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Quantitative analysis of oestrogen receptor α and β gene expression during prolonged culture of pig granulosa cells*

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ABSTRACT

The aim of the present work was to determine the relative transcript abundance of the oestrogen receptor alpha (ERα) and beta (ERβ) gene in long-term cultures (up to 18 days) of pig granulosa cells (GC) obtained from small (1-2 mm; SF-GC) and large (5-7 mm; LF-GC) follicles. GC were cultured in the presence of 10% foetal calf serum (FCS) and one of the following growth factors: leukaemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), or stem cell factor (SCF). The relative transcript abundance of the investigated genes was measured by real time PCR (RT-PCR). In comparison with the control (medium only), LIF significantly increased ($P<0.05$-0.01) ERα gene expression in SF-GC after 3, 15 and 18 days of culture and after 12 days in LF-GC. The ERβ mRNA level in SF-GC was significantly decreased ($P<0.01$) by all of the investigated factors after 3, 6, 9 and 12 days of incubation. In conclusion, expression of both types of oestrogen receptors during prolonged culture of pig GC under different conditions suggests the role of oestrogens acting *via* their receptors in maintaining survivability of porcine granulosa cells *in vitro*.

KEY WORDS: pig, granulosa cells, oestrogen receptor, mRNA, prolonged culture, growth factors

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INTRODUCTION

Oestrogens, acting in a paracrine or autocrine manner, play an essential role in the proliferation and differentiation of granulosa cells (Hewitt et al., 2005). These steroids exert their effect through two subtypes of nuclear receptors: oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ). Slomczynska and Wozniak (2001) and Slomczynska et al. (2001) localized both subtypes of oestrogen receptors in the pig ovary, with ERβ predominating. ERβ was present at all stages of follicular development, whereas ERα mRNA and protein were detected only in preovulatory follicles and early corpora lutea. Previously, we observed higher expression of ERβ and ERα genes in freshly isolated pig SF-GC in comparison with LF-GC (unpublished). The effect of oestrogens acting through their nuclear receptors on granulosa cell function is still not fully elucidated. In our previous study we demonstrated the involvement of ERα and ERβ in pig granulosa cell proliferation, oestrogen synthesis, and telomerase activity (Chronowska et al., 2009, 2010). Oestradiol produced in the developing ovarian follicle is considered to be an antiatretic survival factor. Its production in atretic follicles is significantly decreased. Yamagata et al. (2002) found a decreased level of telomerase activity in experimentally induced atretic rat follicles. Administration of oestradiol to female rats prevented both follicular atresia as well as a decrease of telomerase activity in granulosa cells.

Luteinization of granulosa cells is a terminal phase of their differentiation process (Niswender et al., 2000). In pigs, effective long-term cultivation of granulosa cells was possible for 144 h (Picton et al., 1999). Spontaneous induction of apoptotic cell death is the reason for quick degeneration of primary granulosa cells *in vitro*. Recently, we demonstrated that porcine granulosa cells cultured in the presence of basic fibroblast growth factor (bFGF) and 10% foetal calf serum survived *in vitro* for a period of 18 days and kept their proliferative potential and telomerase activity. Stem cell factor (SCF) and leukaemia inhibitory factor (LIF) also stimulated telomerase activity but showed no effect on proliferation of long-term cultured pig GC (Chronowska and Kott, 2011). LIF has been recently shown to permit the prolonged survival of luteinizing human granulosa cells that progressively lost their major characteristics such as FSHR (follicle stimulating hormone receptor) and aromatase (Kossowska-Tomaszczuk et al., 2009). The effect of LIF on long-term maintenance of embryonic stem cells is well documented in mice but not in humans (Daheron et al., 2004). Additionally, LIF plays an important role in stem cell self-renewal in the brain, gut, and bone marrow (Jiang et al., 2002; Kalabis et al., 2003; Bauer et al., 2006). Basic fibroblast growth factor (bFGF) is an important component of human embryonic stem cell culture medium due to its essential role in keeping the embryonic stem cells in an undifferentiated state (Lysdahl et al., 2006). Stem cell factor (SCF) is expressed

in female primordial germ cells (Rossi et al., 2000). It was also shown to increase the survival rate of haematopoietic stem cells (HSCs) *in vitro* and to contribute to the self-renewal and maintenance of HSCs *in vivo* (Kent et al., 2008).

Taking into account that oestrogens acting *via* oestrogen receptors are important factors regulating granulosa cell function and survivability, in the present study we attempted to determine the level of ERα and ERβ gene expression in porcine granulosa cells cultured over a prolonged period of time in the presence of growth factors known to be involved in maintaining the survivability of pig granulosa cells *in vitro*.

MATERIAL AND METHODS

Isolation of porcine granulosa cells and in vitro culture conditions

Ovaries from prepubertal gilts were collected from a local slaughterhouse and transported within 30 min to the laboratory in a thermo-container filled with phosphate-buffered saline (PBS). In the laboratory the ovaries were placed in sterile PBS supplemented with an antibiotic antimycotic solution (Sigma, UK) for 20 min. Individual follicles of 1-2 mm and 5-7 mm in diameter were isolated by dissection and split-open under a stereomicroscope (Leica MZ6, Switzerland) into DMEM/F12 medium (Gibco, BRL) to obtain granulosa cells. The cell suspension was treated with trypsin and DNase (Sigma, USA) to eliminate dead cells. The number of living cells in suspension was estimated by using 0.25% trypan blue (Sigma, USA) in PBS and by counting in a haematocytometer. The viability of the granulosa cell suspension after trypsin/DNase treatment was 90%. Cells were seeded onto 6-well Nunclon Delta (Nunc, Denmark) culture plates at a density of $4x10^5$ living cells/well and cultured as a proliferating monolayer in a Knockout™ DMEM medium supplemented with an insulin-transferrin-selenium mixture (ITS-X, 1 ml/100 ml) and gentamicin (all Gibco, BRL). The cells were cultured at 37ºC in a humidified atmosphere of 5% CO_2 in a Sanyo MCO-175M incubator. The culture medium was changed every second day and cells were passaged when 80% confluency was reached.

Cell culture medium supplements

Granulosa cells from small and large follicles were cultured for up to 18 days in the presence of 10% of foetal calf serum (FCS, EmbryoMax; Chemicon, USA) and one of the following growth factors: leukaemia inhibitor factor (LIF; Chemicon, USA), basic fibroblast growth factor (bFGF; Sigma, USA), or stem cell factor (SCF; Chemicon, USA). The concentration of experimental factors was: LIF-1.000 IU/ml (Kossowska-Tomaszczuk et al., 2009), bFGF- 10 ng/ml

(Ko et al., 2010), SCF-10 ng/ml (Brankin et al., 2003). Growth factors were added to the culture medium 24 h after the beginning of the incubation. Cells were harvested for mRNA analysis in 72 h intervals.

Preparation of granulosa cells for mRNA analysis

At specified time intervals/points, the culture media from individual culture wells were removed. Cells were washed with serum-free medium and PBS and detached with Accutase (Chemicon, USA). They were then transferred to Eppendorf tubes and centrifuged at 4°C at 6000 rpm (3500 G) for 15 min. Pellets of cells were stored at -80°C until further use.

RNA isolation and reverse transcription

Total RNA was isolated from the cultured cells using 6100 Nucleic Acid PrepStation and Total RNA Chemistry (Applied Biosystems (ABI), USA) and quantified by measuring absorbance at 260 nm. Fifty microlitres of RNA were transcribed into cDNA using the cDNA Archive Kit (ABI) following the instructions of the provider.

Analysis of relative transcript abundance by real time PCR

Glyceraldehyde 3-phosphate dehydrogenase (G3APDH) was used as a reference gene. ERα primers (forward, 5'-ACTAAGAAGAACAGCCCGGT CTT-3'; reverse, CTCCAACAAGGCACTGATCATC-3') and MGB probe (TagMan MGB NED-TCCCTGACAGCCGAC) and glyceraldehyde 3-phosphate dehydrogenase primers (forward, 5'-GAGCATCTCCTGACTTC CAGTTTC-3'; reverse, 5' CCTAAGCCCCTCCCCTTCT-3') and MGB probe (VICATCCCA GACCCCC) were designed using the Primer Express 3.0 Programme (ABI). For ERβ, the primer sequences published in Diaz and Witlbank (2004) were used (forward, 5'- TCCTTTAGCCATCCATTGCC-3'; reverse, 5'-TCCTGACGCATAATCACTGCA-3'). For G3APDH and ERα, the PCR mix composition for one sample consisted of: 1 µl of cDNA, 500 nM of forward and reverse primers, 250 nM of TaqMan®MGB probe, 5 µl of 1XTaqMan®Fast Universal MasterMix, NoAmpErase®UNG (Applied Biosystems, USA), water to a volume of 10 µl. The PCR reaction was continued for 40 cycles after the initial denaturation at 95°C for 20 s. Each cycle of PCR consisted of 1 s of denaturation at 95°C and 20 s of annealing at 60°C. The PCR mix composition for ERβ analysis consisted of: 1 µl of cDNA, 15 µM of forward and reverse primers, 5 µl 2x Power SYBR Green PCR Master Mix (Applied Biosystems, USA), water to a volume of 10 µl. PCR was continued for 40 cycles after initial denaturation at 95°C for 20 s. Each cycle of PCR consisted of 1 s of denaturation at 95°C, 20 s of annealing

at 60°C and 20 s of elongation at 72°C. Quantification of ERα and ERβ was performed using the ABI 7500 fast real-time PCR System.

Statistical analysis

The data were obtained from 3 experiments. Each experiment consisted of three repetitions per treatment. The data are presented as means ± SEM. All data were analysed using the SAS programme (SAS, 2001; SAS System for Windows, Release 8.2; TS2M0). One-way ANOVA was used to determine the significance of differences of the effect of LIF, bFGF and SCF on oestrogen receptor α and β mRNA levels in porcine granulosa obtained from small and large follicles cultured for 18 days. Differences with a probability of $P<0.05$ were considered significant.

RESULTS

LIF significantly increased ($P<0.05$-0.01) ERα gene expression in SF-GC after 3, 15 and 18 days of culture (Figure 1) and after 12 days of culture in LF-GC (Figure 2). SCF increased ($P<0.05$) relative transcript abundance of the ERα gene after 3 days of incubation in SF-GC (Figure 1) and after 9 and 18 days in LF-GC (Figure 2). bFGF increased ($P<0.01$) ERα gene expression in SF-and LF-GC after 18 days of culture (Figures 1 and 2). The relative transcript

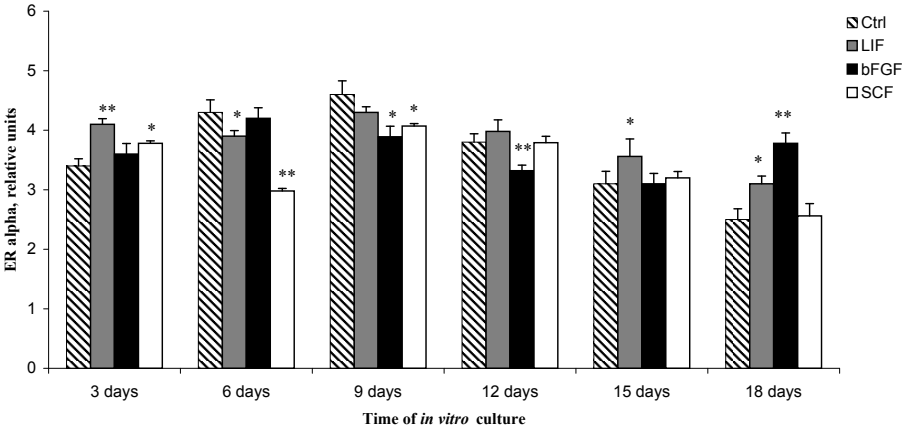

Figure 1. Relative transcript abundance of ERα gene in pig SF-GC assayed by real time PCR after 3, 6, 9, 12, 15 and 18 days of *in vitro* culture in the presence of 10% of FCS and one of the following experimental factors: LIF (1.000 IU/ml), bFGF (10 ng/ml) and SCF (10 ng/ml). Each bar represents mean ± SEM for 3 experiments performed in triplicates. * and ** denotes means significantly different from control ($P<0.05$-0.01)

abundance of the ERβ gene in SF-GC was significantly decreased (P<0.01) by all of the investigated factors after 3, 6, 9 and 12 days of incubation (Figure 3). In LF-GC, LIF, SCF, and bFGF decreased (P<0.05-0.01) ERβ gene expression after 3 and 6 days of incubation. After 18 days of culture, the relative transcript

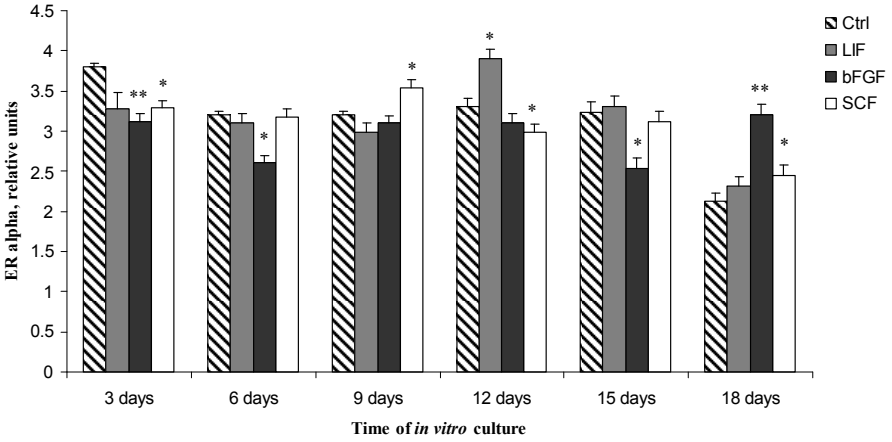

Figure 2. Relative transcript abundance of ERα gene in pig LF-GC assayed by real time PCR after 3, 6, 9, 12, 15 and 18 days of *in vitro* culture in the presence of 10% of FCS and one of the following experimental factors: LIF (1.000 IU/ml), bFGF (10 ng/ml) and SCF (10 ng/ml). Each bar represents mean ± SEM for 3 experiments performed in triplicates. * and ** denotes means significantly different from control (P<0.05-0.01)

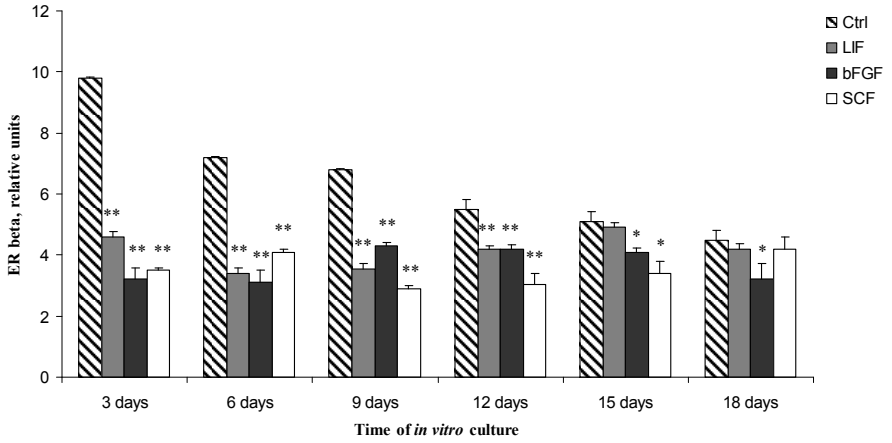

Figure 3. Relative transcript abundance of ERβ gene in pig SF-GC assayed by real time PCR after 3, 6, 9, 12, 15 and 18 days of *in vitro* culture in the presence of 10% of FCS and one of the following experimental factors: LIF (1.000 IU/ml), bFGF (10 ng/ml) and SCF (10 ng/ml). Each bar represents mean ± SEM for 3 experiments performed in triplicates. * and ** denotes means significantly different from control (P<0.05-0.01)

abundance of the ERβ gene in LF-GC was significantly increased (P<0.05-0.01) by all of the investigated growth factors (Figure 4).

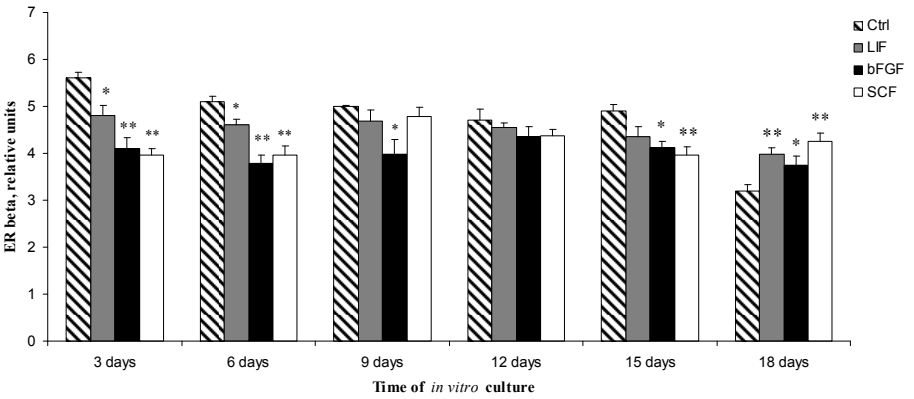

Figure 4. Relative transcript abundance of ERβ gene in pig LF-GC assayed by real time PCR after 3, 6, 9, 12, 15 and 18 days of *in vitro* culture in the presence of 10% of FCS and one of the following experimental factors: LIF (1.000 IU/ml), bFGF (10 ng/ml) and SCF (10 ng/ml). Each bar represents mean ± SEM for 3 experiments performed in triplicates. * and ** denotes means significantly different from control (P<0.05-0.01)

DISCUSSION

Previously, we demonstrated that porcine granulosa cells cultured in the presence of bFGF have the ability to survive *in vitro* over a prolonged period of time (up to 18 days) and maintain their proliferative potential and telomerase activity. In the presence of LIF and SCF, granulosa cells also exhibit telomerase activity during prolonged culture, however, a stimulatory effect of these growth factors on GC proliferation has not been shown (Chronowska and Kott, 2011). In the present study we focused on the expression of genes for two subtypes of oestrogen receptor in pig granulosa cells cultured in the presence of LIF, bFGF, or SCF. The rationale behind undertaking this study is the well-established role of oestrogens in maintaining granulosa cell survivability (Yamagata et al., 2002). Therefore, it was interesting to elucidate if and how the level of oestrogen receptors is changed under the influence of different growth factors during prolonged culture of GC obtained from follicles of different sizes.

Even though the exact role of both types of oestrogen receptors in the porcine ovary is still to be elucidated, their expression on the protein and mRNA levels has been clearly demonstrated under different physiological conditions.

Positive signals for ERβ were found in both granulosa and theca cells of all types of antral follicles, as well as in the corpora lutea at all stages of regression. ERα mRNA was limited exclusively to the granulosa cells of preovulatory follicles and was present in a few cells of the early corpora lutea. Thus, differential expression of ERα and ERβ at the mRNA level was demonstrated (Slomczynska et al., 2001). Large antral follicles and early corpora lutea are sites where both forms of oestrogen receptor are expressed. RT-PCR results indicated that ERα mRNA was expressed only in ovarian follicles of pregnant sows, while that of ERβ, in both follicles and corpora lutea (Knapczyk et al., 2008). In the present study, ERα mRNA was detected in both large and small follicles during the entire period of culture. In SF-GC, LIF stimulated ERα expression at the beginning of the culture (3 days) and at the final stages of cultivation (days 15 and 18), while in the case of LF-GC, this effect was noted only after 12 days of cultivation. ERβ expression was stimulated by LIF only in LF-GC after 18 days of culture. In our previous study (Chronowska and Kott, 2011), LIF showed a stimulatory effect on telomerase activity in pig granulosa cells. To date, the effect of LIF on ER expression in granulosa cells had not been investigated. Indirectly, the effect of this growth factor on oestrogen synthesis in human granulosa cells was studied by Kossowska-Tomaszczuk et al. (2009). The authors found that in the presence of LIF, granulosa cells from preovulatory follicles could be cultured for more than 50 days and that under these conditions they lost their functional properties, such as aromatase expression and FSHR. Thus, LIF apparently contributes to the dedifferentiation of long-term cultured granulosa cells. In human breast cancer *in vitro*, both primary tumours and cultured cell lines, stimulation of growth by LIF acting *via* its receptor (LIFR) was demonstrated. Moreover, the expression of LIFR correlated with the presence of oestrogen receptors (Dhingra et al., 1998). In other studies, LIF stimulated the growth of MCF-7 as well as other oestrogen-dependent and independent breast cancer cell lines, but the effect on normal breast epithelial lines was less significant (Estrov et al., 1995).

In the present study, bFGF stimulated ERα gene expression in SF- and LF-GC only after 18 days of culture. In our previous study (Chronowska and Kott, 2011), we demonstrated that bFGF enabled long-term culture of granulosa cells and decreased their differentiation, as measured by aromatase expression. In light of this, the observed lack of a stimulatory effect of bFGF on ER expression in GC during most of the culture period is not surprising. On the other hand, it was observed (Sharma et al., 2010) that bFGF stimulates survival, growth, antrum formation, and steroidogenesis in buffalo preantral follicles. Similar results were obtained by Garor et al. (2009) who concluded that bFGF apparently plays a role in oestradiol production of early human follicles.

In the present study, SCF stimulated ERα and ERβ in LF-GC after 18 days of culture. The effect of SCF on oestrogen receptor expression in pig GC has not been investigated yet. It is well established that SCF acts in para- and autocrine manners in developing pig ovarian follicles and contributes to enhanced production of progesterone and oestradiol (Brankin et al., 2003). In our previous study (Chronowska and Kott, 2011), SCF reduced aromatase expression in long-term culture of granulosa cells, however, to a lesser extent than observed in the case of bFGF. Aromatase expression is considered to be one of the most important markers of granulosa cell differentiation. Absence of an effect of SCF on ER expression and its role in decreasing aromatase expression may suggest the involvement of this growth factor in keeping granulosa cells in a less differentiated state (or in induction of their dedifferentiation). This observation may be interesting from the point of view of establishing porcine granulosa cell lines, which is an important aspect in elucidation of GC stem cell properties.

CONCLUSIONS

In conclusion, the results of the present study show the expression of oestrogen receptor alpha (ERα) and beta (ERβ) mRNA in porcine small and large granulosa cells cultured long-term. Moreover, expression of ERα and ERβ is regulated by leukaemia inhibitory factor, basic fibroblast growth factor and stem cell factor, which belong to the factors involved not only in maintaining the survivability of different types of cells *in vitro*, but also play a role in the physiology of the pig ovary.

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