Prevention and Epidemiology

Luteolin Inhibits Protein Kinase Cε and c-Src Activities and UVB-Induced Skin Cancer

Sanguine Byun[1,2,3], Ki Won Lee[2], Sung Keun Jung[1,2,3], Eun Jung Lee[1], Mun Kyung Hwang[1,2], Sung Hwan Lim[1], Ann M. Bode[3], Hyong Joo Lee[1], and Zigang Dong[3]

Abstract

Luteolin, a flavonoid present in various vegetables including onion and broccoli, has been reported to possess anticarcinogenic effects. However, its chemopreventive effect on UV-induced skin cancer and its mechanism are not fully understood. Herein, we examined the chemopreventive effect and associated mechanisms of luteolin in the JB6 P+ cell line and the SKH-1 hairless mouse model. Luteolin suppressed UVB-induced cyclooxygenase-2 expression and activator protein-1 and nuclear factor-κB activity in JB6 P+ cells. Immunoblot and kinase assay data showed that luteolin attenuated protein kinase Cε (PKCε) and Src kinase activities and subsequently inhibited UVB-induced phosphorylation of mitogen-activated protein kinases and the Akt signaling pathway. In addition, pull-down assays revealed that luteolin binds directly to PKCε and Src in an ATP-competitive manner. Importantly, luteolin suppressed tumor incidence, multiplicity, and overall size in SKH-1 hairless mice. Analysis of the skin by immunohistochemistry and immunoblotting showed that luteolin-treated groups had a substantial reduction in the levels of cyclooxygenase-2, tumor necrosis factor-α, and proliferating cell nuclear antigen compared with groups treated with only UVB. Further analysis using skin lysates showed that luteolin inhibited PKCε and Src kinase activity. Together, these data suggest that luteolin exerts potent chemopreventive activity against UVB-induced skin cancer mainly by targeting PKCε and Src. *Cancer Res; 70(6); 2415–23. ©2010 AACR.*

Introduction

Skin cancer is the most common type of cancer in the United States with increasing incidence (1). About 90% of nonmelanoma skin cancers and 65% of melanomas are attributable to UV exposure (especially the UVB component; 290–320 nm; refs. 2, 3). UVB functions as a complete carcinogen and causes DNA damage, inflammation, skin aging, and eventually skin cancer (3, 4). In photocarcinogenesis, various signaling pathways of activated tyrosine and serine/threonine kinases and transcription factors are responsible for UVB-induced tumor promotion (5). Thus, a strategy for targeting UVB-induced signaling pathways could be an effective approach for preventing skin cancer and other skin disorders.

Irradiation of the skin with UVB causes the development of hyperplasia, benign papillomas, and carcinomas with consistently increased levels of cyclooxygenase-2 (COX-2) expression (6, 7). COX-2 is a critical enzyme for enhancing cell proliferation, angiogenesis, and tumor promotion, and abnormally increased expression of COX-2 and elevated levels of prostaglandins have been observed in various cancers (7, 8). The *cox-2* promoter contains multiple binding sites for transcription factors, including activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and cyclic AMP response element binding protein (CREB) which are activated by UVB irradiation (9–11). Previous studies have suggested that mitogen-activated protein kinases (MAPK) and Akt signaling act as potential regulators of skin carcinogenesis and COX-2 expression by controlling transcription factors AP-1, NF-κB, and CREB in UVB-induced signaling (11–14).

Protein kinase Cε (PKCε) is a calcium-independent PKC isoform linked to proliferation, differentiation, and carcinogenesis in the skin (15). The oncogenic function of PKCε has been well documented by many studies in skin carcinogenesis. Research using the PKCε transgenic mouse model showed that PKCε is involved in UV-induced skin damage, hyperplasia, elevated tumor necrosis factor-α (TNF-α) levels, and the development of squamous cell carcinoma (16, 17). c-Src is a nonreceptor tyrosine kinase that is reportedly activated or overexpressed in various human tumors including skin, and the activity of c-Src is closely associated with proliferation, metastasis, and angiogenesis (18).

Authors' Affiliations: [1]Department of Agricultural Biotechnology, Major in Biomodulation, Seoul National University, [2]Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul, Republic of Korea; and [3]The Hormel Institute, University of Minnesota, Austin, Minnesota

Note: S. Byun and K.W. Lee contributed equally to this work.

Corresponding Authors: Zigang Dong, The Hormel Institute, University of Minnesota, 801 16th Avenue Northeast, Austin, MN 55912. Phone: 507-437-9600; Fax: 507-437-9606; E-mail: zgdong@hi.umn.edu or Hyong Joo Lee, Department of Agricultural Biotechnology, Major in Biomodulation, Seoul National University, Seoul 151-921, Republic of Korea. Phone: 82-2-880-4853; Fax: 82-2-873-5095; E-mail: leehyjo@snu.ac.kr.

American Association for *Cancer Research*

Byun et al.

Luteolin(3,4,5,7-tetrahydroxyflavone) is a natural flavonoid abundant in various fruits and vegetables including onions, broccoli, and celery (19). Previous studies have shown that luteolin has antiproliferative (20), antimetastatic (20, 21), antioxidative (22), antiangiogenic (23), and anti-inflammatory (24) effects, primarily in cancer cell assay models. One study showed that luteolin inhibits chemically induced skin tumorigenesis in a mouse model (25). However, in that particular study, the molecular mechanisms were not evaluated and the underlying mechanism and target(s) of luteolin in skin cancer development and chemoprevention are not clear. The present study showed that luteolin exerts significant protective effects against UVB-induced skin tumorigenesis in SKH-1 hairless mice by directly suppressing PKCε and c-Src kinase activity.

Materials and Methods

Materials. Luteolin (98%) was purchased from Indofine Chemical Company. Eagle's MEM, gentamicin, and ʟ-glutamine were obtained from Life Technologies. Fetal bovine serum (FBS) and the antibody against β-actin were purchased from Sigma-Aldrich. The antibody against COX-2 was from Cayman. Antibodies to detect phosphorylated and total p38, JNK, MKK3/6, MEK1/2, p90RSK, p70S6K, phosphorylated MKK4, phosphorylated Elk1, phosphorylated c-Jun, and phosphorylated Src were purchased from Cell Signaling Biotechnology. Antibodies against PKCε, phosphorylated ERK1/2 and total ERK, total MKK4, and total c-Jun were obtained from Santa Cruz Biotechnology. The antibody against TNF-α was from R&D Systems. The active c-Src and PKCε proteins and antibodies against c-Src, phosphorylated PKCε, phosphorylated CREB, and total CREB were purchased from Upstate Biotechnology. CNBr-Sepharose 4B, glutathione-Sepharose 4B, [γ-^{32}P]ATP, and the chemiluminescence detection kit were purchased from Amersham Pharmacia Biotech. The protein assay kit was obtained from Bio-Rad Laboratories and G418 and the luciferase assay substrate were purchased from Promega.

Cell culture. The JB6 P+ mouse epidermal cell line was maintained at 37°C in a humidified atmosphere of 5% CO_2 in 5% FBS-MEM, 2 mmol/L of ʟ-glutamine, and 25 μg/mL of gentamicin. The JB6 P+ mouse epidermal cell line was stably transfected with the AP-1, NF-κB, or COX-2 luciferase reporter plasmid and maintained in 5% FBS-MEM containing 200 μg/mL of G418.

Luciferase assay for AP-1, NF-κB, and COX-2 transactivation. AP-1 or NF-κB luciferase reporter–transfected JB6 P+ cells were constructed as described earlier (26). The cells were treated for 1 h with luteolin (10 or 20 μmol/L) before exposure to UVB (0.05 J/cm^2) and then were incubated for 6 h. Cells were disrupted with lysis buffer and luciferase activity was measured using a luminometer (Luminoskan Ascent; Thermo Electron).

Immunoblot assays. Total cell lysates were prepared and subjected to Western blot as described earlier (26). After cell lysis, the protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories) as described

in the manufacturer's manual. Mouse skin lysates were prepared and subjected to Western blot as reported earlier (26).

Kinase assays. The *in vitro* kinase assay was carried out in accordance with the instructions provided by Upstate Biotechnology. In brief, the Src kinase assay was performed using an active c-Src protein, a c-Src substrate peptide contained in the assay buffer, and [γ-^{32}P]ATP solution diluted with magnesium-ATP cocktail buffer. The PKCε kinase assay was performed with an active PKCε protein, PKCtide, and lipid activator contained in the assay buffer, and [γ-^{32}P]ATP solution diluted with magnesium-ATP cocktail buffer. The c-Src and PKCε kinase assay mixtures were each incubated at 30°C and then aliquots were transferred onto p81 paper and washed with 0.75% phosphoric acid. The radioactive incorporation was determined using a scintillation counter. The effect of luteolin (10 or 20 μmol/L) was evaluated by incubating luteolin with the c-Src or PKCε kinase reaction mixtures at 30°C for 10 min. Each experiment was performed three times.

For the *in vivo* Src immunoprecipitation and kinase assay, skin proteins were extracted as described above (see Immunoblot assays) using kinase assay buffer. Mouse skin extracts were mixed with a c-Src or PKCε antibody and then Sepharose-4B beads were added and gently rocked overnight at 4°C. These tubes were centrifuged and the pellets washed. Radioactive incorporation was determined as for the kinase assay described above.
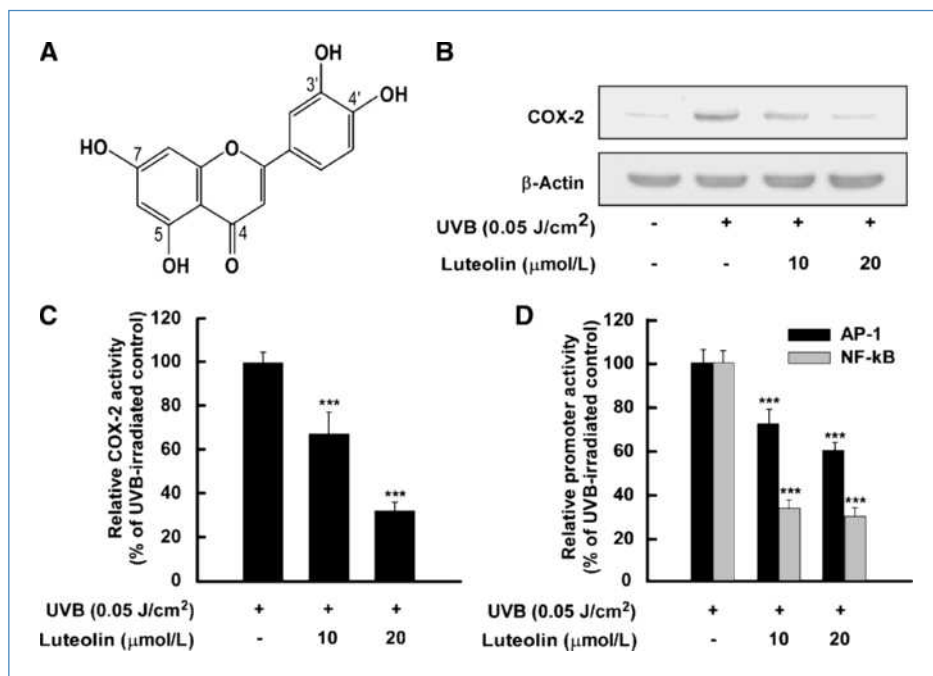
Preparation of luteolin-Sepharose 4B beads. Sepharose 4B powder was suspended in 1 mmol/L of HCl and luteolin was added to the coupling solution (0.1 mol/L NaHCO$_3$ and 0.5 mol/L NaCl) and mixed on a rotary shaker at 4°C overnight. The procedure was performed as reported earlier (26).

Pull-down assays. For the *in vitro* pull-down assay, c-Src or PKCε was incubated with luteolin-Sepharose 4B (or Sepharose 4B alone as a control) beads in reaction buffer. After incubation, the beads were washed five times with washing buffer. Proteins bound to the beads were analyzed by immunoblotting. For the *in vivo* pull-down assay, dorsal skin was prepared as for immunoblotting and proteins were extracted as for the c-Src or PKCε immunoprecipitation and kinase assays described above. The mouse skin extract was incubated with luteolin-Sepharose 4B (or Sepharose 4B alone as a control) beads in reaction buffer and were further processed as for the *in vitro* pull-down assay.

ATP and luteolin competition assay. Active c-Src or PKCε was incubated with 100 μL of luteolin-Sepharose 4B or Sepharose 4B beads in reaction buffer (see Pull-down assays) for 12 h at 4°C, and ATP was added at different concentrations (10 or 100 μmol/L) to a final volume of 500 μL. The samples were washed and proteins were detected by immunoblotting.

Animals. All animal procedures were conducted in accordance with guidelines provided by Seoul National University (Seoul, Korea). Female SKH-1 hairless mice (5 wk old; mean body weight, 25 g) were purchased from the Institute of Laboratory Animal Resources at Seoul National University. Animals were acclimated for 1 wk prior to the study and had free access to food and water. The animals were housed

Figure 1. Effect of luteolin on UVB-induced COX-2 expression and AP-1 and NF-κB activation in JB6 P+ cells. A, structure of luteolin. B, luteolin inhibits UVB-induced COX-2 expression in JB6 P+ cells. Data are representative of three independent experiments that gave similar results. C and D, luteolin suppresses UVB-induced COX-2 promoter activity (C) and AP-1 and NF-κB transactivation (D). Luciferase activity was assayed and COX-2, AP-1, or NF-κB activity in cells is expressed as a percentage of activity in cells treated with only UVB. Columns, mean of respective AP-1, NF-κB, and COX-2 luciferase activity from three independent experiments; bars, SD. Each experiment was replicated in five-well plates (***, $P < 0.001$, significant differences between groups treated with both luteolin and UVB and the group treated with UVB alone).

in climate-controlled quarters (24°C at 50% humidity) with a 12 h light/dark cycle.

Two-stage tumorigenesis experimental design. Skin tumorigenesis was induced in mice using a UVB irradiation system. The UVB light source (Bio-Link Crosslinker; Vilber Lourmat) emitted wavelengths of 254 nm, 312 nm, and 365 nm, with peak emission at 312 nm. Treatment groups were divided into four groups of 10 animals each. In the control group, the dorsal skin of mice were topically treated with 200 μL of acetone alone. In the UVB group, dorsal skin of the mice was topically treated with 200 μL of acetone before UVB ($0.18 J/cm^2$) exposure thrice a week for 27 wk. The mice in the third and fourth groups received topical applications of luteolin (10 or 40 nmol) in 200 μL of acetone 1 h before UVB ($0.18 J/cm^2$) irradiation thrice a week for 27 wk. The incidence of skin tumors was recorded weekly. A tumor was defined as an outgrowth >1 mm in diameter that persisted for 2 wk or more. Tumor incidence, multiplicity, and volume were recorded every week until the end of the experiment at 27 wk.

Short-term in vivo ***kinase assay.*** SKH-1 hairless mice were irradiated with UVB ($0.18 J/cm^2$) for five consecutive days. Treatment groups were divided into four groups of three animals each. In the control group, the dorsal skin of mice was topically treated with 200 μL of acetone alone. In the UVB group, the dorsal skin of the mice was topically treated with 200 μL of acetone after UVB exposure. The mice in the third and fourth groups received topical applications of luteolin (10 nmol in 200 μL acetone) after UVB irradiation. Mice were sacrificed 30 min after the last UVB exposure. Immunoprecipitation and kinase assays were conducted as described above for in vitro kinase assays.

Immunohistochemical analysis. Dorsal skin from the mice was prepared for immunohistochemical analysis of COX-2, proliferating cell nuclear antigen (PCNA), and TNF-α expression. Tissue sections (5 μm thick) from 10% neutral formalin solution–fixed paraffin-embedded tissue were cut on silane-coated glass slides and then deparaffinized with xylene and dehydrated through a graded alcohol bath. The deparaffinized sections were incubated in citric acid buffer (pH 6.0) for the detection of COX-2 and PCNA, and glycine buffer (pH 9.0) for the detection of TNF-α, and then boiled for antigen retrieval. Each section was treated with 3% hydrogen peroxide for 10 min and a blocking solution containing 1% bovine serum albumin for 30 min. The slides were incubated overnight with an affinity-purified primary antibody at 4°C in 1% bovine serum albumin and then developed using an anti-rabbit or anti-mouse Histostain Plus Kit (Zymed Laboratories, Inc.). Peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Mayer's hematoxylin (Sigma-Aldrich) was applied as a counterstain.

Statistical analysis. As necessary, data were expressed as means ± SEM and ANOVA was used to perform statistical analysis for single comparisons. $P < 0.05$ was used as the criterion for statistical significance. All analyses were performed using Statistical Analysis Software (SAS, Inc.).

Results

Luteolin inhibits UVB-induced COX-2 expression and AP-1 and NF-κB activation in JB6 P+ cells. We first examined the effect of luteolin on UVB-induced COX-2 protein expression and promoter activity using JB6 P+ cells stably transfected with a COX-2 luciferase plasmid. Luteolin inhibited UVB-induced COX-2 protein expression in a dose-dependent manner in JB6 P+ cells (Fig. 1B). COX-2 luciferase

promoter activity induced by UVB irradiation was also dose-dependently attenuated by luteolin in JB6 P+ cells (Fig. 1C). JB6 P+ cells pretreated with luteolin significantly reduced UVB-induced activation of AP-1 and NF-κB in JB6 P+ cells, which were stably transfected with AP-1 or the NF-κB luciferase plasmid (Fig. 1D).

Luteolin represses UVB-induced phosphorylation of ERKs, p38, JNKs, and Akt in JB6 P+ cells. MAPKs including ERKs, p38 MAPK, JNKs, and Akt signaling mediate UVB-induced upregulation of AP-1, NF-κB, CREB, and COX-2 (11–13). Luteolin inhibited UVB-induced phosphorylation of ERKs, p38 MAPK, JNKs, and Akt in a dose-dependent manner (Fig. 2A). To identify the upstream molecular target of luteolin in UVB-induced signaling, we assessed the effect of luteolin on upstream kinases of MAPKs. UVB-induced phosphorylation of MEK, MKK4, and MKK3/6 was also inhibited by luteolin (Fig. 2B). However, luteolin increased UVB-induced phosphorylation of c-Src (Fig. 2C) and inhibited the translocation of PKCε. Luteolin also suppressed the

phosphorylation of downstream targets of MAPKs and the Akt signaling pathway (Fig. 2D).

Luteolin attenuates PKCε and c-Src kinase activity and directly binds with PKCε and c-Src in an ATP-competitive manner. Because luteolin inhibited the UVB-induced translocation of PKCε and increased the phosphorylation of c-Src, we hypothesized that luteolin might directly inhibit PKCε and c-Src kinase activity. Results showed that PKCε kinase activity was significantly attenuated by treatment with 10 or 20 μmol/L of luteolin (Fig. 3A, left). Also, c-Src kinase activity was significantly blocked by treatment with 10 or 20 μmol/L of luteolin (Fig. 3A, right). Because luteolin inhibited PKCε and c-Src kinase activity, we conducted pull-down assays to determine whether luteolin interacts directly with PKCε and c-Src kinase. Results indicated that PKCε directly binds to the luteolin-Sepharose 4B beads (Fig. 3B, left, lane 3), but did not bind to the Sepharose 4B beads alone (Fig. 3B, left, lane 2). In addition, luteolin directly interacted with c-Src (Fig. 3B, right). To further investigate how luteolin binds
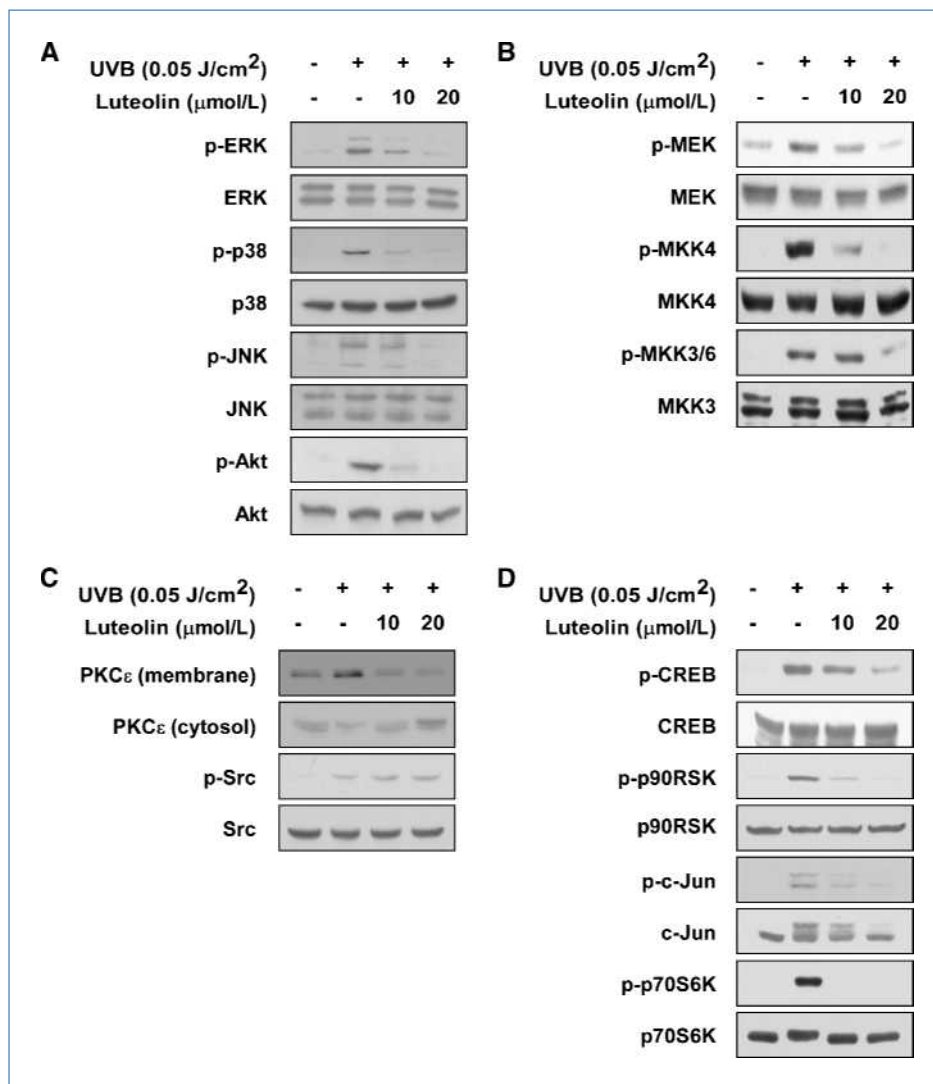

Figure 2. Effect of luteolin on UVB-induced signaling in JB6 P+ cells. A, luteolin inhibits UVB-induced phosphorylation (*p*–) of ERK, p38, JNK, and Akt. B, luteolin inhibits UVB-induced phosphorylation of MEK, MKK4, and MKK3/6. C, luteolin inhibits UVB-induced translocation of PKCε to the membrane fraction and increases phosphorylation of Src. D, luteolin inhibits UVB-induced phosphorylation of CREB, p90^{RSK}, c-Jun, and p70^{S6K}. The cells were starved in 0.1% FBS-MEM and then treated or not treated with luteolin at the indicated concentration for 1 h before they were stimulated with UVB (0.05 J/cm²). Data are representative of three independent experiments that gave similar results.
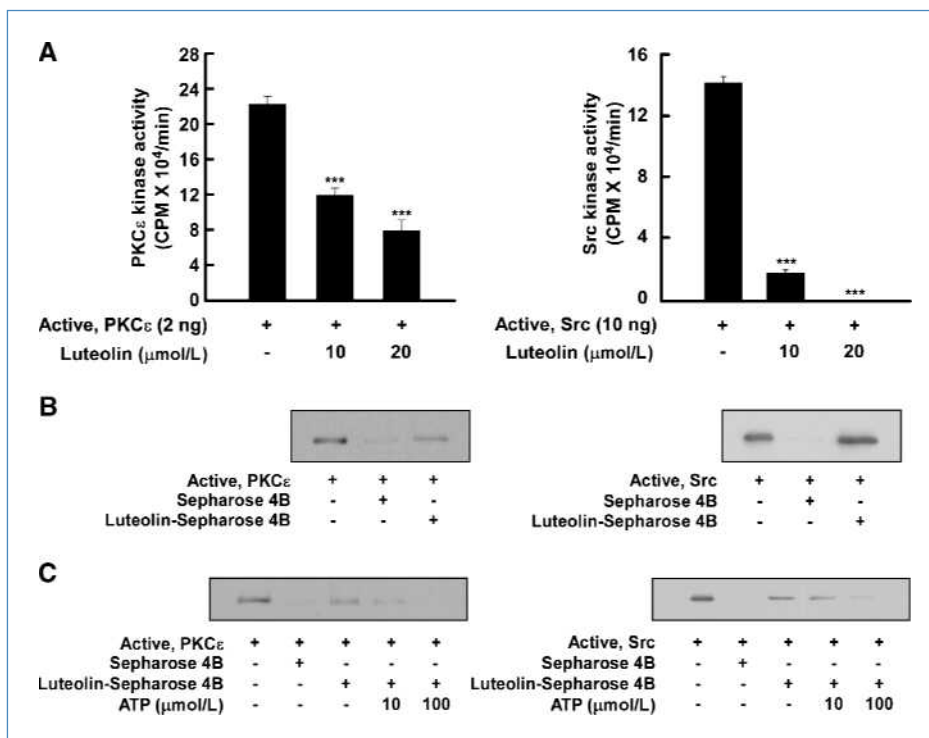
Figure 3. Effect of luteolin on PKCε and Src kinase and binding activities. A, luteolin inhibits PKCε and Src kinase activity. The active PKCε (left) or Src (right) protein was used to assess kinase activity in the presence or absence of luteolin at the indicated concentration *in vitro*. Columns, means determined from three independent experiments; bars, SD (***, $P < 0.001$, significant difference between groups treated with luteolin and the untreated group). B, luteolin directly binds with PKCε or Src. Luteolin binding was assessed by immunoblotting using an antibody against PKCε (left) or against Src (right). Lane 1, active PKCε or Src; lane 2, negative control, PKCε, or Src cannot bind with Sepharose 4B beads; lane 3, PKCε or Src binds with luteolin-Sepharose 4B beads. C, luteolin binds with PKCε or Src in an ATP-competitive manner. Active PKCε or Src was incubated with ATP at different concentrations (10 or 100 μmol/L) and 50 μL of luteolin-Sepharose 4B or Sepharose 4B (as a negative control) in reaction buffer. Lane 1, active PKCε or Src (100 ng); lane 2, negative control, PKCε, or Src cannot bind with Sepharose 4B; lane 3, positive control, PKCε, or Src binds with luteolin-Sepharose 4B; lanes 4 and 5, increasing amounts of ATP suppress luteolin binding with PKCε or Src. Each experiment was performed thrice.

to PKCε and c-Src, we performed the pull-down assay with increasing concentrations of ATP. The binding ability of luteolin with PKCε was altered in an ATP concentration-dependent manner (Fig. 3C, left), suggesting that luteolin competes with ATP for binding to PKCε. In addition, luteolin also competed with ATP for binding to c-Src (Fig. 3C, right). These results showed that luteolin is an ATP-competitive inhibitor that reduces PKCε or c-Src kinase activity.

Luteolin suppresses UVB-induced skin tumorigenesis in mouse skin. To further investigate the chemopreventive effect of luteolin *in vivo*, we used a two-stage tumorigenesis model. Topically applied luteolin on mouse skin resulted in a substantial inhibition of UVB-induced tumor incidence (Fig. 4A) and resulted in a delay in the onset of tumors. Exposure of mice to UVB resulted in the development of skin tumors in all mice in the UVB-only–treated group by 20 weeks, whereas 24 or 25 weeks were required for tumor development in all mice treated with 10 or 40 nmol of luteolin (in 200 μL of acetone), respectively (Fig. 4B). Luteolin treatment also reduced tumor multiplicity. At the end of the study (27 weeks), a 54% or 65% decrease in tumor numbers per mouse was observed in groups treated with 10 or 40 nmol of luteolin, respectively, compared with the UVB-only–treated

group (Fig. 4C). Topical application of luteolin dramatically decreased the average tumor volume per mouse from 17 mm^3 in the UVB-only–treated group to 4.5 or 2.6 mm^3 in the 10 or 40 nmol luteolin-treated groups, respectively (Fig. 4D).

Luteolin decreases COX-2 and TNF-α expression and cell proliferation in UVB-exposed mouse skin. Immunohistochemical staining throughout the skin showed that chronic exposure to UVB strongly increased COX-2 expression compared with the acetone-only–treated group. However, treatment of skin with 10 or 40 nmol of luteolin (in 200 μL of acetone) resulted in a downmodulation of COX-2 levels (Fig. 5A). TNF-α has been reported to be involved in the development of skin cancer and overexpression of PKCε enhances the level of UV-induced TNF-α (16). Chronic UVB exposure caused a significant increase in TNF-α levels compared with nonirradiated mouse skin, and treatment with 10 or 40 nmol of luteolin notably reduced UVB-induced TNF-α levels in mouse skin (Fig. 5B). Consistent inflammation causes hyperproliferation in the skin and enhanced proliferation is a major hallmark of cancer development (27). Therefore, we assessed the effect of luteolin on proliferation in chronic UVB-exposed skin by measuring PCNA levels. Chronic UVB exposure (27 weeks) caused a marked increase in
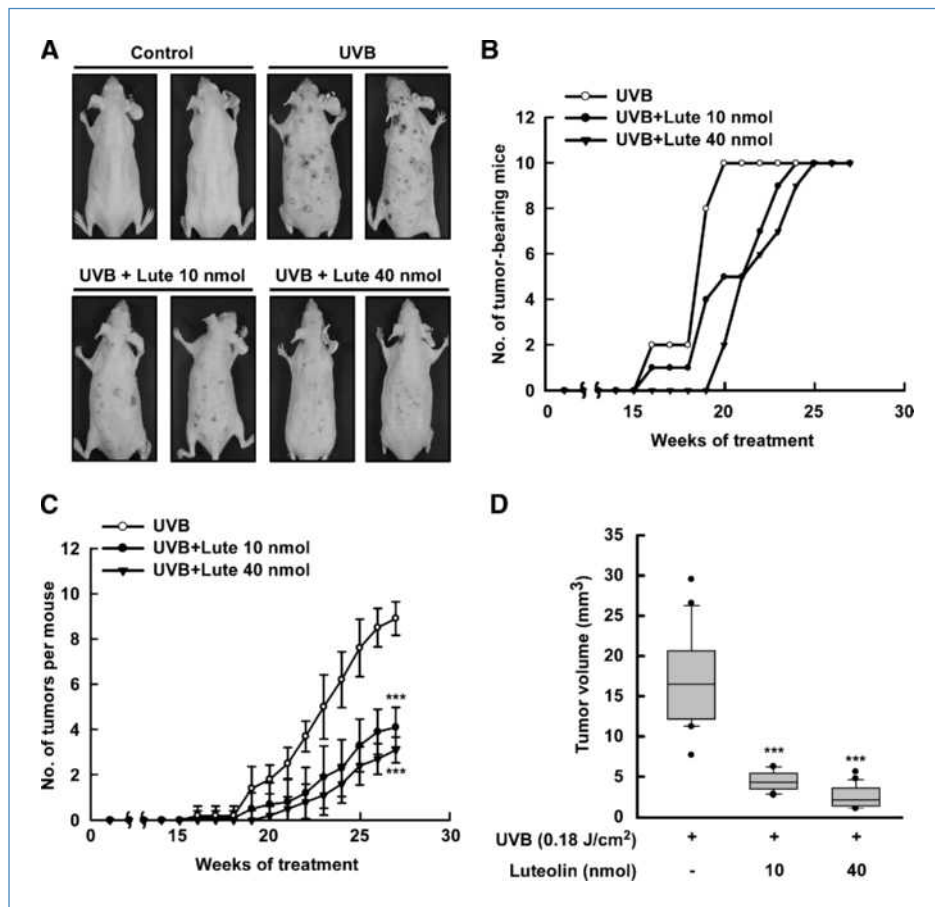
Figure 4. Effect of luteolin treatment on UVB-induced skin tumorigenesis in SKH-1 hairless mice. The treatment groups were as follows: (*a*) not irradiated (control); (*b*) 0.18 J/cm^2 UVB; or (*c*) treated with 10 or 40 nmol of luteolin in 200 μL of acetone. The compound was applied topically to the dorsal surface of each mouse before UVB (0.18 J/cm^2) exposure (UVB + Lute 10 nmol and UVB + Lute 40 nmol) as detailed in Materials and Methods. A, representative pictures of mice. B, number of tumor-bearing mice. C, number of tumors per mouse. D, tumor volume calculated using the following formula: tumor volume = $4\pi/3(l/2)(w/2)(h/2)$, where *l* is length, *w* is width, and *h* is height (***, $P < 0.001$, significant differences between groups treated with both luteolin and UVB and the group treated with UVB alone).

PCNA-positive cells compared with nonirradiated mouse skin, and treatment with 10 or 40 nmol of luteolin significantly reduced UVB-induced PCNA levels in mouse skin (Fig. 5C). Immunoblot analysis of skin tissues showed the same trend of reduction in COX-2, TNF-α, and PCNA expression in 10 or 40 nmol of luteolin-treated groups compared with the UVB-only–treated group (Fig. 5D).

Luteolin inhibits PKCε and c-Src kinase activity in mouse skin. Because luteolin showed significant inhibitory effects against PKCε and c-Src kinase activity *in vitro*, we next examined the effect of luteolin on UVB-induced PKCε and c-Src kinase activity in mouse skin. Hyperactivated PKCε was observed in UVB-only–treated mouse skin extracts and mouse skin extracts from groups treated with 10 or 40 nmol of luteolin showed a significant decrease in UVB-induced PKCε activity (Fig. 6A, left). Chronic UVB irradiation resulted in an increase in c-Src kinase activity compared with the control group, and topical treatment with 10 or 40 nmol of luteolin markedly inhibited UVB-induced c-Src kinase activity in mouse skin extracts (Fig. 6A, right). This showed that luteolin could suppress PKCε and c-Src kinase activity not only *in vitro* but also *in vivo*. To rule out the possibility that luteolin might act as a sunscreen, we treated mouse skin with luteolin after UVB exposure and performed an *in vivo*

kinase assay. Results showed that post-treatment with luteolin also significantly decreased UVB-induced PKCε and c-Src kinase activity in mouse skin (Supplementary Fig. 1).

Discussion

Chronic inflammation is a critical component of tumor development (28). Multiple lines of evidence suggest that COX-2, a rate-limiting enzyme in the synthesis of prostaglandins, is a key link between inflammation and cancer (8, 29). UVB is considered to be a complete carcinogen because it is capable of triggering the initiation, promotion, and progression phases of carcinogenesis (3, 30). The present study revealed that treatment with luteolin significantly suppressed UVB-induced COX-2 expression in epidermal cells and in chronic UVB-exposed SKH-1 hairless mouse skin. In addition to COX-2, TNF-α is also involved in inflammation and skin cancer (16). Activation of PKCε signaling by TPA or UV elevates TNF-α levels during skin carcinogenesis (16). Luteolin treatment decreased the level of TNF-α in UVB-irradiated skin and this could be due to the inhibitory effect of luteolin against PKCε activity. Because chronic inflammation is related to proliferation, we next focused on the associated molecular markers. The level of PCNA was high in the UVB-treated
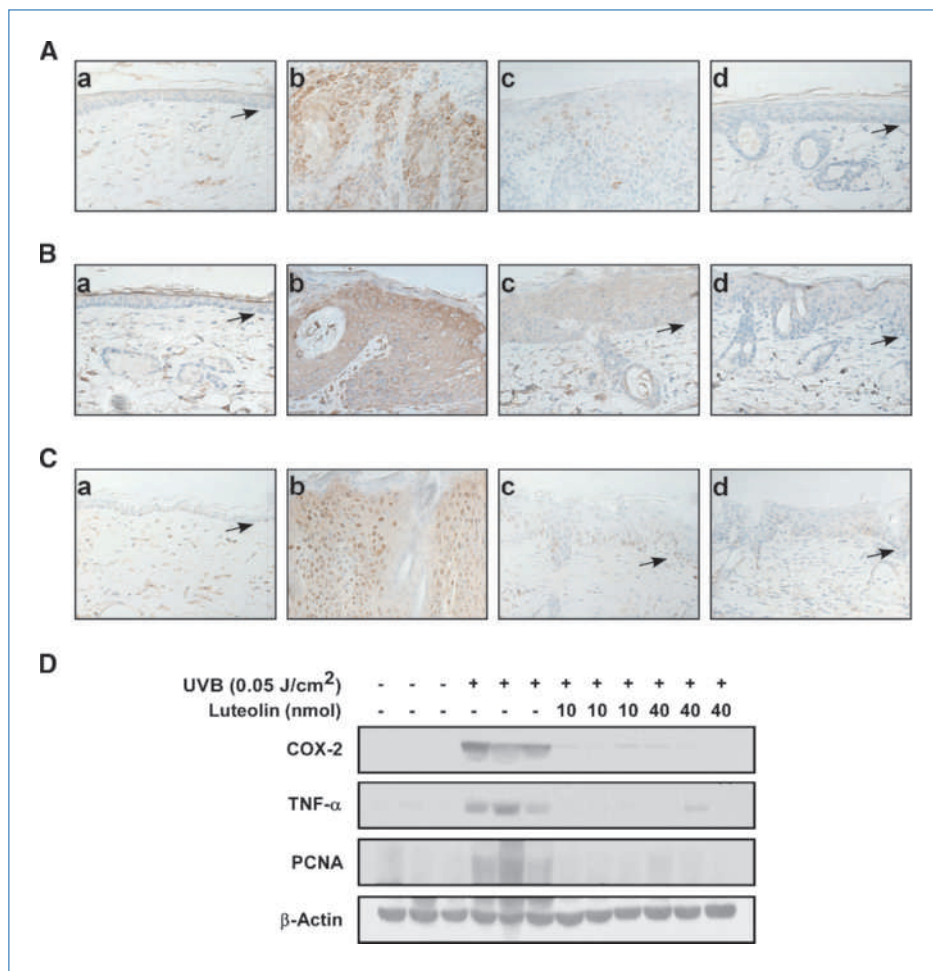
group and was dramatically reduced by luteolin treatment in mouse skin. A reduction in proliferative activity in luteolin-treated skin could explain the suppression of tumorigenesis in UVB-irradiated skin.

Aberrant overexpression of COX-2 is regulated by signal pathways composed of various transcription factors and upstream kinases. The 5′-flanking region of the *cox-2* gene has binding sites for several transcription factors such as AP-1, NF-κB, and CREB (9–11). Our results showed that luteolin inhibited COX-2 expression by regulating *cox-2* promoter activity, and suppressed AP-1 and NF-κB transcription activation. AP-1 and NF-κB are transcription factors that have crucial roles in carcinogenesis, such as cell proliferation, inflammation, metastasis, and transformation (12, 31, 32). In our study, luteolin delayed the development of tumors, inhibited the multiplication of tumors, decreased tumor volume, and suppressed the expression of inflammatory and proliferative biomarkers. These cancer chemopreventive effects of luteolin could be explained by its inhibitory effects on transcriptional activation of AP-1 and NF-κB.

In the present study, we found PKCε and c-Src to be upstream target proteins of luteolin in UVB-induced skin carcinogenesis. Our results clearly showed that luteolin inhibited the UVB-induced activation of ERK, p38 MAPK, JNK, and the Akt signaling pathway. Activated ERK, p38 MAPK, JNK, and Akt are well known as regulators of AP-1 and NF-κB activation and are crucially involved in the development of cancers (12, 33). Because luteolin inhibited UVB-induced MAPK and MAPKK signaling, we hypothesized that the molecular targets of luteolin could be upstream kinases of MAPKKs and Akt. PKCε regulates MAPKs and Akt signaling as downstream targets (34, 35), and experiments using dominant-negative PKCε cells revealed similar signaling pathways compared with those of luteolin treatment (data not shown). Translocation of PKCε to the plasma membrane is essential for subsequent phosphorylation of target substrates (16). The inhibitory effect of luteolin on downstream molecules could occur through the suppression of UVB-induced PKCε translocation. In multiple studies, ERKs, p38, JNKs, and Akt have been shown to be regulated by Src kinase activity (36–40). Our experiments showed that luteolin not only inhibited PKCε and c-Src kinase activity *in vitro* but also *in vivo*. PKCε is known to promote skin cancer development by inducing oncogenic signaling (16), and its crucial role in skin carcinogenesis is well characterized (17, 41). c-Src was originally classified as a proto-oncogene (42, 43) that plays a

Figure 5. Effect of luteolin treatment on UVB-induced COX-2, TNF-α, or PCNA expression in SKH-1 hairless mouse skin. At the end of 27 wk of study (as described in Fig. 4), mouse skin was analyzed for COX-2 (A), TNF-α (B), and PCNA (C) by immunohistochemical staining and for COX-2 (D, top), TNF-α (middle), and PCNA (bottom) by immunoblotting using the whole skin lysate. For immunohistochemistry, paraffin-embedded sections were immunostained for COX-2, TNF-α, and PCNA as described in Materials and Methods. Representative photos were taken from five similar tissue samples. COX-2, TNF-α, or PCNA expression is brown. (a) Vehicle-treated controls, (b) UVB-irradiated (0.18 J/cm²) mice, and UVB plus (c) 10 nmol of luteolin or (d) 40 nmol of luteolin. For immunoblotting, COX-2, TNF-α, or PCNA expression were detected with specific antibodies. Arrows, the junction between the epidermis and dermis.

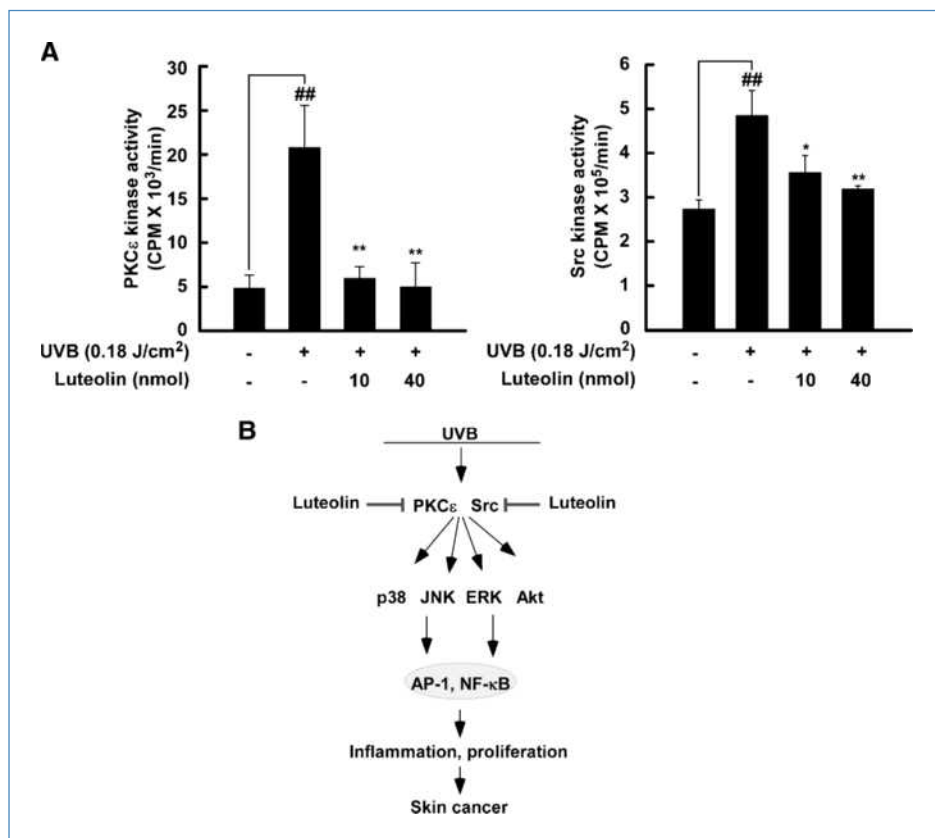**Figure 6.** Proposed mechanism of luteolin in preventing skin cancer. A, luteolin inhibits PKCε and Src kinase activity in mouse skin. Mouse skin lysates were prepared and immunoprecipitated for PKCε (left) or Src (right) kinase assays as described in Materials and Methods. Columns, means determined from three independent experiments; bars, SD (##, $P < 0.01$, significant difference between the control group and the group exposed to UVB alone; *, $P < 0.05$ and **, $P < 0.01$, significant differences between groups treated with both luteolin and UVB and the group exposed to UVB alone). B, simplified depiction of a proposed mechanism for the skin cancer-preventive effect of luteolin.

pivotal role in skin cancer (5, 44). The expression level and kinase activity of Src in skin tumors have been reported to be elevated compared with normal skin (45), and Src activation is suggested as an initial step in the UV-induced signaling cascade (46). Thus, the significant protective effect of luteolin against UVB-induced skin cancer is due to the direct inhibition of PKCε and Src kinase activity and subsequent suppression of downstream signaling.

In the past, inhibitors designed for a specific target with high selectivity were preferred. However, the majority of cancers are derived from many mutations (27). Thus, multitargeted kinase inhibitors are anticipated to have therapeutic advantages and are emerging as new alternatives for cancer treatment (47). Our study showed that the naturally occurring phytochemical luteolin exerted a significant chemopreventive effect on UVB-induced skin carcinogenesis. Furthermore, luteolin showed remarkable inhibitory effects on COX-2, AP-1, NF-κB, and diverse upstream MAPKs and the Akt signaling pathways by directly attenuating PKCε and Src kinase

activities. Thus, luteolin, which is abundant in onions, broccoli, and celery, could be beneficial in preventing skin cancer development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

References

1. Ricotti C, Bouzari N, Agadi A, Cockerell CJ. Malignant skin neoplasms. Med Clin North Am 2009;93:1241–64.
2. Armstrong BK, Kricker A. How much melanoma is caused by sun exposure. Melanoma Res 1993;3:395–401.
3. Melnikova VO, Ananthaswamy HN. Cellular and molecular events leading to the development of skin cancer. Mutat Res 2005;571: 91–106.
4. Matsumura Y, Ananthaswamy HN. Toxic effects of ultraviolet radiation on the skin. Toxicol Appl Pharmacol 2004;195:298–308.
5. Ichihashi M, Ueda M, Budiyanto A, et al. UV-induced skin damage. Toxicology 2003;189:21–39.
6. Athar M, An KP, Morel KD, et al. Ultraviolet B (UVB)-induced COX-2 expression in murine skin: an immunohistochemical study. Biochem Biophys Res Commun 2001;280:1042–7.

7. Buckman SY, Gresham A, Hale P, et al. COX-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. Carcinogenesis 1998;19:723–9.

8. DuBois RN, Abramson SB, Crofford L, et al. Cyclooxygenase in biology and disease. FASEB J 1998;12:1063–73.

9. Balkwill F, Coussens LM. Cancer: an inflammatory link. Nature 2004; 431:405–6.

10. Kim Y, Fischer SM. Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells: regulatory role of CCAAT/enhancer-binding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. J Biol Chem 1998;273:27686–94.

11. Tang Q, Gonzales M, Inoue H, Bowden GT. Roles of akt and glycogen synthase kinase 3β in the ultraviolet B induction of cyclooxygenase-2 transcription in human keratinocytes. Cancer Res 2001;61: 4329–32.

12. Bode AM, Dong Z. Signal transduction pathways: targets for chemoprevention of skin cancer. Lancet Oncol 2000;1:181–8.

13. Chen W, Tang Q, Gonzales MS, Bowden GT. Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. Oncogene 2001;20: 3921–6.

14. Pikarsky E, Porat RM, Stein I, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–6.

15. Breitkreutz D, Braiman-Wiksman L, Daum N, Denning MF, Tennenbaum T. Protein kinase C family: on the crossroads of cell signaling in skin and tumor epithelium. J Cancer Res Clin Oncol 2007;133:793–808.

16. Verma AK, Wheeler DL, Aziz MH, Manoharan H. Protein kinase Cε and development of squamous cell carcinoma, the nonmelanoma human skin cancer. Mol Carcinog 2006;45:381–8.

17. Wheeler DL, Martin KE, Ness KJ, et al. Protein kinase Cε is an endogenous photosensitizer that enhances ultraviolet radiation-induced cutaneous damage and development of squamous cell carcinomas. Cancer Res 2004;64:7756–65.

18. Ishizawar R, Parsons SJ. c-Src and cooperating partners in human cancer. Cancer Cell 2004;6:209–14.

19. Koo Hui M, Mohamed S. Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. J Agric Food Chem 2001;49:3106–12.

20. Huang YT, Hwang JJ, Lee PP, et al. Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. Br J Pharmacol 1999;128:999–1010.

21. Lee WJ, Wu LF, Chen WK, Wang CJ, Tseng TH. Inhibitory effect of luteolin on hepatocyte growth factor/scatter factor-induced HepG2 cell invasion involving both MAPK/ERKs and Pl3K-Akt pathways. Chem Biol Interact 2006;160:123–33.

22. Manju V, Balasubramaniyan V, Nalini N. Rat colonic lipid peroxidation and antioxidant status: the effects of dietary luteolin on 1,2-dimethylhydrazine challenge. Cell Mol Biol Lett 2005;10:535–51.

23. Bagli E, Stefaniotou M, Morbidelli L, et al. Luteolin inhibits vascular endothelial growth factor-induced angiogenesis;inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3′-kinase activity. Cancer Res 2004;64:7936–46.

24. Ueda H, Yamazaki C, Yamazaki M. Luteolin as an anti-inflammatory and anti-allergic constituent of Perilla frutescens. Biol Pharm Bull 2002;25:1197–202.

25. Ueda H, Yamazaki C, Yamazaki M. Inhibitory effect of perilla leaf extract and luteolin on mouse skin tumor promotion. Biol Pharm Bull 2003;26:560–3.

26. Jung SK, Lee KW, Byun S, et al. Myricetin suppresses UVB-induced skin cancer by targeting Fyn. Cancer Res 2008;68:6021–9.

27. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100: 57–70.

28. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420: 860–7.

29. Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta Rev 2000:1470.

30. Black HS, deGruijl FR, Forbes PD, et al. Photocarcinogenesis: an overview. J Photochem Photobiol B 1997;40:29–47.

31. Dong Z, Birrer MJ, Watts RG, Matrisian LM, Colburn NH. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proc Natl Acad Sci U S A 1994; 91:609–13.

32. Greten FR, Eckmann L, Greten TF, et al. IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004;118:285–96.

33. Zhang X, Jin B, Huang C. The Pl3K/Akt pathway and its downstream transcriptional factors as targets for chemoprevention. Curr Cancer Drug Targets 2007;7:305–16.

34. Chen N, Ma W, Huang C, Dong Z. Translocation of protein kinase Cε and protein kinase Cδ to membrane is required for ultraviolet B-induced activation of mitogen-activated protein kinases and apoptosis. J Biol Chem 1999;274:15389–94.

35. Shankar E, Sivaprasad U, Basu A. Protein kinase Cε confers resistance of MCF-7 cells to TRAIL by Akt-dependent activation of Hdm2 and downregulation of p53. Oncogene 2008;27:3957–66.

36. Frey MR, Golovin A, Polk DB. Epidermal growth factor-stimulated intestinal epithelial cell migration requires Src family kinase-dependent p38 MAPK signaling. J Biol Chem 2004;279:44513–21.

37. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 2003;284:31–53.

38. Kasahara K, Nakayama Y, Nakazato Y, Ikeda K, Kuga T, Yamaguchi N. Src signaling regulates completion of abscission in cytokinesis through ERK/MAPK activation at the midbody. J Biol Chem 2007; 282:5327–39.

39. Nagao M, Yamauchi J, Kaziro Y, Itoh H. Involvement of protein kinase C and Src family tyrosine kinase in Gαq/11-induced activation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. J Biol Chem 1998;273:22892–8.

40. Xing J, Zhang Z, Mao H, Schnellmann RG, Zhuang S. Src regulates cell cycle protein expression and renal epithelial cell proliferation via Pl3K/Akt signaling-dependent and -independent mechanisms. Am J Physiol Renal Physiol 2008;295:F145–52.

41. Aziz MH, Manoharan HT, Sand JM, Verma AK. Protein kinase Cε interacts with Stat3 and regulates its activation that is essential for the development of skin cancer. Mol Carcinog 2007;46:646–53.

42. Hunter T, Sefton BM. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci U S A 1980;77: 1311–5.

43. Jove R, Hanafusa H. Cell transformation by the viral src oncogene. Annu Rev Cell Biol 1987;3:31–56.

44. Matsumoto T, Jiang J, Kiguchi K, et al. Targeted expression of c-Src in epidermal basal cells leads to enhanced skin tumor promotion, malignant progression, and metastasis. Cancer Res 2003;63: 4819–28.

45. Barnekow A, Paul E, Schartl M. Expression of the c-src protooncogene in human skin tumors. Cancer Res 1987;47:235–40.

46. Devary Y, Gottlieb RA, Smeal T, Karin M. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell 1992; 71:1081–91.

47. Sebolt-Leopold JS, English JM. Mechanisms of drug inhibition of signalling molecules. Nature 2006;441:457–62.

Cancer Research

AACR American Association for Cancer Research

Luteolin Inhibits Protein Kinase Cε and c-Src Activities and UVB-Induced Skin Cancer

Sanguine Byun, Ki Won Lee, Sung Keun Jung, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-4093

Cited articles	This article cites 46 articles, 14 of which you can access for free at: http://cancerres.aacrjournals.org/content/70/6/2415.full#ref-list-1
Citing articles	This article has been cited by 5 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/70/6/2415.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/70/6/2415. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.