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TERC suppresses PD-L1 expression by downregulating RNA binding protein HuR

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TERC is the RNA component of telomerase, and provides a template for TERT to synthesize telomere repeats at chromosome ends. Increasing evidence has revealed that TERC is involved in other biological processes beyond telomerase. Here, we found that the expression level of TERC is negatively correlated with PD-L1 and that ectopic expression of TERC but not TERT in ALT cells significantly inhibits PD-L1, suggesting that TERC suppresses PD-L1 expression in a telomerase-independent manner. Mechanistically, instead of regulating PD-L1 mRNA directly, TERC accelerates PD-L1 mRNA degradation by inhibiting the expression of HuR, which binds to the 3′UTR of PD-L1 mRNA and maintains its stability. We also found that the small molecule AS1842856, a FoxO1 inhibitor, promotes TERC expression and reverses the PD-L1 upregulation caused by chemotherapy, providing a potential combination cancer therapy that avoids cancer immune escape during chemotherapy.

TERC, PD-L1, HuR, FoxO1 inhibitor

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INTRODUCTION

Programmed death ligand 1 (PD-L1), also known as B7 homolog 1 (B7-H1), is the ligand of programmed cell death-1 (PD-1) which is a pivotal immune checkpoint for controlling the activation of T cells. PD-L1 delivers negative signals to T cells by binding to PD-1 and suppresses the activation of the immune system (Keir et al., 2008; Sharpe et al., 2007). Many types of cancer cells craftly utilize this mechanism to escape T cell attack by expressing high levels of PD-L1 (Blank and Mackensen, 2007). There is evidence indicating that a high level of PD-L1 is correlated with the malignancy and aggressiveness of tumors in several types of cancer (Gao et al., 2009; Hamanishi et al., 2007). Whereas PD-L1 on the tumor cell surface effectively suppresses T cell activation, it also provides a perfect target for cancer therapy. Several cancer therapeutic methods of reactivating T cells by blocking the PD-1/PD-L1 interaction have been proposed, including anti-PD-1/anti-PD-L1 neutralization antibodies and small molecules that block the PD-1/PD-L1 immune checkpoint (Peggs et al., 2008; Sznol and Chen, 2013; Zhai et al., 2021). To date, various immunotherapy drugs have been approved by the FDA and used clinically (Pardoll, 2012; Yang et al., 2020). Further understanding of the regulation of PD-L1 expression could be helpful for cancer treatments targeting the PD-L1/PD-1 immune checkpoint.

Cancer cells maintain telomere length in two ways: one is dependent on telomerase (Shay and Wright, 2011), and the other is independent of telomerase named alternative lengthening of telomeres (ALT) (Bryan et al., 1995). Compared with telomerase-positive cancers, ALT cancers tend to be more aggressive and malignant, associated with poor clinical outcomes (Zhang and Zou, 2020), but the underlying mechanisms remain unclear. TERC is the RNA component of telomerase, acting as a template for telomerase reverse transcriptase (TERT) to synthesize telomeres at the ends of

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chromosomes (Feng et al., 1995). As a lncRNA, TERC is not only expressed in telomerase positive cancer cells and stem cells but also ubiquitously expressed in somatic cells that do not express TERT (Yi et al., 1999). It has been reported that TERC plays different roles independent of telomerase, such as an anti-apoptotic role in human T cells (Gazzaniga and Blackburn, 2014), regulation of the DNA damage response (Ting et al., 2009) and promotion of the cellular inflammatory response by regulating gene transcription (Liu et al., 2019).

LncRNAs regulate gene expression at multiple levels, including epigenetic modification, transcription, post-transcription and translation (Meller et al., 2015; Wang and Chang, 2011; Yao et al., 2019). For instance, B2 RNA can directly inhibit transcription by binding to Pol II in a non-functional complex. Therefore, Pol II fails to contact DNA to start transcription (Long et al., 2017). In addition to direct regulation of target genes, another important way for lncRNAs to regulate gene expression is by binding to RNA binding proteins (RBPs) to modulate the stability of target mRNAs (Huang et al., 2016; Zhao et al., 2018). Human antigen R (HuR), also known as HuA and ELAVL1 (embryonic lethal abnormal vision-like 1), is one of the most extensively studied RBPs (Hinman and Lou, 2008) and is well known for increasing the stability of target mRNA or controlling the translation of mRNA (Barnhart et al., 2013; Brennan and Steitz, 2001; Lebedeva et al., 2011; Yoon et al., 2012). HuR binds to mRNAs containing AU-rich elements (AREs) in the nucleus in the resting state, and then the HuR-mRNA complex is transported to the cytoplasm where HuR stabilizes the mRNA and protects it from rapid degradation by exonucleases (Wang et al., 2013; Zou et al., 2006).

Considering that both ALT cancer and high expression levels of PD-L1 are associated with more aggressive and malignant cancer subtypes, we hypothesized that ALT cancer cells probably express more PD-L1 than telomerase positive cancer cells. Here, we tested this possibility and found that TERC reduces the cytoplasmic distribution of HuR, and decreases the stability of PD-L1 mRNA, resulting in decreased PD-L1 protein. Furthermore, we found that the small molecule AS1842856 could inhibit PD-L1 by upregulating TERC, providing a potential way to avoid cancer immune escape.

RESULTS

Correlation between TERC and PD-L1 expression in telomerase positive and ALT cells

To explore whether the expression levels of PD-L1 are different between ALT and telomerase positive cancer cells, we studied the PD-L1 levels in three pairs of ALT/telomerase positive cancer cells from non-small-cell lung cancer (SKLU-1/A549), osteosarcoma (U2OS/MG63) and neuroblastoma (SK-N-FI/SH-SY5Y), respectively. First, we validated the expression of TERC and TERT in three pairs of cells. The results showed that the expression levels of TERC and TERT were significantly higher in telomerase-positive cells (A549, MG63, SH-SY5Y) than in the corresponding ALT cells (SKLU-1, U2OS, SK-N-FI) (Figure 1A–C). Then, the expression levels of PD-L1 were pairwise compared between ALT cells and telomerase-positive cells. Both the mRNA and protein levels of PD-L1 in telomerase-positive cells were significantly lower than those in the corresponding ALT cells (Figure 1D–I). Therefore, this phenomenon supported the hypothesis that the PD-L1 expression levels were higher in ALT cells, which were more aggressive and malignant than telomerase-positive cells.

TERC but not TERT negatively regulates PD-L1 expression

The principal difference between ALT and telomerase-positive cells is that telomerase is present in the latter but not the former cells. Therefore, it seems that PD-L1 expression is converse to telomerase, as observed in Figure 1. Consequently, the question is whether telomerase negatively regulates PD-L1 expression. To answer this question, telomerase-positive cancer cell lines from osteosarcoma (MG63) and breast cancer (MCF7) were treated with the telomerase inhibitor BIBR1532 for 24 h, and the expression level of PD-L1 was detected. However, there was no difference between BIBR1532-treated and control cells (Figure 2A and B). This result excluded the possibility that telomerase activity affects PD-L1 expression. Since BIBR1532 only inhibits telomerase activity but does not decrease TERC and TERT expression levels (Pascolo et al., 2002), it is possible that TERC or TERT decreases PD-L1 expression independent of telomerase activity. Hence, we detected the PD-L1 expression level in U2OS stable cell lines that overexpress TERC or TERT, with vector pBabe as a control. Fluorescence activated cell sorting (FACS) results showed that the expression of PD-L1 was significantly lower in TERC-U2OS cells than in pBabe-U2OS cells, whereas there was no difference between pBabe-U2OS and TERT-U2OS cells (Figure 2C). This suggested that TERC but not TERT negatively regulates PD-L1 expression. In addition, MG63 and MCF7 cells transiently transfected with TERC also displayed less PD-L1 on the cell surface (Figure 2D–G). Given that the TERC level in U2OS is very low (Figure 1B), we knocked down TERC by siRNA in telomerase-positive cells MG63 and MCF7, and the expression of PD-L1 increased significantly (Figure 2H–K). Altogether, these results revealed that TERC inhibited PD-L1 expression independent of telomerase activity.

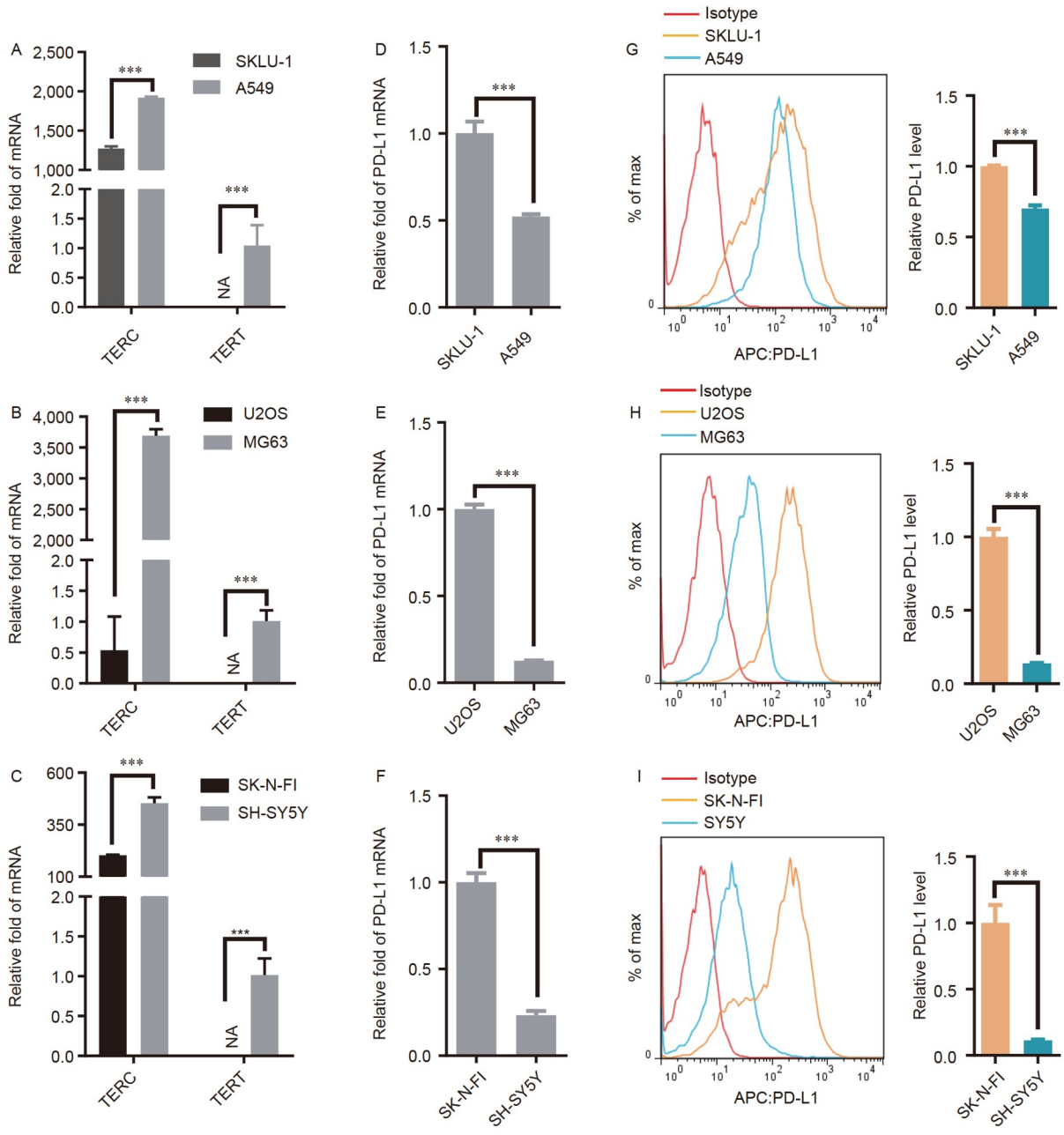

Figure 1 The PD-L1 expression level was compared between three pairs of ALT/telomerase-positive cancer cells from (SKLU-1/A549, U2OS/MG63 and SK-N-FI/SH-SY5Y) respectively. A–C, The mRNA levels of TERC and TERT in the indicated cells. "NA" indicates that TERT was undetectable in ALT cells. D–F, mRNA levels of PD-L1 in the indicated cells. G–I, Protein levels of PD-L1 on the cell surface in the indicated cells. Representative histograms are shown on the left, whereas quantitative data are shown on the right. All values are the means±SEM of more than three independent experiments (***, $P<0.001$).

TERC accelerates PD-L1 mRNA degradation

To explore the mechanism by which TERC decreases PD-L1 levels, we first detected the mRNA and protein levels of PD-L1 in TERC-U2OS cells to determine whether TERC affects the transcription or translation of PD-L1. The results showed that both the mRNA and protein levels of PD-L1 were lower in TERC-U2OS cells than in pBabe-U2OS cells (Figure 3A and B) and knockdown of TERC increased PD-L1 mRNA (Figure 3C and D). These results suggested that TERC decreased PD-L1 at the mRNA level. To figure out how TERC regulates PD-L1 mRNA, the promoter fragments (0.8, 1.4 and 3.3 kb upstream of TSS) and 3′UTR of the PD-L1 gene were cloned into a luciferase reporter construct and co-transfected into U2OS cells with TERC or vector. TERC downregulated the luciferase activity with the 3′UTR but not the promoter fragments of PD-L1 (Figure 3E), indicating that TERC does not impact the transcription of PD-L1, but decreases the PD-L1 expression depending on the 3′UTR of the PD-L1 gene. The 3′UTR of mRNA is important for post-

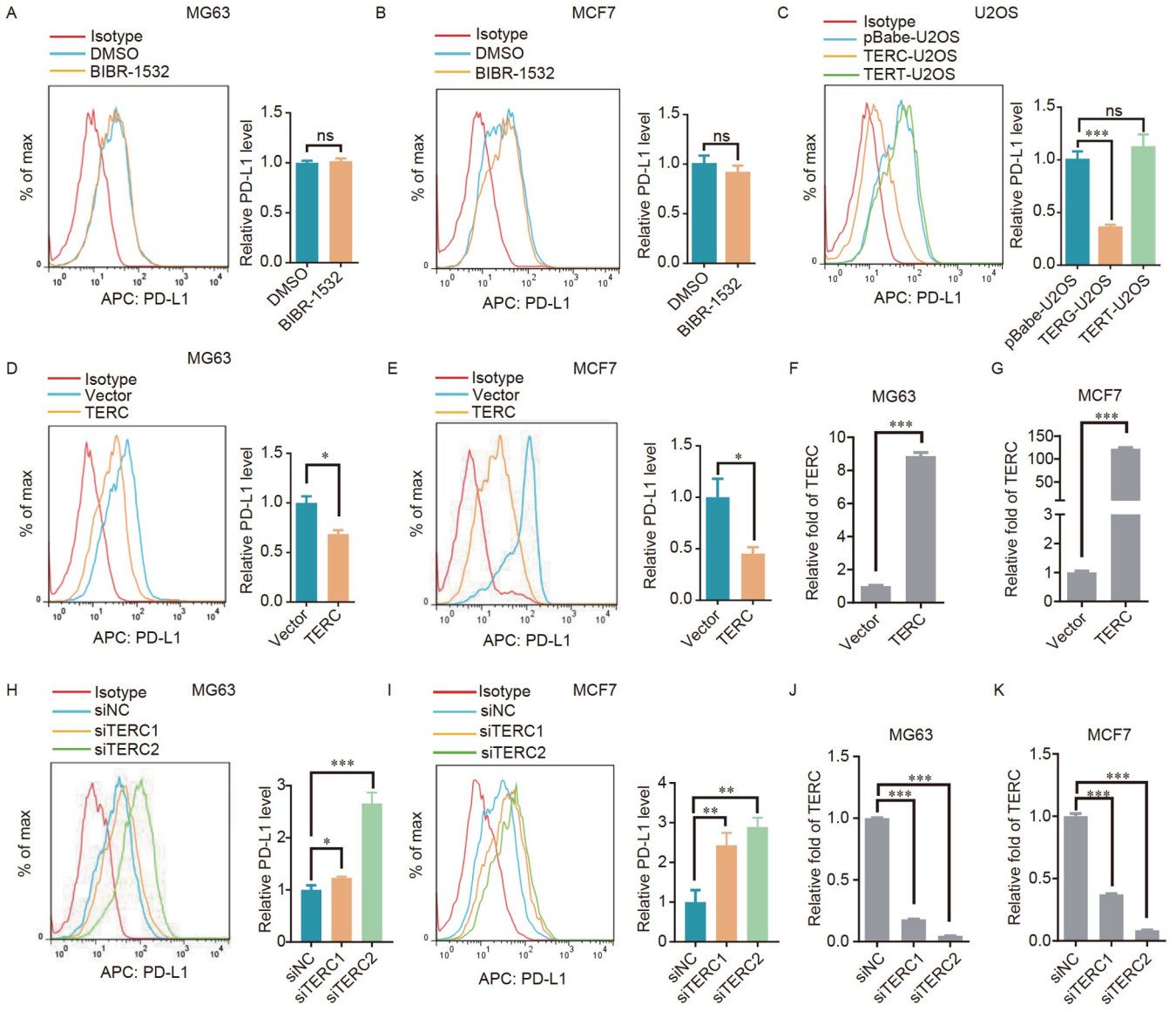

Figure 2 TERC but not TERT negatively regulates PD-L1 expression. A and B, Twenty-four hours after BIBR1532 treatment, cell-surface expression of PD-L1 in MG63 cells or MCF7 cells were detected by FACS. C, Cell-surface PD-L1 levels with TERC and TERT ectopic expression. PD-L1 levels were detected by FACS in TERC-U2OS, TERT-U2OS, and pBabe-U2OS stable cell lines. D and E, The surface PD-L1 levels were detected by FACS 48 h after TERC transfection in MG63 and MCF7 cells. F and G, TERC mRNA levels were detected after TERC transfection in MG63 and MCF7 cells. H and I, The surface PD-L1 levels in MG63 and MCF7 cells were detected by FACS 72 h after siRNA transfection. J and K, TERC mRNA were detected after siRNA transfection in MG63 and MCF7 cells. In the FACS figures, representative histograms are shown on the left, whereas quantitative data are shown on the right. All values are the means±SEM of more than three independent experiments (ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

transcriptional regulation, including mRNA location and degradation (Mayr, 2017). The 3′UTR could also be a target for lncRNA regulation, for example, linc-ROR competes with the 3′UTR of c-Myc mRNA for AUF1 protein, which enhances mRNA degradation, resulting in increased stability of c-Myc mRNA (Huang et al., 2016). We wondered whether TERC decreased PD-L1 mRNA by affecting its stability. Thus, we treated TERC-U2OS and pBabe-U2OS cells with actinomycin D to block RNA synthesis, and then isolated total RNA at 2, 4, and 6 h for PD-L1 mRNA detection. As shown in Figure 3F, the PD-L1 mRNA level was significantly reduced in TERC-U2OS cells as compared with pBabe-U2OS cells, supporting the idea that TERC accelerates PD-L1 mRNA degradation. To explore whether TERC binds to PD-L1 mRNA directly, we performed an RNA pull-down assay with TERC probes in MCF7 whose TERC level is three times of MG63 (data not shown) and detected PD-L1 mRNA in the precipitate using quantitative RT-PCR. However, PD-L1 mRNA could not be pulled down, whereas FLNA mRNA which is reported to bind with TERC (Ivanyi-Nagy et al., 2018) was pulled down by the TERC probe (Figure 3G), suggesting that TERC does not bind to PD-L1

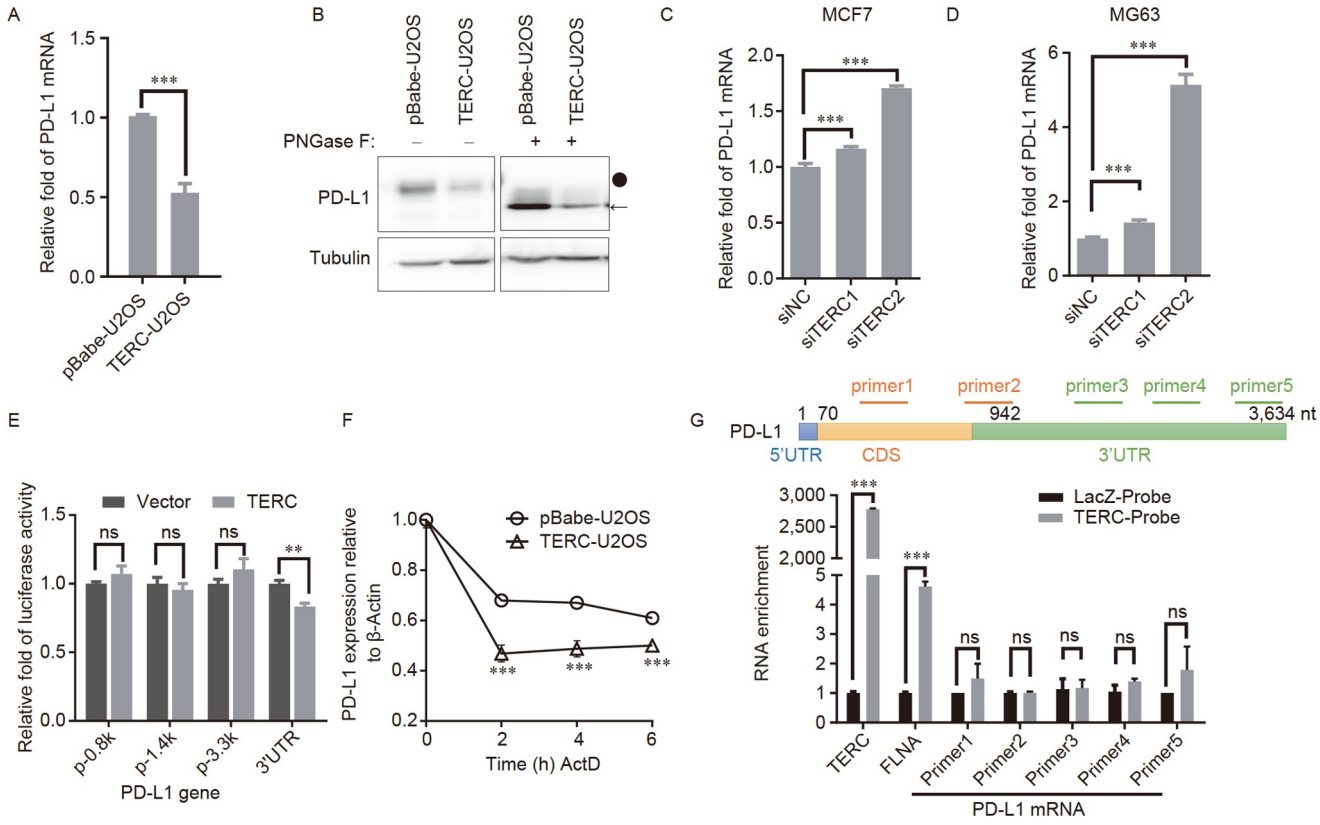

Figure 3 TERC accelerates PD-L1 mRNA degradation. A, PD-L1 mRNA levels were measured in the stable cell lines pBabe-U2OS and TERC-U2OS. B, PD-L1 protein levels were measured in the stable cell lines pBabe-U2OS and TERC-U2OS. Cells were treated with or without glycosidase PNGase F to remove the entire N-glycan. Black circle, glycosylated PD-L1; arrowhead, nonglycosylated PD-L1. C and D, PD-L1 mRNA levels were measured after TERC knockdown by siRNA for 72 h in MCF7 and MG63 cells. E, The effect of TERC on luciferase activity fused with the indicated fragments. The indicated length of the PD-L1 promoter and the 3′UTR of PD-L1 were fused to the luciferase reporter gene and cotransfected into U2OS cells with TERC or vector. Luciferase activity was measured 48 h after transfection. F, Degradation rate of PD-L1 mRNA. TERC-U2OS, and pBabe-U2OS cells were treated with actinomycin D to inhibit RNA synthesis. PD-L1 mRNA was detected at the indicated time points. G, TERC pulldown assay. Top panel: schematic diagram of primer design. Down panel: PCR results of the TERC pulldown assay. RNA pulldown was performed with biotin-labeled TERC probes in MCF7 cells. TERC, mRNA of FLNA (positive control) and PD-L1 were detected by RT-PCR in the products. All values are the means±SEM of more than three independent experiments (ns, not significant; **, P<0.01; ***, P<0.001).

mRNA directly.

The RNA binding protein HuR stabilizes PD-L1 mRNA

According to the results above, TERC is able to regulate PD-L1 expression by affecting its mRNA degradation process. However, TERC does not bind to PD-L1 mRNA directly. Therefore, one or more proteins should have been involved in this process. According to published papers, RBPs are usually essential for lncRNAs to execute various kinds of functions (Astakhova et al., 2018). In addition, it has been reported that there are many AU-rich elements, which are RBP binding sites, in PD-L1 mRNA (Coelho et al., 2017), raising the possibility that PD-L1 mRNA is targeted by RBPs. HuR (human antigen R), one of the RBPs, is reported to be able to associate with and stabilize numerous transcripts (Brennan and Steitz, 2001; Sun et al., 2016; Zhuang et al., 2013). Hence, we studied whether HuR regulates PD-L1 mRNA stability by overexpressing HuR in U2OS cells, and the results showed that PD-L1 expression increased at both the mRNA and protein levels (Figure 4A and B). When HuR was depleted by siRNA in U2OS cells, both the mRNA and protein levels of PD-L1 significantly decreased (Figure 4C and D). It appears that HuR stabilized PD-L1 mRNA and resulted in increased cell-surface PD-L1 protein. To determine whether HuR could bind to PD-L1 mRNA, we performed RNA immunoprecipitation (RIP) using an anti-HuR antibody, and detected different regions of PD-L1 mRNA with multiple primers targeting the CDS and 3′UTR of PD-L1 mRNA in the precipitate. The primers were designed to amplify regions with or without AU-rich elements as shown in Figure 4E. The 3′UTR (with AU-rich elements) but not the CDS of PD-L1 mRNA was accumulated in the anti-HuR group compared with the IgG group (Figure 4E), revealing that HuR interacted with PD-L1 mRNA by binding to its 3′UTR. Next, we researched whether HuR increased gene expression depending on the PD-L1 3′UTR by a dual luciferase reporter assay. A luciferase reporter constructed

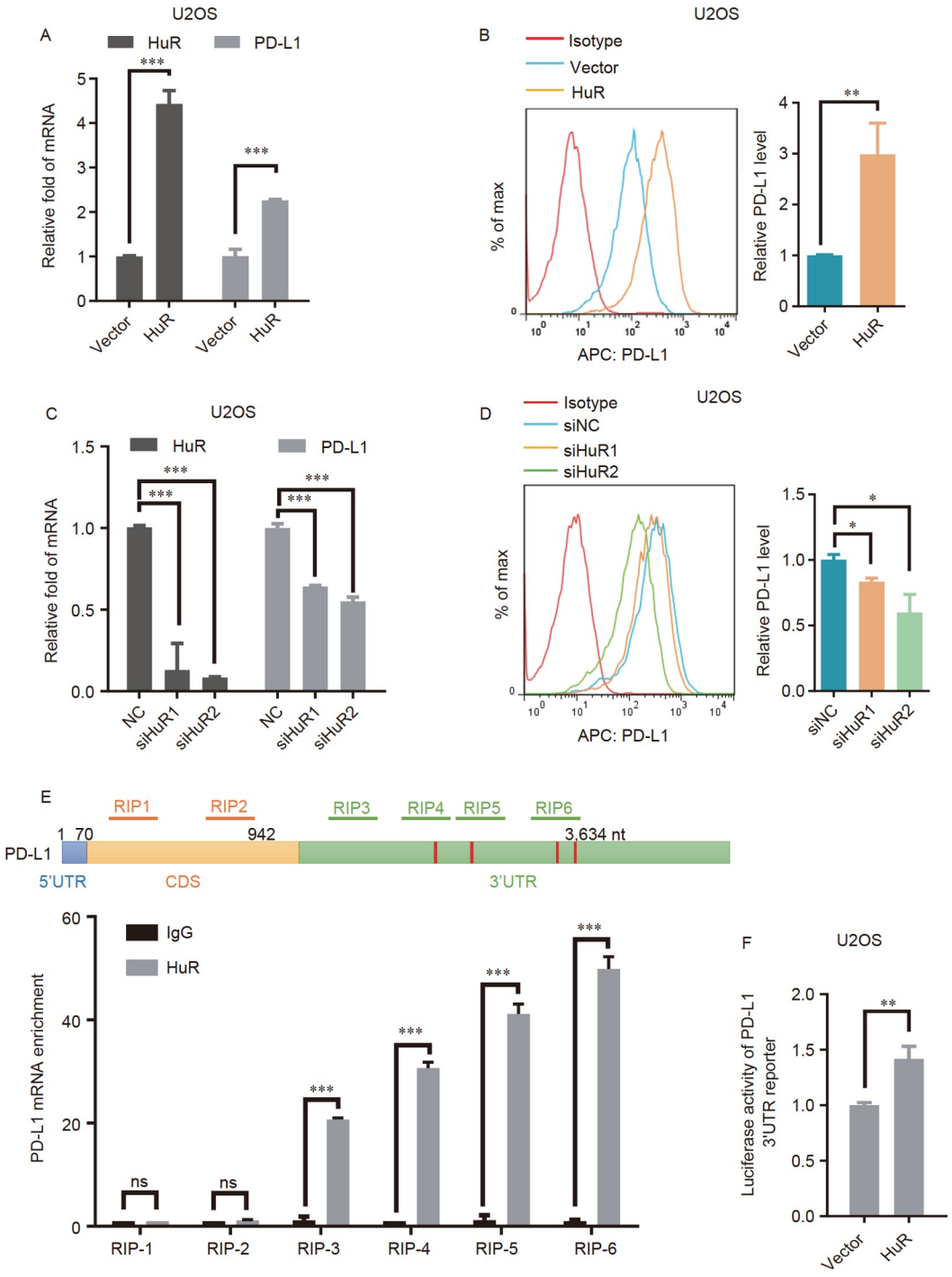

Figure 4 HuR protein stabilizes PD-L1 mRNA. A, The mRNA levels of HuR and PD-L1 after HuR overexpression in U2OS cells. HuR or empty vectors were transfected into U2OS cells. Forty-eight hours after transfection, the cells were collected for HuR and PD-L1 mRNA detection. B, The surface PD-L1 level in (A) was detected by FACS. C, The mRNA levels of HuR and PD-L1 after HuR knockdown in U2OS cells. siRNAs for HuR were transfected into U2OS cells. Seventy-two hours later, the cells were collected for HuR and PD-L1 mRNA detection. D, The surface PD-L1 level in (C) was detected by FACS. E, RIP assay of HuR. Top panel: schematic diagram of RIP primer design. Red lines indicate AU-rich elements (not all). Down panel: PCR results of the RIP assay. HuR antibody was used to pull down HuR-binding RNA in U2OS cells. PD-L1 mRNA was detected by quantitative RT-PCR in the precipitate. F, PD-L1 3′UTR luciferase activity after HuR overexpression in U2OS cells. The 3′UTR of PD-L1 mRNA was fused to the luciferase reporter gene, and cotransfected into U2OS cells with HuR expression or empty vector. Forty-eight hours later, luciferase activity was measured by a dual luciferase reporter assay. In the FACS figures, representative histograms are shown on the left, whereas quantitative data are shown on the right. All values are the means±SEM of more than three independent experiments (ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

with the PD-L1 3′UTR was transfected into U2OS cells together with HuR expression or empty vector. Luciferase activity was detected 48 h later. The 3′UTR luciferase activity increased after HuR overexpression compared to the

empty vector (Figure 4F). Collectively, our results revealed that HuR bound to the 3′UTR of PD-L1 mRNA and stabilized it, resulting in increased PD-L1 expression.

TERC decreases PD-L1 mRNA by reducing HuR

Because both TERC and HuR regulate PD-L1 mRNA levels depending on the PD-L1 3′UTR, we proposed that the effect of TERC on PD-L1 mRNA is mediated by HuR. We detected the total HuR expression level in TERC-U2OS and pBabe-U2OS cells. Both HuR mRNA and protein were decreased in TERC-U2OS cells as compared to pBabe-U2OS cells (Figure 5A and B).

HuR is distributed in both the nucleus and cytoplasm, with

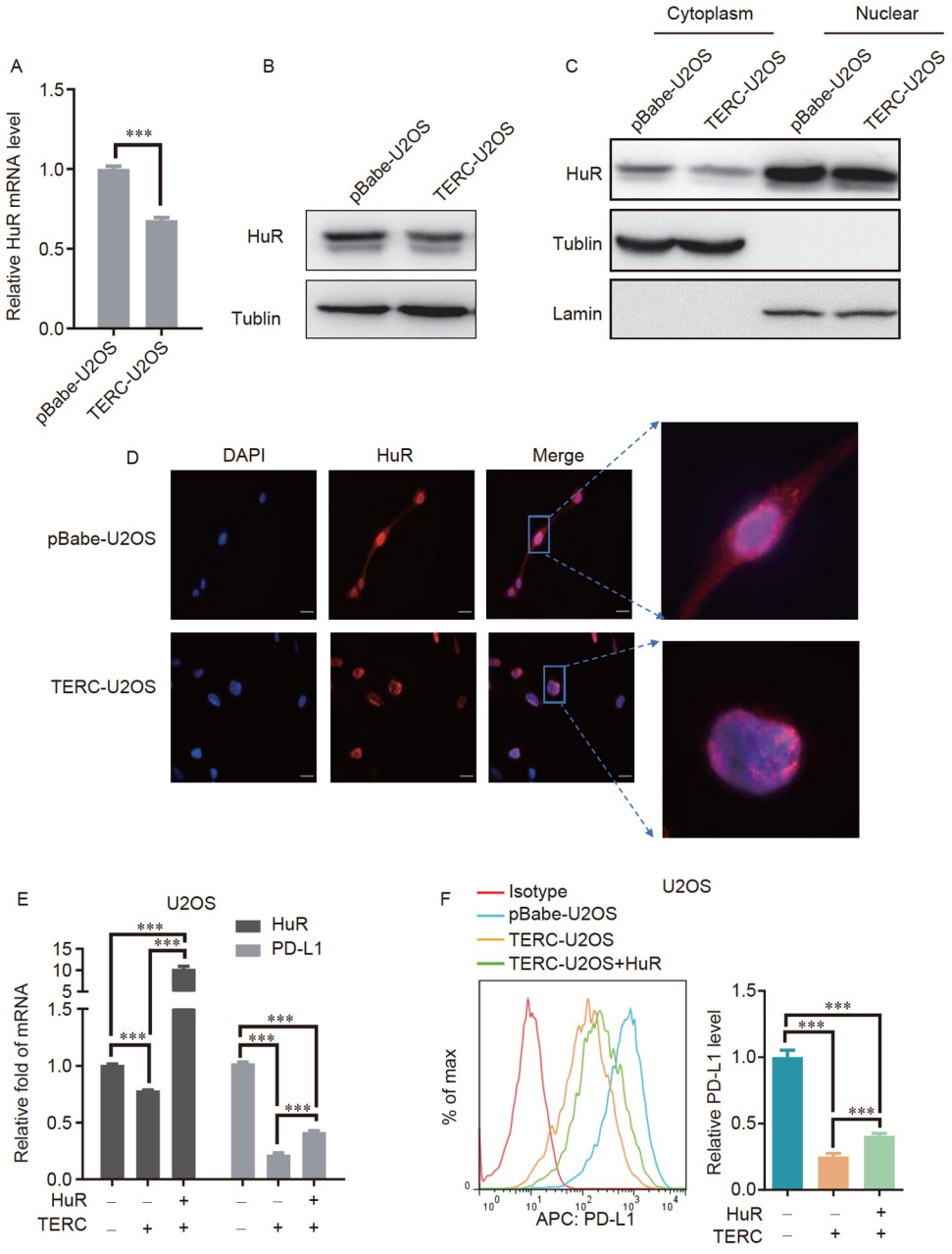

Figure 5 TERC decreases HuR and results in PD-L1 mRNA degradation. A, HuR mRNA expression levels in TERC-U2OS and pBabe-U2OS cells. B, HuR protein levels in TERC-U2OS and pBabe-U2OS cells. C, Cytoplasmic and nuclear distribution of HuR. Cytoplasmic and nuclear proteins were extracted from pBabe-U2OS and TERC-U2OS cells. HuR was detected by Western blot, with tubulin and lamin as markers of the cytoplasm and nucleus, respectively. D, HuR localization in TERC-U2OS and pBabe-U2OS. Endogenous HuR (red) was detected by immunofluorescence using anti-HuR antibody. Nucleus (blue) was stained with DAPI. Scale bars=20 μm. E, HuR and PD-L1 mRNA levels after HuR rescue in TERC-U2OS cells. HuR and PD-L1 mRNAs were detected 48 h after HuR or empty vectors were transfected into TERC-U2OS and pBabe-U2OS cells. F, The surface PD-L1 level in (E) was detected by FACS. In the FACS figure, representative histograms are shown on the left, whereas quantitative data are shown on the right. All values are the means±SEM of more than three independent experiments (***, $P<0.001$).

the cytoplasmic part stabilizing target mRNA (Wang et al., 2013). By separating the nuclear and cytoplasmic proteins, we found that HuR in the cytoplasm decreased significantly after TERC overexpression, while the nuclear HuR change was not obvious (Figure 5C). Immunofluorescence observation also indicated that there was less HuR in the cytoplasm in TERC-U2OS cells than in pBabe-U2OS cells (Figure 5D). Collectively, these results suggested that TERC decreased cytoplasmic HuR, which is important for mRNA stabilization, thus accelerating PD-L1 mRNA degradation. Next, we overexpressed HuR in TERC-U2OS cells to study whether it could rescue the effect of TERC on PD-L1. Both the mRNA and protein levels of PD-L1 partially recovered after HuR expression in TERC-U2OS cells (Figure 5E and F). Therefore, TERC decreased the cytoplasmic level of HuR which stabilized PD-L1 mRNA, resulting in the accelerated degradation of PD-L1.

FoxO1 inhibitor decreases PD-L1 by upregulating TERC

Chemotherapy is widely used in cancer treatment, for example, camptothecin (CPT) is a cancer chemotherapy drug that leads to DNA damage by inhibiting topoisomerase I (Hsiang et al., 1985). However, the DNA damage caused by chemotherapy often stimulates the expression of PD-L1, resulting in the immune escape of cancer cells (Sato et al., 2017). According to our results above, the PD-L1 expression level is negatively associated with TERC, raising the possibility that the TERC expression level impacts the outcome of chemotherapy. Clinical data revealed that a high level of TERC led to a better survival probability for breast invasive carcinoma (BRCA) patients undergoing chemotherapy (Figure 6A). Another study in our group (Wu et al., 2022) found that the transcription factor FoxO1 negatively regulates TERC expression and that the FoxO1 inhibitor AS1842856 (Nagashima et al., 2010) promotes TERC expression. Therefore, we explored whether AS1842856 inhibits PD-L1 expression by upregulating TERC during CPT treatment. The results showed that AS1842856 significantly promoted TERC expression in a dose-dependent manner (Figure 6B and C) and inhibited PD-L1 expression in both U2OS and MCF7 treated with CPT (Figure 6D and E). Furthermore, we knocked down TERC with siRNA in MCF7 cells first and then treated the cells with AS1842856. Quantitative RT-PCR results showed that AS1842856 treatment rescued TERC expression (Figure 6F), and FACS results showed that the enhanced PD-L1 level induced by TERC knockdown was reversed by AS1842856 treatment (Figure 6G), revealing that AS1842856 inhibited the upregulation of PD-L1 during CPT treatment by upregulating TERC. Altogether, these results indicated that elevating TERC is a potential solution for avoiding the immune escape of cancer cells during chemotherapy.

DISCUSSION

The classical function of TERC serves as a structural scaffold for telomerase complex formation, and provides a template for telomere synthesis. However, TERC is broadly expressed in somatic cells without telomerase, and the expression level of TERC is usually in excess of TERT in telomerase-positive cells (Yi et al., 1999), indicating that free TERC exists generally. It has been reported that TERC is involved in various telomerase-independent biological processes (Gazzaniga and Blackburn, 2014; Liu et al., 2019; Ting et al., 2009). In this study, we found that TERC performed a nonclassical function that negatively regulates PD-L1 expression independent of telomerase (Figure 2). The high level of PD-L1 in cancers often correlates with poor prognosis (Tamura et al., 2015). We observed that ALT cells (SKLU-1, U2OS, SK-N-FI), which contain low levels of TERC, expressed more PD-L1 as compared to corresponding telomerase-positive cells (A549, MG63, SH-SY5Y) (Figure 1). This may be one of the reasons why ALT cancers are more aggressive and malignant than telomerase cancers.

Previously, we found that TERC regulates gene expression at the transcriptional level by targeting the promoter containing motif "GGCCACCACCCC" (Liu et al., 2019). However, we did not find this motif in the PD-L1 promoter and therefore TERC did not affect PD-L1 gene transcription from the promoter. TERC did not bind to PD-L1 mRNA either (Figure 3F), but the regulation of PD-L1 expression by TERC required the PD-L1 3′UTR, leading us to find that the RBP, HuR, was required for TERC to regulation PD-L1 expression (Figures 4 and 5). This finding is reasonable based on the following two aspects. On the one hand, during the process of post-transcriptional regulation by lncRNAs, RBPs are always required, which bind to mRNA and change its stability, splicing, and subcellular localization (Peng et al., 2017). On the other hand, according to reported studies, the 3′UTR of PD-L1 mRNA plays an important role in its post-transcription regulation (Sun et al., 2018). There are a number of AU-rich elements, which are binding sites for HuR, located in the 3′ UTR of PD-L1 mRNA (Coelho et al., 2017). We validated that HuR bound to the AU-rich elements in the 3′UTR of PD-L1 mRNA and stabilized it (Figure 4). The expression of TERC in U2OS cells decreased the mRNA and protein levels of HuR (Figure 5). HuR localizes both in the cytoplasm and nucleus, but only cytoplasmic HuR contributes to mRNA stability (Wang et al., 2013; Zou et al., 2006). Although the inhibitory effect of TERC on HuR was limited, almost all of the HuR reduction occurred in the cytoplasm (Figure 5), ultimately leading to a decrease in PD-L1 expression (Figure 2). Considering that HuR can bind to and regulate mRNAs

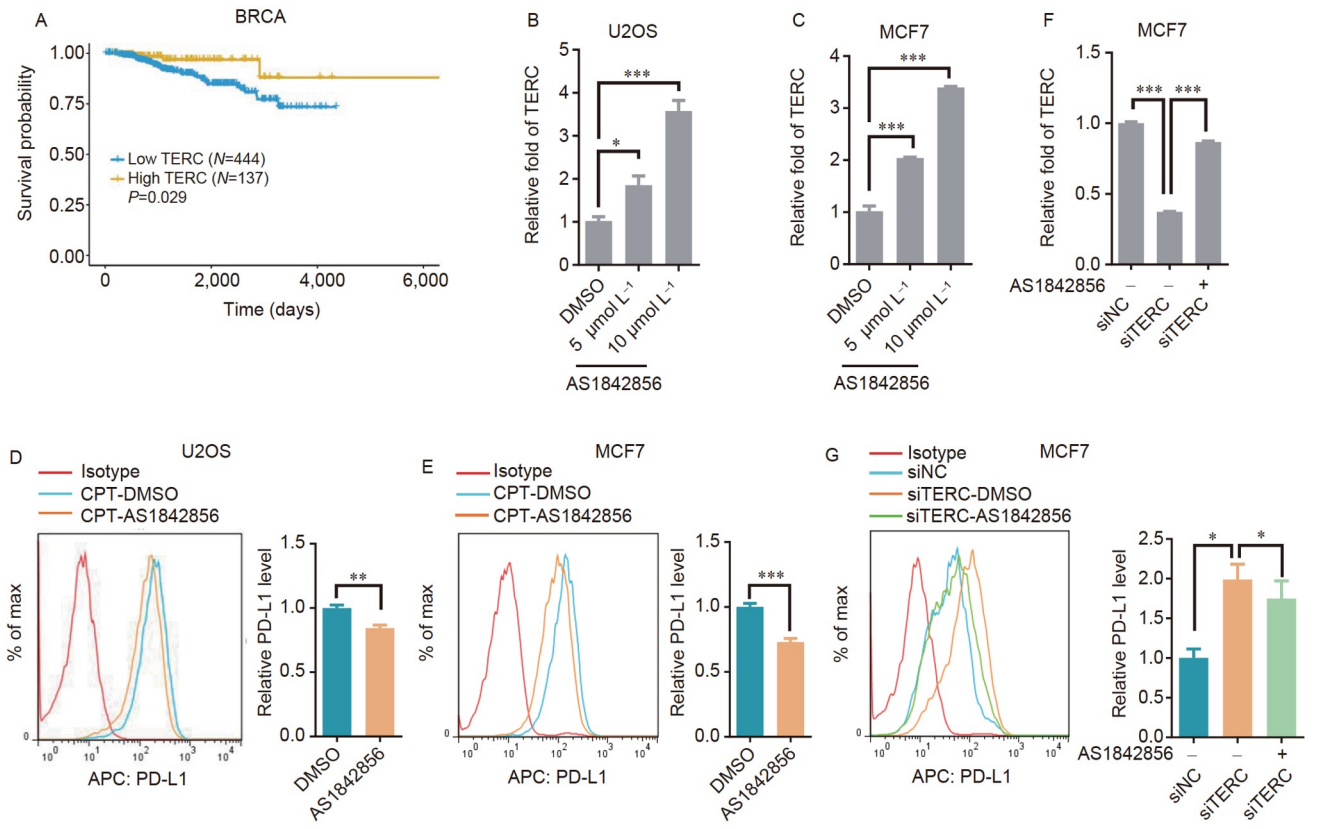

Figure 6 AS1842856 decreased PD-L1 by upregulating TERC. A, Kaplan-Meier curve analysis of the overall survival probabilities of BRCA patients undergoing chemotherapy. Patients were divided to two groups based on TERC expression levels. B and C, AS1842856 upregulated TERC expression. U2OS and MCF7 cells were treated with AS1842856 at the indicated concentrations for 12 h, and TERC was detected by quantitative RT-PCR. D and E, AS1842856 decreased cell surface PD-L1 protein levels in U2OS and MCF7 cells treated with CPT. Cells were treated with AS1842856 (5 μmol L^{-1}) and CPT (0.5 μmol L^{-1}) for 12 h, and PD-L1 was detected by FACS. F, TERC expression level after AS1842856 treatment in combination with TERC knockdown. MCF7 cells were transfected with siTERC or siNC. Forty-eight hours later, AS1842856 (5 μmol L^{-1}) and CPT (0.5 μmol L^{-1}) were added to the cells and maintained for another 12 h. The cells were collected for TERC detection by quantitative RT-PCR. G, The surface PD-L1 level in (F) was detected by FACS. In the FACS figures, representative histograms are shown on the left, whereas quantitative data are shown on the right. All values are the means ±SEM of more than three independent experiments (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

containing AU-rich elements, theoretically, TERC can decrease other mRNAs by decreasing the cytoplasmic HuR. However, this assumption requires further exploration.

In cancer treatment, traditional chemotherapy is widely used to kill cancer cells. However, such therapeutic strategy causes DNA damage in cancer cells and results in the upregulation of PD-L1 expression (Sato et al., 2017), which contributes to the immune escape of cancer cells. Although antibodies of PD-1 or PD-L1 have been developed and used to treat cancers expressing high levels of PD-L1 (Pardoll, 2012), some patients are still resistant to checkpoint blockade immunotherapy (Li et al., 2018; Xia et al., 2021). Therefore, it is meaningful to inhibit the upregulation of PD-L1 during chemotherapy. The new mechanism of PD-L1 regulation discovered in this study provides a theoretical basis for a potential combination therapy of cancer that could include both chemotherapy drugs such as camptothecin and small molecules that can increase the TERC level. We utilized the small molecule AS1842856, which promotes TERC expression in U2OS and MCF7 cancer cells and was able to inhibit PD-L1 expression after camptothecin treatment (Figure 6). Hence, it raises the possibility that combining AS1842856 with chemotherapy drugs, such as camptothecin, for cancer treatment may avoid cancer immune escape. Furthermore, upregulation of TERC will not result in enhanced telomerase activity, because TERC expression levels are always higher than TERT in cancer cells and telomerase activity is dependent on TERT expression levels (Figure 1) (Blasco et al., 1996), thus making TERC a promising candidate for PD-L1 inhibition during cancer therapeutics. Considering that we only used CPT as chemotherapy drug and only in vitro experiments were performed, the effectiveness and safety of this strategy needs further research and investigation.

MATERIALS AND METHODS

Reagents

Antibodies to PD-L1, HuR were from Abcam (UK), antibodies to Tublin and Lamin were from Proteintech (USA).

Camptothecin was purchased from MCE (USA), and the telomerase inhibitor, BIBR-1532 was from Selleck (USA).

Cell culture and transfection

Cells were cultured in Dulbecco's modified Eagles medium (DMEM) (Life Technologies, USA) supplemented with 10% fetal bovine serum (Life Technologies), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Life Technologies) at 37°C in 5% CO_2 incubator.

Plasmid transfection was performed using Lipofectamine 3000 kit (Thermo Fisher Scientific, USA) following the manufacturer's instruction. TERC-U2OS and pBabe-U2OS stable cell lines were generated as described previously (Liu et al., 2019).

siRNAs were used to knockdown genes. SiRNA transfection was carried out with Lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's instructions. Cells were collected for detection 72 h after transfection. The sequences of siRNAs are as below: TERC si1: GUCUAACCCUAACUGAGAAGG; TERC si2: CCGUUCAUUCUAGAGCAAAC; HuR si1: GAACGAAUUUGAUCGUCAATT; HuR si2: GCAGAUGUUUGGGCCGUUUTT.

RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted using RNAiso Plus (TaKaRa, Japan) according to the manufacturer's instruction. cDNA was synthesized with PrimeScript II first-strand cDNA Synthesis Kit (TaKaRa), followed by quantitative PCR amplification with RealStar Power SYBR Mixture (GenStar, Beijing, China). Real-time quantitative PCR was performed in LightCycler 480 (Roche, Switzerland). Primer sequences were listed in Table 1.

Dual-luciferase reporter assays

U2OS cells (1×10^4) were plated in 100 μL medium in 96-well plates. The cells were transfected with 100 ng reporter plasmid with PD-L1 3′UTR and 300 ng TERC expression plasmid by lipo3000 (Invitrogen). 48 h after transfection, cells were harvested and detected using the dual-luciferase reporter assay kit (Promega, USA) according to the manufacturer's instructions.

Immunofluorescence

TERC-U2OS and pBabe-U2OS cells were grown on coverslip, washed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature, and then permeabilized in 0.5% Triton X-100 at room temperature for 30 min. Cells were washed thrice with 1× PBST and blocked with 5% goat serum for 1 h at room temperature. The cells were incubated sequentially with anti-HuR antibody (Abcam) overnight at 4°C and secondary antibody conjugated with DyLight 555

Table 1 PCR primer sequences

Primer name	Forward (5′–3′)	Reverse (5′–3′)
ACTIN	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
PD-L1	TGGCATTTGCTGAACGCATTT	TGCAGCCAGGTCTAATTGTTTT
TERC	TCTAACCCTAACTGAGAAGGGCGTAG	GTTTGCTCTAGAATGAACGGTGGAAG
TERT	AAATGCGGCCCCTGTTTCT	CAGTGCGTCTTGAGGAGCA
HuR	AACTACGTGACCGCGAAGG	CGCCCAAACCGAGAGAACA
	RNA pull-down assay:	
FLNA	CGGTGATCACTGTGGACACTA	ATTCTCCACCACGTCCACATC
Primer1	CACGGTTCCCAAGGACCTAT	GGGAGAGCTGGTCCTTCAAC
Primer2	GGAGGAGACGTAATCCAGCA	CAGGCTCCCTGTTTGACTCC
Primer3	TGTCTGTGCAGTATCTGTTCCA	AAAAGGACAGTGGGTGGCAG
Primer4	GCAACTGCTACTGCCTTTCA	AGAAGGCATGGATCCTCAGC
Primer5	CGAGATTCAGATGCCCTGGG	CAGCCACAATTCTTGCCTGT
	RIP assay:	
RIP1	CCTACTGGCATTTGCTGAACGC	CCAGATGACTTCGGCCTTGGG
RIP2	ACCAGCACACTGAGAATCAACACA	TGGCTCCCAGAATTACCAAGTGAGT
RIP3	CTGAACAAGGAGCCTCCAAGCA	TGTCCCGTTCCAACACTGAGAC
RIP4	GGAGTATTTGTAAGGTGCTTGGTCTCC	GCACAGACACTTGAGGTCTGAGAAT
RIP5	CAAGTGTCTGTGCAGTATCTGTTCCA	GCTTCCTCAGCTGTACGATGGGT
RIP6	AAAGTACCTGTCCTCAAGGAGCTCAT	CAGAACTGTTAAACAATACCAGACACTATA

for 1 h at room temperature. The coverslip was washed with PBST, mounted with DAPI, and visualized using a Zeiss microscope (Zeiss, Germany).

Fluorescence activated cell sorting

Cultured cells were collected with trypsin digestion, washed twice with FACS buffer (PBS supplement with 2% FBS), and then incubated with CD274-APC antibody (LiankeBio, Hangzhou, China) for 30 min on ice. Samples were washed with FACS buffer once and resuspended by PBS for FACS analysis (BD Calibur, USA).

RNA pull-down assay

RNA pull-down assay was performed as previously described with minor modifications (Chu et al., 2011). MCF7 cells were collected and crosslinked with 1% formaldehyde in PBS for 10 min at room temperature. 0.125 mol L^{-1} glycine was used to quench crosslinking. Harvested cells were lysed with lysis buffer (50 mmol L^{-1} Tris 7.0, 10 mmol L^{-1} EDTA, 1% SDS, add DTT, PMSF, Proteinase Inhibitors, and Superase-in before use) on ice for 10 min and sonicated at 4°C to shear the RNA to 200 nt. Biotin-labeled TERC probes (odd probes of TERC from published study (Chu et al., 2011)) and sonicated cell lysate were hybridized in hybridization buffer (500 mmol L^{-1} NaCl, 1%SDS, 100 mmol L^{-1} Tris 7.0, 10 mmol L^{-1} EDTA, 15% Formamide, add DTT, PMSF, Proteinase Inhibitors, and Superase-in fresh) at 37°C for 4 h. Streptavidin-magnetic beads were added to the hybridization tube. Hybridization continued for another 30 min at 37°C. After the hybridization, the magnetic beads were washed with wash buffer (2× SSC, 0.5% SDS, add DTT and PMSF fresh) and reverse crosslinked in RNA elution buffer (Tris 7.0, 1% SDS) and boiled for 15 min. RNA in the products were isolated by Trizol. TERC, mRNA of FLNA and PD-L1 were detected by quantitative PCR. Primer sequences were listed in Table 1.

RNA immunoprecipitation

RIP analysis was performed as previously described with minor modifications (Gagliardi and Matarazzo, 2016). Crosslinked U2OS cells were incubated with cell lysis buffer (50 mmol L^{-1} HEPES-NaOH pH 7, 10 mmol L^{-1} $MgCl_2$. Before use add 1 mmol L^{-1} DTT, 200 units/mL RNase OUT, and EDTA-free Protease Inhibitor) for 30 min on ice and sonicated at 4 °C to shear the RNA to 200 nt. Then DNase was added to the sonicated product and incubated at 37°C for 30 min. Cell lysate was supplemented with reaction buffer (1% Triton X-100, 0.1% sodium deoxycholate, 0.01% SDS, and 140 mmol L^{-1} NaCl) for immunoprecipitation. Before immunoprecipitation, protein A/G beads were incubated with anti-HuR antibody (Abcam) and IgG antibody (Abcam) for 1 h at room temperature, and then cell lysate was incubated with antibody coated beads overnight at 4°C. Then beads were washed and incubated in buffer (10 mmol L^{-1} Tris-HCl pH 7.4, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} $MgCl_2$, 0.05% NP-40, 10% SDS and proteinase K) to reverse crosslink at 55°C for 30 min. RNA in product was then isolated with Trizol. PD-L1 mRNA was detected using reverse transcription quantitative PCR. Primer sequences were listed in Table 1.

Kaplan-Meier survival analysis

TERC expression level and survival data of patients treated with chemotherapy were downloaded from TCGA data portal (https://www.cancer.gov/tcga/). TERC expression and corresponding survival data were used for Kaplan-Meier survival analysis. The median score was used to divide patients into high expression and low expression groups. A log-rank test was applied to compare the survival distributions of the two groups. The *P*-value smaller than 0.05 was considered statistically significant. Survival curves were plotted by an R package "survminer" (http://www.sthda.com/english/rpkgs/survminer).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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