Science is the great antidote to the poison of enthusiasm and superstition

Adam Smith
9. APPENDIX

9.1 List of Publications


7. Osteopontin-regulated signaling network in cancer: redefining the molecular targets. Goutam Chakraborty, Shalini Jain, Reeti Behera, Mansoor Ahmed, Priyanka Sharma,


9.2 Conference Presentations

1. Oral presentation entitled “osteopontin regulates prostate tumor growth, metastasis and angiogenesis by modulating the balance between NF-κB and FOXO3a” was given by Shalini Jain, Goutam Chakraborty and Gopal C. Kundu in “2nd International Symposium on Translational Research, Natural products and cancer” December 9-12, 2007 Lonavala India.

2. Oral presentation entitled “(COX-2) and its metabolites results in drastic reduction of OPN-induced tumor progression: a new dimension in the therapeutics of prostate cancer” was give by Shalini Jain, Goutam Chakraborty, Gopal Kundu in 26th Annual Convention of Indian Association for Cancer Research and International Symposium on Translational Research in Cancer, January 17th-19th 2007, Bhuvaneshwar, India.

3. Poster entitled “cyclooxygenase-2 (COX-2) plays crucial role in osteopontin-induced PKCα/IKKα/β dependent prostate tumor growth and angiogenesis” by Shalini Jain, Goutam Chakraborty, and Gopal C. Kundu was presented in the 3rd SFRR-Asia Conference, Lonavala (MH) January 8-11th 2007. (Awarded as the Best Poster)

4. Poster entitled “Hypoxia/Reoxygenation controls the crosstalk between Syk and Lck leading to regulation of MelCAM-expression: signaling mechanism underlying breast cancer progression and angiogenesis” by Goutam Chakraborty, Shalini Jain, Gopal C. Kundu was presented in 3rd Biennial Meeting of the Society for Free Radical Research-Asia-SFRR-Asia, January 8-11, 2007, Lonavala, India

5. Poster entitled “suppression of cyclooxygenase-2 (COX-2) and its metabolites results in drastic reduction of osteopontin-induced prostate cancer progression” by Shalini Jain, Goutam Chakraborty and Gopal C. Kundu was presented in XXIX All India Cell Biology Conference & Symposium on Gene to Genome: Environment and Chemical Interaction, Lucknow (U.P.), January 17-20, 2006.
The Crucial Role of Cyclooxygenase-2 in Osteopontin-Induced Protein Kinase C $\alpha$/c-Src/IκB Kinase $\alpha$/β–Dependent Prostate Tumor Progression and Angiogenesis

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Abstract

The regulation of tumor progression towards its malignancy needs the interplay among several cytokines, growth factors, and enzymes, which are controlled in the tumor microenvironment. Here, we report that osteopontin, a small integrin-binding ligand N-linked glycoprotein family of calcified extracellular matrix–associated protein, regulates prostate tumor growth by regulating the expression of cyclooxygenase-2 (COX-2). We have shown that osteopontin stimulates the activation of protein kinase C $\alpha$/nuclear factor–inducing kinase/nuclear factor-κB–dependent signaling cascades that induces COX-2 expression, which in turn regulates the prostaglandin E$_2$ production, matrix metalloproteinase-2 activation, and tumor progression and angiogenesis. We have revealed that suppression of osteopontin-induced COX-2 expression by the nonsteroidal anti-inflammatory drug celecoxib or blocking the EP2 receptor by its blocking antibody resulted in significant inhibition of cell motility and tumor growth and angiogenesis. The data also showed that osteopontin-induced mouse PC-3 xenograft exhibits higher tumor load, increased tumor cell infiltration, nuclear polymorphism, and neovascularization. Interestingly, use of celecoxib or anti-EPI2 blocking antibody drastically suppressed osteopontin-induced tumor growth that further indicated that suppression of COX-2 or its metabolites could significantly inhibit osteopontin-induced tumor growth. Human clinical prostate cancer specimen analysis also supports our in vitro and animal model studies. Our findings suggest that blockage of osteopontin and/or COX-2 is a promising therapeutic approach for the inhibition of prostate tumor progression and angiogenesis. (Cancer Res 2006; 66(13); 6638–48)

Introduction

Cancer of the prostate is the most frequently diagnosed cancer and one of the major causes of death in men especially in western world (1). Prostate cancer progression is a series of complex events, which require crosstalk between several oncogenic molecules, and enable the cancer to spread and evoke angiogenesis. A high level of constitutive cyclooxygenase-2 (COX-2) expression has been detected in colorectal, gastric, pancreatic, head and neck, lung, breast, and in other cancers (2). COX is an integral membrane bifunctional enzyme, which metabolizes arachidonic acids to many biologically active eicosanoids (3–5). There are three isoforms of the COX enzyme (COX-1, COX-2, and COX-3). COX-1 is involved in the production of prostaglandin (PG)–mediating cellular and physiologic functions. The functional role of COX-3 in human physiology and pathophysiology remains to be established (6).

COX-2 is induced in many cell types by mitogens, growth factors, cytokines, and tumor promoters, and its increased expression is associated with cancer progression through a PG-dependent manner (2–5). COXs convert free arachidonic acids into PGs and thromboxanes (3). In human prostate carcinoma, significant levels of PGE$_2$ has been detected, which plays an important role in cancer progression (7). PGE$_2$ can contribute to tumor development through several mechanisms, including promotion of angiogenesis, inhibition of apoptosis, increased invasiveness and motility, and modulation of inflammation and immune responses (8–10). Recent reports showed that COX-2 promotes tumor cell proliferation, survival, and angiogenesis through a PGE$_2$–mediated pathway (10, 11).

Osteopontin, a secreted, noncollagenous, chemokine-like small integrin-binding ligand N-linked glycoprotein family of protein plays significant role in determining the oncogenic potential of various cancers and recognized as a key marker in the processes of tumorigenicity and metastasis (12). Osteopontin is an acidic glycoprotein with a molecular mass varying from 44 to 75 kDa, depending on the degree of posttranslational modification (13). It has an NH$_2$-terminal signal sequence, a sialic acid region consisting of nine consecutive aspartic acid residues, a GRGDS cell adhesion sequence that is predicted to be flanked by β-sheet structure (14). Osteopontin interacts with several integrins and CD44 variants in an RGD sequence–dependent and RGD sequence–independent manner (15, 16). Osteopontin is involved in normal tissue remodeling processes, such as bone resorption, wound healing, and tissue injuries as well as restenosis, atherosclerosis, tumorigenesis, and autoimmune diseases (17, 18). Previous studies indicated that osteopontin plays crucial role in chemotaxis and chemoinvasion of PC-3 cells (19). Recent data also showed that osteopontin induces pro-matrix metalloproteinase-2 (pro-MMP-2) and pro-MMP-9 activation, urokinase plasminogen activator (uPA) secretion, cell motility, extracellular matrix invasion, and tumor growth (20, 21).

Cell motility, a major step in cancer metastasis, is often associated with the activation of protein tyrosine or serine kinases, like c-Src and protein kinase C $\alpha$ (PKCo), respectively. Previous studies have indicated that c-Src and PKCo play crucial roles in COX-2 expression and COX-2-dependent tumor progression (22–24). The transcription factor nuclear factor-κB (NF-κB) act as a key molecule in regulating wide range of physiologic and pathologic processes (25). Nuclear factor inducing kinase (NIK), a member of mitogen-activated protein kinase kinase kinase (MAPKKK) family has been reported to activate NF-κB through phosphorylation and degradation of IκBα (26). We have recently...
reported that osteopontin induces uPA secretion and MMP-2/ MMP-9 activation through c-Src/phosphatidylinositol 3-kinase/ MAPK signaling pathways (20, 21, 27). However, the molecular mechanism by which osteopontin regulates PKCo/a-c-Src–dependent I-B kinase (IKK)–mediated NF-κB activation, which ultimately regulates tumor progression and angiogenesis through induction of COX-2 expression in prostate cancer and signaling cascades, underlying these processes are not well defined.

In this study, we provide both in vitro and in vivo experimental evidences, at least in part, the molecular mechanism by which osteopontin regulates PKCo/a-c-Src/IKK/NF-κB signaling cascades leading to COX-2-mediated PGE2 production and MMP-2 activation in prostate cancer. Furthermore, we have shown that osteopontin-induced COX-2 regulates cell motility, angiogenesis, and tumorigenesis of prostate cancer through both autocrine and paracrine pathways. However, suppression of COX-2 activity by nonsteroidal anti-inflammatory drug (NSAID) celecoxib or blocking the interaction between PGE2 and its receptor EP2 by using specific anti-EP2 blocking antibody significantly suppressed osteopontin-induced in vitro cell motility, invasiveness, and in vivo tumor growth. Moreover, the clinical data indicated that the increased expressions of osteopontin and COX-2 correlate with enhanced MMP-2 expression and angiogenesis in prostate cancer specimens of higher grades. Consequently, osteopontin plays important and essential role in two key aspects of tumor progression: COX-2-mediated PGE2 production and MMP-2 activation by tumor cells leading to tumor progression and COX-2/PGE2–stimulated angiogenesis. Our findings suggest that blockade of osteopontin and COX-2 is a promising therapeutic approach for the inhibition of tumor progression by suppressing tumor growth and angiogenesis.

Materials and Methods

Antibodies and reagents. Rabbit polyclonal anti-COX-2, anti-PKCo/a-c-Src, anti-NIK, anti-p-NIKThr599, anti-IKKe/β, anti-NF-κB, p65, anti-MMP-2, anti-c-Src, anti-EP2, and anti-actin, mouse monoclonal anti-phosphotyrosine, goat polyclonal anti-phospho-PKCo/a-c-Src (Ser323) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-p-IKKe/β (Ser276/Ser325) antibody was from Cell Signaling Technology (Beverly, MA); goat polyclonal anti-osteopontin antibody was from R&D Systems (Minneapolis, MN); and rabbit polyclonal anti-human vWF antibody was purchased from Sigma (St. Louis, MO). The [γ32P]ATP was purchased from Board of Radiation and Isotope Technology (Hyderabad, India). All other chemicals were of analytic grade. The human osteopontin was purified from milk as described previously (20), with minor modification, and used throughout this study.

Cell culture. The human prostate cancer (PC-3) cells were obtained from the American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cell line EA.hy-926 was a generous gift from Dr. Christopher Newton (University of Hull, United Kingdom). PC-3 cells were cultured in F-12 Ham Nutrient Mixture (Sigma), and EA.hy-926 cells were cultured in DMEM (Sigma) supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Plasmids and DNA transfection. The full-length osteopontin cDNA was a generous gift from Dr. Ann Chambers (University of Western Ontario, London, Ontario, Canada). The dominant-negative c-Src (pcSrcY527F/S12A) and COX-2–Luc (–1432/−59) constructs were generous gifts from Dr. David Shalloway (Cornell University, NY) and Dr. Hiroyashu Inoue (Osaka, Japan; ref. 28), respectively. The wild-type NIK (wt pcDNA NIK) and kinase-negative NIK (mut pcDNA NIK and NIK-K429A/K430A) in pcDNA3 were obtained from Prof. David Wallach (Weizmann Institute of Science, Rehovot, Israel). The wild-type and dominant-negative constructs of IKKe (wt IKKe and dn IKKe) and IKKβ (wt IKKβ and dn IKKβ) in PKC-2-related kinase were kind gifts from Prof. D.V. Goeddel (Tularik, Inc., San Francisco, CA). The super-repressor form of I-Bα cDNA fused downstream to a FLAG epitope in an expression vector (pCMV4) was a gift from Dr. Dean Ballard (Vanderbilt University School of Medicine, Nashville, TN). PC-3 cells were split 16 hours before transfection and transiently transfected with cdNA using LipofectAMINE 2000 reagent according to manufacturer’s instructions (In vitro, San Diego, CA). The cell viability was detected by trypan blue dye exclusion test. Transfected cells were used for COX-2 expression, COX-2 promoter analysis studies, IKK and p65 phosphorylation, and PKCo/a-c-Src kinase assay and migration and invasion assays.

Small interfering RNA. PC-3 cells were transfected with small interfering RNA (siRNA) that specifically targets the osteopontin gene or nonsilencing control using LipofectAMINE 2000 according to manufacturer’s instructions. siRNA duplexes were synthesized by Dharmacon, Inc. (Lafayette, CO). The sequence targeted for osteopontin is 5'-GUUUCACAGCCCAAGGGCATdTdTdTCTCAAAUGUCCGGUGUCUCG-3′, and the nonsilencing control is 5'-CATGUAACAGCAUCUGGACdATdTdTdTGCUACGUGGUAGACGCU-3′.

Western blot and immunoprecipitation. The level of COX-2, PKCo/a-c-Src, osteopontin, IKKe/β, actin expressions and phosphorylations of IKKe/β and NF-κB, p65 in transfected or treated PC-3 cells were analyzed by Western blot using its specific antibody (20). The tyrosine phosphorylation of IKKe/β and the interaction between PKCo/a-c-Src and were done by immunoprecipitation followed by Western blot.

RNA extraction and reverse transcription-PCR. Total RNA was isolated from osteopontin-treated PC-3 cells and analyzed by reverse transcription-PCR (RT-PCR). The reverse transcription and PCR amplification used 10 μg of total RNA, with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and primers COX-2 forward (5′-TACATGAGATTGTGGGAAAAATTGCT-3′) and COX-2 reverse (5′-AGATCATTCTCGCTCTGATTCCTT-3′) to reverse transcribe sense and antisense RNAs, respectively (29). The amplified cDNA fragments were resolved by 2% agarose gel electrophoresis.

COX-2 luciferase assay. The semiconfluent PC-3 cells were grown in 24-well plates and transiently transfected with COX-2 luciferase reporter construct using LipofectAMINE 2000. In separate experiments, cells were individually transfected with wt and mut NIK and wt and dn IKKe/β along with COX-2 luciferase construct. The transfection efficiency was normalized by cotransfecting the cells with pRL vector (Promega, Madison, WI) containing a full-length Renilla luciferase gene under the control of a constitutive promoter. After 24 hours of transfection, the cells were treated with 0.5 μmol/L osteopontin, and the luciferase activities were measured by luminometer (Thermo Electron) using the dual luciferase assay system according to the manufacturer’s instructions (Promega). Changes in the luciferase activity with respect to control were calculated.

Estimation of PGE2. The levels of PGE2 from the conditioned media of osteopontin-treated PC-3 cells and mice plasma were detected by using the Biortack PGE2 competitive enzyme immunoassay kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions.

In vitro kinase assay by autophosphorylation study. In vitro kinase assay was done as described earlier (30). Briefly, PC-3 cells were either treated with osteopontin (0.5 μmol/L) for 5 minutes or pretreated with 100 nmol/L staurosporine (Calbiochem, La Jolla, CA) for 3 hours or transfected with dn c-Src and then treated with osteopontin. Cell lysates were immunoprecipitated with anti-PKCo/a-c-Src antibodies. Autophosphorylation of PKCo/a-c-Src were measured by incubating the immunoprecipitated samples with 40 μCi of [γ32P]ATP in 40 μL kinase assay buffer (20 mmol/L HEPES (pH 7.7), 2 mmol/L MgCl2, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, 10 mmol/L β-mercaptoethanol, 2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mmol/L DTT) for 30 minutes at 30°C. Reactions were terminated by the addition of sample buffer and analyzed by SDS-PAGE followed by autoradiography.

Zymography. The gelatinolytic activity was measured as described previously (20). Briefly, PC-3 cells were either treated with osteopontin or pretreated with staurosporine and COX-2 inhibitor (Celecoxib) or...
transfected with dn c-Src and then treated with osteopontin. Conditioned medium was collected, and gelatinolytic activity of MMP-2 was detected by zymography.

**Cell migration and invasion assay.** The migration and invasion assays were done using Transwell cell culture chamber (Corning, Corning, NY) and Matrigel coated invasion chambers (Collaborative Biomedical, Bedford, MA), respectively, according to the standard procedure as described previously (27). Briefly, the confluent monolayers of PC-3 (treated or transfected) cells were harvested with trypsin-EDTA and centrifuged at 800 \( \times g \) for 10 minutes. The cell suspension (2 \( \times 10^6 \) per well) was added to the upper chamber. The lower chamber was filled with fibroblast-conditioned medium, which acted as a chemoattractant. In separate experiments, osteopontin-treated conditioned medium of PC-3 cells was incubated with EA.hy-926 cells alone or along with anti-EP-2 blocking antibody and subjected to migration and invasion assays. After 12 hours, cells in the lower chamber were fixed and stained with Giemsa stain and counted in three high-power fields (C/HPF) under an inverted microscope (Olympus). Data are presented as the average of three counts \( \pm SE \).

**Wound assay.** The wound assay was done as described (31). Briefly, the post-confluent PC-3 cells with typical cobblestone morphology were used in this experiment. Wounds with a constant diameter were made. Cells were treated with osteopontin alone or along with EP2 blocking antibody or celecoxib (COX-2 inhibitor). In other experiments, EA.hy-926 cells were incubated with conditioned media obtained from osteopontin-treated PC-3 cells either alone or along with EP2 blocking antibody, and wound assay was conducted. The wound photographs were taken under phase-contrast microscope (Olympus).

**PC-3 xenograft tumor model.** The tumorigenicity experiments were done as described previously (27). Briefly, PC-3 cells (5 \( \times 10^5 \)) were mixed with an equal volume of cold Matrigel and then injected s.c. into the dorsal side of the athymic nude mice (NMRI, nu/nu; NIV, Pune, India). Osteopontin alone or along with anti-EP-2 blocking antibody (20 \( \mu g \)) was injected into tumor sites twice a week for up to 4 weeks. In other experiments, celecoxib (1,500 ppm) was given along with diet of the osteopontin-injected animals as described earlier (32). Three mice were used in each set of experiments. The mice were kept under pathogen-free conditions. Growths of s.c. tumors were monitored weekly by measuring the tumors with calipers. At the termination of the experiment, blood was collected from the retro-orbital plexus under anesthesia from both experimental and control groups. Animals were sacrificed by cervical dislocation, and tumors were excised and weighed. The tumor samples were used for histopathologic and immunohistochemical studies using standard procedure (33). The expressions of COX-2, MMP-2, and vWF (endothelial cell–specific marker) were determined by immunofluorescence using their specific antibodies. The slides were analyzed under confocal microscopy (Zeiss, Jena, Germany).

**Human prostate clinical sample analysis.** Human prostate tumor specimens of different Gleason grades and normal prostate tissues were collected from a local hospital with informed consent. The COX-2, osteopontin, MMP-2, and vWF expressions were detected by immunofluorescence using specific antibodies. Five samples of each group (normal, low grade (prostatic intraepithelial neoplasia or PIN), and malignant) were analyzed. Normal prostate tissues were used as control.

**Statistical analysis.** The data reported in cell migration, wound healing, and invasion are expressed as mean \( \pm SE \). Statistical differences were determined by two-way ANOVA and Student’s \( t \) test. \( P < 0.05 \) was considered significant. All these bands were analyzed densitometrically (Kodak Digital Science, Rochester, NY), and the fold changes were calculated.

## Results

**Osteopontin augments COX-2 protein and mRNA expression.** To determine whether osteopontin augments COX-2 expression, PC-3 cells were treated with 0.5 \( \mu \)mol/L osteopontin for 0 to 24 hours. Expression of COX-2 in cell lysates was detected by Western blot, and the data indicated that osteopontin enhances COX-2 promoter activity and expression in PC-3 cells. A, serum-starved PC-3 cells were treated with 0.5 \( \mu \)mol/L osteopontin for 0 to 24 hours. The level of COX-2 from whole-cell lysates was analyzed by Western blot. Actin was used as loading control. B, cells were treated with osteopontin (0-0.5 \( \mu \)mol/L) for 6 hours; total RNA was isolated; and RT-PCR analysis was done using COX-2-specific primers. Changes in mRNA levels were determined as fold of induction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. C, PC-3 cells were transfected with osteopontin cDNA, osteopontin-specific siRNA, or nonsilencing scrambled siRNA. In separate experiments, osteopontin siRNA–transfected cells were either cotransfected with osteopontin cDNA or treated with 0.5 \( \mu \)mol/L osteopontin. COX-2 was analyzed by Western blot. The level of osteopontin is also detected by Western blot. The data reported in cell migration, wound healing, and invasion are expressed as mean \( \pm SE \). Statistical differences were determined by two-way ANOVA and Student’s \( t \) test. \( P < 0.05 \) was considered significant. All these bands were analyzed densitometrically (Kodak Digital Science, Rochester, NY), and the fold changes were calculated.

![Figure 1. Osteopontin (OPN) induces COX-2 promoter activity and expression in PC-3 cells. A, serum-starved PC-3 cells were treated with 0.5 \( \mu \)mol/L osteopontin for 0 to 24 hours. The level of COX-2 from whole-cell lysates was analyzed by Western blot. Actin was used as loading control. B, cells were treated with osteopontin (0-0.5 \( \mu \)mol/L) for 6 hours; total RNA was isolated; and RT-PCR analysis was done using COX-2-specific primers. Changes in mRNA levels were determined as fold of induction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. C, PC-3 cells were transfected with osteopontin cDNA, osteopontin-specific siRNA, or nonsilencing scrambled siRNA. In separate experiments, osteopontin siRNA–transfected cells were either cotransfected with osteopontin cDNA or treated with 0.5 \( \mu \)mol/L osteopontin. COX-2 was analyzed by Western blot. The level of osteopontin is also detected by Western blot. The data reported in cell migration, wound healing, and invasion are expressed as mean \( \pm SE \). Statistical differences were determined by two-way ANOVA and Student’s \( t \) test. \( P < 0.05 \) was considered significant. All these bands were analyzed densitometrically (Kodak Digital Science, Rochester, NY), and the fold changes were calculated. Columns, mean of triplicate determinations; bars, SE.](Image)
Figure 2. Osteopontin (OPN) induces PKCα activation, PKCα-mediated c-Src and IKK phosphorylation, and COX-2 expression. A, PC-3 cells were treated with osteopontin for 0 to 7 minutes, and the phosphorylation of PKCα was detected by Western blot using anti-phospho-PKCα antibody (p-PKCα; a, top). The cells were treated with osteopontin for 0 and 5 minutes, and cell lysates were immunoprecipitated with anti-c-Src antibody and analyzed by Western blot using anti-phospho-PKCα antibody (b, top). Levels of PKCα and c-Src were detected by reprobing the same blots with their specific antibodies (a and b, bottom). B, cells were either transfected with dn c-Src or pretreated with staurosporine (Stau, a PKC inhibitor, 100 nmol/L) and then treated with osteopontin for 5 minutes. Cell lysates were immunoprecipitated with anti-PKCα or anti-c-Src antibody, and the kinase activity of PKCα (a) and c-Src (b) was determined by autophosphorylation experiment and visualized by autoradiography. C, cells were individually transfected with dn c-Src, wt and mut NIK, or pretreated with staurosporine and then treated with osteopontin. Half of the cell lysates were analyzed by Western blot using anti-phospho-Ser-IKKα/β (Ser180/Ser181) antibody (top), and the remaining part of cell lysates was immunoprecipitated with IKKα/β antibody and detected by Western blot using anti-phosphotyrosine antibody (middle). The level of IKKα/β was also detected by Western blot (bottom). D, cells were pretreated with staurosporine or individually transfected with dn c-Src, h-Ba super-repressor (sup. rep.), and dn IKKα or IKKβ, and wt or mut NIK and then treated with osteopontin. COX-2 expression was detected by Western blot. Actin was used as loading control. Represents three experiments exhibiting similar results. Fold changes were calculated.

induced maximum COX-2 expression at 24 hours (Fig. 1A). The dose-dependent effect of osteopontin (0-1 μmol/L) on COX-2 expression was also detected, and the results indicated that osteopontin at 0.5 μmol/L exhibits maximum level of COX-2 expression in PC-3 cells (data not shown). The COX-2 mRNA level was also determined by RT-PCR, and the data indicated that osteopontin at 0.5 μmol/L enhanced maximum COX-2 expression at transcriptional level (Fig. 1B). Interestingly, our results also showed that osteopontin induced COX-2 expression in other prostate cancer cell lines like DU-145 and LNCaP (data not shown). To examine the specificity of osteopontin on COX-2 expression, cells were transfected with wt osteopontin cDNA alone or cotransfected with osteopontin siRNA duplex and control RNA duplex. The data showed that cells transfected with osteopontin cDNA enhanced the COX-2 expression. Cells transfected with osteopontin cDNA followed by siRNA duplex suppressed the osteopontin-induced COX-2 expression (Fig. 1C). Our results indicated that osteopontin enhanced COX-2 expression both at transcriptional and translational levels.

Osteopontin enhances NIK/IKK/NF-κB–dependent COX-2 promoter activity. The effect of osteopontin on COX-2 promoter activity was determined by transfecting PC-3 cells with COX-2 luciferase construct followed by treatment with osteopontin in a dose-dependent manner. In separate experiments, cells were cotransfected with wt NIK or IKKβ or kinase-negative (mut) NIK or dn IKKβ along with COX-2 luciferase construct and then treated with osteopontin. Further transfecting the cells with pRL construct normalized the transfection efficiency, and COX-2 luciferase activities were measured according to standard procedure. The results showed that cells transfected with wt NIK or IKKβ enhanced the COX-2 promoter activity, whereas mut NIK or dn IKKβ significantly suppressed osteopontin-induced COX-2 promoter activity (Fig. 1D). Cells transfected with wt IKKα enhanced osteopontin-induced COX-2 promoter activity, whereas dn IKKα or the super-repressor form of h-Ba suppressed osteopontin-induced COX-2 promoter activity (data not shown). These results indicated that osteopontin enhanced COX-2 promoter activity through a NIK/IKK–dependent NF-κB-mediated pathway.
loading controls (Fig. 2A, a and b, bottom). The results indicated that osteopontin enhanced PKCα phosphorylation and its interaction with c-Src. To determine the role of αβ3 integrin on osteopontin-induced PKCα phosphorylation, cells were pretreated with anti-human αβ3 integrin blocking antibody, RGDI, or RGE (GpenGRGDSPCA and GRGESp, respectively) peptides and then treated with osteopontin, and the level of PKCα phosphorylation was detected by immunoblotting. The results indicated that anti-αβ3 integrin blocking antibody and GpenGRGDSPCA but not GRGESp suppressed the osteopontin-induced PKCα phosphorylation (data not shown). The data suggested that αβ3 integrin plays an important role in osteopontin-induced PKCα phosphorylation. To delineate whether PKCα is upstream of c-Src or vice versa, cells were pretreated with PKCα inhibitor (staurosporine) and treated with osteopontin, and cell lysates were immunoprecipitated with anti-c-Src antibody. The immunoprecipitated samples were analyzed by in vitro kinase assay. Similarly, in other experiments, cells were transfected with dn c-Src and treated with osteopontin, and cell lysates were immunoprecipitated with anti-PKCα. The immunoprecipitated samples were used for in vitro PKCα kinase assay. The data showed that dn c-Src has no effect on PKCα autophosphorylation, whereas PKCα autophosphorylation by in vitro kinase assay. The data showed that both PKC inhibitor and dn c-Src suppressed osteopontin-induced serine and tyrosine phosphorylations of IKKα/β, whereas mut IKK inhibits only the serine but not tyrosine phosphorylation of IKKα/β, suggesting that NIK plays differential role in osteopontin-induced PKCα/c-Src–mediated IKKα/β activation (Fig. 2C, top and middle). The level of IKKα/β was also detected by Western blot as control (Fig. 2C, bottom).

To further investigate whether PKCα, c-Src, NIK, IKKα, and NF-κB play any role in osteopontin-induced COX-2 expression, cells were pretreated with PKC inhibitor (staurosporine) or individually transfected with dn c-Src, wt and mut NIK, and IκBα super-repressor, wt and dn IKKα or IKKβ followed by treatment with osteopontin. The COX-2 expression was detected by Western blot (Fig. 2D). These results suggested that PKCα and c-Src play an important role in regulating osteopontin-induced COX-2 expression through a NIK/IKK/NF-κB–mediated pathway.

**Osteopontin induces PKCα/c-Src–mediated p65 phosphorylation.** Recent findings suggested that phosphorylation of p65 subunit of NF-κB leads to nuclear translocation and activation of NF-κB (35), and that the promoter region of COX-2 contains the NF-κB response element (36). In Fig. 2D, we showed that suppression of NF-κB by overexpression of IκBα super-repressor significantly inhibits osteopontin-induced COX-2 expression. Therefore, we sought to determine whether osteopontin regulates NF-κB, p65 phosphorylation that ultimately regulates COX-2 expression, and whether PKCα and c-Src are involved in this process. Accordingly, cells were treated with osteopontin from 0 to 180 minutes or pretreated with PKC inhibitor (staurosporine) or transfected with dn c-Src and then treated with osteopontin. The level of p65 phosphorylation in the cell lysates was detected by using anti-phosphoseryl IKKα/β antibody or immunoprecipitated with anti-IKKα/β antibody followed by immunoblotting with anti-phosphotyrosine antibody (Fig. 2C, top and middle). These data showed that both PKC inhibitor and dn c-Src suppressed osteopontin-induced serine and tyrosine phosphorylations of IKKα/β, whereas mut IKK inhibits only the serine but not tyrosine phosphorylation of IKKα/β, suggesting that NIK plays differential role in osteopontin-induced PKCα/c-Src–mediated IKKα/β activation (Fig. 2C, top and middle). The level of IKKα/β was also detected by Western blot as control (Fig. 2C, bottom).

**Figure 3.** Osteopontin (OPN) induces PKCα/c-Src–dependent NF-κB, p65 phosphorylation, MMP-2 activation, and PGE₂ production. A, PC-3 cells were treated with osteopontin for the indicated time, and the level of phospho-p65 (p-p65; Ser536) from cell lysates were detected by Western blot (top). B, cells were transfected with dn c-Src or pretreated with staurosporine (Stau) and then treated with osteopontin. The level of phospho-p65 was analyzed by Western blot. The level of non-phospho-p65 was also detected by Western blot (A and B, bottom). C, cells were transfected with dn c-Src or pretreated with staurosporine or celecoxib (COX-2 inhibitor, 50 μmol/L) and then treated with osteopontin for 24 hours. Conditioned media were collected, and the level of MMP-2 activation was detected by zymography. Recombinant MMP-2 (c MMP-2) was used as positive control. D and E, cells were treated with 0.5 μmol/L osteopontin for 0 to 24 hours. In separate experiments, cells were transfected with dn c-Src or pretreated with staurosporine and celecoxib and then treated with osteopontin for 24 hours. Conditioned media were collected, and the level of PGE₂ was estimated by enzyme immunoassay. Represents three experiments exhibiting similar results.
Western blot using anti-phospho-p65 antibody (Fig. 3A and B). The results showed that inhibition of PKCα and c-Src significantly suppressed osteopontin-induced NF-κB, p65 phosphorylation (Fig. 3B). These results clearly suggested that osteopontin induces p65 phosphorylation through a PKCα/c-Src-dependent pathway, which leads to NF-κB activation that ultimately regulates COX-2 expression.

Osteopontin augments COX-2-mediated PGE_2 production and MMP-2 activation. Earlier reports revealed that overexpression of COX-2 leads to up-regulation of arachidonic acid metabolism, which in turn results in enhancement of PGE_2 production and tumor progression (2, 37–39). Accordingly, to determine whether osteopontin can induce COX-2-mediated PGE_2 production, cells were treated with osteopontin for 0 to 24 hours, and the PGE_2 level in the conditioned media was measured by using PGE_2 enzyme immunoassay kit. To examine whether PKC, c-Src, and COX-2 are involved in osteopontin-induced PGE_2 production, cells were pretreated with PKC inhibitor (staurosporine) or COX-2 inhibitor (celecoxib) or transfected with dn c-Src and then treated with osteopontin. The results indicated that inhibitors of PKC and COX-2 and dn c-Src drastically suppressed the osteopontin-induced PGE_2 production (Fig. 3D and E). To delineate the role of PKCα, c-Src, and COX-2 in osteopontin-induced MMP-2 activation, cells were treated or transfected as described earlier, and the level of active MMP-2 in the conditioned medium was determined by zymography (Fig. 3C). These results showed that COX-2 plays a crucial role in osteopontin-induced PKC/c-Src-mediated PGE_2 production and MMP-2 activation in prostate cancer cells.

COX-2 and PGE_2 play an important role in osteopontin-induced PC-3 cell migration and invasion. Previous data suggested that COX-2 and its metabolite PGE_2 act as crucial molecules in regulating tumor cell motility, invasiveness, and tumor metastasis (2). Therefore, to examine the role of COX-2 and PGE_2 in osteopontin-mediated PC-3 cell migration, the wound assay was done. Cells were either pretreated with PKC inhibitor (staurosporine), celecoxib, or anti-EP2 antibody or transfected with dn c-Src. Wounds with a constant diameter were made, and cells were treated with osteopontin. The wound photographs were taken under phase-contrast microscope (Fig. 4A). These data showed that inhibition of COX-2 or its upstream kinases (PKCα and c-Src) resulted in significant suppression of osteopontin-induced PC-3 cell motility towards the wound and further indicated that COX-2 act as a key molecule in osteopontin-induced tumor cell migration. Interestingly, blocking the interaction between PGE_2 and its receptor EP2 by using specific anti-EP2 blocking antibody also suppressed osteopontin-induced tumor cell motility, indicating that osteopontin regulates wound migration via COX-2-dependent PGE_2 production through interaction with its receptor EP2 in an autocrine fashion. The wound migration data were further confirmed by modified Boyden chamber migration and extracellular matrix invasion assays. The results indicated that the cell migration due to over expression of wt NIK, IKKα, or IKKβ in response to osteopontin was suppressed by the celecoxib, suggesting the potential role of COX-2 in osteopontin-induced NIK/IKK–dependent tumor cell migration and invasion (Fig. 4B and C). To examine the specificity of COX-2 in osteopontin-induced cell migration, NS398 (NSAID), a COX-2 inhibitor, was used, and the data showed that NS398 is also suppressed osteopontin-induced cell migration and invasion. PGE_2 was used as a positive control. Taken together, these data indicated the potential and crucial role of COX-2 and PGE_2 in osteopontin-induced tumor cell motility and invasiveness.

Osteopontin-induced tumor cell–derived PGE_2 enhanced endothelial cell motility and invasiveness through paracrine mechanism. Earlier data indicated that tumor-derived PGE_2...
interacts with the endothelial cell surface receptor and induced endothelial cell motility and invasiveness that leads to tumor angiogenesis (38). It is reported that EP2 acts as one of the main PGE₂ receptor in endothelial cells (39). To determine whether osteopontin-induced PC-3 cell–derived PGE₂ enhances endothelial cell migration and invasion through EP2-mediated paracrine manner, endothelial cells were used on the upper side of modified Boyden or Matrigel-coated invasion chamber. The conditioned media collected from osteopontin-treated PC-3 cells were used as chemoattractant (Fig. 5A and B). In separate experiments, endothelial cells were pretreated with anti-EP2 blocking antibody and used for migration and invasion assays. Our data showed that conditioned media of osteopontin-treated PC-3 cells significantly enhanced endothelial cell migration and invasion, whereas blocking the EP2 receptor in these cells with its antibody significantly suppressed the endothelial cell migration and invasion (Fig. 5A and B). The enhanced migration of endothelial cells was further confirmed by wound assay under the same experimental condition (Fig. 5C). Our results indicated that osteopontin-induced tumor cell–derived PGE₂ enhanced migration and invasion of endothelial cells through an EP2-mediated paracrine mechanism.

**Development of PC-3 xenograft model to study the role of COX-2 in osteopontin-induced tumor progression.** Our in vitro results prompted us to study the role of COX-2 and PGE₂ in osteopontin-induced mouse xenograft tumor progression. Accordingly, PC-3 cells (5 × 10⁵) were mixed with Matrigel and then injected s.c. into the dorsal flanks of the male athymic nude mice. In other experiments, cells were treated with osteopontin alone or along with anti-EP2 blocking antibody and then injected into the nude mice. The osteopontin alone or mixture of osteopontin and anti-EP2 antibody was also injected to the tumor sites twice a week for up to 4 weeks. In another experiments, celecoxib (1,500 ppm) was supplemented to the normal diet of the osteopontin-injected animals. All the mice were kept under pathogen-free conditions.

Figure 6A (a–d) showed typical photographs of tumors grown in nude mice. Blood was collected; mice were sacrificed by cervical dislocation; and tumors were excised, weighed, and measured (Table 1). Tumor samples were used for histopathologic and immunohistochemical studies according to standard procedure. The histopathologic analysis by H&E staining is summarized in Table 2, and these data clearly indicated that osteopontin-induced tumorigenicity in nude mice was suppressed significantly by anti-EP2 blocking antibody or by supplementation of celecoxib in mice diet (Table 2; Fig. 6A, e–h). Moreover, the immunohistochemical studies showed that the enhanced expressions of COX-2 and MMP-2 were detected in mice treated with osteopontin (Fig. 6A, i–p). The expression of vWF (an endothelial cell–specific marker) was also detected (Fig. 6A, q–t). The plasma PGE₂ level was also higher in osteopontin injected mice (Fig. 6B). Taken together, our in vivo xenograft study showed that osteopontin induces prostate tumor growth and angiogenesis in nude mice that is correlated with up-regulation of COX-2 expression, MMP-2 activation, and PGE₂ secretion. Inhibition of COX-2 or blocking of EP2 receptor significantly suppressed osteopontin-induced angiogenesis and tumor progression in nude mice and further showed that COX-2 plays an important role in osteopontin-induced prostate cancer progression.

**Expressions of osteopontin, COX-2, MMP-2, and NF-κB, p65 localization, and their correlation with human prostate cancer progression and angiogenesis in different pathologic grades.** To correlate the in vitro data and in vivo mouse model results with human clinical specimens, human prostate cancer tissues were collected from a local hospital with informed consent. The tumor samples were stained with H&E, and the grades of these samples were determined by Gleason grading system with the help of expert histopathologist (Fig. 7A, a–c). Expression of osteopontin, COX-2, MMP-2, and vWF and cellular localization of p65 were analyzed by immunohistochemistry using their specific
antibodies (Fig. 7A, d-r). These results indicated the higher levels of osteopontin and COX-2 in malignant tumors than normal and PIN, and that further correlated with the enhanced MMP-2 expression and neovascularization (vWF expression). Moreover, there was significant nuclear translocation of p65 in the malignant tumors. All these data correlate with our in vitro and mice xenograft studies.

Discussion

The interaction between osteopontin and prostate cancer cells are likely to be the key determinants in regulating tumor progression and metastatic phenotype of human prostate carcinoma and further indicates that osteopontin may be an important mediator of prostate cancer progression (40). Recent strategies in therapeutics of prostate cancers with inhibitors that target COX-2 (41) and its enhanced expression in prostate carcinoma suggest that understanding the molecular mechanism underlying enhanced COX-2 expression may help in developing the novel therapeutic approach in prostate cancer treatment. The experimental work presented in the study showed for the first time the molecular mechanism that underlies osteopontin-induced COX-2 expression and its potential role in regulating in vitro cell motility and invasion of prostate cancer, which ultimately modulates in vivo tumor growth and angiogenesis. Silencing of osteopontin in prostate cancer cells results drastic reduction, whereas overexpression of osteopontin significantly increased COX-2 level, suggesting the specificity of osteopontin on COX-2 expression. Moreover, we find that COX-2 plays an important role in osteopontin-induced PGE2 production and MMP-2 activation that ultimately regulates tumor progression.

Table 1. Effect of COX-2 inhibition and EP2 receptor blocking on osteopontin-induced prostate tumor growth in nude mice

<table>
<thead>
<tr>
<th>No. mice</th>
<th>Treatments</th>
<th>Tumor wt (fold changes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Control</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>3</td>
<td>Osteopontin</td>
<td>2.83 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>Osteopontin + celecoxib (1,500 ppm)</td>
<td>0.52 ± 0.14</td>
</tr>
<tr>
<td>3</td>
<td>Osteopontin + EP2 antibody (20 µg)</td>
<td>0.12 ± 0.10</td>
</tr>
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</table>

Figure 6. COX-2 and PGE2 play crucial roles in osteopontin (OPN)–induced tumorigenesis and angiogenesis in nude mice. A, photographs of athymic mice showing 4-week-old xenograft tumor growth by PC-3 cells; inset, excised tumors with respective mice (a-d). Experimental details were described in Materials and Methods. Representative H&E-stained sections from PC-3 xenograft tumors and the characteristics of these tumors were analyzed (e-h). The expression of COX-2 (i-), MMP-2 (m-p), and neovascularization (vWF expression; q-t) were visualized by immunofluorescence study using their specific antibodies. COX-2, MMP-2, and vWF were stained with FITC-conjugated IgG (green). B, levels of PGE2 production in these mice were determined from mice plasma by enzyme immunoassay. Three mice were used in each set of experiments.
In prostate cancer, PKCα plays a key role in the regulation of downstream oncogenic molecules (34). Recent reports also revealed that activation of PKCα is required for the survival and growth of androgen-independent human prostate cancer cells (41–43). However, it is not well established how osteopontin regulates PKCα activation and PKCα-dependent downstream signaling events in prostate carcinoma. In this study, we provide evidences that PKCα plays an important role in osteopontin-induced c-Src/NIK-mediated IKKα/β-dependent NF-κB phosphorylation, which ultimately controls COX-2 expression. Recently, we have shown the involvement of the NIK/IKK/NF-κB signaling pathway in osteopontin-induced expression of downstream effector molecules that regulate melanoma and breast tumor progression (21, 27). In this study, we report that osteopontin regulates PKCα/c-Src-mediated

<table>
<thead>
<tr>
<th>Tumor characteristics</th>
<th>Control</th>
<th>Osteopontin (0.5 μmol/L)</th>
<th>Osteopontin (0.5 μmol/L) + celecoxib (1,500 ppm)</th>
<th>Osteopontin (0.5 μmol/L) + EP2 antibody (20 μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor infiltration</td>
<td>Moderate</td>
<td>Very high</td>
<td>Moderate to poor</td>
<td>Poor</td>
</tr>
<tr>
<td>Vessel formation</td>
<td>Poor</td>
<td>High</td>
<td>Moderate to poor</td>
<td>Negligible</td>
</tr>
<tr>
<td>Mitotic features/hpf</td>
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<td>12-16</td>
<td>2-4</td>
<td>1-3</td>
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<tr>
<td>Tumor giant cells</td>
<td>Less</td>
<td>Plenty</td>
<td>Scanty</td>
<td>Very less</td>
</tr>
<tr>
<td>Nuclear polymorphism</td>
<td>Moderate nuclear size variation</td>
<td>Marked nuclear size variation</td>
<td>Less nuclear size variation</td>
<td>Small regular uniform nucleus</td>
</tr>
</tbody>
</table>

**Table 2. Characteristics of the xenograft tumors from experimental mice**

![Figure 7](https://example.com)  
Figure 7. Expressions of osteopontin (OPN), COX-2, MMP-2, and NF-κB: p65 localization, and their correlation with human prostate cancer progression and angiogenesis in different pathological grades. A, prostate tumor specimens were collected from a local hospital with informed consent. The gradations of these specimens were done according to the Gleason grading system by H&E staining (a–c). The levels of osteopontin (d–f), COX-2 (j–l), vWF (m–o), and MMP-2 (p–r) expression and cellular localization of p65 (g–i) were detected by immunohistochemical studies using their specific antibodies. Osteopontin, vWF, and MMP-2 were stained with FITC (green), whereas COX-2 and p65 (NF-κB) were stained with TRITC (red)–conjugated IgG, and the nucleus was counterstained with 4’,6-diamidino-2-phenylindole (blue). B, schematic representation of osteopontin-induced PKCα/c-Src/IKKα/β-mediated COX-2 expression leading to enhanced PGE2 production and MMP-2 activation that further induces tumorigenesis and angiogenesis via autocrine and paracrine mechanisms.
IKK activation followed by NF-κB phosphorylation. Moreover, the results showed that overexpression of super-repressor form of IκBα significantly suppressed osteopontin-induced COX-2 expression, suggesting that NF-κB act as the key transcription factor in regulation of COX-2 expression and further showed the dispensability of the PKCa/c-Src/IKK/NF-κB signaling cascade in osteopontin-induced COX-2 expression.

It is well established that increased level of PGE2 production and MMP-2 activation is associated with enhanced expression of COX-2 in many cancers (44, 45). Administration of COX-2 inhibitors has been reported to suppress PGE2 production and MMP-2 activation in various cancers (46–48). Recent reports also indicated that PGE2 could also induce MMP-2 activation in cancer cells (49). Our data showed that inhibition of upstream signaling pathway or treatment with COX-2 inhibitor (celecoxib) significantly suppressed osteopontin-induced PGE2 production and MMP-2 activation.

Recent findings indicated that COX-2-specific inhibitors significantly suppressed tumor growth in prostate cancer (50). Interestingly, our studies showed that NSAID celecoxib and NS398 (COX-2-selective inhibitor) significantly suppressed osteopontin-induced PC-3 cells migration and invasion. The in vivo xenograft tumor experiment indicated that mice fed with celecoxib showed significant reduction in osteopontin-induced tumor growth, and this study suggested that COX-2 could be an effective target in caner therapy.

Tumor cell–derived PGE2 can contribute tumor progression by regulating the cell motility/invasiveness and inducing angiogenesis (8–10). Among PGE2 receptors, EP2 is expressed in both prostate cancer and endothelial cells (39, 51). Moreover, the development of selective antagonists against the EP2 receptor has the potential to improve antitumor activity, and EP2 receptor antagonist may be more specific than the use of COX-2 inhibitors (52). Sung et al. showed that tumors from wt EP2 mice produced more blood vessels than those of knockout mice (53). In our study, we showed that the blocking the EP2 receptor by specific blocking antibody suppressed osteopontin-induced prostate cancer (PC-3) and endothelial cell motility and invasiveness. Furthermore, we also find that administration of anti-EP2 blocking antibody significantly suppressed osteopontin-induced mice xenograft tumor progression.

Our study showed the detailed molecular mechanism by which osteopontin induces cell migration, invasion, and tumor progression through induction of COX-2 expression and PGE2 production (Fig. 7B). Our results further warrant that the mechanism shown in the mouse model underlies the human pathology and a clear understanding of osteopontin and COX-2 regulation could illuminate cellular changes that accompany prostate cancer progression and may facilitate the development of novel therapeutic approaches to suppress osteopontin-regulated PKCα/IKK/NF-κB–mediated COX-2 expression and thereby controlling tumor progression and angiogenesis.

Acknowledgments

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References

22. Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T. MAL/MyD88-independent NF-κB activation through induction of COX-2 and Tumor Progression and Angiogenesis.


Prostaglandin E₂ Regulates Tumor Angiogenesis in Prostate Cancer
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National Center for Cell Science, Pune, India

Abstract
In cancer management, the cyclooxygenase (COX)–targeted approach has shown great promise in anticancer therapeutics. However, the use of COX-2 inhibitors has side effects and health hazards; thus, targeting its major metabolite prostaglandin E₂ (PGE₂)–mediated signaling pathway might be a rational approach for the next generation of cancer management. Recent studies on several in vitro and in vivo models have revealed that elevated expression of COX-2 correlates with prostate tumor growth and angiogenesis. In this study, we have shown the in-depth molecular mechanism and the PGE₂ activation of the epidermal growth factor receptor and β3 integrin through E prostanoid 2 (EP2)–mediated and EP4-mediated pathways, which lead to activator protein-1 (AP-1) activation. Moreover, PGE₂ also induces activating transcription factor-4 (ATF-4) activation and stimulates crosstalk between ATF-4 and AP-1, which is unidirectional toward AP-1, which leads to the increased expressions of urokinase-type plasminogen activator and vascular endothelial growth factor and, eventually, regulates prostate tumor cell motility. In vivo Matrigel angiogenesis assay data revealed that PGE₂ induces angiogenesis through EP2 and EP4. Human prostate cancer specimen analysis also supported our in vitro and in vivo studies. Our data suggest that targeting PGE₂ signaling pathway (i.e., blocking EP2 and EP4 receptors) might be a rational therapeutic approach for overcoming the side effects of COX-2 inhibitors and that this might be a novel strategy for the next generation of prostate cancer management. [Cancer Res 2008;68(19):7750–9]

Introduction
Treatment of cancer by chemotherapeutic agents is considered one of the most effective approaches in cancer management in recent times. Earlier reports have depicted that reduced apoptosis, increased neovascularization, and immunosuppression are some of the known consequences of cyclooxygenase-2 (COX-2) overexpression, and each effect could have an important role in tumor progression and angiogenesis (1). Several selective and nonselective COX-2 inhibitors have been in use for the treatment of different cancers, but many questions have arisen regarding their side effects (2). Various studies have shown the correlation between COX-2 overexpression and enhanced production of prostaglandin E₂ (PGE₂) by cancer cells (3). It has been reported that the rate of PGE₂ conversion from arachidonic acid is almost 10-fold higher in malignant prostatic tissues than in benign prostatic tissues (4).

Thus, the concerns regarding the safety of these COX-2 inhibitors, as well as the identification of the more effective therapeutic agents, prompted us to understand the downstream signaling events regulated by PGE₂ in prostate cancer, which might help to develop new therapeutic approach in the treatment of prostate cancer.

PGE₂ interacts with the E prostanoid (EP) family of receptors, which consist four different subtypes (EP1–EP4). The enhanced expressions of EP2 and EP4 receptors have been shown in prostate cancer, as well as in endothelial cells (5, 6). In this study, we have examined the role of PGE₂-mediated signaling during prostate cancer progression and suggested that blocking the interaction between PGE₂ and its receptors, rather than global prostaglandin synthesis by using specific COX-2 inhibitors, might circumvent some of the adverse side effects. Recently, we have shown that the chemokine-like protein, osteopontin, induces COX-2–dependent PGE₂-mediated prostate cancer progression (7). However, the molecular mechanism by which PGE₂ directly regulates prostate cancer progression and angiogenesis is not well defined.

Previous studies have shown that PGE₂ augments cyclic AMP (cAMP) production (8), increases cellular growth, and regulates differentiated cell functions by promoting the activation of cAMP-dependent protein kinase A (PKA). The PKA-mediated phosphorylation of cAMP-responsive element binding protein (CREB) and regulation of transcription via interaction between cAMP-response elements with CREB are considered as the major pathways that alter gene expression in cancer cells (9, 10). Earlier studies have revealed that activating transcription factor 4 (ATF-4; also called CREB-2) regulates the expression of genes involved in oxidative stress, amino acid synthesis, differentiation, metastasis, and angiogenesis (11). It has been reported that the expression of ATF-4 is induced by various external stimuli in cancer microenvironment and regulates various processes that control cancer progression (11), but the function of ATF-4 in prostate cancer progression remains unknown.

The overexpression of proteases often correlates with the enhanced tumor cell invasion and metastasis by virtue of degradation of extracellular matrix and basement membranes in almost all malignancies, including prostate cancer (12, 13). Urokinase-type plasminogen activator (uPA), a protease, plays an important role in tumor cell invasion and metastasis (14). Increased expressions of uPA and vascular endothelial growth factor (VEGF) have been reported in malignancies of various organs including prostate (14, 15), and the increased expression of these molecules is associated with an enhanced metastatic and angiogenic potential and poor survival of patients (16). Earlier data have shown that the response elements for activator protein 1 (AP-1) and ATF-4 are present in the promoter region of uPA and VEGF (17–20). Although it has been reported that PGE₂ plays a crucial role in VEGF production in prostate cancer cells (21), the molecular mechanism by which PGE₂ regulates ATF-4/AP-1–mediated uPA and VEGF expressions, which lead to prostate tumor cell motility and in vivo angiogenesis, remains unknown.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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In this study, we have shown that PGE$_2$ triggers mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and augments expression and activation of β3 integrin in prostate cancer cells. Moreover, we have shown that PGE$_2$ induces the activation of ATF-4 and AP-1 via ERGFR-MEK/ERK1/2 or β3 integrin–mediated pathway, which ultimately leads to the increased expressions of uPA and VEGF. Furthermore, we have observed that PGE$_2$ regulates endothelial cell motility and in vivo angiogenesis. Analysis of human prostate clinical samples showed that the expression profiles of EP2 and EP4 receptors correlated with levels of AP-1, ATF-4, uPA, and VEGF. These data suggested that, at least in part, PGE$_2$ plays a crucial role in the oncogenesis and angiogenesis of prostate cancer. Thus, targeting PGE$_2$ receptor–mediated signaling might be a potential approach for the improved prostate cancer therapeutics.

Materials and Methods

Cell culture and transfection. Human prostate cancer cell lines (PC-3, DU-145, and LNCaP) were obtained from American Type Culture Collection. Human umbilical vein endothelial cell (HUVEC) was purchased from Cambrex. COX-2 cDNA (Dr. Stephen Prescott, University of Utah), wild-type (wt) and dominant-negative (dn) ATF-4 (wt, pEF/mATF-4; dn, pEF/mATF-4M; Dr. Javed Alam, Yale University School of Medicine), and A Fos (Dr. Charles Vinson, National Cancer Institute) were transfected in PC-3 cells using Lipofectamine 2000.

Small interfering RNA. PC-3 cells were transfected with small interfering RNA (siRNA) that specifically targets COX-2 (COX-2 siRNA, Santa Cruz Biotechnology), EP2 (ON-TARGET plus SMARTpool PTGER2; D-005712-00), EP4 (siGENOME SMARTpool PTGER4; M-005714-00), human integrin β3 (siGENOME SMARTpool ITGB3; M-004124-02), and control siRNA (siGENOME nontargeting siRNA; D-001206-14-05 and ON-TARGET plus nontargeting pool; D-001810-10-05, Dharmacon) according to the manufacturer's instructions.

Western blot and EMSA. The Western blot and EMSA experiments were performed as described earlier (7, 22).

Immunofluorescence. Immunofluorescence studies were performed using specific antibodies as described earlier (22, 23).

Flow cytometry. Flow cytometry experiments were performed as described (24).

Reverse transcription–PCR. Total RNA was isolated from PC-3 cells and analyzed by reverse transcription–PCR (RT-PCR). The following primers were used: uPA sense, 5′-CAC GCA AGG GGA GAT GAA-3′; uPA antisense, 5′-ACA GCA TTT TGG TGG TGG CTT-3′; VEGF sense, 5′-CCT CGG AAA CCA TGA ACT TT-3′; VEGF antisense, 5′-AGA GAT CTG GTT CCC GAA AC-3′; β-actin sense, 5′-GCC ATC ATC ACC CTG AAG TA-3′; β-actin antisense, 5′-GGG GTG TTG AAG GTC TCA AA-3′. The amplified cDNA fragments were analyzed by 1.5% agarose gel electrophoresis.

Cell migration and comigration assay. The migration and comigration experiments were performed as described (22). Briefly, PC-3 cells, either alone or individually transfected with dn ATF-4, dn c-Jun, and A-Fos or pretreated with PKA inhibitor peptide, were added to the upper chamber of the Boyden chamber. PGE$_2$ was added in the upper chamber. For comigration assay, PC-3 cells, either alone or transfected with COX-2 cDNA or COX-2 siRNA, were used in the lower chamber. Endothelial (HUVEC) cells, either alone or pretreated with EP2 (AH6809, Sigma) or EP4 (AH23848, Sigma) receptor antagonist, were used in the upper chamber. The migrated endothelial cells to the reverse side of the upper chamber were fixed and stained with Giemsa and counted in three high-power fields under an inverted microscope (Nikon). Data are represented as the average of three counts ± SE.

Wound assay. Wound assays were performed using PC-3 and endothelial cells as described earlier (7). Wounds with a constant diameter were made. PC-3 cells were treated with PGE$_2$ alone or pretreated with EP2 or EP4 receptor antagonist or PKA inhibitor peptide, or transfected with dn ATF-4, dn c-Jun, A-Fos and then treated with PGE$_2$. In separate experiments, endothelial cells were treated with PGE$_2$ alone or pretreated with EP2 or EP4 receptor antagonist and then treated with PGE$_2$. After 12 h, wound photographs were taken through a microscope (Nikon).

In vivo Matrigel plug assay. In vivo Matrigel plug angiogenesis assay was carried out, as described previously (22). Briefly, Matrigel, either alone or along with PGE$_2$, was injected s.c. into the ventral groin region of male athymic NMRI (nu/nu) mice. In separate experiments, PGE$_2$ containing Matrigel was mixed with EP2 or EP4 antagonist (30 μmol/L) and injected into the mice. In another experiment, conditioned medium of PC-3 cells, either nontransfected or transfected with COX-2 cDNA, was mixed with Matrigel and injected into the mice. After 21 d, mice were sacrificed, dissected out, and photographed. The Matrigel plugs were excised and used for immunohistochemistry. The paraffin sections were immunostained with anti-vWF (Chemicon), anti-CD31 (Chemicon), anti–phosphorylated p65, nuclear factor-κB (NF-κB; Cell Signaling Technology), and anti–phosphorylated Akt (Santa Cruz) antibodies and visualized under confocal microscope (Ziess).

Human prostate cancer specimen analysis. Specimens of different Gleason grades and normal tissues of prostate were collected from a local hospital with informed consent and analyzed by immunohistochemistry as described (7). The expression profiles of EP2, EP4, ATF-4, c-Jun, Fos, uPA, and VEGF were detected by immunohistochemistry using their specific antibodies. Five specimens from each group [normal, prostata intraepithelial neoplasia (PIN), and malignant] were analyzed.

Statistical analysis. The data reported in cell migration, comigration, in vivo Matrigel plug angiogenesis, and the clinical specimen analysis are expressed as mean ± SE. Statistical differences were determined by Student’s t test. A P value of <0.05 was considered significant. All bands were analyzed densitometrically (Kodak Digital Science), and fold changes were calculated. The in vivo angiogenesis and clinical specimen data were quantified using the Image Pro Plus 6.0 Software (Nikon).

Results

PGE$_2$ augments EP2/EP4-mediated EGFR/MAPK and β3 integrin activation in prostate cancer cells. To examine the effect of PGE$_2$ on EGFR, MEK, ERK1/2, and β3 integrin phosphorylation, serum-starved PC-3 cells were treated with PGE$_2$ in a dose (0–1.0 μmol/L)–dependent and time (0–60 minutes)–dependent manner. The levels of phosphorylation of these signaling molecules were analyzed by Western blots using their phosphorylated-specific antibodies. The data indicated that PGE$_2$ induces phosphorylation of EGFR, MEK, ERK1/2, and β3 integrin, and maximum phosphorylations were observed between 10 and 15 minutes (Fig. 1A) with 0.5 μmol/L of PGE$_2$ (Supplementary Fig. S1A). Moreover, the effect of PGE$_2$ on the activation of these signaling molecules (EGFR, MEK, ERK, and β3 integrin) was examined in other prostate cancer (DU-145 and LNCaP) cells. The data showed significant phosphorylations of these molecules in DU-145 compared with LNCaP cells in response to PGE$_2$ (Supplementary Fig. S1B). Previous reports have shown that PC-3 cells express higher levels of EP2 and EP4 receptors (21). Therefore, to examine the involvement of EP2 and EP4 receptors in PGE$_2$-induced EGFR and β3 integrin phosphorylation, PC-3 cells were pretreated with EP2 (AH6809) or EP4 (AH23848) receptor antagonist in a dose-dependent manner (0–30 μmol/L) for 1 hour and then treated with PGE$_2$, and the levels of phosphorylated EGFR and phosphorylated β3 integrin were analyzed by Western blot. AH6809 or AH23848 at 30 μmol/L concentration showed maximum inhibition of PGE$_2$-induced EGFR and β3 integrin phosphorylation (Fig. 1B, I and II). To examine whether EP2 and EP4 receptor
agonists mimic the effect of PGE₂ and regulate the downstream molecular events. PC-3 cells were treated with butaprost (EP2 agonist) and PGE₁ alcohol (EP4 agonist) and phosphorylated EGFR and phosphorylated β3 integrin were analyzed. The data showed that both the agonists induce the phosphorylation of EGFR and β3 integrin (Supplementary Fig. S2A and B). These data revealed that PGE₂ induces the phosphorylation of EGFR and β3 integrin through EP2 and EP4 receptors–mediated process. Recently, it has been reported that PGE₂ induces β1 integrin expression in hepatocellular carcinoma cells (25). To determine whether PGE₂ regulates the expression of β3 integrin in prostate cancer cells, PC-3 cells were treated with PGE₂ for 12 hours and the expression of β3 integrin was analyzed by flow cytometry (Fig. 1C). To determine the roles of EP2 and EP4 receptors in PGE₂-induced β3 integrin expression, PC-3 cells were pretreated with AH6809 or AH23848 and then treated with PGE₂, and expression of β3 integrin was analyzed by immunofluorescence. The data showed that AH6809 and AH23848 suppressed the PGE₂-induced β3 integrin expression, indicating that EP2 and EP4 receptors play crucial roles in regulating this process (Fig. 1D). These data suggested that PGE₂ does not only stimulate EGFR and β3 integrin phosphorylation but also induces the expression of β3 integrin via EP2/EP4 receptor–mediated pathway.

**EGFR and β3 integrin play crucial roles in PGE₂-induced AP-1 activation.** Earlier studies have shown the role of AP-1 in prostate cancer progression (26, 27). Activation of AP-1 involves the increased expression or activation of Jun and Fos proteins (28–30). To examine the effect of PGE₂ on c-Fos and c-Jun expression/activation, PC-3 cells were treated with PGE₂, and expressions of c-Fos and phosphorylation of c-Jun were analyzed by Western blot and immunofluorescence, whereas AP-1–DNA binding was performed by EMSA. The results revealed that PGE₂ does not only augment the expression of c-Fos and phosphorylation of c-Jun (Fig. 2A and Supplementary Fig. S3A) but also stimulates the AP-1–DNA binding (Supplementary Fig. S3B). Furthermore, to study the role of EGFR/MAPK or β3 integrin on PGE₂-induced AP-1

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**Figure 1.** PGE₂ augments phosphorylation of EGFR, MEK, ERK, and β3 integrin in PC-3 cells. A, PC-3 cells were incubated with 0.5 μmol/L PGE₂ for 0 to 60 min, and the levels of p-EGFR, p-MEK, p-ERK, and p-β3 integrin were analyzed by Western blot using their specific antibodies. Total EGFR, MEK, ERK, and β3 integrin expressions in the cells were used as loading controls. B, roles of EP2 and EP4 receptors in PGE₂-induced phosphorylations of EGFR and β3 integrin. PC-3 cells were pretreated with either EP2 receptor antagonist (AH6809) or EP4 receptor antagonist (AH23848) in a dose-dependent manner (0–30 μmol/L) for 1 h and then treated with PGE₂, and the levels of phosphorylated EGFR and phosphorylated β3 integrin were analyzed by Western blot (I and II). C, PC-3 cells were treated with PGE₂, and β3 integrin expression was analyzed by flow cytometry using anti–β3 integrin antibody. D, PC-3 cells were pretreated with AH6809 or AH23848 and then treated with PGE₂, and the level of β3 integrin was analyzed by immunofluorescence using anti–β3 integrin antibody followed by staining with Cy2-conjugated IgG (green). Nuclei were stained with propidium iodide (PI, red). All figures are representation of three experiments. Fold changes were calculated.
activation, PC-3 cells were pretreated with PD98059 (MEK inhibitor) or AG1478 (EGFR inhibitor) or transfected with β3 integrin siRNA and expression of c-Fos and levels of the phosphorylated c-Jun were analyzed by Western blot. The data showed that inhibition of EGFR-MAPK pathway or down-regulation of β3 integrin suppressed PGE2-induced c-Fos expression and c-Jun phosphorylation, indicating that PGE2 triggers EGFR-MAPK and β3 integrin phosphorylation, indicating that PGE2 triggers EGFR-MAPK and β3 integrin-mediated AP-1 activation (Fig. 2B, I and II). Altogether, these results suggested that EGFR and β3 integrin play crucial roles in PGE2-induced AP-1 activation in PC-3 cells.

**PGE2 stimulates ATF-4-dependent AP-1 activation.** Elevated expression of ATF-4 has been observed in various cancers associated with enhanced malignancy (11). Recent findings have shown that ATF-4 is also involved in the regulation of expression of various oncogenic molecules and plays a crucial role in cancer progression (11). Therefore, we have examined the expression of ATF-4 in PC-3, DU-145, and LNCaP cells by immunofluorescence. The results showed the significant level of ATF-4 expression, particularly in PC-3 and DU-145 cells (data not shown). To investigate the role of PGE2 on ATF-4 activation, PC-3 cells were treated with PGE2 and ATF-4 nuclear localization and DNA binding.
were determined by immunofluorescence and EMSA. The data indicated that PGE2 induces nuclear localization and DNA binding of ATF-4 (Supplementary Fig. S4A and B). Moreover, we have observed the enhanced nuclear colocalization of ATF-4 with phosphorylated c-Jun in response to PGE2 (Fig. 2C). Furthermore, to explore the cross-talk between ATF-4 and AP-1, PC-3 cells were individually transfected with wt or dn ATF-4, followed by treatment with PGE2. The levels of c-Fos and phosphorylated c-Jun expressions were analyzed by Western blot. The data indicated that wt ATF-4 enhances, whereas dn ATF-4 suppresses, PGE2-induced c-Fos and phosphorylated c-Jun expression (Fig. 2D).

PGE2 induces EP2/EP4-mediated uPA and VEGF expressions in prostate cancer cells. To examine the role of PGE2 on uPA and VEGF expressions, PC-3 cells were treated with 0.5 μmol/L PGE2 for 0 to 24 h, and the levels of uPA and VEGF were detected by Western blot using their specific antibodies. Actin was used as loading control. B, cells were treated with PGE2 in a dose (0–1.0 μmol/L)-dependent manner, total RNA was isolated, and the levels of uPA and VEGF mRNA were detected by semiquantitative RT-PCR. β-Actin was used as internal control. C, PC-3 cells were transfected with EP2 or EP4 specific siRNA (EP2i or EP4i) and then treated with PGE2, and the levels of uPA, VEGF, EP2, and EP4 were analyzed by Western blot. Actin was used as loading control. D, roles of ATF-4 and AP-1 in PGE2-induced uPA and VEGF expression. Cells were individually transfected with dn ATF-4, dn c-Jun, and A-Fos followed by treatment with PGE2, and the levels of uPA and VEGF were analyzed by Western blot. All figures are representation of three experiments. Fold changes were calculated.

The levels of uPA and VEGF were analyzed by Western blot. The results indicated that PGE2 with 0.5 μmol/L concentration induced maximum expressions of uPA and VEGF at ~16 hours (Fig. 3A and Supplementary Fig. S5A). Similarly, PGE2 at 0.5 μmol/L concentration stimulated maximum uPA and VEGF expressions at mRNA levels (Fig. 3B). The PGE2-induced uPA and VEGF expressions were also detected in DU-145 and LNCaP cells (Supplementary Fig. S5B). The data indicated that PGE2 up-regulates uPA and VEGF expressions, both at transcriptional and protein levels. Earlier reports have indicated that COX-2 regulates PGE2 production in prostate tumor cells (3). Therefore, to determine the role of tumor-derived PGE2 on uPA and VEGF expressions, PC-3 cells were transfected with COX-2 cDNA or COX-2 siRNA (COX-2i) and expressions of uPA and VEGF were detected by Western blot. All figures are representation of three experiments. Fold changes were calculated.

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treated with PGE2. The levels of uPA, VEGF, EP2, and EP4 were analyzed by Western blot. The data indicated that silencing of EP2 or EP4 receptor suppresses PGE2-induced uPA and VEGF expressions (Fig. 3C). Moreover, to examine the roles of ATF-4 and AP-1 on PGE2-induced uPA and VEGF expressions, PC-3 cells were transfected with dn ATF-4 or dn c-Jun or A-Fos cDNA construct and then treated with PGE2, and the levels of uPA and VEGF were analyzed by Western blot. The data revealed that dn ATF-4, dn c-Jun, or A-Fos suppressed PGE2-induced uPA and VEGF expressions (Fig. 3D), demonstrating the roles of AP-1 and ATF-4 in PGE2-induced uPA and VEGF expressions. Taken together, these data indicated that both exogenous and tumor-derived PGE2 induced uPA and VEGF expressions via EP2 and EP4 receptors–mediated ATF-4–dependent and AP-1–dependent pathway.

**ATF-4 and AP-1 regulate PGE2-induced prostate tumor cell motility.** It has been reported that nonsteroidal antiinflammatory drugs and selective COX-2 inhibitors suppress invasiveness of human prostate cancer cell lines, PC-3 and DU-145, and this effect can be reversed by the addition of PGE2 (31). Although it has been proposed that overexpression of COX-2/PGE2 may enhance the invasive properties of tumors (3), leading to increase in tumor cell migration, the molecular mechanism underlying this process is not well defined. Therefore, to delineate the molecular mechanism of PGE2-regulated tumor cell migration, PC-3 cells were either individually transfected with dn ATF-4, dn c-Jun, and A-Fos or pretreated with AH6809, AH23848, and PKA inhibitor peptide and then treated with PGE2, and wound migration assay was performed. These data showed that antagonists of EP2 and EP4 receptors, PKA inhibitor peptide, dn ATF-4, dn c-Jun, and A Fos significantly suppressed PGE2-induced tumor cell migration (Fig. 4A, I and II). The roles of these molecules in PGE2-mediated PC-3 cell migration were further confirmed by migration assay.
using Boyden chamber (Supplementary Fig. S6A). Taken together, the results showed that 
PGE2 regulates ATF-4/VP-1-dependent prostate tumor cell motility through interaction with EP2 and EP4 receptors.

**PGE2 induces EP2/EP4-mediated Akt/NF-κB activation in endothelial cells, tumor-endothelial cell interaction, and angiogenesis.** NF-κB regulates the expression of various factors that control endothelial and tumor cell motility and invasion (32). The serine/threonine protein kinase Akt is an important component in the migratory and prosurvival signaling pathways (33). Therefore, to examine the effect of PGE2 on the activation of NF-κB and Akt, endothelial cells (HUVEC) were treated with PGE2 in a time-dependent manner and the levels of phosphorylated Akt and phosphorylated p65 and NF-κB were analyzed by Western blot. The data showed that PGE2 induces the phosphorylations of Akt and p65 in these cells (Fig. 4B). To examine the role of EP2 or EP4 receptor on PGE2-induced phosphorylation of Akt and p65, HUVEC were pretreated with AH6809 and AH23848 and then treated with PGE2, and the levels of phosphorylated Akt and phosphorylated p65 were analyzed. The data showed that EP2 and EP4 receptor antagonists suppressed PGE2-induced phosphorylation of Akt and p65, suggesting that EP2 and EP4 play crucial roles in this process (Fig. 4C). To examine the roles of EP2 and EP4 on PGE2-mediated endothelial cell motility, wound migration assay was performed. The data indicated that EP2, as well as EP4 receptor antagonists, suppressed PGE2-induced endothelial cell motility (Supplementary Fig. S6B).

Various studies have indicated that overexpression of COX-2 and PGE2 correlates with tumor angiogenesis (3, 7, 34). To determine the role of tumor-derived PGE2 on endothelial cell motility, direct comigration assay was performed. PC-3 cells, either alone or transfected with wt COX-2 cDNA or COX-2 siRNA, were used in the lower chamber, whereas HUVEC, either alone or pretreated with EP2 or EP4 receptor antagonist, were used in the upper chamber. In separate experiments, PGE2 was used in the lower chamber as positive control. The endothelial cells migrated toward the reverse side of the upper chamber were stained with Giemsa, photographed, counted, and represented in the form of a bar graph (Fig. 5A). The data revealed that overexpression of COX-2 significantly enhanced, whereas silencing COX-2 or antagonists of EP2 or EP4 receptor drastically suppressed, endothelial cell motility toward tumor cells, suggesting that tumor-derived PGE2 plays a crucial role in this process (Fig. 5A).

To examine the effect of PGE2 on *in vivo* tumor angiogenesis, Matrigel plug angiogenesis assay was performed. Accordingly, PGE2 was mixed with growth factor depleted Matrigel alone or along with EP2 or EP4 receptor antagonists and Matrigel and injected into the nude mice. After the termination of the experiments, Matrigel plugs were photographed and analyzed by immunohistochemistry using anti-CD31, anti-vWF, anti-phosphorylated p65, and anti–phosphorylated Akt antibodies. The results showed that PGE2-induced angiogenesis was inhibited by EP2 or EP4 receptor antagonist (Fig. 5B). The expressions of VWF and CD31 (endothelial cell markers) and phosphorylations of p65, NF-κB, and Akt were higher in PGE2-treated plugs compared with the plugs developed with EP2 and EP4 receptor antagonists (Fig. 5B). In other experiments, conditioned medium of PC-3 cells, either nontransfected or transfected with COX-2 cDNA, was mixed with Matrigel and injected into the mice. The Matrigel plugs generated from the conditioned medium of COX-2 overexpressing PC-3 cells showed enhanced tumor angiogenesis compared with the conditioned medium of PC-3 cells alone, suggesting that tumor-derived PGE2 plays a crucial role in regulating tumor angiogenesis (data not shown). The PGE2-induced angiogenesis (vWF positivity) was analyzed and represented in the form of a bar graph (Fig. 5B). These data showed that PGE2-induced EP2 and EP4 receptors mediated angiogenesis via NF-κB and Akt-dependent pathway and further suggested that both EP2 and EP4 receptors might play important roles in regulating PGE2-induced tumor angiogenesis.

**Correlation between expression profiles of EP2 and EP4 with ATF-4, c-Jun, c-Fos, uPA, and VEGF and their significance in prostate tumor progression.** Our *in vitro* and *in vivo* data prompted us to examine the expression profiles of various oncogenic and angiogenic molecules in human prostate cancer specimens by immunohistochemistry. The results showed that the expression levels of ATF-4, c-Jun, c-Fos, uPA, and VEGF were higher in malignant tumors compared with normal and PIN specimens, which further correlated with the enhanced expressions of EP2 and EP4 in human prostate cancer specimens (Fig. 6A). The expressions of these molecules were quantified and represented in the form of a bar graph (Fig. 6A).

**Discussion**

Prostate cancer is considered as one of the most lethal disease for men in the United States and other parts of the world. To date, treatments like androgen deprivation therapy and chemotherapy are two of the major approaches known to increase survival of patients with metastatic prostate cancer; however, some side effects have been observed in patients undergoing these therapies (2, 35). Therefore, identification of novel prognostic marker and development of new therapeutic strategies could be the most promising approaches in the next generation of prostate cancer management.

Recently, several studies have shown the correlation between overexpression of COX-2 with prostate tumorigenesis; however, the molecular mechanism underlying COX-2–induced prostate cancer progression and angiogenesis is still not well understood. Although various reports have revealed the relationship between the elevated levels of PGE2 with malignant cancers (3), the molecular mechanism underlying PGE2-mediated prostate tumor progression is still the subject of intense investigation. In this study, we have shown the in-depth molecular mechanism underlying PGE2–induced tumor cell motility and angiogenesis in prostate cancer via EP2 and EP4 receptor–dependent pathway.

EGFR and MAPK-mediated activation of transcription factor AP-1 has been reported as one of the crucial signaling cascade that affects tumor cell motility in various cancers, including the malignancies in prostate (36). Elevated expression of EGFR has been observed in higher grades of prostate cancer, which further correlate with poor clinical prognosis (37, 38). Earlier studies reported that activation of EGFR in response to PGE2 leads to the phosphorylation of ERK, which, in turn, regulates downstream signaling events (36, 39). Moreover, it has also been observed that PGE2 transactivates EGFR, which ultimately influences cell migration, proliferation, and invasiveness in different cancer models (36, 39, 40). In this paper, we have shown that PGE2 induces the activation of EGFR and MAPK signaling cascade in prostate cancer cells. Recently, Wang and colleagues have shown that among different PGE2 receptors, EP2 and EP4 predominantly express PC-3 cells in androgen-independent prostate cancer (21).
Here, we have shown that blocking of both PGE\(_2\) receptors (EP2 and EP4) by their specific antagonists curbs the PGE\(_2\)-mediated activation of EGFR and downstream signaling cascades, which ultimately suppress the prostate tumor cell motility.

Integrin β3 has been shown to play critical roles in several distinct processes, such as tumor growth, metastasis, and angiogenesis in various cancers, including prostate cancer (41–43). Phosphorylation of β3 integrin is essential for the activation of small GTP-binding proteins (Rho family), and activation of Rho is necessary for invasion and migration in a wide variety of cell types (44). Previous studies have indicated that integrins control activation of AP-1 in prostate cancer cells (26). Furthermore, earlier reports have shown that overexpression of β3 integrin correlates with enhanced metastatic phenotype in LNCaP cells (42). Moreover, it has been observed that stromal cell derived factor-1 transiently increased the expression and activation of β3 integrin in prostate cancer cells, which in turn augmented the aggressiveness of prostate cancer (43). In this study, we have reported that PGE\(_2\) induces the expression and phosphorylation of β3 integrin in PC-3 cells. The EP2 and EP4 receptor antagonists suppressed PGE\(_2\)-induced expression and phosphorylation of β3 integrin, which further showed the involvement of both these receptors in this process. Silencing of β3 integrin expression by its specific siRNA suppresses PGE\(_2\)-induced AP-1 activation, suggesting that PGE\(_2\) via EP2/EP4 controls AP-1 activation through β3 integrin–mediated pathway. Chen and colleagues have shown that arachidonic acid regulates PGE\(_2\)-mediated PKA-dependent expression of c-fos in PC-3 cells (5). In this study, we have shown the molecular mechanism, at least in part, whereby PGE\(_2\) via EGFR-ERK or β3 integrin–mediated pathway augments the expression of c-Fos and phosphorylation of c-Jun, which ultimately regulates AP-1 activation in prostate cancer cells.

Previous data showed that ATF-4 forms heterodimers with either c-Fos or c-Jun subunit of AP-1 and regulates the activation of AP-1 (45). In this study, we have shown that PGE\(_2\) augments ATF-4 activation, controls the interaction between ATF-4 and phosp-
ylated c-Jun, and regulates ATF-4–dependent AP-1 activation in prostate cancer cells.

uPA and its receptor uPAR-mediated signaling has been implicated in tumor cell invasion, survival, and metastasis in a variety of cancers including prostate (20). Both uPA and uPAR are expressed at higher levels in malignant prostate tissues than in benign and normal prostate tissues (20). VEGF acts as one of the key proangiogenic factor responsible for neovascularization in cancer cells (46). Previous studies have indicated that inhibition of VEGF expression is a critical step in arresting prostate tumor growth and progression (47, 48). In this study, we have shown in-depth molecular mechanism underlying PGE2-induced EP2/EP4-mediated expression of uPA and VEGF via activation of AP-1 and ATF-4, which eventually affects tumor cell motility and angiogenesis. Moreover, our data revealed that inhibition of tumor-derived PGE2 by COX-2 siRNA suppressed uPA and VEGF expression, suggesting that PGE2 both tumor-derived and exogenous, regulates this process in prostate cancer cells.

Figure 6. Expression profiles of EP2, EP4, ATF-4, c-Jun, c-Fos, uPA, and VEGF in human prostate cancer specimens and their correlation with human prostate cancer progression in different pathologic grades. A, the levels of EP2, EP4, ATF-4, c-Jun, c-Fos, uPA, and VEGF were detected by immunohistochemical studies using their specific antibodies. EP2, EP4, ATF-4, c-Jun, c-Fos, and uPA were stained with Cy3-conjugated IgG (red). VEGF was stained with Cy2-conjugated IgG (green). Sections stained with anti-rabbit IgG were used as control. Nuclei were stained with DAPI (blue). The expression profiles were quantified by Image Pro Plus 6.0 Software and represented in the form of a bar graph (*, P < 0.003; **, P < 0.006; #, P < 0.02). B, schematic representation of PGE2-induced EP2/EP4-mediated EGFR/MAPK or β3 integrin–dependent ATF-4/AP-1 activation leading to enhanced uPA and VEGF expression, which in turn controls prostate tumor cell motility and angiogenesis. In endothelial cells, PGE2 through EP2/EP4 receptor stimulates Akt and NF-κB activation leading to enhanced endothelial cell motility and angiogenesis.

Angiogenesis is one of the most crucial steps in the development of tumor. The proliferation and migration of endothelial cells play crucial roles in the regulation of tumor-associated angiogenesis (49). We have shown that PGE2 induces the phosphorylation of Akt and NF-κB, p65 in endothelial cells. Moreover, both exogenous and tumor-derived PGE2 augments endothelial cell motility, and blocking of endothelial EP2 and EP4 receptors by their antagonists suppresses endothelial cell motility toward tumor cells. In vivo Matrigel plug angiogenesis assay showed that PGE2 induces angiogenesis whereas blocking EP2 and EP4 receptors suppressed this effect, indicating that PGE2 augments EP2/EP4-mediated in vivo angiogenesis. Our data also revealed that tumor-derived PGE2 induces tumor angiogenesis. Taken together, these results showed that PGE2 via EP2/EP4 receptor promotes in vivo angiogenesis through activation of Akt and NF-κB, suggesting that PGE2 plays an important role in the regulation of tumor angiogenesis. Furthermore, prostate tumor clinical specimen analysis data also
corroborated with in vitro and in vivo findings, indicating higher levels of uPA and VEGF expression in malignant prostate tumors compared with normal and PIN tissues, which further correlated with the enhanced expression levels of ATF-4, c-Fos, c-Jun, EP2, and EP4. These data provide, at least in part, the molecular basis by which PGE2 controls downstream signaling cascades and leads to the expression of various oncogenic and angiogenic molecules that ultimately regulate the prostate cancer progress and angiogenesis (Fig. 6B). Thus, targeting PGE2 by interfering its interaction with EP2 and EP4 receptors might be an alternative therapeutics that may aid in the rational design of therapeutic strategy for the next generation of prostate cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
19. Mira-Y-Lopez R, Jaramillo S, Ying J.Synergic expression of urokinase plasminogen activator (uPA) and cathepsin D: a potential therapeutic target as an alternative therapeutics that may aid in the rational design of the next generation of prostate cancer treatment.
Hypoxia Regulates Cross-talk between Syk and Lck Leading to Breast Cancer Progression and Angiogenesis*

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Hypoxia is a key parameter that controls tumor angiogenesis and malignant progression by regulating the expression of several oncoprogenic molecules. The nonreceptor protein-tyrosine kinases Syk and Lck play crucial roles in the signaling mechanism of various cellular processes. The enhanced expression of Syk in normal breast tissue but not in malignant breast carcinoma has prompted us to investigate its potential role in mammary carcinogenesis. Accordingly, we hypothesized that hypoxia/reoxygenation (H/R) may play an important role in regulating Syk activation, and Lck may be involved in this process. In this study, we have demonstrated that H/R differentially regulates Syk phosphorylation and its subsequent interaction and cross-talk with Lck in MCF-7 cells. Moreover, Syk and Lck play differential roles in regulating Sp1 activation and expressions of melanoma cell adhesion molecule (MelCAM), urokinase-type plasminogen activator (uPA), matrix metalloproteinase-9 (MMP-9), and vascular endothelial growth factor (VEGF) in response to H/R. Overexpression of wild type Syk inhibited the H/R-induced uPA, MMP-9, and VEGF expression but up-regulated MelCAM expression. Our data also indicated that MelCAM acts as a tumor suppressor by negatively regulating H/R-induced uPA secretion and MMP-9 activation. The mice xenograft study showed the cross-talk between Syk and Lck regulated H/R-induced breast tumor progression and further correlated with the expressions of MelCAM, uPA, MMP-9, and VEGF. Human clinical specimen analysis supported the in vitro and in vivo findings. To our knowledge, this is first report that the cross-talk between Syk and Lck regulates H/R-induced breast cancer progression and further suggests that Syk may act as potential therapeutic target for the treatment of breast cancer.

Hypoxia plays a crucial role in regulating breast tumor progression through a multistep process that includes oncoprotein activation or inhibition of tumor suppressor genes (1). Most tumors develop regions of chronically or transiently hypoxic cells during growth (2). Hypoxic tumor regions may show increased expression of many genes because of hypoxia-induced activation of transcription factors (3–5). Low extracellular pH, glucose depletion, high lactate levels, and regions with low oxygen tension (6, 7) characterize most tumors. Low oxygen tension in tumors has been associated with poor outcome, enhanced local or locoregional spread, and enhanced metastatic potential (8). Hypoxia is a key parameter, which modulates the expression of a variety of genes that are involved in tumor angiogenesis, malignant progression, and distant metastasis (9). The signaling properties of reactive oxygen species are because of the reversible oxidation of redox-sensitive target proteins (10). The generated reactive oxygen species act as intracellular second messengers in various signal transduction pathways and hence play a crucial role in regulating disease and stress-induced cellular injuries such as ischemia/reperfusion, UV irradiation, and inflammation (10).

Previous reports have indicated that areas of hypoxia/reoxygenation (H/R)² are a typical feature of rapidly growing and metastasizing tumors (11, 12). It was also demonstrated that both hypoxia and consecutive hypoxia/reoxygenation exert a variety of influence in tumor cell biology that ultimately regulates tumor progression (13). Earlier reports also showed that hypoxia and H/R regulate the expression of various mitogen-activated protein kinase signaling pathways (13) and induce the activation of several transcriptional factors such as HIF-1α, NFκB, AP-1, and Sp1 (13–15). However, very recently it was demonstrated that H/R rather than hypoxia alone appears to induce the expression and activation of several oncoprogenic molecules and plays an important role in tumor progression (13, 16).

The nonreceptor protein-tyrosine kinase Syk is widely expressed in hematopoietic cells (17, 18). It has tandem amino-terminal Src homology 2 domains and a carboxyl-terminal kinase domain (19). The Src homology 2 domains bind phosphorylated immunoreceptor tyrosine-based activation motifs and hence play a significant role in immunoreceptor and cytokine signaling (20). The expression of Syk has also been reported in cell lines of epithelial origin (21), but its function in these cells is not well understood. It has been documented that Syk is commonly expressed in normal human breast tissue, benign breast lesions, and low tumorigenic breast cancer cell lines (22). Previous data indicated that Syk suppresses cell motility and NFκB-mediated urokinase-type plasminogen activator (uPA) secretion by inhibiting phosphorylation and nuclear translocation of NFκB (23). Syk enhances angiogenesis and promotes tumor metastasis by regulating the expression and activity of several proangiogenic factors. The role of Syk in breast cancer progression and angiogenesis is not well understood.

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3 The abbreviations used are: H/R, hypoxia/reoxygenation; Syk, splenic tyrosine kinase; Lck, leukocyte-specific kinase; HIF-1α, hypoxia-inducible factor 1α; EMSA, electrophoretic mobility shift assay; MelCAM, melanoma cell adhesion molecule; uPA, urokinase-type plasminogen activator; MMP-9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor; VWF, von Willebrand factor; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; WT, wild type; Mut, mutant; DN, dominant negative; NMRI, Naval Medical Research Institute.
MelCAM, previously known as MUC18 or MCAM, a newly recognized cell adhesion molecule with an apparent molecular mass of 113 kDa, belongs to the immunoglobulin superfamily (27–29). The presence of putative binding sites for the transcription factors Sp1, AP-1, AP-2, and CAMP-response element-binding protein in the promoter region suggests that MelCAM expression can be modulated by exogenous factors (30). At the cellular level, phorbol ester and cyclic AMP have been shown to modulate MelCAM expression (31). Earlier reports have indicated that MelCAM acts differently in the progression of breast carcinomas. It is expressed in normal and benign proliferative breast epithelium, and its expression is frequently lost in situ and infiltrating breast carcinomas (32, 33). The MelCAM core promoter contains four binding sites for the Sp1 transcription factor, and deletion analyses have indicated that removal of all putative Sp1 sites reduced the promoter activity by 80%, suggesting that Sp1 is an important regulator of MelCAM expression (34). However, the molecular mechanism by which H/R regulates Syk activation and Syk-dependent Lck-mediated Sp1 activation leading to the regulation of MelCAM expression in MCF-7 cells is not well defined.

Degradation of extracellular matrix plays an important role in tumor metastasis. uPA is a member of the serine protease family that interacts with uPA receptor and facilitates the conversion of inert zymogen plasminogen into widely acting serine protease plasmin (35, 36). MMP-9, also referred as gelatinase-B, is not only associated with invasion and metastasis but also has been implicated in angiogenesis, rheumatoid arthritis, retinopathy, and vascular stenosis and hence is considered to be a prioritized therapeutic target (37, 38). Several reports have indicated the positive correlation between uPA/MMP-9 activation and the metastatic potential of tumors (39). However, the molecular mechanism by which H/R regulates Syk/Lck-dependent MelCAM expression and uPA secretion and the uPA-dependent pro-MMP-9 activation in breast carcinoma cells is not well understood. Moreover, the roles of these molecules in regulating H/R-induced tumorigenesis and its clinical implications are not well defined.

Hypoxia-induced VEGF production provides one of the main driving forces that stimulate the angiogenesis, which accompanies tumor progression (40, 41). To date, VEGF is considered as the key factor that guided and regulated tumor angiogenesis (42). Tumor cell-derived VEGF binds to its specific receptors and regulates tumor progression through neovascularization via autocrine and paracrine pathways (43, 44). Recent evidence suggested that the VEGF promoter contains an Sp1-response element (45). However, the role of Lck and Syk in H/R-induced VEGF expression in breast cancer is not defined clearly.

In this study we have demonstrated the differential role of Syk and Lck in the H/R-induced uPA, MMP-9, and VEGF expression. Our findings suggested that H/R down-regulates Syk activation leading to enhanced uPA, MMP-9, and VEGF expression. Furthermore, over-expression of Syk restored H/R-induced down-regulation of MelCAM expression. In hypoxic cells, Lck also physically associates with Syk, and this association plays a crucial role in regulating the downstream signaling. The in vivo relevance of our study was further validated in a xenografted nude mice model, which also supports our in vitro findings. Clinical data also indicated that the higher grades of tumors showed significant HIF-1α expression compared with that of lower grades or normal breast tissue and also demonstrated an inverse correlation between Syk/MelCAM and uPA/MMP-9/VEGF expression, which further correlates with enhanced tumorigenic potential and neovascularization.

Experimental Procedures

Materials—The rabbit polyclonal anti-Syk, anti-Lck, anti-Sp1, anti-MelCAM, anti-uPA, anti-MMP-9, anti-actin, mouse monoclonal anti-Lck, anti-Syk, anti-phosphotyrosine antibodies, and MelCAM blocking peptide were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-VEGF was from Oncogene. Rabbit polyclonal anti-HIF-1α was from Ustate Biotechnology, Inc. Lipofectamine Plus was obtained from Invitrogen. pp2, aminogenistein, and damnacanthal were from Calbiochem. The Sp1 consensus oligonucleotide was purchased from Bangalore Genei. Matrigel was purchased from BD Biosciences. The female nude mice (NMRI, nu/nu) were from National Institute of Virology (Pune, India). All other chemicals were of analytical grade.

Cell Culture—The MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO2 and 95% air at 37 °C.

Hypoxic/Reoxygenation Cultures—The MCF-7 cells grown to 50–70% confluence were made hypoxic in evacuation chambers by intermittent application of vacuum and sparging with 95% N2, 5% CO2. Cells were analyzed at this point or maintained under hypoxic conditions in the presence of 100 μM dithionate (an O2 scavenger) at 37 °C for the indicated time point. These cells were reoxygenated for the indicated periods by replacing the medium with fresh medium and incubating the cultures in humidified atmosphere of 5% CO2 and 95% air at 37 °C.

Immunofluorescence and Immunohistochemistry—To detect the effect of H/R on cellular localization of Syk, cells grown in monolayer on glass slides were induced by hypoxia for 2 h and reoxygenated for 45 min. The cells were fixed and incubated with rabbit polyclonal anti-Syk antibody (1:50 dilution) followed by FITC-conjugated anti-rabbit IgG at room temperature. The role of H/R in regulating Syk-Lck colocalization was determined by immunofluorescence studies using a mixture of mouse monoclonal anti-Syk and rabbit polyclonal anti-Lck antibody followed by a mixture of TRITC- and FITC-conjugated IgG. The cells were washed and mounted with coverslips. All these samples were analyzed under confocal microscopy (Zeiss).

The clinical specimens were analyzed by immunohistopathological studies. Formalin-fixed paraffin-embedded sections (4 μm) were subjected to antigen retrieval, and the Syk-Lck colocalization was determined by immunofluorescence studies using a mixture of rabbit polyclonal anti-Syk and mouse monoclonal anti-Lck antibodies followed by a mixture of FITC- and TRITC-conjugated IgGs. The levels of HIF-1α expression in these clinical samples were determined by Western blot analysis. The levels of MelCAM, uPA, MMP-9, and VEGF expressions in these samples were detected by immunofluorescence studies by using their specific antibodies. The tumor microvessel densities were detected by immunostaining with anti-vWF antibody. All these samples were analyzed under confocal microscopy (Zeiss).

Plasmids and DNA Transfection—The dominant-negative form of Lck (DN Lck, K273R) in pcDNA3 was a kind gift from Dr. D. R. Branch (Canadian Blood Services, Toronto, Ontario). The wild type and kinase-negative Syk cDNA in pcDNA 3.1 were the generous gifts from Dr. Susette C. Mueller (Department of Oncology, Georgetown University Medical School, Washington, D. C.). MCF-7 cells were transfected with specific cDNA using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions as described previously (46). These transfected cells were used for Syk-Lck colocalization and interaction studies, detection of Sp1, Syk, Lck, MelCAM, uPA,
Cross-talk between Syk and Lck in Response to H/R

MMP-9, and VEGF by Western blot analysis, and in vivo tumorigenicity experiments.

Zymography Experiments—The gelatinolytic activity was measured as described (46, 47). To examine whether H/R regulates MMP-9 activation and to investigate the effect of inhibition of MelCAM expression on H/R-induced MMP-9 activation, cells were either induced with H/R for 24 h or pretreated with MelCAM blocking peptide (50 μg) and then exposed with H/R. The conditioned media were collected, and the samples were analyzed by zymography as described (47). Negative staining showed the zones of gelatinolytic activity.

Immunoprecipitation—To delineate the role of H/R in the regulation of tyrosine phosphorylation of Syk, cells were exposed to hypoxia for 2 h followed by reoxygenation for 0–60 min. In separate experiments, cells were pretreated with Lck inhibitors, pp2 (4 nM), aminogenistein (2 μM), and damcanthal (0.8 μM), and then exposed to H/R. Cell lysates were immunoprecipitated with rabbit polyclonal anti-Syk antibody and analyzed by Western blot using anti-phosphotyrosine antibody. The same blots were reprobed with anti-Syk antibody. To analyze whether Syk interacts with Lck and to determine whether H/R regulates this process, cells were exposed to H/R for 45 min. In other experiments, cells were transfected with wild type and kinase-negative Syk and then subjected to H/R. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The lysates containing equal amount of total proteins were immunoprecipitated with rabbit anti-Lck antibody and analyzed by Western blot using anti-Syk antibody. The same blots were reprobed with anti-Lck antibody.

Western Blot Analysis—To analyze the roles of Syk and Lck in regulating MelCAM, VEGF expression, and MMP-9 activation, cells were transfected with wild type and kinase-negative Syk or dominant-negative Lck and then exposed to H/R for 24 h as described earlier. The cell lysates were analyzed by Western blot using anti-MelCAM or anti-VEGF antibody. The levels of Lck and Syk in nontransfected or transfected cell lysates were also detected by Western blot using anti-Lck or anti-Syk antibody. The level of active MMP-9 in conditioned media was also detected by Western blot using anti-HIF-1α antibody. The same blots were reprobed with anti-actin antibody as loading control.

Nuclear Extracts and Western Blot—To check whether H/R regulates Sp1 expression, cells were subjected to H/R for 0–180 min at 37 °C. The nuclear extracts were prepared as described earlier (24). Briefly, cells were incubated in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer. The nuclei were separated by spinning at 3300 × g for 5 min at 4 °C. The supernatant was used as cytoplasmic extract. The nuclear pellet was extracted in nuclear extraction buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and centrifuged at 12,000 × g for 30 min. The supernatant was used as nuclear extract. The nuclear extracts were resolved by SDS-PAGE, and the level of Sp1 was detected by Western blot using rabbit anti-Sp1 antibody. In separate experiments, to examine the effects of Syk and Lck on H/R-induced Sp1 expression, cells were transfected with wild type and kinase-negative Syk or DN Lck and then induced with H/R. The nuclear extracts were prepared and analyzed by Western blot using anti-Sp1 antibody. The levels of Lck and Syk in nuclear extracts were also detected by Western blot using anti-Lck or anti-Syk antibody. The level of expression of HIF-1α in hypoxia or H/R-induced nuclear extracts was detected by Western blot using anti-HIF-1α antibody. Actin was used as loading control.

EMSA—To examine whether H/R induces the Sp1-DNA binding, cells were exposed to H/R for 0–180 min as described earlier. To check whether Syk and Lck play any role in regulating H/R-induced Sp1-DNA binding, cells were transfected with wild type and kinase-negative Syk or pretreated with Lck inhibitors, pp2 (4 nM), aminogenistein (2 μM), and damcanthal (0.8 μM), and then exposed by H/R for 1 h. The nuclear extracts were prepared as described above and incubated with 16 fmol of 32P-labeled double-stranded Sp1 oligonucleotide (5’-ATTCGATCGGCGGGGCGC-3’) in binding buffer (25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl) containing 1 μg of poly(dI-dC). The DNA-protein complex was resolved on a native polyacrylamide gel and analyzed by autoradiography.

In Vivo Xenograft Tumor Model—The tumorigenicity experiments were performed as described (47, 48). Briefly, female athymic nude mice, NMRI, nu/nu (BALB/c) obtained from National Institute of Virology (Pune, India) were housed under specific pathogen-free conditions and used for in vivo tumorigenicity studies. Cells were either exposed to H/R or transfected with wild type or mutant Syk and then exposed to H/R for 24 h. Cell viability was determined by the trypan blue exclusion test, and only a single cell suspension (5 × 10⁶/0.2 ml) of greater than 90% viability were mixed with Matrigel and injected subcutaneously into the flanks of female athymic NMRI (nu/ nu) mice (6–8 weeks old). Five mice were used in each set of experiments. Growth of tumors was monitored weekly by measuring the tumors with calipers. The mice were killed after 4 weeks of injection, and the levels of uPA, VEGF, and MelCAM in the tumors were analyzed by Western blot. The level of Sp1-DNA binding in the nuclear extracts of the tumor samples was determined by EMSA.

The tumor samples were also processed for histopathological studies. Formalin-fixed paraffin-embedded sections (4 μm) were subjected to antigen retrieval and then stained with rabbit polyclonal anti-MMP-9, anti-uPA antibody, or mouse monoclonal anti-MelCAM antibody followed by FITC-conjugated anti-rabbit or anti-mouse IgG. The tumor microvessel densities were detected by immunostaining with anti-vWF antibody. The level of colocalization of Syk and Lck in these tumor specimens was determined by immunofluorescence using a mixture of mouse monoclonal anti-Syk and rabbit polyclonal anti-Lck antibody followed by a mixture of FITC- and TRITC-conjugated IgG. All these samples were analyzed under confocal microscopy (Zeiss).

RESULTS

H/R Regulates the Cellular Localization of Syk—To determine whether H/R regulates the cellular localization of Syk, MCF-7 cells (2 × 10⁵ cells/ml) seeded on 35-mm tissue culture plate were exposed to hypoxia for 2 h and then reoxygenated for 45 min. After that, cells were fixed and incubated with rabbit polyclonal anti-Syk antibody followed by FITC-conjugated anti-rabbit IgG. The data indicated that H/R induces the cytoplasmic translocation of Syk at 45 min in these cells.
were immunoprecipitated with anti-Syk antibody and immunoblotted with anti-Tyr(P) antibody (Fig. 2B). The same blots were reprobed with anti-Syk antibody (Fig. 2B). The results indicated that cells treated with Lck inhibitors restored Syk phosphorylation, which was down-regulated upon exposure to H/R.

**H/R Regulates the Interaction between Syk and Lck**—To examine the role of H/R in regulating the interaction between Syk and Lck, cells were either exposed to H/R or transfected with wild type and mutant Syk and then exposed to H/R. Cell lysates were immunoprecipitated with anti-Lck antibody and analyzed by Western blot with anti-Syk antibody (Fig. 2C, upper panel). Cells transfected with WT Syk but not with Mut Syk resulted in restoration of Syk and Lck interaction in response to H/R. The same blots were reprobed with anti-Lck antibody (Fig. 2C, lower panel). The cellular colocalization between Syk and Lck in response to H/R was further confirmed by immunofluorescence study (Fig. 2D). These results suggested that H/R down-regulates the physical association between Syk and Lck, and this is due to cytoplasmic translocation of Syk in response to H/R at 45 min.

**Syk Negatively Regulates H/R-induced Sp1 Activation**—To determine the role of H/R on Sp1 nuclear translocation and DNA binding, MCF-7 cells were exposed to H/R for 0–180 min as described earlier, and the cytoplasmic and nuclear extracts were prepared. Nuclear extracts were subjected to Western blot using anti-Sp1 antibody (Fig. 3A, upper panel, lanes 1–5). Similarly, cytoplasmic extracts were also used for Western blot using anti-Sp1 antibody (Fig. 3A, 2nd middle panel). Actin was used as loading control (Fig. 3A, 1st middle and lower panels). To examine whether H/R regulates Sp1-DNA binding, nuclear extracts were analyzed by EMSA using Sp1-specific oligonucleotide (Fig. 3C, lanes 1–5). The results showed that H/R induces maximum Sp1 nuclear translocation and DNA binding at 60 min (Fig. 3, A and C). To examine the roles of Syk and Lck in H/R-induced Sp1 nuclear translocation and Sp1-DNA binding, cells were individually transfected with WT and Mut Syk or DN Lck or treated with Lck-specific inhibitors (pp2, aminogenistein, and dammacanthal) and were then exposed to H/R. Nuclear extracts were analyzed by Western blot (Fig. 3B, upper panel, lanes 1–5) and EMSA (Fig. 3, D and E). The blots were reprobed with anti-Syk or anti-Lck antibody in order to detect the expression of Syk and Lck (Fig. 3B, 1st middle and 2nd middle panels). Actin was used as loading control (Fig. 3B, lower panel). The results demonstrated that Syk negatively regulates H/R-induced Sp1 nuclear translocation and DNA binding.
whereas pharmacological or genetic inhibitors of Lck significantly suppressed H/R-induced Sp1 activation, suggesting that Lck and Syk differentially regulate the Sp1 activation in response to H/R.

**Syk and Lck Differentially Regulate MelCAM, VEGF, and MMP-9 Expressions in Response to H/R**—To delineate the roles of Syk and Lck in differentially controlling the expressions of MelCAM and VEGF and the activation of MMP-9 in the presence of H/R, cells were transfected with DN Lck or WT and Mut Syk and then exposed to H/R. The levels of MelCAM, VEGF, and MMP-9 were detected by Western blots using their specific antibodies. The results showed that DN Lck and WT Syk restored the H/R-suppressed MelCAM expression (Fig. 4A, lanes 1–5). The levels of Lck and Syk expression were also detected by Western blot analysis using their specific antibodies (Fig. 4A, 1st and 2nd middle panels). Actin was used as loading control (Fig. 4A, lower panel). In contrast, WT Syk and DN Lck suppressed the H/R-induced VEGF expression and MMP-9 activation (Fig. 4B, lanes 1–4, 6–10).
and C, lanes 1–5). However, cells transfected with Mut Syk further enhanced the H/R-induced VEGF expression and MMP-9 activation (Fig. 4, B, lane 3, and C, lane 5). These data suggested that Syk, which is considered a negative regulator of tumor progression, can also regulate the expression of MelCAM, a breast tumor suppressor molecule.

**MelCAM Negatively Regulates H/R-induced uPA Secretion and MMP-9 Activation**—Our previous study demonstrated that uPA plays a crucial role in MMP-9 activation (47). To examine the role of MelCAM in regulation of uPA secretion and MMP-9 activation, cells were either exposed to H/R or pretreated with MelCAM blocking peptide and then exposed to H/R. Cell lysates were analyzed by Western blot using anti-uPA antibody. Similarly, the conditioned media were used to detect the levels of uPA by Western blot (D) and MMP-9 by zymography (E), respectively. Note that H/R down-regulates MelCAM expression but enhances VEGF expression and MMP-9 activation. Blocking of MelCAM by its blocking peptide further stimulates H/R-induced uPA expression and MMP-9 activation.

**Effect of Syk on Xenograft Tumor Growth**—Our in vitro results prompted us to extend our studies to the in vivo system. Accordingly, MCF-7 cells were transfected with WT or Mut Syk, exposed to H/R, and then implanted into the nude mice. After 4 weeks, mice were sacrificed, and tumor specimens were excised. A portion of tumor samples was used for histopathological studies. The histopathological analysis data indicated that Mut Syk-transfected tumors showed higher infiltration toward the extravasations, nuclear polymorphism, and mitotic features compared with the control or WT Syk-transfected tumors (Fig. 5). The cellular localizations of Syk and Lck in human clinical breast tumor sections were detected by incubating with a mixture of anti-Syk and anti-Lck antibodies. Our results indicated that in normal breast tissue there was colocalization of Syk and Lck, but in higher grades of tumors, the expression of Syk and Lck was significantly lower in higher grades of tumors compared with the increasing grade of tumor specimens, no colocalization was observed. Furthermore, in higher grades of tumors, the expression of Syk was totally diminished (Fig. 6A, panels a–d). The cellular localizations of Syk and Lck in human clinical breast tumor sections were detected by incubating with a mixture of anti-Syk and anti-Lck antibodies. Our results indicated that normal breast tissue was characterized by the presence of MelCAM and the absence of uPA, VEGF, and vWF (vWF expression) were also higher in the Mut Syk-transfected tumor (Fig. 5E, panels a–i) that further correlated with our in vitro study. As expected, the MelCAM expression was higher in WT Syk-transfected tumors (Fig. 5A, panels g–i). This was also confirmed by Western blot analysis (Fig. 5C, lanes 1–3). Western blot data also suggested that the levels of uPA and VEGF are higher in Mut Syk-transfected tumors (Fig. 5B, lanes 1–3). Similar results were obtained in Sp1-DNA binding as detected by EMSA (Fig. 5D, lanes 1–3). Thus, our studies clearly indicated that Syk acts as a tumor suppressor and anti-angiogenic candidate gene that differentially regulates MelCAM expression that ultimately controls uPA-dependent MMP-9 activation. Syk also negatively regulates VEGF expression. These signaling molecules ultimately control the tumor progression and angiogenesis.

**Human Breast Tumor Specimen Analysis**—The in vitro and in vivo mouse model data further prompted us to extend these studies with human clinical breast tumor samples. Human solid breast tumor specimens were collected with the informed consent from a local hospital. The tumor grading was determined by histopathology analysis by hematoxylin and eosin staining using a modified Scarff-Bloom-Richardson system, and photographs were taken with a Nikon microscope (Fig. 6A, panels a–d). The cellular localizations of Syk and Lck in human clinical breast tumor sections were detected by incubating with a mixture of anti-Syk and anti-Lck antibodies. Our results indicated that in normal breast tissue there was colocalization of Syk and Lck, but with the increasing grade of tumor specimens, no colocalization was observed. Furthermore, in higher grades of tumors, the expression of Syk was totally diminished (Fig. 6A, panels e–h). As expected, MelCAM expression was significantly lower in higher grades of tumors compared with lower grades or normal tissue (Fig. 6A, panels i–l). The expression of HIF-1α was detected by Western blot using the same specimens, and the data showed that the level of HIF-1α was significantly higher in grade II and III tumors (Fig. 6B, lanes 1–4), which indicated the hypoxic status in the tumor microenvironment. Higher expressions of MMP-9, uPA, VEGF, and vWF in these tumor specimens further correlated with...
FIGURE 5. H/R regulates tumor growth and angiogenesis through differential interplay between Syk and Lck. A, MCF-7 cells (5 x 10^6/0.2 ml) were either exposed to H/R for 24 h or transfected with WT or Mut Syk and then exposed to H/R. The cell suspension was mixed with cold Matrigel and injected subcutaneously in the dorsal region of mice. After 4 weeks, tumors were excised and subjected to histopathological analysis by hematoxylin and eosin staining (panels a–c). Note that increased infiltration and nuclear polymorphism were observed in a tumor generated by injecting Mut Syk but not WT Syk-transfected cells. The physical association between Syk and Lck in these tumors was detected by immunohistochemistry (panels d–f). Note that tumors generated by injecting WT Syk-transfected cells showedhigher nuclear localization and enhanced interaction between Syk and Lck, whereas Mut Syk-transfected tumors showed enhanced cytoplasmic localization of Syk (panels d–f). Syk was stained with FITC-conjugated IgG (green), and Lck was stained with TRITC-conjugated IgG (red). The nuclei of the cells was visualized by DAPI (blue) staining. The MelCAM expression in these tumors was analyzed by immunofluorescence using anti-MelCAM antibody (panels g–i). B and C, the expressions of uPA, VEGF, and MelCAM in the tumor extracts were also analyzed by Western blot using their specific antibodies. D, the Sp1-DNA binding in the nuclear extracts was performed by EMSA. E, the levels of uPA, MMP-9, and vWF in the above tumor specimens were analyzed by immunofluorescence using their specific antibodies (panels a–i). Note that there was drastic reduction in uPA and MMP-9 levels in a tumor generated by injecting WT Syk but not Mut Syk-transfected cells. The microvessel density was detected by staining with anti-vWF antibody (endothelial cell-specific marker) in these tumors (panels g–i). The nuclei were stained with DAPI (blue). The enhanced vWF expression observed in Mut Syk-transfected tumors indicated higher vascularization compared with control or WT Syk-transfected tumors.
higher grades (Fig. 6C, panels a–p). Taken together, our data indicated that the Syk and MelCAM act as a negative regulator of tumor progression and angiogenesis and can act as a potential therapeutic target in breast cancer.

DISCUSSION

Our study indicated that Syk and Lck play a crucial regulatory factor in hypoxia-induced tumor progression and angiogenesis. The data also demonstrated that Syk acts as a negative regulator of H/R-induced tumor progression, whereas Lck as a positive regulator, and H/R plays a pivotal role in regulating the cross-talk between Syk and Lck. Our previous study indicated that inhibition of Syk activity by Syk-specific antisense S-oligonucleotide resulted in enhanced uPA expression and cell motility in MCF-7 cells (23). In this study, we showed that H/R plays a crucial role in inactivation of Syk by inhibiting its phosphorylation, thereby resulting in its cytoplasmic translocation. Furthermore, the data indicated that the physical association between phosphorylated Syk and Lck in the nucleus resulted in inactivation of Lck. Upon H/R treatment
the phosphorylation of Syk was abrogated, which in turn resulted in the dissociation of Syk from Lck leading to activation of Lck, which ultimately targets downstream signaling events.

Previous studies indicated that MelCAM act as a tumor suppressor in breast carcinoma (32). This prompted us to investigate whether H/R regulates Syk/Lck-mediated MelCAM expression in MCF-7 cells. Our data indicated that H/R down-regulates the expression of MelCAM, which was restored upon transfection of cells with WT Syk or DN Lck. Our findings established a crucial molecular link between the two breast cancer-specific tumor suppressor molecules, Syk and MelCAM. The data clearly indicated that Syk enhances and Lck inhibits MelCAM expression, and thus both ultimately regulate breast cancer progression. Moreover, H/R-induced expressions of MMP-9 and VEGF were suppressed upon overexpression of Syk or inactivation of Lck. Thus Syk exerts its tumor-suppressive effect either through induction of MelCAM expression or suppression of tumor promoters like uPA, MMP-9, and VEGF. On the other hand, Lck activated upon H/R treatment, which enhances breast tumor progression through suppression of MelCAM expression and induction of MMP-9, uPA, and VEGF expression. Interestingly, the data also showed that blocking of MelCAM using its specific blocking peptide also resulted in induction of uPA as well as MMP-9 expression and activation. The data further indicated that Syk suppresses tumor progression through regulation of MelCAM-mediated mechanism.

Sp1 is a ubiquitously expressed transcription factor that recognizes GC-rich sequences present in the regulatory sequences of numerous housekeeping genes and those genes that are involved in growth regulation and cancer (34, 45). The transcription factor Sp1-response element is present in the promoter region of various genes, including uPA, MMP-9, and VEGF. Sp1 is one of the key transcription factors, and its activation occurs under hypoxic conditions in various cancer cells, including breast carcinoma (34, 36, 45). In our study, we have demonstrated that H/R-regulated cross-talk between Syk and Lck controls nuclear localization and DNA binding of Sp1. Furthermore, Syk negatively and Lck positively regulates Sp1 activation. Therefore, H/R through a Syk-Lck-mediated pathway controls Sp1 activation and regulates the expression of downstream molecules such as uPA, MMP-9, VEGF, and MelCAM that promotes tumor growth and angiogenesis (Fig. 7).

Our in vivo and clinical specimen analysis clearly supports our in vitro findings. In a xenograft study, tumors generated by injecting wild type Syk-transfected cells significantly suppressed the tumor growth, whereas mutant Syk-transfected cells showed enhanced tumor growth. The levels of uPA, MMP-9, VEGF, as well as microvessel density were significantly reduced, whereas the physical interaction between Syk and Lck and expression of MelCAM were significantly enhanced in wild type but not in Mut Syk-transfected tumors. In clinical specimen analysis, we observed that in higher grades of tumors (grade II and III), the level of HIF-1α is significantly higher, which indicated the enhanced hypoxic condition in tumor microenvironment. The low level of HIF-1α was observed in normal breast tissue and in lower grades of tumor. There was significant expression of Syk in normal breast tissues and lower grades of tumor. However, the physical association between Syk and Lck was observed in normal breast tissues. Moreover, the increased expression of Lck but not Syk was visualized in higher grades of tumors. Furthermore, the higher grades of tumors showed enhanced expression of uPA, MMP-9, and VEGF, and the increased microvessel density was also characterized in these tumors. In addition, the MelCAM expression was significantly diminished in tumors of higher grades that corroborated with both in vitro and in vivo findings.

In summary, to our knowledge, this is the first report that H/R differentially regulates the cross-talk between Lck and Syk, which ultimately control tumor progression. Previous reports have indicated that an increased hypoxic or consecutive hypoxia/reoxygenation condition in the tumor microenvironment plays a crucial role in determining the oncogenic potential of various cancers. Therefore, an in-depth understanding of the H/R-regulated signaling mechanism may be beneficial in designing a novel therapeutic approach for the treatment of cancer. We have shown that Syk, a tumor suppressor, positively regulates the expression of the breast cancer-specific tumor suppressor MelCAM. Interestingly, overexpression of Syk inhibits H/R-induced expression of several tumorigenic molecules, including uPA, MMP-9, and VEGF. Our results indicate that the mechanism demonstrated in the mouse model underlies human pathology, and a clear understanding of such mecha-
nisms may facilitate the development of novel therapeutic approaches to suppress hypoxia/reoxygenation-regulated Syk/Lck-mediated uPA, MMP-9, and VEGF expression, thereby controlling tumor growth and angiogenesis.

**REFERENCES**

Osteopontin Promotes Vascular Endothelial Growth Factor–Dependent Breast Tumor Growth and Angiogenesis via Autocrine and Paracrine Mechanisms

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Abstract

Angiogenesis is the hallmark of cancer, and development of aggressiveness of primary tumor depends on de novo angiogenesis. Here, using multiple in vitro and in vivo models, we report that osteopontin (OPN) triggers vascular endothelial growth factor (VEGF)–dependent tumor progression and angiogenesis by activating breast tumor kinase (Brk)/nuclear factor–inducing kinase/nuclear factor–κB (NF-κB)/activating transcription factor–4 (ATF-4) signaling cascades through autocrine and paracrine mechanisms in breast cancer system. Our results revealed that both exogenous and tumor-derived OPN play significant roles in VEGF-dependent tumor angiogenesis. Clinical specimen analysis showed that OPN and VEGF expressions correlate with levels of neuropilin-1, Brk, NF-κB, and ATF-4 in different grades of breast cancer. Consequently, OPN plays essential role in two key aspects of tumor progression: VEGF expression by tumor cells and VEGF-stimulated neovascularization. Thus, targeting OPN and its regulated signaling network could be a novel strategy to block tumor angiogenesis and may develop an effective therapeutic approach for the management of breast cancer. [Cancer Res 2008;68(1):152–61]

Introduction

Angiogenesis, the formation of new blood vessels from the existing one is a key step for tumor growth, survival, progression, and metastasis (1). A large number of proangiogenic factors and their cognate receptors have been identified (2–4). To date, the best characterized proangiogenic cytokine to turn on the "angiogenic switch" is vascular endothelial growth factor (VEGF; ref. 5). For over a decade, the role of VEGF in regulation of tumor angiogenesis has been under intense investigation (6). Previous results indicated that VEGF signaling represents a critical rate-limiting step in angiogenesis (7). Alternative exon splicing results in four isoforms of VEGF (VEGF121, VEGF165, VEGF189, and VEGF206), and among them, VEGF165 is the predominant one that plays a major role in tumor angiogenesis (7, 8). Recent data show that the functions of VEGF may not be limited to endothelial cells but also play important roles in survival, proliferation, and migration in tumor cells (9, 10).

Osteopontin (OPN), a secreted noncollagenous, sialic acid–rich, chemokine-like protein and also a member of small integrin-binding ligand N-linked glycoprotein family plays important role in determining the oncogenic potential of various cancers (11, 12). OPN exerts its prometastatic effects by regulating various cell signaling events through interaction with integrins and CD44 receptors that ultimately lead to tumor progression (13). Recent evidences indicated that OPN regulates tumor growth through induction of cyclooxygenase-2 and urokinase-type plasminogen activator expressions and activation of matrix metalloproteinases in various cancer cells (14–17). The role of OPN in various pathophysiologic conditions, particularly in cancer, suggested that the variation in glycosylation, phosphorylation, and sulfation generates the different functional forms that might alter its normal physiologic functions (12, 18). Previous studies have shown that tumor-derived OPN is soluble, and it exhibits close similarity with human milk OPN (19, 20). Earlier reports also suggested that OPN produced either from tumor or stromal cells has been shown to enhance the metastatic ability (21).

Breast tumor kinase (Brk/PTK6/Sik) is a nonreceptor tyrosine kinase and expressed in metastatic breast tumors but not in normal mammary tissue or benign lesions (22). It has been postulated that Brk promotes cell migration, and silencing of Brk resulted in inhibition of migration and proliferation in various cancer cells, including MDA-MB-231 (23, 24). Recent data revealed that activating transcription factor–4 (ATF-4/cAMP-responsive element binding protein-2) regulates VEGF expression (25, 26). Neuropilin-1 (NRP-1) was initially characterized to mediate the chemorepulsive activity of collapsin/semaphorins in neuronal cells (27, 28). NRP-1 is also expressed in endothelial cells and in various tumors, and it functions as isomorph-specific receptor for VEGF165 and plays an important role in tumor progression (29–32). Previous studies have shown that blocking or silencing of NRP-1 resulted in significant reduction of tumor progression (10). However, the role of Brk in regulation of ATF-4-dependent VEGF expression and interaction of VEGF with NRP-1 in response to OPN and how all of these ultimately control breast tumor angiogenesis is not well defined.

In this study, we provide both in vitro and in vivo experimental evidences that show the molecular mechanism by which OPN regulates Brk/nuclear factor–κB (NF-κB)/ATF-4 signaling cascades that ultimately augment the VEGF expression and tumor angiogenesis through autocrine and paracrine mechanisms. We have further substantiated the crucial roles of tumor-derived, endogenous OPN in tumor angiogenesis using short interfering RNA (siRNA) based approach in in vitro and in vivo models. Moreover, our clinical data revealed that the enhanced expressions of OPN and VEGF correlate with NRP-1, Brk, NF-κB and ATF-4 levels in breast carcinoma of higher grades. These data provide new insights into the mechanism underlying the regulation of VEGF expression by OPN in breast tumor angiogenesis and understanding these mechanisms may form the basis of new therapeutic regimens for the management of breast cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell culture and transfection. Human breast adenocarcinoma (MDA-MB-231) cell line was obtained from American Type Culture Collection. Human umbilical vein endothelial cell line EA.hy926 was a generous gift from Dr. Christopher Newton. The super repressor form of I human umbilical vein endothelial cell line EA.hy-926 was a generous gift (Dr. Dean Ballard, Vanderbilt University School of Medicine), wild-type (wt) and kinase negative (mut) nuclear factor–inducing kinase (NIK; K292A/ K340A) in pCDNA3 vector (Prof. David Wallach, Weizmann Institute of Science), VEGF-Luc construct (Dr. Debabrata Mukhopadhyay, Mayo Clinic College of Medicine), VEGF-GFP cDNA construct (Dr. Rakesh K. Jain, Massachusetts General Hospital), wt and kinase mutant (KM) Brk in pRCh CMV vector (Prof. Mark Crompton, Royal Holloway University of London), and human OPN cDNA in pCpDNA 3.1 (Dr. Ann Chambers, University of Western Ontario) were transfected in MDA-MB-231 cells using Lipofectamine 2000.

siRNA. Cells were transfected with OPN siRNA (OPNi) or NRP-1 siRNA (NRP-1i; Dharmacon) using liposome according to manufacturer’s instructions. The sequence targeted for OPN is 5'- GUU UCA CAG CCA CAA GGA CdtTdT/dTdT CAA AGU GUC GGU GUU CCU G 5' and its nonsilencing control is 5'- CAG UAC AAC GCA UCC GGC AdTdT/dTdT CUG AUG UUG CCA AGA CGU U 5'. The NRP-1 siRNA sequence is 5'- GAG AGG UCC UGA AUU CAG CdtTdT/dTdT CUC UCC AGG ACU UAC G 5' and its nonsilencing control is 5'- AGA GAU GUC GCC GGG CdtTdT/dTdT UCUC UAU CAU CAG CAG GCC A 5'. The Brk siRNA sequence is 5'-AAG GUC GCC AUU AAG GUG dAdTdT/dTdT UUC CAC CGG UAA UUC CAC U 5' and its nonsilencing control is 5'-CAC ACU UAG UUG CCA CAG CdtTdT/dTdT GUG UGA UCC AAC GGU GUC C 5'.

Purification of human OPN. The human OPN was purified from human breast milk as described previously with minor modifications and used throughout this study (15).

Coculture assay. The tumor-endothelial cell interaction was studied by coculture experiments using MDA-MB-231 and EA.hy926 cells. EA.hy926 cells were transfected with NRP-1i and cocultured with nontransfected or OPNi or NRP-1i transfected MDA-MB-231 cells. In separate experiments, cocultured cells were either treated with OPN alone or along with anti-VEGF antibody. The level of pDKR in cell lysates was detected by immunoprecipitation followed by Western blot.

Immunoprecipitation and Western blot. The OPN expression and immunoblot experiments were performed using their specific antibodies as described (14).

RNA extraction and reverse transcription–PCR. The RNA was isolated from MDA-MB-231 cells and used for reverse transcription–PCR (RT-PCR). The VEGF primers were used: sense, 5'-GCC TTC GAA ATG AAC ATC TTT-3' and antisense, 5'-AGA GAT CTG GTT CCC GAA AC-3'. The amplified cDNA fragments were resolved by agarose gel electrophoresis.

Reporter gene expression. MDA-MB-231 cells were transfected with VEGF-luciferase reporter construct, either alone or along with OPN cDNA or OPNi. Cell lysates were analyzed by Western blot using antiluciferase antibody and were visualized under confocal microscopy (Zeiss) and represented in the form of bar graph.

Brk kinase assay. Brk kinase assay was performed as described earlier (22). EMSA. MDA-MB-231 cells were either transfected with wt and KM Brk or wt and dn ATF-4 or I;Bo super repressor (sup. rep.) or Brk siRNA and then treated with OPN. The GFP expression was measured by fluorescense microscope (Nikon) and quantified by Image Pro Plus 6.0 Software (Nikon) and represented in the form of bar graph.

Immunofluorescence. To examine which OPN regulates ATF-4 expression and nuclear translocation, MDA-MB-231 cells were treated with 0.5 μmol/L OPN. Nuclear extracts were used for NF-κB and ATF-4–DNA binding.

Immunofluorescence. To examine whether OPN regulates ATF-4 expression and nuclear translocation, MDA-MB-231 cells were treated with 0.5 μmol/L OPN, and immunofluorescence was performed using anti–ATF-4 antibody.

Wound migration. The wound assay was performed using MDA-MB-231 and endothelial (EA.hy926) cells with typical cobblestone morphology as described (14).

Cell comigration assay. The endothelial-breast tumor cell interaction was shown by comigration assays. 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.

In vivo Matrigel-based angiogenesis assay. The in vivo Matrigel angiogenesis assay was performed as described (33). 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) and 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. The paraffin sections were immunostained with antihuman vWF and anti–phosphorylated KDR antibodies.

In vivo tumorigenicity, histopathology, and immunohistochemistry. The tumorigenicity and immunohistochemistry experiments were performed as described earlier (34, 35). Briefly, MDA-MB-231 (5 × 106) cells were injected orthotopically in the mammary fat pad of female athymic nude mice. OPN (0.5 μmol/L) or anti-VEGF (400 μg/kg of body weight per mice) blocking antibody was injected to the site of tumor twice a week until termination of the experiments. Mice were sacrificed and photographed, the tumors were dissected out and weighed, and tumor tissues were used for histopathologic 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. The levels of OPN, VEGF, and CA 15-3 were detected by Western blot using their specific antibodies. The OPN-induced VEGF–NRP-1 interaction in tumor samples was analyzed by immunoprecipitating tumor lysates with anti-VEGF antibody and immunoblotted with anti–NRP-1 antibody. The nuclear extracts of tumor tissues were prepared as described earlier and used for EMSA for detection of NF-κB–DNA and ATF-4–DNA binding (35).

Histologic and immunohistochemical analysis. Human breast tumor specimens analysis. Human breast tumor specimens of different grades and normal breast tissues were collected from local hospital with informed consent and flash frozen. The levels of OPN and VEGF were examined by Western blot. The tumor sections were stained with H&E. The vWF expression and microvessel density were detected by immunofluorescence using anti–vWF antibody. To examine the status of NF-κB localization and NRP-1, Brk, and ATF-4 expression, tumor tissue sections were analyzed by immunohistochemistry using their specific antibodies and were visualized under confocal microscopy (Zeiss).

Statistical analysis. The results of the experimental studies are expressed as mean ± SE. Statistical differences were analyzed by Student’s t test, P < 0.05 was regarded as significant. All these bands were analyzed densitometrically (Kodak Digital Science), and the fold changes were calculated. The in vivo angiogenesis and all other in vivo experimental data were quantified using the Image Pro Plus 6.0 Software (Nikon).

Results

OPN augments the expression of VEGF. To examine whether OPN regulates VEGF expression and control breast tumor angiogenesis, MDA-MB-231 cells were treated with exogenous OPN, and VEGF expression were analyzed by Western blot (Fig. 1A) and by RT-PCR (Fig. 1B). The results indicated that OPN induces VEGF expression both at protein and RNA levels. To examine the specificity of OPN on VEGF expression, cells were individually...
transfected with OPN cDNA or OPNi or treated with anti-OPN blocking antibody (R&D Systems). The data revealed that cells transfected with OPN cDNA enhanced, whereas transfected with OPNi or treated with anti-OPN antibody suppressed, the VEGF expression (Fig. 1C). To examine whether OPN regulates VEGF promoter activity, cells were individually transfected with OPN cDNA or OPNi along with VEGF-luciferase reporter construct. Cell lysates were analyzed by Western blot using anti-luciferase antibody. The data showed that silencing of OPN drastically suppressed, whereas overexpression significantly enhanced, VEGF promoter activity (Fig. 1D). These results suggested that exogenous, as well as tumor-derived, OPN augments VEGF expression both at transcriptional and protein levels.

**OPN stimulates Brk/NIK-dependent NF-κB activation.** Brk is considered as one of the key regulator during breast cancer progression and is expressed moderately in MDA-MB-231 cells (22–24). We sought to determine whether OPN regulates Brk phosphorylation in MDA-MB-231 cells. Accordingly, cells were treated with OPN for 0 to 24 min, and level of pBrk was detected by immunoprecipitation followed by Western blot. The results showed that OPN enhances Brk phosphorylation (Supplementary Fig. S1A). Pretreatment of cells with αvβ3 blocking antibody significantly suppressed, whereas inhibitor of c-Src (pp2) or phosphatidylinositol 3-kinase (PI3K; wortmannin) had no effect on OPN-induced Brk phosphorylation and Brk kinase activation (Fig. 2A). The data are analyzed densitometrically and represented in the form of bar graph (Fig. 2A). Cells treated with RGD but not with RGE peptide also suppressed OPN-induced Brk phosphorylation (data not shown). These data suggested that OPN regulates αvβ3-integrin-dependent Brk activation, which is independent of c-Src/PI3K signaling pathway. Earlier, we have shown that NIK plays a crucial role in OPN-induced signaling, which in turn regulates tumor growth (18). To examine whether Brk plays any role on OPN-induced NIK phosphorylation, cells were individually transfected with wt or KM Brk and then treated with OPN. The data revealed that wt Brk enhanced, whereas KM Brk inhibited, OPN-induced NIK phosphorylation, indicating that Brk plays a crucial role in this process (Fig. 2B). EMSA results revealed that wt Brk enhanced, whereas KM Brk suppressed, OPN-induced NF-κB–DNA binding (Supplementary Fig. S1B). Moreover, Brk siRNA inhibited OPN-induced NF-κB–DNA binding (data not shown). These results define a novel-signaling pathway by which OPN regulates Brk-dependent NIK-mediated NF-κB activation.

**OPN regulates crosstalk between NF-κB and ATF-4 leading to VEGF expression.** Previous reports have indicated that ATF-4 regulates VEGF expression in response to various stimuli (26). To examine whether OPN regulates ATF-4 cellular localization, cells were treated with OPN or transfected with OPNi, and localization of ATF-4 was detected by immunofluorescence (Supplementary Fig. S1C). The results showed that OPN enhanced ATF-4 nuclear localization. To check whether OPN regulates ATF-4–DNA binding and involvement of Brk in this process, EMSA was performed. The data revealed that wt Brk enhanced, whereas KM Brk inhibited, OPN-induced ATF-4–DNA binding. Interestingly, ATF-4–DNA binding was significantly enhanced, whereas wt Brk enhanced, whereas KM Brk inhibited, OPN-induced ATF-4–DNA binding (Fig. 2B). Brk siRNA also suppressed OPN-induced ATF-4–DNA binding (data not shown). To examine whether Brk plays any role on OPN-induced NF-κB–DNA binding, cells were transfected with wt or KM Brk along with Lb-Box sup. rep. or mut NIK inhibited, whereas wt NIK significantly enhanced, OPN-induced ATF-4–DNA binding (Fig. 2C). Cotransfection of cells with wt Brk along with Lb-Box sup. rep. or mut NIK unaffected OPN-induced ATF-4–DNA binding (Fig. 2C). Similar results were obtained

![Figure 1](https://example.com/image1)

**Figure 1.** Exogenous and tumor-derived OPN augments VEGF expression in MDA-MD-231 cells. **A,** serum-starved cells were incubated with 0 to 1 μmol/L OPN for 6 h at 37°C, and conditioned media and cell lysates were analyzed by Western blot using anti-VEGF antibody. Actin was used as loading control. **B,** cells were treated with 0 to 1 μmol/L OPN for 3 h, total RNA was isolated, and the level of VEGF mRNA was detected by semi quantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. **C,** cells were transfected with OPN cDNA, OPNi, or Coni (empty vectors) or treated with OPN blocking antibody. The expressions of VEGF and OPN in cell lysates were analyzed by Western blot using their specific antibodies. Human recombinant VEGF and purified OPN were used as controls. **D,** cells were transfected with VEGF-luciferase reporter construct alone or cotransfected with OPNi, Coni, or OPN cDNA, and luciferase expression was detected by Western blot using antiluciferase antibody. The data represent three experiments exhibiting similar results. Fold changes were calculated.
When cells were cotransfected with KM Brk along with wt NIK (Fig. 2C, lane 11). These data indicated that OPN up-regulates Brk-dependent ATF-4 activation. However, OPN also enhances Brk-dependent NIK/NF-κB-mediated ATF-4 activation. Transfection of cells with wt or dn ATF-4 had no effect on OPN-induced NF-κB–DNA binding, indicating that OPN-regulated crosstalk between NF-κB and ATF-4 is unidirectional toward ATF-4 (Supplementary Fig. S1D). To determine the role of Brk and ATF-4 on OPN-induced VEGF expression, cells were individually transfected with wt/KM Brk, Brk siRNA, or wt/dn ATF-4 and then treated with OPN. The results showed that cells transfected with wt Brk or wt ATF-4 increased VEGF expression upon OPN treatment, whereas transfection with KM Brk, Brk siRNA or dn ATF-4 showed drastic suppression of VEGF expression (Fig. 2D). Moreover, Is-B expression rep., significantly reduced OPN-induced VEGF expression (Fig. 2D). The effect of OPN on VEGF promoter activity was examined either by transfecting the cells with VEGF-GFP reporter construct alone or cotransfecting with wt and KM Brk, wt and dn ATF-4, Is-B expression rep., or Brk siRNA and then treated with OPN. The OPN-induced GFP expression was analyzed by fluorescence microscope (Nikon), quantified by Image Pro Plus 6.0 Software (Nikon), and represented in the form of bar graph (Supplementary Fig. S2). These data clearly suggested that Brk, ATF-4, and NF-κB play important role in OPN-induced VEGF expression both at transcriptional and protein level.

**VEGF and its receptor NRP-1 play crucial roles in OPN-regulated breast tumor cell motility through autocrine mechanism.** Recent data revealed that VEGF acts as survival, migratory and proliferative factor in breast carcinoma cells (10, 36). NRP-1 is the only VEGF-specific receptor that is expressed in MDA-MB-231 cells (29, 30). We therefore speculated that OPN might regulate the interaction between VEGF and NRP-1, which may control the motility of MDA-MB-231 cells. Accordingly, cells were treated with OPN alone or along with anti-VEGF or anti-NRP-1 blocking antibody, and lysates were immunoprecipitated with anti-VEGF antibody and immunoblotted with anti-NRP-1 antibody. The data suggested that OPN-induced VEGF interacts with NRP-1 in MDA-MB-231 cells through autocrine manner (Fig. 3A, I). To examine whether OPN has any effect on breast tumor cell motility and whether VEGF and NRP-1 are involved in this process, wound assay was performed using MDA-MB-231 cells with typical cobblestone morphology. Wounds with a constant diameter were made, and cells were treated with OPN alone or along with anti-VEGF or anti-NRP-1 blocking antibody. In separate experiments, cells were transfected with wt Brk or wt ATF-4 enhanced, whereas KM Brk or dn ATF-4 drastically suppressed, OPN-induced wound migration.

**Figure 2.** OPN regulates VEGF expression via Brk/NF-κB/ATF-4 signaling pathways in MDA-MB-231 cells. A, OPN induces Brk phosphorylation and kinase activity through αvβ3 integrin–dependent but c-Src and PI3K-independent pathway. Cells were treated with 0.5 μmol/L OPN for 8 min or pretreated with anti-αvβ3 antibody or wortmannin or pp2 for 2 h and then treated with OPN. The cell lysate was immunoprecipitated with anti-Brk antibody. Half of the immunoprecipitated samples were analyzed by Western blot using antiphosphotyrosine antibody. Remaining half of the samples was used for detecting Brk kinase activity by autophosphorylation study. Total Brk expression in the cells was used as loading control. The levels of p-Tyr-Brk were quantified by densitometric analysis and represented in the form of bar graph (*, P < 0.03). B, OPN-induced Brk-mediated NIK phosphorylation was determined by transfecting the cells with wt or KM Brk and then treated with 0.5 μmol/L OPN for 15 min. Cell lysates were analyzed by Western blot using anti–phosphorylated NIK antibody. C, Brk regulates OPN-dependent ATF-4–DNA binding and crosstalk between NF-κB and ATF-4. Cells were transfected with wt or KM Brk and treated with OPN. In separate experiments, cells were transfected with wt NIK or mut NIK or sup. rep. Is-B or wt Brk and mut NIK or KM Brk and wt NIK or wt Brk and Is-B sup. rep followed by treatment with OPN. ATF-4–DNA binding activity was analyzed by EMSA. Note that Brk up-regulates ATF-4 activity through NF-κB–dependent/independent pathway. D, roles of Brk and ATF-4 in OPN-induced VEGF expression. Cells were individually transfected with wt and KM Brk or wt and dn ATF-4 or Brk siRNA or Is-B sup. rep. and then treated with OPN. VEGF expressions (secreted and total) were analyzed by Western blot. All figures are representation of three experiments. Fold changes were calculated.
Interestingly, the enhanced wound migration caused by overexpression of wt Brk or wt ATF-4 was suppressed upon treatment of cells with anti-VEGF and anti–NRP-1 blocking antibodies (Fig. 3A, II). These results clearly indicated that OPN regulates VEGF-dependent wound migration through NRP-1–mediated autocrine mechanism.

OPN-induced VEGF controls KDR-dependent endothelial cell motility and in vivo angiogenesis through paracrine and juxtacrine mechanisms. Tumor-derived VEGF interacts with endothelial cell surface VEGF receptor-2 (also called KDR) and induces KDR phosphorylation that leads to tumor angiogenesis (37). Therefore, we sought to determine whether OPN-induced VEGF, which is expressed in MDA-MB-231 cells, can regulate KDR phosphorylation in endothelial (EA.hy-926) cell line through paracrine loop. Accordingly, MDA-MB-231 cells were treated with OPN and CM either alone or mixed with anti-VEGF blocking antibody incubated with endothelial cells. In separate experiments, CM of OPNi transfected MDA-MB-231 cells were incubated with endothelial cells. Cell lysates were analyzed by Western blot for KDR phosphorylation. The data revealed that OPN-induced VEGF enhanced KDR phosphorylation in endothelial cells through paracrine mechanism (Supplementary Fig. S3A). To investigate whether OPN-induced MDA-MB-231 cell-derived VEGF regulates endothelial cell wound motility, MDA-MB-231 cells were either

Figure 3. A, OPN enhances the VEGF–NRP-1 interaction in MDA-MB-231 cells. I, OPN-induced VEGF–NRP-1 interactions were examined by treating the cells with OPN for 6 h or pretreated with anti-VEGF or anti–NRP-1 blocking antibody and then treated with OPN. Cell lysates were immunoprecipitated with anti-VEGF antibody and analyzed by Western blot with anti–NRP-1 antibody. II, VEGF and NRP-1 play crucial roles in OPN-induced tumor cell motility. Wounds with a constant diameter were made, and cells were treated with OPN. In separate experiments, cells were transfected with wt and KM Brk or wt and dn ATF-4 and then treated with OPN in absence or presence of anti-VEGF or anti–NRP-1 blocking antibody, and wound assay was performed. In separate experiments, cells were individually transfected with OPNi, and wound assay was conducted. Cells were also transfected with NRP-1i and then treated with OPN. Wound photographs were taken after termination of the experiments (t = 12 h). B, OPN controls VEGF-dependent endothelial cell (EC) wound migration in a paracrine mechanism. Endothelial (EA-hy.926) cells were incubated with conditioned media obtained from OPN-treated or OPNi transfected MDA-MB-231 cells, and wound assay was performed and represented in the form of bar graph (*, P < 0.007; **, P < 0.01). In separate experiments, endothelial cells were also incubated with OPN-treated conditioned media mixed with anti-VEGF blocking antibody and used for wound assay. C, OPN enhances VEGF-dependent NRP-1/pKDR mediated tumor-endothelial cell interaction through juxtacrine mechanism. Endothelial cells were transfected with NRP-1i (EA NRP-1i) and cocultured with normal or OPNi or NRP-1i transfected MDA-MB-231 cells. OPN alone or along with anti-VEGF blocking antibody were added to the cocultured cells. Cell lysates were immunoprecipitated with anti–NRP-1 antibody and analyzed by Western blot with anti–phosphorylated KDR antibody. D, OPN enhances VEGF/NRP-1–mediated endothelial cell migration through juxtacrine manner. Endothelial cells were added on the upper portion of modified Boyden chamber. MDA-MB-231 cells seeded in lower chamber were either treated with OPN alone or along with anti-VEGF or anti–NRP-1 blocking antibody and used for direct comigration assays. In separate experiments, MDA-MB-231 cells were transfected with OPNi or NRP-1i and seeded in the lower chamber, and comigration assays were performed. Endothelial cells were migrated from upper chamber to the lower chamber and counted under inverted microscope (Nikon). Columns, means of three determinations; bars, SE (*, P < 0.001; **, P < 0.004).
treated or transfected under similar conditions as described above and endothelial cell wound migration was performed. The data are analyzed and represented in the form of bar graph (Fig. 3B). These results showed that OPN enhances VEGF-dependent endothelial cell motility in paracrine mechanism.

The interaction of OPN-induced VEGF with NRP-1 and KDR through juxtacrine mechanism is shown by coculture experiments using MDA-MB-231 and endothelial cells. Accordingly, endothelial cells were transfected with NRP-1i and cocolulturated with MDA-MB-231 cells in the presence of OPN alone or along with anti-VEGF antibody. In separate experiments, MDA-MB-231 cells were also transfected with OPNi or NRP-1i and used for coculture experiments. The cell lysates were immunoprecipitated with anti–NRP-1 antibody, followed by immunoblotting with anti–pKDR antibody. Our data revealed that OPN-induced VEGF acts as bridge between NRP-1, expressed in tumor cells, and KDR, expressed in endothelial cells, and regulates NRP-1–mediated KDR phosphorylation through juxtacrine mechanism (Fig. 3C). To verify whether pKDR is indeed expressed in endothelial but not in MDA-MB-231 cells, endothelial cells were incubated with CM of OPN-treated MDA-MB-231 cells. In separate experiments, MDA-MB-231 cells were also treated with OPN. The level of pKDR was analyzed by Western blot. The results showed that pKDR/KDR is detectable only in endothelial but not in MDA-MB-231 cells (Supplementary Fig. S3B). The endothelial-breast tumor cell interaction was further examined by direct comigration assay. The MDA-MB-231 cells either treated with exogenous OPN or transfected with OPNi or NRP-1i or treated with anti-VEGF or anti–NRP-1 antibody followed by treatment with OPN and used in the lower chamber whereas endothelial cells were used in the upper portion of modified Boyden chamber. The results showed that OPN stimulates VEGF/NRP-1–mediated endothelial cell migration through juxtacrine pathway (Fig. 3D). Taken together, these data showed that OPN-induced VEGF interacts with endothelial cell surface receptor, KDR, and regulates endothelial cell motility through paracrine and juxtacrine mechanisms.

OPN promotes VEGF-dependent in vivo angiogenesis. To assess whether OPN regulates VEGF-dependent in vivo angiogenesis, Matrigel plug assay was performed. The CM obtained from OPN-treated or OPNi-transfected MDA-MB-231 cells was mixed with Matrigel either alone or along with anti-VEGF blocking antibody and injected s.c. (ventral groin region) to the nude mice. Matrigel plugs were excised, and the sections were analyzed by
histopathologic and immunohistochemical studies using anti-vWF and anti-pKDR antibodies. The data revealed that OPN enhances VEGF-dependent in vivo angiogenesis through KDR phosphorylation (Fig. 4A). The neovascularization and KDR phosphorylation were quantified and represented graphically (Supplementary Fig. S4B). The data showed that OPN regulates VEGF-dependent in vivo angiogenesis through paracrine loop, whereas silencing tumor-derived OPN resulted in drastic reduction of tumor angiogenesis.

VEGF plays a crucial role in OPN-induced tumor growth and angiogenesis in orthotopic mouse model. Our in vitro and in vivo data prompted us to examine whether OPN regulates tumor growth in orthotopic nude mouse model and whether VEGF is involved in this process. Accordingly, MDA-MB-231 cells were transfected with OPN cDNA or treated with OPN and injected orthotopically into the mammary fat pad of female nude mice. In separate experiments, OPN or NRP-1i were mixed with transfection reagent and injected to the site of the tumor. Purified OPN and anti-VEGF blocking antibody were injected intratumorally twice a week up to completion of the experiments. After 6 weeks, mice were sacrificed and tumors were collected. The typical photographs of tumors are shown in Fig. 4B. The changes of tumor weight (% to control) were analyzed and represented as bar graph (Supplementary Fig. S4B). Blocking of VEGF with its antibody or NRP-1i significantly suppressed OPN-induced breast tumor growth in nude mice suggesting that VEGF and its receptor NRP-1 play crucial role in this process.

Tumor sections were also analyzed by histopathology. The results showed that tumors generated by OPN exhibit enhanced tumor cell infiltration, poor differentiation, higher nuclear polymorphism, mitotic count (12–16/hpf) and increased vessel formation in mammary fat pad of mice compared with control (Fig. 4D, top). Moreover, well-differentiated tumor structure, little infiltration and nuclear polymorphism, lesser mitotic features (2-5/hpf), and decreased vessel formation were observed in the tumors of the mice injected with anti-VEGF blocking antibody or NRP-1i along with OPN. To examine whether OPN regulates tumor angiogenesis, mice tumors were analyzed by immunohistochemistry using anti-vWF antibody (Fig. 4D, bottom; Supplementary Fig. S4C). The results indicated that OPN enhanced microvessel density, whereas blocking of VEGF or silencing NRP-1 suppressed OPN-induced tumor angiogenesis. Silencing tumor-derived OPN drastically suppressed vWF expression, suggesting the potential role of tumor-derived OPN in tumor angiogenesis. To examine

Figure 5. A and B, analysis of human breast tumor clinical specimens with different pathologic grades. Human breast tumor specimens (1–14) were collected from local hospital with informed consent. The tumor grading was performed by histopathologic analysis using a modified Scarff-Bloom-Richardson system. Tumor samples were analyzed by Western blot using anti-VEGF or anti-OPN antibody (A). The levels of OPN and VEGF were also detected in normal breast epithelial tissues. The expressions of OPN and VEGF were quantified and represented in the form of bar graph (B). C and D, histopathologic micrographs of normal and breast tumor tissues of different grades (specimen 5, grade II; specimens 8 and 9, grade III). The sections of normal and tumor tissues (specimens 5, 8, and 9) of different grades were analyzed by immunohistochemistry using anti-vWF antibody. Note that the enhanced microvessel density was observed in higher grades of tumor (specimens 8 and 9) and correlates with increased OPN and VEGF expressions. The vWF-positive areas indicate the microvessel density (pink arrows). Cellular localization of NF-kB and expression profiles of NRP-1, ATF-4, and Brk were analyzed by immunohistochemical studies using their specific antibodies. Note that NF-κB, ATF-4, and Brk are stained with FITC (green), whereas NRP-1 is stained with TRITC (red).
whether OPN enhances the interaction between VEGF and NRP-1 in vivo, the tumor lysates were immunoprecipitated with anti-VEGF antibody and analyzed by Western blot using anti-NRP-1 antibody. Treatment with anti-VEGF blocking antibody or silencing NRP-1 significantly suppressed OPN-induced interaction between VEGF and NRP-1 (data not shown) and further suggested that OPN augments the interaction between VEGF and NRP-1 that ultimately control the tumor growth and angiogenesis.

Tumor tissues were lysed, and the levels of OPN and VEGF were detected by Western blot (Fig. 4C). The level of CA15-3 from mice serum was analyzed as breast cancer marker. The levels of VEGF and OPN were also detected from mice serum (Fig. 4C). Significant induction of VEGF was observed in tumor generated by OPN. Tumor samples were also analyzed by EMSA (Fig. 4C) and immunofluorescence (Supplementary Fig. S4D), and the data suggested that OPN augments NF-κB and ATF-4–DNA binding and nuclear localization in these tumors. Moreover, silencing tumor-derived OPN significantly suppressed NF-κB and ATF-4–DNA binding and nuclear localization. These data suggested that both tumor-derived and exogeneous OPN regulates NF-κB and ATF-4–dependent VEGF/NRP-1–mediated breast tumor progression and angiogenesis.

**Expressions of OPN and VEGF and their implications in human breast tumor angiogenesis.** The in vitro and in vivo data further prompted us to extend these studies in human clinical breast tumor specimens. Human solid breast tumor specimens and regional lymph nodes were collected with informed consent from a local hospital. Tumor grading was performed with the help of expert oncopathologist using modified Scarff-Bloom-Richardson system, and regional lymph node metastasis was analyzed by histopathology (Supplementary Table S1). The expressions of OPN and VEGF (Fig. 5A) and the interaction between VEGF and NRP-1 (data not shown) were studied in tumor specimens of different grades. The levels of OPN and VEGF were quantified and represented as bar graph (Fig. 5B). The data revealed that there were significantly higher levels of OPN and VEGF expressions (Fig. 5A; 8 of 14 tumor specimens and one normal sample), and these data correlated with tumors of higher grades. The data also indicated that the interaction between VEGF and NRP-1 was also enhanced significantly with tumors of higher grades (data not shown). The immunohistochemical staining with anti-vWF antibody showed the enhanced angiogenic vessel formation in breast tumor specimens of higher grades (Fig. 5C).

**Localization and expression of NF-κB, ATF-4, Brk, and NRP-1 in breast tumors specimens.** To examine the activation status of NF-κB in different grades of tumor specimens, tissue sections were analyzed by immunohistochemistry using anti-NF-κB, p65 antibody. The results indicated that the NF-κB nuclear localization was significantly enhanced in higher grades (grades II and III) of tumors compared with the tumor of lower grades or normal breast tissues (Fig. 5C). The status of expressions of NRP-1, ATF-4, and Brk in different grades of tumors was also analyzed by immunohistochemistry using their specific antibodies. These results indicated that the higher expressions of NRP-1, ATF-4, and Brk correlate with higher grades of tumors (Fig. 5D). The ATF-4 nuclear
localization was also significantly enhanced in higher grades (grades II and III) of tumors and correlated with NF-κB (Fig. 5C and D). The status of OPN and VEGF expressions and its correlation with NF-κB and ATF-4 activation and metastatic potential in various grades of tumors are summarized in Supplementary Table S1. These results showed that tumors with higher grades exhibit significantly higher levels of ATF-4 and NF-κB nuclear localization, and these data correlated with enhanced OPN, Brk, and VEGF expression both in vitro and in vivo systems, which further corroborated with tumor growth and angiogenesis in breast carcinoma.

**Discussion**

We have for the first time shown the molecular mechanism of OPN-induced VEGF expression and its potential role in regulating in vitro cell motility which ultimately modulates in vivo tumor growth and angiogenesis in breast cancer model. Our data also revealed that OPN might be necessary for maximal induction of neovascularization by inducing VEGF expression through activation of Brk/NF-κB/ATF-4 pathways. Several lines of evidences support this conclusion. First, OPN regulates VEGF promoter activity and expression in human breast carcinoma cell lines. Second, OPN stimulates Brk phosphorylation and Brk/NIK-mediated NF-κB–dependent/independent ATF-4 activation and crosstalk between NF-κB and ATF-4 that leads to VEGF expression. Third, in autocrine pathway, OPN-induced VEGF interacts with tumor cell surface receptor NRP-1 and stimulates VEGF–NRP-1–mediated tumor cell motility. Fourth, in paracrine loop, OPN-induced VEGF up-regulates KDR phosphorylation, resulting in enhanced endothelial cell motility and in vivo angiogenesis in nude mice. Fifth, in juxtacrine manner, OPN-induced VEGF regulates tumor-endothelial cell interaction through binding with NRP-1 in breast cancer and KDR in endothelial cells. Sixth, OPN-induced VEGF leads to increased breast tumor growth and angiogenesis in nude mice model and blocking of tumor-derived VEGF or silencing of tumor-derived OPN and NRP-1 significantly suppressed breast tumor progression and angiogenesis. Seventh, analysis of human solid breast tumor specimens showed the significant correlation between the OPN, Brk, NRP-1, and VEGF expressions NF-κB and ATF-4 activations with different pathologic grades of tumors. All these data supported by the fact that OPN and its downstream signaling molecules might be used as therapeutic target for the treatment of breast cancer.

Previous reports indicated that Brk plays important role during breast tumor progression (22, 23). We provide evidence, at least in part, that Brk plays crucial role in OPN-induced NIK-mediated NF-κB activation. Our data also showed that OPN regulates Brk-dependent ATF-4 activation and a crosstalk between NF-κB and ATF-4 that controls VEGF-dependent tumor angiogenesis. NRP-1 acts as key receptor for VEGF-mediated signaling in MDA-MB-231 cells (36, 38). Recent evidence indicated that NRP-1 could independently regulate VEGF-induced cell signaling (39). We sought to determine whether OPN plays any role in regulating the interaction between VEGF and NRP-1 in breast cancer cells. Our results showed that enhanced interaction of VEGF and NRP-1 in response to OPN control the breast cancer cell migration and tumor growth through paracrine mechanism. We have also shown that OPN-induced tumor-derived VEGF regulates KDR/NRP-1–mediated endothelial cell motility and tumor angiogenesis through paracrine and juxtacrine mechanisms.

The role of OPN in the regulation of tumor angiogenesis is under intense investigation (12, 40). Earlier Senger et al. reported that VEGF induces OPN expression in endothelial cells, which in turn regulates angiogenesis (41). However we have shown that OPN regulates VEGF expression, which controls tumor angiogenesis through autocrine, paracrine, and juxtacrine pathways. These data suggested that there could be a positive feedback loop between OPN and VEGF which might play an important role in tumor progression and angiogenesis. It has also been reported that coexpression of VEGF and OPN correlates with angiogenesis in patients with stage I lung adenocarcinoma (42). Earlier reports have shown that cooperation of OPN and VEGF may facilitate tumor progression and angiogenesis in carcinomas derived from other organs (43). Recently, we have showed that OPN regulates cyclooxygenase-2–dependent prostaglandin E2 production, which in turn regulates prostate cancer angiogenesis (14). Although earlier reports indicated that OPN acts as an angiogenic factor but the molecular mechanism by which OPN regulates VEGF-dependent tumor angiogenesis in breast cancer is not well defined. Our study for the first time showed the in-depth signaling pathways by which OPN induces cell migration and angiogenesis through induction of VEGF expression in breast cancer. Moreover, these studies correlate with the data obtained from human breast tumors specimens in Indian scenario.

Suppression of tumor progression by targeting the angiogenic switch is one of the greatest challenges for cancer biologists and medical oncologists. Recent reports on anticancer therapy indicated that suppression of tumor angiogenesis by administration of antiangiogenic molecules is one of the most potent therapeutic approaches for the treatment of cancer (44). However, it is also known that some side effect of these antiangiogenic drugs are considered as the main problem of this therapy (45). Faced with the barrier to the traditional antiangiogenic strategies, we tested an in vivo RNA interference–based approach by targeting OPN and NRP-1. In recent time, in vivo siRNA-based approach has been proposed for treatment of several diseases including cancer (46). Therefore, appropriately designed OPN siRNA, injected intratumorally, exhibit significant suppression of tumor angiogenesis and may help in the surgical excision of tumor and provide considerable therapeutic value for the treatment of cancer.

Finally, we have shown for the first time that OPN induced the potent angiogenic molecule, VEGF expression. OPN regulates tumor growth and angiogenesis by induction of VEGF expression through activation of Brk/NF-κB/ATF-4 pathways. These data showed that OPN-induced VEGF regulates tumor angiogenesis through autocrine, paracrine, and juxtacrine mechanisms (Fig. 6). Our results further warrant that the mechanism shown in the mouse model underlies the human pathology, and a clear understanding of such mechanism(s) may facilitate the development of novel therapeutic approaches to suppress OPN-regulated NF-κB/ATF-4–dependent VEGF expression, thereby controlling tumor growth and angiogenesis.

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References

Curcumin suppresses breast tumor angiogenesis by abrogating osteopontin-induced VEGF expression

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Abstract. The development and progression of malignant tumors depends on the formation of new blood vessels inside the tumor. This phenomenon is termed tumor angiogenesis. Angiogenesis is one of the fundamental processes that occur during cancer progression, and depends on the expression and activation of various angiogenic molecules, cytokines, growth factors, kinases and transcription factors. We recently demonstrated that the chemokine-like ECM-associated protein osteopontin (OPN) turns on the angiogenic switch by upregulating expression of vascular endothelial growth factor (VEGF) in a human breast cancer model. Furthermore, we hypothesize that targeting OPN-induced VEGF might serve as a potential therapeutic approach for the treatment of breast cancer. In this study, we demonstrate that curcumin (diferuloylmethane) abrogates OPN-induced VEGF expression and curbs OPN-induced VEGF-dependent breast tumor angiogenesis in vivo. We also explore the fact that curcumin in combination with anti-VEGF or anti-neuropilin (NRP)-1 antibody exhibits enhanced anti-angiogenic activity compared to curcumin alone. Our results indicate that curcumin suppresses OPN-induced VEGF expression and tumor angiogenesis, and suggest that this study may aid in the development of a curcumin-based OPN-targeted therapeutic approach to the control of breast tumor angiogenesis.

Introduction

Breast cancer is considered to be one of the most common cancer threats worldwide. According to the World Health Organization (WHO), it is the most deadly cancer in females, with more than 500,000 deaths attributed to it globally each year. To date, the major cause of breast cancer is not clearly understood. However, experimental evidence has revealed that the expression profiles of certain oncogenic molecules or biomarkers are significantly associated with breast cancer progression, and have shown great promise in breast cancer therapy (1,2). Osteopontin (OPN), a chemokine-like ECM-associated small integrin binding ligand N-linked glycoprotein (SIBLING), has recently been identified as one of the major markers of breast cancer progression (3,4). Elevated expression of OPN at tumor sites as well as in the serum of breast cancer patients has signalled the prognostic importance of this protein in breast cancer (5,6). Moreover, the targeting of OPN and its downstream signaling pathways has shown great promise in the therapeuticities of various cancers, breast cancer included (7). The role of OPN in breast cancer progression is therefore being intensely investigated.

Angiogenesis, or the formation of new blood vessels from existing ones, is a key step in tumor growth, survival, progression and metastasis (8). Tumor angiogenesis is thought to result from the secretion of "angiogenetic factors" by tumor cells. These include growth factors, cytokines and a number of small molecules (9,10). Of these, vascular endothelial growth factor (VEGF) has been recognized as the most important (11). Highly malignant tumors are characterized by enhanced vascularization, which is further correlated with elevated VEGF expression (12). Previous reports suggest that tumor-derived VEGF interacts with tumor or endothelial cell surface receptors via autocrine or paracrine mechanisms and promotes tumor angiogenesis (13-15). We recently demonstrated that OPN augments VEGF expression and promotes VEGF-dependent breast tumor angiogenesis (16). Therefore, we can hypothesize that targeting OPN-induced VEGF might serve as a potential therapeutic approach for the treatment of breast cancer.

Curcumin (diferuloylmethane) is a polyphenol derived from the rhizomes of Curcuma Longa, traditionally used as an anti-inflammatory compound, and appears to be useful in the prevention and treatment of various cancers (17-19). Curcumin has exhibited a significant inhibitory effect on several malignant cancers, including breast cancer (20). It has also been reported that curcumin downregulates the activation of NF-κB and suppresses tumor growth in various cancer models (21-23). We have previously reported that curcumin suppressed OPN-induced MMP-2 activation and tumor growth in a murine melanoma model (24). However, the role of curcumin in the regulation of OPN-induced VEGF-dependent breast tumor angiogenesis is not well defined. In this study, we demonstrate that curcumin abrogates OPN-induced VEGF expression and suppresses VEGF-dependent breast tumor angiogenesis.

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Materials and methods

Cell culture. The human breast adenocarcinoma cell line MDA-MB-231 was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in L-15 media supplemented with 10% FBS, 100 μg/ml streptomycin and 100 units/ml penicillin. Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Walkersville, MD, USA) and cultured in the EBM bullet kit (Lonza) according to the manufacturer’s instructions.

Western blot analysis and EMSA. Western blot analysis and EMSA were performed as previously described (16,25). Briefly, the cells were treated with 0.5 μM OPN, or pretreated with various doses of curcumin (0-50 μM) and then treated with OPN. Cell lysates were used to detect VEGF expression by Western blot analysis, and the nuclear extracts were used for NF-κB and ATF-4-DNA binding by EMSA.

Immunofluorescence study. Immunofluorescence staining was performed using a standard protocol as described previously (26). Briefly, the cells were treated with 0.5 μM OPN, or pretreated with 20 μM curcumin and then treated with OPN. Cells were fixed in 2% paraformaldehyde by incubation at 4°C for 15 min. After quenching with 0.1% glycine, cells were permeabilized with 0.1% Triton X-100, washed three times with PBS for 5 min and then incubated in 2% BSA in PBS (pH 7.4) for 1 h at room temperature (RT) to block non-specific binding. Subsequently, cells were incubated with specific primary antibody in 0.2% BSA for 2 h at RT, followed by incubation with secondary antibody for 1 h at RT. Nuclei were stained with PI. Cells were washed and mounted in mounting media and analyzed using a confocal microscope (Zeiss).

Wound migration assay. The motility of MDA-MB-231 cells was determined by wound migration assay as previously described (16,27). Cells were treated with 0.5 μM OPN, or pretreated with 0-20 μM curcumin followed by treatment with OPN. In separate experiments, cells were pretreated with 10 μM curcumin along with anti-VEGF or anti-neuropilin (NRP)-1 antibody and then treated with OPN. After 12 h, wound photographs were captured using a phase contrast microscope (Nikon).

Co-migration assay. Tumor-endothelial cell interactions were carried out using a co-migration assay with a modified Boyden chamber. MDA-MB-231 cells were treated with 0.5 μM OPN alone and used in the lower chamber. Endothelial cells (HUVEC) were seeded on the upper chamber. In separate experiments, MDA-MB-231 cells were pretreated with curcumin and then treated with OPN alone or with anti-VEGF or anti-NRP-1 antibody in the lower chamber, and co-migration experiments were performed. The migrated endothelial cells were stained with Giemsa, photographed and then counted in three high-power fields under an inverted microscope (Nikon). They were then analyzed statistically, and the resulting data was represented by a bar graph.

In vivo Matrigel-based angiogenesis assay. The in vivo Matrigel angiogenesis assay was performed as previously described (16). Briefly, Matrigel (0.5 ml) was subcutaneously injected into the ventral groin region of female athymic NMRI (mu/mu) mice. In separate experiments, the conditioned medium (CM) of untreated or OPN-treated MDA-MB-231 cells was mixed with Matrigel and injected into the mice. In other experiments, OPN-treated CM was mixed with curcumin (20 μM) in Matrigel and then injected into the mice. After 3 weeks, Matrigel plugs were excised and processed for histopathology and immunohistochemistry using specific antibodies.

Immunohistochemical study. Immunohistochemical analysis of paraffin-embedded sections was performed using standard procedure as previously described (16,27). After deparaffinization in xylene, the sections were re-hydrated using descending grades of ethanol (100, 95 and 70%), washed thrice in PBS, then used for antigen retrieval. After quenching with 100 mM glycine, tissue sections were permeabilized with 0.1% Triton X-100, washed twice with PBS, then blocked with 2% bovine serum albumin for 1 h. Finally, the sections were incubated with specific primary antibodies overnight at 4°C. Subsequently, the slides were washed in PBS and incubated with specific secondary antibodies for 2 h. The sections were mounted and analyzed using a confocal microscope (Zeiss).

Statistical analysis. Bands were analyzed densitometrically (Kodak Digital Science) and fold changes were calculated. The wound migration and co-migration assays were analyzed statistically, and the data were represented by means of a bar graph. Statistical differences were determined by the paired Student’s t-test. Differences were considered significant at P-values <0.05.

Results

Curcumin abrogates OPN-induced VEGF expression. We previously reported that curcumin suppressed OPN-induced MMP-2 activation in a murine melanoma model (24). More recently, we demonstrated that OPN augments VEGF expression in human breast cancer cells (16). To examine the effect of curcumin on OPN-induced VEGF expression, serum-starved MDA-MB-231 cells were either treated with 0.5 μM OPN or were pretreated with curcumin and then treated with OPN. The results indicate that curcumin significantly abrogates OPN-induced VEGF expression in these cells, with maximum inhibition observed using 20 μM of curcumin (Fig. 1A).

Effects of curcumin on OPN-induced NF-κB and ATF-4-DNA binding. We recently showed that OPN promotes VEGF expression via activation of NF-κB in MDA-MB-231 cells (16). However, it has been reported that curcumin suppresses NF-κB activation in various cancer cells (21-23). Therefore, to examine whether curcumin inhibits OPN-induced NF-κB-DNA binding, cells were pretreated with curcumin and then treated with OPN, and EMSA was performed. The data showed that curcumin significantly suppressed OPN-induced NF-κB-DNA binding (Fig. 1B).

The ATF-4 response element has been reported to be in the promoter region of VEGF (28,29). ATF-4 plays a crucial role in OPN-induced VEGF expression (16). To confirm the effect of curcumin on OPN-induced ATF-4-DNA binding
and nuclear localization, cells were pretreated with curcumin and then treated with OPN. Analysis of ATF-4-DNA binding and nuclear localization was performed by EMSA and immunofluorescence, respectively. The data indicate that curcumin inhibits OPN-induced ATF-4-DNA binding as well as nuclear localization (Fig. 1C and D). This suggests that curcumin curbs OPN-induced NF-κB and ATF-4 activation, and thereby alters OPN-induced VEGF expression in human breast cancer cells.

Curcumin inhibits OPN-induced VEGF-dependent breast tumor cell motility. To ascertain whether curcumin has any effect on OPN-induced breast tumor cell motility, a wound migration assay was performed using MDA-MB-231 cells. Cells were treated with OPN, or pretreated with curcumin alone or in combination with anti-VEGF or anti-NRP-1 antibody and then treated with OPN. Wound photographs were captured and analyzed, and the data represented by a bar graph (Fig. 2A and B). The data indicate that curcumin significantly inhibits OPN-induced tumor cell motility. A low concentration of curcumin in combination with anti-VEGF or anti-NRP-1 antibody also inhibited OPN-induced cell motility; however, the percent of inhibition was higher in this condition compared to curcumin alone (Fig. 2A and B). These data suggest that curcumin suppresses OPN-induced VEGF/NRP-1-dependent breast tumor cell motility.
Curcumin suppresses OPN-induced VEGF-dependent tumor-endothelial interaction. To examine the effect of curcumin on the suppression of OPN-induced tumor-endothelial cell interaction, a co-migration assay was performed using MDA-MB-231 cells and HUVEC in a modified Boyden chamber. After termination of the experiments, HUVEC which had migrated from the upper to the lower chamber were stained with Giemsa, photographed, and counted in three high-power fields. The resulting data were represented by a bar graph (Fig. 3A and B). The data indicate that OPN-induced endothelial cell migration towards tumor cells was suppressed by curcumin (Fig. 3A and B). More importantly, curcumin with anti-VEGF or anti-NRP-1 antibody suppressed OPN-induced migration of endothelial cells towards tumor cells, further suggesting that OPN-induced tumor-derived VEGF promotes tumor-endothelial interaction and that curcumin suppresses this effect.

Inhibition of OPN-induced in vivo Matrigel plug angiogenesis by curcumin. To determine the role of curcumin in the suppression of OPN-induced in vivo angiogenesis, a Matrigel plug angiogenesis assay was performed in nude mice. Conditioned medium (CM) collected from untreated or OPN-treated MDA-MB-231 cells was mixed with Matrigel and injected subcutaneously into the ventral groin region of female nude mice as described previously (16). In separate experiments, curcumin was mixed with the CM of OPN-treated MDA-MB-231 cells along with Matrigel and then injected into mice. After 21 days, the mice were sacrificed and the Matrigel plugs photographed (Fig. 4, panel I). The data indicate that curcumin radically suppressed OPN-induced in vivo angiogenesis as compared to the controls (Fig. 4). The excised Matrigel plugs were analyzed histopathologically (panels II and III; magnification, x10 and x60) and by immunofluorescence using anti-vWF (Von Willebrand Factor, an endothelial cell marker) and anti-pKDR antibodies. The data indicate that curcumin significantly suppressed OPN-induced in vivo angiogenesis (panel IV) and KDR phosphorylation. Taken together, the data demonstrate that curcumin curtails OPN-induced in vivo angiogenesis by inhibiting KDR phosphorylation.

Discussion

In the current study, we have demonstrated that curcumin inhibits OPN-induced VEGF expression, which in turn suppresses tumor and endothelial cell motility and in vivo angiogenesis. Previously, we reported that NF-κB and ATF-4 play a crucial role in OPN-induced VEGF expression (16). In this study, we found that curcumin suppresses OPN-derived VEGF-α and ATF-4-DNA binding and ATF-4 nuclear localization. Thus, curcumin abrogates OPN-induced VEGF expression by suppressing the binding activities of NF-κB and ATF-4. Our data also indicate that OPN-induced VEGF-dependent in vivo angiogenesis was downregulated by curcumin. This clearly suggests that curcumin acts as a potent anti-angiogenic agent in regulating OPN-induced tumor angiogenesis.

The role of OPN in tumor angiogenesis is being intensely investigated (30,31). Previously, OPN was considered to be a metastasis-associated gene (32). Recently, however, its role in tumor angiogenesis has been demonstrated (16,27,30). Angiogenesis-based therapy has emerged as one of the major therapeutic approaches to cancer (33). Thus, identifying novel therapeutic approaches to targeting the angiogenic pathways could prove to be promising for cancer therapy. We previously showed that OPN acts as a potent angiogenic molecule by regulating VEGF expression in a breast cancer model (16). Consequently, targeting OPN-regulated angiogenic pathways could be a promising therapeutic strategy for the treatment of breast cancer.
Figure 4. Curcumin abrogates OPN-induced in vivo tumor angiogenesis. Typical photographs of in vivo Matrigel plug angiogenesis in nude mice. Angiogenic blood vessels are indicated by arrows. Note the significant reduction in blood vessel formation in the plug treated with curcumin and OPN compared with OPN alone (panel I). Matrigel plugs were analyzed histopathologically by H&E staining, and photographs were captured at a magnification of x10 and x60 (panels II and III). Matrigel plug sections were immunohistochemically analyzed using anti-vWF and anti-pKDR antibodies (panel IV). vWF was stained with Cy3 (red) and p-KDR with FITC (green). Nuclei were stained with DAPI (blue).

Figure 5. Schematic representation of the curcumin suppression of OPN-induced VEGF expression leading to the inhibition of tumor growth and angiogenesis.
Curcumin has long been used as a potent anti-inflammatory agent. Recently, though, this molecule has been identified as having anti-cancer properties (17-19). Curcumin also inhibits PMA-induced VEGF mRNA expression and suppresses angiogenesis (19). Previously, we found that it suppressed OPN-regulated murine melanoma growth by inhibiting MMP-2 activation (25). However, the role of curcumin in OPN-induced breast tumor angiogenesis is not well defined. In this study, we demonstrated that curcumin abrogates OPN-induced VEGF expression, thus inhibiting tumor angiogenesis. Moreover, anti-VEGF or anti-NRP-1 antibody alone with curcumin resulted in higher anti-angiogenic activity than curcumin alone. We therefore hypothesize that curcumin along with anti-VEGF or anti-NRP-1 antibody could be a novel approach for the treatment of cancer.

In this study, we have gone some way in demonstrating for the first time that curcumin inhibits OPN-induced NF-κB/ATF4-dependent VEGF expression, ultimately suppressing breast tumor angiogenesis (Fig. 5). As OPN is considered to be a major molecule in the control of breast cancer progression, targeting the OPN-regulated signaling pathway by curcumin could be an emerging approach to the treatment of the disease. This approach may help block the OPN-regulated VEGF-dependent angiogenic switch, and could also contribute to the development of a novel strategy for the treatment of breast cancer.

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References