

## ORIGINAL ARTICLE

# Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1

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High expression of epidermal growth factor receptor (EGFR) is found in a variety of solid tumors, including colorectal cancer. EGFR has been identified as a rational target for anticancer therapy. Curcumin, the yellow pigment of turmeric in curry, has received attention as a promising dietary supplement for cancer prevention and treatment. We recently reported that curcumin inhibited the growth of human colon cancer-derived Moser cells by suppressing gene expression of cyclinD1 and EGFR. The aim of the present study was to explore the molecular mechanisms underlying curcumin inhibition of gene expression of EGFR in colon cancer cells. The generality of the inhibitory effect of curcumin on gene expression of EGFR was verified in other human colon cancer-derived cell lines, including Caco-2 and HT-29 cells. Promoter deletion assays and site-directed mutageneses identified a binding site for the transcription factor early growth response-1 (Egr-1) in *egfr* promoter as a putative curcumin response element in regulating the promoter activity of the gene in Moser cells. Electrophoretic mobility shift assays demonstrated that curcumin significantly reduced the DNA-binding activity of the transcription factor Egr-1 to the curcumin response element. In addition, curcumin reduced the *trans*-activation activity of Egr-1 by suppressing *egr-1* gene expression, which required interruption of the ERK signal pathway and reduction of the level of phosphorylation of Elk-1 and its activity. Taken together, our results demonstrated that curcumin inhibited human colon cancer cell growth by suppressing gene expression of EGFR through reducing the *trans*-activation activity of Egr-1. These results provided novel insights into the mechanisms of curcumin inhibition of colon cancer cell growth and potential therapeutic strategies for treatment of colon cancer.

*Oncogene* (2006) 25, 278–287. doi:10.1038/sj.onc.1209019; published online 19 September 2005

**Keywords:** colon cancer; receptors; phyto-chemical; gene expression; chemo-prevention

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Received 28 February 2005; revised 9 June 2005; accepted 13 July 2005; published online 19 September 2005

## Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the United States. More than 56 000 newly diagnosed colorectal cancer patients die each year in this country (Chauhan, 2002). Epidemiological data suggested that dietary modification could reduce the risk of colorectal cancer by as much as 90% (Plummer *et al.*, 1999; Chauhan, 2002). Curcumin, the active ingredient of the rhizome of the plant turmeric (*Curcuma longa*, Linn), has been widely used for centuries as a spice and a coloring agent in foods in Asian countries without toxic effects. Epidemiological results suggested that curcumin might be responsible for the low rate of colorectal cancer in these countries (Mohandas and Desai, 1999). Besides its dietary uses, turmeric has been used in Chinese herbal medicine for skin and gut diseases and wound healing for thousand years. Curcumin possesses antiproliferative, antioxidant, antiinflammatory, antiangiogenic and antitumor effects (Ruby *et al.*, 1995; Chen *et al.*, 1999; Mohandas and Desai, 1999; Mohan *et al.*, 2000). The anticancer potential of curcumin stems from its ability to suppress cell proliferation and to induce apoptosis of a wide variety of tumor cells, including colon cancer and other cancers (Chauhan, 2002; Zheng *et al.*, 2004). However, underlying molecular mechanism of the actions are largely yet to be defined.

Epidermal growth factor receptor (EGFR/erbB1/HER1) is one of the family of four erbB receptors. Activation of EGFR is initiated by binding of ligands, including epidermal growth factor (EGF) and transforming growth factor- $\alpha$ . This results in formation of homo- or hetero-dimers and activation of receptor tyrosine kinase, which, in turn, leads to signaling cascades and regulating expression of target genes. Overexpression and aberrant function of EGFR have been found in a variety of human tumors, including colorectal cancers (Salomon *et al.*, 1995). Aberrant activation of EGFR and the EGF signal pathway is associated with neoplastic cell proliferation, migration, stromal invasion, resistance to apoptosis and angiogenesis (Dancey and Freidlin, 2003). Studies have shown that interruption of EGF signaling impairs tumor growth (Arteaga, 2002; Dancey and Freidlin, 2003).

Thus, EGFR is an attractive target for the development of cancer therapeutics (Baselga, 2000; Ciardiello, 2000; Mendelsohn, 2002).

We recently reported that curcumin activated the peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) in Moser cells, a human colon cancer-derived cell line, leading to inhibition of cell growth by inhibiting tyrosine phosphorylation of EGFR and suppressing gene expression of EGFR and cyclin D1 (Chen and Xu, 2005). The aim of this study was to explore molecular mechanisms underlying curcumin inhibition of *egfr* expression in colon cancer cells. Our results in the current report demonstrated that curcumin reduced expression of *egfr* in human colon cancer-derived cells, including Moser, Caco-2 and HT-29, which required inhibition of the *trans*-activation activity of the transcription factor early growth response-1 (Egr-1). Interruption of the ERK signal pathway was a necessity in curcumin inhibition of Egr-1 gene expression. These results provided novel insights into the mechanisms of curcumin inhibition of colon cancer cell growth and contributed potential therapeutic strategies to the treatment and prevention of colon cancer.

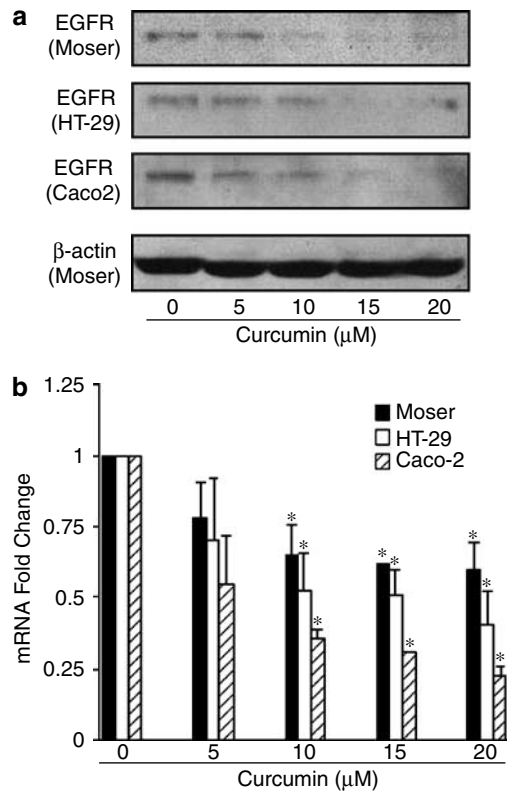
## Results

### *Curcumin inhibits gene expression of EGFR in human colon cancer cells*

We recently observed the inhibitory effect of curcumin on cell proliferation of human colon cancer-derived Moser cells demonstrated by MTS assays (Chen and Xu, 2005) and by colony formation (unpublished data). To elucidate underlying mechanisms, it was hypothesized that curcumin inhibited *egfr* expression in these cells. To test the hypothesis, serum-starved Moser, Caco-2 and HT-29 cells were pretreated with curcumin at indicated concentrations for 30 min prior to the stimulation with fetal bovine serum (FBS) (10%) for an additional 8 h. Whole-cell extracts and total RNA were prepared. Western blotting analyses (Figure 1a) and real-time PCR (Figure 1b) collectively demonstrated that curcumin significantly inhibited gene expression of EGFR in these cells. Immunocytochemical assays confirmed that curcumin reduced the abundance of EGFR in Moser cells (data not shown here). Taken together, these results indicated that curcumin significantly inhibited gene expression of EGFR in human colon cancer cells tested.

### *Curcumin reduces the *egfr* promoter activity in Moser cells, which requires inhibition of the ERK activity*

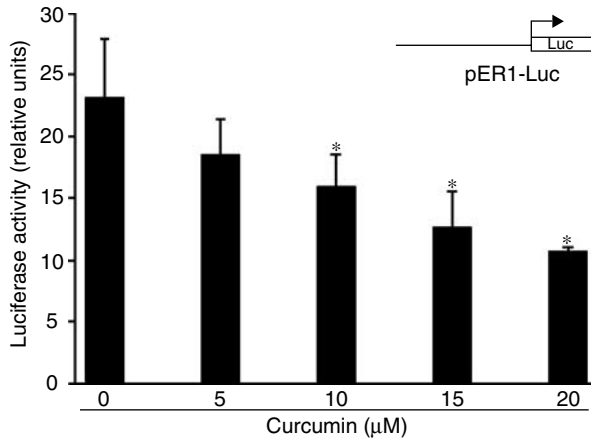
Moser cells were chosen for further exploring molecular mechanisms underlying curcumin inhibition of *egfr* expression. Moser cells were transiently transfected with the *egfr* promoter luciferase reporter plasmid pER1-Luc, in which a fragment of 5'-untranscriptional region of *egfr* promoter (1109 bp nucleotides) was subcloned into the luciferase reporter plasmid pGL3 (Nishi *et al.*, 2002). After serum-starvation for 24 h, cells were



**Figure 1** Curcumin dose dependently suppresses gene expression of EGFR in human colon cancer cells. Serum-starved Moser, Caco-2 and HT-29 cells were pretreated with curcumin at indicated concentrations for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Whole-cell extracts, or total RNA, were prepared for analyses of curcumin effects on expression of EGFR in these cells by Western blotting analyses (a) ( $n = 3$ ), or by real-time PCR (b) ( $n = 3$ ), respectively.  $\beta$ -Actin was used as an internal control for equal loading in all Western blotting analyses.  $\beta$ -Actin in Moser cells was presented here as a representative. For real-time PCR, mRNA fold changes were calculated by using  $\beta$ -actin as an invariant control. Values were expressed as means  $\pm$  s.d. \* $P < 0.05$ , vs cells without curcumin.

pretreated with curcumin at indicated concentrations for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. As shown by luciferase assays in Figure 2, curcumin caused a dose-dependent reduction in luciferase activity, suggesting the inhibitory effect of curcumin on the *egfr* promoter activity.

Others and we have previously demonstrated that curcumin effectively inhibits the extracellular signal-regulated kinase (ERK) in a variety of cells, including colon cancer cells (Chen and Tan, 1998; Chen *et al.*, 1999; Jobin *et al.*, 1999). To determine the role of the ERK signal pathway in curcumin inhibition of the *egfr* promoter activity, Moser cells were cotransfected with the plasmid pER1-Luc and the cDNA expression plasmid pdn-ERK, or pa-ERK, at indicated doses. The plasmids pdn-ERK and pa-ERK contained cDNA encoding the dominant-negative form of ERK (dn-ERK) and the constitutively active form of ERK, respectively, in a plasmid with a human cytomegalovirus (CMV)-driven promoter (Davis *et al.*, 1996). As shown in Figure 3 by luciferase assays, pdn-ERK caused, like

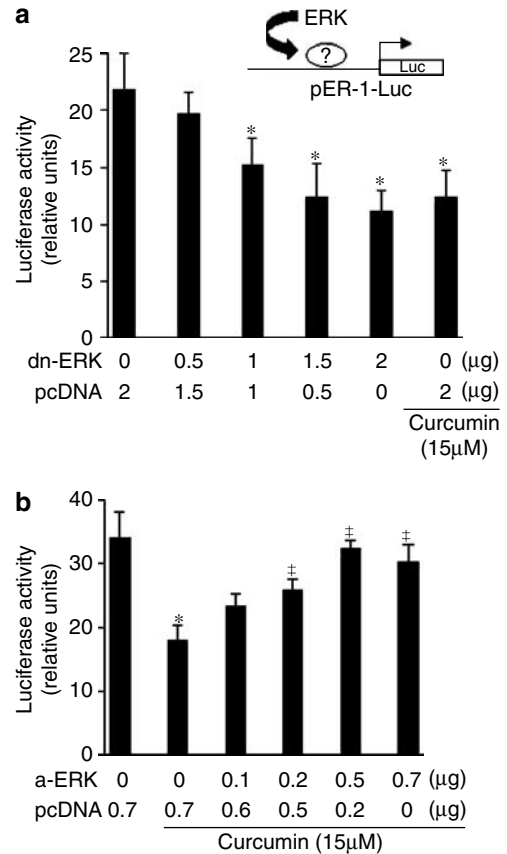


**Figure 2** Curcumin dose dependently reduces the *egfr* promoter activity in Moser cells. Semiconfluent Moser cells were transiently transfected with the *egfr* promoter luciferase reporter plasmid pER1-Luc, in which a fragment of the 5'-untranscriptional region (1109 bp nucleotides) of *egfr* promoter was subcloned into a luciferase reporter plasmid. After serum starvation for 24 h, cells were pretreated with curcumin at indicated concentrations for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Luciferase activity was expressed as relative units after  $\beta$ -galactosidase normalization ( $n=6$ ). Values were expressed as means  $\pm$  s.d. \* $P < 0.05$ , vs cells transfected without curcumin (0).

curcumin, a dose-dependent reduction in luciferase activity (Figure 3a), suggesting that forced expression of dn-ERK mimicked the inhibitory effect of curcumin and inhibited the *egfr* promoter activity in Moser cells. In addition, coupled with an increase in the dose of pa-ERK, the inhibitory effect of curcumin on luciferase activity was progressively abrogated (Figure 3b), suggesting that forced expression of active ERK eliminated the inhibitory effect of curcumin on the gene promoter. Interruption of the ERK signal pathway by the specific MEK inhibitor PD 98059 mimicked the role of curcumin in reducing the *egfr* promoter activity (data not shown). Taken together, these results suggested that curcumin reduced the *egfr* promoter activity in Moser cells, which required inhibition of the ERK activity. Additional experiments in the following would further elucidate the underlying mechanism.

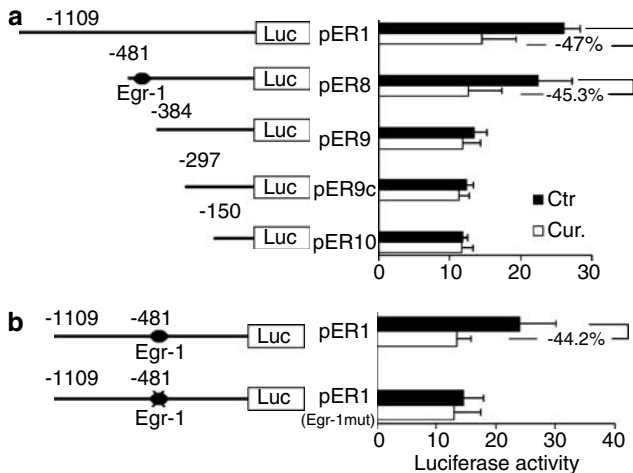
#### The Egr-1-binding site in *egfr* promoter is required for responding to curcumin

To localize curcumin response element(s) in *egfr* promoter, promoter deletion assays were conducted in Moser cells transfected with plasmids containing various length of *egfr* promoter (Figure 4a). After starvation, cells were pretreated with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. As shown in Figure 4a by luciferase assays, curcumin significantly reduced luciferase activity by 47 and 45.3% in cells transfected with pER1-Luc and pER8-Luc, respectively. Loss of the fragment of nucleotides -481 to -384 in pER9-Luc resulted in failure in response to curcumin. These results suggested that the DNA fragment of nucleotides -481 to -384 in the *egfr* promoter might contain curcumin response element(s).



**Figure 3** Inhibition of ERK is required for curcumin to reduce the *egfr* promoter activity. Moser cells in six-well culture plates were cotransfected with the *egfr* promoter luciferase reporter plasmid pER1-Luc (2  $\mu$ g/well), 0.5  $\mu$ g/well of pSV- $\beta$  gal, and the cDNA expression plasmid pdn-ERK (a), or pa-ERK (b) at indicated doses plus the empty vector pcDNA. The amount of DNA of pdn-ERK plus pcDNA was equalized to 2  $\mu$ g/well, while pa-ERK plus pcDNA was equalized to 0.7  $\mu$ g/well. After recovery, cells were serum-starved for 24 h, followed by pretreatment with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Luciferase activity was expressed as relative units after  $\beta$ -galactosidase normalization ( $n=6$ ). Values were expressed as means  $\pm$  s.d. \* $P < 0.05$ , vs cells transfected with no pdn-ERK (a), or pa-ERK (b);  $\ddagger P < 0.05$ , vs cells with no pa-ERK and with curcumin (the second column on the left side in the b).

Computer analyses of the DNA fragment (from -481 to -384) revealed a putative Egr-1-binding site (GCGGGGCC) located within nucleotides from -425 to -433 of the *egfr* promoter. To evaluate the role of the Egr-1-binding site in curcumin inhibition of the gene expression, the plasmid pER1(Egr-1mut)-Luc with site-directed mutations in the Egr-1-binding site was generated from the parental plasmid pER1-Luc. Moser cells were, then, transfected with the two plasmids, respectively. Luciferase assays in Figure 4b demonstrated that curcumin significantly reduced luciferase activity by 44.2% in cells transfected with wild-type pER1-Luc. In great contrast, no significant difference in luciferase activity was observed in cells transfected with mutant pER1(Egr-1mut)-Luc, suggesting that site-directed mutations in the Egr-1-binding site in the plasmid resulted in a loss of the response to curcumin in

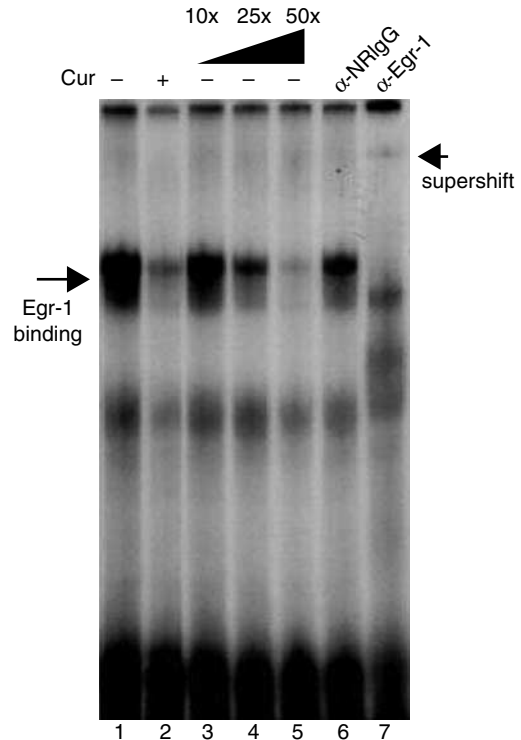


**Figure 4** The Egr-1-binding site in *egfr* promoter is required for responding to curcumin. Semiconfluent Moser cells were transiently transfected with luciferase reporter plasmids with various length of the 5'-untranscriptional region of *egfr* promoter. Cells were then serum-starved for 24 h, followed by pretreatment with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Luciferase activity was expressed as relative units after  $\beta$ -galactosidase normalization ( $n=6$ ). Values were expressed as means  $\pm$  s.d. The numbers next to the bars were the decrease in luciferase activity caused by curcumin treatment. (a) Luciferase assays of cells transfected with the luciferase reporter plasmids containing various length of wild-type *egfr* promoter. (b) Luciferase assays of Moser cells transfected with pER1-Luc or pER1(Egr-1mut)-Luc derived from pER1-Luc with site-directed mutations in the Egr-1-binding site.

Moser cells. These results collectively suggested that the Egr-1-binding site might be the curcumin response element, which played, as a *cis*-activating element, a critical role in regulating the *egfr* promoter activity in response to curcumin.

#### Curcumin significantly reduces the DNA-binding activity of Egr-1 to the curcumin response element

To evaluate the effect of curcumin on the DNA-binding activity of protein(s) to the Egr-1-binding site in *egfr* promoter, electrophoretic mobility shift assays (EMSA) were performed using the  $^{32}$ P-labeled probe P(*egr-1*) with the Egr-1-binding site found in the *egfr* promoter. Nuclear extracts were prepared from serum-starved Moser cells pretreated with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS. As demonstrated in Figure 5 by EMSA, a clear and strong protein-DNA complex was observed in Lane 1. The DNA-binding activity of the protein to the DNA probe was dramatically reduced by curcumin treatment (Lane 2). To examine the DNA-binding specificity of the protein to the probe, competition assays were performed using a 10-, 25- or 50-fold excess of the unlabeled probe P(*egr-1*) (Lanes 3, 4 and 5). It was found that the amount of the protein binding to the  $^{32}$ p-labeled probe P(*egr-1*) was competitively reduced by the cold probe P(*egr-1*) in a dose-dependent manner (Lanes 3, 4 and 5). These results suggested that this protein specifically bound to the Egr-1-binding site found in the *egfr* promoter. Addition of anti-Egr-1 antibodies



**Figure 5** Curcumin significantly reduces the DNA-binding activity of Egr-1 to the curcumin response element. Serum-starved Moser cells were pretreated with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Nuclear extracts were prepared for EMSA using the  $^{32}$ P-labeled probe P(*egr-1*) with the Egr-1-binding site found in *egfr* promoter (Lanes 1–7). A 10-, 25- or 50-fold excess of the unlabeled probe P(*egr-1*) was used in competition assays (Lanes 3–5). In all, 1  $\mu$ M of normal rabbit IgG (NRIgG) or anti-Egr-1 antibodies were used in supershift assays (Lanes 6 or 7, respectively). A representative EMSA was shown from three independent experiments.

caused disappearance of the binding band and a weak supershifting band (Lane 7). However, normal rabbit IgG (NRIgG) had no significant effect on the binding band (Lane 6). These results indicated that the protein binding to the Egr-1-binding site was the transcription factor Egr-1. Taken together, our results suggested that Egr-1 bound, presumably as a *trans*-activating factor, to the Egr-1-binding site, as the curcumin response element, in the *egfr* promoter. The DNA-binding activity of Egr-1 was significantly inhibited by curcumin.

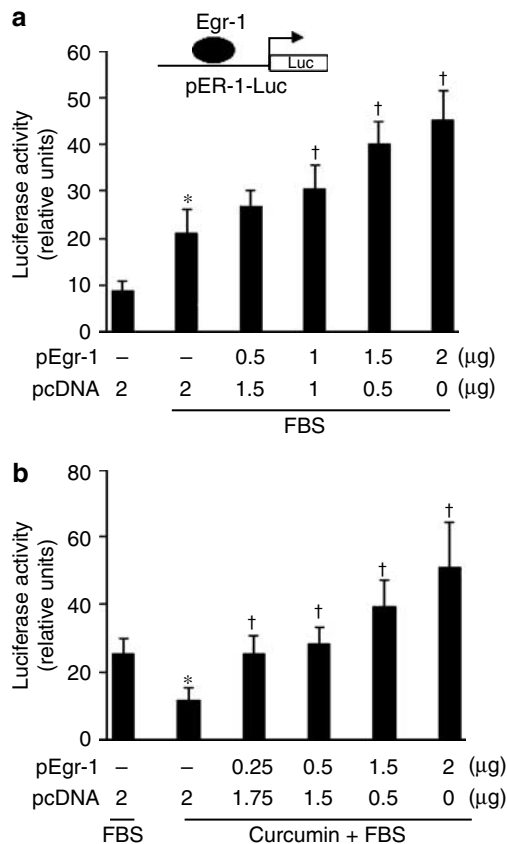
#### Egr-1 acts as a trans-activating factor in stimulating the *egfr* promoter activity in Moser cells

To evaluate the role of Egr-1 in regulating the *egfr* promoter activity, Moser cells were cotransfected with the *egfr* promoter luciferase reporter plasmid pER1-luc and the cDNA expression plasmid pEgr-1cDNA, containing Egr-1 cDNA in a CMV-driven expression vector. After recovery, cells were serum-starved for 24 h followed by pretreatment with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS. Luciferase assays demonstrated that forced expression of Egr-1 cDNA dose-dependently induced luciferase activity in the cells (Figure 6a). Furthermore, forced

expression of Egr-1 cDNA ameliorated, in a dose-dependent manner, the inhibitory effect of curcumin on luciferase activity (Figure 6b). These results collectively indicated that the transcription factor Egr-1 acted as a *trans*-activating factor in stimulating the *egfr* promoter activity in Moser cells. Similar results were observed in Caco-2 and HT-29 (data not shown).

#### Curcumin suppresses gene expression of Egr-1 in colon cancer cells

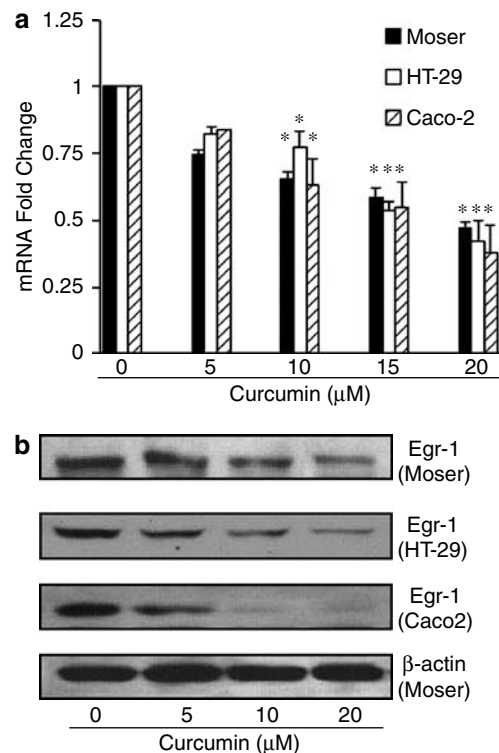
Additional experiments were performed to explore the mechanisms of curcumin reduction of the *trans*-activation activity of Egr-1. Serum-starved Moser, HT-29 and Caco-2 cells were pretreated with curcumin at indicated concentrations for 30 min prior to the stimulation with



**Figure 6** Egr-1 acted as a *trans*-activating factor in stimulating the *egfr* promoter activity in Moser cells. Moser cells in six-well cell culture plates were cotransfected with the *egfr* promoter luciferase reporter plasmid pER-1-Luc and the plasmid pEgr-1, containing Egr-1 cDNA in a CMV-driven expression vector. A total of 4.5  $\mu$ g of plasmid DNA was used in each well for the transfection, including 2  $\mu$ g of pER1-Luc, 0.5  $\mu$ g of pSV- $\beta$  gal, pEgr-1cDNA at indicated doses and the empty vector pcDNA. The amount of DNA of pEgr-1cDNA plus pcDNA was equalized to 2  $\mu$ g. Cells were serum-starved for 24 h, followed by pretreatment with (b), or without curcumin (a) at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Luciferase activity was expressed as relative units after  $\beta$ -galactosidase normalization ( $n=6$ ). Values were expressed as means  $\pm$  s.d. \* $P<0.05$ , vs cells cotransfected with no pEgr-1, the first column on the left-side in each panel; <sup>†</sup> $P<0.05$ , vs cells cotransfected with no pEgr-1, the second column on the left side in each panel.

FBS. Total RNA and whole cell extracts were prepared from the cells. Real-time PCR (Figure 7a) and Western blotting analyses (Figure 7b), respectively, demonstrated that curcumin significantly reduced the steady-state level of Egr-1 mRNA and the abundance of Egr-1 protein in these cells, suggesting that curcumin suppressed gene expression of Egr-1. Taken together, our results demonstrated that curcumin suppressed Egr-1 gene expression in human colon cancer cells, which might lead to reduction of the *trans*-activation activity of Egr-1.

*Interruption of the ERK signal pathway is required for curcumin to reduce the trans-activation activity of Egr-1*  
To elucidate the mechanism underlying the requirement of inhibition of the ERK activity in curcumin suppression of *egfr* expression observed in Figure 3, it was hypothesized that the inhibitory effect of curcumin was mediated by reducing the *trans*-activation activity of Egr-1 through inhibition of the ERK activity. To evaluate the effect of the ERK activity on the *trans*-activation activity of Egr-1, Moser cells were cotransfected with the Egr-1 luciferase reporter plasmid

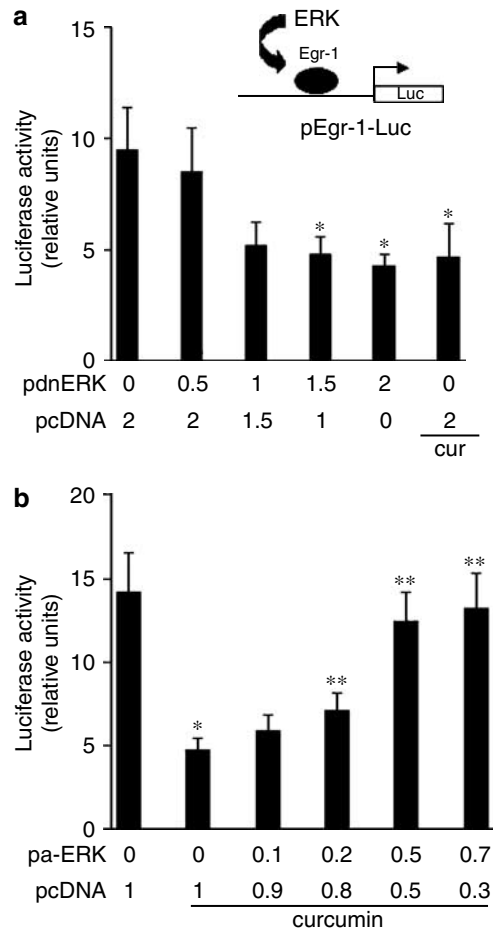


**Figure 7** Curcumin dose-dependently suppresses *egr-1* expression in colon cancer cells. Serum-starved Moser, HT-29 and Caco-2 cells were pretreated with curcumin at indicated concentrations for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Total RNA or whole cell extracts were prepared for analyses of curcumin effects on gene expression of EGFR by real-time PCR (a) ( $n=3$ ), or Western blotting analyses (b) ( $n=3$ ), respectively. Fold changes of mRNA were calculated using  $\beta$ -actin as an invariant control for real-time PCR. Values were expressed as means  $\pm$  s.d. \* $P<0.05$ , vs cells without curcumin.  $\beta$ -actin in Moser cells, as a representative for the others, was used as an internal control for equal loading in Western blotting analyses.

pEgr-1-Luc, containing a DNA fragment with an Egr-1-binding site in a luciferase reporter vector, and the dn-ERK cDNA expression plasmid pdn-ERK, or the active ERK cDNA expression plasmid pa-ERK, at indicated concentrations. After serum starvation for 24 h, cells were pretreated with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%). As shown in Figure 8 by luciferase assays, forced expression of dn-ERK mimicked the inhibitory effect of curcumin and caused a dose-dependent reduction in luciferase activity (Figure 8a), suggesting that interruption of the ERK signal pathway led to reduction of the *trans*-activation activity of Egr-1. In addition, forced expression of active ERK eliminated the inhibitory effect of curcumin and increased luciferase activity in a dose dependent manner (Figure 8b), indicating that activation of the ERK signal pathway increased the *trans*-activation activity of Egr-1. Taken together, these results indicated that inhibition of the ERK activity was required for curcumin to reduce the *trans*-activation activity of Egr-1, leading to suppression of the *egfr* promoter activity previously observed in Figure 3.

*Curcumin dramatically reduces the level of phosphorylation of Elk-1 and its activity in colon cancer cells*

Further experiments were performed to elucidate mechanisms of curcumin inhibition of Egr-1 gene expression. Deletion analyses of the Egr-1 gene promoter previously identified several serum response elements (SRE), which jointly bound the transcription factor Elk-1 and serum response factor (SRF) (Chen *et al.*, 2004; Cohen *et al.*, 1996). Elk-1 is a substrate of ERK and activated by phosphorylation (Treisman, 1994). These prior results prompted us to assume that curcumin suppressed gene expression of Egr-1 observed in Figure 7 by reducing the level of phosphorylation and the activity of Elk-1 through inhibition of the ERK activity. To study the assumption, serum-starved cells of Moser, HT-29 and Caco-2 were pretreated with or without curcumin at indicated concentrations for 30 min followed by the stimulation with FBS (10%) for an additional 1 h. Pilot experiments observed that FBS rapidly induced phosphorylation of ERK in no more than 2 min in these cells, which reached its peak within 30 min (data not shown). Whole cell extracts were prepared for Western blotting analyses. As shown in Figure 9a, curcumin dose dependently reduced the level of phosphorylated Elk-1 in these cells. Luciferase assays in Figure 9b revealed that curcumin reduced, in a dose-dependent manner, luciferase activity in cells transfected with the PathDetect<sup>®</sup> Elk1 *trans*-reporting system. The system contained a construct encoding a fusion protein with the GAL4 DNA-binding domain and the *trans*-activation domain of Elk (GAL4-Elk-1). Phosphorylation of the *trans*-activation domain of the fusion GAL-Elk-1 protein by ERK activated the fusion protein and induced its DNA-binding domain to bind to the GAL4-binding sites in pFR-Luc, leading to expression of the luciferase gene from the reporter plasmid. Taken

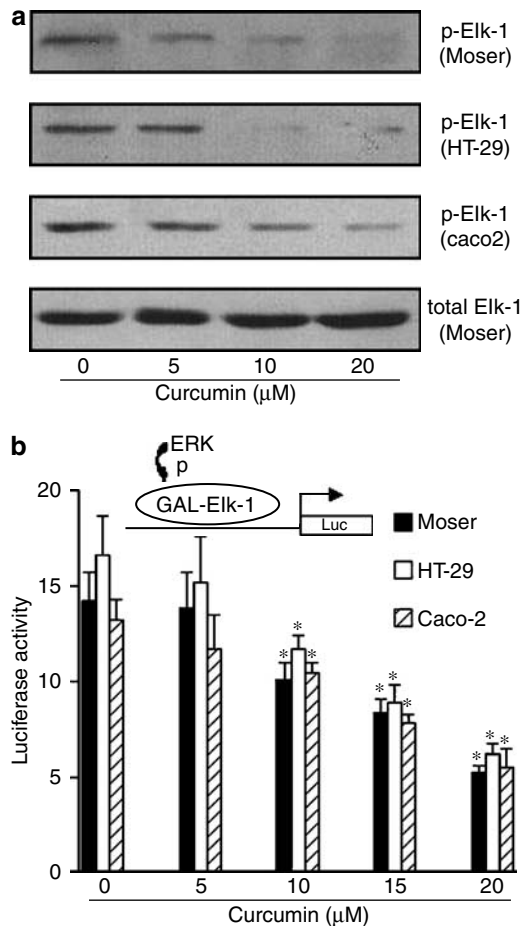


**Figure 8** Interruption of the ERK pathway is required for curcumin to reduce the *trans*-activation activity of Egr-1. Moser cells in six-well culture plates were cotransfected with the Egr-1 luciferase reporter plasmid pEgr-1-Luc (2  $\mu$ g/well), 0.5  $\mu$ g/well of pSV- $\beta$  gal, and the cDNA expression plasmid pdn-ERK (a), or pa-ERK (b) at indicated doses plus the empty vector pcDNA. The amount of DNA of pdn-ERK plus pcDNA was equalized to 2  $\mu$ g/well, while pa-ERK plus pcDNA was equalized to 1  $\mu$ g/well. After recovery, cells were serum-starved for 24 h, followed by pretreatment with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Luciferase activity was expressed as relative units after  $\beta$ -galactosidase normalization ( $n=6$ ). Values were expressed as means  $\pm$  s.d. \* $P < 0.05$ , vs cells transfected with no pdn-ERK (a), or no pa-ERK (b) (the first column on the left side in each a and b); \*\* $P < 0.05$ , vs cells with no pa-ERK and with curcumin (the second column on the left side in b).

together, our results indicated that curcumin dramatically reduced the *trans*-activation activity of Elk-1 in colon cancer cells, presumably by inhibition of the ERK activity.

**Discussion**

EGFR has been identified as a rational target for anticancer therapy (Ciardiello, 2000; Mendelsohn, 2002). Among the classes of agents targeting EGFR in clinical development are monoclonal antibodies against



**Figure 9** Curcumin reduces the level of phosphorylation of Elk-1 and its activity in colon cancer cells. **(a)** To evaluate the effect of curcumin on Elk-1 phosphorylation, serum-starved cells of Moser, HT-29 and Caco-2 were pretreated with curcumin at indicated concentrations for 30 min followed by addition of FBS (10%) for an additional 1 h. Whole cell extracts were prepared for Western blotting analyses of phosphorylated Elk-1 detected by anti-phospho-Elk-1 antibodies. Total Elk-1 was then detected by anti-Elk-1 antibodies as a control for equal loading. Total Elk-1 in Moser cells was presented here as a representative for the others. Representative results from three independent experiments were shown. **(b)** To evaluate the effect of curcumin on the Elk-1 *trans*-activation activity, Moser, HT-29 and Caco-2 cells were transfected with the PathDetect<sup>®</sup> Elk1 *trans*-reporting system. Cells were serum-starved for 24 h, followed by pretreatment with curcumin at indicated concentrations for 30 min prior to the addition of FBS (10%) for an additional 24 h. Luciferase activity was expressed as relative units after  $\beta$ -galactosidase normalization ( $n=6$ ). Values were expressed as means  $\pm$  s.d. \* $P<0.05$ , vs cells with no curcumin treatment.

the extracellular ligand-binding domain of the receptor (Baselga, 2000), and small molecules that inhibit activation of the receptor tyrosine kinase (Baselga and Averbuch, 2000; Hidalgo *et al.*, 2001). We recently observed that the FDA-proved anti-EGFR monoclonal antibodies IMC-C225, used for treatment of colorectal cancer, dramatically inhibited cell growth of Moser, Caco-2 and HT-29 cells (unpublished data), confirming the important role of inhibition of EGFR activation/signaling in treatment of colon cancer. The advantage of

curcumin as a potential agent for cancer therapy is that this phytochemical possesses the capabilities to inhibit receptor tyrosine phosphorylation and suppress gene expression of EGFR in colon cancer cells (Chen and Xu, 2005). These actions lead to instant and long-lasting effects on interrupting the EGF signal pathway. The current study focused on elucidating molecular mechanisms underlying the inhibitory effect of curcumin on regulating gene expression of EGFR in colon cancer cells. Our results demonstrated that curcumin suppressed *egfr* expression in human colon cancer-derived cells by reducing the *trans*-activation activity of the transcription factor Egr-1 through inhibition of the ERK activity. Additional experiments are ongoing in our lab to elucidate the mechanisms of activation of PPAR $\gamma$  in curcumin inhibition of *egfr* expression in colon cancer cells (Chen and Xu, 2005). Most of the inhibitory effects of curcumin, including the ones observed in the present report, are in the range of  $10^{-5}$ – $10^{-4}$  M *in vitro*, which is much higher than those observed in blood and tissues of human and animals (Ammon and Wahl, 1991; Pan *et al.*, 1999). However, it bears emphasis that because the *in vivo* system is multifactorial and more complicated, directly extrapolating *in vitro* conditions and results, such as effective concentrations, to the *in vivo* system might be misleading.

Accumulating evidence has demonstrated the property of curcumin on inhibition of colon cancer development (Chauhan, 2002). However, underlying mechanisms remain largely to be defined. We recently reported that activation of PPAR $\gamma$  by curcumin inhibited Moser cell growth and mediated the suppression of gene expression of cyclin D1 and EGFR (Chen and Xu, 2005). In this report, we further observed that inhibition of the *trans*-activation activity of Egr-1 by curcumin played a critical role in suppressing *egfr* expression in colon cancer cells. Curcumin modulation of the Egr-1 activity has been observed in other cell types (Giri *et al.*, 2004). Egr-1 is a nuclear phospho-protein, which binds to a specific GC-rich sequence in the promoter region of many genes and regulates expression of these target genes, including those involved in regulation of cell growth and differentiation (Liu *et al.*, 1996; Thiel and Cibelli, 2002). We previously observed that *egfr* promoter region between –484 and –389, which contained a putative Egr-1 consensus motif, was crucial for the expression of EGFR (Nishi *et al.*, 2002). Egr-1 mediated upregulation of EGFR expression in human cancer cells during hypoxia (Nishi *et al.*, 2002), suggesting that stimulation of the *egfr* promoter activity by Egr-1 represented a mechanism for cancer cell growth. It was assumed that curcumin inhibition of the Egr-1 *trans*-activation activity stemmed from suppressing *egfr* expression.

The current report demonstrated that curcumin significantly suppressed gene expression of Egr-1 at both transcription and translation levels (Figure 7), which is consistent with previous other reports (Pendurthi *et al.*, 1997; Han *et al.*, 1999; Pendurthi and Rao, 2000). Others and we have previously demonstrated that curcumin effectively inhibits the ERK activity in a

variety of cells, including colon cancer cells (Chen and Tan, 1998; Chen *et al.*, 1999; Jobin *et al.*, 1999). Curcumin inhibition of gene expression of Egr-1 requires interruption of the ERK signal pathway (Fujita *et al.*, 2004; Harja *et al.*, 2004; Schaefer *et al.*, 2004). *egr-1* promoter deletion analyses and gel shift assays identified a *cis*-activating element (nucleotides -376 to -350) binding to the transcription factor Elk-1 (Chen *et al.*, 2004). Expression of *egr-1* is induced by the ternary complex factor Elk-1, which is phosphorylated and activated by ERK1/2 (Janknecht *et al.*, 1993; Gineitis and Treisman, 2001; Andrade *et al.*, 2004). Once phosphorylated by ERK, activated Elk-1 interacts with SRF to bind jointly to SRE in *egr-1* promoter, leading to a rapid transcriptional response to various extracellular stimuli (McMahon and Monroe, 1995; Cohen *et al.*, 1996; Watson *et al.*, 1997). Results in the present report demonstrated that curcumin significantly reduced the level of phosphorylated Elk-1 and its *trans*-activation activity in colon cancer cells tested, presumably by inhibiting the ERK activity (Figure 9), leading to inhibition of *egr-1* expression. The role of phosphorylation of Egr-1 remains controversial (Cao *et al.*, 1993; Jain *et al.*, 1996; Huang *et al.*, 1998; Kamimura *et al.*, 2004). We recently observed that curcumin reduced the level of phosphorylation of Egr-1, which was mimicked by a specific MEK inhibitor (data not shown here). Additional experiments are necessary to determine the role of the phosphorylation in curcumin inhibition of the *trans*-activation activity of Egr-1 in colon cancer cells.

Based on our observations, a model was proposed to elucidate the inhibitory effects of curcumin on *egfr* expression, leading to inhibition of colon cancer cell growth (Figure 10). Curcumin reduces the *trans*-activation activity of the transcription factor Egr-1 by suppressing *egr-1* expression, resulting from interrupting the ERK signal pathway and reducing the activity of Elk-1. Reduction of the Egr-1 activity plays a critical role in inhibition of *egfr* expression in human colon cancer cells. Suppression of the *egfr* expression and inhibition of receptor tyrosine phosphorylation of

EGFR interrupt EGF signaling (Chen and Xu, 2005), which collectively contribute to curcumin inhibition of colon cancer cell growth. It bears emphasis that interruption of EGF signaling, including reducing receptor tyrosine phosphorylation and suppressing *egfr* expression, is not the exclusive mechanism underlying curcumin inhibition of colon cancer cell growth. This model does not exclude any other mechanisms involved. Our results provided novel insights into the mechanisms of curcumin inhibition of cancer cell growth. The characteristics of curcumin with no adverse health effects make it an excellent candidate for prevention and treatment of colon cancer.

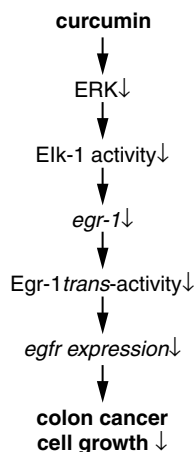
## Materials and methods

### Material and cell culture

Moser cells, a human colon carcinoma-derived cell line, was a gift from Dr Michael G Brattain (Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY, USA) (Levine *et al.*, 1985). Cells were cultured in modified McCoy's 5A medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with FBS (10%). Both Caco-2 and HT-29 cells, human colon carcinoma-derived cell lines, were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). HT-29 cells were incubated in modified McCoy's 5A medium supplemented with FBS (10%), while Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with FBS (10%), as we previously described (Chen *et al.*, 2002a, b). To stimulate gene expression and evaluate the effect of curcumin, semiconfluent cells were rendered quiescent by serum starvation for 24 h in media with no FBS, followed by pretreatment with curcumin for 30 min prior to the stimulation with FBS (10%) in media for an additional indicated time. Curcumin at no more than 20  $\mu$ M is not toxic to Moser cells (Chen and Xu, 2005). Curcumin (purity >94%) was purchased from Sigma (St Louis, MO, USA). The stock solution of curcumin at 10 mM was prepared with ethanol. Media with curcumin was replaced every 24 h without specific indication.

### Plasmids

The *egfr* promoter luciferase reporter plasmids, containing a fragment of 5'-untranscriptional region of *egfr* promoter in a luciferase reporter plasmid, were described previously (Nishi *et al.*, 2002). The cDNA expression plasmids pdn-ERK and pa-ERK contained cDNA encoding the dominant negative form of ERK and the constitutively active form of ERK, respectively (Davis *et al.*, 1996). The Egr-1 luciferase reporter plasmid pEgr-1-Luc contained an Egr-1-binding site in the luciferase reporter vector pGL3 (Nishi *et al.*, 2002). PathDetect<sup>®</sup> Elk1 *trans*-Reporting System was purchased from Stratagene (Cedar Creek, TX, USA). This system includes a GAL-Elk-1 *trans*-activator plasmid and the luciferase reporter plasmid pFR-Luc. The GAL-Elk-1 *trans*-activator plasmid expresses a fusion *trans*-activator protein, consisting of the activation domain of the transcriptional activator Elk-1 fused with the yeast GAL4 DNA-binding domain. pFR-Luc contains a synthetic promoter with five tandem repeats of the yeast GAL4-binding sites that controls expression of the *Photinus pyralis* (American firefly) luciferase gene. Phosphorylation of the *trans*-activation domain of the fusion GAL-Elk-1 protein by ERK activates the fusion protein and induces its DNA-binding domain to bind to the GAL4-binding sites in



**Figure 10** Schema of the putative mechanisms of curcumin suppression of gene expression of EGFR, leading to inhibition of human colon cancer cell growth.

pFR-Luc, leading to expression of the luciferase gene from the reporter plasmid.

#### Transient transfection assays

Semiconfluent cells in six-well plastic plates were transiently transfected using the LipofectAMINE<sup>®</sup> reagent (Life Technologies, Grand Island, NY, USA). Each sample (total 3–4  $\mu$ g DNA/well) was performed in triplicate in every experiment. Luciferase assays were performed as previously described (Xu *et al.*, 2003). After recovery, without specific indication, cells were serum-starved for 24 h, followed by pretreatment with curcumin at indicated concentrations for 30 min prior to the stimulation with FBS (10%) for an additional indicated time. Transfection efficiency was determined by cotransfection of a  $\beta$ -galactosidase reporter, pSV- $\beta$  gal (0.5  $\mu$ g/well) (Promega).  $\beta$ -Galactosidase activity was measured by using a  $\beta$ -galactosidase assay kit (Promega), according to the manufacturer's instructions. Each experiment was independently repeated at least three times. 'Luciferase activity (relative units)' was determined after normalization with  $\beta$ -galactosidase activity based on the same amount of protein ( $\mu$ g/ml), that is, Luminescence per  $\beta$ -galactosidase unit per  $\mu$ g/ml of protein. Transfection results were expressed in relative luciferase activity as means  $\pm$  standard deviation (s.d.).

#### Site-directed mutageneses

The plasmid pER1(Egr-1mut) was derived from pER-1-Luc, with site-directed mutations in the Egr-1-binding site using the GeneEditor<sup>™</sup> *in vitro* site-directed mutagenesis system (Promega). The sequence containing the Egr-1-binding site 5'-GAC TAG GCC CGC **GGG** GGC CAC CGC TG-3' in pER-1-Luc was changed to 5'-GAC TAG GCC CGC **TAT** GGC CAC CGC TG-3' in pER1(Egr-1mut)-Luc. The site-directed mutageneses generated a new restriction enzyme MscI cutting site in pER1(Egr-1mut)-Luc. The mutations were confirmed by DNA sequencing.

#### Electrophoretic mobility shift assay

Serum-starved Moser cells were pretreated with or without curcumin at 15  $\mu$ M for 30 min prior to the stimulation with FBS (10%) for an additional 8 h. Nuclear extracts from cells were prepared and EMSA was performed as previously described (Chen *et al.*, 1999). The probe P(egr-1) used in EMSA contained double-stranded oligonucleotides of the Egr-1-binding site found in *egfr* promoter. The probe P(egr-1) of 5'-GAC TAG GCC CGC GGG GGC CAC CGC TG-3' was synthesized by GIBCOBRL. (Grand Island, NY USA).

#### Western blotting analyses

Semiconfluent cells were rendered quiescent by serum starvation for 24 h in media with no FBS, followed by pretreatment with curcumin at indicated concentrations for 30 min prior to the stimulation with FBS (10%) for an additional indicated time. Whole cell protein extracts were prepared as previously described. SDS/PAGE with 10% resolving gel was used to separate proteins (25  $\mu$ g/lane). Target proteins were detected

by using primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized by using chemiluminescence reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA).

#### RNA isolation and real-time PCR

Total RNA was isolated by TRI-Reagent (Sigma), following the protocol provided by the manufacturer. Real-time PCR was carried out as previously described (Xu *et al.*, 2003). In brief, DNase I-treated total RNA (1  $\mu$ g) was used for synthesis of the first strand of cDNA. Reverse transcription conditions were as follows: 42°C for 15 min, 95°C for 5 min and 5°C for 5 min (one cycle). Real-time PCR was carried out in 25  $\mu$ l of reaction solution (2.5  $\mu$ l of 10  $\times$  buffer, 5 mM dNTPs, 10 mM MgCl<sub>2</sub>, 200 nM primers and 0.75 units of platinum<sup>®</sup> *Taq* polymerase; all from Invitrogen) plus 1  $\mu$ l of SYBR Green (1:2000; BioWhittaker, Richland, ME, USA). No genomic DNA contamination or pseudogenes were detected by PCR without the reverse transcription step in the total RNA used. The reactions started at 95°C for 7 min, followed by 40 cycles of 95°C for 20 s, 54°C for 30 s and 72°C for 30 s. Melting peaks of PCR products were determined by heat-denaturing them over a 35°C temperature gradient at 0.2°C/s from 60 to 95°C. mRNA-fold changes in target genes relative to the  $\beta$ -actin control were calculated as suggested by Schmittgen (Schmittgen *et al.*, 2000). Primers used in real-time PCR were:

EGFR: (F) 5'-GTG ACC GTT TGG GAG TTG ATG A-3', (R) 5'-GGC TGA GGG AGG CGT TCT C-3'; Egr-1: (F) 5'-GCC TGC GAC ATC TGT GGA A-3', (R) 5'-GCC GCA AGT GGA TCT TGG TA-3';  $\beta$ -actin: (F) 5'-GGG GGA AAT GGT GGG TGA CAT-3', (R) 5'-GAT GGA GTT GAA GGT AGT TTC-3'.

#### Statistical analysis

Differences between means were evaluated using an unpaired two-sided Student's *t*-test ( $P < 0.05$  was considered significant). Where appropriate, comparisons of multiple treatment conditions with control were analyzed by ANOVA with the Dunnett's test for *post hoc* analysis.

#### Abbreviations

EGFR, epidermal growth factor receptor; Egr-1, early growth response-1; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; PPAR $\gamma$ , peroxisome proliferator-activated receptor-gamma.

#### Acknowledgements

The work was supported by the grants to A Chen, from Feist-Weiller Cancer Center, LSUHSC-S, DK 47995 from NIH/NIDDK and starting funds from Department of Pathology, LSUHSC-S.

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