“Growth for the sake of growth is the ideology of the cancer cell.”

Edward Abbey
3. INTRODUCTION AND REVIEW OF LITERATURE

Deregulated growth control and metastatic spread due to modulation of tumor cell adhesion, extracellular matrix (ECM) degradation, increased cell invasion and motility are the major characteristics of cancer progression (1). Metastases is considered as the most adverse effect of prostate cancer, and to date there are no reliable serum or plasma biomarkers available or morphologic features observed that can reliably predict which patients are at higher risk of developing malignant prostate cancer (2, 3). Therefore, understanding the differences in the biology of metastatic and organ confined primary tumors is essential for developing new prognostic markers and therapeutic targets (3). Deciphering the molecular events involved in the development of metastatic prostate cancer has the potential to identify biological determinants that can assist in prognosis and development of effective therapies for prostate cancer management.

Prostate cancer is one of the major causes of death in men especially in the western world. Prostate cancer alone accounts for ~25% of all newly diagnosed cancers in men in United States. (4). In recent years the discovery of cancer biomarkers has become a major focus of cancer research. The widespread use of prostate-specific antigen (PSA) in prostate cancer screening and the problems associated with it has motivated researchers to identify suitable markers for screening different types of cancer (5). Biomarkers are also useful for diagnosis, monitoring disease progression, predicting disease recurrence and therapeutic treatment efficacy (5). Osteopontin (OPN) is considered as one such type of biomarker for various types of cancers (6). Extensive research from various laboratories has shown the multifaceted role of osteopontin in human physiological and pathological functions (6-9). The OPN has been shown to regulate the growth, metastasis and angiogenesis of various types of cancer (6, 7). It also protects the cells from apoptosis (10).

3.1 Osteopontin (OPN)

OPN is a chemokine like calcified ECM-associated glyco-phospho-sialo protein that belongs to the SIBLING (Small Integrin Binding LIgand N-linked Glycoprotein) family (11). OPN plays important role in regulating normal physiological processes such as tissue remodeling, bone resorption, wound healing, immunological responses and tissue injuries, as well as human pathophysiology including restenosis, atherosclerosis, and autoimmune diseases.
A large number of studies have shown the significant role of OPN in cancer progression (6-8). Transformed cells are often characterized by abundant secretion of OPN (6, 7). This may indicate the inducible expression of OPN that is secondary to transformation with oncogenes, such as \( v-myce \) or \( ras \) (13). Transformation of cells from OPN knockout mice by transfection with \( ras \) leads to impaired colony formation in soft agar and slower tumor growth \textit{in vivo} as compared to cells from wild-type mice (14).

The importance of OPN in tumor dissemination has been shown by gene transfer experiments as the transfection of cells with OPN increased their malignant phenotype (15), whereas transfection with antisense oligonucleotides yielded population with reduced malignant potential (16, 17). Moreover, organs that physiologically produce OPN isoforms may also provide microenvironment that is favorable for the homing of tumor cells (18).
3.2 Nomenclature and General Characteristics of OPN

OPN was first described as a secreted transformation-specific phosphoprotein by Senger et al (19) and then rediscovered as transformation-associated cell adhesion phosphoprotein in mouse epidermis (20). Later, it was identified together with bone sialoprotein (BSP) as a major sialoprotein in the extracellular matrix of bone (21, 22). The name “osteopontin” was introduced to reflect the potential of the bone protein to serve as a bridge between cells and hydroxyapatite through RGD and polyaspartic acid motifs discovered in the primary sequence of this protein (23). It was also termed as Eta-1 (early T-lymphocyte activation gene 1) as it was identified as a putative lymphokine produced by lymphocytes and macrophages (24). The alternative name secreted phosphoprotein 1(SPP1) was also introduced to reflect the broader functional role of this protein. However, the name “osteopontin” has been retained for the nomenclature (25).

The OPN cDNA and gene have been cloned and characterized in several species (12). The human OPN gene contains 7 exons located on the long arm of chromosome 4 (4q13) (26, 27), whereas mouse OPN gene has been reported to be at the chromosome 5 and the pig gene is on chromosome 8 (28, 29). The OPN promoter contains a TATA box, a CCAAT box and a GC box located at the upstream of transcriptional site (12) and the response element of several transcription factors like Sp1, AP-1, AP-2, AP-4, AP-5, PEA-3, CTF/NF-1, vitamin D response element are also found in the promoter region of OPN gene (12).

3.3 Tissue Distribution of OPN

OPN expression has been detected in a variety of tissues, including bone, dentin, cementum, hypertrophic cartilage, kidney, brain, vascular tissues, cytотrophoblasts of the chorionic villus in the uterus and the decidua, ganglia of the inner ear, the specialized epithelia found in mammary, salivary, and sweat glands, in bile and pancreatic ducts, distal renal tubules, gut and in activated macrophages and lymphocytes (12, 30). In various situations it appears in biological fluids, including blood (31), milk (32) urine (uropontin) (33) and seminal fluid (34). The amount of OPN in normal plasma of women ranges from 22 to 122 μg/L, with a median level of 47 μg/L (12), whereas in urine it ranges from 1.9 to 4.3 μg/mL, with ~4 mg excretion per day (12). The plasma level of OPN significantly increases in patients with increased tumor burden in cancer patients and in patients with auto-immune diseases (35, 36).
3.4 Structural Features of OPN

OPN is rich in aspartic acid, glutamic acid and serine. OPN binds to hydroxyapatite and calcium ions through polyaspartic acid motif. OPN contains N-terminal signal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, and a RGD (arginine-glycine-aspartic acid) cell adhesion sequence predicted to be flanked by the β-sheet structure (12, 30). The GRGDS sequence is also flanked by several highly conserved sequences, including the thrombin cleavage site (12). Studies have shown the high conservation in the amino- and carboxy-terminal regions, polyaspartate segment, GRGDS sequence and thrombin cleavage sites and in several potential phosphorylation sites among the mammalian OPN sequences (12). The OPN contains a protease hypersensitive site that separates the integrin and CD44 binding domains (12, 37). The thrombin cleavage motif in this region has a conserved sequence, RSK present in most species (37). The binding of the C-terminal OPN fragment to CD44v6 occurs through a protein-protein interaction (38). It may also interact with CD44v3 via heparin bridge (39). In contrast, activation of the RGD-containing N-terminal domain is a prerequisite for integrin ligation (37, 40). OPN acts as a substrate for liver transglutaminase as well as the plasma transglutaminase factor XIIIa (41, 42).

The post-translational modification of OPN such as phosphorylation and sulphation increases the anionic surface characteristics, whereas extensive glycosylation can limit flexibility (12). Phosphorylation of OPN is catalyzed by several kinases including, casein kinases I and II, golgi kinases, cAMP- and GMP-dependent kinases, protein kinase C and ectokinases (43, 44). Significant glycosylation constitutes > 33 % weight of the OPN (23). In bovine milk OPN, 3 O-linked oligosaccharides have been identified (45), whereas 5 or 6 O-linked and a single N-linked oligosaccharide are present in rat bone OPN. Sialic acid residues in the OPN molecule are essential for cell binding properties, which may be important for invasive behavior of the OPN expressing cancer cells (46).

Molecular weight of OPN varies from 35 kDa to 80 kDa because of its altered glycosylation and phosphorylation whereas tumor-derived OPN shows molecular weight of ~ 60 kDa (7). Philip et al. have purified ~55 kDa OPN from human milk (47). Rittling et al. have shown that tumor-derived OPN is soluble and not matrix associated (48) and Denhardt et al. reported that whether the OPN is produced from tumor or stromal cells, it enhances the metastatic ability of transformed cells (49). Senger et al demonstrated that tumor-derived OPN
and milk OPN has been found to exhibit close similarity (32). Moreover, studies have suggested that the variation in glycosylation, phosphorylation and sulfation generate the different functional forms of this protein, which might alter its normal physiological functions (12). Recently, several groups have demonstrated that OPN regulates expression of several oncogenic and angiogenic molecules and ultimately play a crucial role in tumor growth, metastasis and angiogenesis (Fig. 2, Ref. 7, 8).

Figure 2, OPN regulates tumor progression. OPN binds with the tumor cell surface receptors and activates downstream signaling cascades, leading to the expression of several oncogenic molecules and helps cancer cells to degrade basement membrane. OPN-induced tumor derived growth factors also regulate angiogenesis and promote the distant migration of tumor cells, which leads to tumor metastasis. Metastasized tumor cells dock at distant site (i.e. lung, liver) of body and generate new tumors (Adopted and modified from Jain et al., Expert Opinion on Therapeutic Targets. 11; 81-90: 2007).
3.5 OPN: a Key Regulator in Cancer Progression and Tumor Angiogenesis

OPN expression is frequently induced during the initiation and progression of cancerous growth by transforming agents. Elevated expression of OPN has been observed in various types of high-grade metastatic cancers (7). The production of OPN can also contribute to the metastatic phenotype through autocrine and paracrine effects (50, 51). The autocrine effects can influence cell proliferation and survival, converting benign tumor cells into highly metastatic cells, whereas paracrine effects can provide protection from cytotoxic macrophages (52) and by inducing tumor angiogenesis.

3.5.1 Integrins and CD44: Receptors for OPN involved in Cancer Progression

The possible mechanism known for OPN-mediated effects on tumor cells is through interaction with cell surface receptors (7, 8). OPN binds with cell surface integrins and CD44 receptors (7, 8). Integrins are a family of cell surface proteins that mediate cell adhesion (53). Adhesion is of fundamental importance to a cell as it provides anchorage, cues for migration, growth and differentiation. Integrins consist of one α and β subunits. To date 24 distinct integrin heterodimers have been described, consisting of 18 α and 8 β subunits (53). OPN binds with integrins in the same and different cells and stimulates different intracellular signals that influence gene expression, affecting the regulation of cell survival, differentiation, proliferation and migration (54, 55). Vitronectin, fibronectin and OPN are known ligands for integrins (56, 57). OPN interacts with αvβ1, αvβ3, αvβ5 via RGD sequence. It binds with α9β1 and α4β1 through the SVVYGLR sequence (18). The expression of αvβ3 is characteristic of several invasive tumor types including melanoma, glioma, ovarian, breast and prostate cancer (6, 7).

CD44 is another cell surface receptor for OPN (55). A wide range of multiple protein isoforms of CD44 are produced from a single gene by both alternative splicing and post-translational modification and various isoforms overexpress in a variety of tumor types including liver carcinoma (58), gastric cancer (59), laryngeal squamous cell carcinoma (60), thyroid carcinoma (61) and breast cancer (62). The principal ligand of CD44 is hyaluronate (HA) and the various isoforms of CD44 bind to OPN (63). The intracellular OPN also binds with CD44 and colocalize with ERM (ezrin/radixin/moesin) complex and regulates migration in embryonic fibroblasts (64). There are reports, which have shown that OPN increases CD44 expression in cancer cell lines, increases their cell adhesion to HA and their metastatic behavior (62, 65).
3.5.2 Role of OPN in Cancer Progression

OPN has been involved a number of physiological and pathophysiological functions (7, 12). Elevated expression of OPN has been observed in most of the tumor tissues (7, 30). It has triggered interest in possible implications of OPN expression as a marker of tumor aggressiveness and patient prognosis (7, 30, 66).

Earlier studies have shown that OPN acts as a master regulator in cancer progression (50, 51, 67-69). The roles of OPN in various cancers are summarized in Figure 3. Investigators from various laboratories have shown that OPN activates multiple signaling pathways in various types of cancer cells and induces cancer progression (Fig. 4).
3.5.2.1 OPN in Regulation of Melanoma Progression

In human melanoma cells, OPN has been shown to interact with αvβ3 integrin that in turn leads to the activation of pp60 c-src kinase (69). OPN acts as a crucial oncogenic molecule for melanoma progression (70).

OPN through αvβ3 integrin mediated pathway induces MT1-MMP dependent MMP-2 activation, which in turn regulates melanoma cell motility, invasiveness and in vivo melanoma growth (67, 68). Moreover, OPN induces IKKα/β mediated activation and ubiquitination of IκBα and augments NF-κB activation (68). Reduced melanoma metastasis in bone has been
reported in the OPN knockout mice than that of wild type mice (70). Recently, Rangaswami et al have demonstrated that OPN regulates MMP-9 activation and \textit{in vivo} tumorigenicity using melanoma, B16F10 cells (68).

\subsection*{3.5.2.2 OPN: a Hallmark for Breast Cancer}

In normal breast, OPN expression is detected mainly in the secretory cells but occasionally in nonsecretory epithelial cells (12), whereas in the clinical conditions considerably higher levels of OPN has been observed in the metastatic breast carcinoma which is associated with poor prognosis and decreased survival (71). Studies have indicated that OPN acts as a metastasis associated protein (72). OPN regulates cell surface receptor CD44s, v6 and v9 expression in breast cancer and stimulates cell migration (62). Recently, He et al. demonstrated that a splice variant of OPN (OPN-c) induces anchorage independent growth in breast cancer cells (73). Moreover, it has been observed that suppression of tumor-derived OPN in MDA-MB-231 cells by its specific antisense S-oligo results in reduction of \textit{in vitro} colonigenicity, proliferation, migration and \textit{in vivo} osteolytic metastasis in nude rats (74, 75). Cook et al showed that OPN also reflects one of the six “hallmarks of cancer” for breast cancer progression (65).

\subsection*{3.5.2.3 Prognostic and Diagnostic Significance of OPN for Prostate Cancer Progression}

Previous data have shown the increased OPN level may be involved in the malignant transformation of prostate epithelial cells and OPN expression level is an important determinant for patient survival (76). OPN plays crucial role in chemotaxis and chemoinvasion of PC-3 prostate cancer cells (77). Thalmann et al. have demonstrated that antibodies to human OPN inhibited the growth stimulatory effects of endogenous OPN, which can be overcome by the addition of exogenous human OPN and suggested that OPN may act as a paracrine and autocrine mediator of prostate cancer growth and progression (78). High expression of OPN has been detected in “benign prostatic hyperplasia” (BPH) and metastatic prostate cancer clinical specimens (79) as well as in the serum (80) and plasma (81) of the patients. Bone-derived OPN triggers the Ca^{2+} transient in human prostate cancer cells and regulates prostate cancer metastasis in bone (82). Studies have indicated that in men with hormone-refractory prostate carcinoma the plasma levels of OPN were associated with the presence of metastases to bone and with other measures of tumor burden, which correlated negatively with survival (83). However, the detailed
molecular mechanism underlying OPN-regulated prostate cancer progression is still the subject of intense investigation.

3.5.2.4 OPN in Other Cancers

Elevated expression of OPN has been detected in the higher grades of various types of cancers, including brain, non-small cell lung carcinoma, hepatocellular carcinoma, colorectal cancer, head and neck cancer etc. suggesting that OPN might act as prognostic marker and therapeutic target for the treatment of various types of cancers (Fig. 5 and Table 1).

Figure 5, Diagrammatic representation of OPN targeted therapeutic approaches for cancer (Adapted & modified from Jain & Chakraborty et al., Expert Opinion on Therapeutic Targets. 11; 81-90: 2007).
3.5.3 Angiogenesis

The process of angiogenesis is defined as the formation of new blood vessels from the existing ones. Angiogenesis is an integrated process that involves basement membrane degradation, endothelial cell proliferation, migration and capillary tubule formation. It is not only
essential for tumor growth but also plays important role in tumor metastasis (84). Angiogenesis facilitates progressive tumor growth by providing adequate oxygenation to the tumor through a series of interrelated steps, including endothelial cell proliferation, motility of endothelial cells through the extracellular matrix toward angiogenic stimuli, and capillary differentiation (85, 86). Under normal circumstances, the microvasculature is maintained in a quiescent state. The acquisition of the angiogenic phenotype depends on the outcome of stimulatory and inhibitory regulation by the tumor and its microenvironment. This process is regulated by the balance between stimulatory and inhibitory factors released by the tumor and its microenvironment (Table 2, 87).

**Table 2, Factors implicated as angiogenesis promoters and inhibitors in prostate cancer.**

<table>
<thead>
<tr>
<th>Angiogenesis promoters</th>
<th>Angiogenesis Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Basic fibroblast growth factor (bFGF)</td>
<td>* Interleukin-10 (IL-10)</td>
</tr>
<tr>
<td>* Vascular endothelial growth factor (VEGF)</td>
<td>* Tissue inhibitor of metalloproteinase-1 (TIMP-1)</td>
</tr>
<tr>
<td>* Interleukin-8 (IL-8)</td>
<td>* Angiostatin</td>
</tr>
<tr>
<td>* Matrix metalloproteinase-9 (MMP-9)</td>
<td>* Interferon (IFN)</td>
</tr>
<tr>
<td>* Matrix metalloproteinase-2 (MMP-2)</td>
<td></td>
</tr>
<tr>
<td>* Transforming growth factor-β1 (TGFβ)</td>
<td></td>
</tr>
<tr>
<td>* Platelet-derived endothelial growth factor (PDGF)</td>
<td></td>
</tr>
<tr>
<td>* Nitric oxide (NO)</td>
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</table>

3.5.3.1 Angiogenic Factors

Studies have demonstrated that the conditioned medium derived from cultures of human prostate cancer cells stimulates endothelial cells indicating that these prostate cancer cells produce proangiogenic factors (88). Higher levels of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been detected in the patients with prostate cancer (89). Among various angiogenic factors VEGF is considered as the most important one for tumor angiogenesis. It has been reported that human prostate cancer cells overexpress VEGF.
and IL-8 (90). These and other proangiogenic factors enhance the tumorigenicity and metastasis of prostate cancer.

### 3.5.3.2 OPN: a Key Angiogenic Factor for Cancer Progression

Till date, OPN has been recognized as a metastasis associated gene but recently several studies have focused on the angiogenic potential of OPN. Takahashi et al have demonstrated that OPN induces angiogenesis by upregulating endothelial cell migration in cooperation with vascular endothelial cell growth factor (VEGF) (91). Recombinant form of OPN induces angiogenic phenotype in human monocytes by inducing the expression of the angiogenic cytokines TNF-alpha and IL-8 and OPN-mediated recruitment of proangiogenic monocytes may represent a mechanism of amplification of fibroblast growth factor (FGF)-induced neovascularization during inflammation, wound healing and tumor growth (92). Senger et al demonstrated that VEGF promotes endothelial cell migration via OPN and integrin mediated pathway (93). This report again stated that the thrombin cleaved fragments of OPN were more potential for migration of endothelial cells (93). Shijubo et al showed the correlation between the expression profile of OPN and VEGF in several cancers indicating that both play crucial role in tumor progression and angiogenesis (94). The same group also reported that the microvessel count in VEGF and OPN positive tumors were significantly higher as compared to OPN and VEGF negative tumors, which was further associated with poor prognosis (94). Recently, we have shown that OPN induces VEGF expression in human breast cancer cells and OPN-induced VEGF in autocrine manner interacts with (Neuropilin-1) NRP-1 receptor and induces tumor cell motility in an autocrine manner, whereas in paracrine manner VEGF binds with the KDR receptor of endothelial cells and induces endothelial cell motility towards tumor cells (51). Moreover, OPN-induced VEGF in juxtacrine manner induces endothelial cells motility towards tumor cells. Taken together, we have demonstrated that OPN-induced VEGF regulates tumor angiogenesis through autocrine, paracrine and juxtacrine mechanisms (51). Therefore, integration of information about the molecular mechanism underlying OPN-regulated tumor angiogenesis and prognostic significance of OPN with tumor angiogenesis might help to understand the unsolved mystery, at least in part, in genesis of angiogenic switch and may help to develop a novel therapeutic approach for the next generation of cancer management.
3.6 Cyclooxygenases (COX)

Cyclooxygenases (COX) are also referred as prostaglandin endoperoxide synthases. They catalyze the rate limiting step in the synthesis of prostaglandins (PGs). COX enzymes perform two enzymatic functions: conversion of arachidonic acids into PGG\(_2\) and as peroxidases they convert PGG\(_2\) into PGH\(_2\), which serves as a common precursor for PGs, prostacyclin and thromboxanes (95). Three isoforms of COX enzyme (COX-1, COX-2 and COX-3) have been identified. COX-1 is constitutively expressed in most tissues and is involved in the production of prostaglandins mediating cellular physiological functions (96). COX-2 enzyme in inducible and its expression is induced in response to various growth factors. Functional role of COX-3 in human physiology and pathophysiology remains to be established (97).

3.7 Prostaglandin E\(_2\) (PGE\(_2\))

Prostaglandins (PGs) are fatty acid derivatives that are found ubiquitously in all tissues (95). It is a potent group of autocrine and paracrine lipid mediators that are implicated in various normal cellular and pathophysiological processes, such as inflammation, edema, bronchoconstriction, platelet aggregation, fever and hyperalgesia.

![Diagram of COX enzyme in prostaglandin synthesis](image)

**Figure 6, Role of COX enzyme in prostaglandin synthesis.** Phospholipases (PLA2) release arachidonic acid from cell membrane. Cyclooxygenases produces prostaglandins and lipooxygenases produce leukotrienes from arachidonic acid. NSAIDs inhibit the COX activity.
Prostaglandins derived from arachidonic acids (AA) are termed series-2 prostaglandins, which consist of PGE2, PGI2, PGD2, PGF2α (95). All of these prostaglandins share a common initial biosynthetic pathway that begins with hydrolysis of cell-membrane phospholipids, mediated by the enzyme phospholipase A2 (PLA2), which is found mostly in cellular membranes including plasma membrane (98). Diverse physiological and pathological stimuli can result in the activation of PLA2 to liberate AA from membrane phospholipids into the cytoplasm (99). AA is then metabolized into prostaglandins by the action of COX enzymes (Fig. 6). Prostanoids are released outside the cell immediately after their synthesis where they exert their biological functions through their interaction with cell surface receptors through autocrine or paracrine mechanism (100).

### 3.8 Prostaglandin Receptors

Prostaglandin receptors belong to the family of rhodopsin-type receptors characterized by their seven transmembrane domains that are intracellularly coupled to different subunits of G proteins (101).

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**Figure 7, Schematic representation of PGE2 receptors and its signaling pathways.** EP1 receptor signaling is coupled to phospholipase C/ inositol triphosphate signaling leading to mobilization of intracellular calcium, whereas EP2 and EP4 receptors couple to Gs and mediate increases in cAMP production. EP3 receptor is coupled to different signaling pathways that result in either a positive or negative cAMP response to PGE2 administration.
Five major types of prostaglandin receptors, namely; D prostanoid (DP), E prostanoid (EP), F prostanoid (FP), I prostanoid (IP) and T prostanoid (TP), which include six subtypes namely; DP1, DP2, EP1, EP2, EP3 and EP4 have been described for the prostanoids PGD$_2$, PGE$_2$, PGI$_2$, PGF$_{2\alpha}$ and TXA$_2$ (102). The cellular membrane receptors for PGE$_2$ are termed EP receptors (EP1-4) (103). They are encoded by different genes and are well conserved throughout the mammalian system from mouse to human (104). EP receptors bind most potently to PGE$_2$ (104). Studies have shown that EP1 receptor signaling is coupled to phospholipase C/ inositol triphosphate signaling leading to mobilization of intracellular calcium (105), whereas EP2 and EP4 receptors couple to G$_s$ and mediate increases in cAMP concentrations (106). There are several splice variants for the EP3 receptor, which are coupled to different signaling pathways that result in either a positive or negative cAMP response to PGE$_2$ administration or increase in intracellular calcium mobilization and accumulation of IP$_3$ (inositol triphosphate), depending on the splice variant and type of cells (107-109, Fig. 7). EP receptors mediate a number of physiological functions in response to PGE$_2$ (104).

3.9 Role of COX-2/PGE$_2$ in Cancer

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 prostaglandin dependent manner (95, 110-114). High-level of constitutive cyclooxygenase-2 (COX-2) expression has been detected in various types of cancers including, colorectal, gastric, pancreatic, head and neck, lung, breast, and in other cancers (95). But, how COX-2 exerts its influence on the malignant phenotype is not yet well understood. Several mechanisms have been proposed by which COX-2 and PGE$_2$ may contribute to cancer progression (95). These include promotion of cell proliferation, inhibition of apoptosis, increased angiogenesis, increased invasiveness, metastasis and immunosuppression (Fig. 8). Tumor cells that lack the ability to express COX-2, proliferate very slowly in vivo (114). Exogenous PGE$_2$ increase cellular proliferation in various cell lines including prostate and breast cancer cells (95). The expression of COX-2 and apoptosis appear to be inversely correlated (95). Dempke et al have demonstrated that COX-2 mediated suppression of apoptosis may be controlled by increased PGE$_2$ levels which modulate pro-apoptotic and anti-apoptotic factors such as Bcl-2, MAKs/Ras, caspase-2, and Par-4 (115). Studies have indicated that selective inhibitors of COX-2 efficiently suppress angiogenesis in in vitro models of many types of cancer (116-119). Tumors grown in COX-2
null mice have shown significant reduction in VEGF expression (120). COX-2 plays important role in tumor invasiveness and metastasis (121). NSAIDS (nonsteroidal anti-inflammatory drugs) and selective COX-2 inhibiting agents reduce invasiveness in human prostate cancer cell lines, PC-3 and DU-145 in vitro (121). Tumor-derived PGE₂ plays a pivotal role in promoting the production of IL-10 (a potent immunosuppressive cytokine) by lymphocytes and macrophages while simultaneously inhibiting IL-12 production (122, 123).

In addition, PGE₂ can inhibit the functional activity of lymphokine-activated killer cells and natural killer cells (124-126). Chaudry et al have shown that the rapid rate of arachidonic
acid turnover is associated with the increase of PGE$_2$ synthesis in malignant prostatic tissues (127). In human prostate carcinoma, significant levels of prostaglandin E$_2$ (PGE$_2$) has been detected, which plays important role in cancer progression (50, 127).

### 3.10 Role of COX-2 and PGE$_2$ in Angiogenesis

COX-2, PGE$_2$ and EP receptors regulate vascular remodeling by promoting angiogenesis. It has been shown that colon carcinoma cells that are forced to overexpress COX-2 stimulate endothelial cell motility and tube formation by increased production of proangiogenic factors (128). Chang et al have reported that COX-2-derived PGE$_2$ promotes the tumor-associated angiogenesis, which is required for the initiation and/or progression of mammary cancer (129). Moreover, they have also observed that PGE$_2$-induces angiogenesis at the earliest stage of tumor development and indomethacin (NSAID) inhibit both angiogenesis and tumor growth, suggesting that NSAIDs suppress tumor development by suppressing angiogenesis (129). In uterine carcinomas, increased COX enzyme expression and PGE$_2$ biosynthesis has been shown to be associated with elevated EP2 and EP4 receptor expression in neoplastic epithelial and endothelial cells (130). Using knockout mouse model, it has been demonstrated that ablation of the EP2 receptor is associated with a decrease in the size of intestinal polyps coincident with a decrease in angiogenic factor expression (131). Similarly, COX-2, EP2 and angiogenic factor expression correlate strongly with an increase in microvessel density in other carcinomas, such as those of the colon (132). Studies have suggested that increased ligand-receptor interaction as a consequence of an increase in the COX-PG biosynthetic pathway, promotes vascular function by enhancing the transcription of target genes involved in angiogenesis (131, 133). In addition, COX-2 can regulate angiogenesis via suppression of production of anti-angiogenic factors (87, 134).

### 3.11. NSAIDs; the COX-2 inhibitors

NSAIDs have been in use over a long period of time as simple painkillers and to reduce inflammation. NSAIDSSs (eg. aspirin, indomethicin, naproxen, ibufen, nabumetone and piroxicam) block the production of prostaglandins (95). These are commonly prescribed medications for the inflammation, pain and arthritis (135). COX-2 inhibitors are newly developed NSAIDs that target mainly the COX-2 enzyme these include celecoxib, rofecoxib, vioxx etc (95). Traditional NSAIDs inhibit both COX-1 and COX-2 enzymes (136). The reduction of COX-1 enzymes decreases the production of prostaglandins that protect the stomach
lining; this allows gastric acids to erode the lining and cause ulcers in users (137). Continual usage of traditional NSAIDs may cause severe side effects in users; most notably stomach irritation and intestinal bleeding (138). COX-2 also produces prostaglandins, but the COX-2 enzyme is located specifically in areas of the body that are responsible for inflammation and not in the stomach (139). When the COX-2 enzyme is blocked, inflammation is reduced. Since the COX-2 enzyme does not play any role in the normal functions of the stomach or intestinal tract, medications which selectively block COX-2 do not present the same risk of injuring the stomach or intestines (140). It is now known that COX-2 enzymes are important for regulation of cardiovascular functioning and specifically blocking this enzyme can lead to serious health issues (141).

### 3.12 Epidermal Growth Factor Receptor (EGFR)

The EGF Receptor (EGFR) is the first transmembrane receptor tyrosine kinase that has been cloned and sequenced, and its closely related family members HER2, HER3, and HER4 have been shown to play myriad roles in mammalian growth and development (142). The EGF receptor activation involves ligand binding to separate receptors followed by formation of active dimers. These receptors can signal as homodimers or they can subtly alter signaling output by heterodimerizing with other family members (142). Few studies have indicated that in androgen dependent tumors, androgens increase the expression of EGFR (143, 144). Mimeault et al have reported that inhibition of protein kinase A (PKA) activity synergizes with EGFR inhibition in suppressing EGF- or serum-driven proliferation in several human prostate cancer cell lines in vitro (145).

### 3.13 β3 Integrin

Studies have shown that among integrin family, β3 integrins have been implicated in a wide variety of functions, including platelet aggregation and thrombosis (146), implantation (147), placentation (148), angiogenesis (149), bone remodeling (150), and tumor progression (151, 152). Bakewell et al have shown that β3 integrin null mice were protected from melanoma metastases to bone suggesting that tumor cells require host β3 integrins to metastasize to bone and induce bone osteolysis (153). β3 integrin plays important role during transendothelial migration of prostate cancer cells (154). The αvβ3 integrin mediate prostate carcinoma cell migration on β3 integrin substrates, such as vitronectin (155). Exogenous expression of αvβ3
induces LNCaP cells to adhere and migrate on vitronectin coated surface (156). 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 (156). Previous studies have indicated that integrins control activation of AP-1 in prostate cancer (157). Furthermore, earlier reports have showed that overexpression of β3 integrin correlates with enhanced metastatic phenotype in LNCaP cells (158). Moreover, it has been observed that stromal cell derived factor-1 (SDF-1) transiently increased the expression and activation of β3 integrin in prostate cancer cells, which in turn augments the aggressiveness of prostate cancer (159).

### 3.14 Protein Kinase C α (PKCα)

The protein kinase C (PKC) family consists of at least 12 isozymes that have distinct and in some cases opposing roles in cell growth and differentiation (160, 161). It has been reported that PKC isozymes are activated by tumor-promoting phorbol esters (162) indicating a key role of PKC in tumor promotion and progression. This led to PKC being considered as a target for anticancer therapy. Studies with antisense PKCα oligonucleotides have demonstrated the inhibition of tumor growth in nude mice bearing implanted human glioblastomas and human bladder, lung and colon carcinomas (163). PKCα plays an essential role in EGFR transactivation by TPA in PC-3 cells (164). It has been revealed that PKCα inhibition may result in apoptosis in androgen independent prostate cancer cell lines (165). Lamm et al demonstrated that reduction in PKCα causes growth impairment in PC-3 cells (166). Liu et al showed that activation of PKCα is associated with increased invasiveness in rat prostate adenocarcinoma cells (167).

### 3.15 c-Src

The c-Src proto-oncogene has been strongly implicated in the development, growth, progression and metastasis of a number of human cancers (168). Increased expression of c-Src has been documented in various types of cancer. Similarly, increased Src protein kinase activity has been observed in numerous human cancer cell lines derived from these tumors (169). The specific activity of Src protein kinase may be increased by direct or indirect interaction with receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) fibroblast growth factor receptor (FGFR), colony simulating factor-1 receptor (CSF-1R), HER2/neu, and hepatocyte growth factor receptor (c-Met) (170).
The effect of Src over-expression and activation appears to be pleiotropic. Src phosphorylates various substrates in tumor cells and many of these Src substrates have been linked to processes that lead to tumorigenicity and metastasis (170). The extensive presence of activated Src in human cancers and its potential role in their development and progression have indicated Src an appealing target for drug discovery efforts.

### 3.16 NF-κB

Nuclear factor-κB (NF-κB) is a transcription factor that was first identified in 1986 by Sen and Baltimore (171). Vertebrate Rel/NF-κB transcription factors include RelA, RelB, c-Rel, p50/p105 and p52/p100 (172). These proteins are structurally-related through an approximately 300 amino acid (aa) N-terminal domain called the Rel homology (RH) domain, which contains sequences important for DNA binding, dimerization and inhibitor (IκB) binding (173). The C-terminal halves of RelA, RelB and c-Rel contain transcriptional activation domains, whereas the C-terminal halves of p105 and p100 contain inhibitory domains (174). Nearly all Rel/NF-κB proteins can form homodimers and heterodimers, which bind to DNA target sites (κB sites) to influence gene expression. The most common dimer is a p50-RelA heterodimer, specifically called NF-κB (172). NF-κB has been detected in numerous cell types that express cytokines, chemokines, growth factors, cell adhesion molecules, and some acute phase proteins (173, 174). In normal cells, these Rel/NF-κB dimers are retained in the cytoplasm as an inactive complex through the direct binding of an IκB protein. Various signals, including many cytokines can lead to degradation of the IκB protein, resulting in translocation of the active Rel/NF-κB complex into the nucleus (173, 174). NF-κB is activated by a wide variety of stimuli such as cytokines, oxidant-free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products. Inappropriate activation of NF-κB has been associated to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, and AIDS (173-175). Constitutive activation of NF-κB has been observed in a high proportion of androgen-independent prostate cancers and many human tumor types (176-178).

Dominant negative NF-κB-inducing kinase (NIK) and tyrosine kinase inhibitors suppress the constitutively elevated NF-κB activity in various prostate cancer cell lines suggesting that NIK, possibly downstream from a tyrosine kinase, may mediate the constitutive activation of IκB kinase (IKK) (179, 180). Several well-characterized IκB kinase (IKK) complexes consist of
IKKα and β and serve as kinases and IKKγ functions as a regulatory subunit (181). Once phosphorylated, IκBs almost immediately undergoes polyubiquitination followed by degradation (182). Several genes that mediate cell proliferation are regulated by NF-κB (183). These include growth factors such as TNF-α, IL-1β, and interleukin-6 (IL-6). Several gene products that negatively regulate apoptosis in tumor cells are controlled by NF-κB activation. These include IAP-1, IAP-2, XIAP, cFLIP, TRAF1, TRAF2, Bcl-2, Bcl-xL, and survivin (177). Various proteases that influence tumor invasiveness (e.g., the matrix metalloproteinases and the serine protease urokinase type plasminogen activator (uPA) are also regulated by NF-κB (184, 185). Moreover, NF-κB controls angiogenesis as it regulates the expression of chemokines (e.g., MCP-1, IL-8) and growth factors (VEGF) by neutrophils, other inflammatory cells and cancer cells (186). Thus, NF-κB plays pivotal role in regulating cell proliferation, migration, invasion and cell survival and angiogenesis leading to neoplastic transformation (177, 186).

3.17 Activating Transcription Factor-4 (ATF-4)

Activating transcription factor-4 (ATF-4) belongs to the ATF/CREB (activating transcription factor/cyclic AMP response element binding protein) family of basic region-leucine zipper (bZip) transcription factors, which have the consensus binding site cAMP responsive element (CRE) (187). Interaction between ATF-4 with several general transcription factors such as TBP, TFIIB and RAP30 have been reported (188). Hai and Curran have reported that ATF-4 forms heterodimers with Jun, Fos, and Fra-1. The ATF-4/Fos heterodimer interact cooperatively with the CRE site: the affinity of this heterodimer to the CRE site was significantly greater than that of either homodimer, however ATF-4/Jun heterodimer associate weakly with the CRE site but do not bind to the AP-1 site (187). ATF-4 is induced by stress signals including anoxia/hypoxia, endoplasmic reticulum stress, amino acid deprivation, and oxidative stress. ATF-4 regulates the expression of genes involved in oxidative stress, amino acid synthesis, differentiation, metastasis and angiogenesis (189). Transgenic mouse studies have demonstrated that ATF-4 is also involved in hematopoiesis, lens and skeletal development, fertility, proliferation, differentiation and long-term memory (189). ATF-4 expression is upregulated in various cancers (189). Since ATF-4 is induced by tumor microenvironmental factors, and regulates processes relevant to cancer progression, it might serve as a potential therapeutic target in cancer management (189)
3.18 Urokinase Plasminogen Activator (uPA)

Tumor cell invasion and metastasis require destruction of the ECM during local invasion, angiogenesis, intravasation and extravasation. These processes are mediated by multiple degradative actions of proteolytic enzymes and these complex events need cooperation of different specificity proteases (190). The functions on ECM degradations are mostly attributed to plasminogen activator system. There are two types of plasminogen activators: the urokinase-type (uPA) and the tissue type (tPA) (191). uPA generates plasmin in events involving degradation of ECM thus uPA participates in cancer invasion and metastasis. uPA is released from cells as a zymogen (pro-uPA), which is converted to active form by plasmin, present in trace quantities in ECM. uPAR, a surface receptor, binds to pro-uPA with high affinity (191). Binding of pro-uPA and plasminogen to uPAR and free lysine groups, on cell surfaces strongly enhances plasminogen activation. This situation facilitates pro-uPA by plasmin and activation of plasminogen by urokinase. Because plasmin is produced in the second reaction, the activation behaves like a closed cycle reaction. Active form of plasmin can degrade most ECM proteins such as fibronectin, vitronectin and fibrin, a notable exception being native collagens (191). It can also indirectly promote matrix degradation through activation of some but not all pro-metalloproteinases (192). Plasmin possibly has functions unregulated in matrix degradation, e.g. activation of proforms of cytokines and growth factors such as pro-TGF-β invasion (193). uPA is overexpressed in various cancer tissues including prostate and it is associated with poor prognosis (194).

3.19 Vascular Endothelial Growth Factor (VEGF)

VEGF has been considered as one of the most important molecule responsible for the regulation of angiogenesis (195). It acts as an endothelial cell-specific mitogen and survival factor (196). Clinical observations have demonstrated that VEGF expression is significantly associated with degree of neovascularization and prognosis in various types of solid tumors (197). It has also been shown that VEGF status is predictive of the resistance to various treatments, including radiotherapy, chemotherapy and hormonal therapy (198). The alternative exon splicing of a single VEGF gene results in the production of 6 isoforms: VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206 (199). VEGF165 is the predominant isoform of VEGF (200). Although VEGF165 is also secreted, a significant fraction remains bound to the cell surface and extracellular matrix (ECM) (201). VEGF189 and VEGF206 are
mostly sequestered in the ECM because of their high binding affinity for heparin (201). The biological activity of VEGF is dependent on its binding with specific receptors. Vascular endothelial growth factor receptors consist of three cell-membrane type receptors that belong to the tyrosine-kinase receptor family (VEGFR-1/Flt-1, VEGFR-2/Flk-1 (KDR), VEGFR-3 (Flt-4)), and a soluble form of VEGFR-1 (sVEGFR-1), an intrinsic negative counterpart of the VEGF (201). VEGF signals mainly through three tyrosine kinase receptors, VEGFR-1, VEGFR-2 and VEGFR-3, which are localized predominantly on vascular endothelial cells (201). VEGF is produced by both malignant cells and non-malignant cells in response to hypoxia, inflammation, growth factors, and cytokines (200).