Osteopontin Induces AP-1-mediated Secretion of Urokinase-type Plasminogen Activator through c-Src-dependent Epidermal Growth Factor Receptor Transactivation in Breast Cancer Cells

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We have recently reported that osteopontin (OPN) stimulates cell motility and nuclear factor κB-mediated secretion of urokinase-type plasminogen activator (uPA) through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells (Das, R., Mahabaleshwar, G. H., and Kundu, G. C. (2003) J. Biol. Chem. 278, 28593–28606). However, the role(s) of OPN on AP-1-mediated uPA secretion and cell motility and the involvement of c-Src/epidermal growth factor receptor (EGFR) in these processes in breast cancer cells are not well defined. In this study we report that OPN induces α3β1 integrin-mediated c-Src kinase activity in both highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. Ligation of OPN with α3β1 integrin kinase activity and tyrosine phosphorylation of EGFR in MDA-MB-231 and wild type EGFR-transfected MCF-7 cells, and this was inhibited by the dominant negative form of c-Src (dn c-Src) indicating that c-Src kinase plays a crucial role in this process. OPN induces association between α3β1 integrin and EGFR on the cell membrane in a macromolecular form with c-Src. Furthermore, OPN induces α3β1 integrin/EGFR-mediated ERK1/2 phosphorylation and AP-1 activation. Moreover, dn c-Src also suppressed the OPN-induced phosphatidylinositol (PI) 3-kinase activity in these cells indicating that c-Src acts as master switch in regulating MEK/ERK1/2 and phosphatidylinositol 3-kinase/Akt signaling pathways. OPN-induced ERK phosphorylation, AP-1 activation, uPA secretion, and cell motility were suppressed when cells were transfected with dn c-Src or pretreated with α3β1 integrin antibody, c-Src kinase inhibitor (pp2), EGFR tyrosine kinase inhibitor (PD155035), and MEK-1 inhibitor (PD98059). To our knowledge, this is the first report that OPN induces α3β1 integrin-mediated AP-1 activity and uPA secretion by activating c-Src/EGFR/ERK signaling pathways and further demonstrates a functional molecular link between OPN-induced integrin/c-Src-dependent EGFR phosphorylation and ERK/AP-1-mediated uPA secretion, and all of these ultimately control the motility of breast cancer cells.

Osteopontin (OPN)1 a non-collagenous, sialic acid-rich, and glycosylated phosphoprotein is a member of the extracellular matrix (ECM) protein family (1, 2). OPN acts both as chemokine and cytokine. It is produced by osteoclast, macrophages, T cells, hematopoietic cells, and vascular smooth muscle cells (3). It has an N-terminal signal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, a GRGDS cell adhesion sequence predicted to be flanked by the β-sheet structure (4). This protein has a functional thrombin cleavage site and is a substrate for tissue transglutaminase (2). It binds with several integrins and CD44 variants in an RGD sequence-dependent and -independent manner (5, 6). This protein is involved in normal tissue-remodeling process such as bone resorption, angiogenesis, wound healing, and tissue injury as well as certain diseases such as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (6–8). OPN expression is up-regulated in several cancers and is reported to associate with tumor progression and metastasis (9–11). The level of OPN was high in the plasma of patients with breast cancer and has been shown to correlate with poor prognosis of breast cancer (10, 12). OPN regulates cell adhesion, cell migration, ECM invasion, and cell proliferation by interacting with its receptor α3β1 integrin in various cell types (6). Previous data indicated that OPN induces pro-matrix metalloproteinase-2 (pro-MMP-2) activation and urokinase-type plasminogen activator (uPA) secretion, cell motility, ECM invasion, and tumor growth (13–15).

Integrins are non-covalently associated, heterodimeric, cell-surface glycoproteins with α- and β-subunits. The various combinations of the α- and β-subunits form integrin dimers with diverse ligand specificity and biological activities. The interaction of cell-surface integrin with ECM proteins can lead to the regulation of cell growth, differentiation, adhesion, and migration. Integrin-dependent signaling includes Ca2+ influx, cytoplasmic alkalinization, potassium channel activation, activation of lipid mediators, tyrosine phosphorylation of cytoplasmic proteins, and activation of mitogen-activated protein kinases (MAPK), ERK1 and ERK2 (16–20). Integrins transduce intracellular signaling by interacting with FAK and Src (21, 22). After activation by integrins, p125 FAK is phosphorylated at Tyr-397 which serves as a binding site for SH2 domain of c-Src and other Src family kinases (23). The Src kinase then phosphorylates Tyr-925 of FAK. This generates binding site of the Grb2-SOS complex that leads to the activation of MAPK cascades. Src family kinase could also activate MAPK pathway independent of FAK (24).

Epidermal growth factor receptor (EGFR) belongs to the

uPA, urokinase-type plasminogen activator; ECM, extracellular matrix; dn c-Src, dominant negative form of c-Src; FAK, focal adhesion kinase; PI, phosphatidylinositol; TGF-α, transforming growth factor-α; FPLC, fast protein liquid chromatography; MMPs, matrix metalloproteinases; EGF, epidermal growth factor; WT, wild type.
ErbB growth factor receptor family. The overexpression of EGFR has been correlated with tumor progression and poor metastasis in breast and colon cancers (25, 26). The oncogenic effects of EGFR include enhanced DNA synthesis, cell growth, invasion, and metastasis. In fibroblasts, c-Src synergistically increases the oncogenic activity of EGFR suggesting that these two kinases may cooperate in the progression of a malignant phenotype (27). c-Src activity has been shown to be required for both the mitogenic effect of EGF in fibroblasts (28) and the migratory effects of EGF in epithelial cells (29). Earlier reports have indicated that OPN induces migration of human mammary epithelial cells through activation of EGFR (30). It has been described that c-Src catalytic activity is required for αβ3 integrin-EGFR macromolecular complex formation followed by EGFR phosphorylation (31). EGFR has been shown to be instrumental in the activation of the MAPK pathway (32). Inhibition of ERK1 and ERK2 by the ERK inhibitor, PD98059, resulted in the significant inhibition of the basal cell migration (33). MEK-1 is a serine/threonine kinase that modulates the ERK1/2 pathway (34). ERK1/2 plays key role in regulation of the transcription factor, AP-1, as its activation leads to the induction of c-Fos, which associates with c-Jun to form an AP-1 heterodimeric complex that can promote targeted gene expression (35). However, the molecular mechanism by which OPN regulates c-Src-dependent EGFR transactivation and whether this leads to the activation of AP-1 through MAPK signaling pathway is not clearly understood.

uPA is a member of the serine protease family that interacts with the uPA receptor and facilitates the conversion of inactive plasminogen into widely acting serine protease plasmin (36). Plasmin regulates cell invasion by degrading matrix proteins such as fibronectin, type IV collagen, and laminin or indirectly by activating matrix metalloproteinases (MMPs) (37). Integrin-EGFR interaction also leads to the activation of AP-1, which is a prerequisite for proteolytic degradation, invasion, and distant metastasis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The rabbit polyclonal anti-EGF, anti-EGFR, anti-ERK1/2, anti-c-Src, anti-c-Jun, anti-actin, mouse monoclonal anti-phosphotyrosine, anti-phospho-ERK1/2, anti-αβ3 integrin, and goat polyclonal anti-TGF-β antibodies were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-uPA antibody was obtained from Oncogene. Mouse monoclonal anti-human αβ3 integrin antibody was from Chemicon International. PD153035, pp2, and PD98059 were obtained from Calbiochem. Rabbit muscle enolase was from ICN. LipofectAMINE Plus and GRGDSP and GRGESP peptides were purchased from Invitrogen. The anti-OPN antibody was purchased from R & D Systems. The dual luciferase reporter assay system and AP-1 consensus oligonucleotide were from Promega. Boyden-type cell migration chambers were obtained from Corning Glass, and Bio- Coat Matrigel invasion chambers were from Collaborative Biomedical. 31P/ATP was purchased from the Board of Radiation and Isotope Technology (Hyderabad, India). All other chemicals were analytical grade.  

**Cell Culture**—The MDA-MB-231 and MCF-7 cells were purchased from the ATCC (Manassas, VA). Both MDA-MB-231 and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium. The medium was supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml glutamine in a humidified atmosphere of 5% CO2, and 95% air at 37 °C.  

**OPN Purification**—OPN was purified from human milk as described previously (13). Briefly, the cleared milk sample was loaded onto a DEAE-Sephadex column. The fraction containing partially purified OPN was purified further on an FPLC Resource-Q column and rechromatographed on the same Resource-Q column. The purity of OPN was checked by SDS-PAGE following Coomassie Blue staining. The purified OPN was characterized by Western blot using anti-OPN antibody. Because EGF and TGF-α are also present in milk, we therefore have checked whether trace amounts of EGF or TGF-α are present in the purified OPN preparation. Accordingly, purified OPN was resolved by SDS-PAGE and analyzed by Western blot using either a mixture of anti-OPN and anti-EFG or anti-TGF-α antibody. This purified OPN was used throughout these studies.  

**Plasmas and DNA Transfection**—Wild type EGFR (EGFR-WT) and kinase-inactive mutant EGFR (EGFR-K) cDNA constructs were generous gifts from Dr. A. N. Habib (Beth Israel Deaconess Medical Center and Harvard Medical School, Boston). Both MCF-7 and MDA-MB-231 cells were transfected with cDNA transfected with cDNA Plus according to the manufacturer’s instructions (Invitrogen). Briefly, cDNA (8 μg) was mixed with Plus reagent, and then cDNA reagent Plus was incubated with LipofectAMINE. The LipofectAMINE Plus cDNA complex was added to the cells and incubated further at 37 °C for 12 h. The control cells received LipofectAMINE Plus alone. The cell viability was detected by a trypan blue dye exclusion test. After incubation, the medium was removed, and the cells were refed with fresh medium and maintained for an additional 12 h. In other experiments, these cells were individually transfected with the dominant negative form of c-Src cDNA (K296R/Y528F, dn c-Src) in pUSEamp (Upstate Biotechnology) under the same conditions as described above. These transfected cells were used for EGFR phosphorylation, ERK1/2 phosphorylation, uPA expression by Western blot analysis, AP-1 activity by luciferase reporter gene assay, cell migration, and ECM invasion assays.

**Western Blot Analysis**—To delineate the role of OPN in regulation of ERK1/2 phosphorylation, both MDA-MB-231 and MCF-7 cells were treated with 5 μM OPN at 37 °C for 0–60 min. In another experiment, both MDA-MB-231 and MCF-7 cells were individually pretreated with anti-αβ3 integrin antibody (20 μg/ml), GRGDSP (10 μM), GRGESP (10 μM), EGFR tyrosine kinase inhibitor (25 μM PD153035), c-Src kinase domain inhibitor (2 mM pp2), or MEK-1 inhibitor (50 μM PD98059) for 1 h and then treated with OPN as described above. In separate experiments, MCF-7 cells were transfected with cDNA for EGFR-EGFR-WT or kinase-inactive mutant of EGFR (EGFR-K) cDNA, or both these cells were transfected with dn c-Src cDNA in the presence of LipofectAMINE Plus and then stimulated with OPN. Cells were lysed in lysis buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA) containing 25 mM NaF and 2 mM Na3VO4. The cleared lysates were collected by centrifugation at 12,000 × g for 15 min at 4 °C. The protein concentration in the lysate was measured by Bio-Rad protein assay. The lysates containing equal amounts of total proteins were resolved by SDS-PAGE. The proteins were electrotransferred from gel to nitrocellulose membrane. The membrane was incubated with mouse monoclonal anti-EGFR, ERK1/2 antibody and incubated further with anti-mouse hors eradish peroxidase-conjugated IgG. The membrane was washed and detected by the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) according to the manufacturer’s instructions. The membrane was probed with rabbit polyclonal anti-ERK1/2 antibody to ensure equal protein loading. To investigate the role of αβ3 integrin, c-Src, EGFR, and ERK1/2 in OPN-induced uPA secretion, both MDA-MB-231 and MCF-7 cells were individually transfected with anti-αβ3 integrin antibody (20 μg/ml), GRGDSP (10 μM), GRGESP (10 μM), PD153035 (25 μM) (MDA-MB-231 cells), pp2 (2 mM), PD98059 (10 μM) for 1 h and then treated with 5 μM OPN for an additional 37 °C. Cell lysates and amounts of total proteins were subjected to Western blot analysis using mouse monoclonal anti-uPA antibody. In separate experiments, both these cells were individually transfected with EGFR-WT, EGFR-K, or dn c-Src cDNA in the presence of LipofectAMINE Plus and then treated...
with 5 μM OPN for 24 h. The level of pUPA in cell lysates was detected by Western blot analysis. The same blots were probed with anti-actin antibody as loading control.

Immunoprecipitation—To examine the effect of OPN in regulation of EGFR phosphorylation, both MDA-MB-231 and MCF-7 cells were treated with 5 μM OPN at 37 °C for 0–60 min. In separate experiments, MCF-7 cells were transfected with EGFR-WT or EGFR-K and then treated with 5 μM OPN for 0–60 min as described above. In other experiments, MDA-MB-231 cells or EGFR-WT transfected MCF-7 cells were either pretreated with anti-α,β3 integrin blocking antibody (20 μg/ml), GRGDSP (10 μM), GRGESP (10 μM), pp2 (2 μM), or transfected with dn c-Src and then treated with 5 μM OPN for 30 min. Cell lysates containing equal amounts of total proteins were immunoprecipitated with mouse monoclonal anti-phosphotyrosine antibody. The same blots were reprobed with rabbit polyclonal anti-EGFR antibody as loading controls.

To delineate whether OPN regulates any direct interactions between α,β3 integrin and EGFR or c-Src in breast cancer cells, non-transfected MDA-MB-231 cells or EGFR-WT transfected MCF-7 cells were individually treated with OPN (5 μM) for 0–30 min. Cell lysates were immunoprecipitated with mouse monoclonal anti-α,β3 integrin antibody. Half of the immunocomplex was immunoblotted with anti-EGFR antibody, and the other half was immunoblotted with anti-c-Src antibody.

c-Src and PI 3-Kinase Assays—To check the role of OPN in regulation of c-Src activity, both MDA-MB-231 and MCF-7 cells were treated with 5 μM OPN at 37 °C for 0–30 min. In separate experiments, cells were pretreated with anti-α,β3 integrin antibody (20 μg/ml), GRGDSP (10 μM), or GRGESP (10 μM) for 1 h and then treated with 5 μM OPN at 37 °C for 5 min. Cells were lysed in Src lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 50 mM NaF, 1 mM phenylmethysulfonyl fluoride, and 0.5 mM dithiothreitol) and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer.

These treated or transfected cells were used for PI 3-kinase assay as described previously (43). Briefly, cells were immunoprecipitated with rabbit polyclonal anti-c-Src antibody. Half of the immunocomplex was incubated with kinase assay buffer (20 mM Hepes (pH 7.4), 10 mM MgCl2,1 0m M KCl, 0.2 mM nyl fluoride, 1% Nonidet P-40, 2 mM orthovanadate, 10 mg/ml pepstatin/H9262 GRGDSP (10 μM), GRGESP (10 μM), PD153035 (25 μM) (MDA-MB-231 alone), pp2 (2 μM), or PD98059 (50 μM) for 1 h and then treated with OPN (5 μM) for an additional 6 h at 37 °C. In other experiments, both these cells were individually cotransfected with EGFR-WT, EGFR-K, or dn c-Src with pAP-1-Luc and then treated with OPN (5 μM) for 6 h. Cells were harvested in passive lysis buffer (Promega). The luciferase activities were measured by a 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.

Cell Migration Assay—The migration assay was conducted using a Transwell cell culture chamber according to the standard procedure as described previously (15, 41). Both MDA-MB-231 and MCF-7 cells were individually pretreated with anti-α,β3 integrin antibody (20 μg/ml), GRGDSP (10 μM), GRGESP (10 μM), PD153035 (25 μM) (MDA-MB-231 alone), pp2 (2 μM), and PD98059 (50 μM) for 3 h. In separate experiments, both these cells were individually transfected with FGFR1 or c-Src M. Cell suspension (5 × 105 cells/mL) for 6 h. Cells were harvested in passive lysis buffer (Promega). The luciferase activities were measured by a 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.

Chemoinvasion Assay—The chemoinvasion assay was performed using Matrigel™-coated invasion chamber as described (14, 15). MDA-MB-231 or MCF-7 cell suspension (5 × 105 cells/mL) was added to the upper portion of the prehydrated Matrigel™-coated chamber. The lower chamber was filled with fibroblast-conditioned medium, which acted as chemointron. Purified human OPN (5 μM) was added to the lower chamber 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 served as a nonspecific control.

OPN Purification and Western Blot Analysis—The OPN was partially purified from human milk by DEAE-Sephadex chromatography followed by final purification on an FPLC Resource-Q column. The final purity of OPN was checked by SDS-PAGE, followed by Coomassie Blue (Fig. 1, panel A, lanes 1–4) and Silver (panel B, lanes 1–4) staining. This was characterized by Western blot analysis using established goat anti-OPN antibody (panel C, lanes 1–3). To confirm further whether any trace amount of EGFR ligands (e.g. EGF or TGF-α) are present in the purified OPN preparation, this was resolved by SDS-PAGE and analyzed by Western blot using a mixture of

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anti-OPN and anti-EGF or anti-TGF-α antibody. The results showed that EGF or TGF-α was absent in purified OPN preparations although these proteins were present in whole milk (panels D and E, lanes 1–3).

**OPN Induces αβ3 Integrin-mediated Autophosphorylation of c-Src and Its Kinase Activity**—To investigate the role of OPN on c-Src kinase activity and to demonstrate the involvement of αβ3 integrin in this activation process, both MDA-MB-231 and MCF-7 cells were treated with 5 μM OPN for 0–30 min or pretreated with αβ3 integrin antibody or RGD peptide and then treated with OPN for 5 min. Cell lysates were immunoprecipitated with anti-c-Src antibody. Half of the immunoprecipitated samples were used for c-Src kinase assay using enolase as a substrate. The maximum c-Src kinase activity and autophosphorylation were observed at 5 min of OPN stimulation in both MDA-MB-231 and MCF-7 cells (Fig. 2, upper panels A and B, lanes 1–5). Pretreatment of cells with anti-αβ3 integrin antibody or GRGDSP but not with GRGESP peptide suppressed the OPN-induced c-Src autophosphorylation and kinase activity in both these cells (upper panels A and B, lanes 6–8), suggesting that OPN induces c-Src kinase activity through αβ3 integrin-mediated pathway. The other half of the immunoprecipitated samples was analyzed by Western blot using anti-c-Src antibody, and the level of c-Src was unchanged in these cells (lower panels A and B, lanes 1–8). The bands were analyzed densitometrically (Kodak Digital Science), and the fold changes were calculated.

**OPN Enhances αβ3 Integrin and c-Src-mediated EGFR Kinase Activity and Tyrosine Phosphorylation**—Previous studies (31) have demonstrated that ligand binding to integrins could lead to transactivation of receptor tyrosine kinase. Therefore, we sought to determine the role of OPN on EGFR transactivation in breast cancer cells. Accordingly, MDA-MB-231 cells were treated with 5 μM OPN for 0–60 min, and cell lysates were immunoprecipitated with anti-EGFR antibody. The immunocomplex was used for kinase assay. The maximum EGFR kinase activity was detected at 30 min of OPN stimulation (Fig. 3, upper panel A, lanes 1–6). The level of non-phospho-EGFR was detected by Western blot analysis, and it remained the same (lower panel A, lanes 1–6).

To examine the role of OPN in regulating tyrosine phosphorylation of EGFR, both MDA-MB-231 and MCF-7 cells were treated with 5 μM OPN for 0–60 min. Cell lysates were immunoprecipitated with anti-EGFR antibody. The immunoprecipitated samples were analyzed by Western blot using anti-phosphotyrosine antibody, and the blots were reprobed with anti-EGFR antibody. The results indicated that maximum OPN-induced tyrosine phosphorylation of EGFR was observed at 30 min in MDA-MB-231 cells (Fig. 3, upper panel B, lanes 1–6). However, no induction of EGFR tyrosine phosphorylation was detected in MCF-7 cells upon OPN stimulation (upper panel C, lanes 1–6). This could be because of less expression of EGFR in MCF-7 cells (45). To examine further the status of OPN-induced tyrosine phosphorylation of EGFR, both MDA-MB-231 and MCF-7 cells were transfected with wild type EGFR (upper panel D, lanes 1–6) but not with EGFR-K' (upper panel E, lanes 1–6), and maximum phosphorylation was observed at 30 min (upper panel D, lane 4) upon OPN stimulation. The level of non-phospho-EGFR remained identical (lower panels B–E, lanes 1–6). These results
suggested that OPN could induce tyrosine phosphorylation of EGFR in MDA-MB-231 cells but not in normal MCF-7 cells. The kinetics of tyrosine phosphorylation of EGFR in wild type EGFR-transfected MCF-7 cells was almost identical with non-transfected MDA-MB-231 cells (panels B and D). No enhanced phosphorylation was detected in MCF-7 cells transfected with kinase-inactive mutant of EGFR (EGFR-K-1) after OPN stimulation (Fig. 3, panel E) suggesting that this domain is crucial for tyrosine phosphorylation.

To check the role of \(\alpha_\beta_3\) integrin and c-Src in OPN-induced EGFR phosphorylation, non-transfected MDA-MB-231 and EGFR-WT-transfected MCF-7 cells were pretreated with \(\alpha_\beta_3\) integrin antibody, GRGDSP, GRGESP, or pp2 and then treated with 5 \(\mu\)M OPN for 30 min. In separate experiments, MDA-MB-

FIG. 2. OPN stimulates the autophosphorylation and kinase activity of c-Src. Both MDA-MB-231 (panel A) and MCF-7 (panel B) cells were either treated with 5 \(\mu\)M OPN for 0–30 min or pretreated with anti-\(\alpha_\beta_3\) integrin blocking antibody or RGD peptide and then treated with OPN. Cell lysates containing equal amounts of total proteins were immunoprecipitated with anti-c-Src antibody, and half of the immunoprecipitated samples was used for kinase assay using enolase as substrate (upper panels A and B, lanes 1–8). The remaining half of the immunoprecipitated samples was analyzed by Western blot using anti-c-Src antibody (lower panels A and B, lanes 1–8). The arrows indicate the specific bands of \(32^P\)-c-Src, \(32^P\)-enolase and c-Src. All these bands were quantified by densitometric analysis, and the fold changes were calculated. The results shown here represent three experiments exhibiting similar effects.

FIG. 3. OPN induces kinase activity and tyrosine phosphorylation of EGFR. Panel A, EGFR kinase activity. MDA-MB-231 cells were stimulated with 5 \(\mu\)M OPN for 0–60 min and immunoprecipitated with anti-EGFR antibody. The half of the immunoprecipitated samples was used for kinase assay (upper panel, lanes 1–6), and the remaining half was used for Western blot using anti-EGFR antibody (lower panel, lanes 1–6). Panels B and C, EGFR tyrosine phosphorylation. Both MDA-MB-231 (panel B) and MCF-7 (panel C) cells were treated with 5 \(\mu\)M OPN for 0–60 min. Cell lysates were immunoprecipitated with anti-EGFR antibody, and the immunocomplexes were analyzed by Western blot with anti-phosphotyrosine antibody (lanes 1–6). Panels D and E, MCF-7 cells were transfected with wild type EGFR (EGFR-WT) (panel D) or with the kinase-inactive mutant of EGFR (EGFR-K-1) (panel E) in the presence of LipofectAMINE Plus and then treated with 5 \(\mu\)M OPN for 0–60 min. Cell lysates were immunoprecipitated with anti-EGFR antibody and immunoblotted with anti-phosphotyrosine antibody (lanes 1–6). These blots were reprobed with anti-EGFR antibody (panels B–E, lower panels). Note that maximum EGFR phosphorylation was observed at 30 min in MDA-MB-231 and EGFR-WT-transfected MCF-7 cells. All these bands were quantified by densitometric analysis, and the fold changes were calculated. The results shown here represent three experiments exhibiting similar effects.
231 and EGFR-WT-transfected MCF-7 cells were further transfected with dn c-Src and then treated with 5 mM OPN for 30 min. Cell lysates were immunoprecipitated with anti-EGFR antibody, and the immunoprecipitated samples were immunoblotted with anti-phosphotyrosine antibody (upper panels A and B, lanes 1–6; upper panels C and D, lanes 1–3). The blots were reprobed with anti-EGFR antibody (lower panels A and B, lanes 1–6; lower panels C and D, lanes 1–3). Panels E and F, effect of OPN on the interaction between αvβ3 integrin, EGFR, and c-Src. Non-transfected MDA-MB-231 (panel E) or EGFR-WT-transfected MCF-7 (panel F) cells were individually treated with 5 μM OPN for 0–30 min. Cell lysates were immunoprecipitated with anti-αvβ3 integrin antibody. Half of the immunocomplexes were immunoblotted with anti-EGFR antibody (upper panels E and F, lanes 1–4) and the remaining half with anti-c-Src antibody (lower panels E and F, lanes 1–4). All these bands were quantified by densitometric analysis, and the fold changes were calculated. The results shown here represent three experiments exhibiting similar effects.

**EGFR Interacts with αvβ3 Integrin and c-Src in the Presence of OPN**—It is reported that αvβ3 integrin associates with c-Src and EGFR on the cell membrane in a macromolecular complex form, which leads to the phosphorylation of EGFR (31). Because we have shown that OPN induces EGFR phosphorylation, we therefore sought to determine whether OPN could regulate this macromolecular complex formation in MDA-MB-231 and MCF-7 cells. Accordingly, MDA-MB-231 and EGFR-WT transfected MCF-7 cells were stimulated with 5 mM OPN for 0–30 min. Cell lysates were immunoprecipitated with anti-αvβ3 integrin antibody, and the half of the immunoprecipitated samples were used for Western blot analysis by using anti-EGFR antibody and the other half of the samples were immunoblotted by anti-c-Src antibody. The results revealed that αvβ3 integrin interacts with EGFR and c-Src in the presence of OPN in MDA-MB-231 (Fig. 4, upper and lower panels E, lanes 1–4) and in EGFR-WT-transfected MCF-7 (upper and lower panels F, lanes 1–4) cells. These interactions were transient, and maximum interactions were observed at 5 min (lane 2) in both these cells.
OPN Induces α,β3 Integrin-mediated c-Src and EGFR-dependent ERK1/2 Phosphorylation—To examine whether OPN regulates ERK1/2 phosphorylation and whether EGFR plays any role in this process, we have treated MDA-MB-231 and MCF-7 cells with 5 μM OPN for 0–60 min. Cell lysates were analyzed by Western blot using anti-phospho-ERK1/2 antibody, and the same blots were reprobed with anti-ERK1/2 antibody. The data indicated that OPN induces ERK1/2 phosphorylation in MDA-MB-231 (Fig. 5, upper panel A, lanes 1–6) and MCF-7 (upper panel B, lanes 1–6) cells. There were 3.4- and 2.5-fold increased in ERK1/2 phosphorylation in MDA-MB-231 and MCF-7 cells, respectively. Maximum phosphorylation was observed at 5 min in both these cells. The level of phosphorylation was retained up to 30 min in MDA-MB-231 cells, while this phosphorylation declined rapidly after 5 min in normal MCF-7 cells. To check whether EGFR is required for OPN-induced long term ERK phosphorylation, MCF-7 cells were individually transfected with EGFR-WT or EGFR-K, treated with OPN for 0–60 min, and the level of phospho-ERK was detected by Western blot analysis. The data demonstrated that the degree of OPN-induced ERK phosphorylation in EGFR-WT-transfected MCF-7 cells was similar to MDA-MB-231 cells (upper panel A and C, lanes 1–6). However, the level of phosphorylation in EGFR-K-transfected MCF-7 cells (upper panel D, lanes 1–6) was almost identical as non-transfected MCF-7 cells (upper panel B, lanes 1–6), indicating that the kinase domain of EGFR is crucial in this process. The expression of non-phospho-ERK remained the same (lower panels A–D).

To investigate the effects of α,β3 integrin antibody and EGFR tyrrosine kinase inhibitor (PD153035) on OPN-induced ERK1/2 phosphorylation, both these cells were pretreated with α,β3 integrin antibody, RGD peptide, or PD153035 and then treated with OPN as described earlier. Cell lysates were analyzed by Western blot using anti-phospho-ERK1/2 antibody. These results showed that α,β3 integrin antibody, GRGDSP but not GRGESP peptide, and PD153035 partially attenuated OPN-induced ERK1/2 phosphorylation in MDA-MB-231 cells (Fig. 6, upper panel A, lanes 1–6). The OPN-induced ERK phosphorylation was completely blocked when MDA-MB-231 cells were pretreated with a mixture of α,β3 integrin antibody and PD153035 suggesting that both α,β3 integrin and EGFR are important in OPN-induced ERK1/2 phosphorylation (lane 7). The α,β3 integrin antibody, GRGDSP but not GRGESP or PD153035, could specifically block OPN-induced ERK phosphorylation in MCF-7 cells (upper panel B, lanes 1–6). These data suggested that OPN induces α,β3 integrin-mediated ERK1/2 phosphorylation through EGFR-dependent (MDA-MB-231 cells) and -independent (MCF-7 cells) pathways.

OPN Modulates AP-1-mediated UPA Secretion—We have shown that c-Src kinase activity is required for OPN-induced α,β3 integrin/EGFR-mediated ERK1/2 phosphorylation. Therefore, we have delineated whether c-Src plays any role in OPN-induced α,β3 integrin-mediated PI 3-kinase activity. Accordingly, cells were either transfected with dn c-Src or pretreated with pp2 or PD98059 and then treated with OPN. The data revealed that both genetic dnc-Src and pharmacological (pp2) inhibitors of c-Src and MEK-1 inhibitor (PD98059) suppressed OPN-induced ERK1/2 phosphorylation in both MDA-MB-231 (upper panel C, lanes 1–5) and MCF-7 (upper panel D, lanes 1–5) cells. These data suggested that OPN-induced ERK1/2 phosphorylation is regulated by both c-Src and MEK. The expression of non-phospho-ERK remained identical (lower panels A–D).

c-Src Regulates PI 3-Kinase Activity in the Presence of OPN—We have shown that c-Src kinase activity is required for OPN-induced α,β3 integrin/EGFR-mediated ERK1/2 phosphorylation. Therefore, we have delineated whether c-Src plays any role in OPN-induced α,β3 integrin-mediated AP-1 transactivation. According to this, cells were either transfected with dn c-Src or pretreated with pp2 and then treated with OPN, and PI 3-kinase activity was measured. The data revealed that both genetic (dn c-Src) and pharmacological (pp2) inhibitors of c-Src suppressed OPN-induced PI 3-kinase activity in MDA-MB-231 (panel E, lanes 1–4) and MCF-7 (panel F, lanes 1–4), cells suggesting that c-Src regulates OPN-induced PI 3-kinase activity.

OPN Induces c-Fos Expression, AP-1-DNA Binding, and AP-1 Transactivation—An earlier report (35) showed that activation of ERK could induce c-Fos expression and leads to AP-1 activation. Therefore, we first examined the level of c-Fos expression in MDA-MB-231 and MCF-7 cells. Accordingly, both these cells were treated with 5 μM OPN for 0–4 h, and nuclear extracts were prepared, and the level of c-Fos expression was detected by Western blot analysis using anti-c-Fos antibody. The results indicated that OPN induced c-Fos expression, and the level of c-Fos was higher at 1 h in MDA-MB-231 (Fig. 7, panel A, lanes 1–5) and MCF-7 (panel B, lanes 1–5) cells.

To check whether OPN induces AP-1 DNA binding, both

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**Fig. 5.** Panels A and B, OPN stimulates ERK1/2 phosphorylation. Both MDA-MB-231 (panel A) and MCF-7 (panel B) cells were treated with 5 μM OPN for 0–60 min. Cell lysates were analyzed by Western blot using anti-phospho-ERK1/2 antibody (upper panels A and B, lanes 1–6). Panels C and D, overexpression of EGFR enhances OPN-induced ERK1/2 phosphorylation. MCF-7 cells were either transfected with EGFR-WT (panel C) or with EGFR-K (panel D) and then treated with 5 μM OPN for 0–60 min. Cell lysates were analyzed by Western blot using anti-phospho-ERK1/2 antibody (upper panels C and D, lanes 1–6). All these blots were reprobed with anti-ERK1/2 antibody (lower panels A–D). The bands were quantified by densitometric analysis, and the fold changes were calculated. The results shown here represent three experiments exhibiting similar effects.
these cells were treated with 5 μM OPN for 0–4 h; nuclear extracts were prepared and used for EMSA using 32P-labeled AP-1 oligonucleotides. The maximum OPN-induced AP-1-DNA binding was observed in 1 hi nMDA-MB-231 (panel C, lanes 1–5) and in MCF-7 (panel E, lanes 1–5) cells. Whether the band (panels C and E) obtained by EMSA in OPN-treated cells is indeed AP-1, the nuclear extracts were incubated with either anti-c-Jun or anti-c-Fos antibody and then analyzed by EMSA. Fig. 7, panel D, showed the shift of AP-1-specific band to higher molecular weight when the nuclear extracts were treated with either anti-c-Jun or anti-c-Fos antibody suggesting that OPN-activated AP-1 complex consisted of c-Jun and c-Fos subunits (lanes 1–3). The AP-1-DNA binding data corroborates with the expression of c-Fos in nuclear extracts of MDA-MB-231 and MCF-7 cells.

To detect whether OPN stimulates AP-1 transcriptional activity and whether αvβ3 integrin, c-Src, EGFR, and ERK are involved in this process, luciferase reporter gene assay was performed. Both MDA-MB-231 and MCF-7 cells were transfected with pAP-1 luciferase reporter construct (pAP-1-Luc) in the presence of LipofectAMINE Plus. Transfected cells were either treated with OPN (5 μM) or pretreated with anti-αvβ3 integrin antibody (20 μg/ml), GRGDSP (10 μM), GREGSP (10 μM), PD153035 (25 μM) (MDA-MB-231 alone), pp2 (2 nM), or PD98059 (50 μM) and then treated with OPN (5 μM). In separate experiments, cells were individually cotransfected with GFR-WT, EGFR-K, or dn c-Src and then treated with OPN (5 μM). Changes in luciferase activity with respect to control were calculated. The data demonstrated that OPN stimulates the AP-1 transcriptional activity, and αvβ3 integrin antibody, RGD peptide, EGFR inhibitor (PD153035), c-Src inhibitor (pp2), or ERK1/2 inhibitor (PD98059) suppressed the OPN-induced AP-1 activity (panels F and G). Similarly, dn c-Src inhibited and EGFR-WT enhanced the OPN-induced AP-1 activity in these cells (panels F and G). EGFR-K has no effect on OPN-induced AP-1 transactivation (panels F and G). These data suggested that OPN induces AP-1 transactivation, and this activation was mediated by αvβ3 integrin, EGFR, c-Src, and ERK in these cells.

**OPN Induces EGFR and ERK1/2-dependent uPA Secretion—** Because the AP-1-binding sequences are present in the promoter...
and we have shown in this paper that OPN induced AP-1-DNA binding and AP-1 transactivation, we therefore sought to determine whether \( /H_{v2} /H_{2} /H_{3} \) integrin, c-Src, and ERK which regulate AP-1 activity are involved in OPN-induced uPA secretion in MDA-MB-231 and MCF-7 cells. Accordingly, both these cells were treated with 5 \( /H_{m} \) OPN or pretreated with anti-\( /H_{v2} /H_{2} /H_{3} \) integrin blocking antibody (20 \( /H_{g} /H_{m} \)), RGD peptide (10 \( /H_{m} \)), PD153035 (25 \( /H_{p} /H_{M} \)) (MDA-MB-231 alone), pp2 (2 \( /H_{n} \)), or PD98059 (50 \( /H_{M} \)) and then treated with OPN (5 \( /H_{m} \)). Cell lysates were analyzed by Western blot using mouse monoclonal anti-uPA antibody. The data indicated that OPN induces the uPA secretion, and OPN-induced uPA secretion was suppressed by \( /H_{v2} /H_{2} /H_{3} \) integrin antibody, GRGDSP but not GRGESP peptide, PD153035, pp2, or PD98059 in MDA-MB-231 (Fig. 8, upper panel A, lanes 1–9) and MCF-7 (upper panel B, lanes 1–7) cells.

In separate experiments, both these cells were individually transfected with wild type EGFR (EGFR-WT), EGFR-K\(^{-} \) or dn c-Src in the presence of LipofectAMINE Plus and then treated with OPN (5 \( /H_{m} \)) as described above. The results indicated that EGFR-WT (panels C and D, lane 3) enhanced and dn c-Src (lane 5) suppressed the OPN-induced uPA secretion compared with cells treated with OPN alone (lane 2). EGFR-K\(^{-} \) had no effect on OPN-induced uPA secretion (lane 4). As loading controls, all these blots were reprobed with anti-actin antibody (lower panels A–D). In all these experiments, the uPA-specific bands were quantified by densitometric analysis, and the values of fold changes are indicated. These results demonstrated that OPN induces uPA secretion via \( /H_{v2} /H_{2} /H_{3} \) integrin/c-Src/EGFR/ERK-mediated pathways and further suggested that AP-1 is involved in this process.

**EGFR, c-Src, and ERK1/2 Play Crucial Roles in OPN-induced \( /H_{v2} /H_{2} /H_{3} \) Integrin-mediated Cell Migration and Chemoinvasion**—We have shown earlier that OPN regulates \( /H_{v3} \) integrin-mediated c-Src and EGFR-dependent ERK1/2 phos-
phorylation and uPA secretion in MDA-MB-231 and MCF-7 cells. Therefore, we have checked whether OPN-induced c-Src/EGFR/ERK-dependent AP-1 activation and uPA secretion play any role in breast cancer cell migration and invasion. Accordingly, both these cells were individually treated with anti-αβ3 integrin antibody, RGD peptide, PD153035 (MDA-MB-231 alone), pp2 (2 nM), and PD98059 (50 μM) for 1 h and then treated with OPN for an additional 24 h. In separate experiments, MDA-MB-231 (panel C) and MCF-7 (panel D) cells were individually transfected with EGFR-WT, EGFR-K, or dn c-Src and then treated with 5 μM OPN. The level of uPA in the cell lysates was detected by Western blot analysis using anti-uPA antibody. The arrow indicates uPA-specific band (upper panels A–D). The same blots were reprobed with anti-actin antibody (lower panels A–D). The bands were quantified by densitometric analysis, and fold changes are calculated. The results shown here represent three experiments exhibiting similar effects.

**DISCUSSION**

In a recent study (15), we have demonstrated that OPN stimulates cell motility and NFκB-mediated secretion of uPA through PI 3-kinase/Akt signaling pathways in breast cancer cells. In this paper, we have delineated the molecular mechanisms by which OPN induces EGFR transactivation and AP-1-mediated uPA secretion in highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. We have shown that OPN induced transient interaction between αβ3 integrin, c-Src, and EGFR which is prerequisite for EGFR transactivation. OPN binding to αβ3 integrin also enhanced ERK1/2 phosphorylation and AP-1 activation, and these were regulated by c-Src and EGFR. OPN also enhanced uPA secretion, cell motility, and ECM invasion through c-Src/EGFR/ERK/AP-1-mediated pathways.

OPN plays significant roles in tissue remodeling processes such as bone resorption, angiogenesis, wound healing, and tissue injury as well as certain diseases such as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (6–8).
Integrins are cell-surface glycoproteins that bind to the extracellular matrix proteins. In fibroblast, Src family kinases are known to regulate integrin-mediated cell attachment, spreading, and cell migration (46). It has been reported that many primary breast tumors showed elevated levels of c-Src and are correlated with breast carcinomas (47). These results prompted us to investigate whether ligation of OPN with \( \alpha_\beta_3 \) integrin regulates c-Src kinase activity in breast cancer cells. In this study, we have demonstrated that OPN induces \( \alpha_\beta_3 \) integrin-mediated c-Src kinase activity in both MDA-MB-231 and MCF-7 cells.

Transactivation of growth factor receptors contributes to integrin-mediated activation of the Ras-ERK pathways (48). Recently Tuck et al. (30) reported that OPN activates EGFR and regulates the migration of breast epithelial cells. However, the molecular mechanism(s) by which OPN regulates EGFR transactivation which leads to breast cancer cell migration is not clearly understood. We have demonstrated that OPN stimulated kinase activity and tyrosine phosphorylation of EGFR in MDA-MB-231 cells, whereas it was almost absent in normal MCF-7 cells. This could be due to the low level of expression of \( \alpha_\beta_3 \) integrin in MCF-7 cells. Accordingly, the level of EGFR was enhanced in MCF-7 cells by transfecting with wild type EGFR (EGFR-WT) and then treated with OPN. These data revealed that OPN enhanced the kinase activity (data not shown) and tyrosine phosphorylation of EGFR in these transfected cells. Furthermore, we have shown that OPN-induced tyrosine phosphorylation of EGFR was suppressed by anti-\( \alpha_\beta_3 \) integrin blocking antibody and RGD peptide indicating that EGFR transactivation is mediated by \( \alpha_\beta_3 \) integrin rather than through direct binding of EGFR with OPN. Moreover, our data suggested that c-Src kinase activity is required for OPN-induced \( \alpha_\beta_3 \) integrin-mediated EGFR phosphorylation. This was confirmed by the fact that both genetic and pharmacological inhibitors of c-Src kinase suppressed the OPN-induced EGFR phosphorylation. Our results also revealed that OPN stimulated the association between \( \alpha_\beta_3 \) integrin and EGFR in MDA-MB-231 cells and wild type EGFR (EGFR-WT)-transfected MCF-7 cells. The association was transient and remained up to 5 min upon OPN stimulation. Moro et al. (31) have recently reported that \( \alpha_\beta_3 \) integrin and EGFR form a complex with tyrosine kinase such as c-Src, p130 Cas, and Crk.

**Fig. 9.** Panels A and B, roles of c-Src, EGFR, and ERK on OPN-induced \( \alpha_\beta_3 \) integrin-mediated cell migration. Panels A and B, the migration assay was performed either by using untreated cells (5 × 10^5 cells/well) or cells pretreated with \( \alpha_\beta_3 \) integrin antibody (20 µg/ml), GRGDSP (10 µM), GRGESP (10 µM), PD153035 (25 pM) (MDA-MB-231 alone), pp2 (2 nM), or PD98059 (50 µM) for 1 h. Panels C and D, in separate experiments, cells were individually transfected with EGFR-WT, EGFR-K, or dn c-Src. The transfected cells were used for migration assay. Purified human OPN was added in the upper chamber. Note that OPN-induced cell migration was blocked by \( \alpha_\beta_3 \) integrin antibody, RGD peptide, PD 153035 (MDA-MB-231 alone), pp2, PD98059, and dn c-Src and enhanced by EGFR-WT (panels A and C, MDA-MB-231 cells; panels B and D, MCF-7 cells). The results are expressed as the means ± S.E. of three determinations.
Our data indicated that in addition to EGFR, c-Src also coimmunoprecipitated with the \(/H9251v/H92523\) integrin antibody in cells stimulated with OPN, suggesting that OPN enhances macromolecular complex formation between \(/H9251v/H92523\) integrin, EGFR and c-Src.

It is well established that MAPKs including ERKs, c-Jun N-terminal kinases, and p38 could be activated by integrin upon ligation with ECM proteins and triggered many cellular responses. For example, \(\alpha_\beta_3\) integrin ligation with vitronectin activates p38 MAPK and increases uPA expression in invasive breast cancer cells (49). Fibronectin stimulates Src kinase activity, which further activates ERK1/2 and promotes cell migration through phosphorylation and enhanced activation of myosin light chain kinase (50). However, it is still not clear whether OPN ligation to integrin can activate MAPKs, especially ERK1/2, which may regulate uPA expression and cell migration in breast cancer cells. Our results indicated that OPN-induced ERK1/2 phosphorylation and the maximum phosphorylation was retained up to 30 min in MDA-MB-231 cells, while it was retained up to 5 min in MCF-7 cells. These results led us to hypothesize that EGFR, which was expressed and phosphorylated in high levels in MDA-MB-231 but not in MCF-7 cells, may be responsible for the retaining of long term ERK1/2 phosphorylation in MDA-MB-231 cells. Accordingly, MCF-7 cells were transfected with EGFR-WT or \(\alpha_\beta_3\) integrin antibody and stimulated with OPN. The data showed that ERK1/2 phosphorylation was also retained up to 30 min in these transfected cells indicating the role of EGFR in OPN-induced ERK phosphorylation.

The OPN-induced ERK phosphorylation was inhibited when both MDA-MB-231 and MCF-7 cells when pretreated with \(\alpha_\beta_3\) integrin-blocking antibody or RGD peptide indicating that OPN induces ERK phosphorylation through \(\alpha_\beta_3\) integrin-mediated pathways. Similarly, treatment of MDA-MB-231 cells with either EGFR inhibitor (PD153035) or in combination of PD153035 and \(\alpha_\beta_3\) integrin antibody blocked the OPN-induced ERK phosphorylation. In contrast, \(\alpha_\beta_3\) integrin antibody inhibited the OPN-induced ERK phosphorylation, whereas PD153035 failed to inhibit it in MCF-7 cells. These results clearly suggested that \(\alpha_\beta_3\) integrin and EGFR can activate the OPN-induced ERK1/2 phosphorylation independently; however, the presence of EGFR can enhanced this \(\alpha_\beta_3\) integrin-mediated phosphorylation process. Our data demon-
strated that both pharmacological and genetic inhibitors of c-Src suppressed the OPN-induced ERK phosphorylation in both MDA-MB-231 and MCF-7 cells, suggesting that c-Src kinase plays crucial roles in OPN-regulated ERK1/2 phosphorylation.

AP-1, a family of transcription factors, consists of homodimers or heterodimers of Jun, Fos, or activating transcription factor protein (51). Previous reports (51) have demonstrated that AP-1 is involved in several cellular processes such as cell growth, apoptosis, and cell motility. In addition, AP-1 activity is elevated in several pathological conditions including cancers. The activation of ERK induces the level of c-Fos, which can associate with c-Jun comprising the transcription factor AP-1 (35). Because we have shown that OPN induced ERK1/2 phosphorylation and because the AP-1-binding site is present in the promoter region of the uPA gene, we therefore sought to determine the level of c-Fos expression upon OPN stimulation. OPN enhances the nuclear level of c-Fos in both MDA-MB-231 and MCF-7 cells. Our results also suggested that OPN induces AP-1-DNA binding activity. The OPN-induced c-Fos expression correlates with AP-1-DNA binding because maximum c-Fos expression and AP-1-DNA binding were observed at same time period in both these cells. OPN also enhanced AP-1 transcriptional activity in both these cells. Both pharmacological and genetic inhibitors of c-Src, α,β3 integrin antibody and MEK kinase inhibitor suppressed the OPN-induced AP-1 transactivation in these cells. However, the level of AP-1 activity was significantly higher in MDA-MB-231 cells compared with MCF-7 cells. These results clearly suggested that OPN induces AP-1 activity through α,β3 integrin/c-Src/ERK-mediated pathways in breast cancer cells.

Several studies have indicated the correlation between uPA expression and metastatic potential and have suggested that uPA plays a major role in controlling cell migration and ECM invasion in various cancer cells (15, 41). Here we have reported that OPN induces uPA secretion. However, the levels of uPA secretion, cell motility, and invasiveness were significantly higher in MDA-MB-231 cells compared with MCF-7 cells. Both pharmacological and genetic inhibitors of c-Src and α,β3 integrin antibody and inhibitors of EGFR and MEK kinase suppressed the OPN-induced uPA secretion, cell motility, and invasion in these cells. Our data revealed that OPN-induced uPA secretion could be regulated by activations of the c-Src kinase, EGFR, and ERK1/2 which ultimately control the α,β3 integrin-mediated cell motility and invasiveness in these cells.

In our earlier study (15), we have demonstrated that OPN stimulates NFκB-mediated secretion of uPA through PI 3-kinase/Akt signaling pathways in breast cancer cells. Previous
OPN Modulates AP-1-mediated UPA Secretion

reports (52, 53) indicated that c-Src could induce PI 3-kinase activity either by directly interacting with the Src homology 3 domain of the p85 subunit of PI 3-kinase or through the FAK-mediated pathway. Here we have shown that the dominant negative form of c-Src (dn c-Src) suppressed the OPN-induced PI 3-kinase activity, suggesting that OPN induces PI 3-kinase activity via c-Src-dependent mechanisms. c-Src kinase activity is also required in OPN-induced ERK1/2 phosphorylation. Our data demonstrated that OPN-induced uPA secretion, cell motility, and ECM invasion were totally blocked by pp2, a c-Src kinase inhibitor. All these results suggested that OPN-induced uPA secretion can be regulated both by PI 3-kinase and MAPK-mediated pathways, and c-Src can act as a master switch in regulating both these pathways.

Previous reports (54, 55) have indicated that nanomolar concentrations of OPN are used in regulation of NO production, NFκB activation, Akt phosphorylation, cell adhesion, and migration in endothelial and other cell types. However, we and other groups (13–15, 30, 56–58) have demonstrated that micromolar concentrations of OPN are required to regulate PI-3-kinase activation, uPA production, pro-MMP-2 activation, cell migration, and ECM invasion in melanoma, breast cancer, and other cell types. Therefore, it is possible that various concentrations of OPN used to regulate these cellular functions may depend on types of cell lines used in these studies.

In summary, we have demonstrated for the first time that OPN induces AP-1 transactivation through phosphorylation of EGFR by inducing the c-Src kinase activity in breast cancer cells. Ligation of OPN with the αβ integrin induces phosphorylation and kinase activity of EGFR, and this was blocked by dn c-Src indicating that c-Src kinase plays a crucial role in these processes. OPN induces αβ integrin/EGFR-mediated ERK1/2 phosphorylation. OPN stimulates c-Fos expression, AP-1-DNA binding, AP-1 transactivation, uPA secretion, cell motility, and invasion in these cells. OPN-induced ERK phosphorylation, AP-1 activation, uPA secretion, and cell motility were suppressed when these cells were transfected with dn c-Src or pretreated with αβ integrin antibody, c-Src kinase domain inhibitor, EGFR tyrosine kinase inhibitor, or MK-1 inhibitor. We have delineated the mechanism by which OPN induces AP-1 activation and uPA secretion by activating c-Src kinase/ERK-mediated and EGFR-dependent or-independent signaling pathways (Fig. 11). These findings may be useful in designing novel therapeutic interventions that block the OPN-regulated c-Src kinase-dependent EGFR phosphorylation and AP-1 activation resulting in reduction of uPA secretion and consequent blocking of cell motility, invasiveness, and metastatic spread of breast cancer.

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Osteopontin Induces AP-1-mediated Secretion of Urokinase-type Plasminogen Activator through c-Src-dependent Epidermal Growth Factor Receptor Transactivation in Breast Cancer Cells

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