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# NDRG2 Controls COX-2/$PGE_2$-Mediated Breast Cancer Cell Migration and Invasion

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**N-myc downstream-regulated gene 2 (NDRG2), which is known to have tumor suppressor functions, is frequently down-regulated in breast cancers and potentially involved in preventing the migration and invasion of malignant tumor cells. In the present study, we examined the inhibitory effects of NDRG2 overexpression, specifically focusing on the role of cyclooxygenase-2 (COX-2) in the migration of breast cancer cells. NDRG2 overexpression in MDA-MB-231 cells inhibited the expression of the COX-2 mRNA and protein, the transcriptional activity of COX-2, and prostaglandin $E_2$ ($PGE_2$) production, which were induced by a treatment with phorbol-12-myristate-13-acetate (PMA). Nuclear transcription factor-κB (NF-κB) signaling attenuated by NDRG2 expression resulted in a decrease in PMA-induced COX-2 expression. Interestingly, the inhibition of COX-2 strongly suppressed PMA-stimulated migration and invasion in MDA-MB-231-NDRG2 cells. Moreover, siRNA-mediated knockdown of NDRG2 in MCF7 cells increased the COX-2 mRNA and protein expression levels and the PMA-induced COX-2 expression levels. Consistent with these results, the migration and invasion of MCF7 cells treated with NDRG2 siRNA were significantly enhanced following treatment with PMA. Taken together, our data show that the inhibition of NF-κB signaling by NDRG2 expression is able to suppress cell migration and invasion through the down-regulation of COX-2 expression.**

## INTRODUCTION

N-myc downstream-regulated gene 2 (NDRG2) belongs to the NDRG family, and it has been reported to function as a tumor suppressor gene (Qu et al., 2002). NDRG2 is highly expressed in skeletal muscle, adult brain, and salivary glands (Deng et al., 2003), whereas it is expressed at low levels in clinically aggressive tumors and tumor cell lines (Melotte et al., 2010). Recently, it has been reported that in contrast to the high expression levels of NDRG2 in normal tissues, breast cancer cells display low or undetectable levels of expression (Lorentzen et al., 2011). In addition, NDRG2 diminishes tumor growth and suppresses the metastasis of mouse breast cancer cells by reducing active autocrine TGF-β production (Oh et al., 2012). In our previous study, BMP-4 induced by NDRG2 led to the attenuation of the metastatic potential in breast cancer cells via the repression of active MMP-9 (Shon et al., 2009). These reports have shown that NDRG2 inhibits tumor development, metastasis, and invasion, suggesting that it may act as a tumor suppressor. However, its precise role in regulating the migration and invasion of malignant breast cancer cells remains unclear.

Cyclooxygenase (COX) is the rate-limiting enzyme that converts arachidonic acid into prostaglandins, and it consists of two isoforms, COX-1 and COX-2 (Takai et al., 2013). COX-1 is constitutively expressed in many tissues, such as kidney, lung, stomach, and colon, whereas COX-2 is inducible under extracellular or intracellular stimuli, such as mitogens, growth factors, pro-inflammatory cytokines, hormones, and infectious agents (Li et al., 2013; Williams et al., 1999). Originally, COX-2 was found to be a component of the cellular response to inflammation (Li et al., 2013). It is well known that COX-2 is abnormally expressed in the majority of lung and colorectal cancers and causes tumorigenesis. Moreover, it produces prostaglandins, which induce cancer cell proliferation, angiogenesis, and invasiveness (Fosslien, 2000). Prostaglandin $E_2$ ($PGE_2$) in particular is a predominant prostaglandin, which is known to contribute to tumor growth and apoptotic resistance (Greenhough et al., 2009). Another study showed that COX-2 expression and the production of $PGE_2$ in breast tumors stimulate bone metastasis in both mouse models and patients with breast cancer (Singh et al., 2007).

Nuclear factor-κB (NF-κB) has been well established as a major transcription factor that plays a critical role in the development of inflammation and cancer (Yang et al., 2010). The constitutive activation of NF-κB up-regulates the expression of genes related to tumor growth and metastasis in many cancers, and one of its key targets is COX-2 (Flowers and Thompson, 2009). The inhibition of NF-κB activation by celecoxib, which is the first COX-2-selective inhibitor, is involved in its anti-inflammatory effects (Funakoshi-Tago et al., 2008). The NF-κB pathway contributes to the unusual phenotype and aggressiveness of inflammatory breast cancer, and COX-2 expression regulated by NF-κB signaling is enhanced in metastatic breast cancer (Lerebours et al.,

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2008). Although COX-2 expression has been closely correlated with NF-κB activity in inflammatory breast cancer, the manner by which NF-κB/COX-2 signaling regulates the migration and invasion of breast cancer cells remains unknown.

In this study, we investigated the effects of NDRG2 expression on the migration and invasion of aggressive MDA-MB-231 breast cancer cells. In addition to the down-regulation of PMA-induced COX-2 expression by NDRG2, its overexpression in breast cancer cells resulted in a significant attenuation of cell migration and invasion through the inhibition of NF-κB signaling.

## MATERIALS AND METHODS

### Cell lines and reagents

Human breast cancer cell lines, highly metastatic MDA-MB-231 cells and non-metastatic MCF7 cells (ATCC, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco/Invitrogen), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco/Invitrogen) at 37°C in a humidified 5% $CO_2$ incubator. The PMA, celecoxib, and curcumin were purchased from Sigma-Aldrich (USA).

### Overexpression and knockdown of NDRG2 gene in MDA-MB-231 cells

MDA-MB-231-NDRG2 clones were generated by the stable transfection of the MDA-MB-231 cells with the plasmid pCMV-Taq2B containing the full length human NDRG2 gene using Lipofectamine™ 2000 (Invitrogen). Stable clones were selected using complete medium containing 1 mg/ml G418 (Duchefa, Netherlands). The control and NDRG2 siRNAs for the knockdown experiment were purchased from Santa Cruz Biotechnology (USA). They were transfected into MCF7 cells using Lipofectamine™ RNAiMAX (Invitrogen).

### Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Total RNA from the harvested cells was isolated using the TRIzol reagent (Invitrogen), and it was reverse transcribed into cDNA using an oligo-(dT) primer, dNTPs (Bioneer, Korea), M-MLV reverse transcriptase, and RT buffer (Promega, USA). The cDNAs were amplified by PCR in a reaction mixture containing cDNA, each specific primer, dNTPs, and Top DNA polymerase. The primers and reagents were purchased from Bioneer. The PCR products were electrophoresed in 1% agarose gels containing ethidium bromide. The following primers were used: 5′-CTGAATGGGGTGATGAGCAG-3′ and 5′-ATTCCTACCACCAG CAACCC-3′ for COX-2; 5′-GGATTCATGGCGGAGCTGCAGGA GG-3′ and 5′-GAATTCTCAACAGGAGACCTCCATGGT-3′ for NDRG2; and 5′-CCACACCTTCTACAATGAGC-3′ and 5′-TGA GGTAGTCAGTCAGGTCC-3′ for β-actin. For the quantitative real-time PCR, the amplification was performed using an ABI StepOnePlus™ Real-Time PCR thermal cycler with Power SYBR Green PCR Master Mix according to the manufacturer's protocol (Applied Biosystems, USA). Target mRNA levels were normalized against GAPDH, and the relative mRNA expression levels were calculated. The experiments were performed in triplicate. The following primers were used: 5′-AGGGTTGCTGGTGG TAGGAA-3′ and 5′-GGTCAATGGAAGCCTGTGATACT-3′ for COX-2; and 5′-CTGGGCTACACTGAGCACCAG-3′ and 5′-CCA GCGTCAAAGGTGGAG-3' for GAPDH.

### Western blot analysis

For the preparation of whole cell lysates, the cells were lysed on ice in the PRO-PREP™ reagent (iNtRON Biotechnology, Korea) for 20 min. Nuclear and cytoplasmic extracts were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, USA), according to the manufacturer's instructions. The proteins were separated in an SDS-polyacrylamide gel and transferred to a PVDF membrane (Amersham Biosciences, UK), which was then blocked with Tris-buffered saline plus 0.05% Tween-20 (TBST) containing 5% skim milk. The membranes were incubated with specific antibodies overnight at 4°C and washed with TBST. The antibody recognizing COX-2 was purchased from Cayman Chemical (USA), and the antibodies against NDRG2, p-IKKα/β (Ser176), IKKβ, NF-κB p65, Akt1/2, Lamin A/C, and actin were purchased from Santa Cruz Biotechnology. The antibodies for p-IκBα (Ser32/36), IκBα, p-Erk1/2 (Thr202/Tyr204), Erk1/2, and p-Akt (Ser473) were purchased from Cell Signaling Technology Inc. (USA). After incubating the membrane with the appropriate secondary antibodies coupled to horseradish peroxidase followed by enhanced chemiluminescence, the blots were visualized using the Ez-Capture MG (ATTO Corporation, Japan).

### Wound healing assay

The cells were seeded in 60 mm dishes and incubated for 12 h in starvation medium with 10 μg/ml mitomycin C (Sigma) to inhibit cell proliferation. The cellular monolayer was wounded with a sterile 200 μl-pipette tip and cultured with starvation medium. Cell migration was monitored with a microscope for 48 h.

### Migration and invasion assays

The migration and invasion assays were performed using a 24-well transwell unit with polycarbonated filters (Corning Costar, USA). For the invasion assay, the cells were starved overnight in serum-free media. The lower part of the transwell was filled with DMEM plus 10% FBS, and the transwell was coated with 20 μl of a 1:2 mixture of Matrigel-DMEM (Matrigel; BD Biosciences, USA). The cells ($5 \times 10^4$) that were suspended in the serum-free DMEM were added to the upper part of the transwell and incubated for 24 h at 37°C. The cells remaining in the upper surface of the transwell were completely removed by wiping with a cotton swap, and those migrating to the lower surface were examined by staining with a 0.1% crystal violet/20% ethanol (w/v) solution. The absorbances of the migrating cells were measured at 590 nm after the extraction of the blue dye using 10% acetic acid. The migration assay was performed using a transwell culture system without a Matrigel coating.

### Luciferase reporter assay

Semi-confluent cells grown in 12-well plates were co-transfected with 1 μg of COX-2 (a kind gift from Dr. Soo-Hwan Lee) or NF-κB (Stratagene, USA) luciferase reporter plasmids and 0.4 μg of pCMV-β-galactosidase reporter plasmid for 18 h using Lipofectamine LTX (Invitrogen). After incubation, the cells were harvested in reporter lysis buffer, and the luciferase activity was measured using the Luciferase Assay System according to the manufacturer's instructions (Promega). The β-galactosidase activity was measured using o-nitrophenyl-β-D-galactopyranoside as a substrate, which was used to normalize the variability in transfection efficiencies.

### Enzyme-linked immunosorbent assay (ELISA)

After exposure of the cells to 80 ng/ml PMA for 24 h, the levels of $PGE_2$ secreted from the cells were determined using the $PGE_2$ high sensitivity ELISA kit (Enzo Life Sciences, USA) according to the manufacturer's instructions. The absorbance at 405 nm was

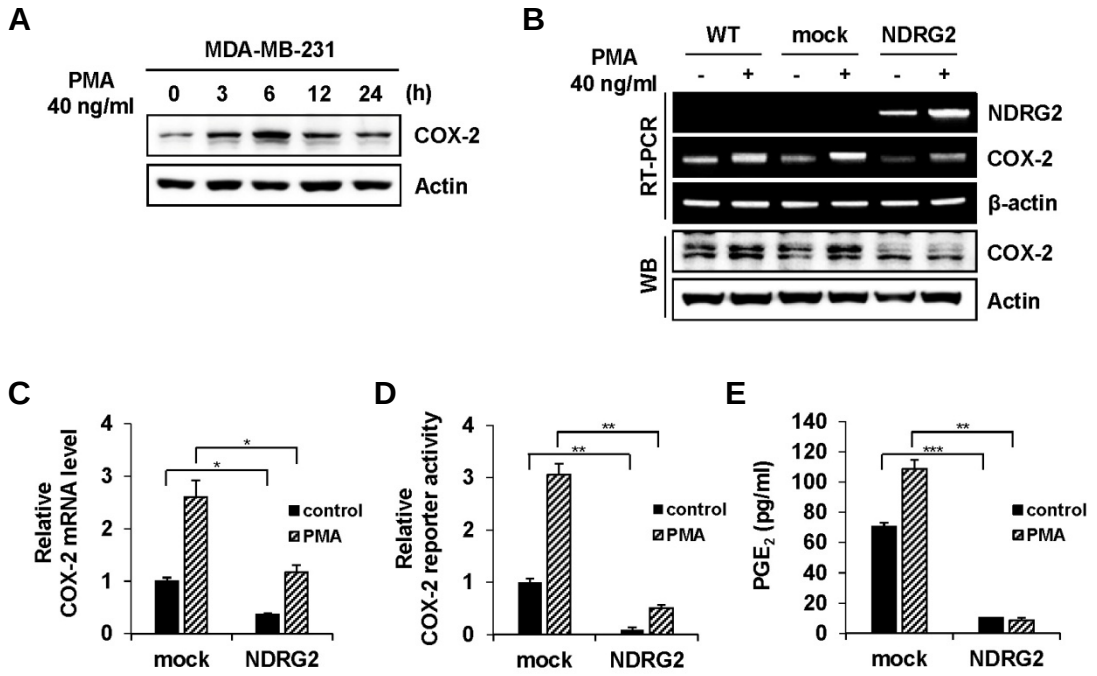


**Fig. 1.** Effects of NDRG2 overexpression on PMA-induced COX-2 expression and $PGE_2$ secretion. (A) MDA-MB-231 cells were exposed to PMA for the indicated time, and the whole-cell extracts were subjected to a Western blot. (B) After 6 h of exposure to PMA, the mRNA and protein levels of COX-2 in MDA-MB-231-WT, -mock, and -NDRG2 cells were detected by RT-PCR and Western blotting, respectively. (C) After PMA treatment, total RNA was isolated from the cultured cells, and the expression of COX-2 was confirmed by quantitative RT-PCR. (D) The semi-confluent cells were transiently transfected with a COX-2 luciferase reporter construct. The transfection efficiency was normalized by co-transfecting the cells with pCMV-β-galactosidase. After 18 h of incubation, the cells were treated with PMA for 6 h and then harvested in lysis buffer, and the luciferase activities were measured with a luminometer using the dual luciferase assay system. The changes in luciferase activity with respect to the mock-control were calculated. (E) After 24 h of treatment with PMA, the supernatants were harvested, and the $PGE_2$ protein secreted from the cells was measured by an ELISA. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

quantified with a VICTOR$^3$ 1420 multilabel counter (PerkinElmer, USA).

### Statistical analysis

Student's *t*-test was used for the statistical analysis. Values are represented as the means ± SD. *P* values of < 0.05 were considered to be significant.

## RESULTS

### NDRG2 overexpression reduces PMA-induced COX-2 expression

To elucidate the effects of NDRG2 overexpression on COX-2 expression in malignant breast cancer cells, we initially established a MDA-MB-231 cell line overexpressing NDRG2 and then treated the cells with PMA to induce COX-2 expression. When MDA-MB-231 cells were treated with PMA, COX-2 expression levels peaked at 6 h, which was followed by a time-dependent decrease up to 24 h (Fig. 1A). As shown in Fig. 1B, mRNA level of COX-2 strongly increased in PMA-treated WT and mock controls, while mRNA expression inhibited by NDRG2 overexpression was only weakly induced under PMA treatment. Likewise, MDA-MB-231-WT and -mock cells stimulated with PMA showed an up-regulation of COX-2 expression compared with the non-treated cells, whereas MDA-MB-231-NDRG2 cells did not show any increases in PMA-induced COX-2 protein expression (Fig. 1B). The COX-2 mRNA levels were confirmed by quantitative real-time PCR (Fig. 1C). To examine whether the effects of NDRG2 overexpression on COX-2 levels contribute to COX-2 promoter activity, we conducted a COX-2 reporter gene assay in MDA-MB-231-mock and -NDRG2 cells. As shown in Fig. 1D, NDRG2 overexpression markedly reduced the activity of COX-2, especially under PMA stimulation. Moreover, PMA-induced $PGE_2$ production was strongly decreased by NDRG2 overexpression (Fig. 1E). Thus, NDRG2 negatively regulates COX-2 expression and activity and $PGE_2$ secretion.

### NDRG2 down-regulates COX-2 expression through NF-κB signaling pathway

To elucidate the mechanism by which NDRG2 regulates COX-2 expression, we examined PMA-stimulated signaling pathways, including NF-κB, MAPK/ERK, and PI3K/AKT. In Fig. 2A, PMA-treated mock controls showed a potent induction of p-IKKα/β and p-IκBα, whereas NDRG2 transfectants were not affected. The p65 subunit was rapidly translocated to nucleus after treatment with PMA in the mock controls, but this was not observed in the MDA-MB-231-NDRG2 cells (Fig. 2B). In the NF-κB reporter gene assay, PMA stimulation of the control cells remarkably induced the promoter activity of NF-κB, while NDRG2 transfectants showed decreased NF-κB promoter activities despite the PMA exposure (Fig. 2C). In addition, MDA-MB-231-mock cells treated with curcumin, which is a NF-κB inhibitor, exhibited reduced COX-2 expression, whereas NDRG2 overexpression, resulting in low levels of COX-2, led to the complete

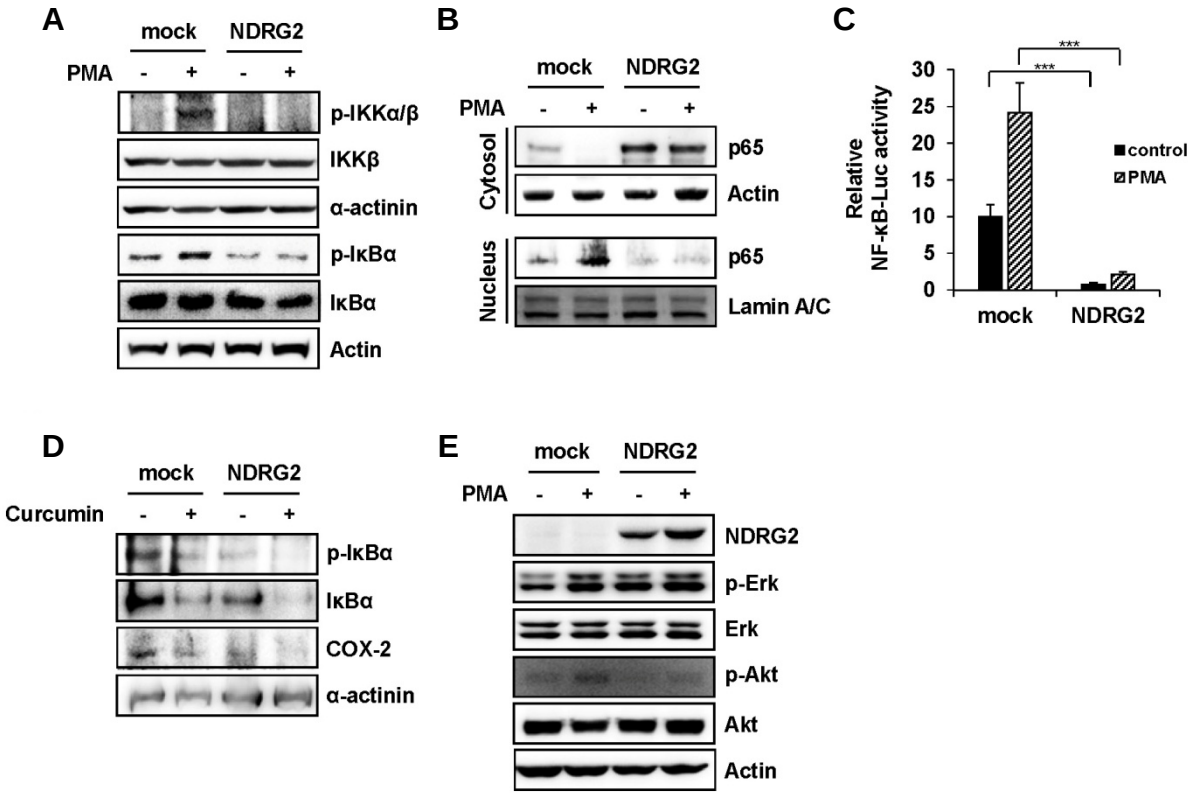


**Fig. 2.** The inhibitory effects of NDRG2 on COX-2 expression through PMA-induced NF-κB. (A) Cells were treated with PMA, and the protein levels of p-IKKα/β and p-IκBα were examined by Western blot analysis. (B) To examine the nuclear translocation of the NF-κB p65 subunit, after 1 h of stimulation with 40 ng/ml PMA, the cell lysates were fractionated into cytosolic and nuclear compartments. (C) NF-κB luciferase activity after PMA stimulation was measured using the luciferase assay system. (D) The cells were treated with 50 μM curcumin for 24 h, and the protein levels of p-IKKα/β and COX-2 were examined by Western blot analysis. (E) The cells were treated with PMA, and the protein levels of p-Akt and p-Erk were examined by Western blotting. ***$P < 0.001$.

suppression of COX-2 expression following the curcumin treatment (Fig. 2D). Similar to the results involving NF-κB signaling, the phosphorylation of AKT, which is an upstream regulator of NF-κB, was significantly induced by PMA treatment in the mock controls; however, the NDRG2 transfectants did not show any increase in PMA-stimulated phosphorylation of AKT. In contrast, there were no differences in the level of Erk phosphorylation following PMA treatment in MDA-MB-231-mock and -NDRG2 cells (Fig 2E). These data indicate that NDRG2 overexpression leads to the induction of COX-2 through the AKT/NF-κB signaling pathway.

### COX-2 inhibition by NDRG2 attenuates PMA-induced migration and invasion in malignant breast cancer cells

Previous reports have found that the migration and invasion of malignant cancer cells are linked to the up-regulation of COX-2 (Chen et al., 2013; Ho et al., 2013; Wu et al., 2013). We determined whether COX-2 inhibition by NDRG2 overexpression affects PMA-stimulated migration and invasion in malignant breast cancer cells. In the cell migration assay, treatment with PMA led to an increase in the migration of control cells, but NDRG2 overexpression reduced cell migration, even after PMA exposure. In particular, MDA-MB-231-NDRG2 cells displayed decreased cell migration in the absence or presence of PMA after the celecoxib treatment (Fig. 3A). Likewise, in the cell invasion assay, the PMA-enhanced invasive potential was suppressed by celecoxib in both the mock controls and NDRG2 transfectants (Fig. 3B). Next, we performed a wound healing assay to further examine the role of NDRG2 in the migration of the MDA-MB-231 cells. The mock controls treated with PMA for 24 h showed increased wound healing abilities compared with the untreated controls, while wound healing in the presence of celecoxib significantly decreased and induction by PMA was also attenuated. Specifically, NDRG2 transfectants showed reduction in the PMA-induced migration compared with the mock controls, and the inhibitory effects of celecoxib on the MDA-MB-231-NDRG2 cells caused them to exhibit increased sensitivity in both the absence and presence of PMA (Fig. 3C). These results indicate that NDRG2 expression down-regulates migration, invasion, and wound healing via the reduction of COX-2 expression.

### siRNA-mediated knockdown of NDRG2 restores COX-2 expression and $PGE_2$ production

To further extend our analysis of the role of NDRG2 in COX-2 expression, we used NDRG2 siRNA in MCF7 cells, which endogenously express NDRG2. The increased COX-2 mRNA and protein levels were confirmed when the expression of NDRG2 was knocked down (Fig. 4A). A qPCR analysis also showed comparable changes in the mRNA levels of COX-2 in the NDRG2-siRNA transfectants (Fig. 4B). Furthermore, NDRG2-

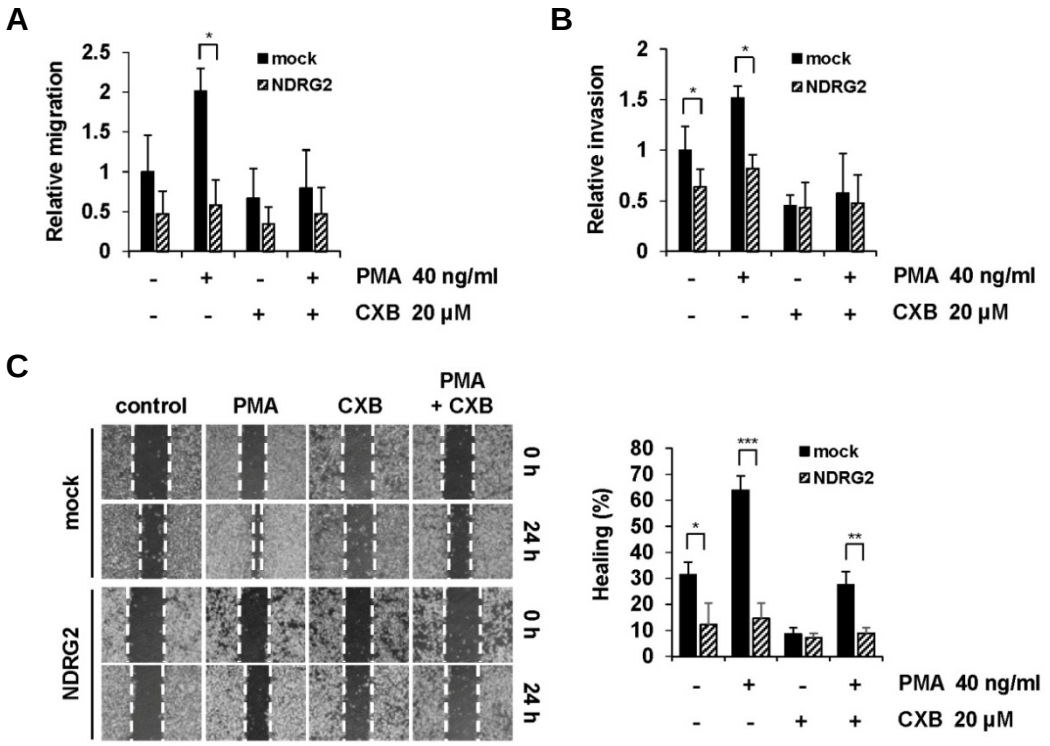


**Fig. 3.** The inhibitory effects of NDRG2 on PMA-stimulated COX-2, wound healing, migration, and invasion. (A) A fixed number of cells were plated into the upper part of the transwell chamber. After treatment with PMA, celecoxib (CXB), or both for 24 h, the cells migrating to the lower surface of the membrane were stained with 0.1% crystal violet/2% ethanol (v/v), and the stained cells obtained from the lower surface of the membrane were dissolved in 10% acetic acid. Absorbance measurements were then obtained at 590 nm. (B) A fixed number of cells were plated into the upper part of a Matrigel-coated transwell chamber. After treatment and incubation, the invasive cells were quantified as described in (A). (C) The cells were grown to reach confluence, scratched with a pipette tip, and photographed immediately following scratching (0 h). After incubation for 24 h, the plates were observed with phase-contrast microscopy. The dotted lines represent the wound margin. The relative closed-wound distance (healing) was calculated after measuring the widths of at least four wounds. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

silenced cells exhibited increased phosphorylation of IκBα and enhancement of COX-2 expression, particularly under PMA stimulation (Fig. 4C). PMA-induced $PGE_2$ production was increased by NDRG2 knockdown, although this occurred at relatively low levels in control MCF7 cells (Fig. 4D).

### siRNA-mediated knockdown of NDRG2 restores ability of migration and invasion

We further investigated whether migration and invasion of tumor cells are affected by siRNA-mediated knockdown of NDRG2. The migration and invasion assay revealed that the NDRG2-siRNA transfectants treated with PMA exhibited strong increases in cell migration and invasion compared with the PMA-treated control (Figs. 5A and 5B). In addition, the wound healing assay showed that the siRNA-mediated knockdown in the MCF7 cells led to markedly increased wound healing abilities compared with the PMA-stimulated control MCF7 cells (Fig. 5C). Collectively, these results support the notion that NDRG2 may function as a negative regulator of cell migration through the suppression of COX-2 expression.

## DISCUSSION

This study investigated the molecular mechanisms by which NDRG2 expression affects migration and invasion in breast cancer cells. Originally, NDRG2 expression was down-regulated in several malignant tumors, such as breast, liver, gastric, and skin cancers (Yao et al., 2008). In addition, the loss of NDRG2 expression is associated with malignant tumor characteristics, such as migration, invasion, and metastasis and poor prognosis (Lee et al., 2008; Shen et al., 2014), suggesting that it acts as a tumor suppressor in many human cancers. In fact, previous reports have shown that NDRG2 suppresses tumor metastasis through the inhibition of active TGF-β production (Oh et al., 2012) and tumor growth and angiogenesis by regulating the expression of VEGF and p53 in breast cancer (Ma et al., 2012). Similarly, we observed that MDA-MB-231-NDRG2 cells had decreased wound healing, migration, and invasion abilities, whereas these abilities were rescued in MCF7-siNDRG2 cells. However, how NDRG2 regulates the migration and invasion of cancer cells remains uncertain. In the present study, we demonstrated that NDRG2 serves as a critical regulator in COX-2/$PGE_2$-mediated cell migration and invasion.

COX-2 is associated with the promotion of tumorigenesis through $PGE_2$ production and the modulation of cancer metastasis and invasion (Imada et al., 2006; Stasinopoulos et al., 2007). As shown in our current study, NDRG2 overexpression could significantly down-regulate COX-2 expression and $PGE_2$ production in malignant breast cancer cells under PMA treatment. Particularly, COX-2 inhibitor treatment resulted in a significant atten-

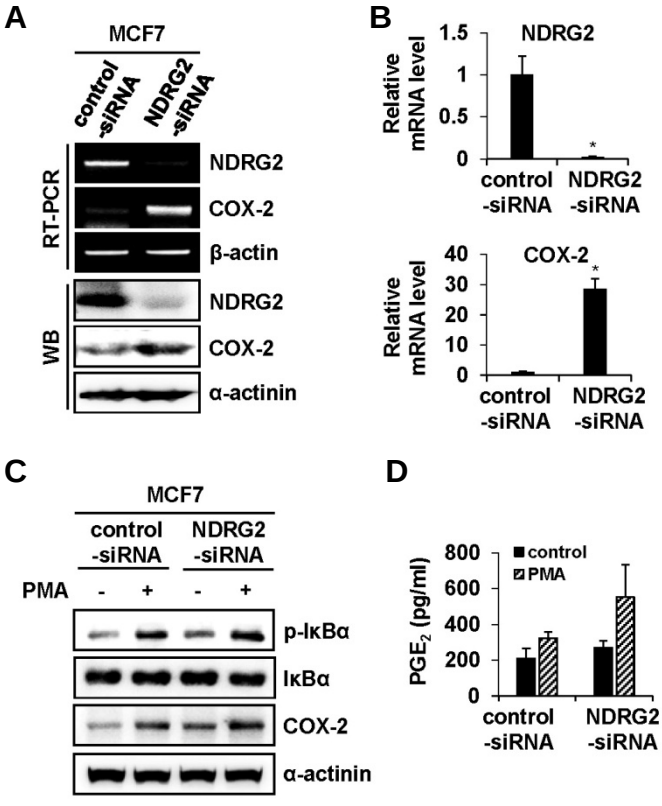


**Fig. 4.** NDRG2 knockdown in MCF7 cells restores COX-2 expression and $PGE_2$ release. (A) MCF7 cells transiently transfected with control or NDRG2 siRNA were cultured and the mRNA and protein levels of NDRG2 and COX-2 were measured by RT-PCR and Western blotting, respectively. (B) mRNA levels of NDRG2 and COX-2 were confirmed by quantitative RT-PCR. (C) After 6 h of exposure to PMA, p-IκBα and COX-2 levels in the MCF7-control-siRNA and -NDRG2-siRNA transfectants were detected by Western blotting. (D) After 24 h of treatment with PMA, the supernatants were harvested, and the $PGE_2$ protein secreted from the cells was measured by an ELISA. *$P$ < 0.05 (values are compared with MCF7-control-siRNA cells).

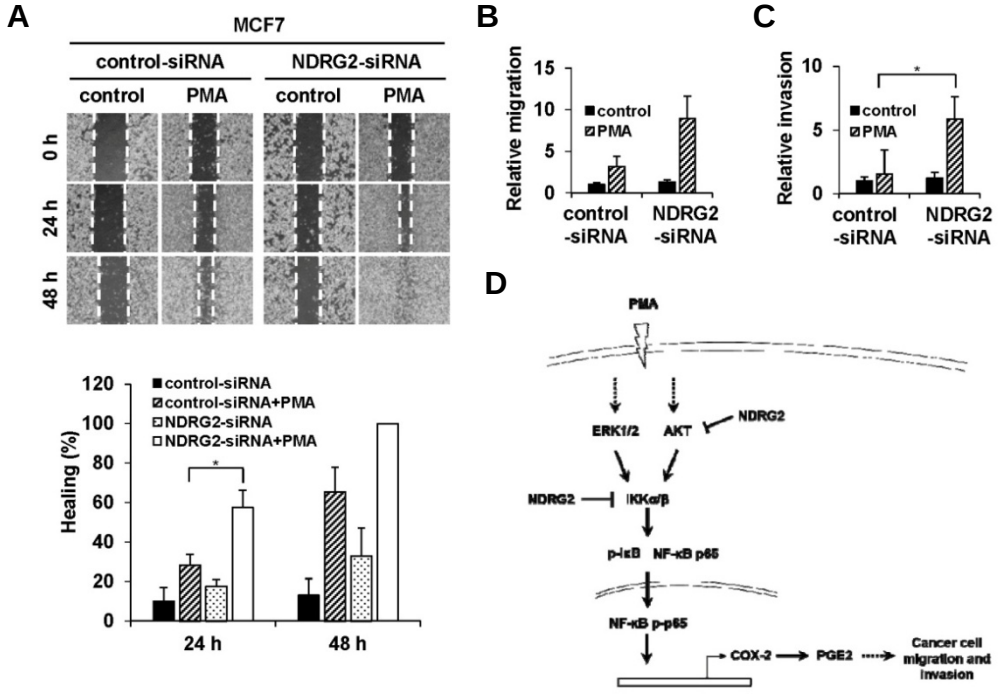


**Fig. 5.** NDRG2 knockdown in MCF7 cells rescues wound healing, migration, and invasion abilities. (A) The cells treated with PMA for 24 h were analyzed by a transwell migration assay. (B) The cells treated with PMA for 24 h were analyzed using a Matrigel-coated transwell chamber. (C) The cells were scratched with a pipette tip (0 h), and after PMA treatment for 48 h, the plates were observed with phase-contrast microscopy. The dotted lines represent the wound margin. The relative closed-wound distance (healing) was calculated after measuring the widths of at least four wounds. (D) Schematic representation of the proposed mechanism of COX-2/$PGE_2$-mediated migration inhibited by NDRG2 in breast cancer cells. *$P$ < 0.05.

uation of migration and invasion in both the resting and PMA-stimulated MDA-MB-231-NDRG2 cells. In addition, among the regulators of COX-2 expression, NF-κB is well known as a key regulator, and activated NF-κB in several types of tumor cells has been demonstrated to play an essential role in cancer proliferation and metastasis (Hao et al., 2014). Thus, the expression of both NF-κB and COX-2 was closely related to the migration, invasion, and metastasis of the tumor cells. We also demonstrated that NDRG2 expression causes the down-regulation of COX-2/$PGE_2$ levels by inhibiting NF-κB signaling in MDA-MB-231-NDRG2 cells (Fig. 5D).

Taken together, we have verified that NDRG2 overexpression not only inhibits COX-2 expression and $PGE_2$ production, but also down-regulates the migration and invasion that is mediated by NF-κB/COX-2/$PGE_2$ in malignant breast cancer cells. Therefore, NDRG2 may be a novel repressor of migration and invasion, acting via the modulation of COX-2/$PGE_2$.

## ACKNOWLEDGMENTS

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