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MiR-29 and *MiR-140* regulate TRAIL-induced drug tolerance in lung cancer

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has chemotherapeutic potential as a regulator of an extrinsic apoptotic ligand, but its effect as a drug is limited by innate and acquired resistance. Recent findings suggest that an intermediate drug tolerance could mediate acquired resistance, which has made the main obstacle for limited utility of TRAIL as an anti-cancer therapeutics. We propose miRNA-dependent epigenetic modification drives the drug tolerant state in TRAIL-induced drug tolerant (TDT). Transcriptomic analysis revealed *miR-29* target gene activation in TDT cells, showing oncogenic signature in lung cancer. Also, the restored TRAIL-sensitivity was associated with *miR-29ac* and *140-5p* expressions, which is known as tumor suppressor by suppressing oncogenic protein RSK2 (p90 ribosomal S6 kinase), further confirmed in patient samples. Moreover, we extended this finding into 119 lung cancer cell lines from public data set, suggesting a significant correlation between TRAIL-sensitivity and RSK2 mRNA expression. Finally, we found that increased RSK2 mRNA is responsible for NF-κB activation, which we previously showed as a key determinant in both innate and acquired TRAIL-resistance. Our findings support further investigation of *miR-29ac* and *-140-5p* inhibition to maintain TRAIL-sensitivity and improve the durability of response to TRAIL in lung cancer.

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MicroRNAs; RSK2; TRAIL-persistence; lung cancer

Introduction

Lung cancer is the leading cause of cancer-related death, and it is often incurable when diagnosed at advanced stages. Over 70% of lung cancers are defined as a non-small cell lung cancer (NSCLC) type, and the rest of them are mainly classified as small cell lung cancer (SCLC) type. The reasons for the high mortality of lung cancer are multifactorial including late diagnosis, but the exact mechanism underlying cancer progression is poorly understood (Molina et al. 2008; Patel et al. 2011; Siegel et al. 2018). TNF-related apoptosis-inducing ligand (TRAIL) known as an extrinsic apoptotic ligand is a promising anti-cancer agent, which has been attractive because of high cancer specificity with minimal cytotoxicity (Wiley et al. 1995; Pan et al. 1997; Ashkenazi et al. 1999; Ichikawa et al. 2001). Unlike the murine system, two distinct receptors, DR4 and DR5 mediate TRAIL-induced cell death signal, and there are either or both expressions in most types of stage III NSCLC tumors, suggesting that there is high therapeutic potential using TRAIL and/or its agonistic antibodies to the receptors (Ashkenazi et al. 1999; Ichikawa et al. 2001; Spierings et al. 2003; Herbst et al. 2010).

Despite its promising therapeutic prospects, preclinical and clinical research indicates that resistance to TRAIL, resulting from either inherent or developed mutations, restricts its effectiveness in chemotherapy. Dulanermin, an early recombinant version of TRAIL, faced failure in clinical trials due to resistance emerging from the activation of pro-survival proteins such as NF-κB after 62 months, confirmed in other observations in

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vitro and in vivo (Chaudhary et al. 1997; Schneider et al. 1997; Ashkenazi et al. 1999; Plantivaux et al. 2009; Subbiah et al. 2012; Jeon et al. 2015). Similarly, Conatumumab, a monoclonal antibody that targets human TRAIL-R2, demonstrated potential in inhibiting tumor growth across various types of tumors both in vivo and in vitro (Kaplan-Lefko et al. 2010). However, even with the preliminary positive effects of these agonists in preclinical studies, antibodies targeting TRAIL-R did not lead to significant improvements in either the objective response rate or overall survival of patients in clinical trials alongside other treatments for different cancers (Kindler et al. 2012; Fuchs et al. 2013; Skelton et al. 2020).

Cancer heterogeneity has been considered a source of drug resistance, and the small subpopulation of cancer cells is resistant to chemotherapeutic agents without obvious actionable mutations (Ke et al. 2022; Li et al. 2022). The intermediate resistant stage has been characterized as a reversible phenotype when the signal is withdrawn to the cells. This drug-tolerant stage possibly gives time to the cells acquiring mutations under such the cell death pressure, which consequently fuels to acquisition of the resistance against a chemotherapeutic agent (Roesch et al. 2010; Sharma et al. 2010; Lee et al. 2014). It has been known that the epigenetic regulation of an oncogenic pathway is responsible for maintaining the intermediate state in response to targeted therapeutic drugs. Moreover, a recent study suggested that O-GlcNacylation on DR4 receptor was dynamically regulated in the drug-tolerant state compared to its naïve control cells (Hata et al. 2016; Smith et al. 2016; Guler et al. 2017). However, the cause and consequence of the drug-persistent stage are largely unknown.

The p90 ribosomal protein s6 kinases encoded by *RPS6KA* gene are serine/threonine protein kinase, which consists of four different isoforms. Although they exhibited functional redundancy with high sequence homology, the expression and roles of each isoform are context-specific in mediating diverse subcellular processes (Lara et al. 2013). A major type of pro-survival MAPK protein, ERK1/2 directly phosphorylates and activates RSKs in response to several external stimuli such as EGFR activation. Like the other isoforms, RSK2-mediated phosphorylation on diverse substrates enhanced cell cycle progression, cell survival, and cell proliferation (Poomakkoth et al. 2016). However, little is known about the oncogenic role of RSK2 in TRAIL-resistance.

Most abundantly expressed small non-coding RNA better known as miRNA plays an important role in cancer progression by targeting subcellular proteins at post-transcriptional or -translational levels (Calin and Croce 2006; Hwang et al. 2023). One of the most interesting features of a miRNA exhibited a context-dependency via regulating multiple targets even in the same cancer types (Genc et al. 2023). *MiR-29* family consists of three members, encoded by two different genomic loci at chromosome 1 and 7. The *miR-29* family was found to be suppressed in lung cancer, which consequently enhanced cancer progression by the loss of suppressing ability to their target oncogenes including DNMT3s (Volinia et al. 2006; Fabbri et al. 2007; Plaisier et al. 2012). Compared to *miR-29* family, the roles of *miR-140* in lung cancer have been less elucidated despite a few studies consistently showing its robust tumor suppressive role in lung cancer. *MiR-140* located on chromosome 16q was found to be suppressed miRNA in NSCLC patients, and its overexpression decreased the metastatic potential of lung cancer cells *in vitro* and *in vivo* (Yuan et al. 2013). In particular, the overexpression of *miR-140-5p* induced resistance of lung cancer cells in response to tyrosine kinase inhibitors or platinum-based chemotherapy (Flamini et al. 2017). However, the roles of both miRNAs in TRAIL-induced persistent resistance are largely unknown.

Materials and methods

Cell culture, cell line generation, and gene delivery

Human lung cancer cells, H460, H292, and A549 cells were purchased from ATCC, and the cell lines were maintained in DMEM supplemented with 10% FBS and 1% Penicillin–streptomycin (Sigma). The generation of TRAIL-resistant H460R cells was described in a previous study (Jeon et al. 2015). The H460 revertant was generated by abolishing TRAIL stimulation for weeks. TRAIL-sensitivity was determined weekly basis to determine revertant phenotype. pcDNA-*RPS6KA3* plasmid was introduced using Lipofectamine 3000 reagent (ThermoFisher Scientific), and miRNAs and siRNAs used in the current study were transfected by Lipofectamine RNAi Max reagent (ThermoFisher Scientific) according to the manufacturer's protocols.

Antibodies

Anti-GAPDH (#5174), anti-PARP-1 (#9532), anti-phosphor p65 (#3033), anti-RSK2 (#5528), and anti-Vinculin (#13901) antibodies were purchased from Cell Signaling Technology. Anti-p65 (#ABE136) was purchased from Sigma-Aldrich. Anti-tubulin (#sc-8035) antibody was purchased from Santa Cruz Biotechnology.

Cell cytotoxicity assay

The cells (1.0×10^3) were cultured into 96 well plates. After 24h, the cells were exposed to TRAIL and/or other drugs as indicated concentration shown in figures or figure legend. Subsequently, the cell survival rate was determined by CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, #G7572) according to the manufacturer's protocol.

NF-κB promoter assay activity

A pGL4.3-luc2p/NF-κB construct harboring five copies of NF-κB binding sites (Promega, #E8491) was co-transfected either alone or with the corresponding miRNAs and/or pcDNA-*RPS6KA3* plasmid. After 24–48h of transfection, the cells were harvested and subject to Dual Luciferase® (Promega, #E1910) as followed by the manufacturer's protocol.

Public data analysis for the expression analysis of miR-29 and -140-associated signatures

The miRNA (GSE55821) and gene expression profiles (GSE55859) of TRAIL-sensitive and -resistant H460 cells were obtained from a previous study (Jeon et al.). A robust multi-array average (RMA) algorithm was used to normalize the data using R package. The hierarchical- and principal component analysis based on 2500 top variable genes were performed using *hclust* and *prcomp* function in R package. Differential expression analysis was performed by fitting linear models using the empirical Bayes method as implemented in the *limma* R package (Smyth 2004) and *P* values were adjusted for multiple testing using FDR method. Genes with an adjusted *P* value smaller than 0.05 and fold change greater than two-fold selected as significant differentially expressed genes (DEGs). DEGs from *limma* were used as input for the implementation in The Molecular Signatures Database (Subramanian et al. 2005) to calculate the overlap for highlighting common processes, pathways, and underlying biological processes. Gene ontology terms with *P* value smaller than 0.05 were selected as significant and used subsequently for visualizations.

The miRNA, RNA-seq expression profiles and corresponding clinical information of Lung Squamous Cell Carcinoma (LUSC) and Lung Adenocarcinoma (LUAD) were obtained from the TCGA data portal (https://portal.gdc.cancer.gov/) with a total of 1143 LUAD samples (1035 tumor- and 108 normal tissue) and 523 LUSC samples (478 tumor- and 45 normal tissue). The data were downloaded in April 2021. Kaplan-Meier curves were plotted for two distinct groups of patients based on miRNA expression, defined by quantile, using the *survfit* function, and the *P* values (log-rank test) were calculated using the *survdiff* function in the survival R package.

Co-expression analyses NF-κB and TRAIL-sensitivity

We collected data on the TRAIL-sensitivity of various lung cancer cell lines from previous literature (Shivapurkar et al. 2004; Wagner et al. 2007; Zitzmann et al. 2011; Yang et al. 2013; Lu et al. 2014; Jeon et al. 2015; Joshi et al. 2015; Li et al. 2017). Additionally, the remaining lung cancer cell lines used in the current study were mined from the Genomics of Drug Sensitivity in Cancer database (Yang et al. 2013). We also acquired data on the *RPS6KA3* mRNA expression in the corresponding lung cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al. 2012). We have finally utilized 119 lung cancer cell lines to investigate the relationship between TRAIL sensitivity and *RPS6KA3* expression (Supplementary Table S1). We categorized the TRAIL sensitivity based on existing literature. Additionally, cell lines exhibiting IC50 values higher than the median value (0.634nM) were classified as TRAIL-resistant, while the remaining cell lines were categorized as TRAIL-sensitive. The significance was determined by Wilcoxon rank sum test.

Migration and invasion assays

The migration and invasion kits were purchased from Calbiochem. The cells were subcultured into 6-well plates. After 24h, the cells were transfected with pre-*miR-29a* or *-140-5p* for 24h, and the media containing reduced serum (1%) was subsequently changed for an additional 24h. The cells subsequently plated into migration (5*10^5 cells/ml) and invasion chamber (1*10^6 cells/ml), and the bottom chambers remained with the serum-reduced media. After 24–48h, the migrated or invasive cells were quantified Calcein AM staining, followed by obtaining fluorescence at an excitation wavelength of 485nm and an emission wavelength of 520nm.

Statistical analysis

Figure 1G-I, Figure 2B-E, and Figures 3 and 4 were confirmed by at least 2 indicated biological replicates. The figures in this manuscript are the representative ones based on technical replicates. Student's t-test or Fisher exact t-test was used to address significance. Error bars are expressed as mean ± standard deviation (SD). Statistical significance assessed by calculating *p*-value was less than 0.05.

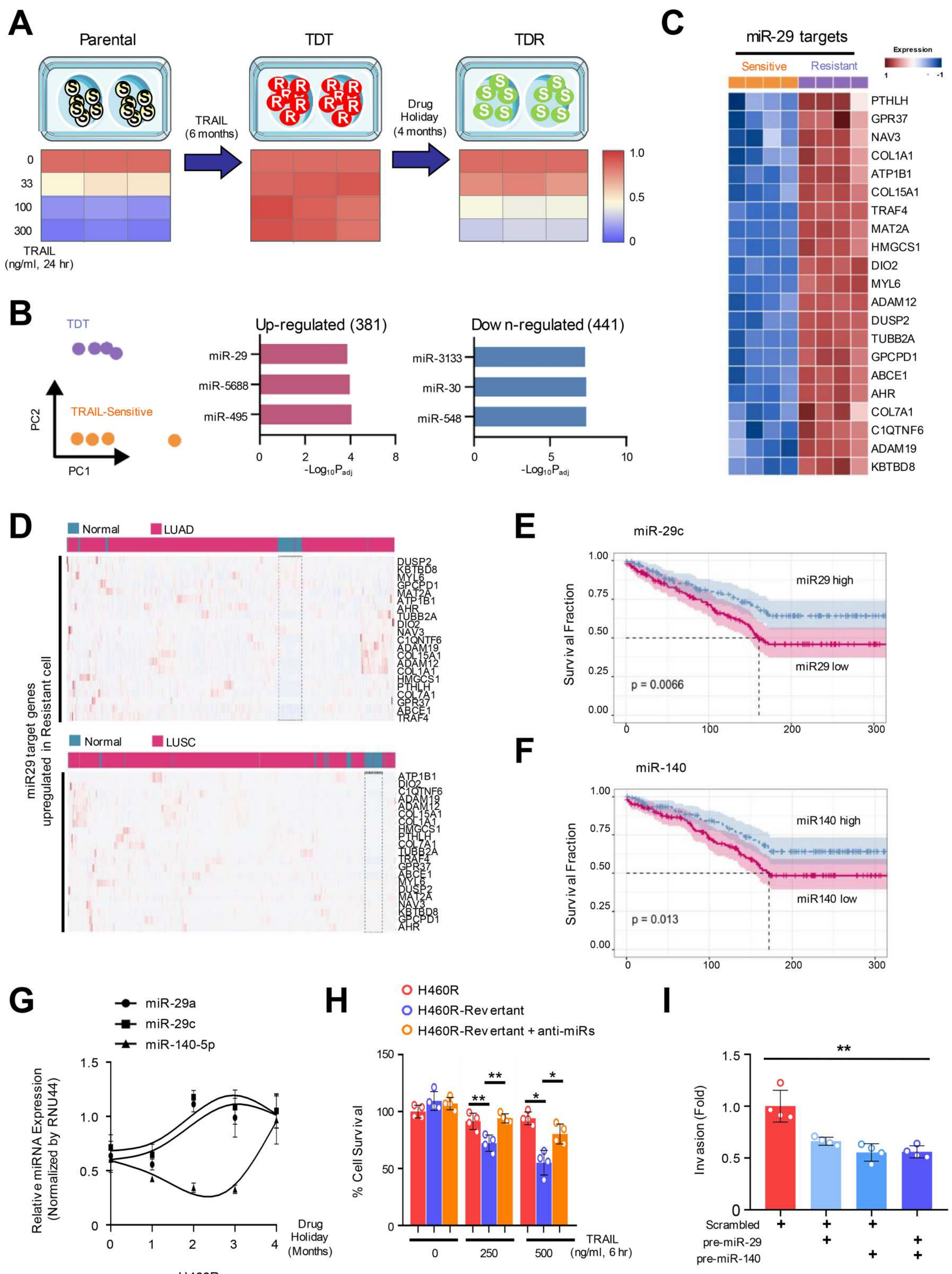

Figure 1. Downregulation of oncogenic *miR-29* and *-140-5p* causes TRAIL-induced Drug-tolerant (TDT) state. (A) A schematic diagram showing the strategy to generate TRAIL-revertant from the resistant cells. (B) Gene Ontology terms associated genes differentially expressed in TRAIL-resistant cell (H460R) compared with sensitive cell. Left panel is a principal component analysis showing clustering between sensitive and resistant quadruplicate. (C) Heatmap showing upregulated *miR-29* target genes in H460R cell. (D) Heatmap of *miR-29* target genes upregulated in TRAIL-resistant cells. Kaplan-Meier curve depicting the survival probability in the patients with LUAD associated with (E) *miR-29* or (F) *miR-140* expression. (G) The restored expression of *miR-29 a/c* and *-140-5p* in H460R in response to drug holiday. (H) Cell survival rate using CellTiter-Glo assay. Error bars indicate mean ±SD ($n = 4$) and the *p*-values were calculated by one-way ANOVA test (**$p < 0.01$). (I) Chamber invasion assay of H460R cell reconstituted by *miR-29* and/or *-140-5p*. Error bars show mean ±SD (n = 6) and the *p*-values were calculated by two-tailed student t-test (* $p < 0.05$, **$p < 0.01$).

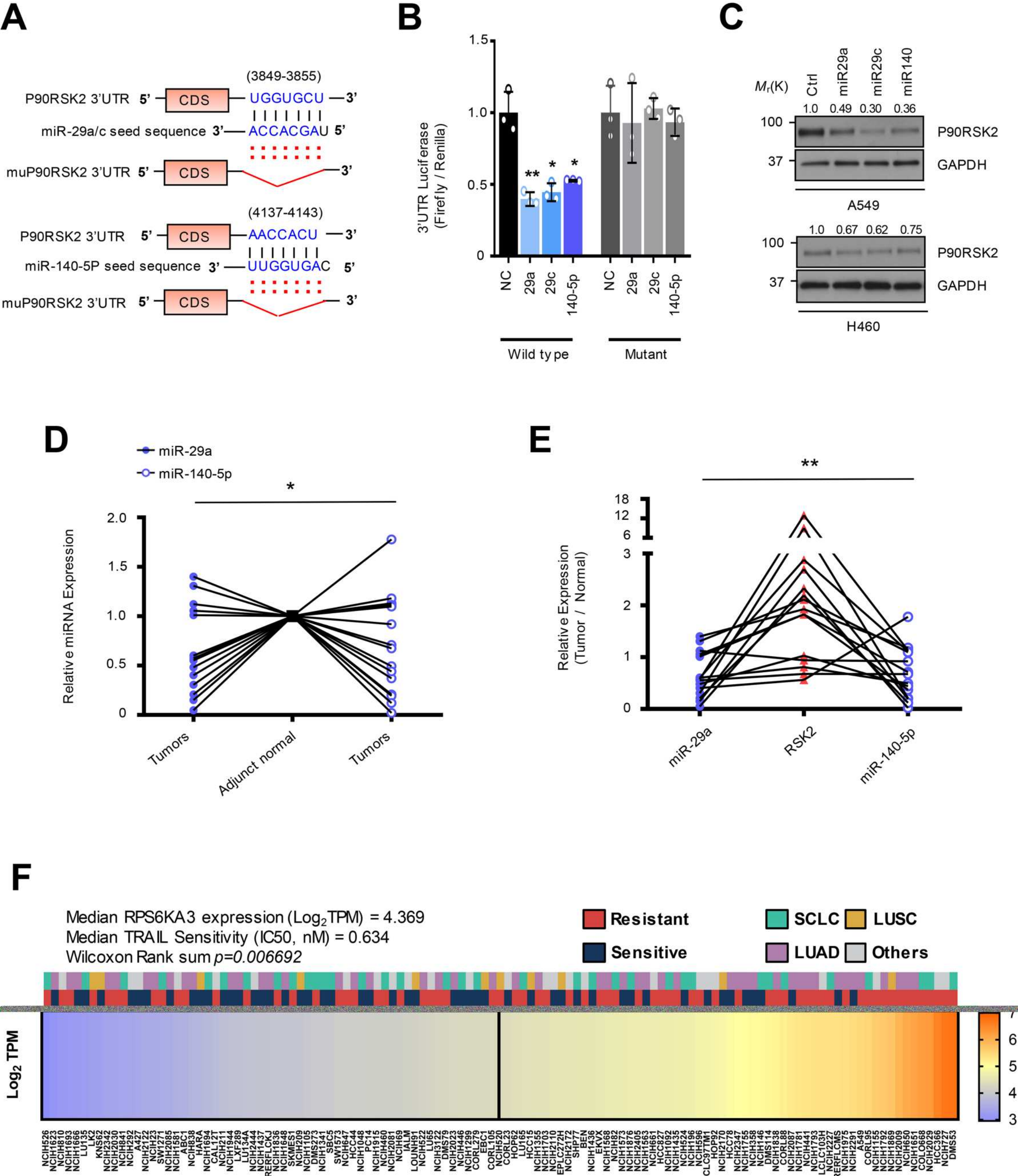

Figure 2. *MiR-29a/c* and *-140-5p* directly suppressed RSK2 protein expression causing TRAIL-sensitivity (A) A schematic diagram showing seed sequence of *miR-29/-140-5p* and the target sequence of *RPS6KA3* 3'UTR. (B) Luciferase reporter assay using 3'UTR of *RPS6KA3* or 3'UTR harboring deletion mutations of the *miR-29a/c* and *-140-5p* in HEK293 cells over-expressing *miR-29a/c* or *-140-5p*. Error bars indicate mean ±SD ($n = 3$). *P*-values were calculated by two-tailed student t-test. (C) Western blot analysis indicating suppressed RSK2 protein level in lung cancer cells. Indicated cells were transfected by indicated miRNAs, respectively. After 48h, cells were harvested and subjected to Western blotting with indicated antibodies. (D) Expressional comparison of the *miR-29a* and *-140-5p* in lung cancer tissues compared to their adjunct normal tissues. Taqman-based qRT-PCR analysis was applied to analyze expression of the target miRNAs, and the expression of the target miRNAs in each cancer sample ($n = 15$) was normalized by their paired control sample ($n = 15$), respectively. *P*-values were obtained by one-way ANOVA test (* $p < 0.05$). (E) Correlation analysis of *miR-29a/-140-5p* and RSK2 protein expression in cancer. Expression of RSK2 protein was quantitated by measuring band intensities using Image J software. The intensity in cancer sample was normalized by paired normal sample. Likewise, relative value for *miR-29a* or *miR-140-5p* was calculated by comparing qRT-PCR values between cancer sample ($n = 15$) and its paired control sample ($n = 15$). *P*-value was obtained by one-way ANOVA test (**$p < 0.01$). (F) Expressional correlation of *RPS6KA3* and TRAIL-sensitivity in NSCLC. TRAIL-sensitivity for each cell line was obtained from previous literatures and colored by their sensitivity as indicated. For statistical analysis, cell lines were divided into two groups with median *RPS6KA3* expression, annotated by TRAIL-sensitivity from literatures, Wilcoxon rank sum test was applied to obtain significance. Detailed information about mRNA expression of *RPS6KA3* gene and TRAIL-sensitivity is described in Material and Methods section. Gene Ontology analysis using upregulated and downregulated genes in H460R cells showing differentially regulated pathways. (*$p < 0.05$, **$p < 0.01$)

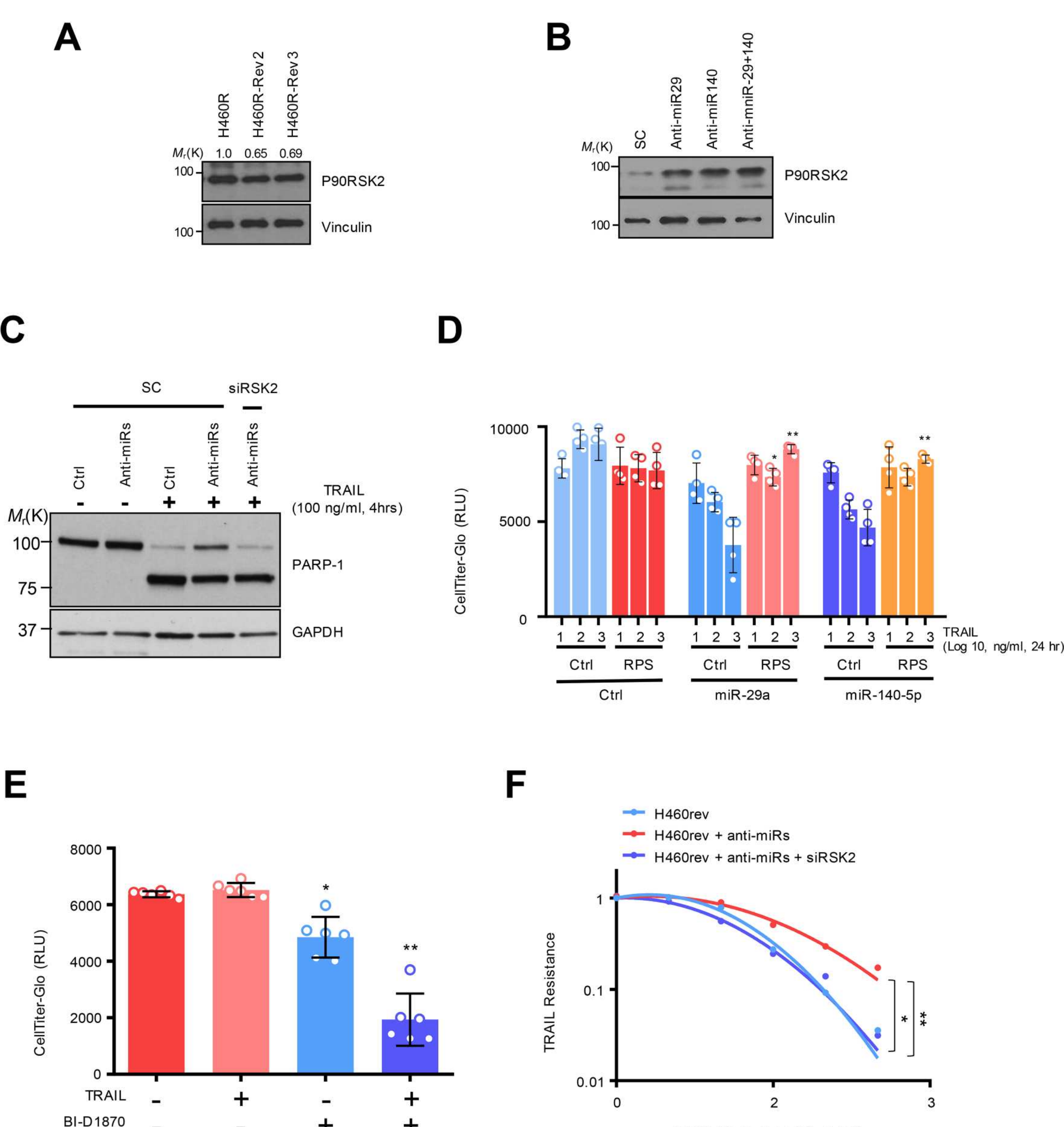

Figure 3. RSK2 is responsible for H460 revertant phenotype. (A and B) Western blot analysis showing the alteration of RSK2 expression in H460R revertant cell. (C) Western blot analysis showing increased PARP-1 cleavage by targeting RSK2. H292 TRAIL-sensitive cells were treated with anti-*miR-29 / -140* with siRSK2 siRNAs for 48h. Subsequently the cells were treated by TRAIL, followed by performing Western blot analysis as indicated antibodies. (D) Cell survival of the H460R cells in RSK2-dependent manner. The precursor of *miR-29a* or *-140-5p* was co-transfected with either empty vector or pcDNA-*RPS6KA3* plasmid for 48h, and the cells were subsequently stimulated by TRAIL as indicated. After that, the CellTiter-Glo assay was performed to determine cell survival rate. *P*-value was calculated by two tailed student t-test (*$p < 0.05$, **$p < 0.01$). (E) Enhanced TRAIL-sensitivity by pharmacologic inhibition of RSK2 activity. BI-D1870 was treated to H460R as indicated, and cell survival rate was determined by CellTiter-Glo assay. Bars shows mean ±SD ($n = 6$) and the *p*-values were calculated by two-tailed student t-test (* $p < 0.01$, **$p < 0.001$). (F) The re-sensitized TRAIL-sensitivity upon RSK2 suppression in cells harboring anti-*miR-29* and *-140-5p*. Bars shows mean ±SD ($n = 4$) and the *p*-values were calculated by two-tailed student t test (*$p < 0.01$, **$p < 0.001$).

Results

Dysregulation of oncogenic miR-29ac and -140-5p is responsible for TRAIL-induced drug-tolerant (TDT) state

To characterize the mechanism underlying TRAIL-resistance, we previously generated TRAIL-resistant cells by treating a subtoxic range of TRAIL to H460 and H292-TRAIL sensitive cells (H460S and H292S) for 6 months, and that resistance was abolished by giving those cells drug-holiday, suggesting that the resistant cells we previously generated showed drug tolerant phenotype referred as TRAIL-induced drug tolerance (TDT) (Figure 1(A)) (Jeon et al. 2015; Joshi et al. 2015). Although we previously characterized how the TDT cell was mechanistically established and its significance in

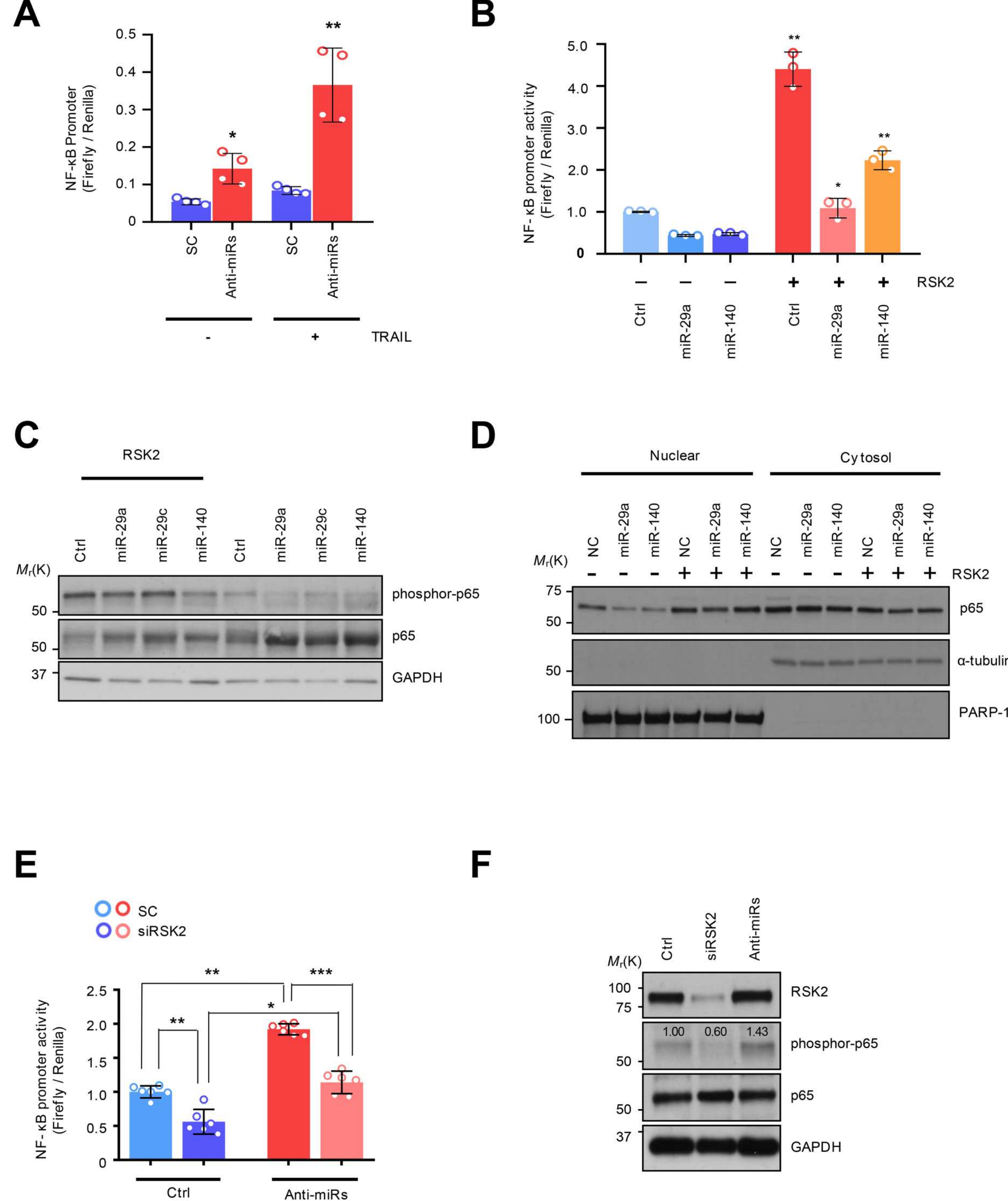

Figure 4. The NF-κB regulation by miRNA-dependent RSK2 suppression: (A) NF-κB promoter measurement using luciferase reporter assay. (B) Restored NF-κB activity by RSK2 overexpression in *miR-29/-140-5p* expressing A549. The cells were co-transfected by *miR-29a/-140-5p* and pcDNA-*RPS6KA3* plasmid for 48h. Subsequently the promoter activity was measured by NF-κB promoter luciferase assay. Error bars indicate mean ±SD ($n = 3$) and the p-value was obtained by fisher exact t-test (*$p < 0.05$, **$p < 0.01$). (C) The phosphorylation of P65 subunit of NF-κB protein was analyzed by Western blot analysis. (D) Subcellular fractionation assay indicating nuclear localization of p65 depending on RSK2. Enhanced NF-κB activity by *miR-29/-140* suppression. The H460 revertant was co-transfected by anti-*miR-29/-140* and si*RPS6KA3* siRNAs for 48h, and the promoter activities were addressed by (E) luciferase assay and (F) Western blot analysis. P-values were calculated by paired student t-test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

lung cancer progression, it has been not sufficiently analyzed as a transcriptome-wide. From the result of our previous study, we found around 400 differentially expressed genes in H460R compared to H460S (Jeon et al. 2015). Subsequent Gene Ontology analysis using those genes suggested that the miRNA signatures

were successfully identified. Of those, the enhanced *miR-29* target genes in H460R brought our interest to further analysis because we initially identified *miR-29* and *-140-5p* as suppressed miRNAs in TDT cells that we established (Figure 1(B); Supplementary Figure S1A). Moreover, the upregulated *miR-29* target genes exhibited lung cancer-specific signatures (Figure 1(C–D)). Additionally, TCGA data showed that *miR-29* was found to be decreased in lung squamous cell carcinoma (LUSC), and *miR-140* was suppressed in both LUSC and lung adenocarcinoma (LUAD), associated with favorable survival prediction of the patients with LUAD (Figure 1E-F; Supplementary Figure S1B-S1C). These data suggest that *miR-29* pathway is suppressed in TDT cells, which could play a significant role in lung cancer progression. To analyze whether these miRNAs could be associated with the reversible sensitivity in TDT cells, we gave 4 months of drug holiday to H460R cells and found both *miR-29ac* and *-140-5p* expression restored in response to drug holiday, referring to it as a TRAIL holiday-induced drug revertant (TDR) cell (Figure 1(G)). Moreover, the revertant was sensitive to TRAIL-induced cell death, rescued by the genetic inhibition of *miR-29ac* and *-140-5p* with anti-miRNA inhibitor treatment (Figure 1H; Supplementary Figure S2A). Conversely, the overexpression of *miR-29* and/or *miR-140* in TRAIL-sensitive H460 and H292 cells decreased cell survival rate, consistently showed an increase in caspase substrate activity and cleaved PARP-1 protein (Supplementary Figure S1D and S2B-S2D). Moreover, these *miR-29* and *-140*-dependent TRAIL-sensitivity seems to be conserved in the Calu-1 cell innately resistant to TRAIL (Supplementary Figure S2E-S2G). Consequently, the H460R overexpressing pre-*miR-29ac* and/or *-140-5p* decreased in migratory and invasive abilities (Figure 1I; Supplementary Figure S3C). These data strongly suggest that the TDT cells exhibit a reversible phenotype referring to a drug-tolerant persistent stage, which could be mainly driven by alteration of *miR-29ac* and *-140-5p* expression.

RPS6KA3 gene is a direct target for both miR-29ac and -140

To elucidate the mechanism underlying the *miR-29/-140*-dependent TRAIL resistance, we investigated the potential target genes using *in silico* tools and found that *RPS6KA3* (RSK2 for protein) could be a potential target for both miRNAs (Figure 2A) (McGeary et al. 2019). The enforced expression of *miR-29ac* and *-140-5p* in HEK293 cells reduced the 3′UTR luciferase activity, whereas the mutants harboring deletion mutations on the binding sites for the miRNAs remained unchanged in the same context (Figure 2B). Consistently, Western blot analysis using two lung cancer cells showed that RSK2 protein was suppressed by the overexpression of the miRNAs (Figure 2(C)). However, we did not see any significant suppression of RPS6KA3 mRNA level in both qRT-PCR and microarray the same context, suggesting that miR-29 and -140-5p regulate RPS6KA3 at translational level without inducing RPS6KA3 mRNA decay (Supplementary Figure S4A-S4B). To analyze whether the *miR-29ac/-140-5p*-dependent *RPS6KA3* suppression could be conserved *in vivo*, we employed 15 cases of LUAD samples and their adjunct normal lung cells. As a result, we found that the miR-29a expression was decreased in 10 cases of tumor samples compared to adjunct tissue samples, whereas 2 cases showed increases and the rest changed minimally. In the analysis of *miR-140*, the 9 cases are found to be decreased, but the remaining 6 cases showed either unchanged or enhanced expression in lung cancer sample (Figure 2D). Next, we analyzed the expression of RSK2 protein and found that RSK2 protein was upregulated in 10 cases of the lung tumor tissues, suggesting that RSK2 protein expression was significantly increased in tumor tissues compared to adjunct control cells (Supplementary Figure S4C-S4D). Collectively, there was a significant inverse correlation between both miRNAs and RSK2 protein in lung cancer, suggesting that *miR-29ac/-140-5p*-dependent *RPS6KA3* suppression is conserved in the patient samples (Figure 2(E)).

Given our current observations of the direct relation between *miR-29/-140* and *RPS6KA3* expression, we hypothesized that *RPS6KA3* expression contributes to the establishment of TRAIL persistent phenotype. To evaluate this hypothesis, we employed public data to compare the bona fide nature of TRAIL sensitivity in lung cancer cells and analyzed any correlation between the expression of *RPS6KA3* gene and TRAIL sensitivity. We identified lung cancer cell lines annotated by TRAIL sensitivity using IC50 score. Also, the lung cancer cell lines containing the feature of TRAIL resistance were additionally analyzed and added to the lists from the previous works of literature. As a result, 119 lung cancer cell lines were classified as either TRAIL-sensitive or TRAIL-resistant cell lines derived from SCLC and two major subtypes of NSCLC such as LUAD and LUSC and others (Shivapurkar et al. 2004; Wagner et al. 2007; Zitzmann et al. 2011; Yang et al. 2013; Lu et al. 2014; Jeon et al. 2015; Joshi et al. 2015; Li et al. 2017). In addition, *RPS6KA3* expression in the corresponding lung cancer cell lines was analyzed from Broad Institute Cancer Cell Line Encyclopedia (CCLE), and was ranked by its abundance (Barretina et al. 2012). As a result, we found that the cells with more *RPS6KA3* expression were more resistant to TRAIL-induced cell death (Figure 2(F)). To

clarify the transcriptional changes in H460R compared to H460S, differently expressed genes were analyzed and annotated to KEGG and Gene Ontology database (Kanehisa and Goto 2000; Thomas et al. 2022). 'MAPK signaling pathway' signature was significantly identified in both up- and down-regulated genes (Supplementary Figure S4E-S4F). All things considered, we not only identified the inverse correlation between *miR-29/ -140* and RSK2, but also figured out there is a relation between RSK2 and TRAIL-resistance.

The expression of RPS6KA3 is responsible for TRAIL-induced drug tolerance

To get better insight into the TRAIL resistance, we first analyzed how the expression of the RSK2 protein is changed in accordance with TRAIL sensitivity. The RSK2 protein abundance was decreased in TDR cells, which showed a rescued expression upon *miR-29* and/or *-140* suppression in the TDR cells, suggesting that *miR-29* and *-140*-dependent *RPS6KA3* regulation is associated with the drug-tolerant status (Figure 3(A,B)). We next sought to determine if the axis of *miR-29/-140-5p* and *RPS6KA3* regulates TRAIL-sensitivity. The suppression of *miR-29a /-140-5p* expression by corresponding anti-miRNA oligonucleotides increased TRAIL resistance in parental H460 and H292 cells with decreased PARP-1 cleavage and/or caspase-3/-7 activation. However, the sensitivity was restored by si*RPS6KA3* treatment in the same context (Figure 3C; Supplementary Figure S5A-S5C). Conversely, decreased cell survival rate and increased caspase-3/-7 substrate activity were observed in TDT H460R cells by overexpressing *miR-29* and *miR-140-5p*, whereas the reconstituted *RPS6KA3* restored cell survival rate in response to TRAIL treatment (Figure 3D; Supplementary Figure S5D). Moreover, pharmacological inhibition of RSK2 activity using a specific inhibitor, BI-D1870 synergistically suppressed cell survival rate with TRAIL in H460R cells (Figure 3E) (Sapkota et al. 2007). Finally, anti-*miR-29* and *-140-5p* treatment to H460 revertant cell enhanced TRAIL-resistance, but the cells were re-sensitized upon *RPS6KA3* suppression (Figure 3F). Taken together, these data strongly suggest that *miR-29ac* and *-140-5p*-dependent *RPS6KA3* suppression is conserved in TRAIL-resistant lung cancer cell, which plays a critical role in establishing TRAIL-persistent phenotype.

RPS6KA3 induces NF-κB activation in TRAIL-induced drug-tolerant (TDT) cell

It has been known that the resistant phenotype could be associated with an aggressive cancer phenotype, mediated by NF-κB activation (Schneider et al. 1997; Kim et al. 2011; Grunert et al. 2012; Jeon et al. 2015; Joshi et al. 2015; Wang et al. 2024). Moreover, our group previously confirmed that NF-κB activation is one of the established resistant markers in innate TDT cells by establishing TDT cells with long-term exposure to TRAIL, showing higher NF-κB activity, which seems to be involved in aggressive cancer phenotype (Sheridan et al. 1997; Wang et al. 1998; Chen et al. 2003; Hall and Cleveland 2007; Plantivaux et al. 2009; Jeon et al. 2015). Moreover, RSK2 induced phosphorylation-dependent proteasomal degradation of IκB-a in TNF receptor pathway (Peng et al. 2010). Therefore, we examined whether this oncogenic pathway could be conserved in the TDR cells. Regarding this, we first sought to analyze the aggressiveness of TRAIL-resistance with NF-κB pathway activation. A series of luciferase reporter assays showed that NF-κB promoter activity was reactivated by *miR-29ac/-140-5p* inhibition (Figure 4(A)). Conversely, the overexpression of the precursor miRNAs diminished TRAIL-dependent NF-κB activation in both H292R and Calu-1 cells (Supplementary Figure S3A-S3B). We then employed a pGL4.3-luc2p/ NF-κB construct to monitor NF-KB promoter activity and found that a decrease in NF-κB promoter activity was observed after overexpressing *miR-29* and *-140-5p*. However, the ectopic expression of *RPS6KA3* rehabilitated the promoter activity (Figure 4 (B)). Also, a major subunit of NF-κB, p65 protein was less phosphorylated in the *miR-29* and *-140-5p* expressing H460R cell, which consistently upregulated in response to *RPS6KA3* overexpression (Figure 4(C)). Moreover, nuclear localization of p65 protein inhibited in *miR-29* and *-140* overexpression condition was dependent on *RPS6KA3* expression (Figure 4(D)) Importantly, H460 revertant has conserved mechanism by which *miR-29ac/-140-5p*-dependent *RPS6KA3* suppression exhibited a decrease in NF-κB promoter activity, similarly conserved in TRAIL sensitive H460 cell (Figure 4(E)). Accordingly, suppressive effect of p65 protein phosphorylation enforced by siRSK2 siRNA treatment was rescued by miR-29ac suppression (Figure 4(F)). Taken these together, RSK2-NF-kB axis is responsible for TDT state.

Discussion

Drug resistance to cancer is a multi-step process, a major obstacle in chemotherapeutic approach. To identify the molecular mechanism underlying acquired TRAIL-resistance, we previously established the resistant cells by treating TRAIL to the sensitive H460 cells for 6 months and reported that the positive feedback loop between miRNAs (*miR-21, -30c* and *-140*) and NF-κB activation is responsible for acquired TRAIL-resistance, consistent with the studies for innate TRAIL-resistance in that NF-

κB is major player for both types of resistance (Chaudhary et al. 1997; Schneider et al. 1997; Plantivaux et al. 2009; Jeon et al. 2015) Despite more robust analysis to identify any mutation associated with TRAIL-resistance, we did not find any actionable mutation in the genes involved in TRAIL-induced signaling pathway (Jeon et al. 2015), which initially motivated us to characterize any mutation or pathway involved in the resistant pathway in the current study. Our current observation suggests that the restored expression of the *miR-29* and *-140-5p* in H460R revertant is responsible for TRAIL-sensitivity, and their novel target, RSK2 protein could mediate *miR-29/-140-5p*-dependent TRAIL-resistance.

Cancer evolution has been thought to be a mechanism underlying drug resistance. In particular, recent studies have suggested that the drug-tolerant stage was driven by epigenetic regulation of the oncogenic pathway, and could mediate the acquired resistance driven by emerging mutations, being consequently an important issue to reduce the chance for the resistance by blocking the intermediated stage (Roesch et al. 2010; Sharma et al. 2010; Lee et al. 2014; Hata et al. 2016; Smith et al. 2016; Guler et al. 2017). Regarding this, we initially observed that *miR-29a/c* and *-140-5p* were restored, associated with recovering TRAIL-sensitivity, suggesting that the established TDT cells were drug-tolerant persister (Figure 1; Supplementary Figure 1-3). Despite the suggested tumor suppressive roles of both *miR-29* family and *miR-140*, recent analysis of circulating plasma miRNAs interestingly suggested that serum *miR-29* family were significantly elevated in the patients with NSCLC, associated with poor 5-year overall survival, raised the feasibility of the *miR-29* as an oncogenic modifier in lung cancer (Yang et al. 2019). However, our current analysis using TCGA data set confirms previous findings that *miR-29* is exclusively suppressed in both LUAD and LUSC patient samples, and its suppression is closely associated with cancer survival probability (Figure 1). Moreover, we also confirmed that *miR-29* overexpression suppressed cell survival rate and NF-κB activity in TRAIL-resistant cells, whereas the inhibition of *miR-29* sensitizes TDR to TRAIL-induced cell death, suggesting *miR-29* as a tumor suppressive miRNA in our system. Therefore, the discrepancy in the studies between lung cancer tissues and plasma should be further analyzed and characterized.

RSK2 is a member of p90 ribosomal S6 kinase, a highly conserved serine/threonine kinase mediating Ras-MAPK signaling pathway. Given their oncogenic roles, interestingly, genetic mutation and amplification were rarely found in many cancers (Roberts and Der 2007; Lara et al. 2013). However, RSK2 encoded by *RPS6KA3* gene activated by ERK1/2 protein enhanced cancer progression through destabilizing a proapoptotic protein, BAD and caspase-8, and by transcriptionally activating anti-apoptotic Bcl-2 as well as inducing DR5 expression, suggesting that RSK2 expression and its kinase activity is likely to be associated with TRAIL resistance (Bonni et al. 1999; Oh et al. 2010; Im et al. 2018). Our current data shows that *miR-29* and *-140-5p* regulate oncogenic RSK2 protein, which consequently plays a significant role in restoring the sensitivity from TDT cells (Figures 2 and 3; Supplementary Figure 4). Given RSK2 expression and annotated TRAIL-sensitivity in 119 different lung cancer cell lines, we observed that there is a significant correlation between RSK2 expression and TRAIL resistance. Moreover, MAPK, as an upstream regulator to RSK2 protein, was dysregulated in TDT cells. Additionally, Gene ontology data following transcriptome analysis suggests that the target genes of the *miR-29* are enriched in TDT cells, consistent with suppressed *miR-29* expression in our qRT-PCR analysis (Figures 1–3). Consistent with previous observations, NF-κB activation was regulated by *miR-29* and *miR-140*, which seems to be dependent on RSK2 regulation (Figure 4) (Peng et al. 2010). Furthermore, NF-κB activation is one of the major determinants of TRAIL resistance (Chaudhary et al. 1997; Schneider et al. 1997; Plantivaux et al. 2009; Jeon et al. 2015). All these data strongly suggest that *miR-29/-140*-RSK2 axis plays an important role in TDT status, which will provide an important insight for chemotherapy using TRAIL in lung cancer.

Conclusions

TRAIL has shown potential as a therapeutic agent for lung cancer, but its efficacy is hindered by the early development of both innate and acquired resistance. To enhance its therapeutic utility, it is crucial to understand the resistance and restoring mechanisms of TRAIL resistance. Our study has revealed that miR-29/-140-depdent RSK2 regulation is a reversal in terms of TRAIL-resistance, which could be an important feature for a TRAIL-tolerant state, suggesting that the inhibition of this pathway plays a critical role in maximizing clinical utility using TRAIL in lung cancer.

Furthermore, TRAIL and its receptors are implicated in various cancer types, underscoring the potential applicability of our findings. For instance, higher expression levels of TRAIL-R1 have been identified as prognostic markers in colon cancer (Strater et al. 2002), hepatocellular carcinoma (Kriegl et al. 2010), and skin cancer (Omran and Ata 2014). Similarly, increased expression of TRAIL-R2 has been associated with improved survival

outcomes in glioblastoma (Kuijlen et al. 2006), renal cell carcinoma (Macher-Goeppinger et al. 2009), breast cancer (Labovsky et al. 2017), and pancreatic cancer. These examples highlight the potential of TRAIL as a universal anticancer agent, capable of targeting a wide spectrum of malignancies.

The implications of our study extend beyond lung cancer, suggesting that the mechanisms of TRAIL resistance we have uncovered could be relevant across a diverse range of cancers. However, our findings are based solely on lung cancer models and in vitro studies. Therefore, further investigation, particularly in vivo studies, is necessary to validate our conclusions and fully understand the therapeutic potential of TRAIL in other cancer types. This future research could pave the way for developing more effective and universally applicable TRAIL-based cancer therapies.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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