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

Hema Rangaswami, Anuradha Bulbule, and Gopal C. Kundu‡

From the National Center for Cell Science, Pune 411 007, India

We have recently demonstrated that osteopontin (OPN) induces nuclear factor κB (NFκB)-mediated promatrix metalloproteinase-2 activation through IκBα/IκB 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 α,β integrin-mediated phosphorylation and activation of nuclear factor-inducing kinase (NIK) and enhanced the interaction between phosphorylated NIK and IκKα/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/IκKβ-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, IκKα/β, and ERK1/2 enhanced and kinase-negative NIK (mut NIK), dominant negative IκKα/β (dn IκKα/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/IκKα/β-mediated pathways, and all of these ultimately control the cell motility, invasiveness, and tumor growth.

Osteopontin (OPN) is a secreted, non-collagenous, sialic acid-rich phosphoglycoprotein (1, 2). OPN acts both as che...

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† To whom correspondence should be addressed: National Center for Cell Science (NCCS), NCCS Complex, Pune 411 007, India. Tel.: 91-20-25690951 (ext. 203); Fax: 91-20-25692259; E-mail: gopalkundu@hotmail.com.

‡ The abbreviations used are: OPN, osteopontin; NIK, nuclear factor-inducing kinase; NFκB, nuclear factor κB; IκBα, inhibitor of nuclear factor κB; IKK, IκB 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 IκB 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) – 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.

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.
kinase reaction mixture was analyzed by Western blot using anti-IKK\(\beta\) antibody.

To investigate the role of NIK in OPN-induced IKK activity, the cells were treated with 5 \(\mu\)M 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 \(\mu\)M 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 gelatin-containing buffer (50 mM Tris-HCl (pH 7.5) containing 100 mM CaCl\(_2\), 1 \(\mu\)M ZnCl\(_2\), 1 \(\mu\)g/ml TNF-
\(\alpha\), 10% (v/v) Triton X-100, and 0.02% (w/v) Na\(_3\)VO\(_4\)) 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 I\(\kappa\)B\(\alpha\) in the presence of LipofectAMINE Plus for 24 h. In separate experiments, cells were treated with wild type or dominant negative IKK\(\alpha\) and IKK\(\beta\) and then treated with 5 \(\mu\)M 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 NF\(\kappa\)B-DNA binding, cells were either treated with 5 \(\mu\)M OPN or transfected with wild type NIK, kinase-negative NIK, or wild type and dn IKK\(\beta\) 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 NF\(\kappa\)B-DNA binding and whether it is regulated by NIK, cells were either pretransfected with PD98059 or cotransfected 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, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) reveals ERK1/2 phosphorylation. The nuclear pellet was extracted in nuclear extract buffer (20 mM Hepes (pH 7.9), 0.4 mM Na\(_3\)VO\(_4\), 1 mM MgCl\(_2\), 0.2 mM EDTA, 25 mM glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) supernatant was used as a nuclear extract. The nuclear extracts (10 \(\mu\)g) were incubated with 16 fmol of \(^{32}\)P-labeled double-stranded NF\(\kappa\)B oligonucleotide (5'-AGT TGA GGC GAC TTT CCC AGG CTT CAG GAG GGC CCA GTC CAC ATT CCG GAC CTT G) as described previously (25). The complexes were resolved by 5% polyacrylamide gel electrophoresis (30 min at 200 V). The gels were dried and autoradiographed. For supershift assay, the NF\(\kappa\)B-binding activity of the nuclear extracts was determined as described previously (25)

**Cell Migration Assay**—To study the role of NIK in OPN-induced cell migration, B16F10 cells were grown in 24-well plates were transiently transfected with a luciferase reporter construct (pNF\(\kappa\)B-Luc) containing five tandem repeats of the NF\(\kappa\)B binding site using LipofectAMINE Plus reagent (Invitrogen). In separate experiments, cells were individually transfected with wild type NIK, kinase-negative NIK, or the super-repressor form of I\(\kappa\)B\(\alpha\), wild type and dn IKK\(\alpha\), and wild type and i NF\(\kappa\)B and ERK2 along with pNF\(\kappa\)B-Luc, and then treated with OPN for 24 h. The non-migrated cells on the upper side of the filter were counted, and the level of active MMP-9 was detected by zymography as described above.

**Western Blot Analysis**—To delineate the role of OPN in regulation of NIK phosphorylation, the cells were treated with 5 \(\mu\)M OPN for 0–30 min. In separate experiments, cells were pretreated with anti-Src, anti-\(\alpha\)-actin, or anti-\(\beta\)-actin antibody. The same blots were reprobed with rabbit anti-Src antibody as loading control. The slight blots were probed with rabbit anti-IKK\(\alpha\) or anti-\(\beta\)-actin antibody as loading controls and detected by the ECL detection system (Amersham Biosciences).

**NIK Regulates OPN-induced MAPK**—To delineate the role of OPN in regulation of NIK phosphorylation, the cells were treated with 5 \(\mu\)M OPN for 0–30 min. In separate experiments, cells were treated with anti-IKK\(\alpha\) or anti-\(\beta\)-actin antibody as loading controls. The same blots were reprobed with rabbit anti-IKK\(\beta\) or anti-\(\beta\)-actin antibody as loading controls and detected by the ECL detection system (Amersham Biosciences).

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**NIK Regulates OPN-induced MAPK**—To delineate the role of OPN in regulation of NIK phosphorylation, the cells were treated with 5 \(\mu\)M OPN for 0–30 min. In separate experiments, cells were treated with anti-IKK\(\alpha\) or anti-\(\beta\)-actin antibody as loading controls. The same blots were reprobed with rabbit anti-IKK\(\beta\) or anti-\(\beta\)-actin antibody as loading controls and detected by the ECL detection system (Amersham Biosciences).
NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation

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 αβ3 integrin-mediated pathways. The cells were individually pretreated with anti-αβ3 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-IKKβ 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.

RESULTS

OPN Induces αβ3 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 analyzed by Western blot using anti-MMP-2 antibody. 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.

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, and photomicrographs were taken under the inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG served as a nonspecific control.

Chemo-invasion Assay—The chemo-invasion assay was performed using a Matrigel-coated invasion chamber as described previously (20–22). The cell suspension (5 × 105 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 chemotacticant. Purified OPN (5 μM) was added to the upper chamber. In another experiment, cells were individually pretreated with anti-MMP-2 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 δN IKKα and IKKδ, or the super-repressor form of 1αβ3α 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.

In Vivo Tumorigenicity Experiments—The tumorigenicity experiments were performed as described previously (20, 21). The mice were treated in the absence or presence of purified human OPN (10 μg) at 37 °C for 20 h. After that, the cells (5 × 105/0.2 ml) were detached and infected 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 μg) 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.

DISCUSSION

NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation

effect. Were analyzed densitometrically, and the -fold changes were calculated. The data shown here represent three experiments exhibiting similar reprobed with anti-NIK (middle panel A) or anti-actin antibody (lower panel A) as loading controls. Panel B, OPN induces NIK phosphorylation through αβ3 integrin-mediated pathways. The cells were individually pretreated with anti-αβ3 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-IKKβ 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.
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 αβ 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 αβ 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 IKK-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-32P]ATP. The samples were resolved by SDS-PAGE and autoradiographed. The radioabeled, 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...
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-32P]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. 3, 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 panels A, lanes 1–4). These data suggested that NIK plays a crucial role in OPN-induced IκBα phosphorylation and degradation.

**OPN Stimulates αβ3 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 αβ3 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 αβ3 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 αβ3 integrin-dependent MEK-1 and ERK1/2 phosphorylations through an NIK-mediated pathway.

**NIK Plays a Crucial Role in OPN-induced ERK1/2-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 32P-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.
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 and defined as 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 and then analyzed by EMSA. The results shown in A–F represent three experiments exhibiting similar effects. SS, supershift; Ab, antibody.

**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 showed the supershift of the 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 and defined as 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 Iκκα, wt or wt IKKα/β and dn IKKα/β, or wt 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 Iκκα 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 αvβ3 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
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 αβ3 integrin or RGD peptide is involved in OPN-induced pro-MMP-9 activation, cells were pretreated with anti-αβ3 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-αβ3 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 αβ3 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 IKK, 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). Similar results were obtained in cells transfected with IKKa (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 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 IKKβ, wild type ERK1/2, or dn ERK1/2 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.

**NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation**
NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation

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 in a concentration-dependent manner (20). In separate experiments, we showed that OPN-induced pro-MMP-2 and pro-MMP-9 activations, cells were transfected with wild type and kinase-negative NIK or wild type and dn ERK1 and ERK2 and then treated with OPN. The conditioned media was collected, and the MMP-9 activities were analyzed by zymography (panel D, lanes 1–5) and by Western blot (panel E, lanes 1–5). The arrows indicate both 92- and 86-kDa MMP-9-specific bands. The results shown here represent three experiments exhibiting similar effects.

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 level of uPA in the cell lysates was detected by Western blot using anti-uPA antibody. The arrow indicates the uPA-specific band. The results shown here represent three experiments exhibiting similar effects. IB, immunoblot; Ab, antibody.
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 (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.

NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation
NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation

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 on 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-αvβ₃ integrin antibody or RGD but not RGE peptide inhibited OPN-induced NIK phosphorylation indicating that αvβ₃ 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. 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 μg/ml 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.
NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation

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

<table>
<thead>
<tr>
<th>No. nude mice</th>
<th>Treatment</th>
<th>Tumor weight (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Control (PBS)</td>
<td>1.0 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>OPN (10 μM)</td>
<td>3.2 ± 0.17</td>
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Signs 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 invasiveness 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-α,β integrin antibody or RGD but not RGE peptide inhibited OPN-induced pro-MMP-9 activation indicating that OPN induces pro-MMP-9 activation through α,β 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/IKKα/β-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/IKKα/β-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-
NF inhibits cell migration, tumor growth, and NIK-dependent uPA secretion, and pro-MMP-9 activation. Taken together, OPN kinase-negative NIK, dn IKK/H9251 IKK pathways. MMP-9 activation through MAPK-mediated uPA secretion and pro-NF phosphorylation, which also activates presence of OPN induces MEK-1/ERK1/2 OPN-induced NIK-regulated NF/H9260 phosphorylation resulting in reduction of NF secretion and pro-MMP-9 activation and consequent blocking of cell motility, invasiveness and melanoma growth.

Acknowledgments—We thank Prof. David Wallach for providing wild type NIK (wt pCDNA NIK) and kinase-negative NIK (mut pCDNA NIK, NIK-K429A/K430A) in pCDNA3 and Prof. D. V. Goeddel for wild type and dominant negative constructs of IKK (wt IKKα and dn IKKα) and IKKβ (wt IKKβ and dn IKKβ) in pRK. We also thank Dr. Melissa Cobb for wt ERK1 and dn ERK1 in pCEP4 and wt ERK2 and dn ERK2 in p3XFLAG-CMV-7.1, Dr. Rainer de Martin for the luciferase reporter construct (pNF B-Luc) containing five tandem repeats of NF binding site, and Dr. Dean Ballard for the super-repressor form of IκB cDNA fused downstream to a FLAG epitope in an expression vector (pCMV4).

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NIK Regulates OPN-induced MAPK/IKK-mediated MMP-9 Activation

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JNK1 Differentially Regulates Osteopontin-induced Nuclear Factor-Inducing Kinase/MEKK1-dependent Activating Protein-1-mediated Promatrix Metalloproteinase-9 Activation

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Hema Rangaswami, Anuradha Bulbule, and Gopal C. Kundu‡
From the National Center for Cell Science, Pune 411 007, India.

We have recently demonstrated that nuclear factor-inducing kinase (NIK) plays a crucial role in osteopontin (OPN)-induced mitogen-activated protein kinase (MAPK) activation. NIK is a member of the mitogen-activated protein kinase kinase (MAPKK) family, is a mammalian serine/threonine protein kinase initiating an upstream kinase in the mitogen-activated protein kinase (MAPK) cascade. Nuclear factor-inducing kinase (NIK) is another member of the mitogen-activated protein kinase (MAPK) family that has been implicated in NFκB activation. Few reports indicate that MEKK1 has the ability to activate ERK, but its effect is less potent (19). These results suggest that MEKK1 is an upstream kinase in the mitogen-activated protein kinase cascade. Nuclear factor-inducing kinase (NIK) is another member of the mitogen-activated protein 3-kinase family that has been implicated in NFκB activation. Few reports indicate that NIK may also be involved in the regulation of transcription factor, AP-1, as its activation leads to the induction of c-Fos.
Cross-talk between OPN-induced JNK and ERK Pathways

that associates with c-Jun to form an AP-1 heterodimeric complex that can promote targeted gene expression (20). However, the molecular mechanism by which OPN regulates NIK and MEKK1-mediated AP-1 transactivation and whether JNK is involved in both these pathways is not clearly understood. Various mitogen-activated protein kinase cascades (e.g. ERK1/2, JNK, p38) are often portrayed as linear cascades, and indications of cross-talk between the various cascades are limited (21, 22). In this respect, the present study also examines whether any cross-talk exists between OPN-induced NIK/ERK- and MEKK1/JNK-signaling pathways.

uPA is a member of serine protease family that interacts with the uPA receptor and facilitates the conversion of inextricable plasminogen into plasmin (23). Plasmin regulates cell invasion by degrading matrix proteins such as type IV collagen, gelatin, fibronectin, and laminin or indirectly by activating MMPs (24, 25). It is established that uPA plays a significant role in tumor growth and metastasis (26–28). It is regulated at the transcriptional level by a number of transcription factors. AP-1 transcriptional factor duplex also plays a major role in the regulation of uPA expression through binding to its promoter (29). However, the molecular mechanism by which OPN regulates NIK and MEKK1-mediated JNK-dependent/independent AP-1 activation and uPA secretion in murine melanoma (B16F10) cells is not well defined.

MMPs are ECM degrading enzymes that play a critical role in embryogenesis, tissue remodeling, inflammation, and angiogenesis (30). We have recently reported that OPN induces NFκB-mediated pro-MMP-2 activation through IkBα/IKK signaling pathways (12, 13). MMP-9, also referred to as type IV collagenase or gelatinase B, efficiently degrades native type IV collagen, gelatin, fibronectin, and, hence, plays a major role in invasion, tumor growth, and metastasis (31–34). The regulation of activation of MMP-9 is more complex than 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 (35–37), 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, is considered to be a prioritized therapeutic target (38). However, the molecular mechanism by which OPN regulates AP-1-mediated pro-MMP-9 activation and controls cell migration and tumor growth is not well defined.

In this paper we have demonstrated that OPN induces αvβ3 integrin-mediated NIK- and MEKK1-dependent c-Jun expression, leading to AP-1 activation and uPA secretion in B16F10 cells. This OPN-induced MEKK1- and NIK-mediated AP-1 transactivation occurs through both JNK-dependent and -independent pathways. OPN also induces a negative cross-talk between NIK/ERK and MEKK1/JNK1 pathways. Moreover, OPN also induces uPA secretion and uPA-dependent pro-MMP-9 activation, cell motility, invasion, and tumor growth. Taken together, these data demonstrated that OPN induces αvβ3 integrin-mediated NIK and MEKK1 kinase activities that ultimately enhance c-Jun expression through JNK-dependent and -independent pathways. OPN regulates cross-talk between JNK and ERK that leads to the induction of uPA secretion and uPA-dependent pro-MMP-9 activation, cell motility, invasion, and tumor growth.

EXPERIMENTAL PROCEDURES

Materials—The rabbit polyclonal anti-NIK, anti-MEKK1, anti-IRKαβ3, anti-JNK1, anti-c-Jun, anti-ERK1/2, anti-uPA, anti-MMP-9 and anti-actin, mouse monoclonal anti-phospho-ERK1/2, anti-phosphotyrosine antibodies, recombinant MEK-1, p42 MAPK, and c-Jun proteins were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-human uPA antibody was from Chemicon International. Lipofectamine Plus, GRGDSP, and GRGESP were obtained from Invitrogen. Mouse monoclonal anti-phosphoserine detection kit and SP600125 were from Calbiochem. Myelin basic protein was from Sigma. The dual luciferase reporter assay system and AP-1 consensus oligonucleotide were purchased from Promega. Boyden type cell migration chambers were obtained from Corning, and BioCoat MatrigelTM invasion chambers were from Collagen Corp. The [γ32P]ATP was purchased from the Board of Radiation and Isotope Technology (Hyderabad, India). The human OPN was purified from milk as described previously and used throughout these studies (12). 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% CO2 and 95% air at 37 °C.

Plasmas and DNA Transfection—The wild type NIK (wt pcDNA NIK) and kinase-negative NIK (mut pcDNA NIK, NIK-K428A/K430A) were generously provided by Prof. David Mann (Department of Science, Behovet, Israel). The wild type and kinase negative constructs of MEKK1 (pcDNA MEKK1 and pcDNA3 FlagMEKK1 K432M) were kind gifts from Prof. Tom Maniatis (Harvard University, Cambridge). The wild type c-Jun in pRJB10B and dominant negative c-Jun in pELF11N were gifts from Dr. Jalam (Ochner Clinic Foundation, New Orleans, LA). The wild type and dominant negative forms of NIK1 and DNA3 were kind gifts from Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA). The luciferase reporter construct (pAP1-Luc) containing seven tandem repeats of the AP-1 binding site was from Stratagene. The B16F10 cells were split 12 h before 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. These transfected cell lysates were analyzed for JNK1- and ERK1/2 phosphorylation and kinase assay, c-Jun expression, AP-1 DNA-binding, AP-1 luciferase reporter gene assay, and detection of MMP-9 by zymography and Western blot, uPA secretion by Western blot, cell migration, chemoinvasion, and in vivo experiments.

Western Blot Analysis—To ascertain the role of MEKK1 and JNK-1 in OPN-induced uPA secretion, the cells were transfected with wild type or kinase negative MEKK1 or wild type and dominant negative JNK-1 or pretreated with 50 µM SP600125 (JNK-1 inhibitor) and then treated with 5 µM OPN for 24 h. To examine whether c-Jun plays any role in OPN-induced uPA secretion, cells were transfected with wild type and dominant negative c-Jun and then treated with OPN. The cell lysates containing equal amount of total proteins were subjected to Western blot analysis using rabbit polyclonal antibody against c-Jun and detected by ECL detection system (Amersham Biosciences). As loading controls, the expression of actin was also detected by reprobing the blots with rabbit anti-actin antibody.

Immunoprecipitation—To examine the effect of OPN in regulation of MEKK1 phosphorylation, cells were treated with 5 µM OPN at 37 °C for 0–80 min. In separate experiments, cells were pretreated with anti-avβ3 integrin antibody (20 µg/ml), GRGDSP or GRGESP peptide (10 µM) and then treated with 5 µM OPN. 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 cell lysates containing equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-MEKK1 antibody. The immunocomplexes were analyzed by Western blot using mouse monoclonal anti-phospho-serine antibody. As loading control, same blots were reprobed with rabbit polyclonal anti-MEKK1 antibody.

To delineate the role of OPN in regulation of JNK-1 phosphorylation, the cells were treated with 5 µM OPN for 0–90 min at 37 °C. In separate experiments cells were pretreated with anti-avβ3 integrin antibody (20 µg/ml), GRGDSP or GRGESP peptide (10 µM) and then treated with 5 µM OPN.

Cell lysates were immunoprecipitated with rabbit polyclonal anti-JNK-1 antibody. The immunocomplexes were analyzed by Western blot using mouse monoclonal anti-phospho-tyrosine antibody. The same blots were reprobed with rabbit anti-JNK-1 antibody as loading control.

To detect whether NIK and MEKK1 are involved in the regulation of OPN induced JNK-1 phosphorylation, cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1...
and then treated with 5 μM OPN for 15 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-JNK-1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody. The same blots were reprobed with anti-NIK antibody.

**Nuclear Extracts and Western Blot**—To check the level of c-Jun expression in the nucleus, cells were treated with 5 μM OPN for 0–4 h at 37 °C. In separate experiments, cells were pretreated with anti-c-JUN polyclonal antibody (20 μg/ml), GRGDSP or GRGESP peptide (10 μM) or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with 5 μM OPN. The nuclear extracts were prepared as described (16). Briefly, cells were incubated in hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer. The nuclei were separated by spinning at 3300 × g for 5 min at 4 °C. The nuclear pellet was extracted in nuclear extraction buffer (20 mM Hepes (pH 7.9), 0.4 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) and centrifuged at 12,000 × g for 30 min. The supernatant was used as nuclear extract. The protein concentration was measured by the Bio-Rad protein assay. The nuclear extracts were resolved by SDS-PAGE, and the level of c-Jun was detected by Western blot using rabbit polyclonal anti-c-Jun antibody.

**Effect of Overexpressed MEKK1 on OPN-Induced JNK and ERK Pathways**—The NIH 3T3 cells were also cotransfected with wild type or dominant negative JNK-1 or treated with 2 μg/ml of myelin basic protein in kinase assay buffer supplemented with 10 μM ATP and 0.5 μM of [γ-32P]ATP. The kinase reactions were stopped by the addition of SDS sample buffer. The samples were resolved by SDS-PAGE and autoradiographed. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-JNK1 antibody.

**NIK-coupled Kinase Assay**—NIK-coupled kinase activity was assayed as described previously (39). Briefly, cells were treated with 5 μM OPN. In separate experiments, cells were transfected with wild type NIK and then treated with OPN. In other experiments the wild type or kinase negative NIK was treated with 5 μM OPN. The conditioned medium was collected, and the samples containing equal amount of total proteins were immunoprecipitated with anti-NIK antibody. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-NIK antibody.

**Cell Migration and Chemoinvasion Assays**—The migration and invasion effect of OPN on NIH 3T3 cells was determined using Transwell cell culture chambers according to the standard procedure as described previously (12–16). Briefly, cells were transfected with wild type and kinase negative MEKK1 or wild type or kinase negative MEK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-NIK antibody. The NIK activity was also assayed under the same conditions using IKK as substrate as described previously (16).

In Vivo Tumorigenicity Experiments—The tumorigenicity experiments were performed as described previously (12, 13, 16). The cells were treated in the absence or presence of purified human OPN (10 μM) at 37 °C for 20 h. In separate experiments cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 in the presence of Lipofectamine plus and then treated with 5 μM OPN. After that, the cells (5 × 10^6/0.2 ml) were detached and

**Zymography Experiments**—The gelatinolytic activity was measured as described (12, 13). To ascertain the role of MEKK1 and NIK on OPN-induced pro-MMP-9 activation, the cells were transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or pretreated with SP600125 (50–50 μM) for 1 h and then treated with 5 μM OPN. The conditioned medium was collected, and the samples containing equal amount of total proteins were mixed with sample buffer and subjected to zymography. In separate experiments cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 in the presence of Lipofectamine plus and then treated with 5 μM OPN. The cell lysates (300 μg) containing equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-JNK1 antibody. Half of the immunoprecipitated samples were incubated with recombinant c-Jun as substrate in kinase assay buffer (20 mM Hepes (pH 7.7), 2 mM MgCl2, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 300 mM Na3VO4, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM 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 (300 μg) containing equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-JNK1 antibody. Half of the immunoprecipitated samples were incubated with recombinant c-Jun as substrate in kinase assay buffer (20 mM Hepes (pH 7.7), 2 mM MgCl2, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 300 mM Na3VO4, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin, 1 mM DTT) containing 10 μM ATP and 3 μC of [γ-32P]ATP at 30 °C. The kinase reactions were stopped by the addition of SDS sample buffer. The samples were analyzed 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-JNK1 antibody. The levels of MEKK1 and NIK expressions in the transfected cells containing equal amount of total proteins were detected by Western blot using anti-MEKK1 or anti-NIK antibody.

To analyze the effect of overexpressed MEKK1 on OPN-induced ERK1/2 activity, cells were transfected with wild type and kinase negative MEKK1 and then treated with OPN. In separate experiments, the NIH 3T3 cells transfected with wild type MEKK1 or wild type and kinase negative NIK were then transfected with 5 μM OPN. In separate experiments, cells were transfected with wild type NIK and then treated with OPN. In other experiments the wild type or kinase negative NIK was treated with 5 μM OPN. The conditioned medium was collected, and the samples containing equal amount of total proteins were immunoprecipitated with anti-NIK antibody. The NIK activity was also assayed under the same conditions using IKK as substrate as described previously (16).
FIG. 1. Panel A, OPN stimulates MEKK1 phosphorylation (\(p\text{MEKK}1\)). B16F10 cells were treated with 5 \(\mu\)M OPN for 0–60 min. Cell lysates were immunoprecipitated (IP) with anti-MEKK1 antibody and analyzed by Western blot (IB) using anti-phosphotyrosine (p-Ser) antibody (Ab) (upper panel A, lanes 1–5), and the same blots were reprobed with anti-MEKK1 antibody (lower panel A). Panel E, OPN induces \(\alpha\beta3\) integrin-mediated MEKK1 phosphorylation. The cells were individually pretreated with anti-\(\alpha\beta3\) integrin antibody, GRGDSP, or GRGESP and then treated with 5 \(\mu\)M OPN. The cell lysates were immunoprecipitated with anti-MEKK1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody (upper panel B, lanes 1–5), and same blots were reprobed with anti-MEKK1 antibody (lower panel B). Panels C and D, OPN stimulates \(\alpha\beta3\) integrin-mediated JNK1 phosphorylation (p-JNK1). Cells were treated with 5 \(\mu\)M OPN for 0–90 min or pretreated with anti-\(\alpha\beta3\) integrin antibody, GRGDSP, or GRGESP and then treated with 5 \(\mu\)M OPN for 15 min. The cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine (p-Tyr) antibody (upper panels C and D). Panel E, OPN-induced JNK1 phosphorylation is enhanced by MEKK1 but not by NIK. Cells were transfected with wild type and kinase negative MEKK1 or wild type and kinase negative NIK and then stimulated with OPN. Cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody (upper panel E, lanes 1–6). Same blots were reprobed with anti-JNK1 antibody (lower panel E). All these bands were analyzed densitometrically, and the fold changes were calculated. The data shown here represent three experiments exhibiting similar effects.
body as loading control (lower panel of E, lanes 1–6). These data suggested that MEKK1 but not NIK plays a crucial role in OPN-induced JNK1 phosphorylation.

**MEKK1 but Not NIK Enhances the OPN-induced JNK1 Activity**—To ascertain the role of OPN on JNK1 activity, the cells were treated with 5 μM OPN, and the cell lysates were immunoprecipitated with rabbit anti-JNK1 antibody. Half of the immunoprecipitated samples were incubated with recombinant c-Jun as substrate in kinase assay buffer. The samples were resolved by SDS-PAGE and autoradiographed. The radiolabeled, phosphorylated c-Jun-specific band is detected in OPN-treated cells, demonstrating that OPN induces JNK1 activity (Fig. 2, upper panel of A, lane 2). The JNK1 activity is not detected in the untreated cells (lane 1). To further check whether MEKK1 and NIK play any direct role in OPN-induced JNK1 activity, in separate experiments cells were transfected with wild type and kinase negative MEKK1 or wild type and kinase negative NIK and then treated with OPN. The cell lysates were immunoprecipitated (IP) with anti-JNK1 antibody, and half of the immunoprecipitated samples were used for JNK kinase assay using recombinant c-Jun as substrate (upper panels A and B, lanes 1–4). The remaining half of the immunoprecipitated samples were immunoblotted (IB) with anti-JNK1 antibody (middle panels A and B, lanes 1–4). The levels of expressions of MEKK1 and NIK in the cell lysates were detected by Western blot using anti-MEKK1 (lower panel A, lanes 1–4) or anti-NIK antibody (lower panel B, lanes 1–4). Panel C, overexpression of MEKK1 attenuates OPN-induced ERK activation. Cells were transfected with wild type and kinase negative MEKK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-ERK1/2 antibody, and half of the immunoprecipitated samples were used for ERK kinase assay using myelin basic protein as substrate (upper panel C, lanes 1–4). Half of the immunoprecipitated samples were immunoblotted with anti-ERK1/2 antibody (lower panel C, lanes 1–4). Panel D, JNK1 inhibition enhances OPN-induced ERK activation. Cells were cotransfected with wild type MEKK1 along with wild type or dn JNK1 and then treated with OPN. In separate experiments cells were transfected with wild type MEKK1 and treated with SP600125 followed by OPN. Cell lysates were immunoprecipitated with anti-ERK1/2 antibody and used for ERK kinase assay (upper panel D, lanes 1–6). Half of the immunoprecipitated samples were analyzed by Western blot using anti-MEKK1 antibody (lower panel D). MBP, myelin basic protein. Panel E, OPN-induced NIK-dependent ERK activation is unaffected by overexpression of MEKK1. Cells were cotransfected with wild type NIK along with wild type or kinase negative MEKK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-NIK antibody and used for NIK coupled kinase assay using MEK and ERK as substrates as described under “Experimental Procedures” (upper panel E, lanes 1–5). Half of the immunoprecipitated samples were immunoblotted with anti-NIK antibody (middle panel E). The NIK activity was also assayed under the same conditions using IKK as substrate (lower panel E, lanes 1–5). The data shown here represent three experiments exhibiting similar effects.

Overexpression of Active MEKK-1 Attenuates OPN-induced ERK1/2 Activation—MEKK-1 functions as a mitogen-activated protein kinase kinase kinase in the JNK pathway; however, several reports have suggested that MEKK-1 may also affect the ERK pathway (21). To determine the effect of MEKK1 on OPN-induced ERK activation, cells were transfected with wild type and kinase negative MEKK1 and then treated with OPN. The cell lysates were immunoprecipitated with anti-ERK1/2 antibody, and kinase activity was measured using myelin basic protein as the substrate. The data indicated that OPN-induced JNK1 activity was unaffected upon overexpression of both wild type and kinase negative NIK (upper panel B, lanes 1–4). The remaining half of the immunoprecipitated samples was analyzed by Western blot using anti-JNK1 antibody (middle panels A and B, lanes 1–4). The levels of MEKK1 and NIK were also analyzed by Western blot using anti-MEKK1 and anti-NIK antibodies, respectively (lower panels A and B, lanes 1–4). These results suggested that MEKK1 but not NIK plays a significant role in modulating OPN-induced JNK activity.

Overexpression of Active MEKK-1 functions as a mitogen-activated protein kinase kinase in the JNK pathway; however,
activation is not affected by kinase negative MEKK-1 (lane 3). Half of the immunoprecipitated samples were immunoblotted with anti-ERK1/2 antibody (lower panel C, lanes 1–4). These results suggested that MEKK1 negatively regulates OPN-induced ERK activation.

**JNK1 Plays a Crucial Role in OPN-induced MEKK1-dependent ERK1/2 Inactivation**—Previous results indicated that JNK−/− mice showed enhanced phosphorylation of ERK leading to tumor growth (40); therefore, we have speculated that activation of JNK1 by OPN may play a role in suppression of ERK1/2 activity. Accordingly, cells transfected with wild type MEKK1 were cotransfected with either wild type or dominant negative JNK1 and then treated with OPN. In separate experiments cells transfected with wild type MEKK1 were treated with JNK1 inhibitor, SP600125, and then treated with OPN. Overexpression of wild type MEKK1 alone or with wild type JNK1 suppressed the OPN-induced ERK activation (upper panel D, lanes 1–4), whereas dominant negative JNK1 or SP600125 along with wild type MEKK1 reversed this effect (lanes 5 and 6). Half of the immunoprecipitated samples were immunoblotted with anti-ERK1/2 antibody (lower panel D, lanes 1–6). These data suggested that JNK1 acts as negative regulator in OPN-induced MEKK1-dependent ERK1/2 activation.

To examine whether NIK plays any role in regulation of OPN-induced MEKK-dependent JNK-mediated ERK1/2 inactivation, cells were transfected with wild type NIK and then cotransfected with either wild type or kinase negative MEKK1 and then treated with OPN. The NIK kinase activity was measured by a coupled kinase assay using MEK and ERK as substrates. The data indicated that expression of active or mutant MEKK-1 had no effect on OPN-induced NIK activity (upper panel E, lanes 1–5), suggesting that overexpressed NIK even in the presence of MEKK up-regulates OPN-induced ERK activation. Half of the immunoprecipitated samples were immunoblotted with anti-NIK antibody (middle panel E, lanes 1–5). The level of NIK activity was also detected by using IKK as substrate (lower panel E, lanes 1–5).

**OPN Induces αvβ3 Integrin-mediated NIK and MEKK1-dependent c-Jun Expression**—Earlier reports have demonstrated that MEKK in the presence of stimulus induces JNK-dependent c-Jun phosphorylation and enhances AP-1 activation (41). Therefore, we sought to determine whether OPN induces c-Jun expression and whether MEKK1/NIK is involved in this process. Accordingly, cells were treated with 5 μM OPN for 0–4 h. The nuclear extracts were prepared, and the level of c-Jun expression was detected by Western blot analysis using anti-c-Jun antibody. These data indicated that OPN induces c-Jun expression, and maximum expression was observed at 1 h (Fig. 3, panel A, lanes 1–5).

To further confirm that this OPN-induced c-Jun expression occurs through αvβ3 integrin-mediated pathway, cells were pretreated with anti-αvβ3 antibody, RGD/RGE peptide, and then treated with OPN for 1 h. The data revealed that αvβ3 antibody and RGD but not RGE suppressed OPN-induced c-Jun expression (panel B, lanes 1–5).

To examine further whether NIK and MEKK1 play important roles in OPN-induced c-Jun expression, cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. The nuclear extracts were prepared and the level of c-Jun expression was detected by Western blot using anti-c-Jun antibody. Wild type NIK enhanced and kinase negative NIK suppressed OPN-induced c-Jun expression (panel C, lanes 1–5). Similarly, kinase negative MEKK1 inhibited and wild type MEKK1 induced the OPN-induced c-Jun expression (lanes 5 and 6). Moreover, overexpression of wild type NIK, which does not affect OPN-induced JNK phosphorylation and kinase activity (Fig. 1, panel E and Fig. 2, panel B) significantly up-regulate c-Jun expression. These data indicated that OPN induces c-Jun expression through both NIK- and MEKK1-dependent pathways; however, NIK-mediated c-Jun expression occurs in a JNK1-independent manner.

**NIK and MEKK1 Play Important Roles in OPN-induced AP-1-DNA Binding**—We have reported earlier that OPN induces AP-1-mediated secretion of uPA through c-Src-dependent transactivation of epidermal growth factor receptor in breast cancer cells (15). Therefore, in this paper we have first examined whether NIK and MEKK1 regulate OPN-induced AP-1-DNA binding in B16F10 cells. Accordingly, cells were either treated with 5 μM OPN or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. The nuclear extracts were prepared and used for EMSA using 32P-labeled AP-1 oligonucleotides. Wild type NIK
Cross-talk between OPN-induced JNK and ERK Pathways

FIG. 4. Panels A and B, OPN induces NIK (panel A) and MEKK1 (panel B)-dependent AP-1-DNA binding. Cells were either treated with OPN or transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. The nuclear extracts were prepared and analyzed by EMSA (lanes 1–4). Panel C, JNK is involved in OPN-induced AP-1-DNA binding. Cells were pretreated with 0–50 µM SP600125 (JNK inhibitor) and then treated with OPN. The nuclear extracts were analyzed by EMSA (lanes 1–4). Panel D, OPN-induced NIK-mediated AP-1-DNA binding is independent of JNK. Cells were either treated with OPN or transfected with wild type NIK, treated with 0–50 µM SP600125 (JNK inhibitor), and then treated with OPN. The nuclear extracts were analyzed by EMSA (lanes 1–4). E, supershift (SS) assay. The nuclear extracts from OPN treated cells were incubated with anti-c-Jun antibody (Ab) and analyzed by EMSA (lanes 1 and 2). The results shown here represent three experiments exhibiting similar effects.

To examine the role of JNK1 on OPN-induced AP-1-DNA binding, cells were pretreated with 0–50 µM SP600125 (JNK inhibitor) and then treated with OPN. The nuclear extracts were prepared and used for EMSA. SP600125 suppressed OPN-induced AP-1-DNA binding in a dose-dependent manner (panel C, lanes 1–4). To ascertain whether OPN-induced JNK1-mediated AP-1-DNA binding is NIK-dependent, cells were transfected with wild type NIK followed by treatment with SP600125 and then stimulated with OPN. The OPN-enhanced AP-1-DNA binding caused by overexpression of wild type NIK was unaltered by SP600125, suggesting that OPN-induced NIK-mediated AP-1-DNA binding is JNK-independent (panel D, lanes 1–4). Whether the band obtained by EMSA is indeed AP-1, the nuclear extracts were incubated with anti-c-Jun antibody and then analyzed by EMSA. The results showed the shift of the AP-1-specific band to a higher molecular weight when the nuclear extracts were treated with anti-c-Jun antibody (panel E, lanes 1 and 2).

**OPN Induces NIK- and MEKK1-regulated JNK1-mediated AP-1 Transactivation**—To further investigate whether NIK and MEKK1 regulate OPN-induced JNK1-mediated AP-1 transactivation, luciferase reporter gene assay was performed. Cells were transiently transfected with AP-1 luciferase reporter construct (pAP-1-Luc) and then treated with OPN. In separate experiments, cells were individually transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 or wild type and dominant negative JNK1 along with pAP-1-Luc and then treated with OPN. In separate experiments, wild type NIK-transfected cells were cotransfected with pAP-1-Luc, treated with SP600125, and then treated with OPN. 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 determinations. The values were also analyzed by Student’s t test (p < 0.002). The data showed that wild type NIK enhanced but kinase negative NIK suppressed OPN-induced AP-1 activity in these cells (Fig. 5, panel A). Similarly, wild type MEKK1 enhanced and kinase negative MEKK1 inhibited OPN-induced AP-1 activity (panel B). Wild type JNK enhanced, whereas dn JNK1 moderately suppressed OPN-induced AP-1 activity (panel C). The enhanced AP-1 transactivation caused by overexpression of wild type NIK followed by OPN treatment was unaffected upon treatment with JNK1-specific inhibitor, SP600125 (panel C). These data indicated that OPN induces AP-1 transactivation through NIK- and MEKK1/JNK-mediated pathways and further suggested that OPN induces a shift in balance toward activation of ERK followed by AP-1 activation.

**OPN Stimulates NIK- and MEKK1-mediated c-Jun-dependent uPA Secretion and uPA-dependent MMP-9 Activation**—We have recently demonstrated that NIK plays a crucial role in OPN-induced uPA secretion and uPA-dependent MMP-9 activation in B16F10 cells (16). Therefore, we have examined whether MEKK1, JNK1, and c-Jun are involved in OPN-induced uPA secretion. Accordingly, cells were transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or wild type and dn c-Jun and then treated with OPN. In separate experiments cells were pretreated with SP600125 (50 µM) and then stimulated 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 wt MEKK1 and wt c-Jun and suppressed when transfected with kinase negative MEKK1 and dn c-Jun (Fig. 6, upper panel A, lanes 1–6). Wild type JNK1 stimulated and dn JNK1 or JNK1 inhibitor (SP600125) moderately reduced OPN-induced uPA secretion due to up-regulation of ERK-mediated c-Jun expression leading to activation of AP-1 (upper panel B, lanes 1–5). All these bands were reprobed with anti-actin antibody (lower panels A and B). All bands were quantified by densitometric analysis, and the fold changes are calculated (panels A and B). These data further demonstrated that OPN induces uPA secretion through both NIK/ERK as well as MEKK1/JNK-mediated pathways.

To examine whether OPN-induced NIK/MEKK1-mediated uPA secretion leads to MMP-9 activation, cells were transfected with wild type and kinase negative MEKK1 or wild type...
FIG. 5. Panels A and B, OPN enhances NIK (panel A)- and MEKK1 (panel B)-dependent AP-1 transactivation. Cells were transiently transfected with luciferase reporter construct (pAP-1-Luc). In separate experiments cells were individually transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 along with pAP-1-Luc. The transfected cells were treated with 5 μM OPN. Cell lysates were used to measure the luciferase activity (panels A and B). The values were normalized to Renilla luciferase activity. Panel C, JNK is differentially regulated in OPN-induced NIK-dependent AP-1 transactivation. Cells were transfected with wild type and dominant negative JNK1 along with pAP-1-Luc and treated with 5 μM OPN. In other experiments cells were transfected with wild type NIK along with pAP-1-Luc and treated with 0–50 μM SP600125 and then with OPN. Cell lysates were used to measure the luciferase activity (panel C). 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 < 0.002).

FIG. 6. Panel A, OPN stimulates MEKK1- and c-Jun-mediated uPA secretion. Cells were either treated with OPN or transfected with wild type and kinase negative MEKK1 or wild type and dn c-Jun and then treated with OPN. The levels of uPA in the cell lysates were analyzed by Western blot (IB) using anti-uPA antibody (upper panel A, lanes 1–6). The same blots were reprobed with anti-actin antibody (lower panel A, lanes 1–6). Panel B, JNK plays a crucial role in OPN-induced uPA secretion. Cells were transfected with wild type and dn JNK1 or pretreated with 50 μM of SP600125 (JNK inhibitor) and then treated with OPN. The level of uPA in the cell lysates was analyzed by Western blot using anti-uPA antibody (upper panel B, lanes 1–5). The same blots were reprobed with anti-actin antibody (lower panel B, lanes 1–5) as the loading control. All these bands were quantified densitometrically. Panels C–E, JNK is differentially regulated in OPN-induced MEKK1-dependent pro-MMP-9 activation. Cells were individually transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or pretreated with SP600125 and then treated with OPN. The conditioned media were collected, and the activity of MMP-9 was examined by gelatin zymography (panels C–E, lanes 1–4). The data shown here represent three experiments exhibiting similar effects.
and dn JNK1 or pretreated with SP600125 and then treated with OPN. The conditioned media were collected and used for gelatinolytic activity by zymography. Increased levels of MMP-9 activation (86 kDa) were observed when cells were treated with OPN. The purified human OPN (5 μM) was added in the upper chamber. The treated or transfected cells were used for migration assay. Note that OPN-induced migration was suppressed by kinase negative MEKK1 and dn c-Jun and enhanced by wt MEKK1, wt JNK1, and wt c-Jun. In separate experiments cells were treated with 0–25 μM SP600125 or transfected with wild type or kinase negative NIK and then treated with 25 μM SP600125. These transfected or treated cells were used for migration assay (panels A, C, and E). dn JNK-1 and SP600125 did not alter OPN-induced cell migration (panel C). Note that JNK1 plays a differential role in OPN-induced NIK/MEKK1-dependent cell migration. The same results were obtained in chemoinvasion assays (panels B, D, and F). The results are expressed as the means ± S.E. of three determinations.

NIK and MEKK1 enhances OPN-induced 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. In separate experiments cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 followed by treatment with OPN and then injected into nude mice. 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 were used as controls.

<table>
<thead>
<tr>
<th>No. nude mice</th>
<th>Transfection/Treatment</th>
<th>Tumor weight (-fold changes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Control (PBS)</td>
<td>1.0 ± 0.17</td>
</tr>
<tr>
<td>4</td>
<td>OPN (10 μM)</td>
<td>3.1 ± 0.15</td>
</tr>
<tr>
<td>4</td>
<td>Wt NIK + OPN (10 μM)</td>
<td>6.1 ± 0.14</td>
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<tr>
<td>4</td>
<td>Mut NIK + OPN (10 μM)</td>
<td>1.4 ± 0.16</td>
</tr>
<tr>
<td>4</td>
<td>Wt MEKK1 + OPN (10 μM)</td>
<td>5.4 ± 0.12</td>
</tr>
<tr>
<td>4</td>
<td>Mut MEKK1 + OPN (10 μM)</td>
<td>1.2 ± 0.15</td>
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MEKK1, JNK1, and c-Jun Play Crucial Roles in OPN-induced αvβ3 Integrin-mediated Cell Migration and Chemoinvasion—We have shown that OPN induces αvβ3 integrin-mediated NIK/ERK and MEKK1/JNK1-dependent c-Jun expression leading to uPA secretion and uPA-dependent MMP-9 activation. Therefore, we have examined whether these OPN-induced NIK/MEKK1-dependent MMP-9 activations play any role in cell migration and chemoinvasion. Accordingly, cells were either transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or wild type and dn c-Jun in the presence of Lipofectamine Plus and then used for migration or chemoinvasion assay. In separate experiments cells were pretreated with JNK1 inhibitor (SP600125) and transfected with wild type and kinase negative NIK and then treated with JNK1 inhibitor. OPN was used in the upper chamber. The data showed that wild type MEKK1, JNK1, and c-Jun enhanced and mutant MEKK1, JNK1, and c-Jun suppressed OPN-induced cell migration (Fig. 7, panels A, C, and E) and chemoinvasion (panels B, D, and F). The data also indicated that dn JNK1 and SP600125 unaltered the OPN-induced cell migration (panel C) and chemoinvasion (panel D). The enhanced migration and invasion caused by overexpression of wild type NIK is unaffected by cells treated with JNK1 inhibitor (panels C and D). However, cells transfected with mutant NIK followed by treatment with JNK1 inhibitor suppressed OPN-induced migration and invasion (panel C and D), suggesting that NIK-regulated migration and invasion are independent of JNK, and both the pathways synergistically contribute the OPN-induced cell migration and chemoinvasion. These data demonstrated that OPN-induced uPA secretion and uPA-dependent pro-MMP-9 activation are regulated by NIK/ERK and MEKK1/JNK1 pathways, and all of these ultimately control the motility and invasiveness of B16F10 cells.

OPN Induces NIK/MEKK1-dependent c-Jun Expression, AP-1-DNA Binding, uPA Secretion, and MMP-9 Activation in Tumor of Nude Mice—The in vitro data prompted us to examine whether NIK and MEKK1 play any role in OPN-induced c-Jun expression, AP-1-DNA binding, uPA secretion, and MMP-9 activation in the tumors of nude mice. Accordingly, cells were either treated with OPN or transfected with wild type and
kinase negative NIK or wild type and kinase negative MEKK1 followed by treatment with OPN and then injected subcutaneously into the flanks of nude mice. Table I shows the fold change of tumor weight growth in 4-week-old nude mice. There were at least 6- and 5.4-fold increased in tumor weight when wild type NIK or wild type MEKK1-transfected cells were injected, respectively. 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). The tumor samples were lysed, and the level of c-Jun expression in the nuclear extract was detected by Western blot using anti-c-Jun antibody. The AP-1-DNA binding in the nuclear extract was performed by EMSA. Both wild type NIK and wild type MEKK1 showed significantly higher levels of c-Jun expression (Fig. 8, panel A, lanes 1–6) and AP-1-DNA binding (panel B, lanes 1–6) compared with cells treated with OPN alone or transfected with kinase negative NIK (mut NIK) or mut MEKK1.

To further examine the levels of uPA and MMP-9 in these tumors, the samples were lysed, and the levels of uPA and MMP-9 were analyzed by Western blot using anti-uPA and anti-MMP-9 antibody, respectively. The results indicated that tumor generated by injecting the mice with wild type NIK and MEKK1-transfected cells showed higher level of uPA expression (panel C, lanes 1–6) compared with cell treated with OPN alone or transfected with mutant NIK or MEKK1. These data demonstrated that OPN induces both NIK- and MEKK1-mediated AP-1 activation leading to uPA secretion and pro-MMP-9 activation through JNK1-dependent pathways in tumor of nude mice and these data corroborates with in vitro study.

**DISCUSSION**

In a recent study (16) we have demonstrated that OPN stimulates NIK-dependent NFκB-mediated uPA secretion and uPA-dependent pro-MMP-9 activation that controls cell motility and tumor growth through both JNK and ERK1/2-mediated pathways in murine melanoma cells. In this paper we have delineated the molecular mechanism by which OPN regulates NIK/MEKK1-dependent c-Jun expression and AP-1 transactivation and the differential role of JNK1 in these activation processes in murine melanoma cells. We have shown that OPN induces ovβ3 integrin-mediated MEKK1 phosphorylation leading to c-Jun activation in a JNK-dependent manner. The data also revealed that OPN induces NIK activation, which further enhances c-Jun expression, leading to AP-1 transactivation in a JNK-independent pathway. Overexpression of MEKK1 leads to sustained activation of JNK, resulting in a negative cross-talk between MEKK1/JNK and NIK/ERK pathways. OPN binding to ovβ3 integrin induced NIK/MEKK1-dependent c-Jun expression, which ultimately stimulates uPA secretion and uPA-dependent pro-MMP-9 activation that enhances cell migration, chemoinvasion, and tumor growth.

OPN plays a significant role in tissue remodeling processes such as bone resorption, angiogenesis, wound healing, and tissue injury as well as certain diseases such as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (6–8). Integrins are cell surface glycoproteins that bind to the extracellular matrix proteins. Recent studies have demonstrated that down-regulation of NIK activation does not affect tumor necrosis factor α-induced JNK activation (42). It has been also reported that ERK and JNK pathways play crucial roles in regulating MMP-9 activation and cell motility in growth factor-stimulated human epidermal keratinocytes (43). These results prompted us to investigate whether binding of OPN to ovβ3 integrin receptors regulates JNK1 activation and whether NIK is involved in this activation process. In this study we have demonstrated that OPN induces ovβ3 integrin-mediated MEKK1 and JNK1 phosphorylations in B16F10 cells. Pretreatment of cells with anti-ovβ3 integrin antibody and RGD but not RGE peptide inhibited OPN-induced MEKK1 and JNK1 phosphorylations, indicating that ovβ3 is involved in this process. Furthermore, OPN-induced JNK1 activation is MEKK1-dependent but NIK-independent. This was confirmed...
by the fact that transient overexpression of wild type MEKK1 enhanced and kinase negative MEKK1 suppressed OPN-induced JNK1 phosphorylation and kinase activity, whereas overexpression of wild type and kinase negative NIK does not affect OPN-induced JNK1 activation.

MEKK1, a Ser/Thr protein kinase has been reported as a mitogen-activated protein kinase kinase 4 (18). Shen et al. (44) have recently reported that sustained activation of JNK blocks ERK activation in response to mitogenic factors like epidermal growth factor and phorbol 12-myristate 13-acetate. Growing evidence also indicated that cross-regulation between JNK and ERK may play an important role in determining cell survival or death. These results prompted us to examine whether overexpression of MEKK1 leads to enhanced JNK activation and whether this activation may affect OPN-induced NIK-mediated ERK activation. Our data demonstrated a negative cross-talk between OPN-induced NIK/ERK and MEKK1/JNK activation and further suggested that sustained activation of JNK resulted in the attenuation of ERK activation. Previous studies have indicated that MEKK1 also has the ability to activate ERK, but the effect is less potent (19). This may be implicated to the short and long phase of MEKK1 activation, which results in a different cellular response; that is, a short phase activation that leads to ERK activation and a long phase activation that results in inhibition of ERK activation. Also, the inhibition of OPN-induced NIK-mediated ERK activation caused by overexpression of wild type MEKK1 involves the ability of MEKK1 to activate the JNK pathway. These implications delineate a mechanism in which treatment with the same agonist may result in a different cellular outcome depending on the duration of treatment. These data are consistent with the recent report that JNK1 deficiency stimulates 12-O-tetradecanoylphorbol-13-acetate-induced ERK phosphorylation, leading to enhanced skin tumorigenesis (40).

It is well established that JNK, a member of the mitogen-activated protein kinase family, could be phosphorylated after exposure to ultraviolet irradiation, growth factors, or cytokines, which in turn phosphorylates specific serine residues (serine 63 and serine 73) of c-Jun and enhances the AP-1 transcriptional activity. AP-1, a family of transcription factors, consists of homo or heterodimers of Jun, Fos, or activating transcription factor protein (44–46). Previous reports have demonstrated that AP-1 is involved in several cellular processes such as cell growth, apoptosis, and cell motility (45). In addition, AP-1 activity is elevated in a number of pathological conditions. Natoli et al. (42) have reported that overexpression of NIK, which does not activate JNK, strongly activates transcription directed by a canonical AP-1 site. Because we have shown that OPN induces MEKK1-dependent but NIK-independent JNK phosphorylation and AP-1 response element is present in the promoter region of MMP-9 gene, we sought to determine the level of c-Jun expression upon OPN stimulation. OPN enhances the expression of c-Jun, resulting in enhancement of AP-1-DNA binding activity. Our data also indicated that OPN induces both NIK- and MEKK1-mediated c-Jun expression, leading to AP-1-DNA binding and AP-1 transactivation. The enhanced AP-1-DNA binding caused by overexpression of wild type NIK was unaffected upon inhibition of JNK activation by SP600125, a specific JNK inhibitor. These data suggested that overexpression of NIK, which does not affect JNK activation significantly, up-regulates AP-1-DNA binding and transcriptional activity, indicating that OPN induces NIK-dependent AP-1 activation, which is independent of JNK.

Signals transduced by cell adhesion molecules play an important role in tumor cell attachment, motility, and invasion, all of which regulate metastasis. OPN, an ECM protein, plays a significant role in cell adhesion, migration, and metastasis. MMPs are a family of Zn²⁺-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 stromal compartment. Elevated levels of MMP-9 have been reported in various cancers. Several studies have shown a correlation between MMP-9 expression and the metastatic potential of tumor (47). Kim et al. (48) also demonstrated that MMP-9 activity but not MMP-2 activity significantly affects tumor extravasation into blood vessel, and uPA is required for pro-MMP-9 activation (49). Several other reports have indicated the correlation between uPA expression and metastatic potential and shown that uPA plays major role in regulating MMPs activation (16, 50). In this study we have reported that OPN induces uPA secretion and uPA-dependent pro-MMP-9 activation through NIK/ERK and MEKK1/JNK-mediated AP-1-dependent pathways. Overexpression of wild type MEKK1 and c-Jun enhanced, and kinase negative MEKK1 and dn c-Jun suppressed the OPN-induced uPA secretion, demonstrating that OPN regulates uPA secretion through MEKK1/c-Jun-mediated pathways. Similarly, overexpression of wild type JNK1 enhanced OPN-induced uPA secretion and MMP-9 activation. Moreover, cells transfected with wild type NIK followed by treatment with JNK1 inhibitor enhanced, whereas cells transfected with kinase negative NIK followed by treatment with JNK1 inhibitor suppressed the OPN-induced cell migration and invasion, indicating that OPN regulates these effects through both NIK- and JNK-mediated pathways. Wild type MEKK1, JNK1, and c-Jun enhanced, and kinase negative MEKK1 and dn c-Jun suppressed OPN-induced cell migration and ECM invasion. However, transfection of cells with the dominant negative form of JNK1 or treatment with SP600125 moderately inhibits OPN-induced uPA secretion or uPA-dependent pro-MMP-9 activation, cell migration, and ECM invasion.

FIG. 9. Molecular mechanism of OPN-induced NIK/MEKK1-dependent AP-1 activation, AP-1-mediated uPA secretion, and MMP-9 activation through differential activation of JNK1. Binding of OPN to αvβ3 integrin induced the phosphorylation and activation of MEKK1 which induces c-Jun-mediated AP-1 activation in a JNK1-dependent manner. In addition, OPN also induced NIK-dependent c-Jun-mediated AP-1 activation through JNK1-independent pathway. These lead to a cross-talk between NIK/ERK and MEKK1/JNK1 pathways. Both NIK and MEKK1 in the presence of OPN regulate AP-1-dependent uPA secretion and MMP-9 activation, and all of these control the cell motility, invasion, and tumor growth.
Cross-talk between OPN-induced JNK and ERK Pathways

This may be due to the cross-talk between MEKK1/JNK and NIK/ERK pathways. These data are consistent with the recent data reported by Chen et al. (40) that disruption of the JNK1 gene resulted in an increase in ERK phosphorylation leading to enhancement of skin tumorigenesis. The in vitro data are also supported by in vivo data which showed that OPN induced both NIK- and MEKK1-mediated c-Jun expression, leading to uPA-dependent pro-MMP-9 activation in tumors of nude mice. These data demonstrated that OPN induces NIK/MEKK1-regulated AP-1-mediated uPA-dependent pro-MMP-9 activation, cell motility, and tumor growth through differential activation of JNK1 in B16F10 cells.

In summary, we have demonstrated for the first time that OPN induces both NIK- and MEKK1-mediated c-Jun expression, leading to AP-1 transactivation in B16F10 cells. Ligation of OPN to αvβ3 integrin receptor induces the phosphorylation and kinase activity of JNK1. This was blocked by kinase negative MEKK1 but unaffected by kinase negative NIK, indicating that OPN-induced NIK-mediated AP-1 transactivation is JNK-independent. OPN also induces a negative cross-talk between MEKK1/JNK and NIK/ERK pathways. Overexpression of wild type MEKK1 attenuates OPN-induced kinase negative NIK-mediated ERK activation, which is restored upon JNK inhibition. Taken together, OPN-induced uPA-dependent pro-MMP-9 activation, cell motility, and tumor growth through both NIK- and MEKK1-mediated c-Jun expression and JNK1 plays a differential role in modulating these processes (Fig. 9). These findings may be useful in designing novel therapeutic interventions that block the OPN-regulated NIK- and MEKK1-dependent c-Jun expression and AP-1 transactivation through differential activation of JNK1, resulting in reduction of uPA secretion and MMP-9 activation and consequent blocking of cell motility, invasiveness, and metastatic spread of malignant melanoma.

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