of *AMHR2* and *LHR* had already dropped below the starting level by day 13 in MGCs, even with FSH, suggesting that its effect on preserving granulosa phenotype is shorter in MGCs. The expression of *CYP19A1* seems to be supported longer in CGCs, while in case of *CYP11A1* there was a clear difference in the expression timing. The positive effect of FSH on the expression of *CYP11A1* was more rapid in MGCs (expression peak on day 7) compared to CGCs (expression peak on day 13), but by day 24 the expression dropped to similarly low levels in both cell types. These results are in concordance with a previous study (Ophir et al., 2014), where FSH was added to MGCs on day 4 and after 3 days they also saw an increase in *LHR*, *CYP19A1* and *CYP11A1* expression levels.

Perhaps the most interesting difference between the two cell types was noted in the *GnRHR* expression pattern. In MGCs *GnRHR* expression seems to be upregulated in both growth media by day 7, but in CGCs the upregulation was more dependent on FSH and the expression stayed high for longer period of time than in MGCs. However, by day 24 its expression was close to the starting levels in both media in both cell types. As the function of *GnRHR* in granulosa cells is not well known, it




*(caption on next page)*

**Fig. 6.** Gene expression of shown genes in MGCs that were cultured in serum-free growth medium with IGF2 and FGF2. FSH (1 U/ml) was added to half of the cells on starting from day 4. Growth medium was exchanged daily and time points were taken on days 0, 4, 7, 13 and 24. qPCR results of GC markers (a) and other targets (b) were analysed using ΔΔCt method, where *SDHA* was used as a reference gene and the results are shown in fold changes relative to day 0, except for *FGFR2* and *FGFR3*, in which case the gene expression levels are normalized to *SDHA*. Results are expressed as mean ± SD; n = 3. *(blue asterisk), $p < 0.05$ gene expression in IGF2 + FGF2 medium between day 0 and the corresponding time point; *(red asterisk), $p < 0.05$ gene expression in IGF2 + FGF2 + FSH medium between day 0 and the corresponding time point; *(black asterisk), $p < 0.05$ gene expression in the corresponding time point between IGF2 + FGF2 medium and IGF2 + FGF2 + FSH medium; Student's t-test after Bonferroni correction for multiple testing. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

is intriguing that our results point to potentially different roles in CGCs and MGCs. One of the FSH-dependent mechanisms where the differential response may be important is the selection of dominant follicle (Mihm and Evans, 2008), where cumulus cells could have a distinct role in fine-tuning the responsiveness to GnRH via FSH-dependent upregulation of GnRHR.

*AMH* expression decreased very rapidly in both media and both cell types in our study. Previously, it has been shown that adding AMH to the growth medium significantly reduces the upregulating effect of FSH to the expression levels of *CYP19A1* and *FSHR* (Pellatt et al., 2011). There is an open discussion about the effect of FSH on the expression of AMH and whether FSH might be inducing or reducing the expression of AMH (Dewailly et al., 2016). Our experiments where *AMH* expression decreased in the medium without FSH and did not increase after addition of FSH, support the findings where FSH does not induce the expression of AMH. At the same time, it is interesting, that FSH seemed to support the expression of *AMHR2* in CGCs, but not much in MGCs under our culture conditions. These findings could be valuable insights for further studies of these effects and we think that our culture conditions are good basis for this. In parallel, the time-dependent expression patterns of other markers allow us to speculate about the differentiation status of the granulosa cells and the fact that we were not able to see increased expression of *AMH* may also reflect cell differentiation status and the limits of the current culture conditions. All in all, our studies indicate that the effect of FSH on GC marker genes is on average stronger in CGCs compared to MGCs, suggesting that CGCs are more sensitive to FSH stimulation.

When looking at the expression patterns of the selected growth factors and their receptors in CGCs and MGCs we also noted some interesting differences. Although not statistically different, the expression of *IGF2* seemed to be increased in CGCs by FSH, but not in MGCs, also suggesting that CGCs are more sensitive to FSH. Likewise, we see similar trends on the expression levels of *IGF1R*, *IGF2R* and *INSR*. Out of the three receptors, *INSR* seems to have most distinctive pattern in the FSH-dependent expression regulation between the two granulosa cell types. The CGCs responded more effectively to FSH with the upregulation of INSR and showed significant difference on day 13. Even though the differences in FSH response for the expression of *IGF2* and *IGF2R* were not statistically significant between CGCs and MGCs, there is still a clear tendency supporting the hypothesis that in the growth medium CGCs are more sensitive to FSH compared to the MGCs. As it is known that oocyte is dependent on CGCs in the glucose metabolism (Russell and Robker, 2007), the increased sensitivity of the *INSR* in CGCs may be important for the final maturation of oocyte. Similarly, the FSH-dependent trend of increased expression of *IGF2* from CGCs has been seen before and was proposed to be important during the final steps of follicular maturation (Baumgarten et al., 2015). Moreover, the oocyte can potentiate the expression of *IGF2* from CGCs via secretion of BMP15 and GDF9 (Hobeika et al., 2018). Interestingly, this effect seems to be at least partially associated with the BMP15-dependent upregulation of FSHR (Shimizu et al., 2019), which shows again the importance of FSH-dependent regulatory mechanisms in follicular maturation. We think that the complex interactions of different growth factors in MGCs and CGCs should be further studied in parallel under more defined culture conditions and the results from current study will help to implement this strategy in future research.

The expression levels of GC markers correlated with cell morphology. When markers' expression levels were high, cells were small and round, similar to their original appearance. When markers' expression levels started decreasing, the cells stared to elongate, became thinner and ended up looking more like fibroblasts. This also correlated to the expression levels of *FGF2* and its main receptor *FGFR1* – the higher the expression, the more similar the cells looked to fibroblasts. Interestingly, the expression of *FGFR1* was also more dependent on FSH in CGCs compared to MGCs. Here the addition of FSH tended to decrease the expression level of *FGFR1* and preserved longer the granulosa cell phenotype.

Since *in vivo* the expression levels of *FSHR*, *LHR*, *CYP19A1* and *CYP11A1* reach their peak in the preovulatory non-luteinized state and are lower after oocyte retrieval (Ophir et al., 2014), we can hypothesize that the cells become more similar to preovulatory GCs in cell culture, using the long-term culture protocol developed in the current study. Thus, the GCs cultured according to our serum-free protocol could be used as a model for studying earlier stages of folliculogenesis with non-luteinized GCs.

## 5. Conclusion

In conclusion, our new cell culture protocol allows cultivating primary human GCs while maintaining the expression of GC markers for at least two weeks of culture. Additionally, using serum-free conditions in GC culture reduces the variability between different experiments, and provides a reliable tool for future research to study the biology of human GCs.

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## CRediT authorship contribution statement

**Kati Hensen:** Investigation, Conceptualization, Formal analysis, Methodology, Writing - original draft. **Martin Pook:** Conceptualization, Formal analysis, Methodology, Writing - original draft. **Anu Sikut:** Resources, Writing - review & editing. **Tõnis Org:** Formal analysis, Writing - review & editing. **Toivo Maimets:** Formal analysis, Writing - review & editing, Resources, Funding acquisition. **Andres Salumets:** Conceptualization, Formal analysis, Methodology, Writing - review & editing, Resources, Funding acquisition. **Ants Kurg:** Conceptualization, Formal analysis, Methodology, Writing - review & editing, Resources, Funding acquisition.

## Declaration of competing interest

The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110816.

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