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Nuclear Factor-inducing Kinase Plays a Crucial Role in Osteopontin-induced MAPK/IκBα Kinase-dependent Nuclear Factor κB-mediated Promatrix Metalloproteinase-9 Activation*

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We have recently demonstrated that osteopontin (OPN) induces nuclear factor κB (NFκB)-mediated promatrix metalloproteinase-2 activation through IκBα/IκBα kinase (IKK) signaling pathways. However, the molecular mechanism(s) by which OPN regulates promatrix metalloproteinase-9 (pro-MMP-9) activation, MMP-9-dependent cell motility, and tumor growth and the involvement of upstream kinases in regulation of these processes in murine melanoma cells are not well defined. Here we report that OPN induced $\alpha_v\beta_3$ integrin-mediated phosphorylation and activation of nuclear factor-inducing kinase (NIK) and enhanced the interaction between phosphorylated NIK and IKKα/β in B16F10 cells. Moreover, NIK was involved in OPN-induced phosphorylations of MEK-1 and ERK1/2 in these cells. OPN induced NIK-dependent NFκB activation through ERK/IKKα/β-mediated pathways. Furthermore OPN enhanced NIK-regulated urokinase-type plasminogen activator (uPA) secretion, uPA-dependent pro-MMP-9 activation, cell motility, and tumor growth. Wild type NIK, IKKα/β, and ERK1/2 enhanced and kinase-negative NIK (mut NIK), dominant negative IKKα/β (dn IKKα/β), and dn ERK1/2 suppressed the OPN-induced NFκB activation, uPA secretion, pro-MMP-9 activation, cell motility, and chemoinvasion. Pretreatment of cells with anti-MMP-2 antibody along with anti-MMP-9 antibody drastically inhibited the OPN-induced cell migration and chemoinvasion, whereas cells pretreated with anti-MMP-2 antibody had no effect on OPN-induced pro-MMP-9 activation suggesting that OPN induces pro-MMP-2 and pro-MMP-9 activations through two distinct pathways. The level of active MMP-9 in the OPN-induced tumor was higher compared with control. To our knowledge, this is the first report that NIK plays a crucial role in OPN-induced NFκB activation, uPA secretion, and pro-MMP-9 activation through MAPK/IKKα/β-mediated pathways, and all of these ultimately control the cell motility, invasiveness, and tumor growth.

Osteopontin (OPN)[1] is a secreted, non-collagenous, sialic acid-rich phosphoglycoprotein (1, 2). OPN acts both as chemokine and cytokine. It has an N-terminal signal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, and a GRGDS cell adhesion sequence predicted to be flanked by the β-sheet structure (3). This protein has a functional thrombin cleavage site and is the substrate for tissue transglutaminase (2). It is produced by osteoclast, macrophages, T cells, hematopoietic cells, and vascular smooth muscle cells (4). OPN binds with type I collagen (5), fibronectin (6), and osteocalcin (7). Several highly metastatic transformed cells synthesize higher levels of OPN compared with non-tumorigenic cells (8). It has been shown that OPN also interacts with several integrins and CD44 variants in an RGD sequence-dependent and -independent manner (9, 10). OPN is involved in normal tissue remodeling processes such as bone resorption, angiogenesis, wound healing, and tissue injuries as well as certain diseases such as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (10–12). OPN causes cell adhesion, migration, extracellular matrix (ECM) invasion, and cell proliferation by interaction with its receptor $\alpha_v\beta_3$ integrin in various cell types (10). Integrins are noncovalently associated, heterodimeric, cell surface glycoproteins with α- and β-subunits.

The NFκB family consists of several members including p65, p50, Rel B, and c-Rel molecules (13). The activity of NFκB is tightly regulated by its inhibitors, the IκB family of proteins (14). These inhibitory proteins bind to NFκB dimers, masking their nuclear localization sequence resulting in cytoplasmic retention of NFκB (15). Upon stimulation, IκB is phosphorylated and degraded via the ubiquitination and proteosome-mediated pathway leading to the nuclear import of NFκB where it binds to cognate sequence in promoter regions of multiple genes. IκBα kinase (IKK) is a multisubunit protein kinase, the activation of which phosphorylates IκB. Nuclear factor-inducing kinase (NIK) is a member of the mitogen-activated protein kinase kinase kinase family that may either directly or indirectly phosphorylate or activate IKKα/β, leading to the phosphorylation and degradation of IκBα followed by NFκB activation (16). Previous data also has indicated that NIK regulates differentiation of PC-12 cells through phosphorylation and activation of MEK-1 (17). The data also show that constitutively expressed or overexpressed wild type NIK in the presence of TNF, interleukin-1, TRAF-2, and TRAF-6 induced, and kinase-negative NIK (mut NIK) in the presence of the same stimuli suppressed, the NFκB activation (18, 19). Our previous results indicate that OPN induces cell motility, tumor growth, NFκB-

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[1] The abbreviations used are: OPN, osteopontin; NIK, nuclear factor-inducing kinase; NFκB, nuclear factor κB; IκBα, inhibitor of nuclear factor κB; IKK, IκBα kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; EMSA, electrophoretic mobility shift assay; Luc, luciferase; uPA, urokinase-type plasminogen activator; MMP, matrix metalloproteinase; ECM, extracellular matrix; mut, kinase-negative; dn, dominant negative; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; p-, phospho-; wt, wild type; DTT, dithiothreitol; MT1, membrane type-1.

mediated urokinase-type plasminogen activator (uPA) secretion, and promatrix metalloproteinase-2 (pro-MMP-2) activation through the phosphatidylinositol 3-kinase/IKK/Akt signaling pathways (20–22). However, the signaling pathway by which OPN regulates NIK activation and NIK-dependent MAPK/IKK-mediated NFκB activation through phosphorylation and degradation of IκBα in B16F10 cells is not well defined.

uPA is a serine protease that interacts with uPA receptor and facilitates the conversion of inert zymogen plasminogen into widely acting serine protease plasmin and activation of MMPs (23, 24). These proteases then degrade the surrounding matrix components (collagen, gelatin, fibronectin, and laminin) and allow cancer cells to migrate to distant sites. It is established that uPA plays a significant role in tumor growth and metastasis (25, 26). NFκB-response element is present in the promoter region of uPA, which plays a key role in cancer metastasis. However, the molecular mechanism by which OPN regulates NIK activation and NIK-dependent MAPK- and IKK-mediated NFκB activation and uPA secretion, which lead to the activation of pro-MMP-9 and control cell migration, ECM invasion, and tumor growth, is not well documented.

MMPs are ECM-degrading enzymes that play a critical role in embryogenesis, tissue remodeling, inflammation, and angiogenesis (27). We have recently reported that OPN induces NFκB-mediated pro-MMP-2 activation through IκBα/IKK signaling pathways (20, 21). MMP-9 (also called type IV collagenase or gelatinase B) is another important contributor to the process of invasion, tumor growth, and metastasis (28–31). MMP-9 can efficiently degrade native type IV and V collagens, fibronectin, ectactin, and elastin. The regulation of activation of MMP-9 is more complex than that of most of the other MMPs because most of the cells do not express a constitutively active form of MMP-9, but its activity is induced by different stimuli depending on cell types (32–34) thereby contributing to the specific pathological events. MMP-9 is not only associated with invasion and metastasis but also has been implicated in angiogenesis, rheumatoid arthritis, retinopathy, and vascular stenosis, and hence MMP-9 is considered to be a therapeutic target of high priority (35). Earlier reports indicate that transgenic mice lacking MMP-9 show reduced keratinocyte hyperproliferation and decreased incidence of invasive tumors (36). NFκB-responsive element is present in the promoter region of MMP-9, which plays a key role in cancer metastasis. However, the molecular mechanism by which OPN regulates NFκB-mediated pro-MMP-9 activation and controls cell migration and tumor growth is not well understood.

In this study, we found that OPN induced phosphorylation and activation of NIK and the interaction between phosphorylated NIK and IKK in B16F10 cells. OPN enhanced NIK- and IKK-dependent NFκB activation. OPN also induced NIK-mediated NFκB activation by modulating the phosphorylation of ERK1/2. Moreover OPN induced uPA secretion and uPA-dependent pro-MMP-9 activation, cell motility, chemoinvasion, and tumor growth. The OPN-induced NIK-dependent ERK/IKK-mediated NFκB transactivation, pro-MMP-9 activation, and cell motility were enhanced when cells were transfected with wild type NIK, IKKα/β, and ERK1/2 and suppressed when cells were transfected with mut NIK, dn IKKα/β, or dn ERK1/2. Taken together, these data demonstrated that OPN enhances cell motility, chemoinvasion, and tumor growth and induces NIK-dependent NFκB-mediated uPA secretion and uPA-regulated pro-MMP-9 activation through MAPK/IKK-mediated signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—The rabbit polyclonal anti-phospho-NIK (Thr-559), anti-NIK, anti-IKKα/β, anti-NFκB p65 X TransCruz, anti-p-MEK-1, anti-MEK-1, anti-ERK1/2, anti-IκBα, anti-uPA, anti-MMP-9, and anti-actin and the mouse monoclonal anti-phospho-ERK1/2, anti-phospho-IκBα antibodies, and IκBα recombinant protein were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-MMP-9 was from Oncogene. Mouse monoclonal anti-human $\alpha_v\beta_3$ integrin antibody was from Chemicon International. LipofectAMINE Plus, GRGDSP, and GRGESP were obtained from Invitrogen. PD98059 was from Calbiochem. TNFα was from R&D Systems. The dual luciferase reporter assay system and NFκB consensus oligonucleotide were purchased from Promega. Boyden type cell migration chambers were obtained from Corning, and BioCoat Matrigel™ invasion chambers were from Collaborative Biomedical. The [γ-^{32}P]ATP was purchased from Board of Radiation and Isotope Technology (Hyderabad, India). The human OPN was purified from milk as described previously and used throughout these studies (20). The nude mice (NMRI, nu/nu) were from National Institute of Virology (Pune, India). All other chemicals were of analytical grade.

Cell Culture—The B16F10 cells were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Plasmids and DNA Transfection—The wild type NIK (wt pcDNA NIK) and kinase-negative NIK (mut pcDNA NIK, NIK-K429A/K430A) in pcDNA3 were generous gifts from Prof. David Wallach (Weizmann Institute of Science, Rehovot, Israel). The wild type and dominant negative constructs of IKKα (wt IKKα and dn IKKα) and IKKβ (wt IKKβ and dn IKKβ) in pRK were kind gifts from Prof. D. V. Goeddel (Tularik Inc., San Francisco, CA). The wt ERK1 and dn ERK1 in pCEP4 and wt ERK2 and dn ERK2 in p3XFLAG-CMV-7.1 were from Dr. Melanie Cobb (Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX). The luciferase reporter construct (pNFκB-Luc) containing five tandem repeats of the NFκB binding site was a generous gift from Dr. Rainer de Martin (University of Vienna, Vienna, Austria). The super-repressor form of IκBα cDNA fused downstream to a FLAG epitope in an expression vector (pCMV4) was a gift from Dr. Dean Ballard (Vanderbilt University School of Medicine, Nashville, TN). B16F10 cells were split 12 h prior to transfection in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. These cells were transiently transfected with cDNA using LipofectAMINE Plus according to manufacturer's instructions. Briefly cDNA (8 μg) was mixed with Plus reagent, and then the cDNA Reagent Plus were incubated with LipofectAMINE. The LipofectAMINE Plus cDNA complex was added to the cells and incubated further at 37 °C for 12 h. The control cells received LipofectAMINE Plus alone. The cell viability was detected by a trypan blue dye exclusion test. After incubation, the medium was removed, and the cells were refed with fresh medium and maintained for an additional 12 h. These transfected cells were used for interaction studies between phosphorylated NIK and IKK, IκBα phosphorylation, IKK assay, MEK-1 and ERK1/2 phosphorylations, EMSA, NFκB luciferase reporter gene assay, and detection of MMP-9 by zymography and Western blot and uPA secretion by Western blot, cell migration, and chemoinvasion assays.

In Vitro Kinase Assay—To examine the effect of OPN on NIK activity, the semiconfluent B16F10 cells were treated with 5 μM OPN for 5 min at 37 °C. The cells were lysed in kinase assay lysis buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.25% Nonidet P-40). The supernatant was obtained by centrifugation at 12,000 × *g* for 10 min at 4 °C. The cell lysates containing an equal amount of total proteins were immunoprecipitated with rabbit anti-NIK antibody. Half of the immunoprecipitated samples were incubated with IKK as substrate in kinase assay buffer (20 mM Hepes (pH 7.7), 2 mM $MgCl_2$, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) containing 10 μM ATP and 3 μCi of [γ-^{32}P]ATP at 30 °C. The kinase reactions were stopped by addition of SDS sample buffer. The samples were resolved by SDS-PAGE, dried, and autoradiographed. The remaining half of the immunoprecipitated samples were subjected to SDS-PAGE and analyzed by Western blot using anti-NIK antibody. A fraction of equal volume of samples from the

kinase reaction mixture was analyzed by Western blot using anti-IKKα/β antibody.

To investigate the role of NIK in OPN-induced IKK activity, the cells were treated with 5 μM OPN for 10 min at 37 °C. In separate experiments, cells were transfected with wild type NIK or kinase-negative NIK in the presence of LipofectAMINE Plus and then treated with 5 μM OPN. The cells were lysed in kinase assay lysis buffer. The cell lysates containing an equal amount of total proteins (300 μg) were immunoprecipitated with anti-IKKα/β antibody. Half of the immunoprecipitated samples were incubated with recombinant IκBα (4 μg) in kinase buffer (20 mM Hepes (pH 7.7), 2 mM $MgCl_2$, 10 μM ATP, 3 μCi of [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) at 30 °C. The kinase reaction was stopped by addition of SDS sample buffer. The sample was resolved by SDS-PAGE, dried, and autoradiographed. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-IKKα/β antibody. A fraction of equal volume of samples from the kinase reaction mixture was analyzed by Western blot using anti-IκBα antibody. The level of NIK in the immunoprecipitated samples was analyzed by Western blot using anti-NIK antibody.

To check whether OPN regulates the interaction between phosphorylated NIK and IKKα/β, cells were treated with 5 μM OPN at 37 °C for 10 min. Cell lysates were immunoprecipitated with rabbit anti-IKKα/β antibody. The immunoprecipitated samples were analyzed by Western blot using rabbit anti-phospho-NIK antibody. The same blots were reprobed with anti-IKKα/β antibody as loading control.

Western Blot Analysis—To delineate the role of OPN in regulation of NIK phosphorylation, the cells were treated with 5 μM OPN for 0–30 min. In separate experiments, cells were pretreated with anti-$\alpha_v\beta_3$ integrin antibody (20 μg/ml) or GRGDSP or GRGESP peptide (10 μM) and then treated with 5 μM OPN for 7 min at 37 °C. In other experiments, cells were treated with TNFα (20 ng/ml) for 5 min as positive control. The cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride), and the lysates containing an equal amount of total proteins were analyzed by Western blot with rabbit anti-phospho-NIK antibody. The same blots were reprobed with rabbit anti-NIK or anti-actin antibody as loading controls and detected by the ECL detection system (Amersham Biosciences).

To examine the effect of NIK on OPN-induced IκBα phosphorylation and degradation, cells were transfected with either wild type NIK or kinase-negative NIK and then treated with 5 μM OPN for 10 min. The cell lysates were analyzed by Western blot using mouse anti-phospho-IκBα antibody. The same blots were reprobed with rabbit anti-IκBα antibody. As loading control, the expression of actin was also detected by reprobing the blot with anti-actin antibody.

To detect whether NIK regulates OPN-induced MEK-1 and ERK1/2 phosphorylations, cells were transfected with wild type or kinase-negative NIK and then treated with 5 μM OPN for 10 min. Cell lysates were analyzed by Western blot using anti-p-MEK-1 or anti-p-ERK1/2 antibody. The same blots were reprobed with anti-MEK-1 or anti-ERK1/2 antibody.

To check whether $\alpha_v\beta_3$ integrin is involved in OPN-induced MEK-1 phosphorylation, the cells were pretreated with anti-$\alpha_v\beta_3$ antibody or RGD peptide (GRGDSP or GRGESP) and then treated with OPN. The cell lysates were subjected to Western blot analysis using anti-p-MEK-1 antibody. The same blots were reprobed with anti-MEK-1 antibody.

To investigate the role of OPN on pro-MMP-9 activation, the cells were treated with 5 μM OPN for 24 h. In separate experiments, cells were pretreated with anti-$\alpha_v\beta_3$ integrin antibody (20 μg/ml), RGD/RGE peptide (10 μM GRGDSP or GRGESP), anti-uPA antibody (25 μg/ml), or anti-MMP-2 antibody (0–50 μg/ml) for 1 h and then treated with 5 μM OPN. The conditioned medium was collected, concentrated, and dialyzed. The samples containing an equal amount of total proteins were analyzed by Western blot using rabbit anti-MMP-9 antibody.

To ascertain the role of NIK in OPN-induced uPA secretion, the cells were either treated with 0–5 μM OPN or transfected with wild type NIK or mut NIK and then treated with 5 μM OPN for 24 h. To examine whether ERK1/2 plays any roles in OPN-induced uPA secretion, cells were transfected with wild type or dn ERK1/2 and then treated with OPN. The cell lysates containing an equal amount of total proteins were subjected to Western blot analysis using rabbit polyclonal anti-uPA antibody. As loading controls the expression of actin was also detected by reprobing the blots with rabbit anti-actin antibody.

Zymography Experiments—The gelatinolytic activity was measured as described previously (20). To further check the effect of OPN on pro-MMP-9 activation, the cells were treated with 0–10 μM OPN for 24 h or pretreated with anti-uPA antibody (25 μg/ml) for 1 h and then treated with 5 μM OPN. The conditioned medium was collected by centrifugation. The samples containing an equal amount of total proteins were mixed with sample buffer in the absence of reducing agent and loaded onto zymography SDS-polyacrylamide gels containing gelatin (0.5 mg/ml) as described previously (20). The gels were incubated in incubation buffer (50 mM Tris-HCl (pH 7.5) containing 100 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% (v/v) Triton X-100, and 0.02% (w/v) NaN_3) for 16 h. The gels were stained with Coomassie Blue and destained. Negative staining showed the zones of gelatinolytic activity. In another experiment, cells were also transfected with wild type NIK, kinase-negative NIK, or the super-repressor form of IκBα in the presence of LipofectAMINE Plus and then treated with OPN for 24 h. In separate experiments, cells were transfected with wild type or dominant negative IKKα and IKKβ and then treated with 5 μM OPN. The conditioned medium was collected, and the level of active MMP-9 was detected by zymography as described above.

EMSA—To check whether NIK and IKK play any role in OPN-induced NFκB-DNA binding, cells were either treated with 5 μM OPN or transfected with wild type NIK, kinase-negative NIK, or wild type and dn IKKβ and then treated with OPN for 6 h at 37 °C, and EMSA was performed as described previously (37). To investigate whether MEK-1 or ERK1/2 is involved in OPN-induced NFκB-DNA binding and whether it is regulated by NIK, cells were either pretreated with PD98059 or transfected with wt NIK or kinase-negative NIK and then treated with PD98059 or transfected with wild type and dn ERK1 and ERK2. Transfected or treated cells were further treated with OPN. The nuclear extracts were prepared as described earlier (37). Briefly the cells were resuspended in hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). The nuclear pellet was extracted in nuclear extraction buffer (20 mM Hepes (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT). The supernatant was used as a nuclear extract. The nuclear extracts (10 μg) were incubated with 16 fmol of ^{32}P-labeled double-stranded NFκB oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) in binding buffer (25 mM Hepes buffer (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl) containing 2 μg of polydeoxyinosinic deoxycytidylic acid (poly(dI-dC)). The DNA-protein complex was resolved on a native polyacrylamide gel and analyzed by autoradiography. For supershift assay, the OPN-treated nuclear extracts were incubated with anti-NFκB p65 (TransCruz) antibody for 30 min at room temperature and analyzed by EMSA.

NFκB Luciferase Reporter Gene Assay—The semiconfluent cells grown in 24-well plates were transiently transfected with a luciferase reporter construct (pNFκB-Luc) containing five tandem repeats of the NFκB binding site using LipofectAMINE Plus reagent (Invitrogen). In separate experiments, cells were individually transfected with wild type NIK, kinase-negative NIK, the super-repressor form of IκBα, wild type and dn IKKα and IKKβ, or wild type and dn ERK1 and ERK2 along with pNFκB-Luc. The transfection efficiency was normalized by cotransfecting the cells with pRL vector (Promega) containing a full-length *Renilla* luciferase gene under the control of a constitutive promoter. After 24 h of transfection, cells were treated with 5 μM OPN for 6 h. Cells were harvested in passive lysis buffer (Promega). The luciferase activities were measured by luminometer (Lab Systems) using the dual luciferase assay system according to the manufacturer's instructions (Promega). Changes in luciferase activity with respect to control were calculated.

Cell Migration Assay—The migration assay was conducted using a Transwell cell culture chamber according to the standard procedure as described previously (20–22). Briefly the confluent monolayers of B16F10 cells were harvested with trypsin-EDTA and centrifuged at 800 × *g* for 10 min. The cell suspension (5×10^5cells/well) was added to the upper chamber of the prehydrated polycarbonate membrane filter. The lower chamber was filled with fibroblast-conditioned medium, which acted as a chemoattractant. Purified OPN (5 μM) was added to the upper chamber. In another experiment, cells were individually pretreated with PD98059 (0–50 μM), anti-uPA antibody (25 μg/ml), anti-MMP-9 antibody (25 μg/ml), anti-MMP-2 antibody (25 μg/ml), or a combination of anti-MMP-2 and anti-MMP-9 antibodies (25 μg/ml) at 37 °C for 6 h. In other experiments, cells were individually transfected with wild type and kinase-negative NIK, the super-repressor form of IκBα, wild type and dn IKKα and IKKβ, or wild type and dn ERK1 and ERK2 and used for migration assays. These treated or transfected cells were incubated in a humidified incubator in 5% CO_2 and 95% air at 37 °C for 16 h. The non-migrated cells on the upper side of the filter

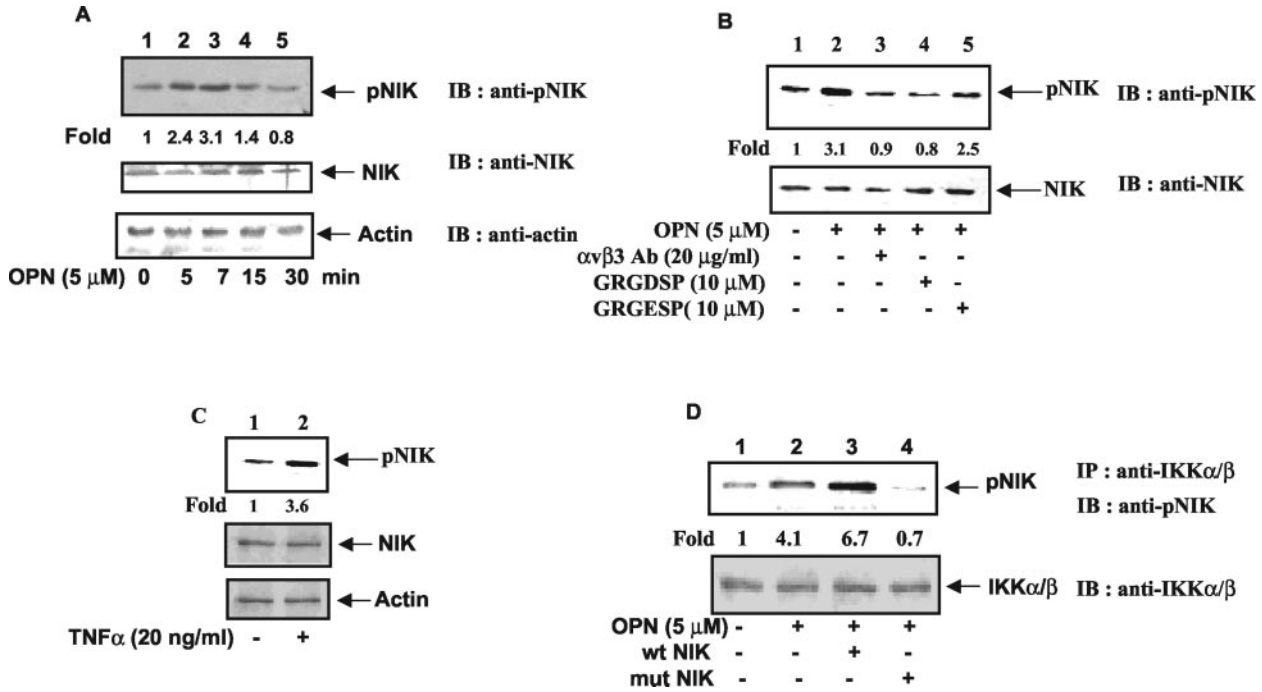

FIG. 1. Panel *A*, OPN stimulates NIK phosphorylation. B16F10 cells were treated with 5 μM OPN for 0–30 min. The cell lysates containing an equal amount of total proteins were analyzed by Western blot using anti-phospho-NIK antibody (*upper panel A*, *lanes 1–5*). The blots were reprobed with anti-NIK (*middle panel A*) or anti-actin antibody (*lower panel A*) as loading controls. *Panel B*, OPN induces NIK phosphorylation through $\alpha_v\beta_3$ integrin-mediated pathways. The cells were individually pretreated with anti-$\alpha_v\beta_3$ integrin antibody, GRGDSP, or GRGESP and then treated with 5 μM OPN. The cell lysates were analyzed by Western blot using anti-phospho-NIK antibody (*upper panel B*, *lanes 1–5*). The same blots were reprobed with anti-NIK antibody (*lower panel B*). *Panel C*, TNFα, as control, enhances NIK phosphorylation. Cells were treated with TNFα, and levels of phospho- and non-phospho-NIK were detected by Western blot (*panel C*). *Panel D*, OPN induces the interaction between phosphorylated NIK and IKK. The cells were treated with 5 μM OPN. In separate experiments, cells were transfected with wt or kinase-negative NIK and then treated with OPN. Cell lysates were immunoprecipitated with anti-IKKα/β antibody. Half of the samples were immunoblotted with anti-phospho-NIK antibody (*upper panel D*, *lanes 1–4*), and the other half were analyzed by anti-IKKα/β antibody (*lower panel D*, *lanes 1–4*). All these bands were analyzed densitometrically, and the -fold changes were calculated. The data shown here represent three experiments exhibiting similar effects. *IB*, immunoblot; *pNIK*, phospho-NIK; *IP*, immunoprecipitation; *Ab*, antibody.

were scraped, and the filter was washed. The migrated cells on the reverse side of the filter were fixed with methanol and stained with Giemsa. The migrated cells on the filter were counted under an inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG served as a nonspecific control.

Chemoinvasion Assay—The chemoinvasion assay was performed using a Matrigel-coated invasion chamber as described previously (20–22). The cell suspension (5×10^5 cells/well) was added to the upper portion of the prehydrated Matrigel-coated chamber. The lower chamber was filled with fibroblast-conditioned medium, which acted as a chemoattractant. Purified OPN (5 μM) was added to the upper chamber. In another experiment, cells were individually pretreated with anti-MMP-9 antibody (25 μg/ml), anti-MMP-2 antibody (25 μg/ml), a combination of anti-MMP-2 and anti-MMP-9 antibodies, and anti-uPA antibody (25 μg/ml) at 37 °C for 6 h. In other experiments, cells were individually transfected with wild type and kinase-negative NIK, wild type and dn IKKα and IKKβ, or the super-repressor form of IκBα as described above and used for invasion assays. OPN (5 μM) was used in the upper chamber. The cells were incubated at 37 °C for 16 h. The non-migrated cells and Matrigel from the upper side of the filter were scraped and removed using a moist cotton swab. The invaded cells on the lower side of the filter were stained with Giemsa and washed with phosphate-buffered saline (pH 7.6). The invaded cells were then counted, and photomicrographs were taken under the inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG served as a nonspecific control.

In Vivo Tumorigenicity Experiments—The tumorigenicity experiments were performed as described previously (20, 21). The cells were treated in the absence or presence of purified human OPN (10 μM) at 37 °C for 20 h. After that, the cells (5×10^6/0.2 ml) were detached and injected subcutaneously into the flanks of male athymic NMRI (nu/nu) mice (6–8 weeks old). Four mice were used in each set of experiments. The mice were kept under specific pathogen-free conditions. OPN (10 μM) was again injected into the tumor sites twice a week for up to 4 weeks. After 4 weeks, the mice were killed, and the tumor weights were measured. The tumor tissues were homogenized and lysed in lysis buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 15 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged at 12,000 × *g* for 10 min. The clear supernatants were collected, and the levels of pro- and active MMP-9 were detected by Western blot analysis. Briefly the samples containing an equal amount of total proteins were resolved by SDS-PAGE and analyzed by Western blot using anti-MMP-9 antibody. The levels of pro- and active MMP-9 in tumor samples were also analyzed by zymography as described above.

RESULTS

OPN Induces $\alpha_v\beta_3$ Integrin-dependent NIK Phosphorylation—Because we have reported earlier that OPN induces NFκB-mediated pro-MMP-2 activation through IKK/IκBα-mediated pathway (20), we first examined whether any upstream kinase(s) such as NIK plays any role in OPN-induced NFκB activation in B16F10 cells. Accordingly these cells were treated with 5 μM OPN for 0–30 min at 37 °C. The cell lysates containing an equal amount of total proteins were resolved by SDS-PAGE, and the level of phosphorylated NIK was detected by Western blot analysis using anti-phospho-NIK (Thr-559) antibody (Fig. 1, *upper panel A*, *lanes 1–5*). The data revealed that the maximum level of OPN-induced NIK phosphorylation occurred at 7 min (*upper panel A*, *lane 3*) and also suggested that Thr-559 of NIK is crucial for OPN-induced NIK phosphorylation. The same blots were reprobed with anti-NIK antibody as loading control, and the data showed that there was no change in expression of non-phospho-NIK in these cells upon treatment with OPN, confirming the equal loading of samples (*middle panel A*, *lanes 1–5*). Actin was also used as a loading control (*lower panel A*, *lanes 1–5*). To further check whether $\alpha_v\beta_3$ integrin or the RGD/RGE peptide is involved in OPN-induced NIK phosphorylation, cells were pretreated with anti-$\alpha_v\beta_3$ in-

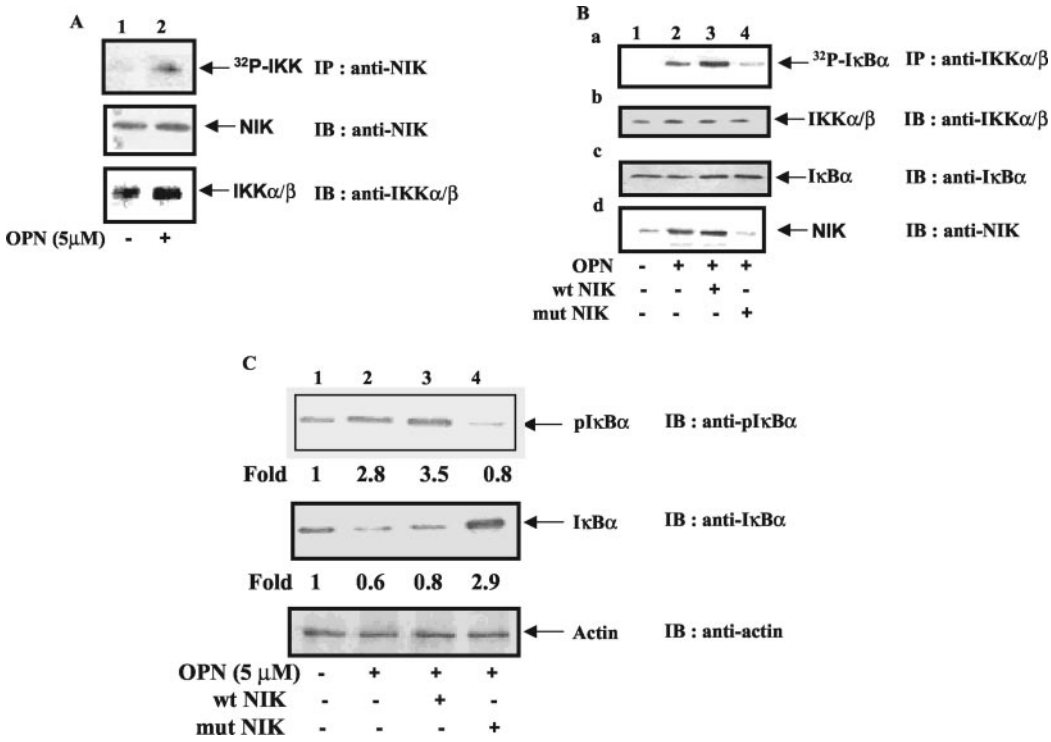

FIG. 2. *Panel A*, OPN induces NIK activity. Cells were treated with 5 μM OPN, cell lysates were immunoprecipitated with anti-NIK antibody, and half of the immunoprecipitated samples were used for the kinase assay using IKK as substrate (*upper panel A*, *lanes 1–2*). The remaining half of the immunoprecipitated samples were immunoblotted with anti-NIK antibody (*middle panel A*). The fraction containing an equal volume of kinase reaction mixture was analyzed by Western blot using anti-IKK antibody (*lower panel A*). *Panel B*, OPN stimulates NIK-dependent IKK activity. The cells were treated with 5 μM OPN or transfected with wild type and kinase-negative NIK and then treated with OPN. Cell lysates were immunoprecipitated with anti-IKKα/β antibody, and half of the immunoprecipitated samples were used for the kinase assay using IκBα as substrate (*panel B*, *a*). The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-IKKα/β antibody (*panel B*, *b*). The fraction containing an equal volume of kinase reaction mixture was immunoblotted with anti-IκBα antibody (*panel B*, *c*). The level of expression of NIK was also analyzed by Western blot using anti-NIK antibody (*panel B*, *d*). *Panel C*, NIK is required for OPN-induced IκBα phosphorylation and degradation. The cells were either treated with 5 μM OPN or transfected with wild type or kinase-negative NIK and then treated with OPN as described under "Experimental Procedures." Cell lysates were analyzed by Western blot using anti-phospho-IκBα (*pIκBα*) antibody (*upper panel C*, *lanes 1–4*). The same blots were reprobed with anti-IκBα (*middle panel C*) or anti-actin (*lower panel C*) antibody. All these bands were analyzed densitometrically, and the -fold changes were calculated. The data shown here represent three experiments exhibiting similar effects. *IP*, immunoprecipitation; *IB*, immunoblot.

tegrin antibody or RGD/RGE peptide (GRGDSP or GRGESP) and then treated with OPN. The level of phosphorylated NIK was detected by Western blot analysis. The data showed that $\alpha_v\beta_3$ integrin antibody and RGD (GRGDSP) but not RGE (GRGESP) peptide suppressed the OPN-induced NIK phosphorylation in these cells (*upper panel B*, *lanes 1–5*). The level of non-phospho-NIK was unchanged (*lower panel B*, *lanes 1–5*). As a control, cells were treated with TNFα, and the levels of phosphorylated and non-phosphorylated NIK were detected by Western blot using anti-p-NIK and anti-NIK antibodies, respectively. The data revealed that OPN stimulates NIK phosphorylation in these cells (*panel C*, *lanes 1* and *2*). Western blot data were quantified by densitometric analysis, and the -fold changes were calculated. These results demonstrated that OPN binds with $\alpha_v\beta_3$ integrin receptor and induces NIK phosphorylation.

OPN Stimulates the Interaction between Phosphorylated NIK and IKK—To delineate whether OPN plays any role in regulating the interaction between phosphorylated NIK and IKK, B16F10 cells were treated with 5 μM OPN. In separate experiments, cells were individually transfected with wild type NIK or kinase-negative NIK in the presence of LipofectAMINE Plus and then treated with OPN. Cell lysates were immunoprecipitated with rabbit polyclonal anti-IKKα/β antibody. Half of the immunoprecipitated samples were analyzed by Western blot using anti-phospho-NIK antibody, and the remaining half of the samples were immunoblotted with anti-IKKα/β antibody. The results indicated that cells transfected with wild type NIK followed by treatment with OPN showed maximum interaction between phosphorylated NIK and IKKα/β compared with cells treated with OPN alone or untreated cells (Fig. 1, *panel D*, *lanes 1–3*). Cells transfected with kinase-negative NIK suppressed the OPN-induced interaction between phosphorylated NIK and IKKα/β in these cells (*lane 4*). All these bands were quantified densitometrically, and the -fold changes were calculated. These results suggested that OPN enhances the interaction between phosphorylated NIK and IKKα/β.

OPN Induces NIK Activity and NIK-dependent IKK Activity through Phosphorylation and Degradation of IκBα—To ascertain the role of OPN on NIK activity, the cells were treated with 5 μM OPN, and the lysates were immunoprecipitated with rabbit anti-NIK antibody. Half of the immunoprecipitated samples were incubated with IKK as substrate in kinase assay buffer containing [γ-^{32}P]ATP. The samples were resolved by SDS-PAGE and autoradiographed. The radiolabeled, phosphorylated IKK-specific band was detected in OPN-treated cells demonstrating that OPN induces NIK activity (Fig. 2, *upper panel A*, *lane 2*). The NIK activity was not detected in the untreated cells (*lane 1*). The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-NIK antibody as loading control (*middle panel A*). A fraction of equal volume of samples from the kinase reaction mixture was

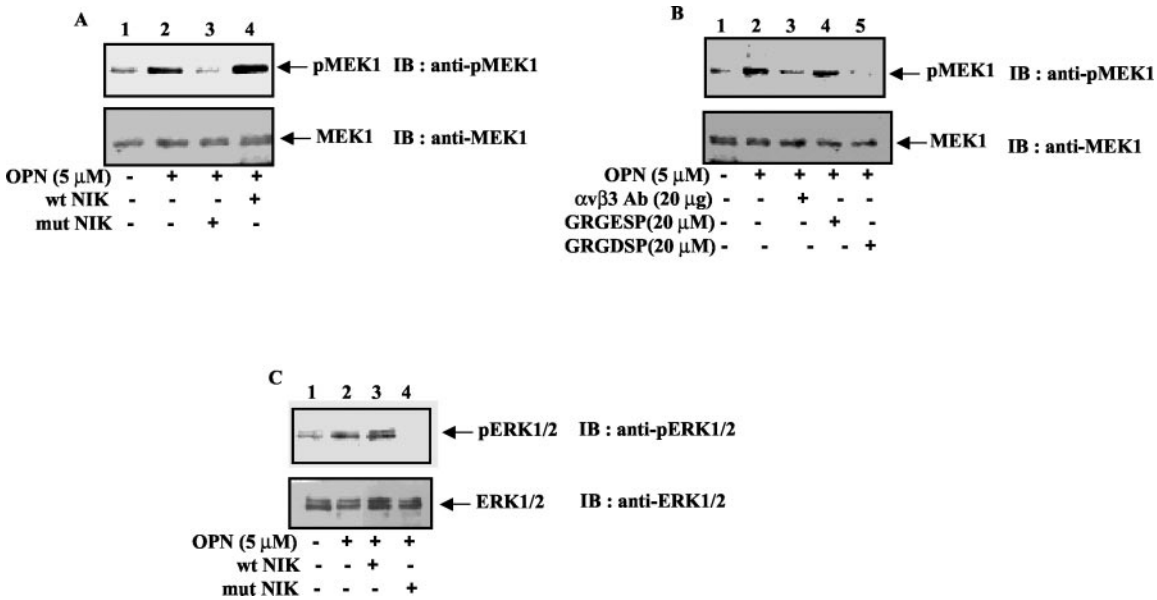

FIG. 3. **OPN induces $\alpha_v\beta_3$ integrin-mediated NIK-dependent MEK-1 and ERK1/2 phosphorylations.** *Panels A–C*, cells were transfected with wild type and kinase-negative NIK or pretreated with anti-$\alpha_v\beta_3$ integrin antibody or RGD/RGE peptide (GRGDSP or GRGESP) and then stimulated with OPN. Cell lysates were analyzed by Western blot using anti-phospho-MEK-1 (*upper panels A* and *B*) or anti-phospho-ERK1/2 (upper *panel C*) antibody. The same blots were reprobed with anti-MEK-1 (*lower panels A* and *B*) or anti-ERK1/2 (*lower panel C*) antibody. The bands were analyzed densitometrically, and the -fold changes were calculated. The data shown here represent three experiments exhibiting similar effects. *IB*, immunoblot; *Ab*, antibody.

analyzed by Western blot using anti-IKKα/β antibody as control (*lower panel A*). These data demonstrated that OPN induces NIK activity in these cells.

To further check whether NIK plays any direct role in OPN-induced IKK activity, cells were transfected with wild type or kinase-negative NIK and then treated with 5 μM OPN. The cell lysates were immunoprecipitated with rabbit anti-IKKα/β antibody. Half of the immunoprecipitated samples were incubated with IκBα as substrate in kinase assay buffer containing [γ-^{32}P]ATP. The samples were resolved by SDS-PAGE and autoradiographed. Cells transfected with wild type NIK followed by treatment with OPN showed maximum IKK activity (Fig. 2, *panel B*, *a*, *lane 3*) compared with untreated cells (*lane 1*) or cells induced with OPN alone (*lane 2*). Cells transfected with kinase-negative NIK followed by treatment with OPN suppressed the IKK activity significantly (*lane 4*) indicating that the kinase domain of NIK plays a crucial role in OPN-induced IKK activity. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-IKKα/β antibody (*panel B*, *b*, *lanes 1–4*). A fraction of equal volume of samples from the kinase reaction mixture was analyzed by Western blot using anti-IκBα antibody (*panel B*, *c*, *lanes 1–4*). The level of NIK was also analyzed by Western blot using anti-NIK antibody (*panel B*, *d*). These results suggested that NIK plays a significant role in OPN-induced IKK activity.

Since we showed that OPN-induced IKK activity was regulated by NIK, we therefore sought to determine whether NIK plays any role in OPN-induced phosphorylation and degradation of IκBα. Accordingly cells were either treated with 5 μM OPN or transfected with wild type or kinase-negative NIK and then treated with OPN. The level of phosphorylated IκBα in cell lysates was detected by Western blot using anti-phospho-IκBα antibody. The data demonstrated that cells transfected with wild type NIK enhanced OPN-induced IκBα phosphorylation compared with untreated cells (Fig. 2, *upper panel C*, *lanes 1–3*). Cells transfected with kinase-negative NIK suppressed OPN-induced IκBα phosphorylation (*lane 4*). The blot was reprobed with anti-IκBα antibody. The low level of IκBα was observed in cells treated with OPN or transfected with wild type NIK followed by OPN treatment indicating the degradation of IκBα (*middle panel C*, *lanes 1–4*). The same blots were reprobed with anti-actin antibody as loading control (*lower panel C*, *lanes 1–4*). These data suggested that NIK plays a crucial role in OPN-induced IκBα phosphorylation and degradation.

OPN Stimulates $\alpha_v\beta_3$ Integrin-mediated NIK-dependent MEK-1/ERK1/2 Phosphorylations—To examine whether NIK plays any role in OPN-induced MEK-1 and ERK1/2 phosphorylations, cells were treated with 5 μM OPN alone, pretreated with $\alpha_v\beta_3$ integrin antibody and RGD/RGE peptide, or transfected with wild type and kinase-negative NIK and then treated with OPN. Cell lysates were analyzed by Western blot using anti-phospho-MEK-1 or anti-phospho-ERK1/2 antibody. Wild type NIK enhanced whereas kinase-negative NIK suppressed the OPN-induced MEK-1 and ERK1/2 phosphorylations in these cells (Fig. 3, *upper panels A* and *C*, *lanes 1–4*). The data also indicated that OPN-induced MEK-1 phosphorylation was inhibited by $\alpha_v\beta_3$ integrin and RGD but not RGE peptide (*upper panel B*, *lanes 1–5*). The same blots were reprobed with anti-MEK-1 and anti-ERK1/2 antibodies as loading controls (*lower panels A–C*). These data suggested that OPN induces $\alpha_v\beta_3$ integrin-dependent MEK-1 and ERK1/2 phosphorylations through an NIK-mediated pathway.

NIK Plays a Crucial Role in OPN-induced ERK/IKK-dependent NFκB-DNA Binding—We have reported earlier that OPN induces pro-MMP-2 activation through activation of NFκB. In this study, we first examined whether NIK regulates OPN-induced NFκB-DNA binding in B16F10 cells. Accordingly cells were treated with 5 μM OPN or transfected with wild type and kinase-negative NIK or wild type and dn IKKβ and then treated with OPN. The nuclear extracts were prepared and used for EMSA using ^{32}P-labeled NFκB oligonucleotides. Wild type NIK enhanced and kinase-negative NIK suppressed OPN-induced NFκB-DNA binding (Fig. 4, *panel A*, *lanes 1–4*). Similarly wild type IKKβ induced and dn IKKβ inhibited OPN-enhanced NFκB-DNA binding (*panel B*, *lanes 1–4*). The same results were obtained in cells transfected with wild type and dn IKKα (data not shown). These data suggested that OPN induces NFκB-DNA binding through NIK/IKK-mediated pathways.

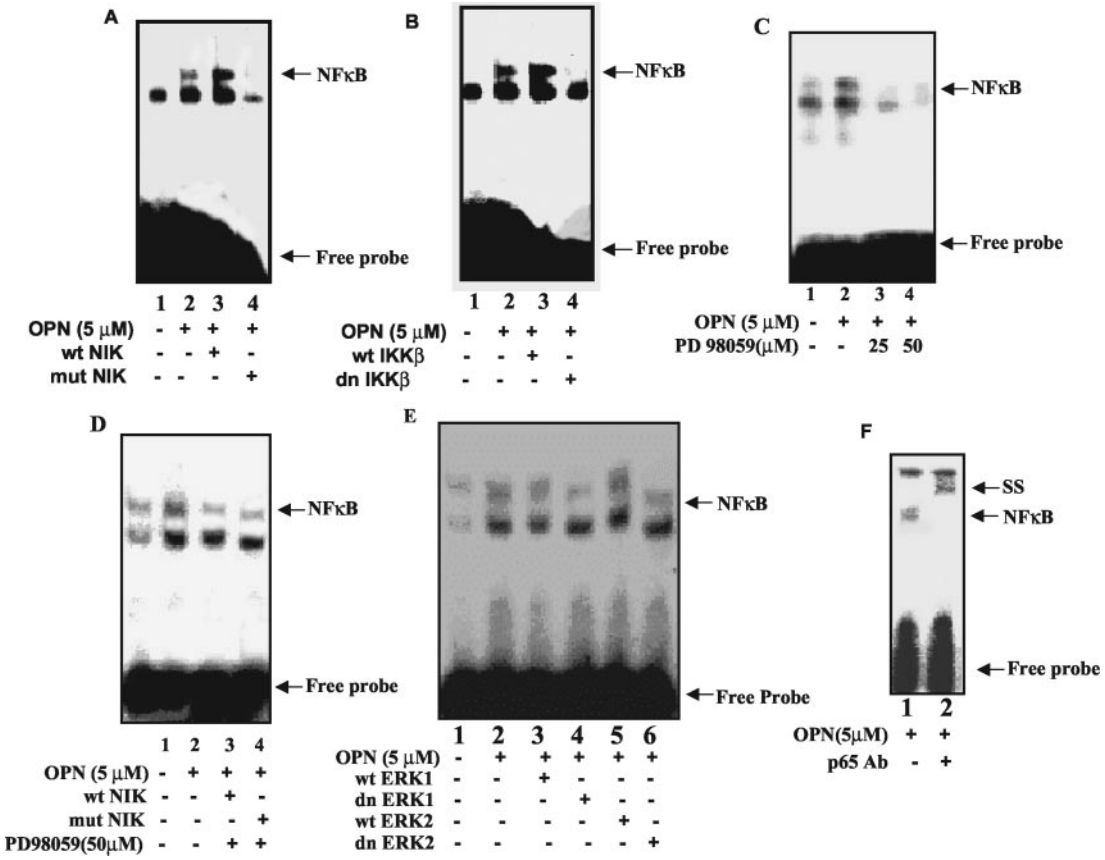

FIG. 4. *Panels A* and *B*, OPN induces NIK/IKK-dependent NFκB-DNA binding. Cells were treated with 5 μM OPN. In other experiments, cells were individually transfected with wild type and kinase-negative NIK or wild type and dn IKKβ and then treated with OPN. The nuclear extracts were prepared and analyzed by EMSA. *Panels C–E*, ERK1/2 is involved in OPN-induced NIK-mediated NFκB-DNA binding. Cells were either pretreated with PD98059 or transfected with wild type and kinase-negative NIK, treated with PD98059, and then treated with OPN. In other experiments, cells were transfected with wt and dn ERK1 and ERK2 and then treated with OPN. The nuclear extracts were analyzed by EMSA. *Panel F*, supershift assay. The nuclear extracts from OPN-treated cells were incubated with anti-p65 antibody and analyzed by EMSA. The results shown in *A–F* represent three experiments exhibiting similar effects. *SS*, supershift; *Ab*, antibody.

To examine the effect of ERK1/2 on OPN-induced NFκB-DNA binding, cells were pretreated with two doses of PD98059 or transfected with wild type and dominant negative constructs of ERK1 and ERK2 and then treated with OPN. The nuclear extracts were prepared and used for EMSA. The data revealed that overexpression of wild type ERK1 or ERK2 resulted in an increase in OPN-induced NFκB-DNA binding, whereas dn ERK1 or ERK2 reduced the OPN-induced NFκB-DNA binding (Fig. 4, *panel E*, *lanes 1–6*). PD98059, an MEK-1 inhibitor, suppressed OPN-induced NFκB-DNA binding (*panel C*, *lanes 1–4*). To ascertain whether OPN-induced ERK-mediated NFκB-DNA binding is NIK-dependent, cells were transfected with wild type and kinase-negative NIK followed by treatment with PD98059 and then stimulated with OPN. The OPN-enhanced NFκB-DNA binding caused by overexpression of wild type NIK was also suppressed by PD98059 (*panel D*, *lanes 1–4*). These results demonstrated that OPN induces NFκB-DNA binding through an NIK/ERK1/2-mediated pathway. To determine whether the band obtained by EMSA was indeed NFκB, the nuclear extracts were incubated with anti-p65 antibody and then analyzed by EMSA. The results showed the shift of the NFκB-specific band to a higher molecular weight when the nuclear extracts were treated with anti-p65 antibody, suggesting that the OPN-activated complex consists of the p65 subunit in these cells (*panel F*, *lanes 1* and *2*).

OPN Induces NIK-dependent ERK/IKK-mediated NFκB Transactivation—To further investigate whether NIK regulates ERK/IKK-dependent OPN-induced NFκB transcriptional activity, a luciferase reporter gene assay was performed. Cells were transiently transfected with NFκB luciferase reporter construct (pNFκB-Luc) and then treated with OPN (5 μM). In separate experiments, cells were individually transfected with wild type and kinase-negative NIK, the super-repressor form of IκBα, wild type IKKα/β and dn IKKα/β, or wild type ERK1/2 and dn ERK1/2 along with pNFκB-Luc and then treated with OPN (5 μM). The transfection efficiency was normalized by cotransfecting the cells with pRL vector. Changes in luciferase activity with respect to control were calculated. The -fold changes were calculated, and the results are expressed as the means ± S.E. of three determination. The values were also analyzed by Student's *t* test ($p < 0.001$). The data showed that wild type NIK enhanced but kinase-negative NIK or the super-repressor form of IκBα suppressed OPN-induced NFκB activity in these cells (Fig. 5, *panel A*). Wild type IKKα and especially IKKβ enhanced OPN-induced NFκB activity (*panel B*). Both dn IKKα and IKKβ suppressed OPN-induced NFκB activity (*panel B*). Similarly wt ERK1 and wt ERK2 enhanced whereas dn ERK1 and dn ERK2 suppressed OPN-induced NFκB activity (*panel C*). These data further suggested that NIK regulates OPN-induced ERK/IKK-dependent NFκB activation.

OPN Stimulates $\alpha_v\beta_3$ Integrin-mediated NIK- and ERK/IKK-dependent Pro-MMP-9 Activation—To examine the effect of OPN on pro-MMP-9 activation, the B16F10 cells were treated with increasing concentrations of OPN (0–10 μM). The conditioned media were collected, and the gelatinolytic activity of MMP-9 was detected by zymography. Increased levels of MMP-9 expression and activation (92-kDa pro- and 86-kDa active forms) were observed when cells were treated with two

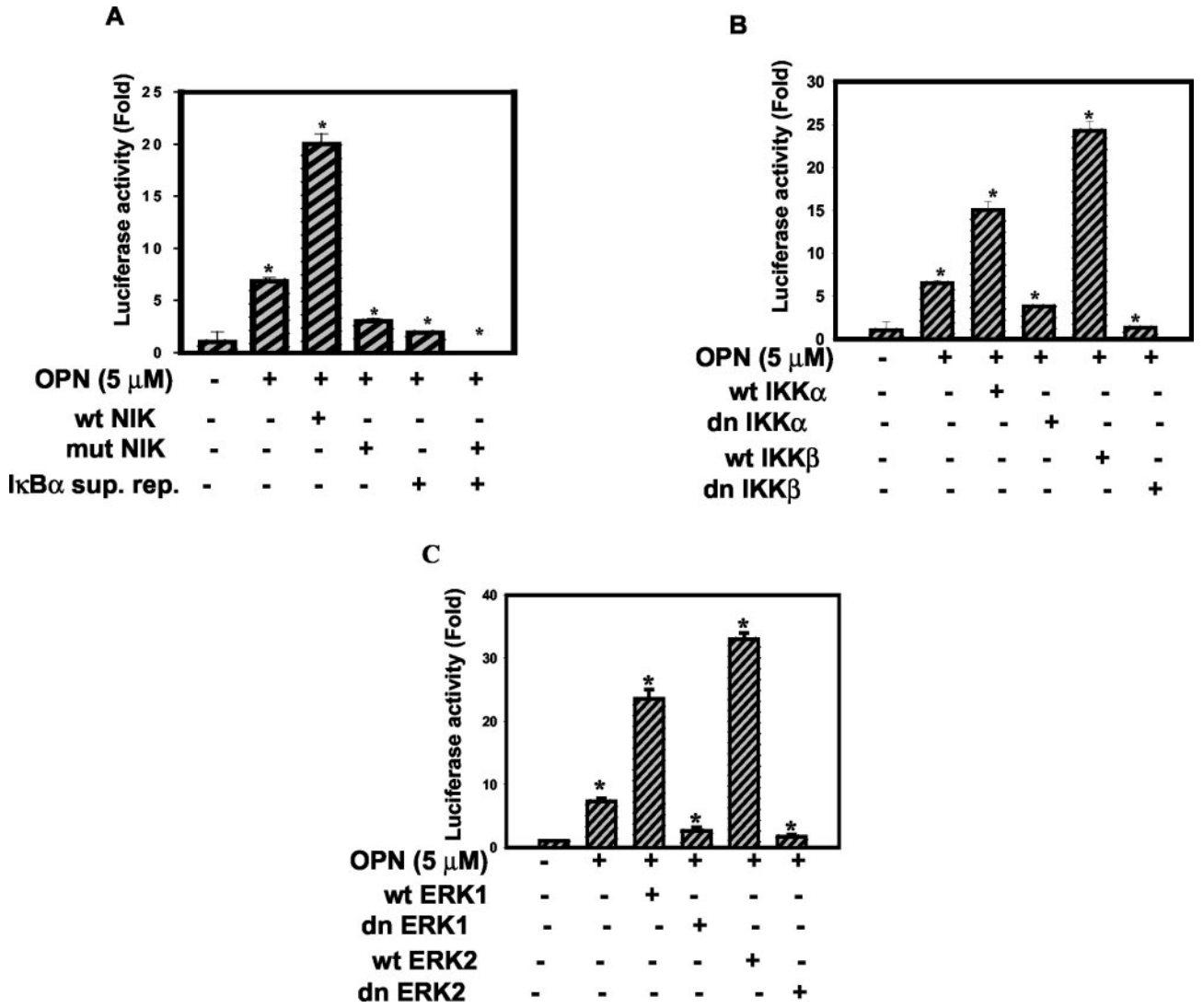

FIG. 5. *Panels A* and *B*, OPN enhances NFκB transactivation through an NIK/IKK-mediated pathway. Cells were transiently transfected with luciferase reporter construct (pNFκB-Luc). In other experiments, cells were individually transfected with wild type and kinase-negative NIK, wild type and dn IKKα/β, or the super-repressor form of IκBα along with pNFκB-Luc. The transfected cells were treated with 5 μM OPN. Cell lysates were used to measure the luciferase activity. The values were normalized to *Renilla* luciferase activity. *Panel C*, ERK1/2 is required in OPN-induced NFκB transactivation. Cells were transfected with wild type and dn ERK1 and ERK2 along with pNFκB-Luc. The transfected cells were treated with 5 μM OPN. Cell lysates were used to measure the luciferase activity. The values were normalized to *Renilla* luciferase activity. The -fold changes were calculated, and means ± S.E. of triplicate determinations are plotted. The values were also analyzed by Student's *t* test (*, $p < 0.001$). *sup. rep.*, super-repressor.

different concentrations of OPN (Fig. 6, *upper panel A*, *lanes 2* and *3*). Almost no pro- and active MMP-9-specific bands were detected in the untreated cells (*lane 1*). The levels of pro- and active MMP-9 expression (gelatinolytic activity) were quantified densitometrically and analyzed statistically. As compared with controls, there were about 3- and 5-fold increases in MMP-9 activation when the cells were treated with 5 and 10 μM OPN, respectively (*lower panel A*).

To check whether $\alpha_v\beta_3$ integrin or RGD peptide is involved in OPN-induced pro-MMP-9 activation, cells were pretreated with anti-$\alpha_v\beta_3$ integrin antibody, GRGDSP, or GRGESP and then treated with 5 μM OPN. The conditioned media were collected, and the levels of pro- and active MMP-9 were detected by Western blot using anti-MMP-9 antibody. The level of OPN-induced MMP-9 activation (Fig. 6, *upper panel B*, *lane 2*) was reduced significantly when cells were individually treated with anti-$\alpha_v\beta_3$ integrin antibody (*lane 3*) or with GRGDSP peptide (*lane 4*) but not with GRGESP peptide (*lane 5*). No detectable level of MMP-9 was observed in OPN-untreated cells (*lane 1*). The intensities of the MMP-9-specific bands were quantified densitometrically and analyzed statistically (*lower panel B*). These data suggested that $\alpha_v\beta_3$ integrin and RGD peptide play an important role in OPN-induced MMP-9 activation.

To investigate the role of NIK, IKK, or ERK1/2 in OPN-induced MMP-9 activation, cells were individually transfected with wild type NIK, kinase-negative NIK, the super-repressor form of IκBα, wild type and dn IKKα and IKKβ, or wild type and dn ERK1/2 and then treated with 5 μM OPN. The level of MMP-9 was detected by zymography as described above. The results indicated that wild type NIK enhanced whereas kinase-negative NIK or the super-repressor form of IκBα suppressed the OPN-induced MMP-9 activation (Fig. 6, *upper panel C*, *lanes 2–5*). Wild type IKKβ enhanced but dn IKKβ suppressed OPN-induced MMP-9 activation (*upper panel D*, *lanes 2–4*). No MMP-9 was detected in OPN-untreated cells (*panels C* and *D*, *lane 1*). Similar results were obtained in cells transfected with IKKα (data not shown). These bands were quantified densitometrically and analyzed statistically (*lower panels* of *panels C* and *D*). Similarly wild type ERK1/2 enhanced and dn ERK1/2 inhibited OPN-induced pro-MMP-9 activation (data not

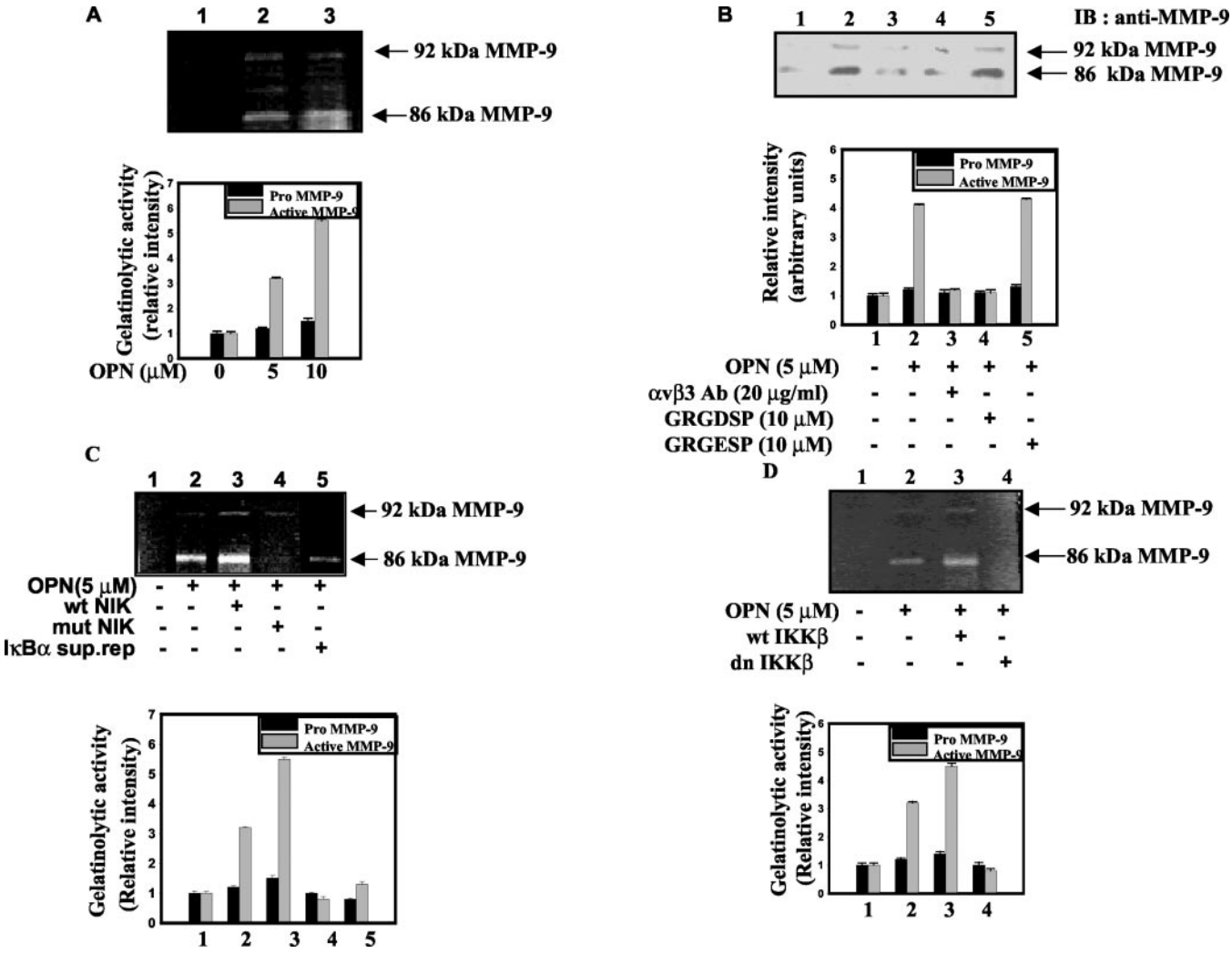

FIG. 6. *Panel A*, OPN induces pro-MMP-9 activity as shown by zymography. Cells were treated in the absence or presence of OPN (0–10 μM). The conditioned media were collected, and MMP-9 activity was analyzed by gelatin zymography. The *arrows* indicate both 92-kDa (pro-) and 86-kDa (active) MMP-9-specific bands (*upper panel A*, *lanes 1–3*). *Panel B*, OPN induces $\alpha_v\beta_3$ integrin-mediated pro-MMP-9 activation. Cells were pretreated with anti-$\alpha_v\beta_3$ integrin antibody (20 μg/ml) or GRGDSP or GRGESP peptide (10 μM) and then treated with 5 μM OPN. The levels of pro- and active MMP-9 in the conditioned media were detected by Western blot using anti-MMP-9 antibody (*upper panel B*, *lanes 1–5*). *Panels C and D*, OPN stimulates NIK/IKK-dependent pro-MMP-9 activation. Cells were individually transfected with wild type and kinase-negative NIK, wild type and dn IKKβ, or the super-repressor form of IκBα and then treated with 5 μM OPN. The conditioned media were collected, and MMP-9 activity was analyzed by gelatin zymography (*upper panels C* and *D*, *lanes 1–5*, *lanes 1–4*). All these bands were quantified by densitometry and are represented in the form of a bar graph (*lower panels A–D*). The data shown here represent three experiments exhibiting similar effects. *sup. rep.*, super-repressor; *Ab*, antibody; *IB*, immunoblot.

shown). These results demonstrated that NIK regulates OPN-induced pro-MMP-9 activation through ERK/IKK-mediated pathways in these cells.

OPN Induces NIK-dependent IKK- and ERK1/2-mediated uPA Secretion and uPA-dependent Pro-MMP-9 Activation—We first examined whether NIK, IKK, and ERK1/2 are involved in OPN-induced uPA secretion in B16F10 cells. Accordingly cells were either treated with various concentrations of OPN (0–5 μM) or transfected with wild type NIK, kinase-negative NIK, wild type and dn IKKα/β, wild type ERK1/2, or dn ERK1/2 and then treated with OPN. The cell lysates were analyzed by Western blot using rabbit polyclonal anti-uPA antibody. The data showed that OPN-induced uPA secretion was enhanced when cells were transfected with wild type NIK and suppressed when transfected with kinase-negative NIK (Fig. 7, *upper panel A*, *lanes 1–5*, and *upper panel B*, *lanes 1–4*). Wild type ERK1/2 stimulated and dn ERK1/2 blocked OPN-induced uPA secretion (*upper panel C*, *lanes 1–6*). Similarly wild type IKKα/β enhanced and dn IKKα/β suppressed OPN-induced uPA secretion (data not shown). All these blots were reprobed with anti-actin antibody (*lower panels A–C*), bands were quantified by densitometric analysis, and the -fold changes were calculated (*panels A–C*). These data suggested that OPN induces NIK-dependent uPA secretion through ERK/IKK-mediated pathways.

To examine whether uPA plays any role in OPN-induced MMP-9 activation, the cells were pretreated with anti-uPA antibody and then treated with OPN (5 μM). The level of MMP-9 was detected by zymography and Western blot analysis. The results indicated that uPA antibody suppressed the OPN-induced MMP-9 activation as shown by zymography (Fig. 7, *panel D*, *lanes 1–3*) and Western blot (*panel E*, *lanes 1–3*), indicating that uPA is involved in OPN-induced MMP-9 activation.

uPA and MMP-9 Play Crucial Roles in OPN-induced NIK-dependent IKK-mediated Cell Migration and Chemoinvasion—Because NIK in the presence of OPN regulates ERK- and IKK-dependent NFκB-mediated uPA secretion and uPA-regulated pro-MMP-9 activation, we therefore sought to determine whether these signaling molecules play any role in OPN-induced cell migration and chemoinvasion. Accordingly cells were

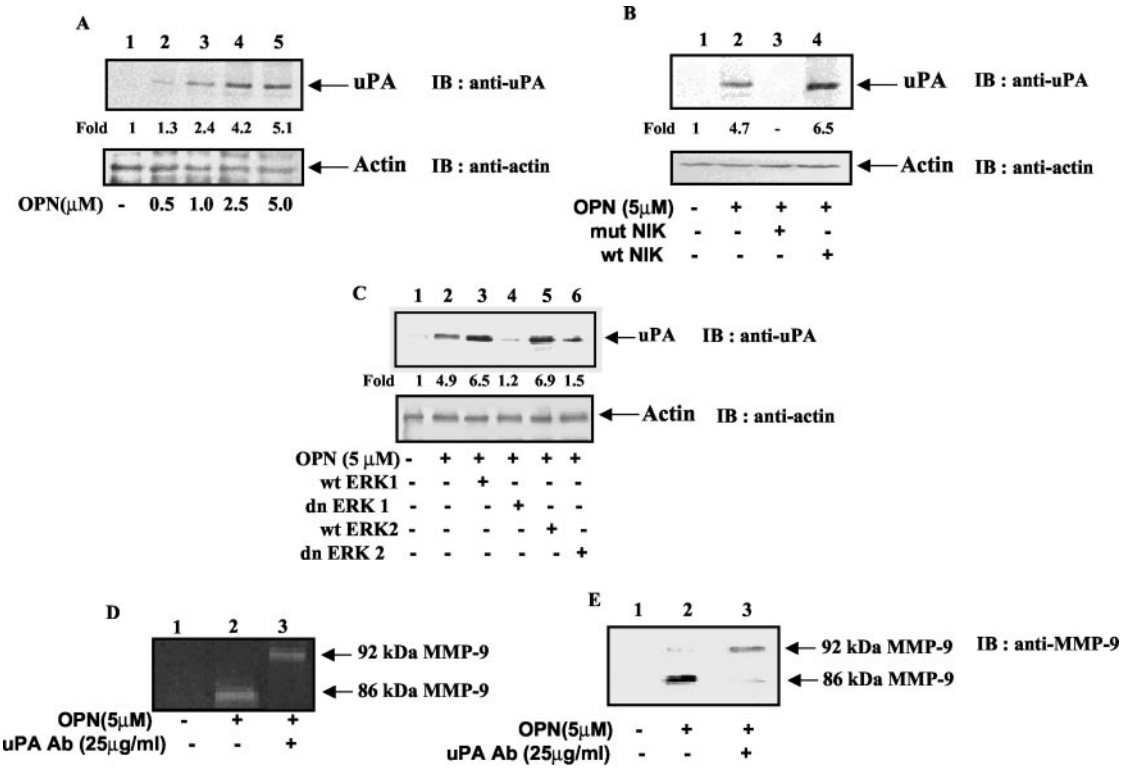

FIG. 7. *Panels A–C*, OPN stimulates NIK- and ERK1/2-dependent uPA secretion. The cells were treated with OPN (0–5 μM). In separate experiments, cells were transfected with wild type and kinase-negative NIK or wild type and dn ERK1 and ERK2 and then treated with OPN. The level of uPA in the cell lysates was detected by Western blot using anti-uPA antibody. The *arrow* indicates the uPA-specific band (*upper panels A–C*). The same blots were reprobed with anti-actin antibody as loading control (*lower panels A–C*). *Panels D* and *E*, uPA is required in OPN-induced pro-MMP-9 activation. Cells were pretreated with anti-uPA antibody (25 μg/ml) and then treated with 5 μM OPN. The conditioned media was collected, and the MMP-9 activities were analyzed by zymography (*panel D*, *lanes 1–3*) and by Western blot (*panel E*, *lanes 1–3*). The *arrows* indicate both 92- and 86-kDa MMP-9-specific bands. The results shown here represent three experiments exhibiting similar effects. *IB*, immunoblot; *Ab*, antibody.

treated with anti-MMP-9 or anti-uPA antibody or transfected with wild type NIK, kinase-negative NIK, the super-repressor form of IκBα, or wild type and dn IKKα and IKKβ and then used for migration or chemoinvasion assay. OPN (5 μM) was used in the upper chamber. The data indicated that anti-MMP-9 or anti-uPA antibody suppressed the OPN-induced cell migration (Fig. 8, *panel A*) and chemoinvasion (*panel B*). Wild type NIK enhanced whereas kinase-negative NIK or the super-repressor form of IκBα suppressed the OPN-induced cell migration (*panel A*) and chemoinvasion (*panel B*). Similarly wild type IKKα and IKKβ stimulated and dn IKKα and IKKβ inhibited the OPN-induced cell migration (*panel C*) and invasion (*panel D*). These data demonstrated that OPN induces uPA- and MMP-9-dependent cell migration and chemoinvasion through NIK/IKK/NFκB-mediated pathways.

ERK1/2 Plays Critical Roles in OPN-induced NIK-regulated Cell Migration—To examine the role of ERK1/2 in OPN-induced NIK-dependent cell migration, the cells were individually pretreated with PD98059, an MEK-1 inhibitor, or transfected with wild type NIK and kinase-negative NIK and then treated with PD98059. In separate experiments, cells were transfected with wild type ERK1/2 or dn ERK1/2. These transfected or treated cells were used for the migration assay. The data indicated that PD98059 suppressed OPN-induced cell migration in the absence or presence of NIK (Fig. 9, *panel D*). Similarly wild type ERK1 and wild type ERK2 enhanced and dn ERK1 and dn ERK2 inhibited OPN-induced cell migration (*panel E*). These data demonstrated that ERK1/2 plays an important role in OPN-induced NIK-dependent cell migration.

Both MMP-2 and MMP-9 Play Important Roles in OPN-induced Cell Migration and Chemoinvasion—We have reported earlier that OPN induces pro-MMP-2 activation, which ultimately regulates cell motility, invasiveness, and tumor growth (20). In this study, we showed that OPN stimulates NIK-dependent uPA secretion and uPA-regulated pro-MMP-9 activation. Therefore, we sought to determine whether both OPN-induced pro-MMP-2 and pro-MMP-9 activations exert any independent roles in regulating OPN-induced cell migration and chemoinvasion. Accordingly cells were pretreated with either anti-MMP-2 antibody or anti-MMP-9 antibody alone or a mixture of both. These cells were used for a migration or invasion assay. The data indicated that MMP-2 or MMP-9 antibody suppressed OPN-induced cell migration and chemoinvasion (Fig. 9, *panels A* and *B*). Pretreatment of cells with a mixture of anti-MMP-2 and anti-MMP-9 antibodies had an additive effect and hence drastically reduced OPN-induced cell migration and chemoinvasion (*panels A* and *B*).

To ascertain whether there is any cross-talk between OPN-induced pro-MMP-2 and pro-MMP-9 activations, cells were pretreated with two different concentrations of anti-MMP-2 antibody followed by treatment with OPN. The conditioned media were collected, and the level of MMP-9 was detected by Western blot analysis using anti-MMP-9 antibody. The data showed that OPN-induced MMP-9 activation was unaffected when cells were pretreated with anti-MMP-2 antibody (Fig. 9, *panel C*, *lanes 1–4*). These data clearly indicated that OPN-induced activations of MMP-2 and MMP-9 occurred through two distinct signaling mechanisms, and both played independent roles in regulating cell migration and chemoinvasion in B16F10 cells.

OPN Induces Pro-MMP-9 Activation in Tumor of Nude Mice—The *in vitro* data prompted us to examine whether

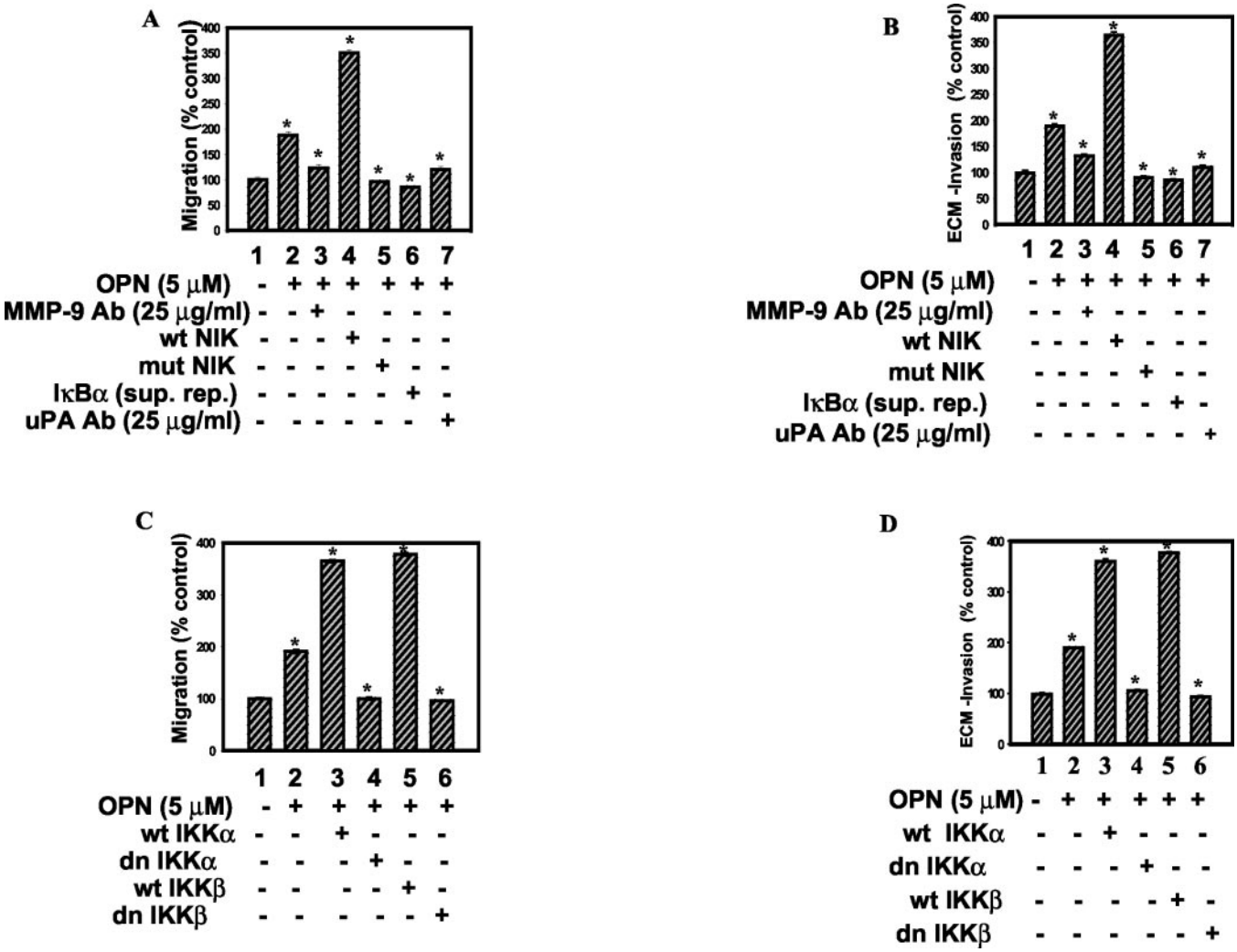

FIG. 8. ***Panels A–D*, NIK and IKK*α*/*β* are involved in OPN-stimulated uPA and MMP-9-dependent cell migration and chemoinvasion.** The migration assay was conducted either by using untreated cells (5×10^6cells/well) or cells pretreated with anti-MMP-9 antibody (25 μg/ml) or anti-uPA antibody (25 μg/ml). In separate experiments, cells were transfected with wild type and kinase-negative NIK, wild type and dn IKKα and IKKβ, or the super-repressor form of IκBα. The purified human OPN (5 μM) was added in the upper chamber. The treated or transfected cells were used for migration assays. Note that OPN-induced cell migration was blocked by anti-MMP-9 antibody, anti-uPA antibody, kinase-negative NIK, and dn IKKα/β and enhanced by wild type NIK and wild type IKKα/β (*panels A* and *C*). The same results were obtained in the chemoinvasion assay (*panels B* and *D*). The results are expressed as the means ± S.E. of three determinations. *Ab*, antibody; *sup. rep.*, super-repressor.

OPN plays any role in MMP-9 activation in tumor of nude mice. Accordingly B16F10 cells were treated with OPN (10 μM) and were injected subcutaneously into the flanks of nude mice. Fig. 10, *panel A*, shows typical photographs of tumors grown in 4-week-old nude mice (*a* and *b*). After 4 weeks, the mice were killed, and tumor weights were measured. The weights of the OPN-induced tumors were increased at least 3-fold compared with the tumors of the non-OPN-injected mice (Table I). Four mice were used in each set of experiments. The changes in tumor weights were analyzed statistically by Student's *t* test ($p < 0.002$). These data are consistent with our previous data.

To examine the levels of pro- and active MMP-9 in tumors, the samples were lysed, and MMP-9 expression was analyzed by zymography (Fig. 10, *panel B*). The levels of both pro- and active MMP-9 in the tumors produced by OPN (10 μM) were significantly higher (*lane 2*) compared with the levels of MMP-9 in the tumors of the non-OPN-injected mice (*lane 1*). The levels of MMP-9 in tumors were further confirmed by Western blot analysis (*panel C*). Both pro- and active MMP-9 expressions were higher in the tumors produced by OPN (*panel C*), and these are corroborated by the zymography data (*panel B*). These data demonstrated that OPN induces pro-MMP-9 activation in tumor of nude mice, and it correlates with tumor growth in nude mice.

DISCUSSION

In this study, we investigated whether OPN regulates pro-MMP-9 activation and MMP-9-dependent cell motility, invasiveness, and tumor growth. Moreover we examined whether any upstream kinase such as NIK is involved in OPN-induced NFκB activation, NFκB-mediated uPA secretion, pro-MMP-9 activation, and cell motility through activation of MAPK/IKK in B16F10 cells. We showed that OPN induced phosphorylation and activation of NIK and enhanced the interaction between phosphorylated NIK and IKKα/β in these cells. The data revealed that OPN induced NIK- and IKK-dependent NFκB activation through phosphorylation and degradation of IκBα. Moreover NIK in the presence of OPN enhanced MEK-1 and ERK1/2 phosphorylations and MEK/ERK-dependent NFκB activation. OPN induced NIK-dependent ERK/IKK-mediated uPA secretion, uPA-dependent pro-MMP-9 activation, cell migration, and chemoinvasion. OPN induced the pro-MMP-9 activation in tumor of nude mice. These data demonstrated that

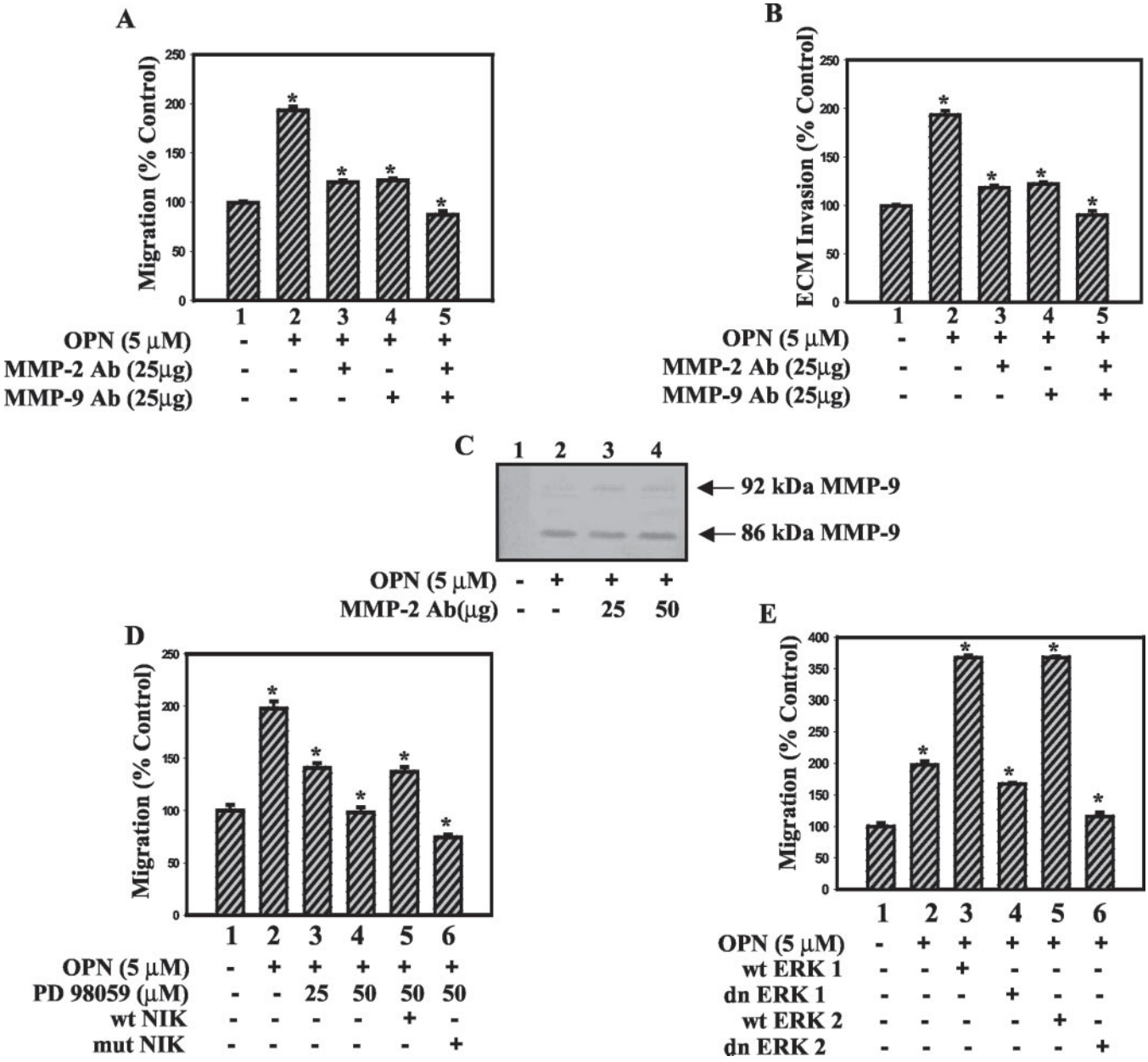

FIG. 9. *Panels A* and *B*, both MMP-2 and MMP-9 play important roles in OPN-induced cell migration and chemoinvasion. The migration assay was performed either by using untreated cells or cells pretreated with anti-MMP-2 antibody (25 μg/ml) or anti-MMP-9 antibody (25 μg/ml) or a combination of both. The purified human OPN was added to the upper chamber. These cells were used for migration assays (*panel A*). The same results were obtained in the invasion assay (*panel B*). *Panel C*, OPN-induced pro-MMP-9 activation is distinct from pro-MMP-2 activation. Cells were pretreated with anti-MMP-2 antibody (0–50 μg/ml) and then treated with 5 μM OPN. The level of MMP-9 was detected by Western blot using anti-MMP-9 antibody (*panel C*, *lanes 1–4*). Note that OPN-induced MMP-9 activation is unaffected by anti-MMP-2 antibody. *Panels D* and *E*, ERK1/2 is involved in OPN-stimulated NIK-dependent cell migration. Cells were either pretreated with PD98059 or transfected with wild type or kinase-negative NIK and then treated with PD98059. In separate experiments, cells were transfected with wild type and dn ERK1 and ERK2. These treated or transfected cells were used for the cell migration assay (*panels D* and *E*). The results are expressed as the means ± S.E. of three determinations. *Ab*, antibody.

OPN induces NIK-dependent cell motility, tumor growth, NFκB-mediated uPA secretion, and uPA-regulated pro-MMP-9 activation by activating ERK/IKK signaling pathways.

Recent studies have demonstrated that mitogen-activated protein kinase kinase kinases including NIK and MEK kinases 1–3 are involved in the activation of IKK complex (38–40). Kouba *et al.* (41) have shown that NIK regulates NFκB activation pathways in epidermal keratinocytes. Thus we examined whether NIK has any effect in OPN-induced NFκB activation in B16F10 cells. The data showed that OPN stimulated NIK phosphorylation and its kinase activity in these cells. Pretreatment of cells with anti-$\alpha_v\beta_3$ integrin antibody or RGD but not RGE peptide inhibited OPN-induced NIK phosphorylation indicating that $\alpha_v\beta_3$ integrin is involved in this process. Previous data has also suggested that NIK strongly interacts with both IKKα and -β (42, 43). NIK also interacts with TRAF proteins including TRAF-3 as shown by yeast two-hybrid systems (44). Our data revealed that OPN enhances the interaction between phosphorylated NIK and IKKα/β in B16F10 cells. Previous studies have also indicated that IKK activation alone could not account for the total NFκB activity in HS294T cells (45). Foehr *et al.* (17) have recently demonstrated that NIK regulates differentiation of PC-12 cells through MEK/ERK pathways. These results prompted us to investigate whether OPN regulates NIK-dependent NFκB activation through the MAPK pathway in B16F10 cells. Our data indicated that transient overexpres-

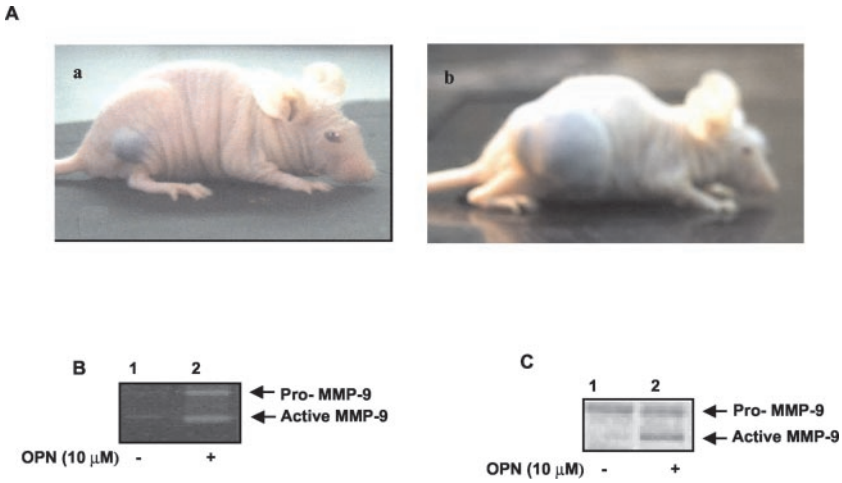

FIG. 10. **OPN induces tumor growth and MMP-9 activation in tumors of nude mice.** *Panel A*, typical photographs of tumors in nude mice. The cells were treated in the absence or presence of OPN (10 μM) and then injected subcutaneously into the flanks of nude mice. *a*, cells with phosphate-buffered saline; *b*, cells with OPN. The results show the representative of four mice used in each set of experiments. *Panels B* and *C*, detection of active MMP-9 in tumors of nude mice by zymography (*panel B*) and Western blot (*panel C*). The tumor samples were lysed, and the levels of MMP-9 in these samples were analyzed by zymography and Western blot as described earlier. The *arrows* indicate the 92- and 86-kDa MMP-9-specific bands.

TABLE I
OPN induces tumor growth in nude mice

B16F10 cells were treated with 10 μM OPN for 16 h and injected into nude mice (NMRI). The injection was performed twice a week for 4 weeks. The mice were killed, and the tumor weights were measured and analyzed statistically by Student's *t* test ($p < 0.002$). Mice injected with cells in phosphate-buffered saline (PBS) were used as controls.

No. nude mice	Treatment	Tumor weight (-fold change)
4	Control (PBS)	1.0 ± 0.15
4	OPN (10 μM)	3.2 ± 0.17

sion of wild type NIK up-regulates OPN-induced MEK and ERK1/2 phosphorylations, whereas kinase-negative NIK abrogated these processes. We also showed that OPN-induced NIK-dependent MEK-1 phosphorylation was suppressed by anti-$\alpha_v\beta_3$ integrin blocking antibody and RGD peptide but not RGE peptide indicating that OPN regulates NIK-mediated MAPK activation through $\alpha_v\beta_3$ integrin-mediated pathways.

Several reports have indicated that NFκB is involved in control of large number of cellular processes such as inflammatory and immune response, developmental processes, cell growth, and apoptosis. In addition, NFκB is activated in several pathological conditions like arthritis, inflammation, asthma, neurodegenerative disease, heart disease, and cancers (46). In our previous report, we have demonstrated that OPN stimulated NFκB activation by inducing the phosphatidylinositol 3-kinase/IKK activity in murine melanoma and breast cancer cells (21, 22). However, it was not clear whether NIK is involved in regulation of OPN-induced NFκB activation and whether MAPK/IKK plays any role in this activation process in B16F10 cells. In this study, we found that OPN induces NIK-dependent IKK activity through interaction between phosphorylated NIK and IKK. Transfection of these cells with wild type NIK and IKKα/β but not with kinase-negative NIK or dn IKKα/β enhanced OPN-induced NFκB-DNA binding and NFκB transactivation. Moreover PD98059, an MEK-1 inhibitor, down-regulated NFκB-DNA binding and NFκB activation (data not shown) in cells transfected with wild type NIK followed by treatment with OPN. Overexpression of wild type ERK1/2 but not dn ERK1/2 dramatically enhanced OPN-induced NFκB activation. These data suggested a link between OPN-induced NIK/IKK/NFκB and NIK/ERK/NFκB pathways in B16F10 cells.

Signals transduced by cell adhesion molecules play an important role in tumor cell attachment, motility, and invasion, all of which regulate tumor metastasis. Cell-matrix interactions play a major role in tissue remodeling, cell survival, and tumorigenesis. OPN, an ECM protein, plays a significant role in cell adhesion, migration, and metastasis. The overexpression of OPN is linked with various cancers and their metastatic potentials (8). MMPs are a family of Zn^{2+}-dependent endopeptidases that are responsible for remodeling of the extracellular matrix and degradation of ECM proteins. MMP-9 is known to degrade basement membrane, which normally separates the epithelial from the stromal compartment. Elevated levels of MMP-9 have been reported in various cancers. Several studies have shown the correlation between MMP-9 expression and metastatic potential of tumor (47). MMP-9 is not only associated with invasion/metastasis but also reported to be involved in a number of angiogenic relevant diseases such as rheumatoid arthritis, retinopathy, and vascular stenosis and is considered to be a therapeutic target of priority (35, 36). Kim *et al.* (48, 49) have also demonstrated that MMP-9 activity but not MMP-2 activity significantly affects tumor intravasation into blood vessel and that uPA is required for pro-MMP-9 activation. Several other reports have indicated the correlation between uPA expression and metastatic potential and shown that uPA plays a major role in regulating MMP activation (22, 50). Therefore, we sought to determine whether OPN regulates uPA secretion and whether uPA plays any role in regulation of pro-MMP-9 activation. In this study, we showed that OPN induces uPA secretion and uPA-dependent pro-MMP-9 activation. Pretreatment of cells with anti-$\alpha_v\beta_3$ integrin antibody or RGD but not RGE peptide inhibited OPN-induced pro-MMP-9 activation indicating that OPN induces pro-MMP-9 activation through $\alpha_v\beta_3$ integrin-mediated pathways. Overexpression of wild type NIK and IKKα/β enhanced and kinase-negative NIK, dn IKKα/β, or the super-repressor form of IκBα suppressed the OPN-induced pro-MMP-9 activation demonstrating that OPN regulates pro-MMP-9 activation through NIK/IKK/NFκB-mediated pathways. Recent data also indicated that the expression of MMP-9 is down-regulated in ERK-mutated stable transfectants; this inhibits glioma invasion *in vitro* (51). In this study, we examined whether MAPK, especially ERK1/2, regulates OPN-induced pro-MMP-9 activation in B16F10 cells. The results showed that overexpression of wild type ERK1/2 but not dn ERK1/2 up-regulated OPN-induced uPA secretion leading to the activation of pro-MMP-9 indicating that OPN induces uPA-dependent pro-MMP-9 activation through NIK/MAPK-mediated pathways.

Previous studies have indicated that uPA and MMP-9 expressions are inversely related to MT1-MMP expression in esophageal carcinoma (52). It has been implicated that there are two pathways involved in esophageal carcinogenesis, one is involved in the MT1-MMP/MMP-2 activation pathway and the other one is the uPA/MMP-9 activation pathway, and both pathways are critical in regulation of cancer cell motility, in-

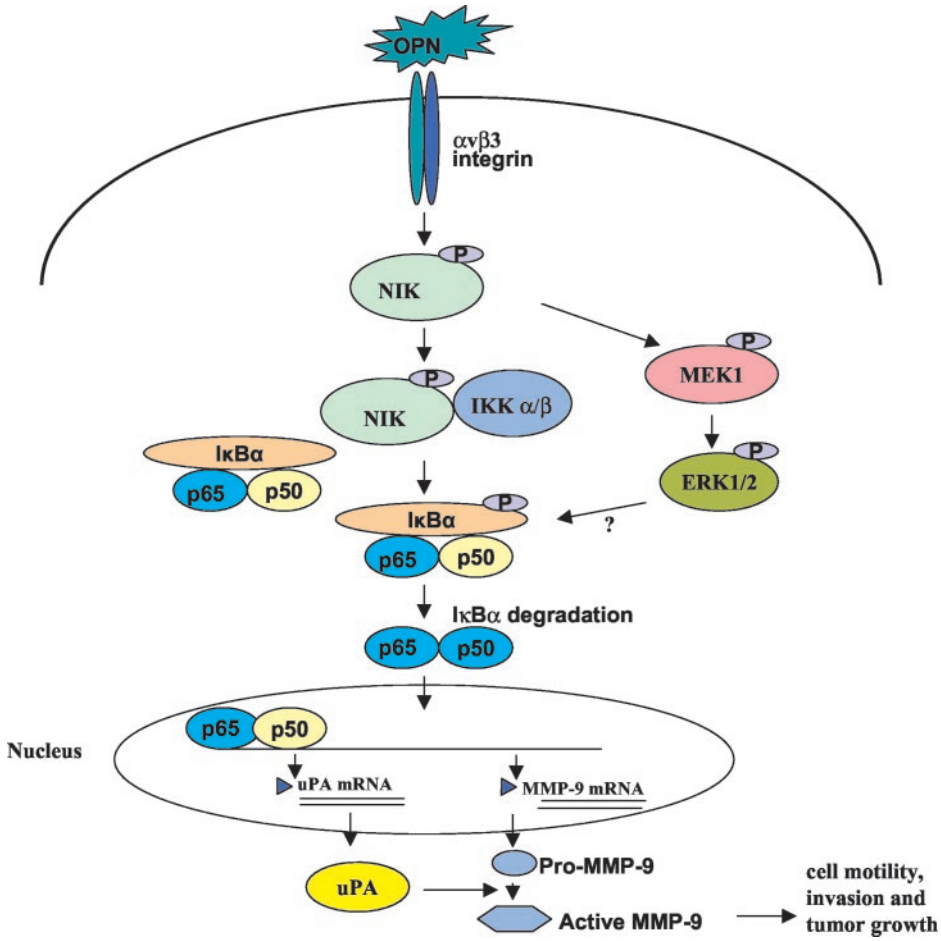

FIG. 11. **Molecular mechanism of OPN-induced NIK-regulated NFκB-mediated uPA secretion and pro-MMP-9 activation through MAPK/IKK pathways.** Binding of OPN to $\alpha_v\beta_3$ integrin receptor induces phosphorylation and activation of NIK and its subsequent interaction with IKKα/β, which activates NFκB through phosphorylation and degradation of IκBα. In addition, NIK in the presence of OPN induces MEK-1/ERK1/2 phosphorylation, which also activates NFκB. OPN stimulates NIK-dependent IKK/MAPK-mediated uPA secretion, which regulates pro-MMP-9 activation, cell motility, invasion, and tumor growth.

vasiveness, tumor growth, and angiogenesis. We have reported earlier that OPN induces pro-MMP-2 activation through NFκB-mediated induction of MT1-MMP in murine melanoma cells (20). However, the mechanism by which OPN regulates uPA-dependent pro-MMP-9 activation and controls cell migration, invasion, and tumor growth are not well defined. Pretreatment of cells with anti-uPA antibody suppressed OPN-induced pro-MMP-9 activation indicating that uPA plays a crucial role in this process. Moreover pretreatment of cells with anti-MMP-2 antibody along with anti-MMP-9 antibody dramatically suppressed the OPN-induced cell migration and chemoinvasion, whereas pretreatment of cells with anti-MMP-2 antibody had no effect on OPN-induced pro-MMP-9 activation. These data suggested that both MMP-2 and MMP-9 are involved in OPN-induced cell migration and chemoinvasion, but OPN-induced MMP-2 activation is distinct from MMP-9 activation. Wild type NIK, IKKα/β, and ERK1/2 enhanced and kinase-negative NIK, dn IKKα/β, and dn ERK1/2 suppressed OPN-induced cell migration and chemoinvasion. OPN also induced the pro-MMP-9 activation in tumor of nude mice. These data demonstrated that OPN induces NIK-regulated NFκB-mediated uPA-dependent pro-MMP-9 activation, cell motility, and tumor growth via ERK/IKK-mediated signaling pathways.

In summary, we demonstrated for the first time that OPN induces activation and phosphorylation of NIK through interaction between IKK and phosphorylated NIK. OPN also enhances NIK-dependent IKKα/β- and ERK1/2-mediated NFκB activation, uPA secretion, and pro-MMP-9 activation. Taken together, OPN induces cell migration, tumor growth, and NIK-dependent NFκB-mediated uPA secretion and pro-MMP-9 activation by activating IKK/ERK signaling pathways (Fig. 11). These findings may be useful in designing novel therapeutic interventions that block the OPN-regulated NIK-dependent IKK- and ERK1/2-mediated NFκB activation resulting in reduction of uPA secretion and pro-MMP-9 activation and consequent blocking of cell motility, invasiveness- and melanoma growth.

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