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NEW RESEARCH HORIZON ***Review***

Telomere length and telomerase activity during oocyte maturation and early embryo development in mammalian species

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ABSTRACT: Telomeres are located at the ends of all eukaryotic chromosomes and protect them from deleterious events such as inappropriate DNA repair, illegitimate recombination or improper segregation of the chromosomes during mitotic or meiotic divisions. However, telomeres gradually shorten primarily due to successive rounds of genomic DNA replication and also as the result of the adverse effects of oxidative stress, genotoxic agents, diseases related to ageing and environmental factors on the nuclear materials of dividing or non-dividing cells. Germline cells, proliferative granulosa cells, early embryos, stem cells, highly proliferative somatic cells and many cancer cells contain the enzyme telomerase so that they are capable of elongating the shortened telomeres. Although numerous studies have revealed the length of telomeres and telomerase activity in oocytes, granulosa cells and early embryos, only a few studies have analyzed and compared the work performed on distinct mammalian species. In this comprehensive review article, we compare and discuss telomere length and telomerase activity in oocytes, granulosa cells and early embryos in different mammalian species including mice, bovines and humans.

Key words: oocyte / granulosa cell / early embryo / telomere / telomerase activity

Introduction

Eukaryotic cells have specialized non-coding DNA sequences at the ends of all chromosomes which are called telomeres. They were first identified and described by Hermann Muller in the fruit fly, *Drosophila melanogaster* (Muller, 1938). Muller named this phenomenon the 'telomere' from the Greek words *telos*, meaning 'end' and *meros* meaning 'part' (Muller, 1938). This unique structure consists of long double-stranded $(TTAGGG)_n$ repeats, and its length varies in mammalian species from 10 to 15 kb in humans, 12 to 23 kb in bovines and 50 to 150 kb in mice (Kipling and Cooke, 1990; O'Sullivan and Karlseder, 2010; Iqbal *et al.*, 2011).

Telomeres show special structural features at their 3′ ends, consisting of a G-rich overhang of ~50–350 nucleotides. This protrusion of single-stranded repeats is called a G-tail or G-overhang (Moyzis *et al.*, 1988; Wellinger and Sen, 1997). The G-overhang folds back and in turn enters into the double-stranded DNA duplex, thereby forming displacement (D-loop) and telomere loops (T-loop) at the ends of all chromosomes (Chan and Blackburn, 2004; Kalmbach *et al.*, 2013). Specifically, telomere-associated proteins that localize at the telomeres contribute to formation of the D- and T-loop structures by inducing bending, looping and pairing of the double-stranded DNA, and by inserting the 3′ single-stranded repeat tail into the duplex telomeric DNA (Bianchi *et al.*, 1997; Griffith *et al.*, 1999). Thus, these loops mask the 3′ overhang to protect it from being recognized as a DNA break by the DNA damage response (DDR) machinery. Additionally, they regulate access of the enzyme telomerase to the telomeric DNA ends so that they regulate the length of telomeres. As is well known, the enzyme telomerase specifically functions in telomere lengthening (Bekaert *et al.*, 2004; O'Sullivan and Karlseder, 2010).

The most important functions of the telomeres are protecting chromosome ends from being recognized as DNA break by the nucleases, preventing illegitimate chromosomal recombination, and properly attaching the chromosome ends to the nuclear matrix or nuclear membrane. They also play central roles in the formation of correct pairing, synapses and chiasmata between homologous chromosomes and proper segregation of the chromosomes/chromatids throughout mitotic or meiotic divisions (Zhao *et al.*, 2008; Aubert *et al.*, 2012; Zhdanova *et al.*, 2012).

Previously, telomeres were considered as transcriptionally silent regions, but recent studies have shown that they are actively transcribed from the subtelomeric foci toward chromosome ends into telomeric repeat-containing RNA (TERRA) molecules (Azzalin *et al.*, 2007; Schoeftner and Blasco, 2008; Kalmbach *et al.*, 2013; Reig-Viader *et al.*,

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2013). TERRA consists of UUAGGG repeats and has heterogeneous lengths, ranging in size from ~100 bp to 9 kb. RNA-FISH (RNA-fluorescent *in situ* hybridization) localizes the TERRA molecules to the ends of each chromosome in mammalian cell nuclei. In the nuclei, as TERRA molecules associate with the telomeres following their transcriptions, it is thought that they may be essential for maintenance of the telomeric stability (Azzalin *et al.*, 2007; Schoeftner and Blasco, 2008). These discoveries have prompted researchers to examine spatio-temporal expression and possible roles of the TERRA molecules in the mammalian somatic and germ cells. Azzalin *et al.* (2007) identified TERRA molecules in different human (HLF, HLF-hTERT, HeLa, HeLa-E1, HEK293T and U2OS), mouse (RenCa and J2–3T3) and Chinese hamster cell lines using northern blot analysis (Azzalin *et al.*, 2007). Then, Reig-Viader *et al.* (2013) observed that TERRA was present at the telomeres of human fetal oocytes at distinct stages of prophase I. Additionally, its co-localization with TERT subunit suggested that TERRA could play a role in maintaining the integrity of the telomeres by regulating the activity of telomerase (Reig-Viader *et al.*, 2013).

Telomeres includes telomere-associated proteins

In addition to telomeric DNA repeats, telomeres also contain telomere-associated proteins involving TRF1, TRF2, POT1, TPP1, TIN2, RAP1 and TRF-linked proteins (Fig. 1). Telomere-associated proteins have two fundamental functions. One of them is to maintain the integrity of chromosome ends by forming nucleoprotein complexes. The other function is to regulate the process of telomere lengthening (reviewed in De Boeck *et al.*, 2009; O'Sullivan and Karlseder, 2010). Note that *the six telomere-associated core proteins* (TRF1, TRF2, POT1, TIN2, RAP1 and TPP1) are collectively defined as the shelterin complex or the telosome, which is exclusively involved in sensing the stability and length of the telomeres (de Lange, 2005; Hug and Lingner, 2006).

Telomeric repeat-binding factor 1 (TRF1 or TERF1) and telomeric repeat-binding factor 2 (TRF2 or TERF2) are telomere-associated proteins that have been intensively studied. TRF 1 and 2 specifically recognize and bind to the double-stranded telomeric DNA. TRF1 is a homodimeric protein, which interacts with the telomeric DNA in the Myb domain at its C-terminus. Notably, this protein reduces telomere elongation by inhibiting access of the enzyme telomerase to the telomeric sites (Chong *et al.*, 1995; Kim *et al.*, 2008). Consistent with this, overexpression of the TRF1 results in significant telomere shortening but dominant-negative expression of the *Trf1* gene causes an increase in the telomere lengthening (van Steensel and de Lange, 1997; Smogorzewska *et al.*, 2000).

TRF2 protects chromosome ends from end-to-end fusion and ensures maintenance of the T-loop structure. Similar to the TRF1 protein, high levels of the TRF2 expression inhibit telomere lengthening (Smogorzewska *et al.*, 2000; Matsutani *et al.*, 2001). TRF2 is also found to play a role in DDR. When DNA damage occurs at telomeres, activated ATM protein kinase leads to phosphorylation of the TRF2. This results in recruitment of certain TRF2-linked proteins to the damaged sites (Tanaka *et al.*, 2005). Dominant negative expression of the *Trf2* allele causes loss of the G-overhang, genomic instability, end-to-end fusion of the chromosomes and induction of the DDR system. As a result, the control of the expressional levels of both TRF1 and TRF2 seems to have critical actions in either telomere length maintenance or telomere stability (reviewed in De Boeck *et al.*, 2009).

In addition to TRF1 and TRF2, other telomere-associated proteins indirectly bind to the telomeres through protein–protein interactions with TRF1, TRF2 or POT1. POT1 (protection of telomeres 1) is constitutively present at telomeres and interacts specifically with the *single-stranded G-overhang*. The binding of POT1 to the G-overhang is carried out by its oligosaccharide or oligonucleotide-binding domain. Its most important functions are suppression of the ATR-mediated telomeric DDR and induction of the WRN (Werner helicase) or BLM (Bloom syndrome protein) helicases to unwind the telomeric duplex and D-loop structure when necessary (Opresko *et al.*, 2005; Denchi and de Lange, 2007). Dysfunction of the *Pot1* gene leads to loss of the G-overhang, rapid increase in the telomere elongation and activation the ATR-mediated telomeric DDR (Churikov *et al.*, 2006; Wu *et al.*, 2006).

Another telomere-associated protein, TIN2 (TRF1 interacting nuclear protein 2), facilitates the formation of an important bridge between TRF1 and TRF2 subcomplexes, and TPP1 (POT1 and TIN2interacting protein; also known as ACD) protein, which associates with POT1. Additionally, TIN2 participates in regulating PARP activity of the tankyrase found at telomeres. The deletion of the Tin2 gene results in decreasing the levels of TRF1 and TRF2, thereby stimulating telomere elongation (Kim *et al.*, 2004; Ye and de Lange, 2004). As RAP1 (repressor activator protein 1) links with the TRF2 and subtelomeric sites, it contributes to telomeric recombination and transcriptional control of the certain genes (Martinez *et al.*, 2010). Moreover, RAP1, like POT1, is capable of activating the WRN or BLM helicases, which unwind telomeric or non-telomeric forked duplexes and D-loop structures (Opresko *et al.*, 2005; Denchi and de Lange, 2007).

In addition to the shelterin complex proteins, DNA repair-related proteins (MRN complex, KU, ERCC1/XPF and ATM) also associate with either TRF1 or TRF2 and play a role in the repair of damaged telomeric sites. The MRN complex consists of one NBS1, two MRE11 and one RAD50 dimer, and it tethers DNA ends (Carney *et al.*, 1998). Basically, this complex (MRE11-RAD50-NBS1) functions in non-homologous DNA double-strand break (NHEJ) repair (Hsu *et al.*, 1999) and also functions in maintenance of the 3′ overhang in telomerase positive cells, but not in telomerase negative cells (Chai *et al.*, 2006). Similarly, KU proteins (KU70 and KU80) accompany recombinational DNA repair process (Takai *et al.*, 2003). On the other hand, the ERCC1/XPF participates in nucleotide excision repair (Zhu *et al.*, 2003) and the Ataxia telangiectasia mutated (ATM) kinase takes place in the damage-induced cell cycle control (Karlseder *et al.*, 2004). Other proteins associated with TRF1 or TRF2 such as WRN, FEN1, PINX1 and TANK1/2 are temporarily present at the telomeres (Nittis *et al.*, 2010) (Fig. 1). The WRN binds to the telomeric sites throughout S phase of the cell cycle and is thought to play role in the DNA replication of the lagging strand (Crabbe *et al.*, 2004). The PINX1 is known to interact directly with TRF1 or telomerase subunits, and thereby inhibits telomere lengthening. The PINX1 localizes at telomeres in the nucleus, as well as in the nucleolus where telomerase subunits (TERT and TERC) reside (Zhou and Lu, 2001).

Tankyrase 1 and 2, the TRF1-interacting ankyrin-related ADP-ribose polymerase, belongs to the poly (ADP-ribose) polymerase (PARP) enzyme family. Tankyrase 1 associates with TRF1 to prevent its binding to telomeric repeats. Overexpression of the tankyrase 1 leads to

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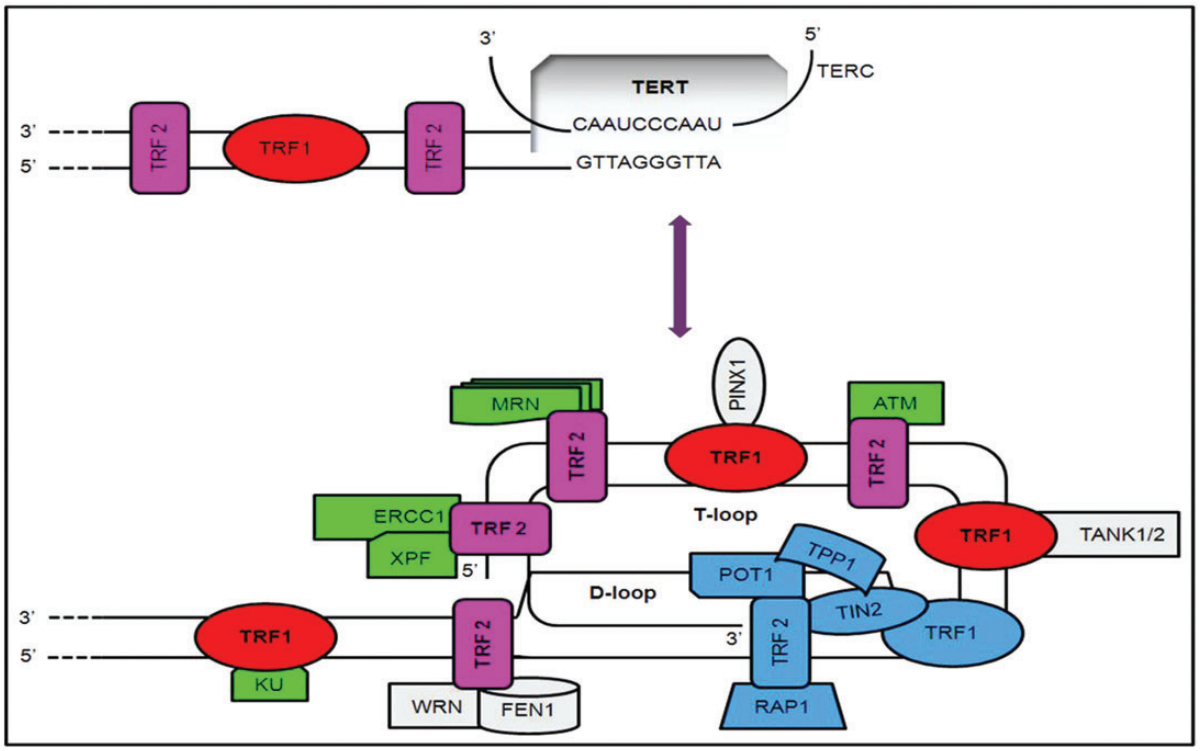


Figure 1 Schematic representation of the telomere structure with telomere-associated proteins. Telomeres can be in two states: an 'open' state in which telomerase is able to lengthen the telomeres and a 'closed' state in which telomerase cannot reach the telomeric site. Shortened telomeres in the 'open' state could be elongated by the enzyme telomerase composed of telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) subunits. In the extended telomeres, the 3′ ends of telomeres fold back and enter into the double-stranded DNA duplex thereby forming displacement (D-loop) and telomere loops (T-loop). In the 'closed' state, telomere and telomere-associated proteins including TRF1, TRF2, POT1, TIN2, TPP1 and RAP1 generate the shelterin complex. Here they are colored *blue*. Telomeric proteins, which play essential roles in the recombination and DNA repair events, are shown in *green*. Other proteins known to play roles in telomere maintenance are colored *gray*.

releasing TRF1 from telomeres, resulting in proteasome-mediated degradation. This degradation is required to allow telomerase easy access to the telomeric sites in order to achieve telomere elongation (Smith *et al.*, 1998; Chang *et al.*, 2003). Tankyrase 2 also associates with TRF1 and plays role in normal growth and development, but not in telomere length maintenance (Hsiao *et al.*, 2006).

Telomeres are mainly elongated by the enzyme telomerase or ALT mechanism

Up to now, studies have revealed that telomere lengthening is carried out by two main mechanisms. One is the enzyme telomerase-based pathway (Liu *et al.*, 2007), and the other is a process called as alternative lengthening of *telomeres* (ALT) (Liu *et al.*, 2007; Cesare and Reddel, 2010; Conomos *et al.*, 2013; Neumann *et al.*, 2013).

The enzyme telomerase initially discovered in the ciliate *Tetrahymena* (Greider and Blackburn, 1985) is evolutionarily conserved from yeast to mammalians. Basically, telomerase is composed of two fundamental subunits: the telomerase reverse transcriptase (TERT) and the telomerase RNA component (TERC) (Fig. 1). TERT, a 127 kDa catalytic subunit, contains ~1122 amino acids, which involves a conserved motif across mammalian species. TERT subunit employs TERC subunit as a template to correctly add telomeric sequences onto the ends of all chromosomes (Morin, 1989; Collins *et al.*, 1995; Greenberg *et al.*, 1998). On the other hand, the TERC subunit consisting of 430 nucleotides is constitutively transcribed in all telomerase positive cells. Additionally, it is expressed in most tissues where there is no telomerase activity; therefore, TERC subunit is not a reliable marker to characterize the telomerase activity (Avilion *et al.*, 1996). Indeed, telomerase activity is strictly controlled by transcriptional and post-transcriptional regulations of the TERT subunit and also by assembly of the telomerase holo-enzyme components (Blasco *et al.*, 1996; Bodnar *et al.*, 1998). When the holo-enzyme telomerase is activated, it adds a tandem array of TTAGGG repeats onto 3′ ends of the chromosomes during the genomic DNA replication process (Greider and Blackburn, 1985; Achi *et al.*, 2000).

Recent studies have shown that telomerase is specifically expressed in highly proliferative cells, including germ cells (Liu *et al.*, 2012; Reig-Viader *et al.*, 2013), proliferative granulosa cells (Goto *et al.*, 2011; Chronowska, 2012), stem cells (Maeda *et al.*, 2011), a majority of the cancerous cells (Liu *et al.*, 2012) and a subset of hematopoietic cells (Ge *et al.*, 2012), but it is not expressed in differentiated or aged somatic cells. Furthermore, telomerase activity was reported in different human tissues

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including endometrium (Kyo et al., 1997), placenta (Rama et al., 2001), liver (Yui et al., 1998) and epidermis (Rea and Rice, 2001), all of which have high regenerative capacity compared with other tissues.

In addition to the critical role of telomerase in the telomere elongation process, it also acts in DNA repair of chromosomal breaks by capping them with telomeric DNA repeats (Masutomi et al., 2005; Chico et al., 2011). As is well known, chromosome breaks and other types of chromosomal impairments may be formed spontaneously or promoted by exogenous agents including ionizing radiation, chemicals or oxygen-free radicals. Most of them are successfully restored in a short time by the DNA repair mechanisms. Collectively termed DDR, these repair mechanisms consist of nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and DNA double-strand break repair (DSBR) (Boulton, 2010; Ozturk and Demir, 2011; Terradas et al., 2012). In addition to the DNA repair function of telomerase, it also enhances proliferation of definite stem cell populations such as epidermal stem cells (Flores et al., 2005) and hair follicle stem cells (Sarin et al., 2005) independent of its basic function in telomere maintenance.

Another mechanism of telomere extension, ALT, includes synthesis of the telomeric sequences by using DNA replication machinery following telomere sister chromatid exchange (T-SCE) (Bryan et al., 1995; Conomos et al., 2013). Although many molecular details of the ALT pathway have remained elusive, it commonly occurs in definite somatic cell types (Herrera et al., 2000; Bailey et al., 2004) and in early cleavage-stage embryos, but interestingly T-SCE rate significantly declines at the blastocyst stages (Liu et al., 2007). In cells with an active ALT pathway, functional defects in the cap structure of telomeres, expressional alterations in some of the shelterin proteins such as TRF2 and abnormal DNA sequences are observed. These defects are considered to be essential for becoming ALT process (reviewed in Conomos et al., 2013).

The ALT mechanism is, in turn, regulated by the DNA methylation status or epigenetic regulation of the telomeric regions (Zhu et al., 2000; Lundblad, 2002). Gonzalo et al. (2006) consistently found that mouse embryonic stem cells that were genetically deficient for the *Dntm1*, or both *Dntm3a* and *Dntm3b* genes had significantly longer telomeres than wild-type counterparts. This suggests that a lack of DNMTs causes a decrease in the methylation status of the embryonic stem cells and also leads to a prominent increase in the telomeric DNA recombination, which results in extended telomeres. Taken together, telomere lengthening in these embryonic stem cells seems to be performed largely by using the ALT mechanism, but the contribution of the telomerase enzyme on this lengthening event remains unclear (Gonzalo et al., 2006).

Methodology for telomere length and telomerase activity

The methods involving telomere restriction fragment (TRF) length analysis, quantitative real-time PCR (qRT–PCR), single telomere length analysis (STELA) and quantitative fluorescent *in situ* hybridization (Q-FISH) are mainly used to measure the length of telomeres. Telomeric repeat amplification protocol (TRAP) and TMA techniques are exclusively employed to determine telomerase activity. The advantages and disadvantages of all methods commonly used to detect the length of telomeres and telomerase activity are summarized in Table I. Note that a recent review article by Aubert et al. (2012) has comprehensively compared these methods (Aubert et al., 2012).

Table I Certain methods commonly used to determine the length of telomeres and telomerase activity are summarized.

Method	Advantage	Disadvantage
TRF	Detects telomere length in each sample Gives average telomere length	Requires large amounts of DNA (0.5–5 μg) Needs 3–5 days Causes 5% variability between individuals May not detect short telomeres Cannot measure telomere length per cell
qRT–PCR	Is simple, practical and reproducible Requires small amount of genomic DNA (20 pg) Analyses multiple samples simultaneously	Is fluorescence-based application Gives only average telomere length have variability of 5.8%
STELA	Measures single telomere Detects short telomeres Provides high resolution and is highly sensitive Needs limited starting material	Requires at least a few chromosomes Is labor intensive Needs unique primer for each chromosome arm Cannot analyze very long telomeres
Q-FISH	Requires low number of cells Measures telomere of individual chromosomes/cells Gives strong signal Is reliable and quantifiably	Needs chromosome spreads Is fluorescence based Needs digital image microscopy Have high variability (11.2%)
TRAP	Gives relative telomerase activity Is highly sensitive and provides fast detection	Is time-consuming procedure Detects only qualitative telomerase activity Cannot give any information related to telomere length
TMA	Gives quantitative telomerase activity Not based on PCR amplification	Needs isothermal amplification system requires exclusive equipments

Advantages and disadvantages of these methods such as Q-FISH, qRT–PCR, STELA, TRF, TRAP and TMA are compared in this table. Note that the TRAP and TMA methods for measuring telomerase activity and others for detecting telomere lengths are employed. Q-FISH, quantitative fluorescent *in situ* hybridization; qRT–PCR, quantitative real-time PCR; STELA, single telomere length analysis; TRF, telomere restriction fragment; TRAP, telomeric repeat amplification protocol; TMA, transcription-mediated amplification.

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TRF was the first technology used to detect an average telomere length by using the Southern blot assay (Moyzis *et al.*, 1988). In brief, this method includes sequential steps involving: genomic DNA extraction, digestion of the genomic DNA with specific restriction enzymes (such as *RsaI*, *NlaIII*, *MboI*, *Sau3A or HinfI*). These restriction enzymes specifically cut the genomic DNA into small fragments, but telomeric repeats remain uncut. Restricted DNA fragments resolved according to their size by agarose gel electrophoresis are transferred to a nitrocellulose or PVDF membrane. Finally, the membrane is hybridized with fluorescently labeled telomere-specific probes and subjected to X-ray film using chemiluminescence. The average TRF length is calculated by comparing smear signals relative to a nuclear weight standard and then normalized to a reference value (Takai *et al.*, 2003; Kimura *et al.*, 2010).

Although this method provides detection of the mean telomere length distribution in each DNA sample and gives the absolute value (kb), it requires high amounts of genomic DNA (0.5–5 μg) and takes 3–5 days. Furthermore, there is a high variability (5%) between individuals due to probable modifications on the subtelomeric restriction sites, including polymorphism, methylation or other nucleotide changes (Cawthon, 2002; Kimura *et al.*, 2010). Importantly, these modifications may cause resistance to the enzymatic digestion, which subsequently leads to produce 2- to 4-kb higher telomeres than are normally found on the gel. The best way to determine the modification type in the subtelomeric fraction is to amplify the digested telomeric sites by using specific primers, and then the nucleotide sequences of PCR products are analyzed (Levy *et al.*, 1992; Steinert *et al.*, 2004).

The potential loss of the short DNA fragments because of their inability to immobilize on the membrane is another disadvantage of this method. Therefore, it cannot detect short telomeres contained in a few telomere repeats, and TRF does not give the telomere length of each chromosome (Gan *et al.*, 2001; Aubert *et al.*, 2012). Moreover, the resolution capacity of this technique is quite low, ranging between 2.5 and 4 kb in length (Counter *et al.*, 1992; Levy *et al.*, 1992). Alternatively, the telomere length of each chromosome can be measured with TRF after DNA isolation of the target chromosomes sorted by flow cytometry (Martens *et al.*, 1998). As expected, this application requires a large amount of input DNA; therefore, slot blot hybridization has been created so that the required starting DNA material is reduced to ∼9 ng. In slot blot strategy, the extracted and subsequently denatured genomic DNA is immobilized onto a membrane by using a vacuum manifold and then hybridized with a labeled telomeric probe (Bryant *et al.*, 1997).

qRT–PCR is a fluorescence-based technique and detects telomere length through PCR amplification by using specific oligonucleotide primers designed specifically to hybridize to the TTAGGG and CCCTAA telomeric DNA repeats. Briefly, this method consists of genomic DNA isolation, followed by amplification of the telomeric regions by using fluorescence signals. SYBR green is widely used as a fluorescent signal which accumulates at the end of each PCR cycle. When the fluorescent intensity reaches detectable levels, the average telomere length of an individual cell or all cells in the sample can be calculated using Ct (cycle threshold) values (Cawthon, 2002). qRT–PCR has the advantage of providing rapid detection, being relatively cheap, requiring a short timeline and low amounts of starting genomic DNA material (∼34 ng/reaction) when compared with non-PCR-based techniques. However, the major drawback of this technique is that it can detect only relative average telomere length, not given the absolute length of individual chromosomes (Gil and Coetzer, 2004; Hewakapuge *et al.*, 2008).

Another PCR-based technology recently defined is STELA. The STELA allows us to accurately determine the length of a single telomere at each chromosome end (Baird *et al.*, 2003). The telomeric end of the target chromosome is amplified using linker and chromosome-specific primers. The linker primer ligates to the 5′ end of the telomere, and the 3′ overhang is used as a template. Chromosome-specific primer anneals to unique subtelomeric sequences of the target chromosome and provides generation of a specific amplicon for individual chromosomes. Amplified PCR products are fractionated by agarose gel electrophoresis. The resultant banding patterns that appear following the Southern hybridization with a telomere-specific probe reveal the telomere length of each chromosome tip after calculated in comparison with a DNA ladder standard (Aubert *et al.*, 2012; Ivankovic *et al.*, 2012).

The biggest advantage of STELA is its ability to measure the single telomere length of each chromosome. It requires limited DNA (<100 pg) and gives a much higher resolution (0.1 kb) than existing methods. However, it is a labor-intensive technology and necessitates a 3′ single-stranded overhang as a template. Since unique chromosome primers are not present for all chromosomes, STELA is only applicable to certain chromosome arms including XpYp, 2p, 11q, 12q and 17p. Moreover, STELA could not detect very long telomeres because its detection capability is restricted to 20 kb (Cheung *et al.*, 2004; Aubert *et al.*, 2012). A new approach related to STELA, universal STELA, has been developed to simultaneously amplify single telomere ends of many chromosomes. However, it is not capable of giving the mean telomere length of a chromosome because each chromosome end may have varying telomere lengths (Bendix *et al.*, 2010).

Q-FISH is a cytogenetic technique and based on measuring fluorescence intensity derived from telomeric sequences of interphase nuclei or metaphase chromosomes (Poon *et al.*, 1999). In brief, this technique includes fixation, denaturation, washing, proteolysis of genomic material and finally probe hybridization to telomeric sites of the genomic DNA. Q-FISH probe, also known as peptide nucleic acid $(CCCTAA)_3$, is directly labeled with a fluorescein fluorophore such as Cy3 (cyanine 3) or FITC (fluorescein isothiocyanate). Finally, Q-FISH slides counterstained with DAPI are analyzed under a fluorescence microscopy. Telomere lengths of the metaphase chromosomes or interphase nuclei are calculated by utilizing the standard of known telomere length found in a software programme specific for Q-FISH image analysis (Smogorzewska *et al.*, 2000; Slijepcevic, 2001).

There are many key advantages of Q-FISH: it can be readily applicable; it gives strong and specific signals; and it facilitates quantitative or relative measurement of individual telomere length of each chromosome in a high resolution (0.3 kb). However, Q-FISH requires metaphase spreads to give correct results for all chromosomes. Therefore, Q-FISH cannot measure telomere length of all chromosomes in terminally senescent or aberrant cells that are not capable of dividing. Q-FISH application on the interphase nuclei of a few cells may not give a reliable result due to potential overlapping of the telomeric signals (Zijlmans *et al.*, 1997; Aubert *et al.*, 2012). Note that the newly introduced Flow-FISH method relies on a combination of flow cytometry (flow) and fluorescent *in situ* hybridization staining protocols and yields quantitative telomere length of particular cell population freshly collected, but it requires adequate cell numbers for flow cytometry (Rufer *et al.*, 1998; Baerlocher *et al.*, 2006).

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TRAP is used specifically to test telomerase activity, but does not give any data related to telomere length. It is dependent on the addition of telomeric repeats $(TTAGGG)_n$ onto oligonucleotide primers by the telomerase enzyme extracted from cells or tissues. The telomerase enzyme in the active state is able to elongate the oligonucleotides by adding dNTPs. Then, the resulting TRAP products serve as a template and are amplified with PCR using specific primers for the telomeric sequences to reveal relative telomerase activity. Generally, immortal cell lines such as human 293 kidney cells are employed to validate the specificity of the assay (Kim *et al.*, 1994; Xin, 2011).

Although the TRAP assay is highly sensitive, it is very time-consuming procedure. Because of using PCR amplification for the synthesized telomeric sequences, only relative level of telomerase activity in the analyzed sample can be determined in a qualified or semi-quantitative way, rather than a fully quantitative way (Zhang *et al.*, 2001). TRAP modifications such as TRAP-ELISA, real-time TRAP, F-TRAP (TRAP with fluorescent primers), stretch PCR and *in situ* TRAP have made classic TRAP more sensitive and more reliably than original TRAP to determine exact telomerase activity (Durusoy and Ozturk, 2001; Fajkus, 2006).

Alternatives to the conventional TRAP technique have also been generated. One of them is TMA (Hirose *et al.*, 1998). This method, in conjunction with hybridization protection assay (HPA), measures the quantitative telomerase activity and does not use PCR amplification. An isothermal amplification system, including a heat block or water bath is used to provide the required temperature. Unlike the original TRAP protocol, the amplified product in this method is RNA, which can be detected by using an HPA system. HPA utilizes acridium ester-labeled probes that bind to the junction of primer and telomeric repeat. The quantitative result of the hybridized probe signal is determined by chemiluminescence measurement in a luminometer. The major advantages are that it is faster than traditional TRAP, but just as sensitive and reproducible. It should be noted that TRAP inhibitors come from the clinical samples minimally affect the TMA/HPA application (Hirose *et al.*, 1998).

Since the telomerase subunit, TERT, mRNA expression has been found to correlate closely with the telomerase activity; analysis of the TERT mRNA is another alternative method for measuring telomerase activity. For this purpose, real-time PCR or *in situ* hybridization techniques are used to determine telomerase activity (Kyo *et al.*, 1999). Fletcher *et al.* (1999) also introduced a new system that provides a natural nuclear environment that imitates *in vivo*, and the activity or function of the telomerase is characterized on the basis of the primer extension in that condition. For this purpose, nuclei from human T cell lymphoblast-like cell line have been used to provide the nuclear environment because these cells have very low telomerase activity and only add up to four TTAGGG repeats. This system is also convenient to evaluate the efficiency of the telomerase inhibitor (Fletcher *et al.*, 1999).

The enzyme telomerase is expressed by different cell types to maintain telomere length

The length of telomeres in all dividing eukaryotic cells including somatic cells, germ cells, stem cell, progenitor cells and early embryos, gradually shortens at the end of each round of genomic DNA replication due to insufficiency of DNA polymerase enzyme in complete synthesis of the chromosome ends. Hence, telomeric DNA inevitably diminishes 50–200 bp per population doubling depending on cell types. For example, a human fibroblast cell looses 48 ± 21 bp from telomeric ends per mitotic division under *in vitro* culture conditions (Harley *et al.*, 1990; Levy *et al.*, 1992). This means that telomere length in a mitotically active somatic cell shortens from 10–15 to 2–5 kb throughout its lifespan (Harley *et al.*, 1990; Liu and Li, 2010). In fact, the initial length of telomeres and the rate of telomere shortening determine the number of cell divisions experienced by a normally differentiated somatic cell. Note that the activity of telomerase in somatic cells is largely diminished after birth so that the shortened telomeres cannot be replenished, and subsequently cellular senescence may be triggered (Bodnar *et al.*, 1998; Hiyama and Hiyama, 2007).

As with the proliferating somatic cells, the development of primordial germ cells into oocytes contains 22 mitotic and then 2 meiotic divisions in humans (Kurahashi *et al.*, 2012). On the other hand, a human male germ cell developed from primordial germ cell into spermatozoon undergoes ~34 mitotic and subsequently 2 meiotic divisions (Kozik *et al.*, 1998; Kurahashi *et al.*, 2012). In contrast to most somatic cells, germline cells have the enzyme telomerase that functions in lengthening of the telomeres that had been shortened during cell division or for other reasons. Spermatozoa and oocytes have consistently higher telomere length than somatic cells (de Lange *et al.*, 1990; Wright *et al.*, 1996). Despite the presence of telomerase activity, if the length of telomeres in germline cells reached the critical points, this would cause mitotic arrest, disjunction and segregation abnormalities in the germline chromosomes (Thilagavathi *et al.*, 2012).

Embryonic stem cells derived from blastocysts are pluripotent and multiply indefinitely. They have the enzyme telomerase and are thereby able to replenish the telomeric DNA loss following each round of DNA replication (Albert and Peters, 2009; Marion *et al.*, 2009; Shay and Wright, 2010). In adult stem and progenitor cells, like embryonic stem cells, telomere shortening can be largely or partially extended by the enzyme telomerase depending on the stem cell types. The catalytic subunit of telomerase, TERT subunit, has been revealed consistently in the stem cell compartment of several somatic tissues (Harrington, 2004). As most stem cells involving hematopoietic, neuronal, skin, intestinal crypt, pancreas, kidney and mesenchymal stem cells have low levels of telomerase activity, their telomeres gradually shorten throughout their lifespans (reviewed in Greenwood and Lansdorp, 2003; Hiyama and Hiyama, 2007). However, when these stem cells such as committed hematopoietic progenitor cells, activated lymphocytes or keratinocytes are induced for rapid expansion, the level of telomerase is up-regulated and thus shortened telomeres can be at least partially elongated (Haik *et al.*, 2000). Based on the stem cell theory of aging, the progressive loss of the telomeres during a typical lifespan could not be compensated for at later decades of life. For this reason, regenerative capacity of the tissues decreases due to increased cell senescence and death of the stem cells (Flores and Blasco, 2010).

In addition to the DNA replication machinery insufficiency, several factors such as certain hormones, growth factors, cytokines, reactive oxygen species, aging, genotoxic insults, genetic predisposition and mitochondrial dysfunction may also enhance telomere attritions in dividing and/or non-dividing cells under different *in vitro* or *in vivo* conditions (Blackburn, 2005; Passos and von Zglinicki, 2005; Keefe *et al.*, 2007). Among these factors, telomeres are more sensitive to the reactive oxygen species generated through normal cellular metabolism and

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exogenous genotoxic insults. Because of high guanine content of the telomeres, reactive oxygen species easily oxidize the telomeric DNA with lipid peroxidation. These modified telomeric repeats can be excised by the DDR system. Consequently, telomeres deplete as a result of exposure to the reactive species in both dividing and non-dividing cells (Liu *et al.*, 2002a, b; Keefe *et al.*, 2007). When the length of telomeres reaches the critical point in a mammalian cell, it can be triggered to p53-dependent cell cycle arrest or apoptosis to prevent unfavorable events. If this control mechanism is not appropriately served, various cancer types, infertility and other defects in the cellular physiology may occur (Bodnar *et al.*, 1998; Hemann *et al.*, 2001; de Lange, 2009).

Telomerase activity and telomere length in oocytes and granulosa cells are different

In mice, the female primordial germ cells (PGCs) that originate from the extra-embryonic yolk sac endoderm migrate to the genital ridge throughout early development. When they arrive at the genital ridge at 10.5 dpc, they undergo a few mitotic divisions and are then classified as oogonia (Pepling, 2006; Brook *et al.*, 2009). Next, oogonia undergo numerous mitotic divisions and develop in clusters of germline cysts or nests (more than 8 oocytes per cluster), and begin to enter meiosis. They are now called oocytes (Borum, 1967; Choi and Rajkovic, 2006). After birth, the germ cell cysts break into individual oocytes enveloped by a single layer of flattened granulosa cells, which are called primordial follicles. In the primordial follicles, each oocyte arrests at the diplotene phase of first meiotic prophase at 17.5 dpc (Pepling, 2006; Brook *et al.*, 2009). Only ~1% of the primordial follicles transform into pre-ovulatory (antral) follicles during reproductive life because most of them undergo atresia via an apoptotic pathway (Sato *et al.*, 1990; Johnson, 2003; Hartshorne *et al.*, 2009).

Folliculogenesis describes the development of multiple primordial follicles into a few antral follicles and is estimated to take ~175 days in humans (Gougeon, 1986). In mice, a subset of primordial follicles is periodically induced to develop into primary follicles that contain oocytes >20 μm, surrounded by cuboidal granulosa cells (Choi and Rajkovic, 2006). Next, the number of granulosa cell layers is increased so that primary follicles become secondary follicles where cytoplasmic and nuclear maturation of the oocytes gradually occur. Ultimately, secondary follicles grow into pre-antral follicles that have small antral spaces. Subsequently, pre-antral follicles transform into antral follicles (also known as Graaf or pre-ovulatory follicle). Each one possesses a large antral space (antrum). In the antral follicles, oocytes (~70 μm in mice) is enveloped by highly differentiated granulosa cells composed of basal, middle and antral layers of the membrana granulosa, and cumulus oophorus (Choi and Rajkovic, 2006) (Fig. 2). Follicular development is largely regulated by the action of different molecules including anti-Mullerian hormone (AMH), AMH receptor-2, activin, follicle-stimulating hormone, Figla, Nobox, Foxl2, Gdf9, FSH receptor, LH (Choi and Rajkovic, 2006). Granulosa cells, of course, play essential functions during oocyte

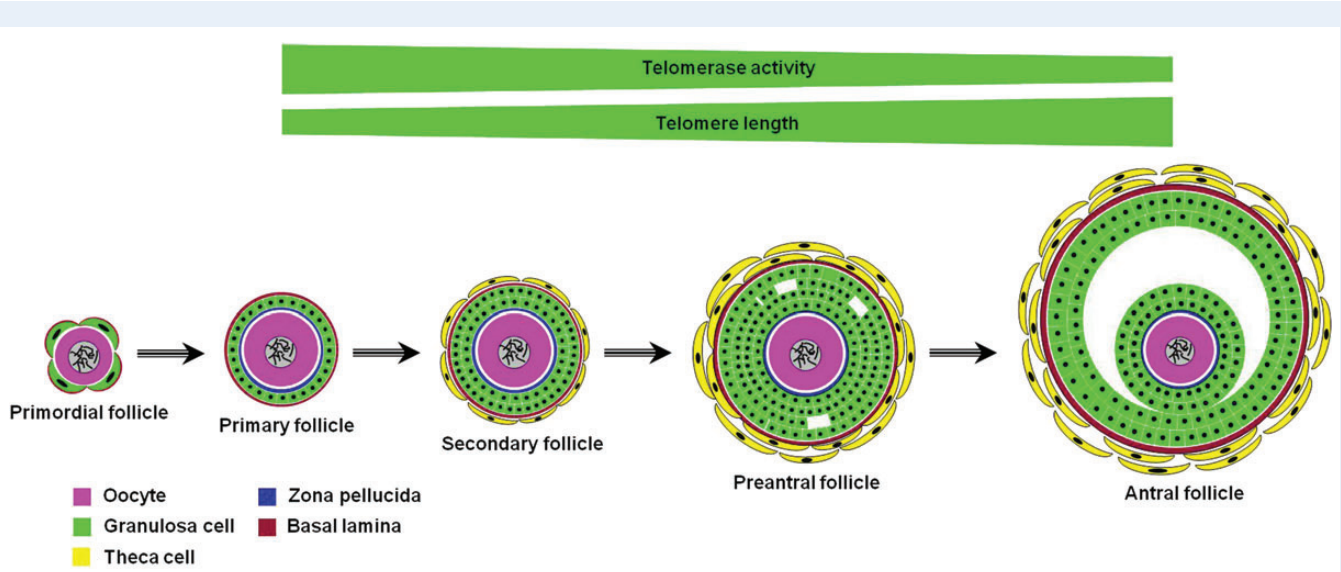


Figure 2 Schematic representation of follicular development from primordial to antral follicle and levels of the telomerase activity and telomere length in the granulosa cells during folliculogenesis. Note that this figure is largely based on the studies performed in the pig and bovine granulosa cells. After sexual maturation, relatively small numbers of primordial follicles are induced to form primary, secondary, pre-antral and antral follicles. Intercellular spaces between granulosa cells begin to appear in the pre-antral follicles. The large antral cavity filled with fluid (shown in white color) can be observed in the antral follicles. Throughout follicular development granulosa cells undergo numerous mitotic divisions that result in telomeric shortening due to the inefficiency of the DNA polymerase to complete the chromosomal ends. These shortened telomeres can be replenished through the enzyme telomerase which has been identified in the granulosa cells. Limited studies performed on different mammalian species have revealed that telomerase activity gradually decreases during follicular development and reaches the lowest level in the large antral follicles (4–5 mm in diameter). Conversely, the length of telomeres increases consistent with follicular growth. Note that both the length of telomeres and telomerase activity in the large antral follicles was found to exhibit differences in the *basal, middle and antral layers of the membrana granulosa, and in the cumulus oophorus cells.*

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development, and following ovulation they differentiate into luteinized cells to synthesize estrogen and progesterone hormones required for proper early embryo development. The proliferative activity of the granulosa cells is at high levels during follicular growth and decreases gradually while follicular development approaches to the antral follicular stage (review in Chronowska, 2012).

As granulosa cells undergo successive mitotic divisions during follicular growth, their telomere lengths are expected to decrease at the end of each DNA replication. The granulosa cells are essential for normal estrus cycle and oocyte development; therefore, those shortened telomeres could be extended with telomerase enzyme or any other mechanism. Decreasing the telomerase activity in the granulosa cells leads to increased rate of apoptosis and number of the atretic follicles (Lavranos et al., 1999; Yamagata et al., 2002). The levels of telomerase activity and localization of its components during follicular development was reportedly demonstrated in rats (Yamagata et al., 2002), pigs (Russo et al., 2006), bovines (Lavranos et al., 1999) and humans (Kinugawa et al., 2000) ovaries.

Telomerase activity in the granulosa cells of immature rats experimentally induced with 15 IU equine CG was characterized by TRAP assay (Yamagata et al., 2002). Although the main purpose of this study was to determine the relationship between follicular atresia and telomerase activity, it also found that small and healthy follicles had higher telomerase activity than large and atretic follicles (Yamagata et al., 2002).

The telomerase activity and length of telomeres have also been studied in the pig ovary (Russo et al., 2006). TERT, one of the most important indicators of the telomerase activity, tends to be localized in the nuclear parts of both the granulosa cells and oocytes of the primary and pre-antral follicles. TERT expression interestingly remained only in the mural granulosa cells of the antral layer and cumulus cells in the medium/large antral follicles (4–5 mm in diameter). It was absent in most of the granulosa cells closed to the basal membrane. Oocytes in the antral follicles had cytoplasmic TERT expression; however, oocytes in primary and pre-antral follicles had TERT localized in the nuclei. In the MII oocytes, although TERT intensively distributed in the subcortical cytoplasm, polar bodies did not show any TERT staining (Russo et al., 2006). When the length of telomeres in the pig granulosa cells was analyzed, the average telomere length per nucleus independent of the follicular stage was found to be 30.13 $\pm$ 5.2 kb. Furthermore, pre-antral and antral follicles obtained from pig ovaries possessed greater telomere length than early follicular stages (Fig. 2). Consistent with the presence of TERT expression in the granulosa cells of the antral and cumulus oophorus layers of the antral follicles, they had higher telomere length than those of the basal one. Oocytes enclosed in the pre-antral follicles exhibited significantly smaller telomere length than those of oocytes found in the primary, small antral ($<$4 mm in diameter) and medium/large (4–5 mm in diameter) antral follicles. When telomere length findings of the oocytes and granulosa cells in the different follicles were taken together, telomere lengths was found to increase from pre-antral to antral follicles, and in the antral follicles, cumulus and antral granulosa cell layers demonstrated higher telomere length than those of the basal layer. These results were found to coincide with the presence of immunopositivity for TERT staining, observed during follicular development in the pig ovary (Russo et al., 2006).

Additionally, Tomanek et al. (2008) displayed telomerase activity in the freshly harvested and *in vitro* cultured granulosa cells from the small antral (1–2 mm in diameter) and large antral (5–7 mm in diameter) follicles of the pig. In this work, the effect of FSH and EGF on stimulation of the telomerase activity in the *in vitro* cultured granulosa was also assayed (Tomanek et al., 2008).

Similar to the studies performed on pig ovarian follicles, telomerase activity was also analyzed in the bovine ovarian follicles including pre-antral follicles (60–100 μm in diameter), small antral follicles (1 mm in diameter) and granulosa cells from medium-sized (3 mm in diameter) and -large antral follicles (6–8 mm in diameter). The levels of telomerase activity were found to decline gradually from pre-antral to large antral follicles (Lavranos et al., 1999) (Fig. 2). Additionally, *in situ* hybridization localized telomerase RNA (TERC subunit) in the granulosa cells of all growing follicles, but not in the primordial follicles. As is well known, each primordial follicle consists of an oocyte enclosed by non-dividing granulosa cells. Therefore, primordial follicles may not necessitate the TERC expression. Similar to the TERT localization in the pig ovary, there were different TERC staining patterns in the membrane granulosa layers of the antral follicles. Specifically, granulosa cells of the middle and antral layers exhibited higher TERC staining than basal layer. Notably, there was no TERC staining in the oocytes of bovine ovarian follicles (Lavranos et al., 1999).

On the other hand, Goto et al. (2011) investigated the effect of aging on the telomerase activity and telomere length in the medium-sized follicles. For this reason, granulosa cells were harvested from the medium-sized follicles on the surface of dominant phase ovaries of young (28.1 months) and from old cows (averaging 151.3 months). Although there were higher telomerase activity and telomere length in the granulosa cells from young cows than from old one, those differences were not found to be significant (Goto et al., 2011).

Studies on telomere length and telomerase activity in the human granulosa cells and cumulus cells obtained from different follicular stages and at the time of oocyte collection are limited. Analyzing the granulosa and cumulus cells is proposed as a predictive value of competent oocytes and good-quality embryos. The importance of cumulus cells in the processes of maturation, ovulation and fertilization of oocytes has been comprehensively evaluated (Eppig, 1982; Tanghe et al., 2002). The relative telomere length in cumulus cells collected from mature human oocytes was found to be greater than cumulus cells from immature oocytes. Similarly, cumulus cells from good-quality embryos had longer telomere length compared with those of cumulus cells from poor-quality embryos. This finding suggests that characterizing the relative telomere length of the cumulus cells obtained at the time of oocyte retrieval may be a potential biomarker of oocyte and embryo quality (Cheng et al., 2013). Additionally, aberrant telomere homeostasis in the granulosa cells may be used to assay ovarian sufficiency. Short telomere length and lack of telomerase activity in women's granulosa cells were observed to be linked with occult ovarian insufficiency (Butts et al., 2009).

In addition to many mitotic divisions in the granulosa cells, their telomerase activity and telomere length are also affected by aging and estrogen level (Bayne et al., 2011; Goto et al., 2011). As is well known, estrogen plays crucial roles in the regulation of telomerase activity in the granulosa cells. High concentration of estradiol-17β (E2) (10 μg/ml) remarkably increased the telomere length of the *in vitro* cultured granulosa cells derived from bovine early antral follicles when compared with the low concentration of estradiol-17β (E2) (0.1 μg/ml) (Endo et al., 2012). Estrogen withdrawals consistently resulted in reduced telomerase activity, which may lead to telomere shortening in the granulosa cells, and subsequently follicular atresia occurs (Yamagata et al., 2002;

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Liu and Li, 2010). To clearly demonstrate the effect of estrogen on telomere length and telomerase expression, the aromatase gene was disrupted to generate estrogen deficient mice models (Bayne *et al.*, 2011). Telomeric lengths in these mice were significantly eroded in the follicular granulosa cells. Additionally, impairments of the proliferation and differentiation events in these cells appeared, and TERT and TERC levels were found to be decreased in this kind of follicular granulosa cells. As expected, estrogen replacement therapy increased the TERT expression and telomerase activity in the granulosa cells and provided telomere lengthening in 4 weeks (Bayne *et al.*, 2011). Aging is another factor that adversely influences telomerase activity and subsequently the length of telomeres in human and bovine ovaries, possibly due to long-term exposure to reactive oxygen species, impairment in cellular physiology and gradual decreasing numbers of follicles (Kinugawa *et al.*, 2000; Goto *et al.*, 2011).

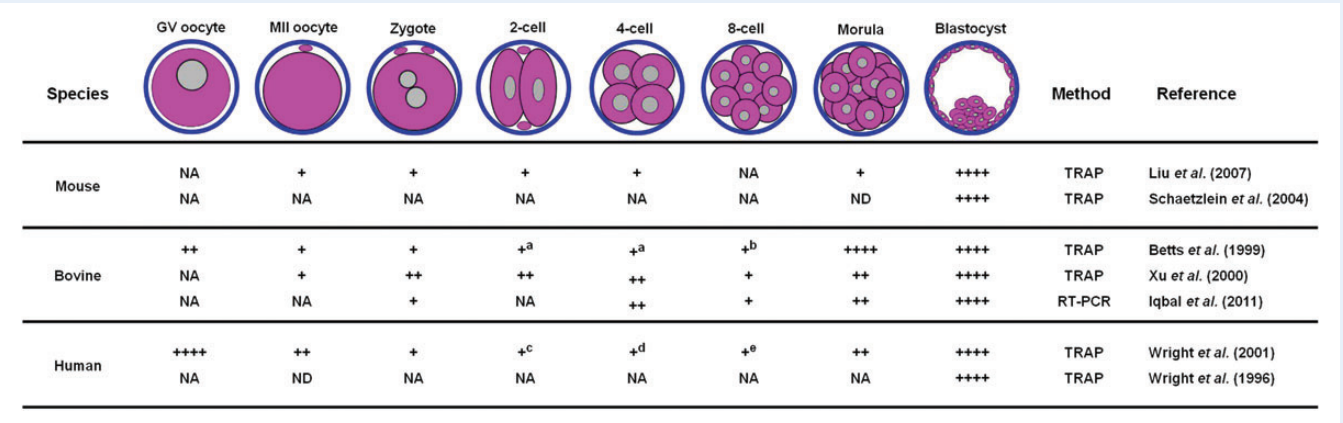
Telomere length and telomerase activity show differences during oocyte and early embryo development

LH surge stimulates resumption of meiosis I and results in ovulation of fertilizable MII oocyte from the antral follicle (Hillier *et al.*, 2010). When the ovulated MII oocyte (secondary oocyte) is fertilized by a competent spermatozoon, it completes meiosis II and then becomes a zygote. The zygote undergoes a series of mitotic divisions (also known as segmentation) to generate 2-cell, 4-cell, 8-cell, morula and blastocyst, collectively known as early embryo development. The blastocyst consists of two main cell types: inner cell mass (ICM) localized on one side of the blastocoel cavity and trophectoderm (TE) found on outer surface of the embryo. While the ICM constitutes embryonic structures, TE gives rise to extra-embryonic membranes such as placenta, yolk sac and amniotic membrane (reviewed in Shi and Wu, 2009).

The length of telomeres and telomerase activity in oocytes and early embryos at distinct developmental stages in mice (Liu *et al.*, 2007), rats (Eisenhauer *et al.*, 1997), bovines (Betts and King, 1999) and humans (Wright *et al.*, 1996) have been analyzed.

In mice, telomerase activity was found to be at low levels in the mature oocytes and early cleavage-stage embryos (zygote, 2-cell and 4-cell embryos), but remarkably enhanced in the blastocyst-stage embryos (Liu *et al.*, 2007) (Fig. 3). Telomere lengths of the mature oocytes, zygote and 2-cell embryos from outbred CD1 mice were measured to be 16.09 ± 0.78, 23.34 ± 1.09 and 26.73 ± 3.07 kb, respectively (Fig. 4). The hybrid B6C3F1 mice had a higher telomere length in the matured oocytes (18.03 ± 0.47 kb) and 2-cell embryos (29.43 ± 1.03 kb) when compared with the CD1 mice. In contrast to the presence of low telomerase activity in the early embryos, telomeres were progressively lengthened in the cleavage-stage embryos (Fig. 4). The numerous telomere sister-chromatid exchanges (T-SCE) throughout early embryo development suggested that a recombination-based mechanism largely contributed to the telomere lengthening during this period of development. The telomere length was found to be 37.40 ± 1.04 kb at the blastocyst-stage embryos and significantly increases when compared with early embryos (Liu *et al.*, 2007). Further, Varela *et al.* (2011) observed that an average telomere length in the mouse blastocyst (32.34 ± 5.77) was significantly longer than morula (24.98 ± 2.72) stage embryos. Additionally, blastocysts had higher telomere length than those of embryos at the ages of E7.5, E10.5 and E13.5 (Varela *et al.*, 2011).

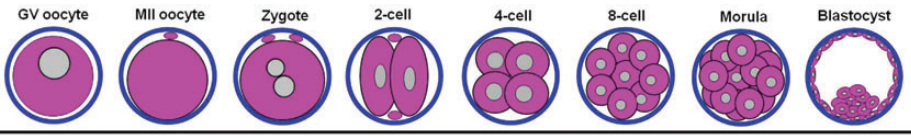
When two cell types, TE and ICM, that had been isolated from mouse blastocysts were analyzed for telomere length, the mean telomere length of the ICM was remarkably higher than TE. Conversely, TE had significantly greater telomeres than ICM in bovine blastocyst (Iqbal *et al.*, 2011). The exact reason of the difference in the length of telomeres of TE and ICM obtained from mouse and bovine blastocysts remains elusive. However, it might relate to the different types of placentation and embryo-specific telomere elongation in bovines when compared with mice.



Species	GV oocyte	MII oocyte	Zygote	2-cell	4-cell	8-cell	Morula	Blastocyst	Method	Reference
Mouse	NA	+	+	+	+	NA	+	++++	TRAP	Liu *et al.* (2007)
	NA	NA	NA	NA	NA	NA	ND	++++	TRAP	Schaetzlein *et al.* (2004)
Bovine	++	+	+	+[a]	+[a]	+[b]	++++	++++	TRAP	Betts *et al.* (1999)
	NA	+	++	++	++	+	++	++++	TRAP	Xu *et al.* (2000)
	NA	NA	+	NA	++	+	++	++++	RT-PCR	Iqbal *et al.* (2011)
Human	++++	++	+	+[c]	+[d]	+[e]	++	++++	TRAP	Wright *et al.* (2001)
	NA	ND	NA	NA	NA	NA	NA	++++	TRAP	Wright *et al.* (1996)

Figure 3 The levels of telomerase activity in oocytes and early embryos obtained from mice, bovines and humans are given. Although there are differences in the telomerase activity in early preimplantation embryos, all species have high telomerase activity in the blastocysts and low activity in the mature oocytes. Note that + and ++++ are used to exhibit the highest and the lowest telomerase activity in oocytes or early embryos. The alphabetic symbols indicate the stages of early embryos characterized in that study. The a and b demonstrate 2–5-cell and 6–8-cell embryos, respectively; the c, d and e show 2–3-cell, 4–5-cell and 8–16-cell embryos, respectively. NA, not analyzed; ND, not detected; TRAP, telomeric repeat amplification protocol; RT–PCR, reverse transcription-polymerase chain reaction.

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Species	GV oocyte	MII oocyte	Zygote	2-cell	4-cell	8-cell	Morula	Blastocyst	Method	Reference
Mouse	NA	16.09 ± 0.78	23.34 ± 1.09	26.73 ± 3.07	NA	NA	NA	37.40 ± 1.04	Q-FISH	Liu *et al.* (2007)
	NA	NA	NA	NA	NA	NA	24.98± 2.72	32.34 ± 5.77	Q-FISH	Varela *et al.* (2011)
Bovine	NA	16.95 ± 2.51	NA	NA	NA	NA	14.36 ± 2.67	19.53 ± 4.6	Q-FISH	Schaetzlein *et al.* (2004)
Human	NA	7.5 ± 1.17	NA	NA	NA	NA	NA	NA	FISH	Keefe *et al.* (2007)
	11.12	NA	NA	8.43 ?	8.43 ?	8.43 ?	8.43 ?	11.27	Q-FISH	Turner *et al.* (2010)
	11.41± 0.81	8.79 ± 0.86	NA	NA	NA	NA	NA	NA	Q-FISH	Turner *et al.* (2013)

Figure 4 The length of telomeres (mean ± SEM or STD) in the oocytes and early embryos of the mice, bovines and humans are given. There are limited studies related to measuring exact telomere length of the oocytes and early embryos obtained *in vivo* or *in vitro* from certain mammalian species. The length of telomeres in the mature oocytes and early embryos is found to exhibit differences between species. NA, not analyzed? There is no information associated with exact stage of the early embryos used in that study. Q-FISH, quantitative fluorescent *in situ* hybridization; FISH, fluorescent *in situ* hybridization. Note that, some studies have not given the standard deviation or standard error of the mean values.

As a result, mouse oocytes and early embryos have different telomere lengths, and telomere lengthening is largely carried out by DNA recombination-based mechanism during early preimplantation embryo development. In the blastocyst-stage embryos, there was a decrease in the rate of T-SCE and DNA recombination protein levels (RAD50 and TRF1). Increased telomerase activity in the blastocysts most likely leads to remarkable telomere elongation and maintenance of the telomeres established in the cleavage-stage embryos (Blasco *et al.*, 1995; Wright *et al.*, 2001; Xu and Yang, 2001; Liu *et al.*, 2007) (Figs 3 and 4).

Although ALT mechanism seems to be an important process in telomere elongation of the cleavage-stage embryos, only a few studies have been undertaken to comprehensively investigate the efficiency of this mechanism during early embryo development (Reddel *et al.*, 1997; Liu *et al.*, 2007; de Frutos *et al.*, 2012). For this purpose, a telomerase-null mice model has been generated and the length of telomeres was measured in the oocytes and early cleavage-stage embryos obtained from telomerase-null or control mice. Increased telomere length independent of telomerase enzyme suggested that the elongation of the telomeres in the early cleavage embryos was mainly performed by the ALT mechanism (Liu *et al.*, 2007). Telomere length measurements of the 8-cell, morula and blastocyst-stage embryos harvested from $mTERC^{-/-}$ or $mTERC^{+/+}$ mouse models revealed that the blastocysts from $mTERC^{+/+}$ mice exhibited a significantly longer telomere length than 8-cell and morula-stage embryos, but $mTERC^{-/-}$ blastocysts did not. This showed that telomerase activity was one of the essential factors for the telomere elongation during morula-blastocyst transition in mice (Schaetzlein *et al.*, 2004; Liu *et al.*, 2007). Furthermore, the researchers also examined that whether telomere elongation occurred during early mouse embryogenesis of Days 8.5, 10.5 and 13.5. Interestingly, there was no telomere lengthening event during those post-implantation developmental days, although numerous mitotic divisions appeared. Consequently, telomere lengthening seems to be restricted to two different periods of early embryogenesis: during early cleavage-stage embryo development and during the morula–blastocyst transition (Schaetzlein *et al.*, 2004; Varela *et al.*, 2011).

In rats, telomerase activity has been observed in the oocytes from early antral and pre-ovulatory follicles as well as in ovulated oocytes. The levels of telomerase activity in oocytes from early antral and pre-antral follicles had considerably higher telomerase activity than in ovulated oocytes. As with the ovulated oocytes, there was very low telomerase activity in the early embryos. Furthermore, several tissues from 27-day-old rats exhibited high telomerase activity in the thymus, ovaries and testes, but low telomerase activity was measured in the liver and brain. The spleen possessed the lowest telomerase activity. These findings indicated that the levels of telomerase activity may largely correlate with the regenerative potential of tissues (Eisenhauer *et al.*, 1997).

In bovines, Betts and King (1999) examined the relative telomerase activity in immature oocytes, mature oocytes, zygotes, 2- to 5-cell embryos, 6- to 8-cell embryos, morulae and blastocysts by using TRAP assay. They found that the telomerase activity decreased during oocyte maturation and further declined in subsequent development to the 8-cell stage, but intriguingly up-regulated significantly at the morula and blastocyst-stage embryos (Betts and King, 1999) (Fig. 4).

Since TERT mRNA expression has been demonstrated to associate closely with telomerase activity, the endogenous levels of TERT mRNA of the bovine preimplantation embryos (zygote, 4-cell, 8-cell, morula and blastocyst) obtained with an *in vitro* culture was characterized with RT–PCR. As expected, the blastocysts had the highest TERT mRNA level. Zygote and 8-cell embryos exhibited TERT transcript levels that were quite low, although a small increase in the 4-cell and morula-stage embryos was observed (Iqbal *et al.*, 2011). Reduced telomerase expression during early embryonic development in bovines is similar to many genes, which are maternally stored in the oocytes for use after transcriptional silence ended at the 8- to 16-cell stages in cows. Additionally, the presence of TTAGGG telomeric DNA repeats on the chromosome ends of bovine embryos was confirmed by using FISH technique (Telford *et al.*, 1990; Betts and King, 1999).

Telomerase activity in *in vitro* fertilized bovine early embryos from zygote to blastocysts has been revealed by using the TRAP method

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(Xu and Yang, 2000). Relatively low telomerase activity in the mature oocytes significantly increased at the zygote stage, but its expression slightly decreased through the 8-cell stage. Next, telomerase activity began to gradually enhance after the 8-cell stage and reached the highest level at the blastocysts stage, which was found statistically significant. However, there was no correlation between the total protein and relative telomerase activity among early embryos, suggesting that higher telomerase activity may originate from increased enzymatic activity of telomerase (Xu and Yang, 2000). Two studies (Betts and King, 1999; Xu and Yang, 2000) have attempted to detect telomerase activity in bovine oocytes and early embryos collected *in vivo* or *in vitro*. They found almost identical results, including increased telomerase activity at the blastocyst stages and low telomerase activity in the mature oocytes and early cleavage-stage embryos. There was, however, a remarkable difference in telomerase activity in the morula-stage embryos, which resembled to the *in vivo*-obtained blastocysts but was considerably lower than *in vitro*-produced blastocysts (Betts and King, 1999; Xu and Yang, 2000). Differences in the morula-stage embryos suggest that *in vitro* conditions may slightly influence telomerase activity in the early embryos.

Telomerase activity in oocytes and early embryos from zygotes to blastocysts derived from parthenogenetic activation or nuclear transfer has also been examined in bovines (Xu and Yang, 2001). Among the oocytes and early embryos obtained from parthenogenetic activation, blastocysts had the highest relative telomerase activity when compared with the oocyte, zygote and cleave- and morula-stage embryos. Similarly, blastocysts derived from IVF or nuclear transfer systems possessed significantly higher relative telomerase activity than zygote and cleavage-stage embryos (Xu and Yang, 2001). Moreover, the telomerase activity of three groups (IVF-, parthenogenic- and nuclear transfer-derived zygote and blastocyst-stage embryos) was also compared in the same study. They found no significant alteration among the groups, but zygotes and blastocysts obtained from parthenogenetic activation showed slightly higher relative telomerase activity than zygotes and blastocysts collected from the other two groups (Xu and Yang, 2001).

The length of telomeres in bovine oocytes and early embryos derived from IVF or *in vivo* fertilization has been analyzed by using Q-FSH in order to show the effect of *in vitro* conditions on the length of telomeres (Schaetzlein *et al.*, 2004). An average telomere length was found to be 16.95 $\pm$ 2.51 kb in *in vitro* maturated oocytes, 14.36 $\pm$ 2.67 kb in the morulae and 19.53 $\pm$ 4.6 kb in the blastocysts obtained from IVF (Fig. 3). However, the mean telomere length of the morulae obtained from *in vivo* fertilization was 12.42 $\pm$ 1.37 kb, that is, even lower than *in vitro*-derived morulae (Schaetzlein *et al.*, 2004). These findings suggest that telomeres were elongated at the blastocyst-stage embryos, and this was most likely carried out with telomerase activity as revealed by Xu and Yang (2000) and Betts and King (1999). However, telomere elongation during early preimplantation embryo development in bovines is carried out by the ALT mechanism, which seems to be affected by *in vitro* culture conditions.

Strikingly, recent studies reported that the length of telomeres can vary based on the gender of the early embryos. For example, female bovine blastocysts had significantly longer average telomere lengths than male blastocysts, this most likely resulting from the differences in the telomerase-dependent genetic program between genders. Furthermore, the expression of *Dnmt3a* and *Dnmt3b* mRNAs was found to be higher in male blastocysts than those in female blastocysts (Bermejo-Alvarez *et al.*, 2008). This is consistent with the study displayed that these methyltransferases influence telomere length negatively (Gonzalo *et al.*, 2006). Similarly, the telomere length on Xqs was revealed to be ~1100 bp shorter in human male newborns than in early female newborns (Perner *et al.*, 2003).

Investigations of telomere length and telomerase activity in human oocytes and preimplantation embryos are carried out with some notable limitations. Only small quantities of DNA can be extracted, for example, and normal human oocytes and preimplantation embryos are quite difficult to obtain due to ethical restrictions. Consequently, telomerase activity has been analyzed only in the discarded or donated human oocytes and early embryos left over from IVF treatment by using the TRAP method (Wright *et al.*, 2001). Telomerase activity was found to be present in all early developmental stages including immature (GV) and mature (MII) oocytes, zygote, 2–3-cell, 4–5-cell, 6–7-cell, 8–16-cell, morula and blastocyst-stage embryos. It is important to note that GV oocytes and blastocyst-stage embryos had significantly higher telomerase activity than others. Interestingly, researchers have not found telomerase activity in the unfertilized mature oocytes, although low telomerase activity has been observed in different mammalian species (Wright *et al.*, 2001). As only three unfertilized human adult oocytes were used to detect the telomerase activity in this study, the amount of extracted DNA obtained from these oocytes may not have been sufficient to characterize the level of telomerase activity by TRAP assay.

In contrast to the absence of telomerase activity in human MII oocytes (Wright *et al.*, 2001), Brenner *et al.* (1999) discovered the expression of the TERT mRNA in human oocytes at the stages of GV (germinal vesicle), MI (metaphase I) and MII (metaphase II) in addition to cleavage-stage embryos (day-3 embryos) or blastocysts by using reverse transcription-polymerase chain reaction (RT–PCR). Moreover, three different TERT subunit isoforms (457-, 421- and 275 bp) were found in all the oocytes and early embryos that were examined (Brenner *et al.*, 1999).

Average telomere lengths were higher in the GV oocytes (11.12 kb) and blastocysts (12.22 kb) than cleavage-stage embryos (8.43 kb) (Turner *et al.*, 2010), which is consistent with having higher telomerase activity in human GV oocytes and blastocysts. In the recently published study by the same group (Turner and Hartshorne, 2013), the lengths of telomeres in individual human oocytes, spermatozoa, and male and female pronuclei have been measured by using Q-FISH to compare their parental contribution to telomere lengths in the human zygote. Findings showed that human mature oocytes (8.79 $\pm$ 0.86) and female pronuclei (8.63 kb) had remarkably higher telomere length than those in individual spermatozoa (6.32 $\pm$ 2.00 kb) and male pronuclei (6.16 kb). Additionally, telomeres in immature oocytes (11.41 $\pm$ 0.81) were significantly longer than in mature oocytes (8.79 $\pm$ 0.86) (Turner and Hartshorne, 2013). Similarly, the mean telomere length of the human mature oocytes aspirated for IVF procedure was found to be 7.5 $\pm$ 1.17 kb (Keefe *et al.*, 2007). Higher telomerase activity in the immature GV oocytes than in the mature oocytes (Wright *et al.*, 1996, 2001) may be related to the existence of longer telomeres in the immature oocytes (Figs 3 and 4).

As women delay child bearing, the quality of oocytes obtained from the aging women inevitably decreases. These oocytes predispose to many chromosome abnormalities involving meiotic non-disjunction, decreased chiasmata and recombination rates, and structural impairments in the meiotic spindle-chromosome association and mitochondrial DNA mutations. Although the mechanisms underlying such

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defects in the older women have not completely been revealed, telomere erosion is proposed as a potential risk factor for these unfavorable defects (reviewed in Keefe et al., 2007). The telomeres in the oocytes from women who have failed IVF cycles and in the fragmented or aneuploid embryos were consistently found in short lengths (Keefe et al., 2005; Treff et al., 2011; Kalmbach et al., 2013).

The possible reason of the telomere shortening in the oocytes from older women may be that oocytes ovulated from these women undergo more DNA replication during fetal oogenesis than the oocytes ovulated from younger women. The number of DNA replications, chronic effects of reactive oxygen species and genotoxic agents on the oocytes destined to ovulate late in life create higher potential risk for shortening of the telomere lengths within oocytes (reviewed in Keefe and Liu, 2009; Kalmbach et al., 2013). A number of chromosomal defects may also appear in the resulting early embryos derived from oocytes that have short telomeres (Keefe et al., 2006, 2007). In contrast to oocytes, telomere length in human individual spermatozoa increases with advancing paternal age possibly due to enhancing telomerase activity in the spermatogonial stem cells (Baird et al., 2006; Kimura et al., 2008). This may contribute to telomere lengthening in the offspring and may also provide compensation for the shortened telomeres in the oocytes from older women (Sartorius and Nieschlag, 2010; Eisenberg et al., 2012; Turner and Hartshorne, 2013). On the other hand, Jorgensen et al. (2013) found no difference in the telomere length and telomerase activity during spermatogenesis between older and younger men (Jorgensen et al., 2013).

Telomerase deficiency adversely affects the female reproductive system

Telomerase (TR) gene knocked out mice models initially exhibited normal fertilization and embryonic development, but at the later generations serious defects including infertility or subfertility, ensued after telomere length reached the critical length (Blasco et al., 1999). Telomerase null oocytes could be fertilized with telomerase null sperm or wild-type sperm with *in vivo* or IVF systems. However, most of the $TR^{-/-}$ oocytes (>50%) showed increased cytofragmentations, impaired fertilization, abnormal cleavages during preimplantation embryo development and decreased blastocyst formation rate. It is important to note that these abnormalities seemed to be derived from telomeric dysfunction since the defects appeared in the late generations (Liu et al., 2002a, b).

Other adverse effects observed in the reproductive system of the telomerase-deficient female mice were a developmental delay in the oocytes and early embryos, an increase of chromosomal abnormities and an impairment of the meiotic spindle formation in the oocytes from fourth generation. Compromised ovulation, a reduction in the ovarian size and atrophic uterus structures were also observed in these mice (Blasco et al., 1995; Herrera et al., 2000; Liu et al., 2002a, b). Most of these unfavorable effects presumably resulted from the fusion of the chromosomes, various translocations and loss of certain chromosomal sites due to progressive loss of the telomeric DNA (Lee et al., 1998; Hande et al., 1999; Herrera et al., 1999). Replacement of telomerase by crossing $Terc^{-/-}$ mice with $Terc^{+/-}$ mice provided lengthening of the shortened telomeres, and thus it reversed the chromosomal abnormalities mentioned above and prevented the premature aging of the progenies (Samper et al., 2001).

Conclusions

Telomeres localize at the ends of all linear chromosomes in the eukaryotic cells. Maintenance and lengthening of their lengths during folliculogenesis, oocyte maturation and early embryo development are very important for successful pregnancy outcomes in the mammalian species including the mouse, rat, pig, bovine and human. Basically, two different mechanisms, defined as the enzyme telomerase- and ALT-dependent, have been reported to act on telomere lengthening in oocytes and early embryos. Notably, these mechanisms also function at different times in early embryogenesis.

Telomere length and telomerase activity analyzed by different methods reveal that the levels of telomerase activity and telomere lengths in these mammalian species demonstrate differences in distinct developmental stages of the oocytes and early embryos. Possible causes of these differences among mammalian species may be derived from (i) the difference in volumes of the oocytes, (ii) distinct times of initiation of the embryonic genome activation, (iii) the use of distinct methods to detect the telomerase expression/activity and the length of telomeres in the germ cells and early embryos and (iv) the presence of evolutionary distance between species.

An analysis of the literature shows that there is limited data on the telomerase activity and length of telomeres during folliculogenesis, oogenesis and early embryo development in the mammalian species. Further studies are required to discover the exact levels and control mechanisms of telomerase activity and length of telomeres in the granulosa cells, oocytes and preimplantation embryos obtained from different mammalian species. The widely varying techniques currently being used in this field should be further evaluated in order to choose the technique best suited to determine the correct telomerase expression/activity and telomere length. Finally, the possible use of telomere length and telomerase activity measurements as fertility diagnostic tools for the oocytes and early embryos should be comprehensively investigated.

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Authors' roles

S.O. wrote the paper and prepared all the figures. N.D. designed and critically read the article. B.S. contributed to writing of the article and created Table I.

Conflict of interest

The authors declare that there is no conflict of interest.

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