Am J Cancer Res 2022;12(7):3373-3389
www.ajcr.us /ISSN:2156-6976/ajcr0143795

Original Article

Dual role of ERK2/NF-κB signaling in TRAIL sensitivity

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Received April 28, 2022; Accepted June 23, 2022; Epub July 15, 2022; Published July 30, 2022

Abstract: Targeting tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling is a promising approach in cancer treatment. Although ERK and/or NF-κB signaling is involved in the expression of TRAIL receptors (TRAIL-R), the exact underlying mechanisms remain unknown. In this study, we evaluated the role of ERK2 and NF-κB in the cytotoxicity of TRAIL during cisplatin treatment. Cisplatin treatment of neuroepithelioma cells (SK-N-MC) significantly induced ERK2 activation and increased TRAIL cytotoxicity via the upregulation of death receptor 5 (DR5) expression. In partial ERK2 knockdown cell lines that maintained only basal levels of ERK2 activity, cisplatin treatment did not increase ERK2 activity or DR5 expression. These findings indicate that induced (rather than basal) ERK2 activity enhances TRAIL susceptibility via DR5 expression. In complete ERK2 knockdown cell lines with no basal ERK2 activity, DR4, DR5, and DcRs expression levels were increased, and additional treatment with cisplatin did not further increase TRAIL-R expression. Chemical inhibition of ERK2 also enhanced TRAIL cytotoxicity by upregulating DR4 and DR5 expression. These findings indicate that basal ERK2 activity suppresses TRAIL-R expression. Both basal and inducible ERK2 activities regulate TRAIL-R expression via the NF-κB signaling pathway. Overall, our findings suggest that the ERK2/NF-κB signaling pathway has a dual role in TRAIL susceptibility by differentially regulating TRAIL-R expression in the same cellular system.

Keywords: TRAIL, ERK2, NF-κB, cisplatin, death receptor

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a potential therapeutic agent for cancer treatment owing to its selective apoptotic activity in cancer cells and minimal cytotoxicity to normal cells. TRAIL, also known as the Apo-2 ligand, is a member of the TNF family that selectively induces apoptosis in tumor cells [1, 2]. TRAIL interacts with two types of receptors: the apoptosis-inducing death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) as well as the non-apoptosis-inducing decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) [3, 4]. By binding to DRs, TRAIL induces receptor trimerization and a conformational change in the intracellular death domain, resulting in the recruitment of Fas-associated death domain (FADD) and pro-caspase-8 and -10 to the death-inducing signaling complex. The recruited caspases are self-activated, and they, in turn, activate downstream effector caspases, such as caspase-3 and caspase-9, which transmit signals that lead to apoptosis. However, when TRAIL binds to DcRs, FADD cannot be recruited and apoptosis is not triggered [5-7].

Although recombinant TRAIL or agonistic DR4/5 antibodies have emerged as promising cancer treatments, their reported clinical efficacy is limited [8-11], which could be attributed to intrinsic TRAIL resistance in many primary tumors [12, 13]. Several studies have demonstrated the synergism of commonly used chemotherapeutic agents to increase the efficacy of TRAIL-induced apoptosis in tumor cells [14]. Cisplatin, a platinum-based agent, sensitizes cancer cells to TRAIL/anti-DR5 antibodies [15-20]. This synergism might be involved in the

Bid-dependent stimulation of the mitochondrial apoptotic pathway in prostate cancer cells [21]. However, the mechanism underlying cisplatin-induced sensitization of these agents remains unclear.

To increase the efficacy of recombinant TRAIL or agonistic DR4/5 antibodies, the modulation of DR4/5 expression should be considered. The upregulation of DR4/5 expression is mediated by NF-κB and ERK signaling. Inhibition of MEK/ERK signaling suppresses DR4/5 expression [22, 23], whereas NF-κB activation increases DR4/5 expression [24, 25]. Thus, elucidating the potential role of cisplatin in DR4/5 upregulation may provide a novel strategy to improve the efficacy of recombinant TRAIL or agonistic DR4/5 antibodies. In this study, we investigated the role of ERK2/NF-κB signaling in the enhancement of TRAIL cytotoxicity in the SK-N-MC cell line.

Materials and methods

Cells and reagents

The human brain neuroepithelioma cell line SK-N-MC was purchased from the American Type Tissue Culture Collection (ATCC; Rockville, MD, USA) and cultured as recommended. This cell line was authenticated by ATCC and was passaged for < 6 months after purchase for use in all experiments. The human glioma cell line C6, glioblastoma cell line T98G, and neuroblastoma cell lines IMR-32 and SK-N-BE were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained in a humidified atmosphere containing 5% CO_2 and 95% humidity at 37°C in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose (Thermo Fisher Scientific, Gibco, Rockville, MD, USA), 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Gibco), 10 mM HEPES (Thermo Fisher Scientific, Gibco), and 1 × antibiotic/antimycotic solution (Thermo Fisher Scientific, Gibco). Stable cell lines with ERK2 and NF-κB knockdown were established using ERK2- and NF-κB-specific shRNA encoded in lentivirus (Santa Cruz Biotechnology, Dallas, TX, USA).

Cell viability assay

SK-N-MC cells were seeded in 96-well plates at a density of 2.5×10^4 or 5×10^4 cells per well. Cells were treated with TRAIL (Thermo Fisher Scientific, Invitrogen, Carlsbad, CA, USA) alone or in combination with cisplatin (Dong-A Pharm, Seoul, Korea) and the DR5:Fc fusion protein (R&D Systems, Minneapolis, MN, USA). Inhibitors specific to MAPK/ERK (U0126), MEK (PD-98059), and IKK were purchased from Calbiochem (San Diego, CA, USA) and Santa Cruz Biotechnology. AlamarBlue (Thermo Fisher Scientific, Invitrogen) was added for the final 3 h of treatment, and absorbances at 570 nm and 600 nm were measured using an ELISA Reader (Molecular Devices, Sunnyvale, CA, USA). Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA) staining was used to visualize nuclear changes and apoptotic body formation, which are characteristic of apoptosis.

Flow cytometry analysis

The expression of TRAIL-R was analyzed using anti-human TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 antibodies (R&D Systems). Normal mouse IgG was used as a control. The fluorescence intensity of cells was determined using a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and then analyzed using the CellQuest software (Becton Dickinson).

Western blot analysis

Cells were treated with cisplatin and/or TRAIL, and cellular lysates were prepared using a cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (Invitrogen). Protein content was measured using the BCA Protein Assay kit (Thermo Fisher Scientific, Thermo Scientific, Rockford, IL, USA). Thereafter, equal amounts of protein from each cell lysate were separated using denaturing gel electrophoresis with reducing conditions and then transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Pittsburgh, PA, USA). Primary antibodies against β-actin (Sigma-Aldrich), pMEK, MEK, pERK1/2, ERK1/2, caspase-3, Mcl-1, Bcl-2, Bid (Cell Signaling Technology, Boston, MA, USA), or ERK2 Ab (Abcam, Cambridge, UK), and peroxidase-conjugated secondary antibodies against rabbit IgG (Cell Signaling Technology) or mouse IgG (Santa Cruz Biotechnology) were used. Proteins were visualized using chemiluminescence (Amersham Biosciences).

Immunocytochemistry

Cells were placed in a fixative solution (4% paraformaldehyde prepared in PBS) for 30 min

at room temperature in the dark, and then washed three times with PBS. To detect intracellular proteins, cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature in the dark. The cells were washed three times and then incubated in a blocking solution (5% FBS prepared in PBS) for 1 h at room temperature. After further washing, antibodies against pERK1/2 (Calbiochem) were added, and the cells were incubated for 1 h at room temperature. The cells were washed three times and incubated with Alexa Fluor 488-conjugated secondary goat anti-mouse IgG (Thermo Fisher Scientific, Invitrogen) for 1 h at room temperature, washed three times, and mounted in 4, 6-diamidino-2-phenylindole (DAPI)-containing anti-fade mounting solution (Vectashield; Vector Lab., Burlingame, CA, USA). Fluorescence imaging of cells was performed using a Carl Zeiss LSM 700 confocal microscope system (Jena, Germany) with a 25 mW laser diode tuned to 405 nm for DAPI staining of cell nuclei and a 30 mW argon laser tuned to 488 nm for visualization of Alexa Fluor 488-labeled pERK1/2. Confocal microscopy results were analyzed using the LSM 700 Zen software.

Analysis of NF-κB activation

Cells were treated with cisplatin and/or TRAIL, and cellular lysates were prepared using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Protein content was measured using a BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's instructions. NF-κB activity was measured using the NFkB (p65) Transcription Factor Assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Inhibitors specific to NF-κB (CAS 545380-34-5) and IKK were obtained from Sigma-Aldrich and Santa Cruz Biotechnology, respectively.

Statistical analyses

Statistical analyses were performed using the Student's t-test. All results are expressed as the mean ± standard deviation. Statistical significance was set at $P < 0.05$.

Results

Pre-treatment with cisplatin enhanced TRAIL cytotoxicity via an increase in DR5 expression

To investigate the underlying mechanism of cisplatin-mediated TRAIL cytotoxicity, we determined whether pre- or cotreatment with cisplatin and TRAIL could increase cytotoxicity in SK-N-MC cells. A single treatment with cisplatin reduced cell viability but a single TRAIL treatment did not; a combination of the two synergistically increased cytotoxicity (**Figure 1A**, **1B**), i.e., cotreatment with cisplatin and TRAIL resulted in a marked synergism. Intriguingly, cotreatment with cisplatin and TRAIL had different effects on the time sequence. Pre-treatment with cisplatin followed by treatment with TRAIL after 12 h or 24 h synergistically enhanced cell death, however, the inverse treatment schedule showed limited synergism. The cytotoxicity of cotreatment was dependent on the exposure time of cisplatin, rather than that of TRAIL. Regardless of the exposure times of TRAIL, the synergistic effects of cisplatin after 48 h of exposure were similar. However, reduced exposure times to cisplatin reduced the synergism of the drug combination (**Figure 1A**, **1B**). These findings indicate that cisplatin treatment modified the cellular environment, which was favorable for TRAIL-mediated cytotoxicity. We next analyzed the changes in TRAIL-R expression after cisplatin treatment using flow cytometry with specific antibodies against DR4, DR5, DcR1, and DcR2. Treatment with cisplatin alone induced the expression of DR5 after 18 h, however, no changes were observed in other TRAIL-Rs (**Figure 1C**, **1D**). To further verify whether an elevated level of DR5 induced by cisplatin treatment was one of the key factors in the synergism, we blocked DR5 using a specific antibody during cisplatin or TRAIL treatment. Although the cytotoxicity of cisplatin alone was not affected by the anti-DR5 antibody, the synergistic elevation of cell death by cotreatment with cisplatin and TRAIL was reduced to a level equal to that with cisplatin-only treatment by the addition of anti-DR5 antibody (**Figure 1E**); this was confirmed by microscopy data (**Figure 1F**). These findings imply that cisplatin elevated the cellular response to TRAIL by inducing the expression of DR5 on the cell surface.

ERK activity modulated DR5 expression

To investigate the signaling pathway involved in the elevation of DR5 expression induced by cisplatin treatment, we analyzed ERK1/2 activation. Cisplatin increased ERK phosphorylation after 12 h of treatment but TRAIL did not (**Figure 2A**). Immunocytochemistry using a phosphorylated ERK-specific antibody revealed that cisplatin induced clear spots in the cytosol; howev-

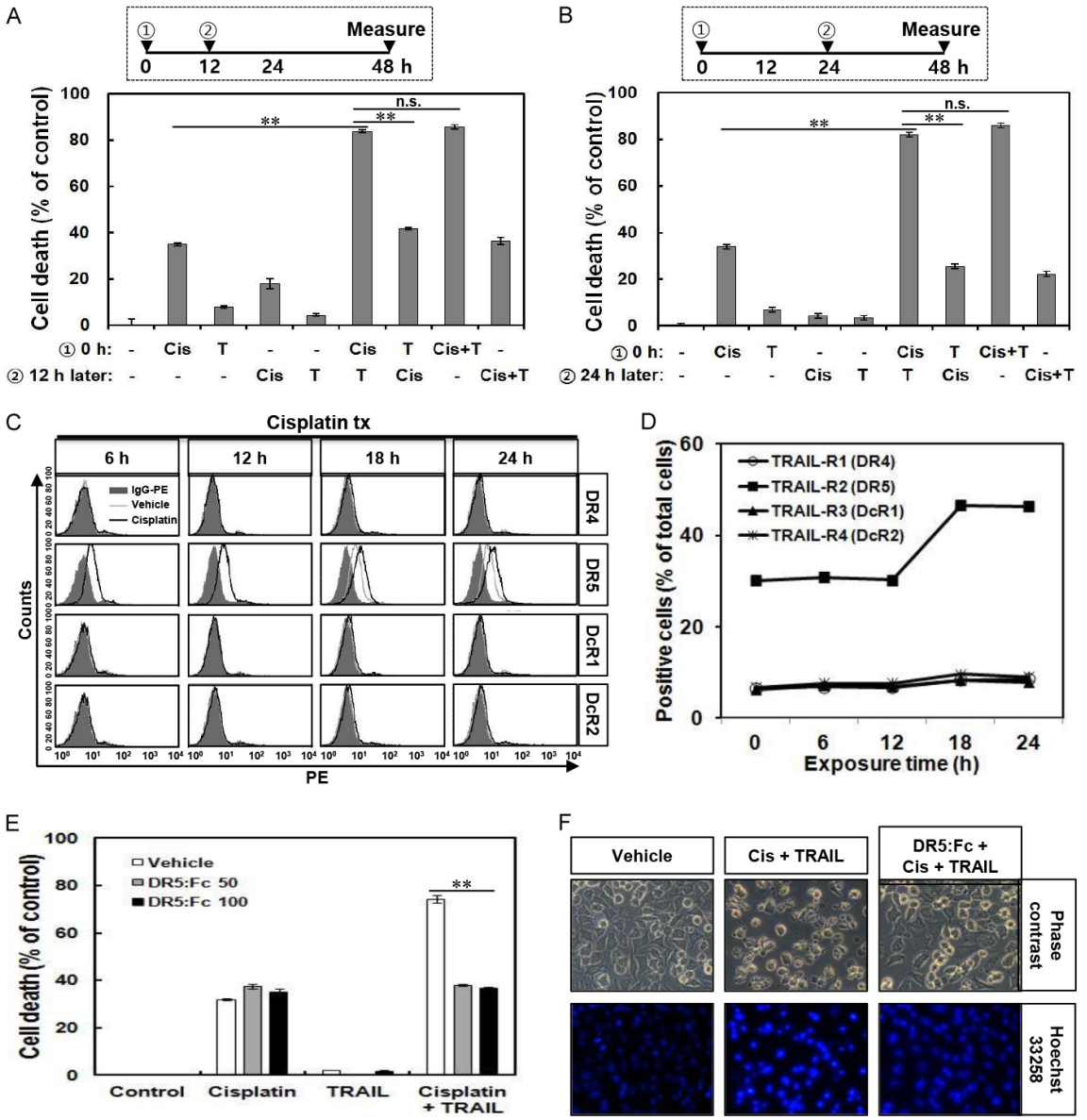

Figure 1. Cisplatin treatment induces cell death via the induction of TRAIL-R2 expression in the neuroepithelioma cell line. A and B. SK-N-MC cells were seeded in 96-well plates and treated with cisplatin or TRAIL for the indicated times. Thereafter, cell viability was assessed using an AlamarBlue assay. C. Surface expression of DR4, DR5, DcR1, and DcR2 in cells treated with cisplatin was analyzed using flow cytometry with receptor-specific antibodies. D. The percentage of TRAIL-R positive cells at each exposure time was determined using flow cytometric analyses. E. Cell viability was assessed after treatment with cisplatin or TRAIL combined with the DR5 antibody (DR5: FC). F. Representative images of Hoechst 33258-positive cells after treatment with cisplatin, TRAIL, or DR5 antibody.

er, the phosphorylated form of ERK was dispersed in the untreated control.

We investigated the correlation between ERK activity and TRAIL-R expression using U0126, an ERK-specific inhibitor. The elevation in phosphorylated ERK levels due to cisplatin treatment was inhibited when U0126 was co-administered (**Figure 2B**, **2C**). Intriguingly, the presence of U0126, the expression levels of all TRAIL-R increased (**Figure 2D**, **2E**). As cisplatin-mediated TRAIL cytotoxicity is dependent on ERK activation, treatment with U0126 should reduce the cytotoxicity of the combination of cisplatin and TRAIL. However, U0126 did not affect the synergistic cytotoxicity of cisplatin and TRAIL (**Figure 2F**). Intriguingly, the inhibition of ERK by U0126 significantly augmented

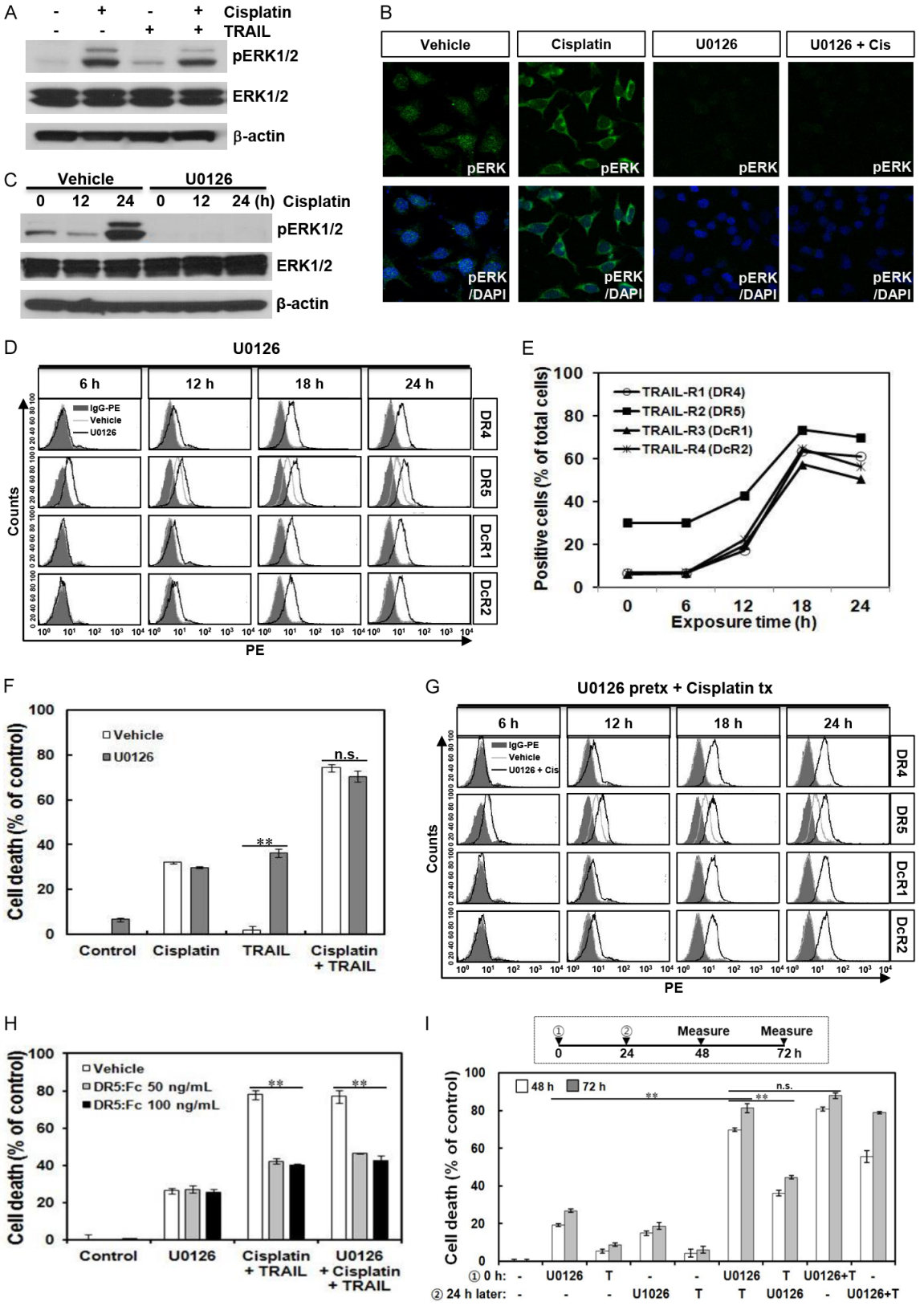

Figure 2. Cisplatin-induced elevation of TRAIL cytotoxicity is mediated by ERK activation. A. Phosphorylated ERK1/2 level was determined using western blotting after treatment with cisplatin or TRAIL. B. Phosphorylated ERK1/2 levels were determined using immunocytochemistry with phosphorylated ERK-specific antibody. C. Western blot-

ting was performed after U0126 treatment to detect ERK1/2 phosphorylation. D. Surface expression of DR4, DR5, DcR1, and DcR2 in U0126 treated cells were analyzed using flow cytometry with receptor-specific antibodies. E. TRAIL-R positive cells at each exposure time were determined using flow cytometric analyses. F. Cell viability was assessed after treatment with cisplatin or TRAIL combined with U0126. G. Surface expression of DR4, DR5, DcR1, and DcR2 in cells treated with cisplatin was analyzed using flow cytometry with receptor-specific antibodies. H. Cell viability was assessed after treatment with U0126, cisplatin, or TRAIL combined with the DR5 antibody (DR5: FC). I. Cell viability assessment was carried out after treatment with U0126 and TRAIL.

the cytotoxicity of TRAIL treatment. Thus, we analyzed the changes in TRAIL-R expression levels using flow cytometry. In the presence of U0126, the increased expression levels of all TRAIL-Rs were maintained by cisplatin treatment (**Figure 2G**). These elevated TRAIL-R levels might explain why the synergism of cisplatin and TRAIL was not affected by U0126 treatment.

We also analyzed the effect of ERK inactivation on DR5-mediated cytotoxicity. A single treatment with U0126 increased levels of cell death; however, DR5 inhibition was not found to be involved in U0126 cytotoxicity (**Figure 2H**). The synergistic elevation of cytotoxicity induced by cisplatin and TRAIL was not affected by U0126 treatment, and inhibition of DR5 significantly reduced cytotoxicity regardless of the presence of U0126. Thus, although ERK phosphorylation was blocked by U0126, cotreatment with cisplatin and TRAIL increased cytotoxicity by elevating DR5 levels. We further investigated the synergistic elevation of TRAIL cytotoxicity when combined with U0126. Pre-treatment or co-treatment with U0126 and TRAIL increased cytotoxicity, whereas pre-treatment with TRAIL followed by U0126 reduced cytotoxicity, indicating that ERK inhibition mediated TRAIL-R expression (**Figure 2I**). Collectively, these data suggest that the inhibition of ERK induced the expression of TRAIL-R.

Activated ERK2 induced TRAIL-R2 expression but ERK1 did not

To further determine the role of ERK activation/phosphorylation during TRAIL-induced cytotoxicity, we evaluated the changes in ERK1 and ERK2 during treatment with cisplatin. The transient knockdown of ERK1 and ERK2 using their specific siRNA revealed that ERK2 was phosphorylated by cisplatin treatment but ERK1 was not (**Figure 3A**). Flow cytometric analysis also showed that DR5 positivity was slightly increased by both ERK1- and ERK2-specific siRNA treatment. However, the strong elevation of DR5 positivity induced by cisplatin was only observed in ERK1 knockdown cells (**Figure 3B**). Thus, the elevation of DR5 expression during cisplatin treatment was mediated by ERK2 and not ERK1.

To validate these findings, we generated several stable ERK2 knockdown cell lines. Western blotting revealed changes in the total and phosphorylated ERK1/2 levels after ERK2 knockdown. Clones 2 and 4 showed complete ERK2 knockdown, whereas the others showed partial ERK2 knockdown as they exhibited only basal levels of ERK2 (**Figure 3C**). In accordance with the western blotting results, immunocytochemistry revealed that clone 8 had limited levels of phosphorylated ERK1/2 in the cytosol, whereas phosphorylated ERK1/2 could not be observed in the completely knocked down cells (clone 4; **Figure 3D**). The effects of ERK2 knockdown on the changes in TRAIL-R expression were analyzed using flow cytometry. The expression of TRAIL-R was not changed in either the negative control (scrambled shRNA) and a partial ERK2 knockdown cell line (**Figure 3E**, **3F**). However, complete knockdown of ERK2 increased the expression levels of all TRAIL-Rs (**Figure 3G**). Therefore, basal ERK2 activity negatively regulates TRAIL-R expression.

We next analyzed the activation of ERK2 by cisplatin in ERK2 knockdown cell lines. In both complete (clones 2 and 4) and partial (clones 3 and 8) ERK2 knockdown cells, no elevation of phosphorylated ERK1/2 induced by cisplatin treatment was observed, despite the detection of basal levels of ERK in the partial ERK2 knockdown cell lines (**Figure 4A**). Immunocytochemistry also revealed changes in ERK2 levels during cisplatin treatment (**Figure 4B**). To verify whether the weak bands observed in the partial ERK2 knockdown cell lines represented ERK1 or ERK2, we performed an immunoprecipitation assay. The lower bands of ERK1/2 in the partial ERK2 knockdown cell lines disappeared after immunoprecipitation with an ERK2-specific antibody, indicating that the

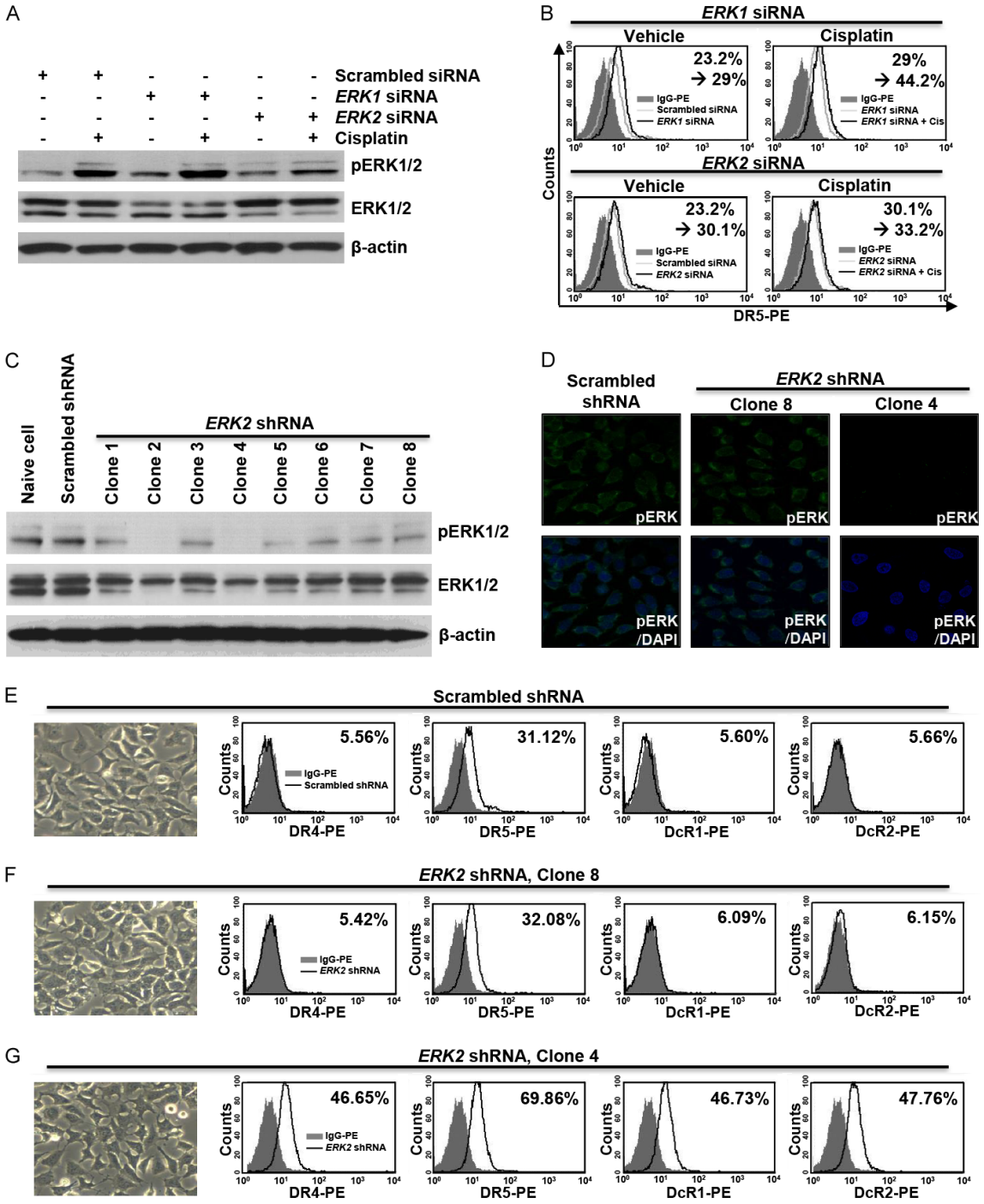

Figure 3. Knockdown of ERK2 changed TRAIL-R expression levels. (A) Western blotting demonstrated ERK1 and ERK2 knockdown using their specific siRNA. (B) Flow cytometry revealed DR5 positivity after ERK1- or ERK2-specific siRNA treatment. (C) ERK2 knockdown cells were screened using western blotting with phosphorylated ERK antibody. (D) ERK2 knockdown was determined using immunocytochemistry with phosphorylated ERK antibody. (E-G) Effects of ERK2 knockdown on the changes in TRAIL-R expression using flow cytometry in the control (E), clone 8 (F), and clone 4 (G).

bands represented ERK1 (**Figure 4C**). After verification of ERK2-specific knockdown in the cell lines, we investigated the changes in DR5 levels after treatment with cisplatin or PMA. In the negative control cell lines, DR5 positivity was elevated by cisplatin or PMA treatment; howev-

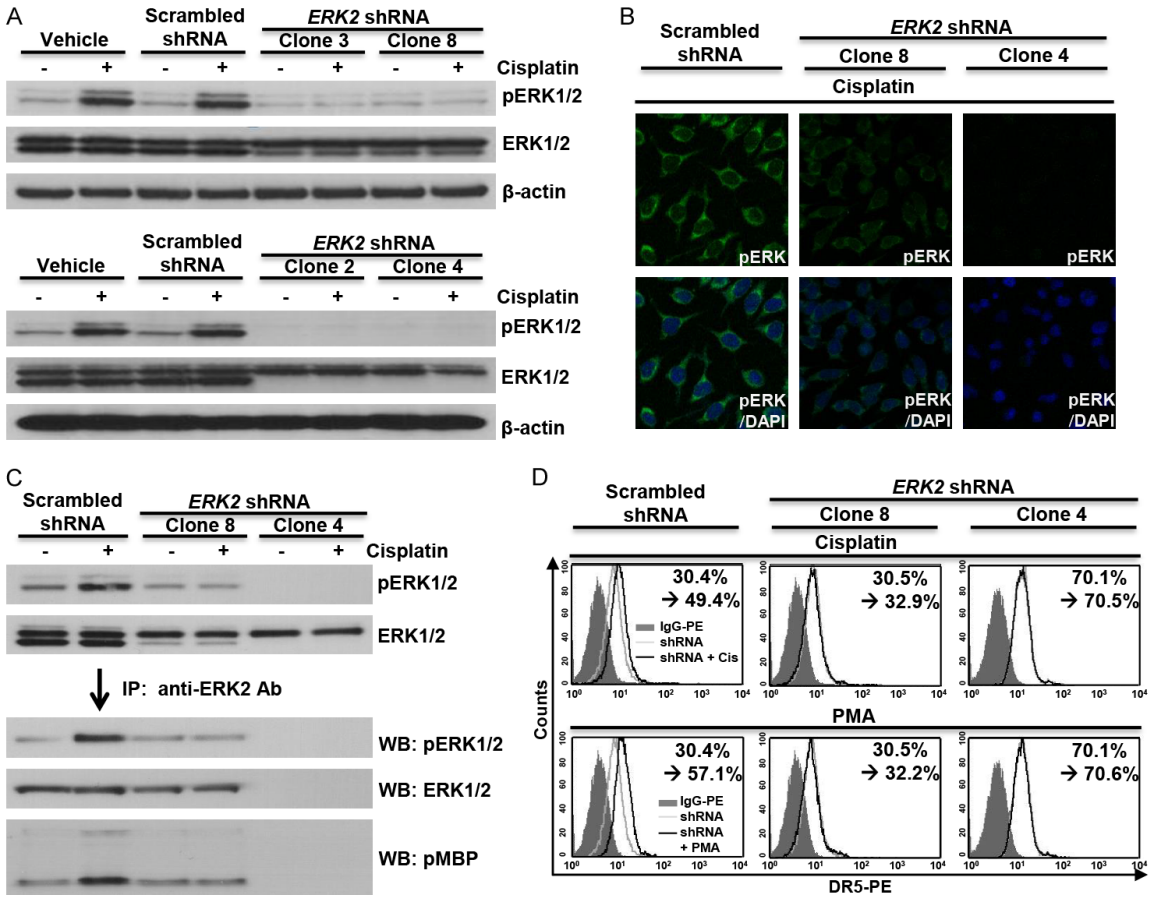

Figure 4. ERK2 phosphorylation does not respond to cisplatin treatment in ERK2 knockdown cells. A. ERK2 phosphorylation induced by cisplatin treatment in ERK2 knockdown cells as determined using western blotting. B. Immunocytochemical analysis of ERK2 phosphorylation in partial (clone 8) and complete (clone 4) ERK2 knockdown cells. C. Immunoprecipitation assay to demonstrate ERK2 level. D. Surface expression of DR5 in partial (clone 8) and complete (clone 4) ERK2 knockdown cells after cisplatin or PMA treatment.

er, there was no change in DR5 intensity in either partial or complete knockdown cell lines in response to cisplatin or PMA treatment (**Figure 4D**). These results suggest that ERK2 activation directed DR5 expression.

Inhibition of basal ERK2 activity enhanced TRAIL cytotoxicity via the upregulation of TRAIL-R, including DR5

U0126 treatment completely abolished basal ERK2 activity in the partial ERK2 knockdown cell lines (clones 3 and 9; **Figure 5A**). In the complete ERK2 knockdown cell lines (clones 2 and 8), phosphorylated ERK2 was not observed following control or U0126 treatment. Immunocytochemistry also demonstrated that ERK2 phosphorylation was abrogated (**Figure 5B**). These results are supported by the immunoprecipitation assay; the bands for ERK1/2 disappeared in the complete ERK2 knockdown cell lines after immunoprecipitation with an ERK2-specific antibody (**Figure 5C**).

We proceeded to analyze TRAIL-R expression in ERK2 knockdown cell lines. In control (scrambled shRNA) and partial ERK2 knockdown cells, U0126 treatment induced TRAIL-R positivity. In the complete ERK2 knockdown cells, TRAIL-R positivity was elevated compared with the control, and these elevated levels were not further increased by U0126 treatment (**Figure 5D**). Additionally, cisplatin- and U0126-induced TRAIL-R expression was evaluated in various brain tumor cell lines. TRAIL-R expression was elevated by ERK2 inhibition in all cell lines, including IMR-32, SK-N-BE (neuroblastoma cell line), C6 (glioma cell line), and T98G (glioblastoma cell

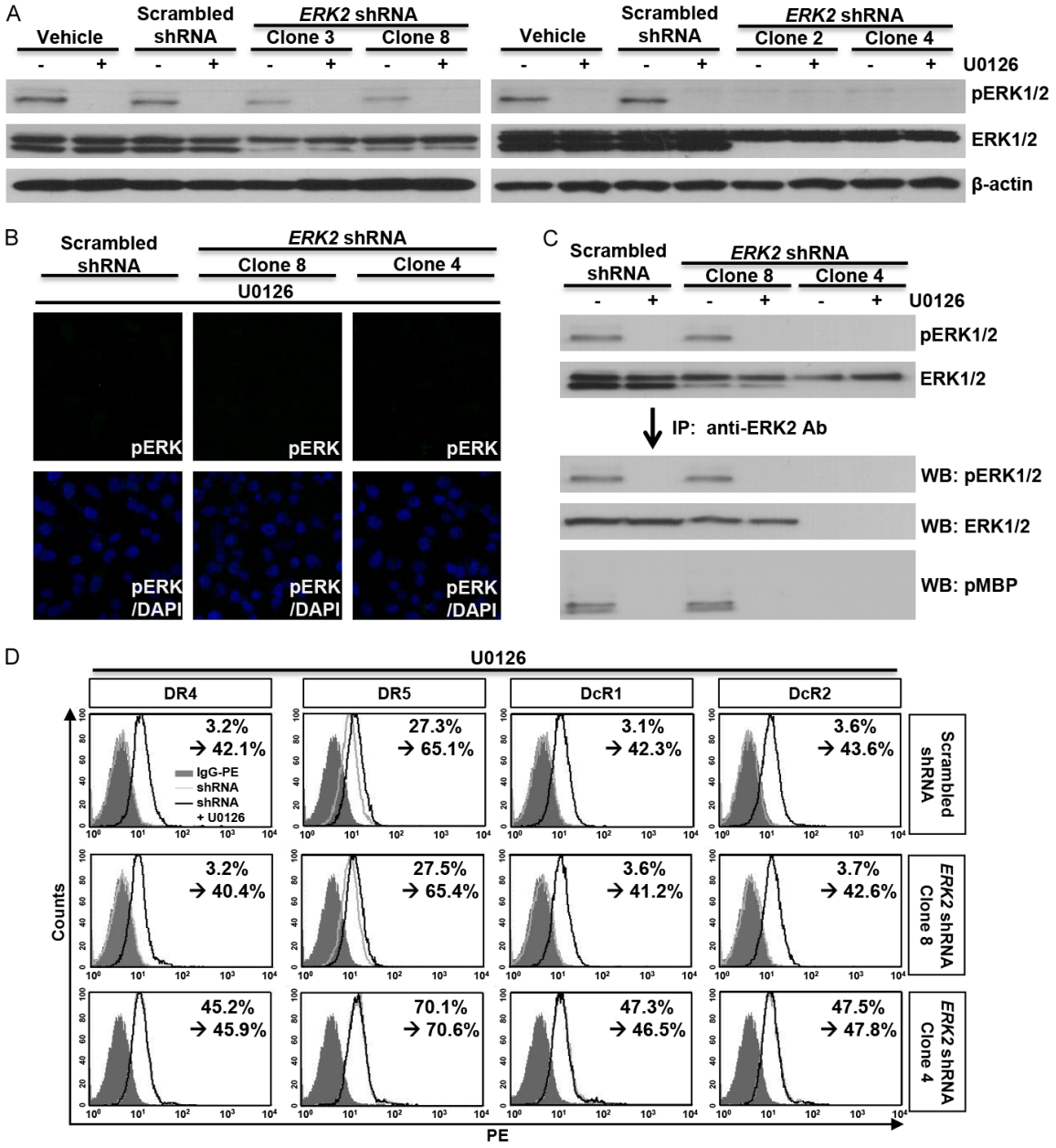

Figure 5. Inactivation of ERK2 elevated TRAIL-R expression levels. A. Inhibition of ERK2 by U0126 in partial (clones 3 and 8) and complete (clones 2 and 4) ERK2 knockdown cells. B. Immunocytochemistry analysis of ERK2 phosphorylation in partial (clone 8) and complete (clone 4) ERK2 knockdown cells after ERK inhibitor treatment. C. Immunoprecipitation assay using anti-ERK2 antibody for detecting the ERK2 band. D. Flow cytometric analysis to determine TRAIL-R expression levels in ERK2 knockdown cells after U0126 treatment.

line, **Figure 6A-D**). Collectively, these data indicate that the abrogation of basal ERK2 activity induced TRAIL-R expression.

DR5 expression was regulated by NF-κB

As both cisplatin-induced ERK2 activity and abrogation of basal ERK2 activity increased TRAIL-R expression, we sought to address the discrepancy in the role of ERK2 during cisplatin-induced cytotoxicity, focusing on NF-κB activation during cisplatin treatment. After 12 h of cisplatin treatment, the levels of IκBα, an inhibitor of NF-κB, were reduced, whereas the levels of phosphorylated ERK2 were increased (**Figure 7A**). The inhibition of IκB kinase (IKK) using IKK inhibitor or a competitor reduced NF-κB activity (**Figure 7B**). Intriguingly, cotreatment with the

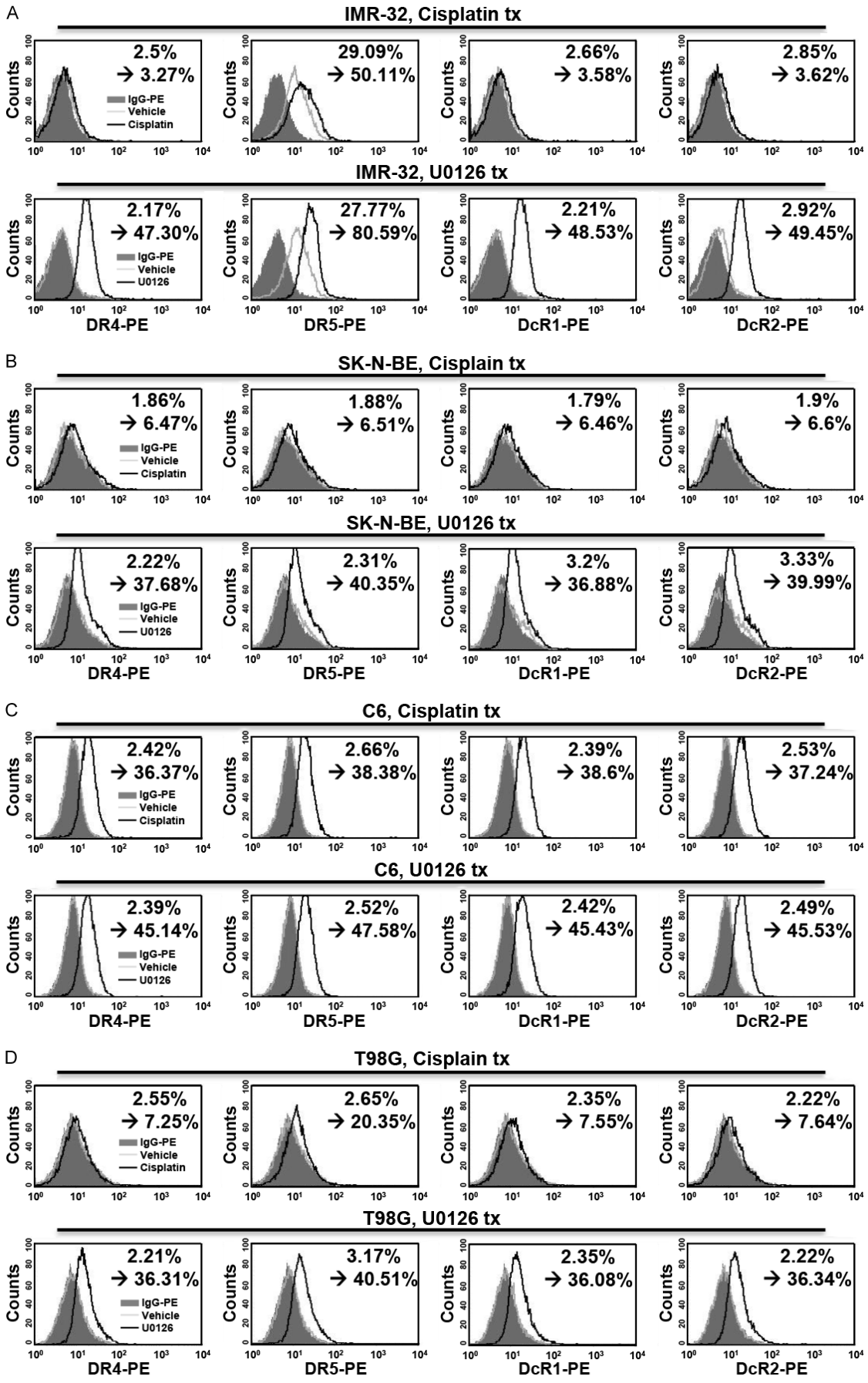
A
IMR-32, Cisplatin tx
2.5%
→ 3.27%
29.09%
→ 50.11%
2.66%
→ 3.58%
2.85%
→ 3.62%
IgG-PE
Vehicle
Cisplatin
IMR-32, U0126 tx
2.17%
→ 47.30%
27.77%
→ 80.59%
2.21%
→ 48.53%
2.92%
→ 49.45%
IgG-PE
Vehicle
U0126
Counts
DR4-PE
DR5-PE
DcR1-PE
DcR2-PE
B
SK-N-BE, Cisplain tx
1.86%
→ 6.47%
1.88%
→ 6.51%
1.79%
→ 6.46%
1.9%
→ 6.6%
SK-N-BE, U0126 tx
2.22%
→ 37.68%
2.31%
→ 40.35%
3.2%
→ 36.88%
3.33%
→ 39.99%
C
C6, Cisplatin tx
2.42%
→ 36.37%
2.66%
→ 38.38%
2.39%
→ 38.6%
2.53%
→ 37.24%
C6, U0126 tx
2.39%
→ 45.14%
2.52%
→ 47.58%
2.42%
→ 45.43%
2.49%
→ 45.53%
D
T98G, Cisplain tx
2.55%
→ 7.25%
2.65%
→ 20.35%
2.35%
→ 7.55%
2.22%
→ 7.64%
T98G, U0126 tx
2.21%
→ 36.31%
3.17%
→ 40.51%
2.35%
→ 36.08%
2.22%
→ 36.34%

Figure 6. TRAIL-R expression levels were elevated by cisplatin treatment or ERK1/2 inhibition. Changes in TRAIL-R expression determined using flow cytometry in (A) IMR-32, a neuroblastoma cell line, (B) SK-N-BE, a neuroblastoma cell line, (C) C6, a glioma cell line, and (D) T98G, a glioblastoma cell line, after treatment with cisplatin and U0126.

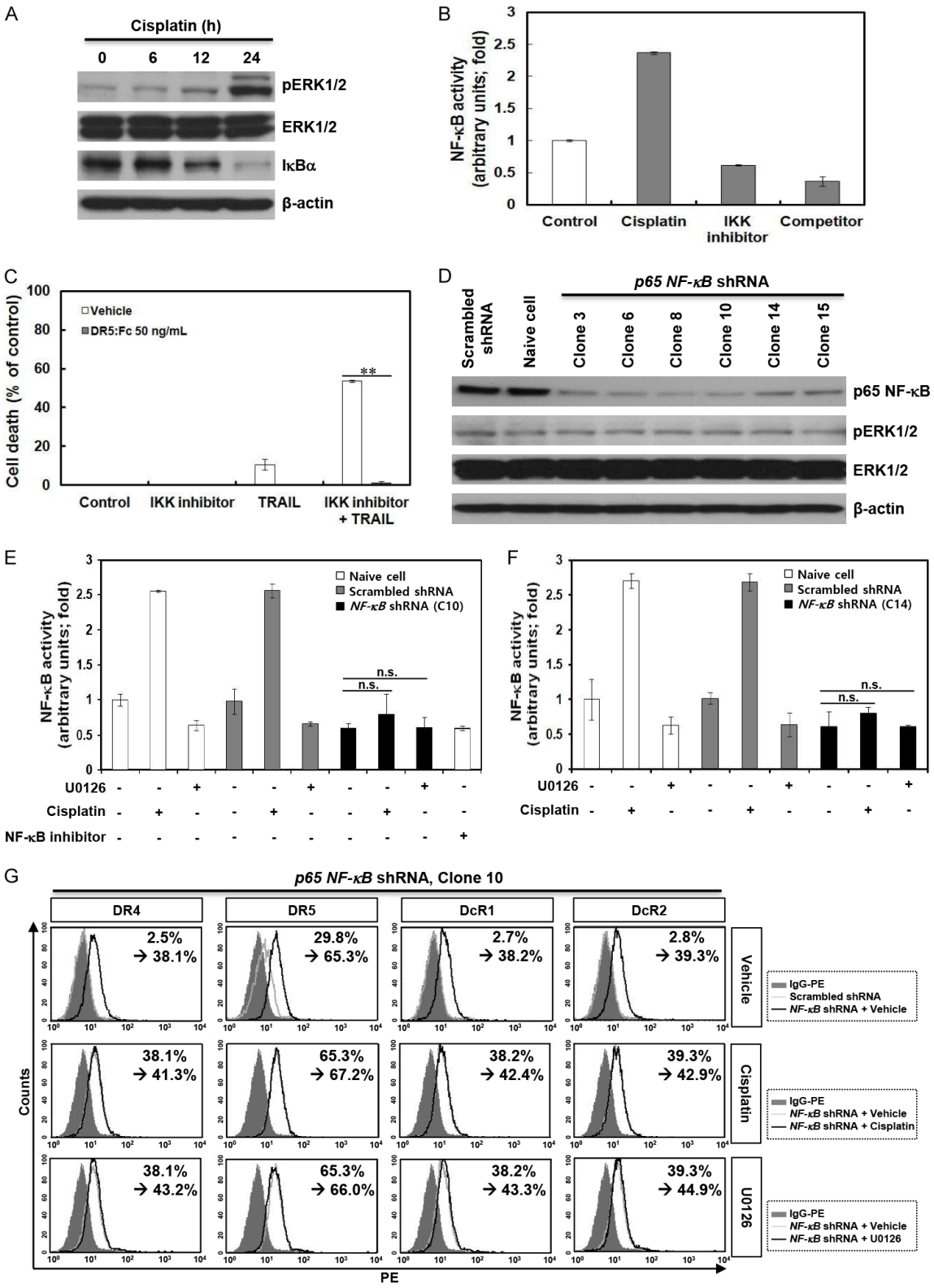

Figure 7. DR5 expression was regulated by NF-κB. A. Western blot analysis to determine ERK1/2 activation and IκBα inactivation induced by cisplatin treatment for the indicated times. B. NF-κB activity assay after cisplatin, IKK inhibitor, or competitor treatment. C. Effect of cotreatment with IKK inhibitor and TRAIL on cell viability. D. Western blotting to determine p65 levels in an NF-κB knockdown cell line using specific shRNA. E. Activity of NF-κB in NF-κB knockdown cells (clone 10) after treatment with U0126, cisplatin, or NF-κB inhibitor. F. Activity of NF-κB in NF-κB knockdown cells (clone 14) after treatment with U0126, cisplatin, or NF-κB inhibitor. G. Flow cytometric analysis to determine TRAIL-R expression in an NF-κB knockdown clone (clone 10).

IKK inhibitor and TRAIL synergistically induced cell death (**Figure 7C**). Although the IKK inhibitor alone (inactivation of NF-κB) did not induce cell death, cotreatment with TRAIL increased levels of cell death, which was mainly dependent on DR5. To further determine the role of NF-κB in DR5 expression, we generated p65 NF-κB knockdown cell lines using specific shRNAs (**Figure 7D**). Using clones 10 and 14, we determined the effect of NF-κB on TRAIL-R expression levels. Abrogation of NF-κB significantly increased the expression of all TRAIL-Rs (**Figure 7E**, **7F**). However, these elevated TRAIL-R levels were not further increased by cisplatin or U0126 (**Figure 7G**). These findings imply that basal NF-κB activity suppressed TRAIL-R expression, whereas NF-κB activity induced by cisplatin increased TRAIL-R expression.

Chemical inhibition of ERK2 using U0126 completely removed the phosphorylated form of ERK2 until day 5; thereafter, the level was restored. However, following cisplatin treatment, ERK2 was reactivated (**Figure 8A**). Accordingly, although U0126 treatment decreased NF-κB activity, cisplatin treatment increased NF-κB activity (**Figure 8B**). The elevated levels of TRAIL-R expression caused by the transient inhibition of ERK2 by U0126 was lowered to basal levels after 7 d. In this setting, cisplatin treatment only increased DR5 expression (**Figure 8C**). NF-κB activity was also found to be regulated by ERK2 as NF-κB activity was not elevated by cisplatin in ERK2 knockdown cells (**Figure 8D**). These findings suggest that both ERK2 and NF-κB play a dual role in TRAIL-R expression. The basal activity of ERK2 and NF-κB suppressed TRAIL-R expression under normal conditions, thereby rendering cells resistant to TRAIL. In contrast, cisplatin-induced ERK2 activity initiates NF-κB signaling, resulting in DR5 overexpression and TRAIL-mediated apoptotic cell death (**Figure 9**). Collectively, our data indicate that cisplatin can sensitize cancer cells to TRAIL by elevating TRAIL-R expression, which is suppressed by basal ERK2 and NF-κB activity.

Discussion

Primitive neuroectodermal tumors (PNETs; also called neuroepitheliomas) are a group of highly malignant tumors composed of small, round cells of neuroectodermal origin that affect soft tissues and bone. They exhibit great diversity in their clinical manifestations and pathologic similarities with other small, round cell tumors. Batsakis et al. [26] divided the PNET family into the following three groups based on their tissue of origin: central PNETs, tumors derived from the central nervous system; neuroblastoma, tumors derived from the autonomic nervous system; and peripheral PNETs, tumors derived from tissues outside the central and autonomic nervous system. Central PNETs originate from the neural tube, whereas peripheral PNETs originate from the neural crest, and both central and peripheral PNETs are deadly solid tumors in infants and children. Despite multimodal therapeutic approaches, including chemotherapy, radionuclide therapy, and immunotherapy, the survival rate of patients with malignant tumors remains poor. Thus, newer therapeutic strategies are urgently needed to successfully treat this devastating childhood malignancy.

TRAIL induces apoptosis in cancer cells but not in normal cells [27]. However, a significant proportion of cancer cells exhibits resistance to the cytotoxic effect of this ligand, suggesting that the use of TRAIL alone may not be sufficient to treat cancers [28]. TRAIL resistance is caused by the deregulated expression of TRAIL-R or intracellular components acting downstream of the receptors. Based on previous reports, conventional chemotherapeutic agents [29, 30], irradiation [31, 32], and histone deacetylase (HDAC) inhibitors [33] enhance the cytotoxicity of TRAIL by upregulating TRAIL-R expression.

In this study, we found that cisplatin induced a cellular environment for TRAIL-mediated cytotoxicity, including the activation of ERK2 and NF-κB activity, as well as the upregulation of TRAIL-R expression. In particular, cisplatin-me-

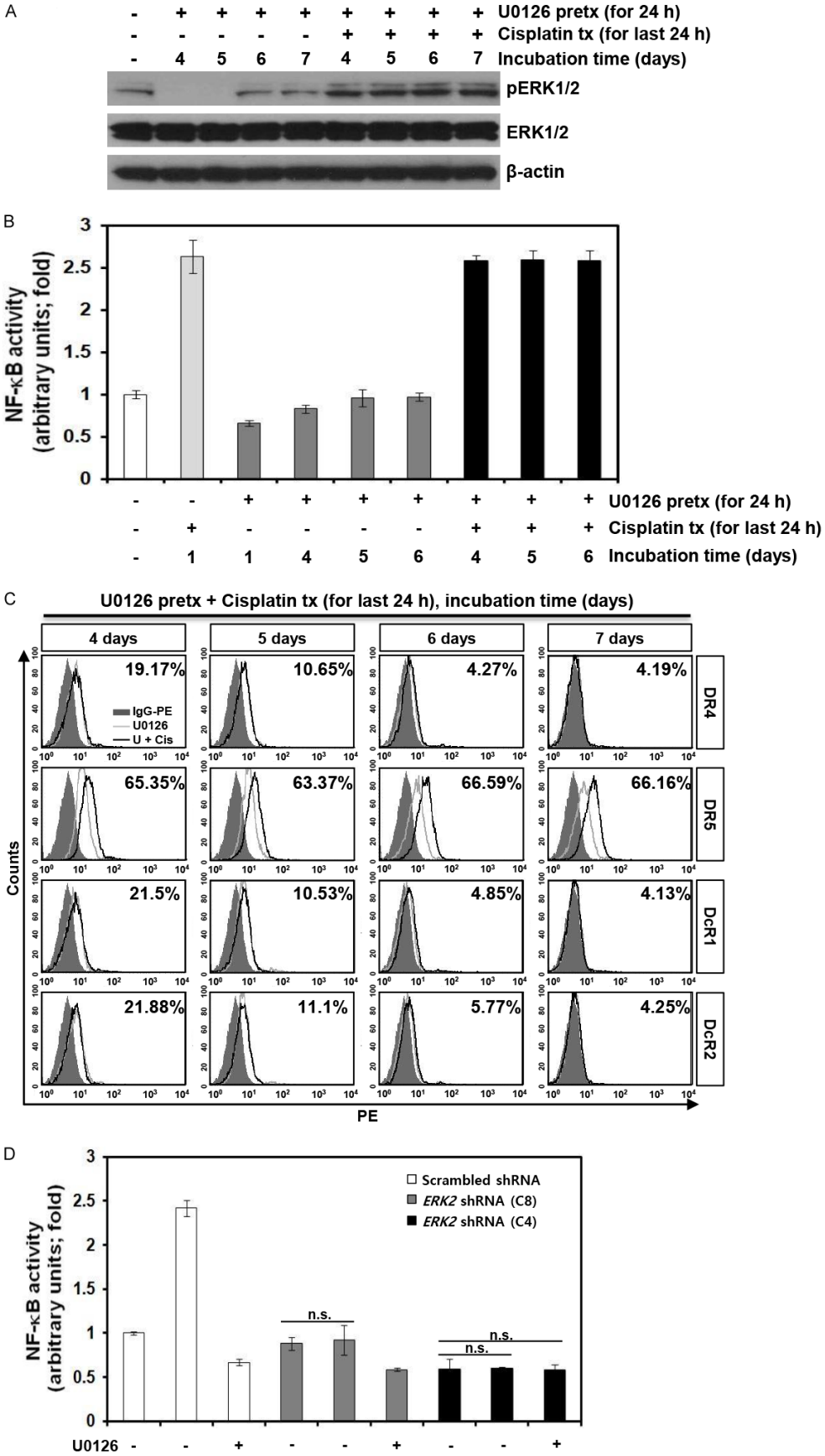
A
U0126 pretx (for 24 h)
Cisplatin tx (for last 24 h)
Incubation time (days)
pERK1/2
ERK1/2
β-actin
B
NF-κB activity (arbitrary units; fold)
U0126 pretx (for 24 h)
Cisplatin tx (for last 24 h)
Incubation time (days)
C
U0126 pretx + Cisplatin tx (for last 24 h), incubation time (days)
4 days
5 days
6 days
7 days
IgG-PE
U0126
U + Cis
19.17%
10.65%
4.27%
4.19%
65.35%
63.37%
66.59%
66.16%
21.5%
10.53%
4.85%
4.13%
21.88%
11.1%
5.77%
4.25%
DR4
DR5
DcR1
DcR2
Counts
PE
D
Scrambled shRNA
ERK2 shRNA (C8)
ERK2 shRNA (C4)
NF-κB activity (arbitrary units; fold)
n.s.
n.s.
n.s.
U0126
Cisplatin

Figure 8. Induction of ERK2 and NF-κB activity by cisplatin directs DR5 expression. A. Western blotting demonstrating the induction of ERK1/2 phosphorylation by cisplatin in the presence of U0126. B. NF-κB activity assay after U0126 and/or cisplatin treatment. C. Flow cytometric analysis to determine the effect of U0126 and cisplatin treatment. D. NF-κB activity in ERK2 knockdown cell lines (clones 4 and 8) after U0126 and cisplatin treatment.

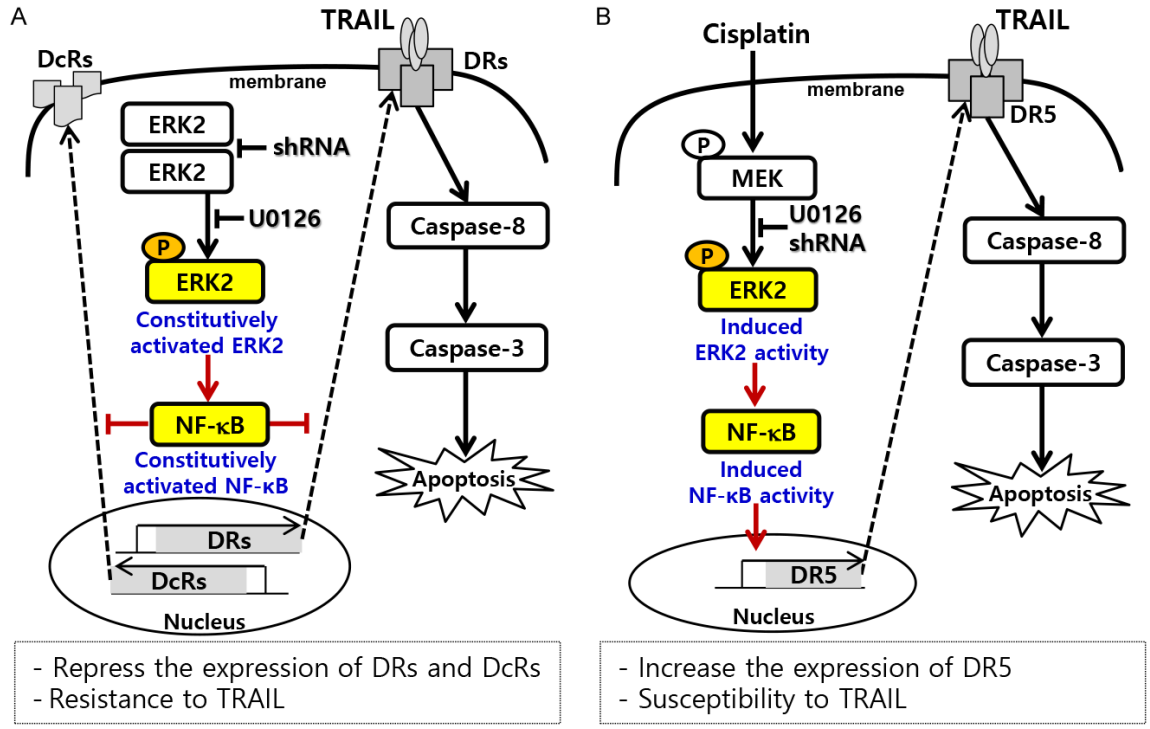

Figure 9. Schematic of ERK2, NF-κB, and DR5. A. Under normal conditions, basal (constitutively activated) levels of both ERK2 and NF-κB suppress TRAIL-R expression, rendering cells resistant to TRAIL. B. During cisplatin stimulation, induced ERK2 activates NF-κB, which results in DR5 overexpression and TRAIL-mediated apoptotic cell death.

diated DR5 expression was found to increase TRAIL cytotoxicity. Previously, chemotherapeutic agent-induced DR upregulation was reported to be dependent on NF-κB or p53 [34-36], which transcriptionally regulates DR expression by binding to sites in the promoter [37, 38]. Overexpression of the p65 subunit of NF-κB upregulates TRAIL-R2 expression in epithelial cell lines [39]. In this study, cisplatin-mediated TRAIL cytotoxicity was dependent on NF-κB activation. In addition, the sequential activation of ERK2 and NF-κB during DR5 expression was revealed. As U0126 treatment decreased NF-κB activity and cisplatin did not elevate NF-κB activity in ERK2 knockdown cells, ERK2 activity regulated NF-κB-mediated DR5 expression. In this context, the signal cascade in ERK2 and NF-κB signaling has received considerable attention for TRAIL therapy. In glioblastoma (GBM), the phosphorylation of ERK is involved in TRAIL resistance, and lovastatin sensitizes GBM cells to TRAIL by inhibiting ERK activity [40]. The inhibition of NF-κB also increases TRAIL-mediated apoptosis in various cancers [41-43]. Therefore, the mechanism involving ERK2 and NF-κB in TRAIL-R expression should be elucidated.

We also found a discrepancy between ERK2 and NF-κB activity during TRAIL-R expression. First, we found that the cisplatin-mediated induction of ERK2 and NF-κB activity directed DR5 overexpression and TRAIL-mediated apoptotic cell death. Furthermore, TRAIL cytotoxicity was found to be dependent on DR5 expression, which was mediated by ERK2, rather than ERK1, during cisplatin treatment. However, the basal activity of ERK2 and NF-κB was found to suppress DR5 expression. In both knockdown and chemical inhibition models, the abrogation of basal levels of ERK2 and NF-κB increased DR5 expression. Collectively, our findings sug-

gest that cisplatin-induced sensitization to TRAIL is mediated by TRAIL-R expression, which is suppressed by basal ERK2 and NF-κB activity. Therefore, both ERK2 and NF-κB play a dual role in TRAIL-R expression, which should be carefully considered in the application of TRAIL-based chemotherapy.

Acknowledgements

This work was supported by the Technological Innovation R&D Program (S3029531) funded by the Ministry of SMEs and Startups (MSS, Korea) and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI21C0168), and supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2022R1A2C1006055).

Disclosure of conflict of interest

None.

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