Aberrant upregulation of TNFRSF21 enhances tumor aggressiveness in lung cancer via activation of the ERK/FOXM1 signaling cascade

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Abstract

Background

Lung cancer is one of the leading causes of cancer-related death worldwide. Identifying alterations in oncogenic drivers are known to be an effective strategy to explore potential druggable targets in the treatment of this disease.

Methods

Integrative analysis of the NCBI Gene Expression Omnibus (GEO) datasets by R language identified TNFRSF21 is upregulated in lung cancers. Using overexpression or knockdown approach to demonstrate the gene effect and mechanisms on lung cancer cells. Immunohistochemical analysis of a commercial lung cancer tissue array showed clinic-pathological correlations.

Results

TNFRSF21 is frequently upregulated and associated with high-grade tumors and is highly correlated with advanced NSCLC. Biochemical studies confirmed that TNFRSF21 overexpression could markedly promote NSCLC cell growth and cell migration/invasion, while suppression of ERK and FOXM1 by U0126 and thiostrepton, respectively, could significantly counteract TNFRSF21-mediated NSCLC cell proliferation and aggressiveness. Mechanistic studies revealed that forced expression or ablation of TNFRSF21 could escalate or attenuate both the phosphorylation of ERK (p-ERK) and the expression of FOXM1, respectively, whereas the levels of TNFRSF21 and p-ERK were not altered when FOXM1 was inhibited by thiostrepton. On the contrary, inhibition of the intensity of p-ERK by U0126 could reduce FOXM1 expression in TNFRSF21-overexpressing NSCLC cells, which suggests that TNFRSF21 is the upstream effector of ERK signaling and that its downstream target is FOXM1.

Conclusions

This study highlights the significance of TNFRSF21 in promoting tumor aggressiveness in lung cancer by increasing ERK/FOXM1 signaling, which suggests that targeting TNFRSF21/MEK/ERK/FOXM1 may represent a potential therapy for lung cancer.

Background

Lung cancer, predominantly non-small cell lung cancer (NSCLC), is the leading cause of cancer-related mortality worldwide [1–3] and accounts for approximately 20% of all cancer-related deaths worldwide [4, 5]. Due to the initial asymptomatic nature of the disease and the unavailability of effective screening methods, lung cancer is typically detected only in advanced stages [1]. While remarkable progress has

been made in recent years in the diagnosis and treatment of lung cancer, the predictive five-year survival rate is only approximately 17% [6], which is mostly a notorious consequence of regional recurrence and lymph node metastasis [4, 7, 8]. Although tobacco smoking has been confirmed to be the predominant risk factor for lung cancer development [2], fewer than a quarter of smokers develop lung cancer, which indicates genetic susceptibility [1, 9]. Thus, a good understanding of the molecular mechanisms implicated in lung cancer development and progression is especially desired. The discovery of oncogenic driver alterations, such as those in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (*EGFR*) gene, has allowed rapid development of FDA-approved inhibitors, such as gefitinib, erlotinib, afatinib, and osimertinib, which have improved the treatment outcomes of lung cancer during the past decade [10]. Broadening our knowledge of additional new genetic alterations from the mining of large high-throughput genetic databases may enable the exploration of novel therapeutic strategies for this cancer.

R language was used to analyze gene expression in four GEO microarray datasets from the NCBI GEO database and to compare lung cancers *vs.* normal lung tissues. Using R language, we found that tumor necrosis factor receptor superfamily member 21 (TNFRSF21), also called death receptor 6 (DR6), is frequently upregulated in lung cancers. Actually, TNFRSF21 has been reported to be involved in apoptotic cell death and to exhibit a potential tumoricidal effect, possibly via a pathway that involves NF-κB activation [11, 12]. However, upregulated TNFRSF21 expression has been observed not only in numerous human tumors [13, 14] but also in the sera of patients with advanced ovarian cancer [14, 15]. In addition, TNFRSF21 overexpression in certain malignant cells typically occurs in conjunction with elevated anti-apoptosis molecules [12–14]. Hence, the biological function of TNFRSF21 is quite controversial, and its role in tumor progression needs to be clarified.

In the present study, we showed that TNFRSF21 overexpression is significantly correlated with advanced and high-grade lung cancers accompanied by poor prognosis. Gain- and loss-of-function *in vitro* assays verified that TNFRSF21 promoted the migration, invasiveness, and viability of lung cancer cells. Mechanistic studies demonstrated that the tumorigenic role of TNFRSF21 in lung cancer at least partly involves activation of the p-ERK/FOXM1 signaling pathway. Therefore, our results identify TNFRSF21 as a novel regulator of lung cancer invasiveness and potentially provide a promising molecular therapeutic strategy for the treatment of lung cancer patients.

Materials And Methods

Cell lines and reagents

The human NSCLC cell lines A549, NCI-H1975, and NCI-H1299 (iCell Bioscience Inc., Shanghai, China) were used in this study. They were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (Gibco) and 100 units/mL penicillin/streptomycin (Gibco) at 37°C in an incubator with a humidified atmosphere of 5% CO_2 and 95%

air. The MAPK/ERK kinase 1/2 (MEK1/2) inhibitor U0126 and the FOXM1 inhibitor thiostrepton were purchased from Calbiochem (La Jolla, CA, USA).

Plasmids and cell transfection

To investigate the effects of forced *TNFRSF21* expression, the human DDK-tagged TNFRSF21-expressing plasmid pCMV6–TNFRSF21 was used for transfection of NSCLC cells. In addition, custom-designed sgRNAs (sgRNA1: 5'-gtatgtgccaatgagattcg-3', sgRNA2: 5'- agtgcattctcggtcagtca-3' and sgRNA3: 5'-ggcatggctgactacagtca-3') for endogenous *TNFRSF21* knockdown were designed by E-CRISP at http://www.e-crisp.org/E-CRISP/. The cloned DNA sequences were then inserted into the mammalian expression vectors pSpCas9(BB)-2A-GFP (PX458) (Addgene, Plasmid #48138) and pSpCas9(BB)-2A-Puro (PX459) (Addgene, Plasmid #48139). Cell transfection was performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. GFP-positive cell sorting and puromycin treatment were adopted to select transfected cells. Expression patterns were analyzed by Western blot.

Cell proliferation and focus formation assays

An XTT cell proliferation kit (Roche, Basel, Switzerland) was used to measure cell viability according to the manufacturer's protocol. Three independent experiments were performed in triplicate. For the focus formation assay, approximately 1000 cells were cultured in each well of a 12-well plate and were treated with different drugs. After incubation at 37°C in an incubator for one week, colonies were stained with crystal violet and counted.

Matrigel cell migration and invasion assays

According to the manufacturer's (Corning, NY, USA) instructions, a cell suspension containing 5×10^5 to 10×10^5 cells in serum-free medium was added to each insert. Medium (500 μL) containing 1% fetal bovine serum was added to the lower chamber as a chemoattractant. After incubation, the migrated/invaded cells were stained and counted by microscopy.

Immunohistochemistry

Immunohistochemistry (IHC) for TNFRSF21 was performed on a lung cancer tissue array (BC041115e) (Pantomics Inc, San Francisco, CA) using primary mouse monoclonal anti-DR6 (E-4) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The percentage of immunopositive cells in tumors and normal epithelia was assessed according to the proportions of immunopositive cells, which ranged from 10 to 100%, while the intensity of staining was scored as 0 (negative), 1 (faint), 2 (moderate), 3 (strong) or 4 (marked). The immunoreactivity in each case was scored as a percentage of the proportion of immunopositive cells multiplied by the staining intensity. The fold change for each stain was obtained by dividing the expression level in each cancer sample by the mean immunoreactivity staining value of normal lung tissues. Immunohistochemical staining was quantified and scored blindly by at least two independent observers.

Western blot analysis

Proteins in cell lysates were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with 5% skimmed milk and subsequently probed overnight at 4°C with primary antibodies specific for p-ERK and ERK (Cell Signaling, Beverly, MA, USA), FOXM1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA) and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Amersham, Uppsala, Sweden). Immunodetection was performed with enhanced chemiluminescent reagent solution (Amersham^{TM} ECLTM) and visualized using medical X-ray films.

Gene expression data analysis

Gene expression was determined in four online lung cancer microarray datasets:

1. Hou: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19188
2. Landi: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10072
3. Selamat: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32863
4. Su: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7670

were downloaded from the NCBI GEO database and analyzed using the R (v3.6.1) package. In contrast, to determine the tissue-specific expression profile of TNFRSF21 in normal and tumor tissues from different organs, as well as the differential expression pattern of TNFRSF21 between normal lung tissues and tumor lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) datasets were explored using Gene Expression Profiling Interactive Analysis (GEPIA) at http://gepia.cancer-pku.cn/index.html. The Kaplan-Meier Plotter at https://kmplot.com/analysis/was applied to assess the effect of TNFRSF21 on survival.

Statistical analysis

Student's *t* test was applied to analyze parametric data, whereas the Mann-Whitney test was used for nonparametric data. Data were expressed as the mean ± SEM. A P value ≤ 0.05 was considered statistically significant.

Results

Identification of TNFRSF21 as a frequently upregulated gene in lung cancer

To identify potential oncogenic driver genes in lung cancers, data mining analysis of the four microarray datasets from the NCBI GEO database was performed using the R language algorithm. The significantly upregulated genes in each dataset were examined, and *TNFRSF21* was found to be one of the 91 overlapping genes among all four datasets (Fig. 1A). Notably, the mRNA expression level of *TNFRSF21* was obviously enhanced in human lung cancer samples compared with normal tissues (Fig. 1B).

TNFRSF21 is highly expressed in a wide variety of human cancers, particularly human non-small cell lung cancers (NSCLCs), such as lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) (Fig. 1C). Analysis of a large cohort of public TCGA and GTEx datasets by GEPIA further revealed the differential expression pattern of TNFRSF21 between normal lung tissues and NSCLC tumor specimens and that TNFRSF21 was predominantly expressed in NSCLC (Fig. 1D). Additionally, the cancer type summary from cBioPortal agreed with the above numbers in that the amplification of TNFRSF21 was the most frequent alteration in NSCLC (Fig. 1E). The Kaplan–Meier survival model showed that, in terms of the overall survival and progression-free survival rates, NSCLC patients in the low TNFRSF21 expression group had a more favorable prognosis than those in the high expression group (Fig. 1F), which suggests an inverse relationship between the TNFRSF21 level and long-term survival among lung cancer patients.

In line with the above *in silico* data, immunohistochemistry (IHC) in a lung cancer tissue array (BC041115e) confirmed that TNFRSF21 was congruently upregulated in lung cancer samples. High TNFRSF21 expression was significantly correlated with high-grade tumors (Grade 3) ($P < 0.05$), 75% of which exhibited TNFRSF21 overexpression, whereas nearly 50% of low-grade tumors (Grades 1 and 2) showed low expression of TNFRSF21 (Table 1). Furthermore, overexpression of TNFRSF21 was observed in 80% of advanced tumors ($P < 0.05$), while nearly 50% of early-stage tumors expressed low levels of TNFRSF21 (Table 1). Regarding the tumor subtype, the expression of TNFRSF21 was highly correlated with LUSC ($P < 0.05$), as 70% of cases demonstrated TNFRSF21 overexpression (Table 1). Concordant pale and strong staining of TNFRSF21 were also differentially seen in early-stage, low-grade (Grade 2) tumors and advanced-stage, high-grade (Grade 3) tumors, respectively, which suggests the importance of this protein in lung cancer progression (Fig. 2).

Table 1
Clinical-pathological analysis of TNFRSF21 expression in lung cancer patients was significantly correlated with features of subtypes, TNM stages, and histological grades. Tumor stages; Early (1A-2B) and Late (3A or above). Tumor Grade; Low (1–2) and High (2/3 or above). Metastasis; Present means tumors found in Lymph node and distal.

TNFRSF21 expression								
Parameters	Total	< 2 folds			≥ 2 folds			P value
All cases	110	50	(45.45%	)	60	(54.55%	)	
Age (y)								
<55	38	18	(47.37%	)	20	(52.63%	)	
≧55	72	32	(44.44%	)	40	(55.56%	)	0.841
Sex								
Male	82	39	(47.56%	)	43	(52.44%	)	
Female	28	11	(39.29%	)	17	(60.71%	)	0.514
Subtypes								
Squamous	40	12	(30.00%	)	28	(70.00%	)	
Others	70	38	(54.29%	)	32	(45.71%	)	0.017*
Adenocarcinoma	52	21	(40.38%	)	31	(59.62%	)	
Others	58	29	(50.00%	)	29	(50.00%	)	0.342
Stage								
Early	80	37	(46.25%	)	43	(53.75%	)	
Late	21	4	(19.05%	)	17	(80.95%	)	0.027*
Grade								
Low	70	34	(48.57%	)	36	(51.43%	)	
High	28	7	(25.00%	)	21	(75.00%	)	0.042*
Metastasis								
Present	56	26	(46.43%	)	30	(53.57%	)	
Absent	42	15	(35.71%	)	27	(64.29%	)	0.309

TNFRSF21 enhances cell proliferation and tumor aggressiveness in lung cancer

Given that lung cancer is highly proliferative and metastatic, it is reasonable to postulate that TNFRSF21 plays a particular role in regulating cell motility and invasion. To investigate the oncogenic roles of

TNFRSF21 in lung cancer, the NSCLC cell line NCI-H1299 with stable expression of TNFRSF21 was established using a human DDK-tagged TNFRSF21-expressing plasmid. Western blot analysis revealed that TNFRSF21 was significantly elevated in four out of six DDK-TNFRSF21 stable clones of NCI-H1299 (C3-C6) cells (Fig. 3A). Functionally, the XTT cell proliferation assay showed that aberrant expression of TNFRSF21 remarkably enhanced the cell proliferation capacity of DDK-TNFRSF21 stable clones of NCI-H1299 cells (C3, C4, and C5) ($P \leq 0.001$) compared with the control (Fig. 3B). In addition, the clonogenic assay demonstrated an approximate 2-fold increase in the number of colonies of NCI-H1299 cells that stably expressed DDK-TNFRSF21 (C3) ($P \leq 0.001$) compared with the control (Fig. 3C). On the contrary, both transient and stable overexpression of TNFRSF21 in NCI-H1975 and NCI-H1299 cells resulted in at least a 2-fold increase in cell migration ability compared with their respective controls (Fig. 3D). Moreover, the Transwell invasion assay revealed that the number of cells that invaded through the Matrigel was markedly increased by approximately 1.5-2.5-fold in DDK-TNFRSF21 stable clones of NCI-H1299 cells (C3 and C4) ($P \leq 0.01$) compared with the control (Fig. 3E). Collectively, these findings indicate that TNFRSF21 promotes lung cancer cell proliferation, cell migration and invasiveness.

Downregulation of TNFRSF21 impairs proliferation and migration/invasiveness of lung cancer cells

As mentioned above, we hypothesized that TNFRSF21 exhibits oncogenic properties that support lung cancer cell growth and aggressiveness. It was therefore of interest to examine whether endogenous TNFRSF21 expression is required to promote lung cancer. The NSCLC cell lines NCI-H1975 and A549 in which endogenous TNFRSF21 was depleted were established using the CRISPR/Cas9 genome-editing system. Western blot analysis revealed that TNFRSF21 was significantly silenced in NCI-H1299 and A549 cells upon CRISPR/Cas9-mediated gene knockdown (Fig. 4A). Growth of NCI-H1975 and A549 cells was significantly hampered by approximately 30% upon ablation of TNFRSF21 ($P \leq 0.01$) compared with the respective controls (Fig. 4B). In addition, a focus formation assay showed that the number of colonies was markedly decreased by 30% and 70% in NCI-H1975 and A549 cells, respectively, upon TNFRSF21 knockdown (Fig. 4C). On the contrary, depletion of endogenous TNFRSF21 remarkably decreased the migration ability of NCI-H1975 and A549 cells by 50% and 30% ($P < 0.001$), respectively, compared with the respective controls (Fig. 4D). Moreover, the Transwell invasion assay demonstrated that the number of NCI-H1975 and A549 cells that invaded through the Matrigel was markedly reduced by approximately 50% and 90%, respectively, after silencing of endogenous TNFRSF21 ($P < 0.001$) compared with the respective controls (Fig. 4E). These results support our hypothesis that endogenous TNFRSF21 is indispensable for lung cancer progression, whereas interference with its expression leads to attenuated growth, migration and invasiveness of lung cancer cells.

Molecular regulation of the ERK/FOXM1 signaling cascade by TNFRSF21 in lung cancer cells

Previous investigations have found that TNFRSF21 is heavily involved in MAPK cascades, especially P38 MAPK signaling, and is usually altered in cancers [12, 16]. As MAPKs have been characterized into various families and are the key signaling pathways that regulate a variety of critical cellular processes, it is believed that other molecular regulatory mechanisms exist within the families with which TNFRSF21

interacts. Therefore, we studied the interaction network of TNFRSF21 and MAPK signaling using bioinformatics online software (http://pathwaynet.princeton.edu). Pathway analysis identified MAPK3/MAPK1 (ERK1/2) and FOXM1 as the primary candidates that exhibited a strong relationship with TNFRSF21 regulation (Fig. 5A). GEPIA further revealed a significant clinical correlation among MAPK3/MAPK1 (ERK1/2), FOXM1 and TNFRSF21 ($P < 0.01$) in lung cancer clinical samples (Fig. 5B). Consistent with these *in silico* findings, forced expression of TNFRSF21 increased ERK phosphorylation and FOXM1 expression in NCI-H1299 cells (Fig. 5C), which confirms the existence of TNFRSF21/ERK/FOXM1 signal transmission in NSCLC cells.

Given that the increased activities of TNFRSF21, ERK, and FOXM1 are significantly correlated with lung cancer, they may be regulated coordinately to mediate oncogenic functions during lung cancer progression. Therefore, it was interesting to investigate the regulatory mechanism among these factors in lung cancer cells. To this end, the human NSCLC cell lines NCI-H1975 and NCI-H1299 were first treated with either U0126, a MEK/ERK inhibitor, or thiostrepton, a FOXM1 inhibitor. Western blot analysis revealed that ERK phosphorylation and FOXM1 expression in NCI-H1975 and NCI-H1299 cells were successfully decreased after treatment with U0126 (10 μM), but TNFRSF21 expression was not obviously disturbed (Fig. 5D). Similarly, treatment with thiostrepton (20 μM) remarkably reduced the level of FOXM1, while TNFRSF21 expression and ERK phosphorylation remained unchanged (Fig. 5D). To exclude the nonspecific action of high-dose thiostrepton, CRISPR/Cas9 was used to knock down endogenous TNFRSF21 in NCI-1975 and A549 cells, which exhibit relatively higher expression of endogenous TNFRSF21 compared with other NSCLC cell lines. This clearly indicated that not only the level of TNFRSF21 but also the phosphorylation of ERK as well as the expression of FOXM1 were concomitantly attenuated (Fig. 5E). This suggests that suppression of TNFRSF21 could decrease p-ERK/FOXM1 signal transduction, whereas the reduction in FOXM1 expression did not affect either TNFRSF21 expression or ERK activity. Furthermore, treatment with U0126 (10 μM) not only inhibited ERK phosphorylation but also reduced FOXM1 expression, similar to thiostrepton (20 μM), in NSCLC cells with ectopic TNFRSF21 expression (Fig. 5F). Collectively, these findings indicate that TNFRSF21 positively upregulates ERK activity, which in turn elevates FOXM1 expression in lung cancer cells.

Targeting inhibition of the TNFRSF21/ERK/FOXM1 signaling cascade attenuates the tumorigenic properties of lung cancer cells

Earlier studies have reported that constitutive activation of ERK or augmented expression of TNFRSF21 and FOXM1 is strongly related to tumorigenicity in several human malignancies [17–21]. Thus, targeting whichever components are in this signaling cascade should tentatively abrogate the tumorigenic properties of lung cancer cells. Indeed, recent studies have reported that ERK and FOXM1 knockdown could decrease cell growth and invasiveness in human cancers [22–24]. In this study, we aimed to investigate tumorigenic alterations using pharmaceutical inhibitors that specifically target ERK/FOXM1 signal transduction. The suppressive effect on the proliferation of the NSCLC cell lines NCI-H1975 and NCI-H1299 was therefore evaluated. The outcomes of the XTT cell proliferation assay revealed that thiostrepton (20 μM) and U0126 (10 μM) resulted in a profound reduction in the proliferation rate of NCI-

H1975 ($P < 0.01$) and NCI-H1299 ($P < 0.01$) cells with ectopic expression of DDK-TNFRSF21 compared with their respective controls (Fig. 6A). These results highlight that TNFRSF21 upregulation enhances lung cancer cell growth, while treatment with either thiostrepton or U0126 efficiently reduces lung cancer progression.

It has been documented that lung tumors exhibit a high capacity for cell migration/invasion [25, 26]. Thus, we wondered whether inhibition of ERK/FOXM1 signaling by specific inhibitors could influence the migration and invasion abilities of lung cancer cells. Transwell migration assays showed that approximately 40% and 30% reductions in the migration abilities of NCI-H1975 and NCI-H1299 cells, respectively, with ectopic expression of DDK-TNFRSF21 were caused by treatment with thiostrepton (20 μM) and U0126 (10 μM), respectively, compared with the DMSO controls (Fig. 6B). Likewise, treatment with thiostrepton (20 μM) and U0126 (10 μM) significantly decreased the invasion rate of NCI-H1975 and NCI-H1299 cells that stably expressed DDK-TNFRSF21 by approximately 2.5-fold and 1.5-fold, respectively, compared with the untreated controls (Fig. 6B). These findings suggest that inhibition of TNFRSF21/ERK/FOXM1 signaling can impair migration and invasiveness of lung cancer cells.

Discussion

We used state-of-the-art data mining of integrative sets of databases to identify commonly upregulated genes and revealed that *TNFRSF21* is a novel candidate oncogene that is overexpressed in lung cancer. Four independent cohorts of clinical lung cancer samples from public TCGA datasets were included, and a high expression level of TNFRSF21 was found to be positively correlated with pathologic grade and poor survival of lung cancer patients, which confirms that upregulated TNFRSF21 plays an oncogenic role in lung tumor growth and progression. It is worth mentioning that tumor necrosis factor (TNF) and its receptors (TNFRs) play a central role in both inflammation and the immune response and are ubiquitously expressed in human malignancies, including lung cancer [27–32]. In terms of oncology, TNF was first recognized as a serum factor that triggers tumor necrosis [27, 33], whereas its therapeutic application has been severely limited by toxicity and endotoxic shock [27]. Many recent studies have indeed characterized a protumorigenic function for TNF in inflammation-related cancers [34–36]. Depending on the cellular context, the outcome of TNFR signaling can therefore either induce cell death or stimulate tumor growth [37–39]. In this study, we provide evidence supporting an oncogenic role of TNFR in cancer with a focus on lung cancer. The results revealed that TNFRSF21 is not only aberrantly upregulated in high-grade lung cancer but that it is also significantly correlated with increased ERK activation and FOXM1 expression in lung cancer cells. Notably, our data demonstrate that these signaling molecules are coordinately regulated in TNFRSF21/ERK/FOXM1 signal transduction in lung cancer cells. Functionally, inhibition of ERK phosphorylation by U0126 and FOXM1 expression by thiostrepton remarkably hampers growth, migration and invasiveness of lung cancer cells with forced expression of TNFRSF21. Taken together, these findings infer that the activated TNFRSF21/ERK/FOXM1 signaling axis plays an important role in the pathogenesis of lung cancer.

Constitutive activation of the ERK signaling pathway has been implicated in cancer development [40, 41]. Numerous reviews have reported that TNFRSF21 is frequently upregulated in lung cancer and can mediate resistance to targeted treatment by modulating the ERK signaling pathway [27, 42, 43]. In concert with this finding, emerging research has revealed that abundant expression of the FOXM1 transcription factor is implicated in the pathogenesis of numerous human cancers including lung cancer [44–47]. Abnormal upregulation of FOXM1 stimulates the proliferation and chemoresistance of malignant cells during lung cancer progression [44] and is associated with poor clinical outcomes of lung cancer patients [48]. Although all these abnormally activated factors are predominantly associated with cancer pathogenesis, the signaling link among TNFRSF21, ERK, and FOXM1 in human cancer cells has not been mentioned. This is a novel report that shows the direct relationship among these factors, among which ERK phosphorylation and FOXM1 expression are concomitantly increased in lung cancer cells upon TNFRSF21 upregulation, while the opposite occurs in lung cancer cells in which TNFRSF21 is downregulated. Intriguingly, in lung cancer cells, inhibition of ERK by U0126 represses the expression of both p-ERK and FOXM1 without disturbing the expression of TNFRSF21, while inhibition of FOXM1 by thiostrepton restricts the expression of FOXM1 and does not affect ERK phosphorylation or TNFRSF21 expression. Not only is this reminiscent of transcriptional suppression of FOXM1 by ERK, which is a plausible downstream effector of the TNFRSF21/ERK signaling pathway, but it also suggests that targeting this signaling cascade is an alternative approach to achieve good outcomes in lung cancer.

As previously mentioned, TNF and its receptors have been reported to be widely expressed in lung cancer [49, 50]. Notably, TNF is known to be released by cancer cells [34] as well as by cells in the tumor microenvironment, and experimental evidence from a variety of models has indicated that TNF can promote tumor progression [51, 52]. To counteract the effects of TNF signaling, therapeutics have been developed to neutralize TNF. Different anti-TNF drugs have been approved for clinical use, such as infliximab, adalimumab, golimumab, and etanercept. Nevertheless, most of these anti-TNF remedies are only tailored to treat autoimmune disorders, such as rheumatoid arthritis, psoriasis, and ankylosing spondylitis [53], but they are seldom used to treat cancer. Although independent studies have suggested that combined inhibition of TNF and EGFR may be a viable therapeutic approach in cancers including glioblastoma [54, 55], restricted responsiveness and adverse side effects, such as opportunistic infections, immunosuppression, and the development of anti-drug antibodies limit the use of anti-TNF drugs [56]. As a result, reagents that selectively target TNFR might be superior to global TNF blockade since they allow differential activation and/or inhibition of TNF receptors. Most of these are still being tested in preclinical studies, and thus, the development of clinical-grade products is necessary for success in clinical trials. Here, our findings illustrate that TNFRSF21 modulates ERK activation and FOXM1 expression in an ordered manner, which confirms that the overexpression of TNFRSF21 escalates cell proliferation and cell aggressiveness in lung cancer by promoting ERK and FOXM1 activities. Hence, we propose that suppression of aberrantly activated ERK and FOXM1 should exert similar effects to anti-TNF or TNFR antagonists in inhibiting the oncogenic properties of lung cancer cells. Indeed, our data clearly show that growth, migration and invasiveness of lung cancer cells are significantly perturbed after treatment with U0126 and thiostrepton, which function by targeting ERK and FOXM1 activities,

respectively. These phenomena seem to offer an alternative therapeutic strategy for the development of targeted therapeutics for lung cancer.

Conclusion

In summary, this study illustrates that aberrant activation of the TNFRSF21/ERK/FOXM1 signaling cascade is extensively correlated with the progression and aggressiveness of lung cancer. Using signal transduction-based therapy, targeting this signaling axis by selectively counteracting the activities of ERK and FOXM1 with respective inhibitors may result in a favorable outcome in lung cancer. Our findings shed light on the application of MEK/ERK and FOXM1 inhibitors in the treatment of lung cancer.

Abbreviations

ATCC: American Type Culture Collection

EGFR: Epidermal Growth Factor Receptor

GEO: Gene Expression Omnibus

GEPIA: Gene Expression Profiling Interactive Analysis

GTEx: Genotype-Tissue Expression

IHC: Immunohistochemistry

LUAD: Lung Tissues and Tumor Lung Adenocarcinoma

LUSC: Lung Squamous Cell Carcinoma

NSCLC: Non-small cell lung carcinoma

PVDF: Polyvinylidene Difluoride

TK: Tyrosine Kinase

TNFRSF21: Tumor Necrosis Factor Receptor Superfamily Member 21

Declarations

Ethical approval and consent to participate: N/A.

Consent for publication: All authors have approved the manuscript for publication.

Availability of data and material: The data supporting the findings of the present study are available from the corresponding author on reasonable request.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions: Fang.S. carried out experiments, Liu J participated all bioinformatic analyses, Zhou C.Z conceived of the study and participated in its design. Fang S. analyzed and interpreted data in this study. Chen Z. helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures

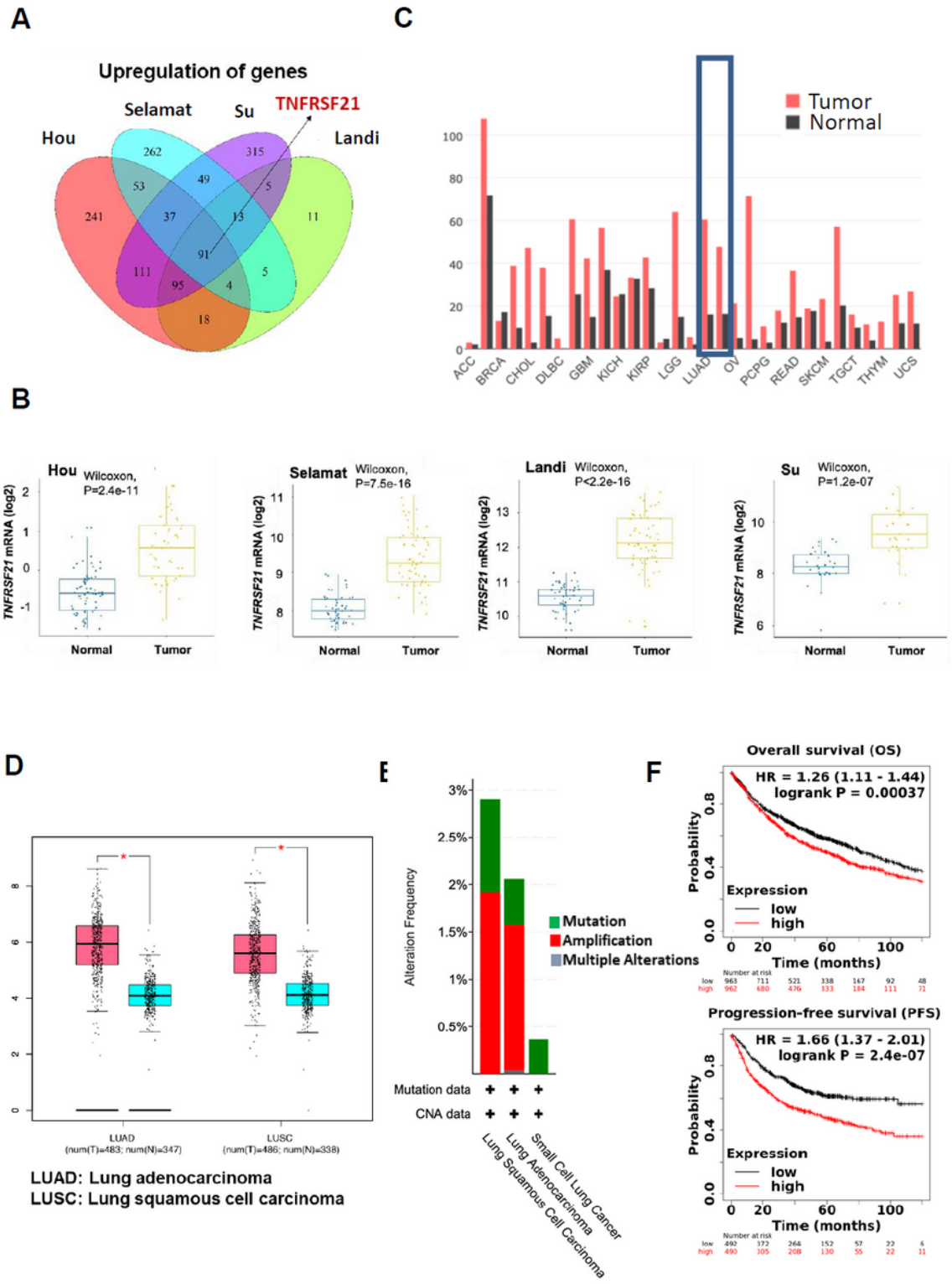

Figure 1

TNFRSF21 expression is upregulated in lung cancer. (A) Venn diagram analysis of the commonly upregulated genes by R (v3.6.1) package, based on the four independent datasets such as Hou, Landi, Selamat and Su from NCBI GEO database. (B) Boxplots with the TNFRSF21 mRNA levels (log2) on the vertical axis and normal tissues versus cancer tissues (lung) on the horizontal axis. Data are collected from datasets used in (A). (C) Bar charts showing the gene expression profile of TNFRSF21 across

different tumor samples, including lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) as well as paired normal tissues. The height of bar in the plot represented the median expression of certain tumor types or normal tissues. (D) TNFRSF21 is predominantly expressed in lung cancer tissues from TCGA database cohorts using GEPIA tool. (E) Among various kinds of gene alternations (mutation, amplification, and multiple alternations), a cancer type summary from cBioPortal revealed that amplified expression of TNFRSF21 was the most frequent alteration in NSCLC. (F) Kaplan-Meier analysis of the overall survival and progression-free survival for lung cancer patients expressing low or high levels of TNFRSF21 expression from the TCGA dataset.

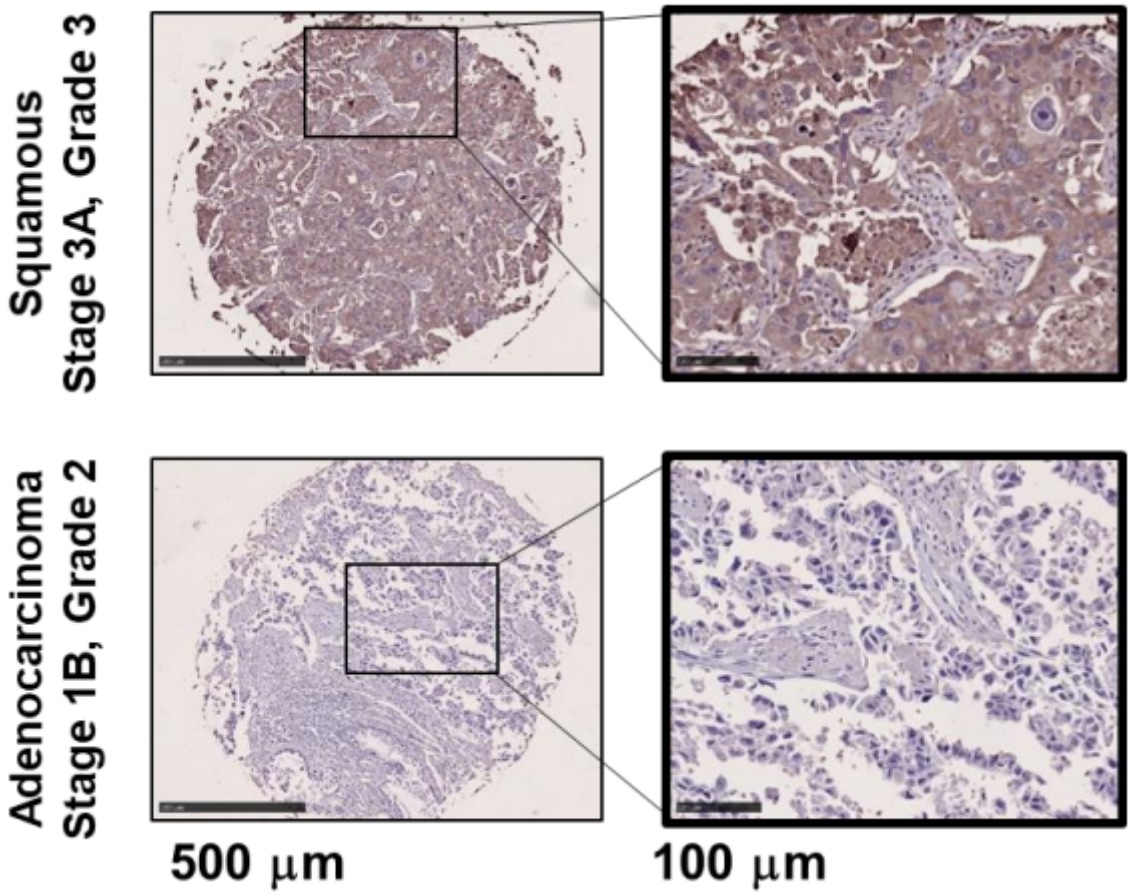

Figure 2

A significant increase in the expression of TNFRSF21 along tumor stage and grade of lung cancer. Representative IHC showed the immunoreactivities of TNFRSF21 between early and advanced stage tumor and also between low- and high-grade tumor on a lung cancer tissue array (BC041115e). Enhanced staining of TNFRSF21 was observed along with the progression of cancer which implicated its potential role in tumorigenesis.

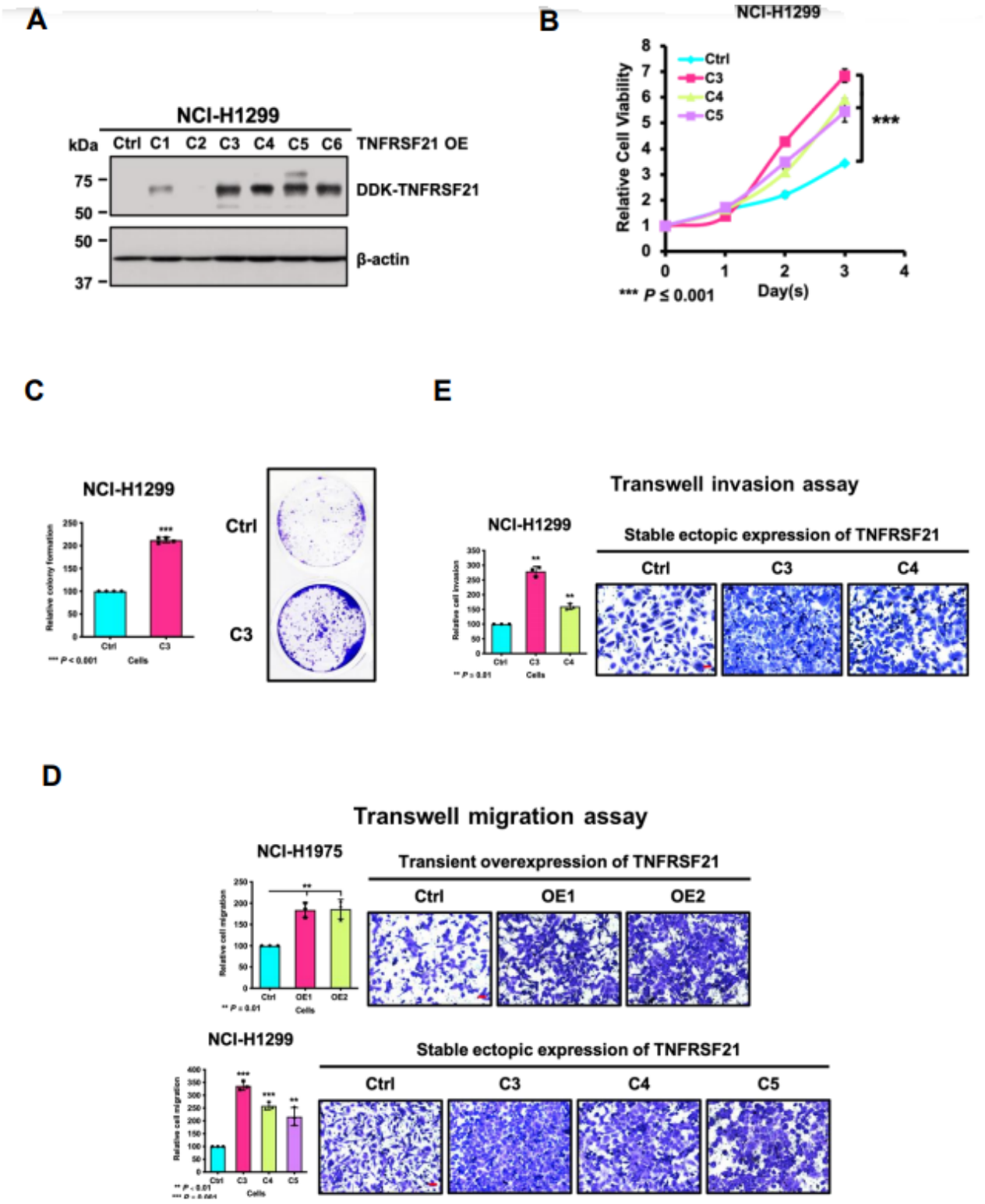

Figure 3

Upregulation of TNFRSF21 induces cell proliferation, migration and invasion in lung cancer cells. (A) Western blot analysis showed the expression pattern of DDK-TNFRSF21 in different clones (C1-6) of NCI-H1299 cells upon overexpression of TNFRSF21. (B) XTT cell proliferation assay demonstrated that overexpression of TNFRSF21 remarkably increased cell growth in NCI-H1299 cells (***P ≤ 0.001) as compared with control. (C) The clonogenic assay showed that about twice the number of colonies were

counted in DDK-TNFRSF21 ectopically expressing NCI-H1299 cells (***P < 0.001) as compared with control. (D) Transwell migration assay with the bar charts illustrated superior migratory rate in both NCI-H1975 cell clones with transient overexpression of TNFRSF21 (OE1-2) and NCI-H1299 cell clones with stable ectopic expression of TNFRSF21 (C3-5) as compared with their respective controls. (E) Transwell invasion assay with the bar chart showed a higher invasion rate through Matrigel-coated membrane in TNFRSF21 ectopic expressing clones (C3-4) of NCI-H1299 cells (**P ≤ 0.01) as compared with control. Scale bar = 20 μm.

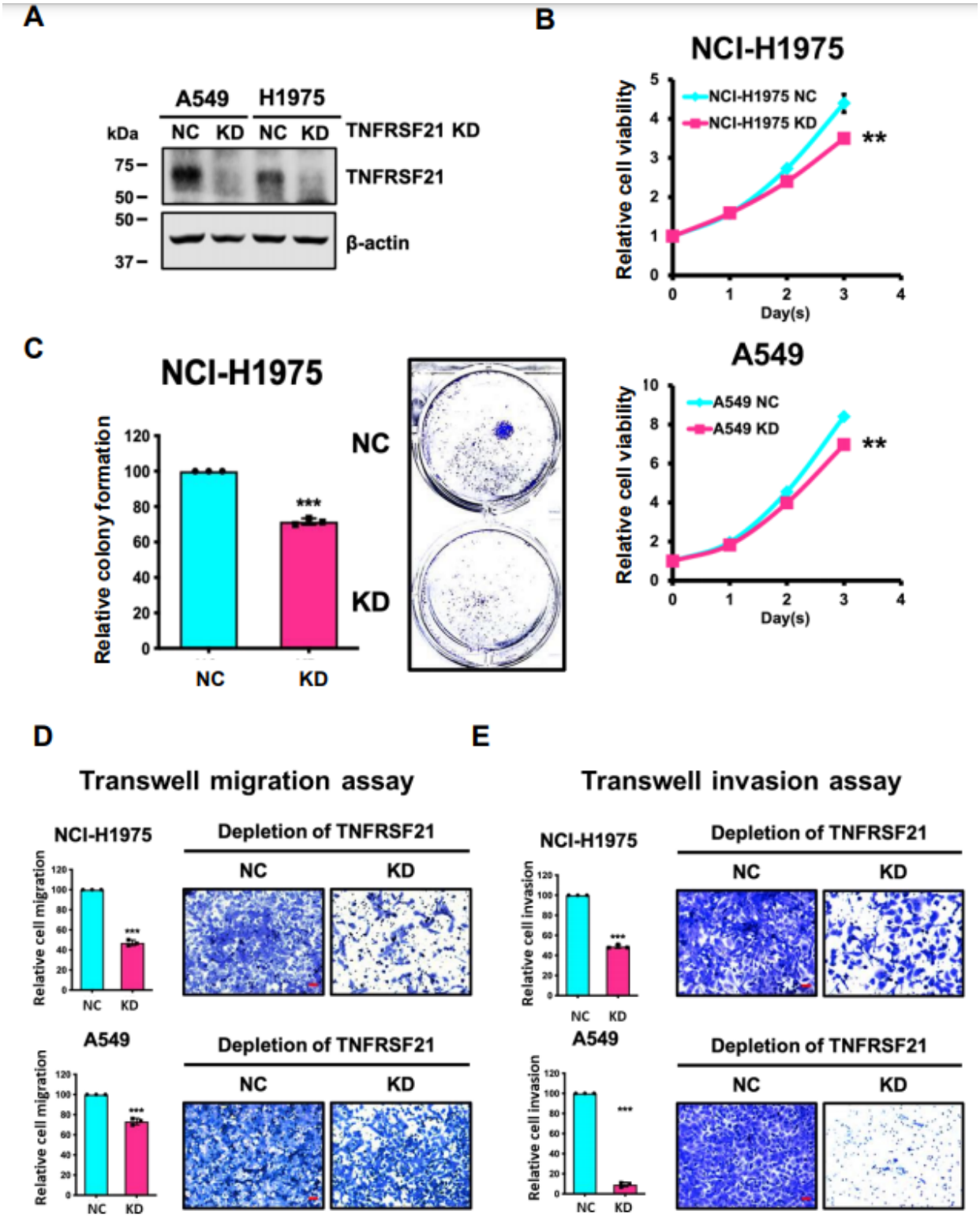

Figure 4

Abatement of TNFRSF21 retards cell proliferation and aggressiveness in lung cancer cells. (A) Western blot analysis validated the successful impediment of endogenous TNFRSF21 in NCI-H1975 and A549 cells upon CRISPR/Cas9-mediated gene knockdown of TNFRSF21 as compared with their respective controls. The scrambled control was used as negative control (NC). (B) XTT cell proliferation assay demonstrated that down-regulation of endogenous TNFRSF21 remarkably reduced cell growth in NCI-H1975 and A549 cells (**$P \leq 0.01$) as compared with their respective controls. (C) Clonogenic assay showed that about one-third the number of colonies were recorded in NCI-H1975 and A549 cells with TNFRSF21 knockdown as compared with their respective controls. (D) Transwell migration assay with the bar charts illustrated lower migratory rate in NCI-H1975 and A549 cells with stable silencing of TNFRSF21 (***$P < 0.001$) as compared with their respective controls. (E) Transwell invasion assay with the bar charts revealed a lower invasion rate through Matrigel-coated membrane in TNFRSF21 depleted clones of NCI-H1975 and A549 cells (***$P < 0.001$) as compared with their respective controls. Scale bar = 20 μm.

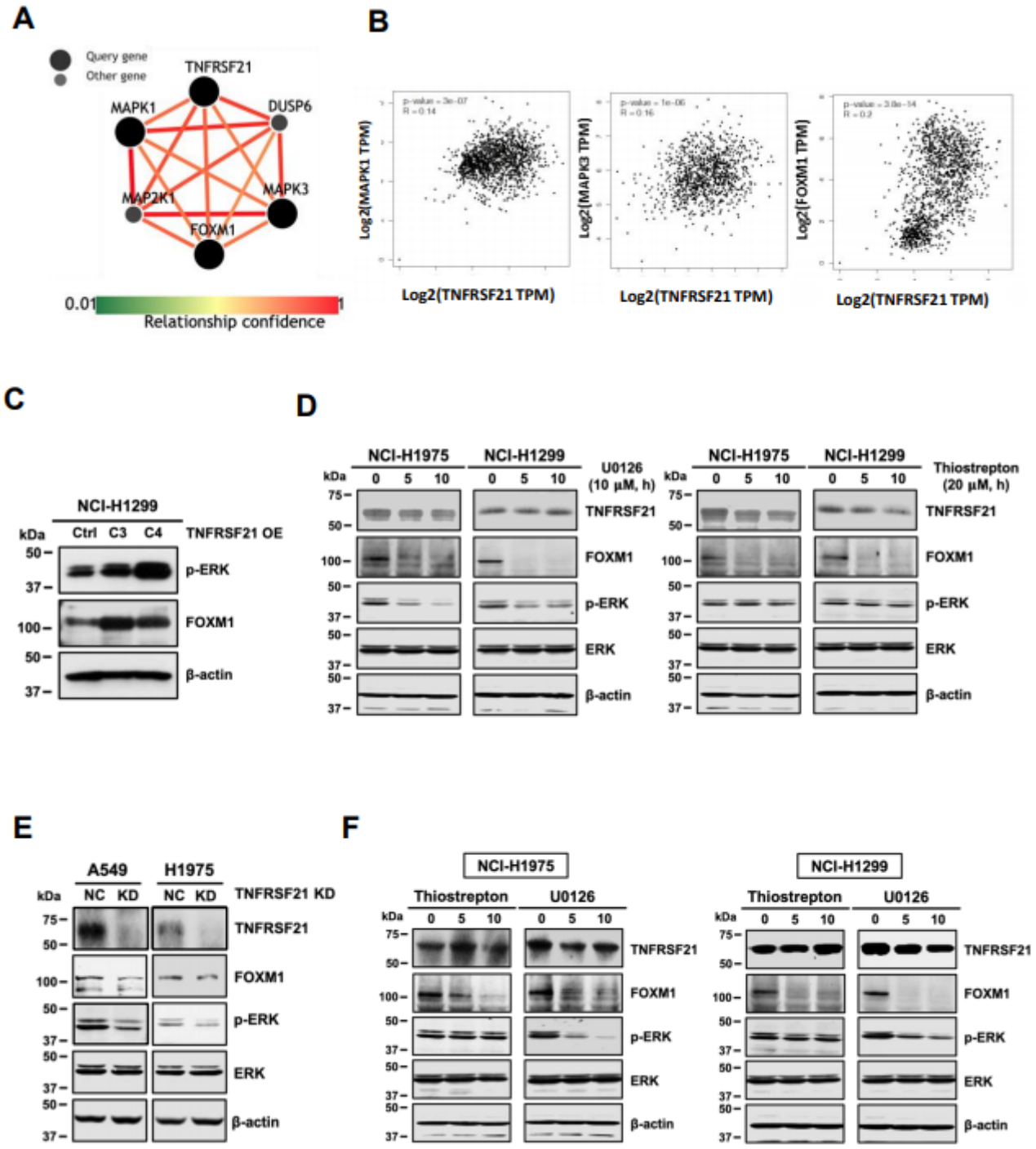

Figure 5

TNFRSF21/ERK/FOXM1 is orchestrated along the same signaling axis. (A) Functional relationship analysis of genes of interest that potentially interacted with TNFRSF21 by PathwayNet analysis program. The candidate genes with the high confidence including MAPK (ERK1/2) and FOXM1 were shown in the network diagram. (B) Correlation analysis of MAPK3/ MAPK1 (ERK1/2) and FOXM1 with TNFRSF21 among patients with lung cancer in TCGA database cohorts using GEPIA tool. (C) Western blot analysis verified that NCI-H1299 cells with overexpression of TNFRSF21 exhibited a much higher activation of ERK/FOXM1 signaling when compared with control. (D) Western blot analysis depicted that treatment of

U0126 (10 μM) in NCI-H1975 and NCI-H1299 cells resulted in a significant reduction in the phosphorylation of ERK accompanied with the expression of FOXM1 in a time-dependent manner, whereas no observable change in expression of TNFRSF21 was found (left). On the other hand, treatment of Thiostrepton (20 μM) remarkably reduced the expression of FOXM1, but no obvious variation was seen in the expressions of TNFRSF21 and phosphorylation of ERK in NCI-H1975 and NCI-H1299 cells (right). (E) CRISPR/Cas9-mediated gene knockdown of endogenous TNFRSF21 was performed in NCI-1975 and A549 cells and depletion of TNFRSF21 accompanied with a reduction in ERK phosphorylation and FOXM1 expressions in both lung cancer cells. The scrambled control was used as negative control (NC). (F) Enforced expression of TNFRSF21 increased ERK phosphorylation and FOXM1. However, treatment with either U0126 (10 μM) or Thiostrepton (20 μM) could suppress the induced ERK phosphorylation and FOXM1 expression in NCI-H1975 and NCI-H1299 cells.

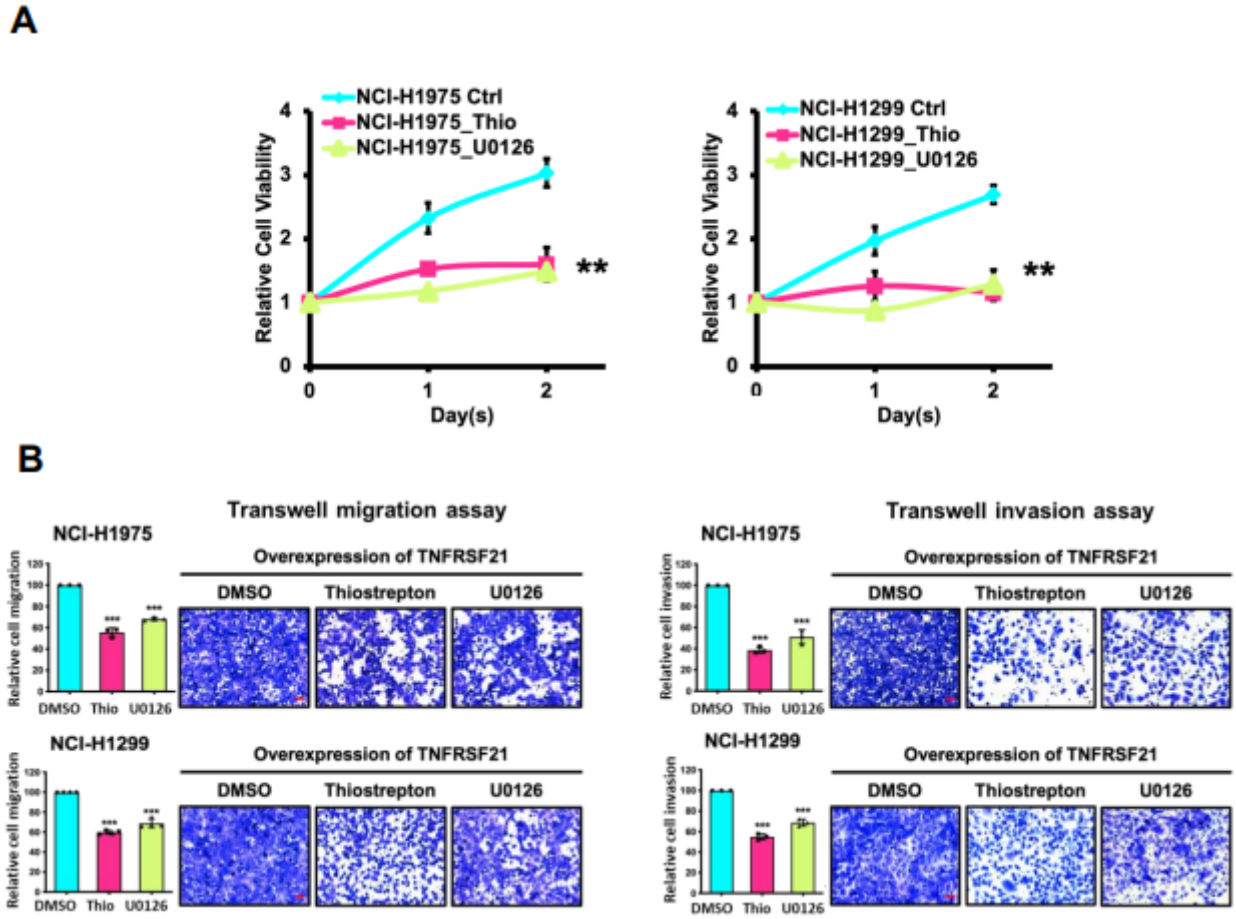

Figure 6

Impairment of ERK phosphorylation or FOXM1 expression inhibits cell growth, migration, and invasion in lung cancer cells. (A) XTT cell proliferation assays showed that the suppression of ERK phosphorylation by U0126 (10 μM) or FOXM1 expression by Thiostrepton (20 μM) significantly abrogated the cell proliferation rate in TNFRSF21 stably expressing NCI-H1975 and NCI-H1299 cells (**P < 0.01) as compared with the respective controls. (B) Transwell migration assay showed that Thiostrepton (20 μM) and U0126 (10 μM) reduced cell migration in NCI-H1975 and NCI-H1299 cells with stable overexpression of TNFRSF21 (***P < 0.001) as compared with control treatment of DMSO for 18 hours (left). Transwell

invasion assay demonstrated that NCI-H1975 and NCI-H1299 cells with overexpression of TNFRSF21 displayed fewer invaded cells through the matrigel upon treatment of Thiostrepton (20 μM) and U0126 (10 μM) for 40 hours when compared with the respective control with DMSO (***P < 0.001) (right). Numbers of migrated or invaded cells in three randomly chosen fields were counted for individual experiments, and the normalized numerical data are presented in bar charts with error bars showing ± SEM. Scale bar = 20 μm.