Aims & Objectives
AIMS AND OBJECTIVES OF THE STUDY

Many transformed cell lines and a variety of cancers including breast cancer, prostate cancer, glioblastoma and squamous cell carcinoma are characterized by abundant secretion of chemokine like ECM protein, osteopontin (OPN). Previous research has implied that OPN exerts its pro-metastatic effects resulting in tumor cell dissemination and metastasis formation by interacting with cell surface receptors such as integrins (αvβ3, αvβ1, αvβ5) or CD44. Thus OPN and its receptors figure prominently in a wide spectrum of malignancies. Another protein that is associated with the transformed phenotype is the matrix degrading matrix metalloproteinases (MMPs). MMP-9 or gelatinase-B is a classical member of the gelatinase family which can degrade a wide range of ECM components. The regulation of activation of MMP-9 is more complex than most of the other MMPs because most of the cells do not express constitutively active form of MMP-9 but its activity is induced by different stimuli depending on cell types. MMPs are known to be highly regulated by several mechanisms and ECM components also play a pivotal role in this regulatory mechanism. Therefore we speculated that ECM components whose expression is altered in transformed cell types may be involved in the regulation of MMP-9 expression and activation. In the past, ECM components such as vitronectin, fibronectin, laminin and collagen have been implicated in the regulation of MMP activity. Therefore, this study is aimed at delineating the molecular mechanism(s) by which OPN regulates MMP-9 activation, its correlation with the tumorigenic and metastatic properties of murine melanoma cells and the clinical implication has been analyzed. Briefly,

- To investigate the role of OPN in the regulation of MMP-9 expression and activation in B16F10, murine melanoma cells
- To study the role of OPN in the regulation of NIK (NFκB inducing kinase) activity and phosphorylation and the subsequent interaction between phosphorylated NIK and IKK α/β.
- To ascertain the role of OPN on NIK mediated IkB kinase (IKK) activity and subsequent serine/threonine phosphorylation of IkBα.
- To study the role of OPN in the regulation of αvβ3 integrin mediated NIK dependent MEK-1 and ERK1/2 phosphorylations.
• To demonstrate the role of NIK in the regulation of OPN induced IKK/ERK1/2 mediated NFκB-DNA binding and transcriptional activity.

• To demonstrate the role of NIK in OPN induced IKK and ERK1/2 mediated NFκB dependent uPA secretion.

• To check whether uPA is involved in OPN induced pro-MMP-9 activation and its role in modulating cell motility, invasion, tumor growth and metastasis in B16F10 cells.

• To analyze whether there exist any crosstalk between OPN induced MT1-MMP mediated MMP-2 activation and uPA mediated MMP-9 activation mechanism.

• To study the role of OPN in regulation of integrin mediated MEKK1 and JNK-1 phosphorylation and kinase activity.

• To investigate whether both MEKK1 and NIK are involved in regulating OPN induced JNK1 activity leading to c-Jun expression and subsequent activation of the transcription factor, AP-1.

• To demonstrate the differential role of JNK1 in regulating OPN-induced NIK/MEKK1 dependent AP-1 mediated uPA secretion leading to MMP-9 activation.

• To investigate whether there exist any crosstalk between OPN induced NIK/ERK1/2 and MEKK1/JNK1 pathways which ultimately control cell motility, invasiveness, tumor growth and metastasis of melanoma.

• To examine the role of OPN in regulating MelCAM expression and any possible interaction between the cell adhesion molecule MelCAM and integrins.

• To examine the activation status of FAK upon OPN stimulation and the role of FAK in modulating NIK phosphorylation.

• To study the role of OPN in the regulation of FAK and NIK mediated Sp1-DNA binding and transcriptional activity and to examine the interplay between the two transcription factors, AP-1 and Sp1.

• To analyze the role of MelCAM in regulating OPN induced uPA secretion and uPA dependent MMP-9 activation which ultimately controls cell motility, invasion, tumor growth and metastasis of malignant melanoma.
Thus, this study is aimed at delineating the molecular mechanism by which the metastatic gene OPN regulates FAK dependent NIK/MAPK mediated NFκB/AP-1/Sp1 regulated uPA expression and uPA dependent MMP-9 activation and how all of these ultimately control cell motility, invasion, tumor growth and metastasis in murine melanoma cells. Moreover the clinical implication of the molecular mechanism by which OPN regulates melanoma progression and its correlation with the pathogenesis and tumor grade has also been analyzed in human malignant melanoma specimens.
Materials & Methods
MATERIALS AND METHODS

5.1. Source of Chemicals

Chemicals, reagents and kits were obtained from the following sources:

Bio-Rad, USA

Acrylamide, N,N- Methylene bisacrylamide, protein assay kit, high, low and broad range pre stained protein markers were obtained from Bio-Rad.

Invitrogen, USA

Tris-base, glycine, sodium dodecyl sulphate (SDS), ammonium persulphate, calcium chloride, N.N,N’,N’–tetramethylethylene diamine (TEMED), Protein-A/G agarose, agarose, T4 polynucleotide kinase, GRGDSP and GRGESP peptides, LipofectAMINE and plus reagent, Opti-MEM transfection media, Dulbecco’s modified Eagle’s medium, sodium chloride, ethylenediamine tetra-acetic acid (EDTA), and buffer saturated phenol were obtained from Invitrogen.

Himedia Labotatories, India

Bacto tryptone, Bacto yeast extract, Miller Luria Bertani agar and Miller Luria Bertani broth were obtained from Himedia Labotatories.

Santa Cruz Biotechnology, USA

Rabbit polyclonal anti-phospho-NIK (Thr-559), anti-NIK, anti-MEKK1, anti-IKKα/β, anti-JNK1, anti-p-MEK-1, anti-MEK-1, anti-ERK1/2, anti-NFκB p65 X TransCruz, anti-c-Jun, anti-1κBα, anti-Sp1, goat polyclonal anti-MelCAM, anti-uPA, anti-MMP-9 and anti-actin, mouse monoclonal anti-phospho ERK1/2, anti-phospho 1κBα antibodies, anti-αvβ3 integrin, anti-phosphotyrosine antibody, 1κBα recombinant protein, recombinant MEK-1, MAP kinase p42 and c-Jun proteins, horse radish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, anti-goat IgG and FITC conjugated anti- rabbit IgG were purchased from Santa Cruz Biotechnology.
**Dharmacon RNA Technologies, USA**

FAK siRNA duplex (5’-GCAUGUGGCCUCUAUGGAdTdT/dTdTCGUA CACCCGGACGAUACC-5’) and the control RNA duplex (5’-GCGCUCUUUGUG AGGAUUCGdTdT/dTdTCGCG-3’-AAACAUCCUAAGC-5’) were synthesized by Dharmacon RNA Technologies, USA.

**Amersham Pharmacia, UK**

ECL™ Western blot detection kit, Hybond-C nitrocellulose membrane, Sephadex G-25, Tween-20 and HEPES ([4-(2-hydroxyethyl)-1-piperazin eethanesulfonic acid] were obtained from Amersham Biosciences.

**Oncogene Research, USA**

Mouse monoclonal anti-uPA antibody, anti-MMP-9 normal rabbit IgG and fluorescent mounting media were obtained from Oncogene Research.

**Chemicon International, USA**

Mouse monoclonal anti-human αvβ3 integrin antibody (LM609) was from Chemicon International.

**BD-Biosciences, USA**

All the laboratory plasticwares for cell and tissue culture were purchased from BD- Biosciences.

**Board of Radiation and Isotope Technology (BRIT), India**

[γ-32P] ATP (5000 Ci/mmol) was obtained from BRIT, Hyderabad.

**Sigma Chemical Co. USA**

Triton-X-100, DTT (Dithiothreitol), Sodium deoxycholate, Bromophenol blue, 2- mercaptoethanol, glutraldehyde, MOPS (3-[N-Morpholino] propanesulfonic acid) and Myelin basic protein were from Sigma.
ICN Biochemicals, USA
Trypan blue, Gelatin, Chloroform, Formaldehyde, Nonidet P-40 (NP-40), Sodium orthovanadate, pNPP, Tri sodium citrate and bovine pancreatic RNAs were from ICN.

Promega, USA
pRL vector (Renilla luciferase reporter construct under CMV promoter), dual luciferase reporter assay system, NFkB and AP-1 consensus oligonucleotide were purchased from Promega.

Calbiochem, USA
Mouse monoclonal anti-phosphoserine detection kit, PD98059 (2’- amino-3’-methoxy flavone), WP 631, Mitramycin and SP600125 were from Calbiochem.

Roche Molecular Biochemicals, Germany
Aprotinin, pepstatin, protease inhibitor cocktail tablet, leupeptin, phenyl methyl sulphonyl fluoride, ethidium bromide were from Roche.

Merck, Germany
Ethanol, methanol, isopropyl alcohol, sodium dihydrogen phosphate, disodium hydrogen phosphate, Giemsa stain, hydrogen peroxide were obtained from Merck.

Miscellaneous
Murine melanoma cell line, B16F10 were obtained from American Type Culture Collection (Manassas, VA) through the Cell Repository, National Centre for Cell Science. Boyden type cell migration chambers were obtained from Corning and BioCoat Matrigel™ invasion chambers were from Collaborative Biomedical. The anti-OPN antibody was purchased from R & D Systems. The nude mice (NMRI, nu/nu) were obtained from the National Institute of Virology (NIV), India. All other chemicals were of analytical grade.
5.2. Maintenance of cell line

The murine melanoma cell line B16F10 were cultured in DMEM (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. The cells were passaged at confluency by trypsinization.

5.3. Trypan Blue Exclusion test for cell viability

Cells were checked for viability by trypan blue exclusion test. The cells were pelleted by centrifugation and resuspended in 1 ml of DMEM. Cell suspension was diluted in trypan blue solution (0.4% trypan blue in PBS) 1:1 and incubated for 3 min at room temperature. A drop of trypan blue/cell mixture were applied to the haemocytometer and unstained (viable) and stained (nonviable) cells were counted separately. Percentage viability was calculated as follows:

\[
\text{Viable cells (\%)} = \frac{\text{Total number of viable cells}}{\text{Total number of cells per ml}} \times 100
\]

5.4. Purification and characterization of osteopontin from human milk

10 mM sodium phosphate buffer pH 7.0

1M Na₂HPO₄ 5.77 ml
1M NaH₂PO₄ 4.23 ml
Deionized water to 1000 ml

Methodology

Human milk was collected from a local hospital. OPN was purified by DEAE-Sephadex chromatography followed by FPLC system. Fat was cleared by centrifugation. Fat free sample was loaded to the DEAE-Sephadex column. The column was washed with 10 mM sodium phosphate buffer pH 7.0 containing 100 mM NaCl and eluted with same buffer containing 500 mM NaCl. Fractions obtained were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. Briefly, the fractions
were resolved by SDS-PAGE and electrotransferred from the gel to nitrocellulose membrane. The membrane was incubated with established goat IgG-purified anti-OPN antibody (R & D Systems 1: 5000 dilution). It was washed, incubated further with HRP-conjugated anti-goat IgG (1: 2000 dilution) and detected by ECL detection system. Partially purified OPN containing fraction was further purified by FPLC Resource-Q column with a linear gradient of NaCl from 0-1 M in 20 mM Tris-HCl buffer, pH 7.6 over a period of 40 min and rechromatographed under the same conditions using Resource-Q column. Final purity of OPN was checked by SDS-gel and Western blot analysis using anti-OPN antibody as described above. The concentration of OPN was measured by Bradford method (Bio-Rad protein assay) according to manufacturer’s instructions.

5.5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot analysis

1) Reagents and solutions for SDS-PAGE

**30% Acrylamide/0.8% Bis-acrylamide**

30.0 g Acrylamide
0.8 g N, N- Methylene bisacrylamide
The volume was made to 100 ml with deionized water. The solution was filtered and stored in dark at 4°C.

**4X Tris-HCl, pH 6.8 (0.5 M Tris-HCl, Stacking solution)**

6.05 g Tris base was dissolved in 40 ml of deionized water, pH was adjusted to 6.8 with 1 N HCl. The volume was made up to 100 ml. The solution was stored at 4°C.

**4X Tris-HCl, pH 8.8 (1.5 M Tris-HCl, Resolving solution)**

18.17 g Tris base was dissolved in 40 ml of deionized water, pH was adjusted to 8.8 with 1 N HCl. The volume was made up to 100 ml. The solution was stored at 4°C.
**10% Sodium Dodecyl Sulphate (SDS) solution**
10 g SDS was dissolved in 80 ml of deionized water and the volume was made upto 100 ml with deionized water and stored at room temperature.

**10% (w/v) Ammonium persulphate**
0.05 g of ammonium persulphate was dissolved in 500 μl of deionized water. The solution was prepared fresh every time.

**3X Lammeli sample buffer (SDS-PAGE sample buffer)**
15 ml 4X- Tris HCl, pH 6.8  
10 g Glycerol  
3 g SDS  
1.5 ml 2- Mercaptoethanol  
0.1 g Bromophenol blue  
Deionized water to 50 ml.

**10 X Electrophoresis buffer**
144 g Glycine  
30.3 g Tris base  
10 g SDS  
Deionized water to 1000 ml.

**Gel Composition**

<table>
<thead>
<tr>
<th>Resolving Gel</th>
<th>7.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide/0.8% Bis-acrylamide</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>4 X Resolving solution</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>6.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>120 μl</td>
</tr>
<tr>
<td>10% Ammonium per sulphate</td>
<td>60 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
</tr>
</tbody>
</table>
Stacking gel

30% Acrylamide/0.8% Bis-acrylamide 2.0 ml
4 X Stacking solution 2.5 ml
Deionized water 6.5 ml
10% SDS 100 µl
10% Ammonium per sulphate 50 µl
TEMED 10 µl

2) Coomassie blue staining solutions

Coomassie blue 0.25%
Acetic acid 10%
Methanol 50%

Made up the volume with deionized water

Destaining solution

Acetic acid 7.5%
Methanol 10%

Made up the volume with deionized water

3) Reagents and solutions for Western Blotting

1X Transfer Buffer

3.03 g Tris
14.4 g Glycine
200 ml Methanol
Deionized water to 1000 ml

Phosphate Buffered Saline (PBS)

8.0 g NaCl
0.2 g KCl
1.44g Na₂HPO₄
0.24 g KH₂PO₄
These chemicals were dissolved in 800 ml deionized water and pH was adjusted to 7.4 with 1 N HCl. The volume was made upto 1000 ml.

**PBS-T**

Tween-20 at a final concentration of 0.1% was added to 1X PBS.

**Blocking Solution**

5% non-fat dry milk in PBS-T

**Stripping Buffer**

62.5 mM Tris-HCl, pH 6.7
2% SDS
100 mM 2- mercaptoethanol

**Methodology**

The protein concentration of the samples was measured by Bio-Rad protein assay kit. The samples containing equal amount of total proteins was mixed with 3 X Laemmeli sample buffer. The samples were boiled for 5 min at 100°C. The proteins were resolved in polyacrylamide gel on discontinuous buffer system of Laemmeli using Bio-Rad mini-gel electrophoresis unit. Electrophoresis was carried out at constant voltage of 60 V till the dye front reaches the bottom of the gel.

For Coomassie blue staining, the protein gel was immersed in Coomassie blue staining solution for 1 h following which gel was washed in tap water and destained with the destaining solution.

For Western blot analysis, the proteins from the polyacrylamide gel were electrophoretically transferred to nitrocellulose membrane at 120 mA for 3 h using a mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad). The membrane was blocked with 5% solution of fat free milk in PBS-T at 4°C overnight. Immunodetection was carried out using ECL detection kit following the manufacturer’s instructions. Briefly, the membrane was rinsed with PBS-T and washed thrice for 15 min. The membranes were incubated with
primary antibody at appropriate dilution in 0.5% milk in PBS-T for 1 h at room temperature. Blot was then washed thrice with PBS-T followed by incubation in HRP-labeled anti-rabbit/anti-mouse/anti-goat IgG antibody. The blot was once again washed as before and exposed to ECL detection reagents (1:1 mixture of solutions 1 and 2) for three minutes. The membrane was autoradiographed for appropriate time period, developed and fixed.

For reprobing, the membranes were incubated in stripping buffer at 50°C for 30 min and then washed twice with PBS-T at room temperature for 10 min. The membrane was then blocked and immunodetected as described.

5.6. Immunoprecipitation

Reagents and solutions

**Lysis buffer-1/wash buffer-1**
50 mM Tris-HCl, pH 7.5
150 mM NaCl
1% Nonidet P-40
0.5% Sodium deoxycholate
1 Tablet of protease inhibitor cocktail was added.

**Wash buffer-2**
50 mM Tris-HCl, pH 7.5
500 mM NaCl
0.1% Nonidet P-40
0.05% Sodium deoxycholate

**Wash buffer-3**
50 mM Tris-HCl, pH 7.5
0.1% Nonidet P-40
0.05% Sodium deoxycholate
**Methodology**

Cells were washed thrice with ice cold 1X PBS to remove any residual serum proteins. Then cells were harvested and resuspended in appropriate amount of ice-cold lysis buffer-1 and incubated on ice for 30 min to allow complete lysis. The cell lysates were clarified by centrifugation at 12000 x g for 10 min at 4°C. The supernatant was transferred to a fresh microfuge tube. The samples containing equal amount of total proteins were incubated with the appropriate amount of specific antibody and gently rocked for 1 h at 4°C. Then 40 µl of protein A agarose suspension was added and incubated further on a rocking platform at 4°C overnight. The immunocomplexes were collected by centrifugation at 12000 x g for 1 min at 4°C. The supernatant was removed and the immunocomplex was resuspended in 1 ml of wash buffer-1. The beads were washed for 20 min at 4°C on a rocking platform. The immunocomplexes were then washed twice with wash buffer 2 and 3 as described above. The 2X SDS-sample buffer was added to the immunocomplex and boiled at 95°C for 5 min. The supernatant was collected and resolved by SDS-PAGE.

5.7. Zymography for detection of MMP-9 activity

**Reagents and solutions**

**Washing buffer**

250 µl Triton X-100 in 100 ml of deionized water

**Incubation buffer**

50 mM Tris-HCl, pH 7.5
100 mM CaCl₂
1% (v/v) Triton X-100
1 µM ZnCl₂
0.02% (w/v) Sodium azide
**Staining solution**

0.1% Coomassie Brilliant Blue in 40% isopropanol

**Destaining solution**

7% glacial acetic acid

**Preparation of conditioned media**

Subconfluent cultures of B16F10 cells were trypsinized, seeded in 6 well tissue culture dishes and grown in DMEM supplemented with 10% serum for 24 h. The serum containing media was replaced with serum free media. In separate experiments, cells were individually pretreated with different inhibitors, blocking antibodies or transiently transfected with different plasmids in the presence of LipofectAMINE Plus followed by treatment with OPN in serum free media for the indicated time period. The conditioned media was cleared by centrifugation, dialyzed and concentrated by lyophilization. The protein concentration was determined by Bio-Rad protein assay reagent.

**Methodology**

The gelatinolytic activity in the conditioned media or the tumor tissue lysates was assessed by zymography. The samples containing equal amount of total proteins were mixed with sample buffer in the absence of reducing agent, incubated at room temperature for 30 min and then loaded onto zymography-SDS-PAGE containing gelatin (0.5 mg/ml). The gels were washed twice in washing buffer at room temperature for 15 min each and incubated in incubation buffer for 16 h at room temperature. The gels were stained with coomassie blue and destained. Negative staining showed the zones of gelatinolytic activity.

**5.8. Cell migration assay**

The migration assay was conducted as described previously (38) using transwell cell culture chamber. Briefly, cells were individually pretreated with different inhibitors, blocking antibodies or transiently transfected with different plasmids in the presence of LipofectAMINE Plus. Cells were harvested using Trypsin-EDTA and collected by centrifugation at 800 x g for 10 min. The cell suspension (5x10^5 cells/well) was added to the
upper chamber of the prehydrated polycarbonate membrane filter. The lower chamber was filled with fibroblast conditioned medium, which acted as a chemoattractant. Purified OPN (5 μM) was added to the upper chamber. The cells were incubated in a humidified incubator in 5% CO₂ and 95% air at 37°C for 16 h. The non-migrated cells on the upper side of the filter were scraped, and the filter was washed. The migrated cells in the reverse side of the filter were fixed with methanol and stained with Giemsa. The migrated cells on the filter were counted under an inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG was used as nonspecific control.

5.9. Extracellular matrix invasion assay

The chemoinvasion assay was performed using Matrigel™-coated invasion chamber as described (38). B16F10 cell suspension (5x10⁵ cells/well) was added to the upper portion of the prehydrated Matrigel™ coated chamber. The lower chamber was filled with fibroblast conditioned medium which acted as a chemoattractant. In separate experiments, cells were individually pretreated with different inhibitors, blocking antibodies or transiently transfected with different plasmids in the presence of LipofectAMINE Plus. OPN (5 μM) was added to the upper chamber. Then, 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, 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).

5.10. Mammalian cell transfection

B16F10 cells were split 12 h prior to transfection in Dulbecco's Modified Eagle's medium containing 10% fetal calf serum. The cells were allowed to grow for 50-60% confluency. Then, 4-8 μg of purified plasmid DNA was diluted in 100 μl Opti-MEM. To this mixture, 9 μl Plus reagent was added, mixed thoroughly and incubated at room temperature for 15 min. The pre-complexed DNA with plus reagent was mixed with 6 μl LipofectAMINE reagent and further incubated for 15 min at room temperature. This entire mixture was added to 800 μl of antibiotic and serum free Opti-MEM media. Cells were
washed twice with serum and antibiotic free Opti-MEM media and DNA-Plus-LipofectAMINE reagent complex was added to tissue culture plates. The cells were further incubated with this complex for 12 h at 37°C, 5% CO2 in humified incubator. After 6 h, the media was replaced with DMEM supplemented with 10% FCS. These cells were allowed to grow for 24-48 h and used for various experiments.

For stable transfection, active G418 (400 μg/ml) containing fresh medium was added to the cultures after 24 h of transfection and was maintained further. Fourteen days later, neo-resistant colonies were isolated and culture was maintained further.

5.11. Nuclear factor inducing kinase (NIK) assay
The NIK kinase assay was performed using IKK as substrate. Briefly, B16F10 cells were washed three times with ice-cold PBS and lysed in 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 Na3VO4, 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) for 15 min. The supernatant was obtained by centrifugation at 12,000 x g for 10 min at 4°C and the protein concentration was determined using Bio-Rad protein assay kit. The cell lysates containing equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-NIK antibody (2 μg) followed by incubation with protein A agarose. Immunocomplex was washed three times with lysis buffer and half of the immunoprecipitated samples were incubated with IKK as substrate in kinase assay buffer (20 mM Hepes, (pH 7.7), 2 mM MgCl2, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM pNPP, 300 μM Na3VO4, 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-32P] ATP at 30°C. The kinase reactions were stopped by addition of 2x SDS-sample buffer and boiled at 95°C for 5 min. The samples were resolved by SDS-PAGE, dried and autoradiographed. The remaining half of the immunoprecipitated samples were subjected to SDS-PAGE and analyzed by Western blot using anti-NIK antibody. A fraction of equal volume of samples from the kinase reaction mixture was analyzed by Western blot using anti-IKKα/β antibody.
5.12. IκB kinase (IKK) assay

IKK assay was performed using IκBα as substrate. B16F10 cells were treated with 5 μM OPN for 10 min at 37°C. In separate experiments, cells were transfected with wild type NIK or kinase negative NIK in presence of LipofectAMINE plus and then treated with 5 μM OPN. The cells were lysed in kinase assay lysis buffer. The cell lysates containing equal amount of total proteins (300 μg) were immunoprecipitated with anti-IKKα/β antibody. Half of the immunoprecipitated samples were incubated with recombinant IκBα (4 μg) in kinase buffer (20 mM Heps, (pH 7.7), 2 mM MgCl₂, 10 μM ATP, 3 μCi of [γ⁻³²P] ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM pNPP, 300 μM Na₃VO₄, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT) at 30°C. The kinase reaction was stopped by addition of SDS-sample buffer. The sample was resolved by SDS-PAGE, dried and autoradiographed. The remaining half of the immunoprecipitated samples was 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.

5.13. JNK kinase assay

JNK kinase assay was performed as described previously (360). To examine the effect of OPN on JNK activity, the semiconfluent cells were treated with 5 μM OPN for 15 min at 37°C. The cells were lysed in kinase assay lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EGTA, 50 mM NaF, 10 mM pNPP, 300 μM Na₃VO₄, 1 mM benzamidine, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 1% NP-40). Lysates were centrifuged at 12,000 rpm 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. The immunocomplexes were washed twice in ice-cold lysis buffer and once with kinase assay buffer (20 mM Heps, (pH 7.7), 2 mM MgCl₂, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM pNPP, 300 μM Na₃VO₄, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT). In vitro JNK activity was performed by incubating half
of the immunoprecipitated samples with recombinant c-Jun as substrate in kinase assay buffer containing 10 μM ATP and 3 μCi of [γ-32P] ATP at 30°C. The kinase reactions were stopped by addition of 2x SDS-sample buffer. The samples were resolved by SDS-PAGE, dried and autoradiographed. The remaining half of the immunoprecipitated samples were subjected to SDS-PAGE and analyzed by Western blot using anti-JNK1 antibody. In separate experiments, the cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 in presence of LipofectAMINE plus and then treated with OPN and JNK kinase assay was performed as described above.

5.14. ERK kinase assay

ERK kinase assay was performed using myelin basic protein (MBP) as substrate. 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 wild type MEKK1 transfected cells were also cotransfected with wild type or dominant negative JNK-1 or treated with 20 μM SP600125 (JNK1 inhibitor) and then treated with OPN. The cells lysates containing equal amount of total proteins were immunoprecipitated with anti-ERK1/2 antibody. Half of the immunocomplexes were incubated with 2 μg of myelin basic protein (MBP) in kinase assay buffer supplemented with 10 μM ATP and 3 μCi of [γ-32P] ATP for 10 min. The samples were resolved on SDS-PAGE and autoradiographed. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-ERK1/2 antibody.

5.15. NIK coupled kinase assay

NIK coupled kinase activity was performed using a slight modification of the method described previously (363). 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 NIK transfected cells were cotransfected with wild type and kinase negative MEKK1 and then treated with OPN. The cell lysates was immunoprecipitated with anti-NIK antibody. Half of the immunocomplexes were incubated with 0.5 μg of recombinant MEK-1 protein in kinase assay buffer containing 100 μM ATP, 10 μCi of [γ-32P] ATP for 20 min at 30°C. After that 2 μg of recombinant kinase inactive
ERK (p42) protein was added to the reaction mixture and the samples were incubated for an additional 30 min at 30°C. The samples were resolved on SDS-PAGE and autoradiographed. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-NIK antibody to ensure equal protein loading.

5.16. Preparation of nuclear and cytoplasmic extracts

Reagents and solutions

Hypotonic buffer
10 mM HEPES, (pH 7.9)
1.5 mM MgCl₂
0.2 mM phenylmethylsulfonyl fluoride
0.5 mM Dithiothreitol (DTT)

Nuclear extraction buffer
20 mM HEPES, (pH 7.9)
1.5 mM MgCl₂
400 mM NaCl
0.2 mM EDTA
25% glycerol
0.5 mM phenylmethylsulfonyl fluoride
5 mM Dithiothreitol (DTT)

Methodology

The nuclear extracts were prepared using a modification of Dignam’s method (374). Cells were grown to 60-70% confluency. The cells were either stimulated with OPN or transfected with different plasmids followed by treatment with OPN for the indicated time points. Cells were washed twice with ice-cold 1X PBS and harvested. The cells were resuspended in hypotonic buffer and allowed to swell on ice for 10 min followed by homogenization using a Dounce homogenizer. The samples were centrifuged at 3300 x g
for 15 min at 4°C and the supernatant obtained was used as cytoplasmic extract. The nuclear pellet was resuspended in ice-cold nuclear extraction buffer and incubated for 30 min at 4°C. The extracted nuclei were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was used as nuclear extract. The protein concentration was measured by the Bio-Rad protein assay. The nuclear extracts were used for Western blot analysis and EMSA.

5.17. Electrophoretic mobility shift assay

**Labeling of Oligonucleotide**

**Reagents and buffers**

**TE Buffer**
10 mM Tris-HCl (pH 8.0)
1 mM EDTA

**10X T4 Polynucleotide Kinase Buffer**
700 mM Tris-HCl (pH 7.6)
100 mM MgCl₂
50 mM DTT

The double stranded NFκB (5’- AGT TGA GGG GAC TTT CCC AGG C-3’), AP-1 (5’-CGCTTGATGACTCAG CCG GAA-3’) and Sp1 (5’-ATTTCGATCGGGCGGGGC-3’) oligonucleotides were labeled using the T4 polynucleotide kinase. The labeling reaction was assembled as follows.

**Labeling reaction**
Consensus oligonucleotide (1.75 pmol/μl) 2 μl
T4 polynucleotide kinase 10 X buffer 1 μl
[ γ-³² P] ATP (5000 Ci/mmol) 1 μl
Nuclease free water 5 µl
T4 polynucleotide kinase (5-10 u/µl) 1 µl

This reaction mixture was incubated at 37°C for 1h. The reaction was stopped by heating at 68°C for 10 min. The reaction mixture was diluted to 50 µl and oligonucleotides were purified by column chromatography by using Sephadex G-25 column.

Reagents and buffers

10X Tris-Glycine EDTA (TGE) Buffer
Tris-base 30.3 g
Glycine 144.0 g
EDTA 1.37 g

The above components were dissolved in 800 ml of deionized water, pH was adjusted to 8.3 and volume was made upto 1000ml.

10X Binding buffer
100 mM Tris-HCl (pH 7.5)
500 mM NaCl
5 mM DTT
5 mM EDTA
10 mM MgCl₂
40% Glycerol

Formulation of 4% Gel (20 ml)

TGE 10X Buffer 1 ml
37.5: 1 acrylamide/bisacrylamide 1.25 ml
40% acrylamide (w/v) 0.75 ml
80% glycerol 0.62 ml
Distilled water                                              16.2 ml  
TEMED                                                        10 μl  
10% APS                                                      150 μl  
The non-denaturing 4% polyacrylamide gel was prepared and allowed to polymerize for 15 min. Pre-electrophoresis was carried for 60 min at 100V at 4°C. Simultaneously, 5-10 μg of nuclear extract was incubated with labeled oligonucleotides under the following condition.

**DNA Binding reaction**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear extract</td>
<td>5-10 μg</td>
</tr>
<tr>
<td>Binding buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>Salmon Sperm DNA</td>
<td>1 μl</td>
</tr>
<tr>
<td>BSA (300 μg/ml)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Labeled probes</td>
<td>5 μl</td>
</tr>
<tr>
<td>Nuclease free water-made up to 20 μl</td>
<td></td>
</tr>
</tbody>
</table>

The binding reaction mixture was incubated at room temperature for 15 min. then the whole reaction mixture was loaded in 4% polyacrylamide gel and electrophoresis was carried out at 4°C for 25 mA. The gel was carefully transferred on to a blotting paper, dried and autoradiographed. For super-shift analysis, the nuclear extracts were incubated with the specific antibody and electrophoresis was carried out as described above.

**5.18. NFκB and AP-1 luciferase reporter gene assay**

The NFκB luciferase reporter gene assay was performed to assess the transcriptional activation of NFκB in the presence of OPN. The semiconfluent cells grown in 24 well plates were transiently transfected with a luciferase reporter construct (pNFκB-Luc) containing five tandem repeats of the NFκB binding site using LipofectAMINE Plus reagent. In separate experiments, cells were individually cotransfected with wild type and mutant forms of various other plasmids 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 (CMV). These cells were grown for an additional 24 h. Cells were serum starved and then 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.

The AP-1 luciferase reporter gene assay was performed to assess the transcriptional activation of AP-1 in the presence of OPN. The semiconfluent cells grown in 24 well plates were individually transfected with a luciferase reporter construct (pAP-1-Luc) containing seven tandem repeats of the AP-1 binding site using LipofectAMINE Plus reagent (Invitrogen). 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. These cells were grown for additional 24 h. Cells were serum starved and then treated with OPN for indicated time periods. In separate experiments, cells were individually cotransfected with various other plasmids along with pAP-1-Luc and then treated with OPN. The luciferase activities were measured as described above.

5.19. Immunofluorescence assay

The cells were trypsinized and resuspended in culture medium and transferred to dishes with sterile cover glasses and grown up to semiconfluency. The cells were then treated with OPN for 24 h. The cover glasses were rinsed with ice cold PBS (pH 7.4). Cells were fixed by incubating in precooled 100% methanol at -20°C. The cells were washed three times for 5 min each with PBS, incubated in 2 % BSA in PBS (pH 7.4) for 30 min to block non specific binding. Following this, cells were incubated with goat polyclonal anti-MelCAM antibody in 1% BSA for 2 h at RT, followed by FITC conjugated anti goat IgG antibody for 1 h at RT. Cells were washed and mounted in mounting media and analyzed under confocal microscopy (Zeiss).
5.20. Three dimensional spheroid culture

Multicellular spheroids were generated by liquid overlay technique (373). Briefly, 24-well tissue culture plates (Costar) were coated with 250 μl of prewarmed 1% Seaplaque agarose solution in serum free DMEM. The agarose was allowed to solidify and form a thin layer on the bottom of the plate. B16F10 cells were either treated with OPN or stably transfected with wild type and kinase negative NIK or pretreated with MelCAM blocking peptide followed by treatment with OPN. A single cell suspension (10^5 cells/well) of treated or transfected cells in complete growth medium was plated into each well. The plates were swirled gently to allow cell to cell contact and incubated undisturbed in a humidified atmosphere at 37°C and 5% CO₂ for 24 h and spheroid formation was determined. Photographs were taken using an inverted microscope.

5.21. In Vivo tumorigenicity experiments

The tumorigenicity experiments were performed with the approval of the animal ethics committee. Cells were grown in monolayer and treated in the absence or presence of purified human OPN in serum free medium and were incubated at 37°C for 24h. After that, the cells were detached, centrifuged, counted with trypan blue staining to exclude any damaged cells and injected (1 x 10^6/0.2 ml) subcutaneously into the dorsal flank region 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. In other experiments, cells stably transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 followed by treatment with OPN (10 μM) were injected to mice. OPN (10 μM) was again injected to the site of tumor twice a week for four weeks.

At the end of four weeks, the mice were sacrificed and tumor weights were measured. The tissue lysates and nuclear extracts from tumor were prepared. The cleared tissue lysates were used for zymography, EMSA and Western blot analysis.
5.21.1. Preparation of lysates from tumor tissue

Reagents and buffers

50 mM Tris-HCl (pH 7.5) 
150 mM NaCl 
1% Nonidet P-40 
15 μg/ml leupeptin 
0.5 mM phenylmethylsulfonyl fluoride

Methodology

Tumors dissected out from the mice were homogenized individually in a Dounce homogenizer. 100 mg tumor tissue from each set of experiment was weighed and homogenized in 500 μl of radioimmune precipitation (RIPA) buffer. The homogenates were subjected to centrifugation at 12000 x g for 15 min at 4°C. The supernatants were collected, protein concentrations determined using Bio-Rad protein assay reagent and analyzed by zymography or Western blot directly or stored as aliquots at −80°C until further use.

5.21.2. Preparation of nuclear extracts from tumor tissue

Reagents and buffers

Buffer A

10 mM Hepes buffer (pH 7.9) 
10 mM KCl
0.1 mM EDTA 
0.1 mM EGTA 
1 mM DTT 
10% Nonidet P-40 
2 μg/ml aprotinin 
2 μg/ml leupeptin
0.5 mg/ml benzamidine
1 Tablet of protease inhibitor cocktail was added.

**Buffer C**

- 20 mM Hepes buffer (pH 7.9)
- 0.4 M NaCl
- 1 mM EDTA
- 1 mM EGTA
- 1 mM DTT
- 1 mM phenylmethylsulfonyl fluoride
- 2 µg/ml aprotinin
- 2 µg/ml leupeptin
1 Tablet of protease inhibitor cocktail was added.

**Methodology**

The tumor tissues were homogenized in buffer A and the nuclei were separated by spinning at 13000 x g for 10 min at 4°C. The nuclear pellet was extracted in Buffer C for 2 h on ice and centrifuged at 13000 x g for 10 min at 4°C. The supernatant was used as nuclear extract. The nuclear extracts were used for EMSA and Western blot analysis.

**5.22. In Vivo metastasis study**

For experimental lung metastasis studies, treated or transfected cells in the absence or presence of OPN (5 X 10^6/0.2 ml) were injected into the lateral tail vein of nude mice. The mice were killed after eight weeks and the lungs were removed, washed with water and fixed in Bouin's solution for 24 h to facilitate counting of tumor nodules. The surface tumor nodules were counted using a dissecting microscope. Sections of the lungs were stained with H & E to confirm that the nodules were melanoma specific. The levels of expressions of pNIK, MelCAM and MMP-9 were detected by immunostaining with their specific antibodies followed by FITC conjugated IgG and analyzed by confocal microscopy (Zeiss).
5.23. Clinical specimens for immunohistochemical studies

Human primary melanoma tissue samples were collected with informed consent, from either diagnostic biopsies or upon surgery at Armed Force Medical College, Pune. Based on the clinical investigations, they were classified as malignant melanoma (MMs) with metastasis and MMs without metastasis. Clinical and histopathological data are summarized in Table III. Immunohistochemical studies were performed on the primary cutaneous lesions.

5.23.1 Paraffin embedding of tissue samples

Tissue was first fixed in 10% formalin for 48 h. After fixing, the samples were dehydrated by successive changes in 50%, 75% and 90% ethanol and finally with three changes of absolute alcohol each 10 min. Tissues were then treated with 1:1 mixture of xylene and ethanol for 15 min and cleared by three changes of xylene/chloroform for 1 h each. Later, tissues were immersed in molten wax at 65°C for 1 h with one more change of wax and kept overnight with complete wax impregnation. After impregnation, the tissues were embedded in paraffin wax and stored at room temperature.

5.23.2 Histopathological grading

For histopathological grading and immunostaining, 4μM thick sections were taken on to poly-L-lysine (Sigma) coated slides and incubated at 37°C overnight. At the time of grading, de-waxing was done by keeping the slides at 58°C for 10 min, followed by three changes of xylene at intervals of 10 min each. Sections were rehydrated in descending series of alcohol changes of 100%, 90%, 75% ethanol and finally rinsed in distilled water. Sections were stained with Harris hematoxylin and later rinsed in differentiation solution of 70% alcohol acidified with 1% HCl for 5 sec. Excess solution was washed off and dehydrated by successive alcohol changes (75%, 90% and 100% ethanol) and three changes of xylene for 30 min duration. Finally slides were mounted in D.P.X mountant and were histopathologically graded by a pathologist.
5.23.3 Immunohistochemistry

Reagents and buffers

**Citrate buffer (0.01M)**

- Citric acid: 2.1g
- Distilled water: 900 ml

The pH was adjusted to 6.0 with 2 N NaOH. The volume was made up to 1000 ml and stored at 4°C.

**TBS Buffer**

- Tris: 6.05g
- NaCl: 8.5 g
- Distilled water: 900 ml

The pH was adjusted to 7.4-7.6 with 1 N HCl. The volume was made up to 1000 ml.

**Diaminobenzidine substrate solution (DAB)**

60 mg of DAB was dissolved in 100 ml of TBS, filtered and 100 ml of 30% H$_2$O$_2$ was added just before use.

**Harris Haematoxylin**

4 gm of haematoxylin powder was dissolved in 40 ml of absolute alcohol over gentle heat. 80 gm of potassium alum was dissolved in 800 ml of boiling distilled water. Both the solutions were mixed thoroughly using a magnetic stirrer. 2 gm of mercuric oxide was added and stirred for 5 min for immediate ripening.

Immunohistochemistry was performed using standard procedure. After deparaffinizing with two changes of xylene, sections were rehydrated using descending
grades of ethanol for 5 min each and washed thrice in TBS buffer. Then the sections were microwaved for 5 min in 10 mM sodium citrate buffer (pH 6.0) to unmask the antigens and allowed to cool. Sections were washed thrice with TBS for 5 min each followed by blocking with 5% bovine serum albumin for 1 h. Finally, the sections were incubated with 1:50 dilution of the indicated primary antibody overnight at 4°C in a humified environment. After incubation with primary antibody, the slides were washed in TBS buffer and incubated with FITC conjugated secondary antibody for 2 h at room temperature. The sections were mounted and analyzed using confocal microscopy (Zeiss).

For DAB staining, the endogenous peroxidase activity was quenched by immersing the sections in 2% H$_2$O$_2$ in methanol. Sections were washed thrice with TBS, blocked followed by incubation with the primary antibody and HRP conjugated secondary antibody. The tissue sections were incubated with DAB substrate at room temperature until appropriate staining develops. A purple reaction product is obtained. The sections were washed with TBS for 5 min. The sections were counterstained with Harris haematoxylin, dehydrated and mounted using D.P.X mountant. Appropriate negative controls were used which were incubated with the secondary antibody alone and used for analysis.

5.24 Densitometric and statistical analysis

All the gels were analyzed densitometrically. Band intensities were determined using the Kodak gel documentation system (Kodak Digital Science). Relative band intensities were calculated and plotted in the form of bar graphs or the fold changes of the test group with respect to the control were indicated in the illustration. In the graphical representations, results indicate means ± S.E of 3 or more experiments. The values of different control and test groups were compared using student’s t-test. A probability level p< 0.05 was considered significant and are marked with asterisk in the illustrations.
Results & Discussion
6.1. Nuclear Factor Inducing Kinase- A Key Regulator in OPN-induced MAPK/IKK
Kinase Dependent NFκB-mediated Pro-MMP-9 Activation

Malignant tumors are characterized by dysregulated growth control, overcoming of
replicative senescence, and metastasis formation. At the molecular level, the metastatic
phenotype is generated by the expression of homing receptors along with associated
signaling molecules, their ligands, and extracellular matrix-degrading proteases.
Osteopontin (OPN), which acts both as chemokine and cytokine is mainly responsible for
the dissemination of various cancers (7). This protein is involved in a number of
pathologies such as tumorigenesis, atherosclerosis and autoimmune diseases and also in
other normal tissue remodeling process such as bone resorption, angiogenesis, wound
healing, and tissue injury (9-12).

Tumor invasion, malignant progression and distant metastasis are complex
multistep processes. One main prerequisite is the ability of tumor cells to initiate
extracellular proteolysis which is required for the tumor cells to cross the tissue barriers,
intravasate and subsequent extravasation leading to metastasis and angiogenesis. Most of
these steps critically depend on the proteolytic activity generated by the matrix
metalloproteinases and the plasminogen activator/plasmin system. uPA is a member of
serine protease that interacts with uPA receptor (uPAR) and facilitates the conversion of
inert zymogen plasminogen into widely acting serine protease plasmin and activation of
MMPs (277). 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 (329).

MMPs are extracellular matrix (ECM) degrading enzymes that play critical role in
embryogenesis, tissue remodeling, inflammation and angiogenesis (324). MMP-9 (also
called type IV collagenase or gelatinase B) is another important contributor to the process
of invasion, tumor growth and metastasis. The regulation of MMP-9 activation is more
complex than most of the other MMPs because most of the cells do not express
constitutively active form of MMP-9 but its activity is induced by different stimuli
depending on cell types thereby contributing to 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 prioritized therapeutic target (345). This study is mainly focused on delineating the various molecular mechanisms by which OPN regulates transcription factor mediated MMP-9 activation which is regarded as one of the potent tumorigenesis and metastasis promoting molecule using B16F10 cells as the model system. We have also delineated the role of specific melanoma associated markers like MelCAM in OPN regulated tumor growth and metastasis through a possible interplay between various transcription factors leading to MMP-9 activation which ultimately controls cell motility, invasion, melanoma growth and metastasis.

**Results**

**6.1.1. OPN Induces αvβ3 Integrin-mediated NIK Phosphorylation**- Since NFκB is one of the principal transcription factor that regulates MMP-9 expression, we were in search of kinases which act upstream of NFκB. In order to examine whether any upstream kinase(s) such as NIK plays any role in OPN induced NFκB activation, B16F10 cells were treated with 5 μM OPN for 0-30 min at 37°C. To further check whether αvβ3 integrin and the RGD/RGE peptide is involved in OPN-induced NIK phosphorylation; cells were pretreated with anti-αvβ3 integrin antibody or RGD/RGE peptide (GRGDSP or GRGESP) and then treated with OPN. The cell lysates containing equal amount of total proteins were resolved by SDS-PAGE, and the level of phosphorylated NIK was detected by Western blot analysis using anti-phospho NIK (Thr-559) antibody (Fig. 6.1.1, upper panel A, lanes 1-5).

The data revealed that maximum level of OPN-induced NIK phosphorylation occurred at 7 min (upper panel A, lane 3) and also suggested that Thr-559 of NIK is crucial for OPN-induced NIK phosphorylation. Further treatment with αvβ3 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 same blots were reprobed with anti-NIK antibody as loading control (lower panels A and B, lanes 1-5). Western blot data was quantified by densitometric analysis and the fold changes were calculated. These results demonstrated that OPN binds with αvβ3 integrin receptor and induces NIK phosphorylation.
**Fig. 6.1.1. A, OPN induces NIK phosphorylation.** Cells were treated with 5 μM OPN for 0-30 min. The cell lysates containing 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-actin antibody (lower panel A) as loading controls. **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).

**6.1.2. 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, cells were treated with 5 μM OPN. In separate experiments, cells were individually transfected with wild type NIK or kinase negative NIK in 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.
**Fig. 6.1.2.** **OPN induces the interaction between phosphorylated NIK and IKK.** The cells were treated with 5 μM OPN. In separate experiments, cells were transfected with wt or kinase negative NIK and then treated with OPN. Cell lysates were immunoprecipitated with anti-IKKα/β antibody. Half of the sample was immunoblotted with anti-phospho-NIK antibody (upper panel, lanes 1-4) and other half was analyzed by anti-IKKα/β antibody (lower panel, lanes 1-4).

The results indicated that cells transfected with wild type NIK followed by treatment with OPN showed maximum interaction between phosphorylated NIK and IKK α/β compared with cells treated with OPN alone or untreated cells (Fig. 6.1.2, 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.

**6.1.3. OPN Induces NIK Activity and NIK Dependent IKK Activity-** 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.
Fig. 6.1.3. A, OPN induces NIK activity. Cells were treated with 5 μM OPN and cell lysates were immunoprecipitated with anti-NIK antibody and kinase assay was performed using IKK as substrate (upper panel A, lanes 1-2). The remaining half of the immunoprecipitated samples was immunoblotted with anti-NIK antibody (middle panel A). The fraction containing equal volume of kinase reaction mixture was analyzed by Western blot using anti-IKK antibody (lower panel A). B, OPN stimulates NIK dependent IKK activity. The cells were treated with 5 μM OPN or transfected with wild type and kinase negative NIK and then treated with OPN. Cell lysates were immunoprecipitated with anti-IKK α/β antibody and were used for kinase assay using IκBα as substrate (upper panel B). The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-IKK α/β antibody (middle panel B). The fraction of equal volume of kinase reaction mixture was immunoblotted with anti-IκBα antibody (lower panel B).

The radiolabeled, phosphorylated IKK specific band was detected in OPN-treated cells demonstrating that OPN induces NIK activity (Fig. 6.1.3, upper panel A, lane 2). The NIK activity is not detected in the untreated cells (lane 1). The remaining half of the immunoprecipitated samples was 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 IkBα 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 (upper panel B, 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 kinase domain of NIK plays crucial role in OPN-induced IKK activity. The remaining half of the immunoprecipitated samples were analyzed by Western blot using anti-IKKα/β antibody (middle panel B). A fraction of equal volume of samples from the kinase reaction mixture was analyzed by Western blot using anti-IκBα antibody (lower panel B). These results suggested that NIK plays significant role in OPN-induced IKK activity.

6.1.4. NIK Plays Crucial Role in OPN-induced IκBα phosphorylation and degradation- Since the earlier data showed that OPN-induced IKK activity is regulated by NIK; we 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. 6.1.4, upper panel, 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, lanes 1-4). The same blots were reprobed with anti-actin antibody as loading control (lower panel, lanes 1-4).
These data suggested that NIK plays crucial role in OPN-induced IκBα phosphorylation and degradation.

Fig. 6.1.4. NIK is required for OPN induced IκBα phosphorylation and degradation. The cells were either treated with 5 μM OPN or transfected with wild type or kinase negative NIK and then treated with OPN. Cell lysates were analyzed by Western blot using anti-phospho-IκBα antibody (upper panel, lanes 1-4). The same blots were reprobed with anti-IκBα (middle panel) or anti-actin (lower panel) antibody. All these bands were analyzed densitometrically and the fold changes were calculated.

6.1.5. OPN Stimulates αvβ3 Integrin-mediated 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 OPN alone, pretreated with αvβ3 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 this cells (Fig. 6.1.5, upper panels A and C, lanes 1-4). The data also indicated that OPN induced MEK-1 phosphorylation was inhibited by αvβ3 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 αvβ3 integrin dependent MEK-1 and ERK1/2 phosphorylations through NIK-mediated pathway.

Fig. 6.1.5. OPN induces αvβ3 integrin mediated NIK dependent MEK-1 and ERK1/2 phosphorylations. A-C, Cells were treated with 5 μM OPN alone, pretreated with anti-αvβ3 integrin antibody, RGD peptide (GRGDSP or GRGESP) or 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 (upper panels A and B) or anti-phospho-ERK1/2 (upper panel C) antibody. The same blots were reprobed with anti-MEK-1 (lower panels A and B) or anti-ERK1/2 (lower panel C) antibody.

6.1.6. OPN induces NIK/IKK mediated NFκB-DNA binding- We have 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, 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. 6.1.6, panel A, lanes 1-4).
Similarly, wild type IKKβ induced and dn IKKβ inhibited OPN-enhanced NFκB-DNA binding (panel B, lanes 1-4). These data suggested that OPN induces NFκB-DNA binding through NIK/IKK-mediated pathways.

**Fig. 6.1.6. 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, wild type and dn IKKβ and then treated with OPN. The nuclear extracts were prepared and analyzed by EMSA.

6.1.7. ERK1/2 is involved in OPN induced NIK mediated NFκB-DNA binding- To examine the effect of ERK1/2 on OPN-induced NFκB-DNA binding, cells were pretreated with PD98059 (0-50 μM) 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 PD98059, a MEK1 inhibitor suppressed OPN-induced NFκB-DNA binding (Fig. 6.1.7, panel A, lanes 1-4). Overexpression of wild type ERK1 or ERK2 showed an increase in OPN-induced NFκB-DNA binding whereas dn ERK1 or ERK2 reduced the OPN-induced NFκB-DNA binding (panel B, lanes 1-6). 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 C, lanes 1-4).
These results demonstrated that OPN induces NFκB-DNA binding through NIK/ERK1/2-mediated pathway.

**Fig.6.1.7. A-C, ERK1/2 is involved in OPN induced NIK mediated NFκB-DNA binding.** Cells were either pretreated with PD98059 or transfected with wt and dn ERK1 and ERK2 and then treated with OPN. In other experiments, 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.

6.1.8. OPN Induces NIK Dependent ERK/IKK-mediated NFκB Transactivation- To further investigate whether NIK regulates OPN-induced ERK/IKK dependent NFκB transcriptional activity, 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, super-repressor form of IκBα, wild type IKKα/β and dn IKKα/β, 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 determinations. The values were also analyzed by Student’s t test (p<0.001).
Fig. 6.1.8. A-C, OPN enhances NFκB-transactivation through both NIK/IKK and NIK/ERK mediated pathways

The data showed that wild type NIK enhanced but kinase negative NIK or super-repressor form of IκBα suppressed OPN-induced NFκB activity in these cells (Fig. 6.1.8, 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) suggesting that NIK regulates OPN-induced ERK/IKK dependent NFκB activation.

6.1.9. OPN Stimulates αvβ3 Integrin-mediated 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. 6.1.9, 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 was about 3- and 5- fold increase in MMP-9 activation when the cells were treated with 5 and 10 μM OPN respectively (lower panel A).

**Fig. 6.1.9.** A, OPN induces pro-MMP-9 activity as shown by zymography. A, cells were treated in absence or presence of OPN (0-10 μM). The conditioned media were collected and MMP-9 activity was analyzed by gelatin zymography. The arrows indicate both 92-kDa (Pro) and 86-kDa (active) MMP-9 specific bands (upper panel A, lanes 1-3). B, OPN stimulates αvβ3 integrin-mediated pro-MMP-9 activation. Cells were pretreated with anti-αvβ3 integrin antibody (20 μg/ml), GRGDSP or GRGESP peptide (10 μM) and then treated with 5 μM OPN. The levels of pro and active MMP-9 in the conditioned media were detected by Western blot using anti-MMP-9 antibody (upper panel B, lanes 1-5).
To check whether αvβ3 integrin or RGD peptide is involved in OPN-induced pro-MMP-9 activation, cells were pretreated with anti-αvβ3 integrin antibody, GRGDSP or GRGESP and then treated with 5 μM OPN. The conditioned media was 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 (upper panel B, lane 2) was reduced significantly when cells were individually treated with anti-αvβ3 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 αvβ3 integrin and RGD peptide play important role in OPN-induced MMP-9 activation.

6.1.10. OPN induces NIK/IKK dependent Pro-MMP-9 Activation- To investigate the role of NIK, IKK or NFκB 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 IKKα and IKKβ, wild type and dn ERK1/2 and then treated with 5 μM OPN. The level of MMP-9 activity was detected by zymography. The results indicated that wild type NIK enhanced whereas kinase negative NIK or super repressor form of IκBα suppressed the OPN-induced MMP-9 activation (Fig 6.1.10, upper panel A, lanes 2-5). Wild type IKKβ enhanced but dn IKKβ suppressed OPN-induced MMP-9 activation (upper panel B, lanes 2-4). No MMP-9 was detected in OPN-untreated cells (panels A and B, lane1). These bands were quantified densitometrically and analyzed statistically (lower panels of A and B). These results demonstrated that NIK regulates OPN-induced pro-MMP-9 activation through ERK/IKK-mediated pathways in these cells.
**Fig.6.1.10.** A and B, 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 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 A and B, lanes 1-5, lanes 1-4). All these bands were quantified by densitometry and are represented in the form of bar graph (lower panels A-B).

6.1.11. OPN Induces NIK dependent IKK and ERK1/2-mediated uPA Secretion- We have first examined whether NIK, IKK and ERK1/2 are involved in OPN-induced uPA secretion in B16F10 cells. Accordingly, cells were either treated with various concentrations of OPN (0-5 μM) or transfected with wild type NIK, kinase negative NIK, wild type and dn IKKα/β, wild type ERK1/2, dn ERK1/2 and then treated with OPN. The cell lysates were analyzed by Western blot using rabbit polyclonal anti-uPA antibody. The data showed that OPN-induced uPA secretion was enhanced when cells were transfected with wild type NIK and suppressed when transfected with kinase negative NIK (Fig. 6.1.11, upper panel A, lanes 1-5 and upper panel B, lanes 1-4). Wild type ERK1/2 stimulated and dn ERK1/2 blocked OPN-induced uPA secretion (upper panel C, lanes 1-6). All these blots were reprobed with anti-actin antibody (lower panels A-C) and bands were quantified by densitometric analysis and the fold changes are calculated (panels A-C).
These data suggested that OPN induces NIK dependent uPA secretion through ERK/IKK-mediated pathways.

**Fig. 6.1.11. 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, 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 same blots were reprobed with anti-actin antibody as loading control (lower panels A-C).

**6.1.12. uPA is required for OPN induced pro-MMP-9 activation**-To examine whether uPA plays any role in OPN-induced MMP-9 activation, the cells were pretreated with anti-uPA antibody and then treated with OPN (5 μM). The level of MMP-9 was detected by zymography and Western blot analysis. The results indicated that uPA antibody suppressed the OPN-induced MMP-9 activation as shown by zymography (Fig 6.1.12, panel A, lanes 1-3) and Western blot (panel B, lanes 1-3), indicating that uPA is involved in OPN induced MMP-9 activation.
Fig. 6.1.12. **A - B, uPA is required in OPN-induced pro-MMP-9 activation.** Cells were pretreated with anti-uPA antibody (25 μg/ml) and then treated with 5 μM OPN. The conditioned media was collected and the MMP-9 activities were analyzed by zymography (panel A, lanes 1-3) and by Western blot (panel B, lanes 1-3). The arrows indicate both 92- and 86-kDa MMP-9 specific bands.

6.1.13. **uPA and MMP-9 Play Crucial Roles in OPN-induced NIK Dependent IKK/ERK1/2-mediated Cell Migration** - Because NIK in presence of OPN regulates ERK and IKK dependent NFκB-mediated uPA secretion and uPA regulated pro-MMP-9 activation, therefore we sought to determine whether these signaling molecules play any role in OPN-induced cell migration. Accordingly, cells were treated with anti-MMP-9 or anti-uPA antibody or transfected with wild type NIK, kinase negative NIK, the super-repressor form of IκBα, wild type and dn IKKα or IKKβ and then used for migration assay. OPN (5 μM) was used in the upper chamber. The data indicated that anti-MMP-9 or anti-uPA antibody suppressed the OPN-induced cell migration (Fig. 6.1.13, panel A). Wild type NIK enhanced whereas kinase negative NIK or super repressor form of IκBα suppressed the OPN-induced cell migration (panel A). Similarly, wild type IKKα and IKKβ stimulated and dn IKKα and IKKβ inhibited the OPN-induced cell migration (panel B). These data demonstrated that OPN induces uPA and MMP-9 dependent cell migration through NIK/IKK/NFκB-mediated pathways.
6.1.14. Both MMP-2 and MMP-9 Play Important Roles in OPN-induced Cell Migration and Chemoinvasion- It has been earlier reported that OPN induces pro-MMP-2 activation which ultimately regulates cell motility, invasiveness and tumor growth. Our data indicated 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 migration or invasion assay. The data indicated that MMP-2 or MMP-9 antibody suppressed OPN-induced cell migration and chemoinvasion (Fig. 6.1.14, panels A and B). Pretreatment of cells with a mixture of anti-MMP-2 and anti-MMP-9 antibodies in combination exerted an additive effect and hence drastically reduced OPN-induced cell migration and chemoinvasion (panels A and B).

To ascertain whether there is any cross talk between OPN-induced pro-MMP-2 and pro-MMP-9 activations, cells were pretreated with two different concentrations of anti-MMP-2 antibody followed by treatment with OPN. The conditioned media was 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 suggested that OPN-induced activations of MMP-2 and MMP-9 occurred through two distinct signaling mechanisms and both played independent roles in regulating cell migration and chemoinvasion in B16F10 cells.

**Fig.6.1.14. A -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 (25µg/ml) or anti-MMP-9 antibody (25 µg/ml) or in combination of both. The purified human OPN was added to the upper chamber. **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/µl) 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)

6.1.15. **OPN Induces Pro-MMP-9 Activation in Tumor of Nude Mice**- The *in vitro* data prompted us to examine whether OPN plays any role in MMP-9 activation in tumor of nude mice. Accordingly, B16F10 cells were treated with OPN (10 µM) and injected
subcutaneously into the flanks of nude mice. Fig. 6.1.15 A shows typical photographs of tumors grown in 4-week old nude mice. 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).

To examine the levels of pro- and active MMP-9 in tumors, the samples were lysed, and MMP-9 expression was analyzed by zymography (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, lanes 1 and 2) and these are corroborated by the zymography data (panel B). This data demonstrated that OPN induces pro-MMP-9 activation in tumor of nude mice and it correlates with tumor growth in nude mice.
Table-I

**OPN induces tumor growth in nude mice**

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

<table>
<thead>
<tr>
<th>No. Nude mice</th>
<th>Treatment</th>
<th>Tumor weight (-fold changes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Four</td>
<td>control (PBS)</td>
<td>1.0 ± 0.15</td>
</tr>
<tr>
<td>Four</td>
<td>OPN (10 μM)</td>
<td>3.2 ± 0.17</td>
</tr>
</tbody>
</table>

**6.1.16 Discussion**

In this study, we have investigated whether OPN regulates pro-MMP-9 activation and MMP-9 dependent cell motility, invasiveness and tumor growth. Moreover, we have also 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. Our data indicated that OPN induced phosphorylation and activation of NIK and enhanced the interaction between phosphorylated NIK and IKKα/β in these cells. OPN also induced NIK and IKK dependent NFκB activation through phosphorylation and degradation of IκBα. Moreover, NIK in presence of OPN enhanced MEK-1 and ERK1/2 phosphorylations and MEK/ERK dependent NFκB activation. OPN induced NIK dependent ERK/IKK-mediated uPA secretion, uPA dependent pro-MMP-9 activation, cell migration and chemoinvasion. The level of pro-MMP-9 activation is higher in OPN induced tumors when compared to the control. These data demonstrated that OPN induces NIK dependent cell motility, tumor growth and 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 (MAPKKKs) including NIK and MEKK 1-3 are involved in the activation of IKK
complex (346). Kouba et al have shown that NIK regulates NFkB activation pathways in epidermal keratinocytes (347). Thus we have examined whether NIK has any effect in OPN-induced NFkB 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 also suggested that NIK strongly interacts with both IKKα and β (348). NIK also interacts with TRAF proteins including TRAF-3 as shown by yeast two hybrid systems (349). Our data revealed that OPN enhances the interaction between phosphorylated NIK and IKKα/β in B16F10 cells. Previous studies also indicated that IKK activation alone could not account for the total NFkB activity in HS294T cells (350). Foehr et al have recently demonstrated that NIK regulates differentiations of PC-12 cells through MEK/ERK pathways (351). These results prompted us to investigate whether OPN regulates NIK dependent NFkB activation through MAPK pathway in B16F10 cells. Our data indicated that transient over expression of wild type NIK upregulates OPN-induced MEK and ERK1/2 phosphorylations whereas kinase negative NIK abrogated these processes. Further OPN-induced NIK dependent MEK-1 phosphorylation was suppressed by anti-αvβ3 integrin blocking antibody and RGD peptide but not RGE peptide indicating that OPN regulates NIK-mediated MAPK activation through αvβ3 integrin mediated pathway.

Several reports have indicated that NFκB is involved in control of large number of cellular processes such as inflammatory and immune response, developmental processes, cell growth and apoptosis. In addition, NFκB is activated in several pathological conditions like arthritis, inflammation, asthma, neurodegenerative disease, heart disease and cancers (352). It has been earlier reported in our laboratory that OPN stimulated NFκB activation by inducing the PI 3-kinase/IKK activity in murine melanoma and breast cancer cells (353-355). However, it was not clear whether NIK is involved in regulation of OPN-induced NFκB activation and whether MAPK/IKK plays any role in this activation process in B16F10 cells. Our data indicated that OPN induces NIK dependent IKK activity through interaction between phosphorylated NIK and IKK. Transfection of these cells with wild type NIK and IKKα/β but not with kinase negative NIK or dn IKKα/β enhanced OPN-
induced NFκB-DNA binding and NFκB transactivation. Moreover, PD98059, a MEK-1 inhibitor down regulated NFκB-DNA binding and NFκB activation in cells transfected with wild type NIK followed by treatment with OPN. Over expression of wild type ERK1/2 but not dn ERK1/2 dramatically enhanced OPN-induced NFκB activation. These data suggested a link between OPN-induced NIK-IKK-NFκB and NIK-ERK-NFκB pathways in B16F10 cells.

Signals transduced by cell adhesion molecules play an important role in tumor cell attachment, motility and invasion, all of which regulate tumor metastasis. Cell-matrix interactions play a major role in tissue remodeling, cell survival and tumorigenesis. OPN, an ECM protein plays a significant role in cell adhesion, migration and metastasis. The overexpression of OPN is linked with various cancers and their metastatic potentials (8). MMPs are a family of Zn$^{2+}$ dependent endopeptidases that are responsible for remodeling of the extracellular matrix and degradation of ECM proteins. MMP-9 is known to degrade basement membrane, which normally separates the epithelial from 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 (335). Kim et al also demonstrated that MMP-9 activity but not MMP-2 activity significantly affects tumor intravasation into blood vessel and uPA is required for pro-MMP-9 activation (356, 357). Several other reports have indicated the correlation between uPA expression and metastatic potential and shown that uPA plays major role in regulating MMPs activation (288). 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 have shown that OPN induces uPA secretion and uPA dependent pro-MMP-9 activation. Pretreatment of cells with anti-αvβ3 integrin antibody or RGD but not RGE peptide inhibited OPN-induced pro-MMP-9 activation indicating that OPN induces pro-MMP-9 activation through αvβ3 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 pro-MMP-9 activation demonstrating that OPN regulates pro-MMP-9 activation through NIK/IKK/NFκB-mediated pathways. Recent data also indicated that the expression of MMP-9 is down regulated in ERK-mutated stable transfectants that inhibits glioma invasion in vitro (358). We have 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 upregulated OPN-induced uPA secretion leading to the activation of pro-MMP-9 indicating that OPN induces uPA dependent pro-MMP-9 activation through NIK/MAPK-mediated pathways.

Previous studies have indicated that uPA and MMP-9 expressions are inversely related to MT1-MMP expression in esophageal carcinoma (359). 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, invasiveness, tumor growth and angiogenesis. Pretreatment of cells with anti-uPA antibody suppressed OPN-induced pro-MMP-9 activation indicating that uPA plays crucial role in this process. Moreover, pretreatment of cells with anti-MMP-2 antibody along with anti-MMP-9 antibody dramatically suppressed the OPN-induced cell migration and chemoinvasion whereas pretreatment of cells with anti-MMP-2 antibody had no effect on OPN-induced pro-MMP-9 activation. These data suggested that both MMP-2 and MMP-9 are involved in OPN-induced cell migration and chemoinvasion but OPN-induced MMP-2 activation is distinct from MMP-9 activation. Wild type NIK, IKKα/β and ERK1/2 enhanced and kinase negative NIK, dn IKKα/β and dn ERK1/2 suppressed OPN-induced cell migration and chemoinvasion. OPN also induced the pro-MMP-9 activation in tumor of nude mice. These data demonstrated that OPN induces NIK regulated NFκB-mediated uPA dependent pro-MMP-9 activation, cell motility and tumor growth via ERK/IKK-mediated signaling pathways.

Taken together, OPN induces cell migration, tumor growth and NIK dependent NFκB-mediated uPA secretion and pro-MMP-9 activation by activating IKK/ERK signaling pathways (Fig.6.1.17). These findings may be useful in designing novel therapeutic interventions that block the OPN regulated NIK dependent IKK and ERK1/2-mediated NFκB activation resulting in reduction of uPA secretion and pro-MMP-9 activation and consequent blocking of cell motility, invasiveness and melanoma growth.
Fig. 6.1.17. Molecular mechanism of OPN-induced NIK regulated NF-κB mediated uPA secretion and Pro-MMP-9 activation through MAPK/IKK pathways.
6.2. The Differential role of JNK1 in Regulating OPN-induced NIK/MEKK1-dependent AP-1 mediated Pro-MMP-9 Activation

Mitogen Activated Protein Kinases (MAPK) are the principal family of kinases which are frequently altered during cancer progression. Extensive studies have analyzed the role of MAPK as valuable therapeutic target for the treatment of cancer. In this regard, in this part of the study, we have mainly analyzed on the molecular mechanism by which OPN regulates the various MAPK pathways particularly the ERK and JNK leading to transcription factor mediated MMP-9 activation which ultimately controls melanoma progression. Our data indicated that OPN induces \( \alpha_v \beta_3 \) 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.

Results

6.2.1. OPN Induces \( \alpha_v \beta_3 \) Integrin Dependent MEKK1 and JNK1 Phosphorylations-

To investigate the role of OPN on MEKK1 and JNK1 phosphorylations and to demonstrate the involvement of \( \alpha_v \beta_3 \) integrin in this activation process, B16F10 cells were treated with 5 \( \mu \)M OPN at 37°C or pretreated with anti-\( \alpha_v \beta_3 \) integrin antibody or RGD/RGE peptide (GRGDSP or GRGESP) and then treated with OPN. The cell lysates were immunoprecipitated with anti-MEKK1 antibody and analyzed by Western blot using anti-phosphoserine antibody. For JNK phosphorylation studies, cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody.

The data revealed that maximum level of OPN-induced MEKK1 and JNK1 phosphorylations occurred at 5 min and 15 min respectively (Fig. 6.2.1, upper panel A and C, lanes 1-5). As loading controls, same blots were reprobed with anti-MEKK1 or anti-
JNK1 antibody (lower panels A and C, lanes 1-5). Pretreatment of cells with anti-αβ₃ integrin antibody or RGD (GRGDSP) but not RGE (GRGESP) peptide suppressed the OPN-induced MEKK1 and JNK1 phosphorylations in these cells (upper panel B and D, lanes 1-5). Same blots were reprobed with anti-MEKK1 or anti-JNK1 antibody (lower panels B and D, lanes 1-5). All the bands were quantified by densitometric analysis and the fold changes were calculated. These results demonstrated that OPN induces MEKK1 and JNK1 phosphorylations through αβ₃ integrin-mediated pathway.

Fig.6.2.1. A-D, OPN stimulates αβ₃ integrin-mediated MEKK1 and JNK1 phosphorylation. Cells were treated with 5 μM OPN or pretreated with anti-αβ₃ integrin antibody, GRGDSP or GRGESP and then treated with OPN. Cell lysates were immunoprecipitated with anti-MEKK1 antibody and analyzed by Western blot using anti-phosphoserine antibody (upper panels A and B) and same blots were reprobed with anti-MEKK1 antibody (lower panels A and B). For JNK1 phosphorylations, cells were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody (upper panels C
and D) and same blots were reprobed with anti-JNK1 antibody (upper panels C and D)

6.2.2. MEKK1 but not NIK is required for OPN-induced αvβ3 Integrin-mediated JNK1 Phosphorylation- Since our earlier data indicated that OPN induces NIK-dependent NFκB mediated pro-MMP-9 activation through ERK/IKK mediated pathways, we sought to examine whether NIK/MEKK1 plays any role in OPN-induced JNK1 phosphorylation in B16F10 cells. Accordingly, cells were transfected with wild type and kinase negative NIK or wild type and kinase negative MEKK1 and then treated with OPN. Cell lysates were immunoprecipitated with anti-JNK1 antibody and analyzed by Western blot using anti-phosphotyrosine antibody. Wild type MEKK1 enhanced whereas kinase negative MEKK1 suppressed the OPN-induced JNK phosphorylation (Fig. 6.2.2, upper panel, lanes 1-4). The data also indicated that OPN-induced JNK1 phosphorylation was unaffected upon transfection of cells with both wild type and kinase negative NIK (lanes 5 and 6). The same blots were reprobed with anti-JNK1 antibody as loading control (lower panel, lanes 1-6). These data suggested that MEKK1 but not NIK plays crucial role in OPN-induced JNK1 phosphorylation.

Fig. 6.2.2. 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, lanes 1-6). Same blots were reprobed with anti-JNK1 antibody (lower panel). All these bands were analyzed densitometrically and the -fold changes were calculated.

6.2.3. 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 used for JNK kinase assay using recombinant c-Jun as substrate. The radiolabeled, phosphorylated c-Jun specific band is detected in OPN-treated cells demonstrating that OPN induces JNK1 activity (Fig. 6.2.3, upper panel 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 and JNK kinase assay was performed. Cells transfected with wild type MEKK1 followed by treatment with OPN showed maximum JNK1 activity (lane 3) compared with untreated cells (lane 1). The cells transfected with kinase negative MEKK1 followed by treatment with OPN showed reduced level of JNK1 activity (lane 4) indicating that kinase domain of MEKK1 plays crucial role in OPN-induced JNK1 activity. The data also indicated that OPN-induced JNK1 activity was unaffected upon over expression 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 significant role in modulating OPN-induced JNK activity.
Fig. 6.2.3. A and B, OPN induces MEKK1 (panel A) but not NIK (panel B) dependent JNK1 activity. Cells were treated with 5 μM OPN or 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 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 was immunoblotted 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).

6.2.4. JNK1 Plays Crucial Role in OPN-induced MEKK1-dependent ERK1/2 Inactivation- MEKK-1 functions as a MAPKKK in the JNK pathway; however several reports have suggested that MEKK-1 may also affect the ERK pathway. 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 MBP as substrate. The data indicated that overexpression of wild type MEKK1 almost completely attenuates OPN-induced ERK activation (Fig. 6.2.4, upper panel A, lanes 1-4). This abrogation depends on MEKK-1 kinase activity because ERK 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 A, lanes 1-4). These results suggested that MEKK1 negatively regulates OPN-induced ERK activation.

![Figure 6.2.4](image)

**Fig. 6.2.4. Panel A, 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 MBP as substrate (upper panel A, lanes 1-4). Half of the immunoprecipitated samples were immunoblotted with anti-ERK1/2 antibody (lower panel A, lanes 1-4). **Panel B, 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 B, lanes 1-6). Half of the immunoprecipitated samples were analyzed by Western blot using anti-ERK1/2 antibody (lower panel B).

Previous results indicated that JNK<sup>−/−</sup> mice showed enhanced phosphorylation of ERK leading to tumor growth (365), therefore we speculated that activation of JNK1 by OPN may play role in suppression of ERK1/2 activation. Accordingly, cells transfected with wild type MEKK1 were cotransfected with either wild type or dn 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 B, 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 B, lanes 1-6). These data suggested that JNK1 acts as negative regulator in OPN-induced MEKK1 dependent ERK1/2 activation.

6.2.5. Overexpression of MEKK1 does not affect the OPN-induced NIK dependent ERK activation- To examine whether NIK plays any role in regulation of OPN-induced MEKK-dependent JNK1-mediated ERK1/2 inactivation, cells were transfected with wild type NIK or 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 as described above.

![Image of experiment results](image)

**Fig.6.2.5.** 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 (upper panel). Half of the immunoprecipitated samples were immunoblotted with anti-NIK antibody (middle panel). The NIK activity was also assayed under the
The data indicated that expression of active or mutant MEKK-1 had no effect on OPN-induced NIK activity (Fig. 6.2.5, upper panel, lanes 1-5), suggesting that overexpressed NIK even in presence of MEKK upregulates OPN-induced ERK activation. Half of the immunoprecipitated samples were immunoblotted with anti-NIK antibody (middle panel, lanes 1-5). The level of NIK activity was also detected by using IKK as substrate (lower panel, lanes 1-5).

6.2.6. OPN Induces αvβ3 Integrin-mediated NIK and MEKK1 Dependent c-Jun Expression- Earlier reports have demonstrated that MEKK in presence of certain stimuli induces JNK-dependent c-Jun phosphorylation and enhances AP-1 activation (364). 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 level of c-Jun expression in the nuclear extracts was detected by Western blot analysis using anti-c-Jun antibody. The results indicated that OPN induces c-Jun expression and maximum expression was observed at 1h (Fig. 6.2.6, panel A, lanes 1-5).

To further confirm that this OPN-induced c-Jun expression is occurred through αvβ3 integrin-mediated pathway; cells were pretreated with anti-αvβ3 antibody, RGD/RGE peptide and then treated with OPN for 1h. 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 for 1h. The level of c-Jun expression in the nuclear extracts was determined by Western blot. Wild type NIK enhanced and kinase negative NIK suppressed OPN induced c-Jun expression (panel C, lanes 1-4). 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 unaffects OPN-induced JNK1 phosphorylation and kinase activity significantly upregulates 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.

**Fig. 6.2.6. Panels A and B, OPN enhances αvβ3 integrin-mediated c-Jun expression.** Cells were treated with 5 μM OPN for 0-4 h or pretreated with anti-αvβ3 integrin antibody, RGD/RGE peptide (GRGDSP or GRGESP) and then stimulated with OPN. The level of c-Jun in the nuclear extracts was analyzed by Western blot (panels A and B, lanes 1-5). **Panel C, NIK and MEKK1 play independent 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 analyzed by Western blot using anti-c-Jun antibody (panel C, lanes 1-6). All these bands were analyzed densitometrically and the fold changes were calculated. The data shown here represent three experiments exhibiting similar effects.

**6.2.7. NIK and MEKK1 Play Important Roles in OPN-induced AP-1-DNA Binding**

To further examine whether NIK and MEKK1 regulate OPN-induced AP-1-DNA binding, 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 used for EMSA using $^{32}$P-labeled AP-1 oligonucleotides. Wild type NIK enhanced and kinase negative NIK suppressed OPN-induced AP-1-DNA binding (Fig. 6.2.7, panel A, lanes 1-4). Similarly, wild type MEKK1 induced and kinase negative MEKK1 inhibited OPN-enhanced AP-1-DNA binding (panel B, lanes 1-4). These data suggested that OPN induces AP-1-DNA binding through both NIK and MEKK1 mediated pathways.

![Fig. 6.2.7. OPN induces NIK (panel A) and MEKK1 (panel B) dependent AP-1-DNA binding.](image)

**Fig. 6.2.7. 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 analyzed by EMSA (panels A and B, lanes 1-4).

### 6.2.8. OPN-induced NIK-mediated AP-1-DNA binding is independent of JNK-

To examine the role of JNK1 on OPN-induced AP-1 DNA binding, cells were pretreated with 0-50 μM SP600125 (JNK1 inhibitor) and then treated with OPN. The nuclear extracts were used for EMSA. SP600125 suppressed OPN-induced AP-1-DNA binding in a dose dependent manner (Fig. 6.2.8, panel A, 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 B, 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 C, lanes 1 and 2).

**Fig. 6.2.8.** Panel A, JNK is involved in OPN-induced AP-1-DNA binding. Cells were pretreated with 0-50 μM of SP600125 (JNK inhibitor) and then treated with OPN. The nuclear extracts were analyzed by EMSA (lanes 1-4). Panel B, 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 of SP600125 (JNK inhibitor) and then treated with OPN. The nuclear extracts were analyzed by EMSA (lanes 1-4). Panel C, supershift assay. The nuclear extracts from OPN treated cells were incubated with anti-c-Jun antibody and EMSA was performed (lanes 1 and 2). The results shown here represent three experiments exhibiting similar effects.

6.2.9. **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 cotransflecting 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).

**Fig. 6.2.9. 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 OPN. Cell lysates were used to measure the luciferase activity (panels A and B). The values were normalized to Renilla luciferase activity. Panel C, JNK1 is
**differentially regulated in OPN-induced NIK dependent AP-1 transactivation.**

Cells were transfected with wild type and dn 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, 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 mean ± S.E. of triplicate determinations are plotted. 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. 6.2.9, 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 suppressed moderately 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 MEKK/JNK-mediated pathways and further suggested that OPN induces a shift in balance towards activation of ERK followed by AP-1 activation.

**6.2.10. OPN Stimulates NIK and MEKK1-mediated c-Jun Dependent uPA Secretion and uPA-dependent MMP-9 Activation**- To further analyze whether MEKK1, JNK1 and c-Jun are involved in OPN-induced uPA secretion, 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. 6.2.10, upper panel A, lanes 1-6).
Fig. 6.2.10. **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 level of uPA in the cell lysates was analyzed by Western blot (upper panel A, lanes 1-6). The same blots were reprobed with anti-actin antibody (lower panel A, lanes 1-6). **Panel B, JNK1 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 (upper panel B, lanes 1-5). The same blots were reprobed with anti-actin antibody (lower panel B, lanes 1-5) as loading control. All these bands were quantified by densitometrically.

Wild type JNK1 stimulated and dn JNK1 or JNK1 inhibitor (SP600125) moderately reduced OPN-induced uPA secretion due to upregulation of ERK-mediated c-Jun expression leading to activation of AP-1 (upper panel B, lanes 1-5). All these blots 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.

6.2.11. **Roles of NIK, MEKK1 and JNK1 in OPN-induced uPA dependent MMP-9 activation** - 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 and dn JNK1 or pretreated with SP600125 and then treated with OPN. The conditioned media were collected and zymography was performed. Increased level of MMP-9 activation (86 kDa) was observed when cells were treated with OPN (Fig. 6.2.11, panels A-C, lane 2). Almost no MMP-9 specific band was detected in the untreated cells (lane 1). Wild type MEKK1 enhanced whereas kinase negative MEKK1 suppressed OPN-induced MMP-9 activation (panel A, lanes 1-4). Wild type JNK1 enhanced and dn JNK1 moderately reduced OPN-induced MMP-9 activation (panel C, lanes 1-4). JNK1 inhibitor, SP600125 retained OPN-induced MMP-9 activation due to upregulation of ERK activation (panel B, lanes 1-4). These data suggested that OPN induces uPA secretion and uPA-dependent MMP-9 activation through both NIK/ERK1/2 and MEKK1/JNK1-mediated pathways and further demonstrated that OPN induces a shift in balance towards activation of ERK and thereby retaining the uPA secretion and MMP-9 activation.

**Fig. 6.2.11.** Panels A-C, JNK1 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 activity of MMP-9 was examined by gelatin zymography (panels A-C, lanes 1-4). The data shown here represent three experiments exhibiting similar effects.
6.2.12. MEKK1, JNK1 and c-Jun Play Crucial Roles in OPN-induced α,β₃ Integrin-mediated Cell Migration and Chemoinvasion- Since our data indicated that OPN induces α,β₃ integrin-mediated NIK/ERK and MEKK1/JNK1 dependent c-Jun expression leading to uPA secretion and uPA-dependent MMP-9 activation, we examined whether OPN-induced NIK/MEKK1 dependent MMP-9 activation 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 presence of LipofectAMINE Plus and then used for migration and invasion assay. In separate experiments, cells were pretreated with JNK1 inhibitor (SP600125) or transfected with wild type and kinase negative NIK and then treated with JNK1 inhibitor. The data showed that wild type MEKK1, JNK1 and c-Jun enhanced and mutant MEKK1 and c-Jun suppressed OPN-induced cell migration (Fig.6.2.12, 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 and chemoinvasion (panels C and 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 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.
Fig. 6.2.12. Panels A-C, Roles of MEKK1, JNK1 and c-Jun in OPN-stimulated cell migration and chemoinvasion. The migration assay was conducted either by using untreated cells or cells transfected with wild type and kinase negative MEKK1 or wild type and dn JNK1 or wild type and dn c-Jun. 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 unaltered OPN-induced cell migration (panel C). Note that JNK1 plays differential role in OPN-induced NIK/MEKK1 dependent cell migration. Same results were obtained in chemoinvasion assay (panels B, D and F). The results are expressed as the means ± S.E. of three determinations.
6.2.13. 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 tumor 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 II shows the fold change of tumor weight grown in 4-week old nude mice. There were at least 6- and 5.4-fold increased of 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 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 level of c-Jun expression (Fig. 6.2.13, panel A, lanes 1-6) and AP-1-DNA binding (panel B, lanes 1-6) compared to cells treated with OPN alone or transfected with mut NIK or mut MEKK1.

**Fig. 6.2.13. Panels A and B, OPN enhances NIK/MEKK-dependent c-Jun expression and AP-1-DNA binding in tumors of nude mice.** The samples obtained from tumor generated by wild type and kinase negative NIK or wild type and kinase negative MEKK1 were lysed and nuclear extracts were prepared and
subjected to Western blot using anti-c-Jun antibody (panel A, lanes 1-6). The same nuclear extracts were used for EMSA assay (panel B, lane 1-6).

To further examine the levels of uPA and MMP-9 in these tumors, the tumor lysates were prepared 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) and MMP-9 activation (panel D, lanes 1-6) compared to cells treated with OPN alone or transfected with mutant NIK or MEKK1. These data demonstrated that OPN induces both NIK and MEKK-1-mediated AP-1 activation leading to uPA secretion and pro-MMP-9 activation through JNK1 dependent/independent pathways in tumor of nude mice and these data corroborates with in vitro data.

Fig. 6.2.13. Panels C and D, OPN induces NIK/MEKK-dependent uPA expression and MMP-9 activation in same tumors of nude mice. The samples obtained from tumor generated by wild type and kinase negative NIK or MEKK1 were lysed and level of uPA was analyzed by Western blot using anti-uPA antibody (panel C, lanes 1-6). The level of MMP-9 in these samples was also analyzed by Western blot using anti-MMP-9 antibody (panel D, lanes 1-6). The data shown here represent three experiments exhibiting similar effects.
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 PBS 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>Four</td>
<td>Control (PBS)</td>
<td>1.0 ± 0.17</td>
</tr>
<tr>
<td>Four</td>
<td>OPN (10 μM)</td>
<td>3.1 ± 0.15</td>
</tr>
<tr>
<td>Four</td>
<td>Wt NIK+ OPN (10 μM)</td>
<td>6.1 ± 0.14</td>
</tr>
<tr>
<td>Four</td>
<td>Mut NIK+ OPN (10 μM)</td>
<td>1.4 ± 0.16</td>
</tr>
<tr>
<td>Four</td>
<td>Wt MEKK1+ OPN (10 μM)</td>
<td>5.4 ± 0.12</td>
</tr>
<tr>
<td>Four</td>
<td>Mut MEKK1+ OPN (10 μM)</td>
<td>1.2 ± 0.15</td>
</tr>
</tbody>
</table>

6.2.14 Discussion

In this part of the study, 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. Our studies demonstrated that OPN induces αvβ3 integrin-mediated MEKK1 phosphorylation leading to c-Jun activation in a JNK dependent manner. The data also revealed that OPN induces NIK activation that 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 crosstalk between MEKK1/JNK and NIK/ERK pathways. OPN binding to αvβ3 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 (9-12). Recent studies have demonstrated that downregulation of NIK activation does not affect TNFα-induced JNK activation (360). It has been also reported that ERK and JNK pathways play crucial role in regulating MMP-9 activation and cell motility in growth factor stimulated human epidermal keratinocytes (361). These results prompted us to investigate whether binding of OPN to αvβ3 integrin receptor regulates JNK1 activation and whether NIK is involved in this activation process. In this study, we have demonstrated that OPN induces αvβ3 integrin-mediated MEKK1 and JNK1 phosphorylations in B16F10 cells. Pretreatment of cells with anti-αvβ3 integrin antibody and RGD but not RGE peptide inhibited OPN-induced MEKK1 and JNK1 phosphorylations indicating that αvβ3 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 MAPKKK that activates JNK via phosphorylation of its downstream kinase MKK4 (362). Shen et al have recently reported that sustained activation of JNK blocks ERK activation in response to mitogenic factors like EGF and PMA (363). 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 leading to enhanced JNK activation may affect OPN-induced NIK-mediated ERK activation. Our data demonstrated a negative crosstalk 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 (364). This may be implicated to the short and long phase of MEKK1 activation which results in different cellular response, a short phase activation which leads to ERK activation and long phase activation 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. This data is consistent with the recent report that JNK1 deficiency stimulates TPA-induced ERK phosphorylation leading to enhanced skin tumorigenesis (365).

It is well established that JNK, a member of the MAPK 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. Previous reports have demonstrated that AP-1 is involved in several cellular processes such as cell growth, apoptosis, cell motility (240) and also in a number of pathological conditions. Natoli et al have reported that overexpression of NIK which does not activate JNK, strongly activates transcription directed by a canonical AP-1 site (360). Since our data indicated 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. The 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 unaffects JNK activation significantly upregulates AP-1-DNA binding and transcriptional activity indicating that OPN induces NIK dependent AP-1 activation which is independent of JNK.

Our data indicated 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, JNK1 and c-Jun enhanced and kinase negative MEKK1, JNK1 and dn c-Jun suppressed the OPN-induced uPA dependent MMP-9 activation, cell motility and chemoinvasion. 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. However, transfection of cells with 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. This may be due to the crosstalk between MEKK1/JNK and NIK/ERK pathways. This data is consistent with the recent data reported by She et al that disruption of the JNK1 gene resulted in increase in ERK phosphorylation leading to enhancement of skin tumorigenesis (365). The \textit{in vitro} data is also supported by \textit{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.

In summary, the data demonstrated that 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.6.2.14). 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.
Fig. 6.2.14. 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 integrin induced the phosphorylation 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 presence of OPN regulate AP-1 dependent uPA secretion and MMP-9 activation and all of these control cell motility, invasion and tumor growth.
6.3. MelCAM, a Melanoma Specific Marker Plays Crucial Role in Osteopontin-induced AP-1 dependent Sp-1 activation, uPA secretion, MMP-9 activation and melanoma progression

**Results**

6.3.1. OPN Stimulates αvβ3 Integrin-mediated NIK Dependent MelCAM Expression-
To examine the effect of OPN on MelCAM expression, B16F10 cells were treated with increasing concentrations of OPN (0-5 μM) for 16h at 37°C. The cell lysates were analyzed by Western blot using goat polyclonal anti-MelCAM antibody. The data revealed that OPN induces MelCAM expression in dose dependent manner (Fig.6.3.1, panel A, lanes 1-6). To examine whether αvβ3 integrin and NIK are involved in OPN-induced MelCAM expression, cells were pretreated with anti-αvβ3 integrin antibody, GRGDSP or GRGESP and then treated with 5 μM OPN. In separate experiments, cells were stably transfected with wild type or kinase negative NIK in the presence of LipofectAMINE Plus and then treated with OPN. The level of MelCAM expression was determined by Western blot analysis. The data demonstrated that αvβ3 integrin antibody (upper panel B, lane 3) and GRGDSP peptide (lane 4) but not with GRGESP peptide (lane 5) suppressed the OPN-induced MelCAM expression in these cells. Similarly wild type NIK enhanced and kinase negative NIK suppressed OPN-induced MelCAM expression (upper panel C and D) suggesting that NIK is involved in OPN-induced MelCAM expression.
**Fig. 6.3.1. A-D, OPN stimulates αvβ3 integrin-mediated NIK dependent MelCAM expression** - The role of OPN in regulation of MelCAM expression was analyzed by treating the cells with 0-5 μM OPN at 37°C for 16h. In separate experiments, cells were pretreated with anti-αvβ3 integrin antibody (20 μg/ml), GRGDSP or GRGESP peptide (10 μM) and then treated with OPN. The cell lysates were analyzed by Western blot using anti-MelCAM antibody (upper panels A-D, lanes 1-5). The same blots were reprobed with anti-actin antibody (lower panels A-D, lanes 1-5). All these bands were analyzed densitometrically and the fold changes were calculated. The data shown here represent three experiments exhibiting similar effects.

The same blots were reprobed with anti-actin antibody as loading control (lower panels A-D). All these bands were analyzed densitometrically (Kodak Digital Science), and the fold changes were calculated. The OPN-induced MelCAM expression is further confirmed by immunofluorescence studies.
Effect of OPN on MelCAM expression by immunofluorescence. Cells grown on glass slides were treated with 5 μM OPN for 16h. The cells were fixed and incubated with anti-MelCAM antibody, followed by incubation with FITC-conjugated anti-rabbit IgG, and analyzed under confocal microscopy.

6.3.2. OPN Induces the Interaction Between MelCAM and αvβ3 Integrin- To delineate whether OPN induces the interaction between MelCAM and αvβ3 integrin and whether NIK plays any role in this process, cells were treated with 5 μM OPN or transfected with wild type NIK or kinase negative NIK and then treated with OPN. Cell lysates were immunoprecipitated with goat polyclonal anti-MelCAM antibody. Half of the immunoprecipitated samples were analyzed by Western blot using anti-αvβ3 integrin antibody, and the remaining half of the samples were immunoblotted with anti-MelCAM antibody. The results indicated that OPN induces the interaction between MelCAM and αvβ3 integrin and cells transfected with wild type NIK enhanced OPN-induced interaction compared to non-transfected cells or cells transfected with kinase negative NIK (Fig. 6.3.2, upper and lower panels, lanes 1-4). These data were further confirmed by reverse pull down experiments and essentially similar results were obtained. These results suggested that OPN enhances the NIK-dependent interaction between MelCAM and αvβ3 integrin.
Fig. 6.3.2. OPN induces the interaction between MelCAM and αvβ3 integrin. The cells were treated with OPN. In separate experiments, cells were transfected with wt or kinase negative NIK and then treated with OPN. Cell lysates were immunoprecipitated with anti-MelCAM antibody. Half of the samples were immunoblotted with anti-αvβ3 antibody (upper panel, lanes 1-4) and other half was analyzed by anti-MelCAM antibody (lower panel, lanes 1-4). The data shown here represent three experiments exhibiting similar effects.

6.3.3. OPN Enhances FAK Phosphorylation and FAK Dependent NIK Activation - To ascertain the role of OPN in regulating FAK phosphorylation, cells were treated with 5 μM OPN for 0-30 min at 37°C. The cell lysates were immunoprecipitated with rabbit polyclonal anti-FAK antibody and analyzed by Western blot using anti-phosphotyrosine antibody (Fig. 6.3.3, upper panel A, lanes 1-5). The data revealed that maximum level of OPN induced FAK phosphorylation occurred at 5 min (lane 2). The same blot was reprobed with anti-FAK antibody as loading control (lower panel A, lanes 1-5).

To examine whether FAK plays any role in OPN induced NIK phosphorylation, cells were treated with 5 μM OPN alone or transfected with FAK siRNA and then treated with OPN. Cell lysates were analyzed by Western blot using rabbit polyclonal anti-phospho-NIK antibody (upper panel B, lanes 1-4). The data revealed that silencing of FAK expression completely abrogates OPN-induced NIK phosphorylation (upper panel B, lanes 1-4) indicating that FAK plays crucial role in OPN-induced NIK phosphorylation. The same blots were reprobed with anti-NIK antibody (lower panel B, lanes 1-4).
**Fig. 6.3.3.** A, OPN stimulates FAK phosphorylation. B16F10 cells were treated with 5 μM OPN for 0-30 min. The cell lysates were immunoprecipitated with anti-FAK antibody and analyzed by Western blot using anti-phospho-tyrosine antibody (upper panel A, lanes 1-5). The blots were reprobed with anti-FAK (lower panel A) as loading controls. B, Silencing of FAK abrogates NIK phosphorylation. Cells were treated with 5 μM OPN alone or transfected with FAK siRNA and then treated with OPN. Cell lysates were analyzed by Western blot using anti-phospho-NIK antibody (upper panel B, lanes 1-4). The level of NIK was also analyzed by Western blot using anti-NIK antibody (lower panel B, lanes 1-4).

6.3.4. OPN Regulates a Bidirectional Signaling Between FAK and MelCAM- MelCAM possesses conserved protein kinase recognition motifs in its cytoplasmic domain, suggesting its potential involvement in intracellular signaling (83). To examine whether MelCAM reciprocally regulates OPN-induced FAK followed by NIK phosphorylation, cells were treated with MelCAM blocking peptide (0-20 μg/ml) and then treated with OPN. Cell lysates were immunoprecipitated with anti-FAK antibody and the level of phosphorylated FAK was analyzed by Western blot using anti-pTyr antibody. Similarly, cell lysates were immunoblotted with anti-pNIK antibody. The data revealed that the levels of OPN-induced phosphorylated FAK and NIK were significantly downregulated upon treatment with MelCAM blocking peptide (Fig 6.3.4, upper panels A and B, lanes 1-4). The same blots were reprobed with anti-FAK or anti-NIK antibody as loading controls (lower
panels A and B, lanes 1-4). These data suggested that OPN induces a reciprocal regulation loop between MelCAM and FAK.

![Diagram](image)

**Fig. 6.3.4.** A and B, OPN induces a reciprocal regulation loop between MelCAM and FAK. To examine whether MelCAM reciprocally regulates OPN induced FAK and NIK phosphorylation, cells were treated with MelCAM blocking peptide (0-20 $\mu$g/ml) and then treated with OPN. Cell lysates were immunoprecipitated with anti-FAK antibody and analyzed Western blot using anti-pTyr antibody or anti-pNIK antibody (upper panels A and B, lanes 1-4). The same blots were reprobed with anti-FAK and anti-NIK antibody as loading controls (lower panels A and B, lanes 1-4). The bands were quantified by densitometric analysis, and the fold changes were calculated.

### 6.3.5. OPN Induces FAK and NIK Dependent Sp1 Expression

Earlier report indicated that Sp1 is an important transcription factor involved in the regulation of MelCAM expression and this was confirmed by deletion analysis where removal of all putative Sp1 sites reduced the promoter activity of MelCAM by 80% (367, 368). To examine whether OPN regulates Sp1 expression and to ascertain the role of FAK and NIK in this process, cells were either treated with 5 $\mu$M OPN or transfected with wild type or kinase negative NIK or FAK siRNA and then treated with OPN. To further ascertain the role of MelCAM in OPN-induced Sp1 expression, cells were pretreated with MelCAM blocking peptide (0-20 $\mu$g/ml) and then treated with OPN. The nuclear extracts were prepared and the level of
Sp1 expression was detected by Western blot using anti-Sp1 antibody. The data indicated that wild type NIK enhanced and kinase negative NIK suppressed OPN-induced Sp1 expression ((Fig. 6.3.5, panel A, lanes 1-6). Treatment with MelCAM blocking peptide dose dependently suppresses OPN-induced Sp1 expression (panel B, lanes 1-4). Silencing of FAK expression resulted in complete suppression of Sp1 expression (panel C, lanes 1-4). These data further suggested that OPN induces FAK and NIK dependent Sp1 expression which is also bidirectionally regulated by MelCAM.

![Western Blot Image](image)

**Fig. 6.3.5. A and C, OPN induces FAK/NIK mediated AP-1 dependent Sp1 expression.** Cells were either treated with OPN or transfected with wild type and kinase NIK or wild type and dn c-Jun or FAK siRNA and then stimulated with OPN. The level of Sp1 in the nuclear extracts was analyzed by Western blot using anti-Sp1 antibody (panels A and C). **B, MelCAM is involved in OPN induced Sp1 expression.** Cells were pretreated with MelCAM blocking peptide (0-20 μg/ml) and then treated with OPN. The level of Sp1 expression in the nuclear extracts was detected by Western blot using anti-Sp1 antibody (panel C, lanes 1-4). All these bands were analyzed densitometrically and the fold changes were calculated. The results shown here represent three experiments exhibiting similar effects.
6.3.6. FAK, NIK and MelCAM Play Crucial Roles in OPN-induced Sp1-DNA Binding. To further examine whether FAK and NIK regulate OPN-induced Sp1-DNA binding, cells were treated with 5 μM OPN or transfected with wild type or kinase negative NIK or FAK siRNA and then treated with OPN. In separate experiments, cells were treated with MelCAM blocking peptide (0-20 μg/ml) and then treated with OPN. The nuclear extracts were prepared and analyzed by EMSA using 32P-labeled Sp1 oligonucleotide. Transfection with FAK siRNA and kinase negative NIK suppressed and wild type NIK enhanced OPN-induced Sp1-DNA binding (Fig. 6.3.6, panels A-B). Treatment with MelCAM blocking peptide dose dependently suppressed OPN-induced Sp1 expression (panel B).

**Fig. 6.3.6. A-B, FAK, NIK and MelCAM are involved in OPN induced Sp1 DNA binding.** B16F10 cells were treated with 5 μM OPN or transfected with FAK siRNA, wild type and kinase negative NIK and then stimulated with OPN. In other experiments, cells were pretreated with MelCAM blocking peptide (0-20 μg/ml) and then treated with OPN. **C. OPN induces AP-1 dependent Sp1 DNA binding.** Cells were transfected with wild type and dn c-Jun and then treated with OPN. The nuclear extracts were analyzed by EMSA.
OPN-stimulated Sp1 activation is Dependent on AP-1 Activation- Previous reports have indicated that both AP-1 and Sp1 are regulated by the same intracellular signal transduction cascades (368). Furthermore, the response to Sp1 is strikingly enhanced when AP-1 subunits are present and vice versa. We examined whether AP-1 activity is required for OPN-induced Sp1 activation. Accordingly, cells were transfected with wild type and dominant negative c-Jun and then treated with OPN. The nuclear extracts were prepared and the level of Sp1 expression was analyzed by Western blot using anti-Sp1 antibody and EMSA using \(^{32}\)P-labeled Sp1 oligonucleotide. Transfection with wild type c-Jun enhanced and dn c-Jun suppressed the OPN induced DNA-binding (Fig. 6.3.6, panel C, lanes 1-4). These data indicated that AP-1 activity is required for OPN-induced Sp1 expression and activation.

6.3.7. OPN Induces FAK Dependent Sp1-mediated MelCAM Expression, uPA secretion and MMP-9 activation- To examine whether FAK is involved in OPN-induced MelCAM expression and MelCAM dependent uPA expression and MMP-9 activation, cells were transfected with FAK specific siRNA and then treated with OPN. The cell lysates were subjected to Western blot analysis using anti-MelCAM or anti-uPA antibody. The conditioned media was used for the detection of MMP-9 by Western blot using anti-MMP-9 antibody. The data showed that cells transfected with FAK siRNA completely suppresses OPN-induced MelCAM expression, uPA secretion and MMP-9 activation (Fig. 6.3.7, upper panels A-C, lanes 1-4). In order to ascertain the role of Sp1 in OPN-induced uPA secretion, the cells were treated with Sp1 specific inhibitors (WP631 and Mitramycin) and then treated with OPN. The cell lysates were subjected to Western blot analysis using anti-uPA antibody. The data indicated that OPN-induced uPA secretion was suppressed by Sp1 inhibitors in a dose dependent manner (upper panel D, lanes 1-6). All these blots were reprobed with anti-actin antibody as loading control (lower panels A-D). These data indicate that OPN induces FAK dependent Sp1 mediated MelCAM expression, uPA secretion and MMP-9 activation.
Fig. 6.3.7. A-D, OPN induces FAK dependent Sp1 mediated MelCAM expression leading to uPA dependent pro-MMP-9 activation - Cells were transfected with FAK siRNA and then treated with OPN. The cell lysates were subjected to Western blot analysis using anti-MelCAM or anti-uPA antibody. The conditioned media was concentrated and analyzed by Western blot using anti-MMP-9 antibody (upper panels A-C). In separate experiments, the cells were treated with specific Sp1 inhibitors, WP631 and Mitramycin (0-25 μM) and then treated with OPN. The cell lysates were subjected to Western blot analysis using anti-uPA antibody (upper panel D). The same blots were reprobed with anti-actin antibody as loading control (lower panels A-D).

6.3.8. MelCAM is required for OPN-induced uPA secretion and MMP-9 activation - The metastatic potential of tumor cells depends on proper vascularization of the tumor and the ability to degrade ECM components. Accordingly, to examine whether MelCAM plays any role in modulating OPN-induced uPA secretion and MMP-9 activation, cells were pretreated with MelCAM blocking peptide (0-50 μg/ml) and then treated with OPN. The cell lysates or the conditioned media were analyzed by Western blot using anti-uPA or anti-MMP-9 antibody. The data indicated that MelCAM peptide suppressed OPN-induced uPA secretion (Fig. 6.3.8, upper panel A, lanes 1-5) and MMP-9 activation (panel B, lanes 1-4).
These data indicated that MelCAM plays crucial role in modulating OPN-induced uPA secretion and MMP-9 activation.

**Fig. 6.3.8. A-B, MelCAM is involved in OPN induced uPA secretion and MMP-9 activation**- Cells were pretreated with MelCAM blocking peptide (0-50 µg/ml) and then treated with OPN. The cell lysates or the conditioned media were analyzed by Western blot using anti-uPA or anti-MMP-9 antibody. The results shown here represent three experiments exhibiting similar effects.

**6.3.9. FAK, Sp1 and MelCAM play crucial roles in OPN-stimulated cell migration**- Our data indicated that OPN regulates FAK-mediated AP-1 dependent Sp1 expression leading to MelCAM expression, uPA secretion and MMP-9 activation. To examine whether FAK, Sp1 and MelCAM play any role in modulating OPN-induced cell migration, cells were transfected with FAK siRNA or pretreated with Sp1 inhibitors (WP 631 and Mitramycin) or MelCAM blocking peptide and then used for migration assay. OPN (5 µM) was used in the upper chamber. The data showed that silencing of FAK expression or Sp1 inhibitors suppressed the OPN-induced cell migration (Fig. 6.3.9, panels A and B). Similarly, MelCAM blocking peptide also suppressed OPN-stimulated cell migration (panel A). These results indicated that OPN-induced FAK dependent Sp1-mediated MelCAM enhances the capacity of melanoma cells to penetrate through the basement membrane and suggested the specific role of these signaling molecules in regulating the cell migration.
**Fig. 6.3.9. Roles of FAK, Sp1 and MelCAM in OPN induced cell migration**- The migration assay was performed either by using untreated cells (5 x 10^6 cells/well) or cells pretreated with MelCAM blocking peptide (0-50 µg/µl), Sp1 inhibitors, WP631 and Mitramycin (0-25 µM). In separate experiments, cells were transfected with FAK siRNA. The purified human OPN (5 µM) was added in the upper chamber. The treated or transfected cells were used for migration assay. Note that treatment with MelCAM blocking peptide downregulated the OPN induced cell migration. The results are expressed as the means ± S.E. of three determinations.

**6.3.10. Roles of NIK and MelCAM in OPN-induced Homotypic Aggregation**- Clumping or emboli formation of tumor cells is an important step prior to extravasation and metastasis. Recent evidence has indicated that melanoma cells adhere to MelCAM suggesting its role in homotypic aggregation. To delineate whether OPN induces this aggregation and whether NIK and MelCAM play any role in this process, B16F10 cells were treated with OPN alone or stably transfected with wild type and mutant NIK or pretreated with MelCAM blocking peptide and then treated with OPN. These transfected or treated cells were allowed to grow in a three-dimensional culture system, i.e., as multicellular spheroids. The results showed that wild type NIK enhanced OPN-induced spheroid formation (Fig. 6.3.10, panel a-c) However, mutant NIK or MelCAM peptide disrupted the OPN-induced spheroid formation (panel d and e). These data suggested that NIK and MelCAM in presence of OPN play major role in homotypic cell to cell interaction.
Fig. 6.3.10. Spheroid generation by B16F10 cells alone, cells treated with OPN and cells stably transfected with wild type NIK but spheroid formation was disrupted in mut NIK stably transfected cells and cells pretreated with anti-MelCAM antibody followed by treatment with OPN.

6.3.11. OPN Induces NIK Dependent MelCAM Expression in Tumor of Nude Mice-
The *in vitro* data prompted us to examine whether OPN-induced NIK dependent MelCAM expression play any role in tumor of nude mice. Accordingly, B16F10 cells were treated with OPN (10 μM) and injected subcutaneously into the flanks of nude mice. In separate experiments, cells were stably transfected with wild type and kinase negative NIK and then treated with OPN. These cells were injected subcutaneously into nude mice. After 4 weeks, the mice were killed and the tumor weights were measured. Four mice were used in each set of experiments. The tumor samples were further processed for immunohistochemical studies. The level of MelCAM expression in these primary tumor samples was determined by immunostaining using anti-MelCAM antibody followed by incubation with FITC-conjugated anti-goat IgG and then analyzed under confocal microscopy (Zeiss). The level of expression of MelCAM was significantly higher in tumor generated by injecting nude mice with wild type NIK transfected cells compared to the mutant NIK transfected cells (Fig. 6.3.11, panels a-d). This data demonstrated that NIK and MelCAM play pivotal role in regulating OPN-induced tumor growth in nude mice.
Fig. 6.3.11. Expression of MelCAM in tumors of nude mice. B16F10 cells treated with OPN (10 μM) or cells stably transfected with wild type and kinase negative NIK and then treated with OPN were injected subcutaneously into the flanks of nude mice. Immunofluorescence staining of MelCAM in B16F10 melanoma primary tumor sections (3 weeks post-inoculation). Note that MelCAM expression was significantly higher in wt NIK stably expressing OPN treated tumors compared with mut NIK expressing tumors.

6.3.12. Role of NIK on melanoma metastasis to lungs- Earlier reports have indicated that silencing of MMP-9 expression blocks metastasis in a rat sarcoma model system (344). To examine the role of NIK in regulating OPN-induced MMP-9 expression in lung metastasis of melanoma, cells were stably transfected with wild type and kinase negative NIK and then treated with OPN and injected through lateral tail vein in nude mice. The mice were killed after 60 days and the lungs were removed and fixed in Bouin's solution for 24 h to facilitate counting of tumor nodules. The surface tumor nodules were counted using a dissecting microscope. Fig. 6.3.12, panel A shows that wild type NIK increased the formation of metastatic nodules (lung colonies) compared to mutant NIK or OPN injected lungs. To further ascertain the role of NIK in OPN-induced lung metastasis, the level of NIK was analyzed in lung by immunohistochemistry. The data indicated that the level of
pNIK was significantly higher in OPN-treated metastasized lung compared to control (panel B). The level of MMP-9 in metastasized lung was also examined by immunohistochemistry and the data showed that the MMP-9 expression was significantly higher in wild type NIK transfected samples compared to mutant NIK or OPN alone (panel C). These data demonstrated that NIK plays crucial role in OPN regulated melanoma metastasis.

Fig. 6.3.12. Pivotal role of NIK in melanoma cell metastasis to lungs. A. The role of NIK in the development of OPN induced melanoma metastasis to lungs after tail vein inoculation of (5 X 10^6 cells) B16F10 cells alone, cells treated with OPN, wt and mut NIK expressing cells treated with OPN in nude mice. Note that large metastatic nodules were formed in both OPN injected and wild type NIK expressing cells treated with OPN when compared to the mice injected with mut NIK transfected cells (10X objective). Photograph of H and E sections. B, Immunofluorescence staining of control and OPN treated metastasized lung sections indicated that NIK was significantly high in OPN treated metastasized lung when compared to the control (panel B). C, Immunofluorescence staining of MMP-9 in the control (a), OPN treated (b), wt NIK and mut NIK stably transfected OPN
treated lung sections (c and d) (63 X Objective). Note that dark spots in the H & E stained sections show melanin pigments produced by melanoma cells.

6.3.13. Expression of OPN, pNIK and MelCAM in human MM specimens- In order to correlate the in vitro data and in vivo mouse model results to human clinical specimens, primary melanoma tissues were collected with informed consent, from either diagnostic biopsies or upon surgery. The level of expression of OPN, NIK and MelCAM was determined by immunohistochemical analysis. The immunohistochemical staining of the human malignant melanoma specimens revealed that OPN was present in the melanoma cells from all 4 patients (3 MM with metastasis and 1 MM without metastasis). There was a significant positive correlation between the expressions of OPN, pNIK and MelCAM in all the four cases (Fig. 6.3.13 A-D). Clinical and histopathologic data are summarized in Table III.
Fig. 6.3.13 A-D: Correlation between OPN, pNIK and MelCAM expressions in human melanoma specimens. Human primary melanoma tissue specimens were collected with informed consent either from diagnostic biopsies or upon surgery. Immunohistochemical studies were performed on the primary cutaneous lesions and Clark's level and Breslow thickness were determined. The levels of OPN, pNIK and MelCAM expressions were determined by immunofluorescence staining using the respective primary antibodies followed by either FITC or HRP conjugated IgG and analyzed under confocal microscopy. Note that there was a significant positive correlation between OPN, pNIK and MelCAM expression and metastatic potential (Clark’s level and Breslow thickness) in different grades of melanoma tissue specimens. These clinical and histopathological data are summarized in Table III.

Table III

Analysis of OPN, pNIK and MelCAM expressions and its correlation with metastatic potential in various grades of malignant melanoma specimens

<table>
<thead>
<tr>
<th>Case</th>
<th>Location</th>
<th>Type of MM</th>
<th>Breslow Thickness (mm)</th>
<th>Clark's Level</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Left foot</td>
<td>NM³</td>
<td>4</td>
<td>IV</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Left second finger</td>
<td>NM</td>
<td>6</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Left foot</td>
<td>NM</td>
<td>3</td>
<td>III</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Lower lip</td>
<td>SSM³</td>
<td>2</td>
<td>IV</td>
<td>-</td>
</tr>
</tbody>
</table>

¹MM: malignant melanoma; ²NM: nodular melanoma and ³SSM: superficial spreading melanoma.
6.3.14 Discussion

In this study, we have investigated the role of OPN in modulating MelCAM expression in B16F10 cells and further delineated the molecular mechanism by which OPN regulates FAK/NIK dependent AP-1 mediated Sp1 expression that ultimately regulates MelCAM expression. Moreover, the roles of these molecules in regulating melanoma growth and lung metastasis in nude mice and its clinical implication have been investigated.

Our data indicated that OPN induces αvβ3-mediated NIK dependent MelCAM expression. Moreover, OPN also induces FAK phosphorylation and silencing of FAK expression completely abrogated the OPN-induced NIK phosphorylation indicating that FAK acts upstream of NIK. OPN induced FAK dependent NIK activation was suppressed upon pretreatment of cells with MelCAM blocking peptide indicating that there is a reciprocal regulation loop between the cell adhesion molecule, MelCAM and FAK in presence of OPN which forms a bidirectional signaling network. Our findings also demonstrated that OPN-induced AP-1 activity also positively regulates Sp1 expression and activation leading to MelCAM expression, uPA secretion and MMP-9 activation. NIK plays a pivotal role in OPN-stimulated melanoma metastasis to lungs. These data demonstrated that OPN binding to αvβ3 integrin induced FAK/NIK dependent AP-1 mediated Sp1 expression which ultimately stimulates MelCAM expression, uPA secretion and uPA dependent MMP-9 activation. In addition, MelCAM also exerts a reciprocal regulation in these process and all of these ultimately control cell motility, tumor growth and metastasis.

Previous studies have indicated that MUC18/MelCAM expression correlates with tumor thickness and metastatic potential of human melanoma cells in nude mice (101, 102). These results prompted us to investigate whether OPN regulates MelCAM expression and whether NIK is involved in this process. In this study, we have demonstrated that OPN induces αvβ3 integrin mediated NIK dependent MelCAM expression in B16F10 cells. Pretreatment of cells with anti-αvβ3 integrin antibody and RGD but not RGE peptide inhibits OPN-induced MelCAM expression. Moreover, overexpression of wild type NIK enhanced and kinase negative NIK suppressed OPN-induced MelCAM expression. This
was also confirmed by using both wild type and kinase negative NIK stably expressing cells indicating that OPN induces NIK dependent MelCAM expression.

FAK, a protein tyrosine kinase plays an important role in both growth factor and integrin stimulated cell motility in both normal and transformed cells. FAK expression and its tyrosine phosphorylation are elevated as a function of human tumor cell malignancy and these changes are associated with increased tumor metastasis (113). It has been reported that integrin mediated activation of FAK is required for signaling to JNK and progression through the G1 phase of the cell cycle (110). These results prompted us to investigate whether FAK plays any role in regulating OPN induced NIK phosphorylation. Our data showed that OPN induces FAK phosphorylation and silencing of FAK expression also completely abrogated the OPN-stimulated NIK phosphorylation indicating that FAK plays a crucial role in regulating this process. In this study, we provide evidence that OPN also controls a reciprocal regulation loop between MelCAM and FAK. This was confirmed by the fact that pretreatment with MelCAM blocking peptide completely abrogates OPN induced FAK and NIK phosphorylation.

AP-1 is a class of basic leucine zipper (bZIP) transcription factors consisting of the Fos (c-Fos, Fos B, Fra-1 and Fra-2) and Jun (c-Jun, Jun B and Jun D) families (225, 366). A number of studies have shown that stimuli such as serum and other growth factors induce AP-1 by activating ERK, a subgroup of mitogen activated protein kinase (MAPK) (238). Sp1 is another ubiquitously expressed transcription factor that recognizes GC rich sequences present in the regulatory sequences of numerous house-keeping genes and those genes which are involved in growth regulation and cancer (367). As we have shown that OPN induces MelCAM expression and Sp1 is an important regulator of MelCAM expression as proved by deletion analysis in which removal of all putative Sp1 sites reduced the promoter activity by 80% (368), we sought to determine the level of Sp1 expression upon OPN stimulation. OPN enhances the Sp1 expression and activation in B16F10 cells. Our data also suggested that OPN-induced Sp1 DNA-binding was completely abrogated by FAK siRNA. Similarly wt NIK enhanced and mut NIK suppressed the OPN-induced Sp1 DNA-binding indicating that FAK and NIK play important role in OPN regulated Sp-1 activation.
Earlier report indicated that MAPK and JNK signaling pathways activate Sp1 leading to the stimulation of the uPA promoter activity (369). Thus, the activation of both AP-1 and Sp-1 are regulated by different mechanisms, they appear to be regulated by the same intracellular signal transduction pathways. Recently, Fujioka et al have demonstrated the crosstalk between two distinct transcription factors, NF-κB and AP-1 and shown that NF-κB plays an essential role in regulating elk-1 expression which in turn activates ERK leading to AP-1 activation (370). These observations led us to examine whether AP-1 plays any role in regulating OPN-induced Sp1 activation. Our data indicated that down regulation of AP-1 especially c-Jun resulted in suppression of Sp1 expression and DNA-binding indicating that OPN induces AP-1 dependent Sp1 expression in B16F10 cells. Furthermore, the role of NIK in regulating OPN induced AP-1 dependent Sp1 expression was also studied in both wild type and kinase negative NIK stably expressing cells. Our data indicated that OPN activates both AP-1 and Sp1 in a time dependent manner in wild type but not mutant NIK expressing cells. The OPN induced Sp1 expression was greatly reduced and delayed in mutant NIK expressing cells. These results further suggested that AP-1 activity is essential for OPN-induced Sp1 activation in these cells.

The process of hematological metastasis includes detachment of cells from the matrix of the primary tumor mass, degradation of the surrounding connective tissue and migration of the cells into the vascular system followed by extravasation (247). MMPs are a family of Zn$^{2+}$ dependent endopeptidases that are responsible for the remodeling of the extracellular matrix and degradation of the ECM proteins. Several studies have shown the correlation between uPA and MMP-9 expression and metastatic potential of tumor (277, 324). Our data indicated that OPN induces FAK/NIK dependent AP-1 mediated Sp1 activation which leads to MelCAM expression, uPA secretion and MMP-9 activation. Further, transfection with FAK siRNA completely abrogated OPN induced cell migration and ECM invasion. Similarly, pre-treatment with specific Sp1 inhibitor, WP631 and MTR (0-25 μM) and MelCAM blocking peptide (0- 50 μg/ml) suppressed OPN-induced cell migration and ECM invasion indicating that FAK, Sp1 and MelCAM play crucial role in OPN induced cell migration and ECM invasion. However, MelCAM blocking peptide suppressed the OPN-induced uPA secretion and MMP-9 activation. Our data also indicated that OPN enhances the interaction between MelCAM and αvβ3 integrin. Recent data also
suggested that the expression and activation of one receptor may influence the transcription and activation of other adhesion receptors leading to downstream signaling (371). Scaffidi et al have reported that αvβ3 integrin interacts with TGF β type II receptor to potentiate the proliferative effects of TGF β 1 in human lung fibroblast (372). These results further substantiate the reciprocal signaling network between FAK and MelCAM that is regulated by OPN.

The ability of tumor cells to detach from the primary site and produce metastases in a distant organ is due to expression of various cell adhesion molecules which mediate cell to cell and cell to matrix interaction. Clumping (emboli formation) of tumor cells is an important step prior to extravasation and metastasis (373). Since our earlier data indicated that OPN induces NIK dependent MelCAM expression, we have specifically analyzed the role of OPN induced NIK in melanoma metastasis. Accordingly, cells either treated with OPN or transfected with wild type and mutant NIK followed by treatment with OPN were analyzed for the ability to grow in three dimensional culture (multicellular spheroids) system. The data indicated that wild type NIK expressing cells treated with OPN formed spheroids with greater efficiency. However spheroid formation was disrupted in mutant NIK stably expressing cells or cells pretreated with MelCAM blocking peptide. These data clearly demonstrated the crucial role of both NIK and MelCAM in OPN regulated homotypic aggregation which is the prerequisite of metastasis.

The in vivo tumorigenicity and metastasis studies indicated that OPN regulated NIK plays a crucial role both in primary tumor growth and lung metastasis. The numbers of lung nodules were significantly higher in the metastasized lung generated by injecting wild type NIK expressing cells compared to mutant NIK. These also correlated with the levels of MelCAM expression and MMP-9 activation in both the primary tumor and metastasized lung. The in vitro data and in vivo mouse model was also correlated to human clinical specimens. The level of expression of OPN, pNIK and MelCAM in the human malignant melanoma specimens was determined by immunohistochemical analysis which indicated a significant positive correlation between the expressions of OPN, pNIK and MelCAM in all the four cases. These results warrant further investigations to determine whether the mechanism demonstrated in the mouse models may underlie the NIK and MelCAM expressions in human clinical conditions. A clear understanding of such mechanism(s) may
facilitate the development of rational therapeutic approaches to suppress NIK activation, thereby treating melanoma growth and metastasis.
Summary & Conclusions
SUMMARY AND CONCLUSIONS

The major alterations in the cell physiology that collectively dictate malignant growth are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tumor invasion and metastasis. Thus the regulation of the cell-cell and cell-matrix interaction in the tumor microenvironment plays a crucial role in tumor progression. One of the key family of enzymes responsible for degradation of the ECM and hence contribute to cancer cell motility, tumor growth and metastasis are the matrix metalloproteinases (MMPs). Of the various MMPs which are reported till date, MMP-9, a classical member of the gelatinase family is involved in a wide range of pathologies such as angiogenesis, rheumatoid arthritis, retinopathy and hence is considered to be a prioritized therapeutic target. MMPs are highly regulated by several growth factors, cytokines and ECM components. OPN, a chemokine like calcified ECM protein has been recognized as a potential marker in the process of tumorigenicity and metastasis. The level of OPN expression is correlated to the metastatic potential of various cancers. However the molecular mechanism by which OPN regulates transcription factor mediated MMP-9 activation that ultimately controls melanoma growth and metastasis is less well understood. This study provides a valuable insight into the potential role of OPN in regulating melanoma progression and the molecular mechanism by which it regulates MMP-9 expression and activation using murine melanoma cells (B16F10) as the model system.

In this study the following points have been addressed

- The effect of OPN on MMP-9 expression, activation, cell migration, invasion, tumor growth and metastasis.
- Does the OPN induced MMP-9 activation play any direct role in the enhanced cell motility, tumor growth and lung metastasis?
- What is the molecular mechanism by which OPN induces MMP-9 activation, invasiveness and melanoma progression?
- To delineate whether OPN regulates any crosstalk between signaling pathways leading to activation of target gene expression and tumor progression.
Can the OPN induced effects be modulated using various strategies like blocking antibodies, overexpression or suppression of specific key signaling molecules using transfection experiments, silencing or knock-down of specific gene expression through a siRNA based approach.

The effect of OPN on MelCAM expression, a melanoma specific marker and the molecular basis of its contribution to melanoma growth and lung metastasis.

The clinical implication of the OPN induced signaling events which ultimately controls melanoma growth using human malignant melanoma specimens through immunohistochemical studies

The major findings of the present study are as follows

- Purified human OPN induces cell motility, ECM invasion and tumor growth which correlates with enhanced expression of pro- and active MMP-9 in vitro and in vivo.
- Increased MMP-9 activation reflects enhanced NFκB DNA binding and transactivation in the OPN treated cells. Moreover transfection of cells with the super repressor form of IκBα abrogated OPN induced MMP-9 expression. Therefore OPN increases the gelatinolytic activity through activation of NFκB.
- In an attempt to identify the kinases which act upstream of NFκB, we identified NIK as a potential candidate. 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).
- NIK is involved in OPN-induced phosphorylations of MEK-1 and ERK1/2 in these cells.

Thus OPN induces NIK dependent NFκB activation through both ERK and IKKα/β-mediated pathways.

- OPN enhances NIK regulated uPA secretion, uPA dependent pro-MMP-9 activation, cell motility and tumor growth.
- Pretreatment of the cells of anti-uPA blocking antibody completely suppresses the OPN induced activation of pro- to active MMP-9.
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.

These results indicated that OPN induced MT1-MMP mediated MMP-2 activation and uPA dependent MMP-9 activation occurs through two distinct signaling mechanisms and both play independent roles in regulating the melanoma cell motility and chemoinvasion.

Transfection of cells with wild type NIK, IKKα/β and ERK1/2 enhanced and kinase negative NIK, dominant negative IKKα/β and dn ERK1/2 suppressed the OPN-induced NFκB activation, uPA secretion, pro-MMP-9 activation, cell motility and chemoinvasion.

The level of active MMP-9 in the OPN-induced tumor was higher compared with control.

These data showed that NIK plays crucial role in OPN-induced NFκB activation, uPA secretion and uPA dependent pro-MMP-9 activation through MAPK/IKKα/β-mediated pathways and all of these ultimately control the cell motility, invasiveness and tumor growth.

Mitogen Activated Protein Kinases (MAPK) is one of the principal family of kinases which are activated during tumor progression. Various MAPK cascades (eg. ERK1/2, JNK, p38) are often portrayed as linear cascades and indications of crosstalk between the various cascades are limited. In this respect, the present study examines the molecular mechanism by which OPN regulates NIK and MEKK1-mediated AP-1 transactivation leading to MMP-9 activation and whether there exists any cross-talk between OPN-induced NIK/ERK and MEKK1/JNK signaling pathways.
OPN induces αvβ3 integrin-mediated MEKK1 phosphorylation and MEKK1 dependent JNK1 phosphorylation and activation. However OPN-induced JNK1 phosphorylation is not affected by NIK.

Transfection of cells with wild type NIK and MEKK1 enhances and kinase negative NIK and MEKK1 suppresses OPN-induced c-Jun expression.

*These data suggested that OPN induces both NIK and MEKK1 mediated c-Jun expression which occurs through both JNK1 dependent and independent pathways.*

Sustained activation of JNK1 by overexpression of wild type MEKK1 resulted in suppression of ERK1/2 activation. But this did not affect the OPN-induced NIK-dependent ERK1/2 activation as indicated by the NIK coupled kinase assay.

*These results showed that OPN induces a negative crosstalk between NIK/ERK and MEKK1/ JNK pathways.*

OPN stimulates both NIK and MEKK1-dependent c-Jun expression leading to AP-1 activation whereas NIK-dependent AP-1 activation is independent of JNK1.

OPN also enhanced JNK1-dependent/independent AP-1-mediated uPA secretion, uPA dependent pro-MMP-9 activation, cell motility and invasion.

OPN stimulates tumor growth and the levels of c-Jun, AP-1, uPA and MMP-9 were higher in OPN-induced tumor compared to control.

*These data indicated that OPN induces NIK/MEKK1-mediated JNK1-dependent/independent AP-1-mediated pro-MMP-9 activation and regulates the negative crosstalk between NIK/ERK1/2 and MEKK1/JNK1 pathways that ultimately control the cell motility, invasiveness and tumor growth.*
OPN induces MelCAM expression in both dose and time dependent manner. Transfection of cells with wt NIK enhanced and kinase negative NIK suppressed the OPN induced MelCAM expression.

OPN also induces FAK phosphorylation. Silencing of FAK expression by transfection of cells with FAK siRNA resulted in complete suppression of NIK phosphorylation.

These data suggested that OPN induces FAK phosphorylation and FAK dependent NIK activation leading to MelCAM expression in these cells.

OPN induces Sp1 expression and DNA binding which is enhanced upon overexpression of wild type NIK. Similarly transfection of cells with kinase negative NIK or FAK siRNA completely abrogates the OPN induced Sp1 activity.

Transfection of cells with dominant negative c-Jun resulted in suppression of OPN induced Sp1 expression and DNA binding indicating that AP-1 activity is required for OPN induced Sp1 activity.

Treatment of cells with MelCAM blocking peptide resulted in suppression of OPN induced FAK phosphorylation and FAK dependent Sp1 activation.

Pull down studies indicated that OPN induced MelCAM also physically associates with αvβ3 integrin which is regulated by NIK in B16F10 cells.

These results indicated that OPN induces αvβ3 integrin-mediated FAK/NIK dependent AP-1 activation and AP-1 dependent Sp1 activation and further suggest that OPN induces a reciprocal regulation loop between MelCAM and FAK.

Pretreatment of cells with MelCAM blocking peptide suppresses OPN induced uPA secretion and MMP-9 activation.

This data suggested that MelCAM is required for OPN induced uPA secretion and pro-MMP-9 activation.
OPN induced FAK/NIK also plays a crucial role in cell migration, invasion, homotypic aggregation (spheroid formation).

The level of expression of MelCAM was significantly higher in tumor generated by injecting nude mice with wild type NIK transfected cells compared to the mutant NIK transfected cells.

This data demonstrated that NIK and MelCAM play pivotal role in regulating OPN-induced tumor growth in nude mice.

OPN induces lung metastasis in nude mice and the number of tumor nodules was significantly higher when wild type NIK stably expressing cells were injected when compared to OPN treated cells or B16F10 cells alone.

The level of phospho-NIK was higher in OPN induced metastasized lung when compared to the control. This also correlated with the MMP-9 levels in the metastasized lung.

There was a significant positive correlation between the levels of OPN, pNIK and MelCAM in human malignant melanoma specimens that also correlated with the Clark’s level and Breslow thickness which ultimately reflected on the melanoma grade.

These data demonstrated that NIK plays crucial role in OPN-induced tumor growth and lung metastasis that ultimately correlated with the melanoma grade in human malignant melanoma specimens.

This work was initiated with the objective of understanding the molecular mechanism by which OPN regulates melanoma progression. Our work has led to some interesting findings and a better understanding of the oncogenic potential of OPN. Drugs that target OPN dependent molecular mechanisms could support clinical intervention against various malignancies. An attempt has been made to delineate the various molecular mechanisms by which OPN regulates melanoma growth and metastasis. Some of the key signaling molecules like NIK, MelCAM and MMP-9 seem to be valuable therapeutic targets. Our studies reveal
that NIK acts as a crucial regulator of melanoma progression by controlling the various
downstream kinases leading to transcription factor mediated MMP-9 activation that
ultimately controls cancer cell motility, tumor growth and metastasis (375, 376). Analysis of
human malignant melanoma specimens substantiated the mouse model data and hence
potentiates its clinical significance for melanoma therapy. Thus our study highlights the
potential role of OPN and the molecular mechanism underlying the genesis of metastasis
signature in melanoma and hence may be useful in developing novel molecular diagnostics
and targeted therapy for the treatment of malignant melanoma.