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Suppression of ERCC1 and Rad51 expression through ERK1/2 inactivation is essential in emodin-mediated cytotoxicity in human non-small cell lung cancer cells

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ABSTRACT

Emodin, a tyrosine kinase inhibitor, is a natural anthraquinone derivative found in the roots and rhizomes of numerous plants. Emodin exhibits anticancer effects against a variety of cancer cells, including lung cancer cells. ERCC1 and Rad51 proteins are essential for nucleotide excision repair and homologous recombination, respectively. Furthermore, ERCC1 and Rad51 overexpression induces resistance to DNA-damaging agents that promote DNA double-strand breaks. Accordingly, the aim of this study was to determine the role of ERCC1 and Rad51 in emodin-mediated cytotoxicity in human non-small cell lung cancer (NSCLC) cells. Both ERCC1 and Rad51 protein levels as well as mRNA levels were decreased in four different NSCLC cell lines after exposure to emodin. These decreases correlated with the inactivation of the MKK1/2-ERK1/2 pathway. Moreover, cellular ERCC1 and Rad51 protein and mRNA levels were specifically inhibited by U0126, a MKK1/2 inhibitor. We found that transient transfection of human NSCLC cells with si-ERCC1 or si-Rad51 RNA and cotreatment with U0126 could enhance emodin-induced cytotoxicity. In contrast, overexpression of constitutively active MKK1/2 vectors (MKK1/2-CA) was shown to significantly recover reduced phospho-ERK1/2, ERCC1, and Rad51 protein levels and to rescue cell viability upon emodin treatment. These results demonstrate that activation of the MKK1/2-ERK1/2 pathway is the upstream signal regulating the expressions of ERCC1 and Rad51, which are suppressed by emodin to induce cytotoxicity in NSCLC cells.

1. Introduction

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a naturally occurring anthraquinone present in the roots and rhizomes of numerous plants and Chinese herbs [1,2]. A number of studies have demonstrated that emodin is capable of inducing cell apoptosis and growth arrest in various cancer cells, such as human lung cancer cells [3], HER-2/neu – overexpressed breast cancer cells [4,5], cervical cancer cells, leukemia cells, hepatoma cells, and prostate cancer cell lines [6–8]. In addition, emodin induces sub-G1 accumulation and G2/M phase arrest in hepatoma cells [7]. Emodin, as a tyrosine kinase inhibitor, can inhibit the kinase activity of HER-2/neu and suppress the proliferation of HER-2/neu – overexpressed non-small cell lung cancer (NSCLC) and breast cancer cells [5,9]. Emodin has also been shown to inhibit p56lck [9] and casein kinase 2 (CK2) activity [10]. In addition, emodin can repress epidermal growth factor receptor (EGFR) tyrosine kinase activity at high concentrations [5]. The molecular mechanism by which emodin induces cell apoptosis is through inactivation of ERK and AKT and the decrease of anti-apoptotic protein Bcl2 levels in human A549 cells [3]. However, the effect of emodin on the ability of DNA repair to regulate cell survival in NSCLC cells has not yet been fully defined.

Excision repair cross-complementary 1 (ERCC1) has a leading role in the nucleotide excision repair (NER) process because of its involvement in the excision of DNA adducts [11]. The damaged DNA strand is cleaved by ERCC1-XPF (xeroderma pigmentosum-F) on the 5' side during NER in human cells [12]. ERCC1 RNA levels are highly correlated with NER activity in blood lymphocytes [13]. The increased expression of ERCC1 is associated with clinical resistance to platinum-based chemotherapy in human NSCLC [14]. Moreover, patients with ERCC1-negative tumors had a longer survival time than those with ERCC1-positive tumors in NSCLC [15].

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Homologous recombination repair (HRR), a major pathway for the repair of DNA double-strand breaks (DSBs) in eukaryotic cells, is mediated by Rad51. The HRR process consists of assembly of Rad51 onto DNA substrates at the site of a DNA break to form a helical nucleoprotein filament, which catalyzes homologous pairing and joins in a hetero-duplex formation with the sister chromatid [16]. Rad51 is overexpressed in many tumors [17,18], and its overexpression is related to the resistance of the tumor to chemotherapeutic agents or radiation [19,20]. In NSCLC, high expression of Rad51 in tumor tissue is associated with an unfavorable prognosis [21,22]. On the other hand, inhibition of Rad51 expression has been shown to sensitize cancer cells to radiotherapy and chemotherapeutic agents [23,24]. However, whether Rad51 and ERCC1 are targeted by emodin to induce cell death in human lung cancer cells is still unclear and remains to be determined.

Lung cancer remains the leading cause of cancer-related deaths in the world, and more than 85% of lung cancers have NSCLC [25]. In this study, we investigated the role of emodin in suppressing cell viability in four NSCLC cell lines and examined possible molecular mechanisms for this activity. We also determined the roles of repair proteins ERCC1 and Rad51 in emodin-induced cytotoxicity. Emodin may be a novel and improved therapeutic modality for advanced lung cancer in the future, especially for patients in whom lung cancer cells are resistant to chemotherapeutic agents.

2. Materials and methods

2.1. Cell culture and chemical reagents

Human lung adenocarcinoma H1650 (CRL-5883), human bronchioloalveolar cell carcinoma A549 (CCL-185), lung squamous cell carcinoma H520 (HTB-182), and H1703 (CRL-5889) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and fetal calf serum (10%).

Emodin, cycloheximide, and actinomycin D were purchased from Sigma–Aldrich (St. Louis, MO). MG132, lactacystin, and U0126 were purchased from Calbiochem-Novabiochem (San Diego, CA). Emodin, actinomycin D, MG132, lactacystin, and U0126 were dissolved in dimethyl sulfoxide (DMSO). Cycloheximide was dissolved in MilliQ-purified water (Millipore, Billerica, MA).

2.2. Western blot analysis

After different treatments, equal amounts of proteins from each set of experiments were subjected to western blot analysis, as previously described [23]. Specific phospho-ERK1/2 (Ser217/221) and phospho-MKK1/2 (Thr202/Tyr204) antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies against ERCC1 (FL-297) (sc-10785), Rad51 (H-92) (sc-8349), ERK2 (K-23) (sc-153), and Actin (I-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Small interfering RNA (siRNA) transfection assay

The sense-strand sequences of siRNA duplexes used for ERCC1, Rad51, and scrambled (as a control) were 5′-GGAGCUGGCUAAGAUGUGU-3′, 5′-UGUAGCAUAUGCUCGAGCG-3′, and 5′-GCGCGCUUUGUAGGATTCG-3′ (Dharmacon Research, Lafayette, CO). H1650, A549, H520, or H1703 cells were transfected with siRNA duplexes (200 nM) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 h and then treated with emodin for 24 h.

2.4. Plasmids and treatments

Plasmid expressions of MKK1-CA (a constitutively active form of MKK1, ΔN3/S218E/S222D) and MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) were produced as previously described [23]. Exponentially growing human lung cancer cells (1×10^6) were plated for 18 h before exposure to emodin for 24 h in RPMI-1640 complete medium. To determine the effect of MKK1/2-ERK1/2 signaling on ERCC1 levels, MKK1/2-CA expression vectors were transfected into lung cancer cells using Lipofectamine (Invitrogen) before emodin treatment.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was isolated from cultured cells using TRIzol (Invitrogen), according to the manufacturer's instructions. RT-PCR was performed with 2 μg of total RNA by using random hexamers following the Moloney Murine Leukemia Virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs. ERCC1 was amplified by using the primers with the sequence 5′-CCCTGGGAATTTGGCGACGTAA-3′ (forward) and 5′-CTCCAGGTACCGCCCAGCTTCC-3′ (reverse) in conjunction with a thermal cycling program consisting of 26 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 60 s. The Rad51 primers were 5′-CTTTGGCCCACAACCCATTTC-3′ (forward) and 5′-ATGGCCTTTCCTTCACCTCCAC-3′ (reverse) in conjunction with a thermal cycling program consisting of 26 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 60 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The GAPDH primer was 5′-CTACATGGTTTACATGTTCC-3′ (forward) and 5′-GTGAGCTTCCCGTTCAGCTCA-3′ (reverse). The samples were loaded in triplicate, and the results of each sample were normalized to that of GAPDH.

2.6. Cell viability assay

The cell viability of lung cancer cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the cells (1×10^4) were plated in 96-well cell culture plates with RPMI containing 10% fetal calf serum in a final volume of 0.2 mL. When the cells reached 60% confluence, they were treated with emodin (25–100 μM) for 24 h. Cell survival was assessed by directly adding 100 μL of MTT (500 μg/mL) to the medium. After 3 h, the cells were solubilized for 15 min in DMSO (100 μL/well) on a shaker at room temperature before reading the absorbance at 562 nm with a microplate reader (Biorad Technologies, Hercules, CA).

2.7. Determination of cell death

Cells were treated with emodin for 24 h. In each preparation, cell death was assessed by the trypan blue dye exclusion test; living cells exclude the dye, while dead cells do not. The proportion of dead cells was determined by using a hemocytometer for counting the cells stained with trypan blue.

2.8. Statistical analyses

For each protocol, three or four independent experiments were performed. Results were expressed as the mean ± standard error of the mean (SEM). Statistical calculations were performed using SigmaPlot 2000 (Systat Software, San Jose, CA). Differences in measured variables between experimental and control groups were assessed by using an unpaired *t*-test. $P < 0.05$ was considered statistically significant.

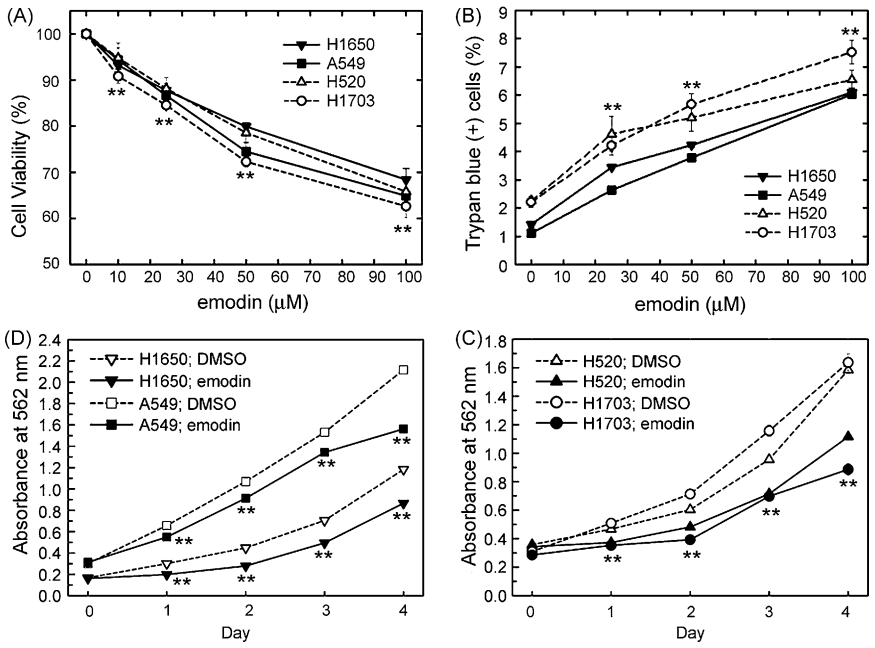

Fig. 1. Dose–response curves of emodin for cell viability in four different NSCLC cell lines. (A) H1650, A549, H520, and H1703 cells were treated with various concentrations of emodin (10–100 μM) for 24 h. Cell viability was determined by MTT assay. (B) After treatment as in (A), both unattached and attached cells were collected and stained with trypan blue dye, and the numbers of dead cells were manually counted in triplicate. Columns, percentage of trypan blue-positive cells representing a population of dead cells; error bar, standard error (SE) from four independent experiments. (C) Cells were treated with emodin (50 μM) for 1–4 days, and cell growth was determined by MTT assay. $^{**}P < 0.01$ using Student's *t*-test for comparison between the cells treated with or without emodin.

3. Results

3.1. Emodin inhibits cell viability in different human lung cancer cell lines

We first examined the cytotoxic effect of emodin on different NSCLC cell lines, including adenocarcinoma (H1650), bronchioloalveolar cell carcinoma (A549), and squamous cell carcinoma (H520 and H1703). As shown in Fig. 1A, emodin induced a dose-dependent decrease of cell viability in all four different NSCLC cell lines, as analyzed by MTT assay. Cell viability in the different lung cancer cell lines was approximately 65% after emodin (100 μM) treatment for 24 h. Moreover, assessment of NSCLC cell death after emodin treatment using the trypan blue exclusion assay also revealed that emodin (25–100 μM) could decrease cell viability (Fig. 1B). The antiproliferative effect of emodin, as examined by MTT assay, is demonstrated in Fig. 1C; emodin potently inhibited cell growth in these four NSCLC cell lines.

3.2. Emodin decreases phosphorylated protein levels of MKK1/2 and ERK1/2

To evaluate the molecular mechanisms of emodin-induced cytotoxicity in different NSCLC cell types, the cell lines were exposed to various concentrations of emodin (25–100 μM) for 24 h. Phosphorylated protein levels of MKK1/2 and ERK1/2 were determined by western blot analysis. In Fig. 2A, emodin dose-dependently suppressed basal protein levels of phosphorylated MKK1/2 and ERK1/2 (Fig. 2A).

3.3. Emodin inhibits the protein and mRNA levels of ERCC1 and Rad51 in human NSCLC cell lines

Previous studies have indicated that immunohistochemical staining patterns for ERCC1 or Rad51 showed no significant relationship with sensitivities to chemotherapy in NSCLC [22,26]. In this study, we wanted to know whether emodin could decrease the expression of ERCC1 and Rad51 to induce a cytotoxic effect in NSCLC cells. Emodin significantly and dose-dependently inhibited the basal protein levels of ERCC1 and Rad51; e.g., 25–100 μM emodin decreased cellular ERCC1 protein levels in H1650 cells to 20–90% of untreated cells (Fig. 2A). To elucidate whether the downregulation of ERCC1 and Rad51 induced by emodin occurs at the transcriptional level, various concentrations of emodin were added to NSCLC cell lines for 24 h. Total RNA was isolated and subjected to RT-PCR analysis for ERCC1 and Rad51, which revealed that both ERCC1 and Rad51 mRNA levels were suppressed by emodin (Fig. 2B).

3.4. Influence of emodin on mRNA and protein stability of ERCC1 and Rad51 in human NSCLC cell lines

To examine whether emodin regulates mRNA of ERCC1 and Rad51 at the posttranscriptional level, actinomycin D (an inhibitor of RNA synthesis) was added during the final 3–9 h when the cells were exposed to emodin. Both ERCC1 and Rad51 mRNA levels additively decreased with time in the presence of actinomycin D and emodin in NSCLC cells, indicating that the mRNA of ERCC1 and Rad51 was unstable upon treatment with emodin (Fig. 2C).

We next measured the protein degradation rate of ERCC1 and Rad51 in four different NSCLC cell lines after treatment with emodin for 0–9 h via cycloheximide (an inhibitor of *de novo* protein synthesis) chase analysis. In Fig. 3A, emodin markedly promoted ERCC1 and Rad51 protein degradation. These results suggested that the mechanism of emodin-induced downregulation of ERCC1 and Rad51 protein levels was through enhancement of mRNA and protein instability.

3.5. Emodin influences the protein stability of ERCC1 and Rad51 through the 26S proteasome

To investigate whether the 26S proteasome was involved in the emodium-induced degradation of ERCC1 and Rad51 proteins, the 26S proteasome inhibitors MG132 and lactacystin were co-added

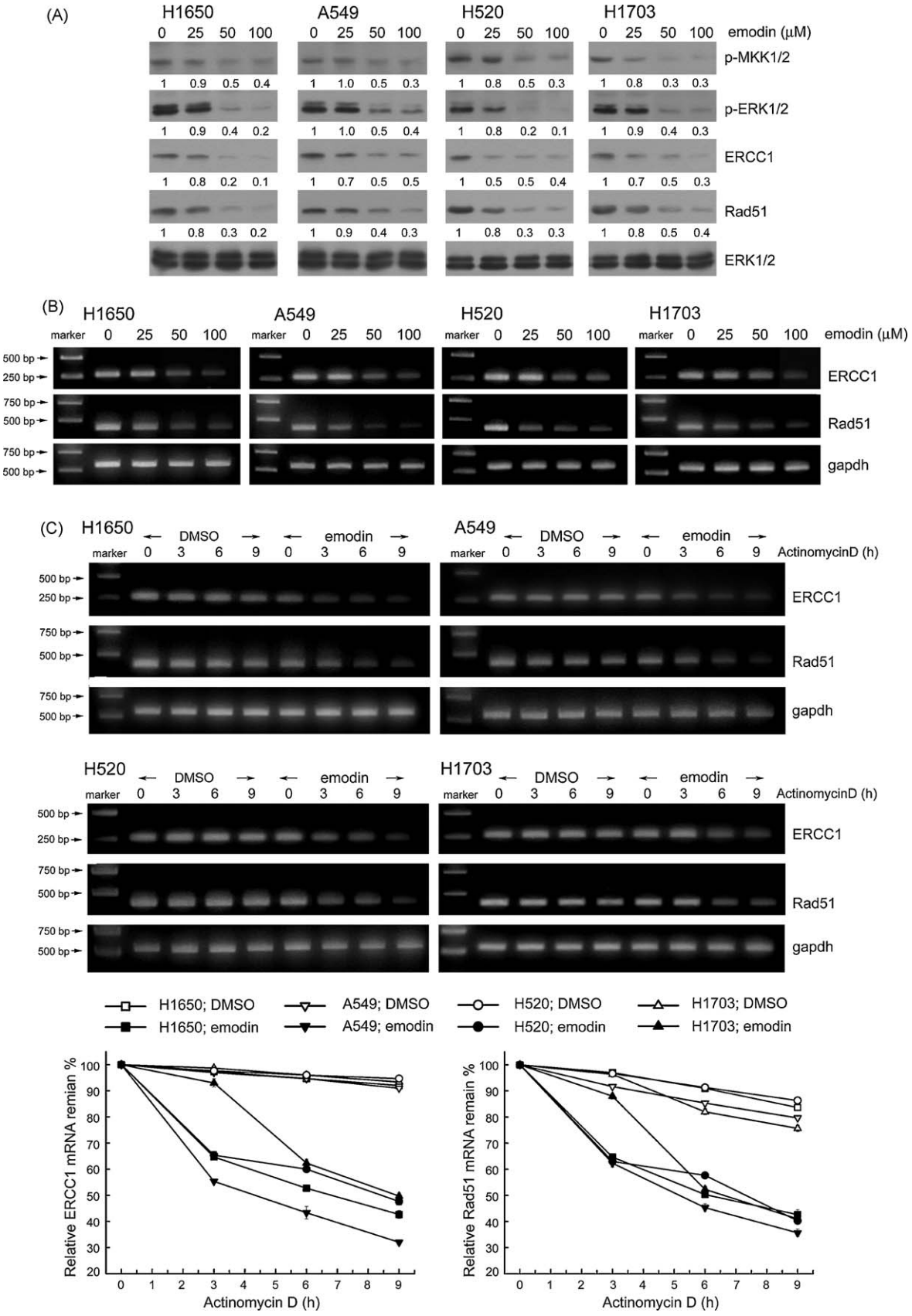

Fig. 2. Emodin decreased the cellular phospho-MKK1/2, phospho-ERK1/2, ERCC1, and Rad51 protein levels. (A) H1650, A549, H520, or H1703 cells were exposed to emodin (25–100 μM) for 24 h. After treatment, cell extracts were examined by western blotting for determination of phospho-MKK1/2, phospho-ERK1/2, ERCC1, Rad51, and ERK1/2 protein levels. (B) Emodin decreased ERCC1 and Rad51 mRNA levels in lung cancer cells. Cells were exposed to various concentration of emodin for 24 h. (C) Cells were exposed to emodin (50 μM) for 9 h, followed by the addition of actinomycin D (2 μg/mL) for 3–9 h. After the treatment, total RNA was isolated and subjected to RT-PCR for ERCC1 and Rad51.

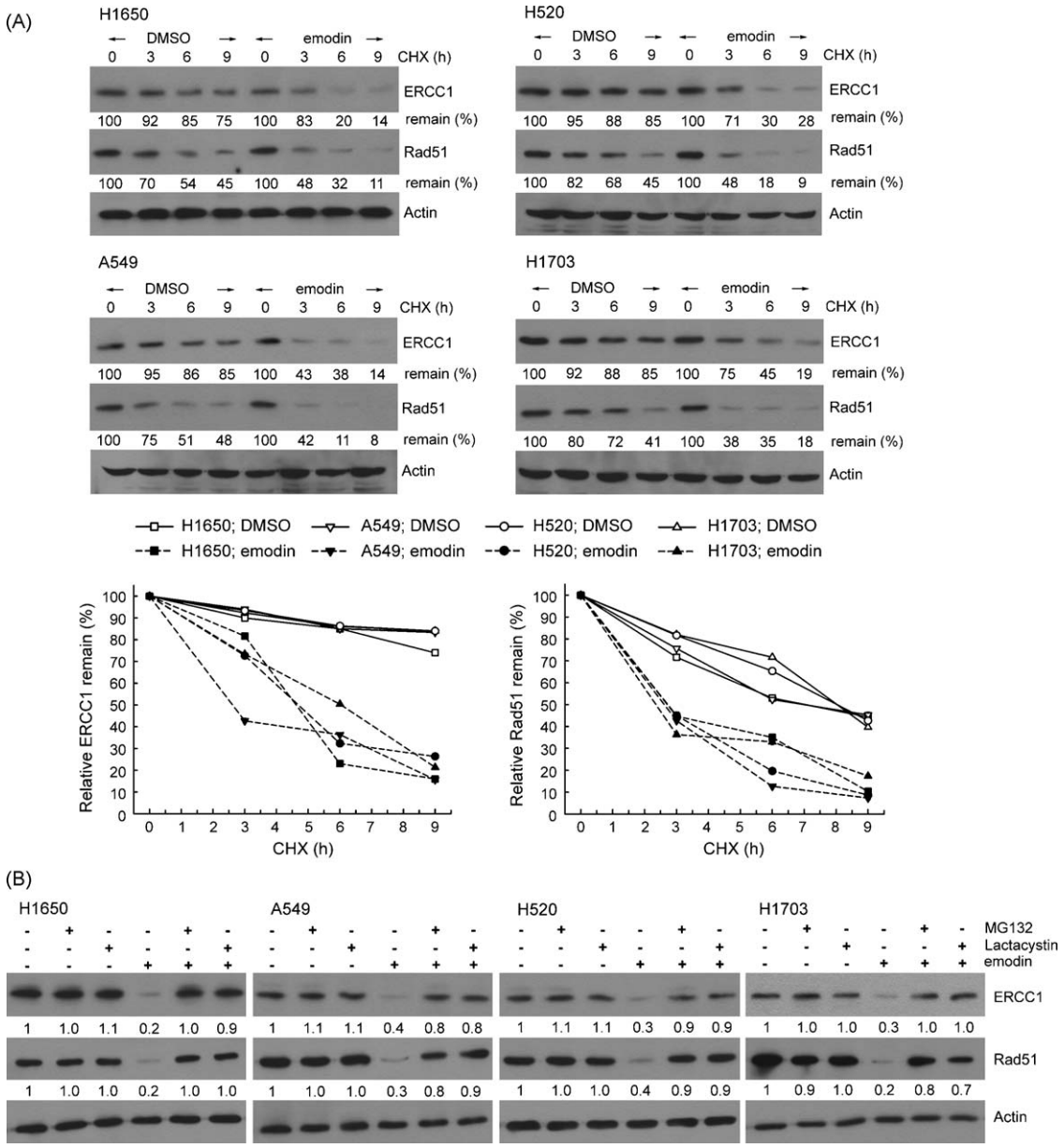

Fig. 3. Emodin decreased cellular ERCC1 and Rad51 protein stability through the 26S proteasome pathway. (A) H1650, A549, H520, and H1703 cells were treated with emodin (50 μM) for 9 h, and then with cycloheximide (50 μg/mL) for 3–9 h. (B) Emodin (50 μM) was added to cells for 18 h, and then the cells were cotreated with MG132 (10 μM) or lactacystin (10 μM) for 6 h. Whole-cell extracts were collected for western blot analysis of ERCC1 and Rad51 protein expression.

with emodin to NSCLC cells. As shown in Fig. 3B, both MG132 and lactacystin restored the ERCC1 and Rad51 protein levels that had been decreased by emodin. These results revealed that 26S proteasome-mediated proteolysis is the mechanism of ERCC1 and Rad51 protein instability enhanced by emodin in NSCLC cells.

3.6. ERK1/2 inactivation is correlated with ERCC1 and Rad51 mRNA and protein downregulation

Our previous studies showed that the regulation of Rad51 mRNA and protein expression was through the MKK1/2-ERK1/2 signaling pathway [27]. In this study, we determined whether the ERK1/2 signaling pathway was also involved in the regulation of ERCC1 RNA and protein expression in NSCLC cells. The four lung cancer cell lines were treated with various concentrations of the MKK1/2 inhibitor U0126 (2.5–10 μM) for 12 h. As shown in Fig. 4A, ERK1/2 phosphorylation and ERCC1 and Rad51 protein levels significantly decreased in U0126-treated NSCLC cells. Moreover, the ERCC1 and Rad51 mRNA levels were also downregulated on treatment with various concentrations of U0126 (Fig. 4B). In addition, the ERCC1 and Rad51 protein stability in U0126-treated cells were analyzed by cycloheximide chase analysis. As shown in Fig. 4C, U0126 treatment markedly decreased the ERCC1 and Rad51 protein stability. Taken together, these data show that the activation of the MKK1/2-ERK1/2 signaling pathway is the upstream signal for maintaining the expression of ERCC1 and Rad51 proteins and mRNA in NSCLC cells.

3.7. Overexpression of the MKK1/2-ERK1/2 signaling pathway enhances the expression of ERCC1 and Rad51 proteins and cell viability after decrease by emodin

To determine whether the ERK1/2 signaling pathway was involved in regulation of ERCC1 and Rad51 protein levels in NSCLC

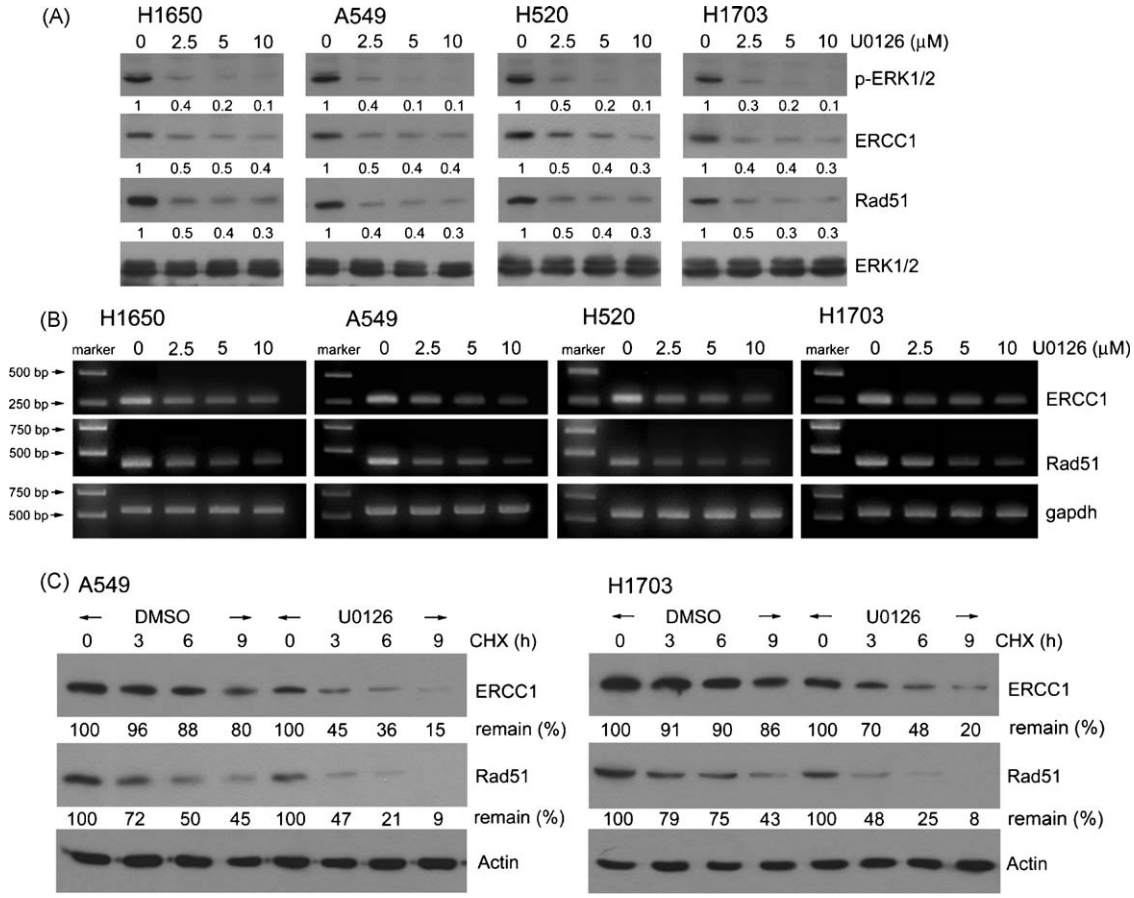

Fig. 4. U0126 (MKK1/2 inhibitor) transfection decreased ERCC1, Rad51 protein, and mRNA levels in NSCLC cells. (A) H1650, A549, H520, or H1703 cells were exposed to U0126 (2.5–10 μM) for 12 h. After treatment, the cell extracts were examined by western blot for determination of phospho-ERK1/2, ERCC1, Rad51, and ERK1/2 protein levels. (B) After treatment as in (A), RNA was isolated and subjected to RT-PCR analysis for ERCC1 and Rad51. (C) A549 and H1703 cells were treated with U0126 (2.5 μM) for 9 h, and followed by the addition of cycloheximide (50 μg/mL) for 3–9 h. Whole-cell extracts were collected for western blot analysis of ERCC1 and Rad51 protein levels.

cells, these cell lines were transiently transfected with plasmids carrying MKK1-CA or MKK2-CA, a constitutively active form of MKK1/2. We found that overexpression of MKK1-CA or MKK2-CA could increase cellular ERK1/2 phosphorylation and ERCC1 and Rad51 protein levels that had been previously decreased by emodin in NSCLC cells (Fig. 5A).

To evaluate the effects of ERK1/2 activation on cell survival suppressed by emodin, the lung cancer cells lines were transfected with MKK1/2-CA vectors, followed by treatment with various concentrations of emodin. Cell viability was assessed by MTT assay. Transfection with MKK1/2-CA vectors enhanced cell survival suppressed by emodin in four different NSCLC cell lines (Fig. 5B).

3.8. MKK1/2 inhibitor enhances cytotoxicity induced by emodin in NSCLC cells

To evaluate the effects of ERK1/2 inactivation on cytotoxicity induced by emodin in NSCLC cells, we examined the influence of U0126 (an MKK1/2 inhibitor) on cell viability inhibited by emodin in each cell line. The cells were treated with U0126 first, followed by treatment with various concentrations of emodin (25–100 μM). As shown in Fig. 5C and D, U0126 markedly enhanced the downregulation of phospho-ERK1/2, ERCC1, and Rad51 expression in H520 and H1703 cells treated with emodin. This indicated that emodin-induced cytotoxicity was correlated with MKK1/2-ERK1/2 inactivation in NSCLC cells.

3.9. Knockdown of ERCC1 or Rad51 enhances cytotoxicity and cell death in NSCLC cells treated with emodin

To determine the effect of ERCC1 or Rad51 downregulation on the augmentation of cytotoxicity in emodin-treated NSCLC cells, ERCC1 or Rad51 was knocked down using specific siRNA duplexes. As shown in Fig. 6A, transfection of si-ERCC1 or si-Rad51 RNA duplex suppressed ERCC1 or Rad51 protein levels without affecting ERK1/2 phosphorylation in emodin-treated H1650 or A549 cells. The cytotoxicity induced by emodin and si-ERCC1 or si-Rad51 RNA transfection in NSCLC cell lines was determined by MTT assay (Fig. 6B). Interestingly, transient knockdown of ERCC1 or Rad51 by siRNA sensitized lung cancer cells to cell death induced by emodin as compared to that by si-scramble RNA transfection (as control) in NSCLC cells (Fig. 6B).

In conclusion, our results are summarized and depicted in the diagram in Fig. 6C, which illustrates the other molecular mechanism involved in emodin-elicited cytotoxicity in NSCLC cells. Emodin decreased the basal levels of phospho-MKK1/2 and phospho-ERK1/2. Furthermore, MKK1/2-ERK1/2 signaling was an upstream signal for ERCC1 and Rad51 expression. Thus, ERCC1 and Rad51 can protect NSCLC cells from cytotoxicity induced by emodin.

4. Discussion

To the best of our knowledge, this study is the first report to elucidate the inhibitory effect and action mechanism of emodin on

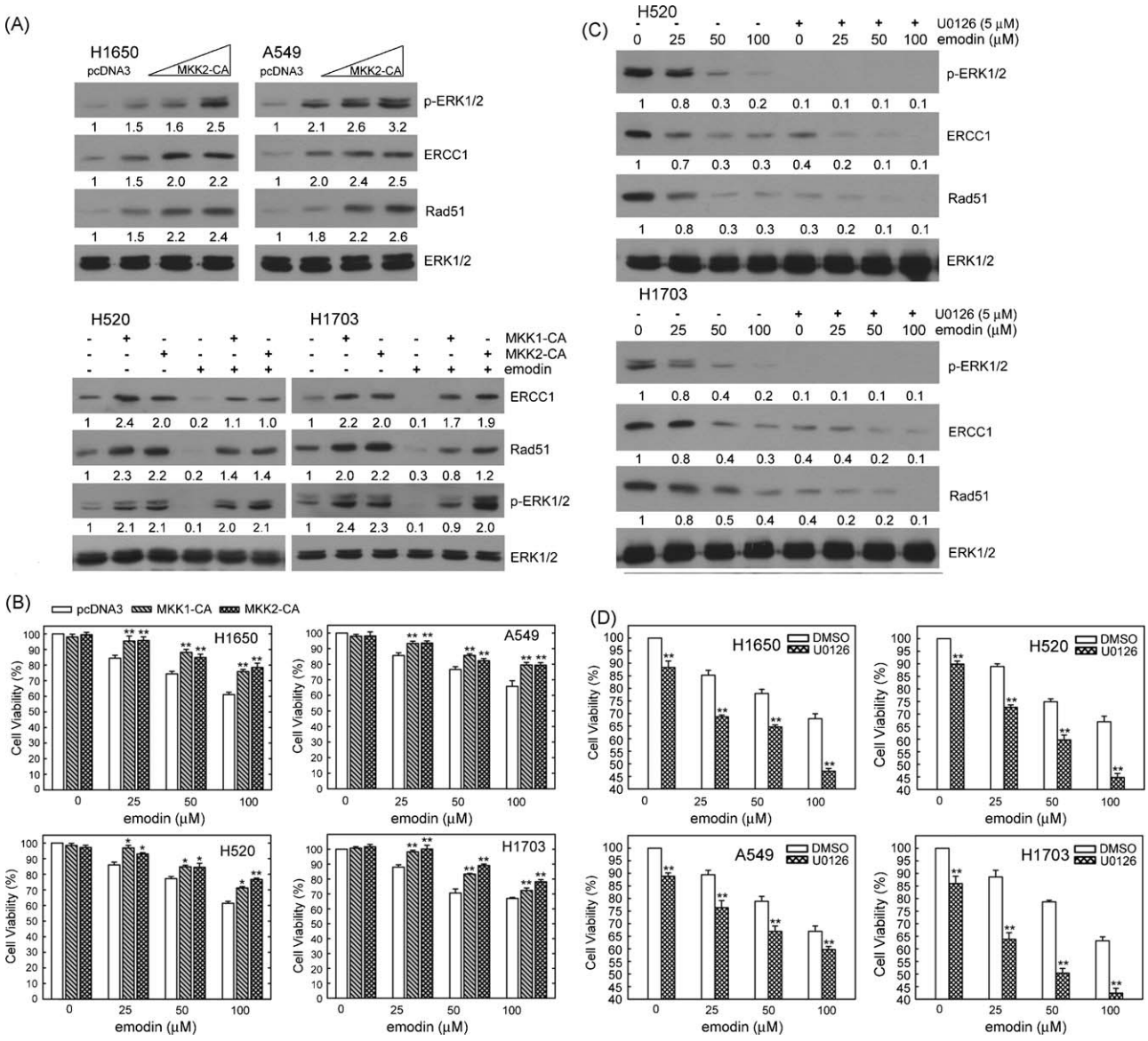

Fig. 5. The influence of the ERK1/2 signaling pathway on ERCC1 and Rad51 protein levels in the cells treated with emodin. (A) pcDNA3 or MKK1-CA (1–5 μg) expression vector was transfected into H1650, A549, H520, or H1703 cells using Lipofectamine. After 24 h expression, cells were treated with emodin (50 μM) for 24 h. Whole-cell extracts were collected for western blot analysis of ERCC1 and Rad51 protein expression. (B) MKK1/2-CA vector- or pcDNA3 vector-transfected cells were treated with emodin (25–100 μM) for 24 h. The cytotoxicity affected by MKK1/2-CA vector transfection in these treatments was determined by MTT assay. The results (mean ± standard error of the mean [SEM]) were obtained from three independent experiments. $^{**}P < 0.01$ using Student's *t*-test for comparisons between the cells transfected with MKK1/2-CA or pcDNA3 vectors. (C) Cells were exposed to U0126 (5 μM), a MKK1/2 inhibitor, and emodin (25–100 μM) for 24 h. Whole-cell extracts were collected for western blot analysis of ERCC1 and Rad51 protein expression. (D) After treatment as in (C), cell viability was determined by MTT assay. $^{**}P < 0.01$ using Student's *t*-test for comparisons between the cells cotreated with emodin and U0126 or emodin alone.

ERCC1 and Rad51 expression. We have demonstrated that emodin-induced cytotoxicity occurs via ERK1/2 inactivation and via ERCC1 and Rad51 downregulation in human lung cancer cells.

Emodin is an anthraquinone derivative from the rhizome of *Rheum palmatum*, an herb widely used in traditional Chinese medicine [28]. Emodin possesses a variety of biological activities, such as those related to immunosuppressive [2], hepatoprotective [29], and antitumor actions [30]. In the present study, we found that emodin could decrease the viability and cellular phospho-MKK1/2 and phospho-ERK1/2 levels in NSCLC cells and have clearly demonstrated that emodin-induced cytotoxicity could be reversed by genetic manipulation of MKK1/2-CA overexpression. Therefore, the inactivation of ERK1/2 induced by emodin was responsible, at least in part, for cell death in lung cancer cells. In accord with our findings, a recent report has shown that emodin could antagonize the signaling pathways ERK and AKT, which are essential for cell survival in A549 lung carcinoma cells [3], and could block the phosphorylation of HER2/neu and ERK in prostate cancer cells [31]. Moreover, emodin has been found to decrease TPA-induced phosphorylation of ERK and JNK, but not p38 kinase [32].

DNA repair capacity has been linked to a genetic predisposition for lung cancer and to treatment outcome in platinum-based chemotherapy [33]. ERCC1 is one of the key enzymes in the NER pathway [34]. Previous studies have shown that high ERCC1 levels in several cancers are associated with resistance to platinum-based treatment [14,35] and that transfection of ERCC1 into an ERCC1-deficient Chinese hamster ovary cell line could restore the repair ability of DNA adducts, conferring platinum-resistance [36]. On the contrary, depletion of ERCC1 by antisense oligonucleotide trans-

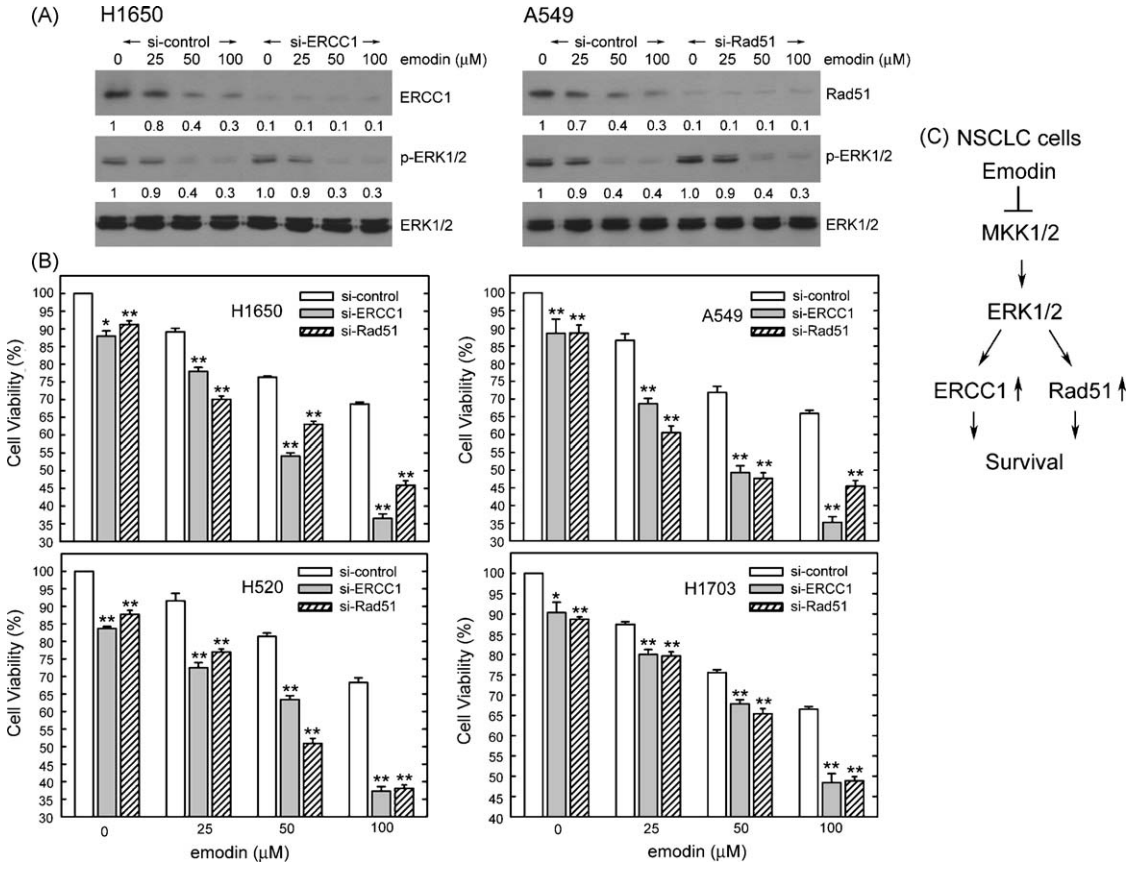

Fig. 6. The influence of ERCC and Rad51 protein levels on cell viability in the cells treated with emodin. (A) NSCLC cells were transfected with siRNA duplexes (200 nM) specific to ERCC1, Rad51, or scrambled (control) in complete medium for 24 h prior to treatment with 25–100 μM emodin for 24 h. Whole-cell extracts were collected for western blot analysis using specific antibodies against ERCC1 or Rad51. (B) After treatment as in A, cytotoxicity was determined by MTT assay. The results (mean ± standard error of the mean [SEM]) were obtained from three independent experiments. $^{**}P < 0.01$ using Student's *t*-test for comparisons between the cells treated with emodin and si-ERCC1/si-Rad51 RNA or si-scramble RNA transfection in lung cancer cells. (C) Schematic of roles of ERCC1 and Rad51 in emodin-elicited cytotoxicity in NSCLC cells.

fection restored sensitivity to cisplatin in the ovarian cancer cell line [37]. In this study, knockdown of ERCC1 expression by specific siRNA transfection enhanced the cytotoxic effect induced by emodin in NSCLC cell lines.

The present study has revealed that inactivation of MKK1/2-ERK1/2 is correlated with protein and mRNA downregulation of ERCC1 and Rad51. Previous studies have indicated that ERCC1 induction depends on the MKK-ERK pathway, which is involved in the activation of transcriptional factor GATA-1 [38]. In NIH3T3 and MCF-7 cells, activated Ras could enhance the transcriptional activity of AP-1 and then increase the expression of ERCC1 [39]. Moreover, the MKK1/2 inhibitor, PD90859, can attenuate EGF-induced ERCC1 protein expression in prostate carcinoma cell lines [40]. In addition, a previous study has shown that radiation-induced ERCC1 and XRCC1 protein expression is also dependent on the ERK1/2 pathway in DU145 prostate carcinoma cells [41]. Similarly, our findings have demonstrated that MKK1/2-ERK1/2 signals participate in maintaining ERCC1 protein expression in human lung cancer cells.

In this study, emodin suppressed NSCLC cell viability through ERK1/2 inactivation. This suppression of cell growth was correlated with significant decreases in ERCC1 and Rad51 protein levels and suppression of ERK1/2 activation. In this study, emodin-induced decrease of ERCC1 and Rad51 protein levels was through downregulation of protein and mRNA stability in NSCLC cell lines (Figs. 2 and 3). In addition, emodin suppressed viability and induced apoptosis of small cell lung cancer NCI-H446 cells and affected the expression of genes involved in apoptosis, tumor metastasis and chemotherapy-resistance, which suggested emodin might have chemopreventive or chemotherapeutic effects for small cell lung cancer [42]. Emodin has also been reported to sensitize different tumor cells, including human esophageal carcinoma cells, cervical cancer cells, and myeloid leukemic cells, to arsenic trioxide [43,44]. Another report revealed that emodin sensitizes cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through suppression of CK2 activity and subsequent NF-κB mediated expression of Bcl-xL and c-FLIP [45].

Rad51 upregulation may play a role in the increased risk of tumorigenesis [18]. It has been suggested that overexpression of Rad51 in nude mice does not result in increased tumor formation; however, cells carrying dominant-negative Rad51 constructs increase tumorigenesis [46]. Thus, Rad51 is likely to control genomic stability in eukaryotic cells exposed to various DNA-damaging agents. Our previous studies have shown that MKK1/2-ERK1/2 signaling maintains the Rad51 protein and mRNA expression levels in NSCLC cells [27]. In this study, we first showed that emodin could decrease Rad51 expression and lung cancer cell viability through inactivation of ERK1/2 and that its action is very similar to that in gefitinib, which is a small molecular tyrosine kinase inhibitor [27]. We also showed that blockage of Rad51 by specific siRNA transfection could enhance emodin-induced cytotoxicity. Overexpression of Rad51 has been shown to be associated with resistance to chemotherapeutic agents in cancer cells [23]. PD-321852 (checkpoint kinase 1 inhibitor) can decrease the Rad51 protein levels and sensitize pancreatic cancer cells to gemcitabine [47]. In this study, exogenous Flag-Rad51 vector transfection could rescue the cytotoxicity in emodin- and/or

MKK1/2 inhibitors (U0126)-treated A549 and H1703 cells (Supplementary Fig. 1). Studies have also revealed that p53 could modulate homologous recombination by transcriptional repression of the *Rad51* gene [48,49], and impaired *Rad51*-repression in mutant p53 proteins may contribute to malignant transformation [49]. Additionally, it has been shown that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is necessary for the basal expression of Rad51 and that *PTEN*-null mouse embryonic fibroblasts exhibit spontaneous DNA double-stranded breaks [50]. Moreover, the decrease of Rad51 gene expression by hypoxia was mediated by the formation of repressive E2F4/p130 complexes at the E2F site in the Rad51 promoter [51]. Therefore, Rad51 plays an important role in the prevention of NSCLC cell death by maintaining DNA integrity and stability.

In summary, our results have revealed that MKK1/2-ERK1/2 inactivation by emodin can decrease the protein levels of DNA repair proteins ERCC1 and Rad51 in human lung carcinoma cells, leading to reduced cell viability. Consequently, emodin can be viewed as a possible new agent for lung cancer treatment. Targeting the ERK1/2 pathway and directly interrupting the downstream expressions of ERCC1 and Rad51 may be another modality in the management of advanced lung cancer in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.09.024.

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