ARTICLE
Journal of Cellular Biochemistry 113:1217–1223 (2012)

Journal of Cellular Biochemistry

Upregulation of Recepteur d'Origine Nantais Tyrosine Kinase and Cell Invasiveness via Early Growth Response-1 in Gastric Cancer Cells

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ABSTRACT

Abnormal accumulation and activation of the recepteur d'origine nantais (RON) has been implicated in carcinogenesis of epithelial tumors. RON expression was induced by the tumor promoter, phorbol 12-myristate 13-acetate (PMA), in gastric adenocarcinoma AGS cells. Studies with deleted and site-directed mutagenesis of Egr-1 promoter and with expression vectors encoding Egr-1 confirmed that Egr-1 is essential for RON expression. In addition, AGS cells pretreated with PMA showed remarkably enhanced invasiveness, which was partially abrogated by siRNA-targeted RON and Egr-1. These results suggest that tumor promoter induces RON expression via Egr-1, which, in turn, stimulates cell invasiveness in AGS cells. J. Cell. Biochem. 113: 1217–1223, 2012.

KEY WORDS: RON; Egr-1; GASTRIC CANCER

Despite decreasing incidence over the last few decades, gastric cancer remains the most common gastrointestinal tract cancer [Ganesh et al., 1996; Yamashita et al., 2011]. Its prognosis is poor and mortality is high. Due to local tissue invasion and metastasis, radiotherapy or chemotherapy do not significantly affect the length or quality of life in advanced gastric cancer patients. Thus, novel therapies are needed to target molecular aberrations that lead to gastric cancer progression.

Recepteur d'origine nantais (RON), a member of the c-Met family of scatter factor receptors, plays an important role in the development, progression, and metastasis of gastric carcinoma [Park et al., 2011]. RON can be activated through ligand-dependent or -independent mechanisms, which leads to cancer development and metastasis [Feres et al., 2009].

Macrophage-stimulating protein (MSP) is the only ligand identified for RON [Wang et al., 1994]. Upon ligand binding, RON dimerizes, autophosphorylates, and transduces a variety of signals that regulate different downstream pathways including Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), c-Jun N-terminal kinase (JNK), β-catenin, and nuclear factor-κB (NF-κB) [Chen et al., 2000; Wang et al., 2009]. Several human malignancies show aberrant expression and activation of RON, including cancers of the breast, colon, and prostate [Leonis et al., 2007].

It was previously suggested that multiple regulatory elements are needed for full RON promoter activity and gene expression [Thangasamy et al., 2009]. Like many tyrosine kinase receptor gene promoters, the RON promoter also lacks a distinct TATA box or CCAAT sequences. However, it contains several GC boxes, seven Sp1-binding sites, four retinoblastoma control elements, three IL-6 response, and two AP-2 elements [Thangasamy et al., 2008]. However, how this important oncogene is regulated remains unelucidated.

The aims of this study were to identify an additional *cis*-element. A putative Egr-1-binding motif in the RON gene promoter region was identified in gastric cancer cells.

Grant sponsor: National Cancer Center; Grant number: 0720570; Grant sponsor: Basic Science Research Program (National Research Foundation of Korea); Grant number: 2010-0009910; Grant sponsor: Medical Research Center (Korean Science and Engineering Foundation); Grant number: R13-2002-013-06001-0.

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Received 5 August 2011; Accepted 4 November 2011 • DOI 10.1002/jcb.23454 • © 2011 Wiley Periodicals, Inc.
Published online 17 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

MATERIALS AND METHODS

CELL CULTURE AND CULTURE CONDITIONS

Human gastric cancer AGS cells were obtained from the American Type Culture Collection (Manassas, VA) and MKN28 and SNU638 cells were from Korean Cell Line Band (Seoul, Korea). The cells were cultured with RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in an atmosphere containing 5% CO_2. To determine the effect of the tumor promoter, phorbol 12-myristate 13-acetate (PMA), on RON expression, cells were harvested at various intervals and the protein and messenger RNA (mRNA) levels of RON were determined by Western blot and reverse transcription-polymerase chain reaction (PCR) analyses, respectively.

REVERSE TRANSCRIPTION-PCR

Total RNA was extracted from AGS cells using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was used for first-strand complementary DNA synthesis using random primers and superscript reverse transcriptase (Invitrogen). The complementary DNA was subjected to PCR amplification with the primer sets for RON and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The specific primers sequences were RON sense, 5′-ACGGCTTAGCGC-CACTGAGC-3′ and RON antisense, 5′-CATGTGTGCCACTGTGACGT-3′ (550 bp); and GAPDH sense, 5′-TTGTTGCCATCAATGACCCC-3′ and GAPDH antisense, 5′-TGACAAAGTGGTCGTTGAGG-3′ (836 bp). PCR involved denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 50 s.

REAL-TIME REVERSE TRANSCRIPTION-PCR (qRT-PCR)

Extracted total RNA was then reverse-transcribed to cDNA with a cDNA synthesis kit (Takara Biochemicals, Kyoto, Japan) according to the manufacturer's instructions. The RON and 18S primers used in the reaction were purchased from Qiagen (Valencia, CA). Briefly, qRT-PCR for indicated genes was carried out using SYBR Premix Ex Taq (Takara Biochemicals) by an iCycler (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The program was run with an initial predenaturation step at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 55°C for 15 s, and 72°C for 15 s. All cDNA samples were synthesized in parallel, and PCR reactions were run in triplicate. mRNA levels were derived from standard curves and were expressed as relative changes after normalization versus 18S mRNA levels.

CONSTRUCTION OF RON PROMOTER REPORTER CONSTRUCT

An approximately 3 kb construct of RON promoter fragment was synthesized from human genomic DNA (Promega, Madison, WI) by PCR using the primers 5′-GGTACCTAGCTGACC-3′ (forward primer) and 5′-GGGCCAAATTTAAGC-3′ (reverse primer). The amplified PCR products were ligated into a T&A vector (RBC Bioscience, New Taipei, Taiwan) and then digested with *Kpn*I and *Bgl*II. The products were ligated into the *Kpn*I and *Bgl*II sites of the pGL3-basic vector (Promega). A series of deletion constructs of human RON promoter fragments was synthesized by PCR using the pRON–Luc plasmid as template. Forward primer sequences were 5′-CCAAGGGCCG-GAAGA-3′ (−128/+173, pGL3–RON-301), 5′-TCGGCTGAGCGC-TAA-3′ (−20/+173, pGL3–RON-193), and 5′-TCGTGCGTCC-GCAGG-3′ (+50/+173, pGL3–RON-123). One reverse primer, 5′-GGGCCAAATTTAAGC-3′, was used to generate all three deletion constructs. The amplified PCR products were ligated into the T&A vector and then digested with *Kpn*I and *Bgl*II. The products were ligated into the *Kpn*I and *Bgl*II sites of the pGL3-basic vector. Site-directed mutagenesis was utilized to mutate potential transcription Egr-1 elements in the promoter region. Mutant promoter constructs were generated using pGL3–RON-301 construct as template. The primers used for mutagenesis (mutations underlined) were as follows: CCCGCCCCA to CCCAATTCCA (pGL3-Mt1), TCCGCCCGCC to TCCATATGCC (pGL3-Mt2). The mutated nucleotide sequences of all mutant constructs were confirmed by DNA sequencing.

RON PROMOTER REPORTER ASSAY

The transcriptional regulation of RON was examined by the transient transfection of a RON promoter–luciferase reporter construct (pGL3–RON). AGS cells (5×10^5) were seeded and grown until they reached 60–70% confluence, pGL3–RON wild type and deletion mutants were transfected into the cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. The importance of Egr-1 in the induction of RON was examined by co-transfecting the cells with pGL3–RON and Egr-1 expression plasmids containing a full-length cDNA coding for human Egr-1 (generous gifts from Dr. Young Han Lee, Konkuk University, Seoul, Korea). To monitor transfection efficiency, the pRL-null plasmid encoding Renilla luciferase was included in all samples. At 24 h posttransfection, the levels of firefly and Renilla luciferase activity were measured sequentially from a single sample using the Dual-Glo Luciferase Assay System (Promega). The firefly luciferase activity was normalized to Renilla activity, and the relative amount of luciferase activity in the untreated cells was designated 1.

SMALL INTERFERING RNA TRANSFECTION

Gene silencing was performed using human Egr-1 (sc-29303; Santa Cruz Biotechnology, Santa Cruz, CA) and human RON sequence-specific duplex small interfering RNA (siRNA; sc-36434). Briefly, for each transfection reaction in two separate tubes, 20 nM siRNA oligonucleotides and 2 μl of Lipofectamine RNAiMAX (Invitrogen) was mixed with 100 μl of the serum-free medium Optimem (Hyclone, Logan, UT) and incubated for 5 min at room temperature. After this time, the contents of the two tubes were combined and allowed to form siRNA–Lipofectamine complexes for 30 min at room temperature. A 900 μl volume of AGS cells (5×10^5) cultured in serum-free medium was combined with the siRNA–Lipofectamine mix, plated in a six-well tissue culture dish and placed in an incubator at 37°C, 5% CO_2 incubator for 5 h. After this time, the medium was replaced with normal growth medium.

WESTERN BLOT ANALYSIS

The cells were suspended in ice-cold RIPA-M buffer with 1% NP-40 and cell lysates were prepared as previously described [Park et al., 2011]. The cell lysate protein (100 μg) was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). The blots

were blocked for at least 1 h at room temperature in a blocking buffer (5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20; TBST). The anti-RONβ (Santa Cruz Biotechnology) and anti-Egr-1 antibodies (Cell Signaling Technology, Beverly, MA) were diluted in a blocking buffer and incubated with the blots overnight at 4°C. The bound antibodies were detected with a 1:3,000 dilution of horseradish peroxidase-conjugated secondary antibody according to the instructions provided with the ECL kit (Amersham, Franklin Lakes, NJ).

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

AGS cells (2×10^6) grown in a six-well plate were cross-linked with 0.5% (v/v) formaldehyde at 37°C for 5 min. The cells were sonicated for 3×20 s before centrifugation at 16,000*g* for 15 min at 4°C. Specific DNA-bound transcription factor complexes were precipitated with 20 μl of anti-Egr-1 at 4°C overnight before the addition of Protein A-agarose beads. The proteins were removed from DNA by digestion with 10 μg/ml proteinase K at 65°C for 30 min. The DNA was recovered from the solution by Qiaquick PCR purification kit (Qiagen), and eluted in 50 μl of sterile water. Eluted DNA (20 μl) was subjected to PCR with F-5′-AGGAGCCAGGCCTCCAAGGGC-3′ and R-5′-TCCCGACAGCCCCAAGATAGC-3 primers, which flank the binding sites of Egr-1.

MATRIGEL INVASION ASSAY

The cell invasion assay was carried out using the BIOCOAT matrigel invasion chambers (Becton-Dickinson, Bedford, MA) with 10% fetal bovine serum as chemo-attractant in the lower chamber. AGS cells (10^5) in 300 μl were allowed to invade the matrigel for 24 h. The non-invading cells on the upper surface of each membrane were removed from the chamber, and the invading cells on the lower surface of each membrane were stained with the Quick-Diff stain kit (Becton-Dickinson, Franklin Lakes, NJ). After two washes with water, the chambers were allowed to air-dry. The number of invading cells was counted using a phase-contrast microscope.

RESULTS

INDUCTION OF RON BY PMA IN HUMAN GASTRIC CANCER AGS CELLS

To determine the effect of PMA on RON expression in human gastric cancer AGS cells, the cells were incubated with PMA for various time periods and the levels of RON mRNA and protein in the cells were determined by RT-PCR and Western blotting analyses, respectively. As shown in Figure 1A,B, the level of RON mRNA and protein increased in a time-dependent manner with PMA treatment for 0–12 h. Next, we verified the transcriptional activity of the RON gene promoter by PMA. To this end, we isolated the 5′-regulatory region of human RON gene and subcloned it into the pGL3-Luc luciferase reporter vector, yielding pRON-Luc (−128/+173). This construct was transfected into AGS cells and the reporter gene activity was measured. Promoter activity increased 4 h after incubating the cells with PMA, with the highest level being observed at 12 h in AGS cells (Fig. 1C).

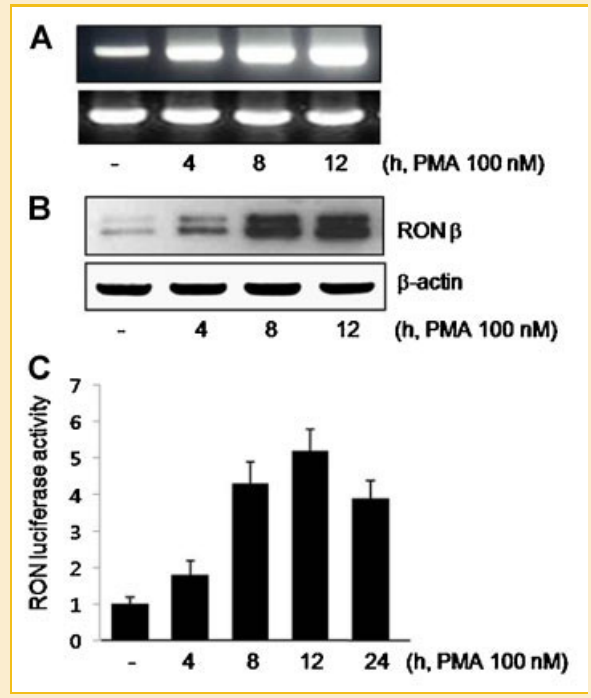

Fig. 1. Induction of RON by PMA in human gastric cancer AGS cells. A,B: RT-PCR and Western blot analyses were performed to determine the effect of PMA on RON mRNA and protein expression in AGS cells, respectively. Cells were incubated with 100 nM PMA for 0–12 h. C: AGS cells were transiently transfected with pGL3-RON reporter (pRON-Luc, −128/+173) construct. The transfected cells were incubated with 200 nM PMA for 0–24 h and luciferase activity was determined using a luminometer. Data represent the mean ± standard deviation from triplicate measurements.

IDENTIFICATION OF AN Egr-1-BINDING SEQUENCE REQUIRED FOR RON PROMOTER ACTIVATION

An analysis of the RON promoter sequences spanning −128/+173 performed using MatInspector (http://www.genomatix.de/) revealed six putative Egr-1-binding motifs located at nucleotides −64, −61, −45, +18, +39, and +124. To identify the role of Egr-1 in the response to PMA, a deletion construct of the RON promoter was generated and transfected into AGS cells. Removal of the 3 Egr-1 binding sites (Egr-1-1 and -2 and Egr-1–3) up to nucleotide position −20 resulted in dramatic loss of PMA responsiveness (Fig. 2A,B). A similar reduction was observed in the construct deleted up to position +50 bp. To study the importance of the Egr-1 sites in RON induction by PMA, site-directed mutagenesis of the −128/+173 construct was performed. A construct containing a mutation in the core sequence of the Egr-1-1 and -2-binding motif (CCCGCCCCA to CCCAATTCCA) and Egr-1–3-binding motif (TCCGCCCGCC to TCCATATGCC) reduced the PMA responsiveness to approximately 58% and 66%, respectively, of that of the wild-type construct. A double-mutant promoter (pGL3-Mt3), containing mutations of both Egr-1-1 and -2 and Egr-1–3, resulted in much loss of PMA inducibility (35% vs. control). These data suggest that both Egr-1-1 and -2 and Egr-1–3 sites are necessary for full PMA-induced RON promoter activation.

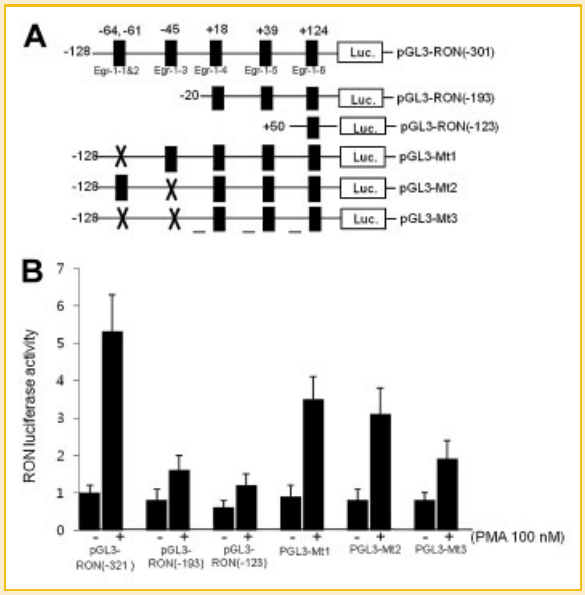

Fig. 2. Identification of the Egr-1-binding sequence required for PMA-induced RON promoter activation. A: Schematic representation of Egr-1 binding sites and deleted and mutated regions in human RON promoter. B: AGS cells were transiently transfected with a series of 5′-deletion constructs of RON promoter reporter plasmid or site-specific mutants for Egr-1 (Mt1, Mt2, and Mt3) derived from the −128/+173 construct. The transfected cells were incubated with or without 200 nM PMA for 8 h and luciferase activity was determined using a luminometer. Data represent the mean ± standard deviation from triplicate measurements.

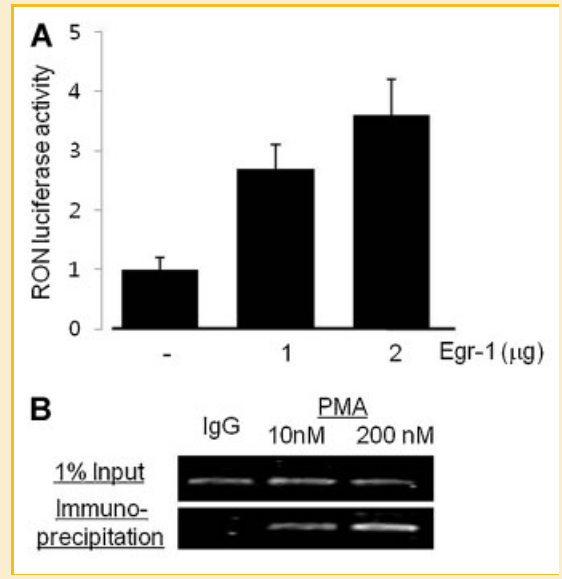

Fig. 3. Activation of transcription factors Egr-1 in PMA-induced RON expression. A: AGS cells were transiently cotransfected with RON promoter reporter plasmids and different concentrations of expression plasmids for Egr-1. After incubation for 48 h, luciferase activity was determined using a luminometer. Data represent the mean ± standard deviation from triplicate measurements. B: ChIP was performed with anti-Egr-1 antibodies at RON promoter in cells treated with 10 and 200 nM PMA for 8 h. The AGS cells treated with PMA were cross-linked, lysed, and immunoprecipitated with anti-Egr-1 along with normal rabbit IgG (for negative control). The precipitated DNA was subjected to regular PCR with primers specific for the Egr-1 (−407/−112).

INVOLVEMENT OF Egr-1 IN RON EXPRESSION

To establish whether Egr-1 alone could trans-activate the RON promoter, an Egr-1 expression plasmid was cotransfected into AGS cells with the pRON–Luc reporter and luciferase reporter activity was measured. Expression of Egr-1 enhanced RON promoter activity in a dose-dependent manner (Fig. 3A). These results suggested that transcription factor Egr-1 alone up-regulates RON expression in gastric cancer AGS cells. To further determine whether Egr-1 binds directly to the putative Egr-1-binding sequence in the RON promoter, a chromatin immunoprecipitation (ChIP) assay was done. Chromatin of AGS cells was immunoprecipitated with rabbit anti-Egr-1 antibodies and the resulting immunoprecipitates were analyzed by PCR using primers flanking the Egr-1-binding sequences (−407 to −112) of the RON promoter. A noticeable increase in the intensity of DNA band was observed when anti-Egr-1 antibodies, but not when normal rabbit IgG, were used (Fig. 3B).

DEPLETION OF ENDOGENOUS Egr-1 EXPRESSION REDUCES RON EXPRESSION

To assess whether endogenous Egr-1 expression is required for PMA-induced RON expression, an RNA interference approach was used. Reduction of Egr-1 expression due to transient expression of siRNA directed against Egr-1 was confirmed by Western blot (Fig. 4A). Western blot and promoter studies showed that RON protein levels and promoter activity induced by PMA were attenuated in AGS cells transfected Egr-1siRNA (Fig. 4B,C). Furthermore, real-time RT-PCR analyses showed that cellular RON mRNA levels induced by PMA were also attenuated in AGS cells transfected Egr-1siRNA, compared to that in control cells (Fig. 5A). Next, to determine if the Egr-1 activation contributed to the expression of RON in different gastric cancer cells, siRNA targeted Egr-1 was transfected into MKN28, SNU638, and AGS cells. As shown in Figure 5B, all the cell lines used displayed a decrease in PMA-induced RON mRNA as determined by RT-PCR.

EFFECT OF PMA, RON siRNA, AND siEgr-1 siRNA ON AGS CELL INVASIVENESS

It has been suggested that the expression of RON is essential for the invasive phenotype of cancer cells. The role of PMA-induced RON in AGS cell invasion was evaluated in a modified Boyden invasion chamber. As shown in Figure 6A,B, cell invasiveness was increased twofold by incubation with PMA. However, cells transfected with RON siRNA and Egr-1 siRNA partially lost the matrigel invasiveness induced by PMA. These results suggest that activation of RON induced by PMA plays an important role in the invasiveness of gastric cancer cells and via Egr-1 signals pathways.

DISCUSSION

Tyrosine kinase receptors regulate multiple processes involved in tumor progression and metastasis and, hence, are attractive targets for molecular therapy. An understanding of the molecular

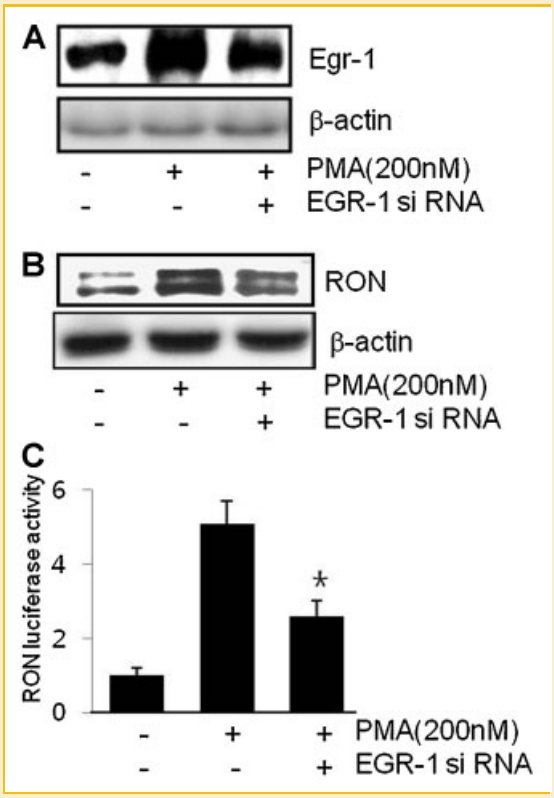

Fig. 4. Effect of siRNA targeted on Egr-1 on PMA-induced RON expression. AGS cells transiently transfected with Egr-1 siRNA were incubated with 200 nM PMA for 8 h. Western blot analysis was performed to determine the effect of Egr-1 siRNA on PMA-induced Egr-1 (A) and RON (B) protein expression in AGS cells. C: AGS cells were transiently cotransfected with Egr-1 siRNA and RON promoter reporter (pRON-Luc, −128/+173) construct. The transfected cells were incubated with 200 nM PMA for 8 h and luciferase activity was determined using a luminometer. Data represent the mean ± standard deviation from triplicate measurements. $^{*}P < 0.05$ versus PMA.

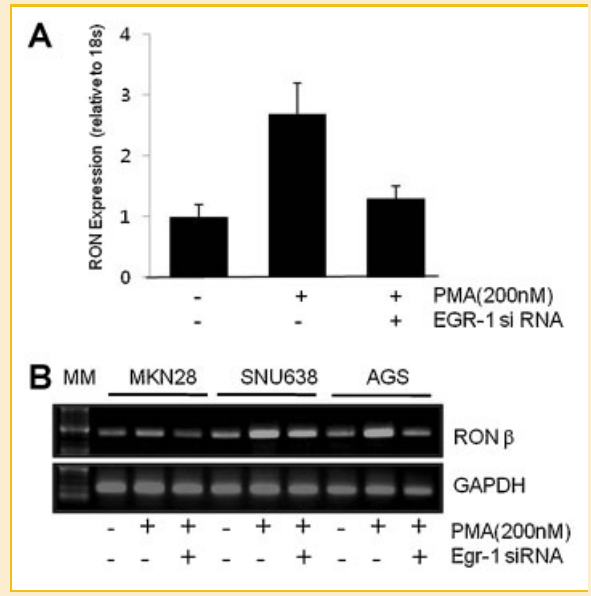

Fig. 5. Involvement of Egr-1 in PMA-induced RON expression in different gastric cancer cells. A: AGS cells transiently transfected with a Egr-1 siRNA were incubated with 200 nM PMA for 8 h. Real-time RT-PCR analysis was performed to determine the effect of Egr-1 siRNA on PMA-induced RON expression. B: MKN28, SNU638, and AGS cells transiently transfected with a Egr-1 siRNA were incubated with 200 nM PMA for 8 h. RT-PCR analysis was performed to determine the effect of Egr-1 siRNA on PMA-induced RON expression.

aberration that leads to tumor progression and metastasis will provide insight into optimizing targeted therapies. The RON receptor tyrosine kinase, also known as the MSP receptor, is a member of the MET proto-oncogene family [Wang et al., 2010]. Upon the binding of MSP, RON is activated via autophosphorylation within its kinase catalytic domain, and transduces a variety of signals such as Ras/MAPK, PI3K, JNK, β-catenin, and NF-κB [Wang et al., 2009; Chen et al., 2000]. The pleiotropic effects of RON include proliferation, tubular morphogenesis, angiogenesis, cellular motility, invasiveness, and resistance to cell death (anoikis) [Camp et al., 2005].

In this study, we delineated the role of RON in conferring the invasive phenotype of gastric cancer cells and the critical regulatory elements necessary for oncogenic RON tyrosine kinase promoter activity and gene expression. RON is mainly transcribed at relatively low levels in normal epithelial cells. However, the levels of RON expression in malignant epithelial cells are increased several fold in comparison with benign epithelium. Aberrant expression and activation of RON have been observed in human cancers and are responsible for various malignant behaviors in breast, colon, and ovarian cancers [Leonis et al., 2007; Thangasamy et al., 2008]. Elevated RON expression and its splice variant have been strongly correlated with tumor occurrence, progression, and metastasis of gastric carcinoma, suggesting that increased RON expression plays a role in the progression of carcinomas to invasive-metastatic phenotypes. [Zhou et al., 2008]. Multiple regulatory elements may be needed for full RON promoter activity and gene expression [Thangasamy et al., 2009]. The RON promoter contains several GC boxes, seven Sp1-binding sites, four retinoblastoma control elements, three IL-6 response, and two AP-2 elements [Thangasamy et al., 2008]. However, the regulation of RON remains unclearly defined.

The results of the present study suggest that Egr-1 is a new regulator of RON transcription that plays an essential role in cell invasion. The transcription factor Egr-1 is an immediate to early response gene rapidly induced by various growth factors, cytokines, and DNA-damaging agents, which modulates cell proliferation, differentiation, apoptosis, and inflammation in a variety of cell types [Thiel and Cibelli, 2002; Shin et al., 2010ab]. Even the role of Egr-1 in tumor development might be largely dependent on tissue type; Egr-1 is highly expressed and plays an essential role in tumor growth and survival in various cancers [Keates et al., 2005; Ma et al., 2009]. Interestingly, Egr-1 levels have been found to be elevated in gastric cancer tissues and it has been suggested that Egr-1 may play an important role in carcinogenesis and cancer progression in the stomach [Kobayashi et al., 2002]. The importance of Egr-1 in tumor promoter-induced RON expression is based on several lines of supporting evidence. An Egr-1-binding sequence in the RON

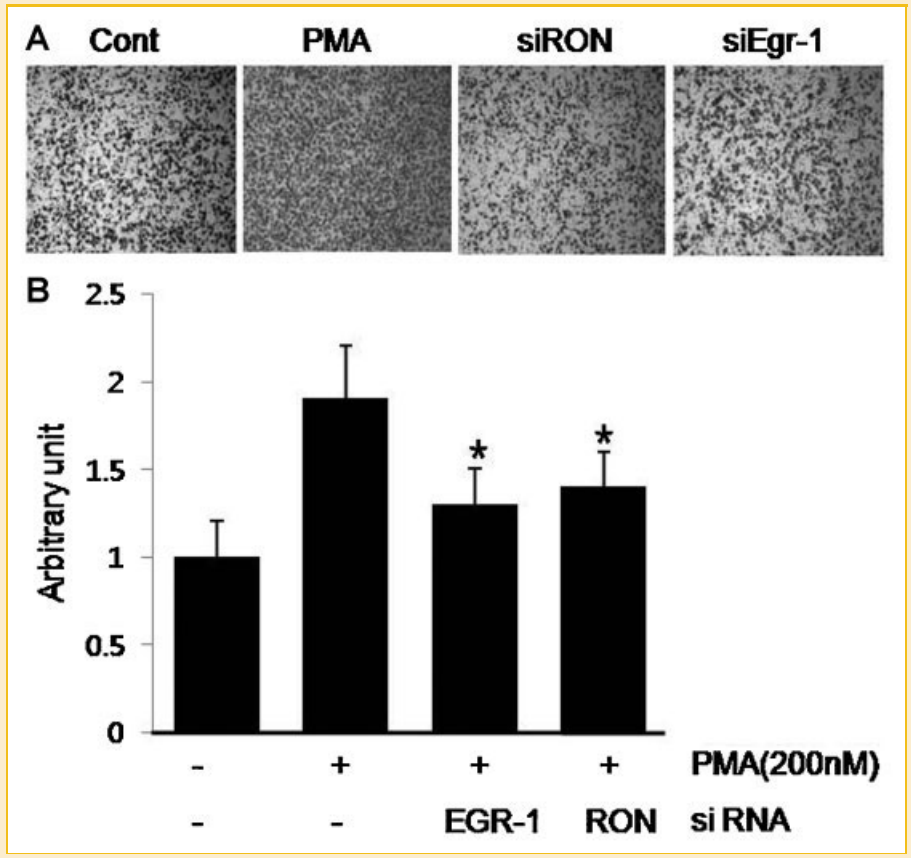

Fig. 6. Effect of siRNA targeted on Egr-1 and RON on PMA-induced cell invasion. AGS cells (10^5) transfected with RON siRNA and Egr-1 siRNA were incubated with 200 nM PMA in a Boyden matrigel apparatus for 24 h. After incubation, the cells having invaded the undersurface of the chambers were counted using a phase-contrast light microscope after staining with a Diff-Quick stain kit (A). Data represent the mean ± standard deviation from triplicate measurements (B). $^*P < 0.05$ versus PMA.

promoter region is necessary for maximal inducible promoter activity in response to PMA, Egr-1 is rapidly induced by PMA and directly binds to the RON promoter region, exogenous Egr-1 transactivates the RON promoter, and siRNA-mediated knockdown of Egr-1 reduces PMA-induced RON expression. The functional importance of the Egr-1-binding site within the RON promoter was analyzed by transient transfection studies using deleted and mutated reporter constructs. We found that the Egr-1-binding element is necessary for induction of maximal RON expression by PMA.

This is the first report to demonstrate that Egr-1 is required for the induction of RON by tumor promoter based on a variety of molecular data in gastric cancer cells. A complete understanding of the molecular mechanisms involved in RON regulation and cell invasion process is crucial for devising better strategies for cancer therapy.

ACKNOWLEDGMENTS

This work was supported by a Research Grant (0720570) from the National Cancer Center, by a Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0009910), and by a Medical Research Center (R13-2002-013-06001-0) from the Korean Science and Engineering Foundation.

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