Regulation of the mTERT telomerase catalytic subunit by the c-Abl tyrosine kinase in mouse granulosa cells

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Abstract

Background: DNA double-strand breaks activate c-Abl protein tyrosine kinase and c-Abl has functional role in repairment of DNA and control of telomere. In this study we hypothesized that c-Abl has regulative role on mTERT in mouse ovarian granulosa cells (GCs) and we aimed to detect c-Abl and mTERT interaction in mouse primary culture of GCs. We showed c-Abl and mTERT immunolocalization in vivo and in vitro mouse GCs. Results: c-Abl presented more intense expression in granulosa cells than mTERT expression. The interaction of the c-Abl–mTERT is supported by the exhibition that c-Abl siRNA knockdown cells show decreased mTERT expression. In addition, our results indicated that the downregulation of c-Abl was also accompanied by reduced expression of proliferating cell nuclear antigen (PCNA) in GCs. We also present interaction between c-Abl and mTERT by immunoprecipitation. Conclusions: We suggest that mTERT may associate with the c-Abl in mouse GCs and the interactions between c-Abl and mTERT suggest a role for c-Abl in the regulation of telomerase function and genomic stability in mouse granulosa cells during folliculogenesis.

Background

Abelson Tyrosine Kinase (c-Abl) is a non-receptor protein tyrosine kinase (Laneuville, 1995) that encodes a nuclear and cytoplasmic protein tyrosine kinase which has known be involved in processes of cell proliferation, differentiation, adhesion, and stress response (O'Neill et al., 1997; Shaul and Ben-Yehoyada, 2005). The c-Abl tyrosine kinase have a nuclear-import and a nuclear-export signals and present a nucleo-cytoplasmic shuttling during cell proliferation. c-Abl protein tyrosine kinase is activated when double strand DNA breaks are comprised. Nuclear isoform of c-Abl orchestrate cell cycle during G1-S transition and relations with several proteins involved in DNA repair as a response to oxidative stress or ionizing radiation (Shaul and Ben-Yehoyada, 2005) beside regulates cytoskeletal structure, cell division, cell growth, and cell proliferation (Hantschel and Superti-Furga, 2004; Plattner et al., 1999). Previous studies have shown that c-Abl has been involved in the regulation of gene transcription during embryonic development (Ahmad and Naz, 1994; Yaba et al., 2011) and homozygous mutations in the *c-Abl* gene have caused increase in perinatal mortality, reduced fertility (Li et al., 2000) and defects in embryonic development (Hantschel and Superti-Furga, 2004; Hernandez et al., 2004).

Telomerase is a ribonucleoprotein complex that synthesizes repeat sequences to 3’ end of telomeres, maintain the length of chromosomes during cell divisions. Telomerase consist of RNA component subunit (Telomerase RNA, TR) and telomerase reverse transcriptase (TERT) catalytic subunit (Chronowska, 2012). Mouse telomerase reverse transcriptase (mTERT) is an imperative protein component of the telomerase complex. There is a significant correlation between telomerase activity and the expression levels of gene that codes hTERT (Brenner et al., 1999). Telomerase undergoes a reversible phosphorylation by forming a complex with various protein kinases. This feature is necessary for modulation of telomerase activity and signal pathways. However, regulation of telomerase activity is clearly undefined. Findings until now show that regulation of telomerase occurs in two ways: protein kinase C/B dependent activation and c-Abl dependent inhibition of the enzyme (Bakalova et al., 2004). c-

Abl has role in the repair of these breaks during control of telomere (Meltser et al., 2011) and Bakalova et al. showed that activation of telomerase is suppressed by activation of c-Abl (Bakalova et al., 2003).

c-Abl is an essential non-receptor tyrosine kinase involved in various cellular functions. We have previously shown the expression patterns of c-Abl suggest that c-Abl plays a role during mouse estrus cycle (Ucar et al., 2012), embryonic and placental development (Yaba et al., 2011). Telomerase has important roles in DNA damage during cell survival or apoptosis, telomere maintenance during tumorigenesis, aging, gene expression, cell survival or apoptosis (Cong and Shay, 2008). But the function of telomerase in these pathways is still not well known. The nuclear localization of telomerase is regulated by DNA damage and oncoproteins which suggests very important findings about telomere biology and cancer. During this process we suggested that c-Abl may be associated with telomerase activity and therefore we hypothesized that c-Abl tyrosine kinase may play crucial role during mouse folliculogenesis via controlling mTERT expression in mouse GCs. GCs play an important role in the oocyte maturation, growth and development of the follicle in the procedure known as folliculogenesis. The objective of this study was to determine the effects of *c-Abl* gene silencing with siRNA knockdown on the mTERT activity of mouse GCs.

Results

Detection of localization of c-Abl and mTERT *in vivo* and *in vitro* mouse granulosa cells

We first examined the expression of c-Abl and mTERT proteins *in vivo* in the adult mouse ovary and *in vitro* in primary granulosa cell culture. Immunohistochemical analysis in mouse ovary showed that c-Abl and mTERT are primarily expressed in mouse ovarian granulosa cells and oocyte (Figure 1). c-Abl showed strong cytoplasmic expression for oocyte and moderate expression for granulosa cells (Figure 1A). mTERT presented cytoplasmic expression for oocyte and granulosa cells (Figure 1B). c-Abl cytoplasmic expression for oocyte is more intense than mTERT.

After 72 h culture of GCs, we took the cells for immunocytochemistry. We detected intense c-Abl expression in cytoplasm and perinuclear area of GCs (Figure 2A). mTERT showed cytoplasmic localization in mouse GCs (Figure 2B). When we compaire these two expession, c-Abl presents stronger expression than mTERT in mouse GCs (Figure 2). c-Abl and mTERT were constitutively expressed in mouse granulosa cells.

siRNAs against c-Abl decrease expression of mTERT level

siRNA-mediated knockdown approach was used to knockdown c-Abl expression to confirm the specificity of the c-Abl.

As assessed by Western blotting, after the transfection of GCs with *c-Abl* siRNA, the expression of the full length 135-kDa c-Abl significantly downregulated the basal levels of c-Abl expression by transfection of

mouse GCs (Figure 3A). Moreover, the c-Abl-induced regulation of mTERT expression was decreased by c-Abl knockdown (Figure 3B). We used mouse ovary and granulosa cell samples for positive control of both c-Abl and mTERT expression (Fig. 3A and B). As our western results shown in Figure 3, treatment with c-Abl siRNA down-regulated mTERT protein levels in mouse granulosa cells and siRNAs against c-Abl decrease expression of mTERT level.

c-Abl regulate mTERT expression in mouse GCs

The IP results revealed an interaction between c-Abl and mTERT in mouse GCs (Figure 4) suggesting that mTERT is regulated by kinase activity of c-Abl.

Knockdown of c-Abl regulates cell proliferation in mouse GCs

Poliferating Cell Nuclear Antigen (PCNA) has protein an essential role in DNA replication and cell proliferation, was significantly downregulated in mouse GCs. To identification of time-dependent knockdown effect of *c-Abl* siRNA on mouse granulosa cell proliferation, we evaluated the PCNA protein expression and we showed that PCNA level decreased from 24h to 96 h (Figure 5).

Discussion

Granulosa cells are crucial for the meiotic maturation of oocytes and development of follicles during ovarian folliculogenesis. These cells are responsible to secrete the hormones required for follicular growth, ovulation and endometrial proliferation. Current experiments examined the expression of c-Abl and mTERT in mouse ovary and granulosa cells. In addition to that, our experimental model transfection of *c-Abl* siRNA, is the first study to demonstrate that the expression of *mTERT* in mouse granulosa cells was down-regulated by *c-Abl* and mTERT levels showed correlation with c-Abl. At the same time, proliferation of GCs decreased time dependently after *c-Abl* knockdown. These results indicate that the regulation of mTERT expression in GCs may be under control of c-Abl tyrosine kinase. Therefore, the present study showed that the knockdown of *c-Abl* induced down-regulation of *mTERT* expression and granulosa cells' proliferation.

Thus far, only few studies have investigated the regulation of mTERT in granulosa cells; additionally, the underlying molecular mechanisms of this regulation are poorly understood for c-Abl. Studies with c-Abl homozygous knockout cells shown that c-Abl regulates telomere length and c-Abl deficient cells have a critical role in the negative regulation of human TERT (Tybulewicz et al., 1991). Some defects at pachytene stage during spermatogenesis were observed in a study with c-Abl knockout mice (Kharbanda et al., 1998). c-Abl protein is located at the ends of pachytene chromosomes. Therefore this is probably why c-Abl protein can contact with telomerase in mitotic cells (Kharbanda et al., 1998). Extension of telomere in c-Abl homozygous knockout cells show that c-Abl has a very important role in the regulation of telomerase function in the relation between c-Abl and human TERT.

Lavranos et al. isolated bovine granulosa cells from the follicles, which are in different stage of follicular development, showed telomerase activity (Lavranos et al., 1999). Studies have shown that smallest preantral follicles and proliferative granulosa cells in growing follicles present high telomerase activity and declines gradually during follicular development (Chronowska, 2012; Lavranos et al., 1999; Yamagata et al., 2002). After ovulation, granulosa cells differantiate to granulosa lutein cells which have significantly less telomerase activity (Yamagata et al., 2002). Luteinised granulosa cells also have potential for proliferation and telomerase activity in human. So far we know that if a women has high level of telomerase activity, she has more possibility of becoming pregnant (Chen et al., 2011). The small and large follicle granulosa cells from pig ovary have proliferation and differentiation potential and express higher levels of telomerase activity in response to Epidermal Growth Factor (EGF) and Follicle Stimulating Hormone (FSH) treatment (Tomanek et al., 2008). A study showed that Estrogen deficient mice presented decreased gene expression level of mTERT and showed impairment of cell proliferation and tissue growth (Bayne et al., 2011). Samantha et al. suggested that telomeres were shorter in women with unexplained ovarian insufficiency than in controls who exhibited normal telomerase activity. They suggested that telomeric shortening of granulosa cells may be associated with ovarian insufficiency in human ovary (Butts et al., 2009).

Therefore the regulation and maintenance of ovarian telomere homeostasis has very curicial role during oocyte maturation, follicle growth and ovarian follicular development in women fertility. It will be important to investigate the possible signal pathways of DNA damage–response which regulates TERT intranuclear dynamics (Maser and DePinho, 2002). Wong et al. suggested that catalytically active human telomerase has a regulated intranuclear localization that is dependent on the cell-cycle stage, transformation and DNA damage. They showed that transformation and DNA damage have opposite effects on the cellular regulation of active telomerase (Wong et al., 2002). c-Abl is known as an important mediator of the DNA damage response, inhibits TERT activity after radiation (Maser and DePinho, 2002).

Conclusion

In the present study, we primarily showed c-Abl and mTERT expression in mouse ovary and GCs. Secondly, our data demonstrate for the first time that the stimulatory effect of c-Abl on mTERT expression and ovarian granulosa cell proliferation in vitro, are involved in the c-Abl-induced down-regulation of mTERT expression. In this point, our study may provide a crucial role of c-Abl in the regulation of mTERT expression during folliculogenesis in mouse ovary. Thence, the identification of c-Abl-mTERT interaction may provide new treatment strategies for infertility and ovarian cancer. Telomeres in the mammalian granulosa cells are progressively expressed during folliculogenesis. Characterization of these mechanisms and future studies on this subject would help us understand the importance of telomerase activity for proper progression of follicular development so that the relationship between c-Abl/mTERT, reproductive senescence and female infertility.

Methods

Animals

All female mice used in this study were BalbC mice 6 weeks of age. The mice were obtained from the animal center of Yeditepe University Medical School Experimental Research Center (YUDETAM) where they were housed in a 23–25 °C temperature room and on a 12 h light/12 h dark (on 7 am, off 7 pm, respectively) light cycle, and water *ad libitum*. The animal protocols used in this study were approved by the Institute Animal Care and Use Committee of Yeditepe University.

Granulosa cell culture

The mice were euthanized by cervical dislocation. Ovaries were disected from female mice 48 h after injection of 5 units pregnant mare's serum gonadotropin (PMSG, Sigma, USA). Large antral follicles were then punctured with a sterile 26-gauge needle to obtain granulosa cells. After removal of oocytes, granulosa cells were washed with RPMI (Invitrogen, USA) and seeded at a density of 1×10^6 cells with RPMI medium containing 10% (v/v) fetal bovine serum and penicillin/streptomycin for 72 h at 37 ^{o}C in the presence of 5% CO2.

Small interfering RNA (siRNA) transfection

After 72 h of culture, the granulosa cell, culture medium was changed and siRNA transfection was carried out according to Dharmafect (Thermo Scientific, USA) transfection reagent protocol. The siRNAs were designed by and purchased from Thermo Scientific. Cells were treated with 0.5 ml of culture medium containing DharmaFECT transfection reagent (Thermo Scientific) containing 10 μM ds-siRNAs (Dharmacon, Chicago, IL) against: c-Abl– 5'AGGUGAAAAGCUCCGGGUC3'. siCONTROL NON-TARGETING *pool* siRNA (Dharmacon) against cyclophilin B was used as the transfection control. We also used siRNA non-treated cells. After 24 h of incubation, we detected c-Abl siRNA transfected cells by fluorescence imaging.

Immunofluorescence

Mouse ovaries were fixed with 4% paraformaldehyde for 6 hr, dehydrated, and embedded by paraffin. Paraffin-embedded mouse ovary samples were cut into 5 μm sections and incubated overnight at 56°C. Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol's while antigen retrieval was performed by microwaving in EDTA (pH: 8.0). To block endogenous peroxidase activity, sections were immersed in 3% hydrogen peroxide for 10 minutes in methanol. Antigen retrieval was performed in microwaving in EDTA (pH: 8.0) and Slides were then incubated in a humidified chamber with TBS-T (Tris-buffered saline containing 0.1% Tween-20 and 5% normal goat serum; Sigma, St Louis, MO) for 1 hour at room temperature. Anti-c-Abl and anti-mTERT antibodies were used at 1/250 dilution for overnight incubation at 4^{o}C in 5% normal goat serum (NGS)/PBS. Control sections were incubated with normal rabbit IgG serum (Vector Laboratories, Burlingame, CA) at the same concentration. Following steps were performed at room temperature, with PBS washes between incubations. Primary antibody binding was detected using anti-rabbit Alexa Flour-488-conjugated secondary antibodies (Thermo Fisher

Scientific) diluted 1:250 in 5% NGS in PBS with 0.01% Tween-20 for 1 h at room temperature and incubated in DAPI for 5min at room temperature before imaging. Images were captured following confocal microscopy.

For immunocytochemistry, granulosa cell culture medium was removed, and cells were fixed with 4% PFA for 20 minutes at room temperature. Afterward cells were incubated with 5% NGS for blocking. Anti-c-Abl and anti-mTERT antibodies were applied at 1:250 dilution for overnight incubation at 4^{o}C in 5% normal goat serum (NGS)/PBS. Primary antibody binding was detected using anti-rabbit Alexa Flour-488-conjugated secondary antibodies (Invitrogen) diluted 1:250 in 5% NGS in PBS with 0.01% Tween-20 for 1 h at room temperature and incubated in DAPI for 5min at room temperature before imaging. Images were captured following confocal microscopy.

Western Blot

Total protein from each granulosa cell culture plate was extracted using T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA), supplemented with protease inhibitor cocktail (1 mM Na3VO4, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM phenylmethylsulphonylfluoride; Calbiochem, San Diego, CA, USA). The protein concentrations of granulosa cells from each group was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Westen blot analysis were performed as previously described (Yaba and Demir, 2012). Shortly, 20-µg of protein was loaded into each lane. To reduce the non-specific binding, membrane was blocked with 5% non-fat dry milk in TBS-T buffer (0.1% Tween-20 in Tris-buffered saline) for 1 h. The membrane was then incubated with rabbit polyclonal c-Abl antibody (1:1000 dilution; Thermo Scientific, USA) and then, incubated in peroxidase-labeled goat anti-rabbit IgG (Pierce; USA) and subsequently washed and chemiluminescence detecting reagents were used for detection of c-Abl protein expression. We repeated same procedure for mTERT (1:500 dilution; Thermo Scientific, USA) and PCNA (Proliferating Cell Nuclear Antigen) (1:1000 dilution; Cell Signalling Technology, USA). We used β-actin (1:1000 dilution; Thermo Scientific, USA) for internal control. Each experiment repeated 3 times.

Immunoprecipitation

Mouse granulosa cells were freshly isolated and directly subjected to protein extraction by using RIPA lysis buffer system (Santa Cruz) supplemented with protease inhibitor cocktail (Santa Cruz), sodium orthovanadate (Santa Cruz), phenylmethylsulfonyl fluoride (Santa Cruz) and Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific) according to manufacturers' instructions. Protein concentration from the total lysate was determined by using Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Scientific). Then, 100 µg total lysate was subjected to immunoprecipitation (IP) by using Pierce™ Crosslink IP Kit according to manufacturer's instructions. Briefly, anti-c-Abl (ab15130, Abcam; 10 µg) or anti-TERT (MA5-16034 (Clone: 2C4), Thermo Scientific, 1:100) antibodies were coupled and crosslinked with Protein A/G resin. Uncoupled resin was used as negative control. Then, 100 µg total granulosa cell lysate was precleared and either with uncoupled resin or coupled resin overnight at 4°C. The flow-through (FT) from

the IP reaction was collected to confirm the IP and the antigen was eluted. The eluate (E) and FT fractions were then resolved on 4-12 % Bis-Tris gel (Thermo Scientific) and subjected to western blot. Membranes were incubated with either anti-c-Abl (ab15130, Abcam; 2 µg) or anti-TERT (ab191523, Abcam; 10 µg) overnight at 4°C, washed, and incubated for 1 h at room temperature. The blot images were acquired by using a ChemiDoc™ XRS+ System (Bio-Rad).

Statistical analysis

Groups were compared by Student T-tests. All experiments were performed in at least three replicates. Statistical calculations were performed using SPSS program ($p<0.05$).

Abbreviations

c-Abl, Abelson Tyrosine Kinase; GC, Granulosa cell; mTERT, mouse Telomerase reverse transcriptase

Declarations

Ethics approval and consent to participate: The animal protocols used in this study were approved of Yeditepe University Ethical Committee of Animal Experiments by the Institute Animal Care and Use Committee of Yeditepe University.

Consent for publication: All authors have consent for publishing this research.

Availability of data and material: All datas are avalible.

Competing interests: The authors declare no conflict of interest.

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Authors' contributions

A.Y. conceived and coordinated the study, performed and analyzed experiments, and wrote the paper. S.A., C.E. and E.Y. performed experiments. B.Y. contributed to analyze the experiments.

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Figures

Figure 1

Confocal representative photomicrographs showing c-Abl and mTERT immunoreactivity in cross sections of mouse ovary. A, c-Abl presents strong cytoplasmic expression in oocyte and moderate expression in granulosa cells during follicular development. B, mTERT showed cytoplasmic expression in oocyte and granulosa cells. Experiments were replicated at least three times. DAPI, 4',6-diamidino-2-phenylindole. (*, oocyte).

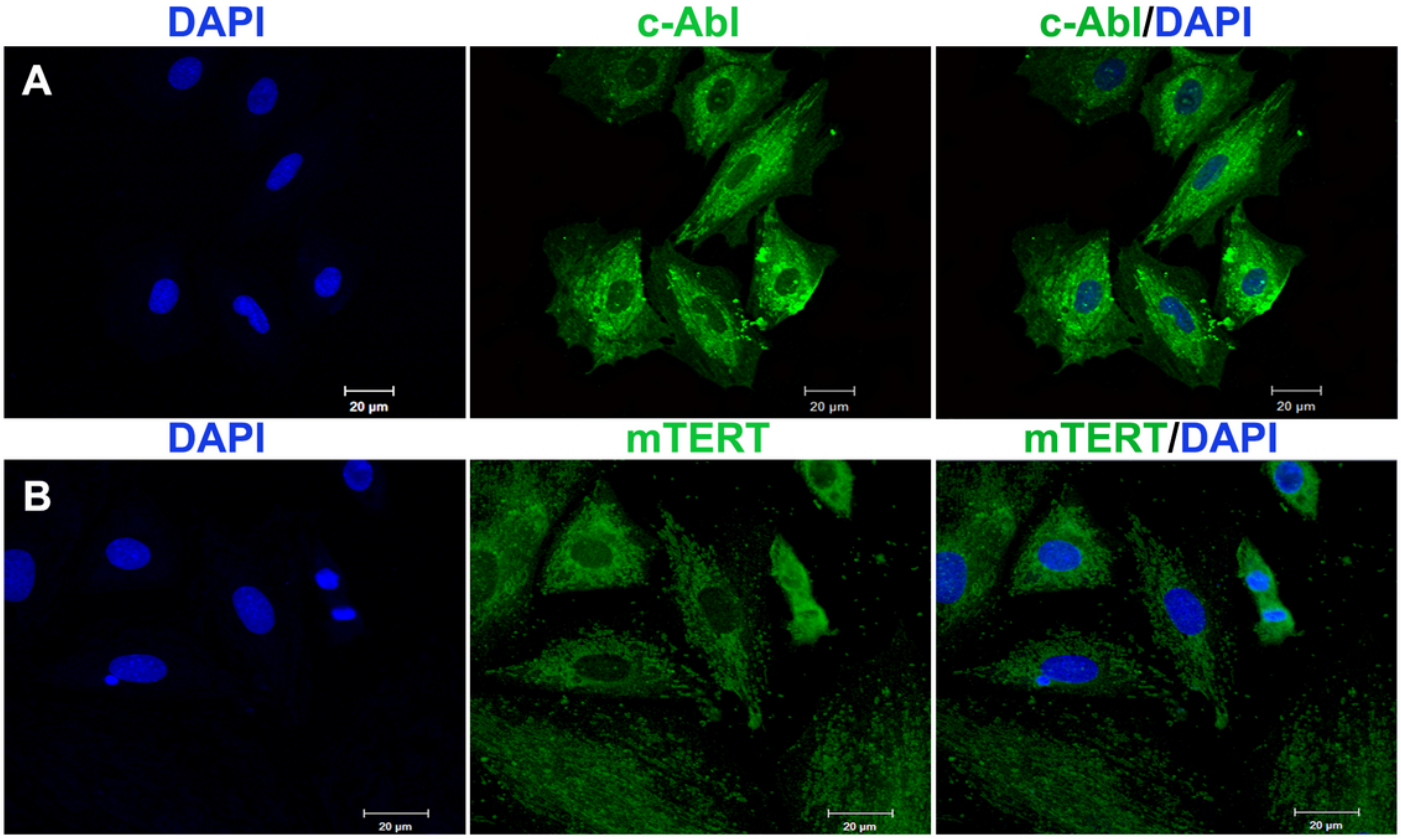
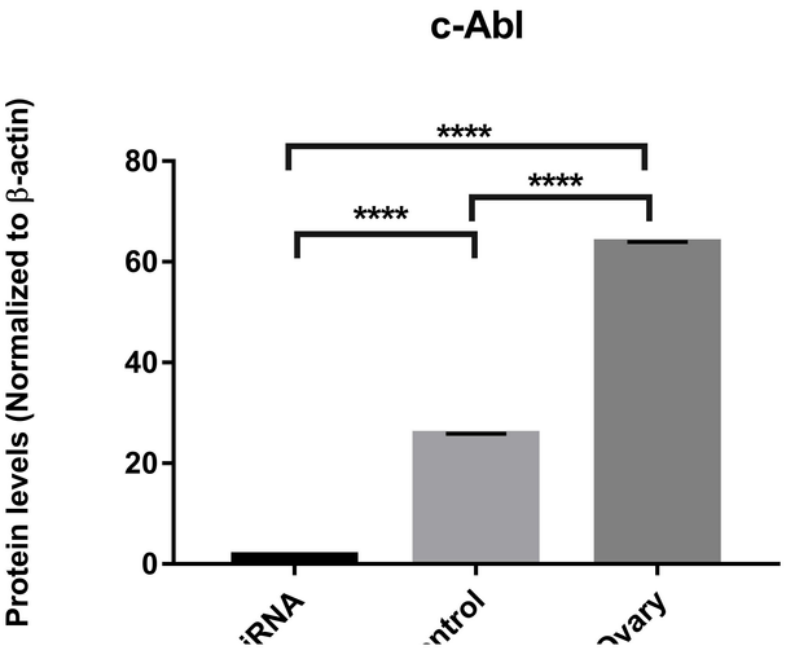


Figure 2

Identification of c-Abl and mTERT expression on mouse granulosa cells after 72 hours culture. A, c-Abl expression was found in perinuclear and cytoplasmic area in GCs. cytoplasmic intense expression detected in oocyte. B, mTERT presents cytoplasmic localization in mouse GCs and oocyte. Experiments were replicated at least three times. DAPI, 4',6-diamidino-2-phenylindole.



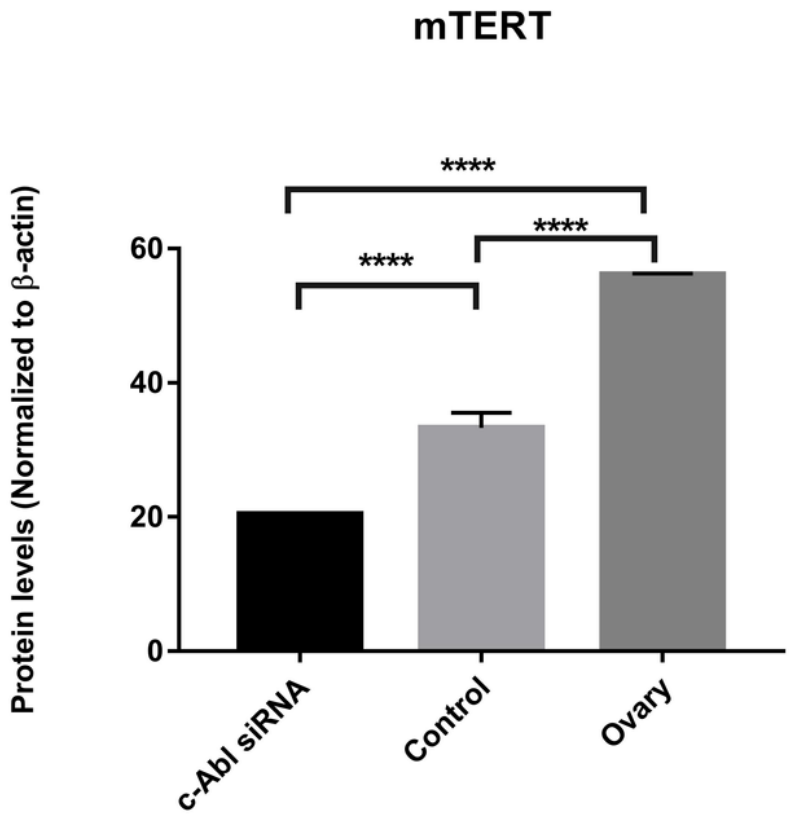


Figure 3

c-Abl down-regulates mTERT expression in mouse granulosa cells. c-Abl (A) and mTERT (B) protein levels. mTERT protein level was significantly decreased by Abl kinase inhibition ($p<0.05$). c-Abl siRNA : c-Abl siRNA transfected mouse granulosa cells; (+) Control: cyclophilin B transfection; (-) Control: No c-Abl siRNA transfection, gc: granulosa cell.

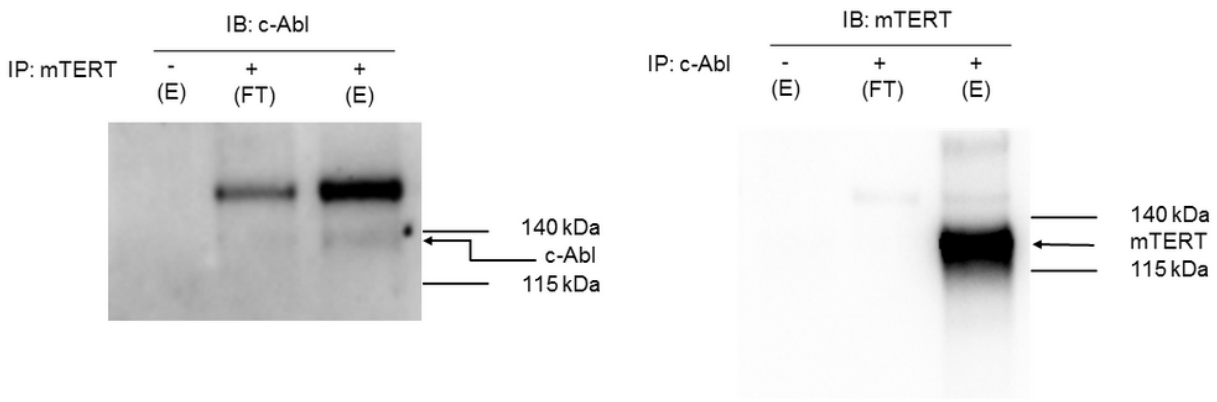


Figure 4

c-Abl and mTERT interaction analyzed by immunoprecipitation in mouse GCs.

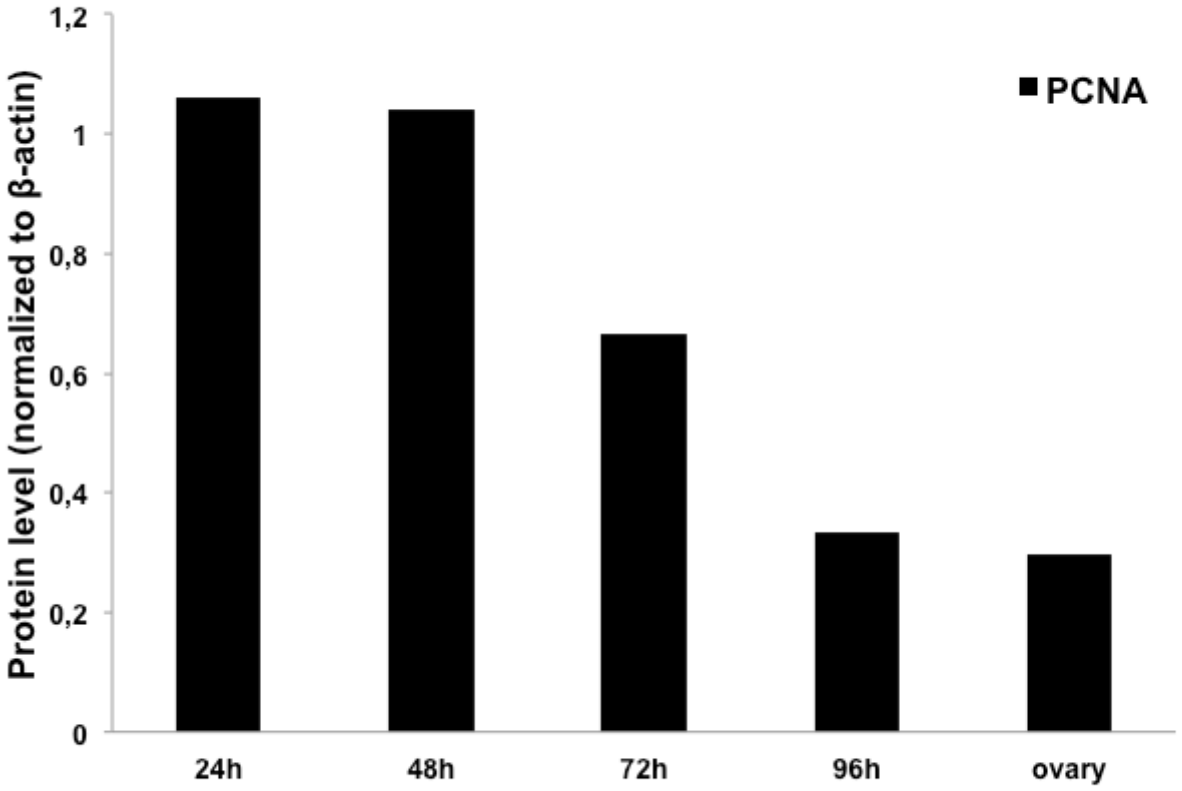


Figure 5

Time-dependent knockdown effect of c-Abl siRNA on mouse GCs proliferation. Poliferating cell nuclear antigen (PCNA) level decreased after 48h incubation.