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Molecular Basis of Co-Targeting Prostate Tumor and Stroma

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Abstract Prostate cancer is one of the leading causes of cancer death in Northern American men. The lethal phenotypes of human prostate cancer are characterized by progression to androgen-independence (AI) and a propensity to form osseous metastases. In ~80% of cases, prostate cancer colonizes bone and elicits a characteristic osteoblastic reaction. The bone metastases are initially sensitive to androgen deprivation treatments, but with time the cancer will eventually progress into an AI stage for which there is currently no effective treatment. Once initial hormonal therapy has failed, median survival of prostate cancer patients with bone metastases is less than 1 year (Tu et al. [2001] Lancet 357:336–341). Novel therapeutic and preventive strategies are needed to decrease morbidity and mortality of this disease. J. Cell. Biochem. Suppl. 38: 65–72, 2002. © 2002 Wiley-Liss, Inc.

Key words: gene therapy; prostate neoplasm; cell-cell interaction; co-target tumor and stroma; transcription factors; cbfa1; Fra2; AP-1; SP-1

In this review, we describe a novel gene therapy strategy in which tissue-specific and tumor-restrictive promoters are employed to drive the expression of therapeutic genes to co-target both the stromal and epithelial compartments in the treatment of prostate cancer bone metastases [Matsubara et al., 2001]. The rationale behind this strategy is based on the evidence that reciprocal stromal-epithelial interactions are important for prostate tumorigenesis and that the osteomimetic properties of prostate cancer cells contribute to prostate cancer progression [Koeneman et al., 1999].

REGULATION OF TISSUE-SPECIFIC AND/OR TUMOR-RESTRICTIVE PROMOTERS, PROSTATE-SPECIFIC ANTIGENS AND HUMAN OSTEOCALCIN, IN ANDROGEN-INDEPENDENT BONE METASTATIC PROSTATE CANCER CELLS

Despite the common occurrence of prostate cancer, little is known about the underlying mechanisms responsible for prostate cancer growth, androgen-independent progression, and acquisition of bone metastatic potential. To gain new insights into the molecular mechanisms of AI progression, our lab focused on defining the transcriptional regulation of human osteocalcin (hOC) and prostate specific antigen (*PSA*) expressions in AI prostate cancer cell lines. A strategy was outlined to identify the transcription factors involved in regulating the critical regions of the promoters of these genes,

Abbreviations: AI, androgen independent; hOC, human osteocalcin; BSP, bone sialoprotein; OPG, osteoprotegerin; ON, osteonectin; OPN, osteopontin; PSA, prostate-specific antigen; IGFBP, insulin-like growth factor binding protein; TGFβ, transforming growth factor beta; PTHrP, parathyroid hormone related protein; IGF, insulin-like growth factor; AREs, androgen responsive elements; AREc, androgen responsive element enhancer core; AR, androgen receptor; EMSA, electromobility shift assay; OSE, osteoblast-specific element; ECM, extracellular matrix; BMPs, bone morphogenic proteins; MMPs, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinase.

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and their roles in tumorigenesis [Yeung et al., 2000, 2001]. The ultimately goal of our study is to devise strategies for expressing therapeutic genes under the control of tissue-specific promoters to treat prostate cancer bone metastasis effectively.

PSA

Due to its tissue-specific expression pattern, *PSA* is the most widely used serum marker for the diagnosis and management of prostate cancer. Since the expression of *PSA* is tightly regulated by androgen, an increase in serum *PSA* in androgen-depleted patients could indicate the development AI tumors. In addition to being dysregulated during cancer progression, *PSA*, a serine protease, is believed to contribute significantly at the molecular level to prostate cancer progression and metastasis via its protease activity. For example, the cleavage of IGFBP [Cohen et al., 1992] and TGFβ by *PSA* could increase the bioavailability of the mitogen IGF; and activating the latent form of growth factor TGFβ, enhancing matrix turnover, tumor angiogenesis, and immune evasiveness. Moreover, the inactivation of PTHrP by *PSA* [Cramer et al., 1996] could tip the balance toward osteoblastic reactions in prostate cancer bone metastasis.

PSA is one of the most widely studied prostate genes. Numerous groups focused on the androgen regulation of PSA have identified several androgen-responsive elements (AREs) [Cleutjens et al., 1996; Huang et al., 1999] and a tissue-specific enhancer core (AREc) [Cleutjens et al., 1997] in the promoter. To identify transcription factors that activate PSA promoter independent of androgen, we performed promoter deletion studies and identified two AI regulatory regions (AREc and pN/H) which suggested the involvement of two distinct pathways in androgen-independent regulation of PSA expression in AI C4-2 cells [Yeung et al., 2000]. One pathway clearly involved androgen receptor (AR) because the binding of AR to the AREs within the AREc appears to be prerequisite for the high activity of the AREc and PSA promoter in C4-2 cells. It is plausible that AR is activated through growth factor mediated signaling pathways independent of androgen. Growth factors like IGF-1, EGF, and KGF were reported to induce AR-mediated gene transactivation in the absence of androgen [Culig et al., 1994]. These growth factors could signal through the PKA [Nazareth and Weigel, 1996] and/or PKC [Sadar, 1999] cascade in which AR activity is enhanced either by modification of AR itself [Ikonen et al., 1994] or by enhancing the interactions between AR and its coactivators [Yeh et al., 1999]. The fact that AREc is highly tissue specific and contains other *cis*-elements suggests [Cleutjens et al., 1997] that in addition to AR, other prostate specific co-activators are also involved in the AI regulation of AREc in C4-2 cells. Activated AR is one of the key transcription factors that interact with other prostate-specific transcription factor(s), and together they associate with the AREc and assemble into a highly active AREc enhanceosome complex [Huang et al., 1999] in C4-2 cells. The aberrant activation of AR and/or it co-activators by growth factors could provide growth advantage to cancer cells in an androgen-depleted environment, and this could be one of the important mechanisms that contribute to prostate cancer progression.

The other pathway mediated by pN/H appears to involve an unknown 45-kDa prostate specific transcription factor (p45). Through DNaseI footprinting and linker-scanning mutagenesis approaches, we showed that p45 binds to a 17 bp site (RI) in the pN/H region and regulates the basal PSA promoter activity. Even though the RI element shares high homology with the SP-1 consensus site, p45 migrates differently from the Sp-1 transcription factors in EMSA, so it does not seem to belong to the Sp-1 transcription factor family. Furthermore, the observed absence of RI-p45 complex in PC3 cells and a higher amount of p45 complex in C4-2 than in LNCaP suggest that the expression of p45 is cell type-specific. Since the addition of androgen could not enhance pN/H activity, p45 does not seem to be regulated by androgen or AR. The fact that C4-2 nuclear extract consistently showed a higher level of RI-p45 complex in EMSA implies that increased association of p45 to RI site is a possible mechanism by which PSA promoter is activated in C4-2 cells in an AR and androgen independent manner [Yeung et al., 2000]. Further efforts to identify p45 may provide additional information on the AI growth and metastasis of prostate cancer cells. For example, P45 may regulate prostate cancer growth by binding to the promoters of the growth-related genes that contain the RI *cis*-element. The identification of p45 could also be of great clinical significance. Since there is a

higher level of p45 in AI prostate cancer cells, new diagnostic approaches based on screening for the level of p45 in tumor cells could provide valuable information for staging the cancer and determining the progression and metastatic potential of the disease.

hOC

Osteocalcin (OC) is one of the major non-collagenous bone matrix proteins expressed in bone. OC expression is transcriptionally regulated by vitamin D and was thought to be limited to cells of the osteoblast lineage. Many regulatory elements have been mapped to the proximal region of the OC promoter. These include OSE1, OSE2, and AP-1/VDRE. OSE1 and OSE2 were first identified in mouse OC promoter and they are associated with osteoblast-specific transcription factors, OSF1 and Runx2, respectively. Vitamin D receptors were shown to bind the VDRE in the proximal promoter, and its activity is tightly regulated by the members of the AP-1 family that bind the contiguous AP-1 site. In proliferating osteoblasts, c-Fos and c-Jun heterodimers were shown to block the binding of VDR and suppress the rat OC promoter activity, while the expression of Fra2 and JunD in the post-proliferated osteoblasts induces rat OC promoter activity by facilitating VDR/RXR binding [Lian et al., 1998].

Human prostate cancer cells with a propensity to metastasize to the skeleton and prostate cancer tissue specimens with increased Gleason scores reveal that prostate cancer cells synthesize, secrete, and/or deposit large amounts of non-collagenous bone matrix proteins such as OC, osteopontin (OPN), osteoprotegerin (OPG), osteonectin (ON), and bone sialoprotein (BSP). In a recent study, we demonstrated that OC protein was prevalently expressed in primary prostate cancer (85%), in prostate cancer lymph node (100%), and in bone metastasis specimens (100%) [Chung and Zhau, 2001; Matsubara et al., 2001]. Since OC was not expressed in normal human prostate gland, the predominant expression of OC and other bone matrix proteins in advanced prostate cancer imply a role in prostate cancer survival and growth in the bone environment. OC secreted by prostate cancer cells can complex with ECM and calcium, serving as a chemoattractant for recruiting osteoblasts and/or osteoclasts, which initiate bone remodeling [Glowacki and Lian, 1987]. This may contribute to the osteotrophic characteristics of prostate cancer bone metastasis. The discovery of hOC expression in prostate cancer specimens has opened new windows on biology and therapy of prostate cancer bone metastasis. In an effort to understand the osteomimetic properties of prostate cancer cells, we used PC3 cells to investigate the regulation of OC expression. PC3 cells are AI, obtained from bone metastatic lesions of a prostate cancer patient. PC3 cells share with mature osteoblasts the unique feature of synthesizing and depositing a large amount of OC. In our study, we demonstrated three groups of transcription factors, Runx2, JunD/Fra-2, and Sp-1, responsible for the high hOC promoter activity in PC3 cells by binding to the OSE2, AP-1/VDRE, and OSE1 elements, respectively. Furthermore, the functional hierarchy of OSE1, OSE2, and AP-1/VDRE was established in the regulation of hOC promoter activity (OSE1>AP-1/VDRE>OSE2) in PC3 cells. We also generated an artificial hOC promoter consisting of dimers of the three elements with significantly higher activity than the wild type promoter. Among the three groups of transcription factors, the expression levels of Runx2 and Fra-2 are higher in the OC-positive PC3 cells and osteoblasts, compared to the OC-negative LNCaP cells. Interestingly, unlike the mouse OC promoter, the OSE1 site in hOC promoter is regulated by the members of Sp-1 family instead the osteoblast specific factor, Osf1 [Yeung et al., 2001]. Therefore, by expressing the osteoblast-specific transcription factor Runx2 and differentially up-regulating the prominent Ap-1 factor, Fra2, in mature osteoblasts, PC3 cells have acquired the phenotypes of osteoblasts. The interplay and co-ordination among these transcription factors provides the molecular basis for AI prostate cancer cells behaving like mature osteoblasts. The balance and activity of these transcription factors is significant in conferring the osteolytic/osteoblastic phenotype of prostate cancer cells frequently observed in metastatic skeletal lesions.

Osteoblast-Specific Transcription Factors and Prostate Cancer Bone Metastasis

Besides OC, the expression of other bone matrix proteins such as OPG, OPN, BSP was also found in bone met prostate cancer cells. It appears that a switch of gene transcription occurs in prostate cancer bone metastasis that allows the cancer cells to acquire an osteoblast

phenotype and presumably leads to their colonization in the skeleton. The roles of these bone matrix proteins are unclear, but based on their functions in bone development and remodeling, one can postulate that OPN expression in prostate cancer cells could facilitate their adhesion and migration [Denhardt et al., 2001] to the bone matrix and participate in subsequent bone "pitting" and steps involved in osteoid mineralization. Since BSP has been shown to be crucial for the expression of osteoblastic phenotypes in cultured bone marrow cells [Mizuno et al., 2000], overexpression of BSP by metastatic prostate cancer cells could enhance their attachment to osteoblasts and osteoclasts, and stimulate osteoblast differentiation. The expression of OPG in prostate cancer cells may lead to the overall repression of osteoclast activity and a shift of bone remodeling toward osteoblast activity in bone metastasis [Simonet et al., 1997]. Therefore, the osteomimetic properties of prostate cancer cells in theory could allow them to invade, adhere, survive, and grow better in the bone microenvironment [Koeneman et al., 1999]. Recent study using the osteotropic prostate cancer cells (C4-2B) demonstrated that in addition to having an osteoblastic phenotype, C4-2B cells could produce hydroxyapatite mineral in vitro; and stimulate osteoblasts to initiate mineralization in the bone [Lin et al., 2001]. The increased expression of bone matrix proteins by prostate cancer cells in skeletal metastatic sites may underlie the predilection of prostate cancer for bone and explain the mineral formation found in osteoblastic lesions.

Even though tumor metastases in patients with prostate cancer are predominantly osteoblastic, evidence from osteoblastic metastasis animal model indicates that an initial phase of bone resorption precedes new bone formation [Yi et al., 2000]. The importance of osteoclast activity in osteoblastic metastasis was further supported by recent findings that bisphosphonates (an inhibitor of osteoclast activity) could effectively alleviate bone pain in patients with metastatic prostate cancer [Adami, 1997]. Since bone is an abundant source of growth factors such as TGFβ, bFGF, IGFs, PDGFs, and BMPs; osteoclastic bone resorption could release and/or activate growth factors from the bone matrix which could serve as paracrine mediators to stimulate tumor growth [Nakase et al., 1994]. Conceivably, prostate cancer cells could modify the bone microenvironment in a reciprocal manner by secreting soluble factors to promote osteoclasts activity, which in turn makes the bone stroma conducive for the growth and survival of prostate cancer cells. Therefore, the reciprocal interactions between prostate cancer cells and their supporting stroma are essential in promoting cancer progression, invasion, and metastasis.

Fra2 is one of the transcription factors that may be involved in bone metastasis. We showed that a higher level of Fra2 was found in AI, bone metastatic prostate cancer cells compared to non-tumorigenic LNCaP prostate cancer cells. It is not clear how Fra2 is upregulated in prostate cancer cells. However, growth factor like TGFβ was known to increase the expression and activity of AP-1 factors. The binding of TGFβ to its cell surface receptor could trigger a cascade of signaling pathways, including the MAPK pathways. MAPK pathways were indicated to regulate both the amounts and transactivating activity of the AP-1 factors in a stimulus-specific manner. As an oncoprotein, high level of Fra2 could contribute in numerous ways to increase proliferation, invasion, and metastasis of cancer cells during disease progression. For example, overexpression of Fra2 reportedly represses the tissue inhibitor of metalloproteinases (TIMP1) promoter activity by forming heterodimers with JunD, which are less active than JunD homodimers [Smart et al., 2001]. The repression of TIMP1 could allow the accumulation of MMPs and lead to increased invasiveness of the tumor. Furthermore, avian primary cells transformed by Jun/Fra2 showed increased anchorage independent growth by their ability to form colonies in soft agar [van Dam et al., 1998]. Therefore, these results are consistent with the notion that enhanced level of Jun/Fra2 confers cell motility and invasiveness.

Runx2 is an osteoblast-specific factor highly expressed in the bone metastatic prostate cancer cells. The expression of this osteoblast-specific factor is believed not only to impart the osteomimetic characteristics of prostate cancer cells but it also has a significant role in carcinogenesis. BMPs and TGFβ are two of the growth factors shown to activate the transcription of Runx2 in bone cells [Bae et al., 2001]. As a member of the TGFβ family, BMPs are known to bind receptors and to induce a cascade of events leading to phosphorylation of Smad proteins.

Upon phosphorylation, Smad translocate into the nucleus to interact with numerous transcription factors, which then regulate gene expression. Smad5 is believed to be responsible for activating the Runx2 gene transcription [Bae et al., 2001]. Other local factors such as hedgehogs [Yamaguchi et al., 2000] and IGF-1 [Yeh et al., 1997] were shown to synergistically enhance BMPs actions. For example, recombinant Shh and conditioned media collected from Shh-or Ihh-overexpressing chicken embryonic fibroblasts can increase BMP2, which induces the downstream OC mRNA level [Kinto et al., 1997]. Therefore, hedgehog-signaling pathway may act co-operatively with the BMP signaling pathway in regulating the transcription of Runx2.

Some of the early gene targets of BMP signaling pathway include the N- and E-cadherins. Recombinant human BMP-2 (rhBMP2) rapidly and transiently increases N- and E-cadherin mRNA and protein levels in human osteoblasts. In addition, the N- and E-cadherin antibodies have inhibitory effects on both the basal and induced Runx2 mRNA levels, and abolish the rhBMP2-induced OC mRNA levels [Hay et al., 2000]. The importance of cell-matrix interaction in the regulation of Runx2 expression is further demonstrated by the fact that α2-integrin-collagen interaction is required for activation of Runx2 and induction of osteoblast-specific gene expression. Induction of matrix synthesis is accompanied by a dramatic increased in the binding of Runx2 to OSE2, suggesting that ECM synthesis upregulates and/or activates Runx2. Furthermore, blocking of integrin-type I collagen binding prevents activation of the OC promoter by ascorbic acid and suppresses binding of Runx2 to OSE2 site [Xiao et al., 1998]. It is not clear how ECM activates Runx2; it is possible that a post-translational pathway or accessory factor(s) are involved in the regulation. Like the AP-1 factors, the MAPK pathway also regulates Runx2. An activated recombinant MAPK was shown to phosphorylate a Runx2 fusion protein in vitro [Xiao et al., 2000]. Therefore, the phosphorylation of Runx2 through the MAPK pathway is essential for responsiveness of osteoblasts to ECM signals and contributes for osteoblast-specific gene expression

Evidence from tooth development study indicated that Runx2 regulates key epithelial-mesenchymal interactions that control advancing morphogenesis and differentiation of the epithelium. Runx2 expression in the mesenchyme is intimately associated with epithelial-mesenchymal interactions during tooth development and is affected by epithelial signals. Runx2 expression in the mesenchymal is controlled by signals emanating from the epithelium. In turn, Runx2 regulates the expression of mesenchymal molecules that act reciprocally on epithelium to control the differentiation of the enamal organ [D'Souza et al., 1999]. It is plausible that the growth factors release from the bone matrix upregulate the expression of Runx2 in prostate cancer cells, as a result triggering a "ping-pong" mechanism (Fig. 1) in which the high level of Runx2 allows expression of gene products in the cancer cells that could modify the surrounding stroma and eventually lead to enhance tumor growth.

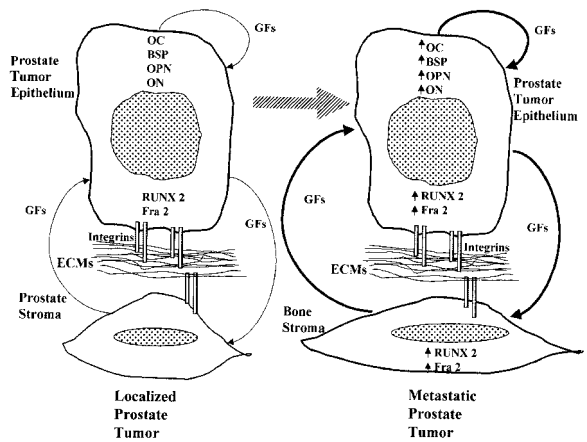

Fig. 1. A ''Ping-Pong'' mechanism of transcription factor activation through stromal-epithelial interaction: potential contributors to prostate cancer bone metastasis. The homeostasis of localized prostate tumor is maintained by the constant stimulation from the prostate stroma and growth factors released from ECMs which determines the proliferative and differentiative status of tumor epithelium. At this stage, tumor epithelium is considered as androgen-dependent and expressed low levels of OC transcription factors, RUNX2 and Fra2, and bone matrix proteins, OC, OPN, BSP, and ON. Upon prostate cancer progression to androgen-independent and metastatic state (such as bone metastasis), a surge of intracellular autocrine and paracrine growth factor signaling results in an elevation of OC transcription factor activity (e.g., increased RUNX2 and Fra2) and elevated level of OC, BSP, OPN, and ON. The enhanced RUNX2 and Fra2 is responsible for an overall stimulation of osteoblast proliferation, which then supports tumor growth and survival in the skeleton through greatly increased autocrine and paracrine loops. The co-targeting concept is building upon the observation of increased osteomimetic properties by AI and metastatic prostate cancer cells so that replication-competent Ad vectors can be designed to exert cytotoxic effects on both tumor epithelial and bone stromal cell compartments.

Potential Use of Tissue-Specific and Tumor-Restrictive Promoters in Co-Targeting Stromal-Epithelial Interactions in Prostate Cancer

Due to the poor response rate of previously treated patients with relapsed prostate cancer to conventional radiotherapy, surgery, or chemotherapy, our laboratory has examined some of the unique biological characteristics associated with prostate cancer and its relationship with prostate or bone stromal cells in the effort to formulate novel targeting strategies. Traditional therapy for prostate cancer has targeted only the malignant epithelial cell. Because of the osteomimetic properties of prostate tumor epithelial cells in bone, we proposed a novel co-targeting strategy incorporating an adenoviral gene therapy approach to the treatment of both localized and metastatic prostate cancers. This approach involves the use of bone matrix protein promoters, such as OC to drive the expression of therapeutic genes co-targeting tumor epithelium and its supporting stroma to maximize tumor cell-kill.

Gene therapy is a newly developed technology based on understanding the genetic and molecular defect of disease. In dealing with cancer, toxic gene therapy is most commonly employed clinically. This kind of therapy involves suicide gene/prodrug systems, in which a suicide gene is delivered to cancer cells to activate a non-toxic prodrug in cells and thus selectively kill the tumor cells while sparing surrounding normal cells from tissue damage.

The concept of delivery and expression of therapeutic genes to tumor cells through the use of tissue-specific promoters has been well recognized. This approach decreases the adverse effects of the therapeutic genes on normal cells and increases the specificity and efficiency of gene transfer to tumor cells. Attractive approaches for the treatment of bone metastasis could be developed through our understanding of the molecular mechanism underlying the acquisition of osteomimetic properties by prostate cancer cells. The discovery of common bone matrix proteins synthesized by both prostate cancer cells and osteoblast cells raise the possibility of employing the promoters of these proteins to drive the expression of therapeutic genes in both prostate cancer and bone stromal compartments for therapeutic gains. In this context, our laboratory has developed an OC-based toxic gene therapies for the treatment of prostate cancer metastasis. We envision the use of OC promoter to drive the expression of therapeutic genes in proliferative cellular compartments to eradicate the growth of both prostate cancer cells and their supporting stroma. This approach could achieve a higher degree of efficacy than the conventional approaches of blocking bone proliferation by bisphosphonate or halting the growth of prostate cancer cells with non-selective chemotherapeutic agents or radiation. The effectiveness of this form of gene therapy can be explained by the uniqueness of the OC promoter, which presumably drives the expression of toxic genes in at least three cellular compartments: tumor epithelium, bone stroma, and vascular endothelial pericytes [Chung and Zhau, 2001]. The rationale for co-targeting bone stroma and epithelium was supported by our preliminary data showing that the growth of prostate cancer cells was enhanced when they were co-cultured with bone stroma cells. The destruction of bone stromal cells alone was sufficient for killing the co-cultured prostate cancer cells. Furthermore, a recent study by Tu et al. [2001] demonstrated that bone targeting with chemoinduction plus Strontium 89 (Sr89) significantly improved patient survival in comparison to Sr89 or chemoinduction alone. The improved survival of patients subjected to co-targeting of bone stroma and epithelium by chemoinduction and Sr89 dramatizes the advantages of co-targeting, and supports our strategy of co-targeting tumor epithelium and bone stroma by gene therapy, which could eventually result in improved survival of patients with metastatic bone disease.

SUMMARY

The study of tissue-specific promoters such as PSA and hOC in AI prostate cancer cells has led to the identification of some of the transcription factors (AR, p45, Runx2, and Fra2) that are dysregulated during cancer progression. Although the roles of these transcription factors in tumorigenesis are unclear, it is feasible to use approaches like ribozyme and antisense to block the expression of these transcription factors and determine their respective effects on tumor growth and invasion. Once the functions are established, we believe effective strategy employing tissue-specific and tumor-restrictive promoters to control gene expression in selec-

tive cell types could be designed to target these transcription factors for cancer gene therapy.

In the past few years, our laboratory has successfully developed a human prostate cancer skeletal metastasis model for the study of the biology and therapeutic targeting stromal-epithelial interactions. We believe the inter- and intra-cellular communication loops between prostate epithelium and its supporting stroma provide additional attractive therapeutic targets for prostate cancer treatment. Results from preliminary animal experiments support the concept that maximum prostate tumor destruction may be achieved by targeting both tumors and their supporting stroma compartments. The development of bone matrix protein promoters, such as OC, driving gene expression in both prostate epithelial and stromal compartments has allowed future explorations into prostate cancer/stroma interactions and signal cascades involving growth factor/growth factor receptor and cell-matrix interactions. Since bone matrix proteins have been implicated in prostate cancer progression, OC promoter could be valuable for delivering genes into both epithelium and stroma compartments to block the expression/secretion of critixcal factors that affect the growth and survival of cancer cells in vivo. Refining our understanding of the regulation of PSA and bone matrix proteins in prostate cancer cells at the molecular level could facilitate the future development of new molecular targets for the prevention and treatment of not only prostate cancer skeletal metastasis but also localized and invasive prostate cancers.

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