

The Crucial Role of Cyclooxygenase-2 in Osteopontin-Induced Protein Kinase C α /c-Src/I κ B Kinase α / 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 α /nuclear factor- κ B-dependent signaling cascades that induces COX-2 expression, which in turn regulates the prostaglandin E₂ 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 mice PC-3 xenograft exhibits higher tumor load, increased tumor cell infiltration, nuclear polymorphism, and neovascularization. Interestingly, use of celecoxib or anti-EP2 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 PGE₂ has been detected, which plays an important role in cancer progression (7). PGE₂ 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 PGE₂-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 NH₂-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 α (PKC α), respectively. Previous studies have indicated that c-Src and PKC α play crucial roles in COX-2 expression and COX-2-dependent tumor progression (22–24). The transcription factor nuclear factor- κ B (NF- κ B) 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- κ B through phosphorylation and degradation of I κ B α (26). We have recently

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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 PKC α /c-Src-dependent I κ B kinase (IKK)-mediated NF- κ B 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 PKC α /c-Src/IKK/NF- κ B 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-PKC α , anti-NIK, anti-p-NIK(Thr⁵⁵⁹), anti-IKK α / β , anti-NF- κ B, p65, anti-MMP-2, anti-c-Src, anti-EP2, and anti-actin, mouse monoclonal anti-phosphotyrosine, goat polyclonal anti-phospho-PKC α (Ser⁶⁵⁷) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-p-IKK α / β (Ser¹⁸⁰/Ser¹⁸¹) 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 [γ -³²P]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 a 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 μ g/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. Hiroyasu Inoue (Osaka, Japan; ref. 28), respectively. The wild-type NIK (wt pcDNA NIK) and kinase-negative NIK (mut pc DNA 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 IKK α (wt IKK α and dn IKK α) and IKK β (wt IKK β and dn IKK β) in

PKC-related kinase were kind gifts from Prof. D.V. Goeddel (Tularik, Inc., San Francisco, CA). The super-repressor form of I κ B α 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 (Invitrogen, 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 PKC α 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'-GUUUCACAGCCACAAGGACdTdT/dTdTCAAAGUGUCGGUGUUCUG-5', and the nonsilencing control is 5'-CAGUACAACGCAUCUGGCAdTdT/dTdTGUCAUGUUGCGUAGACCGU-5'.

Western blot and immunoprecipitation. The level of COX-2, PKC α c-Src, osteopontin, IKK α / β , actin expressions and phosphorylations of IKK α / β and NF- κ B, p65 in transfected or treated PC-3 cells were analyzed by Western blot using its specific antibody (20). The tyrosine phosphorylation of IKK α / β and the interaction between PKC α and c-Src 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 μ g of total RNA, with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and primers COX-2 forward (5'-TTCAAATGAGATTGTGGGAAAATTGCT-3') and COX-2 reverse (5'-AGATCATCTCTGCCCTGAGTATCTT-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 IKK 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 μ mol/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 Biotrack 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 μ mol/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-PKC α and anti-c-Src antibodies. Autophosphorylation of PKC α and c-Src were measured by incubating the immunoprecipitated samples with 40 μ Ci of [γ -³²P]ATP in 40 μ L 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 pNPP, 300 μ mol/L Na₃VO₄, 1 mmol/L benzamide, 2 μ mol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 μ g/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 (2×10^5 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 (5×10^5) 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 μ 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 μ 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

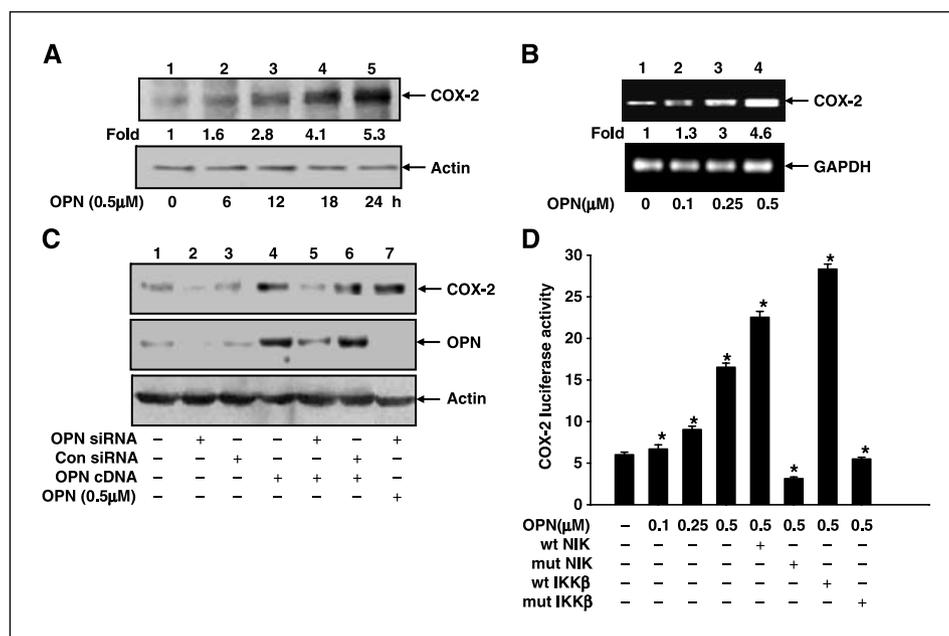


Figure 1. Osteopontin (OPN) induces COX-2 promoter activity and expression in PC-3 cells. *A*, serum-starved PC-3 cells were treated with 0.5 μ mol/L osteopontin for 0 to 24 hours. The level of COX-2 from whole-cell lysates was analyzed by Western blot. Actin was used as loading control. *B*, cells were treated with osteopontin (0–0.5 μ 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 μ mol/L osteopontin. COX-2 was analyzed by Western blot. The level of osteopontin is also detected by Western blot. Actin was used as loading control. *D*, osteopontin enhances NIK/IKK-mediated COX-2 promoter activity. Cells were transfected with luciferase reporter construct (COX-2-Luc) and then treated with 0 to 0.5 μ mol/L osteopontin or cotransfected with wt and mut NIK or wt and dn IKK β and then treated with 0.5 μ mol/L osteopontin. Cell lysates were used to measure the luciferase activity. The values were normalized to *Renilla* luciferase activity. The fold changes were calculated. *Columns*, mean of triplicate determinations; *bars*, SE.

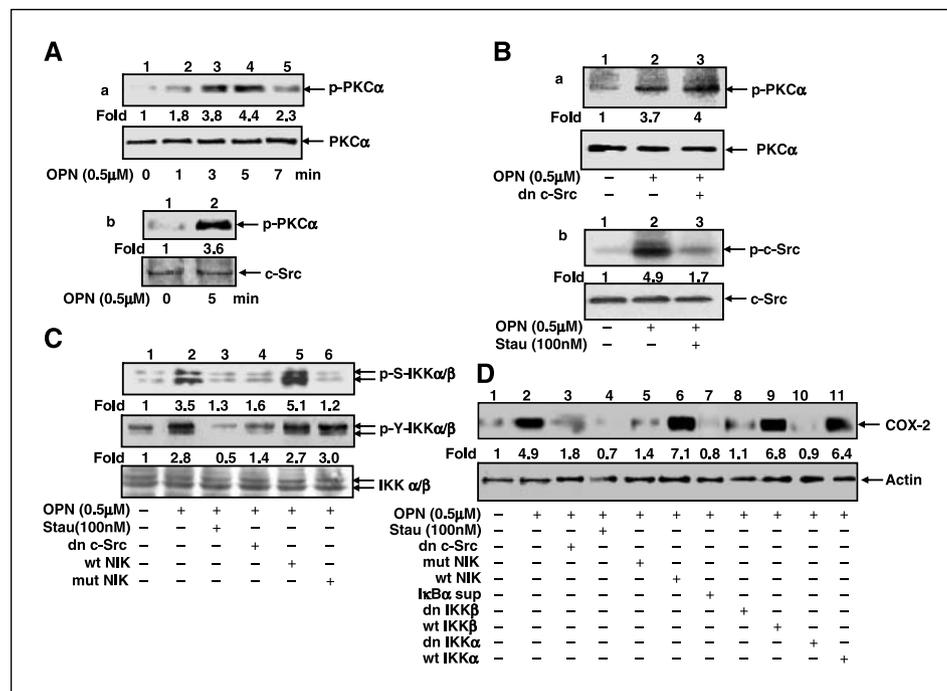


Figure 2. Osteopontin (OPN) induces PKC α activation, PKC α -mediated c-Src and IKK phosphorylation, and COX-2 expression. **A**, PC-3 cells were treated with osteopontin for 0 to 7 minutes, and the phosphorylation of PKC α was detected by Western blot using anti-phospho-PKC α antibody (*p-PKC α* ; **a**, *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-PKC α antibody (**b**, *top*). Levels of PKC α 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-PKC α or anti-c-Src antibody, and the kinase activity of PKC α (**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-IKK α/β (Ser¹⁸⁰/Ser¹⁸¹) antibody (*top*), and the remaining part of cell lysates was immunoprecipitated with IKK α/β antibody and detected by Western blot using anti-phosphotyrosine antibody (*middle*). The level of IKK α/β was also detected by Western blot (*bottom*). **D**, cells were pretreated with staurosporine or individually transfected with dn c-Src, I κ B α super-repressor (*sup. rep.*), wt and dn IKK α or IKK β , 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 μ mol/L) on COX-2 expression was also detected, and the results indicated that osteopontin at 0.5 μ mol/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 μ mol/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- κ B-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 IKK β or kinase-negative (mut) NIK or dn IKK β 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 IKK β enhanced the COX-2 promoter activity, whereas mut NIK or dn IKK β significantly suppressed osteopontin-induced COX-2 promoter activity (Fig. 1D). Cells transfected with wt IKK α enhanced osteopontin-induced COX-2 promoter activity, whereas dn IKK α or the super-repressor form of I κ B α 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- κ B-mediated pathway.

Osteopontin regulates PKC α /c-Src-dependent IKK/NF- κ B-mediated COX-2 expression. Recent reports revealed that PKC α plays important role in the survival and growth of androgen-independent human prostate cancer (PC-3) cells (34). It is well established that PKC α plays crucial role in COX-2 expression (23). Therefore, to delineate the effect of osteopontin on PKC α phosphorylation and its interaction with c-Src, cells were treated with osteopontin for 0 to 7 minutes, and one part of the cell lysates was analyzed by Western blot using anti-phospho-PKC α antibody (Fig. 2A, *a*, *top*). The remaining part of lysates was immunoprecipitated with anti-c-Src antibody, and the interaction between phosphorylated PKC α and c-Src was determined by Western blot using anti-phospho-PKC α antibody (Fig. 2A, *b*, *top*). The same blots were reprobed with anti-PKC α and c-Src antibody, respectively, as

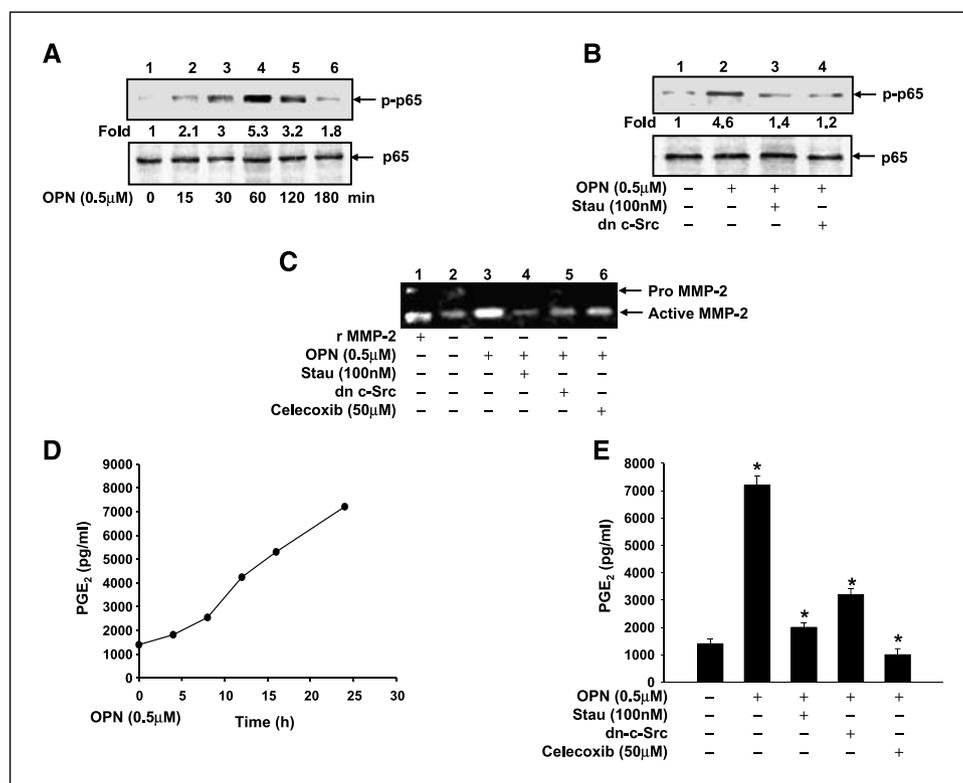


Figure 3. Osteopontin (OPN) induces PKC α /c-Src-dependent NF- κ B, p65 phosphorylation, MMP-2 activation, and PGE₂ production. **A**, PC-3 cells were treated with osteopontin for the indicated time, and the level of phospho-p65 (*p-p65*; Ser⁵³⁶) from cell lysates were detected by Western blot (*top*). **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 μ mol/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 (r MMP-2) was used as positive control. **D and E**, cells were treated with 0.5 μ mol/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.

loading controls (Fig. 2A, *a and b, bottom*). The results indicated that osteopontin enhanced PKC α phosphorylation and its interaction with c-Src. To determine the role of $\alpha_v\beta_3$ integrin on osteopontin-induced PKC α phosphorylation, cells were pretreated with anti-human $\alpha_v\beta_3$ integrin blocking antibody, RGD, or RGE (GpenGRGDSPCA and GRGESP, respectively) peptides and then treated with osteopontin, and the level of PKC α phosphorylation was detected by immunoblotting. The results indicated that anti- $\alpha_v\beta_3$ integrin blocking antibody and GpenGRGDSPCA but not GRGESP suppressed the osteopontin-induced PKC α phosphorylation (data not shown). The data suggested that $\alpha_v\beta_3$ integrin plays an important role in osteopontin-induced PKC α phosphorylation. To delineate whether PKC α is upstream of c-Src or vice versa, cells were pretreated with PKC α inhibitor (staurosporine) and treated with osteopontin, and cell lysates were immunoprecipitated with anti-c-Src antibody. The immunoprecipitated samples were analyzed for c-Src autophosphorylation 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-PKC α . The immunoprecipitated samples were used for *in vitro* PKC α kinase assay. The data showed that dn c-Src has no effect on PKC α autophosphorylation, whereas PKC α inhibitor suppressed c-Src phosphorylation, suggesting that osteopontin regulates PKC α -dependent c-Src activation (Fig. 2B, *a and b, top*). The levels of PKC α and c-Src in the same lysates were detected by Western blot using their specific antibodies as controls (Fig. 2B, *a and b, bottom*). Furthermore, suppression of PKC α and c-Src resulted in inhibition of osteopontin-induced NIK phosphorylation (data not shown). To determine whether these upstream kinases (PKC, c-Src, and NIK) play any role in osteopontin-induced IKK α/β phosphorylation, cells were individually transfected with dn c-Src or wt and mut NIK or pretreated with PKC α inhibitor (staurosporine) and then treated with osteopontin. Cell lysates were analyzed either by Western blot

using anti-phosphoserine IKK α/β antibody or immunoprecipitated with anti-tyrosine 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 IKK α/β , whereas mut NIK inhibits only the serine but not tyrosine phosphorylation of IKK α/β , suggesting that NIK plays differential role in osteopontin-induced PKC α /c-Src-mediated IKK α/β activation (Fig. 2C, *top and middle*). The level of IKK α/β was also detected by Western blot as control (Fig. 2C, *bottom*).

To further investigate whether PKC α , c-Src, NIK, IKK, and NF- κ B 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 I κ B α super-repressor, wt and dn IKK α or IKK β followed by treatment with osteopontin. The COX-2 expression was detected by Western blot (Fig. 2D). These results suggested that PKC α and c-Src play an important role in regulating osteopontin-induced COX-2 expression through a NIK/IKK/NF- κ B-mediated pathway.

Osteopontin induces PKC α /c-Src-mediated p65 phosphorylation. Recent findings suggested that phosphorylation of p65 subunit of NF- κ B leads to nuclear translocation and activation of NF- κ B (35), and that the promoter region of COX-2 contains the NF- κ B response element (36). In Fig. 2D, we showed that suppression of NF- κ B by overexpression of I κ B α super-repressor significantly inhibits osteopontin-induced COX-2 expression. Therefore, we sought to determine whether osteopontin regulates NF- κ B, p65 phosphorylation that ultimately regulates COX-2 expression, and whether PKC α 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

Western blot using anti-phospho-p65 antibody (Fig. 3A and B). The results showed that inhibition of PKC α and c-Src significantly suppressed osteopontin-induced NF- κ B, p65 phosphorylation (Fig. 3B). These results clearly suggested that osteopontin induces p65 phosphorylation through a PKC α /c-Src-dependent pathway, which leads to NF- κ B activation that ultimately regulates COX-2 expression.

Osteopontin augments COX-2-mediated PGE₂ 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 PGE₂ production and tumor progression (2, 37–39). Accordingly, to determine whether osteopontin can induce COX-2-mediated PGE₂ production, cells were treated with osteopontin for 0 to 24 hours, and the PGE₂ level in the conditioned media was measured by using PGE₂ enzyme immunoassay kit. To examine whether PKC, c-Src, and COX-2 are involved in osteopontin-induced PGE₂ 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 PGE₂ production (Fig. 3D and E). To delineate the role of PKC α , 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 PGE₂ production and MMP-2 activation in prostate cancer cells.

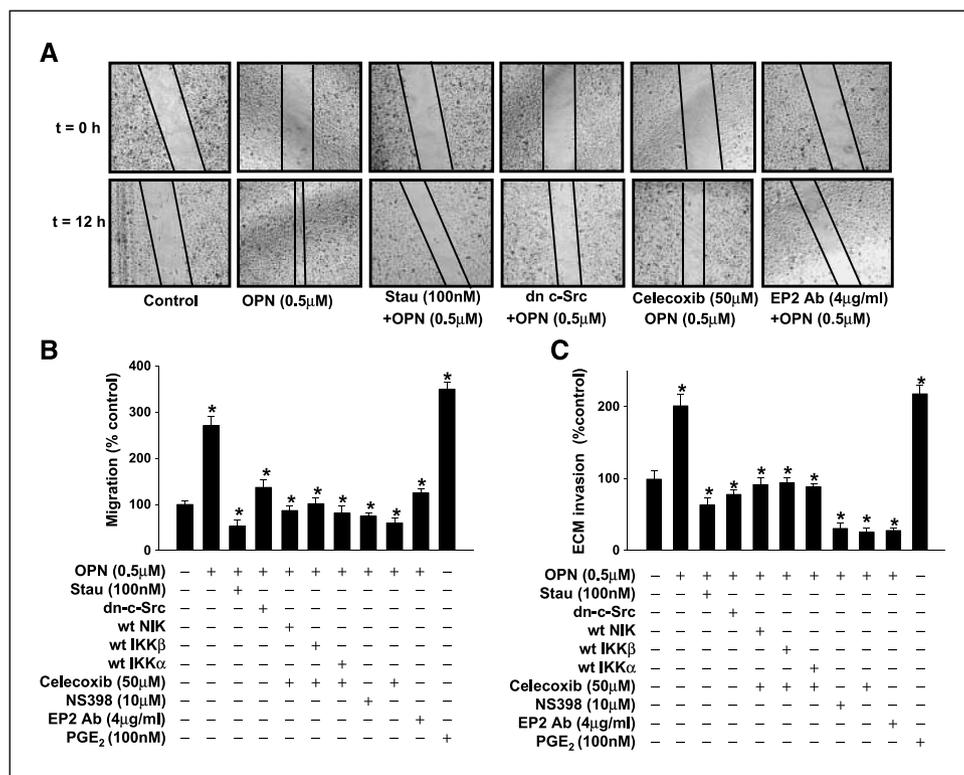
COX-2 and PGE₂ play an important role in osteopontin-induced PC-3 cell migration and invasion. Previous data suggested that COX-2 and its metabolite PGE₂ act as crucial molecules in regulating tumor cell motility, invasiveness, and

tumor metastasis (2). Therefore, to examine the role of COX-2 and PGE₂ 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 (PKC α 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 PGE₂ 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 PGE₂ 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, IKK α , or IKK β 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. PGE₂ was used as a positive control. Taken together, these data indicated the potential and crucial role of COX-2 and PGE₂ in osteopontin-induced tumor cell motility and invasiveness.

Osteopontin-induced tumor cell-derived PGE₂ enhanced endothelial cell motility and invasiveness through paracrine mechanism. Earlier data indicated that tumor-derived PGE₂

Figure 4. Inhibition of the PKC, c-Src, and COX-2 or blocking of EP2 receptor suppressed osteopontin (OPN)-induced PC-3 cell motility and invasiveness.

A, cells were transfected with dn c-Src, or pretreated with staurosporine (Stau), celecoxib, or anti-EP2 blocking antibody (4 μ g) then treated with osteopontin for 12 hours. Wound assays were done as described earlier. Wound photographs were taken at initial time (0 hour) and the termination of the experiments (12 hours). The experiments were repeated in triplicate. B, cells were individually transfected with wt NIK and IKK α and IKK β and treated with celecoxib. In separate experiments, cells were transfected with dn c-Src or treated with staurosporine, celecoxib, NS398, and EP2 blocking antibody and used for migration assay. Osteopontin was used in the upper chamber. PGE₂ was used as positive control. After 12 hours, migrated cells were counted from the lower chamber and represented graphically. C, experiments paralleling those of (B) but measuring invasion rather than migration. Columns, means of three determinations; bars, SE.



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 act 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 mice xenograft tumor progression. Accordingly, PC-3 cells (5×10^5) 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- κ B, 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

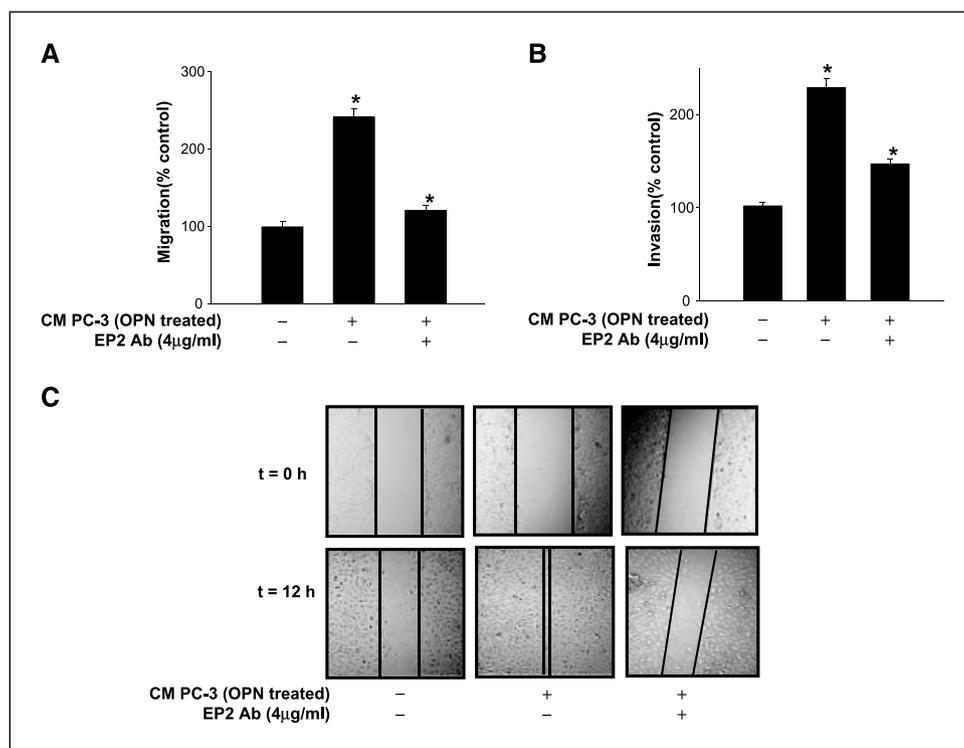


Figure 5. Osteopontin (OPN) stimulates EP2-mediated endothelial cell migration, invasion, and wound healing via paracrine mechanism. A, human endothelial (EA.hy-926) cells were added in the upper chamber. The conditioned medium (CM) collected from PC-3 cells treated with osteopontin was used as chemoattractant in the lower chamber. In separate experiments, EA.hy-926 cells were treated with anti-EP2 blocking antibody and used for migration assay. B and C, experiments paralleling those of (A) but measuring invasion and wound healing rather than migration. Columns, means of three determinations; bars, SE.

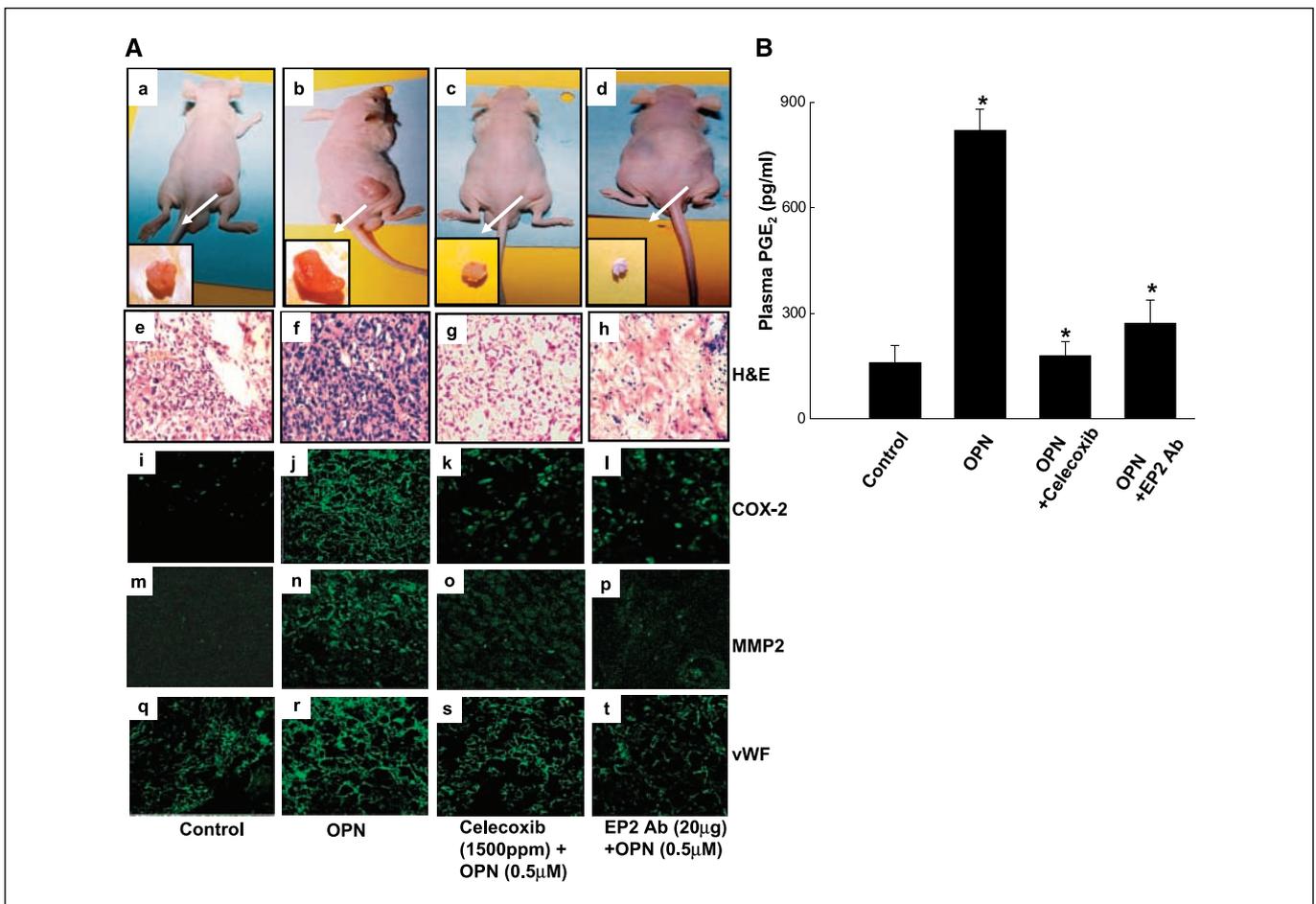


Figure 6. COX-2 and PGE₂ play crucial roles in osteopontin (OPN)-induced tumorigenesis and angiogenesis in nude mice. *A*, photographs of athymic mice showing 4-week-old xenograft tumor growth by PC-3 cells; *inset*, excised tumors with respective mice (*a-d*). Experimental details were described in 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-l*), 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.

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 2. Characteristics of the xenograft tumors from experimental mice

Tumor characteristics	Control	Osteopontin (0.5 μmol/L)	Osteopontin (0.5 μmol/L) + celecoxib (1,500 ppm)	Osteopontin (0.5 μmol/L) + EP2 antibody (20 μg)
Tumor infiltration	Moderate	Very high	Moderate to poor	Poor
Vessel formation	Poor	High	Moderate to poor	Negligible
Mitotic features/hpf	4-6	12-16	2-4	1-3
Tumor giant cells	Less	Plenty	Scanty	Very less
Nuclear polymorphism	Moderate nuclear size variation	Marked nuclear size variation	Less nuclear size variation	Small regular uniform nucleus

In prostate cancer, PKC α plays a key role in the regulation of downstream oncogenic molecules (34). Recent reports also revealed that activation of PKC α 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 PKC α activation and PKC α -dependent downstream signaling events in prostate carcinoma. In this study, we provide evidences

that PKC α plays an important role in osteopontin-induced c-Src/NIK-mediated IKK α/β -dependent NF- κ B phosphorylation, which ultimately controls COX-2 expression. Recently, we have shown the involvement of the NIK/IKK/NF- κ B 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 PKC α /c-Src-mediated

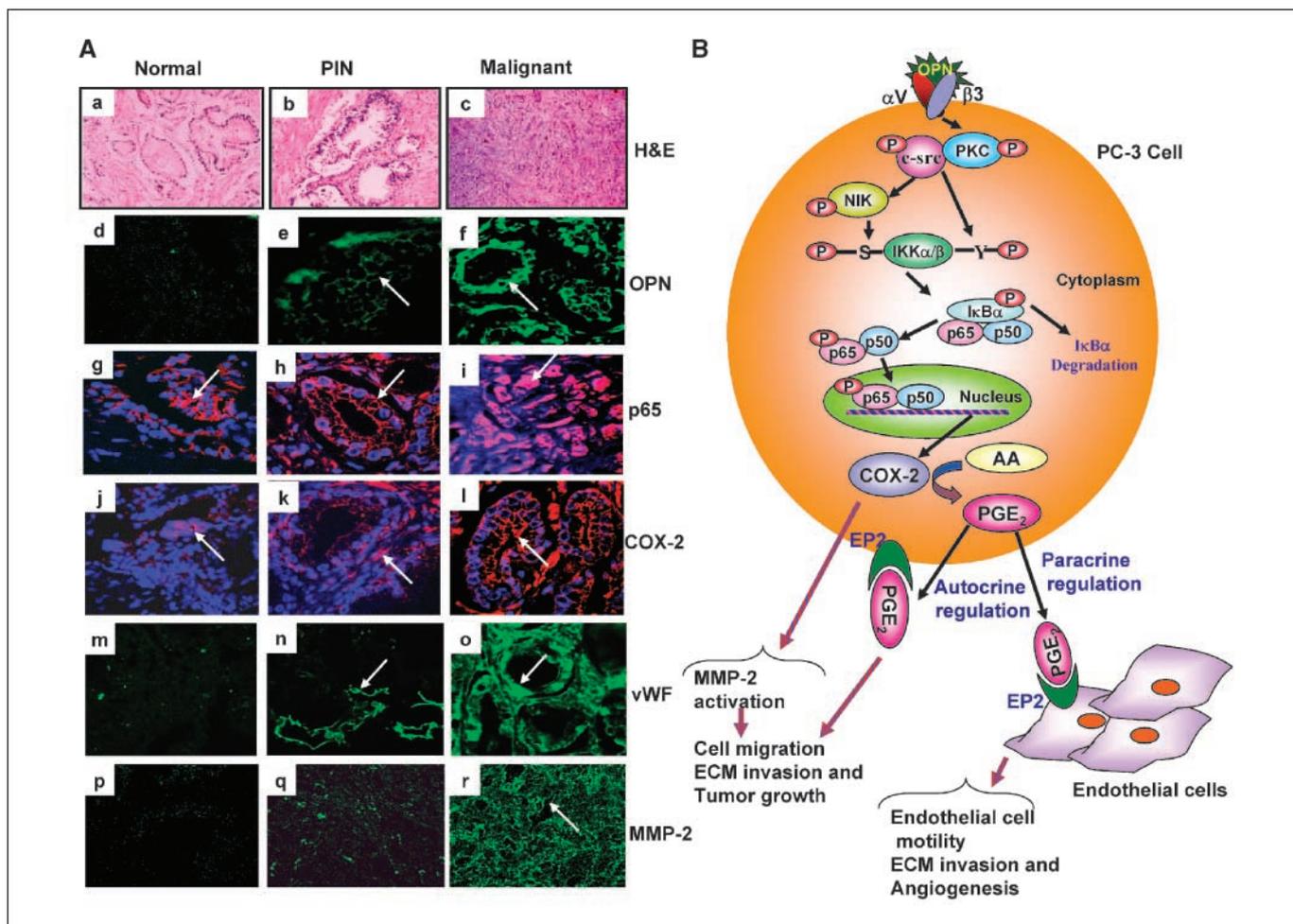


Figure 7. Expressions of osteopontin (OPN), COX-2, MMP-2, and NF- κ B; p65 localization; and their correlation with human prostate cancer progression and angiogenesis in different pathological grades. A, prostate tumor specimens were collected from a local hospital with informed consent. The gradations 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- κ B) 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 PKC α /c-Src/IKK/NF- κ B-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- κ B phosphorylation. Moreover, the results showed that overexpression of super-repressor form of I κ B α significantly suppressed osteopontin-induced COX-2 expression, suggesting that NF- κ B act as the key transcription factor in regulation of COX-2 expression and further showed the dispensability of the PKC α /c-Src/IKK/NF- κ B 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 cancer 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 PKC α /IKK/NF- κ B-mediated COX-2 expression and thereby controlling tumor progression and angiogenesis.

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The Crucial Role of Cyclooxygenase-2 in Osteopontin-Induced Protein Kinase C α /c-Src/I κ B Kinase α / β -Dependent Prostate Tumor Progression and Angiogenesis

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