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Nuclear factor inducing kinase: A key regulator in osteopontin-induced MAPK/IκB kinase dependent NF-κB-mediated promatrix metalloproteinase-9 activation

Hema Rangaswami · Anuradha Bulbule · Gopal C. Kundu

Abstract Osteopontin (OPN) is a secreted, non-collagenous, sialic-acid rich, glycosylated adhesive phosphoprotein. Several highly metastatic transformed cells synthesized a higher level of OPN compared with non-tumorigenic cells. We have recently reported that OPN induces nuclear factor-κB (NF-κB)-mediated promatrix metalloproteinase-2 activation through IκBα/IKK signaling pathways. However, the molecular mechanism(s) by which OPN regulates pro-matrix metalloproteinase-9 (pro-MMP-9) activation and involvement of upstream kinases in regulation of these processes that ultimately control cell motility and tumor growth in murine melanoma cells are not well defined. Here we report that OPN induces αvβ3 integrin-mediated phosphorylation and activation of nuclear factor inducing kinase (NIK) and enhances the interaction between phosphorylated NIK and IκBα kinase α/β (IKKα/β) in B16F10 cells. Moreover, NIK is involved in OPN-induced phosphorylations of MEK-1 and ERK1/2 in these cells. OPN induces NIK-dependent NF-κB activation through ERK/IKKα/β-mediated pathways. Furthermore, OPN enhances NIK-regulated urokinase-type plasminogen activator (uPA) secretion, uPA-dependent pro-MMP-9 activation, and cell motility. 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. Taken together, NIK acts as crucial regulator in OPN-induced MAPK/IKK-mediated NF-κB-dependent uPA secretion and MMP-9 activation thereby controlling melanoma cell motility and chemoinvasion.

Keywords Osteopontin · NIK · NF-κB · MMP-9 · Cell migration

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Introduction

Osteopontin (OPN) is a secreted, non-collagenous, sialic acid-rich, glycosylated adhesive phosphoprotein [1]. It has a GRGDS cell-adhesion sequence at the centre of the single chain which is highly conserved [2]. Earlier reports have indicated that OPN plays a major role in cell attachment, cell spreading, and cell migration by interacting with several integrins and CD44 variants in a RGD sequence-dependent and independent manner [3]. Previous studies have indicated that altered sialylation of OPN prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells [4]. Several highly metastatic transformed cells synthesized a higher level of OPN compared to non-tumorigenic cells [5,6]. Furthermore, the role of OPN in tumorigenesis has also been proved by studies using an antisense OPN cDNA construct designed to eliminate the secretion of OPN in transformed cells. Cells transfected with antisense OPN construct exhibit reduced ability to form tumors and to metastasize *in vivo* [7,8]. Our previous results indicated that OPN induces cell motility, tumor growth, NF-κB-dependent MT1-MMP- mediated promatrix metalloproteinase-2 (pro MMP-2) activation, and urokinase-type plasminogen activator (uPA) mediated-pro-MMP-9 activation through PI 3-kinase/IKK/Akt signaling pathways [9–13].

The NF-κB family of transcription factors consists of several members including p65, p50, Rel B, and c-Rel molecules. It is involved not only in a wide number of

pathologies like arthritis, inflammation, asthma, neurodegenerative diseases, and cancer but also in a number of normal cellular process such as cell growth, inflammatory and immune response, cell growth, and apoptosis [14–16]. The activity of NF-κB is tightly regulated by its inhibitor, the IκB family of proteins. In unstimulated cells, NF-κB is retained in the cytoplasm as an IκB-bound complex [17]. Upon stimulation, IκB becomes phosphorylated and degraded via the ubiquitination and proteosome-mediated pathway leading to nuclear translocation of NF-κB and activation of target gene expression [18]. Most signals induce the activity of a large multi-subunit protein kinase called IκB kinase (IKK). Nuclear factor-inducing kinase (NIK) is a member of MAP kinase kinase kinase (MAPKKK) family which may either directly or indirectly phosphorylate IKKα/β, leading to the phosphorylation and degradation of IκBα followed by NF-κB activation [19]. Since NF-κB inducible genes play a vital role in a number of disorders including cancer, the identification of various signaling molecules that regulate NF-κB activity may serve as valuable therapeutic targets for treatment of a wide range of pathologies.

uPA is a member of serine protease which interacts with uPA receptor (uPAR) and facilitates the conversion of inert zymogen plasminogen into widely acting serine protease plasmin and activation of matrix metalloproteinases (MMPs) [20]. These proteases degrade a wide range of ECM components including collagen, gelatin, fibronectin, etc. thereby facilitating the tumor cells to become detached from the primary site and metastasize. NF-κB response- element is present in the promoter region of uPA which plays a key role in cancer metastasis. MMPs belong to a family of zinc-dependent neutral endopeptidases that degrade extracellular matrix components. It plays a critical role in embryogenesis, tissue remodeling, inflammation, and angiogenesis. Due to matrix degrading properties, MMPs are highly regulated. An important aspect in the regulation of MMP activity is that they are produced in an inactive pro-form in which a Cys residue prevents the Zn^{2+} binding domain from becoming catalytically active [21]. Elimination of this prodomain is a prerequisite for MMPs to become active. *In vitro,* activation of pro-MMPs occurs in the presence of destabilizing agents such as organomercurial APMA, initiating an autocatalytic cleavage of the pro-domain. However, in *in vivo* system, the mechanisms involved in pro-MMP activation are more complex and are less well understood. They include the participation of serine proteases such as plasmin, furins, and also other MMPs.

MMP-9 or gelatinase B is secreted as a 92 kDa-inactive proenzyme which is processed into an active form (86 kDa). 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 thereby contributing to the specific pathological events [22]. 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 prioritized therapeutic target. However the exact molecular mechanism by which OPN regulates MMP-9 expression and activation is not well defined.

In this study, we have delineated the molecular mechanism by which OPN regulates pro-MMP-9 activation and hence controls cell motility and invasion in murine melanoma cells. Our data indicated that OPN regulates NIK activation and NIK-dependent MAPK and IKK-mediated NF-κB activation. This enhances uPA secretion leading to the activation of pro-MMP-9 which ultimately controls cell migration and ECM invasion in murine melanoma cells.

Experimental procedures

Western blot analysis

To check whether OPN induces NIK phosphorylation and to determine whether αvβ3 integrin and RGD peptide are involved in this process, B16F10 cells were either treated with 5 μM OPN for 5 min at 37°C or pretreated with anti-αvβ3 integrin antibody (20 μg/ml), GRGDSP or GRGESP peptide (10 μM), and then treated with OPN. The cells were lysed in Ripa 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 PMSF) at 4°C for 2 h.

To determine whether NIK regulates OPN-induced MEK1 and ERK1/2 phosphorylations, cells were transiently transfected with wild type or kinase-negative NIK and then treated with OPN. Cell lysates were analyzed by Western blot using anti-p-MEK-1 or anti-p-ERK1/2 antibody. The same blots were reprobed with anti-MEK-1 or anti-ERK1/2 antibody.

To ascertain the role of NIK and ERK1/2 in OPN-induced uPA secretion, cells were either treated with 0-5 μM OPN or transfected with wild-type NIK and kinase-negative NIK or wild-type and dn ERK1/2 and then treated with OPN. The cell lysates were subjected to Western blot analysis using rabbit polyclonal anti-uPA antibody. As loading controls, the same blots were reprobed with anti-actin antibody.

To check whether there is a crosstalk between OPN-induced MT1-MMP-mediated MMP-2 activation and uPA-dependent MMP-9 activation, cells were pretreated with anti-MMP-2 antibody (0-50 μg/ml) and then treated with OPN. The conditioned media were collected and the level of MMP-9 was analyzed by Western blot using anti-MMP-9 antibody and detected by ECL-detection system.

In vitro kinase assay

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

To investigate whether NIK plays any role in the regulation of OPN-induced IKK activity, the cells were treated with 5 μM OPN for 10 min. In separate experiments, cells were transfected with wild-type NIK or kinase-negative NIK in presence of LipofectAMINE plus and then treated with OPN. The cells were lysed in kinase assay lysis buffer. The cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-IKKα/β antibody. Half of the immunoprecipitated samples were incubated with recombinant IκBα (4 μg) in kinase buffer supplemented with 10 μM ATP, 3 μCi of [γ-^{32}P] ATP at 30°C. The kinase reaction was stopped by addition of SDS-sample buffer. The sample was resolved by SDS-PAGE and autoradiographed. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-IKKα/β antibody. A fraction of equal volume of samples from the kinase reaction mixture was analyzed by Western blot using anti-IκBα antibody.

To investigate whether OPN regulates the interaction between phosphorylated NIK and IKK$\tilde{\alpha}/\beta$, cells were treated with 5 μM OPN at 37°C for 10 min. Cell lysates were immunoprecipitated with rabbit anti-IKK$\tilde{\alpha}/\beta$ antibody. The immunoprecipitated samples were analyzed by Western blot using rabbit anti-phospho NIK antibody. The same blots were reprobed with anti-IKK$\tilde{\alpha}/\beta$ antibody as loading control.

Nuclear and cytoplasmic fractionation and EMSA

The nuclear extracts were prepared using a modification of the Dignam method [23]. Briefly, cells were treated with 5 μM OPN or transfected with wild-type and kinase-negative NIK, wild type and dn IKKβ and then treated with OPN for 6 h at 37°C. To investigate whether NIK is involved in OPN-induced ERK1/2-mediated NF-κB-DNA binding, cells were transfected with wild-type and kinase-negative NIK and then treated with PD98059. The cells were resuspended in hypotonic buffer (10 mM HEPES, (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). The nuclear pellet was extracted in nuclear extraction buffer (20 mM HEPES, (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM DTT). The supernatant was used as nuclear extract. The nuclear extracts (10 μg) were incubated with 16 fmol of ^{32}P labeled double-stranded NF-κB oligonucleotide (5′- AGT TGA GGG GAC TTT CCC AGG C-3′) in binding buffer (25 mM Hepes buffer (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl) containing 2 μg of polydeoxyinosinic deoxycytidylic acid (poly(dIdC)). The DNA-protein complex was resolved on a native polyacrylamide gel and analyzed by autoradiography.

NF-κB luciferase reporter gene assay

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

Zymography for detection of MMP-9 activity

OPN was purified as described previously [9]. B16F10 cells were treated with OPN (0-10 μM) for 24 h at 37°C. In separate experiments, cells were transfected with wild-type NIK, kinase- negative NIK, wild-type and dominant-negative IKKβ or super-repressor form of IκBα in presence of

Lipofect AMINE Plus or pretreated with anti-uPA antibody (25 μg/ml) for 1 h and then treated with OPN. The gelatinolytic activity in the conditioned media was assessed by zymography. The samples containing equal amounts of total proteins were mixed with sample buffer in the absence of reducing agent and loaded to the zymography-SDS-gel copolymerized with gelatin (0.5 mg/ml). The gels were washed and incubated in incubation buffer (50 mM Tris-HCl, (pH 7.5) containing 100 mM $CaCl_2$, 1 μM $ZnCl_2$, 1% (v/v) Triton-X100, and 0.02% (w/v) NaN_3) for 16 h. The gels were stained with Coomassie blue and destained. Negative staining showed the zones of gelatinolytic activity.

Cell migration assay

The migration assay was performed using transwell cell culture chamber as described previously [24]. Briefly, a subconfluent monolayer of cells was harvested with trypsin-EDTA and centrifuged at 800 × g for 10 min. The cells were washed in DMEM and cell suspension (5×10^5 cells/well) was added to the upper chamber of a prehydrated polycarbonate membrane filter. Cells in the upper chamber were treated with purified intact OPN (5 μM) and the lower chamber was filled with fibroblast-conditioned media which served as a chemoattractant. In separate experiments, cells were individually transfected with wild-type and kinase-negative NIK, super-repressor form of IκBα, wild-type and dn IKKα and IKKβ and used for migration assay. In another experiment, cells were individually pretreated with PD98059 (0–50 μM), anti-uPA antibody (25 μg/ml), anti-MMP-9 antibody (25 μg/ml) at 37°C for 6 h. To check whether MMP-2 and MMP-9 act synergistically in regulating OPN-induced cell migration, cells were treated with anti-MMP-2 antibody or anti-MMP-9 antibody (25 μg/ml) or in combination of both (25 μg/ml). Purified OPN (5 μM) was added to the upper chamber. Following incubation at 37°C for 16 h, non-migrated cells were scraped from the upper side of the filter, the filter was fixed in 70% methanol, stained with Giemsa, and washed with PBS. The cells were counted under an inverted microscope. Experiments were performed in triplicate, and preimmune-IgG served as non-specific control.

Results

OPN induces αvβ3 integrin dependent NIK phosphorylation

To delineate whether OPN regulates NIK phosphorylation and activation and to determine whether αvβ3 integrin or RGD/RGE peptide are involved in this process, B16F10 cells were pretreated with 5 μM OPN or pretreated with anti-$\alpha_v\beta_3$ integrin antibody or RGD/RGE peptide (GRGDSP or GRGESP) and then treated with OPN. The level of phosphorylated NIK was detected by Western blot analysis using anti-pNIK antibody. The results showed that OPN induces NIK phosphorylation, and $\alpha_v\beta_3$ integrin antibody and RGD (GRGDSP) but not RGE (GRGESP) peptide suppressed the OPN-induced NIK phosphorylation in these cells (Fig. 1, upper panel A, lanes 1–5). The level of non-phospho NIK was unchanged (lower panel A, lanes 1–5) indicating that OPN induces NIK phosphorylation through $\alpha_v\beta_3$ integrin-mediated pathway.

OPN stimulates the interaction between phosphorylated NIK and IKKα/β

To examine whether OPN plays any role in regulating the interaction between phosphorylated NIK and IKKα/β, B16F10 cells were treated with 5 μM OPN. In separate experiments, cells were individually transfected with wild-type NIK or kinase-negative NIK 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 data indicated that OPN induces the interaction between phosphorylated NIK and IKKα/β, and cells transfected with wild-type NIK followed by treatment with OPN showed maximum interaction (Fig. 1, upper panel B, lanes 1–4). All these bands were quantified densitometrically and the -fold changes were calculated. These results suggested that OPN enhances the interaction between phosphorylated NIK and IKKα/β.

OPN induces NIK activity and NIK-dependent IKK activity

Previous reports have indicated that IKK plays a major role in the cytokine induced IκBα phosphorylation at serine residues 32 and 36 leading to NF-κB activation. Therefore, to determine the role of OPN on NIK activity and to check whether NIK plays any direct role in OPN-induced IKK activity, the cells were treated with 5 μM OPN. The lysates were immunoprecipitated with rabbit anti-NIK antibody. Half of the immunoprecipitated samples were used for NIK kinase assay using IKK as substrate. The radiolabeled, phosphorylated IKK specific band is detected in OPN-treated cells demonstrating that OPN induces NIK activity (Fig. 1, upper panel C, lane 2). The NIK activity is 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 C). A fraction of equal volume of samples from the kinase reaction mixture

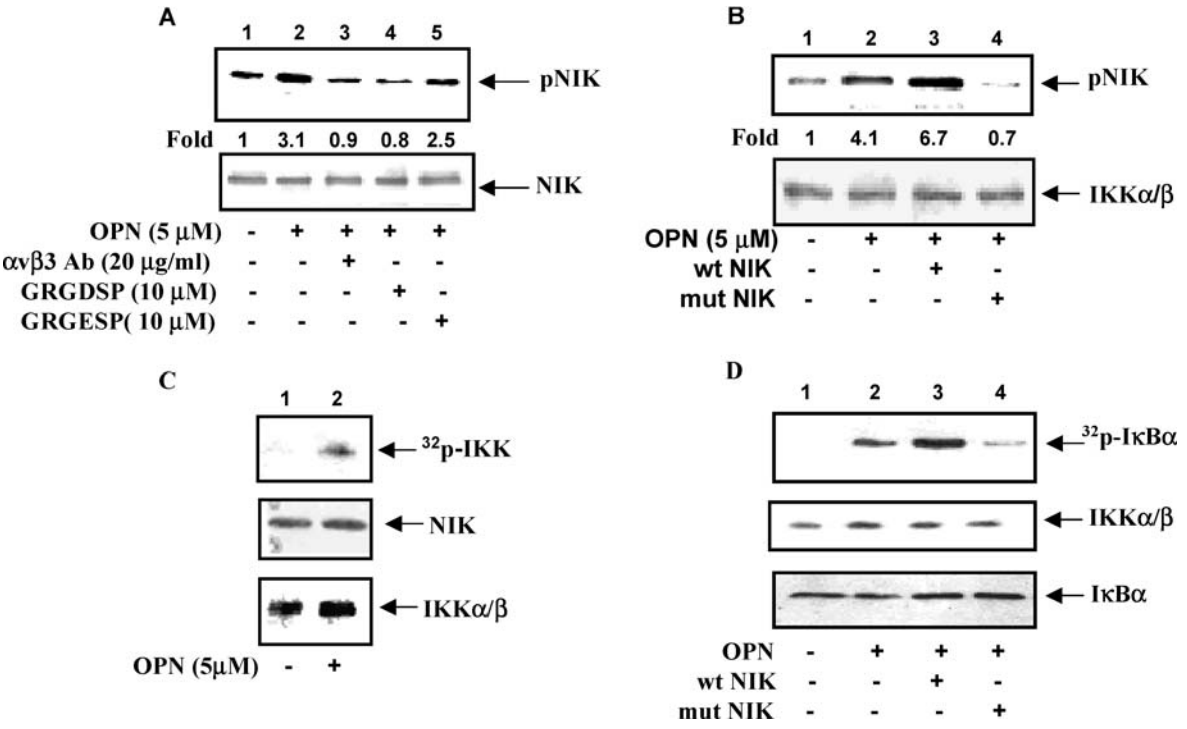

Fig. 1 Panel A: OPN induces $\alpha v\beta 3$ integrin-dependent NIK phosphorylation. B16F10 cells were treated with 5 μM OPN or pretreated with anti-$\alpha_v\beta 3$ integrin antibody, GRGDSP or GRGESP, and then treated with OPN. The cell lysates were used for the detection of pNIK by Western blot analysis (upper panel A). The same blots were reprobed with anti-NIK antibody (lower panel A) as loading controls. Panel B: OPN enhances the interaction between phosphorylated NIK and IKK. The cells were treated with 5 μM OPN. In separate experiments, cells were transfected with wt or mut NIK and then treated with OPN. Cell lysates were immunoprecipitated with anti-IKKα/β antibody. Half of the samples were immunoblotted with anti-phospho-NIK antibody (upper panel B) and other half was analyzed by anti-IKKα/β antibody (lower panel B). All these bands were analyzed densitometrically and the fold changes were calculated. Panels C and D: OPN induces NIK activity and NIK-dependent IKK activity. Cells were treated with 5 μM OPN, and cell lysates were immunoprecipitated with anti-NIK antibody and used for NIK kinase assay using IKK as substrate (upper panel C). The immunoprecipitated samples were immunoblotted with anti-NIK antibody (middle panel C) and anti-IKK antibody (lower panel C). In separate experiments, cells were treated with 5 μM OPN or transfected with wild-type and kinase-negative NIK and then treated with OPN. Cell lysates were immunoprecipitated with anti-IKKα/β antibody and the immunoprecipitate was used for IKK kinase assay using IκBα as substrate (upper panel D). The immunoprecipitated samples were analyzed by Western blot using anti-IKKα/β antibody (middle panel D) and anti-IκBα antibody (lower panel D). The bands were analyzed densitometrically and fold changes were calculated. The data shown here represent three experiments exhibiting similar effects

was analyzed by Western blot using anti-IKKα/β antibody as control (lower panel C).

To further check the role of NIK 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, and IKK assay was performed using IκBα as substrate. The data demonstrated that cells transfected with wild type NIK followed by treatment with OPN showed maximum IKK activity (upper panel D, 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). The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-IKKα/β antibody (middle panel D, lanes 1–4). A fraction of equal volume of samples from the kinase reaction mixture was analyzed by Western blot using anti-IκBα antibody (lower panel D, lanes 1–4). These results suggested that NIK plays a crucial role in OPN-induced IKK activity.

OPN stimulates NIK-dependent MEK1/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 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. The level of OPN-induced MEK-1 and ERK1/2 phosphorylations were enhanced in wild-type NIK transfected cells whereas kinase-negative NIK suppressed the OPN-induced MEK-1 and ERK1/2 phosphorylations (Fig. 2, upper panels A and B, lanes 1–4). The same blots were reprobed with anti-MEK-1 and anti-ERK1/2 antibodies as loading controls

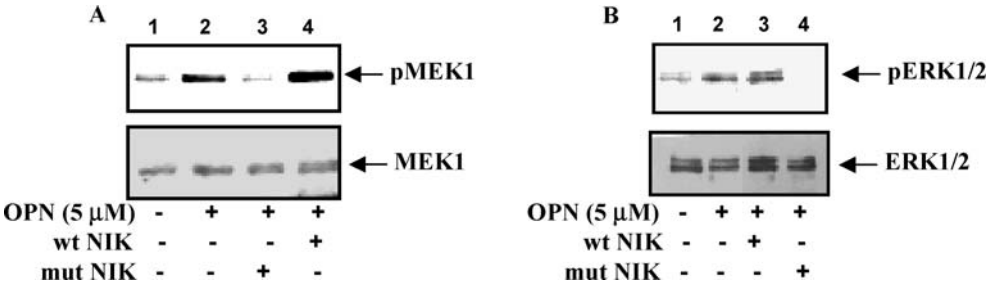

Fig. 2 Panels A and B: OPN Induces NIK dependent MEK and ERK phosphorylations**.** Cells were transfected with wild type and kinase negative NIK and then stimulated with OPN. Cell lysates were analyzed by Western blot using anti-phospho-MEK-1 or anti-phospho-ERK1/2 (upper panels A and B) antibody. The same blots were reprobed with anti-MEK-1 or anti-ERK1/2 antibody (lower panels A and B). The bands were analyzed densitometrically and the fold changes were calculated. The data shown here represent three experiments exhibiting similar effects

(lower panels A and B). These data suggested that OPN induces MEK-1 and ERK1/2 phosphorylations through NIK-mediated pathway in B16F10 cells.

NIK plays crucial role in OPN-induced ERK/IKK-mediated NF-κB-DNA binding and transactivation

The above results indicate that OPN induces NIK-dependent IKK activity leading to IκBα phosphorylation and degradation. In this paper, we have first examined whether NIK regulates OPN-induced NF-κB-DNA binding in B16F10 cells. Accordingly, cells were treated with 5 μM OPN or transfected with wild-type and kinase-negative NIK, wild-type and dn IKKβ and then treated with OPN. To examine, whether ERK is involved in OPN-induced NIK-mediated NF-κB-DNA binding, cells were transfected with wild-type and kinase negative NIK, followed by treatment with PD98059 and then stimulated with OPN. The nuclear extracts were prepared and used for EMSA using ^{32}P-labeled NF-κB oligonucleotides. The results indicated that wild-type NIK enhanced and kinase negative NIK suppressed OPN-induced NF-κB-DNA binding (Fig. 3, panel A, lanes 1–4). Similarly, wild-type IKKβ induced and dn IKKβ inhibited OPN-induced NF-κB-DNA binding (panel B, lanes 1–4). The OPN-enhanced NF-κB-DNA binding caused by overexpression of wild-type NIK was also suppressed by PD98059 (panel C, lanes 1–4). To further confirm that the band obtained by EMSA in OPN treated cells is indeed NF-κB, the nuclear extracts were incubated with anti-p65 antibody and then analyzed by EMSA. There was a shift of the NF-κB-specific band to a higher molecular weight when the nuclear extracts were treated with anti-p65 antibody, suggesting that OPN-activated complex consists of the p65 subunit of NF-κB (data not shown).

The effect of OPN on NF-κB transcriptional activity was monitored by luciferase reporter-gene assay. Cells were transiently transfected with NF-κB luciferase reporter construct (pNF-κB-Luc) and then treated with OPN (5 μM). In separate experiments, cells were individually transfected with wild-type and kinase-negative NIK, super-repressor form of IκBα, wild-type ERK1/2, and dn ERK1/2 along with pNFκB-Luc and then treated with OPN. The transfection efficiency was normalized by cotransfecting the cells with pRL vector (Promega) containing a full length renilla luciferase gene under the control of a constitutive promoter. The cell lysates were used to measure luciferase activity. The data demonstrated that wild-type NIK enhanced, but kinase-negative NIK or super-repressor form of IκBα suppressed OPN-induced NF-κB activity in these cells (Fig. 3, panel D). Similarly, wt ERK1 and wt ERK2 enhanced whereas dn ERK1 and dn ERK2 suppressed OPN-induced NF-κB activity (panel E). The values were normalized to renilla luciferase activity. The fold changes were calculated and means $\pm$ S.E. of triplicate determinations were plotted. The values were also analyzed by student's t test ($^{*}p < 0.001$). These results demonstrated that NIK regulates OPN-induced NF-κB-DNA binding and transactivation through both IKK/ERK1/2-mediated pathways.

OPN stimulates NIK-dependent NF-κB mediated MMP-9 expression and activation

The effect of OPN on pro-MMP-9 expression and activation was analyzed by treating the cells with 5 μM OPN. To investigate the role of NIK, IKK, and NF-κB in OPN-induced MMP-9 activation, cells were individually transfected with wild-type NIK, kinase-negative NIK, super-repressor form of IκBα, wild-type and dn IKKβ and then treated with 5 μM OPN. The conditioned medium was collected and the MMP-9 activity was detected by zymography. The results showed that OPN induces the expression and activation of MMP-9 (Fig. 4, panel A, lanes 1 and 2). The level of OPN induced MMP-9 expression was significantly enhanced when the cells were transfected with wild-type NIK and downregulated upon transfection with kinase-negative

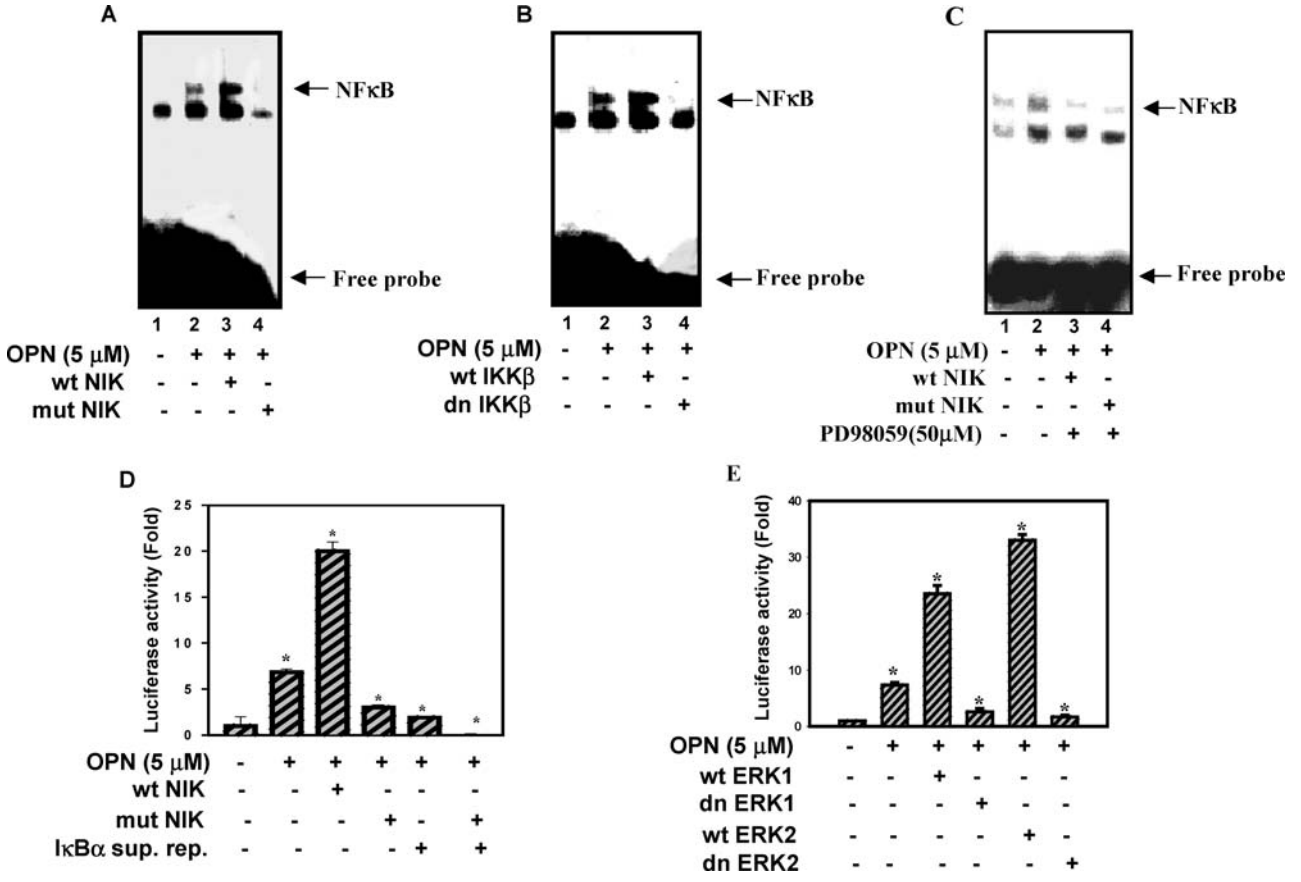

Fig. 3 Panels A and B: OPN induces NIK/IKK dependent NF-κB-DNA binding. Cells were treated with 5 μM OPN. In other experiments, cells were individually transfected with wild-type and kinase-negative NIK, wild-type and dn IKKβ and then treated with OPN. The nuclear extracts were prepared and analyzed by EMSA. Panel C: ERK1/2 is involved in OPN-induced NIK mediated NF-κB-DNA binding. Cells were transfected with wild-type and kinase-negative NIK, treated with PD98059 and then treated with OPN. The nuclear extracts were analyzed by EMSA. Panels D and E: OPN enhances NIK-dependent NF-κB transactivation through IKK- and ERK- mediated pathways. Cells were transiently transfected with luciferase reporter construct (pNF-κB-Luc). In other experiments, cells were individually transfected with wild-type and kinase-negative NIK, or super repressor form of IκBα along with pNF-κB-Luc. In another experiment, cells were transfected with wild-type and dn ERK1 and ERK2 along with pNF-κB-Luc. The transfected cells were treated with 5 μM OPN. Cell lysates were used to measure the luciferase activity. The values were normalized to Renilla luciferase activity. The fold changes were calculated and mean $\pm$ S.E. of triplicate determinations is plotted. The values were also analyzed by Student's t test ($^*p < 0.001$)

NIK and super repressor form of IκBα (panel A, lanes 3–5). Similarly, wild-type IKKβ enhanced and dn IKKβ suppressed the OPN-induced MMP-9 expression (panel B, lanes 1–4). These results demonstrated that OPN induces MMP-9 expression and activation through NIK/NF-κB-mediated pathway.

OPN induces NIK-dependent IKK/ERK1/2-mediated uPA-secretion and uPA-dependent MMP-9 activation

To examine whether NIK and ERK1/2 are involved in OPN-induced uPA secretion, cells were either treated with OPN (5 μM) or transfected with wild type NIK, kinase negative NIK, or wild-type ERK1/2, dn ERK1/2 and then treated with OPN. The cell lysates were analyzed by Western blot using rabbit-polyclonal anti-uPA antibody. The data showed that OPN-induced uPA secretion was enhanced when cells were transfected with wild-type NIK and suppressed when transfected with kinase-negative NIK (upper panel C, lanes 1–4). Wild-type ERK1/2 stimulated and dn ERK1/2 blocked OPN-induced uPA secretion (upper panel D, lanes 1–6). The blots were reprobed with anti-actin antibody as loading controls (lower panels C and D). These data indicated that OPN induces NIK-dependent uPA secretion through ERK1/2-mediated pathways.

Earlier reports have indicated the correlation between uPA expression and metastatic potential and have shown that uPA plays a major role in regulating MMPs activation. To determine whether uPA plays any role in OPN-induced MMP-9 activation, cells were pretreated with anti-uPA antibody and then treated with OPN (5 μM). The level of MMP-9 was detected by zymography as described above. The data indicated

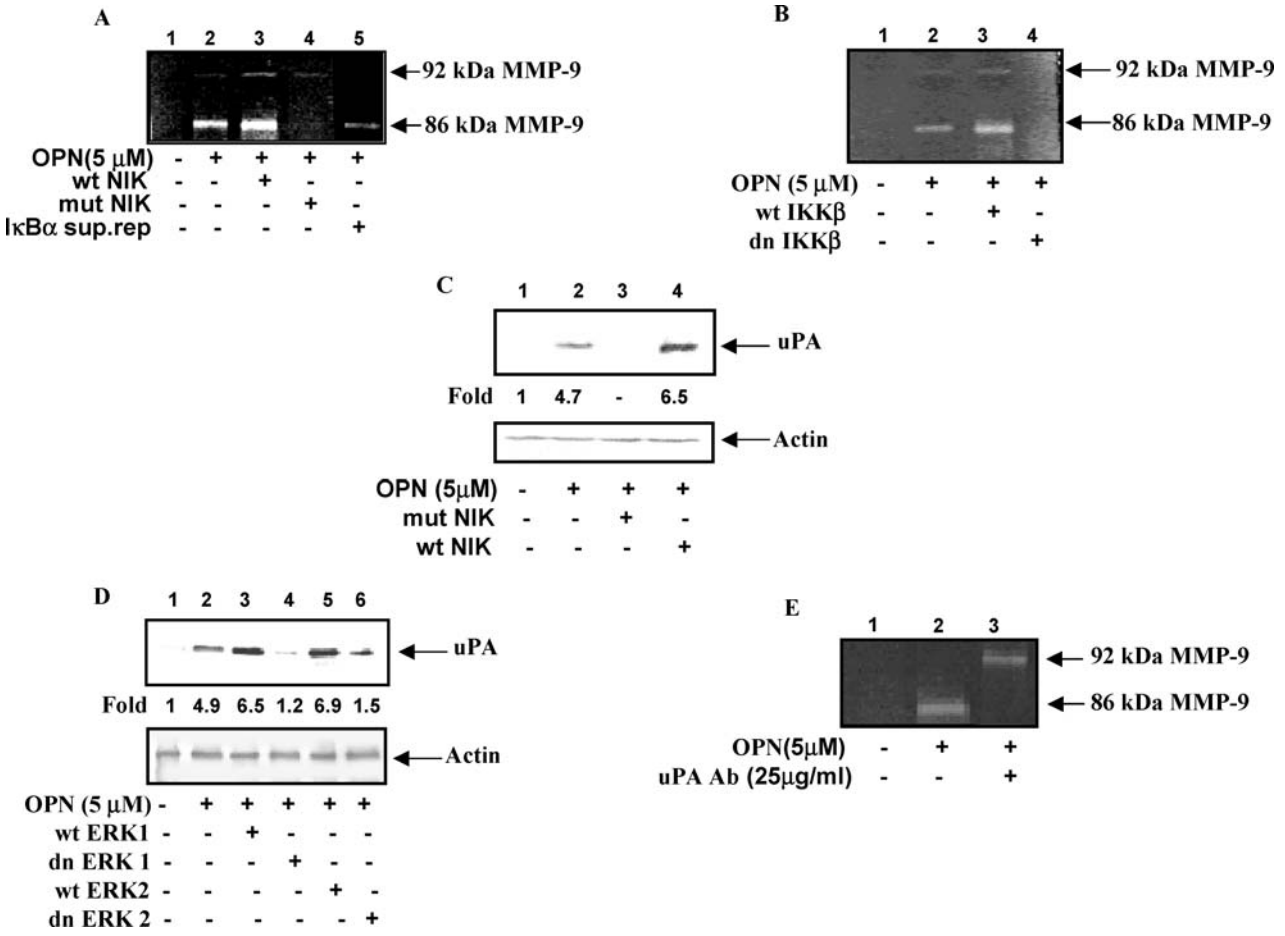

Fig. 4 Panels A and B: OPN stimulates NIK/IKK-dependent MMP-9 activation. Cells were individually transfected with wild-type and kinase-negative NIK, wild type and dn IKKβ or super repressor form of IκBα and then treated with 5 μM OPN. The conditioned media were collected and MMP-9 activity was analyzed by gelatin zymography (panels A and B). Panels C and D: OPN induces NIK- and ERK1/2-dependent uPA secretion. Cells were treated with OPN (5 μM). In separate experiments, cells were transfected with wild-type and kinase-negative NIK, 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 (upper panels C and D). The same blots were reprobed with anti-actin antibody as loading control (lower panels C and D). Panel E: uPA is required in OPN-induced MMP-9 activation. Cells were pretreated with anti-uPA antibody (25 μg/ml) and then treated with 5 μM OPN. The conditioned media were collected and the MMP-9 activity was analyzed by zymography (panel E, lanes 1–3). The results shown here represent three experiments exhibiting similar effects

that uPA antibody suppressed the OPN-induced pro-MMP-9 activation (panel E, lanes 1–3), indicating that uPA is required for OPN-induced MMP-9 activation.

Roles of NIK, IKK and NF-κB in OPN-stimulated uPA- and MMP-9-dependent cell migration

Since NIK plays a major role in the regulation of OPN-induced ERK and IKK dependent NF-κB mediated uPA secretion and uPA-dependent MMP-9 activation, therefore we sought to determine the role of these signaling molecules in OPN-induced cell migration. This was evaluated by using established *in vitro* cell migration assay systems. The results showed that cells transfected with wild-type NIK enhanced the OPN-induced cell migration compared with control (Fig. 5, panel A). However, the OPN-induced cell migration was suppressed upon transfection with kinase negative NIK or super repressor form of IκBα (panel A). Similarly, wild-type IKKα/β enhanced and dn IKKα/β suppressed OPN-induced cell migration (panel B). Pretreatment of cells with anti-uPA or anti-MMP-9 antibody drastically reduced the OPN- induced cell migration (panel A). These data indicate that OPN induces uPA and MMP-9 dependent cell migration through NIK/IKK/NF-κB-mediated pathways.

ERK1/2 plays a crucial role in OPN-induced NIK regulated cell migration

To examine the role of ERK1/2 in OPN-induced NIK-dependent cell migration, cells were treated with PD98059 (MEK-1 inhibitor) or transfected with wild type NIK and kinase-negative NIK followed by treatment with PD98059.

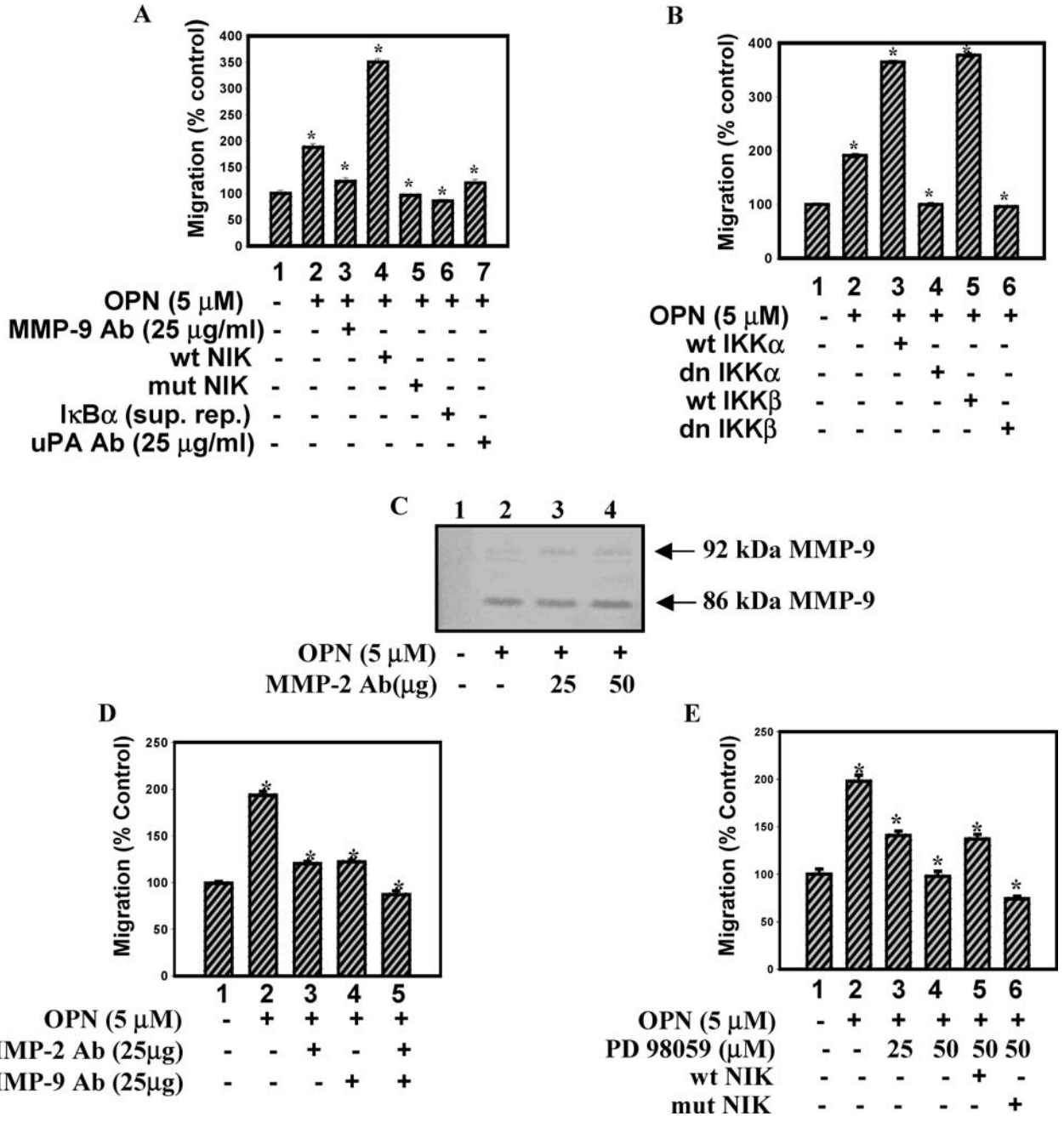

Fig. 5 Panels A and B: NIK and IKKα/β are involved in OPN-stimulated uPA- and MMP-9-dependent cell migration. The migration assay was conducted either by using untreated cells (5×10^5 cells/well) or cells pretreated with anti-MMP-9 or anti-uPA antibody. In separate experiments, cells were transfected with wild-type and kinase-negative NIK, wild-type and dn IKKα and IKKβ or super-repressor form of IκBα. The purified human OPN (5 μM) was added in the upper chamber. The treated or transfected cells were used for migration assays (panels A and B). Panel C: OPN-induced MMP-9 activation is distinct from MMP-2 activation. Cells were pretreated with anti-MMP-2 antibody and then treated with 5 μM OPN. The level of MMP-9 was detected by Western blot using anti-MMP-9 antibody (panel C, lanes 1–4). Panel D: MMP-2 and MMP-9 play independent roles in regulating OPN-induced cell migration. The migration assay was performed either by using untreated cells or cells pretreated with anti-MMP-2 or anti-MMP-9 antibody or in combination of both. These cells were used for migration assays (panel D). Panel E: ERK1/2 is involved in OPN-stimulated NIK dependent cell migration. Cells were either treated with PD98059 or transfected with wild-type or kinase-negative NIK and then treated with PD98059 and used for cell migration assay (panel E). The results are expressed as the means ± S.E. of three determinations

These treated or transfected cells were used for migration assay. PD98059 dose dependently suppressed OPN-induced cell migration (panel E). Moreover, PD98059 also inhibited the migration in wild-type NIK-transfected cells (panel E). These data suggested that NIK regulates OPN-induced cell migration through both IKK- and ERK 1/2-mediated pathways.

MMP-2 and MMP-9 play independent roles in regulating OPN-induced cell migration

We have earlier reported that OPN induces MT1-MMP mediated MMP-2 activation which ultimately controls cell motility, invasion, and tumor growth in murine melanoma cells [10]. Therefore, we sought to determine whether there is any crosstalk between OPN-induced MT1-MMP-mediated MMP-2 activation and uPA-dependent MMP-9 activation, or whether both MMP-2 and MMP-9 play independent roles in regulating cell migration. Accordingly, cells were pretreated with either anti-MMP-2 or anti-MMP-9 antibody or a combination of both and used for migration assay. The OPN-induced cell migration was suppressed when the cells were pretreated with anti-MMP-2 or anti-MMP-9 blocking antibody. However, treatment with a combination of both exerted an additive effect and hence drastically reduced OPN-induced cell migration (panel D).

To further investigate whether MMP-2 plays any direct role in OPN-induced MMP-9 activation, cells were pretreated with anti-MMP-2 antibody followed by treatment with OPN. The conditioned media were collected and the level of MMP-9 was detected by Western blot analysis using anti-MMP-9 antibody. The data showed that OPN-induced MMP-9 activation was unaffected when cells were pretreated with anti-MMP-2 antibody (panel C, lanes 1–4). These data clearly indicated that OPN-induced activations of MMP-2 and MMP-9 occurred through two distinct signaling mechanisms and both played independent roles in regulating cell migration (panel D) and chemoinvasion (data not shown) in B16F10 cells.

Discussion

Signals transduced by cell adhesion molecules play an important role in tumor cell attachment, motility, and invasion, all of which regulate tumor metastasis. Cell-matrix interactions play a major role in tissue remodeling, cell survival, and tumorigenesis. Osteopontin and its receptors figure prominently in a wide spectrum of malignancies. MMPs are a family of Zn^{2+} dependent endopeptidases responsible for remodeling of the extracellular matrix through alteration of cell-ECM interactions. MMP-9 is an important member of the gelatinase family which degrades the basement membrane normally separating the epithelial from stromal compartment. Several studies have shown the correlation between MMP-9 expression and metastatic potential of tumor [25]. Therefore we investigated the potential role of OPN in regulation of MMP-9 activation and the molecular mechanism involved in this process in murine melanoma cells.

The number of functions attributed to being regulated by the transcription factor NF-κB is rapidly increasing. Several reports have indicated the involvement of NF-κB in a large number of cellular processes such as inflammatory and immune response, developmental processes, cell growth, and apoptosis. In addition, it is also activated in several pathological conditions like arthritis, asthma, heart disease, and cancers [15]. NF-κB activity is regulated by an endogenous inhibitor IκBα; interaction of NF-κB with IκBα blocks the nuclear localization signal and hence NF-κB is retained in the cytoplasm. Upon stimulation, IκBα is phosphorylated at serine residues 32 and 36 leading to ubiquitination and proteolytic degradation. This results in nuclear translocation of NF-κB and activation of target gene expression. Inducible phosphorylation of NF-κB is mediated by a multisubunit complex of kinase, IKK [26]. As NF-κB response element is present in the promoter region of MMP-9, we sought to identify the various upstream kinases involved in this pathway. Recent studies have demonstrated that mitogen-activated protein kinase kinase kinases (MAPKKKs) including NF-κB-inducing kinase (NIK) and MEKK 1–3 are involved in the activation of IKK complex [27,28]. We provide evidence to show that OPN induces NIK phosphorylation and enhances the subsequent interaction between phosphorylated NIK and IKKα/β in B16F10 cells. Foehr *et al.* have demonstrated that NIK regulates the differentiation of PC12 cells by modulating MEK/ERK pathways [29]. These results prompted us to investigate whether NIK regulates OPN-induced NF-κB activation through the MAPK pathway in B16F10 cells. Our data indicated that overexpression of wild-type NIK significantly enhances the OPN-induced MEK and ERK phosphorylations whereas transfection of kinase negative NIK abrogated these processes. The data presented here demonstrate that overexpression of wild-type NIK and IKKα/β but not with kinase-negative NIK, and IKKα/β enhanced the OPN-induced NF-κB-DNA binding and NF-κB transactivation. Further, PD98059, a MEK-1 inhibitor suppressed the enhanced NF-κB-DNA binding caused by overexpression of wild-type NIK indicating that OPN induces NF-κB-DNA binding and transactivation through NIK/ERK1/2-mediated pathway.

Cell migration and degradation of extracellular matrix are crucial steps involved in tumor metastasis. It is well established that serine proteinases, such as uPA and MMPs, play essential roles in cell invasion. Previous studies have indicated the role of ECM components in regulating the expression of MMPs. Maquoi *et al.* demonstrated that type IV collagen induces MMP-2 activation in human fibrosarcoma cells

[30,31]. These data indicated cell microenvironment plays an important role in regulating its behavior. These studies delineate a potentially important mechanism by which increased OPN expressions in cancer lead to MMP-9 activation resulting in enhanced-cell migration and ECM invasion. In this study, we have shown that OPN induces uPA secretion and uPA-dependent MMP-9 activation through $\alpha v\beta 3$ integrin mediated pathways. Overexpression of wild-type NIK and IKKα/β-enhanced and kinase-negative NIK, dn IKKα/β, or super-repressor form of IκBα suppressed the OPN-induced MMP-9 activation. Recent data also indicated that the expression of MMP-9 is down regulated in ERK-mutated stable transfectants inhibiting glioma invasion *in vitro* [32]. Accordingly, we have also examined whether MAPK particularly ERK1/2 regulates OPN-induced MMP-9 activation in B16F10 cells. The results showed that overexpression of wild type ERK1/2 but not dn ERK1/2 upregulated OPN-induced uPA secretion. These data clearly indicated that OPN induces NF-κB-mediated uPA dependent pro-MMP-9 activation through both NIK/IKK- and NIK/ERK-mediated pathways.

We have earlier reported that OPN induces pro-MMP-2 activation through NF-κB-mediated induction of MT1-MMP in murine melanoma cells [10]. It has been reported that both MT1-MMP/MMP-2 activation and uPA/MMP-9 activation pathways are critical in the regulation of cell motility, tumor growth, and angiogenesis in esophageal carcinoma. Pretreatment of cells with anti-uPA antibody suppressed the OPN-induced pro-MMP-9 activation suggesting that uPA is the key molecule involved in the MMP-9 activation. To further examine whether OPN-induced MMP-2 and MMP-9 contribute independently to cell motility, cells were pretreated with anti-MMP-2 and anti-MMP-9 blocking antibodies. This resulted in a drastic reduction of OPN-induced cell migration. Moreover, pretreatment of cells with anti-MMP-2 antibody had no effect on OPN-induced MMP-9 activation. These data clearly suggested that MMP-2 activation is distinct from MMP-9 activation and both play independent roles in regulating OPN-induced cell motility and chemoinvasion. Wild-type NIK, IKKα/β, and ERK1/2 enhanced and kinase-negative NIK, dn IKKα/β, and dn ERK1/2 suppressed OPN-induced cell migration and chemoinvasion. Taken together, our data demonstrated that OPN induces NIK-regulated NF-κB-mediated uPA dependent pro-MMP-9 activation through ERK/IKK-mediated signaling pathways, and all of these ultimately control cell motility and chemoinvasion of melanoma cells.

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