Research Article

# Curcumin, a Dietary Component, Has Anticancer, Chemosensitization, and Radiosensitization Effects by Down-regulating the *MDM2* Oncogene through the PI3K/mTOR/ETS2 Pathway

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## Abstract

**The oncoprotein MDM2, a major ubiquitin E3 ligase of tumor suppressor p53, has been suggested as a novel target for human cancer therapy based on its p53-dependent and p53-independent activities. We have identified curcumin, which has previously been shown to have anticancer activity, as an inhibitor of MDM2 expression. Curcumin down-regulates MDM2, independent of p53. In a human prostate cancer cell lines PC3 (*p53$^{null}$*), curcumin reduced MDM2 protein and mRNA in a dose- and time-dependent manner, and enhanced the expression of the tumor suppressor p21$^{Waf1/CIP1}$. The inhibitory effects occur at the transcriptional level and seem to involve the phosphatidylinositol 3-kinase/mammalian target of rapamycin/erythroblastosis virus transcription factor 2 pathway. Curcumin induced apoptosis and inhibited proliferation of PC3 cells in culture, but both MDM2 overexpression and knockdown reduced these effects. Curcumin also inhibited the growth of these cells and enhanced the cytotoxic effects of gemcitabine. When it was administered to tumor-bearing nude mice, curcumin inhibited growth of PC3 xenografts and enhanced the antitumor effects of gemcitabine and radiation. In these tumors, curcumin reduced the expression of MDM2. Down-regulation of the *MDM2* oncogene by curcumin is a novel mechanism of action that may be essential for its chemopreventive and chemotherapeutic effects. Our observations help to elucidate the process by which mitogens up-regulate MDM2, independent of p53, and identify a mechanism by which curcumin functions as an anticancer agent.** [Cancer Res 2007;67(5):1988–96]

## Introduction

MDM2, the cellular ubiquitin E3 ligase of the tumor suppressor p53, is considered to be an oncoprotein because of its activity in promoting p53 ubiquitination and proteasomal degradation (1). Further, MDM2 binds to the $NH_2$ terminus of p53 and blocks its transactivational activities (1). The activation of *p53* target genes induces apoptosis, cell cycle arrest, and senescence, which are important to tumor suppression (2, 3). Recently, p53-independent tumorigenic mechanisms for MDM2 have been identified (4). MDM2 also binds to other proteins with a wide range of functions (4). For example, MDM2 promotes cell cycle progression by binding to and modulating the activities of p21$^{Waf1/CIP1}$ (5, 6) and E2F1 proteins (7). Both animal studies with transgenic mice and clinical observations have established the role of MDM2 in cancer development and the response to treatment, both dependent and independent of p53 (8, 9).

Because overexpression of *MDM2* in human cancers is associated with a poor prognosis (10), *MDM2* is considered to be a target for human cancer therapy (11–13). To test this hypothesis, we have developed a second generation of antisense oligonucleotides specifically targeting human *MDM2*. These oligonucleotides have anticancer, chemosensitization, and radiosensitization effects *in vitro* and *in vivo* in a wide range of human cancer models, regardless of their *p53* status (14–20).

Cancer prevention has been stressed increasingly because human cancers are frequently at late stages and are incurable when detected. Considering the role of MDM2 in the development and treatment of cancer, we evaluated various dietary components for inhibition of *MDM2*. Our initial studies identified genistein, an isoflavone from soybeans, as having anticancer effects through down-regulation of *MDM2* expression (21). We have now found that another dietary component reduces MDM2 levels in human cancer cells.

Curcumin (diferuloylmethane), a component of the spice tumeric, has numerous medicinal properties and seems to be useful in the prevention and treatment of cancer (22, 23). This compound has the capacity to reduce proliferation of a variety of malignant and normal cells; to induce apoptosis; and to suppress tumor initiation, promotion, and metastasis. It also has antiangiogenic, anti-inflammatory, and anti-oxidant properties. Although inhibition of tyrosine kinases, sensitization to tumor necrosis factor–related apoptosis-inducing ligand–mediated apoptosis, and other mechanisms have been implicated in the biological effects of curcumin (22, 23), its primary molecular target and mechanism of action remain to be clarified.

In the present study, we have shown that curcumin down-regulates *MDM2* expression in cells with either wild-type (WT) or nonfunctional p53 and that this effect is at the transcriptional level. *MDM2* transcription is regulated by the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR)/erythroblastosis virus transcription factor 2 (ETS2) pathway, which is modulated by curcumin. Further, curcumin has anticancer, chemosensitization, and radiosensitization effects in human cancer models *in vitro* and *in vivo*, independent of p53. Inhibition of *MDM2* expression seems to be important for these effects of curcumin. Our studies help to elucidate the nature of regulation of *MDM2* expression and the mechanism by which curcumin functions as an anticancer agent.

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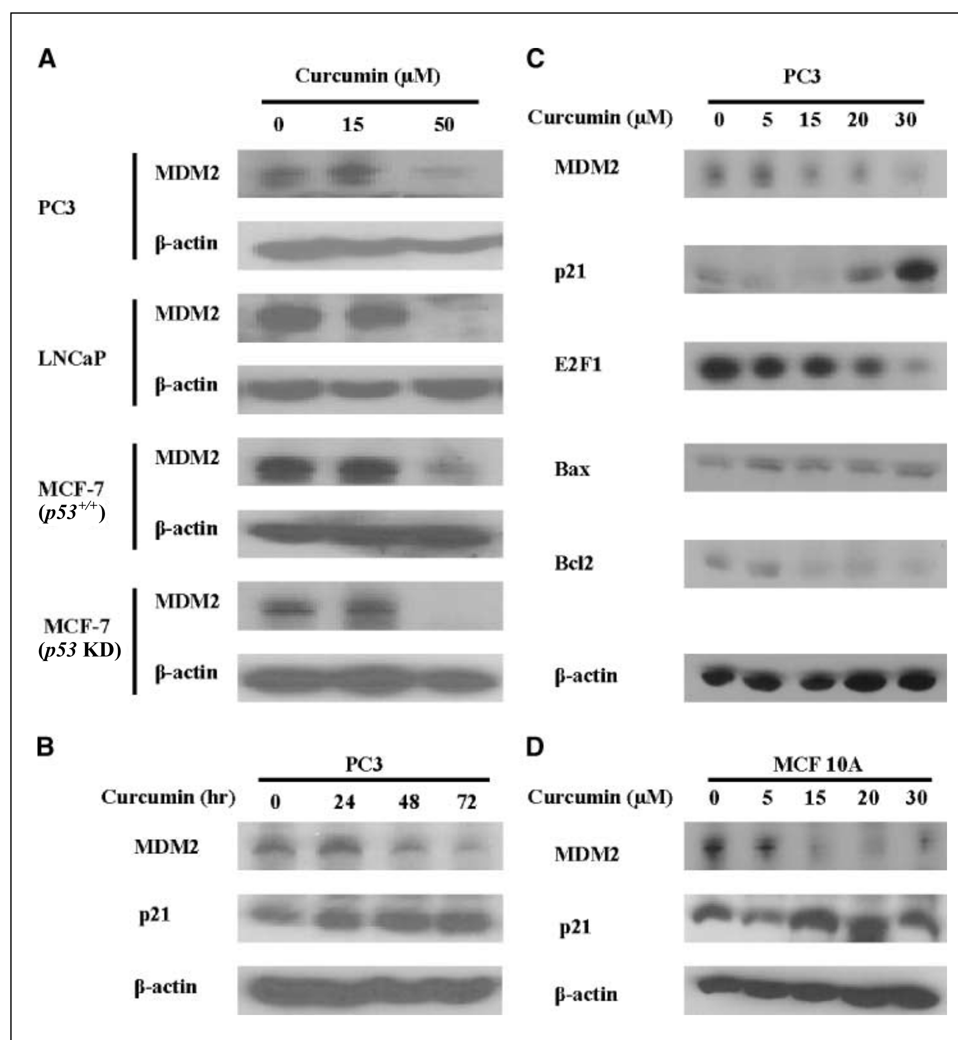


**Figure 1.** Changes in expression of *MDM2* and related genes in cells exposed to curcumin. *A,* PC3, LNCaP, MCF-7 (*p53*$^{+/+}$), and MCF-7 (*p53* KD) cells were exposed to various concentrations of curcumin for 24 h, and target proteins (MDM2, p21, Bcl2, E2F1, Bax, and β-actin) were detected by immunoblotting with specific antibodies. *B,* PC3 cells were exposed to curcumin (15 μmol/L) for various times, and the protein expression levels of MDM2 and p21 were assessed. *C,* PC3 cells were exposed to various concentrations of curcumin for 24 h, and the expression levels of various proteins were assessed. *D,* human normal breast cells, MCF10A, were exposed to various concentrations of curcumin for 24 h, and MDM2 and p21 levels were determined.

## Materials and Methods

**Chemicals, plasmids, animals, and cell culture.** Curcumin and gemcitabine were purchased from LKT Laboratories (St. Paul, MN) and Eli Lilly (Indianapolis, IN), respectively. (−)-Deguelin, PD98059, rapamycin, wortmannin, propidium iodide, and crystal violet were obtained from Sigma (St. Louis, MO). ETS2 overexpression plasmids and control plasmids were provided by Dr. D.K. Watson (Hollings Cancer Center, Charleston, SC; ref. 24). ETS2 double-stranded small interfering RNA pool, control nontargeting siRNA pool, and the transfection reagent DharmaFECT 1 were purchased from Dharmacon (Lafayette, CO). WT, constitutive-active, and dominant-negative Akt vectors were gifts from Dr. A. Nicolin (University of Milan, Milan, Italy; ref. 25). The control vector of Akt expression plasmids, pUSEamp(+), was purchased from Upstate (Lake Placid, NY). Male athymic nude mice (nu/nu), 4 to 6 weeks old, were obtained from Frederick Cancer Research and Development Center (Frederick, MD). Human prostate cancer PC3 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD) and cultured in Ham's F-12 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. Stable PC3 cell lines with MDM2 overexpression or knockdown were described previously (21).

**Reverse transcription-PCR.** Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA), quantified by UV spectrophotometry, and used to create cDNA with the SuperScript reverse transcription-PCR (RT-PCR) kit from Invitrogen. The PCR coamplification of MDM2 with β-actin was accomplished by a method described previously (19).

**Real-time quantitative PCR.** Expression levels were determined using an ABI 7900 Sequence Detection System as previously described (26). The real-time quantitative PCR primers and fluorophore-labeled probe (Hs00234753_m1) were purchased from Applied Biosystems (Foster City, CA). The sequence for the primers and probes for human S9 ribosomal have been described previously (26). Expression levels were calculated using the relative standard curve method (26). All reactions were run in triplicate, and standard curves with correlation coefficients <0.98 were repeated. Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template-control reactions were done.

**Luciferase assay.** With the *Renilla* luciferase reporter as an internal control, cells were cotransfected for 12 h with full-length or deleted human MDM2 promoter vectors. The cells were then exposed to curcumin for 24 h. The luciferase activity of the *MDM2* promoter reporters was determined with the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol. *MDM2* reporter activity was normalized to that for the *Renilla* luciferase reporter.

**Assays for apoptosis, cell proliferation, and clonogenicity.** The methods used were described previously (19, 21).

**Evaluation of curcumin as a chemotherapeutic, chemosensitizing, and radiosensitizing agent *in vivo*.** The PC3 xenograft model was established by methods described previously (17, 19). Briefly, cultured cells were washed with and resuspended in serum-free medium. Portions of the suspension ($5 \times 10^6$ cells in 0.2 mL) were injected into the left inguinal area of nude mice. The mice were monitored by measuring tumor growth and body weight and by general clinical observation (16, 19). Mice bearing

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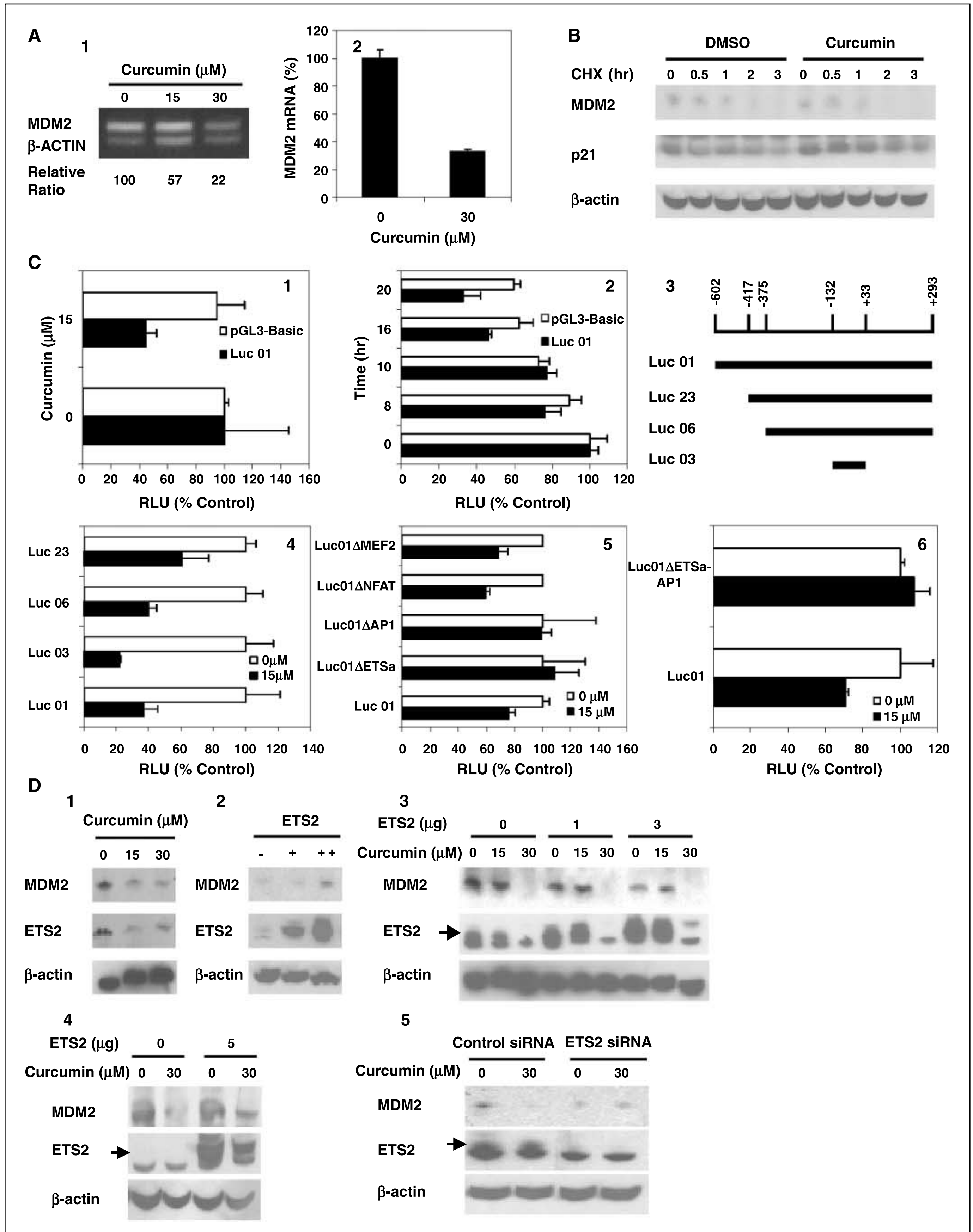


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tumors of ~100 mg were randomly divided into multiple treatment and control groups (five mice per group). Curcumin, dissolved in cottonseed oil, was given by p.o. gavage at doses of 5 mg/d, 5 days per week for 4 weeks. The control group received cottonseed oil only. Alone or in combination with curcumin, gemcitabine (160 mg/kg) was given by i.p. injection on days 7, 14, and 21, and radiation (3 Gy) was administered on days 4, 6, and 10. Tumor monitoring and body weight data were expressed as mean and SD of tumor mass, and the significance between various treatment groups was analyzed by ANOVA.

## Results

**Curcumin inhibits *MDM2* expression in both normal and cancerous human cell lines, independent of p53 activity.** In the PC3 human prostate cancer cell line, MDM2 levels were decreased by curcumin in a dose-dependent (Fig. 1*A*) and time-dependent (Fig. 1*B*) manner. Consistent with the idea that the activity was not through p53, curcumin inhibited *MDM2* in cell lines with either WT *p53* (LNCaP and MCF-7) or with *p53* knockdown (*p53* KD; MCF-7 *p53* KD; ref. 21; Fig. 1*A*). In addition, $p21^{Waf1/CIP1}$ and Bax were induced, whereas E2F1 and Bcl2 were decreased in PC3 cells, likely as a result of MDM2 inhibition (Fig. 1*C*). These results are similar to those observed with other MDM2 inhibitors (19, 21). The inhibitory effect of curcumin on *MDM2* was also noted in the human normal breast cell line MCF10A (Fig. 1*D*).

**Curcumin inhibits MDM2 through the transcription factor ETS2 by modulation of the PI3K/mTOR signaling pathway.** The expression of MDM2 mRNA in PC3 cells, measured by semiquantitative RT-PCR, was decreased by curcumin in a dose-dependent manner (Fig. 2*A1*). The expression changes of the *MDM2* gene were also confirmed by real-time PCR (Fig. 2*A2*). There was, however, no appreciable difference in MDM2 protein stability between PC3 cells exposed to curcumin and those exposed only to the solvent, DMSO (Fig. 2*B*). In contrast, the p21 protein was stabilized, probably as a result of MDM2 down-regulation (5, 6).

To establish how curcumin affects *MDM2* transcription, a human full-length *MDM2* P2 promoter reporter (Luc01; ref. 27) was transfected into PC3 cells. These cells were then exposed to curcumin (15 μmol/L), which decreased the *MDM2* transcriptional activity (Fig. 2*C1*). This activity generally decreased with time of exposure to curcumin (Fig. 2*C2*).

To characterize the curcumin-responsive element in the *MDM2* promoter, a series of deletions of Luc01 (Fig. 2*C3*; ref. 27) were evaluated. The shortest segment (−132 to +33, Luc03) still retained the capacity to respond to curcumin (Fig. 2*C4*). Interestingly, Luc03 is the fragment that responds best to curcumin among the *MDM2* promoter fragments, suggesting that additional *cis*- or *trans*- elements in the full-length *MDM2* promoter modulate the response of the *MDM2* promoter to curcumin. There are several response sites for transcription factors in this region of the *MDM2* promoter, including those for ETS, activator protein-1 (AP1), myocyte enhancer factor-2 (MEF2), and nuclear factor for activated T-cells (NFAT). The composite ETS/AP1 site is responsible for activation of the promoter by growth factors dependent on the ras–raf–extracellular signal-regulated kinase (ERK) kinase (MEK)–mitogen-activated protein kinase pathways (27, 28).

To define the site involved in the effects of curcumin more specifically, four luciferase vectors with ETSa, AP1, MEF2, and NFAT mutations were transfected into PC3 cells, which were then exposed to curcumin. Those with mutations at the MEF2 and NFAT sites retained the capacity to respond to curcumin (Fig. 2*C5*). Mutations at the AP1 and ETSa sites eliminated the response of the promoter to curcumin, and a Luc01 promoter with a mutation in the composite AP1/ETSa site was also not responsive (Fig. 2*C6*).

The involvement of transcription factor ETS2 in the transcription of *MDM2* and the inhibitory effects of curcumin on *MDM2* expression were then confirmed. Curcumin inhibited ETS2 expression in PC3 cells in a concentration-dependent manner (Fig. 2*D1*); overexpressed ETS2 resulted in the accumulation of MDM2 (Fig. 2*D2*). Reconstituted ETS2 rescued *MDM2* expression in PC3 cells exposed to 15 μmol/L curcumin (Fig. 2*D3*). With the highest concentration of curcumin (30 μmol/L), however, the ectopically expressed ETS2 was reduced and, as a result, the inhibition of MDM2 was not reversed (Fig. 2*D3*). To confirm this observation, ETS2/control plasmid–transfected PC3 cells were treated with curcumin (30 μmol/L) for a shorter period (3 h). As shown, with the compensation of ETS2 expression, *MDM2* inhibition by curcumin was reversed (Fig. 2*D4*). To further establish the role of ETS2 in the curcumin-mediated *MDM2* inhibition, ETS2 was transiently knocked down by double-stranded siRNA (Dharmacon; Fig. 2*D5*). Under these conditions, curcumin did not down-regulate the MDM2 level any further (Fig. 2*D5*).

Several signaling pathways activated by growth factors have been implicated in the p53-independent regulation of *MDM2* (27–34). To determine the mechanism(s) by which curcumin down-regulates *MDM2*, PC3 cells were exposed to (−)-Deguelin, an inhibitor of Akt; PD98059, an inhibitor of MEK; wortmannin, an inhibitor of PI3K; or rapamycin, an inhibitor of mTOR, for 60 min. The cells were then exposed to curcumin for 24 h. Down-regulation

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**Figure 2.** Curcumin down-regulates *MDM2* transcription through the ETS2 transcription factor, independent of p53. *A,* PC3 cells were exposed to curcumin (0, 15, or 30 μmol/L) for 24 h and then total RNA was extracted. *MDM2* mRNA was coamplified with β-actin mRNA (*A1*) or quantified by real-time PCR (*A2*). *B,* PC3 cells were exposed to curcumin or solvent (DMSO) followed by addition of the protein synthesis inhibitor, cycloheximide (*CHX*, 10 μg/mL). At selected times after cycloheximide exposure, cell lysates were collected for determination of the MDM2 and p21 protein levels by immunoblotting. *C,* curcumin acts on the ETS2 site in the *MDM2* promoter. *C1,* PC3 cells were transfected with the *MDM2* P2 promoter luciferase construct, Luc01, or with the corresponding control vector, pGL3-Basic, for 12 h, followed by incubation with curcumin (15 μmol/L) for an additional 24 h, at which time luciferase activities were measured. *Columns,* mean; *bars,* SD. *C2,* PC3 cells were transfected with Luc01 or with the corresponding empty vector (pGL3-Basic) for 12 h followed by exposure to curcumin (15 μmol/L) for various times. Luciferase activities were plotted as percentages of the control. *Columns,* mean; *bars,* SD. *C3,* structures of plasmids with full-length and deleted MDM2 promoters. *C4,* PC3 cells were transfected with MDM2 deletion reporters (Luc 03, Luc 06, and Luc 23) or with the full-length reporter (Luc 01) for 12 h followed by incubation with 0 or 15 μmol/L curcumin for an additional 24 h. The luciferase activity of each reporter exposed to curcumin is a percentage of that obtained for the control (no curcumin). *Columns,* means; *bars,* SD. *C5* and *C6,* PC3 cells were transfected with the plasmids with mutant *MDM2* promoters (Luc01ΔMEF2, Luc01ΔNFAT, Luc01ΔAP1, or Luc01ΔETSa-AP1) or with the full-length reporter (Luc01) for 12 h followed by incubation with 0 or 15 μmol/L curcumin for an additional 24 h. The luciferase activity for each reporter exposed to curcumin is a percentage of that for the control (no curcumin). *Columns,* mean; *bars,* SD. *D,* curcumin down-regulates MDM2 through ETS2. *D1,* PC3 cells were exposed to various concentrations of curcumin for 24 h. MDM2 and ETS2 proteins were detected by immunoblotting. *D2,* MDM2 levels in PC3 cells transfected with various amounts of ETS2 were examined by immunoblotting. *D3,* PC3 cells were transfected with ETS2 for 8 h followed by exposure to curcumin for an additional 24 h. MDM2 and ETS proteins in the cell lysates were detected by immunoblotting. *D4,* ETS2 or control vector–transfected PC3 cells were treated with curcumin for 3 h. MDM2 level was determined by immunoblotting. *D5,* PC3 cells were transfected with ETS2 siRNA or control siRNA for 24 h followed by exposure to curcumin for 1 h. MDM2 and ETS2 levels were detected by immunoblotting.

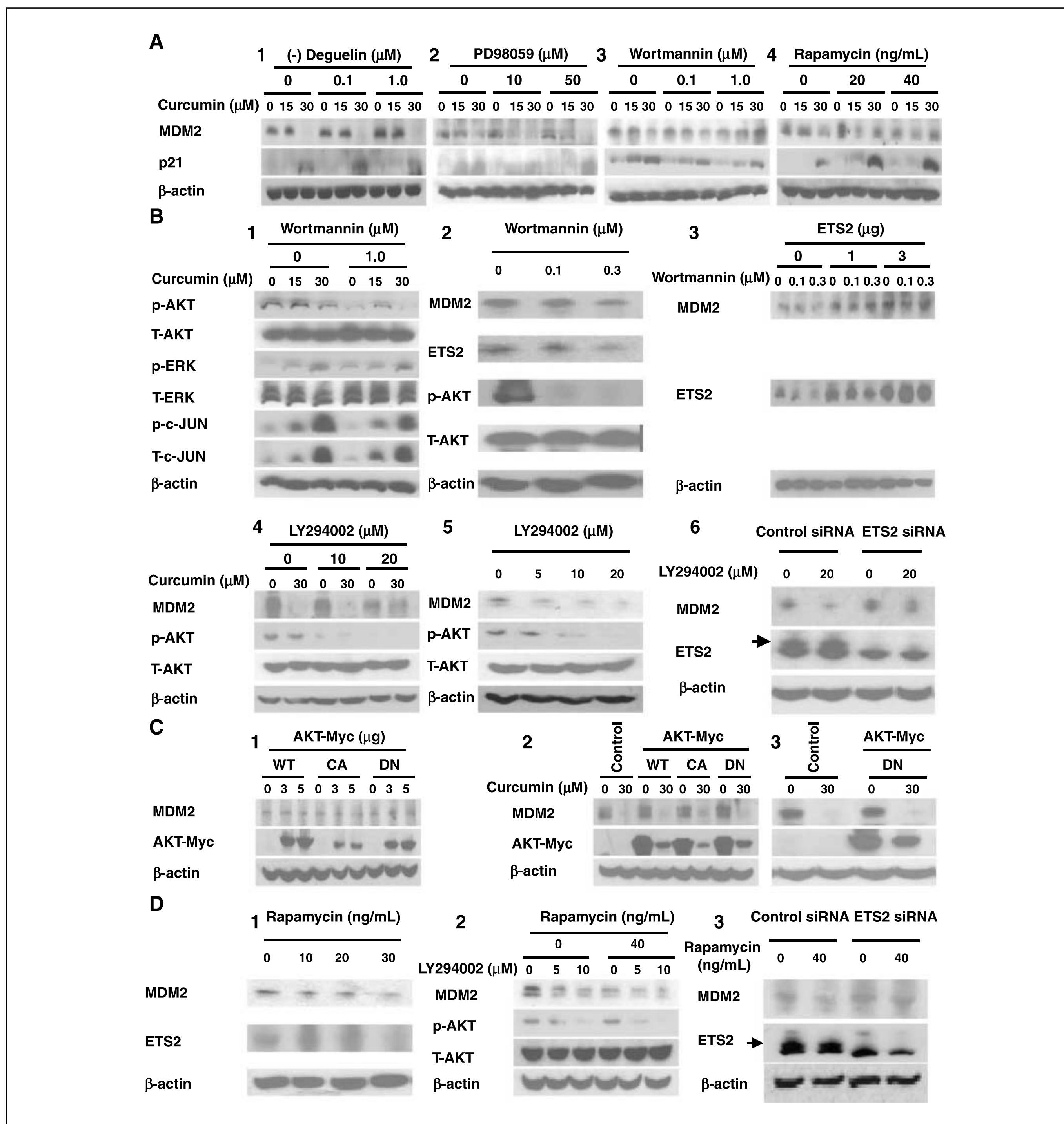


**Figure 3.** Down-regulation of MDM2 by curcumin through the PI3K/mTOR pathway. *A,* PC3 cells, exposed to various concentrations of (−)-Deguelin (*A1*), PD98059 (*A2*), wortmannin (*A3*), or rapamycin (*A4*) for 60 min, were further exposed to curcumin for an additional 24 h. MDM2, p21, and β-actin proteins were detected by immunoblotting. *B,* curcumin down-regulates MDM2 through PI3K. *B1,* PC3 cells, exposed to wortmannin (0 or 1 μmol/L) for 60 min, were further exposed to curcumin (0, 15, or 30 μmol/L) for an additional 24 h. The proteins, phosphorylated Akt (*p-Akt*), total Akt (*T-Akt*), phosphorylated ERK (*p-ERK*), total ERK (T-ERK) phosphorylated c-Jun (*p-c-Jun*), and total c-Jun (*T-c-Jun*) were detected by immunoblotting. *B2,* PC3 cells were exposed to wortmannin for 2 h, and target proteins were detected by immunoblotting. *B3,* PC3 cells with ectopically expressed ETS2 were exposed to wortmannin for 2 h. MDM2, ETS2, and β-actin proteins were examined by immunoblotting. *B4,* PC3 cells were pretreated with LY294002 for 2 h followed by the exposure to curcumin for additional 2 h. Target proteins were detected by immunoblotting. *B5,* MDM2 levels in LY294002-treated cells were determined by immunoblotting. *B6,* changes in MDM2 and ETS2 levels in ETS2 knockdown cells, which were further treated with LY294002 for 3 h, were examined by immunoblotting. *C,* Akt is not involved in MDM2 down-regulation by curcumin. *C1,* various doses of WT, constitutively active (*CA*), and dominant-negative (*DN*) Akt were overexpressed in PC3 cells. The MDM2 level was detected by immunoblotting. *C2* and *C3,* PC3 cells were transfected with the plasmids expressing different kinds of Akt followed by exposure to curcumin for different times, 24 h (*C2*) or 4 h (*C3*). The MDM2 level in the cell lysates was detected by immunoblotting. *D,* mTOR is the downstream protein kinase of PI3K that regulates MDM2 expression. *D1,* changes in the expression of MDM2 and ETS2 proteins in PC3 cells exposed to rapamycin for 2 h were determined by immunoblotting. *D2,* PC3 cells were pretreated with rapamycin for 2 h followed by the exposure to LY294002 for an additional 6 h. MDM2 expression was detected by immunoblotting. *D3,* MDM2 and ETS2 levels in ETS2 knockdown PC3 cells, which were further treated with rapamycin for 4 h, were examined by immunoblotting.

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of MDM2 was not affected by (−)-Deguelin or PD98059 (Fig. 3*A1* and *A2*), suggesting that inhibition of Akt or MEK is not responsible for the effect of curcumin on MDM2. Wortmannin and rapamycin, inhibitors of PI3K or mTOR, the downstream target of PI3K, however, eliminated the effects of curcumin on *MDM2* expression (Fig. 3*A3* and *A4*). The expression levels of p21 were not greatly affected by either of the kinase inhibitors.

To evaluate the involvement of PI3K/mTOR in the activity of curcumin, PC3 cells were pretreated with wortmannin or its solvent (DMSO) followed by exposure to various concentrations of curcumin. Curcumin inhibited PI3K activity, as manifested by changes in the phosphorylation status of Akt (Fig. 3*B1*). Pretreatment with wortmannin, however, blocked the effects of curcumin on PI3K activity (Fig. 3*B1*). In contrast, curcumin had no effects on the activities of MEK and c-Jun-$NH_2$-kinase, as represented by the phosphorylation status of ERK and c-Jun, respectively (Fig. 3*B1*). Additionally, blocking the activity of PI3K by wortmannin resulted in decreases in ETS2 and MDM2 (Fig. 3*B2*); this effect was reversed by overexpression of ETS2 (Fig. 3*B3*). Because wortmannin is not a specific PI3K inhibitor, a more specific PI3K inhibitor, LY294002 (35), was included to confirm the involvement of PI3K in *MDM2* regulation. Similar to above results, *MDM2* inhibition by curcumin was abrogated by PI3K blockade when PC3 cells were pretreated with various doses of LY294002 (Fig. 3*B4*). Consistently, blocking the activity of PI3K by LY294002 resulted in a decrease in *MDM2* expression (Fig. 3*B5*). The ensuing knockdown experiment in PC3 cells suggested that the downstream target of PI3K responsible for regulating *MDM2* expression is ETS2 (Fig. 3*B6*).

To confirm that Akt was not involved in the *MDM2* regulation, WT, constitutively active, and dominant-negative Akt were overexpressed in PC3 cells; no appreciable changes in MDM2 expression were evident (Fig. 3*C1*). Consistently, overexpression of WT, constitutively active, or dominant-negative Akt did not reverse the effects of curcumin in inhibiting *MDM2* expression (Fig. 3*C2*). Similarly, short time exposure (4 h) of dominant-negative Akt–transfected PC3 cells to curcumin resulted in apparent *MDM2* down-regulation.

In contrast, inhibition of mTOR by rapamycin decreased cellular levels of ETS2 and MDM2 (Fig. 3*D1*), and the blockade of mTOR by pretreatment of PC3 cells with rapamycin abrogated *MDM2* down-regulation resulting from LY294002 treatment (Fig. 3*D2*). Similarly, a knockdown experiment indicated that ETS2 was the downstream mediator of rapamycin in its inhibition of *MDM2* expression (Fig. 3*D3*).

**The p53-independent anticancer effects of curcumin are related to *MDM2*.** Control PC3 cells, PC3 cells with stable knockdown of *MDM2*, PC3 cells with stable overexpression of *MDM2*, or cells exposed to the corresponding control vector (pCMV) were incubated with curcumin (0, 15, or 30 μmol/L) for 24 h followed by immunoblotting for MDM2, p21, and β-actin (Fig. 4*A*). In control cells, curcumin decreased the expression of MDM2; this effect was blocked in PC3 cells with *MDM2* overexpression. In all cells exposed to curcumin, there was increased expression of p21.

Curcumin reduced viability (Fig. 4*B*) and proliferation (Fig. 4*B*) of PC3 cells in a dose-dependent manner. It also induced apoptosis (Fig. 4*B*) and reduced the surviving fraction of cells (Fig. 4*B*). Cells with *MDM2* knockdown or overexpression, however, were less susceptible to the anticancer effects of curcumin than control cells.

**Curcumin sensitizes human cancer cells to chemotherapy and radiation through *MDM2*, independent of p53.** PC3 cells were exposed to 0 or 15 μmol/L of curcumin for 24 h followed by exposure to 10 Gy of γ-irradiation. Although levels of MDM2 were decreased initially after irradiation, the expression of MDM2 was increased in later time points (Fig. 5*A*). This irradiation-related increase of MDM2 was blocked by curcumin (Fig. 5*A*). Likely resulting from the inhibition of *MDM2*, combining curcumin and irradiation had potent effects on the expression of p21, Bax, and E2F1 proteins (Fig. 5*A*). Similar results have been observed in studies with other MDM2 inhibitors, such as MDM2 antisense oligonucleotides (19, 20) and genistein (21).

In separate studies, control PC3 cells and PC3 cells with *MDM2* knockdown or overexpression were exposed to curcumin (5 μmol/L) for 24 h, then irradiated with various doses of radiation (0, 5, or 10 Gy). Combining curcumin and irradiation led to lower viability

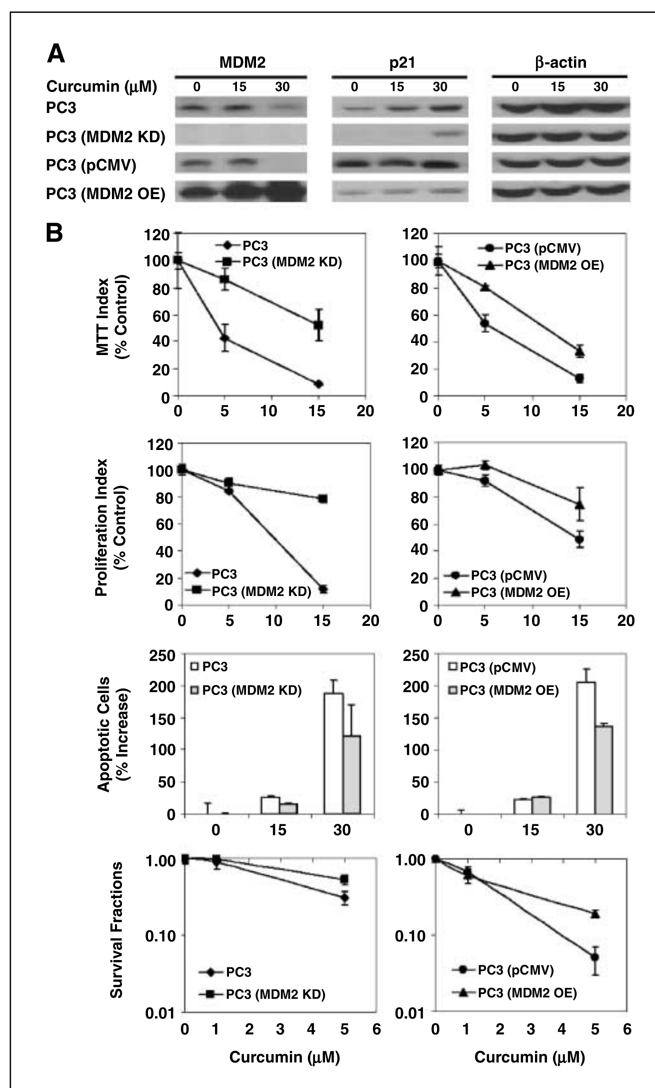


**Figure 4.** The role of the *MDM2* expression level in the response of PC3 cells to curcumin. *A,* wild-type PC3 cells, or PC3 cells with stable knockdown (*KD*) or overexpression (*OE*) of *MDM2* were exposed to various concentrations of curcumin for 24 h followed by Western blot analyses for MDM2, p21, and β-actin. *B,* the stable PC3 cell lines were exposed to various concentrations of curcumin for 48 h followed by MTT assay for cell viability, bromodeoxyuridine incorporation assay for cell proliferation, and apoptosis assay or clonogenic assay.

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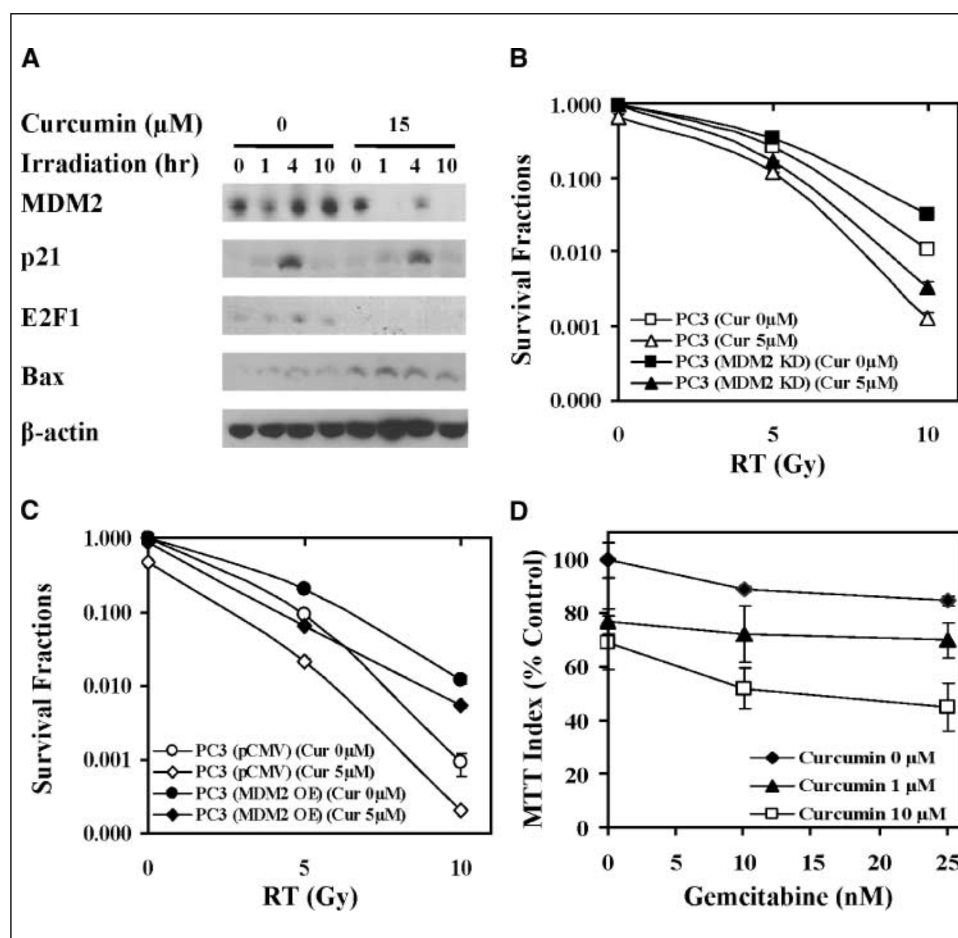


**Figure 5.** *In vitro* radiosensitization and chemosensitization effects of curcumin through inhibition of MDM2 expression. *A,* PC3 cells were exposed to 15 µmol/L of curcumin or to the solvent DMSO for 24 h and then exposed to 10 Gy of radiation administered with a $^{60}$Co-Picker unit irradiator (1.56 Gy/min). Time 0 was the end of radiation exposure. Protein levels were quantified by immunoblotting. *B* and *C,* the stably transfected PC3 cells with MDM2 knockdown or overexpression and control PC3 cells were exposed to curcumin (5 µmol/L) for 24 h followed by irradiation (0, 5, or 10 Gy), and then cultured for an additional 14 d. At the end of the experiments, cell colonies were fixed, stained, and counted. *Points,* fraction of corresponding control; *bars,* SD. *D,* PC3 cells were exposed to curcumin (1 or 10 µmol/L) for 24 h and then to gemcitabine for an additional 48 h followed by MTT assay. Relative levels were expressed as percentages of the control.

compared with cells exposed to irradiation alone (Fig. 5*B* and *C*). Cells with *MDM2* overexpression or knockdown were less sensitive to the effects of curcumin and radiation (Fig. 5*B* and *C*).

To evaluate the possible chemosensitization effects of curcumin, PC3 cells were incubated with various concentrations of curcumin for 24 h and then exposed to gemcitabine for an additional 24 h. In accordance with the previous studies, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed that curcumin (1 or 5 µmol/L) plus gemcitabine had more potent cytotoxic effects than gemcitabine or curcumin alone (Fig. 5*D*).

When administered by p.o. gavage to tumor-bearing nude mice, curcumin (5 mg/d) inhibited the growth of PC3 xenografts by ~50% (Fig. 6*A* and *B*). Curcumin also enhanced the antitumor effects of gemcitabine (Fig. 6*A*) and irradiation (Fig. 6*B*). Analysis of tumors collected at the end of the experiment showed that curcumin reduced the expression of *MDM2* in xenografts treated with curcumin alone, and in xenografts treated with combinations of curcumin plus gemcitabine or irradiation (Fig. 6*C*).

## Discussion

Our observations suggest that a novel signaling pathway, PI3K/mTOR/ETS2, regulates *MDM2* transcription, independent of p53. Although MDM2 is transactivated by p53 and regulates p53 (1), the mechanism by which *MDM2* expression is regulated independent of p53 is not clear. Because overexpression of *MDM2* is observed in human cancers and is related to a poor prognosis (10), and >50% of human cancers harbor nonfunctional p53, the mechanism by which *MDM2* is regulated in the absence of p53 needs to be defined. Our finding that PI3K/mTOR/ETS2 signaling is involved in *MDM2* regulation has implications for carcinogenesis and cancer progression. First, the described p53-independent up-regulation of *MDM2* by growth factors through PI3K may result in p53 repression; this process could be a critical step in tumorigenesis. Moreover, in the majority of human cancers, mutant p53 is present at high levels and is incapable of transactivating *MDM2*. Under these circumstances, p53-independent pathways regulate the expression of MDM2.

PI3K-mediated signaling is one of the most frequently targeted pathways in human cancers (36). Activated PI3K promotes cell survival and proliferation through mechanisms that are not fully understood (36). mTOR, a highly conserved serine/threonine kinase activated by PI3K, is involved in cancer initiation and progression (37). Again, the mechanisms are not clear. PI3K/Akt has been implicated in MDM2 protein stabilization and subcellular localization (29, 33, 34). Here, we provide evidence that PI3K induces *MDM2* transcription through mTOR/ETS2, suggesting that these proteins may govern the mechanisms by which mitogens promote cell proliferation and inactivate p53. Because MDM2 is tumorigenic even in the absence of p53, the presented results provide a mechanism by which mitogens promote cancer progression independent of functional p53. Moreover, our findings could facilitate development of more rational and effective therapy

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for human cancers. A remaining concern is the fact that cells have redundant mechanisms for PI3K-mediated regulation of MDM2. This can be explained by the Akt-mTOR feedback loop (37). In this loop, activated Akt leads to stabilized MDM2 and activated mTOR, which, in turn, inhibits Akt. Thus, the level of MDM2 would decrease over time if mTOR were incapable of inducing MDM2 up-regulation. Future studies clarifying the differential regulation of Akt and mTOR with respect to MDM2 would be informative.

Although we have shown that the transcription factor ETS2 is important for *MDM2* transcription, the involvement of other ETS family members cannot be excluded. For example, ETS transcription factor Fli-1 regulates *MDM2* transcription (38). The transcription of the *MDM2* P2 promoter through the composite AP1-ETS responsive site requires the cooperation of both activated members of the AP1 and ETS families. These families have multiple members, which form heterodimers with other family members. Further studies are needed to determine whether other members, in addition to ETS2 and Fli-1, are capable of up-regulating MDM2. Results of such studies would help in understanding the p53-independent regulation of *MDM2* expression.

In rodent model systems, curcumin, a dietary component, inhibits the formation of carcinogen-induced cancers of the colon (39), oral cavity (40), forestomach (41), esophagus (42), stomach (43), lung (44), liver (45), and skin (46). In a small clinical study, p.o. administered curcumin had beneficial effects on several types of premalignant lesions (47). Although several oncogenes and tumor suppressors have been suggested to be modulated by curcumin (22, 23), the main cellular target(s) of curcumin is not known. Our present study identified a novel activity of curcumin, inhibition of *MDM2* expression.

*MDM2* has been suggested as a drug target for human cancer therapy (14–20). To target *MDM2*, we have generated a second-generation antisense oligonucleotide that showed effective and efficient knockdown of *MDM2 in vitro* and *in vivo*. This inhibition of *MDM2* led to substantial *in vitro* and *in vivo* activity against human cancer cells and tumors (14–20). In our continuing search for *MDM2* inhibitors, we have found that genistein, a component of soybeans, inhibits *MDM2* expression, and this inhibition is at least partially responsible for the anticancer activities of genistein (21). We now show that another dietary component with antitumor activity, curcumin, down-regulates *MDM2*. This compound has entered clinical trials for certain human cancers (47, 48). Our studies help to elucidate the mechanism by which curcumin acts as an anticancer agent and thus substantiates the role of curcumin in cancer therapy. Future studies focused on the structure-activity relationship of known *MDM2*-inhibiting compounds, including genistein and curcumin, could allow for development of more potent molecules for cancer therapy.

In conclusion, our observations support the finding that PI3K/mTOR/ETS2 is a previously unrecognized pathway, leading to up-regulation of *MDM2* transcription (Fig. 6*D*). Considering the numerous activities of MDM2 in cancer growth and progression, we propose that curcumin can be used as a therapeutic agent,

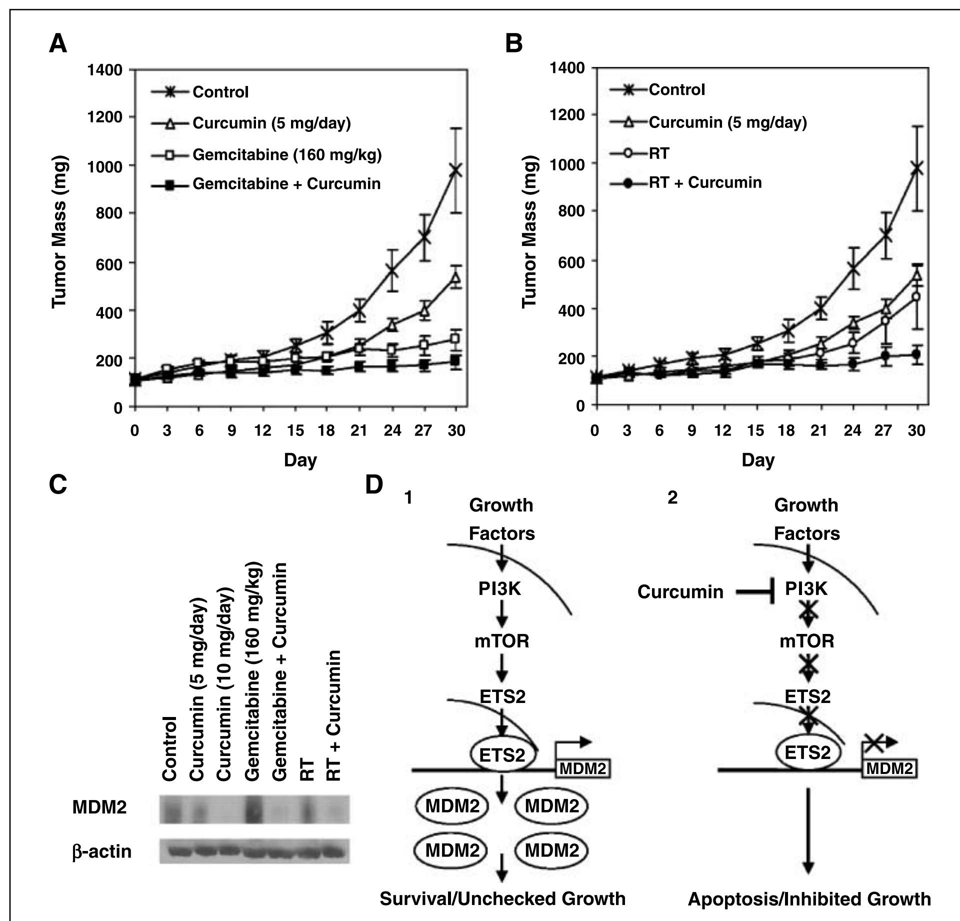


**Figure 6.** *In vivo* antitumor activity of curcumin administered alone or in combination with gemcitabine (*A*) or radiation (*B*) to nude mice bearing PC3 xenografts, and a simplistic model for *MDM2* regulation through the PI3K/mTOR/ETS2 pathway, which is modulated by curcumin (*D*). Curcumin treatment (5 mg/d by p.o. gavage) was initiated on day 0 and continued 5 d/wk for 4 wk. The mice were dosed with gemcitabine (160 mg/kg) i.p. on days 7, 14, and 21; radiation (*RT*; 3 Gy) was administered on days 4, 6, and 10. *C,* at the end of the experiment, tumors were removed and proteins (MDM2 and β-actin) present in tumor homogenates were measured by immunoblotting. *D1,* growth factors activate ETS2 through PI3K and mTOR. Activated ETS2 transactivates the *MDM2* gene, the products of which promote cell proliferation and/or inhibit apoptosis through both p53-dependent and p53-independent pathways. *D2,* curcumin down-regulates *MDM2* expression by acting on PI3K, which leads to inhibition of cell proliferation and/or apoptosis.

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alone or in combination with other conventional agents, for the treatment of human cancers.

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