Basic research is what I’m doing when I don’t know what I’m doing

Werner von Braun
5. MATERIALS AND METHODS.

5.1. Sources of Chemicals and cDNA.

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

**American Type Culture Collection (ATCC), USA.**

Human breast adenocarcinoma MDA-MB-231, MCF-7, human melanoma A375, human prostate carcinoma LNCaP, human osteosarcoma HT1080 and mouse breast adenocarcinoma C127I (209,210) cell lines were obtained from ATCC and maintained at National Centre for Cell Science (NCCS) Cell Repository.

**Amersham Pharmacia (GE Healthcare), UK.**

Hybond-C nitrocellulose membrane, Sephadex G-25, Tween-20, Resource S column and HEPES ([4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] were obtained from Amersham Pharmacia.

**BD-Biosciences, USA.**

Matrigel, invasion chambers, FITC-conjugated anti-rabbit, anti-mouse and anti-goat IgG, and all the laboratory plastic materials for cell and tissue culture were purchased from BD Biosciences.

**Bio-Rad, USA.**

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

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

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

**Calbiochem, USA**

Rabbit polyclonal anti-VEGF antibody, mouse monoclonal anti-phosphoserine detection kit, anti-MT1-MMP, pp2 and Wortmannin were obtained from Calbiochem.

**Cell Signaling Technologies, USA.**

Rabbit anti-phospho KDR antibody was obtained from Cell Signaling Technologies.

**Chemicon International, USA.**

Mouse monoclonal anti-human αvβ3 integrin antibody (LM609) and anti-rabbit-Cy2, -Cy3 conjugated IgG were purchased from Chemicon International.
Dharmacon RNA Technologies, USA.

All siRNAs (Table 3) were synthesized from Dharmacon RNA Technologies.

| OPNi 1  | 5’ AUU UCA CAG CCA UGA AGA UdTdTdTdTdT UAA AGU GUC GGU ACU UCU A 5’ |
| Coni 1  | 5’ CAG UAC AAC GCA UCU GGC AdTdT/ dTdT GUC AUG UUG CGU AGA CCG U 5’ |
| OPNi 2  | 5’-CCA GUU GUC CCC ACA GUA GdTdT/ dTdT GGU CAA CAG GGG UGU CAU C-5’ |
| Coni 2  | 5’- UCU UUC GAG CAA UCA GGU CdTdT/ dTdT AGA AAG CUC GUU AGU CAA G-5’ |
| OPNi 3  | 5’-GCA GAC CUC UCC AAG UAA GdTdT/ dTdT CGU CUG GGA AAG UUC AUU C-5’ |
| Coni 3  | 5’-GGA AUG UUC GGC UGC UAA UdTdT/ dTdT CCU UAC AAG CCG ACG GAU A-5’ |
| OPNi 1 Single mismatch (SM) | 5’AUU UCA CAG GCB UGA AGA UdTdTdTdTdT UAA AGU GUC C CB ACU UCU A 5’ |
| OPNi 1 Double mismatch (DM) | 5’AUU UCA CAG CBG UGA AGA UdTdTdTdTdT UAA AGU GUC CCA ACU UCU A 5’ |
| NRP-1 siRNA (NRP-1) | 5’ GAG AGG UCG UGA AUG UUC C dTdT/ dTdT CUC UCC AGG ACU UAC AAG G 5’ |
| Con NRP-1i | 5’ AGA GAU GUA GUC GCU CGC U dTdT/ dTdT UCU CUA CAU CAG CGA GCG A 5’ |
| Brk siRNA (Brk i) | 5’AAG GUG GCC AUU AAG GUG AdTdT/ dTdT UUC CAC CGG UAA UUC CAC U 5’ |
| Con Brk i | 5’CAC ACU AGG UUG CCA GAG CgdTdT/ dTdT GUG UGA GCC AAC GGU GUC C 5’ |

Table 3, List of various siRNA used in this study.

Difco Laboratories, USA.

Bacto Tryptone, Bacto Yeast extract, Miller Luria Bertani agar and Miller Luria Bertani broth were obtained from Difco Laboratories.

ICN Biochemicals, USA.

Tris-Base, glycine, sodium dodecyl sulphate (SDS), ammonium persulphate, trypan blue, gelatin, chloroform, formaldehyde, Nonidet P-40 (NP-40), calcium chloride, sodium chloride, paraformaldehyde, formaldehyde, glycerol, DMSO, sodium orthovanadate, pNPP, tri-sodium citrate, citric acid, ethylenediamine tetra-acetic acid (EDTA), and bovine pancreatic RNase were from ICN.

Invitrogen, USA.

N,N,N’,N’-tetramethylethylenediamine (TEMED), Protein-A/G agarose, agarose, T4 polynucleotide kinase, GRGDSP and GRGESP peptides, LipofectAMINE 2000, Dulbecco’s modified Eagle’s medium, L15 medium, RPMI 1640 medium, fetal bovine serum (FBS), see
blue plus pre-stained protein marker, and rabbit anti-CA-15-3 antibody were obtained from Invitrogen.

**Jackson Laboratories, USA.**

Wild type C57 and OPN knockout (OPN<sup>−/−</sup>) (C57BL/6Jx129/SvJ, Strain name: B6.Cg-Spp1tm1Blh/J) mice were obtained from Jackson laboratory, and maintained in NCCS Experimental Animal Facility.

**Merck, Germany.**

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

**R & D System, USA.**

Mouse monoclonal anti-VEGF, anti-NRP-1, goat polyclonal anti-OPN antibodies were purchased from R & D system.

**Roche Molecular Biochemicals, Germany.**

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

**Santa Cruz Biotechnology, USA.**

Rabbit polyclonal anti-phospho-NIK (Thr-559), anti-NIK, anti-Brk, anti-ATF-4, anti-NF-κB p65, anti- PI 3-kinase (p85), anti-Akt, anti-ERK, anti-MMP-9, anti-uPA, goat polyclonal anti-actin, mouse monoclonal anti-phospho-ERK1/2, anti-phospho-Akt (Ser-473) anti-KDR and anti-MMP-2 antibodies, horse radish peroxidase conjugated anti-rabbit IgG, antirabbit IgG, anti-goat IgG, Western blot luminol reagent, fluorescent mounting media, bovine serum albumin and siRNA transfection reagent were purchased from Santa Cruz Biotechnology.

**Sigma Chemical Co., USA.**

Rabbit polyclonal anti-vWF antibody, mouse monoclonal anti-VEGF, goat polyclonal anti-OPN antibodies, Triton-X-100, DTT (Dithiothreitol), sodium deoxycholate, bromophenol blue, 2- mercaptoethanol, HEPES, PIPES, gluteraldehyde, propidium idodide and pristane were obtained from Sigma.

**Miscellaneous.**

Boyden type cell migration chambers were obtained from Corning. Plasmid isolation kit was purchased from Qiagen. Rabbit polyclonal anti-luciferase antibody was from Upstate and
the nude mice (NMRI, nu/nu) were obtained from the National Institute of Virology (NIV), India. Human OPN was isolated from human milk as described earlier with minor modification (28). All other chemicals were of analytical grade.

**cDNA Constructs.**

The super repressor form of IκBα in pCMV4 was a generous gift from Dr. Dean Ballard, Vanderbilt University School of Medicine. Wild type (wt) and kinase negative (mut) nuclear factor–inducing kinase (NIK; K249A/K430A) cDNA construct in pcDNA3 vector were obtained from Prof. David Wallach, Weizmann Institute of Science. VEGF-Luc construct was a kind gift of Dr. Debabrata Mukhopadhyay, Mayo Clinic College of Medicine. VEGF-GFP cDNA construct was provided by Dr. Rakesh K. Jain, Massachusetts General Hospital. Wt (pEF/mATF-4) and dominant-negative (dn; pEF/mATF-4M) ATF-4 were a generous gift from Dr. Javed Alam, Yale University School of Medicine. Wt and kinase mutant (KM) Brk in pRC CMV vector were obtained from Prof. Mark Crompton, Royal Holloway University of London, and human OPN cDNA in pcDNA 3.1 was a kind gift from Dr. Ann Chambers, University of Western Ontario.

**5.2 Maintenance of Cell Lines.**

**Mammalian Cell Culture.**

The Human breast adenocarcinoma MDA-MB-231, MCF-7, human melanoma A-375, human osteosarcoma HT-1080, human endothelial cell line, EA.hy-926 (generous gift from Dr. Christopher Newton) mouse breast adenocarcinoma, C127I, and human prostate carcinoma, LNCaP, were cultured in L15, DMEM and RPMI 1640 respectively 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.

**Generation of Mouse Embryonic Fibroblast Cells.**

Mouse Embryonic Fibroblast (MEF) was isolated from 13.5 days embryos. Pregnant mice were sacrificed and embryos were removed in sterile PBS containing 1% glucose. After removal of head and internal organs (i.e. liver), embryos were rinsed twice in PBS. Embryos were minced, digested in Trypsin-EDTA (0.5% trypsin, 50 mM EDTA) for 45 minutes in a rotating magnetic stirrer. Trypsin was inactivated by adding DMEM medium, supplemented with 10% FBS, 40 μg/ml Ciprofloxacin, 0.1% Fungizone and cultured in 100 mm tissue culture disc.
Co-culture Assay.

The tumor-endothelial cell interaction was studied by co-culture experiments using MDA-MB-231 and EA.hy-926 cells. EA.hy-926 cells were transfected with NRP-1i and co-cultured with non-transfected or OPNi or NRP-1i transfected MDA-MB-231 cells. In separate experiments, co-cultured cells were either treated with OPN alone or along with anti-VEGF antibody. The level of pKDR in cell lysates was detected by immunoprecipitation followed by Western blot.

5.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis.

I. 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 1N HCl. The volume was made upto 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 1N HCl. The volume was made upto 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 freshly 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  
Volume adjusted to 50 ml. with deionized water.  

**10 X Electrophoresis buffer**  
144 g Glycine  
30.3 g Tris base  
10 g SDS  
Volume adjusted to 1 lit. with deionized water  

**II. Gel Composition**  

**Resolving Gel 7.5%**  
30% Acrylamide/0.8% Bis-acrylamide 3.0 ml  
4 X Resolving solution 3.0 ml  
Deionized water 6.0 ml  
10% SDS 120 μl  
10% Ammonium per sulphate 60 μl  
TEMED 20 μl  

**Stacking gel**  
30% Acrylamide/0.8% Bis-acrylamide 1.0 ml  
4 X Stacking solution 1.25 ml  
Deionized water 3.25 ml  
10% SDS 50 μl  
10% Ammonium per sulphate 40 μl  
TEMED 10 μl  

**III. Coomassie Blue Staining Solutions**  
Coomassie blue 0.25%  
Acetic acid 10%  
Methanol 50%  

**Distaining solution**  
Acetic acid 7.5%  
Methanol 10%  

**IV. Reagents and Solutions for Western Blotting**  

**1X Transfer Buffer**  
3.03 g Tris
14.4 g Glycine
200 ml Methanol
Volume adjusted to 1 lit. with deionized water

**Phosphate Buffered Saline (PBS)**
- 8.0 g NaCl
- 0.2 g KCl
- 1.44g Na$_2$HPO$_4$
- 0.24 g KH$_2$PO$_4$

These chemicals were dissolved in 800 ml deionized water and pH was adjusted to 7.4 with 1N HCl. The volume was made upto 1000 ml.

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

**Stripping Buffer**
- 62.5 mM Tris-HCl, pH 6.7
- 2% SDS
- 100 mM 2- mercaptoethanol.

**Methodology**

The concentration of protein (obtained from cell lysate, conditioned medium or tumor tissue lysate) was measured by Bio-Rad protein assay kit. The samples containing equal amount of total proteins was mixed with 3 X sample buffer. The samples were boiled at 95°C for 10 minutes. The proteins were resolved in polyacrylamide gel on discontinuous buffer system of Lammeli using Bio-Rad mini-gel electrophoresis unit. Electrophoresis was carried out at constant voltage.

For Coomassie blue staining, the protein gel was stained with Coomassie blue staining solution for 1 h followed by distaining with the distaining solution.

For Western blot analysis, the proteins were electrotransferred to nitrocellulose membrane using a mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad). The membrane was blocked with 5% milk or BSA in PBS-T at 4°C overnight. After that, 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/BSA in PBS-T for 1 h at room temperature or overnight at 4°C. 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 again washed and exposed to luminol reagents and detected by autoradiography.
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 by using separate primary antibodies as described above.

5.4. 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 to 25 ml of lysis buffer

**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. After that, 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 × g for 10 min at 4°C. 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 for 16 h. The immunocomplexes were collected by centrifugation at 12000 × g for 2 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 and detected by Western blot as described above.
5.5 RNA Extraction and Reverse Transcription (RT)-PCR.

Total RNA was isolated from OPN-treated MDA-MB-231 cells and analyzed by RT-PCR. The reverse transcription and PCR amplification was performed by using 10 μg total RNA, with MMLV reverse transcriptase (Invitrogen) and primers (VEGF-sense; 5'-CCC TCC GAA ACC ATG AAC TTT-3' and VEGF-antisense; 5'-AGA GAT CTG GTT CCC GAA AC-3'). The amplified cDNA fragments were resolved by agarose gel electrophoresis.

5.6 Mammalian Cell Transfection.

For *in vitro* transfection, cells were trypsinized 16 h prior of transfection, split and seeded in the tissue culture plate. The cells were allowed to grow for 50-60% confluency. Then, 1μg of purified plasmid DNA or 5μg of siRNA was diluted in 50μl tissue culture medium and incubated for 5 mins. 5μl of LipofectAMINE 2000 were also mixed in 100 μl of cell culture medium and incubated separately for 5 mins. The above solutions were then mixed together and further incubated for 20 mins. This entire mixture was added to 850 μl of serum free media and added to tissue culture plates. The cells were further incubated with this complex for 6 h at 37°C, 5% CO₂ in humified incubator. After 6h, transfection was terminated by adding 10% FCS. These cells were allowed to grow for 24-48h and used for various experiments.

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

5.7 Reporter Gene Expression.

MDA-MB-231 cells were transfected with VEGF-luciferase reporter construct alone or along with OPN cDNA or OPNi. Cell lysates were subjected to Western blot using anti-luciferase antibody.

The VEGF promoter activity was also measured using a reporter construct with *A. victoria* GFP which was located immediately downstream of VEGF promoter. The effect of OPN on VEGF promoter activity was detected either by cells transfected with VEGF-GFP alone or co-transfected with IκBα super repressor (sup. rep.) or wild type (wt) and mutant (mut) NIK or wt and dominant negative (dn) IKKα/β along with VEGF-GFP and then treated with OPN. The OPN-induced GFP expression was measured by using fluorescence microscopy (Nikon).
5.8  *In vitro* Brk kinase Assay.

Brk kinase assay was performed as described earlier (138). Briefly, serum-starved MDA-MB-231 breast cancer cells treated with 0.5 μM OPN alone or pretreated with αvβ3 blocking antibody or Wortmannin (PI 3-kinase inhibitor) or pp2 (c-Src inhibitor) followed by OPN treatment. Endogenous Brk was immunoprecipitated from cell lysates using anti-Brk-specific antibodies. Brk immunoprecipitates were washed twice with 1 ml each in buffer EB (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.1% bovine serum albumin, 200 μM Na3VO4, 0.1% (v/v) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin), twice in PAN Nonidet P-40 buffer (10 mM PIPES, pH 7.0, 100 mM NaCl, 0.5% Nonidet P-40, and 20 μg/ml aprotinin), and twice in PAN buffer minus Nonidet P-40. Autophosphorylation of Brk was measured by incubating immunoprecipitated Brk in 50 μl of kinase 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 for 30 min at 30 °C. Reactions were terminated by the addition of 10 μl 5x SDS-PAGE sample buffer. Samples were boiled for 10 min and analyzed by SDS-PAGE on 10% polyacrylamide gels followed by autoradiography.

5.9  Gelatin Zymography.

**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.
Methodology

The metalloproteinase activity in the tumor tissue lysates was determined by gelatin zymography. Samples containing equal amount of total proteins were mixed with zymography sample buffer under non-reducing condition and incubated at room temperature for 30 min and then loaded in zymography-SDS-PAGE containing gelatin (0.5 mg/ml). The gels were washed thrice 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 Comassie blue and destained. Negative staining showed the zones of gelatinolytic activity.

5.10 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 (211). Cells were grown to 60-70% confluency. For preparation of cytoplasmic and nuclear extract, 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.

5.11 Electrophoretic Mobility Shift Assay (EMSA).

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 NF-κB (5’- AGT TGA GGG GAC T TT CCC AGG C-3’), ATF-4 (5’-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3’) and AP-1 (5’-CGC TTG ATG ACT CAG CCG GAA-3’) oligonucleotides were labeled using the T4 polynucleotide kinase. The labeling reaction was performed as follows.

**Labeling reaction**

- Consensus oligonucleotide (1.75 pmol/μl) 2 μl
- T4 polynucleotide kinase 10 X buffer 1 μl
- [γ-32P] ATP (5000 Ci/mmol) 1 μl
- Nuclease free water 5 ml
- T4 polynucleotide kinase (5-10 μg/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 1000 ml.

**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 out 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**
- Nuclear extract 5-10 μg
- Binding buffer 2 μl
- Salmon Sperm DNA 1 μl
- BSA (300 μg/ml) 1 μl
- Labeled probes 5 μl
- Nuclease free water-made up to 20 μl

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.
5.12 Wound Migration Assay.

The motility of tumor and endothelial cells were determined by wound migration assay. Post confluent MDA-MB-231 cells with the typical cobblestone morphology was used for this experiment. Wounds with a constant diameter were made. Cells were treated with OPN alone or along with specific blocking antibodies. In separate experiments, MDA-MB-231 cells were transfected with wt and KM Brk or wt and dn ATF-4, followed by treatment with OPN alone or along with specific blocking antibodies and subjected to wound assay. In another experiment, MDA-MB-231 cells were transfected with either OPNi or NRP-1i or OPN cDNA alone or OPN cDNA transfected cells were treated with anti-NRP-1 or anti-VEGF blocking antibodies and wound assays were performed. In other experiments, EA.hy-926 cells were treated with conditioned media obtained from MDA-MB-231 cells alone or along with specific blocking antibodies and wound assays were conducted. The conditioned media were prepared by treating MDA-MB-231 cells with OPN or transfected MDA-MB-231 cells with OPN i. Cells were incubated at 37°C for 12 h, wound photographs were taken under phase contrast microscope (Nikon).

5.13 Cell Migration and Co-migration Assays.

The migration assay was conducted in MDA-MB-231 cells using transwell cell culture chamber (modified Boyden chamber) according to the standard procedure as described previously (50, 57). Briefly, cells were individually pretreated with different blocking antibodies or transfected with different plasmids or siRNA. Cells were harvested using Trypsin-EDTA and collected by centrifugation. The cell suspension (1x10^5 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 was added to the upper chamber. The cells were incubated in a humidified incubator in 5% CO₂ and 95% air at 37°C for 12 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 cells on the filter were counted under an inverted microscope (Nikon) analyzed statistically and represented in the form of bar graph.

The endothelial-tumor cell interaction was shown by direct comigration assays (Fig 10.1). MDA-MB-231 cells were treated with either OPN alone or along with anti-VEGF or anti–NRP-1 blocking antibody and used in lower chamber. EA.hy-926 cells were seeded on the upper chamber.
In separate experiments, MDA-MD-231 cells were transfected with OPNi or NRP-1i and used for comigration assay. The migrated cells were stained with Giemsa and counted in three high-power fields under an inverted microscope, analyzed statistically and represented in the form of bar graph.


Reagents

1 x cold PBS (pH 7.4)
2% paraformaldehyde in 100mM HEPES (pH 7.9)
0.1% glycine in PBS
0.1% Triton X 100 in PBS
BSA

Methodology

The cells were trypsinized and resuspended in culture medium and transferred to dishes with sterile cover glasses and grown up to semi-confluency. After treatment with OPN or transfection with OPN siRNA, cover glasses were rinsed with ice cold PBS (pH 7.4). Cells were fixed by incubating in 2% paraformaldehyde at 4°C for 15 mins. After quenching with 0.1%
glycine, cells were permialized by incubating with 0.1% Triton X 100. The cells were washed three times for 5 min each with PBS, and incubated in 2% BSA in PBS (pH 7.4) for 1 h at room temperature (RT) to block non-specific binding. After that, cells were incubated with specific primary antibodies in 0.2% BSA for 2 h at RT, followed by FITC-conjugated secondary antibodies for 1 h at RT. Cells were washed and mounted in mounting media and analyzed under confocal microscopy (Zeiss).

5.15 In Vivo Matrigel Based Angiogenesis Assay.

The in vivo Matrigel angiogenesis assay was performed as described (212). Briefly, Matrigel (0.5 mL) was injected s.c. in the ventral groin region of female athymic NMRI (nu/nu) mice. In separate experiments, the conditioned medium (CM) of untreated or OPN-treated MDA-MB-231 cells were mixed with Matrigel and injected to the mice. In other experiments, OPN-treated CM was mixed with anti-VEGF blocking antibody (400 μg/kg of body weight per mice) in Matrigel or CM collected from OPNi-transfected cells were mixed with Matrigel and then injected to the mice. After 3 weeks, Matrigel plugs were excised and processed for histopathology and immunohistochemistry using specific antibodies.

5.16 In Vivo Tumorigenicity Studies.

5.16.1 Generation of orthotopic breast tumor in nude mice.

MDA-MB-231 (5x10^5) cells were implanted orthotopically in the mammary fat pad of female athymic nude mice (8-12 weeks old). OPN (0.5 μM) or anti-VEGF (400μg/kg of body weight/mice) antibody was injected to the site of tumor twice a week upto 6 weeks. In other experiments, OPN cDNA transfected cells alone or along with anti-VEGF antibody were injected into the nude mice. In separate experiments, OPNi or NRP-1i (250 μg/kg of body weight/mice) mixed with transfection reagent were administered to the site of tumor twice a week until termination of the experiments. Mice were sacrificed, photographed, the tumors were dissected out, weighed, tumor tissues were used for histopathological and immunohistochemical studies with their specific antibodies. Tumor samples were lysed in lysis buffer. Mice blood was collected from retro-orbital plexus and serum was isolated and used for further studies.

In separate experiments, MDA-MB-231 (5 x 10^6) cells were injected orthotopically into the left inguinal mammary fat pad of female nude mice (NMRI). After one week, OPN specific siRNA (250 μg/kg of body weight/mice) was mixed with siRNA transfection reagent and
injected intratumorally as described (187, 188), thrice a week until the completion of experiments. Control group of mice was injected with Coni mixed with transfection reagent. Animals were kept in pathogen free condition and monitored regularly. After eight weeks, mice were sacrificed by cervical dislocation and photographed. Primary breast tumors were dissected out and weighed. One part of the tumors were fixed in 10% formalin solution and used for histopathology and immunohistochemistry. The other part was snap frozen and used for EMSA.

5.16.2 Generation of breast tumor in wild type and OPN knockout mice.

Mouse breast adenocarcinoma C127I (5 X 10⁶) cells were mixed with 500μl cold growth factor depleted Matrigel and injected orthotopically to the left inguinal mammary fat pad of female wild type (OPN +/+) and knockout (OPN−/−) mice (n=6/group). The mice were kept in the pathogen free condition for 8 weeks. The care of the animals was provided as per National Centre for Cell Science (NCCS) Experimental Animal Facility (EAF) ethical guidelines. After termination of experiments, mice were sacrificed; photographed, primary breast tumors were dissected out, weighed and analyzed.

5.16.3 Generation of pristane-induced mammary tumorigenesis in mice.

Pristane (200 μl stock solution) was injected in the left inguinal mammary fat pad of female nude mice, thrice in a week upto six weeks. After that, mouse OPN siRNA (mOPNi; 250μg/kg of body weight/mice) was mixed with transfection reagent and injected directly into the pristane-induced tumor thrice in a week for subsequent six weeks as described above. After 12 weeks, mice were sacrificed and mammary fat pad was dissected out and used for histopathology and immunohistochemical analysis.

In separate experiments, pristane was injected (thrice in a week) to the left inguinal mammary fat pad of female wild type (OPN +/+) and knockout (OPN−/−) mice (n=6/group). Mice were kept until development of tumors (16 weeks) and then sacrificed, photographed and tumors in mammary fat pad were dissected out for further analysis.

5.17 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 Western blot or EMSA or stored as aliquots at –80°C until further use.

5.18 Immunohistochemical Study of Tumor Tissue.

Reagents

Xylene
Descending ethanol gradation
1 X PBS (pH 7.4)
100 mM citric acid (stock solution)
100 mM glycine in PBS
0.1% Triton X 100 in PBS
BSA

Methodology

Immunohistochemical analysis was performed using standard procedure. After deparaffinizing the tissue sections in xylene, sections were rehydrated using descending grades of ethanol (100%, 95% and 70%) for 2 x 2 mins each and washed thrice in PBS. Then the sections were kept in fumigated citric acid for antigen retrieval until the solution come down to room temperature. After quenching with 100 mM glycine, tissue sections were permeabilized by incubating in 0.1% Triton X 100. Sections were washed twice with PBS for 2 x 2 mins followed by blocking with 2% bovine serum albumin for 1 h at room temperature. Finally, the sections were incubated with specific primary antibody overnight at 4°C in a humified environment. After incubation with primary antibody, the slides were washed in PBS and incubated with specific secondary antibody for 2 h at room temperature. The sections were mounted and analyzed using confocal microscopy (Zeiss).
5.19 Human Breast Cancer Clinical Specimens Analysis.

Human female breast carcinoma and normal breast tissues were obtained from a local hospital after surgical removal with informed consent. Tissues were fixed in formaldehyde and paraffin blocks were prepared using standard protocols. Paraffin sections were used for histopathology and immunohistochemistry studies. Tumor grading was carried out by a modified Scarff-Bloom-Richardson system with the help of an expert oncopathologist. Fresh tissues of various grades were also collected and used for EMSA.

5.20 Statistical Analysis.

The bands were analyzed densitometrically (Kodak Digital Science) and fold changes were calculated. The migration and invasion assays were analyzed statistically and represented in the form of bar graph. The expressions of various molecules in cell lines, mice tissue sections and human clinical specimens were quantified using the Image Pro Plus 6.0 software (Nikon) and expressed as mean ± SE. The mouse tumor weights were measured and plotted in the form of bar graph. Statistical differences were determined by Student's t test. Differences were considered significant when the $P$-value was less than 0.05.