Every science begins as philosophy and ends as art.

*Will Durant*
5. MATERIALS AND METHODS

5.1 Sources of chemicals, antibodies and plasmid constructs

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

Human breast cancer cell lines (MCF-7 and MDA-MB-231), B16F10 and NIH3T3 were obtained from ATCC, USA.

**Lonza, USA.**

HUVEC and EGM-2 bullet kit were procured from Lonza, USA.

**Xenogen Corporation**

MDA-MB-231-Luc, B16F10-luc cells and D-Luciferin were purchased from Xenogen Corporation (Alameda, CA).

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

Hybond-C nitrocellulose membrane

**BD-Bioscienes, USA.**

FITC-conjugated anti-rabbit, anti-mouse and anti-goat IgG, Annexin V-FITC, rat anti-CD31 matrigel, invasion chambers and all the laboratory plastic materials for cell and tissue culture were obtained from BD Biosciences.

**Bio-Rad, USA.**

N,N,N,N-tetramethylethylenediamine (TEMED), and Bradford reagent were obtained from Bio-Rad.

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

($\gamma$-32P) ATP (5000 Ci/µmol) was purchased from BRIT, Hyderabad.

**Plasmid constructs.** The human full length OPN promoter fragments cloned in pGL3-basic vector were generous gift from Dr Chen (Tzu Chi University, Taiwan), (55).

**Santa Cruz Biotechnology, USA.**

Rabbit polyclonal anti-PARP, anti-phospho-Akt, anti-p85α, anti-p110α, anti-c-Fos, anti-c-Jun, anti-cyclinD1, anti-ABCG2, anti-phospho-ERK1/2, goat polyclonal anti-Akt, anti-Cox-2, anti-actin, mouse polyclonal anti-Flk1, anti-ERK1/2, anti-phospho-p65 and Western blotting luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
Chemicon International.
Rabbit Cy3, mouse Cy3 and anti-OPN antibodies were obtained from Chemicon International.

Calbiochem, USA.
Wortmannin, U0126, SN50 and Normal mouse IgG were obtained from Calbiochem.

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

ICN Biochemicals, USA.
Glycine, Tris, sodium dodecyl sulphate (SDS), ammonium persulphate, trypan blue, Tween-20, Nonidet P-40 (NP-40), formaldehyde, chloroform, paraformaldehyde, formaldehyde, glycerol, DMSO, sodium orthovanadate, ethylenediamine tetra-acetic acid (EDTA), trisodium citrate, citric acid, and bovine pancreatic RNase were purchased from ICN.

Invitrogen, USA.
Agarose, Protein-A/G agarose, T4 polynucleotide kinase, MMLV reverse transcriptase, LipofectAMINE 2000, Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), penicillin & streptomycin and see blue Plus pre-stained protein marker were obtained from Invitrogen.

Merck, Germany.
Isopropyl alcohol, sodium dihydrogen phosphate, disodium hydrogen phosphate, hydrogen peroxide and Giemsa stain were obtained from Merck.

R&D System, USA.
Mouse anti-OPN antibody, Z-VAD and Z-DEVD were purchased from R & D system.

Roche Molecular Biochemicals, Germany.
Protease inhibitor cocktail, phenyl methyl sulphonyl fluoride, ethidium bromide were purchased from Roche.

Sigma Chemical Co., USA.
Acrylamide, N, N-methylene bisacrylamide, DMEM, DTT (Dithiothreitol), sodium deoxycholate, bromophenol blue, β-mercaptoethanol, HEPES, Triton-X-100, PIPES,
propidium iodide, Hoechst 33342, Reserpine, mouse anti-VEGF and phalloidin FITC were obtained from Sigma.

**Dharmacon International (Lafayette, CO).**

Small interfering RNA (siRNA) specifically targeting OPN (siGENOME SMARTpool human SPP1) was obtained from Dharmacon International (Lafayette, CO).

**The Jackson Laboratory, USA**

OPN knockout mice were procured from Jackson laboratories, USA and were maintained in the experimental animal facility (EAF) of NCCS

**Miscellaneous.**

C57BL/6 and NOD/SCID mice were obtained from the Experimental Animal Facility (EAF), NCCS. Plasmid isolation kit was purchased from Qiagen. Boyden type cell migration chambers were purchased from Corning. All other chemicals were of analytical grade.

### 5.2 Maintenance of cell lines

Human breast adenocarcinoma (MDA-MB-231 and MCF-7), mouse fibroblast (NIH-3T3) and B16F10 cell lines were maintained in L-15 (MDA-MB-231), DMEM (MCF-7, NIH-3T3) and RPMI (B16F10) media (Sigma) supplemented with 10% FBS, 100 units penicillin and 100 µg/ml streptomycin. MDA-MB-231-Luc and B16F10-Luc cells were maintained according to the manufacturer’s instructions. Human umbilical vein endothelial cells (HUVEC) were cultured as per supplier’s instructions.

### 5.3 Extraction, Isolation and Characterization of Andro

The plants were collected from suburbs of Eastern India, identified and authenticated as *Andrographis paniculata* from the registered facility of Agharkar Research Institute (ARI), Pune, India. The whole plants were air dried, powdered, soaked overnight in acetone at room temperature and filtered. The filtrate was evaporated and resuspended in dichloromethane (DCM). The DCM precipitates obtained were further treated with charcoal to remove pigments. The fractions were then chromatographed on silica gel column (mesh size 60-120) using MeOH /DCM. Gradient elution was carried out with MeOH/DCM and different fractions were collected and analyzed by TLC. The fractions giving similar spots on TLC were pooled and concentrated. The purified compound was further chemically
characterized by $^1$H, $^{13}$C- NMR, DEPT, COSY, HSQR, HMBC, NOESY and LC-MS under the same conditions as described earlier (210).

**Andrographolide:** colorless needles; mp 230-232°C; ESI-MS (pos) m/z : 351 (M + H)$^+$, 373 (M+Na)$^+$, 389 (M+K)$^+$, $^1$H NMR (DMSO-<sub>d6</sub>, 400 MHz) δ: 5.75 (1H, d, $J$ = 15.9 Hz; H-12), 4.87 (1H, d, $J$= 1.8 Hz; OH-14), 4.21 (1H, d, $J$ = 5.0 Hz; OH-3), 4.04 (1H, t, $J$= 1.8 Hz; H-14), 3.94 (1H, s; 17a), 3.75 (1H, s; 17b), 3.51 (1H, dd, $J$ = 6.02 Hz, 9.70 Hz; 15a), 3.29 (1H, dd, $J$ = 7.3, 2.6 Hz; OH-19), 3.17 (1H, dd, $J$ = 1.5, 9.79 Hz; H-15b), 2.95 (1H, d, $J$ = 11.0, 2.6 Hz; H-19<sub>a</sub>), 2.38 (1H, dd, $J$=11.0, 7.3; H-19<sub>b</sub>), 2.29 (1H, ddd, $J$=10.3, 5.0, 5.0 Hz; H-3), 1.63 (2H, dd, $J$= 15.9, 5.02 Hz; H-11), 1.45 (1H, m; H-7<sub>eq</sub>), 1.05 (1H, ddd, $J$=12.6, 5.0, 5.0 Hz; H-7<sub>ax</sub>), 0.98 (1H, m; H-9), 0.84 (1H, 1H, ddd, $J$=13.5, 13.0, 13.0, 4.1 Hz; H-6<sub>eq</sub>), 0.80 (1H, s; H-1<sub>eq</sub>), 0.75 (2H, m; H-2), 0.46 (1H, m; H-6<sub>ax</sub>), 0.34 (1H, m; H-1<sub>ax</sub>), 0.31 (1H, m; H-5), 0.21 (3H, s; H-18), 0.22(3H, s; H-20). $^{13}$C NMR (DMSO-<sub>d6</sub>, 400 MHz ) δ: 170.26 (C-16), 147.85(C-8), 146.63 (C-12), 129.21 (C-13), 108.52 (C-17), 78.70 (C-3), 74.61 (C-10), 64.77 (C14),62.91 (C-19), 55.73 (C-9),54.60 ( C-5), 42.51 (C-4), 40.33 (C-10), 39.07 (C-7), 37.75 (C-1), 36.75 (C-11), 28.13 (C-2), 24.23 (C-6) 23.32 (C-18), 15.00 (C-20).IR Spectra (cm<sup>-1</sup>): 1724.42, 1674.27, 3394.83, 3294.53, 2924.18.

5.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

5.4.1 Reagents and Solutions for SDS-PAGE

30% Acrylamide/0.8% Bis-acrylamide

| Acrylamide | 30.0 g |
| N,N- Methylene bisacrylamide | 0.8 g |

The volume was made to 100 ml with deionized water. The solution was filtered and stored in dark at 4°C.

1M Tris-HCl, pH 6.8 (stacking solution)

12.11 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.

1.5 M Tris-HCl, pH 8.8 (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 (APS)
0.05 g of ammonium persulphate was dissolved in 500 µl of deionized water.

5x Lammeli sample buffer (SDS-PAGE sample buffer)
1M Tris HCl, pH 6.8   5.0 ml
Glycerol 2.6 ml
SDS 1.0 g
β-Mercaptoethanol 0.5 ml
Bromophenol blue 0.05 g
Volume adjusted to 10 ml with deionized water.

10x Electrophoresis buffer
Glycine 144 g
Tris base 30.3 g
SDS 10.0 g
Volume adjusted to 1 litre with deionized water.

5.4.2 Gel Composition

Resolving Gel 10% (for 10 ml)
30% Acrylamide/0.8% Bis-acrylamide 3.0 ml
1.5M Tris (pH-8.8) 2.5 ml
Deionized water 3.75 ml
10% SDS 100 µl
10% APS 100 µl
TEMED 20 µl

Stacking gel (for 5 ml)
30% Acrylamide/0.8% Bis-acrylamide 0.5 ml
1M Tris (pH-6.8) 1.25 ml
Deionized water 3.4 ml
10% SDS 50 µl
10% APS 50 µl
TEMED 10 μl

5.4.3 Coomassie Blue Staining Solutions

Staining solution
Coomassie blue 0.25%
Acetic acid 10.0%
Methanol 50.0%

Destaining solution
Acetic acid 75.0%
Methanol 10.0%

5.4.4 Reagents and Solutions for Western Blotting

1x Transfer Buffer
Glycine 3.03 g
Tris 14.42 g
200 ml Methanol
Volume adjusted to 1 litre with deionized water

10x Phosphate Buffered Saline (PBS) (pH-7.4)
KCl 2.0 g
NaCl 80 g
Na₂HPO₄ 14.4 g
KH₂PO₄ 2.4 g
pH adjusted to 7.4 with conc. HCl and volume to 1 litre with deionized water

20x TBS (pH-7.6)
Tris 60.0 g
NaCl 160 g
KCl 1.0 g
These chemicals were dissolved in 800 ml deionized water and pH was adjusted to 7.4 with conc. HCl. The volume was made upto 1 litre.

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

Stripping Buffer
1M Tris HCl, pH 6.8 12.5 ml
β-Mercaptoethanol  0.7 ml
2% SDS
The volume was made upto 100 ml.

**Methodology**

The concentration of total proteins in cell lysates was measured by Bradford reagent. The samples containing equal amount of total proteins was mixed with 5x sample buffer. The samples were boiled at 95°C for 10 min. 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 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 TBS-T at 4°C overnight. Membrane was rinsed with TBS-T and washed thrice for 15 min each. The membranes were incubated with primary antibody at appropriate dilution in 0.5% milk/BSA in TBS-T for 4 h at room temperature or overnight at 4°C. Blots were then washed thrice with TBS-T followed by incubation in HRP labeled anti-rabbit or anti-mouse or anti-goat IgG antibody. The blots were again washed, incubated with a luminol reagent and detected by autoradiography. For reprobing, the membranes were incubated in stripping buffer at 50°C for 30 min and then washed twice with TBS-T at room temperature for 10 min. The membrane was then blocked and immunodetected by using separate primary antibodies as described above.

**5.5 Preparation of Nuclear and Cytoplasmic Extracts**

**Reagents and Solutions**

**Hypotonic buffer (50 ml)**

- 1 M HEPES, (pH 7.9)  0.5 ml
- 3 M MgCl₂  25 µl
- 2.5 M KCl  0.2 ml
- 0.1 M PMSF  0.1 ml
- 1 M DTT  25 µl

**Nuclear extraction buffer (50 ml)**

- 1 M HEPES, (pH 7.9)  1.0 ml
- 3 M MgCl₂  25.0 µl
Methodology

MDA-MB-231 cells were grown to 70-80% confluency. For preparation of cytoplasmic and nuclear extracts, cells were washed twice with ice-cold 1x PBS and harvested. The cells were resuspended in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT) and incubated on ice for 10 min followed by homogenization. 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 (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.4 M NaCl, 0.2 mM EDTA, 2.5% Glycerol, 0.3 mM PMSF, 0.5 mM DTT) 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.

5.6 Electrophoretic mobility shift assay (EMSA)

5.6.1 Labeling of Oligonucleotide

Reagents and buffers

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

The double stranded AP-1 and NF-κB 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: 2 μl
(γ-³²P) ATP (5000 Ci/mmol): 3 μl
Nuclease free water: 11 μl
T4 polynucleotide kinase (5-10 μg/μl): 2 μl
This reaction mixture was incubated at 37°C for 1 h. 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.

5.6.2 DNA-protein binding

Reagents and buffers

10 x Tris-Glycine EDTA (TGE) Buffer
Tris-base: 30.3 g
Glycine: 144.0 g
EDTA: 3.9 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.

10 x Binding buffer
250 mM HEPES (pH 7.9),
500 mM NaCl
5 mM DTT
5 mM EDTA
10% Nonidet P-40,
50% Glycerol

Components of 8% native gel (10 ml)
40% acrylamide 2.0 ml
2% bisacrylamide 0.32 ml
10X TGE Buffer 0.5 ml
50% glycerol 1.0 ml
Distilled water 6.2 ml
10% APS 100.0 μl
TEMED 10.0 μl
The non-denaturing 8% polyacrylamide gel was prepared and allowed to polymerize for 15 min. Pre-electrophoresis was carried out for 1 h at 80V at 4°C. Simultaneously, 5 μg of nuclear extract was incubated with labeled oligonucleotides under the following condition.
**DNA Binding reaction (20 µl)**

<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>Sonicated Salmon Sperm DNA</td>
<td>1 µl</td>
</tr>
<tr>
<td>BSA (1 mg/ml)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Labeled probes</td>
<td>5 µl</td>
</tr>
<tr>
<td>Nuclease free H₂O-make upto</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

**Methodology**

EMSA was performed as described previously (15). In brief, MDA-MB-231 cells were treated with Andro (0-150 µM), nuclear extracts (5 µg each) were incubated with γ³²P-labeled double-stranded oligonucleotide containing either AP-1 or NF-κB consensus sequences in binding buffer containing 2 µg of polydeoxyinosinic deoxycytidylic acid (poly dIdC). The DNA-protein complex was resolved on 8% native polyacrylamide gel and analyzed by autoradiography.

**5.7 Preparation of LB/Amp**

Luria Bertani (LB) agar was prepared by dissolving 35 g Luria agar in 1 litre of distilled water and sterilized by autoclaving at 121°C for 20 min. Once the temperature of LB agar has reached 42°C (approx.), 50 µg/ml of ampicillin was added; the plates were poured and allowed to solidify at room temperature. The plates were incubated at 37°C overnight (14-18 hours) to check the sterility and then used.

**5.8 Preparation of competent cells**

**Reagents and buffers**

**Buffer A (100 ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Potassium acetate</td>
<td>3 ml</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>10 ml</td>
</tr>
<tr>
<td>1 M CaCl₂</td>
<td>1 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15 ml</td>
</tr>
<tr>
<td>1 M MnCl₂</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

**Buffer B (50 ml)**
1 M Sodium MOPS 0.5 ml
1 M KCl 0.5 ml
1 M CaCl₂ 3.75 ml
Glycerol 7.5 ml

**Methodology**

A single colony of E. coli strain DH5α was inoculated in 5 ml of Luria-Bertani (LB) medium containing 20 mM MgSO₄ and allowed to grow overnight at 37°C, 220 rpm. 1 ml of the primary culture was inoculated in 100 ml of LB medium containing 20 mM MgSO₄ and incubated at 37°C, 220 rpm till optical density of 0.3 to 0.6 at A₆₀₀ is reached. The growth of the cells was arrested by keeping them in ice for 30 min while continuously stirring to maintain uniform temperature. The culture was aseptically transferred to oak ridge centrifuge tube which was sterile and prechilled at 4°C. It was centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 20 ml of chilled buffer A (30 mM potassium acetate, 100 mM KCl, 10 mM CaCl₂, 15% glycerol and 50 mM MnCl₂) by gentle vortexing. It was incubated on ice for 60-90 min. The cells were pelleted at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 2 ml of chilled buffer B (10 mM sodium MOPS, 10 mM KCl, 75 mM CaCl₂ and 15% glycerol). Aliquots of 50 µl into sterile cryovials were made and stored at -80°C.

### 5.9 Transformation

1-5 µg of plasmid DNA or ligation mix was added to 100 µl aliquot of competent cells in ice. This was mixed well by tapping and incubated in ice for 30 min followed by heat shock for 90 sec at 42°C. The mixture was incubated in ice for 2 min without shaking. 850 µl of LB without antibiotic was added to the cells and incubated at 37°C for 1 h with shaking at 220 rpm. The cells were pelleted at 5,000 rpm at 4°C and plated on LB agar with antibiotic. The plate was incubated at 37°C for overnight incubation and colonies were monitored the next day.

### 5.10 Plasmid preparation

**Reagents and buffers**
**Buffer P1 (50 ml)**

50 mM Tris (pH 8.0) 0.303 g  
0.5 M EDTA 1 ml  
100 μg/ml RNase A

**Buffer P2 (50 ml)**

10% SDS 5 ml  
10 N NaOH 1 ml

**Buffer P3 (50 ml)**

3 M potassium acetate (pH-5.5)

**Methodology**

5 ml of LB medium containing the appropriate antibiotic was inoculated with a single bacterial colony with the appropriate plasmid/clone and incubated for 16 h at 37°C with shaking at 220 rpm. The culture was pelleted by centrifugation at 5000 rpm for 5 min, RT). The supernatant was discarded and the pellet was resuspended in 200 μl of buffer P1 (50 mM Tris pH 8.0, 10 mM EDTA, 100 μg/ml RNase A) by gentle vortexing. The mixture was incubated at RT for 5 min followed by addition of 400 μl of buffer P2 (1% SDS, 0.2 N NaOH). It was mixed by slow inversion and incubated in ice for 10 min. 300 μl of chilled buffer P3 (3 M potassium acetate, pH 5.5) was added followed by slow inversion and incubated in ice for 10 min. The sample was centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was transferred to another tube to which 500 μl of isopropanol was added for DNA precipitation. The sample was centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was washed with chilled 70% ethanol. Pellet was air dried and resuspended in 100-200 μl of sterile TE buffer.

**5.11 Agarose Gel Electrophoresis**

**Reagents and buffer**

**5x TBE (Tris-borate buffer)**

Tris 54 g  
Boric acid 27.5 g  
0.5 M EDTA 20 ml

The volume was made upto 1 litre with distilled water.

**Casting 1.2% agarose gel**
1.2 g of agarose was added to 100 ml of 0.5x TBE buffer. The mixture was heated for melting and poured into the casting tray with the combs.

5.12 Preparation of Glycerol Stocks

The transformed *E. coli* cells containing plasmid were inoculated in LB ampicillin broth, and were grown overnight till the O.D. reaches 0.6. To 0.85 ml of culture, 0.15 ml of sterile glycerol was added to a final concentration of 15 % and 1 ml of the same was aliquoted into sterile cryovial. The cryovial were tightly closed, labeled and vortexed thoroughly for proper mixing. It was snap-frozen in liquid nitrogen and stored at -80°C. Whenever required, the glycerol stocks were revived by streaking (without melting) on Luria agar plate and single isolated colonies were obtained from the plate.

5.13 Transfection

For *in vitro* transfection, MDA-MB-231 or MCF-7 cells were trypsinized 16 h prior of transfection and seeded in tissue culture plate. Cells were allowed to grow for 80-90% confluency. Then, 3 μg of purified plasmid DNA or 25 nM of siRNA was diluted in respective dilution medium and incubated for 5 min. 4 μl of Lipofectamine 2000 was also mixed in 50 μl of cell culture medium and incubated separately for 5 min. The above solutions were then mixed together and further incubated for 20 min. This entire mixture was added to 900 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 humidified incubator. After 6 h, transfection was terminated by adding 10% FBS. Cells were allowed to grow for 24-48 h and used for various experiments. For stable transfection, G418 or hygromycin containing fresh medium was added to the cultures after 24 h of transfection and maintained. Fourteen days later, resistant colonies were isolated and culture was maintained further.

5.14 Luciferase Reporter Assay

The Luc-reporter assay was performed with MCF-7 cells cotransfected with hOPN promoter containing Luc reporter gene in pGL3 basic and renilla Luc (pRL) reporter construct as described previously (55). Briefly, MCF-7 cells were co-transfected with 3 μg each of hOPN promoter containing Luc reporter gene in pGL3 basic and renilla Luc vector (pRL) using lipofectamine 2000 as per manufacturer’s instruction. Cells were treated with either Andro (0-150 μM) or vehicle for 24 h and luciferase activity was measured using a
luciferase reporter assay kit (Promega). Each luciferase activity was normalized to the renilla activity. Fold change of luc activity was calculated and analyzed with respect to control.

5.15 RNA isolation and reverse transcription-PCR (RT-PCR)

RNA isolation and reverse transcription-PCR were performed as described earlier (55). Briefly, MDA-MB-231 cells were treated with Andro for 24 h, total RNA was extracted using Trizol reagent (GIBCO BRL, Grand Island, NY) and reverse transcription-PCR was performed using following sets of primers. The following sets of primers for OPN and actin were used for PCR amplification:

OPN forward 5’ ATG AGA ATT GCA GTG ATT TG 3’
OPN reverse 5’ GCT GTG GGC TTC AGC ACT C 3’
actin forward 5’ GGC ATC CTC ACC CTG AAG TA 3’
actin reverse 5’ GGG GTG TTG AAG GTC TCA AA 3’

**Reaction Mixture (for cDNA synthesis) Total volume-25 µl**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>4-8 µg</td>
</tr>
<tr>
<td>Oligo dT primers</td>
<td>1 µl</td>
</tr>
<tr>
<td>5x RT buffer</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1 µl (20 U/ µl)</td>
</tr>
<tr>
<td>Reverse transcriptase enzyme</td>
<td>1 µl (20 U/ µl)</td>
</tr>
<tr>
<td>Water made upto</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 37°C for 1 h.

**PCR reaction mixture (20 µl)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>13 µl</td>
</tr>
<tr>
<td>10X buffer (for enzyme)</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer (forward)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer (reverse)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq DNA pol (3U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Template DNA (cDNA)</td>
<td>1 µl</td>
</tr>
</tbody>
</table>
PCR reaction cycles
95°C for 5 min
95°C for 30 sec
56°C for 30 sec x 30 cycles
72°C for 30 sec
72°C for 10 min

Aliquots of PCR products were analyzed by electrophoresis using 1.2% agarose gel.

5.16 MTT Cell Viability Assay

The cell viability assay was performed as described previously (211). Briefly, 2x10⁴ cells/well were plated in a 96-well flat-bottom microplate. Cells were treated with Andro (0-500 µM) for 24 h. 200µl of MTT (0.5 mg/ml) was added into each well and incubated at 37°C for 4 h. In another experiments, sorted SP and non-SP cells were seeded into 96 well plate and SP cells were treated mitoxantrone in absence or presence of reserpine and incubated for 24 h. After incubation, formazan crystals were dissolved with 200 µl of isopropanol. The optical density of formazan solution, as a measure of cell viability, was taken using a microplate reader at 570 nm (Molecular Devices). Experiments were performed in triplicates.

5.17 Wound Healing Assay

The wound migration assay was performed using MDA-MB-231, HUVEC and B16F10 melanoma cells as described earlier (113). Briefly, cells were grown in monolayer and synchronized for 24 h in serum-free medium. Wound with uniform size was made using sterile tip and the cells were treated with Andro (0-30 μM). After 12 h, wounds photographs were captured using phase contrast microscope (Nikon). Distance migrated was measured by Image-Pro plus software, analyzed and represented in the form of bar graph.

In separate experiments, B16F10, B16-WT and B16-KO were grown to confluency and wound with constant size were made. Cells migrated towards wounds were monitored, photographed and analyzed using Image-Pro plus software.

5.18 Cell Migration Assay

The migration assay was performed using Transwell cell culture chamber (Corning, NY) according to the standard procedure as described (79). Briefly, MDA-MB-231 cells were incubated with Andro (0-30 µM) for 24 h; cells were harvested and counted. The cell suspensions (1x10⁴ cells/200µl) either untreated or pretreated with Andro was seeded to the
upper part of Boyden chamber. In separate experiment B16F10, B16-WT and B16-KO cells were seeded on upper chamber of transwell, and at the lower chamber, 5% FBS was used as chemoattractant. After incubating at 37 °C for 12 h or 24 h, the cells that migrated to the lower surface of the membrane were fixed with methanol, and stained with Giemsa. Migrated cells were photographed at three high power fields (hpf) under inverted microscope at a magnifications of 20x (Nikon). Cells were counted from three different images; mean was calculated, analyzed statistically and represented graphically.

5.19 Tumor-Endothelial Cell Comigration/Coinvasion Assay

Comigration/coinvasion assays were performed to demonstrate endothelial-breast tumor cell interaction as described (79). Briefly, conditioned media collected from Andro treated MDA-MB-231 cells were used in lower chamber. HUVEC (1×10^4 cells) were added at the upper chamber (Boyden Chamber, BD Bioscience) and incubated for 12 h. In separate experiments, conditioned media were treated with either Andro or VEGF blocking antibody or OPN blocking antibody or in combination of OPN and VEGF blocking antibodies or in combination of Andro, OPN and VEGF antibodies and used in the lower chamber for comigration and coinvasion experiments. Coinvasion assay were carried out using matrigel coated invasion chamber (BD Bioscience).

In additional experiment B16F10, B16-WT and B16-KO cells were seeded on lower chamber and HUVEC were added on upper side of transwell for comigration/coinvasion experiment. The migrated or invaded cells to the other side of the membrane were stained with Giemsa and photographed at three high-power fields under an inverted microscope (Nikon) at magnification of 20x. Cells were counted from three different images; mean was calculated, analyzed statistically and represented graphically.

5.20 In Vitro Tube Formation Assay

HUVEC were maintained in EGM-2 medium (Lonza) according to supplier’s instructions. Tube formation assay was performed as described earlier (212). Briefly, 50 µl of growth factor depleted matrigel was coated on 96 well plates for polymerization and HUVEC (1x10^4 cells/well) were added. Cells were treated either with DMSO or Andro (0-30 µM). After 12 h, photographs were captured using inverted microscope (Nikon), and the effect of Andro was assessed by measuring the number of cords/tube formed and analyzed statistically.
For Vasculogenic mimicry experiments B16F10, B16-WT and B16-KO (1x10⁴ cells/well) cells were seeded on matrigel containing plate. Plates were incubated at 37°C for 8 h and photographs were captured, tubes formed were analyzed statistically and represented as mean ± SE. All the experiments were performed in triplicates.

5.21 Matrigel Colony Formation Assay

The matrigel colony formation assay was carried out to assess the anti-tumor activity of Andro as described earlier (213). Briefly, 50 µl of growth factor depleted matrigel was coated on 96 well plates. Equal number of MDA-MB-231 cells were plated on matrigel coated plates and incubated at 37 °C for 24 h. Cells were either treated with Andro (0-30 µM) or vehicle for 24 h. In separate experiments B16F10, B16-WT, B16-KO cells or Sorted SP and non-SP cells were seeded on matrigel containing plate and incubated at 37 °C for 10 days. Existing media was replaced with fresh media containing 10% FBS on every alternate day. After 10 days of incubations, colonies were visualized under microscope (Nikon) and photographed; colonies were counted, analyzed statistically and represented graphically.

5.22 Cell Cycle Analysis

Cell cycle analysis was performed as described elsewhere (77). The proportion of cells at different phases of the cell cycle was monitored by FACSCalibur (BD Biosciences). To analyze the cell-cycle distribution, MDA-MB-231 cells were first treated with Andro (0-150 µM), trypsinized and fixed with ice cold 70% ethanol. After incubating at 4 °C for 30 min, cells were washed and resuspended in 100 µl of PBS containing 0.5 mg/ml ribonuclease (RNase) A and incubated at 37 °C for 30 min. Cells were resuspended in PBS containing 50 µg/ml propidium iodide and incubated for 15 min. Stained cells were then filtered through nylon mesh to ensure a single cell suspension and immediately subjected to analysis at an excitation wavelength of 488 nm and an emission wavelength of 630 nm on FACS Calibur. Ten thousand cells in each sample were analyzed and the cell cycle distribution was calculated by using CellQuest software (BD Immunocytometry System).

5.23 Annexin V/Propidium Iodide Staining

To determine whether Andro induces apoptosis in breast cancer cells, MDA-MB-231 cells were treated with Andro (0-150 µM) for 36 h and stained with annexin V-FITC using apoptosis detection kit (BD PharMingen) according to the manufacturer's instructions. Briefly, cells were treated with Andro, stained with Annexin V-FITC followed by propidium
iodide and analyzed by FACSCalibur cytometer (BD Biosciences). Annexin V and PI double-negative cells are defined as live cells; annexin V-positive and PI-negative cells are defined as early apoptotic cells; and Annexin V and PI double-positive cells are defined as late apoptotic and necrotic cells.

5.24 Detection of Oxidative Stress

Oxidative stress or ROS were detected as described (214). Briefly, MDA-MB-231 cells were treated with Andro (0–150 µM) and then with 2 µM dihydroethidine (DHE) (Molecular Probes) for 15 min and washed. The fluorescence intensity of ethidine, the oxidation product of dihydroethidine was subsequently visualized under confocal microscope (Lieca) and quantified by FACSCanto flow cytometer (BD Biosciences).

5.25 Hoechst 33342 Staining and SP analysis

Flow cytometry with Hoechst 33342 was undertaken in a manner similar to that previously described with a few modifications (207). Briefly, either B16F10 cells or cells dissociated from the tumors of (OPN+/+ or OPN−/− mice) were resuspended at 10⁶ cells/ml in Hank’s balanced salt solution (HBSS) containing 2% fetal bovine serum (FBS) and 25 mM HEPES. Cells were preincubated at 37°C for 15 min with 50 µM reserpine (Sigma-Aldrich, St. Louis) to inhibit ABC transporters and incubated for 90 min at 37°C with 5 µg/ml Hoechst 33342 (Sigma-Aldrich). Cells were incubated on ice for 10 min and washed with ice-cold HBSS. Hoechst dye was excited at 407 nm by trigon violet laser, and its dual wavelengths were detected using 450/40 (Hoechst 33342-Blue) and 695/40 (Hoechst 33342-Red) filters. Dead cells were excluded by gating on forward and side scatter and eliminating PI-positive population. Cells were analyzed on a FACSAria with Diva Option cell sorter (BD Biosciences, San Jose, CA) and the data were processed using FACS Diva software.

5.26 Tumor Xenografts and Bioluminescence Analysis

All experimental protocols involving mice were approved by Institutional Animal Care and Use Committee (IACUC) of National Centre for Cell Science. MDA-MB-231-luc cells (1x10⁶) were injected into mammary fat pad of 6-week old female NOD/SCID mice. Once tumors formed, Andro (25 mg/Kg and 100 mg/Kg body weight) was injected intraperitoneally twice a week. Tumors were measured every week with vernier caliper. In vivo tumor growth was also studied in real time manner for 5 weeks using In Vivo Imaging System (Xenogen Corp.) as described earlier (215). Briefly, mice were anesthetized with
xylazine/ketamin (75 mg/kg ketamin and 3 mg/kg xylazine). D-luciferin was injected through i.p. into mice at a dose of 150 mg/kg and image was captured after 10 min. At the end of the experiments, mice were sacrificed and tumor samples were removed, weighed and fixed in formalin. Photon flux was calculated using Living Image acquisition and analysis software (version 3) and represented as photons/s/cm²/sr. Tumor volume and weight were measured and analyzed statistically as described (114).

5.27 Development and Establishment of Murine Primary culture from the Tumors of OPN⁺/⁺ and OPN⁻/⁻ mice

Murine melanoma B16F10 cells was injected (1 x 10⁶) subcutaneously into the dorsal flank region of OPN⁺/⁺ and OPN⁻/⁻ mice (6-8 weeks old). Tumor length was measured every week. At the end of five weeks, mice were sacrificed, photographed; tumors were dissected out, weighed and used for further studies. Tumor isolated from OPN⁺/⁺ and OPN⁻/⁻ mice were rinsed twice in sterile PBS. Tumors were minced and digested in Trypsin-EDTA (0.5% trypsin, 50mM EDTA) for 30 minutes and the cells obtained were incubated in humidified atmosphere of 5% CO₂ and 95% air at 37°C with 10% FBS in DMEM. The cells derived from the tumors of OPN⁺/⁺ and OPN⁻/⁻ mice denoted as B16-WT and B16-KO respectively.

In separate experiment, B16F10, B16-WT and B16-KO cells (1 x 10⁶) were injected subcutaneously into OPN⁻/⁻ mice and tumor kinetics were studied for 5 weeks.

In additional experiment, sorted SP and non-SP cells (1 x 10³) injected subcutaneously into OPN⁺/⁺ and OPN⁻/⁻ mice and tumor incidence were studied for five weeks of duration.

5.28 In Vivo Metastasis Study by Intra-Cardiac Injection

B16F10 cells was intracardiacaly injected (1 x 10⁵) directly into anesthetized wild OPN⁺/⁺ and OPN⁻/⁻ mice (6-8 weeks old). After 21 days mice were sacrificed, photographed and internal organs i.e. lung and liver were dissected out for histopathological studies.

In separate experiments, sorted SP or non-SP from B16F10-Luc cells (1 x 10³) were injected at intracardiac position of NOD/SCID mice and kept for 21 days. Lung and liver metastasis were studied using In Vivo Imaging System.

5.29 Immunofluorescence.

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

Methodology

MDA-MB-231 cells were trypsinized and resuspended in culture medium and transferred to dishes with sterile cover slips and grown to semiconfluency. After the termination of the experiments, cover slips were rinsed with ice cold PBS (pH-7.4). Cells were fixed by incubating in 2% paraformaldehyde at 4°C for 10 min. After quenching with 0.1% glycine, for intracellular staining cells were permeabilized 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 the non-specific binding. After that, cells were incubated with specific primary antibody in 0.2% BSA for 2 h at RT, followed by fluorochrome conjugated secondary antibody for 1 h at RT. Cells were washed and mounted in mounting media containing DAPI and analyzed under confocal microscopy (Zeiss).

5.30 Immunohistochemistry

Reagents

Xylene
Ethanol (Descending gradation)
1x PBS(pH 7.4)
100 mM citric acid (stock solution)
100 mM glycine in PBS
0.1% Triton X 100 in PBS
2% BSA in PBS
Super Sensitive Polymer-HRP IHC Detection System (BioGenex, CA, USA)

Methodology

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tumor tissue (5 μm sections mounted on poly-L-lysine–coated slides) as previously described (215). Briefly; slides were baked at 55°C for 30 min (deparaffination) and then soaked in xylene, passed through graded alcohols (rehydration), endogenous peroxidase were blocked with 3%
hydrogen peroxide in methanol for 10 min and then pretreated with boiling citrate buffer (pH 6.0) and allowed to cool gradually (antigen retrieval). Slides were quenched with quenching sol (glycine 10 mg/ml in PBS) for 10 min and then treated with 0.1% Triton X to permeabilize tissue followed by wash with PBS. After blocking with 2% bovine serum albumin, the sections were incubated with rabbit anti-OPN, anti-p-Akt, anti-c-Jun or mouse anti-p-p65 antibodies (1:50 dilution) followed by HRP conjugated secondary antibodies (1:200 dilutions). After washing, immunoperoxidase staining was developed using a diaminobenzidene (DAB) chromogen (Biogenex) as per the manufacturer’s instruction and counterstained with hematoxylin. The cryo-sectioned tumor tissues were used for CD-31 staining.

5.3.1 Statistical Analysis

The results of MTT, luciferase reporter and tumorigenicity assays are expressed as mean ± SE or ± SD. Statistical differences were analyzed by ANOVA using Sigma-Stat software. $P$ value of $< 0.05$ was considered as significant.