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) 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; Wellingher 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.,
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 nuclease protein 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. Telomere-associated proteins are also involved in telomere dysfunction and length of the telomeres (de Lange, 2005; Hug and Lingner, 2006).

Telomeric repeat-binding factor 1 (TRF1 or TRF1) and telomeric repeat-binding factor 2 (TRF2 or TERT) are telomere-associated proteins that have been intensively studied. TRF1 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 due to dominant-negative expression of the TRF1 gene. In addition to telomere lengthening, the TRF1 protein is also thought to be involved in telomere maintenance (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 expression 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 helicase) 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 TIN2-interacting 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 a role in the DNA replication of the lagging strand (Crawb 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).
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 mammals. 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 (Aтив 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...
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, expression 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 Dnmt1, or both Dnmt3a and Dnmt3b 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 1. Note that a recent review article by Aubert et al. (2012) has comprehensively compared these methods (Aubert et al., 2012).

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Disadvantage</th>
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<tr>
<td>TRF</td>
<td>Detects telomere length in each sample</td>
<td>Requires large amounts of DNA (0.5–5 μg)</td>
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<td></td>
<td>Gives average telomere length</td>
<td>Needs 3–5 days</td>
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<td></td>
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<td>Causes 5% variability between individuals</td>
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<td>qRT–PCR</td>
<td>Is simple, practical and reproducible</td>
<td>Is fluorescence-based application</td>
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<td></td>
<td>Requires small amount of genomic DNA (20 pg)</td>
<td>Gives only average telomere length</td>
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<td></td>
<td>Analyses multiple samples simultaneously</td>
<td>have variability of 5.8%</td>
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<td>STELA</td>
<td>Measures single telomere</td>
<td>Requires at least a few chromosomes</td>
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<td></td>
<td>Detects short telomeres</td>
<td>Is labor intensive</td>
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<td></td>
<td>Provides high resolution and is highly sensitive</td>
<td>Needs unique primer for each chromosome arm</td>
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<td></td>
<td>Needs limited starting material</td>
<td>Cannot analyze very long telomeres</td>
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<td>Q-FISH</td>
<td>Requires low number of cells</td>
<td>Needs chromosome spreads</td>
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<td></td>
<td>Measures telomere of individual chromosomes/cells</td>
<td>Is fluorescence based</td>
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<td></td>
<td>Gives strong signal</td>
<td>Needs digital image microscopy</td>
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<td></td>
<td>Is reliable and quantifiably</td>
<td>Have high variability (11.2%)</td>
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<td>TRAP</td>
<td>Gives relative telomerase activity</td>
<td>Is time-consuming procedure</td>
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<td>Is highly sensitive and provides fast detection</td>
<td>Detects only qualitative telomerase activity</td>
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<td>TMA</td>
<td>Gives quantitative telomerase activity</td>
<td>Cannot give any information related to telomere length</td>
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<td></td>
<td>Not based on PCR amplification</td>
<td>Needs isothermal amplification system</td>
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<td>requires exclusive equipments</td>
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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.
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, Mbol, 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 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 (cya nine 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 program 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 FlowFISH 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; Bae rlocher et al., 2006).
activity. For this purpose, real-time PCR or TERT mRNA is another alternative method for measuring telomerase found to correlate closely with the telomerase activity; analysis of the nuclear environment that imitates the efficiency of the telomerase inhibitor (Fletcher et al., 1998). This system is also convenient to evaluate 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 (Durusoym 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 attrition 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...
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](image-url)
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 Chronowonska, 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 (Lavrano
nos 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 (Lavrano
nos 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 activity than large and atretic follicles (Yamagata et al., 1999). 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 (Lavrano
nos et al., 1999) and humans (Kinugawa et al., 2000) ovaries.

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 MI 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 ± 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, Tomane
k 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 (Tomane
k 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; Yamagata et al., 2003).

Ozturk et al.
Telomere length and telomerase activity in oocytes and early embryos (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 levels were found to be decreased in this kind of 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. 

**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 shortening 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.

**Table 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.
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 mouse 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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**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.
Telomere length and telomerase activity in oocytes and early embryos

(Keefe et al., 2005). 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 blastocyst 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-, parthenogenetic- 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 vivo conditions on the length of telomeres (Schaetzlein et al., 2004). An average telomere length was found to be 16.95 ± 2.51 kb in in vivo matured oocytes, 14.36 ± 2.67 kb in the morulae and 19.53 ± 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 ± 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 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 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), MII (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 ± 0.86) and female pronuclei (8.63 kb) had remarkably higher telomere length than those in individual spermatozoa (6.32 ± 2.00 kb) and male pronuclei (6.16 kb). Additionally, telomeres in immature oocytes (11.41 ± 0.81) were significantly longer than in mature oocytes (8.79 ± 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 ± 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
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 replication, 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 spermatzoa 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<sup>−/−</sup> 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 abnormalities 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 Ter<sup>−/−</sup> mice with Ter<sup>+/−</sup> 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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