Telomerase in the ovary

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Abstract

Telomerase, an enzyme complex that binds the chromosome ends (telomeres) and maintains telomere length and integrity, is present in germ cells, proliferative granulosa cells, germline stem cells, and neoplastic cells in the ovary, but it is absent in differentiated or aged cells. Activation of telomerase in the ovary underpins both benign and malignant cell proliferation in several compartments, including the germ cells, membrana granulosa, and the ovarian surface epithelium. The difference in telomerase operation between normal and abnormal cell proliferations may lie in the mechanisms of telomerase activation in a deregulated manner. Recent studies have implicated telomerase activity in ovarian cancer as well as oogenesis and fertility. Inhibition of telomerase and the shortening of telomeres are seen in occult ovarian insufficiency. Studies of how telomerase operates and regulates ovary development may provide insight into the development of both germ cells for ovarian reproductive function and neoplastic cells in ovarian cancer. The current review summarizes the roles of telomerase in the development of oocytes and proliferation of granulosa cells during folliculogenesis and in the process of tumorigenesis. It also describes the regulation of telomerase by estrogen in the ovary.


Introduction

Cell proliferation in the ovary is critical to the ovarian functions of producing and releasing germ cells (oocytes) and hormones for reproduction. During the development of oocytes, folliculogenesis is accompanied by significant proliferation of granulosa cells, which is tightly controlled in a temporal and spatial manner. As the overall reproductive lifespan in mammals is predetermined at birth by the number of eggs in each ovary, the ovarian aging manifestations of menopause occur when the finite supply of eggs is depleted. The timing when ovarian aging begins is critical and often reflected by the speed of primordial follicle depletion. The magnitude of the follicular reserve is determined by a discontinuous balance between follicular recruitment, sustainment, and exhaustion (McGee & Hsueh 2000, Adhikari & Liu 2009). In follicular recruitment and sustainment, ovarian cell proliferation plays a primary role, with granulosa cell proliferation, differentiation, and subsequent senescence (aging) and apoptosis (death) to underpin oogenesis and the production of estrogen and progesterone. Recent studies have shown the presence of the cells in adult ovaries with germline stem cell properties of proliferative potential in the postnatal mammalian ovary (Virant-Klun et al. 2009, Zou et al. 2009). Implantation of a cell line established from the germline stem cells of adult mouse ovary into the ovaries of infertile mice reinitiates oogenesis and fertility (Zou et al. 2009). The presence of germline stem cells in adult ovary may reflect suggesting the presence of a potentially renewal mechanism in support of the ovary reproduction, and release of eggs and hormones.

Several lines of evidence indicate that specific genetic mechanisms mediate production by the ovary of germ cells and the hormones needed to support oogenesis. The ovary is surrounded by a single layer of ovarian surface epithelium (OSE) that is a layer of cuboidal cells derived from mesoderm during embryonic development (Auersperg et al. 2001). As a source of ovarian stem cells in humans (Virant-Klun et al. 2009) and mice (Lee et al. 2008), OSE undergoes hyperplasia in response to estrogen treatment to potentially lead to tumorigenesis (Laviolette et al. 2010). Estrogen accelerates tumorigenesis initiated by the simian virus 40 (SV40) T antigens, resulting in an early onset and reduced overall survival time (Laviolette et al. 2010). In the follicular compartment, estrogen is released by and acts on folliculogenesis. In contrast, recent studies showed that phosphatase and tensin homolog (PTEN) that binds and dephosphorylates the phosphatidylinositol (3,4,5)-trisphosphate has a chief function in suppressing folliculogenesis and ovulation (Fan et al. 2008, Marx 2008, Reddy et al. 2008, Adhikari & Liu 2009). Lacking PTEN in oocytes causes the entire primordial follicle pool to become activated, followed by depletion of all primordial follicles in young adult mice (Reddy et al. 2008, Gleicher et al. 2009).
whereas inactivating PTEN in the granulosa cells results in not only enhanced ovulation but also persistent non-steroidogenic luteal structures in the adult mouse ovary (Fan et al. 2008). To date, however, neither the downstream signaling or effect of PTEN, nor the opposing positive regulatory pathway in follicular activation is known.

One of the major genetic mechanisms determining cell proliferative capacity is the maintenance of telomeres. Work from several independent laboratories has shown that forced expression of the telomerase reverse transcriptase (TERT) gene to maintain telomeres in cultured OSE cells results in increased proliferative potential of the cells, causing them to resemble stem cells (Davies et al. 2003, Kasukari et al. 2003, Alvero et al. 2004, Maeda et al. 2005, Li et al. 2007, Yang et al. 2007a, 2007b). This fundamental effect of telomere remodeling on OSE cells demonstrates proof of principle that the regulation of telomere remodeling is closely linked to OSE cell proliferative capacity. However, substantial in vivo evidence also shows that telomere remodeling is regulated by various factors, including hormones, growth factors, and cytokines, under diverse conditions. Intriguingly, during folliculogenesis, ovarian aging, and neoplastic development, the pattern of TERT-induced telomerase activity is dependent on cell type, time, and location. This review presents the current understanding of the fundamental roles of telomerase and telomere maintenance in ovarian cell proliferation.

**Telomere and telomerase: a mechanism for cell regeneration**

Telomeres, the specialized structures at the ends of all eukaryotic chromosomes, are formed from a unique DNA nucleotide sequence and specific coat proteins. Telomeres from different species assume various tertiary and quaternary structures. The telomeric DNA sequence in humans comprises extended arrays of a few thousand tandem repeats of TTAGGG complementary double strands. However, telomeres end with a single-stranded 3'-end overhang of ~150 bp. This 3’ single-stranded overhang is protected against nuclease by protein complexes or within the double-stranded duplex of the telomere (de Lange 2005, Zhao et al. 2008, Lue 2009; Fig. 1). Entry of the telomere 3’ overhang into the duplex results in a displacement loop and a larger telomere loop at each end of the chromosome (Griffith et al. 1999). The specialized configuration of telomeric DNA has a critical role in protecting chromosomes. During each round of telomeric DNA replication, telomeres are shortened due to the end replication problem (Olovnikov 1996). With each round of chromosome replication, 100–200 bp of telomeric DNA are lost; so, telomeres shorten from 10–15 to 2–5 kb in a lifespan of human cells. When telomeres shorten to a critical length, the cell exits the cell cycle, which is characteristic of cell senescence (Counter et al. 1992, Shay 1999, Blackburn 2001, Wong & Collins 2003). Thus, the initial size of telomeres and the extent of telomere shortening during cell divisions define a cell’s replicative lifespan.

The telomere end replication problem is evolutionarily solved by the enzyme complex telomerase (Szostak & Blackburn 1982, Greider & Blackburn 1985). Telomerase binds, protects, and positively regulates telomere length (Fig. 1). Telomerase is conserved from yeast to higher eukaryotes to synthesize telomeres by RT and to maintain telomeres for continuous renewal and proliferation, particularly of highly proliferative cells, such as germ lines and stem cells in mammals (Shay 1999, Blackburn 2001). As a ribonucleoprotein complex, telomerase contains the catalytic subunit TERT, the telomerase RNA components (TERC), and binding proteins (Cohen et al. 2007, Venteicher et al. 2009). Murine TERT comprises 1122 amino acid residues encoded by a gene on chromosome 13 (D13Mit8; Greenberg et al. 1998). Murine TERC comprises 430 nucleotides (Blasco et al. 1995). Reconstitution of telomerase demonstrates that TERT is the rate-limiting factor capable of lengthening telomeric DNA when expressed in cells (Bodnar et al. 1998). Expression of TERT enables telomerase to function in telomere lengthening and length maintenance; conversely, deficiency of either TERT or TERC abolishes telomerase activity and results in short telomeres (Fig. 2).

**Telomerase is required for oocyte development**

Telomerase is present in oocytes from early antral and preovulatory follicles, as well as in ovulated oocytes, and at markedly decreased levels during oocyte maturation.
High levels of telomerase activity are present in germline stem cells of ovarian origin (Virant-Klun et al. 2009, Zou et al. 2009), which have the capacity to differentiate into oocytes on implantation into the mouse ovary and confer fertility on infertile ovaries (Zou et al. 2009). Consistently, human ovarian germline stem cells with high telomerase activity (scraped from 21 post-menopausal women) proliferate in vitro to produce oocyte-, blastocyst-, and embryo-like structures (Virant-Klun et al. 2009). The human embryonic stem cell lines H9 and hES-NCL1 have been used for differentiation to primordial germ cells (Tilgner et al. 2008). Similarly, mouse embryonic stem cells have been shown to form ovarian follicles and oocyte-like cells (Qing et al. 2007, Virant-Klun et al. 2009). A stem cell-like line with high telomerase activity has been established from preantlal follicle cultures from 35 mouse blastocyst from 193 parthenotes (Lee et al. 2008). Mouse embryonic bodies cultured in testis-conditioned medium developed into ovarian structures containing putative oocytes that expressed oocyte-specific markers such as FIGLA (Fig-x) and ZP3, and were surrounded by one to two layers of flattened cells without a visible zona pellucida (Lacham-Kaplan et al. 2006). Collectively, these studies demonstrate that telomerase activity is important in the processes of oocyte development and parthenogenesis.

In determining the effects of telomerase deficiency on germ cell development, researchers have investigated the cleavage and pre-implantation development of embryos derived from in vivo and IVF of sperm with oocytes with or without TERC (Liu et al. 2002). Fertilization of oocytes containing short telomeres by TERC wild-type sperm leads to aberrant cleavage and development of the embryos. More than 50% of the fertilized eggs developed only one pronucleus, and had a high incidence of cytofragmentation (Liu et al. 2002). These data suggest that short telomeres in the oocytes contribute to anomalous fertilization of gametes and abnormal cleavage of embryos. The defects of short telomeres from oocytes appear not to be reset sufficiently following fertilization. So despite significant telomere lengthening during early cleavage development, the data showing that telomere repair is insufficient in the absence of telomerase argue in favor of a significant role for telomerase in the reprogramming of telomere length after fertilization. Under normal conditions, oocytes have shorter telomeres than somatic cells; so
telomere reprogramming for lengthening must take place following fertilization. Telomeres are reprogrammed by a recombination-based mechanism during early cleavage cycles, and from the blastocyst stage onwards, telomerase maintains the telomere length established by this alternative mechanism (Liu et al. 2007). Telomerase activity is present at low levels in ovulated oocytes, significantly increased in four-cell rat embryos following fertilization, bovine zygotes, and two- to five-cell embryos, but decreased in six- to eight-cell embryos, morulae, and blastocysts (Eisenhauer et al. 1997, Betts & King 1999, Xu & Yang 2000). Thus, following fertilization, the dramatic telomere remodeling involves both telomerase activity and telomere homologous recombination to enable embryonic development.

Role of telomerase in granulosa cell proliferation during folliculogenesis

Granulosa cells share many characteristics with other epithelia, including their origin as epithelial stem cells. In developing ovarian follicles, where intense cell division is crucial to sustain the estrous cycle and drive oocyte development, the formation and regression of granulosa cells determines the development of germ cells and also the timing and the amount of hormones secretion. Studies have consistently shown that high telomerase activity is present in the smallest preantral follicles, and telomerase activity declines gradually during the transition from small- to medium-sized follicles (Lavranos et al. 1999). In situ hybridization and in situ assay of telomerase activity demonstrate the association of high levels of telomerase activity with the highly proliferative granulosa cells in growing follicles (Lavranos et al. 1999, Yamagata et al. 2002), suggesting that the high proliferative activity of granulosa cells depends on telomerase activity to maintain telomeres. Following ovulation, the follicle develops into the corpus luteum with the granulosa cells to exit the cell cycle undergoing differentiation to luteal cells and apoptosis. Recently, luteinizing granulosa cells isolated from the ovarian follicles of infertile patients have been shown to survive in cultures for a long period of time, and a subset of the differentiated granulosa cells shows in vitro differentiation into other cell types such as neurons, chondrocytes, and osteoblasts (Kossowska-Tomaszczuk et al. 2009). In the large follicles undergoing atretic changes, however, telomerase activity is significantly less, and the inhibition of telomerase is prevented by estrogen administration in rats, suggesting that telomerase withdrawal plays an integral role in granulosa cell apoptosis and follicular atresia induced by estrogen withdrawal (Yamagata et al. 2002).

Analysis (by immunohistochemistry and fluorescence in situ hybridization) of the distribution of TERT during folliculogenesis and its correlation with telomeres in porcine ovarian primary and preantral follicles showed that TERT is expressed in granulosa cells with a typical nuclear location, and that its expression correlates with the size of telomeres. Long telomeres were seen from preantral to antral follicles (Russo et al. 2006). Telomerase activity was detected in two distinct populations of porcine granulosa cells: relatively undifferentiated granulosa cells isolated from small (1–2 mm) antral follicles and differentiated cells with advanced functionality obtained from large (5–7 mm) antral follicles (Tomanek et al. 2008). However, epidermal growth factor stimulated telomerase activity to a greater extent in the granulosa cells isolated from small follicles (Tomanek et al. 2008), suggesting that telomerase activity and telomere lengthening are needed for granulosa cell proliferation, particularly in small, rapidly growing follicles. A cross-sectional study on 54 women ≤37 years of age undergoing IVF with classified occult ovarian insufficiency investigated telomerase activity and telomere length in the granulosa cells acquired at the time of oocyte retrieval (Butts et al. 2009). Lack of granulosa cell telomerase activity and short telomeres were found, suggesting that aberrant telomere homeostasis is associated with occult ovarian insufficiency in young women (Butts et al. 2009).

Thus, abnormalities in telomere length and telomerase activity in human granulosa cells may serve as molecular markers for occult ovarian insufficiency (Butts et al. 2009). Compromised telomerase maintenance of telomeres in granulosa cells might play a causal role in infertility due to occult ovarian insufficiency. The age-related decline in ovarian follicle number has been attributed to estrogen deficiency, but the cause of failed maintenance of telomeres in occult ovarian insufficiency in young women is poorly understood.

Regulation of telomerase activity by estrogen

The ovary is both a major source of estrogen and an important target organ of the hormone. By autocrine mechanisms, estrogen exerts profound effects on the ovary (Weihua et al. 2003). Withdrawal of estrogen induces structural atrophy and dysfunction of the ovaries. Conversely, prolonged exposure to estrogen leads to the risk of certain types of tumors, such as ovarian cancer (Lacey et al. 2002). Estrogen acts by binding to estrogen receptors (ESR) that dimerize and bind to estrogen response elements (ERE) in the promoters of estrogen target genes to regulate gene transcription (Bjornstrom & Sjoberg 2005). The estrogen–ESR complex also transactivates gene expression through interactions with other DNA-binding elements (Huang et al. 2004). Estrogen acts mainly through ESR1 (ERα) and ESR2 (ERβ; Couse et al. 2000), two members of the nuclear receptor superfamily. Both ESR forms are present in granulosa cells of the rodent ovary, with ESR2 suggested to be the predominant form (Palter et al. 2001). While ESR2 is essential for granulosa
cell proliferation, ESR1 has specific roles in ovulation, corpus luteum formation, and interstitial glandular cell development (Couse et al. 2000, Dupont et al. 2000). In addition, both ESR1 and ESR2 are implicated in regulating TERT gene transcription in cancer and healthy differentiated cells (Kondoh et al. 2007, Grasselli et al. 2008).

Estrogen activates TERT transcription by a direct action on the gene in ESR-positive human cancer cell lines (Kyo et al. 1999, Misiti et al. 2000, Ling et al. 2006). Moreover, in human OSE cells, a putative ERE identified in the TERT gene promoter binds ESR1 (Misiti et al. 2000). In vivo DNA footprinting showed an ESR-dependent remodeling of the TERT gene promoter in OSE cells (Misiti et al. 2000). Estrogen and ESR1, but not ESR2, markedly stimulate TERT gene promoter activity in an ERE-dependent manner; the estrogen-induced telomerase activity occurs within 3 h of treatment of OSE cells (Misiti et al. 2000).

Although both ESR1 and ESR2 are present in murine ovary, the mouse TERT promoter does not appear to possess a canonical ERE. Estrogen stimulates the expression of transcription factor MYC (c-myc) in rat granulosa cells in vivo (Piontkewitz et al. 1997). The MYC gene is likely to be a direct target of ESR, as stimulation of MYC gene transcription occurs within 1 h of estrogen exposure (Santos et al. 1988). Estrogen stimulation of telomerase activity is dependent on the integrity of the MYC gene in human choriocarcinoma cells (Sarkar et al. 2006). These data suggest that estrogen positively regulates TERT gene transcription by both a direct action on the TERT gene promoter and an indirect mechanism involving the MYC gene. In ovarian cancers, MYC mediates aurora-A kinase-induced telomerase activity (Yang et al. 2004), and the actions of MYC on the TERT gene are further controlled by the tumor suppressor BACR1 (Zhou & Liu 2003) and the transforming growth factor-β family signaling transducer SMAD3 (Li & Liu 2007, Cassar et al. 2009, Liu et al. 2010).

Evidence suggests that prolonged exposure of the ovary to high levels of estrogen is associated with the development of ovarian tumors. Estrogen replacement therapy may also be involved in promoting ovarian cancer (Lacey et al. 2002). The mitogenic effects of estrogen have been studied in various ESR-positive ovarian cancer cell lines, and anti-estrogens, such as tamoxifen and glyceollins, suppress ovarian cancer cell growth in vitro and in animal models. Moreover, estrogen induces ovarian papillary neoplasms in several species, including dogs (Jabara 1962), rabbits (Bai et al. 2000), and pigs (Silva et al. 1998). Expression of ESR even at low levels has been associated with an earlier-stage, higher tumor differentiation and better prognosis (Geisler et al. 1996, Munstedt et al. 2000). These in vitro and in vivo studies support the notion that estrogen plays a significant part in ovarian tumorigenesis by regulating the genes underlying tumor cell immortalization and transformation. Further studies are required to investigate the mechanism by which estrogen induces tumorigenesis. During multi-step ovarian carcinogenesis, many alterations occur in the interactive network of estrogen-responsive genes, including the proto-oncogene MYC, anti-apoptotic protein BCL2, and TERT (Chow et al. 1996). These estrogen-regulated genes may play important roles during ovarian tumorigenesis, such as aberrant epithelial cell proliferation, immortalization, transformation, invasion, and metastasis (Li et al. 2008; Fig. 1).

**Reactivation of telomerase in OSE and the role in ovarian regeneration and oncogenesis**

The replicative lifespan is limited in cells lacking telomerase activity by replication-associated telomere shortening. Therefore, in the context of ovarian regeneration, telomerase activation might extend the lifespan of replicating OSE and other ovarian progenitor cells. On the other hand, high levels of telomerase activity are a hallmark of cancer, including ovarian epithelial carcinoma. Accumulating data indicate that telomerase activation is an early event in ovarian carcinogenesis. At present, it is unclear whether telomerase activation preserves the non-malignant phenotype and replicative longevity of ovarian reproductive cells or constitutes an early alteration needed for subsequent unlimited proliferation and malignant transformation.

Increasing telomerase activity by overexpressing TERT alone (Alvero et al. 2004), TERT plus SV40 large T antigen (Davies et al. 2003, Kusakari et al. 2003), or TERT plus human papillomavirus-16 antigens (Maeda et al. 2005) extended the proliferative lifespan of human OSE cells toward immortality. These studies independently showed the requirement for inactivation of the p53 and RB1 (Rb) tumor suppressor mechanisms in TERT-induced cell immortalization. To establish whether a single disruption of either the p53 or RB1 pathway would promote telomerase-induced human OSE cell immortalization, Liu et al. used RNAi technology to knock down either RB1 or p53 in combination with ectopic expression of the TERT in human OSE cells. Significantly, knockdown of either RB1 (Yang et al. 2007a) or p53 (Yang et al. 2007b) in the presence of TERT is sufficient to immortalize human OSE cells. It was later shown that human OSE cells could be immortalized by TERT expression without functional disruption of RB1 and p53 (Li et al. 2007). Thus, while telomerase activity is indispensable in the immortalization step of human OSE, p53 and RB1 are not, at least under certain conditions. Furthermore, TERT-immortalized human OSE cells have lengthened telomeres, are diploid without chromosomal instability, and maintain epithelial characters without tumorigenicity (Kusakari et al. 2003, Yang et al. 2007a, 2007b, Sasaki et al. 2009).

The importance of telomerase in tumorigenesis has been fully recognized (Shay & Bacchetti 1997, 2000).
with tumor suppressor p53, proto-oncogenes demonstrate that the presence of telomerase, together in vitro the development of ovarian epithelial cancer (Counte et al. 1994, Baykal et al. 2004). Telomerase is not present in normal OSE and premalignant lesions, but is up-regulated in 90–97% of ovarian carcinomas (Gorham et al. 1997, Yokoyama et al. 1998, Baykal et al. 2004, Bayne & Liu 2005, Bayne et al. 2007, Shay & Wright 2007). By in situ hybridization, 90% (28 of 31) of ovarian carcinomas expressed TERT. However, TERT gene expression has also been found in benign epithelial tissues, so it is unlikely to be a useful marker for differentiating between benign and malignant tumors (Baykal et al. 2004).

Ovarian cancer is highly lethal. Based on the pattern of epithelial differentiation and morphological criteria, there are four major subtypes of ovarian epithelial cancer: serous, mucinous, endometrioid, and clear cell. Serous tumors are most common, representing about 50% of all ovarian cancers derived from epithelial cells. Endometrioid adenocarcinomas account for 20–25% of ovarian cancer, and clear cell and mucinous adenocarcinomas account for <10% of ovarian cancer (Soslow 2008). Aggressive ovarian tumors result from the uncontrolled proliferation of ovarian stem cells (Bapat et al. 2005, Szotek et al. 2006), and epithelial ovarian cancer is thought to arise from OSE (Young et al. 2004). Consistently, TERT- and SV-40 large T antigen-immortalized human OSE cells undergo anchorage-independent growth in vitro and tumorigenesis in mice upon forced expression of either oncogene ERBB2 (c-erbB-2) or mutant HRAS (Ha-Ras; Kusakari et al. 2003). Recently, oncogenic transformation of human OSE cells has been investigated with defined cellular oncogenes (Sasaki et al. 2009). Non-viral transduction of primary human OSE cells with human genes encoding mutant cyclin-dependent kinase 4, cyclinD1, and TERT establishes normal diploid OSE cells with extended proliferation without chromosomal instability (Sasaki et al. 2009). Inactivation of p53 plus oncogenic transduction of the KRAS oncogene did not lead to tumor formation, but the additional transduction of AKT, or combined transduction of MYC with BCL2, resulted in tumor formation (Sasaki et al. 2009). The findings of Sasaki et al. (2009) demonstrate that the presence of telomerase, together with tumor suppressor p53, proto-oncogenes RAS and MYC, and the anti-apoptotic pro-survival BCL2, causes the development of ovarian epithelial cancer in vitro.

**The future**

The discoveries from various studies indicating that telomerase has an indispensable role in ovarian germ cell development highlight a regulatory mechanism through which telomerase determines different cell proliferative potentials by regulating ovarian telomere homeostasis. The regulation of telomerase activity may thus provide a means to modulate germ cell development in the ovary. The regulation of telomerase activity by estrogen implies a pathway through which telomere length and cell proliferative potential can be modulated to support the various functions of the ovary. The regulation of the telomerase–telomere axis by estrogen may also reflect how granulosa cells facilitate germ cell development and how aging occurs when estrogen is withdrawn. Further studies are required to determine a potential intermediate role of telomerase activity in estrogen deficiency-induced ovarian aging. Elucidation of a temporal and spatial mechanism of estrogen regulation of telomerase activity (and the roles of other hormones in telomere remodeling) would provide a basis for the design of interventions to treat ovarian aging and infertility.

However, it is clear that telomerase is a double-edged sword in germ cell development and neoplastic immortalization. The ability to control telomerase activity in OSE could allow the prevention and treatment of ovarian cancers derived from these cells. Multiple signaling molecules and pathways could be involved in telomerase activation during ovarian cancer cell immortalization. They warrant investigation of their relationships to OSE cell immortalization and transformation as seen in ovarian cancer.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This work was supported by grants from the National Health and Medical Research Council of Australia and Cancer Council of Victoria, Australia.

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Received 7 January 2010
First decision 12 February 2010
Revised manuscript received 1 June 2010
Accepted 18 June 2010


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