

# A Superoxide-Mediated Mitogen-Activated Protein Kinase Phosphatase-1 Degradation and c-Jun NH<sub>2</sub>-Terminal Kinase Activation Pathway for Luteolin-Induced Lung Cancer Cytotoxicity<sup>[S]</sup>

Lang Bai, Xiuling Xu, Qiong Wang, Shanling Xu, Wei Ju, Xia Wang, Wenshu Chen, Weiyang He, Hong Tang, and Yong Lin

*Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, Albuquerque, New Mexico (L.B., X.X., Q.W., S.X., W.J., W.C. W.H., Y.L); Infectious Disease Center, West China Hospital, Sichuan University, Sichuan, China (L.B., H.T.); and Laboratory of Molecular and Translational Medicine, West China Second University Hospital, Sichuan University, Chengdu, China (Q.W., X.W.)*

Received November 2, 2011; accepted January 5, 2012

## ABSTRACT

Although luteolin is identified as a potential cancer therapeutic and preventive agent because of its potent cancer cell-killing activity, the molecular mechanisms by which its cancer cell cytotoxicity is achieved have not been well elucidated. In this report, luteolin-induced cellular signaling was systematically investigated, and a novel pathway for luteolin's lung cancer killing was identified. The results show that induction of superoxide is an early and crucial step for luteolin-induced apoptotic and nonapoptotic death in lung cancer cells. The c-Jun N-terminal kinase (JNK) was potently activated after superoxide accumulation. Suppression of superoxide completely blocked luteolin-induced JNK activation, which was well correlated to alleviation of luteolin's cytotoxicity. Although luteolin slightly stimulated the JNK-activating kinase mitogen-activated protein

kinase kinase 7, the latter was not dependent on superoxide. We further found that luteolin triggers a superoxide-dependent rapid degradation of the JNK-inactivating phosphatase mitogen-activated protein kinase phosphatase-1 (MKP-1). Introduction of a degradation-resistant MKP-1 mutant effectively attenuated luteolin-induced JNK activation and cytotoxicity, suggesting that inhibition of the JNK suppressor MKP-1 plays a major role in luteolin-induced lung cancer cell death. Taken together, our results unveil a novel pathway consisting of superoxide, MKP-1, and JNK for luteolin's cytotoxicity in lung cancer cells, and manipulation of this pathway could be a useful approach for applying luteolin for lung cancer prevention and therapy.

## Introduction

Lung cancer is a major health concern that affects approximately 160,000 people each year in the United States (Jemal et al., 2010). Because lung cancer is mostly diagnosed at a late disease stage when surgery is not a viable option and

because chemotherapy and radiation therapy are usually ineffective for lung cancer, the prognosis is very poor for many patients (Onn et al., 2004). Therefore, development of effective preventive and therapeutic agents against lung cancer is critical for reducing morbidity and mortality.

The flavonoid luteolin (3',4',5,7-tetrahydroxyflavone) has been suggested as a potential lung cancer chemoprevention and chemotherapy agent (Lin et al., 2008). Luteolin belongs to the flavone class of flavonoids, a large class of polyphenols found ubiquitously in vegetables, fruits, and medicinal plants. In the past two decades, flavonoids have been shown to have antioxidative, antiviral, antitumor, and anti-inflammatory activities (Birt et al., 2001; Ueda et al., 2003; Matsuo

This work was supported in part by the National Institutes of Health National Cancer Institute [Grant R03-CA125796] and Department of Energy Low Dose Radiation Research Program [Grant DE-SC0001173].

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.

<http://dx.doi.org/10.1124/mol.111.076653>.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

**ABBREVIATIONS:** ROS, reactive oxygen species; TNF, tumor necrosis factor; NF- $\kappa$ B, nuclear factor  $\kappa$ B; JNK, c-Jun N-terminal kinase; MKP-1, mitogen-activated protein kinase phosphatase; BHA, butylated hydroxyanisole; z-VAD, N-benzyloxycarbonyl-Val-Ala-Asp(O-Met); NAC, N-acetylcysteine; SP600125, 1,9-pyrazoloanthrone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; ERK, extracellular signal-regulated kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; IKK, I $\kappa$ B kinase complex; Ro 31-9549, 2-[1-3-(aminopropyl)indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; MG132, N-benzyloxycarbonyl (Z)-Leu-Leu-leucinal; MKK, mitogen-activated protein kinase kinase; DHE, dihydroethidium; PCR, polymerase chain reaction; MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKK, mitogen-activated protein kinase kinase; CHX, cycloheximide.

et al., 2005; Lin et al., 2008). Epidemiological studies suggest that dietary flavonoid intake is inversely associated with risk of lung, prostate, stomach, and breast cancer (Le Marchand et al., 2000; Hirvonen et al., 2001; Wright et al., 2004). Because of many confounding factors, the preventive potential of luteolin for lung cancer is not clear (Garcia-Closas et al., 1998; Hirvonen et al., 2001). However, the chemopreventive potential of luteolin has been seen in a 20-methylcholanthrene-induced fibrosarcoma mouse model, and the anticancer activity of luteolin has been well documented (Ko et al., 2002; Lee et al., 2002; Ueda et al., 2003; Osakabe et al., 2004). The antitumor activity of luteolin was attributed to its ability to induce DNA damage, cell cycle arrest, and apoptosis and to suppress angiogenesis and cell survival capacity (Ueda et al., 2003; Ju et al., 2007; Lin et al., 2008; Bai et al., 2009a). As for other flavonoids, luteolin is able to modulate the redox status of the cells. Depending on the cell contexts, luteolin functions as either an antioxidant or a prooxidant (Matsuo et al., 2005; Michels et al., 2005; Ju et al., 2007). Reactive oxygen species (ROS) are a diverse group of reactive, short-lived, oxygen-containing species, such as superoxide and H<sub>2</sub>O<sub>2</sub>. Besides damaging the cellular components by oxidizing DNA, protein, and lipids, ROS also serve as a mediator for cell signaling (Lin et al., 2004; Starkov, 2008; Trachootham et al., 2009). We found that luteolin-induced ROS, specifically superoxide, suppress TNF-induced NF- $\kappa$ B while potentiating JNK activation, which promotes TNF-induced apoptosis in lung cancer cells (Ju et al., 2007).

Although luteolin has been shown to induce and potentiate apoptosis in cancer cells, the precise mechanisms by which luteolin kills lung cancer cells is not well elucidated. Understanding the cell signaling mechanisms of luteolin will undoubtedly facilitate the application of this flavonoid for lung cancer chemoprevention and chemotherapy. In this report, we identify a novel pathway that involves superoxide production, MKP-1 degradation, and JNK activation as the main mechanism for luteolin's cytotoxicity in lung cancer cells. Modulation of this pathway could be a useful approach for applying this agent for lung cancer prevention and therapy.

## Materials and Methods

**Reagents and Antibodies.** Luteolin was from Cayman Chemical (Ann Arbor, MI). Butylated hydroxyanisole (BHA), *N*-acetylcysteine (NAC), necrostatin-1, z-VAD, and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO). The JNK inhibitor 1,9-pyrazoloanthrone (SP600125), p38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580), ERK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126), IKK inhibitor II, protein kinase C inhibitor, 2-[1-3-(aminopropyl)indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl) maleimide (Ro 31-9549), 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole (Gö6976), and proteasome inhibitor *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132) were from Calbiochem (La Jolla, CA). The following antibodies were used for Western blots: anti-phospho-JNK (Invitrogen, Carlsbad, CA), anti-MKP-1, and anti-SKP-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-MKK7 (Abcam, Cambridge MA), anti-JNK1 (BD Biosciences, San Jose, CA), and anti- $\beta$ -tubulin (Sigma-Aldrich). Dihydroethidium (DHE) was purchased from Invitrogen. The pRSV-LacZ plasmid was described previously (Lin et al., 1999). The MKP-1EE plasmid was constructed by PCR-directed mutagenesis using the pLenti6/V5-MKP-1 plasmid as a template (a gift from Dr. C. Chen, Albany Medical College, Albany, NY) to generate a MKP-1 mutant, in which the two serine residues at

positions 359 and 364 were replaced by glutamic acid residues (Liu et al., 2009). The PCR primers were 5'-ttggatcccATGGTCATG-GAAGTGGGCAC-3' and 5'-ttctcgagTCAGCAGCTGGGTTCCGGTCTAATGGGTTCTGAAGGTAGCTCGCGCAC-3'. The PCR products were digested by BamHI/XhoI and cloned into the pcDNA 3.1/HisB vector (Invitrogen), resulting in pcDNA-MKP-1EE. The construct was verified by DNA sequencing.

**Cell Culture, Transfection, and X-Gal Staining.** The human lung cancer cell lines H460 and A549 were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 with 10% fetal bovine serum, 1 mM glutamate, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells grown in 12-well plates were transfected with pcDNA-MKP-1EE with FuGENE HD according to manufacturer's instruction (Roche Diagnostics, Indianapolis, IN). Twenty-four hours after transfection, cells were treated as indicated in each figure legend. For X-gal staining, cells were cotransfected with pRSV-LacZ and pcDNA-MKP-1EE or empty vector with FuGENE HD. Twenty-four hours after transfection, the cells were treated as indicated in each figure legend. Cells were washed with phosphate-buffered saline once and fixed in 1% paraformaldehyde and stained as described (Lin et al., 1999). Cells were visualized and photographed under a microscope.

**Cytotoxicity Assay.** Cytotoxicity was determined using a lactate dehydrogenase (LDH) release-based cytotoxicity detection kit (Promega, Madison, WI). Cells were seeded in 48-well plates at 70 to 80% confluence. After culture overnight, cells were treated as indicated in each figure legend. LDH release was determined, and cell death was calculated as described previously (Wang et al., 2006). To morphologically study cell death, H460 cells were cultured on cover slides and pretreated with luteolin (40  $\mu$ M) for 36 h or remained untreated. Cells were stained with 50  $\mu$ g/ml acridine orange and 50  $\mu$ g/ml ethidium bromide and immediately visualized and photographed under a fluorescent microscope (Chen et al., 2007).

**Western Blot.** Cells were harvested and lysed in M2 buffer (20 mM Tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM  $\beta$ -glycerophosphate, 1 mM sodium vanadate, and 1  $\mu$ g/ml leupeptin). Equal amounts of protein extracts were resolved using 12% SDS-polyacrylamide gel electrophoresis, and the proteins of interest were probed by Western blot and visualized by enhanced chemiluminescence according to the manufacturer's instructions (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) (Bai et al., 2009b).

**Detection of Superoxide.** Cells were seeded in 12-well plates, cultured overnight, and then treated as indicated in each figure legend. Thirty minutes before cells were collected, DHE (5  $\mu$ M) was added. Superoxide was detected by flow cytometry (FACSCalibur; BD Biosciences) and analyzed with the CellQuest program (BD Biosciences) as reported previously (Ju et al., 2007).

**Reverse Transcription-PCR.** Total RNA was extracted with an RNeasy Kit (QIAGEN, Valencia, CA). One microgram of RNA from each sample was used as a template for cDNA synthesis with a reverse transcription kit (Promega). An equal volume of cDNA product was used in the PCR. The primers used were the following: MKP-1, 5'-CCCCGGATC-CAATGGTCATGGAAGTGGGCACC-3' and 5'-CCCCGAATTCGGAGCTGGGTTCCGGTCTGTAATGGGTTCTGAAGGTAGCT-3'; and  $\beta$ -actin, 5'-CCAGCCTTCCTTCTGGGCAT-3' and 5'-AGGAGCAATGATCTTGATCTTATT-3'. The reaction condition was 94°C for 45 s, 55°C for 40 s, and 72°C for 45 s. For MKP-1 and  $\beta$ -actin, the cycles for PCR were 27 and 21, respectively. PCR products were run on 2% agarose gel with 0.5  $\mu$ g/ml ethidium bromide, visualized, and photographed (Bai et al., 2009a).

**Statistical Analysis.** Data are expressed as means  $\pm$  S.D. Statistical significance was examined by one-way analysis of variance pairwise comparison.  $P < 0.05$  was considered statistically significant.

## Results

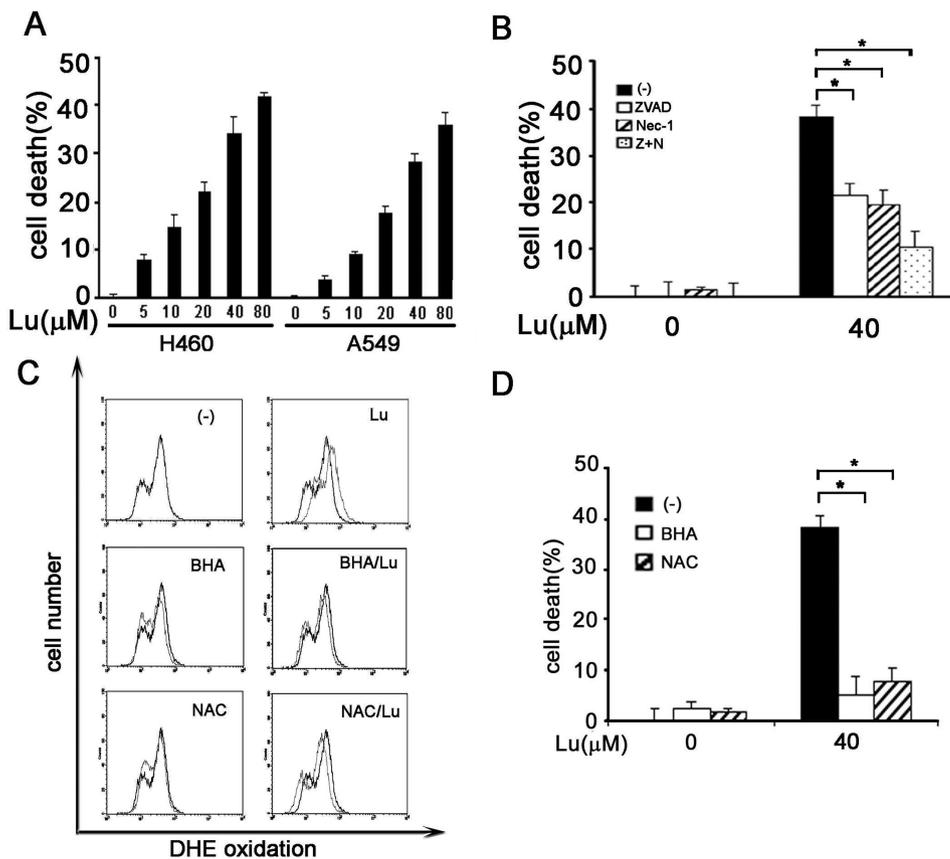
**Luteolin-Induced Cytotoxicity Is Dependent on Superoxide.** Luteolin-induced cytotoxicity in a dose-dependent manner was detected in H460 and A549 cells, confirming its anticancer activity in lung cancer cells (Fig. 1A). The dying and dead cells showed both apoptotic and necrotic morphologies, suggesting that luteolin-induced cancer cell death was associated with both apoptosis and necrosis (Supplemental Fig. 1). Pretreatment of the cells with either the pan-caspase inhibitor z-VAD, which suppresses apoptosis, or the necrosis inhibitor necrostatin-1 effectively suppressed luteolin-induced cytotoxicity, and the combination of these two inhibitors caused a potentiated inhibition of cell death (Fig. 1B). These results confirmed that luteolin is capable of killing lung cancer cells and determined that induction of both apoptosis and necrosis contributes to the anticancer property of luteolin.

Our previous results showed that luteolin induces superoxide, which is pivotal for blocking TNF-induced NF- $\kappa$ B and promoting TNF-induced apoptosis in lung cancer cells (Ju et al., 2007). To investigate whether induction of superoxide contributes to luteolin-induced cancer cell death, ROS scavengers BHA and NAC were used to block luteolin-induced superoxide accumulation. Both reagents potently suppressed luteolin-induced cytotoxicity, which was associated with effective scavenging of luteolin-induced superoxide (Fig. 1, C and D). These results establish that luteolin induces lung cancer cell death through induction of cellular superoxide accumulation.

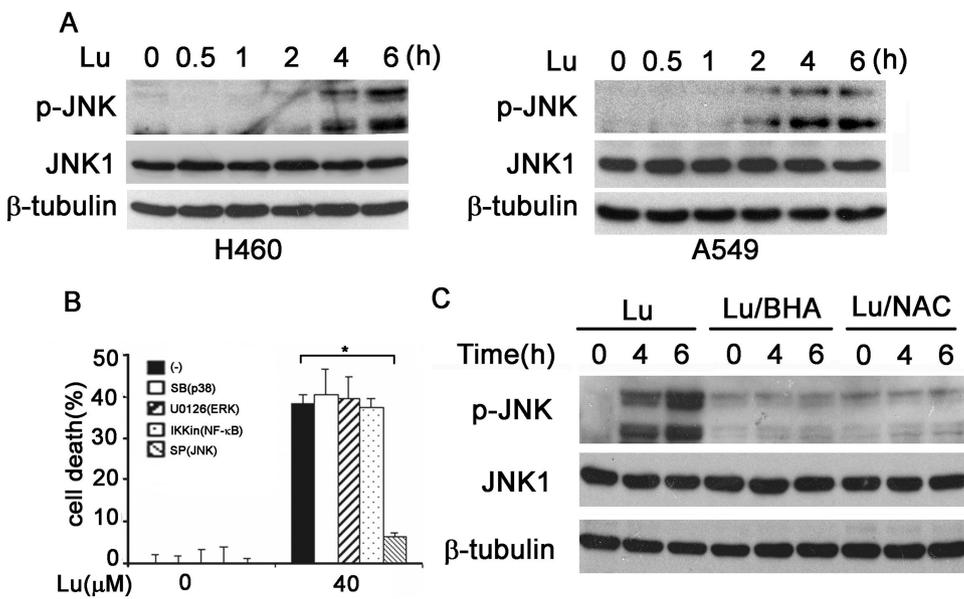
**Superoxide-Dependent JNK Activation Is Required for Luteolin-Induced Cytotoxicity.** Because ROS are po-

tent JNK activators and our previous studies showed that luteolin potentiates TNF-induced lung cancer cell death involving JNK (Ju et al., 2007), we investigated whether luteolin by itself kills lung cancer cells through JNK activation. Indeed, luteolin potently induced JNK activation in both A549 and H460 cells, although with relatively slow kinetics; starting at 2 h after luteolin exposure (Fig. 2A). The pivotal role of JNK in luteolin-induced cytotoxicity was demonstrated by JNK inhibition with the selective JNK inhibitor SP600125, which almost completely blocked luteolin-triggered cell death (Fig. 2B). As controls, the inhibitors for p38 (SB203580), ERK (U0126), and NF- $\kappa$ B (IKK inhibitor II) had no detectable effect on luteolin-induced cytotoxicity (Fig. 2B). Scavenging superoxide with either BHA or NAC completely suppressed luteolin-induced JNK activation, suggesting that luteolin stimulates JNK through superoxide generation (Fig. 2C). These results unveil a pathway involving superoxide and JNK through which luteolin kills lung cancer cells.

**Luteolin Activates JNK through Suppressing MKP-1 Expression.** To investigate the mechanism by which luteolin activates JNK in lung cancer cells, we first investigated whether luteolin activates the MAPKKs and MAPKKs upstream of JNK. Under the conditions that luteolin strongly induced JNK, there was no detectable activation of MAPKKs including mitogen-activated protein kinase kinase 1, apoptosis signal-regulating kinase1, and tousel-like kinase. Likewise, MKK4, the MAPKK that is able to mediate JNK activation, was not stimulated by luteolin either (data not shown). Another MAPKK, MKK7, was moderately activated by luteolin in both A549 and H460 cells (Fig. 3A). However, the ROS scavengers BHA and NAC, which com-



**Fig. 1.** Superoxide is required for luteolin-induced apoptosis and necrosis in lung cancer cells. A, H460 and A549 cells were treated with the indicated concentrations of luteolin (Lu) for 36 h. Cell death was measured by LDH release assay. Data shown are means  $\pm$  S.D. B, H460 cells were pretreated with z-VAD (20  $\mu$ M), Nec-1 (30  $\mu$ M), or z-VAD plus necrostatin-1 (Nec-1) for 1 h, followed by luteolin (40  $\mu$ M) treatment for 36 h. Cell death was measured as indicated in A. \*,  $p < 0.01$ . C, H460 cells were pretreated with BHA (100  $\mu$ M) or NAC (500  $\mu$ M) for 30 min and then treated with luteolin (40  $\mu$ M) for 30 min. Cells were stained with DHE (5  $\mu$ M) for 30 min before collecting. The histogram overlays show the results of treated cells (gray lines) compared with untreated cells (dark lines).  $x$ -axis, fluorescent intensity;  $y$ -axis, cell number. D, H460 cells were pretreated with BHA (100  $\mu$ M) or NAC (500  $\mu$ M) for 30 min, followed by luteolin (40  $\mu$ M) for 36 h. Cell death was measured as indicated in A. \*,  $p < 0.01$ .



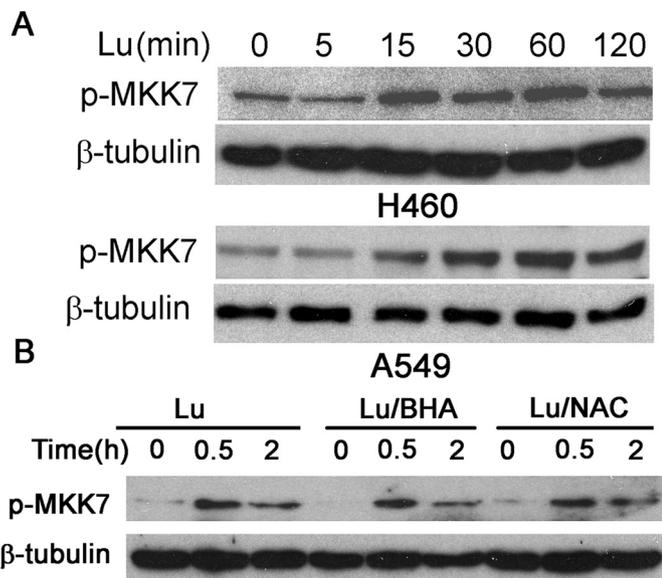
**Fig. 2.** Luteolin (Lu)-induced and superoxide-dependent JNK activation is essential for luteolin-induced cytotoxicity in cancer cells. **A**, H460 and A549 cells were treated with luteolin (40  $\mu$ M) for the indicated times. Phospho (p)-JNK and JNK1 proteins were detected by Western blot.  $\beta$ -Tubulin was detected as an input control. **B**, H460 cells were pretreated with SB203580 (SB; 10  $\mu$ M), U0126 (10  $\mu$ M), IKK inhibitor II (IKKIn; 10  $\mu$ M), and SP600125 (SP; 20  $\mu$ M) for 1 h followed by luteolin (40  $\mu$ M) treatment for 36 h or left untreated. Cell death was measured by a LDH release assay. **C**, H460 cells were pretreated with BHA (100  $\mu$ M) or NAC (500  $\mu$ M) for 30 min, followed by luteolin (40  $\mu$ M) treatment for the indicated times. Phospho-JNK and JNK1 proteins were detected by Western blot.  $\beta$ -Tubulin was detected as an input control.

pletely blocked luteolin-induced JNK activation (Fig. 2C), had no effect on luteolin-stimulated MKK7 activation (Fig. 3B). These results suggest that MKK7 has a subtle contribution to luteolin-induced JNK activation in lung cancer cells, and luteolin stimulation of JNK is unlikely through direct stimulation of the MAPKKK-MAPKK-JNK cascade.

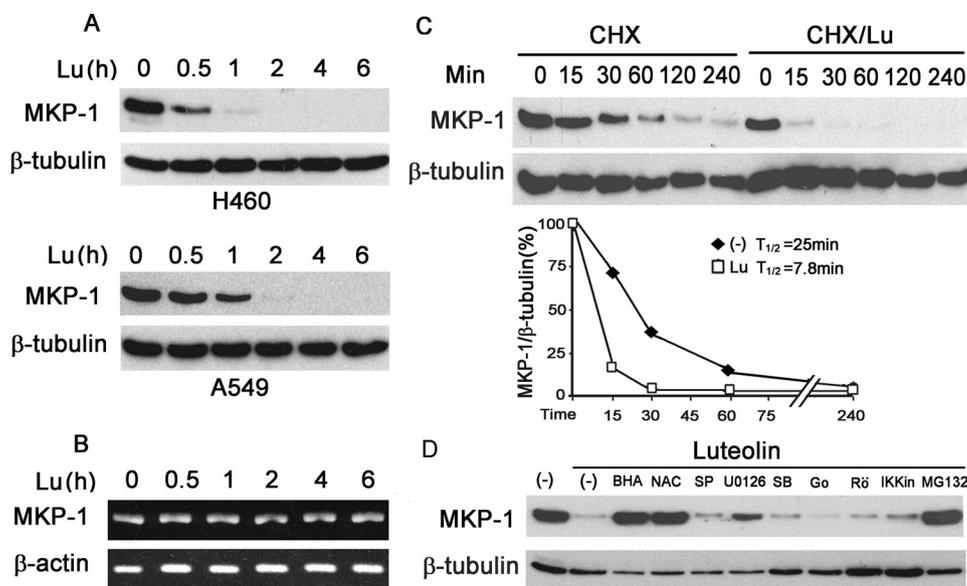
We next investigated whether the JNK-inactivating mechanism is interfered with by luteolin. Because MKP-1 is the main phosphatase that inactivates JNK and is implicated in the resistance of lung cancer to therapy (Wu, 2007), we examined the effect of luteolin on MKP-1 protein expression in lung cancer cells. The MKP-1 protein levels rapidly decreased in luteolin-exposed A549 and H460 cells (Fig. 4A). It is remarkable that the MKP-1 decrease preceded JNK activation in both A549 and H460 cells. MKP-1 protein declined at 30 min and diminished at 1 h (Fig. 4A), whereas JNK was activated at 2 h (Fig. 2A) in H460 cells. A similar trend was also clearly seen in A549 cells, in which MKP-1 was reduced at 1 h and diminished at 2 h, whereas JNK activation began at 2 h (Figs. 2A and 4A). These results strongly suggest that suppression of MKP-1 contributes to luteolin-induced JNK activation.

**Luteolin Suppresses MKP-1 Expression through Destabilizing the MKP-1 Protein.** To address the mechanism by which luteolin induces MKP-1 down-regulation, we first checked mRNA expression levels in luteolin-treated cells. As shown in Fig. 4B, there was no detectable effect of luteolin on MKP-1 mRNA levels, suggesting that it is unlikely that luteolin regulates MKP-1 expression via an effect on transcription or RNA stability. Then we examined protein stability by shutting off protein synthesis with cycloheximide (CHX) and examining the MKP-1 levels at different time points. In A549 cells, after CHX treatment, the MKP-1 level declined and showed a half-life of 25 min. However, when the cells were treated with CHX combined with luteolin, the MKP-1 levels decreased much faster, showing a half-life of 7.8 min, which suggests that luteolin suppresses MKP-1 expression through destabilization of the MKP-1 protein (Fig. 4, C and D).

**Luteolin Triggers Superoxide-Mediated Proteasomal Degradation of MKP-1 Protein.** The above results suggest that MKP-1 protein degradation accounts for the major mechanism underlying luteolin-induced MKP-1 down-regulation. Then we investigated whether MKP-1 down-regulation is mediated by superoxide. Indeed, the ROS scavengers BHA and NAC completely blocked the effect of luteolin on MKP-1 expression, strongly suggesting that ROS are the main mediators for luteolin-induced MKP-1 degradation (Fig. 4D). In contrast, the inhibitors for ERK (U0126), p38 (SB203580), JNK (SP600125), protein kinase C (Ro 31-9549 and Gö6976), and NF- $\kappa$ B (IKK inhibitor II) had a marginal effect on luteolin-induced MKP-1 decrease, suggesting that the corresponding signaling pathways play a minor role, if any, in this effect of luteolin (Fig. 4D). All the inhibitors are effective in blocking their respective pathways (Li et al., 2011) (data not shown). Together with the



**Fig. 3.** Superoxide-independent MKK7 activation induced by luteolin (Lu). **A**, H460 and A549 cells were treated with luteolin (40  $\mu$ M) for the indicated times. Phospho (p)-MKK7 was detected by Western blot.  $\beta$ -Tubulin was detected as an input control. **B**, H460 cells were pretreated with BHA (100  $\mu$ M) or NAC (500  $\mu$ M) for 30 min, followed by luteolin (40  $\mu$ M) treatment for the indicated times. Phospho-MKK7 was detected by Western blot.  $\beta$ -Tubulin was detected as an input control.



**Fig. 4.** Luteolin (Lu)-induced and superoxide-dependent proteosomal MKP-1 degradation. A, H460 and A549 cells were treated with luteolin (40  $\mu$ M) for the indicated times. MKP-1 was detected by Western blot.  $\beta$ -Tubulin was detected as an input control. B, H460 and A549 cells were treated with luteolin (40  $\mu$ M) for the indicated times. MKP-1 mRNA was detected by reverse transcription-PCR.  $\beta$ -Actin was detected as an input control. C, top, H460 cells were pretreated with cycloheximide (CHX, 10  $\mu$ g/ml) and then with luteolin (40  $\mu$ M) for the indicated times. MKP-1 was detected by Western blot.  $\beta$ -Tubulin was detected as an input control. Bottom, quantification of the band densities and normalized with that of  $\beta$ -tubulin. D, H460 cells were pretreated with BHA (100  $\mu$ M), NAC (500  $\mu$ M), SP600125 (SP; 20  $\mu$ M), U0126 (10  $\mu$ M), SB203580 (SB; 10  $\mu$ M), G66976 (Go; 10  $\mu$ M), Ro 31-9549 (Ro; 10  $\mu$ M), IKK inhibitor II (IKKIn; 10  $\mu$ M), and MG132 (10  $\mu$ M) for 1 h, followed by luteolin (40  $\mu$ M) treatment for 3 h. MKP-1 was detected by Western blot.  $\beta$ -Tubulin was detected as an input control.

results that luteolin induced superoxide accumulation and BHA and NAC completely blocked luteolin-induced JNK activation (Figs. 1D and 2C), these findings strongly suggest that luteolin stimulates JNK activation predominantly through superoxide-mediated degradation of MKP-1. The pretreatment with the proteasome inhibitor MG132 fully restored MKP-1 expression in luteolin-treated cells (Fig. 4D), suggesting that the MKP-1 protein is degraded in the proteasome. Taken together, these results suggest that luteolin triggers superoxide-mediated proteasomal degradation of MKP-1 protein.

**Overexpression of a Degradation-Resistant MKP-1 Mutant Suppresses Luteolin-Induced Cytotoxicity in Lung Cancer Cells.** To further validate the role of MKP-1 in luteolin-induced cytotoxicity in lung cancer cells, a MKP-1 mutant that is resistant to proteasomal degradation was constructed and used for rescuing luteolin-induced cell death in A549 cells. To this end, we substituted the serine residues at positions 359 and 364, which are critical for ERK-mediated stabilization (Brondello et al., 1999; Liu et al., 2009), with two glutamic acidic residues to create MKP-1EE to mimic the phosphorylated conformation. The replacement of serine with glutamic acidic residues is a well accepted approach to mimic a phosphorylated conformation of a protein (Puri et al., 2000). The MKP-1EE protein was confirmed to be much more stable than the endogenous wild-type MKP-1 in luteolin-treated cells (Fig. 5A). Overexpression of MKP-1EE significantly suppressed luteolin-induced JNK activation (Fig. 5B), which was well correlated to effective attenuation of luteolin-induced cell death (Fig. 5C). These results strongly substantiate the conclusion that MKP-1 degradation is the main mechanism by which luteolin activates JNK and kills lung cancer cells.

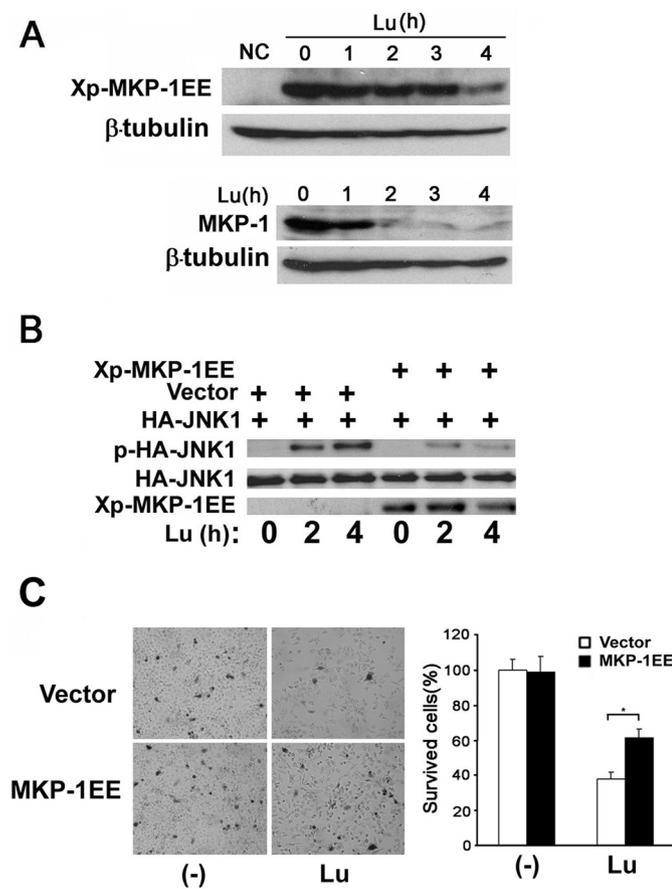
## Discussion

In an attempt to explore the mechanisms for the cancer prevention and therapy potential of luteolin, we have systematically examined the main pathways that are involved in luteolin-induced cancer cell cytotoxicity. We found that luteolin-induced cytotoxicity (apoptosis and necrosis) in lung cancer cells is associated with induction of superoxide, and

the subsequent activation of JNK and the rapid MKP-1 degradation triggered by superoxide underlie the main mechanism of JNK activation. Thus, our results identify a novel luteolin-activated pathway consisting of superoxide, MKP-1, and JNK for lung cancer cell death (Fig. 6), and manipulation of this pathway could be a useful approach for applying luteolin for lung cancer prevention and therapy.

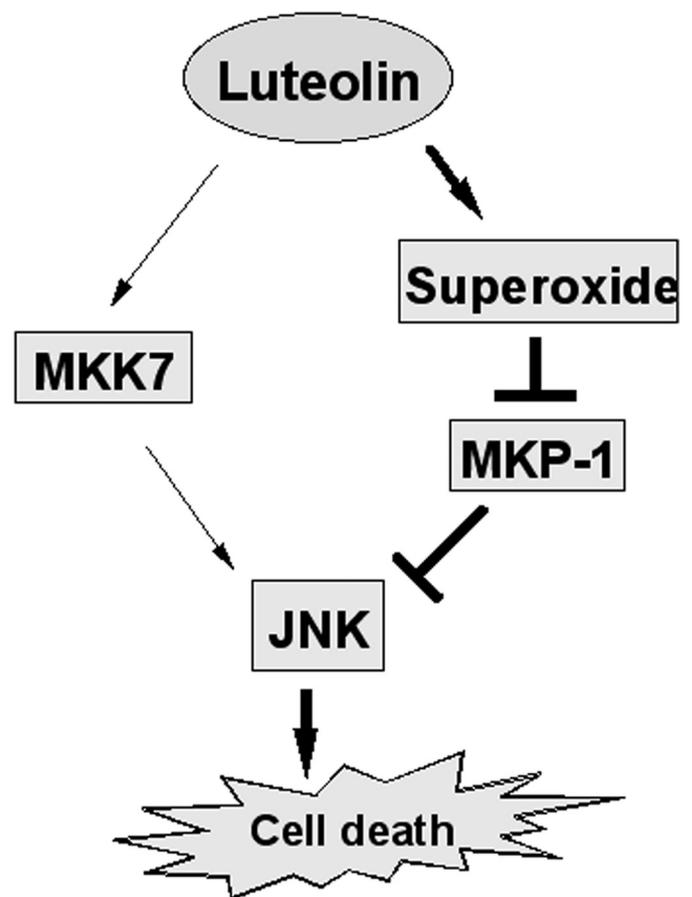
Luteolin-induced cytotoxicity in lung cancer cells is associated with both apoptosis and necrosis, which was demonstrated by morphological (acridine orange/ethidium bromide staining, Supplemental Fig. 1), pharmacological (chemical inhibitors, Fig. 1B), and biochemical (caspase activation, data not shown) experiments. Removal of superoxide by ROS scavengers BHA or NAC dramatically protected cells against luteolin-induced cell death, clearly showing that the pro-oxidative but not the antioxidative property of luteolin is involved in lung cancer cell death. Indeed, luteolin's antioxidative activity mainly underlies the mechanisms of its cytoprotective effect in normal tissues (Lin et al., 2008; López-Lázaro, 2009). This finding is not surprising, because under certain conditions many polyphenols act as either pro-oxidants or antioxidants. For example, quercetin is an antioxidant for brain cell survival, whereas it is a pro-oxidant for cancer cell death (Thangasamy et al., 2007; De Marchi et al., 2009). Other reports provided clear evidence for luteolin's pro-oxidant property (Matsuo et al., 2005; Ju et al., 2007). In particular, we found that luteolin induces superoxide accumulation to block TNF-induced NF- $\kappa$ B activation and potentiate apoptosis (Ju et al., 2007).

Our results further reveal that JNK plays a key role downstream of superoxide in mediating luteolin's cytotoxicity. As a main mitogen-activated protein kinase in response to a variety of extracellular stimulations and intracellular stresses, JNK is usually activated through the activation of a MAPKKK-MAPKK-JNK kinase cascade (Lin and Dibling, 2002). In this study, we did not detect any effect of luteolin on apoptosis signal-regulating kinase 1, mitogen-activated protein kinase kinase kinase 1, and mixed-lineage kinase, the MAPKKKs that are involved in ROS-mediated JNK activation (data not shown). Although we detected a moderate



**Fig. 5.** Overexpression of a degradation-resistant MKP-1 mutant protects cancer cells from luteolin (Lu)-induced cell death. **A**, top, A549 cells were transfected with pcDNA-MKP-1EE. Twenty-four hours after transfection, cells were treated with luteolin (40  $\mu$ M) for the indicated times. MKP-1 was detected by Western blot.  $\beta$ -Tubulin was detected as an input control. Bottom, A549 cells treated with luteolin (40  $\mu$ M) for the indicated times. MKP-1 was detected by Western blot.  $\beta$ -Tubulin was detected as an input control. **B**, A549 cells were cotransfected with HA-JNK1 and pcDNA-MKP-1EE or empty vector (pcDNA3.1B). Twenty-four hours after transfection, the cells were treated with or without luteolin (40  $\mu$ M) for the indicated times. Phospho (p)-JNK1, HA-JNK1, and MKP-1EE proteins were detected by Western blot with anti-phospho-JNK, -HA and -Xpress (Xp), respectively. **C**, A549 cells were cotransfected in duplicate with pRSV-lacZ and pcDNA-MKP-1EE or empty vector (pcDNA3.1B). Twenty-four hours after transfection, the cells were treated with or without luteolin (40  $\mu$ M) for 36 h and then were fixed and stained with X-gal. Top, cells were photographed under a light microscope (100 $\times$ ). Bottom, quantification of cell survival. Blue cells from nine randomly selected fields were counted. The total cell counts in nontreated groups were regarded as 100%. The percentages of cell counts in the luteolin-treated group related to the control groups show cell survival rates after luteolin challenge. \*,  $p < 0.01$ .

activation of MKK7, the JNK-activating MAPKK, it appears that the JNK activation cascade contributes subtly to luteolin-induced JNK activation. This view is supported by the fact that scavenging superoxide completely blocked luteolin-induced JNK activation but had an undetectable effect on MKK7 activation in lung cancer cell lines. Instead, we determined that luteolin causes an early and dramatic reduction of the main JNK-inactivating phosphatase MKP-1 preceding JNK activation. The reduction in MKP-1 is mediated by superoxide, because removal of superoxide with BHA and NAC completely blocked luteolin-induced MKP-1 reduction. Luteolin did not affect MKP-1 mRNA expression, whereas the proteasome inhibitor MG132 fully restored MKP-1 pro-



**Fig. 6.** A model of luteolin-induced cell death in lung cancer cells. Whereas luteolin activates MKK7, ROS-mediated suppression of MKP-1 underlies the main mechanism for luteolin-induced JNK activation and cytotoxicity.

tein expression in luteolin-treated cells, suggesting that transcription of the MKP-1 gene is probably not involved in the inhibiting effect of luteolin on MKP-1, whereas enhancement of proteasomal degradation underlies the main mechanism of this function of luteolin. Of importance, the introduction of a degradation-refractory MKP-1 mutant effectively suppressed luteolin-induced cell death. Although the defined mechanism by which luteolin induces MKP-1 degradation requires further investigation, the results from this study establish a novel pathway that involves superoxide, MKP-1, and JNK in luteolin-induced cytotoxicity in lung cancer cells. It is worthy noting that MKP-1 has been suggested to play an important role in cancer cell survival, inhibition of which is implicated in cancer prevention and chemosensitization (Liao et al., 2003; Chen et al., 2005; Wang et al., 2007; Wu, 2007).

In summary, our results unveil a novel pathway consisting of superoxide, MKP-1, and JNK for luteolin's cytotoxicity in lung cancer cells. This property of luteolin renders it a candidate not only for lung cancer therapy but also for lung cancer chemoprevention, which warrants further studies in animal models.

#### Acknowledgments

We thank Dr. Ceshi Chen from Albany Medical College for providing the pLenti6/V5-MKP-1 plasmid.

## Authorship Contributions

Participated in research design: Tang and Lin.

Conducted experiments: Bai, X. Xu, Q. Wang, S. Xu, Ju, X. Wang, Chen, and He.

Performed data analysis: Bai and Lin.

Wrote or contributed to the writing of the manuscript: Bai and Lin.

## References

- Bai L, Chen W, Wang X, Ju W, Xu S, and Lin Y (2009a) Attenuating Smac mimetic compound 3-induced NF- $\kappa$ B activation by luteolin leads to synergistic cytotoxicity in cancer cells. *J Cell Biochem* **108**:1125–1131.
- Bai L, Chen W, Chen W, Wang X, Tang H, and Lin Y (2009b) IKK $\beta$ -mediated nuclear factor- $\kappa$ B activation attenuates smac mimetic-induced apoptosis in cancer cells. *Mol Cancer Ther* **8**:1636–1645.
- Birt DF, Hendrich S, and Wang W (2001) Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol Ther* **90**:157–177.
- Brondello JM, Pouyssegur J, and McKenzie FR (1999) Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science* **286**:2514–2517.
- Chen W, Wang X, Zhuang J, Zhang L, and Lin Y (2007) Induction of death receptor 5 and suppression of survivin contribute to sensitization of TRAIL-induced cytotoxicity by quercetin in non-small cell lung cancer cells. *Carcinogenesis* **28**:2114–2121.
- Chen YW, Huang SC, Lin-Shiau SY, and Lin JK (2005) Bowman-Birk inhibitor abates proteasome function and suppresses the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1. *Carcinogenesis* **26**:1296–1306.
- De Marchi U, Biasutto L, Garbisa S, Toninello A, and Zoratti M (2009) Quercetin can act either as an inhibitor or an inducer of the mitochondrial permeability transition pore: a demonstration of the ambivalent redox character of polyphenols. *Biochim Biophys Acta* **1787**:1425–1432.
- Garcia-Closas R, Agudo A, Gonzalez CA, and Riboli E (1998) Intake of specific carotenoids and flavonoids and the risk of lung cancer in women in Barcelona, Spain. *Nutr Cancer* **32**:154–158.
- Hirvonen T, Virtamo J, Korhonen P, Albanes D, and Pietinen P (2001) Flavonol and flavone intake and the risk of cancer in male smokers (Finland). *Cancer Causes Control* **12**:789–796.
- Jemal A, Siegel R, Xu J, and Ward E (2010) Cancer statistics, 2010. *CA Cancer J Clin* **60**:277–300.
- Ju W, Wang X, Shi H, Chen W, Belinsky SA, and Lin Y (2007) A critical role of luteolin-induced reactive oxygen species in blockage of tumor necrosis factor-activated nuclear factor- $\kappa$ B pathway and sensitization of apoptosis in lung cancer cells. *Mol Pharmacol* **71**:1381–1388.
- Ko WG, Kang TH, Lee SJ, Kim YC, and Lee BH (2002) Effects of luteolin on the inhibition of proliferation and induction of apoptosis in human myeloid leukaemia cells. *Phytother Res* **16**:295–298.
- Le Marchand L, Murphy SP, Hankin JH, Wilkens LR, and Kolonel LN (2000) Intake of flavonoids and lung cancer. *J Natl Cancer Inst* **92**:154–160.
- Lee LT, Huang YT, Hwang JJ, Lee PP, Ke FC, Nair MP, Kanadaswam C, and Lee MT (2002) Blockade of the epidermal growth factor receptor tyrosine kinase activity by quercetin and luteolin leads to growth inhibition and apoptosis of pancreatic tumor cells. *Anticancer Res* **22**:1615–1627.
- Li Z, Xu X, Bai L, Chen W, and Lin Y (2011) Epidermal growth factor receptor-mediated tissue transglutaminase overexpression couples acquired tumor necrosis factor-related apoptosis-inducing ligand resistance and migration through c-FLIP and MMP-9 proteins in lung cancer cells. *J Biol Chem* **286**:21164–21172.
- Liao Q, Guo J, Kleeff J, Zimmermann A, Büchler MW, Korc M, and Friess H (2003) Down-regulation of the dual-specificity phosphatase MKP-1 suppresses tumorigenicity of pancreatic cancer cells. *Gastroenterology* **124**:1830–1845.
- Lin A and Dibling B (2002) The true face of JNK activation in apoptosis. *Aging Cell* **1**:112–116.
- Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS, Tran JH, Nedospasov SA, and Liu ZG (2004) Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *J Biol Chem* **279**:10822–10828.
- Lin Y, Devin A, Rodriguez Y, and Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev* **13**:2514–2526.
- Lin Y, Shi R, Wang X, and Shen HM (2008) Luteolin, a flavonoid with potential for cancer prevention and therapy. *Curr Cancer Drug Targets* **8**:634–646.
- Liu R, Zheng HQ, Zhou Z, Dong JT, and Chen C (2009) KLF5 promotes breast cell survival partially through fibroblast growth factor-binding protein 1-pERK-mediated dual specificity MKP-1 protein phosphorylation and stabilization. *J Biol Chem* **284**:16791–16798.
- López-Lázaro M (2009) Distribution and biological activities of the flavonoid luteolin. *Mini Rev Med Chem* **9**:31–59.
- Matsuo M, Sasaki N, Saga K, and Kaneko T (2005) Cytotoxicity of flavonoids toward cultured normal human cells. *Biol Pharm Bull* **28**:253–259.
- Michels G, Wätjen W, Niering P, Steffan B, Thi QH, Chovolou Y, Kampkötter A, Bast A, Proksch P, and Kahl R (2005) Pro-apoptotic effects of the flavonoid luteolin in rat H4IIE cells. *Toxicology* **206**:337–348.
- Onn A, Tsuboi M, and Thatcher N (2004) Treatment of non-small-cell lung cancer: a perspective on the recent advances and the experience with gefitinib. *Br J Cancer* **91** (Suppl 2):S11–S17.
- Osakabe N, Yasuda A, Natsume M, and Yoshikawa T (2004) Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of *Perilla frutescens* extract in the murine two-stage skin model. *Carcinogenesis* **25**:549–557.
- Puri PL, Wu Z, Zhang P, Wood LD, Bhakta KS, Han J, Feramisco JR, Karin M, and Wang JY (2000) Induction of terminal differentiation by constitutive activation of p38 MAP kinase in human rhabdomyosarcoma cells. *Genes Dev* **14**:574–584.
- Starkov AA (2008) The role of mitochondria in reactive oxygen species metabolism and signaling. *Ann NY Acad Sci* **1147**:37–52.
- Thangasamy T, Sittadjody S, Lanza-Jacoby S, Wachsberger PR, Limesand KH, and Burd R (2007) Quercetin selectively inhibits bioreduction and enhances apoptosis in melanoma cells that overexpress tyrosinase. *Nutr Cancer* **59**:258–268.
- Trachootham D, Alexandre J, and Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov* **8**:579–591.
- Ueda H, Yamazaki C, and Yamazaki M (2003) Inhibitory effect of *Perilla* leaf extract and luteolin on mouse skin tumor promotion. *Biol Pharm Bull* **26**:560–563.
- Wang J, Zhou JY, and Wu GS (2007) ERK-dependent MKP-1-mediated cisplatin resistance in human ovarian cancer cells. *Cancer Res* **67**:11933–11941.
- Wang X, Ju W, Renouard J, Aden J, Belinsky SA, and Lin Y (2006) 17-Allylamino-17-demethoxygeldanamycin synergistically potentiates tumor necrosis factor-induced lung cancer cell death by blocking the nuclear factor- $\kappa$ B pathway. *Cancer Res* **66**:1089–1095.
- Wright ME, Mayne ST, Stolzenberg-Solomon RZ, Li Z, Pietinen P, Taylor PR, Virtamo J, and Albanes D (2004) Development of a comprehensive dietary antioxidant index and application to lung cancer risk in a cohort of male smokers. *Am J Epidemiol* **160**:68–76.
- Wu GS (2007) Role of mitogen-activated protein kinase phosphatases (MKPs) in cancer. *Cancer Metastasis Rev* **26**:579–585.

**Address correspondence to:** Dr. Yong Lin, Molecular Biology and Lung Cancer Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr., SE, Albuquerque, NM 87108. E-mail: ylin@lrri.org