Curcumin Blocks RON Tyrosine Kinase–Mediated Invasion of Breast Carcinoma Cells

Madhusudhanan Narasimhan¹ and Sudhakar Ammanamanchi¹²

¹Department of Medicine, Division of Hematology and Medical Oncology, ²Cancer Therapy and Research Center, The University of Texas Health Science Center at San Antonio, San Antonio, Texas

Abstract

We have recently shown that macrophage-stimulating protein (MSP) promotes the invasion of receptor d’origine nantais (RON), a tyrosine kinase receptor–positive MDA-MB-231, MDA-MB-468 breast cancer cells, and also identified the regulatory elements required for RON gene expression. In this report, we have analyzed the efficacy of a chemopreventive agent, curcumin, in blocking RON tyrosine kinase–mediated invasion of breast cancer cells. Reverse transcription-PCR and Western analysis indicated the down-regulation of the RON message and protein, respectively, in MDA-MB-231 and MDA-MB-468 cells. Significantly, curcumin-mediated inhibition of RON expression resulted in the blockade of RON ligand, MSP-induced invasion of breast cancer cells. We have identified two putative nuclear factor–κB p65 subunit binding sites on the RON promoter. Using chromatin immunoprecipitation analysis and site-directed mutagenesis of the RON promoter, we have confirmed the binding of p65 to the RON promoter. Our data show that curcumin reduces RON expression by affecting p65 protein expression and transcriptional activity. Treatment of MDA-MB-231 cells with pyrrolidine dithiocarbamate, an inhibitor of p65, or small interfering RNA knockdown of p65, blocked RON gene expression and MSP-mediated invasion of MDA-MB-231 cells. This is the first report showing the regulation of human RON gene expression by nuclear factor–κB and suggests a potential therapeutic role for curcumin in blocking RON tyrosine kinase–mediated invasion of carcinoma cells. [Cancer Res 2008;68(13):5185–92]

Introduction

The central role played by receptor tyrosine kinases in the control of cell proliferation, differentiation, and metastasis of various types of cancers renders these proteins an attractive target for molecular-based cancer therapy. The human receptor d’origine nantais (RON) gene encodes a receptor tyrosine kinase (RTK) of 1,400 amino acids that belong to the MET gene family. RON is initially synthesized as a single-chain precursor, 170 kDa pro-RON, which is subsequently cleaved into the 40 kDa α-chain and 150 kDa β-chain. The α-chain traverses the cell membrane and contains the intracellular tyrosine kinase (1). Amplification and/or overexpression of RON was reported in various epithelial cancers (2–5). Macrophage-stimulating protein (MSP) is the only known ligand for RON. MSP is an 80 kDa heterodimer consisting of a 53 kDa α-chain and a 30 kDa β-chain linked by a disulfide bond. The β-chain of MSP binds to RON (1). The RON receptor activates an array of downstream signaling cascades such as phosphoinositide-3-kinase/Akt, focal adhesion kinase, and mitogen-activated protein kinases which are involved in cell proliferation, tubular morphogenesis, cell motility, migration, and invasion (6–9). RON overexpression in patients with breast cancer is associated with a worse clinical outcome (10), and is recognized as a target for molecular therapy.

Curcumin (diferuloylmethane) is a naturally occurring yellow pigment extracted from the rhizomes of the plant Curcuma longa (Linn). Curcumin is one of the promising chemopreventive and chemotherapeutic agents (11) and inhibits the proliferation of tumor cells in vitro (12), and suppressing tumor formation in various animal models (13–15). Numerous studies show that curcumin’s anti-inflammatory and anticarcinogenic effects have been attributed to its ability to inhibit transcription factors such as nuclear factor–κB (NF-κB), activator protein-1 (AP-1), EGR1, ETS2, signal transducers and activators of transcription, etc., and serine/threonine protein kinases, i.e., phospholipase kinase, protein kinase C, protamine kinase, pp60c-src tyrosine kinase, p44/42 mitogen-activated protein kinase, and c-Jun-NH2-kinase (16–21). Phase I human trials have been performed showing that curcumin is well-tolerated and lacks toxicity (22, 23).

We have recently shown that the RON ligand, MSP, promotes the invasive phenotype of MDA-MB-231 and MDA-MB-468 breast cancer cells, and also identified the regulatory elements that are required for basal RON promoter activity and RON expression (24). The aim of our current study is to investigate the efficacy of curcumin on the regulation of RON tyrosine kinase in the invasive MDA-MB-231 and MDA-MB-468 cells. Our data shows that curcumin could down-regulate RON expression significantly in breast cancer cells. Furthermore, curcumin-mediated RON down-regulation inhibited the MSP-induced invasion of both MDA-MB-231 and MDA-MB-468 cells. We have identified two novel NF-κB p65 subunit-binding sites on the RON promoter. Curcumin blocks RON kinase expression by inhibiting the p65 protein expression and the consequent inactivation of p65-mediated transactivation of the RON promoter activity. Using pyrrolidine dithiocarbamate (PDT), a pharmacologic inhibitor of p65 as well as p65 small interfering RNA (siRNA), we have further confirmed the role of p65 in the regulation of RON tyrosine kinase and the MSP-associated invasion of breast cancer cells. In summary, the present study identified NF-κB as a novel regulator of RON tyrosine kinase and RON could be a novel pharmacologic target of curcumin in the blockade of invasion of carcinoma cells.
Materials and Methods

Cells, treatment, and reagents. MDA-MB-231 and MDA-MB-468 breast carcinoma cells were cultured as described (24). Cells were treated with DMSO or various concentrations (10, 20, 40 and 80 μM/L) of curcumin for 24 h before the assays. For PDTC experiments, MDA-MB-231 cells were seeded in six-well plates (2.5 × 10⁵ cells/well) and treated with 25, 50, and 100 μM/L of PDTC for 24 h. Anti-RON and anti-actin polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-p65 polyclonal antibody was purchased from Abcam. Anti-pIkKα/β (Ser180/181), IkK-α, anti-IκB-β, anti-p-IkK-α, and anti-IκB-α antibodies were purchased from Cell Signaling Technology. Curcumin was obtained from MP Biomedicals. Recombinant MSP was purchased from R&D Systems. PDTC was obtained from Sigma-Aldrich. Reagents for SDS-PAGE were from Bio-Rad Laboratories. All other reagents used were from Sigma-Aldrich.

Reverse transcription-PCR. Total RNA was extracted from the cells treated with DMSO or curcumin using the Trizol method (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed as described previously (24). Primers for RON generate a 246-bp fragment as follows: sense primers, 5'-AGG CCA GCC GTA TCT AT-3' and antisense primers, 5'-GGG CAC TAG GAT CAT CTG TCA-3'. Primers for actin generate a 621-bp fragment as follows: sense primers, 5'-ACA CTG TGC CCA TCT AGC AGG-3' and antisense primers, 5'-GGG CCC GGG ACT GTG ACT-3'.

Western blot analysis. Equal amounts of cell lysates from cells treated with DMSO, different concentrations of curcumin, or PDTC were used for Western analysis.

Electrophoretic mobility shift assay. The oligonucleotide containing the consensus NF-κB-binding site was end-labeled using 32P-ATP and electrophoretic mobility shift assays (EMSA) were performed on the nuclear extracts from controls and varying concentrations of curcumin-treated MDA-MB-231 cells as described previously (24).

Construction of −1.2 kb and −400 bp wild-type RON promoter-luciferase reporter constructs. The −1.2 kb and −400 bp RON promoter-luciferase reporter vectors were described previously (24).

Site-directed mutagenesis. Mutations in the RON promoter were created in putative NF-κB binding sites at nucleotides −133 to −140 (site 1) and −156 to −162 (site 2) using the Quickchange site-directed mutagenesis kit (Stratagene). For construction of site-directed mutants of the RON promoter, the reporter vector containing the wild-type 1.2 kb and 400 bp RON promoter sequence was used as a template. To generate a site 1 mutant, we used primer A, 5’-GAA CTG GGG GGG CCA ATG TGT CCG CTA TCT GTG-3’; and primer B, 5’-ACA AGG GAT GGG ACT GGC-3’. The mutant nucleotides are shown in italicized boldface. The same protocol was used to create mutations in the site 2 region of the RON promoter. For this PCR reaction, we used primer C, 5’-CGGG AGA GGT TTT CTG TTA TCT CAC AGG AAC CTG G-3’ and primer D, 5’-CAAGG TCG TCT GGT GAA TGA AAT CAA ATC CTC TCG G-3’. The authenticity of mutation of all constructs was confirmed by nucleotide sequencing analysis.

Cell transfection and reporter gene assays. MDA-MB-231 and MDA-MB-468 cells were seeded in 12-well plates at a density of 1.25 × 10⁵ cells/well the day before transfection. Individually, 1 μg of plasmid DNA was transfected into the cells with Lipofectamine 2000 (Invitrogen). For p65-mediated RON reporter activity experiments, both the pCMV-p65 and pGL3 RON Luc reporter vectors were cotransfected. Twenty-four hours following transfection, the cells were treated with either DMSO or various concentrations of curcumin (10, 20, 40, and 80 μM/L). After an additional 24 h of incubation, the cells were lysed and luciferase activity was assayed. Cell transfection vectors were dually transcribed for target promoter-luciferase values and expressed as relative units following normalization to protein levels.

siRNA knockdown experiments. NF-κB p65 siRNA oligos were purchased from Dharmaco Inc. Briefly, MDA-MB-231 cells cultured in six-well plates were transfected with 50, 100, and 200 nmol/L of p65 siRNA or scrambled siRNA oligos using HiPerFect transfection agent (Qiagen) following the manufacturer’s instructions. Cells were then incubated for 65 h to allow maximum knockdown, after which they were harvested for Western blot analysis as described above.

Invasion assay. Matrigel invasion experiments were performed as described previously (24).

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assay was performed as described elsewhere (25). Oligonucleotide primers specific for the RON promoter spanning the −65 binding sites (forward, 5’-CTC CAA GGG CCG GAA GAG TC-3’; reverse, 5’-TAA AGC GGC GTT CCC GAC GAC CCC A-3’) were used. The PCR conditions were described previously (24).

Results

Curcumin decreases RON protein expression. To determine if the chemopreventive/chemotherapeutic agent curcumin could block RON protein expression, we treated MDA-MB-231 and MDA-MB-468 cells with varying concentrations of curcumin for 24 hours. We carried out Western analysis on equal amounts of total cell lysates from control and curcumin-treated MDA-MB-231 and MDA-MB-468 cells using rabbit anti-human RON and actin polyclonal antibodies. RON antibody recognizes the 170 kDa pro-RON and the intracellular 150 kDa β-chain of mature RON receptor. The results in Fig. 1A show that curcumin can down-regulate the expression of RON in a dose-dependent manner. In the present study, we observed that 40 μM/L of curcumin was able to abolish the RON protein expression almost completely when compared with untreated control in MDA-MB-231 cells. Importantly, curcumin-mediated RON down-regulation also occurred in another breast cancer cell line, MDA-MB-468, suggesting that this effect might not be a cell line–specific phenomenon. Although the maximum effect of down-regulating RON in MDA-MB-231 cells was achieved at 40 μM/L of curcumin, it is achieved at 80 μM/L in MDA-MB-468 breast cancer cells. Under these conditions, the levels of β-actin protein were unaffected, suggesting that the down-regulation was specific to RON protein.

Curcumin blocks RON-mediated invasion of breast cancer cells. We have recently shown that RON ligand, MSP, promotes the invasive phenotype of MDA-MB-231 and MDA-MB-468 cells (24). Our Western analysis indicated that 80 μM/L of curcumin completely inhibited RON protein expression in both MDA-MB-231 and MDA-MB-468 cells (Fig. 1A). To determine if curcumin-mediated inhibition of RON protein expression blocks the RON ligand, MSP-stimulated invasion, we carried out in vitro Matrigel assays. We treated MDA-MB-231 and MDA-MB-468 cells for 24 hours either with 2.5 ng/mL of RON ligand MSP or 80 μM/L of curcumin alone or 2.5 ng/mL of MSP plus 80 μM/L of curcumin. Twenty-four hours later, the migrated cells were stained with crystal violet and photographed (Fig. 1B). MSP induced the invasion through Matrigel of both MDA-MB-231 and MDA-MB-468 cells. However, curcumin almost completely blocked the MSP-mediated invasion of both the cell lines.

Curcumin down-regulates RON mRNA. We have carried out RT-PCR analyses to determine if RON mRNA levels reflect RON protein expression. The cell lines, MDA-MB-231 and MDA-MB-468, were treated with different doses of curcumin for 24 hours. Total RNA from control and curcumin-treated cells was reverse-transcribed into cDNA. We have performed a PCR analysis using RON and actin primers (Fig. 1C, lane 1). Curcumin treatment dose-dependently decreased RON mRNA expression in both cells (Fig. 1C). When cells were treated with 10 μM/L of curcumin, an ~45% and 25% decrease in RON mRNA levels was detected in MDA-MB-231 and MDA-MB-468, respectively, and reached a
maximum of nearly 3.8-fold at 80 μmol/L in both cells (Fig. 1C). These results indicate that the decrease in RON protein expression observed in MDA-MB-231 and MDA-MB-468 cells was due to the decrease in RON message levels following curcumin treatment. Actin message levels were shown as a control.

**Effect of curcumin on RON promoter activity.** To determine whether the decrease in RON expression levels following curcumin treatment in the invasive breast cancer cells was due to decreased RON transcription, we analyzed RON promoter activities using the −1.2 kb full-length RON promoter in the absence/presence of curcumin in MDA-MB-231 and MDA-MB-468 cells. We have recently described the RON promoter construct (24). The RON promoter lacks a distinct TATA box or CCAAT sequences. However, it contains several GC boxes and consensus sequences for seven Sp1-binding sites and we have shown that Sp1 is required for basal RON promoter activity and RON gene expression. Now, using two different software analyses (TFsearch, Genomatix) of the RON promoter region, we have identified two putative NFκB p65-binding sites at −133 bp and −156 bp relative to the transcription start site. We refer to them as NFκB1 and NFκB2, respectively (Fig. 1D). We have transiently transfected pGL3 control vector or −1.2 kb full-length RON promoter into MDA-MB-231 and MDA-MB-468 cells. Twenty-four hours following transfection, we treated the cells with varying concentrations of curcumin for 24 hours and analyzed the RON promoter activity. The luciferase activity of the −1.2 kb full-length RON promoter was approximately 12 times and 7 times higher than that of the pGL3 basic vector in MDA-MB-231 and MDA-MB-468 cells (Fig. 1D; compare lane 1 versus lane 2). Furthermore, cells transfected with −1.2 kb of RON promoter, when incubated with varying amounts of curcumin for 24 hours,

![Figure 1](image-url)
curcumin for 24 h and luciferase activity was measured following normalization to protein levels. Reporter vectors were transiently transfected into breast cancer cells. Twenty-four hours following transfection, cells were treated with varying concentrations of curcumin for 24 h and luciferase activity was measured following normalization to protein levels. C. EMSA: the oligonucleotide-containing NF-κB consensus binding site was end-labeled with \( ^{32}P \)-ATP, and gel shift analysis using nuclear extracts from control and varying concentrations of curcumin-treated MDA-MB-231 cells was performed.

displayed a gradual inhibition of the luciferase activity in a dose-dependent manner and a maximum inhibition of 95\% was achieved at 80 \( \mu \)mol/L concentration in both cells. These results show that the decrease in RON expression in curcumin-treated cells was due to the decrease in RON transcription.

**Effect of curcumin on NF-κB p65 protein expression/activity.**

One of the well-established biological effects of curcumin that is potentially associated with its chemoprevention ability is the inhibition of the NF-κB pathway. Constitutive NF-κB activity is associated with enhanced proliferation and survival of malignant cells. We have identified two putative NF-κB p65 subunit binding sites on the RON promoter. We have analyzed p65 protein levels and p65 transcriptional activity to determine if a curcumin-mediated decrease in p65 protein expression/transcriptional activity was contributing to a decrease in RON protein levels in the cells. We have treated MDA-MB-231 and MDA-MB-468 cells with varying concentrations of curcumin for 24 h and carried out Western analyses on equal amounts of cell lysates from control and curcumin-treated cells (Fig. 2A). A curcumin dose-dependent decrease in the p65 protein expression was observed in both cell lines. To determine the effect of curcumin on NF-κB p65 subunit transcriptional activity, we have transiently transfected NF-κB cis-responsive element luciferase reporter construct or empty control vector into MDA-MB-231 and MDA-MB-468 cells. Twenty-four hours following transfection, we treated the transfectants with varying concentrations of curcumin for 24 h and analyzed the activity of NF-κB cis-responsive element. A curcumin dose-dependent decrease in NF-κB cis-responsive element transcriptional activity was observed in both cell lines (Fig. 2B). We have performed gel shift analysis (EMSA) to determine the effects of curcumin on NF-κB binding activities. The oligonucleotide-containing consensus NF-κB binding site was end-labeled with \( ^{32}P \)-ATP, and EMSA was performed using nuclear extracts from control and varying concentrations of curcumin-treated MDA-MB-231 cells (Fig. 2C). A curcumin dose-dependent decrease in the NF-κB binding activities was observed.

**Effect of curcumin on ectopic p65-mediated RON expression.**

We next investigated whether forced expression of p65 would stimulate the RON promoter activity as well as NF-κB protein expression. MDA-MB-231 and MDA-MB-468 cells were cotransfected with −1.2 kb RON promoter and increasing doses of pCMV p65 and were assayed for RON promoter luciferase activity 48 h following transfection. The activity of −1.2 kb RON promoter in both cells was effectively induced in a dose-dependent manner by pCMV p65 (Fig. 3A). Even at a very low p65 concentration of 5 ng, the expression of −1.2 kb RON promoter luciferase reporter activity was induced by ~5-fold and 7-fold in MDA-MB-231 and MDA-MB-468 cells, respectively (Fig. 3A, compare lane 1 versus lane 2). Furthermore, increasing concentrations of p65 dramatically increased the RON promoter activity and it peaked at a p65 concentration of 250 ng. In contrast, when cells were treated with curcumin for 24 h following cotransfection of −1.2 kb RON promoter 250 ng of p65, nearly the 17-fold induction of the 1.2 kb RON promoter mediated by p65 in both cells was almost completely abolished (Fig. 3A, compare lane 5 versus lane 6). This finding suggests that p65, a component of NF-κB, could be a key player in trans-activating the RON promoter and curcumin may well down-regulate the p65-mediated RON promoter activity.

Because ectopic p65 dose-dependently increased RON promoter activity, we investigated whether the transcriptional activation of RON by p65 could affect RON protein expression. We have transiently transfected MDA-MB-231 and MDA-MB-468 cells with CMV-control vector or varying amounts of pCMV p65 expression plasmid. Twenty-four hours following transfection, one set of transfectants was treated with 80 \( \mu \)mol/L of curcumin for 24 h. Forty-eight hours following transfection of ectopic p65,
the p65 and RON protein expression levels in MDA-MB-231 and MDA-MB-468 cells were detected by Western blot (Fig. 3B). As expected, p65 protein levels were increased in cells transfected with the p65 expression plasmid compared with the levels in cells transfected with mock plasmid (Fig. 3B, lane 2). Interestingly, although the basal level of RON expression in these cell lines is high, the ectopic p65 at the tested concentrations of 500 ng and 1 μg increased the RON expression significantly. Furthermore, we examined whether curcumin could modulate the expression of RON induced by ectopic p65. As shown in Fig. 3B, curcumin attenuated the p65-induced increase in RON protein which parallels the increased p65 expression. These results highlight the fact that curcumin may perhaps be a potent pharmacologic agent which is capable of down-regulating RON through its ability to interfere with the p65 component of NFκB binding to RON promoter.

**In vivo binding of NFκB p65 to RON promoter.** To show that the NFκB p65 subunit binds to RON promoter in vivo, we have carried out ChIP analysis with control IgG or p65 antibodies on the chromatin fragments from MCF-7, MDA-MB-231, and MDA-MB-468 cells (Fig. 4A). DNA from the immunoprecipitates was isolated and PCR using primers covering the p65 binding sites on RON promoter was performed on the isolated DNA and nonimmunoprecipitated starting material (input). RON was detected in the input chromatin fragments from all three cell lines. However, an accumulation of RON was detected in the p65 immunoprecipitates from MDA-MB-231 and MDA-MB-468 cells but not in MCF7 cells, which have been recently shown to be null for RON expression (24). RON was not detected in the control IgG immunoprecipitates, demonstrating the specificity of p65-binding to the RON promoter.

**Effect of p65-binding site mutations on RON promoter activities in the invasive breast cancer cells.** Using two different computer analysis tools (TFSEARCH, Genomatix) on the promoter region of RON, we have identified two putative binding sites for NFκB p65: one at −133 bp and the other at −156 bp relative to the transcription start site. We have referred to them as NFκB1 RON and NFκB2 RON, respectively. To better evaluate the contribution of NFκB to the activity of the RON promoter, the −1.2 kb full-length and −400 bp RON promoter deletion constructs which retains the two putative NFκB binding sites were mutated either alone or in combination as given in Materials and Methods. The wild-type, deletion, and mutant RON constructs were transiently transfected individually into MDA-MB-231 and MDA-MB-468 cells, and the RON promoter activity was evaluated after 48 hours. It is interesting to note that the deletion of 800 bp upstream of the −400 bp proximal promoter region did not affect the basal RON promoter activity. This data suggests that the −400 bp RON promoter contains all the necessary regulatory elements. Mutation of the NFκB1 site in both the −1.2 kb full-length and −400 bp RON promoter resulted in ~30 and 35 times decrease in the promoter activity against the basal transcriptional activity of RON in MDA-MB-231 and MDA-MB-468 cells, respectively (Fig. 4B, compare lane 2 versus lane 3). In addition, mutating the NFκB2 site also dramatically decreased the basal promoter activity (Fig. 4B, compare lane 2 versus lane 4). The transfection of a double-mutant construct almost totally abolished the basal promoter activity of RON constructs, suggesting the fact that both NFκB sites could be clearly involved in basal RON promoter activation in the breast cancer cells.

**PDTC or siRNA p65 knockdown blocks RON gene expression and MSP-induced invasion of breast cancer cells.** We have shown that the NFκB p65 subunit could transcriptionally regulate RON gene expression. We next determined whether NFκB p65 is indeed necessary for regulating RON expression. PDTC, a potent chemical inhibitor of activation of NFκB or siRNAs targeting p65, was used to decrease p65 expression and the effect of down-regulating p65 on RON protein expression was examined. PDTC was shown to be specific in the inhibition of NFκB expression (26). When MDA-MB-231 cells were treated with different

---

**Figure 3.** A, effect of ectopic p65 on RON promoter activity: cells were transfected with −1.2 kb of full-length RON promoter and varying amounts of CMV-p65 expression plasmid. Twenty-four hours following transfection, one set of transfectants was treated with 80 μmol/L curcumin for 24 h. Forty-eight hours following transfection, luciferase activity was measured following normalization to protein levels. B, effect of ectopic p65 on RON expression: cells were transfected with CMV-control vector or varying amounts of CMV-p65 expression plasmid. Twenty-four hours following transfection, one set of transfectants was treated with 80 μmol/L of curcumin for 24 h. Forty-eight hours following transfection, p65, RON, and actin expression levels were measured by Western blot.
concentrations of PDTC, p65 levels were dose-dependently decreased by PDTC, and to our interest, RON protein levels were also significantly reduced when compared with the untreated control cells (Fig. 5A). Similarly, when MDA-MB-231 cells were transfected withchemically synthesized siRNA pools directed against p65, not only were the cellular p65 levels decreased effectively, but the RON protein levels were also decreased, and this decrease was dose-dependent as well (Fig. 5B). However, neither DMSO (Fig. 5A) nor scrambled siRNA (Fig. 5B) modulated RON expression. Consequently, these data strongly suggest that RON tyrosine kinase receptor could be down-regulated by blocking the p65 component of NFκB. To show the specificity of p65 siRNA-mediated RON knockdown on the blockade of MSP-mediated invasion of MDA-MB-231 cells, we have expressed control scrambled siRNA or p65 siRNA in MDA-MB-231 cells. Forty-eight hours following transfection, cells were trypsinized and plated along with untreated control cells for the Matrigel assay, either in the presence or absence of 2.5 ng of the RON ligand, MSP. MSP promoted the invasion of untransfected control or scrambled siRNA-transfected cells (Fig. 5C). However, p65 siRNA-transfected MDA-MB-231 cells exhibited almost complete blockade in invasion because RON expression was inhibited in these cells.

**Discussion**

In general, tumors exhibit higher tyrosine kinase activity in comparison to normal tissues. This correlation is also reflective in tumor cells versus normal epithelial cells. Receptor tyrosine kinases modulate diverse processes involved in tumor progression and metastasis. Consequently, receptor tyrosine kinases are viewed as attractive targets for molecular therapy. An understanding of the molecular alterations that facilitate tumor progression and metastasis will provide insight into approaches to optimize targeted therapies. In this context, the role of the receptor tyrosine kinase RON in human epithelial cell malignancies is currently under active investigation. We have recently shown that the RON ligand, MSP, promotes the invasive phenotype of MDA-MB-231 and MDA-MB-468 cells and also identified the critical regulatory elements that are required for basal RON promoter activity and RON gene expression (24). We have followed up those studies in this article and showed that a chemopreventive agent, curcumin (diferuloylmethane) can effectively block RON tyrosine kinase-mediated invasion of MDA-MB-231 and MDA-MB-468 cells.

RON is expressed at relatively low levels in normal epithelial cells and is absent in fibroblasts. The levels of RON expression in...
malignant epithelial cells was shown to be increased by several fold in comparison to benign epithelium (27). RON expression was an independent predictor of distant relapse in node-negative breast cancer (28). Mammary-specific RON receptor overexpression induced metastatic mammary tumors, which has been shown to involve β-catenin activation (29). Our recent data clearly showed that RON promotes MSP-stimulated invasion of MDA-MB-231 and MDA-MB-468 cells (24). The acquisition of invasive phenotype is directly correlated to the expression of oncogenic RON tyrosine kinase. These studies suggest that blocking RON expression will have therapeutic benefits. RT-PCR and Western analysis indicated that curcumin, a chemopreventive and chemotherapeutic agent, reduces RON message and RON protein in MDA-MB-231 and MDA-MB-468 cells, respectively (Fig. 1A and C). However, the decrease in RON message was not due to the decrease in RON mRNA stability (Supplemental Fig. S1). Most significantly, curcumin-mediated down-regulation of RON expression resulted in the abrogation of RON ligand, MSP-induced invasion of these breast cancer cells. Previous reports indicated that curcumin also blocks the invasion and metastasis of MDA-MB-231 cells through the inhibition of matrix metalloproteinases (30, 31).

Overexpression and activation of RON in epithelial cancers in contrast to normal epithelium suggests that cellular mechanisms that control RON expression are dysregulated in primary tumors and tumor cell lines. The RON gene promoter has been partially characterized (24). The RON promoter lacks a distinct TATA box or CCAAT sequence. However, it is GC-rich and contains consensus sequences for seven Sp1-binding sites. We have recently shown that out of these seven Sp1 sites, Sp1 site at −113 bp and an overlapping Sp1 site at −94 bp relative to the transcription start site are involved in the basal RON promoter activity and RON gene expression (24). However, curcumin reduced RON expression without affecting Sp1 expression levels or Sp1 transcriptional activity (data not shown). This result was not surprising because it was previously reported that curcumin did not affect Sp1 activity (16, 32). Our data indicated that curcumin shows a dose-dependent decrease in the RON promoter activity (Fig. 1D). Numerous studies indicate that curcumin’s anticarcinogenic effects have been attributed to its ability to inhibit transcription factors such as NFκB and AP-1 (16, 33–35). Curcumin inhibits NFκB activation and NFκB-regulated gene expression through inhibition of IKK activation (21). We have seen similar results (Supplemental Fig. S3).

Using two different software programs (TFSearch, Genomatix), we have identified two putative NFκB-binding sites at −133 bp and −156 bp relative to the transcription start site on the RON promoter. There is one AP-1 binding site at −1183 bp relative to the transcription start site. In vivo ChIP analysis clearly indicated the binding of the NFκB p65 subunit to native RON promoters in MDA-MB-231 and MDA-MB-468 cells which express RON protein (Fig. 4A). MCF-7 cells that do not express RON protein and are noninvasive when stimulated with RON ligand, MSP, do not exhibit the binding of NFκB p65 subunit to the RON promoter, thus suggesting the authenticity of the two novel NFκB p65 subunit binding sites on the RON promoter in the RON expression–positive MDA-MB-231 and MDA-MB-468 cells. We have recently shown that the −1.2 kb full-length and −400 bp RON promoter deletion constructs exhibited similar activities in MDA-MB-231 and MDA-MB-468 cells, suggesting that the −400 bp region of the promoter contains all the necessary regulatory elements required for the RON promoter activity and RON gene expression (24). The −400 bp RON promoter region does not contain the AP-1 element, thus ruling out the involvement of AP-1 in the regulation of RON gene expression. Site-directed mutagenesis revealed that both the NFκB binding sites at −133 bp and −156 bp are equally important in the regulation of RON promoter activity (Fig. 4B). Phorbol ester, TPA, an inducer of NFκB activity stimulated RON promoter activity.

Figure 5. PDTC or p65 siRNA blocks RON expression and invasion. Equal amounts of cell lysates from control and PDTC-treated cells (A) or control (B), scrambled siRNA, or p65 siRNA were resolved by 7.5% SDS-PAGE, and Western analysis using anti-human RON, p65, and actin polyclonal antibodies was performed. C, control, scrambled siRNA, or p65 siRNA–transfected cells were plated in Matrigel invasion chamber (top) and treated with 2.5 ng/mL of MSP for 24 h. The migratory cells on the undersurface of the membrane were fixed in 70% methanol and stained with crystal violet. Images of migratory cells were captured by a photomicroscope (Nikon Eclipse-TE 2000-U; magnification, X10).
Curcumin decreased the p65 protein expression as well as p65-dependent NFκB cis-element transcriptional activity in both MDA-MB-231 and MDA-MB-468 cells (Fig. 2A and B). The mechanism of p65 protein down-regulation following curcumin treatment is unclear. One possibility is that curcumin treatment directly or indirectly leads to ubiquitination and proteasome-mediated degradation of p65. Another possibility is that curcumin affects the transcriptional regulation of p65 promoter. The decrease in p65 protein levels was a consequence of the decrease in p65 mRNA as reported previously in the curcin-treated MDA-MB-231 cells (33). Studies using PDTC, a specific inhibitor of NFκB p65 subunit activity or siRNA-mediated p65 knockdown, confirmed the requirement of p65 for RON gene expression (Fig. 5A). The loss of p65 protein expression in the presence of PDTC might be the consequence of the effects of PDTC on the inhibition of IκB activity (Supplemental Fig. S4) or due to the decrease in p65 mRNA as previously reported (36, 37). Most significantly, p65 siRNA-mediated inhibition of RON expression abrogated RON ligand, MSP-mediated invasion of MDA-MB-231 cells (Fig. 5C). To surmise, the results presented in this report identified the NFκB p65 subunit as a regulator of the human RON gene and suggests a potential therapeutic role for curcumin in the blockade of RON tyrosine kinase-mediated invasion of carcinoma cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 12/31/2007; revised 3/24/2008; accepted 4/10/2008.
Grant support: NCI CTBC grant P00 CA 54174.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Amalraj Thangasamy for the construction of the −400 bp RON promoter-luciferase reporter, Dr. Rysani Chandrasekar for providing NFκB cis-response element, Jessica Rogge and Dr. Lenin Mahmaintainath for technical assistance, and Dr. Senlin Li for the photomicroscope facilities.

References
16. Hour TC, Chen J, Huang CY, Guan JY, Lu SH, Pu YS. Curcumin enhances cytotoxicity of chemotherapeutic agents in prostate cancer cells by inducing p21(WAF1/ CIP1) and C/EBPβ expression and reducing chemoresistance. Prostate 2005;65:211–18.
22. Cancer Res 2008;68: (13), July 1, 2008 5192 www.aacrjournals.org
Cancer Research
5192
Cancer Research
in MDA-MB-231 and MDA-MB-468 cells, which was effectively blocked by curcumin (data not shown). Also, ectopic p65 induced RON promoter activity, RON mRNA, and RON protein expression, which was effectively abrogated by the curcumin treatment (Fig. 3A and B; Supplemental Fig. S2).