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Ectopic over-expression of tristetraprolin in human cancer cells promotes biogenesis of *let-7* by down-regulation of Lin28

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ABSTRACT

Tristetraprolin (TTP) is a AU-rich element (ARE) binding protein and exhibits suppressive effects on cell growth through down-regulation of ARE-containing oncogenes. The *let-7* microRNA has emerged as a significant factor in tumor suppression. Both TTP and *let-7* are often repressed in human cancers, thereby promoting oncogenesis by derepressing their target genes. In this work, an unexpected link between TTP and *let-7* has been found in human cancer cells. TTP promotes an increase in expression of mature *let-7*, which leads to the inhibition of *let-7* target gene *CDC34* expression and suppresses cell growth. This event is associated with TTP-mediated inhibition of Lin28, which has emerged as a negative modulator of *let-7*. *Lin28* mRNA contains ARE within its 3′-UTR and TTP enhances the decay of *Lin28* mRNA through binding to its 3′-UTR. This suggests that the TTP-mediated down-regulation of Lin28 plays a key role in *let-7* miRNA biogenesis in cancer cells.

INTRODUCTION

MicroRNAs (miRNAs) are a class of short (~21–25 nt), single-stranded, non-coding RNAs. They bind to the 3′-untranslated regions (3′-UTRs) (1) or protein-coding exons of specific messenger RNAs (mRNAs) (2) and inhibit translation or promote degradation of the transcript (3). The expression of miRNA can be controlled at both the transcriptional and post-transcriptional level. This regulation is crucial, as aberrant miRNA expression has been linked to human diseases including cardiovascular disorders and cancer (4). Most miRNAs are transcribed by RNA polymerase II as long primary transcripts, termed pri-miRNAs. However, post-transcriptional regulation occurs at multiple steps of miRNA biogenesis, and thus mature miRNA expression does not always correlate with expression of pri-miRNA (5,6).

The miRNA *let-7* was initially discovered as a critical regulator of stem-cell differentiation in *Caenorhabditis elegans* (7), and is highly conserved across diverse animal species (8). It functions as a tumor suppressor by targeting multiple oncogenes and a reduction of *let-7* level is strongly associated with increased tumorigenicity and poor patient prognosis (9). It has been reported that mature *let-7* appears only after differentiation in embryonic stem (ES) cells, while the levels of *pri-* and *pre-let-7* are comparable between undifferentiated and differentiated ES cells, suggesting post-transcriptional control in *let-7* biogenesis (10,11). Recently, the Lin28 proteins have been identified as regulatory factors for *let-7* biogenesis (12–14). Mammals have two Lin28 homologs, Lin28a and Lin28b, which are indistinguishable from each other in their biochemical activities (12,14). Lin28 has been reported to interfere with the Drosha processing of *pri-let-7* (14) and Dicer processing of *pre-let-7* (12,13). In the case of the *pre-let-7*, Lin28 binds to the terminal loops of the pre-miRNA, recruits a poly (U) polymerase, terminal (U) transferase (TUT4) which adds a uridine tail to the 3′-end of the pre-miRNA and

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triggers its degradation (15–17). In human tumors, Lin28 is up-regulated and reactivated to function as an oncogene promoting malignant transformation and tumor progression (18,19). Thus, down-regulation of Lin28 in tumors that overexpress this gene may provide a means to reactivate the expression of *let-7* tumor suppressor (18). However, the mechanisms that regulate Lin28 expression are still largely unknown.

Post-transcriptional regulation of gene expression is also mediated by AU-rich elements (AREs) located in the 3′-UTR of a variety of short-lived mRNAs such as cytokines and proto-oncogenes (20). The destabilizing function of AREs is believed to be regulated by ARE binding proteins (21). One of the best-characterized ARE-binding proteins is tristetraprolin (TTP) that promotes degradation of ARE-containing transcripts (22,23). TTP expression was significantly decreased in various cancers (24), which correlates with increased expression of proto-oncogenes (25–27) and, as a result, may lead to abnormalities that contribute to cancer processes.

Here, we show that TTP acts as a positive regulator of *let-7* biogenesis by down-regulating Lin28 expression in human cancer cells. TTP expression is significantly reduced and negatively correlates with Lin28a expression in human cancer cells. *Lin28a* mRNA contains ARE within the 3′-UTR and TTP operates its destabilizing activity through binding to the first ARE in the *Lin28a* 3′-UTR. Through this activity, TTP increases *let-7* biogenesis, decreases expression of *let-7* target genes, and consequently suppresses the proliferation of ovarian cancer cells. These novel findings suggest that TTP serves as a positive regulator of *let-7* biogenesis and provides a mechanism for the coordinate expression of TTP and *let-7* observed in human cancers.

MATERIALS AND METHODS

Cell culture

Human cancer cell lines, AGS, Colo320, HCT116, MCF7, K562, HepG2, NT2 and PA1 were purchased from the Korean Cell Line Bank (KCLB-Seoul, Korea). PA1 cells were cultured in Eagle's Minimum Essential Medium (EMEM). AGS, Colo320, MCF7 and K562 cells were cultured in RPMI 1640 media. HepG2, HCT116 and NT2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). All cell lines were supplemented with 10% FBS (heat-inactivated fetal bovine serum) (WELGENE, Korea) and were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Immunohistochemistry

Immunohistochemical detection of TTP and Lin28a was performed on a tissue array slide constructed with paraffin sections from 192 cases of ovarian adenocarcinoma, 10 adjacent normal and 6 normal tissues (OV20810, US Biomax Inc., Rockville, MD, USA). After deparaffinization, primary antibody anti-human TTP antibody (sc-14030, Santa Cruz Biotechnology) or anti-human Lin28a antibody (ab46020, Abcam) at a 1:100 dilution was applied for 2°h at room temperature. Primary antibodies were detected using EnVision™+/HRP kits (DAKO, Carpinteria, CA, USA). Peroxidase activity was visualized with 3-amino-9-ethyl carbazole (Sigma). The sections were counterstained with Mayer's hematoxylin. Negative controls, in which the primary antibody incubation step was omitted, were also included for each staining. The expression of TTP and Lin28a was scored semi-quantitatively based on the intensity and proportion of staining. Staining intensity was subclassified as 0, negative; 1, weak; 2, moderate and 3, strong. The proportion of staining was scored as 1, 0–25%; 2, 26–50%; 3, 51–75% and 4, 76–100%. Staining scores were obtained by multiplying staining intensity by the proportion of staining. Two pathologists, with no prior clinical or pathological information, scored the expression at 100× magnification under light microscopy. All available areas in the section were evaluated.

Plasmids, siRNAs, transfections and dual-luciferase assay

The pcDNA3/BRF1 and the pcDNA6/V5-TTP constructs were described previously (28,29). The pcDNA3/Flag-Lin28a construct was a gift from Dr V. Narry Kim (Seoul National University, Seoul, Korea). Full-length cDNA of human *CDC34* was amplified from the cDNA of PA1 cells using the following PCR primers: 5′-CGGGATCCATGGCTCGGCCGCTAGTG-3′, 5′-CCGCTCGAGTCAGGACTCCTCCGTGCC-3′. The underlined sequences are restriction enzyme sites. PCR product was ligated into the BamHI/XhoI sites of pCMVT2B (Stratagene). PA1 cells were transfected with pcDNA6/V5-TTP, pcDNA3/Flag-Lin28a and pCMVT2B/CDC34 using the FuGENE6 *in vitro* transfection reagent (Roche) or TurboFect™ *in vitro* transfection reagent (Fermentas).

Small interfering RNAs (siRNAs) against human TTP (TTP-siRNA, sc-36761), human Lin28a (Lin28a-siRNA, sc-106829) and control siRNA (scRNA, sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz). Mature *let-7b*, mature miR26a, antisense oligonucleotides against *let-7b* (AS-let-7b, AACCACACAACCUACUACCUCA) and scrambled oligonucleotides (scRNA, AGUAGUAUGUUCCGCUUAUUGAU) were purchased from ST Pharm. Co., Ltd. (Korea). PA1 cells were transfected 24 h after plating using Lipofectamine™ RNAiMAX (Invitrogen) and were harvested 48 h. The expression levels of *TTP* or *Lin28* mRNA and protein were analyzed by RT-PCR or western blots. To monitor transfection efficiency, the GFP expression vector pEGFP-N1 (Clontech) was co-transfected with the plasmid construct or each oligonucleotide. After confirming transfection efficiency (>80%), cells were used for further study.

Two fragments of *Lin28a* 3′-UTR containing AUUUA pentamer (the first and the second AUUUA, respectively) were PCR amplified from the cDNA of PA1 cells using the following primer sets: Frag-AUUUA1 and Frag-AUUUA 2 (Supplementary Table S1). PCR products were inserted into the XhoI/NotI sites of the psiCHECK2 Renilla/firefly dual-luciferase expression vector (Promega, Madison, WI, USA). One oligonucleotide containing the first AUUUA (ARE1) within the *Lin28a* 3′-UTR (Oligo-ARE1W) was

synthesized at Integrated DNA Technologies (Coralville, IA, USA). A mutant oligonucleotides in which the AUUU A pentamer was substituted with AGCA (Oligo-ARE1M) (Supplementary Table S1) was also synthesized. The oligonucleotides were ligated into the XhoI/NotI site of the psiCHECK2 vector.

Full-length of *CDC34* 3′-UTR was amplified from the cDNA of PA1 cells using the following PCR primers: 5′-CCGCTCGAGCACCACCAGAATAAACT T-3′, 5′-TAGTTTAGCGGCCGCCTCATAAAGTAGT TTTAT-3′. The underlined sequences are restriction enzyme sites. PCR products were inserted into the XhoI/ NotI sites of the psiCHECK2 to generate psiCHECK2-CDC34 3′-UTR WT. A mutant of *CDC34* 3′-UTR in which the UACCUCA sequences within two let-7b target sites (27 and 69 bases from the start of CDC34 3′-UTR) were substituted with UGGGAGG was generated using psiCHECK2-CDC34 3′-UTR WT as a template and using a QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction. Mutagenic primers used for generation of site-directed mutant of CDC34 3′-UTR were as follows: CDC34 3′-UTR-27: 5′-GAATAAACTTGCCGAGTTTG GGAGGCTAGGGCCGGACCC-3′, 5′-GGGTCCGGC CCTAGCCTCCCAAACTCGGCAAGTTTATTC-3′; CDC34 3′-UTR-69: 5′-CTCCTTAGACGACAGACTGG GAGGCGGAGGTTTTGTGCTG-3′, 5′-CAGCACAAA ACCTCCGCCTCCCAGTCTGTCGTCTAAGGAG.

For luciferase assays, cells were cotransfected with various psiCHECK2-Lin28 3′-UTR constructs and pcDNA6/V5-TTP using the TurboFect™ *in vitro* transfection reagent (Fermentas). Transfected cells were lysed with lysis buffer and mixed with luciferase assay reagent (Promega) and the chemiluminescent signal was measured in a SpectraMax L Microplate (Molecular Devices, Sunnyvale, CA, USA). Firefly luciferase was normalized to Renilla luciferase in each sample. All luciferase assays reported here represent at least three independent experiments, each consisting of three wells per transfection.

RNP immunoprecipitation assay

RNP complexes were immunoprecipitated after reverse crosslinking between target RNA and proteins as previously described (25). Briefly, 1×10^7 cells of PA1 cells were cotransfected with 10 μg of pcDNA6/V5-TTP and psiCHECK2-Lin28a-Oligo-ARE1W or psiCHECK2-Lin28a-Oligo-ARE1M. At 24 h after transfection, the cell suspension was incubated in 1% of formaldehyde for 20 min at room temperature. The reaction was stopped with 0.25 M of glycin (pH 7.0), and cells were sonicated in modified RIPA buffer containing protease inhibitors (Roche, Indianapolis, IN, USA). RNP complexes were immunoprecipitated using protein G-agarose beads preincubated with 1 μg of anti-V5 (Invitrogen) or 1 μg of isotype control (Sigma, St Louis, MO, USA). After crosslinking reversion at 70°C for 45 min, RNA was isolated from the immunoprecipitates and treated with DNAse I (Qiagen, Valencia, CA, USA). cDNA was synthesized from the RNA and Renilla luciferase gene was amplified by PCR using Taq polymerase (Solgent, Daejeon, Korea) and Renilla luciferase specific primers (Up-ACGTGCTGGACTCCTT CATC, Down-GACACTCTCAGCATGGACGA). TTP proteins in the immunoprecipitated samples were detected by western blot analysis using an anti-V5 antibody (Invitrogen).

Electrophoretic mobility shift assay

The biotinylated RNA probes for the wild-type (Lin28a-ARE1-WT, 5′-CCCCAUUCUGGGCCAAUGU GAUUUUAUUUAUUUGCUCCCUUGGAUACUGC ACCUU-3′) and mutant (Lin28a-ARE1-MUT, 5′-CCCC AUUCUGGGCCAAUGUGAUUUUAGCAUUUGCU CCCUUGGAUACUGCACCUU-3′) constructs were generated by ST Pharm. Co., Ltd (Korea). A mutant RNA probe in which one AUUUA pentamer was substituted with AGCA was used as a negative control. Cytoplasmic extracts were prepared from HCT116 cells and TTP-transfected PA1 cells using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Pierce Biotechnology Scientific, Rockford, IL, USA). Electrophoretic mobility shift assay (EMSAs) were performed using the LightShift™ Chemiluminescent EMSA kit (Thermo Pierce Biotechnology Scientific). Binding reactions were conducted using 80.0 fmol of biotinylated RNA and 3.0 μg of cytoplasmic protein cellular extract in a binding buffer. For EMSA supershifts, anti-TTP antibody (ab36558, Abcam), anti-V5 antibody (20-783-70389, GenWay) or control antibody (I-5381, Sigma) was added to the reaction mixture. After addition of antibodies, reaction mixtures were incubated overnight on ice and resolved on 5% non-denaturing polyacrylamide gels in 0.5× Tris borate/EDTA buffer. Gels were transferred to nylon membrane (Hybond™-N^+) in 0.5× Tris borate/EDTA at 70 V for 40 min. Transferred RNAs were cross-linked to the membrane and detected using horseradish peroxidase-conjugated streptavidin (LightShift™ Chemiluminescent EMSA kit) according to the manufacturer's instructions.

SDS–PAGE analysis and immunoblotting

Proteins were resolved by SDS–PAGE, transferred onto Hybond-P membranes (Amersham Biosciences Inc.), and probed with appropriate dilutions of rabbit anti-human TTP antibody (ab36558, Abcam), anti-human Lin28a antibody (ab46020, Abcam), anti-Lin28b (sc-130802, Santa Cruz Biotechnology), anti-Drosha antibody (#3364 s, Cell Signaling), anti-Dicer antibody (sc-30226, Santa Cruz Biotechnology), anti-Ago2 antibody (#2897s, Cell Signaling), anti-BRF1 antibody (ab42473, Abcam), anti-CDC34 antibody (sc-5616, Santa Cruz Biotechnology), anti-COX-2 antibody (160106, Cayman), anti-c-Fos antibody (PC38, Calbiochem), anti-c-Myc antibody (1472-1, Epitomics), anti-K-Ras antibody (sc-30, Santa Cruz Biotechnology) and anti-cyclin D antibody (sc-246, Santa Cruz Biotechnology). Immunoreactivity was detected using the ECL detection system (Amersham Biosciences Inc.). Films were exposed at multiple time points to ensure that the images were not saturated. If required, the band densities were analyzed

with NIH image software and normalized by comparison with the densities of internal control β-actin bands.

RNA kinetics, quantitative real-time PCR and semi-qRT-PCR

For RNA kinetic analysis, we used actinomycin D and assessed *Lin28a* mRNA expression by quantitative real-time PCR (qRT-PCR). Three micrograms of DNase I-treated total RNA was reverse transcribed using oligo-dT and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed by monitoring the increase in fluorescence in real-time of the SYBR Green dye (QIAGEN, Hilden, Germany) using StepOnePlus™ Real-time PCR systems (Applied Biosystems). Semi-qRT-PCR was performed using Taq polymerase (Solgent, Daejeon, Korea) and PCR primer pairs (Supplementary Table S1). mRNA half-life was calculated from the non-linear regression of the mRNA levels at 0-, 30- and 60-min time points following addition of actinomycin D using GraphPad Prism 4.00 software based on a one-phase exponential decay model.

miRNA analysis

Total RNA was isolated with Trizol reagent (Ambion). Quantitative levels of mature *let-7a*, *let-7b, let-7f, let-7 g, miR-16, miR302b* and *RNU6B* were determined using TaqMan® MicroRNA Assay kits (let-7a-000377, let-7b-002619, let-7f-000382, let-7 g-002282, miR-16-000391, miR-302b-000531 and RNU6B-001093, Applied Biosystems) according to the manufacturer's protocol. Mature microRNAs and *RNU6B* were detected using StepOne Plus by monitoring in real-time the increase in fluorescence of TaqMan probe-based detection (Applied Biosystem). To determine the expression levels of *pri-let-7* and *pre-let-7*, qRT-PCR was performed using EvaGreen qPCR Mastermix (Applied Biological Materials Inc.), StepOne Plus™ Real-time PCR systems (Applied Biosystems) and the primers sets for pri-let-7a, pre-let-7a, pri-let-7b, pre-let-7b, pri-let-7f, pre-let-7f, pri-let-7 g and pre-let-7 g (Supplementary Table S1).

Cell viability/proliferation

For the MTS cell proliferation assay, PA1 cells were plated in triplicate at 1.0×10^4 cells/well in 96-well culture plates in EMEM media. At 24 h after plating, CellTiter 96® AQueous One solution reagent (Promega) was added to each well according to the manufacturer's instructions, and absorbance at 490 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Statistical analysis

For statistical comparisons, *P* values were determined using Student's *t*-test or one-way ANOVA.

RESULTS

TTP positively regulates let-7 expression in human cancer cells

Both the TTP ARE-binding protein and the *let-7* miRNA contribute to the post-transcriptional regulation of genes as the expression of both TTP and *let-7* are often repressed in human cancers (9,24,30). Thus, it is highly possible that there are links between their expressions in cancer cells. We initially sought to determine whether overexpression of TTP could affect the biogenesis of *let-7* in human cancer cells. TTP expression levels were examined by western blotting in four human cancer cell lines including PA1 (ovarian cancer), HCT116 (colorectal cancer), Colo320 (colorectal cancer) and AGS (stomach cancer) cells. The expression level of TTP was high in HCT116 and AGS and low in PA1 and Colo320 cells (Figure 1A). The ovarian cancer PA1 cells with low expression level of endogenous TTP were transfected with pcDNA6/V5-TTP (PA1/TTP) or the control pcDNA6/V5 vector (PA1/pcDNA) and we analyzed the levels of endogenous *let-7* in PA1/TTP and PA1/pcDNA cells by quantitative RT-PCR (qRT-PCR). Overexpression of TTP in PA1/TTP cells was confirmed by western blots (Figure 1B), and overexpression of TTP increased the levels of mature *let-7a*, *let-7b*, *let-7f* and *let-7 g* miRNAs in PA1/TTP cells (Figure 1C). Notably, these changes were not attributed to different rates of *let-7* transcription, as the levels of both *pri-* and *pre-let-7* transcripts were comparable between PA1/TTP and PA1/pcDNA cell lines (Figure 1C). To determine whether TTP overexpression affects the level of other miRNAs, we analyzed the levels of mature *miR-16* and *miR-302b* in PA1/TTP and PA1/pcDNA cells by quantitative RT-PCR (qRT-PCR). TTP overexpression did not affect the levels of both *miR-16* and *miR-302b* (Supplementary Figure S1), suggesting the specific effect of TTP on *let-7*. These results indicate that TTP regulates *let-7* biogenesis in PA1 cells at the post-transcriptional level.

TTP regulates the expression level of the let-7 target gene CDC34

Our next goal was to determine whether TTP controls the expression levels of *let-7* target genes. It has been reported that *let-7* functions as a tumor suppressor by down-regulating *K-Ras*, *cyclin D, CDC34* and c-*Myc*, as well as several genes involved in cell cycle and cell division control (31–34). To determine the effects of TTP overexpression on the expression of *let-7* target genes, we analyzed the expression level of *K-Ras*, *cyclin D, CDC34* and *c-Myc* by RT-PCR and western blots in PA1 cells transfected with pcDNA6/V5-TTP or the control pcDNA6/V5 vector. We also analyzed the expression level of TTP-target genes including *VEGF*, *COX2* and *c-Fos* genes (26,27,29). Interestingly, while overexpression of TTP resulted in significant decrease in the expression level of *CDC34* (Figure 2A and B), it did not affect the expression level of any of the other genes (Supplementary Figure S2). Down-regulation of *CDC34* by TTP overexpression was confirmed by semi-quantitative

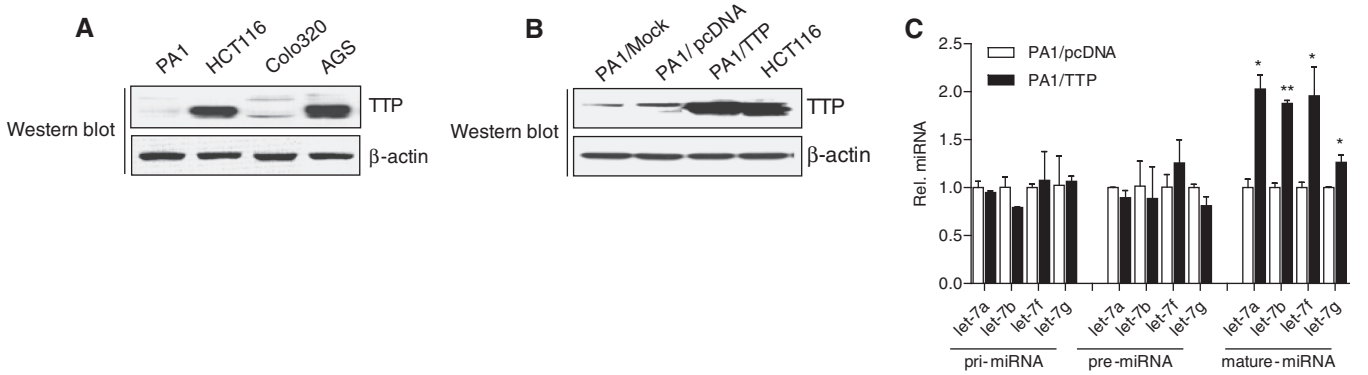

Figure 1. TTP positively regulates the level of mature *let-7* in cells. (**A**) TTP protein level in human cancer cell lines measured by western blot assays. PA1 cells with low TTP expression and HCT116 cells with high TTP expression were selected for further study. (**B** and **C**) Overexpression of TTP increases the levels of *let-7*. PA1 cells were transiently transfected with pcDNA6/V5-TTP (PA1/TTP) or empty vector pcDNA6/V5 (PA1/pcDNA) for 24 h. (B) TTP protein levels determined by western blot assays. Cell lysate from HCT116 cells was included as a positive control. (C) Levels of pri-, pre- and mature-miRNA of *let-7* were determined by quantitative RT-PCR (qRT-PCR). The levels obtained from PA1/pcDNA were set to 1.0. Results shown represent the means ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$).

RT-PCR (semi-qRT-PCR) (Figure 2A, top panel), western blots (Figure 2A, bottom panel) and qRT-PCR (Figure 2B). Coinciding with a previous report (34), transfection of *let-7b* decreased the level of *CDC34* (Figure 2C and D). On the contrary, down-regulation of *let-7b* by treatment with *anti-let-7b* oligonucleotides (AS-let-7b) (Figure 2E), which act as competitive inhibitors of *let-7b*, attenuated the TTP-induced decrease of *CDC34* (Figure 2F). To determine whether the inhibitory effect of TTP on CDC34 is mediated by increased *let-7b*, we prepared a luciferase reporter gene linked to the wild-type *CDC34* 3′-UTR (CDC34 3′-UTR WT) or mutant *CDC34* 3′-UTR (CDC34 3′-UTR MUT) with point mutations in *let-7b* target sites in the CDC34 3′-UTR. *Let-7b* was also used as a control. While TTP overexpression or let-7b treatment significantly decreased the luciferase activity of luciferase reporter gene containing wild-type *CDC34* 3′-UTR, it did not affect that containing mutant CDC34 3′-UTR (Figure 2G). Collectively, these results indicate that TTP inhibits *CDC34* expression and *let-7b* mediates the effect.

Overexpression of CDC34 attenuates the inhibitory effect of TTP on the growth of PA1 cells

We next examined whether TTP overexpression affects the proliferation of PA1 cells *in vitro*. PA1 cells were transfected with pcDNA6/V5-TTP or pcDNA6/V5 and we evaluated the effects of TTP overexpression on cellular metabolic activity using MTS cell proliferation assay. The results showed that TTP overexpression decreased the MTS value (Figure 3A). It has been reported that TTP overexpression can induce apoptosis (35). However, overexpression of TTP did not induce apoptosis in PA1 cells (Supplementary Figure S3), indicating that decrease in the MTS value is resulted from TTP-induced suppression of the cell growth. TTP expression can suppress cell growth through destabilization of *VEGF*, *COX2*, and *c-Fos*, *c-Myc*, and *cyclin D* mRNAs (26,27,29). However, as shown in Supplementary Figure S2, there was no difference in expression of these genes between PA1/pcDNA and PA1/TTP cells. Also, TTP overexpression did not lead to a change in the expression level of several genes involved in the control of cell cycle such as *CCNA1*, *CCNB1*, *CCNB2*, *CCND1*, *CCND2*, *CCND3* and *CDK2* (Supplementary Figure S2). CDC34 is an E2 ubiquitin-conjugating enzyme that supports cell growth by facilitating the proteasome-mediated degradation of multiple cell cycle regulators (36). To determine whether the TTP-induced inhibition of cell growth was mediated by a reduction in CDC34 levels, we transfected PA1 cells with *CDC34* cDNA, whose expression is not affected by TTP and *let-7b* because the transcript does not contain a *CDC34* 3′-UTR. Overexpression of CDC34 (Figure 3B and C) increased the growth of PA1/TTP to 92% of PA1/pcDNA (Figure 3D). These results show that TTP expression suppresses the growth of PA1 cells through down-regulation of CDC34.

TTP down-regulates the expression of Lin28

TTP overexpression did not affect the levels of *pri-* and *pre-let-7b* but increased mature *let-7b* level (Figure 1C). Thus, we hypothesized that TTP controls *let-7b* biogenesis by modulating the expression level of proteins involved in the post-transcriptional regulation of *let-7b* biogenesis. The core components involved in the post-transcriptional regulation of miRNA biogenesis are Drosha, Dicer, Argonaute (AGO) and Lin28 (6); as such, we analyzed the levels of these known *let-7b* regulatory factors in PA1/TTP and PA1/pcDNA cells. Western blot analysis revealed that while TTP overexpression did not affect the levels of Drosha, Dicer and AGO, it decreased the expression of the negative regulatory factor Lin28a (Figure 4A). TTP-induced reduction of *Lin28a* level was confirmed by semi-qRT-PCR (Figure 4B) and qRT-PCR (Figure 4C). TTP also decreased Lin28b level in PA1 cells (Figure 4D).

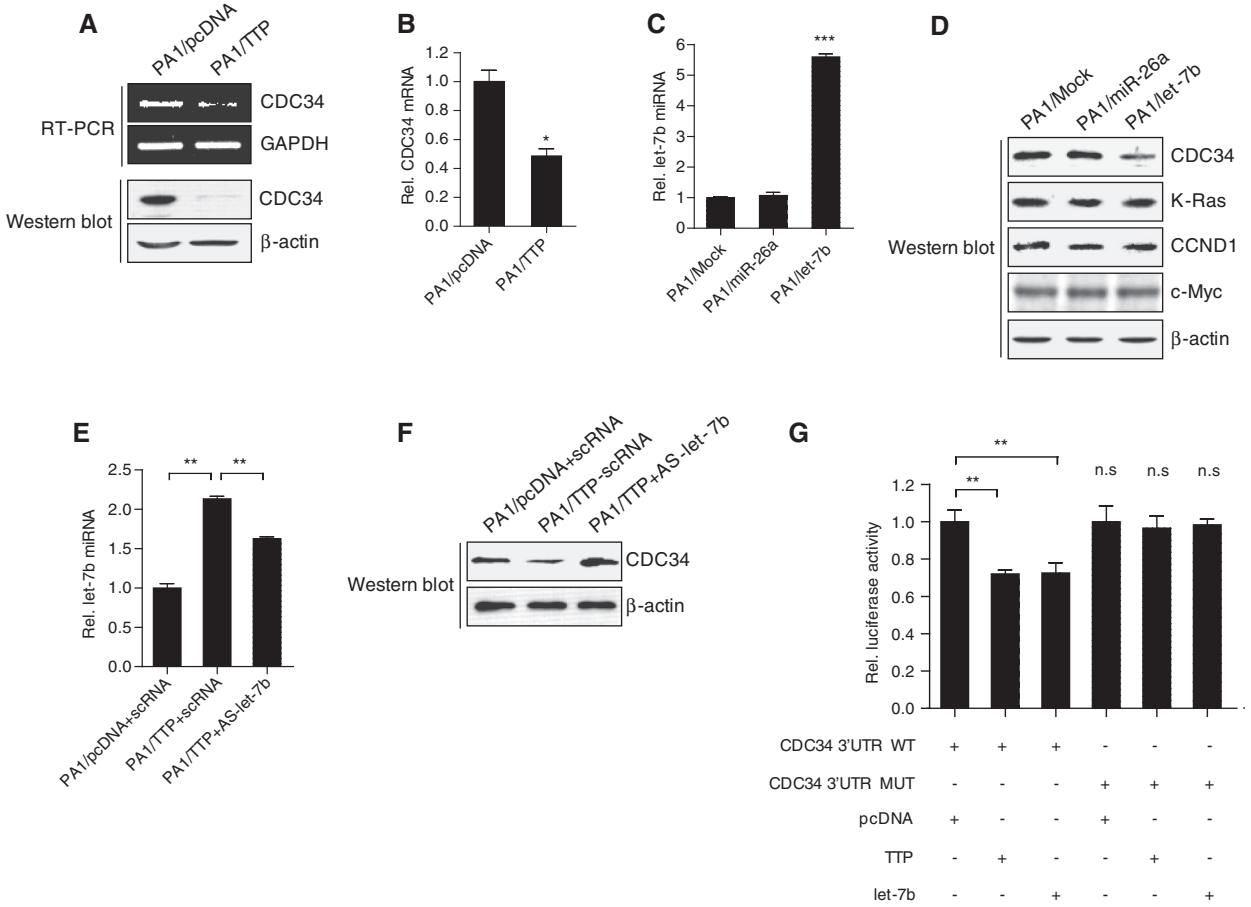

Figure 2. TTP negatively regulates the expression of the *let-7b* target gene *CDC34*. (**A** and **B**) Overexpression of TTP downregulates *CDC34* levels. The levels of *CDC34* in PA1 cells transfected with pcDNA6/V5-TTP and pcDNA6/V5 was determined by semi-qRT-PCR (A, top panel), western blots (A, bottom panel) and qRT-PCR (B). The data represent the mean ± SD of 3 independent experiments ($*P < 0.05$). (**C** and **D**) Overexpression of *let-7b* suppresses *CDC34* expression. PA1 cells were transfected with 50 nM of *let-7b* or scrambled miRNA oligonucleotides for 24 h. (C) The levels of *let-7b* was determined by qRT-PCR. The levels obtained from mock-treated cells (PA1/Mock) were set to 1.0. Data are presented as the mean ± SD ($n = 3$) ($***P < 0.001$). (D) CDC34 protein levels were measured by western blot assays. (**E** and **F**) Knockdown of *let-7b* abolishes the suppression of CDC34 expression induced by TTP. PA1 cells were cotransfected with pcDNA6/V5-TTP, anti-let-7b oligonucleotides (AS-let-7b) or scrambled oligonucleotides (scRNA) for 24 h. (E) The level of *let-7b* was determined by qRT-PCR. The levels of *let-7b* in PA1 cells transfected with pcDNA and scRNA oligonucleotides were set to 1.0. Data are presented as the mean ± SD ($n = 3$) ($**P < 0.01$). (F) The level of CDC34 was determined by western blot assays. (**G**) Down-regulation of CDC34 by TTP is mediated by let-7b. PA1 cells were transfected with various combination of pcDNA6/V5, pcDNA6/V5-TTP, psiCHECK2-CDC34 3′-UTR WT, psiCHECK2-CDC34 3′-UTR MUT and *let-7b* for 24 h. After normalizing luciferase activity, the luciferase activity obtained from PA1 cells co-transfected with the pcDNA6/V5 and psiCHECK2-CDC34 3′-UTR WT was set to 1.0. Results shown represent the means ± SD of three independent experiments ($**P < 0.01$). ns, not significant.

We next determined the effect of TTP down-regulation on the expression level of Lin28a. The expression level of TTP in PA1 cells was extremely low (Figure 1A) and thus this cell line was not suitable for TTP knockdown studies. Instead, the human colon cancer HCT116 cell line that expressed high levels of TTP was used for TTP knockdown experiments. The inhibition of TTP by siRNA (Figure 4E and G) increased *Lin28a* level in HCT116 cells (Figure 4F and G). We also determined the effects of TTP inhibition on the expression levels of *let-7b*, *let-7b* target genes and cell growth. Down-regulation of TTP decreased the *let-7b* level (Figure 4H), increased the *let-7b* target gene, CDC34 and cell growth (Figure 4G and I). Collectively, our results suggest that TTP down-regulates Lin28a expression in cancer cells.

Expression of TTP is inversely correlated with Lin28a in human cancer cell lines and ovarian adenocarcinoma tissues

To determine if TTP expression is inversely correlated with Lin28a expression in human cancer cell lines, we analyzed the expression of TTP and Lin28a in seven human cell lines: PA1, MCF7 (breast adenocarcinoma), AGS, K562 (erythroleukemia), HepG2 (hepatocellular carcinoma) and NT2 (neuron-committed teratocarcinoma). While cell lines with low TTP levels showed high expression levels of Lin28a, those with high TTP levels showed a low level of Lin28a (Figure 4J), suggesting an inverse correlation between TTP expression and Lin28a expression in human cancer cell lines.

Next, we investigated the expression of TTP and Lin28a proteins in 192 ovarian adenocarcinoma, 10 adjacent

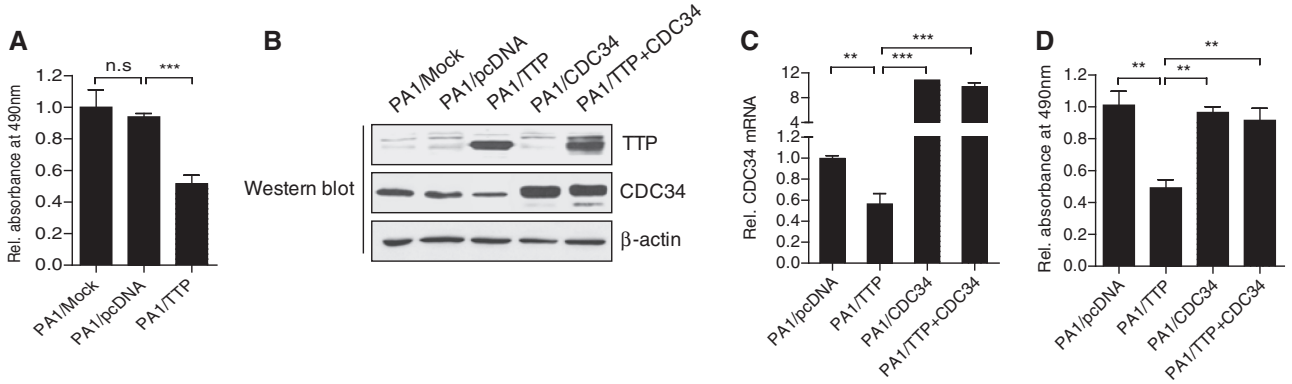

Figure 3. Transfection of *CDC34* cDNA without the 3′-UTR (or overexpression of CDC34) attenuates the inhibitory effect of TTP on the growth of PA1 cells. (**A**) Overexpression of TTP suppresses the growth of PA1 cells. PA1 cells transfected with pcDNA6/V5-TTP or pcDNA6/V5 were seeded at 1.0×10^4 cells per well in 96-well plates. Cell viability was assessed at 24 h after seeding by measuring absorbance at 490 nm using a MTS cell proliferation assay. The values obtained from PA1/Mock cells were set to 1.0. The data represent the mean ± SD of three independent experiments (***$P < 0.001$). ns, not significant. (**B–D**) Transfection of *CDC34* cDNA without 3′-UTR abrogates the suppressive effect of TTP on PA1 growth. PA1 cells were transfected with a combination of pcDNA6/V5-TTP and pCMVT2B/CDC34 for 24 h. (B) The levels of TTP and CDC34 proteins were measured by western blot assays. (C) *CDC34* levels were measured by qRT-PCR. The levels obtained from PA1/pcDNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (**$P < 0.01$; ***$P < 0.001$). (D) Cell viability was assessed by measuring absorbance at 490 nm using a MTS cell proliferation assay. The levels obtained from PA1/pcDNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (**$P < 0.01$).

normal and 6 normal tissues by immunohistochemical staining. TTP expression was high in normal ovarian tissues (mean staining score, 6.23 ± 0.40) but low in ovarian adenocarcinoma (mean staining score, 2.66 ± 0.18; $P < 0.0001$). On the contrary, Lin28a expression was extremely low in normal ovarian tissues (mean staining score, 0.52 ± 0.03) and high in ovarian adenocarcinoma (mean staining score, 5.43 ± 0.26; $P < 0.0001$) (Figure 4K and Table 1). There were no significant differences in TTP and Lin28a expression according to tumor stage (Table 1). These results show that Lin28a expression is inversely correlated with TTP in ovarian adenocarcinoma.

TTP destabilizes Lin28a mRNA

To determine whether decreased expression of Lin28a resulted from changes in the stability of *Lin28a* mRNA, the half life of this mRNA was calculated from the mRNA levels measured by qRT-PCR in PA1 cells transfected with pcDNA6/V5-TTP or control pcDNA6/V5 vector. Overexpression of TTP protein in PA1/TTP cells was confirmed by western blot analysis (Figure 5A). In the control PA1/pcDNA cells, *Lin28a* mRNA was stable for 2 h after actinomycin D treatment. However, in PA1/TTP cells, the half-life of *Lin28a* mRNA was 1 h 30 min (Figure 5B). We also determined the half-life of *Lin28a* mRNA in HCT116 cells treated with siRNA against TTP (HCT116/TTP-siRNA) or control siRNA (HCT116/scRNA). In the control HCT116 and HCT116/scRNA cells, the half-life of *Lin28a* mRNA was 40 min. However, in HCT116/TTP-siRNA cells, *Lin28a* mRNA was stable until 3 h after actinomycin D treatment (Figure 5C). To determine whether regulation of *Lin28a* mRNA decay by TTP is biologically important, we compared the *Lin28a* mRNA half-life between HCT116 with high TTP levels and PA1 with low TTP level. As shown in Figure 5D, the half-life of *Lin28a* mRNA in PA1 cells was longer ($t^{1/2}$ = 4 h 50 min) than that in AGS cells ($t^{1/2}$ = 35 min). These results indicate that the TTP expression contributes to a decrease in Lin28a levels through destabilization of *Lin28a* mRNA.

TTP decreases the expression of luciferase mRNA containing the Lin28 3′-UTR

TTP protein regulates mRNA stability through binding AREs within the mRNA 3′-UTR (22,23,29). Analysis of the 3259-bp human *Lin28a* 3′-UTR revealed the presence of two pentameric AUUUA motifs (Figure 5E). To determine whether down-regulation of Lin28a expression by TTP is mediated through the *Lin28a* mRNA 3′-UTR, we made use of a luciferase reporter gene linked to the *Lin28a* 3′-UTR fragments containing each pentameric AUUUA motif; Frag-AUUUA1 (containing the first pentameric AUUUA) and Frag-AUUUA2 (second pentameric AUUUA) in the plasmid psiCHECK2 (Figure 5E). When PA1 cells were transfected to overexpress TTP (Figure 5A), luciferase activity from Frag-AUUUA1 was inhibited; however, Frag-AUUUA2 did not respond to TTP (Figure 5F, left panel). The results suggest that the Frag-AUUUA1 within the *Lin28a* 3′-UTR is responsible for destabilization of *Lin28a* mRNA by TTP. To determine the importance of the first AUUUA pentamer (ARE1) within Frag-AUUUA1, we prepared luciferase reporter genes containing wild-type (Oligo-ARE1W, containing wild-type ARE1) and mutant (Oligo-ARE1M, containing AUUUA sequences substituted with AGCA) oligonucleotides (Figure 5E). While Oligo-ARE1W responded to TTP (60% inhibition), Oligo-ARE1M did not respond to TTP (Figure 5F, right panel). These results strongly suggest that the first AUUUA pentamer within the *Lin28a* 3′-UTR is involved in TTP inhibitory activity in PA1 cells. *Lin28b* mRNAs also contain AUUUA pentamers within their 3′-UTR and TTP can

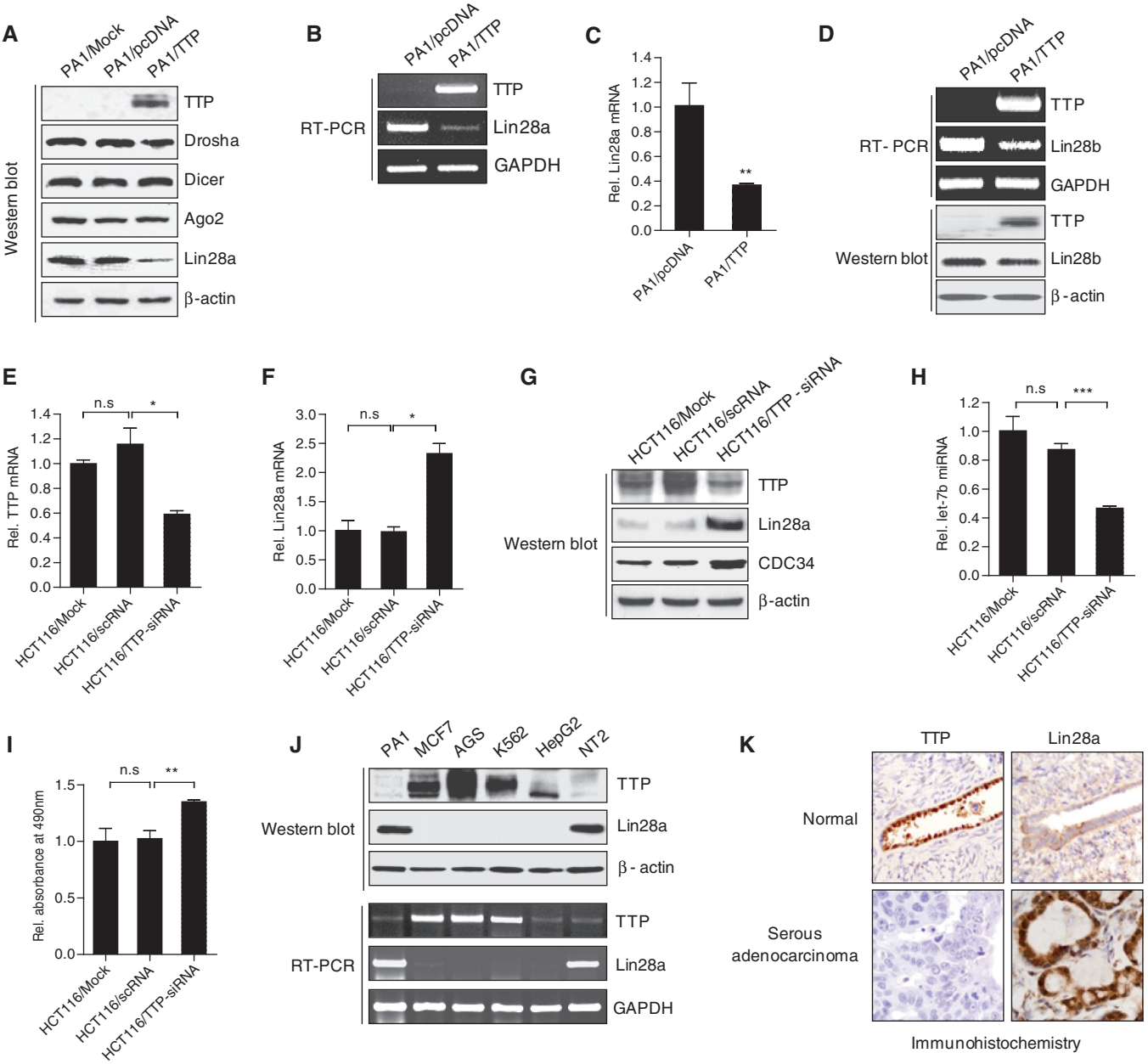

Figure 4. TTP negatively regulates the levels of Lin28a in human cancer cells. (**A–D**) Overexpression of TTP inhibits Lin28a levels in PA1 cells. PA1 cells were transfected with pcDNA6/V5-TTP or pcDNA6/V5 for 24 h. (A) The levels of TTP, Drosha, Dicer, Ago2 and Lin28a proteins were determined by western blot assays. (B) The expression levels of *TTP* and *Lin28a* were determined by semi-qRT-PCR. (C) The level of *Lin28a* was determined by qRT-PCR. The levels obtained from PA1/pcDNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (**$P < 0.01$). (D) Overexpression of TTP decreases *Lin28b* levels in PA1 cells. The level of TTP and *Lin28b* were determined by RT-PCR (top panel) and western blot (bottom panel). (**E–I**) Downregulation of TTP by siRNA increases Lin28a levels and decreases let-7b in HCT116 cells. HCT116 cells were transfected with TTP-specific (TTP-siRNA) or scRNA. After 24 h, the levels of TTP and Lin28a were determined by qRT-PCR (E and F) and western blot assays (G). The level of *let-7b* was determined by qRT-PCR (H) and cell viability was assessed by measuring absorbance at 490 nm using a MTS cell proliferation assay (I). The levels obtained from mock-transfected HCT116 cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (**$P < 0.01$; ***$P < 0.001$). ns, not significant. (**J**) The level of TTP protein is inversely correlated with those of Lin28a protein within several human cell lines. Levels of TTP and Lin28a proteins were determined by western blot analysis in PA1 (ovarian teratocarcinoma), MCF7 (breast adenocarcinoma), AGS (gastric adenocarcinoma), K562 (erythroleukemia), HepG2 (hepatocellular carcinoma) and NT2 (neuronally committed teratocarcinoma) cells. β-Actin was detected as the loading control. (**K**) TTP expression level is inversely correlated with that of Lin28a in human ovarian tissues. Representative TTP and Lin28a immunohistochemical staining in normal and ovarian adenocarcinoma tissues. Normal ovarian tissues showed strong immunoreactivity for TTP, where as the ovarian adenocarcinoma showed strong positive staining for Lin28a.

Table 1. TTP and Lin28a expression and clinicopathologic features of patients with ovarian adenocarcinoma

	N	TTP		Lin28a	
		Staining score[a]	*P*-value	Staining score[a]	*P*-value
Disease index					
Adenocarcinoma	182	2.66 ± 0.18	<0.0001	5.43 ± 0.26	<0.0001
Normal	16	6.23 ± 0.40		0.52 ± 0.03	
Tumor stage					
I	132	2.79 ± 0.22	0.306**	5.60 ± 0.31	0.594**
II	26	2.15 ± 0.39		5.04 ± 0.68	
III	15	3.06 ± 0.76		4.67 ± 0.84	
IV	9	1.56 ± 0.47		5.33 ± 1.15	

[a]Mean ± SEM.
***P*-values were determined using one-way ANOVA.

down-regulate a luciferase reporter gene containing the *Lin28b* 3′-UTR at its 3′-end (Figure 5G and H).

TTP binds to the first AUUUA pentamer within the Lin28a mRNA 3′-UTR

To determine whether TTP interact with ARE1 of *Lin28a* 3′-UTR, PA1 cells were cotransfected with pcDNA6/V5-TTP (V5-TTP) and psiCHECK2-Lin28a-Oligo-ARE1W (wild-type ARE1) or psiCHECK2-Lin28a-Oligo-ARE1M (mutant ARE1). After immunoprecipitation with anti-V5 or control antibody, the presence of TTP was determined by western blot using an anti-V5 antibody (Figure 5I). Total RNA was extracted from the immunoprecipitates and the presence of luciferase mRNA was analyzed by RT-PCR using PCR primers specific to the luciferase gene. The amplified PCR product was observed in immunoprecipitates from cells transfected with psiCHECK2-Lin28a-Oligo-ARE1W and pcDNA6/V5-TTP (Figure 5I). However, PCR product was not detected in samples from cells transfected with psiCHECK2-Lin28-Oligo-ARE1M and pcDNA/TTP (Figure 5I). PCR product was not also detected in immunoprecipitates obtained using control antibody. These results demonstrate that TTP interacts specifically with the *Lin28a* ARE1.

To confirm the interaction of TTP with *the ARE1 of the Lin28a 3′-UTR, RNA EMSA* was conducted using a biotinylated RNA probe containing the wild-type or mutant ARE1 of *Lin28a* . The RNA probes used for RNA EMSA were the same as those used for the luciferase assay. Cytoplasmic extracts were prepared from PA1 cells transfected with pcDNA6/V5-TTP and incubated with biotinylated RNA probe containing the wild-type or mutant ARE1 of *Lin28a* 3′-UTR. When RNA EMSA was conducted using the wild-type *Lin28a-ARE1* probe, a dominant RNA-protein complex was observed; however, mutant *Lin28a-ARE1* prevented the formation of this complex. Complex formation was supershifted with an anti-V5 antibody (Figure 5J). Next, we determined the association between endogenous TTP and *Lin28a-ARE1*. For this purpose, we used high TTP-expressing HCT116 cells (Figure 1A). When the wild-type *Lin28a-ARE1* probe was mixed with cytoplasmic extracts from HCT116 cells, a dominant probe-protein complex was observed (Figure 5K) while the mutant *Lin28a-ARE1* probe failed to form the complex. Formation of the *Lin28a-ARE1* probe-protein complex was reduced by preincubation of the reaction mixture with an anti-TTP antibody but not with a control antibody (Figure 5K). Taken together, these data strongly suggest that repression of Lin28a occurs through interaction of TTP with the ARE1 of *Lin28a* 3′-UTR.

Overexpression of Lin28a attenuates the inhibitory effects of TTP on let-7b expression and cell growth

Based on our results, it was speculated that TTP induces *let-7b* level through down-regulation of Lin28a. To confirm this hypothesis, we first determined whether knockdown of *Lin28a* by siRNA enhanced *let-7b* expression in PA1 cells. Western blot analysis confirmed the inhibition of Lin28a expression in PA1 cells (PA1/Lin28a-siRNA) by siRNA treatment (Figure 6A). As expected, *let-7b* expression level in PA1/Lin28a-siRNA was increased compared to PA1 cells treated with control siRNA (PA1/scRNA) (Figure 6B). In addition, inhibition of *Lin28a* decreased the level of CDC34 (Figure 6C). To confirm the involvement of Lin28a for the TTP-induced increase of *let-7b* level, we co-transfected PA1 cells with pcDNA6/V5-TTP and pcDNA3/Flag-Lin28a, which does not contain the *Lin28a* 3′-UTR. At 24-h post-transfection, cells were analyzed for the expression of *let-7b*, CDC34 and proliferation. Overexpression of Lin28a (Figure 6D) abrogated the effect of TTP on the expression of *let-7b* and CDC34 (Figure 6E). In addition, overexpression of Lin28a restored the growth of PA1/TTP to 87% of PA1/pcDNA (Figure 6F). These results indicate that TTP affects the expression level of *let-7b* and CDC34 and the cell growth through down-regulation of Lin28a in PA1 cells.

DISCUSSION

The miRNA *let-7* controls the expression of multiple oncogenes in cancer cells. As such, understanding the regulation of *let-7* expression is crucial as alteration of *let-7* expression has been linked to human diseases, including several cancers (9). TTP is an ARE-binding protein that promotes destabilization of ARE-containing mRNAs (22,23,29). Here, we described a role for TTP in the regulation of *let-7* expression. When we overexpressed TTP in cancer cells, expression level of *let-7* was increased. On the contrary, down-regulation of TTP decreased the *let-7* level. Thus, our data indicate that TTP is a positive regulator for *let-7* expression.

Regulation of *let-7* expression occurs at both the transcriptional and post-transcriptional levels. It is likely that TTP regulates *let-7* expression post-transcriptionally, as TTP did not affect the level of *pri-let-7* but affected the level of mature *let-7*. Post-transcriptional regulation

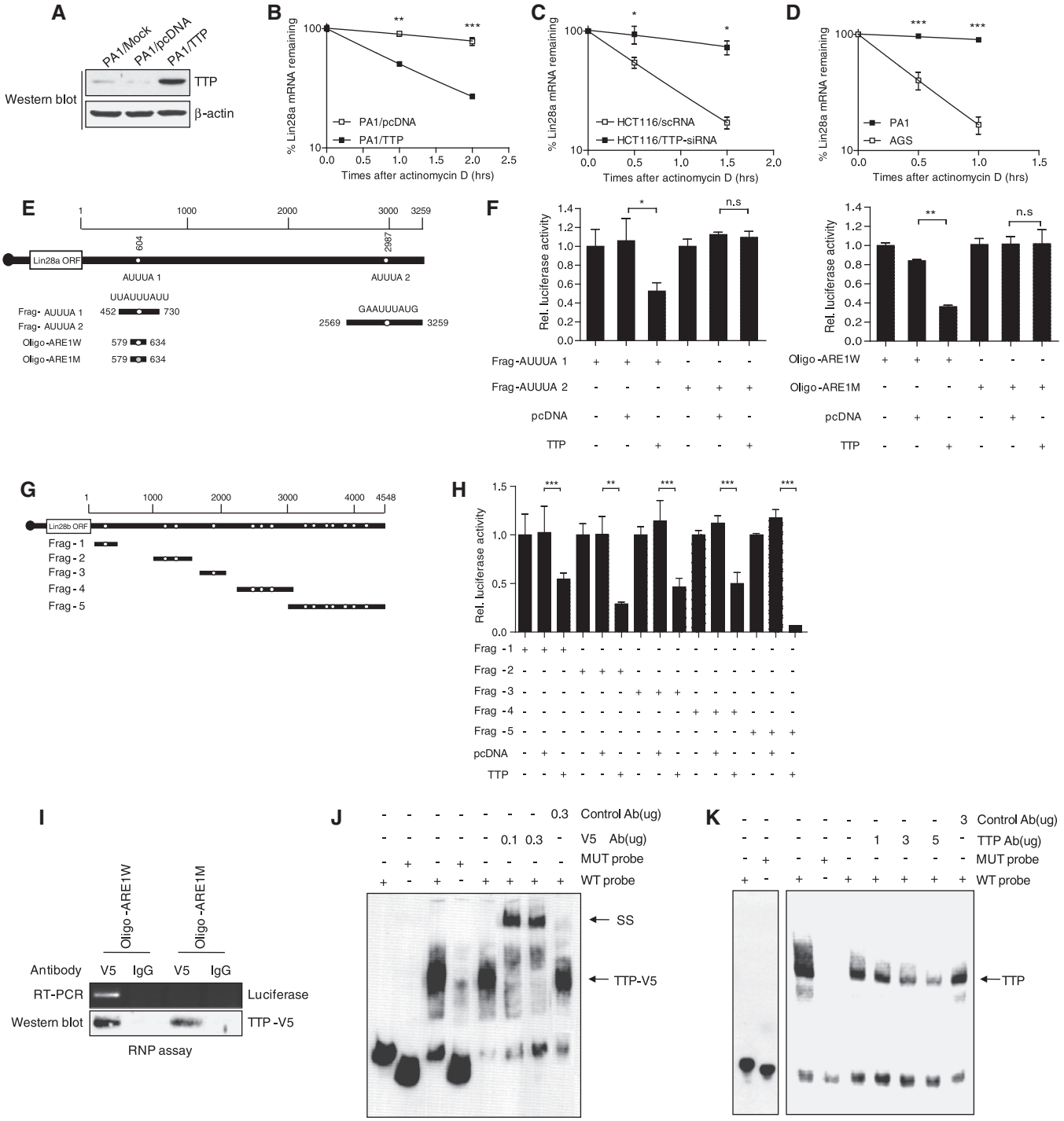

Figure 5. TTP enhances the decay of *Lin28a* mRNA through interaction with an ARE within the *Lin28a* mRNA 3′-UTR. (**A** and **B**) TTP destabilizes *Lin28a* mRNA. PA1 cells were transfected with pcDNA6/V5-TTP or pcDNA6/V5 for 24 h. (A) The level of TTP protein was determined by western blot assays. (B) Expression of *Lin28a* mRNA in PA1 cells was determined by qRT-PCR and mRNA half-life was calculated from the non-linear regression of the mRNA levels at the indicated times after the addition of 5.0 μg/ml actinomycin D. A one-phase model of exponential decay was used to derive the indicated mRNA decay curves. Results shown on the graph represent the means ± SD of three independent experiments (**$P < 0.01$; ***$P < 0.001$). (**C**) Inhibition of TTP by siRNA enhances Lin28a mRNA stability. HCT116 cells were transfected with siRNA against TTP-specific (TTP-siRNA) or scRNA for 24 h. Expression of *Lin28a* mRNA in HCT116 cells was determined by qRT-PCR and mRNA half-life was calculated as described in (B). Results shown on the graph represent the means ± SD of three independent experiments (*$P < 0.05$). (**D**) Lin28a mRNA half-life in PA1 cell with low TTP level is longer than that in AGS cells with high TTP level. Expression of *Lin28a* mRNA in PA1 and AGS cells was determined by qRT-PCR and mRNA half-life was calculated as described in (B). Results shown on the graph represent the means ± SD of three independent experiments (***$P < 0.001$). (**E** and **F**) The first AUUUA pentamer (ARE1) within the *Lin28a* 3′-UTR is essential for the inhibitory effect of TTP. (E) Schematic representation of the luciferase reporter constructs used in this study. Fragments (Frag) and oligonucleotides (Oligo) derived from the *Lin28a* mRNA 3′-UTR were cloned downstream of the luciferase reporter gene in the psiCHECK2 luciferase expression vector. White circles, wild-type (W) pentameric motif AUUUA; gray circles, mutated (M) motif AGCA. (F) PA1 cells were co-transfected with

(continued)

occurs at multiple steps of miRNA biogenesis (5,6). Recently, the Lin28 proteins have been identified as negative regulatory factors for *let-7* biogenesis (12–14). We provided several lines of evidence that TTP enhances *let-7b* expression by down-regulation of Lin28a expression. First, *Lin28a* mRNA contains ARE within its 3′-UTR and TTP binds to the ARE of *Lin28a* mRNA; second, TTP enhances the decay of *Lin28a* mRNA and a luciferase reporter gene containing the *Lin28a* 3′-UTR at its 3′-end. Overexpression of Lin28a, whose transcript does not contain a *Lin28a* 3′-UTR, attenuates the increase of *let-7b* induced by TTP. So, our data suggest that TTP interacts with the 3′-UTR of *Lin28a* mRNA to enhance the decay of *Lin28a* mRNA, leading to increased expression of *let-7b*. An inverse correlation was noted between the expression levels of TTP and Lin28a in tissues of ovarian adenocarcinoma, indicating that the mechanism by which TTP inhibits Lin28a is fundamentally important in cancer.

Based on our results, TTP-induced down-regulation of Lin28 was followed by the inhibition of cancer cell growth. In addition to blocking *let-7* biogenesis (12–14), Lin28 has been reported to bind to a specific subset of mRNAs including those for *IGF-2*, *Oct4* and several cell cycle-related factors, thereby modulating their translation (37–39). The observed growth inhibition following TTP-mediated Lin28a repression is likely the result of restoration in *let-7b* miRNA expression, which blocks CDC34 translation, a target mRNA involved in promoting cell cycle progression (34). We provided evidence supporting this hypothesis as TTP overexpression decreased the expression level of CDC34 and inhibition of TTP by siRNA increased that inhibition. Overexpression of Lin28a without a 3′-UTR blocked the increase of *let-7b*, and decrease of CDC34 and inhibition of cell growth induced by TTP overexpression. Inhibition of *let-7b* by antisense treatment attenuated the decrease of CDC34 induced by TTP overexpression and overexpression of the *CDC34* ORF without a 3′-UTR attenuated the inhibitory effect of TTP on the cell growth. Interestingly, antisense oligonucleotide in TTP-overexpressing cells did not reduce the *let-7b* level to that of mock-transfected cells (Figure 2E), suggesting that TTP might inhibit the antisense-mediated silencing of let-7b by regulating the expression and/or activity of factors involved in *let-7b* degradation. *CDC34* may not be the only gene whose expression is affected by TTP, as the expression level of another *let-7b* target gene, *HMGA2* (40) was affected by TTP level (Supplementary Figure S4). These data indicate that TTP can control cell growth by regulating miRNA target genes as well as ARE-containing genes.

There are many ARE-binding proteins that can affect the decay of ARE-containing mRNA (41,42). However, it is not likely that all ARE-binding proteins affect the stability of *Lin28* mRNA, as overexpression of ARE-binding protein BRF1 did not affect the expression level of the *Lin28a* (Supplementary Figure S5). The precise mechanism of TTP-specific targeting of *Lin28a* mRNA remains elusive. Further studies on proteins required for the function of TTP and other ARE-binding proteins will reveal the mechanism(s) for this specificity.

Recent studies have indicated a connection between ARE-binding proteins and miRNAs (43,44). A few cases of cooperative (43,45) and competitive (46) binding have been described as involved in ARE-containing mRNA decay. TTP was shown to interact with the RNA-induced silencing complex (RISC) to facilitate *miR-16* targeting of ARE, leading to ARE-mRNA decay (43). When *let-7* binds to adjacent sites of ARE, HuR cooperates with the *let-7* miRNA to repress the expression of *MyC* transcripts (45). The *miR-122* miRNA binds to the 3′-UTR of *CAT-1* mRNA and induces mRNA degradation, but binding of HuR to the *CAT-1* 3′-UTR protects *CAT-1* mRNA from *miR-122*-induced degradation (46). The KH-type splicing regulatory protein (KSRP), which is an ARE-binding protein, binds to the terminal loop of the precursors of a subset of miRNAs as a component of both Drosha and Dicer to promote their maturation (47). Here, our results provide a novel mechanism by which TTP regulates *let-7* biogenesis and, combined with previous reports, suggests that ARE-binding proteins are closely connected with miRNAs in post-transcriptional regulation of gene expression.

Figure 5. Continued
pcDNA6/V5-TTP and a psiCHECK2 luciferase reporter constructs containing fragments (left panel) or oligonucleotides (right panel) derived from the *Lin28a* mRNA 3′-UTR as described in (E) for 24 h. After normalizing luciferase activity, the luciferase activity obtained from PA1 cells transfected with the Frag-ARE1 luciferase construct alone or Oligo-ARE1W alone were set to 1.0. Results shown represent the means ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$). ns, not significant. (**G** and **H**) TTP decreases the luciferase activity of luciferase reporter gene containing the *Lin28b* 3′-UTR. (G) Schematic representation of the luciferase reporter constructs used in this study. Fragments (Frag) derived from the *Lin28b* mRNA 3′-UTR were cloned downstream of the luciferase reporter gene in the psiCHECK2 luciferase expression vector. White circles, wild-type pentameric motif AUUUA. (H) PA1 cells were co-transfected with pcDNA6/V5-TTP and a psiCHECK2 luciferase reporter constructs containing fragments derived from the *Lin28b* mRNA 3′-UTR as described in (G) for 24 h. After normalizing luciferase activity, the luciferase activity obtained from PA1 cells transfected with each Frag-ARE luciferase construct alone was set to 1.0. Results shown represent the means ± SD of three independent experiments (**$P < 0.01$; ***$P < 0.001$). (**I**) Ribonucleoprotein immunoprecipitation assay. PA1 cells were cotransfected with pcDNA6/V5-TTP and psiCHECK2 luciferase reporter constructs containing *Lin28a* Oligo-ARE1W. psiCHECK2 luciferase reporter construct containing mutant ARE1, Oligo-ARE1M was used as a negative control. At 24 h after transfection, the ribonucleoprotein complexes containing TTP were immunoprecipitated with protein G-agarose and anti-V5 or a control antibody. The luciferase mRNA in the immunoprecipitates was amplified by RT-PCR. The presence of TTP in the immunoprecipitates was detected by western blot with anti-V5 antibody. (**J** and **K**) RNA EMSA was performed by mixing cytoplasmic extracts containing 3.0 μg of total protein from pcDNA6/V5-TTP-transfected PA1 cells (J) or HCT116 cells (K) with 80 fmol biotinylated wild-type Oligo-ARE1W (WT) or mutant Oligo-ARE1M (MUT) probe. Anti-V5 (J), anti-TTP (K) or control antibody was added to the reaction mixtures. Position of the TTP containing bands (TTP) and super-shifted bands (SS) are indicated.

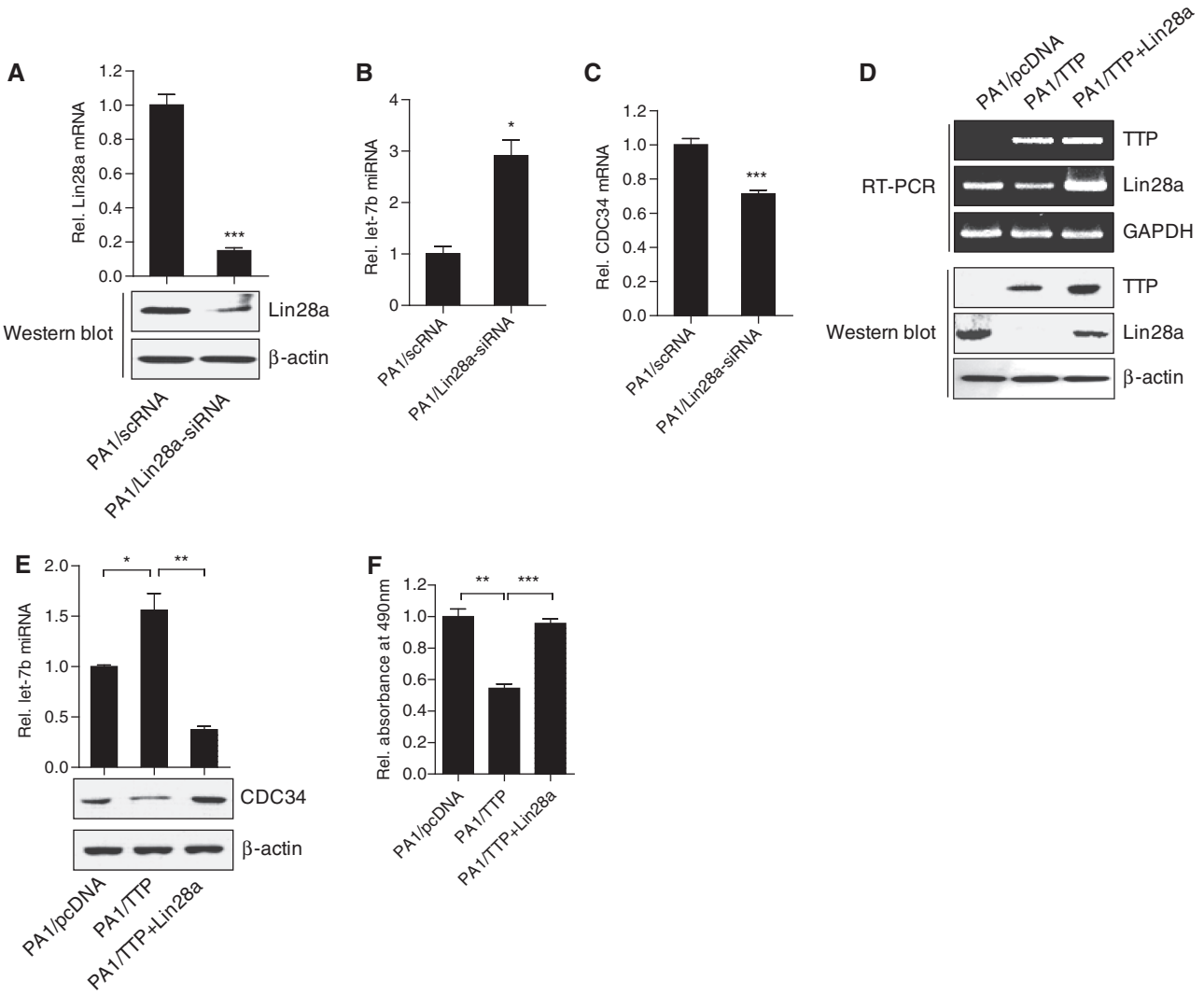

Figure 6. Overexpression of Lin28a attenuates the effects of TTP on let-7 b levels, CDC34 levels and PA1 cell growth. (**A–C**) Downregulation of Lin28a by siRNA increases *let-7b* levels but decreases CDC34 levels in PA1 cells. PA1 cells were transfected with *Lin28a*-specific (Lin28a-siRNA) or scRNA for 24 h. (A) The level of Lin28a was determined by qRT-PCR (top panel) and western blot assays (bottom panel). The levels obtained from PA1/scRNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (***$P < 0.001$). (B) The level of mature *let-7b* was measured by qRT-PCR. The levels obtained from PA1/scRNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (*$P < 0.05$). (C) The level of *CDC34* was determined by qRT-PCR. The levels obtained from PA1/scRNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (***$P < 0.001$). (**D–F**) Transfection of *Lin28a* cDNA without the 3′-UTR abolishes the effects of TTP on expression of *let-7b*, *CDC34* and PA1 cell growth. PA1 cells were transfected with a combination of pcDNA6/V5-TTP and pcDNA3/Flag-Lin28a for 24 h. (D) The levels of TTP and Lin28a were measured by semi-qRT-PCR (top panel) and western blot assays (bottom panel). (E) The level of *let-7b* was measured by qRT-PCR. The levels obtained from PA1/pcDNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (*$P < 0.05$; **$P < 0.01$). (F) Cell viability was assessed by measuring absorbance at 490 nm using a MTS cell proliferation assay. The levels obtained from PA1/pcDNA cells were set to 1.0. Data are presented as the mean ± SD ($n = 3$) (**$P < 0.01$; ***$P < 0.001$).

In conclusion, we have identified the unique characteristic of TTP as a positive regulator of *let-7* biogenesis. Our study provides a molecular basis for TTP-mediated regulation of let-7 biogenesis in human cancer cells and extends our current understanding of ARE-binding proteins in miRNA biogenesis by revealing a role for TTP functioning upstream of *Lin28*. These findings that TTP not only mediates the down-regulation of ARE-containing mRNAs but is also involved in the upstream regulation of miRNA target genes further highlights the important role of TTP in human tumors. There is emerging evidence supporting the role of Lin28 in stem cell development and differentiation (48,49). It will therefore be of interest to explore whether this type of regulatory mechanism is also important in ES cell biology, cell reprogramming and development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online: Supplementary Table S1 and Supplementary Figures S1–S5.

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Conflict of interest statement. None declared.

REFERENCES

1. Filipowicz,W., Bhattacharyya,S.N. and Sonenberg,N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.*, **9**, 102–114.
2. Rigoutsos,I. (2009) New tricks for animal microRNAS: targeting of amino acid coding regions at conserved and nonconserved sites. *Cancer Res.*, **69**, 3245–3248.
3. Lewis,B.P., Burge,C.B. and Bartel,D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15–20.
4. Slack,F.J. and Weidhaas,J.B. (2006) MicroRNAs as a potential magic bullet in cancer. *Future Oncol.*, **2**, 73–82.
5. Kim,V.N., Han,J. and Siomi,M.C. (2009) Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.*, **10**, 126–139.
6. Siomi,H. and Siomi,M.C. (2010) Posttranscriptional regulation of microRNA biogenesis in animals. *Mol. Cell*, **38**, 323–332.
7. Reinhart,B.J., Slack,F.J., Basson,M., Pasquinelli,A.E., Bettinger,J.C., Rougvie,A.E., Horvitz,H.R. and Ruvkun,G. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, **403**, 901–906.
8. Pasquinelli,A.E., Reinhart,B.J., Slack,F., Martindale,M.Q., Kuroda,M.I., Maller,B., Hayward,D.C., Ball,E.E., Degnan,B., Muller,P. *et al.* (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature*, **408**, 86–89.
9. Esquela-Kerscher,A. and Slack,F.J. (2006) Oncomirs - microRNAs with a role in cancer. *Nat. Rev. Cancer*, **6**, 259–269.
10. Suh,M.R., Lee,Y., Kim,J.Y., Kim,S.K., Moon,S.H., Lee,J.Y., Cha,K.Y., Chung,H.M., Yoon,H.S., Moon,S.Y. *et al.* (2004) Human embryonic stem cells express a unique set of microRNAs. *Dev. Biol.*, **270**, 488–498.
11. Thomson,J.M., Newman,M., Parker,J.S., Morin-Kensicki,E.M., Wright,T. and Hammond,S.M. (2006) Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes Dev.*, **20**, 2202–2207.
12. Heo,I., Joo,C., Cho,J., Ha,M., Han,J. and Kim,V.N. (2008) Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol. Cell*, **32**, 276–284.
13. Rybak,A., Fuchs,H., Smirnova,L., Brandt,C., Pohl,E.E., Nitsch,R. and Wulczyn,F.G. (2008) A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat. Cell. Biol.*, **10**, 987–993.
14. Viswanathan,S.R., Daley,G.Q. and Gregory,R.I. (2008) Selective blockade of microRNA processing by Lin28. *Science*, **320**, 97–100.
15. Hagan,J.P., Piskounova,E. and Gregory,R.I. (2009) Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat. Struct. Mol. Biol.*, **16**, 1021–1025.
16. Heo,I., Joo,C., Kim,Y.K., Ha,M., Yoon,M.J., Cho,J., Yeom,K.H., Han,J. and Kim,V.N. (2009) TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell*, **138**, 696–708.
17. Lehrbach,N.J., Armisen,J., Lightfoot,H.L., Murfitt,K.J., Bugaut,A., Balasubramanian,S. and Miska,E.A. (2009) LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in Caenorhabditis elegans. *Nat. Struct. Mol. Biol.*, **16**, 1016–1020.
18. Viswanathan,S.R., Powers,J.T., Einhorn,W., Hoshida,Y., Ng,T.L., Toffanin,S., O'Sullivan,M., Lu,J., Phillips,L.A., Lockhart,V.L. *et al.* (2009) Lin28 promotes transformation and is associated with advanced human malignancies. *Nat. Genet.*, **41**, 843–848.
19. Iliopoulos,D., Hirsch,H.A. and Struhl,K. (2009) An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell*, **139**, 693–706.
20. Shaw,G. and Kamen,R. (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*, **46**, 659–667.
21. Shyu,A.B. and Wilkinson,M.F. (2000) The double lives of shuttling mRNA binding proteins. *Cell*, **102**, 135–138.
22. Carballo,E., Lai,W.S. and Blackshear,P.J. (1998) Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science*, **281**, 1001–1005.
23. Lykke-Andersen,J. and Wagner,E. (2005) Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. *Genes Dev.*, **19**, 351–361.
24. Brennan,S.E., Kuwano,Y., Alkharouf,N., Blackshear,P.J., Gorospe,M. and Wilson,G.M. (2009) The mRNA-destabilizing protein tristetraprolin is suppressed in many cancers, altering tumorigenic phenotypes and patient prognosis. *Cancer Res.*, **69**, 5168–5176.
25. Lee,H.H., Vo,M.T., Kim,H.J., Lee,U.H., Kim,C.W., Kim,H.K., Ko,M.S., Lee,W.H., Cha,S.J., Min,Y.J. *et al.* (2010) Stability of the LATS2 tumor suppressor gene is regulated by tristetraprolin. *J. Biol. Chem.*, **285**, 17329–17337.
26. Marderosian,M., Sharma,A., Funk,A.P., Vartanian,R., Masri,J., Jo,O.D. and Gera,J.F. (2006) Tristetraprolin regulates Cyclin D1 and c-Myc mRNA stability in response to rapamycin in an Akt-dependent manner via p38 MAPK signaling. *Oncogene*, **25**, 6277–6290.
27. Young,L.E., Sanduja,S., Bemis-Standoli,K., Pena,E.A., Price,R.L. and Dixon,D.A. (2009) The mRNA binding proteins HuR and tristetraprolin regulate cyclooxygenase 2 expression during colon carcinogenesis. *Gastroenterology*, **136**, 1669–1679.
28. Lee,S.K., Kim,S.B., Kim,J.S., Moon,C.H., Han,M.S., Lee,B.J., Chung,D.K., Min,Y.J., Park,J.H., Choi,D.H. *et al.* (2005) Butyrate response factor 1 enhances cisplatin sensitivity in human head and neck squamous cell carcinoma cell lines. *Int. J. Cancer*, **117**, 32–40.
29. Lee,H.H., Son,Y.J., Lee,W.H., Park,Y.W., Chae,S.W., Cho,W.J., Kim,Y.M., Choi,H.J., Choi,D.H., Jung,S.W. *et al.* (2010) Tristetraprolin regulates expression of VEGF and tumorigenesis in human colon cancer. *Int. J. Cancer*, **126**, 1817–1827.
30. Lu,J., Getz,G., Miska,E.A., Alvarez-Saavedra,E., Lamb,J., Peck,D., Sweet-Cordero,A., Ebert,B.L., Mak,R.H., Ferrando,A.A. *et al.* (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.
31. Johnson,S.M., Grosshans,H., Shingara,J., Byrom,M., Jarvis,R., Cheng,A., Labourier,E., Reinert,K.L., Brown,D. and Slack,F.J. (2005) RAS is regulated by the let-7 microRNA family. *Cell*, **120**, 635–647.
32. Yu,F., Yao,H., Zhu,P., Zhang,X., Pan,Q., Gong,C., Huang,Y., Hu,X., Su,F., Lieberman,J. *et al.* (2007) let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell*, **131**, 1109–1123.
33. Mayr,C., Hemann,M.T. and Bartel,D.P. (2007) Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science*, **315**, 1576–1579.

34. Legesse-Miller,A., Elemento,O., Pfau,S.J., Forman,J.J., Tavazoie,S. and Coller,H.A. (2009) let-7 Overexpression leads to an increased fraction of cells in G2/M, direct down-regulation of Cdc34, and stabilization of Wee1 kinase in primary fibroblasts. *J. Biol. Chem.*, **284**, 6605–6609.
35. Johnson,B.A. and Blackwell,T.K. (2002) Multiple tristetraprolin sequence domains required to induce apoptosis and modulate responses to TNFalpha through distinct pathways. *Oncogene*, **21**, 4237–4246.
36. Willems,A.R., Schwab,M. and Tyers,M. (2004) A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim. Biophys. Acta*, **1695**, 133–170.
37. Polesskaya,A., Cuvellier,S., Naguibneva,I., Duquet,A., Moss,E.G. and Harel-Bellan,A. (2007) Lin-28 binds IGF-2 mRNA and participates in skeletal myogenesis by increasing translation efficiency. *Genes Dev.*, **21**, 1125–1138.
38. Xu,B. and Huang,Y. (2009) Histone H2a mRNA interacts with Lin28 and contains a Lin28-dependent posttranscriptional regulatory element. *Nucleic Acids Res.*, **37**, 4256–4263.
39. Xu,B., Zhang,K. and Huang,Y. (2009) Lin28 modulates cell growth and associates with a subset of cell cycle regulator mRNAs in mouse embryonic stem cells. *RNA*, **15**, 357–361.
40. Lee,Y.S. and Dutta,A. (2007) The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev.*, **21**, 1025–1030.
41. Barreau,C., Paillard,L. and Osborne,H.B. (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res.*, **33**, 7138–7150.
42. Wilusz,C.J., Wormington,M. and Peltz,S.W. (2001) The cap-to-tail guide to mRNA turnover. *Nat. Rev. Mol. Cell. Biol.*, **2**, 237–246.
43. Jing,Q., Huang,S., Guth,S., Zarubin,T., Motoyama,A., Chen,J., Di Padova,F., Lin,S.C., Gram,H. and Han,J. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell*, **120**, 623–634.
44. Vasudevan,S., Tong,Y. and Steitz,J.A. (2007) Switching from repression to activation: microRNAs can up-regulate translation. *Science*, **318**, 1931–1934.
45. Kim,H.H., Kuwano,Y., Srikantan,S., Lee,E.K., Martindale,J.L. and Gorospe,M. (2009) HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev.*, **23**, 1743–1748.
46. Bhattacharyya,S.N., Habermacher,R., Martine,U., Closs,E.I. and Filipowicz,W. (2006) Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell*, **125**, 1111–1124.
47. Trabucchi,M., Briata,P., Garcia-Mayoral,M., Haase,A.D., Filipowicz,W., Ramos,A., Gherzi,R. and Rosenfeld,M.G. (2009) The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature*, **459**, 1010–1014.
48. Yang,D.H. and Moss,E.G. (2003) Temporally regulated expression of Lin-28 in diverse tissues of the developing mouse. *Gene Expr. Patterns*, **3**, 719–726.
49. Yu,J., Vodyanik,M.A., Smuga-Otto,K., Antosiewicz-Bourget,J., Frane,J.L., Tian,S., Nie,J., Jonsdottir,G.A., Ruotti,V., Stewart,R. *et al.* (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science*, **318**, 1917–1920.