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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Androgen receptor inhibitor stimulates telomerase activity of pig granulosa cells *in vitro**

Ewa Chronowska[1], Tomas Kott[2]**

[1] Department of Biology of Reproduction, Institute of Animal Science, Přátelství 815, 104 00 Prague Uhříněves, Czech Republic

[2] Department of Molecular Genetics, Institute of Animal Science, Přátelství 815, 104 00 Prague Uhříněves, Czech Republic

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The aim of the study was to investigate the effect of testosterone (T) and dihydrotestosterone (DHT) applied alone or in combination with antiandrogen (flutamide) on proliferation, progesterone secretion and telomerase activity (TA) of pig granulosa cells (GC) derived from small (1-3 mm; SF-GC) and large (5-7 mm; LF-GC) ovarian follicles. Cells were treated with investigated factors for 48 h. 5α-DHT stimulated (P<0.05-0.01) proliferation of SF and LF granulosa cells. Flutamide applied individually and in a combination with testosterone and DHT stimulated (P<0.05-0.01) proliferation of pig GC from small and large antral follicles. Flutamide had no effect on progesterone synthesis in small as well as in large follicle GC. Antiandrogen applied individually and in a combination with testosterone and DHT enhanced (P<0.05-0.01) telomerase actitvity in SF- and LF-GC. The results of the study suggest the involvement of androgen receptor in telomerase activity regulation in pig GC and a link between telomerase and the proliferation status of GC.

KEY WORDS: androgen / antiandrogen / granulosa cells / pig / progesterone / proliferation / telomerase

Ovarian androgens are gonadal steroids which regulate follicular development through modulation of gonadotropin and growth factors (such as GDF9 and IGF-1) action [Hillier and Tetsuka 1997, Orisaka *et al*. 2009, Lokman *et al*. 2010, Hickey *et*

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Corresponding author:** chronowska.ewa@gmail.com

al. 2004]. In the early growth phase, granulosa cells preferentially metabolize these androgens to 5a-dihydrotestosterone (DHT), a nonaromatizable androgen with the highest affinity to the androgen receptor (AR). During antral follicle growth, theca cells predominantly secrete aromatizable androgens (androstenedione, testosterone) which accumulate in follicular fluid [McNatty *et al*. 1979].

In the porcine ovary, AR was found to be highly expressed in the nuclei of granulosa cells of preantral and antral follicles. However, the amount of androgen receptor in granulosa cells of small antral follicles (<2 mm) was significantly higher than that in the largest follicles [Cardenas and Pope 2002]. Slomczyńska *et al*. [2006] localized AR in the pregnant gilt ovarian follicles with limited number of apoptotic cells, and Duda and Slomczynska [2007] detected AR immunostaining in antral and mural granulosa cells derived from porcine follicles at all stages of development.

It has been shown that androgen stimulates the pig small (1-3 mm) antral follicle mural granulosa cell proliferation *in vitro* by enhancing growth promoting effect of oocytes and growth differentiation factor 9 [Hickey *et al*. 2005]. On the other hand, DHT inhibits FSH-induced progesterone secretion in small antral follicles. In case of large antral follicles, androgen alone or in combination with FSH or IGF-1 has little proliferative effect on mural granulosa cells and does not influence the progesterone secretion [Hickey *et al*. 2004].

In our earlier studies the telomerase activity (TA) was found in pig granulosa cells derived from small and large antral follicles [Tomanek *et al*. 2008]. It was also shown that TA in granulosa cells is under the control of estrogens and it has been suggested that the activity of this enzyme is not only related to proliferative potential of GC, but may also be involved in differentiation related to estrogen synthesis [Chronowska *et al*. 2009, 2010].

The role of androgens in stimualtion of telomerase actvity in cancer cells has been well evidenced. In prostate cancer cells it was shown that using anti-androgen to compete with androgens for binding to AR resulted in down regulation of human telomerase reverse transcriptase (hTERT) at the transcriptional level and that this effect was mediated by reduced occupancy of androgen receptor to the promoter region of hTERT [Liu *et al*. 2010]. Recently, it was shown that testosterone and androstendione not only increased viability of ovarian cancer cells, but also induced expression of telomerase and phosphorylation of its catalytic subunit [Nourbakhsh *et al*. 2010].

To our knowledge, there are no data concerning effect of androgen acting *via* androgen receptor on telomerase activity in porcine granulosa cells. Facing the above, in the present study we used testosterone, dihydrotestosterone and androgen receptor inhibitor – flutamide – to study the androgen influence on TA of porcine granulosa cells derived from small and large antral follicles. As telomerase activity is tightly correlated with proliferation and differentiation status of the cell [Bayne and Liu 2005] we also investigated the effect of androgens and androgen receptor antagonist on proliferation and progesterone secretion of pig granulosa cells *in vitro*.

Material and methods

Isolation of granulosa cells and *in vitro* culture conditions

Porcine ovaries were collected from local slaughterhouse and within 30 min transported to the laboratory in a thermo-cointainer 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 desired size (small antral 1-2 mm and large antral 5-7 mm) were isolated by dissection and split-opened under a stereomicroscope (LEICA MZ6, Switzerland) into DMEM/F12 medium (GIBCO, BRL) to obtain granulosa cells (GC). The number of living cells in suspension was estimated by the use of 0, 25% trypan blue (SIGMA, USA) in PBS and by counting in a hematocytometer. Cells were seeded onto 24-well culture microplates Nunclon Delta (NUNC, Denamrk) with a density of 1-1,5x10^5 of living cells/well and cultured as a proliferating monolayer in a DMEM/F12 medium with phenol red supplemented with an insuline-transferrin-selenium mixture (ITS-X, 1ml/100ml) and gentamycin (all GIBCO, BRL). Two per cent of foetal bovine serum (FBS, GIBCO) were added in order to facilitate plating. Cell cultures were performed at 37°C in a humified atmosphere of 5% CO2 in a SANYO MCO-175M incubator.

Hormonal supplements

Hormonal supplements were added to the culture medium 24 h after the start of the incubation. GC were cultured for 48 h in the presence of testosterone (STERALOIDS Inc., USA; 10^{-8}M), 5a-dihydrotestosterone (SIGMA, USA; 10^{-8}M) and non-steroidal androgen receptor antagonist – flutamide (2-Methyl-N-(4-nitro-3-[trifluoromethyl]phenyl) propanamide; SIGMA, USA; 10^{-7}M) applied alone or in a combination. Each experiment consisted of three repeations per treatment.

Cell proliferation assay

Radiochemical analysis was used to determine the GC proliferation activity *in vitro*. Thymidine (Methyl-^{3}H-thymidine, 6-7 Ci/mmol, ICN, USA in a final concentration of 1 μCi/ml) was added 12 h before the termination of the culture. DNA synthesis was measured by incorporation of ^{3}H-thymidine using the technique of TCA precipitation and liquid scintillation counting.

Measurement of progesterone synthesis

The level of progesterone synthesis in GC *in vitro* was evaluated by radio-immunoassay of progesterone in the culture medium using 11-alfa -hemisuccinate -125I-tyrosin methylester-progesterone (AMERSHAM, A.P.Czech, Prague, Czech Republic). The lower detection limit for progesterone RIA was 0.154 ng/ml. The intra- and inter-assay coefficient of variation was 5.16 and 9.33%, respectively.

Telomerase activity assay

Preparation of granulosa cells. *In vitro* cultured GC were prepared as follows: after 48 hours of incubation, the culture media from individual culture wells were removed and stored at -20°C for RIA. 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. Pelletes of cells were stored at -80°C for further analysis.

Telomeric Repeat Amplification Protocol (TRAP) assay. Before the telomerase activity assay, cell lysates from *in vitro* cultured cells were prepared in a CHAPS buffer. Briefly, cell pellets of cultured GC (2×10^5) and the positive control provided in the kit were resuspended in 200 µl of 1X CHAPS Lysis Buffer. The protein concentration was measured spectrophotometrically. A modified protocol based on the Telomerase Detection Kit (TRAPEZE, CHEMICON, USA) was applied to evaluate telomerase activity. Used was an internal telomerase assay standard and the fluorescent type-specific TS primer AATCCGTCGAGCAGAGTT-6-FAMTM (APPLIED BIOSYSTEMS, USA). Telomerase extension and PCR were both performed in a Biometra T gradient PCR thermocycler (BIOMETRA, Germany). The PCR was continued for 30 cycles. Each cycle consisted of 30 s denaturation at 94°C and 30 s annealing at 59°C. Telomerase activity was expressed as Total Product Generated (TPG) per mg protein.

Statistical

The data were obtained from four experiments with different pools of small- and large-follicle GC, and presented as means and standard errors (SEM). All data were analysed using the SAS Programme of 2001, SAS System for Windows, Release 8.2 (TS2M0), SAS Inst., Inc., Cary, NC, USA). Two-way ANOVA was used to identify the significance of differences of testosterone, dihydrotestosterone and flutamide effect as applied alone or in a combination on proliferation, progesterone production, and telomerase activity of small- and large-follicle GC *in vitro*. Differences with a probability of $P<0.05$ were considered significant.

Results and discussion

5α-dihydrotestosterone stimulated ($P<0.05$-0.01) proliferation of SF and LF granulosa cells (Fig. 1A and 1B, respectively). Testosterone applied alone stimulated ($P<0.01$) proliferation of GC derived from large follicles (Fig. 1B). Flutamide applied alone and in a combination with testosterone and DHT stimualted ($P<0.05$-0.01) proliferation of GC from small and large antral follicles (Fig. 1A and 1B, respectively).

DHT stimulated ($P<0.05$) progesterone synthesis in small-follicle GC (Fig. 2A). Flutamide applied alone and in a combination with testosterone and DHT had no

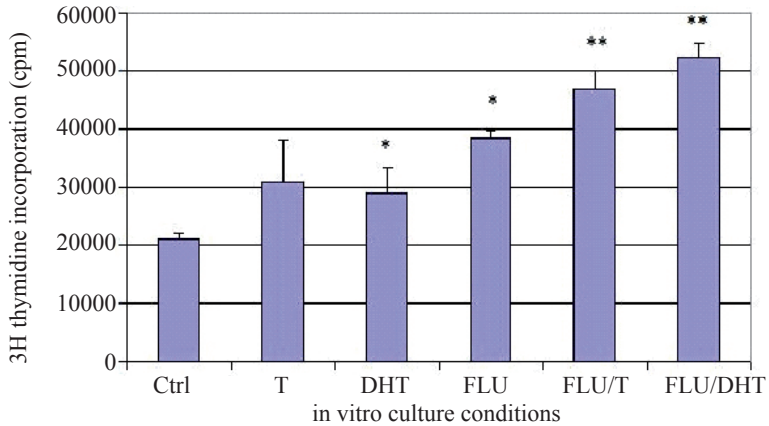

Fig. 1A. Proliferation of porcine granulosa cells derived from small (1-2 mm) follicles and assayed by incorporation of ^{3}H thymidine over 48 h of *in vitro* culture in the presence of testosterone (T; 10^{-8}M), 5α-dihydrotestosterone (DHT; 10^{-8}M) and flutamide (FLU; 10^{-7}M) applied individually and in a combination. Each bar represents mean ± SEM for 4 experiments performed in triplicates. Means bearing 1 or 2 asterisks are significantly different from control (Ctrl) at P<0.05 and 0.01, respectively.

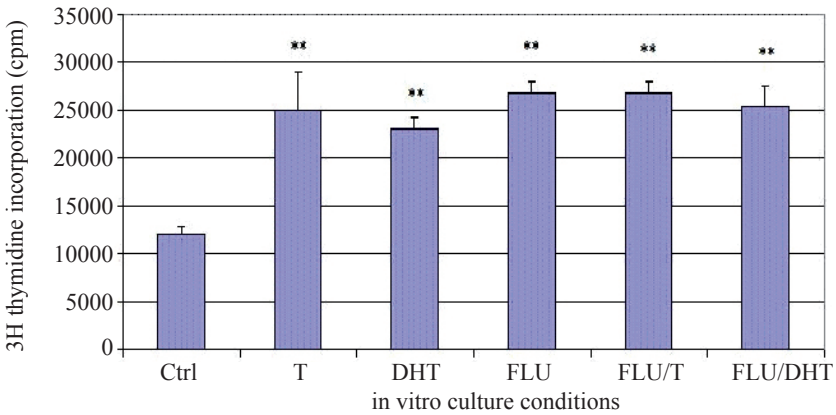

Fig. 1B. Proliferation of porcine granulosa cells derived from large (5-7 mm) follicles. Explanations as for Fig. 1A.

effect on progesterone synthesis in either small- or large-follicle GC (Fig 2A. and 2B, respectively).

Testosterone and DHT as applied alone did not stimulate telomerase activity in small- and large-follicle GC (Fig 3A and 3B, respectively). Flutamide applied individually and in a combination with testosterone and DHT enhanced (P<0.05-0.01) telomerase actitvity in small- and large-follicle GC (Fig 3A and 3B, respectively).

Androgen plays a role in follicular development in two ways: as a substrate for aromatase activity and as a ligand for androgen receptor. In the present study the

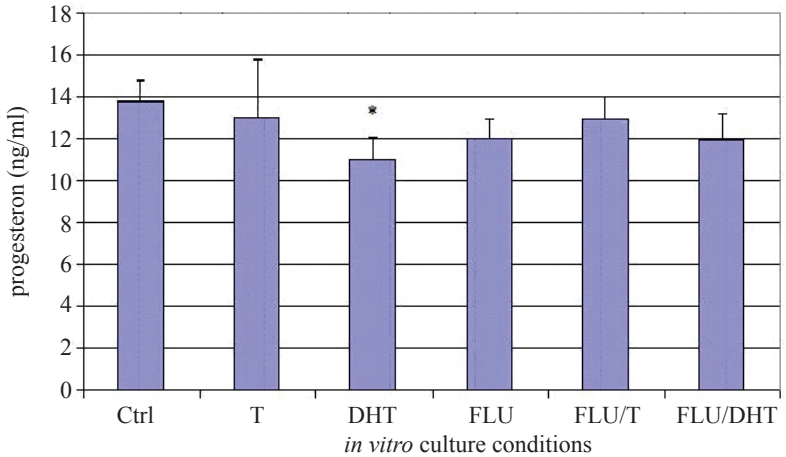

Fig. 2A. Progesterone secretion by porcine granulosa cells derived from small (1-2 mm) follicles and assayed by RIA after 48 h of *in vitro* culture in the presence of testosterone (T; 10^{-8}M), 5α-dihydrotestosterone (DHT; 10^{-8}M) and flutamide (FLU; 10^{-7}M) applied individually and in a combination. Each bar represents mean±SEM for 4 experiments performed in triplicates. Means bearing an asterisk are significantly different from control (Ctrl) at $P<0.05$.

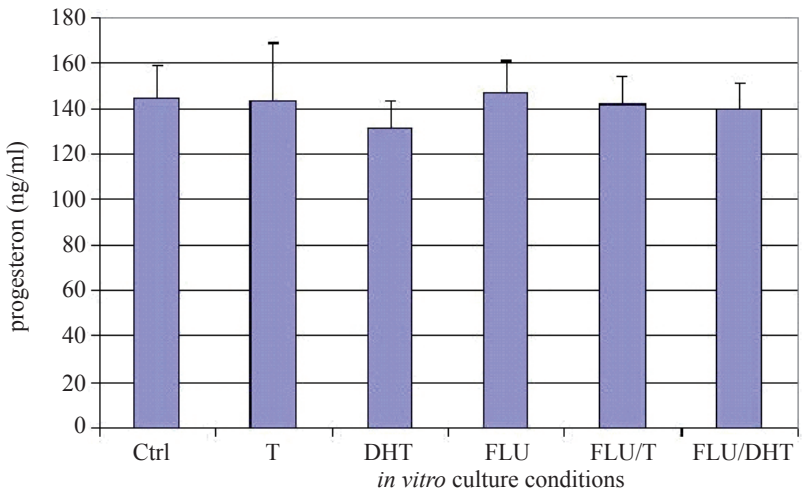

Fig. 2B. Progesterone secretion by porcine granulosa cells derived from large (5-7 mm) follicles. Explanations as for Fig. 2A.

aromatizable (testosterone) and non-aromatizable (dihydrotestosterone) androgens as well as non-steroidal antagonist of androgen receptor (flutamide) were applied to investigate the role of androgens in proliferation, progesterone secretion and telomerase activity of the porcine GC derived from antral follicles of different size.

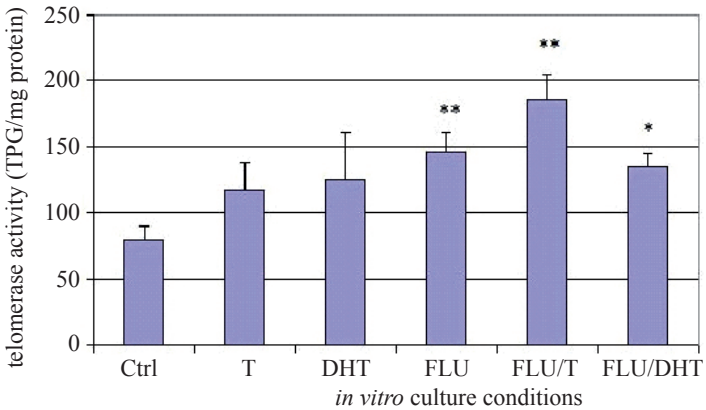

Fig. 3A. Telomerase activity in porcine granulosa cells derived from small (1-2 mm) follicles assayed using TRAPEZE Telomerase® Detection Kit after 48 h of *in vitro* culture in the presence of testosterone (T; 10^{-8}M), 5α-dihydrotestosterone (DHT; 10^{-8}M) and flutamide (FLU; 10^{-7}M) applied individually and in a combination. Each bar represents mean ± SEM for 4 experiments performed in triplicates. Means bearing 1 or 2 asterisks are significantly different from control (Ctrl) at $P<0.05$ and 0.01, respectively.

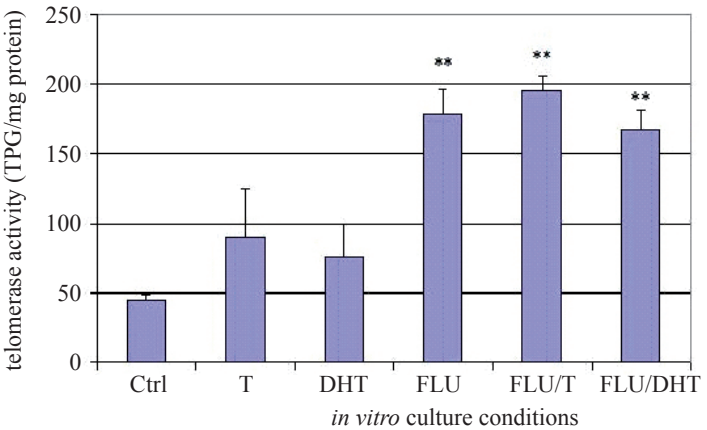

Fig. 3B. Telomerase activity in porcine granulosa cells derived from large (5-7 mm) follicles. Explanations as for Fig. 3A. Means bearing an asterisk are significantly different from control (Ctrl) at $P<0.05$.

GC of small ovarian follicles are characterized by significantly higher number of androgen receptors compared to large follicle GC [Cardenas and Pope 2002]. In study of Cardenas and Pope [2002] testosterone had a proliferation stimulatory effect only in case of large-follicle GC. Earlier, we found out [Tomanek *et al*. 2008, Chronowska *et al*. 2010] much higher level of estradiol synthesis in large-follicle granulosa cells. Those cells were cultured in the presence of testosterone as a substrate for aromatase acitvity. The higher proliferative potential of large-follicle GC cultured with testosterone observed in the present study might be caused by indirect effect of

estradiol as a product of androgen aromatization. In another study by Chronowska *et al*. [2009], a much higher level of aromatase expression in pig large-follicle GC was observed in comparison to GC cultured under basic conditions (medium only) derived from small antral follicles. Similar results were previously obtained with human granulosa cells which expressed higher aromatase activity in later stages of follicular development [McNatty *et al*. 1979].

Interestingly, in the present study, the DHT showed proliferation stimulatory effect not only in small- but also in large-follicle GC. It was previously shown that DHT enhanced the IGF-1 stimulated proliferation of mural GC from porcine small antral follicles [Hickey *et al*. 2004]. On the other hand, the same study revealed that DHT had no effect on proliferation of large follicle granulosa cells. Differences between the present results and those of Hickey's group might be explained by lack of additional experimental factors used in the former work, while in the latter investigated was the effect of DHT in a combination with IGF-1 and FSH. What is more, in the present study used was mixed population of granulosa cells whereas Hickey and colleagues focused on mural and cumulus oophorus cells separately. It may also be speculated that DHT stimulates porcine large follicle GC proliferation *via* some other mechanism, without involvment of androgen receptor [Bagchi *et al*. 2008]. Data obtained in humans provide evidence for the presence in granulosa cells of a novel, short term mechanism of androgen action involving voltage-dependent Ca2+ channels in the plasma membrane and phospholipase C activation *via* a pertussis toxin-sensitive G protein [Machelon *et al*. 1998]. On the other hand, studies in rats revealed inhibitory effect of DHT on insulin-stimulated proliferation of GC [Kayampilly and Menon 2006].

In the present study, flutamide alone and in a combination with androgens caused increase in a proliferative activity of small and large follicle GC. Androgens play an important role in a human prostate cancer development. Therefore, antiandrogens have been widely used to treat this type of cancer [Labrie 2010]. However, recent studies have shown that pharmacological androgen deprivation using antiandrogens leads to persistence of some viable tumour cells capable of proliferation [Mercader *et al*. 2007]. The results reported in the present study differ from those by Hickey *et al*. [2004] who noticed that hydroxyflutamide reversed proliferative effect of DHT on mural GC. Again, these differences may be explained by mixed population of GC used in the present study and additional factors investigated by Hickey and his group.

Recent studies showed the regulation of the FSHR mRNA and protein in neonatal porcine ovaries (oocytes, granulosa cells and surface epithelium) after maternal exposure to flutamide which confirms that androgens play a key role in porcine folliculogenesis at the early stages [Durlej *et al*. 2010]. It was also suggested that flutamide through blocking androgen action, causes delayed gonadal maturation in later postnatal life and may be involved in the regulation of Cx43 gene expression in pig gonads [Kopera *et al*. 2010]. In the present study, androgens and flutamide showed no effect on progesterone secretion in large-folicle GC. Moreover, DHT diminished progesterone secretion in small-follicle GC. These observations are in accordance with

those made earlier by Hickey *et al*. [2004]. On the contrary, however, it was shown that testosterone directly induces progesterone synthesis in preovulatory follicle granulosa cells of laying hens [Rangel *et al*. 2007]. This testosterone stimulation of granulosa cells is specific since the testosterone antagonist (flutamide) decreased testosterone stimulatory action. In mouse ovarian follicles progesterone production was slightly elevated by day 12 of culture in the highest concentration of hydroxyflutamide tested (50 μM) – Lenie and Smitz [2009]. In rat, cultured follicular cells flutamide had no effect on progesterone production [Akgul *et al*. 2008].

It is well documented that telomerase activity remains under the control of androgens in tissues being the target of these steroids [Meeker *et al*. 1996]. As already mentioned, prostate cancer treatment with antiandrogen resulted in down-regulation of telomerase expression and activity [Liu *et al*. 2010]. In the present study antiandrogen applied alone or in a combination with androgens resulted in a significant increase of telomerase activity in small- and large-follicle granulosa cells. Recently Chronowska *et al*. [2010], using antiestrogens to elucidate the role of estrogen receptor in telomerase activity regulation in pig GC identified similar pattern of telomerase activity which correlated with estradiol secretion rather than with proliferative potential of GC, suggesting the role of telomerase in differentiation processes of granulosa cells. In the present study the enhanced proliferation of GC was accompanied by enhanced telomerase activity in both populations of granulosa cells. These observations are in accordance with those of Lavranos *et al*. [1999] who reported the higher proliferative potential of bovine small follicle granulosa cells to be accompanied by increased telomerase activity. On the contrary, recent studies by Tomanek *et al*. [2008] revealed that higher proliferative potential of pig small-follicle GC was not reflected by enhanced telomerase activity during the culture in the presence of EGF and FSH. The results reported here deal with these species-specific observations and indicate that in addition to the potential role in differentiation processes, telomerase is also important in pig granulosa cell proliferation.

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