Regulation of telomerase activity in ovarian granulosa cells

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Follicular development is characterized by intensive proliferation and differentiation of granulosa cells. It was reported that during follicular growth granulosa cells arise from the population of stem cells. One of the main evidences for stem characteristics of the cell is the ability to express telomerase – an enzyme complex responsible for integrity and stability of chromosome ends (telomeres). It was demonstrated that telomerase activity in granulosa cells is linked to their proliferation and differentiation status and is under the control of growth factors and steroid hormones. In this review current knowledge on the existence and regulation of telomerase activity in granulosa cells has been presented.

Keywords: Differentiation, Follicle, Granulosa cells, Ovary, Proliferation, Telomerase

Introduction
During folliculogenesis less differentiated cells form heterogeneous tissues consisting of several cell types (epithelial, mesenchymal and other). These tissues undergo growth and maturation and form functionally and morphologically complex structure[1]. It was postulated that folliculogenesis is based on continuous renewal of cell population where differentiated cells are replaced by proliferation of more primitive cells[2]. During follicular growth marked proliferation and differentiation of granulosa cells (GC) takes place. To examine the proliferation process during follicular development in pig the expression of proliferating cell nuclear antigen protein (PCNA) was determined in the ovary[3]. Advanced preantral and actively growing small and large antral follicles showed extensive PCNA labeling in the layers of granulosa and theca cells and in the cumulus cells surrounding the oocyte.

Proliferation and differentiation events during follicular development are controlled by gonadotropins, growth factors and steroid hormones and complex interplays between all these factors. Follicle stimulating hormone (FSH) is necessary for granulosa cell differentiation and final growth of antral follicles[4,6]. Growth factors produced in the ovary - such as epidermal growth factor (EGF)- are considered as important regulators of granulosa cell proliferation and function[7,8]. Estrogens produced in the ovary are now considered to be potent regulators of follicular growth and development[9,10]. It was postulated that during follicular growth granulosa cells arise from the population of stem cells[11,12]. Expression of telomerase in granulosa cells was indicated as one of the most important evidences to support this hypothesis.

Cell proliferative abilities highly depend on the maintenance of telomeres. It has been shown that forced expression of the telomerase reverse transcriptase (TERT) gene in cultured ovarian surface epithelium cells results in enhanced proliferative potential of the cells[13].

Role, structure and expression of telomerase
Mammalian cells are equipped in a complex mechanism controlling their lifespan. Healthy, normal (non-transformed) cells in the body have a limited potential of their growth and undergo aging after passing a certain number of cell cycles. On the other hand, cancer cells and stem cells have the capacity of almost unlimited cell divisions and hence are called "immortal". The aging of the cells is associated with shortening of chromosome ends, called telomeres, which include non-coding, tandem repeats of DNA sequences and associated proteins[14,15]. Chromosomes with damaged telomeres undergo fusion with other chromosome ends or are eliminated during cell division. Telomere DNA consists of six nucleotide tandem repeats which include mainly guanine and thymidine. In humans and other vertebrates telomeric repeats have following nucleotide sequence: 5’-TTAGGG-3’[16] (Fig. 1). In pig telomere length is 10-30 kb[17].
In addition to stabilization and protection of the ends of chromosomes, telomeres play an important role in the process of cell proliferation. All chromosomes lose telomeric DNA during each cell division. This phenomenon is a consequence of the nature of the process of DNA replication. DNA polymerase moves in the direction 3' to 5', building a new strand from 5' to 3'. Leading strand is replicated continuously, while the lagging strand synthesis begins from several RNA primers that are extended. In this way, so called Okazaki fragments are created. RNA fragments are then eliminated and replaced by DNA sequences. Because there is no template for the "last" Okazaki fragment beyond the 5' end of the chromosome one strand cannot be synthesized to its very end. As a result, in each mitotic cycle shortening of the chromosome DNA on the 3' end occurs. This phenomenon is called "end replication problem" and is the reason why non-stem and non-cancer cells can divide only a limited number of times.

Telomere shortening functions as a "molecular clock". When specific telomere length is reached, cells stop dividing and enter into the stage ending in their death\textsuperscript{15}. However, cancer cells and stem cells are characterized by a relatively constant telomere length, in spite of intensive proliferation they undergo. Such a possibility is get through the expression of the enzyme telomerase. Activation of telomerase leads to unlimited proliferative capacity and thus potentially to cell immortality. The cells having active telomerase are able to avoid the "end replication problem", through synthesis and adding of nucleotide repeats to the 3’ end of DNA\textsuperscript{18}.

Telomerase consists of two basic subunits: the RNA component (Telomerase RNA, TR), which is the template for the synthesis of telomeric DNA, and the catalytic subunit with the activity of reverse transcriptase (Telomerase reverse transcriptase, TERT). RNA subunit is expressed at relatively high levels in all tissues, regardless of whether these tissues have active telomerase or not and therefore it cannot be considered as a marker of enzyme activity\textsuperscript{19}. In contrast, there is a significant correlation between the expression of TERT and telomerase activity (TA) hence the quantitative measurement of catalytic subunit mRNA gives reliable information about the level of enzyme activity\textsuperscript{20}. Expression of the catalytic subunit is greatly reduced or absent in normal differentiated cells\textsuperscript{21} while its high level is detected in malignant transformed cells, which gives them unlimited proliferative capacity\textsuperscript{22,20}. Active telomerase is also found in some tissues having high regenerative need and actively dividing stem cells such as bone marrow\textsuperscript{23,24}. In addition, telomerase activity was detected in human germ cells\textsuperscript{25} endometrium\textsuperscript{26}, placenta\textsuperscript{27}, in the liver in humans\textsuperscript{28} and in mice\textsuperscript{29} and in the epidermis\textsuperscript{30} so apparently it is expressed in intensively dividing cells and tissues which have the capacity of regeneration. It was observed that the expression of telomerase is associated with increased life span of human bone marrow cells and promotes their developmental capabilities\textsuperscript{31}. In contrast, telomere shortening and a decrease in telomerase activity occurs during aging and differentiation of mesenchymal stem cells and chondrocytes\textsuperscript{32}. The specific telomerase expression pattern occurs in mice where the enzyme is present in almost all tissues\textsuperscript{33}. In case of pig telomerase activity was detected in lungs, kidneys and lymph nodes but it was not present in heart and brain\textsuperscript{17}.

**Expression of telomerase in granulosa cells**

Little is known about the expression and the role of telomerase in granulosa cells. In the bovine ovary RNA component was localized to granulosa cells of growing follicles but was not detected in primordial follicles. Telomerase activity was the highest in the smallest examined follicles (60-100 µm) decreasing...
significantly as the follicles enlarged. Recently, it was demonstrated that the relative telomere lengths and telomerase activity of granulosa cells showed the tendency to be shorter with the age of donor cows. Significantly decreased telomerase activity was observed in large and atretic follicles in comparison to small and healthy follicles in rat. In the human, low level of telomerase activity in the ovary was found to be related to primordial follicle depletion with age and it was speculated that telomerase activity could be used as a marker of the ovarian functional age. It was shown that luteinized human granulosa cells have a certain potential to proliferate and that telomerase activity of luteinized GC may predict the clinical outcomes of in vitro fertilization treatment. In primary and preantral follicles of pig ovary, TERT was localized in granulosa cells and in germ cells, with a typical nuclear location. During antral differentiation, only somatic cells close to the antrum (antral layer) and cumulus cells maintained TERT expression. In pig, telomerase activity was detected in GC obtained from small (1-2 mm) and large (5-7 mm) follicles. TA was observed in freshly isolated granulosa cells as well as in cells cultured in the presence of FSH and EGF.

Role of epidermal growth factor (EGF) in the regulation of telomerase activity

EGF acts on target cells via glycoprotein, transmembrane receptor with tyrosine kinase domain. Effect of EGF on telomerase activity has been documented in case of malignant, transformed cells. The increase in telomerase activity in these cells is associated with an increased level of expression of the gene encoding the catalytic subunit of telomerase which indicates the transcriptional effect within the TERT gene promoter. The results of studies on mechanisms of regulation of TERT subunit and thus telomerase activity by EGF suggest the involvement of MAPK (mitogen activated protein kinase) in this process. On the other hand, EGF has no effect on telomerase activity in normal (non-transformed) cells such as skin fibroblasts or endometrial cells. In pig EGF was shown to stimulate telomerase activity in small and large follicle granulosa cells however its effect on SF-GC telomerase activity was greater. This observation leads to conclusion that telomerase activity plays an important role particularly in small, actively growing follicles.

Role of steroid hormones in the regulation of telomerase activity

The ovary is a main source of estrogens and also an important target organ of these steroids. Withdrawal of estrogen induces structural atrophy and dysfunction of the ovaries. Conversely, prolonged exposure to estrogen leads to the risk of certain types of tumors, such as ovarian cancer. Estrogen acts by binding to estrogen receptors (ERs) that dimerize and bind to estrogen response elements (ERE) in the promoters of estrogen target genes to regulate gene transcription. Two subtypes of ERs have been localized in mammalian ovary: ERα and ERβ. High amount of ERβ protein and mRNA was detected in the granulosa

![Fig. 2—Schematic illustration of estradiol and estrogen receptor interaction. Upon estrogen (E)-estrogen receptor (ER) binding, receptor undergoes dimerization and subdomains AF-1 and AF-2 become active. As a result receptor binds to the estrogen responsive element (ERE) localized in the promoter region of the target gene which leads to the recruitment of coactivators taking part in RNA polymerase complex organization.](image-url)
cells of primary, secondary and antral follicles in rodents\textsuperscript{45,46} and humans\textsuperscript{47,48}. On the other hand low amounts of mRNA and protein of ER\textalpha were detected in human ovary\textsuperscript{48,49}. Under physiological conditions, significant correlation between estrogen levels and telomerase activity in cells being under the control of these hormones has been shown. It was demonstrated that the epithelial cells of human endometrium exhibit a certain level of telomerase activity, which varies depending on the stage of the menstrual cycle, reaching the highest values with the increase in estrogen production in the follicular phase of the cycle\textsuperscript{50,26}. Rapid increase in the level of TERT gene expression and telomerase activity in tumor cell lines with estrogen receptor alpha was shown\textsuperscript{51}. Induction of TERT expression in these cell lines was associated with estrogen receptor interaction with an element located within the TERT gene promoter region. These data suggest that estrogen receptor is an important molecular mediator through which estrogens regulate telomerase activity in the studied cells. It was found out that estrogens induce the expression of catalytic subunit of telomerase in cells of human ovarian surface epithelium\textsuperscript{52}. In this type of cells estrogen receptor is expressed\textsuperscript{53}. In case of cells lacking the estrogen receptor, there was no effect of estrogen on the expression of the catalytic subunit, and thus the activity of telomerase\textsuperscript{52}. The results of this study confirmed earlier observations on the basis of which arose the hypothesis that telomerase activity is hormonally regulated in the tissues under the control of estrogens, such as the endometrium\textsuperscript{26} or prostate\textsuperscript{54}. Still little is known about the role of estrogen in the regulation of telomerase activity in ovarian follicle cells. Significantly lower levels of telomerase activity were found in rat large ovarian follicles in comparison to TA level in small, healthy follicles\textsuperscript{36}. It was shown that telomere maintenance is the primary mechanism mediating the mitogenic effect of estrogen on mouse ovarian granulosa cell proliferation\textsuperscript{9}. Recent study demonstrated the involvement of androgen acting via androgen receptor in the regulation of telomerase activity in pig granulosa cells\textsuperscript{55}.

**Effect of antiestrogens on telomerase activity**

A possible way to study the role of estrogens acting via the estrogen receptors in the cells may be \textit{in vitro} experiments using estrogen receptor antagonists (antiestrogens) and the observation of induced effects in this way. Antiestrogens are often used in breast cancer therapy as the development of tumor is dependent on estrogen\textsuperscript{56}. ICI 164.384, belonging to the group of pure antiestrogens, is \(7\alpha\)-alkilamid analogue of estradiol. It has the ability to block the estrogen receptor through inhibition of subdomains AF-1 and AF-2\textsuperscript{57} (Fig. 3). In addition, biochemical studies have shown that ICI 164.384 also affects the process of estrogen receptor dimerization\textsuperscript{58} and its movement between the cytoplasm and nucleus\textsuperscript{59}. Cyclofenil is a non-steroid selective modulator of estrogen receptor \(\beta\). It shows a fivefold greater affinity for the estrogen receptor \(\beta\) compared with estrogen receptor \(\alpha\)\textsuperscript{60}. Due to the ability to block

![Fig. 3—Schematic illustration of “pure” antiestrogen (ICI 164.384) and estrogen receptor (ER) interaction. In the cell nucleus antiestrogen blocks receptor dimerization and inhibits the action of subdomains AF-1 and AF-2. As a result receptor does not bind to the estrogen responsive element (ERE) localized in the promoter region of target gene which leads to the lack of recruitment of coactivators taking part in RNA polymerase complex organization.](image-url)
estrogen receptor β, cyclofenil is an important tool in studies aimed at determining the functions of estrogen receptor subtypes.

It was observed that antioestrogens (ICI 164.384 and cyclofenil) applied individually and in a combination significantly decreased proliferative potential of cultured pig granulosa cells. On the other hand, antioestrogens increased GC estradiol production and telomerase activity. Results of the study indicated the involvement of estrogen receptor α and β in the control of proliferation, differentiation and telomerase activity in pig GC. Furthermore, it was pointed out that telomerase activity does not have to be linked only to pig GC proliferation but may also be involved in the differentiation process related to estrogen synthesis.

**Effect of aromatase inhibitors on telomerase activity**

Blocking of estrogen synthesis with aromatase inhibitors is a solution often used in the treatment of breast cancer. These substances are chemically divided into steroid and non-steroid. Fadrozole, belonging to the second group, is a competitive inhibitor. It exerts the inhibitory effect by competing with a natural substrate to bind aromatase. After binding to the enzyme fadrozole reversibly blocks its catalytic site. Disconnecting from the aromatase inhibitor restores the enzyme activity. It was found out that the treatment of human prostate epithelial cells with aromatase inhibitor – letrozole resulted in reduced telomerase activity. Based on this observation it was suggested that the intracellular conversion of androgens to estrogen, resulting in activation of telomerase, may contribute to the development of prostate cancer. It was shown that telomerase activity decreased in pig large follicle granulosa cells incubated in the presence of an aromatase inhibitor (fadrozole) for 72 h. Fadrozole also caused a decrease of 3H-thymidine incorporation and in estrogen synthesis in granulosa cells derived from small (1-2 mm) and large follicles (5-7 mm).

**Telomerase activity in long-term cultured granulosa cells**

Recently, it was demonstrated that human luteinizing granulosa cells isolated from the ovarian follicles of infertile patients can be maintained in culture over prolonged periods of time in the presence of the leukemia-inhibiting factor (LIF). A subpopulation of these cells shows a potential to differentiate into other types of cells (chondrocytes, neurons, osteoblasts). In the recent study it was shown that in the presence of basic fibroblast growth factor (bFGF) porcine granulosa cells were able to survive in vitro for a period of 18 days maintaining their proliferative potential and telomerase activity and showing reduced aromatase expression. LIF and stem cells factor (SCF) also stimulated telomerase activity during prolonged culture however they had no effect on proliferation of GC. These findings are important for further efforts to establish the conditions for long term culture of granulosa cells. Telomerase activity seems to be critical factor controlling survivability and proliferation potential of long term cultured granulosa cells.

**Conclusion**

The presence of telomerase activity in granulosa cells indicates their stem cells properties. However, in spite of extensive research stem cells properties of granulosa cells were not yet completely proved. Results of studies on regulation of telomerase activity in granulosa cells contribute to deeper understanding of the follicular growth and development both from scientific and clinical point of view. Ovarian tumors arise from the uncontrolled proliferation of the ovarian stem cells. Better understanding of mechanisms of telomerase activity regulation, with a special focus on estrogen action, may lead in the future to development of more efficient strategies to treat ovarian cancer, infertility and may help to understand the mechanisms of ovarian aging. Furthermore, additional information about cellular and molecular background of stem cell character of granulosa cells may contribute to increase in the efficiency of some biotechnological methods in which granulosa cells can be used, such as somatic cell nuclear transfer or induced pluripotent stem cells generation.

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**References**


