OPTIMIZATION OF CONDITIONS OF PROLONGED CULTURE OF PIG GRANULOSA CELLS IN VITRO*

Ewa Chronowska, Tomas Kott

1Department of Biology of Reproduction, 2Department of Molecular Genetics, Institute of Animal Science, Práteleství 815, 104 00 Prague-Uhříněves, Czech Republic

Abstract

The object of the present study was to establish conditions (optimal serum concentration and growth factor supplement) for prolonged culture of pig granulosa cells (GC). GC were cultured in the presence of different fetal calf serum (FCS) concentrations (10% and 20%) and one of the following growth factors: leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and stem cell factor (SCF). GC proliferation potential was measured by ³H-thymidine incorporation and telomerase activity whereas cell differentiation by relative transcript abundance of the aromatase gene. In the presence of 10% FCS GC proliferative potential was significantly higher (P<0.01) in comparison to control (medium only) after 9 days of culture. Basic fibroblast growth factor significantly increased (P<0.01) ³H-thymidine incorporation at all investigated time intervals/points, up to 18 days of culture. Telomerase activity was stimulated (P<0.01) by LIF, bFGF and SCF after 3, 6, 9, 12, 15 and 18 days of cultivation while aromatase expression was decreased (P<0.05) by all of the applied growth factors, at all investigated time intervals. In conclusion, in the presence of 10% of FCS and bFGF GC can be maintained in culture over prolonged period of time without losing their proliferative potential, telomerase activity and with decreased degree of differentiation measured by aromatase expression.

Key words: pig, granulosa cells, proliferation, telomerase, prolonged culture

Rapid proliferation of the granulosa cells (GC) in the growing ovarian follicle and the exertion of such a large variety of specialized functions can only be possible by accepting the fact that the population of GC in a healthy follicle is not uniform but rather consists of subpopulations of differentiated and less differentiated cells (Kossowska-Tomaszczuk et al., 2009). It was previously postulated that during follicular growth GC arise from the population of stem cells (Rodgers et al., 1999). Stem cell
characteristics of GC were verified by their ability to express telomerase (Tomanek et al., 2008; Chronowska et al., 2010). The telomerase activity was higher in bovine GC derived from small follicles characterized by higher proliferative potential and decreased in large follicle GC (Lavranos et al., 1999). One of the most important characteristics of stem cells is their ability to go through numerous cell cycles while maintaining the undifferentiated state (Wobus and Boheler, 2005).

Luteinization of granulosa cells is considered a terminal phase of their differentiation process (Niswender et al., 2000). Human granulosa cells obtained from preovulatory follicles quickly luteinize in vitro and their culture has not been possible beyond 10 days (Kossowska-Tomaszczuk, 2009). In pigs, effective, long-term cultivation of granulosa cells was possible for 144 h (Picton et al., 1999). Spontaneous onset of apoptotic cell death is the reason for rapid degeneration of primary granulosa cells in vitro. Earlier attempts to circumvent this limitation included GC transformation with oncogenes, spontaneous immortalization of primary cultures, and chemical carcinogenesis (Horisberger, 2006). Tajima et al. (2002) reported that forskolin, 8-Br-cAMP and FSH modulate cell growth and steroidogenesis in human granulosa cells immortalized by transfection of primary cells with p53 mutated gene in combination with Harvey-ras oncogene. Nishi et al. (2001) established steroidogenic human granulosa-like tumour cell line that expressed functional follicle stimulating hormone receptor (FSHR). Recently, leukemia inhibitory factor (LIF) has been shown to permit the prolonged survival of luteinizing human granulosa cells which progressively lost their major characteristics such as FSHR and aromatase (Kossowska-Tomaszczuk et al., 2009). LIF is a glycoprotein with a remarkable range of biological actions in different tissues. Its effect on long-term maintenance of embryonic stem cells is well documented in mouse but not in human (Daheron et al., 2004). In brain (Bauer et al., 2006), the gut (Kalabis et al., 2003) and bone marrow (Jiang et al., 2002) LIF was shown to play an important role in stem cell self-renewal.

Basic fibroblast growth factor (bFGF) is a critical component of embryonic stem cell culture medium for human as it is necessary to keep the embryonic stem cells in undifferentiated state (Lysdahl et al., 2006). Stem cells factor (SCF) is expressed in female primordial germ cells (Rossi et al., 2000). It also plays a role in the regulation of hematopoietic stem cells (HSCs) in the stem cell niche in the bone marrow. SCF has been shown to increase the survival of HSCs in vitro and to contribute to the self-renewal and maintenance of HSCs in vivo (Kent et al., 2008).

To our knowledge there are no data demonstrating effective culture of porcine GC for a period exceeding 6 days. Therefore, in the present work we studied the effect of LIF, bFGF and SCF on proliferation, telomerase activity and aromatase expression of pig granulosa cells cultured long-term (up to 18 days). At the first stage of experimental protocol we focused on establishment of appropriate serum concentration for prolonged GC culture. Thereafter, in combination with optimal serum concentration, we applied one of the growth factors to study its effect on proliferation and differentiation of pig GC.
Material and methods

Isolation of porcine granulosa cells and in vitro culture conditions

Ovaries from prepubertal gilts were collected from local slaughterhouse and transported to the laboratory in a thermo-container filled with phosphate-buffered saline (PBS) within 30 min. In the laboratory ovaries were placed in sterile PBS supplemented with an antibiotic antimycotic solution (Sigma, UK) for 20 min. Individual follicles of 3–5 mm in diameter were isolated by dissection and split-opened under a stereomicroscope (Leica MZ6, Switzerland) into DMEM/F12 medium (Gibco, BRL) to obtain granulosa cells. Cell suspension was treated with trypsin and DNase (Sigma, USA) to eliminate dead cells. The number of living cells in suspension was estimated by use of 0.25% trypan blue (Sigma, USA) in PBS and by count in a hematocytometer. Viability of granulosa cell suspension after trypsin/DNase treatment was 90%. Cells were seeded onto 6-well culture plates Nunclon Delta (Nunc, Denmark) in a density of \(4 \times 10^5\) living cells/well and cultured as a proliferating monolayer in a Knockout™ DMEM medium supplemented with an insulin-transfer- rin-selenium mixture (ITS-X, 1 ml/100 ml) and gentamicin (all Gibco, BRL). Cell cultures were performed at 37ºC in a humified atmosphere of 5% CO\(_2\) in a Sanyo MCO-175M incubator.

Cell culture medium supplements

At the first stage of experimental protocol, granulosa cells were cultured for 9 days in the presence of 10% or 20% of fetal calf serum (FCS, EmbryoMax; Chemicon, USA) to study the granulosa cell proliferative response to different serum concentrations during prolonged culture in vitro. Cells were screened for \(^3\)H-thymidine incorporation every third day. When optimal serum concentration was established cells were cultured with experimental factors for up to 18 days. The concentration of experimental factors was as follows: LIF-1,000 IU/ml (Kossowska-Tomaszczuk et al., 2009; Chemicon, USA), bFGF-10 ng/ml (Ko et al., 2010; Sigma, USA), SCF-10 ng/ml (Brankin et al., 2003; Chemicon, USA). During the culture cells were screened for their proliferative potential, telomerase activity and aromatase expression in 72 h intervals (every third day). Hormonal supplements were added to the culture medium 24 hours after the beginning of the culture.

Cell proliferation assay

To study the granulosa cell proliferation potential in vitro, the newly synthesized DNA in cell cultures was measured by incorporation of \(^3\)H-thymidine using the technique of TCA precipitation and liquid scintillation counting.

Telomerase activity assay

Preparation of granulosa cells

In vitro cultured granulosa cells were prepared as follows: at the end of in vitro culture, the culture media from individual culture wells were removed. Cells were washed with a serum free medium and PBS and detached by Accutase (Chemicon,
USA). The cells were then transferred to Eppendorf tubes and centrifuged at 4°C and 6000 rpm (3500 G) for 15 min. Pellets of cells were stored at –80°C until further analysis.

TRAP (Telomeric Repeat Amplification Protocol) assay

For telomerase activity assay, lysates from in vitro cultured cells were prepared in a CHAPS buffer. Briefly, cell pellets of cultured granulosa cells \((2 \times 10^5)\) and the positive control provided in the kit were resuspended with 200 μl of 1X CHAPS Lysis Buffer. The protein concentration was measured spectrophotometrically. A modified protocol based on the TRAPEZE Telomerase Detection Kit (Chemicon, USA) was applied to evaluate telomerase activity. We used an internal telomerase assay standard and the fluorescent type-specific TS primer AATCCGTCGAGCAGAGTT-6-FAMTM (Applied Biosystems, USA). Telomerase extension and PCR were performed in a Biometra T gradient PCR thermocycler (Biometra, Germany). Telomerase activity was expressed as TPG/mg protein (Total Product Generated per milligram of protein).

Analysis of relative transcript abundance of P450 aromatase gene by real time PCR

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. 50 μl of RNA was transcribed into cDNA using the cDNA Archive Kit (ABI). Glyceraldehyde 3-phosphate dehydrogenase was used as a reference gene. Aromatase primers (forward, 5′-CTG ACCGTCTCTTGCCAGTT-3′; reverse, 5′GCCGACCTCTCT-3′) and MGB probe (TET – AATCA CCAAGCACCTT-GGA) and glyceraldehyde 3-phosphate dehydrogenase primers (forward, 5′-GAGCACCTCCTGACTCTCCAGTTCTT-3′; reverse, 5′ CCTAAGCCCCTCCCCCTTCTT-3′) and MGB probe (VIC- ATCCCAGACCCCC) were designed using the Primer Express 3.0 Program (ABI). The PCR mix composition for one probe 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 5 s of denaturation at 95°C and 30 s of annealing at 60°C. Quantification of aromatase was performed using the ABI 7500 Fast Real-Time PCR System.

Statistical analysis

The data were obtained from 4 experiments. Each experiment consisted of three repetitions per treatment. The data are presented as means±SEM. All data were analysed using the SAS Program (SAS 2001, SAS System for Windows, Release 8.2 (TS2M0), SAS Inst., Inc., Cary, NC, USA). One-way ANOVA was used to determine the significance of differences for different serum concentrations, LIF, bFGF and SCF effect on proliferation, telomerase activity and aromatase mRNA level of porcine granulosa cells cultured for 18 days. Differences with a probability of P<0.05 were considered significant.
Results

Porcine granulosa cells cultured in the presence of 10% FCS showed higher (P<0.01) proliferative activity in comparison to the control after 3, 6 and 9 days of cultivation. Proliferative potential of GC cultured in the presence of 20% FCS did not differ from control at all investigated time intervals (Figure 1).

![Figure 1. Proliferation of porcine granulosa cells assayed by incorporation of $^3$H-thymidine after 3, 6 and 9 days of in vitro culture in the presence of 10% and 20% of fetal calf serum (FCS). Each bar represents mean ± SEM for 4 experiments performed in triplicates. ** denotes means significantly different from control (P<0.01)](image1)

![Figure 2. Proliferation of porcine granulosa cells assayed by incorporation of $^3$H-thymidine after 3, 6, 9, 12, 15 and 18 days of in vitro culture in the presence of 10% of fetal calf serum (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 4 experiments performed in triplicates. * and ** denotes means significantly different from control (P<0.05–0.01)](image2)
Figure 3. Telomerase activity in porcine granulosa cells assayed using TRAPEZE Telomerase® Detection Kit after 3, 6, 9, 12, 15 and 18 days of in vitro culture in the presence of 10% of fetal calf serum (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 4 experiments performed in triplicates. ** denotes means significantly different from control (P<0.01)

Figure 4. Aromatase gene expression in porcine granulosa cells assayed by Real Time RT-PCR after 3, 6, 9, 12, 15 and 18 days of in vitro culture in the presence of 10% of fetal calf serum (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 4 experiments performed in triplicates. ** denotes means significantly different from control (P<0.01)

Basic fibroblast growth factor significantly increased (P<0.01) the level of $^3$H-thymidine incorporation after 3, 6, 9, 12, 15 and 18 days of culture. LIF stimulated (P<0.05) GC proliferation only after 9 days of culture (Figure 2). LIF, bFGF
and SCF significantly increased (P<0.01) telomerase activity in pig GC cultured for 3, 6, 9, 12, 15 and 18 days (Figure 3). When compared to control, after 3 days of culture aromatase relative transcript abundance was decreased only by bFGF. At all other time intervals, bFGF, LIF and SCF significantly decreased (P<0.01) the level of aromatase mRNA (Figure 4).

Discussion

During in vitro culture granulosa cells (GC) quickly reach the luteal phenotype characterized by enhanced progesterone synthesis and decreased proliferative potential (Savion et al., 1981). Therefore, cultivation of granulosa cells from growing and preovulatory follicles is possible only for a period of time which usually does not exceed 10 days (Kossowska-Tomaszczuk et al., 2009). In the present study we attempted to find the proper culture conditions which would allow to culture pig granulosa cells for longer period of time without losing their proliferative potential and telomerase activity and with reduced differentiation measured by aromatase expression. To achieve this aim we tested two different serum concentrations (10 and 20%) and three growth factors (LIF, bFGF and SCF).

In the present work, granulosa cells cultured with 10% of FCS expressed higher proliferative potential in comparison to control than cells cultured in the presence of 20% FCS did. In our previous studies (Tomanek et al., 2008; Chronowska et al., 2009; Chronowska et al., 2010) we used only 2% of FCS for GC culture. However, this concentration was suitable only for short-term cultivation. GC cultured with 2% of serum showed signs of apoptosis after 72 h. Apoptotic changes in GC were probably linked not only to luteinization but were also induced by lack of nutrient factors. Granulosa cells cultured for 9 days in the presence of 10% FCS were characterized by highest proliferative potential and showed normal morphology (data not shown). On the contrary, presence of 20% of FCS in culture medium resulted not only in decreased proliferation of GC but also increased number of dead cells. The reason for this is not clear. Possibly, some undefined factors present in serum have an unfavourable effect on granulosa cells when applied in higher concentration for prolonged time. In most studies focused on embryonic and adult stem cell culture 10% or 15% of serum in culture medium was used (Ko et al., 2010; Kossowska-Tomaszczuk, 2009). Therefore, based on our results, for the further experiments we chose 10% of FCS as a component of culture medium.

In the present study bFGF significantly increased the level of $^3$H-thymidine incorporation at all investigated time intervals. The favourable effect of bFGF on survivability of granulosa cells has been confirmed before. It was shown that bFGF inhibits primary granulosa cells from undergoing apoptosis and that this process is mediated by regulation of intracellular free calcium levels through protein kinase C delta-dependent pathway (Lynch et al., 2000). In our studies LIF showed a stimulatory effect on GC proliferation only after 9 days of incubation. Our results differ from those obtained earlier by Kossowska-Tomaszczuk et al. (2009). Their study
brought interesting results for LIF effect on long-term culture of luteinizing human granulosa cells isolated from follicles of infertile patients. In the presence of LIF GC survived in the culture for more than 50 days.

In our study all of the investigated factors decreased aromatase expression in porcine granulosa cells cultured for 18 days. Aromatase expression is one of the most important markers of GC differentiation (Rodgers et al., 1999). Similar results were previously obtained by Kossowska-Tomaszczuk et al. (2009). In the studies mentioned above, GC cultured in the presence of LIF for more than 50 days gradually lost functional markers, such as follicle stimulating hormone receptor and aromatase. At the same time typical stem cell marker POU5F1 (POU domain, class5, homebox 1) was expressed throughout the culture. Down-regulation of telomerase activity is directly linked to cell terminal differentiation (Sharma et al., 1995). In our study bFGF, LIF and SCF stimulated telomerase activity in GC at all of investigated time intervals. Similar results were obtained in rheumatoid synoviocytes where bFGF also upregulated the activity of the enzyme (Tsumuki et al., 2000). Moreover, LIF and bFGF are necessary components of embryonic stem cells culture media to keep embryonic stem cells in undifferentiated state (Lysdahl et al., 2006).

In the present study bFGF significantly enhanced proliferation of pig granulosa cells and increased telomerase activity level. At the same time it decreased aromatase expression. We conclude that 10% of FCS and bFGF used in the concentration of 10 ng/ml are important components of culture medium for long-term culture of pig granulosa cells. The results of our study might have a great impact on further research aimed at elucidation of the stem cell characteristics of granulosa cells and application of this knowledge in patient treatment. Cancer can be a debilitating disease in its own right but therapies to treat cancers can also have major long-term consequences. Chemo or radiation therapies often leave young women infertile following treatment. These therapies damage follicles in the ovaries but little is known about this process. Stem cells in adult tissues are important for repairing and renewing tissues throughout life. The results of our study may contribute to the understanding of the mechanisms of chemo and radiation therapy damage caused to stem cells of ovarian follicles. Moreover, the results of the present study contribute to the development of an optimal experimental model for studying ovarian cell interactions and response to investigated factors.

Acknowledgements

The authors thank Mrs. Petra Pacesova for technical support.

References


Optymalizacja metody wydłużonej hodowli komórek granulozy świń w warunkach in vitro

STRESZCZENIE

Celem niniejszej pracy było ustalenie optymalnych warunków (optymalna koncentracja surowicy i obecność odpowiedniego czynnika wzrostu) wydłużonej hodowli komórek granulozy świń. Komórki granulozy hodowano w obecności surowicy płodów cięciowych dodawanej w dwóch różnych koncentracjach: 10% i 20% oraz jednego z następujących czynników wzrostowych: LIF (czynnik hamujący białaczkę), bFGF (bazalny czynnik wzrostu fibroblastów) i SCF (czynnik komórek macierzystych). Reakcje komórek granulozy na obecność danego czynnika eksperymentalnego sprawdzano za pomocą pomiaru potencjału proliferacji, poziomu aktywności telomerazy oraz ekspresji aromatazy. Komórki granulozy charakteryzowały się zwiększym (P<0,01) poziomem proliferacji w obecności 10% surowicy w porównaniu do kontroli po 9 dniach hodowli. Bazalny czynnik wzrostu fibroblastów spowodował zwiększenie (P<0,01) poziomu inkorporacji trytowanej tymidyny we wszystkich badanych przedziałach czasowych, aż do 18. dnia hodowli. LIF, bFGF i SCF stymulowały (P<0,01) aktywność telomerazy po 3, 6, 9, 12, 15 i 18 dniach hodowli, natomiast ekspresja aromatazy uległa obniżeniu (P<0,05) w efekcie działania każdego z wymienionych czynników wzrostu, we wszystkich badanych przedziałach czasowych. Reasumując, komórki granulozy świń mogą być hodowane przez dłuższy okres czasu w obecności 10% surowicy płodów cięciowych oraz bazalnego czynnika wzrostu fibroblastów. W tych warunkach komórki granularne zachowują swój zwiększony potencjał proliferacji, aktywność telomerazy oraz wykazują zmniejszony poziom zróżnicowania wyrażony poziomem ekspresji aromatazy.