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—Review—

Telomere length and telomerase activity during folliculogenesis in mammals

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Abstract. Telomeres are repetitive non-coding DNA sequences located at the ends of chromosomes in eukaryotic cells. Their most important function is to protect chromosome ends from being recognized as DNA damage. They are also implicated in meiosis and synapse formation. The length of telomeres inevitably shortens at the end of each round of DNA replication and, also, as a consequence of the exposure to oxidative stress and/or genotoxic agents. The enzyme telomerase contributes to telomere lengthening. It has been reported that telomerase is exclusively expressed in germ cells, granulosa cells, early embryos, stem cells, and various types of cancerous cells. Granulosa cells undergo many mitotic divisions and either granulosa cells or oocytes are exposed to a variety of genotoxic agents throughout folliculogenesis; thus, telomerase plays an important role in the maintenance of telomere length. In this review article, we have comprehensively evaluated the studies focusing on the regulation of telomerase expression and activity, as well as telomere length, during folliculogenesis from primordial to antral follicles, in several mammalian species including mice, bovines, and humans. Also, the possible relationships between female infertility caused by follicular development defects and alterations in the telomeres and/or telomerase activity are discussed.

Key words: Female infertility, Granulosa cell, Oocyte, Telomerase, Telomere

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Introduction

Telomeres are specialized nucleoprotein structures located at the ends of all eukaryotic chromosomes and are composed of repetitive DNA sequences and associated proteins [1]. They were first identified in *Drosophila melanogaster* by Herman Muller [2]. He named them "telomeres" from the Greek words *telos*, meaning 'end', and *meros*, meaning 'part' [2]. Telomeric repetitive DNA sequences exhibit differences among organisms, e.g., TTTTGGGG in *Oxytricha* [3], TTGGGG in *Tetrahymena* [4], and TTAGGG in mammals [5]. Moreover, the length of telomeres differs in mammals, ranging from 50 to 150 kb in mice [6], 12–23 kb in bovines [7], and 10–15 kb in humans [8].

Telomeres contain two main DNA sequences: the subtelomere and the main telomere [9]. The subtelomere (1–200 kb in length), adjacent to the main telomere, includes either a low copy number of telomeric DNA repeats or duplicated DNA strands [10]. It has been shown that the subtelomeric region plays an important role in maintaining telomeric integrity, as well as in telomere lengthening, by a mechanism known as alternative lengthening of telomeres (ALT) [11, 12]. The main telomere region consists of single and double DNA strands, mostly comprising specific DNA repetitive TTAGGG sequences, in mammals. At the 3' end of the telomere region, within the single-stranded part, there is a guanine-rich sequence, 50 to 500 nucleotides in length, called G overhang or G-rich region [13]. The G overhang region invades the double-stranded telomeric DNA to protect the single-stranded DNA from being recognized as a DNA break, forming both D-loop and T-loop structures at the ends of all chromosomes [14] (Fig. 1).

Telomeres were previously considered as transcriptionally silent regions; however, later studies have shown that telomeres are transcribed into telomere repeat-containing RNAs (TERRAs) [15–17]. TERRA molecules have been characterized in rodents and humans [15, 18]. They contain UUAGGG repeats and range from 100 bp to 9 kb in length [15]. Interestingly, TERRAs possess multiple promoter regions at the subtelomeric site [19]. Since they reside at telomeres during the S phase of the cell cycle, the main function of TERRAs is thought to be the maintenance of telomeric stability [20, 21]. Moreover, they participate in the re-extension of telomeres by organizing the recruitment of telomerase to the shortened telomeres [20]. Consistently, TERRA molecules interact with telomerase reverse transcriptase (TERT), telomeric repeat factor 1 (TRF1), and telomeric repeat factor 2 (TRF2) [16, 17]. TERRAs are found to be expressed in the human fetal oocytes throughout the germinal vesicle stage [17] and in the human spermatogenic cells [22].

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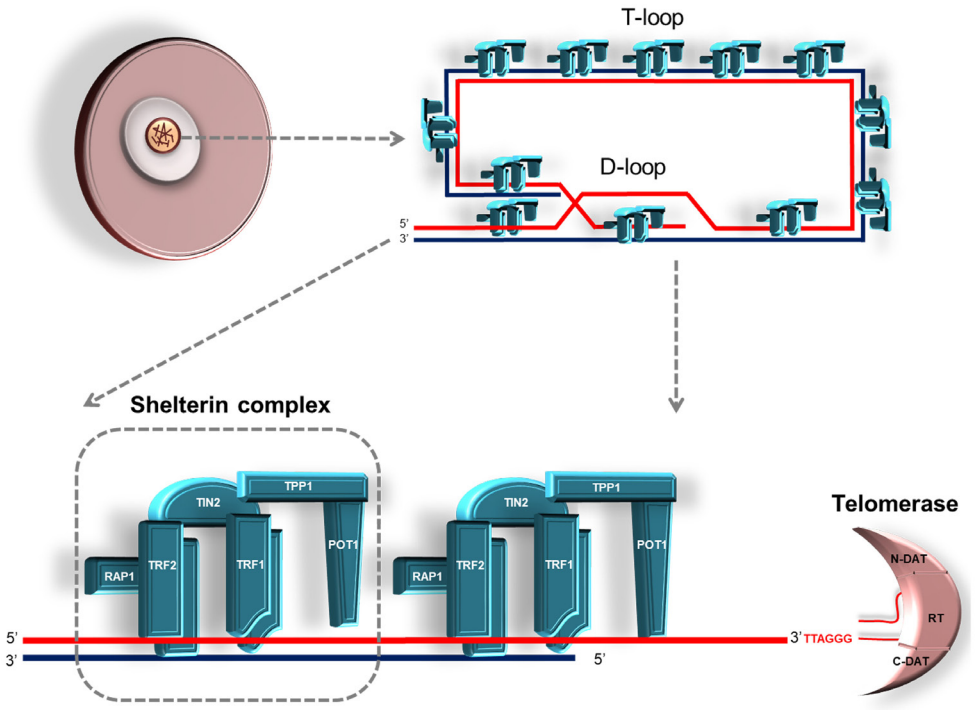

Fig. 1. Schematic diagram showing telomere structure, telomere-associated proteins (TRF1, TRF2, POT1, TIN2, TPP1, and RAP1; forming the shelterin complex), and telomerase in a mammalian oocyte. The 3' overhang of telomere end enters the doubled-stranded DNA to create a displacement (D-loop) and a telomere (T-loop) loop. The shortened telomeres are elongated by the telomerase enzyme, composed of a TERT and a TERC subunits, as well as by other components.

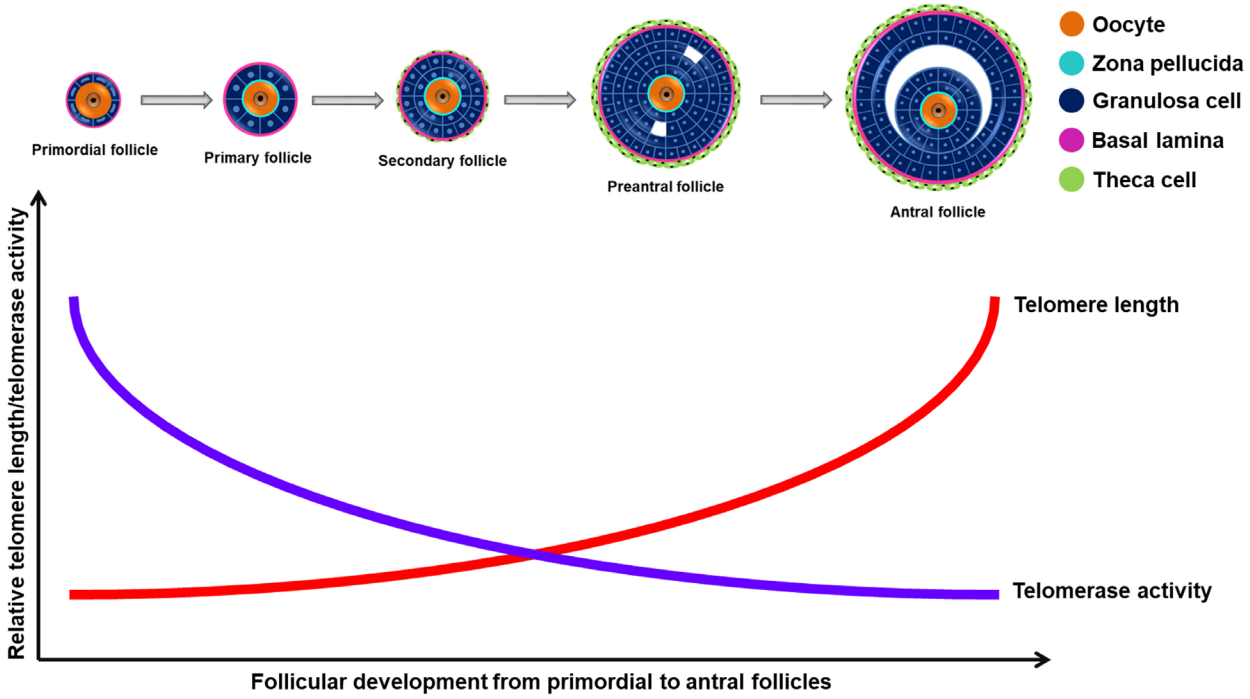

Fig. 2. Telomerase activity and telomere length during folliculogenesis. The studies performed in pigs and bovines suggest that the telomerase activity gradually decreases and the length of telomeres progressively increases from primordial follicles to antral follicles. The blue line represents telomerase activity, whereas the red line represents telomere length.

Telomere-associated Proteins

In addition to DNA, telomeres include telomere-associated proteins including TRF1, TRF2, protection of telomeres 1 (POT1), TRF1-interacting nuclear protein-2 (TIN2), TIN2 interacting protein (TPP1), repressor-activator protein 1 (RAP1), as well as TRF-interacting proteins (Fig. 1). The primary role of these proteins is the maintenance of chromosomal end integrity and the regulation of the telomere lengthening process [23]. A complex of six telomere-associated proteins (TRF1, TRF2, POT1, TIN2, TPP1, and RAP1) are known as shelterin or telosome [24] (Fig. 1).

The most extensively studied telomere-associated proteins, TRF1 and TRF2, specifically bind to the double-stranded telomeric DNA [25] (Fig. 1). TRF1 forms homodimers that bind to the telomeric DNA and thereby control telomerase activity, preventing further telomere elongation [26, 27]. Moreover, TRF1 also interacts with TRF2 in cooperation with TIN2, contributing to the shelterin complex stability [28]. Notably, the absence of TRF1 causes replication fork-dependent defects, telomeric fusion, and altered genome packaging [29]. TRF2 also associates with double-stranded DNA upon formation of TRF2-TRF2 homodimers [25]. TRF2 is structurally similar to TRF1, except that its N-terminal end contains more basic amino acid residues [30]. RAP1 interacts with TRF2, and both play a critical role in regulating the expression of specific genes [31]. As TRF2 is implicated in the protection of the G-overhang stability, its lack leads to loss of the G-overhang structure [32].

Another member of the shelterin complex, TPP1, interacts with either TIN2 or POT1 and this event facilitates the interaction between telomere-binding proteins and the G-overhang which, in turn, prevents the activation of the DNA damage response [33]. In addition, both POT1 and TPP1 are involved in the regulation of telomere length [34]. Notably, although TPP1 does not directly bind to telomeric DNA, it increases the binding affinity of POT1 to telomeric DNA by 5–10 fold [35]. Moreover, TPP1 promotes telomerase activity [34]; however, POT1 masks the 3' telomeric end and prevents telomerase access to the telomeric area [36]. Consistently, POT1 knockdown by RNA interference causes loss of G-overhangs, chromosomal instability, senescence, and induction of apoptosis [37]. In summary, the telomere-binding proteins preserve telomeric integrity and ensure the formation and maintenance of D-loop and T-loop structures. Interestingly, these proteins may combine with each other forming various homo- or heterodimers, reflecting their multifaceted regulation of telomeric functions. Examples of these complexes are TRF1-TRF1, TRF2-TRF2, TRF1-TIN2-TPP1/POT1, TRF2-RAP1-TIN2-TPP1/POT1, and TRF2-RAP1 [38].

Telomerase is Mainly Responsible for Telomere Lengthening

Telomere lengthening is carried out by two different mechanisms: the telomerase-based pathway and the ALT process. The telomerase enzyme was first discovered in the ciliate "*Tetrahymena thermophila*" [4] and it is evolutionarily conserved in mammals. Telomerase consists of two main subunits: TERT and the telomerase RNA component (TERC) [39] (Fig. 1). Moreover, mass spectrometric analysis of affinity-purified telomerase from HeLa cells revealed that the telomerase complex comprises accessory proteins such as dyskerin, NHP2, NOP10, pontin/reptin, and TCAB1 [40, 41]; nevertheless, these proteins are not required for telomerase activity [42].

The TERT subunit of telomerase contains three main structural elements, i.e., a long N-terminal (N-DAT) domain including conserved DNA- and RNA-binding regions, a central catalytic reverse transcriptase domain (RT domain), and a C-terminal C-DAT domain [43] (Fig. 1). The N-DAT region of TERT involves two subdomains: the telomerase essential N-terminal (TEN) domain and the telomerase RNA-binding domain (TRBD), both of which are responsible for binding to nucleic acids [44]. The RT domain of TERT consists of two subdomains, called 'fingers' and 'palm' [45, 46]. These subdomains are the functional regions of the enzyme facilitating the direct interaction with the RNA-DNA hybrids [47]. It is important to note that the C-DAT domain exhibits poor sequence conservation, unlike other regions of TERT [35]. The TERC subunit of telomerase has three conserved regions: the pseudoknot/template core, the CR4/CR5, and the box H/ACA domains [48]. While the core domain is only required for telomerase activity [49], the box H/ACA domain is important for the stability and the nuclear localization of the TERC subunit [49, 50]. The exact function of the CR4/CR5 domain remains elusive.

In addition to telomerase-driven elongation, telomeres can be lengthened by an alternative mechanism known as ALT, especially in the absence of telomerase activity [51]. Although the ALT mechanism has not been fully elucidated, it is mainly based on homologous recombination (HR) between telomeres of sister chromatids [52]. The Werner syndrome helicase (WRN) and the Bloom syndrome helicase (BLM) are necessary for the proper functioning of the ALT process [53]. Further molecular and functional studies are required to characterize the mechanisms underlying ALT activation in eukaryotic cells.

Telomerase Activity and Telomere Length During Folliculogenesis in Mammals

Female primordial germ cells (PGCs) originate from the extra-embryonic yolk sac endoderm and migrate to the genital ridge [54], after which they undergo additional mitotic divisions and become oogonia [55]. Once they have entered meiosis, the oogonia in the genital ridges are defined as oocytes. Before birth, primordial follicles are formed. These are composed of oocytes arrested at the diplotene stage of prophase I of the first meiotic division, as well as of squamous follicular cells [56]. Folliculogenesis may be defined as the development of the primordial follicles into antral follicles by consecutive gonadotropin-independent and -dependent mechanisms [57, 58]. First, primordial follicles, with squamous follicular cells surrounding the oocyte, mature into primary follicles, covered by one layer of cuboid follicular cells [57]. Thereafter, the cuboidal follicular cells undergo multiple mitotic divisions, resulting in the formation of secondary follicles containing a few layers of granulosa cells [57]. The secondary follicles develop into early antral follicles with small antral spaces among the granulosa cells [57]. Small antral spaces eventually join to form single large antrum at the antral follicular stage. In the antral follicles, oocytes remaining at the prophase I stage of the first meiosis undergo luteinize hormone-dependent nuclear

and cytoplasmic maturation [58].

It is known that granulosa cells undergo many mitotic divisions during folliculogenesis, which result in telomeric shortening. Additionally, granulosa cells are exposed to many agents, including hormones and other factors, accumulating in the follicular fluid. This microenvironment most likely affects the length of telomere in these cells. The granulosa cells also possess telomerase activity capable of restoring the shortened telomeres [59, 60]. To date, a few studies have addressed the mechanisms controlling telomere length, as well as the expression and activity of telomerase, in the granulosa cells and the oocytes during folliculogenesis in rodents, bovines, pigs, and humans. It is important to note that telomerase activity is largely controlled through the expression levels of TERT protein and mRNA [61]. However, the TERC subunit of telomerase also affects telomerase activity since the lack of TERC ($TERC^{-/-}$) results in telomeric loss at late stages of mouse embryogenesis [62]. Thus, although telomerase activity is largely dependent on TERT expression, the TERC subunit seems to be a limiting factor for sustained telomerase activity.

Telomerase activity as a function of follicular development was initially evaluated in rats by Eisenhauer *et al*. [63]. Enzyme activity was found to be higher in small and healthy follicles than in large and atretic follicles [63]. High telomerase activity was detected in oocytes from both preantral and preovulatory follicles, whereas it was relatively low in ovulated oocytes [63]. A key role of estrogen in the regulation of telomerase activity in the granulosa cells, was suggested by the observation that cell exposure to estrogen causes increased telomerase activity, most likely as a result of enhanced TERT expression [64]. Consistently, a recent *in vitro* study showed that high concentrations of estradiol-17b (E2) facilitate telomere elongation in granulosa cells obtained from early antral follicles [65]. As it is known, luteinizing hormone surges resume meiotic activity, resulting in mature metaphase II (MII) ovulating oocytes from antral follicles. In mice, telomerase activity is lower in MII oocytes in comparison with germinal vesicle (GV) oocytes [66]. Telomere length was also measured in different mouse strains such as hybrid B6C3F1 and outbred CD1 by quantitative fluorescent *in situ* hybridization (Q-FISH) [66]. The length of telomeres was found to be 18.03 ± 0.47 kb and 16.09 ± 0.78 kb in the MII oocytes of hybrid B6C3F1 and CD1 mice, respectively [66]. The difference between these values suggests the presence of distinct telomere lengthening mechanisms in the MII oocytes of the two strains. However, the potential impact of telomere length on the lifespan of these mice still remains elusive.

In bovines, similar to that observed in rats, high telomerase activity was found in the smallest pre-antral follicles (60–100 μm in diameter), which gradually declined during the transition from small- (1 mm diameter) to medium-sized (3 mm diameter) follicles [67]. In the same study, the TERC subunit of telomerase was found to localize to the granulosa cells of growing follicles, but it was not detected in the primordial follicles [67]. In pigs, TERT protein expression was detected by immunohistochemistry in the primary, preantral, small antral, and medium/large antral ovarian follicles [68]. Notably, the subcellular distribution of TERT exhibited maturation stage-dependent differences: the protein was localized in the cytoplasm of oocytes in the antral follicles, whereas it was in the nuclei of oocytes in either primary or pre-antral follicles [68] (Fig. 2). In the MII oocytes, TERT was abundant in the subcortical cytoplasm, while no TERT expression was found in the polar bodies [68]. When Russo *et al.* (2006) analyzed the telomere length in porcine granulosa cells by using fluorescence *in situ* hybridization (FISH), longer telomeres were found in the preantral and antral follicles with respect to early-stage follicles [68] (Fig. 2). However, oocytes in the preantral follicle had shorter telomeres compared to the oocytes of the primary, small-antral, and medium/large antral follicles [68]. Thus, regulation of telomere length was somehow divergent in granulosa cells and oocytes. This may result from a different activation timing of telomere lengthening processes during developmental maturation. Newly designed studies, analyzing telomerase activity and telomere length during folliculogenesis in either the oocytes or granulosa cells are required to directly address this issue.

Telomerase activity was also evaluated during oocyte maturation in bovines by using the TRAP method [69]. The activity was high in the immature oocytes, gradually decreased during oocyte maturation, and reached the lowest level in the mature oocytes [69] (Fig. 2). When the expression of TRF1, TRF2, and *TERC* was examined in the bovine GV and MII oocytes, as well as in the early embryos, no significant differences were detected [70]. Notably, measurement of the relative telomere length by quantitative real time PCR (qPCR) revealed the presence of longer telomeres in GV oocytes compared to MII oocytes, in bovines [70] (Fig. 2). On the other hand, Meerdo *et al.* (2005) observed no differences in telomere length between immature and mature oocytes in bovines [71]. This discrepancy may derive from the use of different techniques for telomere measurement or the different samples employed.

Ovarian Function and Telomeres/Telomerase Activity in Humans

A few studies have explored telomerase expression and telomere length in human oocytes. Generally, the surplus human oocytes donated by couples undergoing treatment with assisted reproductive technologies (ARTs) are used in this kind of investigations. Relative telomerase activity was measured in the GV and MII oocytes, as well as in the early embryos, using the TRAP assay [72]. Telomerase activity is significantly higher in the GV oocytes compared to that in the MII oocytes [72], as also found in mouse [66] and bovine oocytes [69]. The length of telomeres in human GV oocytes obtained from patients undergoing *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), was found to be 11.12 kb, as assessed by Q-FISH [73]. In another study conducted by the same group, telomere lengths were measured in human oocytes, spermatozoa, male and female pronuclei [74]. The average telomere length of the human spermatozoa and mature oocytes was 6.32 ± 2.00 kb and 8.79 ± 0.86 kb, respectively, whereas it was 6.16 kb and 8.63 kb in the male and female pronuclei, respectively [74]. It is important to note that telomere length in the mature oocytes (8.79 ± 0.86) was significantly shorter than in the immature oocytes (11.41 ± 0.81) [74]. Consistently, there is no telomerase activity in the unfertilized mature human oocytes [75]. Most likely, in the absence of telomerase activity, progressive telomere shortening during oogenesis results in shorter telomeres in the mature oocytes. On the other hand, genetically induced telomeric shortening in mice triggers cytoplasmic fragmentation and

Table 1. The studies in humans, focusing on the relationship between alteration of telomeres/telomerase activity and polycystic ovary syndrome (PCOS)/premature ovarian failure (POF) development, are summarized. qPCR, quantitative polymerase chain reaction; TRAP, telomere repeat amplification protocol

Ovarian disease	Analyzed parameter	The finding	Sample	Method	Species	Reference
PCOS	Telomere length	Increased telomere length	Follicles	qPCR	Human	Wei D *et al*., 2017
	Telomere length	Decreased telomere length	Follicles	qPCR	Human	Li Y *et al*., 2017
POF	Telomere length	Decreased telomere length	Follicles	qPCR	Human	Butts S *et al*., 2009
	Telomerase activity	High in the patients with dysfunctional follicles Low in the patients with follicular depletion	Follicles	TRAP	Human	Kinugawa C *et al*., 2000

disrupts meiosis and chiasm formation [76], resulting in defective oocyte fertilization and abnormal embryo cleavage [77]. Telomeric shortening has also been demonstrated to induce apoptosis in human preimplantation embryos [78]. Interestingly, successful conception was associated with the presence of longer telomeres in the oocytes [79]. Consistently, Keefe *et al*. (2003) found that IVF patients with relatively long telomeres in their oocyte polar bodies (7.5 ± 1.17 kb) had higher chances of becoming pregnant as compared to patients with shorter telomeres (6.2 ± 1.69 kb) [80]. Importantly, pregnancy seems to be precluded to patients with telomere lengths below 6.32 kb [80]. In the granulosa cells, telomerase activity is a more suitable parameter, as compared to telomere length, for predicting the pregnancy outcome following IVF treatment [81]. Consistently, the length of telomeres in the mature oocytes and early embryos seems to be crucial for successful pregnancy outcome. Ovarian granulosa cells surround the oocytes and support their development. It has been suggested that IVF treatments carried out in the presence of high levels of telomerase activity in granulosa cells may result in increased chances of pregnancy [82]. However, in another study, no differences were found in the telomere length of granulosa cells between the different IVF patient groups, albeit telomerase activity was higher in the transferred blastocysts that actually resulted in pregnancy [81]. This study suggested that the level of telomerase activity in granulosa cells is a more effective indicator of pregnancy success, as compared to other parameters including telomere length, age, FSH, and estrogen levels. On the other hand, estrogen deficiency leads to the inhibition of TERT gene expression and telomerase activity and results in telomere shortening in mouse ovarian granulosa cells [64]. Estrogen is produced by granulosa cells in cooperation with theca cells and luteinizing cells in the corpus luteum [83] and its level during folliculogenesis seems to be important for the development of high-quality oocytes and for successful pregnancy.

As telomeres play important roles in many cellular events including meiosis, nuclear organization, and genomic integrity throughout the lifespan of eukaryotic cells, their dysfunctions associate with various types of diseases including Werner syndrome [84], dyskeratosis congenita [85], Bloom syndrome [86], Fanconi anemia [87], and cancer [88]. Furthermore, there is a close relationship between telomeres/telomerase activity and female infertility caused by ovarian aging [89], polycystic ovary syndrome (PCOS) [90], and premature ovarian failure (POF) [23].

Telomere length and telomerase activity have been investigated in the granulosa cells of 54 women aged 37 years or less, suffering from ovarian insufficiency and under treatment with ARTs [89]. This study revealed that the granulosa cells from the patient group lacked telomerase activity and displayed shorter telomeres when compared to those in their control counterparts [89]. Therefore, telomerase activity and telomere length may be used as molecular markers for ovarian insufficiency, a common condition in infertile women [89].

PCOS is one of the commonly encountered reproductive disorders exhibiting menstrual irregularity, hyperandrogenism, and polycystic ovaries as prominent clinical symptoms. Moreover, 74% of the PCOS patients experience infertility [6, 91]. The relative telomere length in peripheral blood samples from PCOS patients [0.764 ± 0.016 T/S (telomere/single copy gene)] was found be significantly shorter than that of the control group (0.876 ± 0.023 T/S) [92]. Hormonal alterations and increased oxidative stress in the PCOS patients may negatively affect the structural integrity of telomeres, leading to telomere attrition in the blood cells. It is important to note that, in order to determine the precise impact of the histopathological/hormonal changes on telomere length, telomeres should be measured in the granulosa cells of PCOS patients. In recent studies, telomere lengths were measured by qPCR in the leukocytes obtained from either PCOS patients or control subjects and no remarkable differences were detected [93–95]. However, the length of telomeres in the granulosa cells was found to be significantly longer in the PCOS group (1.57 ± 0.67) than in the controls (1.00 ± 0.37) [93] (Table 1). Most likely, this was due to a decreased number of mitotic divisions in granulosa cells and also to altered hormonal levels in the PCOS patients. Wang *et al*. (2017) also analyzed telomere length and TERRA levels in peripheral blood leukocytes of patients with PCOS and control subjects [96] and found that the telomeres were significantly longer in the patient-derived leukocytes [96]. Of note, the level of TERRAs, contributing to the regulation of telomere length, was found to be lower in the PCOS patients [96]. Previous studies have shown that, after aromatization, androgens may regulate telomerase expression and activity, acting through ERα [97, 98]. Consistently, the increased testosterone concentration observable in patients with PCOS might lead to enhanced telomerase activity, resulting in extended telomeres. In contrast, Li *et al*. (2017) reported that telomeres in the granulosa cells were shorter in the PCOS patients (0.971) than in the controls (1.118) (Table 1), whereas they could not detect any difference in telomerase activity [90]. Further studies with larger numbers of samples will be necessary to comprehensively evaluate the mechanisms underlying the altered regulation of telomeric length characterizing patients with PCOS.

POF is an irreversible pathologic loss of the ovarian function occurring before age 40 and leading to early female infertility [99].

The predominant clinical symptoms include premature menopause, primary ovarian failure, hypergonadotropic hypogonadism, and gonadal dysgenesis [37, 93]. In the ovary of POF patients, two main defects are observed: follicle dysfunction and follicle depletion [99]. Thus, reduced ovarian function and infertility result from a declined follicle reserve and/or poor follicular response to the gonadotropins [99]. To date, only a few studies have addressed the question of whether alterations in telomere length and/or telomerase activity play a role in POF development. Kinugawa *et al*. (2000) first compared telomerase activity in POF patients and normal subjects by using the TRAP method [100]. Whereas the patients with follicular dysfunction displayed high ovarian telomerase activity, those with follicle depletion possessed low telomerase activity [100] (Table 1). The interpretation of these findings was that decreased telomerase activity in the ovary may arise from age-related primordial follicle depletion and that telomerase activity may represent a marker of ovarian function [100]. It has been found that women lacking telomerase activity in the granulosa cells have an 11-fold risk of experiencing POF as compared to those with normal telomerase activity [89]. Consistently, the length of telomeres was remarkably shorter in the granulosa cells of POF patients (1.88) in comparison to the controls (3.15) [89] (Table 1).

In conclusion, telomeres are conserved structures located at the ends of eukaryotic chromosomes and play a critical role in protecting genomic integrity during the cellular lifespan. Telomeres can be extended by two main pathways: the telomerase-dependent pathway and the ALT process. In addition to telomere-associated proteins, recently identified TERRA molecules have been discovered to contribute to telomere reorganization. A limited number of studies have addressed the expression of telomerase, telomere-associated proteins, and TERRA molecules, as well as telomere length, during folliculogenesis in mammals. We think that additional studies are required to evaluate the relationship between the altered expression of telomerase and telomere-associated proteins, on the one hand, and the development of female infertility, on the other. Finally, the cellular mechanisms regulating telomerase activity and telomere structure in oocytes and granulosa cells during folliculogenesis should be the focus of extensive research for the elucidation of the events underlying female infertility.

Conflict of interest: The authors declare that there is no conflict of interest.

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