# Resveratrol inhibits Lin28A expression and induces its degradation via the proteasomal pathway in NCCIT cells

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**Abstract.** Lin28A is an oncoprotein overexpressed in several cancer types such as testicular, ovarian, colon, breast and lung cancers. As a pluripotency factor that promotes tumorigenesis, Lin28A is associated with more undifferentiated and aggressive tumors phenotypes. Moreover, Lin28A is a highly stable protein that is difficult to downregulate. The compound resveratrol (RSV) has anticancer effects. The present study aimed to elucidate the mechanisms underlying the downregulation of Lin28A protein expression by RSV in the NCCIT cell line. NCCIT cells were treated with different concentrations of RSV to investigate its effects on Lin28A expression. The mRNA expression levels of Lin28A and ubiquitin-specific protease 28 (USP28) were assessed using reverse transcription-quantitative PCR. Western blot analysis was employed to evaluate the protein levels of Lin28A, USP28 and phosphorylated Lin28A. In addition, in some experiments, cells were treated with a MAPK/ERK pathway inhibitor, and other experiments involved transfecting cells with small interfering RNAs targeting USP28. The results demonstrated that RSV significantly reduced Lin28A expression by destabilizing the protein; this effect was mediated by the ability of RSV to suppress the expression of USP28, a deubiquitinase that normally protects Lin28A from ubiquitination and degradation. Additionally, RSV inhibited phosphorylation of Lin28A via the MAPK/ERK pathway; this phosphorylation event has previously been shown to enhance the stability of Lin28A by increasing its half-life. This resulted in Lin28A degradation through the proteasomal pathway in NCCIT cells. The results provide further evidence of the anticancer activity of RSV, and identified Lin28A and USP28 as promising therapeutic targets. As a stable oncoprotein, downregulating Lin28A expression is challenging. However, the present study demonstrated that RSV can overcome this hurdle by inhibiting USP28 expression and MAPK/ERK signaling to promote Lin28A degradation. Furthermore, elucidating these mechanisms provides avenues for developing targeted cancer therapies.

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## Introduction

Lin28 is an RNA-binding protein that regulates key cellular processes, including cell development, glucose metabolism, differentiation, pluripotency and stem cell self-renewal (1-3). Lin28 has been identified as a central factor in reprogramming mammalian somatic cells to a pluripotent state and maintaining pluripotency (4,5). Additionally, Lin28 overexpression in cancer is associated with more undifferentiated and aggressive tumor phenotypes as well as resistance to conventional therapies (6,7).

There are two paralogs of the Lin28 gene in vertebrates, Lin28A and Lin28B, which share high DNA sequence homology (8). Both paralog proteins negatively regulate the tumor suppressor micro (mi)RNA Let-7 (3,9,10). The present study assessed the effects of resveratrol (RSV) on Lin28A protein stability using the NCCIT cell line, which overexpressed the Lin28A protein (11).

The mechanisms underlying the post-translational overexpression and stabilization of Lin28A have been previously described. In 2017, Tsanov *et al* (12) demonstrated that Lin28A is phosphorylated at serine 200 (S200) by the MAPK/ERK pathway in pluripotent stem cells, leading to

increased expression through post-translational stabilization. Additionally, in 2019, Haq *et al* (11) reported that Lin28A undergoes ubiquitination, destabilization and degradation through the 26S proteasome pathway in human embryonal carcinoma cells. However, the deubiquitinase ubiquitin-specific protease 28 (USP28) was reported to reverse Lin28A ubiquitination, preventing its degradation and increasing stability. In contrast, several studies have reported that therapeutic inhibition, genetic inactivation or silencing of Lin28 reverses the invasive phenotype in cancer cells (13,14). Together, these findings provide insight into potential molecular mechanisms regulating Lin28A stability at the post-translational level.

Extensive research has documented the anticancer effects of RSV and its ability to modulate key signaling pathways in several cancer cell types (15). Regarding the Lin28A oncoprotein, a luciferase assay in one study demonstrated that RSV decreased Lin28A gene expression in colorectal cancer cells (16). However, the impact of RSV on Lin28A protein stability is yet to be elucidated, and understanding the regulatory mechanisms of oncogenes such as Lin28A is essential for developing improved cancer treatments. Furthermore, a deeper understanding of how therapies modulate oncogenic drivers could enable more targeted and effective combination strategies.

Natural compounds, such as polyphenols, phytosterols, triterpenoids and saponins, also show promise when combined with conventional cancer treatments (17). Used together, they can enhance anticancer impacts, reduce side effects, boost immunity and promote cancer cell death (18). RSV in particular has demonstrated mixed benefits (19,20) and further investigation in to the effects of RSV on the Lin28A oncoprotein may reveal new therapeutic opportunities. For example, downregulating critical cancer drivers like Lin28A through pharmaceutical or dietary interventions represents an attractive strategy, and expanding the knowledge of the molecular targets of RSV could support its inclusion in rational, multitargeted regimens designed to maximize clinical impact.

## Materials and methods

*Reagents and antibodies*. Resveratrol (RSV) was purchased from Sigma-Aldrich (Merck KGaA; cat. no. R5010) and suspended in ethanol absolute (vehicle). MAPK/ERK inhibitor PD0325901 was purchased from Sigma-Aldrich (Merck KGaA; cat. no. PZ0162) and suspended in DMSO. Proteasomal inhibitor-MG132 (cat. no. M8699) and cycloheximide (CHX; cat. no. 01810) were also purchased from Sigma-Aldrich (Merck KGaA). Monoclonal anti-Lin28-HRP antibodies (cat. no. sc-293120) were purchased from Santa Cruz Biotechnology, Inc. Monoclonal anti-β-actin-peroxidase antibodies (cat. no. A3854) were purchased from Sigma-Aldrich (Merck KGaA). Polyclonal anti-phosphorylated-Lin28A (pLin28) (Ser200) antibodies (cat. no. PA5-105696) were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Mouse anti-rabbit IgG-HPR antibodies (cat. no. sc-2357) were purchased from Santa Cruz Biotechnology, Inc. Polyclonal anti-USP28 HRP antibodies (cat. no. orb480432) were purchased from Biorbyt, Ltd.

*Cell culture*. The pluripotent embryonal carcinoma (testicular teratocarcinoma) NCCIT cell line was purchased from the American Type Culture Collection (cat. no. CRL-2073). This cell line was cultured in RPMI 1640 medium (Biowest SAS) supplemented with 10% fetal bovine serum (cat. no. S1810-500; Biowest SAS) at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*Cell viability assay*. Cell viability was assessed using the MTT assay, which measures cellular metabolic activity as an indicator of viability. Cells were seeded at a density of $8x10^4$ cells/well in 24-well plates and cultured overnight at 37˚C. The following day, the cells were treated with several concentrations of RSV (0, 25, 50, 100, 150 and 200 $\mu$M). After 48 h of treatment, MTT reagent (Sigma-Aldrich; Merck KGaA) was added to each well at a final concentration of 0.5 mg/ml and incubated at 37˚C for 30 min to allow formazan crystal formation by metabolically active cells. The supernatant was then removed and the formazan crystals were dissolved in 500 $\mu$l acidified isopropanol. Absorbance was measured at 570 nm using a Tecan Sunrise™ absorbance microplate reader (Tecan Group, Ltd.). Cell viability was expressed as a percentage relative to vehicle-treated control cells, which were set at 100%. Each treatment was performed in triplicate and the experiment was repeated three times.

To assess the viability of cells treated with the ERK inhibitor PD0325901, the MTT assay was performed following the aforementioned procedure, with cells exposed to a range of PD0325901 concentrations (0, 1, 2, 5, 7.5 and 10 $\mu$M).

*Treatment of cells with RSV and ERK inhibitor*. For RSV treatments, the following doses were used in the experiments: 25, 50, 100 and 150 $\mu$M. The 150 $\mu$M dose, which was close to the calculated half-maximal inhibitory concentration ($IC_{50}$), was a particular focus of the analysis due to the strong decrease in the Lin28A oncoprotein with this treatment dose. For ERK inhibitor treatments, doses close to or below the $IC_{50}$ were tested, specifically 5 and 7.5 $\mu$M. The concentration of 7.5 $\mu$M was revealed to be the most effective in inhibiting Lin28A phosphorylation at S200.

For the vehicle controls, the amount of absolute ethanol corresponding to the highest RSV concentration tested was used, which was 150 $\mu$M. This ethanol vehicle was suspended in the cell culture medium (RPMI 1640 medium supplemented with 10% fetal bovine serum) at a final concentration of 0.18% by adding 1.8 $\mu$l of ethanol per ml of medium.

Similarly, for the ERK inhibitor treatments, the highest concentration used was 7.5 $\mu$M, which was suspended in DMSO. This resulted in a final DMSO concentration of 0.05% in the medium, achieved by adding 0.5 $\mu$l of DMSO per ml of medium.

In prior experiments, the present study assessed the maximum tolerable concentrations of DMSO and ethanol in MTT assays performed on untreated or vehicle-free control cells, to ensure that these solvents did not alter the proliferation of the cells under study.

*Small interfering (si)RNA transfection*. NCCIT cells were transfected with pre-designed siRNAs targeting USP28 (cat. nos. 4392420, s33508, s33509 and s33510) or a non-targeting control siRNA (cat. no. 4390843); all purchased from Ambion® (Thermo Fisher Scientific, Inc.). The siUSP28

sequences are provided in Table I. siRNAs were transfected at a concentration of 2 nmol/ml using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol for 24 h at 37˚C. After 24 h, cells were lysed and protein extracts were collected. Western blot analysis was performed to assess the silencing of USP28 protein expression after transfection with siRNAs targeting USP28 compared with the non-targeting control. siRNA concentrations were optimized in separate experiments to achieve maximum USP28 knockdown without inducing cytotoxicity, as determined using previously established criteria in the instructions provided by Ambion.

*Reverse transcription-quantitative PCR (RT-qPCR).* Cells were seeded at a density of $8x10^4$ cells/dish in p60 culture dishes and incubated for 24 h at 37˚C prior to treatment. Following treatment, total RNA was extracted from cells using TRIzol® (cat. no. 15596026; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA integrity and concentration were determined by agarose gel analysis and quantified using the Thermo Scientific™ NanoDrop™ One/One$^C$ (Thermo Fisher Scientific, Inc.). Complementary (c)DNA was synthesized from 1 $\mu$g total RNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol; the incubation time was 1 h at 37˚C and 5 min at 70˚C. qPCR was then performed using the 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.). PCR thermocycling conditions were as follows: 1 cycle at 94˚C for 10 min for initial denaturation, 35 repetitions of a 10-sec denaturation step at 94˚C, a 30-sec annealing step at 60˚C and a 30 sec extension step at 72˚C. Final extension was at 72˚C for 5 min. The specificity of each PCR was assessed using the melting temperature profiles of the final products. Assays were performed in triplicate and the relative expression of target genes was normalized to the reference gene β-2 microglobulin (β2M) using the $2^{-\Delta\Delta Cq}$ method (21). The pre-designed primers, Lin28A, USP28 and β2M were purchased from Integrated DNA Technologies, Inc. and the sequences are provided in Table II.

*Western blotting.* Cells were seeded at a density of $8x10^4$ cells/dish in p60 cell culture dishes and incubated for 24 h at 37˚C prior the treatment. Following treatment, cells were lysed in RIPA buffer (150 mM NaCl; 0.5% sodium deoxycholate; 0.1% SDS; 50 mM Tris; pH 7.4) supplemented with 1X complete™, Mini, EDTA-free Protease Inhibitor Cocktail (cat. no. 11836170001; Sigma-Aldrich; Merck KGaA) containing phosphatase inhibitors (1 mM sodium fluoride and 0.5 mM sodium orthovanadate; Sigma-Aldrich; Merck KGaA). Cell lysates were sonicated for 1 min and were centrifugated at 16,800 x g and 4˚C for 5 min to collect supernatants containing proteins of interest. Protein integrity and concentration were determined on a polyacrylamide gel using Coomassie staining and were quantified using the NanoDrop One/One$^C$. Equal amounts of protein (30 $\mu$g) were separated by 12% (w/v) SDS-PAGE and were transferred onto nitrocellulose membranes (cat. no. 1620115; Bio-Rad Laboratories, Inc.). Membranes were blocked for 1 h at room temperature with 5% skimmed milk or 5% BSA (cat. no. 30063721; Gibco; Thermo Fisher Scientific, Inc.) for phosphorylated protein detection. Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies against β-actin (1:20,000), pLin28A (S200; 1:1,000), Lin28A (1:750) and USP28 (1:1,000). Membranes were washed with TBS-0.05% Tween (cat. no. P7949; Sigma-Aldrich; Merck KGaA) and incubated with the secondary anti-rabbit antibodies (1:20,000) for 1 h at room temperature in the case of pLin28A. For the other antibodies, it was not necessary to use a secondary antibody since the primary antibodies were coupled to HRP. Protein bands were detected using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and quantified by densitometry using Image Studio™ Lite 5.2 software (LI-COR Biosciences). Band intensity was normalized to β-actin as a loading control. Western blots shown are representative of ≥3 independent experiments.

*Statistical analysis.* All statistical analyses including correlation and half-life tests were performed using GraphPad Prism software (version 8; Dotmatics). Differences between experimental groups were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test, as appropriate. The unpaired Student's t-test was used for comparisons between two groups. A two-way ANOVA followed by Tukey's multiple comparisons test was used for a control analysis of the Pearson's correlation coefficient analysis as 2 time points and 2 concentrations were evaluated. Data are presented as the

Table I. Sequences of select pre-designed small-interfering ubiquitin-specific protease 28.

| siRNA | Strand | Sequence (5'-3') |
|---|---|---|
| 1 | Sense | GAUUAUAGUUUGUUCCGAAtt |
| | Antisense | UUCGGAACAAACUAUAAUCtt |
| 2 | Sense | GUGAUUGCUUUAUACCGAAtt |
| | Antisense | UUCGGUAUAAAGCAAUCACgg |
| 3 | Sense | GGCCUAGAACUCUAUCAAAtt |
| | Antisense | UUUGAUAGAGUUCUAGGCCtg |

siRNA, small-interfering RNA.

Table II. Sequences of primers used for reserve transcription-quantitative PCR.

| Gene | Direction | Sequence (5'-3') |
|---|---|---|
| Lin28A | F | CATCTGTAAGTGGTTCAACGTG |
| | R | CCCTTCCATGTGCAGCTTA |
| USP28 | F | TGGCCAGGCTGATCTCTAACTC |
| | R | GAAGGCCGGGTACGATGA |
| β2M | F | GGACTGGTCTTTCTATCTCTTGT |
| | R | ACCTCCATGATGCTGCTTAC |

F, forward; R, reverse; USP28, ubiquitin-specific protease 28; β2M, β-2 microglobulin.

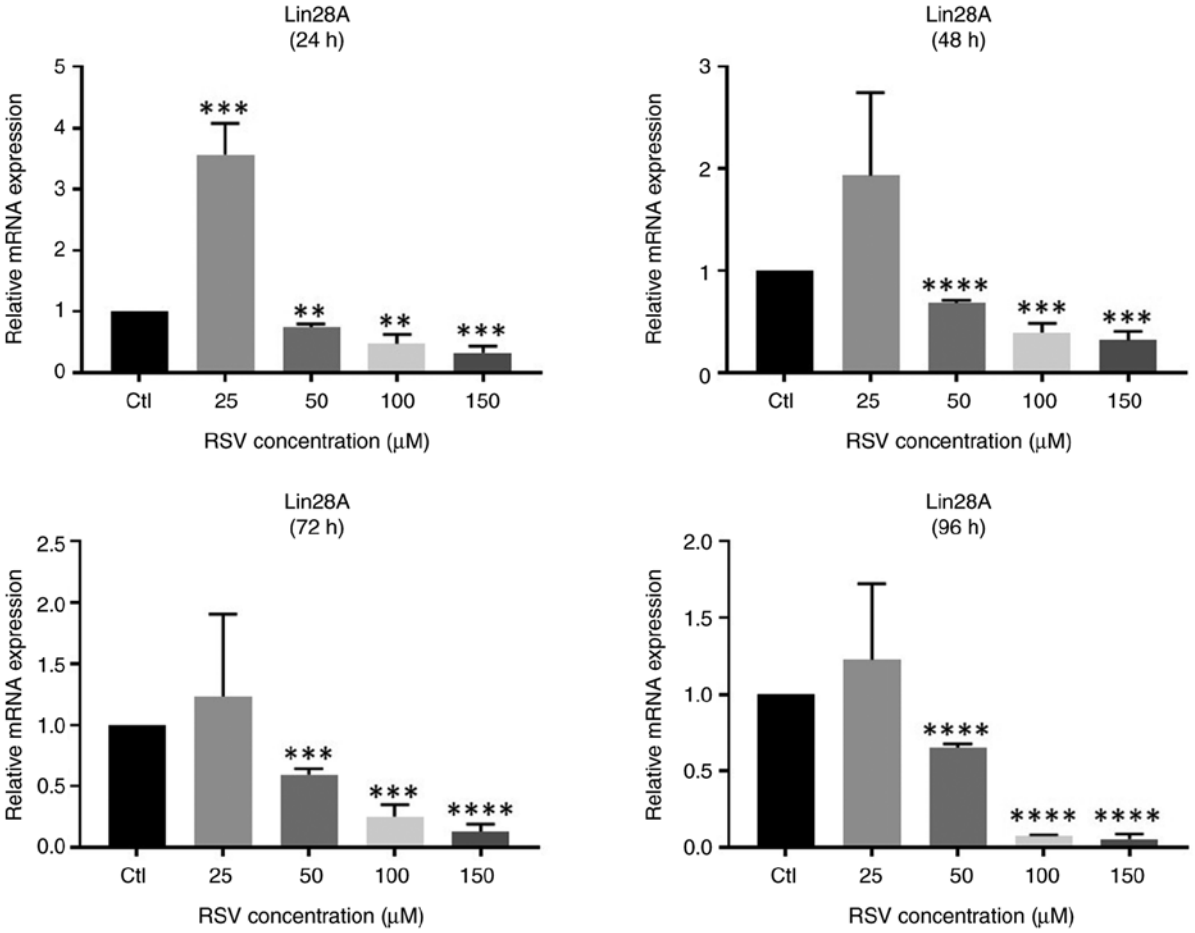


Figure 1. RSV decreases expression of Lin28A mRNA in NCCIT cells in a time-concentration manner. **P<0.01; ***P<0.001; ****P<0.0001 vs. Ctl. RSV, resveratrol; Ctl, control.

mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

## Results

*RSV decreases the cell viability of NCCIT cells*. To assess the effect of increasing doses of RSV (25-200 $\mu$M) on NCCIT teratocarcinoma cell proliferation, MTT assays were performed. The results demonstrated that RSV exerted a marked dose-dependent inhibition of NCCIT cell viability (Fig. S1). Analysis of the data revealed that the $IC_{50}$ of RSV in NCCIT cells was 148 $\mu$M. Based on this finding, the subsequent experiments were restricted to a dose range of 25-150 $\mu$M RSV, which encompassed concentrations below and near the $IC_{50}$ to adequately assess concentration-dependent responses. In summary, this initial analysis established the ability of RSV to suppress NCCIT cell viability in a dose-dependent manner and informed the selection of appropriate treatment doses for further mechanistic evaluation in the present study.

*RSV decreases the mRNA expression of Lin28A in NCCIT cells*. The effect of RSV on Lin28A mRNA expression in NCCIT was evaluated using RT-qPCR. Notably, the results revealed that treatment with the lowest RSV concentration (25 $\mu$M) was associated with a significant increase in Lin28A mRNA expression levels. However, further increases (≥50 $\mu$M) in the RSV dose were associated with significant decreases in Lin28A mRNA expression in a concentration-dependent manner (Fig. 1). These results indicate that higher RSV doses downregulate Lin28A expression at the transcriptional level. The present study then sought to assess whether this inhibitory effect persisted at the protein level.

*RSV at high concentrations decreases Lin28A protein expression levels in NCCIT cells*. The effect of RSV on the expression of the Lin28A protein was evaluated in NCCIT cells using treatment with RSV at different concentrations (25, 50, 100 and 150 $\mu$M). Western blot analysis was performed and the results demonstrated that low concentrations of RSV (25 and 50 $\mu$M) had no significant effect on the Lin28A protein expression level. However, the protein expression of Lin28A began to significantly decrease compared with in the control group in response to 100 and 150 $\mu$M RSV; with a more pronounced decrease in Lin28A protein detected at 72 and 96 h compared with at earlier time points (Fig. 2).

Pearson's correlation coefficient analysis was performed between Lin28A mRNA and protein expression. A total of two time points and two concentrations were chosen: i) 48 h, as in the $IC_{50}$, and ii) 96 h, corresponding to the maximum effect of RSV over Lin28A protein. Low (50 $\mu$M) and high (150 $\mu$M) concentrations of RSV were also chosen. The results demonstrated that there was a strong negative correlation at

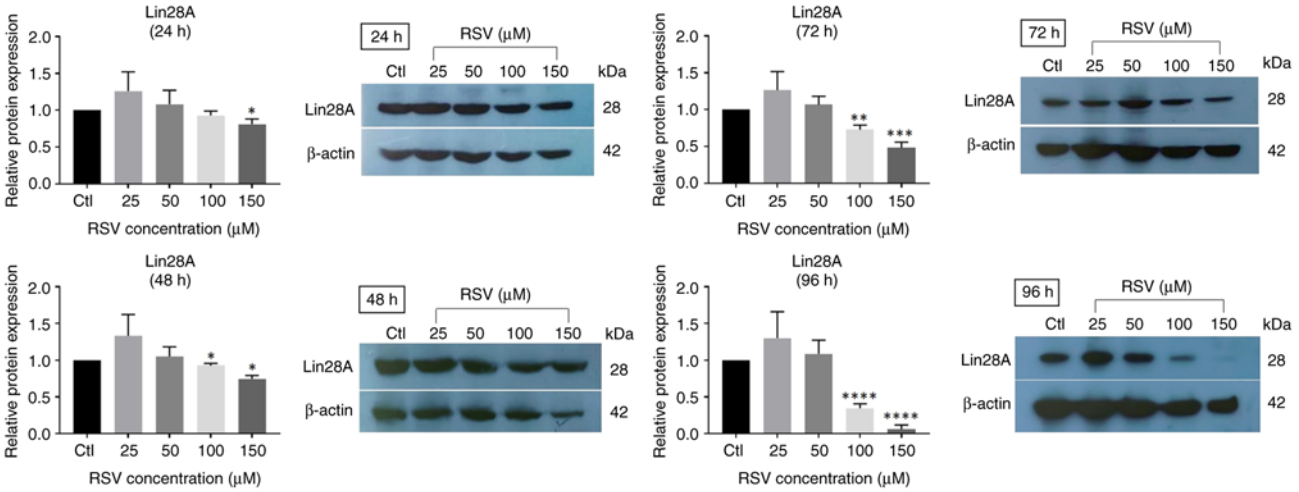


Figure 2. Lin28A protein is more stable than mRNA and is affected only by the highest concentrations of RSV. NCCIT cells were treated with different concentrations of RSV (25, 50, 100 and 150 $\mu$M) at different time periods. $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001; $^{****}$P<0.0001 vs. Ctl. RSV, resveratrol; Ctl, control.

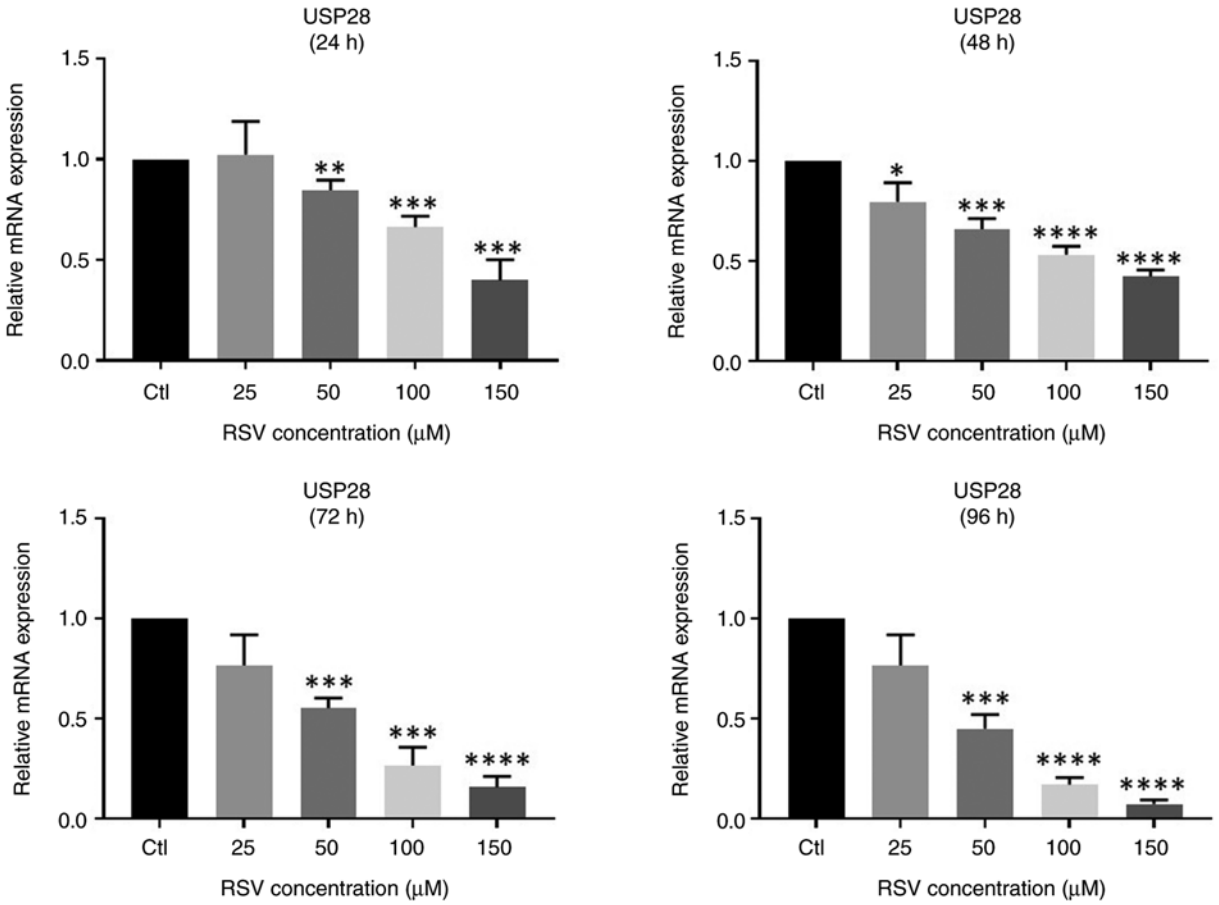


Figure 3. RSV decreases USP28 mRNA expression at different concentrations and treatment times in NCCIT cells. $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001; $^{****}$P<0.0001 vs. Ctl. RSV, resveratrol; USP28, ubiquitin-specific protease 28; Ctl, control.

48 h for 50 and 150 $\mu$M (r=-0.982 and r=-0.8841, respectively); however, this was not significant. This indicates that, for both concentrations (50 and 150 $\mu$M), mRNA expression was downregulated, while protein was upregulated or slightly downregulated. This demonstrated that RSV can produce a hormetic effect (dose-response phenomenon) for both mRNA and protein. Notably, this hormetic effect varies between transcript and protein at the same dose. This discrepancy can be attributed to the high stability of the Lin28A protein and the distinct mechanisms, such as USP28 and phosphorylation by the MAPK/ERK pathway, that protect it from degradation. At 96 h, there was a not significant moderate negative correlation for 50 $\mu$M RSV (r=-0.4039) and a very strong positive correlation for 150 $\mu$M (r=0.7669). Although the results were not

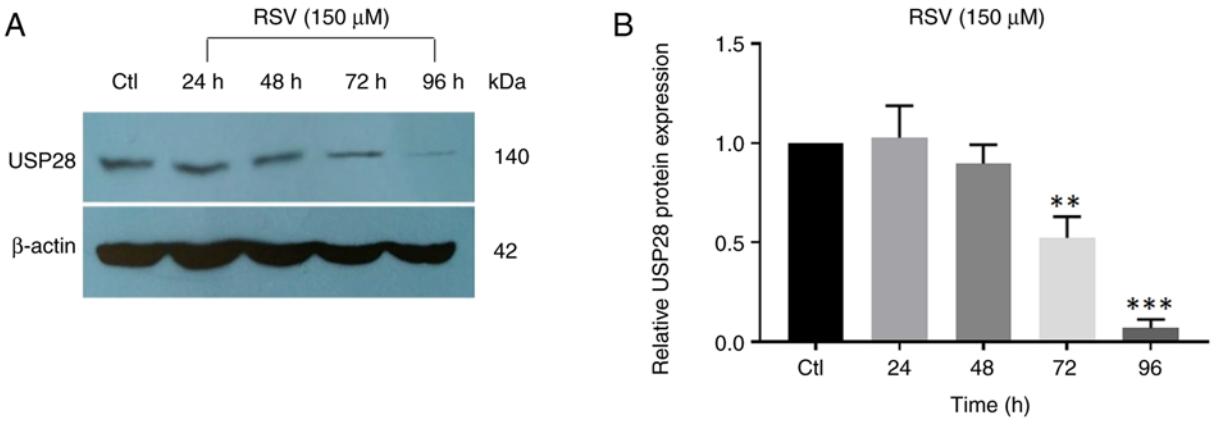


Figure 4. RSV at 150 $\mu$M can decrease the expression level of the USP28 protein. NCCIT cells were treated with 150 $\mu$M RSV at different time periods (24, 48, 72 and 96 h). (A) Representative western blot of USP28 protein expression. (B) Analysis of the western blot. $^{**}$P<0.01; $^{***}$P<0.001 vs Ctl. RSV, resveratrol; USP28, ubiquitin-specific protease 28; Ctl, control.

significant, these results confirm that the protein expression must be being stabilized post translationally by the aforementioned mechanisms, and mRNA and protein expression only demonstrated a positive correlation at the maximum time and concentration. The concentration of 150 $\mu$M RSV proved to be the best treatment option to reduce both Lin28A mRNA and protein levels in this cell model.

Since RSV decreased Lin28A expression, the present study subsequently evaluated the expression of USP28, a deubiquitinase that has been reported to be capable of keeping Lin28A stable, preventing its degradation (11).

*RSV decreases USP28 mRNA expression*. RT-qPCR was performed to evaluate the effect if RSV on USP28 mRNA expression in the NCCIT cell line (Fig. 3). The results revealed that as the concentration of RSV increased, the expression of USP28 mRNA significantly decreased, similar to that of Lin28A. Therefore, it was decided to also evaluate the USP28 protein.

*RSV decreases the expression level of the USP28 protein*. The $IC_{50}$ concentration at which there was a greatest decrease in Lin28A protein expression was 150 $\mu$M RSV (Fig. S1). To assess the effect of 150 $\mu$M RSV on USP28 protein expression, western blot analysis was performed (Fig. 4). The administration of 150 $\mu$M RSV significantly reduced the protein level of USP28 deubiquitinase, with the greatest effect observed at 72 and 96 h of treatment, similar to that of the Lin28A protein. Once the decrease in USP28 was demonstrated with RSV treatment, the present study continued to evaluate whether the degradation of Lin28A by RSV occurred via the proteasomal pathway.

*RSV degrades the Lin28A protein via the proteasomal pathway*. To assess if RSV treatment could induce the Lin28A protein degradation by the proteasomal pathway, the protein level of Lin28A was evaluated in the presence of the proteasomal inhibitor MG132 (M) in NCCIT cells treated with 150 $\mu$M RSV (R) at different time points (Fig. 5). The results demonstrated that the presence of MG132 in the RSV treatment significantly inhibited the degradation of Lin28A.

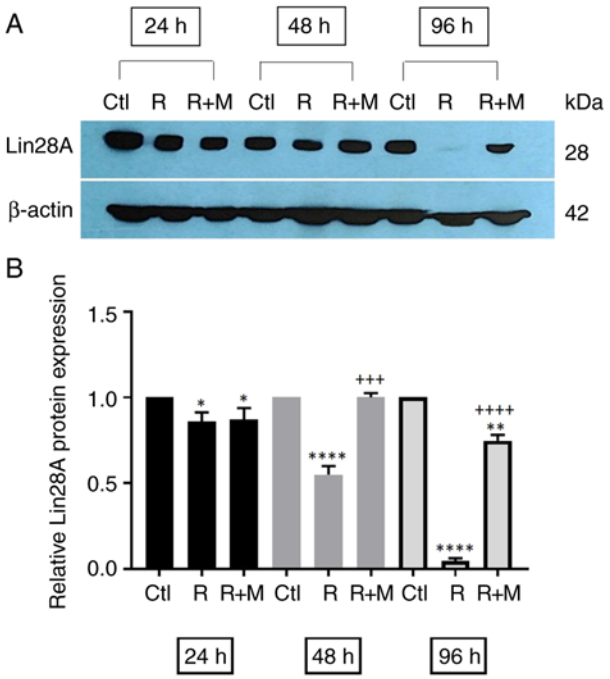


Figure 5. RSV induces Lin28A proteasomal degradation. NCCIT cells were treated with 5 $\mu$M of MG132 and 150 $\mu$M of RSV for different periods of time (24, 48 and 96 h) and then western blotting was performed. (A) Representative western blot of Lin28A protein expression. (B) Representative graph of western blotting analysis. $^{*}$P<0.05; $^{**}$P<0.01; $^{****}$P<0.0001 vs. Ctl. $^{+++}$P<0.001; $^{++++}$P<0.0001 vs. R group. RSV/R, resveratrol; M, MG132; Ctl, control.

Given that the degradation of Lin28A was by the proteasomal pathway, this could explain the stability of Lin28A protein as USP28 deubiquitinase was also downregulated at high RSV concentrations, and at 72 and 96 h.

*Inhibition of USP28 using siUSP28 decreases the levels of Lin28A, but the reduction is less pronounced compared to the decrease observed after RSV treatment*. To evaluate the expression of Lin28A when USP28 is silenced, NCCIT cells were transfected with different specific siUSP28s using lipofectamine (Fig. S2). The results were evaluated using western

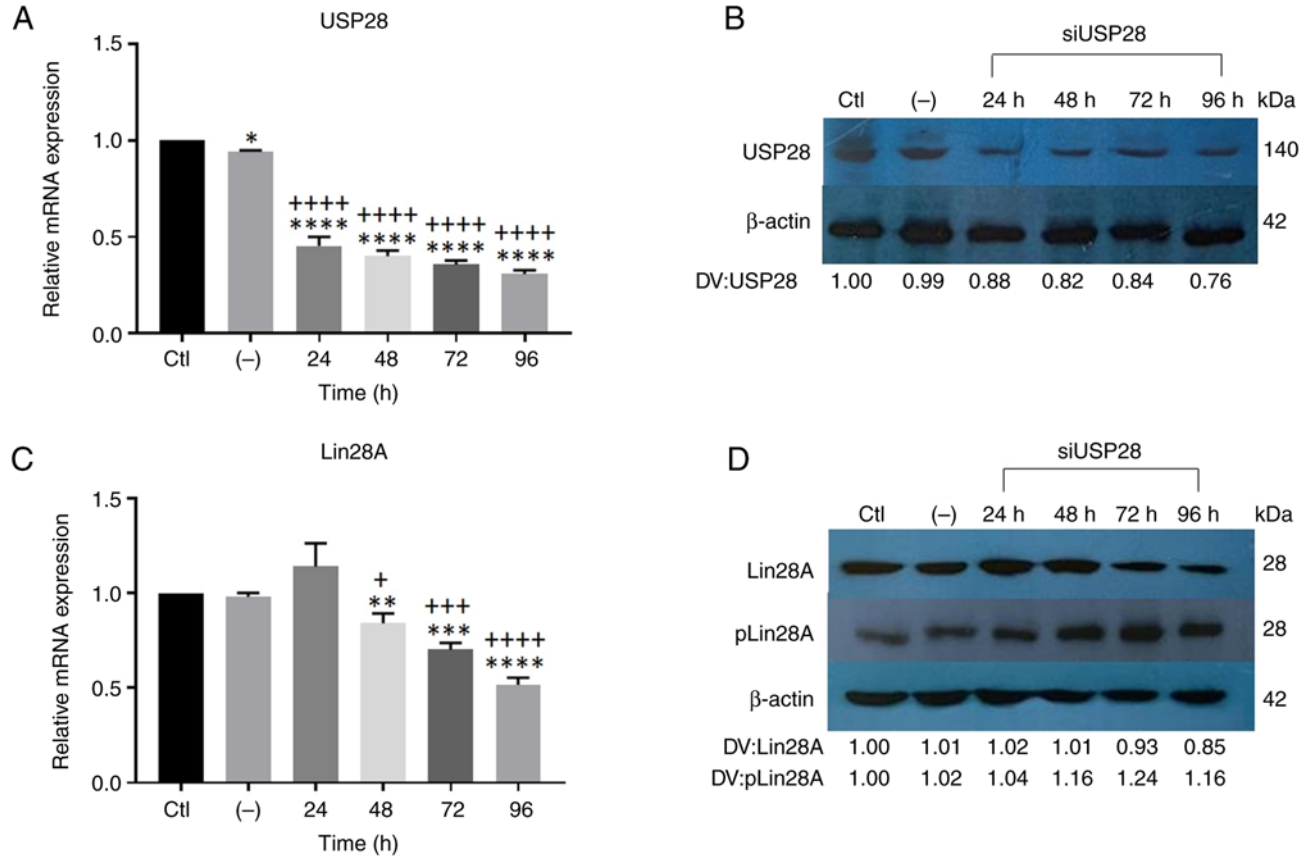


Figure 6. Inhibition of USP28 with specific siRNAs is not sufficient to totally silence Lin28A protein expression. (A) Expression of USP28 mRNA in cells transfected with siUSP28, quantified using RT-qPCR. The controls were transfected NCCIT cells and the transfection of a nonspecific siRNA that did not affect the gene of interest (USP28). (B) Representative western blot of USP28 protein expression in cells transfected with siUSP28 at different times (24, 48, 72 and 96 h). (C) Expression of Lin28A mRNA in cells transfected with siUSP28, quantified by stem-loop RT-qPCR. (D) Representative western blot of the expression of Lin28A protein and pLin28A by MAPK/ERK (serine 200) in cells transfected with siUSP28 at different times (24, 48, 72 and 96 h). $^{*}$P<0.05; $^{**}$P<0.01; $^{***}$P<0.001; $^{****}$P<0.0001 vs. Ctl. $^{+}$P<0.05; $^{+++}$P<0.001; $^{++++}$P<0.0001 vs. (-). DV, densitometry value; USP28, ubiquitin-specific protease 28; si, small-interfering; RT-qPCR, reverse transcription-quantitative PCR; Ctl, control; (-), nonspecific siRNA; pLin28A, phosphorylated Lin28A.

blotting, noting that for each of the siRNAs used, silencing of the expression of USP28 was observed. However, the present study used a combination of the three siRNAs to obtain a better result for the following experiments. Fig. 6A shows the expression of USP28 mRNA in cells transfected with siUSP28 at different times, and how expression significantly decreased compared with in the group without siRNAs. Concordantly, a significant decrease in USP28 protein expression was observed when NCCIT cells were transfected with siUSP28 (Fig. 6B). Notably, when evaluating the mRNA expression of Lin28A in NCCIT cells, it decreased significantly after 48 h of transfection with siUSP28 (Fig. 6C). By contrast, Lin28A protein expression remained stable at the same time (48 h) in response to siUSP28 transfection in NCCIT cells (Fig. 6D); however, a decrease in Lin28A protein levels was observed after 72 and 96 h of siUSP28 transfection, although this reduction was smaller compared with in response to RSV treatment (150 $\mu$M) alone (Fig. S3). This result is one of the key findings that confirms the importance of the USP28 mechanism in stabilizing the Lin28A protein. However, when one mechanism was knocked down (e.g., using siRNA against USP28), the other mechanism appeared to contribute to the stabilization of the protein by increasing its half-life, as seen with phosphorylation at S200 by the MAPK pathway. Fig. S3 shows the effect of 150 $\mu$M RSV on decreasing protein levels of Lin28A at different treatment times; this demonstrates that RSV at that concentration substantially reduces Lin28A protein levels, even more so than siUSP28 treatment. The minor reduction in Lin28A protein expression in response to siUSP28 transfection (Fig. 6D) could be explained by the fact that siUSP28 did not completely ablate USP28 mRNA and protein expression. Therefore, the remaining expression of USP28 may have been sufficient to protect the Lin28A protein. Alternatively, an additional mechanism for Lin28A stability is in place. Given that Lin28A phosphorylation is a key protective mechanism, the present study examined the expression of pLin28A in cells transfected with siUSP28. As expected, no downregulation of pLin28A was demonstrated, given that USP28 has no direct phosphorylating activity (12). Notably, the results demonstrated a marked increase in pLin28A expression upon knockdown of USP28 with siRNAs (Fig. 6D). This increase may also have helped in the protection of Lin28A and may be due to a negative feedback loop between USP28 and ERK signaling.

*RSV decreases the expression of Lin28A S200 phosphorylation*. The effect of RSV on the phosphorylation of S200 in the Lin28A protein was evaluated. NCCIT cells were treated with

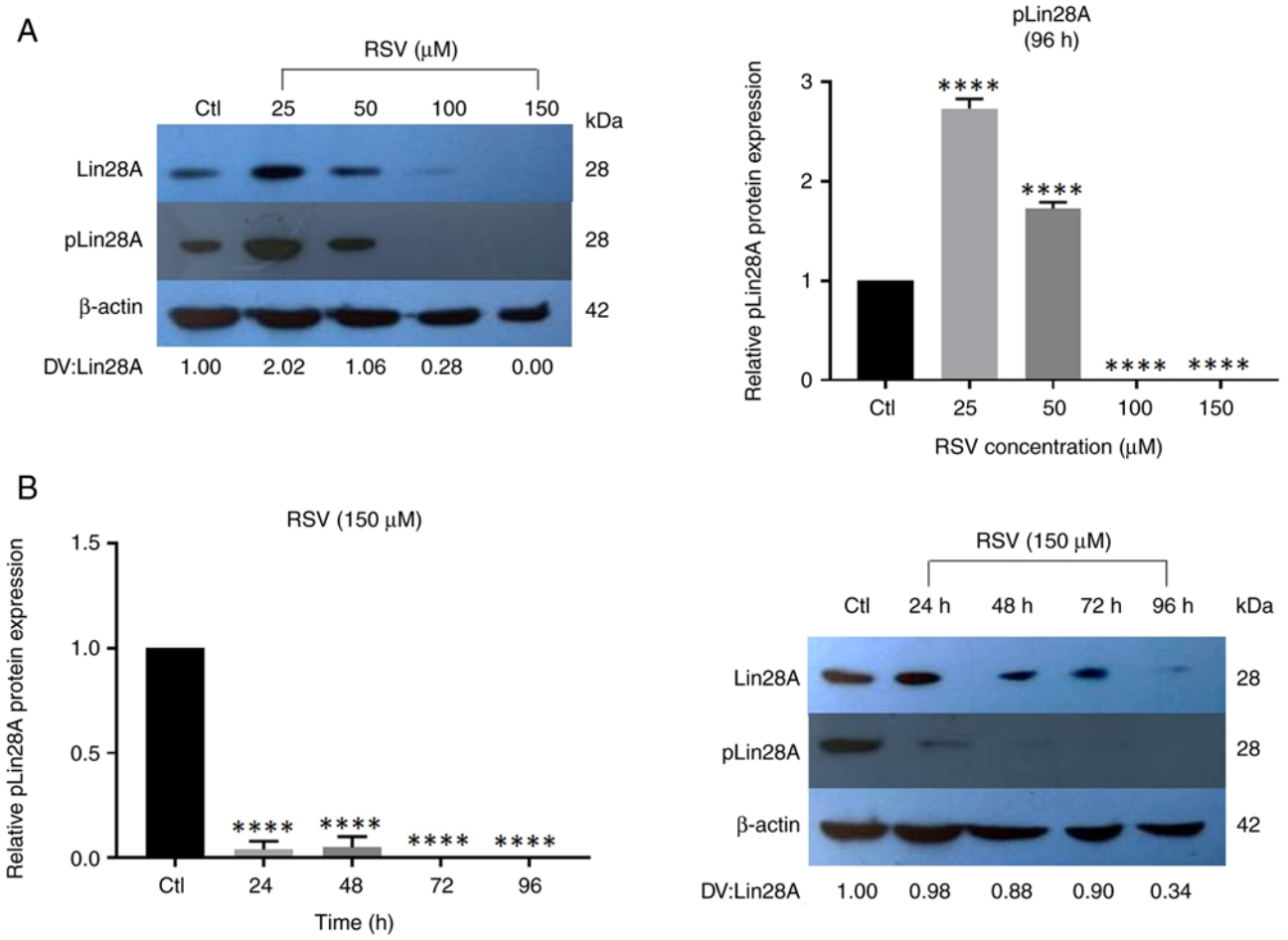


Figure 7. RSV decreases the expression of pLin28A in serine 200 caused by MAPK/ERK. Western blot and representative graph of western blot analysis of NCCIT cells treated with (A) different concentrations of RSV for 96 h and (B) 150 $\mu$M of RSV at different times (24, 48, 72 and 96 h). $^{****}$P<0.0001 vs. Ctl. DV, densitometry value; RSV, resveratrol; pLin28A, phosphorylated Lin28A; Ctl, control.

different concentrations of RSV for 96 h (a time for which a greater effect on the reduction of Lin28A protein was demonstrated). The results revealed that RSV at low concentrations (25 and 50 $\mu$M) significantly increased pLin28A expression, whereas concentrations of 100 and 150 $\mu$M RSV significantly decreased pLin28A expression compared with those in the control group (Fig. 7A). The effect of 150 $\mu$M RSV was also evaluated at different time periods. The findings demonstrated that treatment with 150 $\mu$M RSV significantly decreased pLin28A expression from 24 h compared with that in the control group (Fig. 7B). This suggests that RSV can reduce Lin28A phosphorylation via the MAPK/ERK pathway, which was reported by Tsanov *et al* (12). The observed decrease in Lin28A phosphorylation following RSV exposure provides experimental evidence explaining why siUSP28 alone did not impact Lin28A protein stability. By contrast, RSV, by simultaneously reducing USP28 levels and affecting the MAPK/ERK pathway (both mechanisms involved in Lin28A stabilization), was able to destabilize Lin28A.

*Inhibition of the MAPK/ERK pathway reduces the level of pLin28A protein*. The effect of inhibiting only the activation of ERK1/2 on the phosphorylation of Lin28A at S200 (pLin28A) was evaluated, as well as if the stability of total Lin28A and USP28 expression in the NCCIT cell line was affected. The cells were treated with a specific inhibitor of the activation of ERK1/2, a member of the MAPK pathway responsible for the phosphorylation of Lin28A at S200 (12). First, an MTT assay was performed to assess the safe dose of inhibitor that could be used to treat the cells; in this experiment, an $IC_{50}$ of 9 $\mu$M was obtained (Fig. S4). Subsequently, the following experiments were performed with 5 or 7.5 $\mu$M of the inhibitor, which were concentrations that had minimally effects on cell viability. It was demonstrated that both concentrations were sufficient for a marked decrease in pLin28A level. Furthermore, neither concentration notably affected the expression of the Lin28A and USP28 proteins (Fig. 8). This indicates that both stabilization pathways (USP28 and Lin28A phosphorylation by MAPK/ERK) can be blocked by RSV to reduce Lin28A protein expression.

*RSV strongly decreases Lin28A protein expression*. Finally, the present study evaluated the kinetics of Lin28A protein degradation in NCCIT cells using different treatments (RSV, siUSP28 and inhibitor/ERK) in a CHX condition assay (Figs. S5 and 9). In this experiment, RSV treatment significantly reduced Lin28A protein expression compared with that in the other treatment groups, such as cells treated with siUSP28, the ERK inhibitor, or a combination of siUSP28 and ERK inhibitor. Furthermore, the half-life of the Lin28A protein

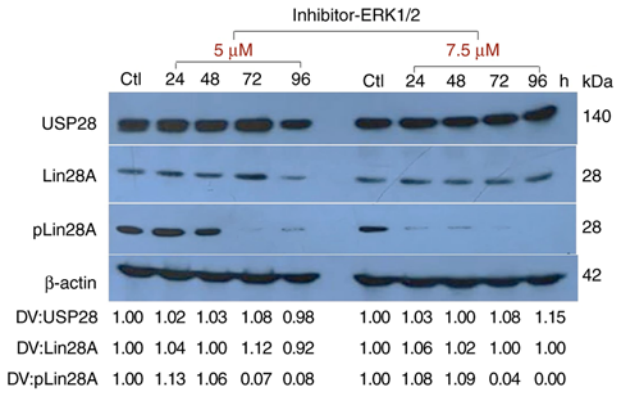


Figure 8. MAPK/ERK inhibitor decreased the expression of pLin28A (serine 200), and the total protein expression of Lin28A, similar to treatment with resveratrol. Representative western blot of the NCCIT cells treatment with the MAPK/ERK inhibitor (5 or 7.5 $\mu$M) at different times (24, 48, 72 and 96 h). DV, densitometry value; pLin28A, phosphorylated Lin28A; USP28, ubiquitin-specific protease 28; Ctl, control.

was 12.4 h with siUSP28 treatment, 17.9 h with inhibitor/ERK treatment, 14.9 h with the combined treatment of siUSP28 + inhibitor/ERK, and 10.8 h with RSV treatment. This suggests that RSV may regulate other factors that could be involved in Lin28A stability.

## Discussion

The present study aimed to elucidate the potential mechanisms by which RSV treatment downregulates the oncoprotein Lin28A in NCCIT cells. The NCCIT cell line was used, which is classified as a germ cell tumor and served as a convenient *in vitro* model of cancer stem-like cells (21-23). Furthermore, the NCCIT line is known to have high expression of both Lin28A and USP28 (11). Given the high basal expression of Lin28A and USP28 in the NCCIT cells, this made the NCCIT line the most appropriate choice to assess whether the natural compound RSV could effectively reduce Lin28A expression, and to elucidate the specific mechanisms by which it does so, including the role of USP28.

The results of the present study indicate that RSV inhibits Lin28A mRNA expression in a dose-dependent manner between concentrations 50-150 $\mu$M. Notably, at the lowest dose of RSV tested (25 $\mu$M), there was an increase in Lin28A mRNA expression levels. This biphasic effect is likely due to the hormesis phenomenon exhibited by certain compounds, in which low doses stimulate activity but higher doses reverse this effect (24). Whilst the exact reason for hormesis remains unclear, evaluating this dose-response behavior is important to determine the most effective concentration for a given positive or negative outcome, regardless of the substance. The RSV-mediated decrease in Lin28A expression may be linked to reduced levels of transcription factors such as SP1 and STAT3, both of which have been shown to bind the Lin28A promoter and promote its transcription (14,25,26). In this regard, the present study assessed SP1 and STAT3 expression following RSV treatment (50-150 $\mu$M) and demonstrated that both were downregulated (data not shown).

Whilst the present study provides useful insights, further research is needed to validate certain findings. For example, experiments assessing whether the aforementioned transcription factors directly bind to the Lin28A promoter in this model, and whether RSV specifically impacts SP1 and STAT3 protein levels, would strengthen the conclusions. It would also be valuable to investigate whether RSV uniformly affects long noncoding RNAs known to promote Lin28A transcription, such as H19, ZNFX1 antisense RNA 1 and long intergenic non-protein coding RNA 1451 (27-29). Additionally, exploring the expression of several oncoproteins previously reported to form positive feedback loops with Lin28A, such as c-MYC, octamer-binding transcription factor 4 and NANOG, could further elucidate the molecular mechanisms of RSV (29,30). Moreover, further targeted validation studies are required to fully characterize the multi-level effects of RSV on Lin28A regulation and contextualize the findings within existing knowledge of transcriptional and epigenetic control of this critical cancer-related gene. Overall, whilst promising, additional experimentation would help substantiate certain aspects of the present work.

When assessing Lin28A protein expression in NCCIT cells, the results of the present study revealed that its stability was not directly associated with mRNA downregulation, as protein levels only decreased at the highest RSV concentrations tested (100 and 150 $\mu$M). These results indicate that the Lin28A protein is highly stable, likely due to post-translational regulatory mechanisms previously described. For example, Haq *et al* (11) reported that Lin28A interacts with USP28 and deubiquitinates it, maintaining the protein stability and prolonging its half-life. Conversely, Tsanov *et al* (12) reported that Lin28A is phosphorylated by a MAPK (ERK1/2) at S200, allowing post-translational stabilization. Considering these studies, the present study evaluated whether RSV treatment could interfere with these post-translational regulatory pathways. Specifically, the present study assessed whether RSV would disrupt the interactions between Lin28A and USP28 or prevents ERK1/2-mediated phosphorylation at S200.

The MTT assay results in the present study agreed with previous reports (31,32), indicating that RSV inhibited NCCIT cell viability in a dose-dependent manner with an $IC_{50}$ of 148 $\mu$M. All experiments were performed using the same concentrations (25-150 $\mu$M). Notably, only treatments with 100 or 150 $\mu$M RSV resulted in a significant decrease in Lin28A protein expression levels over time, with the protein expression almost completely absent after 96 h at 150 $\mu$M. The present study also assessed USP28 protein expression following treatment with 150 $\mu$M RSV. The findings demonstrated a significant reduction in USP28 protein levels similar to the decrease observed for Lin28A protein expression. These findings support the hypothesis that disruption of the Lin28A-USP28 interaction, which stabilizes Lin28A, could underlie the mechanism of RSV of reducing Lin28A protein expression levels at higher doses.

USP28 is an important regulator of proteins involved in proliferation and metastasis, such as c-MYC, lysine-specific histone demethylase 1, hypoxia-inducible factor 1α, c-JUN and mediator of DNA damage checkpoint protein 1; moreover, USP28 is usually upregulated in several cancers including pancreatic, squamous, gastric and colorectal (33-37), marking it as an important therapeutic target (33,37). The results of the present study demonstrated that RSV downregulated USP28

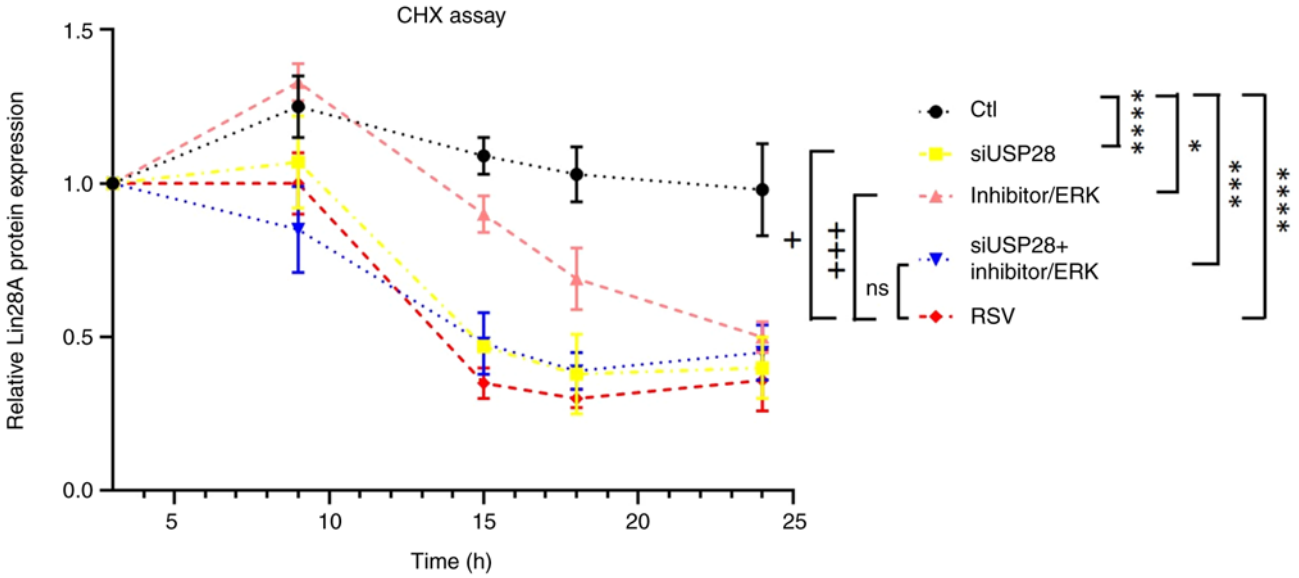


Figure 9. CHX protein stability assay. Comparative graph of the degradation kinetics of the Lin28A protein with the different treatments (siUSP28, Inhibitor/ERK and RSV) in NCCIT cells with 11 $\mu$g/ml CHX. The assay was performed over a time period of 3-24 h. The time of 15 h was considered to perform the comparison tests as, during this time, the most drastic decrease in the treatments was observed. $^{*}$P<0.05; $^{***}$P<0.001; $^{****}$P<0.0001 vs. Ctl. $^{+}$P<0.05; $^{+++}$P<0.001 vs. RSV. ns, non-significance; si, small-interfering; RSV, resveratrol; CHX, cycloheximide; Ctl, control.

at the protein level. Furthermore, analysis revealed that RSV also reduced USP28 mRNA expression in a dose- and time-dependent manner, suggesting transcriptional regulation. This effect may occur through downregulation of transcription factors that bind the USP28 promoter or upregulation of inhibitory micro (mi)RNAs. Potential candidates affected by RSV include oncogenic regulators c-JUN and c-MYC, which activate USP28 transcription (38-40). Previous studies have reported RSV inhibits these factors (40-42). Additionally, RSV may increase miR92b-3p expression, reported to repress USP28 by binding its 3'-untraslated region (43). RSV could also boost miR-216b expression, which inhibits USP28 mRNA in hepatocellular carcinoma cells (44). Increased miR-622 expression is also possible as it acts as a tumor suppressor against KRAS, an oncogene that upregulates USP28 (38). Whilst speculative, future experiments could assess whether RSV modulation of these transcriptional and post-transcriptional regulators underlies USP28 mRNA reduction. Overall, downregulating both USP28 protein and mRNA expression may contribute to the anticancer effects of RSV in the model in the present study. In addition, the present study demonstrated that RSV induces Lin28A degradation by the proteasomal pathway, consistent with the findings of Haq *et al* (11), who reported that Lin28A is degraded by this route. On the other hand, comparing the expression of Lin28A and pLin28A after USP28 inhibition using siRNAs revealed a small decrease in Lin28A protein expression, while pLin28A levels increased. We hypothesize that constant Lin28A mRNA expression may compensate for loss of USP28-mediated protection, whilst decreased USP28 may elevate ERK activation and consequently pLin28A levels (12,45). Notably, USP28 knockout alone in the NCCIT cells was insufficient to completely reduce Lin28A protein expression, indicating additional regulatory mechanisms. Phosphorylation of Lin28A at S200 is another critical post-translational modification that enhances stability (12); therefore, the effects on RSV on the phosphorylation of Lin28A mediated by the MAPK/ERK pathway was analyzed. A biphasic effect was observed, with low RSV concentrations (25-50 $\mu$M) increasing pLin28A expression, and higher doses (100-150 $\mu$M) significantly decreasing it. Low doses of RSV (25 $\mu$M) appeared to stimulate the MAPK/ERK pathway, as demonstrated by the increased Lin28A phosphorylation. This likely increased Lin28A mRNA and protein synthesis at early time points under normal conditions and it could be possible that the stability of the Lin28A protein influences in the auto-regulation of Lin28 mRNA. Previous studies have reported that phosphorylation can enhance the activity of Lin28 on its mRNA targets (12,46). Moreover, there is evidence that Lin28 binds to the mRNA of several targets, including its own mRNA, increasing the steady-state levels of its own transcript and consequently enhancing its expression (47,48). Additionally, increased Lin28 expression has been reported to boost the translation of its targeted mRNAs by recruiting RNA helicase A and LINE-1 type transposase domain containing 1 to polysomes (49,50). Therefore, it seems plausible that the interaction of Lin28 with its own mRNA could also promote its own translation.

On the other hand, higher doses of RSV (100-150 $\mu$M) inhibited the MAPK/ERK pathway and decreased Lin28A synthesis. As RSV can inhibit the MAPK/ERK signaling cascade (51-53), we hypothesize that this polyphenol can reduces phosphorylation at S200, favoring Lin28A degradation. Notably, a recent study by Tan *et al* (54) described the significance of phosphorylation at S200 for Lin28A stability and function, reporting that mutation of this region reduced stability and impaired function. The results of the present study demonstrated that whilst RSV at 150 $\mu$M decreases S200 phosphorylation as early as 24 h, total Lin28A protein levels remain stable through 96 h of treatment. This indicates that additional protective mechanisms may sustain Lin28A

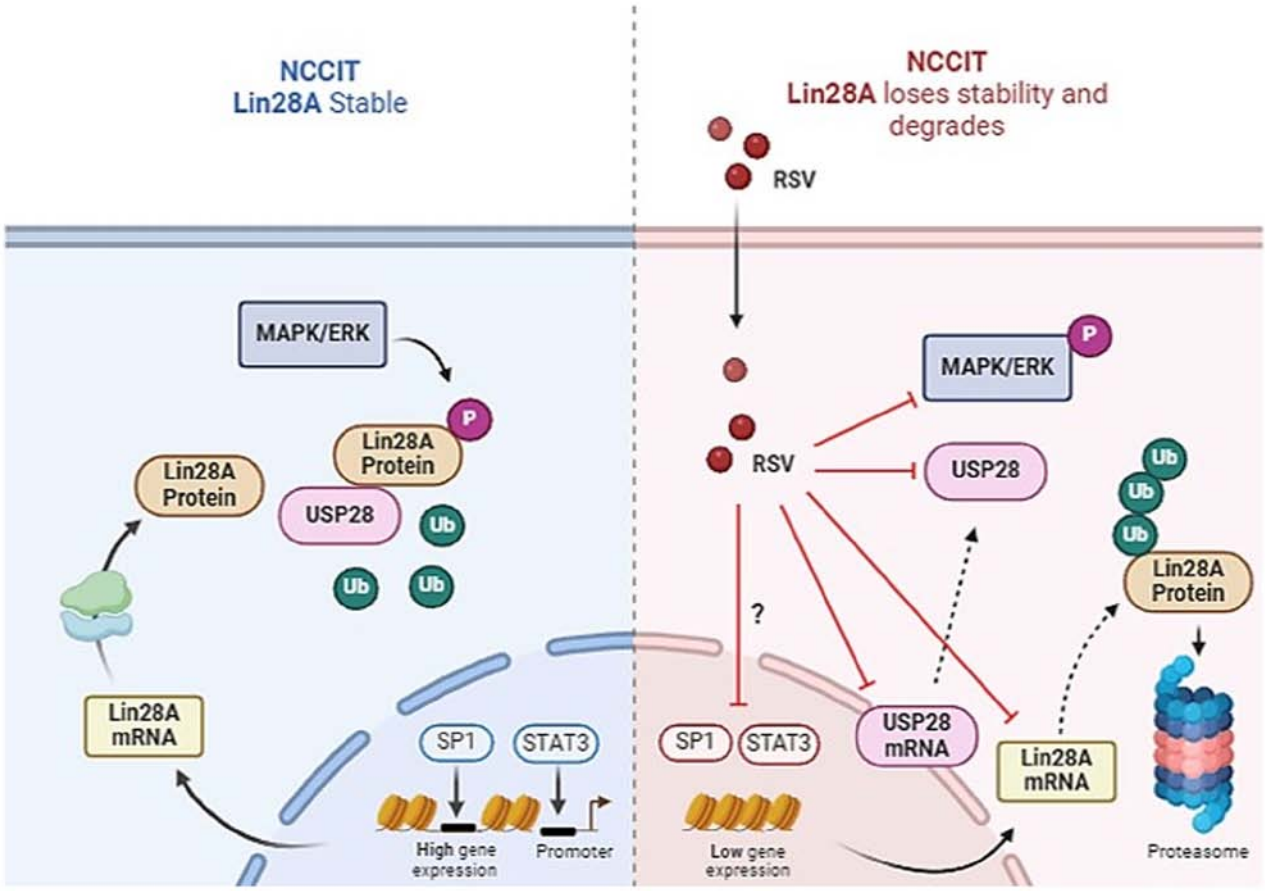


Figure 10. RSV inhibits Lin28A mRNA expression in NCCIT cells, potentially through inhibiting Lin28A mRNA synthesis via the decrease in the transcription factors SP1 and STAT3; however, this has not been completely proven in the model. Conversely, it was revealed that RSV inhibits the expression of the USP28 deubiquitinase and the MAPK/ERK pathway, which is responsible for phosphorylation at serine 200, which allows its stability. This causes the Lin28A protein to lose its stability mechanisms and begin its degradation through the proteasomal pathway. Image produced in Biorender. RSV, resveratrol; USP28, ubiquitin-specific protease 28; P, phosphorylation at serine 200; Ub, ubiquitin marks. Created with BioRender.com.

stability over prolonged periods, despite diminished S200 phosphorylation. It would be worthwhile to elucidate these stabilizing mechanisms more precisely using the model in the present study. Specifically assessing how Lin28A stability is maintained for lengthy durations when S200 phosphorylation is suppressed by RSV could yield valuable insights into the complex regulation of this important oncoprotein. Overall, the present study provides new context regarding Lin28A phosphorylation and complements prior findings on phosphorylation's role in Lin28A turnover.

Subsequently, the present study assessed whether inhibiting the MAPK/ERK pathway was sufficient to decrease both pLin28A and total Lin28A protein levels. pLin28A and total Lin28A were analyzed in NCCIT cells treated with only the ERK inhibitor (PD0325901), which was also used in the study by Tsanov *et al* (12). These experiments demonstrated that whilst phosphorylation of Lin28A was reduced, total Lin28A protein remained stable. USP28 protein expression with the ERK inhibitor treatment was also analyzed and it was demonstrated that USP28 expression was maintained, suggesting that Lin28A protein stability depends on both pathways (USP28 and MAPK/ERK). Inhibiting only one pathway may not be sufficient for complete Lin28A degradation, at least in a model with continuous Lin28A mRNA expression. Furthermore, using CHX, the production of new proteins was inhibited. Therefore, at the highest RSV concentration of 150 $\mu$M, the degradation of Lin28A was most effectively observed under these assay conditions.

In contrast, the experiments presented in Figs. 1 and 2 measured steady-state protein and mRNA levels for 24 h, allowing both the synthesis of the new Lin28A protein and the production of other proteins to affect stability and degradation to influence protein levels. It was demonstrated that inhibiting USP28 was more important for Lin28A degradation than inhibiting ERK activation. The most significant finding was that RSV was the most effective treatment at inhibiting Lin28A protein levels. Notably, the degradation kinetics observed with combined USP28 siRNA and ERK inhibitor treatments mimicked those seen with RSV alone, suggesting these pathways are key mediators of the negative regulation of the Lin28A protein by RSV. However, it is possible RSV may also impact other pathways. For example, RSV could affect Kruppel-Associated Box Domain-Associated Protein 1 (KAP1), a protein reported to bind Lin28A and inhibit its ubiquitination similar to USP28 (55). Additionally, RSV may directly upregulate miRNA Let-7 expression, indirectly downregulating Lin28A in turn (9,56). However, further study is needed to confirm whether RSV does indeed act through these or other potential mechanisms. Specifically, targeted experiments investigating the direct effects of RSV on KAP1

and Let-7 levels could provide insight into additional pathways of Lin28A regulation affected by this polyphenol. Whilst the findings of the present study implicate USP28 and ERK signaling, more research is warranted to fully elucidate the multidimensional impact of RSV on Lin28A expression and turnover at the post-transcriptional and translational levels.

The primary limitation of the present study is that human cancer samples were not assessed as obtaining human cancer samples that include treatment with RSV to observe the relationship between the decrease in Lin28A expression and patient survival is not feasible in Mexico. However, the findings of the present study could be validated through further investigations using other types of cancer cells, such as ovarian, gastric, prostate and breast cancer, where previous research has established that both Lin28A and USP28 are overexpressed. Although the results of the present study contribute to the general knowledge of RSV pathways and mechanisms to regulate Lin28A and USP28 that may help to develop new strategies to combat cancer, it is acknowledged that the study lacks data on human tissue experimentation and *in vivo* models, which are the most important counterpart of *in vitro* first approach experiments.

In conclusion, the present study demonstrated that RSV inhibits Lin28A expression through multiple mechanisms. RSV targets both USP28-mediated post-translational regulation of Lin28A as well as MAPK/ERK phosphorylation of Lin28A, promoting Lin28A degradation through the proteasomal pathway (Fig. 10). Notably, the finding that RSV inhibited USP28 is significant, as USP28 stabilizes several oncoproteins and facilitates EMT and metastasis (57). The ability of RSV to modulate the USP28 pathway implies it may have broader anticancer effects beyond Lin28A suppression. Collectively, RSV exerts multidirectional inhibitory effects on Lin28A expression and stabilization, supporting its potential as an attractive adjunctive therapy. Moreover, RSV simultaneously targets both Lin28A and USP28, which likely enhances its antitumor efficacy. This multifaceted mechanism supports further development of RSV as part of comprehensive cancer treatment regimens.

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**Availability of data and materials**

The data generated in the present study may be requested from the corresponding author.

**Authors' contributions**

SCN, SCR and EGV performed all experiments. SCN and EGV performed the data interpretation and analysis of the results. SCN, EGV, PG and JDC conceptualized and proposed the study. SCN wrote the manuscript and generated the figures. SCN, EGV, SCR, PG and JDC confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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