

## Review

# Molecular therapeutics in prostate cancer

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**Summary.** The purpose of this review is to provide information on the molecular basis of prostate cancer biology and to identify some of the targets for therapy, and highlight some potential strategies for molecular treatment. Here we give a synopsis of what we have learned regarding molecular biology of cancer in general and the directions research might take in the future in order to impact prostate cancer specifically. This work is certainly not encyclopedic in nature and we apologize in advance to colleagues whose work we were not able to include. Hope lies in learning to utilize some of these molecular workings for better prevention, diagnosis, and treatment of the most common solid organ cancer in men. Prostate cancer is a formidable disease and at current rates of diagnosis will affect one-in-six men living in the United States (Greenlee et al., 2000) Many of these men are diagnosed at an early stage of the disease and can be effectively treated by surgery or radiation. However, a significant fraction of men are diagnosed with later stage disease or progress despite early curative therapeutic attempts. Unfortunately, many of these men succumb to prostate cancer, as management options are limited and not always successful. Through an understanding of the molecular processes that occur in the development and progression of prostate cancer, novel therapies will arise that will provide longer survival, better quality of life, and a chance for cure in men afflicted with this disease.

**Key words:** Prostate cancer, Therapy, Molecular therapeutics, Metastasis, Angiogenesis

### The life of the cell

#### *Cell growth*

Normal cells abide by an internal clock; they progress through a sequence of events known as the cell cycle. Different cells have varying time on this clock and

the body regulates which cells divide into new (daughter) cells and how long it takes them to do so. It is in this manner that the body replaces worn out cells or makes more cells when needed, for example the production of liver cells in response to injury and the turnover of gastrointestinal epithelial lining cells. Cancer cells, in contrast, escape the body's regulatory system and multiply despite being unwanted and unnecessary. Cells have a period when duplication of DNA occurs (synthesis phase), and a period when chromosomal copies are segregated to opposite ends of the cell (mitosis) permitting subsequent cellular division. These two phases are separated by checkpoints wherein cells determine that there are no mistakes in the replication procedure. If errors are found they are either repaired, or if the mistake is irreparable the cell is programmed to stop replication and will die, thus preventing the production of faulty progeny. Therefore, it is a balance between mitosis and programmed cell death (apoptosis) that gives the body a relatively constant number of healthy cells throughout life (Fig. 1).

The cell is usually in a quiescent state during which time it performs the functions for which it was created; this is termed the  $G_0$  phase. Growth factors and steroid hormones can stimulate the cell to enter into the beginning of the growth cycle. The initial cell-cycle phase is termed the  $G_1$  phase (first gap phase) and represents the pre-synthesis period where the cell accumulates the building blocks necessary for replication. There is a checkpoint between the  $G_1$  phase and the synthesis phase (S phase) known as the  $G_1/S$  checkpoint. Once preparations are completed, the S phase begins and each chromosome is copied. Following the complete replication of the DNA content, there is a second gap period or  $G_2$  phase during which time the cell prepares for mitosis. When all is in order, confirmed at the  $G_2/M$  checkpoint, mitosis occurs in the M phase. The cell divides into two daughter cells; the nuclei reorganize, cytoskeletal components are rebuilt, and cell membranes are sealed, thus completing the cell cycle.

Checkpoint regulators play a large role in preventing the replication of faulty cells and thus the formation of cancer. Cells utilize a number of brakes at these checkpoints and the master brake is the retinoblastoma

(Rb) protein. Rb is present in all cells and works in the nucleus at the G1/S checkpoint to halt progression. When the cell is ready to proceed, Rb is modified by phosphorylation via the regulatory protein cyclin-dependent kinase (CDK). CDK, as the name implies, requires the protein cyclin for its activation. Phosphorylation of Rb removes the brake by inactivation and allows the cell to enter the S phase. Some tumor suppressor genes also act as brakes, the best known being p53. CDK itself can be inactivated by cyclin-dependent kinase inhibitors (CDKI), which in turn are induced by the protein p53. When a cell recognizes damage to its DNA the p53 protein is activated, thus indirectly preventing the Rb brake from removal (Fig. 1).

**Programmed cell death (apoptosis)**

The decision for a cell to enter programmed cell death, or apoptosis, can be initiated by the detection of irreparable DNA damage. The mechanisms by which cells identify damaged DNA is incompletely described, but may involve the addition of a special polymer to the ends of broken DNA onto which poly-ADP chains are then added. This poly-ADP string sets off a signal that begins the cell death pathway. In addition to DNA damage, cells can be disrupted from the extracellular matrix anchorage or the internal cytoskeleton may be damaged, which also set a course for cell destruction. Furthermore, there are antagonists to growth factors such as tumor necrosis factor (TNF) and Fas-ligand that can signal the start of apoptosis. Negative growth factors such as Transforming Growth Factor-beta (TGF-β) are also known to activate cell death in epithelial cells.

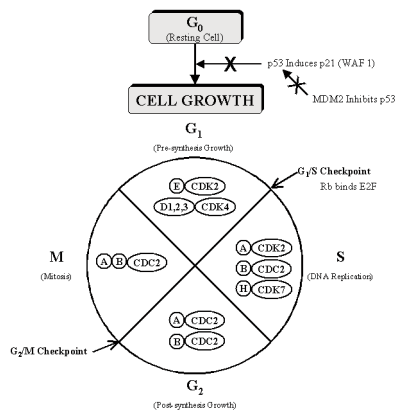
The absence of “survival factors” may also induce

cell death. A notable example in prostate cancer is how the absence of androgen can induce cell death. Similarly, loss of Epidermal Growth Factor (EGF) or fibroblast growth factors (FGF2 and FGF7) will promote cell death. Interestingly, Vascular Endothelial factor (VEGF), a potent angiogenic factor in many developmental and malignant processes including prostate cancer, is also a “survival factor” for tumor endothelial cells (Benjamin et al., 1999). Loss of this factor not only prevents new angiogenesis but also destroys many pre-existing tumor endothelial vessels by this mechanism resulting in necrosis of the tumor cells previously supplied by these vascular arcades.

Just as there are brakes for cell growth, there are also brakes for cell death. It is these regulators that are manipulated by cancer cells to prevent apoptosis allowing for unchecked tumor growth. Manipulation of these factors, in order to force the cell into a suicidal death, is under active investigation. Moreover, the survival factor B-cell chronic lymphocytic leukemia/lymphoma 2 gene (Bcl-2) was originally identified in follicular cell lymphoma and inhibits apoptosis (Bissonnette et al., 1992) The Bcl-2 binding protein bax forms heterodimer with Bcl-2 and is required to remove the brake and allow apoptosis to proceed (Oltvai et al., 1993) (Table 1).

Once positive signals are activated, or negative signals removed, a series of events take place that result in death of the cell. Early events include the induction of the protease interleukin-converting enzyme (ICE), which activates one cascade of events, and the activation of several genes including the receptor for TGF-β and a nuclease that degrades DNA. Nuclear fragmentation ensues, which is the irreversible step, followed by destruction of the nucleus and finally by phagocytosis of the fragmented cell by macrophages and other scavengers.

The DNA itself has a mitotic clock. Normal cells accomplish approximately 50 doublings before they are no longer able to divide. This is due to a quirk in the replication of the DNA; DNA polymerase requires a template for attachment in order to replicate chromosomes. The lagging strand of bi-directional replication is incompletely copied and the cell loses a



**Fig. 1.** Regulation of the cell cycle depends largely on cyclin (A, B, D, E, and H) that are produced and degraded during specific phases of the cell cycle. Cyclins activate cyclin-dependent kinases (CDK2, CDK4, CDK7, and CDC2) that remains at a relatively constant level throughout the cell cycle resulting in phosphborylation of key regulation proteins. For example, cyclin-CDK complexes in G<sub>1</sub> phase phosphorylate Rb protein releasing the transcription factor E2F, which then induces proteins required to enter S phase.

**Table 1.** Factors involved in cell growth and cell death.

GROWTH PROMOTING FACTORS	GROWTH INHIBITING FACTORS	DEATH PROMOTING FACTORS	DEATH INHIBITING FACTORS
Androgen	Retinoblastoma protein (Rb)	Tumor Necrosis Factor (TNF)	Bcl-2
Fibroblast Growth Factor 2	p53	Fas (CD95)	
Fibroblast Growth Factor 7	p21	Transforming Growth Factor Beta (TGFβ)	
Epithelial Growth Factor	p16		

small portion of the chromosome each division. Therefore, the end of chromosomes consists of a repeating segment of DNA that is not used for encoding messages, called telomeres. The telomeres are designed to be clipped and may be measured experimentally to indicate the age of the cell, akin to rings in a tree trunk. When the telomeres become too short the cell assumes a pathway to death. Immortal cells, including cancer cells and stem cells such as bone marrow precursors, intestinal cells and spermatogonia, avoid this problem by expressing an enzyme called telomerase. New telomere length is obtained through the action of telomerase, which synthesizes the DNA repeating segment on its own template. In this way they avoid a major pathway to apoptosis. As one would expect, telomerase activity is one of the best markers available to delineate cancer cells from differentiated epithelial cells of the same organ as been shown for prostate cancer cells (Sommerfeld et al., 1996). An excellent recent review in the current journal addressing apoptosis in prostate cancer further delineates these processes (Kyprianou et al., 2000).

### **Mechanisms of cancer development and growth of the primary tumor**

#### *DNA changes*

##### Mutation

If only one of the cells in any given organ fails the checkpoint mechanisms and divides with faulty DNA the birth of cancer may ensue. One major way DNA is damaged is by reactive oxygen species, such as superoxide, hydrogen peroxide, and hydroxyl radicals. These are formed as byproducts of mitochondrial aerobic oxidation incompletely reducing oxygen to water, as well as by carcinogens, ionizing radiation, and ultraviolet light. These by-products are termed free radicals because they lack the ability to be paired; they have the propensity to attack proteins, lipids, and DNA within the cell. Specifically, the nucleotide guanine is converted to 8-oxoguanine, which leads to mutations in the DNA as 8-oxoguanine preferentially mispairs with adenine. Cells attempt to repair this error by excising the 8-oxoguanine and replacing it with the proper guanine, excreting 8-oxoguanine in the urine. Since reactive oxygen species are always being created the cell has a number of enzymes that protect against damage from these species as well as damage from other harmful agents that attack DNA. Antioxidants act as scavengers for reactive oxygen species. These include the lipid-soluble vitamin E, Selenium, Lycopene and others.

##### Methylation

In contrast to changing the composition of the DNA code (mutation), modulation of the DNA in the form of methylation can occur. The pattern of DNA methylation

can affect the expression of genes by altering protein interaction with DNA. The state of methylation of the nucleotide cytosine is inheritable and is termed DNA imprinting. Genes have a promoter region that regulate their activity and contain repetitive units containing cytosine termed CpG islands. These islands change the local three-dimensional structure of the DNA containing the gene causing them to remain in an inactive state. Clearly, if heavy methylation of these CpG islands within the promoters of tumor suppressor genes occurs, as reported in nearly one-half of tumor suppressor genes in cancers, protection against the development of tumors is diminished (Rountree et al., 2001) The most common genetic change in prostate cancer is hypermethylation of the CpG islands of glutathione-S-transferase  $\pi$  one of the major enzymes protecting against reactive oxygen species damage (Lee et al., 1994). Through this link, one can see how methylation abnormalities can enhance mutation risk.

#### *Prostate cancer genetics*

Multiple insults are required to form cancer as cells have backup mechanisms of protection. As proposed by Alfred Knudson, an individual inherits two copies of genes and both copies must be affected to induce cancer (Knudson, 1971) While most prostate cancers appear to be sporadic, 10% are inherited. The first reported prostate cancer susceptibility gene is found on chromosome 1 (HPC1) (Smith et al., 1996). Families that carry the HPC1 gene have multiple affected members and prostate cancer tends to be diagnosed at an earlier age (less than 65). There are other genes that contribute to familial prostate cancer and further characterizations of these abnormalities are active areas of research. Further evidence for a genetic predisposition towards prostate cancer is the difference between racial groups. For example, black men have a greater incidence of the disease that also tends to present earlier than in white men. However, unlike previously believed, these men do not have more aggressive cancers than their Caucasian counterparts (Merrill and Lyon, 2000). Sporadic, or non-inherited, prostate cancer then represents 90% of the men with this disease. These men tend to harbor genetic abnormalities and chromosomes 7, 8, 13, and 17 have all been described to have changes in prostate cancer. Some of the mutated genes include Rb, bcl-2, p53, androgen receptor, PTEN, p16, p27, and ras. For example the mutation of the tumor suppressor gene p53 is very common in advanced disease, but not frequent in localized prostate cancer and thus serve as good predictors of tumor behavior (Theodorescu et al., 1997b; Krupski et al., 2000).

#### *Signal transduction*

There is a tremendous amount of communication that occurs between cells within an organ and between different organs. A signal that operates within the cell in

which it was made is termed an “intracrine” factor. Autocrine factors are secreted by a cell and in turn signal the very same cell. Cells also signal neighboring cells via a “paracrine” mechanism and signals can be transported through the circulation to affect distant cells in an endocrine fashion (the latter signals being known as hormones). Nerves can transport signals (“neurocrine”) and immune cells can secrete “cytokines” as signals. Moreover, cells can communicate directly by utilizing pores formed by cell adhesion molecules (CAMs) connected to the cytoskeleton, akin to adjoining hotel rooms. Additionally, cells make contact with extracellular matrix (ECM) by binding integrins that also attach to the cytoskeleton. In fact, what a cell touches largely determines what a cell does, from the embryonic stages of organogenesis to cancer cells trying to attach in foreign sites in the process of “metastasis”. This is an active area of research and finding ways to modulate cell signaling is a major effort.

As discussed, growth factors affect the regulation of cell growth and death. This is accomplished via interactions with receptors on the cell surface, which leads to cellular events or a cascade of cellular events that will eventually transmit the signal to the nucleus and effect the expression of genes. At least five families of growth factors influence prostate cancer in an autocrine or paracrine fashion: TGF- $\beta$ , EGFs, FGFs, insulin-like growth factors (IGFs), and platelet-derived growth factors (PDGFs). Additionally, hormones acting in an endocrine manner, such as androgen, can also alter the growth characteristics of prostate cancer.

The TGF- $\beta$  family contains at least five forms with the TGF- $\beta$ 1 form being the only one inappropriately expressed in prostate cancer and benign prostatic hyperplasia (BPH) (Truong et al., 1993). The usual function of TGF- $\beta$ 1 is inhibition of normal epithelial cell growth, but it has been shown to be stimulatory in several epithelial cell lines as a function of tumor progression (Theodorescu et al., 1991; Huang et al., 1995). In addition, it can stimulate growth of prostatic supporting stromal cells under some conditions. TGF- $\beta$ 1 can also encourage tumor progression by promoting angiogenesis and inhibiting the immune response. Prostate cancer is often insensitive to the growth inhibition normally affected by TGF- $\beta$ 1. Of the FGF family, the FGF-1 and FGF-2 subtypes are able to promote angiogenesis and can be mitogenic in animal models. FGF-2 has been demonstrated to increase expression in highly malignant experimental prostate cancer cell lines as opposed to less aggressive prostate cancer cells (Nakamoto et al., 1992). The EGF family includes EGF, tumor growth factor-alpha (TGF- $\alpha$ ), and amphiregulin, all of which are expressed in both prostate cancer and normal prostatic tissue. EGF and TGF- $\alpha$  are mitogens and both interact with the EGF receptor. Significant epithelial changes occur in response to TGF- $\alpha$  including dysplastic changes in the epithelium resembling prostatic epithelial neoplasia (PIN). The role of the IGF axis in prostate carcinogenesis is gaining attention. Elevated plasma levels of IGF-1 predict an

increased prostate cancer risk of up to six-fold, though direct evidence that IGF-1 causes the initiation of prostate cancer is lacking. Ongoing work to delineate the role of the IGF axis in prostate cancer development and progression should provide important information on these growth factors and their binding proteins (IGFBPs). Generally, IGFs promote prostate cancer growth while IGFBPs are largely inhibitory (Grimberg and Cohen, 2000). IGFBPs are overexpressed in prostate cancer cells, with the exception of IGFBP-3, while IGFs are usually downregulated. IGFs along with the IGF receptors and IGFBPs are regulated by androgens and the IGF axis is involved in the evolution towards androgen independence. Although IGFBPs are predominately inhibitory to prostate cancer cell growth, the enzyme prostatic specific antigen (PSA) is capable of cleaving IGFBP-3 resulting in mitotic activity (Cohen et al., 1992).

### **Androgen regulation**

Androgen regulates both the development of the prostate and many aspects of prostate cancer growth. Eunuchs do not get prostate cancer therefore demonstrating that this hormone is necessary for the development of this disease. Androgens have been shown to increase the level of oxygen free radical formation in androgen-dependent LNCaP cells but not androgen-independent DU145 cells by altering the balance between pro-oxidant and anti-oxidant states. In large part this is secondary to increased mitochondrial activity in response to mitogenic stimulus and can be inhibited by anti-oxidants such as ascorbic acid (Ripple et al., 1997).

Prostate cancers are initially composed of a majority of cells which require androgen for proper growth and thus lack of androgen can cause these cells to die. The Nobel Prize was awarded to Charles Huggins in 1966 for this discovery, which led to present day hormonal therapy. However, the larger and more advanced the cancer is prior to chemical or surgical castration the lower the response rate, namely the number of cells which are going to die and thus tumor shrinkage in response to androgen withdrawal. In addition, after castration, the cancer continues to grow, albeit more slowly, and during this time often becomes androgen-independent (AI), no longer requiring androgens for proliferation.

The androgen receptor (AR) is encoded on the X chromosome and is responsible for binding androgen and taking it to the nucleus where it affects a cell response. The action of androgen on target cells is mediated through the androgen receptor (AR), which exists in the cytoplasm in an inactive hypophosphorylated form in complexes with heat shock proteins (hsp). Androgen enters the cell and binds the ligand-binding domain of AR resulting in dissociation with heat shock proteins and phosphorylation of AR. These effects result in conformational changes in AR producing ligand-activated AR that from homodimers

and are translocated in to the nucleus. In the nucleus, AR dimers associate with the androgen-response elements (AREs) in the promoters of hundreds of target genes thereby regulating transcription (Koivisto et al., 1998). A number of co-activator proteins, such as TIF-2 and GRIP-1, and co-repressor proteins bind the AR-DNA complex and influence gene transcription. The AR is upregulated after androgens are withdrawn by both surgical and pharmacologic castration. Complicating the story is the ability of AR to respond to peptide growth factors even in the absence of androgen. IGF-I, EGF, and keratinocyte growth factor (KGF) have been demonstrated to activate AR in this manner (Culig et al., 1994). During tumor progression and development of the AI phenotype, spontaneously occurring mutations in the AR can lead to promiscuous activation of this receptor by progesterone or antiandrogen medications used in the initial treatment of this cancer. This may allow estrogens or antiandrogen therapy to activate the AR, perhaps explaining the improvement seen in some patients after withdrawing antiandrogen therapy (Sartor et al., 1994). The frequency of androgen receptor mutations in AI prostate cancer is unknown, though an increase in the X chromosome region containing AR has been demonstrated in some men unsuccessfully treated with hormonal therapy. Thus, it is possible that instead of becoming insensitive to androgen prostate cancer may become supersensitive or even respond to non-androgen steroid hormones. In addition to mutations in the AR, androgen-independent prostate cancers demonstrate a heterogeneous loss of AR expression in 20-30% of tumors. Regulation of AR occurs at least in part through DNA methylation of a specific region of the minimal promoter, similar to silencing due to methylation in many other genes (Jarrard et al., 1998; Kinoshita et al., 2000). The androgen receptor therefore certainly plays an important role in the subset of prostate cancers that are hormone refractory.

Vascular endothelial growth factor (VEGF) is one of the major regulators of angiogenesis in prostate cancer and is downregulated by anti-androgen therapy. In both *in vitro* and *in vivo* mouse xenograft human prostate cancer models androgen withdrawal results in decreased VEGF expression, and reversal of neovascularization in xenografts. This was found to be an early event in the tumor response to therapy (Stewart et al., 2001). Furthermore, complete androgen blockade before radical prostatectomy is demonstrated to downregulate the expression of VEGF and decrease vascularization in tumor specimens, except in areas with neuroendocrine (NE) features (Mazzucchelli et al., 2000). These data suggest that the acute response to androgen withdrawal resulting in decreased vascularization could inhibit primary tumor growth and prevent the formation of micrometastasis.

### Progression of disease

Two histologic lesions are considered malignant

precursors of invasive prostate cancer. Prostatic intraepithelial neoplasia (PIN) is the histologic abnormality most commonly associated with prostate cancer and is proposed as a precursor of invasive prostate cancer. This lesion is the prostate equivalent of "carcinoma in situ" in other cancers. PIN is segregated into high-grade (HGPIN) and low-grade (LGPIN) classifications and it is the HGPIN variety that is considered to be the most likely precursor to invasive carcinoma (Graham et al., 1992). Autopsy studies indicate a relationship between HGPIN and prostate carcinoma with HGPIN development occurring as early as the fourth decade of life and 5-10 years prior to clinically detectable cancer (Sakr et al., 1994). In addition, HGPIN is frequently found in conjunction with carcinoma in radical prostatectomy specimens, and reported to be present in up to 86% of specimens in one study (Qian et al., 1997). Furthermore, the present of HGPIN alone on prostate needle biopsy portends a higher likelihood of adenocarcinoma and is an indication for repeat biopsy (Davidson et al., 1995). What is still unclear is the exact risk of harboring invasive cancer in such a situation. As early as 1954 Franks proposed that epithelial hyperplasia was a precursor to prostatic carcinoma (Franks, 1954). Ten years later McNeal described PIN ("atypical hyperplasia") and published the classic paper associating PIN and adenocarcinoma in 1986 (McNeal, 1965; McNeal and Bostwick, 1986). Prostate carcinoma and PIN share multifocality and zonal similarities, and a majority of carcinomas have adjacent HGPIN within 2 mm (Qian and Bostwick, 1995). Atypical adenomatous hyperplasia (AAH) has characteristics that are intermediate between BPH and low-grade carcinoma but has far less evidence for acting as a precursor than HGPIN (Helpap et al., 1997).

### Metastasis

#### Metastatic sites

Metastasis is the battle line in prostate cancer as localized prostate cancer can often be effectively treated while metastatic prostate cancer is currently incurable. Prostate cancer grows at varying rates, but most men diagnosed with metastatic prostate cancer die over a period of months to years (Plesnicar, 1985). Therefore, the forefront of prostate cancer research is dedicated to preventing the development or curing patients with metastasis. Understanding the changes that occur at the molecular level during the progression to the metastatic phenotype is a necessary precursor to the development of novel therapeutic approaches. Prostate most frequently metastasizes to lymph nodes (pelvic and abdominal) and to bone (60-70% of patients suffering metastasis having bony involvement) with the spine, pelvis, sternum, ribs, and femurs being the most common skeletal sites (Mintz and Smith, 1934). Lymphatics originating from the prostate travel to the pelvis (iliac lymph nodes) upwards retroperitoneally

along the great vessels towards the heart.

The first report of prostate cancer bone metastasis was made by sir Thompson in 1854 and is responsible for much of the morbidity associated with this condition. Batson first described the venous plexus that drains the prostate and proposed a mechanical mechanism of prostate cancer seeding to the spine (Batson, 1942). In contrast, Paget proposed the "seed-and-soil" theory for metastasis, which highlights the importance of host factors at the metastatic site ("soil") that determines the preferential growth of metastatic prostate cancer cells ("seed") to sites such as bone (Paget, 1889). The former hypothesis is no longer thought to be valid.

#### *Development of metastasis*

The steps in the progression to metastasis include angiogenesis, cell attachment, invasion (basement membrane degradation), migration to a suitable environment, and proliferation (Woodhouse et al., 1997). Each of these steps is regulated and is therefore a potential target for molecular treatment. Interfering with any or all of these steps should impede the ability of cancer cells to metastasize.

As tumors grow they require new blood vessels from which they obtain oxygen and nutrients, without which they are unable to grow beyond 2 mm (Folkman and Klagsbrun, 1987). Furthermore, the addition of new vessels increases the odds for tumor cells reaching the circulation through which they may metastasize. In particular, prostate cancer has a negative correlation between progression and the degree of angiogenesis independent of Gleason score (Silberman et al., 1997). Angiogenesis can be divided into several steps: proliferation of endothelial cells, breakdown of ECM, migration of endothelial cells toward the chemotactic angiogenic stimulus (such as VEGF) and finally tube formation followed by blood circulation through the lumen (Denijn and Ruiter, 1993). Studies suggest that tumor cells release soluble factors that induce an angiogenic response (Gimbrone et al., 1972). Angiogenic factors include vascular endothelial growth factor (VEGF), members of the FGF family, angiogenin, TNF- $\alpha$ , IGF-I, hepatocyte growth factor (HGF), and others. Members of the TGF- $\beta$  family have been shown to have both angiogenic and anti-angiogenic effects depending on the systems studied probably by affecting different tumor and host populations. There are other inhibitors of angiogenesis and modification or removal of these factors is a future therapeutic possibility. These inhibitors include interferon- $\alpha$  (IFN- $\alpha$ ), platelet factor-4, thrombospondin, angiostatin, pigment epithelium-derived factor, and tissue inhibitors of metalloproteinases (TIMPs). Angiogenesis is a highly regulated event balancing pro- and anti-angiogenic factors.

Cells within organs are generally attached to a foundation (ECM) and cancer cells must disassociate with the ECM in order to escape the primary tumor.

Integrins are membrane proteins that bind a variety of ECM molecules including laminin, fibronectin, vitronectin, and collagens. Furthermore, integrins have a crucial role in the attachment of tumor cells to ECM (Goldbrunner et al., 1996). The vitronectin receptor v<sub>3</sub> integrin is involved in the bone attachment mechanism of osteoclasts and is important in tumor metastasis to bone (Zheng et al., 1999). Cadherins are proteins that anchor cells to one another. For example, epithelial cadherins (E-cadherins) link to E-cadherins on adjacent cells through interactions with the catenin family of proteins (such as  $\beta$ -catenin). E-cadherin function is often lost during progression of many cancers, including prostate cancer (Umbas et al., 1994). Additionally, mutations in  $\beta$ -catenin occur in prostate cancer, or the promoter for E-cadherin can be hypermethylated, and E-cadherin can be disrupted by the matrix metalloproteinase (MMP) family member stromelysin-1 (Lochter et al., 1997). Other types of cell adhesion actually promote metastasis; these include immunoglobulins and vascular cell adhesion molecule-1, which as the name implies is involved in cell attachment to vascular endothelium.

The ECM forms a barrier through which cancer cells must traverse to escape the primary tumor. Invasion involves proteolysis of the ECM, pseudopodial extension, and cell migration (Stetler-Stevenson et al., 1993). The matrix metalloproteinases are a zinc binding family of proteins that disrupt ECM components; they are secreted in a proenzyme form and must be activated in the extracellular spaces. There are three classes of these molecules: interstitial collagenases, stromelysins, and gelatinases (type IV collagenases). Metalloproteinases inhibition occurs by the action of TIMPs. Tumor cell motility is stimulated by hepatocyte growth factor/scatter factor (HGF/SF), IGF-II, and autotaxin (ATX). Additionally, ECM proteins vitronectin, fibronectin, laminin, type I collagen, type IV collagen, and thrombospondin promote motility by chemotaxis or through interactions with integrin receptors (Leavesley et al., 1992). Host-secreted factors, sometimes called homing factors, cause tumor cells to move towards the organs in which they are produced and include IGF-I, interleukin-8 (IL-8), and histamine (Woodhouse et al., 1997).

Tumor establishment at the metastatic site occurs under the influence of paracrine and autocrine growth factors. However, as tumors progress and become increasingly malignant, they become decreasingly dependent on exogenous factors for growth. In the case of bone metastasis, bone marrow-derived growth factors include TGF- $\beta$ , IGF-I, and IGF-II. Furthermore, osteoblastic lesions arising from prostate cancer have increased growth in response to basic fibroblast growth factor (bFGF) secreted by osteoblasts (Gleave et al., 1991). IL-6 is another stimulatory factor secreted by osteoblasts and prostate cancer cells may respond to this interleukin as they express the IL-6 receptor (Siegall et al., 1990).

## Molecular targets in the treatment of prostate cancer

### *Targeting cellular processes: rationally based therapeutics*

As mentioned above, androgen withdrawal is the only effective treatment modality for advanced prostate cancer and will provide an objective response in the majority of patients. Unfortunately, progression to androgen independent (AI) disease, often causing death, occurs in many of these cases within a few years (Denis and Murphy, 1993). Hormone refractory prostate cancer (HRPC) therefore, is the main cause of the demise of patients with advanced disease. Until very recently, strategies that involved cytotoxic agents were the mainstay of investigative efforts in prostate cancer research. Despite the tremendous efforts to find an effective combination of chemotherapeutic agents to combat prostate cancer, results have been disappointing. Although the primary goal in the management of prostate cancer should be prevention of clinically significant disease, by alterations in lifestyle and diet for example, the likelihood of significantly affecting these changes is low. Therefore, research efforts are needed to aim at all phases of prostate cancer, from prevention to treatment of advanced localized disease to management of metastatic disease. The promise of molecular therapeutics resides with the potential of increased efficacy and decreased morbidity for all stages of prostate cancer by virtue of increased specificity.

A paradigm for translation of basic science research and therapeutic development may be found in the discoveries leading to the effective use of imatinib mesylate (Gleevec™) in leukemia (Garber, 2001). After thirty years of basic science research imatinib mesylate was the first drug approved by the Food and Drug Administration (FDA) that directly inhibits a protein known to cause cancer (Arnold, 2001). The FDA approved imatinib mesylate in a very quick two and one-half years (a process which often lasts ten years) primarily because the drug was extremely effective in Phase I and Phase II trials, but also because the basic science research provided a clear rationale for this treatment. Genetic studies identified a translocation event in chronic myeloid leukemia (CML) patients resulting in the Philadelphia chromosome (Ph+). Biochemical studies then demonstrated that in Ph+ patients, production of a Bcr-Abl protein-tyrosine kinase fusion protein causes CML. This abnormality in the constitutively active Bcr-Abl protein-tyrosine kinase was targeted for therapeutic intervention and led to the discovery that the protein-tyrosine kinase inhibitor protein imatinib mesylate can constrain Bcr-Abl *in vitro* and leads to inhibition of tumor growth and induction of apoptosis *in vivo* (Buchdunger et al., 1996). In phase I trials, 53 of 54 patients treated with a dose of 300mg or greater had a complete hematologic response (Druker et al., 2001b). Additionally, in advanced disease, a 55% response rate (19% complete response) was observed in

CML patients with myeloid blast crisis and a 70 % response rate (20% complete response) in acute lymphocytic leukemia (ALL) patients harboring the Philadelphia chromosome combined with patients suffering a lymphoid blast crisis (Druker et al., 2001a). The rational step-wise research approach resulting in imatinib mesylate approval serves as a model to cancer investigators and accentuates the value of careful characterization of tumor mechanisms before therapeutic investigation.

### *Chemoprevention*

As mentioned above, generation of free radicals has significant potentially detrimental effects on cellular processes that may lead to carcinogenesis and tumor progression. The epidemiology of prostate cancer is incredibly different in Asian countries as compared to Western Europe and the United States. Additionally, men who migrate from Asia to the United States acquire an epidemiologic profile similar to other Americans (Whittemore et al., 1995). Although the reason for these changes is not completely delineated, dietary factors, particularly the “Western diet” high in saturated fats, have been touted as a positive risk factor of prostate cancer incidence and mortality (Kolonel et al., 1988). A putative protective effect of soy proteins, particularly the isoflavones has been postulated (Blumenfeld et al., 2000). Furthermore, selenium also has been postulated to be a protective agent, though more studies are needed to confirm this hypothesis (Clark et al., 1998).

The antioxidant lycopene, present in tomatoes, was shown in the Health Professionals Follow-up Study (HPFS) to lower the risk of developing prostate cancer (Giovannucci et al., 1995). Oxidative stress, in combination with faulty cellular defenses, has been suggested from both epidemiologic and molecular biology studies. The ability to inhibit free-radical damage by modulation of oxidative mechanisms has a promising outlook as a prostate cancer chemoprevention strategy. The aforementioned Phase 2 enzyme GSTP 1 is inactivated in prostate cancer and is not expressed in PIN as well. Replacing the function of this enzyme by gene therapy or agents that induce a large amount of other Phase 2 enzymes may be an effective method of stopping prostatic carcinogenesis or progression of prostate cancer (Wang et al., 1999).

### *Hormonal activity*

Dihydrotestosterone (DHT), the 5- $\alpha$ -reduced product of testosterone, binds to androgen receptors with 2.5 fold higher affinity than testosterone itself, and serves as the major regulator of prostatic tumor growth. Androgen mediated cancer proliferation occurs by direct effects of androgens, indirectly by growth factors stimulated by androgens, and by a combination of these mechanisms (Cuif et al., 2000). Approximately 7,000 mg of testosterone is secreted daily by the testes of

which only 7% is converted into DHT in peripheral tissues (Cuif et al., 2000). The testes produce 95% of androgens in men, while the adrenal gland accounts for the remaining 5% of hormone (Sanford et al., 1977). Testosterone, as well as precursors to androgens such as androstenedione, dehydroepiandrosterone (DHEA), and DHEA sulfate, originate in the adrenal gland and are likewise converted to DHT in peripheral tissues. Approximately 40% of prostatic DHT originates from steroids of adrenal origin (Geller et al., 1984). Androgen dependent prostate cancer contains androgen receptors that bind DHT and transmit proliferative signals to the nucleus (Newmark et al., 1992).

The responsiveness of the prostate gland and most prostate cancers to androgen indicates the importance of this hormone, as well as the ability to manipulate its action, in prostate cancer treatment. Many abnormalities in AR expression occur during prostate cancer progression and the AR gene is amplified in 30% of hormone refractory prostate cancers and multiple copies of chromosome X are found in 20% of such tumors (Visakorpi et al., 1995; Koivisto et al., 1997). Additionally, mutations of the androgen receptor occur, but the frequency in primary prostate cancer is controversial (Taplin et al., 1995). An early study found a 30% incidence of androgen receptor mutations in primary prostate cancers, while others have found a much lower frequency ranging from 0-5%. However, all investigators find mutations in metastatic disease ranging from 21% to 50% (Avila et al., 2001). The frequency and type of mutations appear to be influenced by selective pressure exerted by anti-androgens (Taplin et al., 1999). As previously discussed, these mutations may be the reason we see the flutamide withdrawal response (Richie, 1999). Drawing a parallel with breast cancer, in which estrogens are known to play an important role and the antiestrogen tamoxifen has been shown to decrease risk, the Prostate Cancer Prevention Trial (PCPT) is studying 18,000 men to determine if the 5- $\alpha$  reductase inhibitor finasteride will effect the development of clinically significant prostate cancer (Thompson et al., 1997; Fisher et al., 1998). In time the PCPT should provide insight regarding the efficacy of finasteride on preventing invasive prostate cancer.

In contrast to breast cancer, where estrogen receptor (ER) and progesterone receptor (PR) expressions are lost in hormone refractory disease, AR mRNA is upregulated *in vitro* in androgen-independent prostate cancer cell lines (Dai et al., 1996). Furthermore, *in vivo* studies have demonstrated high levels of AR expression as well as increased expression of androgen-regulated genes in castrate versus hormonally intact human prostate cancer xenografts (Gregory et al., 1998). The maintenance of androgen-regulated genes in the absence of androgen could occur via an AR-independent mechanism, however, the importance of androgen-independent activation of AR itself is becoming increasingly recognized. In the absence of androgen, cytosolic AR can be phosphorylated and activated by alternative

kinase pathways. For example, the protein kinase A (PKA) activator, forskolin, was shown to activate AR *in vitro* in the absence of androgen; this effect could be blocked by a PKA inhibitor protein and partially blocked by the competitive inhibitors flutamide and bicalutamide. The authors demonstrated that AR activation in this manner is dependent on a functional AR DNA binding domain by mutational studies (Nazareth and Weigel, 1996). Other studies have shown androgen-independent activation of AR involving androgen-activated protein (MAP) kinase, HER-2/neu receptor tyrosine kinase, and cyclin-dependent kinases (Abreu-Martin et al., 1999; Craft et al., 1999; Yeh et al., 1999; Gregory et al., 2001). A separate mechanism of androgen-independent AR activation occurs through direct binding of growth factors to cytosolic AR. As previously mentioned, IGF-1, KGF, and EGF all are capable of activating the AR in the absence of androgen (Culig et al., 1994). FGF-1 and FGF-2 however, were shown to lack this ability *in vitro* (Shain, 2001). Interestingly, blockade of the EGF receptor stimulated pathway with the specific inhibitor of PKA (H89) in DU145 cells was found to inhibit not only the action of EGF on the MAP kinase system, but also IGF-1 activation of MAP kinase as well as the interaction between the kinase pathways PKA and MAP kinase (Putz et al., 1999). PKA pathway inhibition with H89 in DU145 cells has also been shown to abolish the neuropeptide calcitonin, which is secreted in neuroendocrine variants of androgen-independent tumors, mediated activation of MAP kinase (Segawa et al., 2001). Similarly, the epidermal growth factor receptor (HER)-2/neu inhibitor typhostin AG825, a cell-permeable tyrosine kinase inhibitor, preferentially induced apoptosis in androgen-independent C4-2 cells but not androgen-dependent LNCaP cells (Murillo et al., 2001). This complex and convergent activation of AR, along with mutations in AR that allow activation by anti-androgens, likely plays an important role in androgen-independent mitogenic stimulation by AR in hormone refractory disease. Strategies inhibiting AR activation in the absence of androgen could be utilized to reduce autonomous tumor growth. A monoclonal antibody against AR has been developed (F52.24.4) against the C-terminal portion of the DNA binding domain (Veldscholte et al., 1992). It may be possible that disruption of AR interaction with DNA by such an antibody could inhibit the penultimate step in AR-responsive genes. Similarly, any strategy to knockout AR in hormone independent prostate cancers may prove to alleviate the mitogenic stimulus by AR in these cancers. Otherwise, delineating the relative importance of the aforementioned mechanisms of AR activation could narrow the approach to designing treatment alternatives.

### Angiogenesis

Agents that have the ability to impede a tumor's



ability to form new blood vessels will decrease the size to which a tumor may grow and perhaps inhibit a tumor's ability to metastasize by decreasing the number of vessels to which it has access. The ability of a tumor to grow beyond 2 mm in diameter depends on both tumor cell proliferation and inducing the growth of new capillary blood vessels from the host, a process called angiogenesis. This occurs via secretion of soluble factors such as vascular endothelial growth factor (VEGF). This may permit a tumor to expand by as much as 1,000-16,000 times its primary volume in weeks or months (Gimbrone et al., 1972). Thus, no matter how strong a growth stimulus a cancer cell has, further tumor growth does not occur unless a tumor becomes vascularized (Folkman, 1992).

During angiogenesis, endothelial cells move from a resting state to one of rapid growth when exposed to diffusible factors secreted by tumor cells. VEGF was the first selective angiogenic growth factor to be purified, and is still a preeminent molecule in this area. Many human tumor biopsies exhibit enhanced expression of VEGF by malignant cells and VEGF receptor in adjacent endothelial cells. Abrogation of VEGF function with monoclonal anti-VEGF antibodies results in complete suppression of prostate cancer induced angiogenesis, and prevents tumor growth beyond the initial prevascular growth phase (Borgstrom et al., 1998). Tissue staining has demonstrated that human prostate cancer is positive for VEGF, while BPH and normal prostate cells displayed little VEGF staining and vascularity (Ferrer et al., 1998). Other studies have demonstrated that castration inhibits prostate cancer VEGF production, but had no effect on other angiogenic factors (Joseph and Isaacs, 1997). Since surgical or chemical castration is a mainstay of prostate cancer therapy, this finding would suggest that VEGF plays an important role in this process. Additionally, increased VEGF expression has been related to neuroendocrine differentiation in prostate cancer, a known poor prognostic factor for survival (Harper et al., 1996; Theodorescu et al., 1997a). Taken together, these data suggest that the prostate tumor growth advantage conferred by VEGF expression appears to be a consequence of stimulation of angiogenesis. One therapeutic design might include utilizing anti-sense-VEGF cDNA with gene therapy to disrupt angiogenesis in prostate cancer.

The various VEGF forms bind to two tyrosine-kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), which are expressed almost exclusively in endothelial cells. Endothelial cells express in addition the neuropilin-1 and neuropilin-2 coreceptors, which bind selectively to the 165 amino acid form of VEGF (VEGF<sub>165</sub>). Fetal liver kinase 1 (Flk-1) receptor tyrosine kinase associates with VEGF as a high affinity ligand and is suggested to have a major role in angiogenesis (Millauer et al., 1993). Analysis of VEGF and Flk-1 receptor expression in benign prostate glands, PIN and prostatic carcinomas of different Gleason scores, was performed on 21 radical prostatectomy

specimens. In all benign glands, VEGF and Flk-1 expression were confined almost exclusively to the basal cell layer; PIN labeling was no longer confined to the basal cell layer, but also was seen in all neoplastic secretory cells. All carcinomas stained positive for both markers and there was a trend for increasing labeling intensity with increasing cellular dedifferentiation (Kollermann and Helpap, 2001). The drug SU5416 decreases Flk-1 phosphorylation and inhibits vascular endothelial growth factor (VEGF)-driven neovascularization. Phase I and Phase II trials of SU5416 in patients with intermediate to hormone-refractory prostate cancer are currently active. In addition, a small molecular weight inhibitor of KDR and Flt-1 and compatible with chronic oral administration was recently developed (Wedge et al., 2000). ZD4190, a substituted 4-anilinoquinazoline, is a potent inhibitor of VEGF stimulated HUVEC proliferation in vitro. Chronic once-daily oral dosing of ZD4190 to mice bearing established human tumor xenografts (breast, lung, prostate, and ovarian) elicited significant antitumor activity and at doses that would not be expected to have any direct antiproliferative effect on tumor cells. Prolonged tumor cytostasis was further demonstrated in a PC-3 xenograft model with 10 weeks of ZD4190 dosing, and upon withdrawal of therapy, tumor growth resumed after a short delay consistent with its purported effect on angiogenesis.

The fungus *Aspergillus fumigatus* secretes an antibiotic fumagillin, and along with its synthetic analog TNP-470, inhibits endothelial cell growth in vitro but is not toxic (Ingber et al., 1990). TNP-470 has been found to be effective *in vivo* against renal tumors, rhabdomyosarcomas, and hepatomas (Morita et al., 1994; Kalebic et al., 1996; Yoshida et al., 1998). Additionally, PC-3 cell xenograft tumor growth is inhibited by TNP-470, which is also synergistic when used with cisplatin despite the fact that PC-3 cells in monolayer culture were insensitive to this agent (Yamaoka et al., 1993). Studies of the transcriptional activation of androgen receptor and PSA in prostate cancer cells in vitro revealed a 1.2-fold and a 1.4 induction with TNP-470, respectively, indicating that using PSA as an endpoint in clinical trials might be misleading (Horti et al., 1999). A phase I trial of TNP-470 in 33 patients with metastatic and androgen-independent prostate cancer has been completed. Dose escalation was performed and the dose-limiting toxic effect was a characteristic neuropsychiatric symptom complex that resolved after discontinuation of the drug. The authors report no definite antitumor effect in their trial (Logothetis et al., 2001). Recently, a CKD-731 analog has been developed and is reported to have 1000-fold more inhibition of endothelial cell growth than TNP-470 (Han et al., 2000). Fumagillin analogs show promise and may provide the specificity needed to suppress endothelial mitogens without being prohibitively toxic.

Platelet factor 4 (PF4) is a chemokine derived from

the precursor  $\beta$ -Thromboglobulin; it is produced in megakaryocytes and platelets and released from alpha granules in activated platelets. PF4 release from platelets results in its rapid binding to endothelial cells where it is then released by heparin in a time-dependent manner. Immunological functions of PF4 include chemotaxis for monocytes and neutrophils, promoting neutrophil attachment to endothelial cells, and activating neutrophils causing degranulation. PF4 also has pro-coagulation properties: neutralizing the anti-coagulatory activity of heparin sulfate in the extracellular matrix of endothelial cells, inhibiting local antithrombin III activity, and accelerating the formation of blood clots after injury (Zucker and Katz, 1991). An additional potential therapeutic action of this molecule is its ability to inhibit collagenase *in vitro*, though no *in vivo* confirmation of this effect has been reported (Hiti-Harper et al., 1978). A recombinant form of human PF4 (rHuPF4) inhibited blood vessel proliferation in the chicken chorioallantoic membrane in a dose-dependent manner and *in vitro* studies suggested that the angiostatic effect was due to specific inhibition of growth factor-stimulated endothelial cell proliferation. This antiangiogenic effect could be abrogated by adding heparin to the assay (Maione et al., 1990). Further studies demonstrated that rHuPF4 inhibited the migration of human endothelial cells *in vitro* and suppressed tumor growth in murine melanoma and human colon carcinoma cell lines *in vivo* (Sharpe et al., 1990). This group then designed another PF4 analogue (rPF4-241) that lacked affinity for heparin, but retained antitumor properties and inhibited angiogenesis in the chicken chorioallantoic membrane. Daily intralesional injections of rPF4-241 significantly inhibited the growth of both murine melanoma and human colon carcinoma tumors in mice, but showed no effect on these cell lines *in vitro*, suggesting that the effect is due to inhibition of angiogenesis and not secondary to direct tumor toxicity (Maione et al., 1991). The use of platelet factor 4 and its analogues have not yet been reported in prostate cancer studies but this molecule is attractive due to its multiplicity of actions, namely antiangiogenesis, pro-coagulation, and immune cell modulation.

Thalidomide was marketed in Europe as a sedative, but was withdrawn 30 years ago because it has potent teratogenic effects that cause stunted limb growth (dysmelia) in humans. *In vitro* data suggested that thalidomide has antiangiogenic activity induced by basic fibroblast growth factor in a rabbit cornea assay (D'Amato et al., 1994). A report on a randomized Phase II study of thalidomide in patients with androgen-independent prostate cancer has recently been released. A total of 63 patients were enrolled in the study; 50 patients were on the low-dose arm and received a dose of 200 mg/day, while 13 patients were on the high-dose arm and received an initial dose of 200 mg/day that escalated to 1200 mg/day. A serum PSA level decline of greater than or equal to 50% was noted in 18% of patients on the low-dose arm, but in none of the patients

on the high-dose arm. Also, a total of 27% of all patients had a decline in PSA of greater than or equal to 40%, often associated with an improvement of clinical symptoms. Only four patients were maintained for greater than 150 days and the most prevalent complications were constipation, fatigue, and neurological disorders. The authors note that the decline in prostate-specific antigen in these patients may be particularly important as pre-clinical studies showed thalidomide increasing PSA levels (Figg et al., 2001).

Endostatin was discovered as an angiogenesis inhibitor produced by hemangioendothelioma, and was determined to be a 20 kDa C-terminal fragment of collagen XVIII. Endostatin was demonstrated to specifically inhibit endothelial proliferation and was found to be a potent inhibitor of angiogenesis and tumor growth. Primary tumors treated with endostatin regressed to dormant microscopic lesions similar to those found in the angiostatin treated tumors (O'Reilly et al., 1996) with immunohistochemistry revealing high proliferation balanced by apoptosis in tumor cells and blocked angiogenesis without apparent toxicity (O'Reilly et al., 1997). A transgenic mouse model developed by insertion of an SV40 early-region transforming sequence under the regulatory control of a rat prostatic steroid-binding promoter was used to evaluate the effects of endostatin treatment on spontaneous prostate cancer tumorigenesis. The SV40 Tag functionally inactivates p53 and Rb through the direct binding to these proteins and appears to interfere with cell cycle regulation. Adenomas develop in about one-third of animals between 6 and 8 months of age and approximately 40% of male mice develop invasive prostate adenocarcinomas by 9 months of age. Mouse endostatin expressed in yeast was administered to mice 7 weeks prior to the expected visibility of tumors. While the authors do not report a decrease in tumor burden as seen with mammary adenocarcinomas in transgenic females with this model, they did demonstrate prolonged their survival time for an additional 74 days (Yokoyama et al., 2000). In human patients with prostate cancer, a single nucleotide polymorphism (D104N) may have impaired the function of endostatin in 13 men heterozygous for the polymorphism D104N and 13 men homozygous for the allele men diagnosed with prostate cancer. Serum ELISA analysis demonstrated endostatin levels were similar both in carriers and non-carriers of this mutation. The results of statistical analysis predict that individuals heterozygous for N104 have a 2.5 times greater chance of developing prostate cancer when compared with men containing two wild-type endostatin alleles. Based on sequence comparison and structural modeling, this polymorphism in endostatin may inhibit the ability to interact with other molecules (Iughetti et al., 2001).

Interestingly, angiostatin, an internal fragment of plasminogen, is a potent inhibitor of angiogenesis, which selectively inhibits endothelial cell proliferation (Ms, 1997). When given systemically, angiostatin potently inhibits tumor growth and can maintain metastatic and

primary tumors in a dormant state defined by a balance of proliferation and apoptosis of the tumor cells. Angiostatin was identified while studying the phenomenon of inhibition of tumor growth by tumor mass. In the original animal model, a primary tumor almost completely suppresses the growth of its remote metastases. However, after tumor removal, the previously dormant metastases neovascularize and grow. Hence when the primary tumor is present, metastatic growth is suppressed by a circulating angiogenesis inhibitor. Serum and urine from tumor-bearing mice, but not from controls, specifically inhibit endothelial cell proliferation. The activity copurifies with a 38 kD plasminogen fragment which was named angiostatin. Human angiostatin, obtained from a limited proteolytic digest of human plasminogen, has similar activities. Systemic administration of angiostatin, but not intact plasminogen, potently blocks neovascularization and growth of metastases and primary tumors.

Supplementing agents of endogenous origin, such as plasminogen, which subsequently is cleaved into angiostatin by proteolysis by tumors, and endostatin may prove useful to reduce primary tumor growth and the establishment of metastasis that requires neovascularization (Gately et al., 1996, 1997; O'Reilly et al., 1997). It is important to determine appropriate endpoints for anti-angiogenesis trials as this mode of therapy may inhibit tumor growth with variable degrees of apoptosis. It is therefore probable that effective use of these therapeutic interventions would best be used in combination with other treatment modalities.

### *Invasion*

Proximity to blood vessels is paramount to a tumor's ability to reach the circulation, the step to metastasis is attachment and invasion of cells into the vasculature. Attachment of epithelial cells involves several junctional structures including desmosomes and tight junctions. These contacts are mediated by calcium-dependent interactions with the cadherin cell-adhesion molecule family, the classic cadherin being E-cadherin that binds to the cytoskeleton via catenins (e.g.  $\beta$ -catenin). Disruption of the cadherin-catenin complex decreases cell-cell adhesion and low levels of E-cadherin have been associated with a more aggressive phenotype of prostate cancer. Replacing E-cadherin in a rat model deficient in this protein has been shown to decrease the invasiveness of cancer cells (Luo et al., 1999) In contrast, CD44 is a protein involved in cell adhesion to the extracellular matrix protein hyaluronic acid (HA) and high cell surface expression correlates with poor outcomes. Forced expression of CD44 variants transform a rat pancreatic carcinoma cell line from non-metastatic to metastatic (Gunthert et al., 1991). Additionally, blocking CD44 with antibodies can inhibit metastatic formation (Seiter et al., 1993).

Integrins are proteins that interact with the extracellular matrix to initiate signal transduction

pathways. This interaction with the extracellular matrix results in focal adhesions, which forms complexes with the cytoskeleton. Focal adhesion kinase (FAK) can autophosphorylate resulting in activation of the mitogen-activated protein (MAP) kinase pathway, which has been linked to the induction of cell migration (Klemke et al., 1997). PTEN is a tumor suppressor gene encoding a protein tyrosine phosphatase. PTEN interacts with FAK and is sometimes mutated in prostate cancer; loss of PTEN function results in alterations in the FAK pathway and leads to an invasive phenotype (Tamura et al., 1999). Inhibition of an integrin-linked kinase directed PTEN-mutant prostate cancer cell lines towards apoptosis in one report (Persad et al., 2000). The integrins are comprised of *alpha* and *beta* subunits in heterodimers. Normal basal cells of the prostate contain multiple combinations of these subunits to bind ECM proteins, such as laminin receptors ( $\alpha 3\beta 1$  and  $\alpha 6\beta 1$ ), and an  $\alpha 6\beta 4$  dimer that forms hemidesmosomes that are downregulated in PIN and prostate cancer specimens (Allen et al., 1998) Expression of the integrins  $\alpha 2$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha v$  and  $\beta 4$  is lost in carcinoma, however, the laminin receptors  $\alpha 3\beta 1$  and  $\alpha 6\beta 1$  are retained even in invasive prostate carcinoma. The predominate laminin receptor is  $\alpha 6\beta 1$  and tumor cells with high levels of  $\alpha 6$  integrin are more invasive when injected into immuno-deficient mice using a diaphragm invasion assay indicating that  $\alpha 6$  integrin may confer an invasive phenotype. Effective strategies to combat invasiveness could include inhibiting the presence or function of  $\alpha 6\beta 1$  integrin, blocking the expression or function of laminin, or preventing the loss of  $\beta 4$  integrin (Cress et al., 1995).

Protease inhibitors that inhibit basement membrane proteases are a class of molecules that may impede the tumor cell ability to penetrate the vasculature as the basement membrane forms a barrier. Matrix metalloproteinases (MMPs) are proteins that break down basement membranes, while the tissue inhibitors of matrix metalloproteinases (TIMPs) are being studied in many types of cancer as invasion inhibitors. There is some evidence that TIMP-3 may have the additional property of causing apoptosis in some cells (Baker et al., 1999). Immunohistochemical studies in human prostate cancer tissues show a correlation between increased levels of MMP-2 and MMP-9 and absence of TIMP-1 and TIMP-2 in higher Gleason sum tumors (8-10) as compared to lower Gleason sum specimens. Additionally, TIMP-1 and TIMP-2 expression was high in organ-confined disease while absent in locally advanced cancers (Wood et al., 1997). Several MMP inhibitors, including derivatives of doxycycline and tetracycline, have been shown to be inhibitory to prostate cancer metastasis in model systems. For instance, the tetracycline derivative CMT-3 inhibited both tumor growth and metastasis in a rat model (Lokeshwar, 1999). IL-10 treatment of PC-3ML cell tumors in the severe combined immunodeficiency (SCID) mouse model was an effective inhibitor of spinal metastasis and increased tumor-free survival rates. IL-10

treatment of the PC-3 ML cells and the SCID mice reduced the number of spinal metastases from 70% seen in the natural progression of the model to 5% of the mice. Additionally, following discontinuation of IL-10 treatment after 30 days, the mice remained tumor-free and mouse survival rates increased dramatically, from less than 30% in untreated mice to about 85% in IL-10-treated mice. To further delineate the mechanism behind these findings, the authors measured expression of MMPs and TIMPs by ELISA assay in IL-10 treated PC-3ML cells. IL-10 treatment of the PC-3 ML cells down-regulated MMP-2 and MMP-9 while up-regulating TIMP-1, but not TIMP-2, expression. IL-10-treated mice exhibited similar changes in MMP-2, MMP-9, and TIMP-1 expression. Lastly, IL-10 receptor antibodies blocked the IL-10 effects on PC-3ML cells (Stearns et al., 1997). Alendronate, a potent bisphosphonate compound has been shown to inhibit TGF- $\beta$ 1 induced MMP-2 secretion in PC-3ML cells, while TIMP-2 secretion was unaffected. The relative imbalance between the molar stoichiometry of TIMP-2 to MMP-2 resulted in decreased collagen solubilization (Stearns, 1998). Several well tolerated, orally active MMP inhibitors (MMPIs) have been generated that demonstrate efficacy in mouse cancer models. Marimastat (BB-2516) was the first matrix metalloproteinase inhibitor to have entered clinical trials in the field of oncology and has completed phase I and phase II trials in prostate and colon cancer patients (Nemunaitis et al., 1998). Marimastat was generally well tolerated in Phase I trials and Phase II trials used serum prostate specific antigen as a marker in patients with prostate cancer. The authors reported a 58% response rate (no increase in serum prostatic specific antigen over the course of the study plus partial response defined as 0-25% increase in serum prostatic specific antigen per four weeks) using doses of greater than 50mg twice daily (Steward, 1999). Other MMPIs have been developed, are in various stages of pre-clinical and clinical trials, and include inhibitor batimastat (BB-94), Bay 12-9566, and prinomastat (Ag3340).

The urokinase-type plasminogen activator (uPA) likely plays a key role in tissue degradation in both normal and cancerous tissues. Increased expression of uPA has been reported in many cancers, including prostate, and gene amplification has been identified in a portion of hormone refractory prostate cancers and may play a role in the high expression of uPA. Prostate cancer cell lines that contain this gene amplification (i.e. PC-3) are more sensitive to the urokinase inhibitor amiloride as compared to prostate cancer cell lines which lack uPA gene amplification (e.g. LNCaP) (Helenius et al., 2001). Over-expression of urokinase type plasminogen activator (uPA) by the rat prostate-cancer cell line Dunning R3227, Mat-LyLu, results in increased tumor metastasis to several sites. Histological examination of skeletal lesions has shown them to be primarily osteoblastic. A selective inhibitor of uPA enzymatic activity, 4-iodo benzo(b)thiophene-2-carboxamide (B-428) was used

in this model resulted in a marked decrease in primary tumor volume and weight as well as in the development of tumor metastases when compared with controls (Rabbani et al., 1995). In a similar study, a mutant recombinant murine uPA, that retains receptor binding but not proteolytic activity, was made by polymerase chain reaction mutagenesis and transfected into the highly metastatic rat Dunning MAT-LyLu prostate cancer cell line. A clone stably expressing uPA was injected into Copenhagen rats and tumors found in these animals were significantly smaller with fewer metastases than in control animals. Additionally, mean microvessel density in transfected tumors was 4-fold lower than that in animals with tumors derived from the control tumor cell line (Evans et al., 1997). These studies demonstrate that uPA-specific inhibitors can decrease primary tumor volume and invasiveness as well as metastasis in a model of prostate cancer. To determine the effect bone cells have on prostate cancer cell expression of basement membrane degrading proteins, serum-free conditioned medium harvested from osteoblast cultures was used to stimulate the *in vitro* chemotaxis of prostate cancer cells and invasion of a reconstituted basement membrane (Matrigel). This enhanced invasive activity was due to osteoblast cell conditioned media stimulated secretion of uPA and matrix MMP-9. Additionally, inhibition of these matrix-degrading proteases by neutralizing antibodies or by inhibitors of their catalytic activity reduced Matrigel invasion. Thus demonstrating that factors produced during osteogenesis by bone cells stimulates prostate cancer cell chemotaxis and matrix proteases expression, thus representing potential targets for alternative therapies deterring the progression of prostate cancer metastasis to bone (Festuccia et al., 1999).

## Cell-cell interactions and metastasis

### Cell-cell interactions

Interactions between prostate cells and stromal cells are important in every aspect of prostate regulation. Beginning with development of the prostate *in utero*, continuing with post-pubertal growth and differentiation, through the development of BPH and prostate cancer later in life, stromal-epithelial interactions are a driving force determining how a prostate behaves. Furthermore, these interactions can accelerate local prostate cancer growth, stimulate distant metastatic tumors, and is involved in the development of hormone-independence (Camps et al., 1990; Gleave et al., 1992). Interactions may occur by multiple means involving communication directly or indirectly. The extracellular matrix (ECM) forms connections with cells providing one route of cross talk, otherwise cells communicate directly between one another and by paracrine factors interacting with cellular receptors.

To modulate the integrin receptor mediated communication with the ECM a number of molecules may be used. Integrin-specific antibodies, such as

integrin  $\alpha_v\beta_3$  antagonist antibodies has been shown to cause tumor regression, with induction of apoptosis of angiogenic blood vessels (Brooks et al., 1994). Cell surface peptides attached to chemotherapeutic agents may home to tumor blood vessels; a peptide with an  $\alpha_v$  integrin binding motif can target tumors and when attached to toxic agents can act with some specificity (Pasqualini et al., 1997; Arap et al., 1998). Based on three-dimensional structures of cell surface receptor molecules designer molecules can be synthesized to recognize these receptors specifically. Laminin-like peptides designed to inhibit degradation of the  $\beta_1$  chain of laminin can promote cell attachment and although not shown to inhibit metastasis *in vivo* demonstrate an alternate strategy for fighting metastatic spread of tumors (Zhao et al., 1994). Chemokines, or pro-inflammatory mediators that control leukocyte migration and upregulation of adhesion receptors may be antagonized by synthetic inhibitors again with the goal of stopping metastasis (Saunders and Tarby, 1999).

#### *Implantation at metastatic sites*

In contrast to the mechanisms for invasion into the vasculature, extravasation into organs does not seem to rely heavily on cellular attachment schemes. As previously noted, the milieu of potential metastatic sites plays a major role in the ability of a cancer cell to multiply, utilize angiogenesis, and avoid the immune system to survive and form metastases. Bone is the best-studied system because of the proclivity of prostate cancer to metastasize skeletally. Prostate cancer cells have osteomimetic properties which likely support metastasis within the bone environment and reciprocal interactions between prostate cancer and bone stromal growth factors leading to gene expression of osteopontin (OPN), osteocalcin (OC), and bone sialoprotein (BSP) may occur. Furthermore, prostate cancer metastases in the bone are frequently osteoblastic and likely due to the secretion of soluble factors by prostate cancer cells, which stimulate bone production (Charhon et al., 1983). In the mouse model, the prostate cancer cell line PC-3 localized preferentially to human bone implanted in the hind-legs, specifically to the reconstituted bone marrow cavity. PC-3 cells found in the human bone stained strongly for parathyroid hormone-related protein (PTHrP), tumor necrosis factor-alpha (TNF alpha) and interleukin-6 (IL-6), which is consistent with osteoclast recruitment and activity (Tsingotjidou et al., 2001). PC-3 tumors found in the bone have been found to be osteolytic in nature, consistent with the recruitment of osteoclasts (Yonou et al., 2001). Osteocalcin is expressed in some prostate cancer specimens by RT-PCR and immunohistochemical staining. Expression in transiently transfected prostate cancer cell lines show upregulation in androgen-independent lines as compared to androgen-dependent lines. Gene delivery with Ad-OC-TK (OC promoter-driven herpes-simplex virus thymidine kinase) was shown to be effective at destroying prostate-cancer

cell lines *in vitro* and prostate tumor xenografts *in vivo* in both subcutaneous and bone sites (Koeneman et al., 2000). Characterization of the OC promoter in PC-3 cells shows activation by transcription factors (Runx2, JunD/Fra-2 and Sp-1) that are responsible for the high OC promoter activity in PC3 cells (Yeung et al., 2001). Despite the observed tendency for prostate cancer metastasis to bone, and some understanding of the osteomimetic properties seen in advanced prostate cancer, characterization of the processes responsible for the prostate cancer-bone interactions requires further development to fully implicate targets for therapy. However, interventions to disrupt these interactions by gene therapy or small designer molecules may provide effective treatment to prevent or treat bony metastasis.

#### **Growth factors the cell cycle and apoptosis**

Growth factors that interact with receptors can be targeted with receptor-specific antibodies attached to therapeutic molecules such as toxins and radioactive isotopes (Baselga et al., 1998). This strategy has been used in Phase II trials in breast cancer. Miyake et al. have developed anti-sense oligodeoxynucleotides (ODN) against some genes upregulated in prostate cancer after androgen withdrawal and in progression to androgen independence. These genes, which have anti-apoptotic or mitogenic activity, are felt to confer resistance to androgen withdrawal and cytotoxic chemotherapy. The authors find a delay in the progression to androgen independence by enhancing apoptotic cell death induced by androgen ablation with ODNs as well as an additive or synergistic effect with ODNs and chemotherapy in prostate cancer models (Miyake et al., 2001).

Suramin is an anthelmintic drug that has been used in clinical trials in the treatment of patients with hormone-refractory prostate cancer since the late 1980's. It has been shown to have some efficacy and is commonly employed in combination with multiple treatment modalities, for example, synergistic action has been seen with hydrocortisone, doxorubicin and TNF- $\alpha$  (Fruehauf et al., 1990). The mechanism of action of suramin is incompletely elucidated though evidence exists to suggest anti-hormonal and direct anti-proliferative effects. *In vitro* inhibition of the growth of PC-3 cells by suramin may be caused, at least in part, by growth factor antagonism (including, but not exclusively bFGF) by the drug (La Rocca et al., 1991; Pienta et al., 1991; Ewing et al., 1993). A recent Southwest Oncology Group Study evaluated the feasibility of administering a combination of suramin and hydrocortisone in addition to androgen deprivation in 62 patients (59 assessed after the first cycle) with newly diagnosed metastatic prostate cancer. Suramin was administered on a 78-day fixed dosing schedule (one cycle), and treatment were repeated every 6 months for a total of four cycles. Thirty-two (54%) of 59 patients received a second cycle, 13 (22%) of 59 patients received a third cycle, and only five patients (8%) received a fourth cycle. There was one

therapy-related death; grade 4 toxicities were noted in fourteen patients during first and second courses, and neurotoxicity of grade 3 or higher was observed in sixteen patients during the first and second cycles. Overall, only 54% of the patients demonstrated acceptable limits of toxicity. The authors conclude that suramin plus hydrocortisone and androgen deprivation has limited applicability in the treatment of patients with newly diagnosed metastatic prostate cancer. Although response was not an end point for this study, of the 57 patients who were assessable for response, no patient had a complete or partial response: 30 patients (53%) had stable disease, and 9 patients (16%) had progressive disease. Of the 59 patients, 40 (68%) failed therapy and 34 (58%) patients were dead, including one early death (Hussain et al., 2000).

The epidermal growth factor receptor interacts with transforming growth factor alpha (TGF $\alpha$ ) secreted by androgen-independent prostate cancer cells to stimulate growth by an autonomous feedback loop. Phosphorylation and activation of EGFR in androgen-independent PC-3 and DU145 cells was decreased with anti-EGFR antibody resulting in inhibited proliferation. Additional studies showed that anti-EGFR enhanced the sensitivity of PC3 cells to the cytotoxic and cytostatic effects of tumor necrosis factor alpha (Fong et al., 1992). Examination of the mechanisms involved in EGFR stimulated proliferation demonstrated that treatment with mAb225 (anti-EGFR) induced G1 cell cycle arrest. This was accompanied by a marked decrease in cyclin-dependent kinase 2, cyclin A, and cyclin E-associated histone H1 kinase activities, as well as a sustained increase in cell cycle inhibitor p27KIP1 by both transcriptional and translational activation (Peng et al., 1996). Utilizing an *in vivo* system, treatment with C225 (anti-EGFR) alone or in combination with doxorubicin significantly inhibited tumor progression of well-established DU145 and PC-3 xenografts in nude mice (Prewett et al., 1996). Using XenoMouse technology, ABX-EGF, a human IgG2 monoclonal antibody, has recently been developed. ABX-EGF binds specifically to human EGFR with high affinity and blocks the binding of both EGF and TGF $\alpha$ ; a Phase I trial has been initiated in patients with advanced prostate cancer (Yang et al., 2001).

Small bioactive peptides offer another promising approach to the problem of HRPC. The G-protein coupled peptides bombesin/gastrin-releasing peptide (GRP), endothelin-1 (ET-1), and neurotensin have been shown to have significant effects in prostate cancer (Nelson and Carducci, 2000). Bombesin/GRP secretion by neuroendocrine-type prostate cancer cells may be partially responsible for progression, androgen independence, and hence a poor prognosis (Aprikian et al., 1998). Synthetic bombesin/GRP receptor antagonists have been shown to inhibit the growth of androgen-independent prostate cancer cell lines and are undergoing clinical trials (Jungwirth et al., 1997). Somatostatin analogs interact with receptors in prostate

cancer and one analog significantly reduced androgen-independent prostate tumor growth in mice and had some effect of stabilizing disease in men with advanced prostate cancer (Pinski et al., 1993).

Cell-cycle inhibitors and agents that disrupt cell division are another strategy for potential utilization against prostate cancer. Most of these agents are in the pre-clinical stage of investigation. They include inhibitors of microtubules, which are critical for segregation of chromosomes during mitosis, tyrosine kinase inhibitors, cyclin-dependent kinase inhibitors, DNA synthesis inhibitors, and thymidylate synthase inhibitors. Because tumor cells generally replicate more quickly than normal somatic cells, these agents could provide some specificity towards cancer, with a goal to restore normal life span to cancerous cells.

Estramustine phosphate, a nitrogen mustard derivative of 17 *beta*-estradiol, has been used for treatment of prostate cancer patients since the 1960's. Mechanisms of action include inhibiting microtubule assembly and promoting disassembly of polymerized microtubules by interacting with microtubule-associated binding proteins, in addition to decreasing serum testosterone levels (Karr et al., 1980; Wallin et al., 1985). Cytotoxic effects derive mainly from microtubule polymer inhibition required in the formation of the spindle pole apparatus during mitosis. Multiple agents that inhibit microtubule polymers have been used together and colchicine with estramustine phosphate is cytotoxic *in vitro* in PC-3 cells (Fakih et al., 1995). Clinically, estramustine phosphate has been used in combination with another microtubule inhibitor, vinblastine, without additive side effects and modest results were observed (Hudes et al., 1992). One video microscopy study suggests that estramustine phosphate mostly stabilizes microtubules in an attenuated state, perhaps explaining its additive effect with more direct microtubule depolymerizing agents such as taxol and vinblastine (Panda et al., 1997). Estramustine phosphate in combination with chemotherapeutics and other microtubule polymer inhibitors have been evaluated in many clinical trials. The results have been modest but encouraging (PSA decreases greater than 50% in 25-75% which is usually predictive of symptomatic improvement) and estramustine continues to be a reasonable treatment option in hormone-refractory advanced prostate cancer.

Tyrosine kinase (TK) receptors are the major form of growth factor receptors in cells, regulating cell proliferation, cell differentiation, and signaling processes. Tyrosine kinase inhibitor, RG-13022 (tyrphostin), was demonstrated to inhibit TGF-phosphorylation of the EGFR and proliferation in LNCaP and PC-3 cells as well as stimulation by EGF itself. Inhibition of androgen-stimulated growth was also seen, suggesting that androgen-induced regulation involves TK pathways (Kondapaka and Reddy, 1996). A similar study using an EGF-R selective tyrosine kinase inhibitor, ZM252868, inhibited basal growth in DU145

cells in addition to EGF- and TGF $\alpha$ -stimulated growth. Interestingly, only TGF alpha-stimulated PC-3 cell growth was inhibited, and the distribution of EGFR by immunohistochemistry varied between DU145 and PC-3 cells, with EGFR being predominately located on the cell membrane and in the cytoplasm, respectively (Jones et al., 1997). The high-affinity tyrosine kinase-linked receptor for nerve growth factor, trkA, has been implicated in prostatic cancer growth; the trk tyrosine kinase inhibitor, CEP-751 (KT6587), was demonstrated to inhibit prostatic cancer growth in nine different animal models. Inhibition was independent of the tumor growth rate, androgen sensitivity, metastatic ability, or state of tumor differentiation. CEP-751 was found to be selective for cancerous versus normal prostate cells, affected the growth of only a limited number of non-prostate tumors, and induced cell death in a cell cycle-independent fashion implying that it could be used in both slowly and quickly dividing tumors (Dionne et al., 1998)

Bcl-2 has been the target of several oligodeoxynucleotide (ODN) studies; in one such report, LNCaP cells *in vitro* treated with Antisense Bcl-2 (ODN) treatment reduced Bcl-2 messenger RNA and protein levels by >90% in a sequence-specific and dose-dependent manner and Bcl-2 mRNA levels returned to pretreatment levels by 48 hours after discontinuing treatment. Athymic male mice bearing subcutaneous LNCaP tumors were castrated and injected with Bcl-2 ODN or controls; LNCaP tumor growth and serum PSA levels were 90% lower in mice treated with antisense Bcl-2 ODN compared with mismatch or reverse polarity ODN controls (Gleave et al., 1999). Antisense Bcl-2 oligodeoxynucleotides after castration have been shown to decrease the progression to androgen-independence in the mouse model and androgen-independent LNCaP tumor regression in mice occurred with a novel method of administering paclitaxel with antisense Bcl-2 oligodeoxynucleotide (Miyake et al., 1999; Leung et al., 2001).

### Novel therapeutic delivery systems in the treatment of prostate cancer

Gene therapy involves delivering recombinant genetic material, in the form of DNA or RNA to combat disease. Major strategies involve modifying gene expression to correct deficiencies or block inappropriate gene expression, inducing "suicide" genes to promote cancer cell death, and modulating the interactions of the immune system with cancer cells. This is performed either by removing tissue and genetically altering it *ex vivo*, or delivering the genetic material *in vivo*. *In vivo* gene therapy has been limited largely by inefficient delivery systems and improvements in gene vectors and delivery will allow practical use of clinical gene therapy for a variety of conditions. The ideal delivery system would be non-toxic to normal cells, deliver the genetic information efficiently with specificity to the targeted

system, and be inexpensive yet easily administered.

Tumor cell vaccines are a typical *ex vivo* gene therapy strategy for cancer and rely on the ability of cancer specific antigens to elicit an immune response. An approach is to harvest tumor cells from the patient, genetically modify the cells (usually with retroviral transfection) so that they can stimulate the immune system, irradiate the cells so that they are non-tumorigenic, and re inject the cells into the patient. The first tumor vaccine trial for prostate cancer began in 1994 when Granulocyte-colony macrophage stimulating factor (GM-CSF) was transfected using retrovirus into a patients prostate cancer cells *in vitro*. The cells were then injected subcutaneously and found to be safe in Phase I trials (Simons et al., 1999b). Data from this trial indicate vaccination activated new T-cell and B-cell immune responses against PCA antigens. T-cell responses, evaluated by assessing delayed-type hypersensitivity (DTH) reactions against untransduced autologous tumor cells, were evident in two of eight patients before vaccination and in seven of eight patients after treatment. These data are the first to suggest that both T-cell and B-cell immune responses to prostate cancer can be generated by treatment with irradiated, GM-CSF gene-transduced prostate cancer vaccines. A limitation in the trial by Simons, et al. was generating vaccine cells in culture. This group has embarked on a new trial to estimate the efficacy of using the *ex vivo*, granulocyte macrophage colony stimulating factor transduced, prostate cancer cell lines PC-3 and LNCaP as a vaccine. They reported results on 21 patients treated and 1 had a partial PSA response of greater than 7 months in duration, 14 had stable disease and 6 patients underwent progression. By 3 months PSA velocity or slope decreased in 71% of cases. Additionally, numerous new post-vaccination IgG antibodies were identified in these patients, indicating that immune tolerance to prostate cancer associated antigens may be broken (Simons et al., 1999a).

The first report to demonstrate anticancer activity of gene therapy in human prostate cancer, by Herman and colleagues, used intratumoral injection of replication-deficient adenovirus carrying the thymidine kinase gene from the herpes simplex virus, followed by parenteral administration of the prodrug gancyclovir. They report promising results with some patients with local recurrence after radiotherapy obtaining a greater than 50% decrease in PSA lasting six weeks to one year (Herman et al., 1999). These data support the usefulness of cyto-reductive gene therapy, by "suicide" genes, or by increasing tumor cell sensitivity to chemotherapeutics or. Replication deficient adenovirus containing the herpes simplex virus thymidine kinase (HSV-tk) gene ("suicide" gene therapy) has also been used in men with localized prostate cancer. Multiple and/or repeat intraprostatic injections was followed by intravenous gancyclovir or oral valaciclovir 14 days after injection. A total of 52 patients were treated, with a total of 76 gene therapy cycles; toxic events were recorded in 16 of 29

patients (55.2%) who were given multiple viral injections into the prostate, 7 of 20 (35%) who received 2 cycles of "suicide" gene therapy and 3 of 4 (75%) who received a third course of gene therapy. All toxic events were mild (grades 1 to 2) and resolved completely once the therapy course was terminated. Mean follow-up was 12.8 months and preliminary results for 28 patients indicated a mean decrease of 44% in serum PSA in 43% of patients (Shalev et al., 2000).

A current study evaluating the effect of p53 replacement therapy utilizing intraprostatic Ad5CMVp53 injection in men with localized prostate cancer prior to radical prostatectomy. Patients are followed for tumor volume response by transrectal ultrasound and magnetic resonance imaging (MRI) and injections are continued every two weeks until tumor shrinkage abates, after which time they undergo radical prostatectomy. Results are not available at this time as this trial is still open for enrollment (Sweeney and Pisters, 2000). Logothetis et al. reported initial results using a similar gene replacement strategy of adenovirus containing wild-type p53 (AdCMVp53) driven by the CMV promoter before radical prostatectomy in 17 patients with locally advanced prostate cancer. AdCMVp53 was administered via a transperineal route under ultrasound guidance. In this phase I-II study, three patients who completed a repeat course of therapy had a greater than 25% decrease in tumor size, as measured by endorectal coil MRI after the initial treatment course and there was no grade 3 or 4 toxicity in the 14 evaluable patients. Further results on efficacy due to the apparent radiological responses, correlations with gene expression, pathological findings at prostatectomy, and surgical outcomes are pending (Logothetis et al., 1999).

Investigators have recently completed a Phase I trial in twenty-four patients with locally advanced prostate cancer using gene-based immunotherapy. A functional DNA-lipid complex encoding the interleukin 2 (IL-2) gene was administered intraprostatically, under transrectal ultrasound guidance, into the hypoechoic tumor lesion. Patients were stratified into two groups: group 1 included patients who underwent radical prostatectomy after the completion of the treatment regimen, and group 2 consisted of patients who had failed a prior therapy. IL-2 gene therapy was well tolerated, with no grade 3 or 4 toxic reactions occurring. Post-treatment prostate specimens were attained and compared with the transrectal biopsies performed prior to therapy. Evidence of systemic immune activation after IL-2 gene therapy included an increase in the intensity of T cell infiltration seen on immunohistochemistry of tissue samples from the injected tumor sites, and increased proliferation rates of peripheral blood lymphocytes that were co-cultured with patient serum collected after treatment. Transient decreases in serum PSA were seen in 16 of 24 patients (67%) on day 1. Fourteen of the patients persisted in this decrease to day 8 (58%). However, in eight patients the PSA level rose after therapy. More patients (9 to 10) in group 2

responded to the IL-2 gene injections and 6 of the 9 also had lower than baseline PSA levels at week 10 after treatment (Belldegrun et al., 2001). This group has also reported on novel chimeric PSA enhancers that exhibit increased activity in prostate cancer gene therapy vectors. They addressed the problem of low transcriptionally active native PSA enhancer and promoter, which otherwise confers prostate-specific expression when inserted into adenovirus vectors. The investigators exploited the mechanism by which androgen receptor (AR) molecules bind cooperatively to androgen response elements (AREs) in the PSA enhancer core, and act synergistically with AR bound to the proximal PSA promoter to regulate transcriptional output. They generated chimeric enhancer constructs by inserting four tandem copies of the proximal ARE1 element, duplicating the enhancer core, or by removing intervening sequences between the enhancer and promoter. They obtained chimeric PSA enhancer constructs, which were highly androgen inducible and retained a high degree of tissue specificity even in an adenoviral vector (Ad-PSE-BC-luc). They also demonstrated that augmented activity of the chimeric constructs *in vivo* correlated with their ability to recruit AR and critical co-activators *in vitro*. Furthermore, systemic administration of Ad-PSE-BC-luc into SCID mice harboring the LAPC-9 human prostate cancer xenografts showed that this prostate specific vector retained tissue discriminatory capability compared with a comparable cytomegalovirus (CMV) promoter driven vector (Wu et al., 2001).

Kwon and colleagues have utilized regulation of T lymphocyte proliferation by the stimulatory HLA-B7 family with T-cell surface inhibitory receptor CTLA4 in a transgenic mouse model of metastatic prostate cancer growth after tumor resection. They modified their tumor vaccine with the addition of anti-CTLA4 antibodies and showed metastatic relapse dropped from 97.4% in controls to 44% in animals treated immediately after tumor resection (Kwon et al., 1999). Additionally, anti-CTLA4 antibody treatment can delay tumor growth in a transgenic mouse model (Hurwitz et al., 2000).

*In vivo* gene therapies for prostate cancer utilize vectors to deliver genetic information to tissues. Viruses are commonly used and types include adenovirus, retrovirus, adeno-associated virus, herpes virus, and poxvirus. Non-viral vectors include liposomes, DNA coated gold particles, plasmid DNA, and polymer DNA complexes. Overcoming the obstacle for gene therapy vector inefficiency is being approached with development of less immunogenic viral vectors and "stealth liposomes" which evade the first-pass destruction by the reticular endothelial system. Furthermore, developing more specific therapies by targeting specific cell receptors or using prostate specific gene promoters (e.g. prostate-specific membrane antigen and human kallekrein II) may allow for lower effective doses and perhaps decreasing the side effects commonly seen with viral vectors.



Correction of genetic alterations of tumor suppressor genes and cancer promoting oncogenes can target a multitude of genes in the pathways to cancer development or progression. Restoration of one of the tumor suppressor genes (Rb, p53, p21, and p16) have been shown to alter malignant phenotype, but would have to be delivered to every cancer cell to eradicate the cancer. The Retinoblastoma (Rb) gene has been shown to be mutated in human prostate cancer specimens, and gene therapy has shown some effectiveness in model systems of neuroendocrine and lung tumors (Kubota et al., 1995; Nikitin et al., 1999). Mutations in p53 are reported at a wide range of rates, however, p53 gene therapy suppressed tumorigenesis and induced apoptosis in vitro (Yang et al., 1995). Furthermore, *in vivo* studies have shown some benefit of p53 gene therapy in prostate tumor models (Eastham et al., 2000). Similarly, p21 gene therapy has been shown to suppress growth in prostate tumor cells in vitro and in vivo (Eastham et al., 1995; Steiner et al., 2000).

A concept that is being expanded is restoration of genes that confer increased chemosensitivity or radiosensitivity to prostate cancer tumors. One study has looked at proliferation of tumor cells after incubation with various combinations of p53 Adenovirus (p53 Ad) and chemotherapeutic drugs. The authors found p53 Ad combined with cisplatin, doxorubicin, 5-fluorouracil, methotrexate, or etoposide inhibited cell proliferation more effectively than chemotherapy alone in multiple human tumor cell lines, including DU-145 prostate cells. Human tumor xenografts in scid mice dosed with intraperitoneal or intratumoral p53 Ad with or without chemotherapeutic drugs showed greater anticancer efficacy with combination therapy in four human tumor xenograft models in vivo (Gurnani et al., 1999). Another study evaluated gene therapy enhancement of prodrugs and radiation therapy. Prostate tumor cells (PC-3) were transduced with adenovirus containing a fusion gene encoding the *E. coli* cytosine deaminase and herpes simplex virus type-1 thymidine kinase under the control of a cytomegalovirus promoter. PC-3 cells expressing this protein were sensitized to killing by the normally innocuous prodrugs 5-fluorocytosine and ganciclovir. In addition, radiation-induced killing was enhanced in virally infected cells in the presence of the prodrugs (Blackburn et al., 1999). Additionally, some evidence exists that gene therapy may be cooperative with androgen deprivation. Castration was combined with HSV-tk plus GCV using an androgen-sensitive mouse prostate cancer cell line, which led to markedly enhanced tumor growth suppression in both subcutaneous and orthotopic models compared with either treatment alone. An enhanced survival was also observed, in which combination-treated animals lived twice as long as controls in the subcutaneous model and over 50% longer than controls in the orthotopic model (Hall et al., 1999). A very interesting study utilized a constructed recombinant adenovirus containing *E. coli* cytosine deaminase and herpes simplex virus type 1

thymidine kinase fusion gene under the control of a human inducible heat shock protein 70 promotional sequence. Heating at 41 degrees Celsius for 1 hour induced strong expression of the fusion gene product. Heat-induced expression of the CD-TK protein significantly reduced the survival of PC-3 cells in the presence of both 5-fluorocytosine and ganciclovir prodrugs. These data represent a novel form of gene therapy involving the transduction and regulation of a double suicide gene in tumor cells and may provide a unique application for hyperthermia in cancer therapy (Blackburn et al., 1998). In the future, perhaps hsp promoters could be used in combination with thermal energy modalities.

Gene transfer trials sponsored by the National Institute of Health (NIH) are listed on the World Wide Web at <http://www4.od.nih.gov/oba/rac/clinicaltrial.htm> and currently list forty trials.

## Conclusions

The observation of phenotypic differences between normal cells and cancer cells lead to basic science experiments to discover why these changes occur. A cascade of discoveries brought about some answers and many more questions. Utilization of the rapidly growing wealth of basic science knowledge leads to therapeutic intervention, first at the level of the test tube or Petri dish, then to animal experimentation, and finally in clinical trials. The outlook for prostate cancer patients is promising. Better methods of screening and diagnosis, as well as recognition of tumor markers, are improving the survival of patients afflicted with prostate cancer. New treatments for advanced prostate cancer are being evaluated at an increasing rate and significant advances for the therapy of prostate cancer should come sooner rather than later. It is likely that a combination of the strategies reviewed here will provide the most effective weapon in the battle against prostate cancer.

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