Protein Cell 2011, 2(4): 333–346
DOI 10.1007/s13238-011-1033-2

Protein & Cell

RESEARCH ARTICLE

Estrogen deficiency reversibly induces telomere shortening in mouse granulosa cells and ovarian aging *in vivo*

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Received October 25, 2010 Accepted March 21, 2011

ABSTRACT

Estrogen is implicated as playing an important role in aging and tumorigenesis of estrogen responsive tissues; however the mechanisms underlying the mitogenic actions of estrogen are not fully understood. Here we report that estrogen deficiency in mice caused by targeted disruption of the aromatase gene results in a significant inhibition of telomerase maintenance of telomeres in mouse ovaries in a tissue-specific manner. The inhibition entails a significant shortening of telomeres and compromised proliferation in the follicular granulosa cell compartment of ovary. Gene expression analysis showed decreased levels of proto-oncogene c-Myc and the telomerase catalytic subunit, telomerase reverse transcriptase (TERT), in response to estrogen deficiency. Estrogen replacement therapy led to increases in TERT gene expression, telomerase activity, telomere length and ovarian tissue growth, thereby reinstating ovary development to normal in four weeks. Our data demonstrate for the first time that telomere maintenance is the primary mechanism mediating the mitogenic effect of estrogen on ovarian granulosa cell proliferation by up-regulating the genes of c-Myc and TERT *in vivo*. Estrogen deficiency or over-activity may cause ovarian tissue aging or tumorigenesis, respectively, through estrogen regulation of telomere remodeling.

KEYWORDS estrogen, telomerase reverse transcriptase (TERT), telomeres, ovary, mice

INTRODUCTION

Estrogen has profound effects on tissue development, growth, differentiation and function in a range of target tissues and organs, including reproductive organs, cardiovascular system, bone, immune system, liver and brain. Withdrawal of estrogen induces atrophic changes and dysfunction in many estrogen-dependent tissues, including the ovaries. Conversely, prolonged exposure to estrogen is associated with an increased risk of developing certain types of tumors, such as breast and ovarian cancers (Lacey et al., 2002; Miller et al., 2007a). Estrogen exerts its effects via ligand-dependent estrogen receptors (ER). Upon the binding of estrogen, ER dimerizes and the estrogen-ER complex binds to estrogen response elements (ERE) in the promoters of estrogen target genes to regulate gene transcription (Björnström and Sjöberg, 2005; Fox et al., 2009). It is estimated that one third of human genes that are regulated by ER do not contain an ERE or ERE-like sequence (Björnström and Sjöberg, 2005). Recently, the plasma membrane-associated ER (Segars and Driggers, 2002; Revankar et al., 2005) and estrogen G-protein-coupled receptor 30 (GRP30) (Filardo et al., 2007) have been identified as mediating some functions of estrogen.

The ovary is a major source of estrogen and a primary target organ of estrogen action. Ovaries undergo cyclical changes of folliculogenesis, growth and ovulation, which requires the proliferation and differentiation of follicular cells. In each cycle, primordial follicles are recruited into the growth phase during which granulosa cells proliferate and differenti-

ate to gain an increasing capacity to produce estradiol. Granulosa cell proliferation is stimulated by exposure to estrogen, resulting in preovulatory follicles containing multiple layers of proliferating granulosa cells. At this stage, proliferating granulosa cells acquire luteinizing hormone receptors and the subsequent luteinizing hormone surge triggers granulosa cells of preovulatory follicles to exit the cell cycle (Robker and Richards, 1998). Estrogen maintains granulosa cells in experimental mice (Britt et al., 2002) and stimulates granulosa cell proliferation in small preantral follicles in hypophysectomized rats (Payne and Hellbaum, 1955); however, the molecular mechanisms underlying the estrogen regulation of cell proliferation remain unclear (Britt et al., 2002). The estrogen-deficient aromatase knockout (ArKO) mouse model has been useful for studies of the requirement for estrogen function in the ovary (Miller et al., 2007b). Female ArKO mice have undetectable estradiol in blood (< 10 pg/mL *vs* normal levels of ~24 pg/mL), are infertile and anovulatory and develop hemorrhagic cysts on the ovary (Britt et al., 2001; Toda et al., 2001). The ovaries of young female ArKO mice contain primordial, primary, secondary and antral follicles, but no corpora lutea, indicating an inability to ovulate. With increasing age, the antral follicles of ArKO ovaries become increasingly atretic and there is an infiltration of red blood cells and macrophages within the antra. It has been postulated that the defect in estrogen regulated folliculogenesis and ovulation involves impaired granulosa cell proliferation and differentiation (Britt et al., 2001).

Estrogen regulates several target genes that control cell proliferation, including the proto-oncogene c-Myc (van der Burg et al., 1989; Greenberg et al., 1999), p53 (Toda et al., 2001), BRCA2 (Jin et al., 2008), LRP16 (Meng et al., 2007), cyclin D1, proliferating cell nuclear antigen, surviving, and cdc-2 protein kinase (Frasor et al., 2003). Recent evidence suggests that estrogen may regulate telomerase, the enzyme that controls the proliferative lifespan of cells by maintaining telomeres (reviewed in (Bayne et al., 2008; Liu and Li, 2010)). *In vitro*, estrogen stimulates telomerase activity in human breast cancer cells (Kyo et al., 1999), ovarian cancer cells (Misiti et al., 2000) and vascular endothelial cells (Ling et al., 2006). However, little is known about the effect of endogenous estrogen on telomerase activity *in vivo* and the consequence of estrogen regulation of telomerase for telomeres and cell proliferation under physiological conditions. While absent from most human somatic tissues, telomerase is present in some mouse tissues (Blasco, 2007), although little is known of how telomerase is regulated and the role of estrogen in regulating telomerase in mice (Bayne et al., 2008; Liu and Li, 2010). In the present study, we used estrogen-deficient ArKO mice to investigate the roles of estrogen in regulating telomerase activity and granulosa cell proliferation in the ovary *in vivo*. We found that telomere length in mouse ovary is subject to estrogen regulation, and that telomeres shorten significantly in the absence of estrogen. Telomerase activity in the ovary is inhibited during estrogen deficiency due to repressed gene expressions of c-Myc and telomerase reverse transcriptase (TERT). Moreover, we found that the loss of telomerase activity and shortening of telomeres occur specifically in the granulosa cell compartment and is associated with impaired granulosa cell proliferation.

RESULTS

Depletion of estrogen and decrease of telomerase activity in mouse ovary

To determine whether telomerase is regulated by estrogen *in vivo*, telomerase activity was analyzed in a range of tissues from age-matched female wild-type (WT) and ArKO mice. The levels of telomerase activity were scored as high, moderate, low or undetectable, where 'high' activity is comparable with that in the HeLa cervical cancer cell line. In wild-type mice, telomerase activity was high in the ovary and liver; moderate in kidney; low in pancreas, lung and uterus; and undetectable in forebrain, cerebellum, skeletal muscle, spleen, heart and fat (not shown), findings consistent with previous reports on general levels and distributions of telomerase activity across different tissues and organs in mice (Chadeneau et al., 1995; Prowse and Greider, 1995). Detected telomerase activity in ovary was compared to that in HeLa cells (positive control), and the data showed a linear increase in telomerase activity with increasing telomerase extract from ovary and liver (not shown). The only significant differences in the levels of telomerase activity between wild-type and ArKO mice were in the ovaries and adrenal glands. Telomerase activity in the ovaries of ArKO mice was about 40% that of ovaries from age-matched WT mice (Fig. 1B). Autoradiography of the synthesized telomere fragments spaced in six nucleotides in each lane showed consistent inhibition of the telomerase activity from ArKO ovaries compared with the WT controls (Fig. 1A).

Decreased gene expression of TERT and c-Myc in the ovaries of estrogen-deficient mice

To determine the mechanisms of telomerase inhibition in the ovaries of estrogen-deficient mice, we assessed by RT-PCR the gene expression levels of TERT in the ovaries of WT and ArKO mice, as well as proto-oncogene c-Myc and the tumor suppressor p53, which are involved in regulating TERT gene expression (Flores and Blasco, 2009). Consistent with the high telomerase activity, expression of the TERT gene was high in the ovaries of WT mice. However, TERT gene expression was about 60% less in the ovaries of ArKO mice than in those of WT ($p < 0.05$, $n = 3$; Fig. 1C). In addition, while p53 gene expression was the same in the ovaries of ArKO and WT mice, the level of c-Myc gene expression was

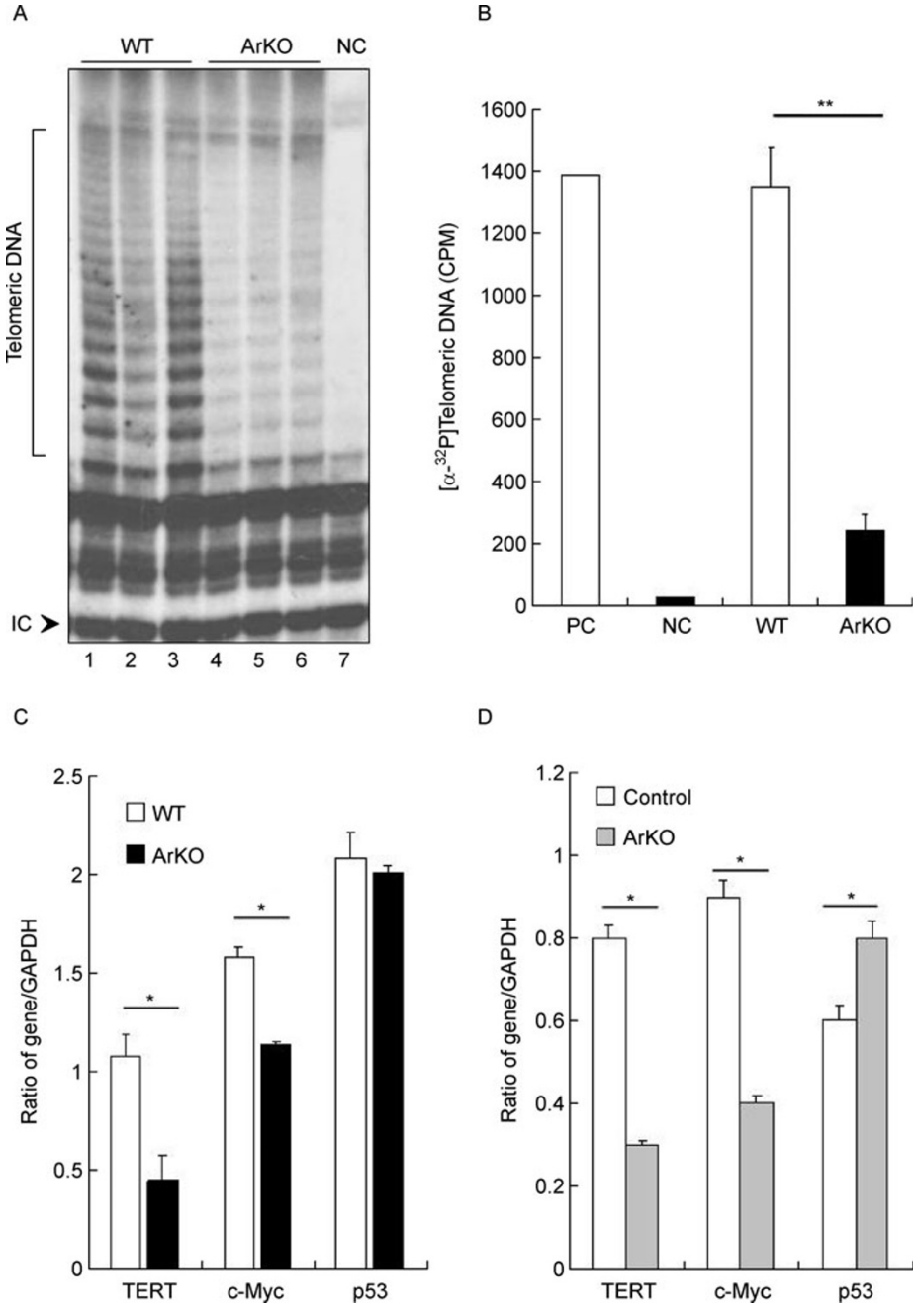


Figure 1. Telomerase inhibition in the ovaries of ArKO mice. (A) Telomerase activity was determined by TRAP (telomere repeat amplification protocol) in equal amounts of nuclear protein extracts from wild type (WT) and ArKO ovaries. The image is representative of telomerase activity in the ovaries of WT and ArKO mice. The negative controls (NC) were lysis buffer and the positive controls (PC) were telomerase extracts from HeLa cancer cell line. Equal amounts of nuclear extracts were used, and a loading and PCR control was included—internal control (IC). Lanes 1–6 show telomerase activity from individual mice. (B) Telomerase activity was quantified by the amount of [α-^{32}P]dATP incorporated into newly produced telomeric DNA and expressed as counts per minute (CPM). The data are presented as mean ± SE from at least three mice per group. ** $p < 0.01$. (C) Gene expression levels of TERT, c-Myc and p53 in mRNA extracts by RT-PCR. Quantification of TERT and c-Myc gene expressions was conducted by densitometry from three similar experiments, adjusted to GAPDH expression levels to normalize for input and expressed as the mean ± SE ratio of gene/GAPDH. * $p < 0.05$. (D) Real time PCR determination of gene expressions of TERT, c-Myc and p53 in the ovaries of WT and ArKO mice at the ages of 3–4 weeks. Data were adjusted to GAPDH expression levels to normalize for input and are expressed as the mean ± SE ratio of gene/GAPDH. * $p < 0.05$.

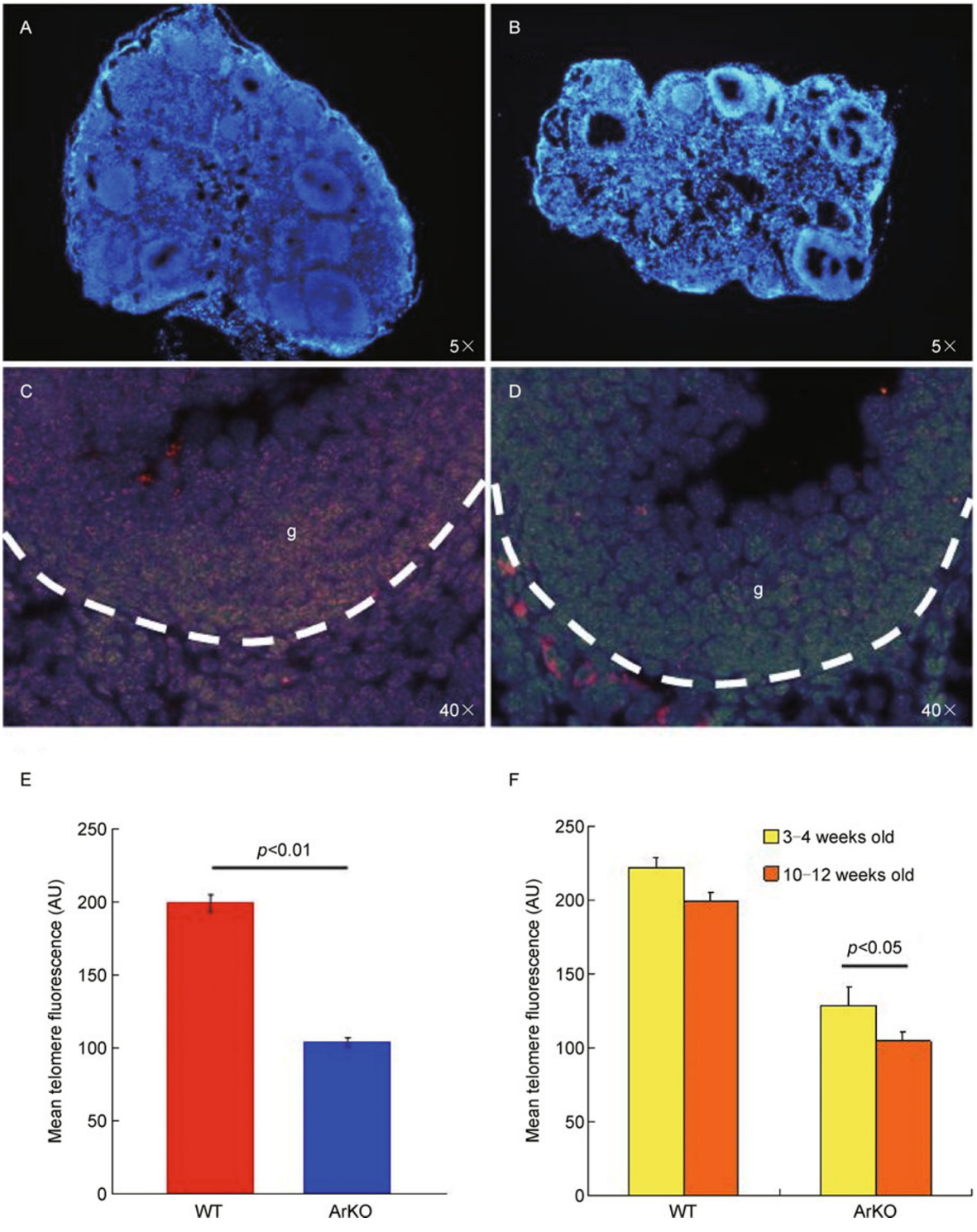


Figure 2. Estrogen deficiency induces telomere shortening in ovarian granulosa cells *in vivo*. Ovaries from wild type (WT) and ArKO mice were sectioned at ~50 μm thickness and examined for telomeres by Q-FISH. (A and B) WT (A) and ArKO (B) ovaries stained with DAPI. (C) Representative image of merged micrographs for telomere Q-FISH showing telomeres (red dots) and DNA (blue) from a WT follicle. The dotted line represents the follicle boundary and granulosa cells used for mean telomere fluorescence measurement are indicated as 'g'. (D) Representative image of merged micrographs for telomere Q-FISH showing telomeres (red dots) and DNA (blue) from an ArKO follicle. (E) Histogram of quantified mean telomere fluorescence intensity (± SEM) of at least 50 granulosa cells randomly chosen for measurement from WT and ArKO follicles. The *p*-value is the difference of the mean telomeres between the WT and ArKO granulosa cells. (F) Comparison of telomere length between two different age groups in the ovarian follicular granulosa cell compartment. Results are expressed as mean ± SEM from at least 50 cells.

reduced by approximately 25% in ArKO mice compared to WT ($p < 0.05$, $n = 3$; Fig. 1C). We confirmed the data by conducting real time PCR using mRNA extracts from the ovaries of 3–4 week-old WT and ArKO mice. Consistent decreases of TERT and c-Myc gene expressions were observed (Fig. 1D). However, we noted an increase of p53 gene expression in the immature ovaries deficient of estrogen. These data suggest a specific regulation of TERT gene expression in association with changes in the expression of c-Myc and p53 genes by estrogen in the ovaries of mice *in vivo*.

Telomere shortening in the follicular granulosa cell compartment of ovary in the absence of endogenous estrogen

To determine if estrogen deficiency induces telomere shortening in the ovary of adult mice in association with TERT gene repression and telomerase inhibition (Fig. 1), we analyzed telomere length in ovarian tissue sections from 10–12 week-old WT and estrogen-deficient ArKO mice by *in situ* hybridization (Q-FISH). Consistent with previous findings (Britt and Findlay, 2003), estrogen deficiency induced ovarian tissue atrophy with extensive interstitial tissue remodeling (Fig. 2A and 2B). Examinations of telomere length in ovarian follicles of similar sizes, however, revealed that the telomeres in the granulosa cell compartment of the ovarian follicles in ArKO mice were approximately 50% shorter than those in WT controls (Fig. 2C–E). These data suggest that telomere shortening is a major consequence of estrogen deficiency in the ovary and that it is caused by inhibition of telomerase under the conditions described.

Comparison of the average telomere length in the granulosa cell compartments of 10–12-week and 3–4-week WT ovaries showed no significant difference. However, a trend toward shorter telomeres in the 10–12-week mice (221.5 ± 7.2 *vs* 199.6 ± 12.4, mean ± SE, $n = 50$; Fig. 2F) suggests that studies in older mice are warranted. In contrast, the mean telomere length in 10–12 weeks old ArKO ovaries was significantly shorter than that of the 3–4 weeks old ArKO ovaries (128.8 ± 5.2 *vs* 104.5 ± 6.1, mean ± SE, $n = 50$, Fig. 2F). Our data show that in the absence of endogenous estrogen, which is associated with decreased telomerase activity in the ovaries, telomeres are significantly shorter in the granulosa cell compartment of 10–12 weeks old than 3–4 weeks old mice, suggesting accelerated telomere shortening under these conditions.

Decreases of telomerase activity in the granulosa cells of ovarian follicles in estrogen-deficient mice

Since telomere shortening occurred predominantly in the granulosa cell compartment of estrogen-deficient ovarian follicles, we hypothesized that telomerase activity in WT granulosa cells may be targeted in estrogen deficiency. In granulosa cells isolated by puncturing follicles from 4-week-old WT ovaries, telomerase activity was significantly enriched, accounting for approximately 70% of total ovarian activity (Fig. 3A and 3B). Consistently, the high levels of telomerase activity correlated with high levels of TERT gene expression in the granulosa cells (not shown). Comparison of telomerase activity in granulosa cells between 4-week-old WT and ArKO mice revealed that the ArKO mice had less than 20% the activity of WT (Fig. 3C).

Effects of 17β-estradiol on telomerase activity in the ovary of mice

To determine whether estrogen had a causal effect on the reduced telomerase activity in ArKO mice, we treated 10–12 week old ArKO and WT mice with exogenous 17β-estradiol (E2, 50 μg) for 21 days via a slow-release pellet implanted subcutaneously. Control mice received a placebo pellet. After E2 treatment, uterine weight returned to normal levels, indicating responsiveness to E2. Assessment of average telomerase activity in the ovaries after hormone replacement therapy showed no difference between WT and ArKO mice (data not shown). The poor response to estrogen in the 10–12 weeks old ArKO mice is likely due to chronic lesions at cellular and tissue levels, including a loss of estrogen receptors in the absence of estrogen. To determine if E2 up-regulated telomerase activity in the ovary of young ArKO mice, we repeated the E2 treatment in 3–4 week old female ArKO mice for 3 weeks, and measured telomere length in the ovaries of treated and control mice. ArKO mouse ovary increased in size in response to E2 (Fig. 4A–C) to reach an ovary section area similar to that of WT (Fig. 4D). Using Q-FISH, we showed that telomeres in the granulosa cell compartment of untreated ArKO mice were associated with about 25% less fluorescence intensity than those of WT (Fig. 4E). However, after E2 treatment for 3 weeks the telomere size in ArKO mice was comparable to that in the WT (Fig. 4E). Thus, these data suggest that E2 stimulates the lengthening of telomeres in the granulosa cell compartment of developing ovaries in mice.

Impairment of cell proliferation and tissue growth in the ovaries of estrogen-deficient mice

To determine if the effects of estrogen removal and replacement on telomerase activity and telomere length correlated with cell proliferation, we determined the status of ovarian cell proliferation in WT and ArKO mice by staining for incorporation of BrdU. The majority of BrdU-positive cells in WT mice were granulosa cells in the follicles (Fig. 5A, and the enlarged view in panel (a)). Larger follicles also showed some BrdU-positive cells in the surrounding thecal cell layer. BrdU-positive cells were occasionally observed in the interstitium and some corpora luteum (Fig. 5A). As for WT ovary, most of

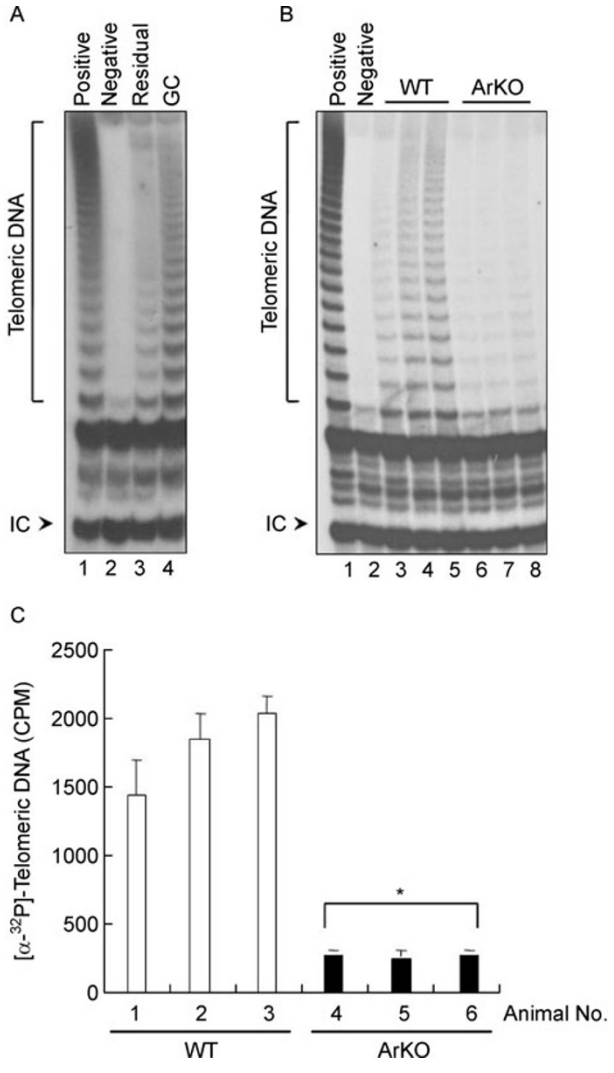


Figure 3. Telomerase activity and TERT gene expression are localized in the granulosa cells of the ovary. Granulosa cells (GC) were isolated from four-week-old wild type (WT) ovary. (A) Telomerase activity was assessed by TRAP from GC and the residual ovaries. The negative controls were lysis buffer and the positive controls were telomerase extracts from HeLa cancer cell line. Equal amounts of nuclear extracts were used, and a loading and PCR control was included — internal control (IC). (B) Telomerase activity is inhibited in ovarian granulosa cells of ArKO animals. GC were isolated from four-week-old WT and ArKO ovaries. Telomerase activity in the GC isolated from the ovaries of three animals in each group was assessed individually by TRAP. The negative controls were lysis buffer and the positive controls were telomerase extracts from HeLa cancer cell line. Equal amounts of nuclear extracts were used, and a loading and PCR control was included – internal control (IC). (C) Telomerase activity in the GC was quantified by the amount of [α-^{32}P]dATP incorporated into newly produced telomeric DNA and expressed as CPM. Data from at least three telomerase assays of different samplings are expressed as mean ± SE. * $p < 0.05$.

the BrdU-positive cells in ArKO ovary were in the granulosa cell compartment. However, ArKO ovaries showed overall fewer BrdU-positive cells compared with WT, especially in the granulosa cell compartment (Fig. 5B, and the enlarged view in panel (b)). The number of BrdU-positive cells correlated with the number of follicles in ArKO ovaries. Moreover, within each BrdU-positive follicle in ArKO ovaries, the proportion of proliferating granulosa cells was significantly reduced in association with the apparently atretic nature of many follicles, as observed by the presence of uneven layers of granulosa cells (Fig. 5B). E2 replacement did not re-instate the compromised cell proliferation (Fig. 5C, and the enlarged view in panel (c)).

About 15% of the cells in follicles from ArKO ovaries were proliferative compared with 45% in WT follicles (Fig. 5D and 5E). Consistent with fewer follicles (Fig. 5D) and decreased cell proliferation (Fig. 5E), ArKO ovary wet weight was also significantly less than that of WT ovary. E2 treatment of ArKO mice was unable to restore the number of follicles, cell proliferation or ovarian weight (Fig. 5D–F).

DISCUSSION

Telomerase is required for the normal development of the ovary (Lee et al., 1998), brain (Herrera et al., 1999) and cardiovascular system (Ling et al., 2006; Grasselli et al., 2008); yet aberrant reactivation of telomerase is observed in more than 85% of human cancers (Shay and Bacchetti, 1997). However, there is limited information on how extracellular cues regulate telomerase activity *in vivo* under physiological conditions (Liu et al., 2010). Neither is it known if telomere shortening with age is under endocrine regulation (Liu et al., 2010; Liu and Li, 2010). The present study examined the involvement of estrogen in regulating telomerase activity and telomere length *in vivo* using an aromatase knockout (ArKO) mouse model, in which estrogen synthesis is specifically blocked. We found for the first time that estrogen-deficiency leads to inhibition of telomerase activity in the ovary, which is associated with reduced gene expression of the telomerase catalytic subunit TERT, and c-Myc, a transcription factor of TERT. We showed that estrogen deficiency-induced telomerase inhibition occurs in the granulosa cells of ovarian follicles in association with compromised granulosa cell proliferation. Furthermore, our data show that in the absence of estrogen, telomere shortening in the granulosa cell compartment is accelerated in mice from 3–4 weeks to 10–12 weeks of age, and is reversed by three weeks of E2 replacement therapy. These data suggest that estrogen plays an indispensable role in telomere maintenance in the follicular granulosa cell compartment of ovary.

The removal of estrogen from the entire body of ArKO mice has no impact on telomerase activity in the liver, kidney, lung and pancreas. Since the ER is present in the liver (Ciana et al., 2003) and kidney (Bullock and Bardin, 1975), where

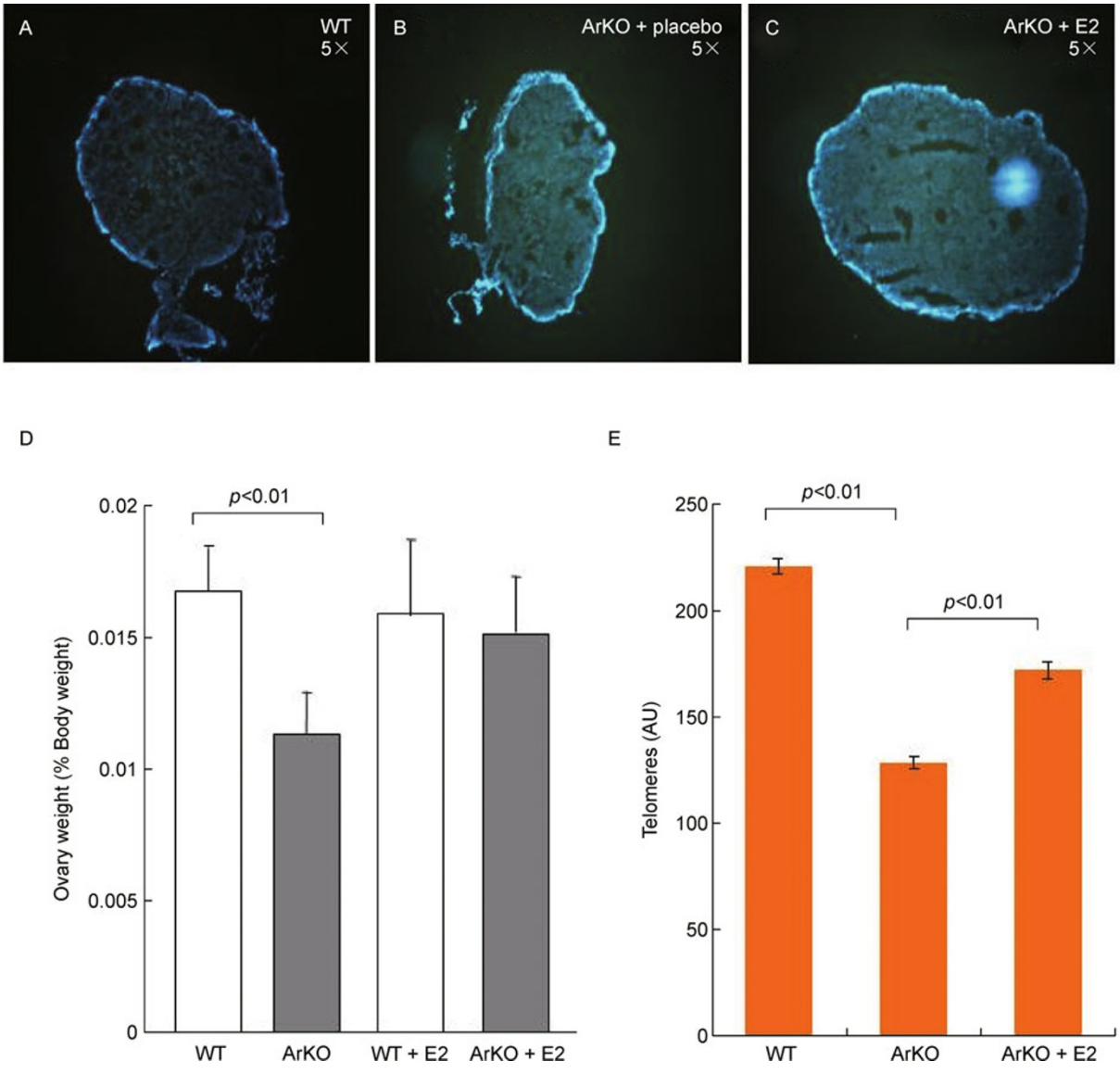
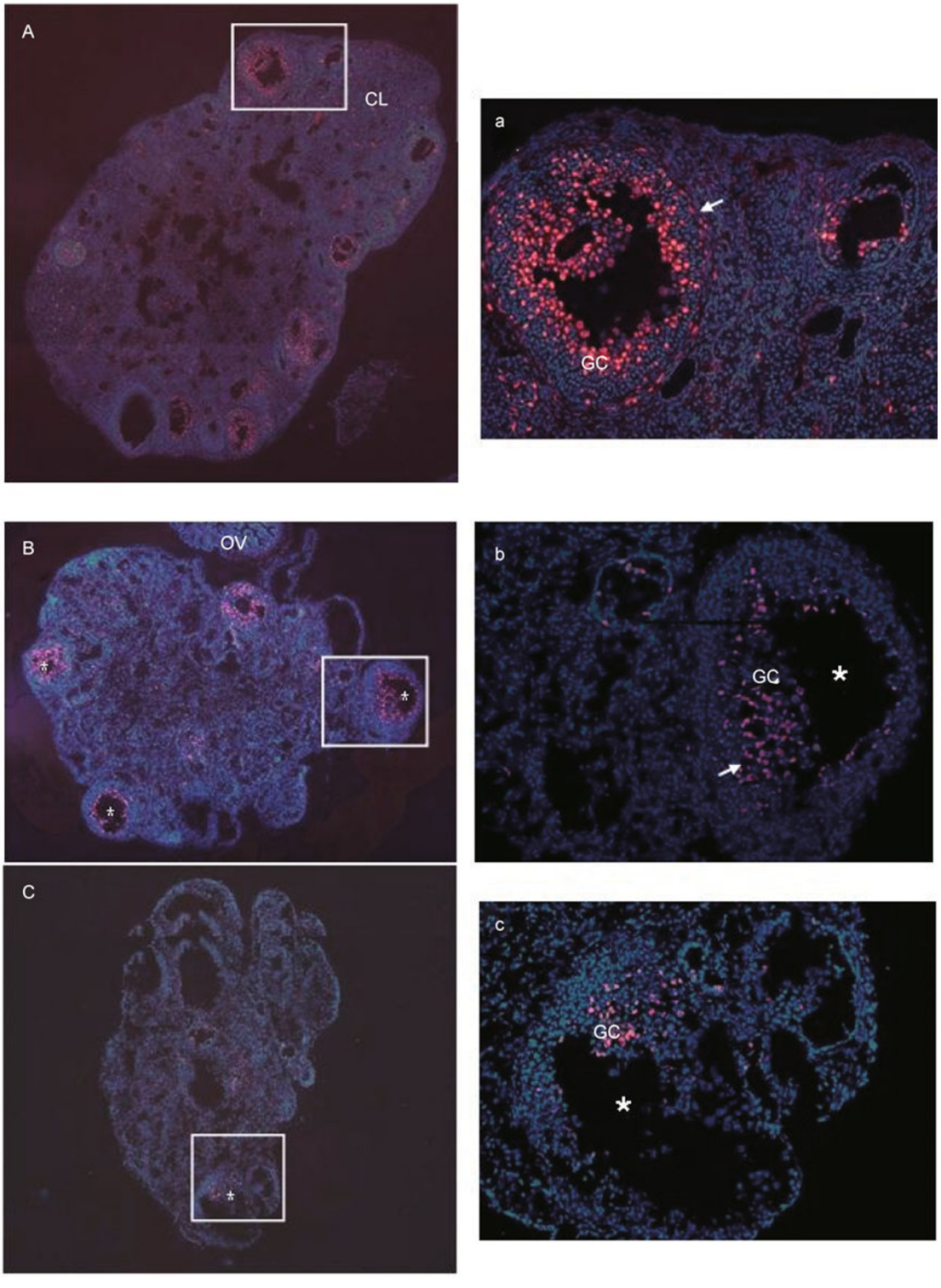


Figure 4. Estrogen replacement therapy stimulates telomerase activity, telomere lengthening and tissue growth in the ArKO ovary. Three-week-old wild type (WT) and ArKO female mice were implanted with estrogen or placebo pellets for 21 days. Ovarian tissues from the WT, ArKO and estrogen-treated ArKO mice were dissected, fat tissues removed, and ovaries collected for telomere length measurement by Q-FISH. (A–C) Sizes of ovarian tissue sections from WT, ArKO and estrogen-treated ArKO mice. (D) Wet ovary weight was measured from placebo- and 17β-estradiol (E2)-treated WT and ArKO mice. Data were collected from 4–6 mice per group and expressed as a percentage of total body weight. Results are expressed as mean ± SE. * $p < 0.05$. (E) Telomere staining in the ovarian follicles of WT, ArKO, and estrogen-treated ArKO mice. Data were collected from each of multiple sections from each group of 3–5 mice. Pooled data of telomere length is presented as mean ± SD. Values are fluorescence intensity from FISH using specific telomeric DNA probe.



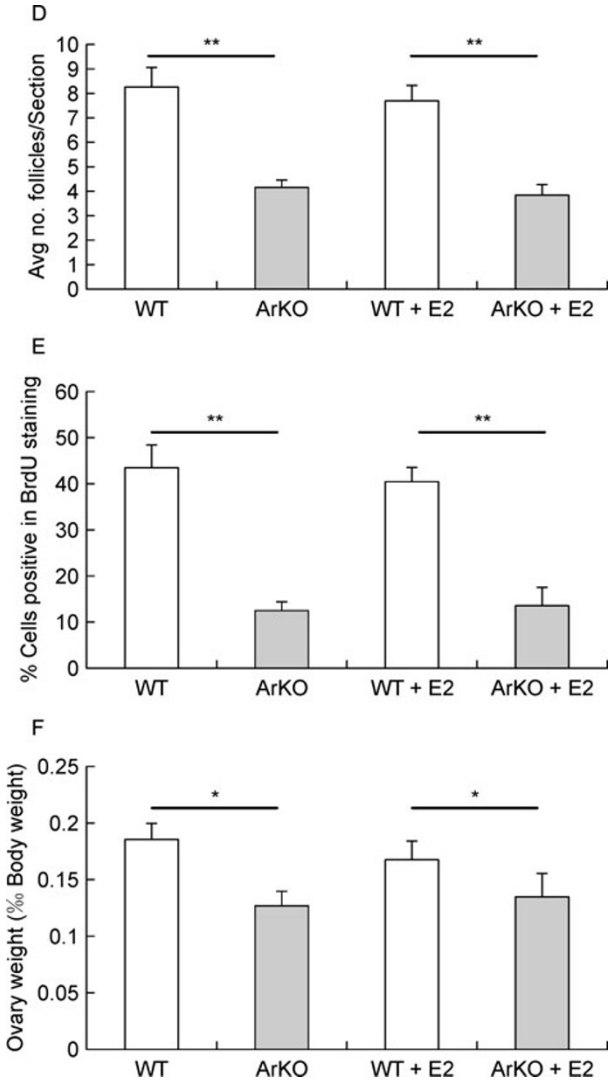


Figure 5. Decreased granulosa cell proliferation and folliculogenesis in the ovaries of ArKO mice. Mice were injected with BrdU and sections were stained for incorporation of BrdU (red) and nuclei (blue). At least three sections approximately 50 μm apart were stained for 3–5 mice per group. (A, B, and C) Representative images from WT (A and a), ArKO (B and b) and E2-treated ArKO mice (C and c). The images are shown at 5× (upper case) and 20× (lower case) magnifications, respectively. CL, corpora lutea; arrow, thecal cell layer; GC, granulosa cells; asterisk, atretic follicle; ov, oviduct. (D) Average follicle numbers per ovarian sections in WT and ArKO mice treated with or without E2. Follicles were counted in multiple sections from three randomly chosen cross-sections per mouse and three mice per group for each group. ** $p < 0.01$. (E) The percentages of BrdU positive cells per follicle were calculated for follicles with three or more layers of granulosa cells, from three randomly chosen cross-sections per mouse and three mice per group. ** $p < 0.01$. (F) Wet ovary weight of placebo- and E2-treated WT and ArKO mice. Data were collected from 3–6 mice per group and expressed as a percentage of total body weight. Results are expressed as mean ± SE. * $p < 0.05$.

telomerase activity is readily measurable, our findings that estrogen deficiency results in telomerase inhibition in the ovary highlight a tissue-specific stimulatory effect of estrogen on telomerase. Our recent studies showing that estrogen deficiency caused telomerase inhibition in the adrenal gland (Bayne et al., 2008) suggest that estrogen links the regulation of telomere homeostasis in steroidogenic organs in mice. The regulation of ovarian telomerase, present in a number of species including mice, rats, cows, pigs and humans (Lavranos et al., 1999; Rodgers et al., 2001; Yamagata et al., 2002; Baykal et al., 2004; Russo et al., 2006), is likely to be physiological, underlying major functions of the ovary. Our findings that estrogen increases telomere length in 3–4-week-old ArKO mice suggest that estrogen deficiency-associated telomerase inhibition is due to the lack of estrogen stimulation of telomerase, since the inhibition is

reversed by estrogen replacement therapy. The lack of response to estrogen in adult mice could be due to compromised ER and/or post-receptor signaling. The chronic lack of estrogen in the atrophic ovary of adult ArKO mice might also cause loss of responsiveness to estrogen of granulosa cell compartment proliferation through mechanisms involving the DNA damage response and apoptosis associated with telomere shortening.

Our data also demonstrate that estrogen replacement alone is sufficient to cause complete recovery of lengthening of telomeres in estrogen-deficient animals *in vivo*, suggesting that estrogen plays an obligatory causal role in the regulation of telomere maintenance. Estrogen deficiency is the primary factor responsible for telomerase inhibition and telomere shortening in the granulosa cells of the ArKO mouse ovary, which possibly contributes to aging of the ovary. These findings are consistent with previous data showing that estrogen treatment stimulates follicular growth in 4-week-old mice (Toda et al., 2001) and point to a physiologically relevant relationship for estrogen in stimulating telomerase activity in ovarian granulosa cells. Since telomerase activity is essential for cell proliferation (Flores et al., 2005), the inhibition of telomerase is likely to contribute to the defects of granulosa cell proliferation, retardation of folliculogenesis and infertility seen in estrogen-deficient animals. This suggestion is supported by the significantly shortened telomeres observed in the granulosa cell compartment of ArKO mice (Fig. 2 and 4), as telomere deregulation underpins cell proliferative lifespan (Harrington and Robinson, 2002; Blasco, 2007; Schoeftner and Blasco, 2009). Consistently, late-generation female telomerase-negative $mTR^{-/-}$ mice, which lack the RNA template for telomerase, are infertile and have smaller ovaries than their wild-type counterparts (Lee et al., 1998). The reduction of egg yield and blastocyst formation (Lee et al., 1998) is consistent with the loss of telomerase activity and telomeres in the ovary, where the rate of cell turnover is high. Thus, our data provide a novel link between estrogen deficiency and the inhibition of telomerase, telomere shortening, inhibition of granulosa cell proliferation, and defective ovaries.

We measured significantly reduced gene expression of the telomerase catalytic subunit TERT in the ArKO ovary, suggesting that the estrogen effect occurs at the level of TERT gene expression. Previous reports have shown that estrogen stimulates TERT promoter activity and gene transcription in ER-positive cancer cell lines (Kyo et al., 1999; Misiti et al., 2000; Ling et al., 2006) and endothelial cells (Ling et al., 2006; Grasselli et al., 2008). Although mouse ovary expresses both α and β ER isoforms, the mouse TERT promoter appears to lack a canonical ERE. It is therefore likely that estrogen regulates TERT gene expression indirectly, via other gene products. One candidate for repressing TERT is the proto-oncogene c-Myc, for which we found gene expression to be decreased with estrogen-deficiency. Estrogen stimulates *in vivo* gene expression of c-Myc in rat granulosa cells (Piontkewitz et al., 1997). Stimulation of c-Myc gene in ER-positive cell lines occurs within one hour (Santos et al., 1988), suggesting that the c-Myc gene is a direct downstream target of ER. Moreover, E2 stimulates telomerase activity in human choriocarcinoma BeWo cells, but not in c-Myc-null HO15.19 cells (Sarkar et al., 2006).

We found no significant change in 10–12-week-old ArKO mouse ovaries of the gene expression of tumor suppressor p53, previously implicated in telomerase inhibition (Kanaya et al., 2000; Shats et al., 2004). The significant increase observed in the 3–4-week-old ArKO mice is consistent with previous findings of increased p53 gene expression in ovarian tissues of ArKO mice (Toda et al., 2001). The increase in p53 expression in the younger group may be due to a DNA damage response rather than a direct response to estrogen withdrawal, which is supported by the finding that increased p53 gene expression in 8-week-old ArKO mice was resistant to estrogen replacement therapy (Toda et al., 2001). On the other hand, with the relatively profound repression of the TERT gene relative to that of c-Myc, mechanisms independent of c-Myc might also mediate the estrogen regulation of telomerase activity, including nuclear signaling from the membrane-associated ER (Segars and Driggers, 2002; Revankar et al., 2005) and GRP30 (Filardo et al., 2007) in the ovary.

A major activity of the ovary is folliculogenesis for cyclic ovulation. In each ovulatory cycle, primary follicles are induced to undertake progressive cell proliferation and differentiation (Hirshfield, 1991). We confirm here that the majority of cell proliferation in the ovary occurs in the granulosa cell compartment in ovary follicles, and that a drastic disruption in the structure and morphology of granulosa cells occurs in the absence of estrogen, consistent with previous findings that estrogen stimulates the proliferation of granulosa cells in order to support developing oocytes during follicular development (Britt et al., 2001). Furthermore, we demonstrate that approximately 70% of total telomerase activity of the wild type ovary is found in isolated granulosa cells. Thus, with 60% of ovarian telomerase activity inhibited in the absence of estrogen, telomerase activity in granulosa cells is a major target of estrogen-deficiency. Indeed, we have demonstrated that granulosa cells in the ovaries of ArKO mice have the remarkably reduced telomerase activity and telomere length. These data, together with the lack of healthy follicles and reduced granulosa cell proliferation in the ArKO ovary (Fig. 5), strongly suggest that estrogen stimulates granulosa cell proliferation under physiological conditions by a mechanism involving telomerase activity and telomere homeostasis. Thus, telomere length in somatic tissues of mice may also be subject normally to hormonal regulation of telomerase in a tissue- and cell-type-specific manner.

In conclusion, we present the first report that estrogen

deficiency induces telomere shortening in mouse ovary under physiological conditions *in vivo*. The shortening was significant relative to wild type mice and between the two age cohorts of 3–4 and 10–12 weeks, suggesting that telomere shortening contributes to ovarian ageing associated with estrogen deficiency. In addition, telomere shortening was reversible upon estrogen replacement therapy, reflecting the plasticity of estrogen reprogramming of telomere remodeling. Estrogen deficiency down regulates the *in vivo* expression of TERT and c-Myc genes to mediate telomere shortening. Telomerase activity is selectively inhibited in the ovarian granulosa cells of estrogen-deficient mice in a tissue- and cell-type-specific manner and the resulting telomere shortening is associated with compromised granulosa cell proliferation and folliculogenesis. Thus, under physiological conditions in mice, estrogen regulation of the maintenance of telomeres by telomerase in the ovary affects productive cell proliferation, and ovarian ageing under conditions of estrogen deficiency involves telomere shortening.

MATERIALS AND METHODS

Mice

All animal procedures have been approved by Monash University Animal Ethics Committee. All mice were treated according to institutional policies of animal welfare and ethical use. Aromatase knockout (ArKO) mice were generated by targeted disruption of the Cyp19 gene required for the conversion of androgens to estrogens (Fisher et al., 1998). Mice were bred, genotyped and maintained as previously described (Jones et al., 2000). A 21-day release 17β-estradiol (0.05 mg 17β-estradiol per pellet; Innovative Research of America) or placebo pellet was subcutaneously implanted into 10–12-week-old female WT and ArKO mice, or 3–4-week-old female animals. Mice used for cell proliferation studies were injected subcutaneously with 1 mg BrdU approximately 2 h before tissue collection. On day 22, mice were killed by CO_2 inhalation and tissues were collected, cleaned of surrounding fat and weighed. The right ovary was embedded and frozen in O.C.T. compound for immunofluorescent staining and the left ovary was snap frozen for either protein or RNA extraction. The work was approved by the Monash University Animal Ethics Committee.

Granulosa cell isolation

Four-week-old WT and ArKO mice were killed by CO_2 asphyxiation. The ovaries were dissected, surrounding fat and tissue removed, and ovaries quickly transferred to a cell culture dish containing 2 mL McCoy's 5C medium and 300 μg/mL DNase (Sigma Aldrich). Ovaries were pooled according to genotype and treatment. Once all ovaries were collected, samples of granulosa cells were collected from each by manual puncture with a 25-gauge needle and the application of slight pressure with a sterile spatula. Granulosa cells were isolated randomly to represent the overall granulosa cell compartment. Follicular debris was removed manually and the granulosa cell suspension filtered through a 150-μm nylon membrane. Ovaries were removed, rinsed with 1×PBS, snap frozen on dry ice and stored at −80°C. The dispersed granulosa cells were pelleted by centrifugation at approximately 600 *g* for 10 min. The supernatant was removed and replaced with fresh McCoy's 5C medium without DNase. Cells were washed with PBS by resuspension and centrifugation as above and cell pellets were snap frozen on dry ice and stored at −80°C.

Telomerase activity assay

Telomerase activity was determined by a TRAP assay performed essentially as described (Bayne et al., 2008). Whole frozen ovaries were homogenized in 10 μL pre-chilled TRAP lysis buffer (0.5% CHAPS, 10 mmol/L Tris-HCl pH 7.5, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 5 mmol/L β-mercaptoethanol, 10% glycerol and 1 mmol/L AEBSF) per mg tissue. Nuclei were pelleted by centrifugation at 3000 *g*, resuspended in 5 μL TRAP lysis buffer per mg tissue and nuclear proteins were isolated by centrifugation at 600 g. Equal amounts (0.4 μg) of nuclear proteins were incubated at 30°C for 20 min in a 30 μL reaction containing 20 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L $MgCl_2$, 63 mmol/L KCl, 0.05% Tween-20, 1 mmol/L EDTA, 2 μg BSA, 50 μmol/L each dNTP and 0.1 μg telomerase substrate. *De novo* synthesized telomeric repeats were amplified in the presence of 0.1 μg ACX, 2 U Taq DNA polymerase (GeneWorks) and 0.05 μCi [α-^{32}P]dATP (PerkinElmer) using the following PCR conditions: 94°C for 3 min, followed by 25 cycles of 94°C 30 s, 60°C 30 s, and 72°C 45 s. To monitor non-specific PCR effects, an additional primer pair was added to the reaction. Telomeric products were resolved on a non-denaturing polyacrylamide gel, followed by overnight autoradiography. Semi-quantitative analysis of telomerase activity was performed by counting ^{32}P activity in each lane using a β-radiation counter. All assays included CHAPS lysis buffer as a negative control and telomerase extracts from HeLa cells as a positive control.

Reverse transcription and real time PCR

Total RNA was extracted using a High Pure RNA Tissue kit (Roche). Equal amounts of RNA were reverse transcribed with oligo dT using the ThermoScript system (Invitrogen) according to the manufacturer's instructions. RT-PCR and real time PCR were used to measure gene expression of TERT, c-Myc and p53. Expression of each gene was normalized to the housekeeping gene GAPDH. Optimization experiments were conducted to ensure that amplification was within the logarithmic linear range of the reactions. PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. Products were measured using densitometry and each gene expressed relative to GAPDH expression. The primers used for PCR amplification of TERT were CTCTCTGCTGCGCAGCCCATAC (JP359) and CCTCGTTAAGCAGCTCAAAG (JP360). The PCR primers for c-Myc were TCACTGGAACTTACAATCTGCGAGC (JP417) and TCCTGTTGGTGAAGTTCACGTTGAG (JP418), and the PCR primers for p53 were ACCATGAGCGCTGCTCCGATGGTG (JP421) and AGACTCCTCTGTAGCATGGGCATCC (JP422).

BrdU incorporation and staining for cell proliferation

Serial 50 μm fresh-frozen sections were cut from 3 to 5 mice per group. Approximately every sixth section was mounted onto poly-L-lysine coated slides for analysis of BrdU incorporation. Sections were

fixed for 15 min in 4% paraformaldehyde/10% sucrose and washed once in phosphate-buffered saline (PBS). DNA was denatured with 20 mmol/L HCl for 30 min at 37°C and the acid neutralized with three changes of 0.1 mol/L borate buffer over 12 min. Sections were blocked with 10% normal rabbit serum (Pierce Biotechnology) for 15 min and incubated with rat anti-BrdU (ab6326, Abcam, UK) at 4°C overnight at a 1:200 dilution. Primary antibody was detected with rabbit anti-rat IgG-Alexa Fluor 594 (Molecular Probes, US) for 90 min at room temperature at a 1:200 dilution. Nuclei were stained with 0.5 μg/μL Hoechst 33258 (Sigma Aldrich) and visualized by fluorescence microscopy. The percentage of BrdU positive granulosa cells per follicle was determined by counting the number of BrdU reactive cells in follicles with at least three layers of granulosa cells, from 3 randomly chosen ovary cross-sections comparable in size from the 3 animals per group.

Telomere Q-FISH

The slides of ovarian tissue sections were fixed in 4% formaldehyde before treatment with acidified 1% pepsin solution, and hybridized with probe solution (0.3 μg/mL Cy3-conjugated [CCCTAA]3 PNA probe (Panagene, Daejeon, South Korea), 70% formamide, 20 mmol/L Tris-HCl, pH 7.0, 1% BSA). Washing was conducted in PBS/Tween-20 with one high stringency wash at 57°C. DNA was counterstained with DAPI and visualized and captured using a Nikon Eclipse TE2000 microscope, Plan Fluor 40× objective, DS-5MC CCD camera and NIS-Elements F 2.20 software (Nikon). Telomere images were captured with a Plan Fluor 100× oil-emersion objective, and individual telomere fluorescence was integrated using spot IOD analysis in the TFL-Telo 2.2 program (a gift from Dr. Peter Lansdorp, Vancouver) (Rufer et al., 1998). Images from at least 13 tissue sections were quantified before assembly of data in a standard spreadsheet program. At least 50 nuclei from each condition were analyzed.

Statistical analysis

Data were analyzed using Student *t*-tests and ANOVA Post Hoc tests. A probability (*p*) value of less than 0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Health and Medical Research Council of Australia, Australia Research Council, and Cancer Council of Victoria, Australia. S.B. is a recipient of an Australian Postgraduate Award.

ABBREVIATIONS

ArKO, aromatase knockout; ER, estrogen receptor; TERT, telomerase reverse transcriptase; RT-PCR, reverse transcription and polymerase chain reaction

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