The role of fibroblast growth factors and their receptors in prostate cancer

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Abstract

Prostate cancer is the most common malignancy in men in the USA and the second leading cause of cancer deaths. Fibroblast growth factors (FGFs), including FGF1 (acidic FGF), FGF2 (basic FGF), FGF6 and FGF8 are all expressed at increased levels in prostate cancer as paracrine and/or autocrine growth factors for the prostate cancer cells. In addition, increased mobilization of FGFs from the extracellular matrix in cancer tissues can increase the availability of FGFs to cancer cells. Prostate cancer epithelial cells express all four types of FGF receptors (FGFR-1 to -4) at variable frequencies. Expression of FGFR-1 and FGFR-4 is most closely linked to prostate cancer progression, while the role of FGFR-2 remains controversial. Activation of FGF receptors can activate multiple signal transduction pathways including the phospholipase C

Introduction

Prostate cancer is the most common visceral malignancy in men in the USA and the second leading cause of cancer deaths in this population. There is a large body of literature linking alterations of the fibroblast growth factor (FGF) system to initiation and progression of a wide variety of malignancies, including prostate cancer. There have been a number of excellent reviews of both the biology of FGFs and FGFR receptors (Basilico & Moscatelli 1992, Johnson & Williams 1993, Dow & deVere White 2000, Powers et al. 2000, Ornitiz & Itoh 2001) and their role in neoplastic transformation (Cronauer et al. 2003, Munro & Knowles 2003). This review will therefore focus more narrowly on the role of FGFs and their receptors in normal prostate and prostate cancer since there is an extensive and growing literature in this area.

The FGF signaling system

The human FGF gene family consists of at least 23 different genes encoding related polypeptides. FGFs are expressed in almost all tissues and play important roles in a variety of normal and pathological processes, including development, wound healing and neoplastic transformation. The FGFs are mitogenic for many cell types, both epithelial and mesenchymal. Some FGFs, like FGF2,
FGFs have potent angiogenic activity and have been implicated as promoters of tumor angiogenesis. FGFs have also been shown to increase the motility and invasiveness of a variety of cell types. Finally, it has been shown that FGFs can inhibit cell death in the appropriate context. Thus FGFs have a broad range of biological activities that can play an important role in tumorigenesis.

FGFs interact with a family of four distinct, high-affinity tyrosine kinase receptors, designated FGFR-1 to -4 (Johnson & Williams 1993). The receptors consist of an extracellular portion containing three immunoglobulin-like domains and an intracellular tyrosine kinase domain and have variable affinities for the different FGFs. In addition, FGFRs1 to -3 all undergo an alternative splicing event in which two alternative exons (IIIb and IIIE) can be used to encode the carboxy terminal portion of the third immunoglobulin-like loop, which results in receptor isoforms with dramatically altered binding specificity. The IIIa alternatively spliced isoform is secreted. A variety of other alternative splicing events have been described, including alternative splicing that results in loss of the first extracellular immunoglobulin-like domain.

The third component of the FGF system is extracellular matrix (ECM) and secreted proteins that help mobilize FGFs from the ECM. Heparin and heparin sulfate proteoglycans play a critical role in facilitating FGF signaling via FGF receptors, although there is controversy regarding the exact manner by which this occurs (Powers et al. 2000). FGFs are bound in the extracellular matrix and can be released by the activity of degradative enzymes such as proteases. In addition, FGF-binding protein (FGF-BP), a 17 kDa secreted polypeptide, can reversibly bind FGF1 and FGF2 and can facilitate release of FGFs from the extracellular matrix and interaction of these growth factors with cellular receptors (Aigner et al. 2001).

Binding of FGFs to the extracellular domains of FGF receptors results in receptor dimerization and transphosphorylation of tyrosine residues in the intracellular domain that is required for FGF receptor kinase activation. Ultimately, activation of FGF receptors leads to signal transduction through multiple pathways including phospholipase Cγ (PLCγ) (Burgess et al. 1990, Mohammadi et al. 1991), phosphatidylinositol 3-kinase (PI3K) (Hart et al. 2001), mitogen-activated protein kinases (MAPK) (Hadari et al. 2001) and signal transducers and activators of transcription (STATs) (Hart et al. 2000, Deo et al. 2002, Udayakumar et al. 2002). These effectors in turn disseminate the receptor tyrosine kinase signals by activating many target proteins, including transcription factors in the nucleus (Fig. 1). All of these pathways have been shown to be upregulated in prostate cancer and there is strong evidence linking each of these pathways to prostate cancer initiation and progression.

The docking proteins, FRS2α and FRS2β, play a critical role in mediating the intracellular signals that are generated at the cell surface by activation of the FGF receptors. Both FRS2α and FRS2β contain myristyl anchors and phosphotyrosine-binding sites in their C-terminal tails that serve as binding sites for the adaptor protein, Grb2, and for the Src homology (SH) 2 domain containing protein tyrosine phosphatase, Shp2 (Kouhara et al. 1997, Hadari et al. 1998). In response to FGF stimulation, Grb2 can also be recruited indirectly to FRS2α through its interaction with tyrosine-phosphorylated Shp2 molecules bound to the docking protein (Hadari et al. 1998). FGF-induced tyrosine phosphorylation of FRS2α results in complex formation with the adaptor protein Grb2 bound to Cbl by means of its SH3 domains. FGF-induced ternary complex formation among FRS2α, Grb2 and Cbl results in ubiquitination and degradation of FRS2α and FGF receptor (Wong et al. 2002). Thus, FRS2α functions as a central platform for recruitment of multiprotein complexes that are responsible for both signal activation and attenuation.

A new family of regulators of FGF activity has recently been identified. Sprouty inhibits signaling mediated by the FGF receptor and the epidermal growth factor (EGF) receptor during eye development and oogenesis in Drosophila (Casci et al. 1999, Kramer et al. 1999, Reich et al. 1999). Four mammalian genes (Sprouty1–4) have been identified with sequence similarity to Drosophila Sprouty (Tefft et al. 1999). In vitro studies have demonstrated that after growth factor stimulation, Sprouty1 and Sprouty2 translocate to the plasma membrane, become tyrosine phosphorylated, and interact with components of the Ras/MAPK and Ras/Raf/ERK pathways and other proteins including c-Cbl, Grb2, Raf1, FRS2, Caveolin-1, dual specificity kinase TESK1, the protein tyrosine phosphatase PTP1B and the Drosophila Ras-GAP, Gap1 (Dikic & Giordano 2003) but the precise molecular mechanism by which the FGF receptor signal is blocked remains controversial.

**FGFs and FGF receptors in normal prostate**

The prostate is a mixed epithelial and stromal organ that requires androgenic stimulation for its development, maintenance and growth. There is a considerable body of evidence indicating that interaction between the stroma and epithelium plays a crucial role in the growth and development of the prostate. The mesenchymal elements in the prostate appear to mediate the development of this organ in response to androgens (Chung et al. 1991,
Hayward et al. 1997). In addition, it has been shown that prostatic stromal cells in culture secrete factors into their medium that stimulate or inhibit prostatic epithelial growth in a paracrine manner (Kabalin et al. 1989, Yan et al. 1992). Thus the accepted paradigm is that in vivo the stromal cells secrete paracrine factors, some of which are under the control of androgens, which are responsible for the maintenance and growth of the epithelium. The major source of FGFs in human prostate is the prostatic stromal cells and they can act as paracrine growth factors for the epithelial cells. FGF2 (basic FGF), FGF7 (KGF) and FGF9 are expressed by the stromal cells of the prostate in biologically significant quantities. The mean FGF2 content of normal peripheral zone tissue as measured by ELISA is 110 ng/g wet weight (Giri et al. 1999a). FGF2 does not contain a classical signal peptide and is not efficiently secreted (Basilico & Moscatelli 1992). Traditionally, it was thought that FGF2 is released by cellular damage. However, a number of investigations have indicated that FGF2 is actively (but inefficiently) secreted and recent evidence indicates that FGF2 can be actively transported across the plasma membrane (Schafer et al. 2004) and/or can be released by extracellular vesicle shedding (Taverna et al. 2003). FGF7 is present at approximately 28 ng/g wet weight in normal prostate (Giri et al. 1999a). FGF7 contains a classic signal peptide

Figure 1 FGF signaling pathways in the prostate. Ligand binding initiates receptor dimerization and activation, promoting binding of the adaptor molecule FRS2 and the subsequent recruitment of adapter proteins Grb2 and Son of sevenless (Sos) to the FGF receptor complex. Sos facilitates guanine nucleotide exchange to activate Ras, which stimulates the Raf-to-MEK (MAPK)-to-extracellular signal-related kinase (ERK). These effectors, in turn, disseminate the receptor tyrosine kinase signals by phosphorylating many target proteins, including transcription factors in the nucleus. The PI3K, PLCγ and STAT pathways are also activated by FGF receptor signaling. Regulators of FGF signaling include Sprouty. Cbl mediates downregulation of receptor protein. FGF receptor dominant negative (DN) proteins can be used to block FGF receptor signaling experimentally. MT1-MMP, membrane-type-I matrix metalloproteinase; P indicates phosphorylation.
and is actively secreted. It has been reported that FGF7 is induced by androgens in cultures of rat (Yan et al. 1992) and human (Planz et al. 1998) prostatic stromal cells, but observations on castrated rats indicated that FGF7 expression in the prostate may not be androgen regulated in vivo (Nemeth et al. 1998) so the effect of androgens on FGF7 production is controversial. Our laboratory has shown a small increase in FGF7 secretion in organ cultures of normal prostate tissue in response to androgens (authors' unpublished data), supporting the idea that FGF7 expression by stromal cells can be regulated at least partially by androgens, but other factors may be more important (Giri & Ittmann 2000). We have also found that FGF9 is expressed by prostatic stromal cells and is present in normal prostate tissue at approximately 51 ng/g wet weight by ELISA (authors' unpublished data). FGF9 is actively secreted and acts as a growth factor for both prostatic epithelial and stromal cells in culture (Giri et al. 1999b). All of these FGFs are potent growth factors for primary epithelial cells in culture at 1–10 ng/ml, so it is clear that the concentrations of FGFs present in normal prostate are biologically significant. FGF10 is expressed by stromal cells, but is present at low levels in normal adult prostate and probably does not act as a significant growth factor in this context, although it is important for prostatic development (Ropiquet et al. 2000a). In addition to these stromal FGFs, epithelial-derived FGFs are present in the normal prostate. Small amounts of FGF6 can be observed in basal cells of normal prostate gland (Ropiquet et al. 2000b) on immunohistochemistry (IHC). We have recently found that FGF17 is expressed in relatively small amounts by epithelial cells (Polnaszek et al. 2004). Both of these FGFs are actively secreted and, given that they are expressed by the epithelial cells in an autocrine manner, they may have biological importance that is disproportionate to their relatively low expression level, since they would not have to diffuse across the ECM in order to interact with epithelial FGF receptors.

The expression of the other FGFs that we have evaluated to date in normal human prostate are substantially less than FGF2, FGF7 and FGF9. We have shown that expression of FGF1 (acidic FGF) is barely detectable by RT-PCR in normal prostate and in primary cultures of prostatic stromal cells, but cannot be detected by Northern blotting (Ittmann & Mansukhani 1997). We have also detected FGF5 and FGF8 mRNA in human prostate by RT-PCR but neither of these was detectable by Northern blotting (Ittmann & Mansukhani 1997). Our observations regarding the expression of FGF8 in normal human prostate are supported by similar observations by other laboratories (Ghosh et al. 1996). No expression of FGF3, FGF4 or FGF16 was detected by RT-PCR of normal peripheral zone tissue (Ittmann & Mansukhani 1997, authors' unpublished observations). Expression of FGF15, FGF19 and FGF20-23 in normal prostate has not been examined to date, to our knowledge. FGFs 11–14 do not appear to be secreted and at least two of these FGF family members act as intracellular signal transduction scaffolding molecules rather than as growth factors (Schoorlemmer & Goldfarb 2001). We have detected expression of FGF13 but not FGF14 in normal prostate by RT-PCR (authors' unpublished data).

Prostate epithelial cells express multiple FGF receptors. FGFR-1 and FGFR-2 are expressed in the basal epithelial cells of the prostate but not the luminal cells (Giri et al. 1999a). Based on studies of primary epithelial cells in culture, FGFR-1 is present exclusively as the IIIc isoform, while FGFR-2 is present exclusively as the IIIB (FGF7 specific) isoform in the epithelium (Ittmann & Mansukhani 1997). FGFR-3 is also present in prostatic epithelium, predominantly, but not exclusively, as the IIIB isoform (Kwabi-Addo et al. 2001). Finally, FGFR-4 is also expressed in prostatic epithelium and IHC has revealed that it is expressed in the luminal epithelial cells (Wang et al. 2004b). Based on the known properties of the various FGF receptor isoforms (Ornitz et al. 1996), prostatic epithelial cells express appropriate receptors to respond to the FGFs present in normal prostate, i.e. FGFR-1 IIIc binds FGF2 and FGF6, FGFR-2 IIIB binds FGF7, FGFR-3 IIIB binds FGF9, and FGFR-4 binds FGF2, FGF6 and FGF9.

Expression of FGFs in prostate cancer

FGF1 (acidic FGF) is a potentially important mitogen in prostate cancer due to the fact that it is mitogenic when it binds any type and isoform of FGF receptor (Ornitz et al. 1996). More than 80% of prostate cancers express FGF1 in the cancer cells by IHC and that strong expression was correlated with increased Gleason score (Dorkin et al. 1999a). FGF1 was also increased in prostatic intraepithelial neoplasia (PIN). Both the PC3 and LNCaP prostate cancer cell lines express FGF1 (Payson et al. 1998). Expression of FGF1 is detected by RT-PCR (Foster et al. 1999) or Western blotting (Polnaszek et al. 2003) in a significant fraction of prostate cancer tissues in the TRAMP mouse model of prostate cancer. FGF1 has a nuclear localization motif and has been detected in the nucleus in other systems (Klingenberg et al. 2000) but the role of nuclear FGF1 in prostate cancer is unclear.

FGF2 (basic FGF) is expressed in many human malignancies, including prostate cancer. Using ELISA we have shown that FGF2 is present at significantly higher concentrations in clinically localized cancer tissue (almost 2.5-fold) when compared with normal prostate (Giri et al. 1996).
Immunohistochemical analysis revealed that FGF2 was present in stromal cells within the prostate cancer, consistent with a paracrine effect of FGF2 in localized prostate cancer from radical prostatectomy specimens. We have also shown that prostate cancer expresses increased levels of interleukin-8 (IL-8) and that IL-8 can induce expression of FGF2 (Giri & Ittmann 2001), so one candidate mediator for the induction of FGF2 expression in stromal cells is IL-8 secreted by the cancer cells. In contrast, two groups, using primarily tissues from more advanced cancers than our laboratory has analyzed, have examined expression of FGF2 in prostate cancer by IHC and detected expression of FGF2 in prostate cancer epithelial cells in the majority of these cases (Cronauer et al. 1997, Dorkin et al. 1999a). Most of the prostate cancers in these studies were locally advanced or metastatic and/or poorly differentiated and thus are much more aggressive than the cancers from the radical prostatectomy specimens studied by our group. High levels of expression of FGF2 are present in PC3 and DU145 prostate cancer cells (Cronauer et al. 1997) and both of these cell lines were derived from metastatic prostate cancer. In addition, it has been demonstrated that prostate cancer patients have elevated levels of serum FGF2 (Cronauer et al. 1997). Thus it seems likely that initially FGF2 is expressed as a paracrine factor by stromal cells in localized prostate cancer and during tumor progression there is a switch to autocrine expression. In support of this hypothesis, there is increased expression of FGF2 during progression of TRAMP prostate cancers to a poorly differentiated phenotype (Huss et al. 2003). When TRAMP mice were crossed with FGF2 knockout mice, there was a significant increase in survival and decreased metastasis in mice bearing even one FGF2 knockout allele, which supports the hypothesis that the increased FGF2 expression seen during progression in the TRAMP model is biologically important in tumor progression (Polnaszek et al. 2003). One interesting aspect of FGF2 biology is the production of high molecular weight forms of FGF2 (22 and 25 kDa) that arise from alternative translation initiation from CUG codons that preferentially localize directly to the nucleus and can promote growth in low serum in some cell types (Arese et al. 1999). These higher molecular weight forms are present in TRAMP prostate cancers (Huss et al. 2003). If FGF2 is expressed as autocrine growth factors by the cancer cells, these higher molecular weight intranuclear forms may be biologically significant.

Our laboratory has demonstrated that FGF6 is increased in prostate cancer (Ropiquet et al. 2000b) and high grade PIN. ELISA of tissue extracts of normal prostate, PIN and prostate cancer for FGF6 showed that this growth factor was undetectable in normal prostate but was present at elevated levels in four of nine PIN lesions and in 15 of 24 prostate cancers. Immunohistochemical analysis with anti-FGF6 antibody revealed weak staining of prostatic basal cells in normal prostate that was markedly elevated in PIN. FGF6 may play a unique role in prostate cancer by acting as a paracrine factor secreted by residual basal cells in PIN that supports the growth of the dysplastic luminal epithelial cells. In the prostate cancers, IHC revealed autocrine expression of FGF6 by the prostate cancer cells in the majority of the cases.

The role of FGF7 in prostate cancer is unclear. We have measured expression of FGF7 in prostate cancer tissue from radical prostatectomy specimens by ELISA and have found that expression of FGF7 is similar in normal and cancer tissue and that, by IHC, as in normal tissue FGF7 is expressed by stromal cells (Giri et al. 1999a). We have recently confirmed that there is no increase in expression of FGF7 mRNA is prostate cancer tissue using quantitative RT-PCR (authors’ unpublished data). In contrast, Planz et al. (1999) have reported expression of FGF7 in cancer cells on IHC of sections from radical prostatectomies using a different antibody. Serum FGF7 levels are lower in men with prostate cancer than in men with benign prostatic hyperplasia (BPH) (Mehta et al. 2000a), and we have shown that FGF7 is substantially elevated in BPH tissue (Ropiquet et al. 1999b), consistent with our observation that FGF7 is not elevated in prostate cancer. Further studies are needed to determine if there is increased expression of FGF7 in prostate cancer. In addition, as will be discussed below, it is unclear whether activation of FGFR-2, the only receptor that binds FGF7, promotes prostate cancer progression, so that even if FGF7 is increased it is not clear whether it would promote tumor progression.

Expression of FGF8 in human prostate cancer cells, both by in situ hybridization and IHC, has been well documented, while normal prostate expresses little detectable FGF8 (Leung et al. 1996, Tanaka et al. 1998, Dorkin et al. 1999a,b, Valpe et al. 2001, West et al. 2001, Gnanapragasm et al. 2003). Overall, about 50% of clinically localized cancers express increased FGF8 while 80% or more of advanced cancers express increased FGF8. Dorkin et al. (1999b) observed strong correlations of FGF8 expression with tumor grade, stage and patient survival, although FGF8 did not appear to be an independent predictor of survival on multivariate analysis. An interesting aspect of FGF8 is that its mRNA undergoes alternative splicing, yielding multiple isoforms designated FGF8a, b, e and f (Ghosh et al. 1996). FGF8b is apparently the major isoform expressed in prostate cancer, although expression of the other alternatively spliced isoforms has also been reported in prostate cancers (Valpe et al. 2001). FGF8b is
expressed by LNCaP, DU145 and PC3 cells and it has transforming activity in NIH3T3 cells (Tanaka et al. 1995, Ghosh et al. 1996). Increased expression of FGF8b in LNCaP cells using a lentivirus vector resulted in increased growth, colony formation in soft agar, invasion and tumorigenesis in vivo and facilitated growth-promoting stromal–epithelial interactions (Song et al. 2000a). Taking the opposite approach, Rudra-Ganguly et al. (1998) showed that antisense inhibition of FGF8 expression in DU145 cells decreased soft agar colony formation and tumorigenicity in vivo. Prostate-specific expression of FGF8b in transgenic mice under the control of an enhanced probasin promoter results in progressive epithelial hyperplasia and ultimately PIN, although invasive carcinoma was not reported (Song et al. 2002). Taken together, the finding of expression in human tissues that correlates with clinical and pathological parameters of aggressive disease and the biological observations in vitro and in vivo are convincing for the importance of FGF8 in human prostate cancer progression.

We have detected expression of FGF17 in normal prostatic epithelium and this expression is maintained in cancer cells (Polnaszek et al. 2004). Although, in samples from clinically localized cancers, the amount of FGF17 per cancer cell is similar to that in normal epithelial cells, the cancer cells constitute most of the tissue volume within the cancer tissue, leading to increased local FGF17 concentration. The FGF17 present in such cancers can act as an autocrine growth factor for the prostate cancer cells. We also observed increased expression of FGF17 in the DU145 cell line, suggesting that FGF17 may be expressed at increased levels in advanced prostate cancers. Further studies of FGF17 expression in advanced cancer tissues are needed to clarify this question.

In summary, multiple FGFs are expressed either as autocrine or paracrine growth factors in PIN and prostate cancer tissues. Many questions remain to be answered. Due to the fragmentary and relatively small scale of most the studies reported to date, the extent to which the expression of the different FGFs overlap is unclear, although Dorkin et al. (1999a) did examine FGF1, FGF2 and FGF8 by IHC in relatively advanced cancers and found that the expression of these growth factors was only partially overlapping. Expression of any one FGF has not been shown to be an independent prognostic factor on multivariate analysis, but the relatively small number of specimens involved in the published studies limits the power of this analysis and the possibility that the FGFs may act synergistically should be examined. The advent of tissue microarrays should greatly facilitate simultaneous analysis of multiple FGFs in large numbers of prostate cancers. In addition, not all FGFs have been studied quantitatively, so it is difficult to determine the relative expression levels of the different FGFs in cancer tissues. Finally, the most important question is the mechanism by which expression of FGFs is regulated in prostate cancer cells. FGF expression can be modulated by transcriptional, post-transcriptional and translational mechanisms, but little is known about what underlies the expression of FGFs as autocrine growth factors in prostate cancer cells. Although androgen may modulate expression of some FGFs, it seems unlikely that androgen receptor alone can selectively increase expression in cancer cells, since androgen receptor is also active in the benign cells. Further studies are needed to determine how FGF expression is upregulated in prostate cancer.

**Expression of FGF receptors in prostate cancer**

Our laboratory has shown that FGFR-1 is expressed in approximately 20% of moderately differentiated cancers and 40% of poorly differentiated clinically localized cancers based on IHC and Western blotting of prostate cancer extracts, but was not detected in well-differentiated cancers (Giri et al. 1999a). Prostate cancer cells most closely resemble prostatic luminal epithelial cells in their differentiation, in that they express cytokeratins that are similar to these cells and prostate-specific antigen (PSA). Given that luminal epithelial cells do not express FGFR-1, it appears that with transformation and progressive loss of differentiation, there is increasing expression of FGFR-1. Takahashi (1998) found increased expression of FGFR-1 mRNA in poorly differentiated prostate cancer, with which our results are consistent. Naimi et al. (2002) found relatively equal expression of FGFR-1 IIIc in normal prostate and cancer tissues by quantitative RT-PCR. However, FGFR-1 IIIc is expressed in stromal cells and as the cancer epithelial cells replace stroma in the cancer tissues (which were 90% cancer in this study), there would be a marked decrease in FGFR-1 IIIc unless the cancer epithelial cells express FGFR-1 IIIc. Thus, these quantitative RT-PCR results are also consistent with our IHC observations. FGFR-1 is also expressed preferentially in poorly differentiated TRAMP prostate cancers (Huss et al. 2003). In the Dunning rat prostate carcinoma model, FGFR-1 expression promotes tumor progression (Feng et al. 1997). Transgenic models that express constitutively active FGFR-1 in the prostate epithelium develop hyperplasia and PIN (Wang et al. 2002, 2004a) and increased expression accelerates the appearance of this phenotype (Jin et al. 2003). Transgenic mice in which FGFR-1 kinase is activated by a chemical dimerizer also develop PIN when treated with dimerizer drug (Freeman et al. 2003a). Finally, activation of FGFR-1 in TRAMP cell lines using chemical dimerizer enhanced tumor
proliferation in vitro and in vivo (Freeman et al. 2003b). It should be noted that FGFR-1 IIIc binds FGF2 and FGF6, both of which are increased in prostate cancer tissues. Thus, all evidence to date strongly supports the hypothesis that FGFR-1 can promote prostate cancer progression.

In contrast to the consistent evidence linking FGFR-1 to prostate cancer progression, the role of FGFR-2 in prostate cancer is far less clear. Activation of FGFR2-2 IIIb by FGF7 can enhance proliferation of primary or immortalized prostate epithelial cells in vitro (Ropiquet et al. 1999a,b) and prostate-specific expression of FGF7 expression promotes hyperplasia of prostatic epithelium in a transgenic model in vivo (Foster et al. 2002). As described above, FGF7 is expressed in prostate cancer tissues at levels similar to those in normal prostate. However, McKeehan and his colleagues have shown that in the Dunning rat prostate cancer model FGFR-2 IIIb expression inhibits neoplastic progression (Feng et al. 1997, Matsubara et al. 1998). Furthermore, when FGFR-2 kinase is activated in a transgenic mouse prostate epithelium using a chemical dimerizer, mice do not develop PIN (Freeman et al. 2003a), while activation of FGFR-2 by chemical dimerizer in TRAMP cell lines does not enhance tumor proliferation as observed for FGFR-1 (Freeman et al. 2003b). Finally, decreased FGFR-2 activity enhances the progression to PIN in mice expressing activated FGFR-1 (Jin et al. 2003). Thus most in vivo studies have found that FGFR-2 either inhibits or does not promote prostate cancer initiation and progression. Another complication is that FGFR-2 is expressed in normal prostate epithelium as the IIIb isoform, which binds almost exclusively to FGF7 and FGF10. During progression in the Dunning model there is a change in alternative splicing of FGFR-2, with increased expression of the IIIc isoform (Yan et al. 1993). In the DU145 cell line and in one of three prostate cancer xenografts studied, there was predominant expression of the FGFR-2 IIIc isoform, suggesting that exon switching to the FGFR-2 IIIc isoform by changes in alternative splicing may occur in human cancers (Carstens et al. 1997).

Using a PCR-based approach we found that such isoform switching occurs in a subset of clinically localized cancers in vivo (Kwabi-Addo et al. 2001). TRAMP prostate cancers express increasing amounts of FGFR-2 during progression to the poorly differentiated phenotype and express the IIIc isoform even at the PIN stage (Huss et al. 2003). Such isoform switching would allow FGFR-2 to be activated by FGF2, FGF6 and FGF9 in human cancer tissue. However, if FGFR-2 activation does not promote or even inhibits tumor progression, it is difficult to see any selective advantage to the tumor cells in this isoform switching, unless the FGFR-2 IIIc is forming heterodimers with other FGF receptors such as FGFR-1 and activating them to promote cancer progression. It is also possible that such isoform switching is a manifestation of an epithelial to mesenchymal transition and does not in itself yield a selective advantage for tumor cells. Our laboratory has shown that FGFR-2 is expressed in approximately 30% of clinically localized cancers by IHC (Giri et al. 1999a). By quantitative RT-PCR, Naimi et al. (2002) found a decreased mean expression of both FGFR-2 IIIb and IIIc in prostate cancer tissues. However, if 70% of cancers do not express FGFR-2 and the cancer tissue is replacing normal epithelium and stroma (which expresses FGFR-2 IIIb and IIIc), one might expect a marked decrease in average FGFR-2 mRNA expression and this might not be compensated for by the 30% of cancers that do express FGFR-2, particularly if they express relatively low levels of the FGFR-2 mRNA. Further investigations are necessary to establish the role of FGFR-2 in prostate cancer, bearing in mind that it is possible that human prostate cancer may be heterogeneous in its response to FGFR-2 activation depending on the presence of other genetic alterations. A major goal is to understand the underlying differences in signal transduction between FGFR-1 and FGFR-2 that could lead to enhancement or inhibition of tumor progression by these two related receptors. In this regard, Freeman et al. (2003b) have shown that FGFR-1 but not FGFR-2 activation can induce osteopontin, which is known to facilitate tumor growth, although the basis for this difference in expression is still unclear.

FGFR-3 is expressed in normal prostate epithelium predominantly as the IIIb isoform and, based on PCR studies, it continues to be expressed in prostate cancer tissues predominantly as the IIIB isoform (Kwabi-Addo et al. 2001), which will bind FGF1 and FGF9. To date no immunohistochemical studies of FGFR-3 expression in prostate cancer have been reported. Using ELISA we have found that cancer tissues contain FGF9 at levels similar to normal prostate. Thus, FGFR-3 may have a role in FGF signaling in prostate cancer.

FGFR-4 is expressed in normal human prostate, in prostate cancer cell lines and in the immortalized human prostate epithelial cell line PNT1A by RT-PCR (Kwabi-Addo et al. 2001). Of note is the observation that FGF2, FGF6, FGF8 and FGF17, which are all present in human prostate cancer tissues, are potent activators of FGFR-4 (Ornitz et al. 1996). We have recently reported that FGFR-4 is expressed in luminal epithelial cells, PIN and in all of the prostate cancers examined (Wang et al. 2004a). A germline polymorphism in the FGFR-4 gene, resulting in expression of FGFR-4 containing either glycine (Gly388) or arginine (Arg388) at codon 388 has been identified and the presence of the FGFR-4 Arg388 allele is associated with decreased disease-free survival in breast cancer patients with lymph node metastasis as well
as with metastasis and poor prognosis in colon cancer (Bange et al. 2002). We have found that the presence of homozygosity for the FGFR-4 Arg<sup>388</sup> allele is significantly associated with prostate cancer incidence. In addition, the presence of the FGFR-4 Arg<sup>388</sup> polymorphism is correlated with the occurrence of pelvic lymph node metastasis and PSA recurrence in men undergoing radical prostatectomy. Expression of the FGFR-4 Arg<sup>388</sup> in immortalized prostate epithelial cells results in increased cell motility and invasion and upregulation of the urokinase-type plasminogen activator receptor, which is known to promote invasion and metastasis (Sidenius & Blasi 2003). This may explain, in part, the increased aggressiveness of prostate cancers in men bearing this polymorphism. These findings indicate that FGFR-4 plays a significant role in prostate cancer initiation and progression.

There are several known mutations that activate FGF receptor signaling and play a role in genetic disorders of bone formation (Naski & Ornitz 1998). Mutations of FGFR-3 that are associated with thanatophoric dysplasia have been found to occur in a significant fraction of bladder cancers (Cappellen et al. 1999). However, these mutations do not occur in prostate cancers (Naimi et al. 2000, Sibley et al. 2001). A similar activating mutation in FGFR-2 also does not occur in prostate cancer (Mehta et al. 2000b). In addition to point mutations, fusion transcripts can lead to aberrant activity and fusions involving FGF receptor genes have been detected in hematopoietic malignancies (Xiao et al. 1998, Demiroglu et al. 2001, Li et al. 2001, Grand et al. 2004, Roumiantsev et al. 2004). Although such fusion transcripts have not been reported in prostate cancer, they may be quite hard to detect in a solid malignancy in which the cancer cells are difficult to grow in culture. Thus the major alteration that has been observed to date in FGF receptors during prostate cancer progression is increased expression.

Many questions remain regarding the role of FGF receptors in prostate cancer. As described above, the role of FGFR-2 and changes in alternative splicing of this receptor in prostate cancer progression are still not clear. The different FGF receptors display differences in signaling and biological activities in various systems, but whether these differences are fundamentally quantitative or qualitative in nature is unclear. FGFR-1 appears to activate the MAPK pathway more robustly than other FGF receptors (Shaoul et al. 1995, Raffioni et al. 1999). FGFR-4, in particular, appears to activate MAPK weakly and yet it can promote proliferation. In addition, FGFR-4 is the only FGFR that can promote membrane ruffling when transfected into COS-7 cells (Johnston et al. 1995). Such membrane ruffling is associated with changes in the actin cytoskeleton related to increased motility. Thus, FGFR-4 activation may be more important in altering motility or other properties when compared with similar stimulation by other FGFs. Further work is needed to understand these differences in FGF receptor activities in the context of prostate cancer. In this regard, the chemical dimerizer system for FGF receptor activation used by Spencer and his colleagues is attractive (Freeman et al. 2003a,b), since multiple FGF receptors and FGFs are expressed in the prostate cancer cell lines examined to date, making clean analysis of the effects of any single receptor difficult using FGF ligands. Finally, the basis for increased expression of FGF receptors is not clear. If one considers that the differentiation displayed by prostate cancers is more luminal than basal, it is not surprising that FGFR-4 which is expressed in normal luminal cells is widely expressed in prostate cancer. In contrast, expression of FGFR-1 and FGFR-2 is normally in basal cells and tends to be increased in more advanced and poorly differentiated cancer, implying that additional genetic alterations are needed to increase expression of these receptors. The nature of these alterations is unknown. Amplification of FGFR-1 and FGFR-2 has been reported in advanced prostate cancers (Edwards et al. 2003) but the observed amplifications are relatively low level (less than two-fold). It is likely that other genetic alterations enhance expression of FGF receptors in cancer cells. For example, overexpression of cyclin D1 in fibroblastic cells leads to upregulation of FGFR-1 (Tashiro et al. 2003). Interestingly, translation of cyclin D1 in fibroblastic cells is enhanced by expression of eIF4e, a cap-binding protein that can regulate protein synthesis, which is overexpressed in prostate cancer. Thus eIF4e could potentially have synergestic effects on proliferation by increasing both FGF ligand and receptor (de Benedetti & Harris 1999). Thus genetic alterations that are known to affect prostate cancer cells can affect FGF receptor expression but further work is needed to determine if there is a linkage of these alterations to changes in FGF receptor expression in prostate cancer.

The extracellular matrix, proteoglycans and FGF activity in prostate cancer

Heparin and heparin sulfate proteoglycans play a critical role in FGF signaling, but little is known of how these molecules are modulated during prostate cancer initiation and progression. Syndecan-1 is a heparin sulfate proteoglycan that can potentially modulate FGFR-1 activity. Immunohistochemical analysis has revealed overexpression of syndecan-1 in prostate cancer and such overexpression is associated with increased Gleason score, early recurrence and decreased survival (Zellweger et al. 2003). However, syndecan-1 can interact with both matrix proteins and a number of different growth factors so that
biological activities of FGFs. The extent to which FGF-BP is expressed in clinical prostate cancer specimens has not been reported to date. Overall, how the ECM and factors releasing FGFs from the ECM contribute to FGF receptor activation modulation in prostate cancer is not well understood and warrants further investigation.

**Alterations of modulators of FGF signal transduction in prostate cancer**

As described above, members of the Sprouty gene family negatively regulate FGF signaling in a variety of systems and could potentially limit the biological activity of FGFs in prostate cancer. Recently Kwabi-Addo et al. (2004) have established that decreased Sprouty1 expression may play a role in prostate cancer. Immunohistochemical analysis of normal and neoplastic prostate tissues using tissue microarrays revealed that Sprouty1 protein is downregulated in approximately 40% of prostate cancers. By quantitative real-time PCR, Sprouty1 mRNA levels were significantly decreased in prostate cancers in vivo in comparison with normal prostate. In prostate cancer cell lines there was loss of the normal upregulation of Sprouty1 mRNA in response to FGFs. The decrease in Sprouty1 expression in the human prostate cancer, despite elevated levels of FGF ligands and FGF receptors, implies a loss of an important growth-regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGF receptor expression in prostate cancer. Alterations of expression of other Sprouty family members and other proteins that may regulate FGF signal transduction in prostate cancer are currently being investigated.

**Biological effects of FGFs and FGF receptors in prostate cancer**

As described above, there is extensive evidence that FGF receptor signaling is enhanced in prostate cancer by multiple mechanisms including increased expression of FGF ligands and receptors, increased mobilization of FGFs from the ECM and loss of negative regulation of FGF signaling. This increased FGF receptor signaling results in activation of multiple signal transduction pathways. The effects of such increased FGF receptor signaling are wide ranging and involve both the cancer cells and surrounding stroma, particularly the vasculature. For prostate cancer, the biological consequences of increased FGF signaling include enhanced proliferation, resistance to cell death, increased motility and invasiveness, increased angiogenesis, enhanced metastasis, resistance to chemotherapy and radiation and androgen independence, all of which can enhance tumor progression and clinical aggressiveness.

Exogenous FGFs can promote proliferation of normal, immortalized and fully transformed prostatic epithelial cells. Our laboratory has shown that FGF2, FGF6, FGF7, FGF9 and FGF17 can all enhance normal and/or neoplastic prostatic epithelial cell proliferation. Song et al. (2000a) investigated the biological effects of overexpression of FGF8b in prostate cancer and demonstrated increased growth rate in comparison with controls. In addition, FGF-BP depletion reduces proliferation (Aigner et al. 2002). Activation of FGF signaling in vivo in multiple transgenic mouse models also leads to enhanced proliferation. Thus one major effect of FGF signaling in prostate cancer cells is to enhance cell proliferation.

It has been shown in different systems that FGFs can inhibit cell death in the appropriate context (Fox & Shanley 1996, Fujiwara et al. 2003, Erez et al. 2004). Our laboratory has demonstrated that FGF receptor signaling may act to provide an important survival signal in prostate cancer cells. Adenovirus-mediated expression of dominant negative FGF receptors, which blocks FGF signaling, leads to an arrest in G2 in prostate cancer cells followed by cell death (Ozen et al. 2001). Primary cultures of epithelial cells show reduced growth after infection with adenovirus-expressing dominant negative FGF receptors but not increased cell death, suggesting that cancer cells are particularly dependent on FGF signaling. We have made similar observations in melanoma cells and primary melanocytes (Ozen et al. 2004). As prostate cancers progress, the amount of stroma decreases and the amount of epithelium increases, particularly as cancers develop Gleason patterns 4 and 5, in which large areas of fused, cribriform tumor or sheets of cells are seen, so that there is far less stroma per unit volume to provide stromal FGFs to the epithelial cells. If the cancer cells require FGF signaling for survival, there would be a strong selective pressure for the emergence of cells that have genetic or epigenetic alterations that enhance FGF signaling, and such changes are common in human prostate cancers, as...
described above. Based on these observations, disruption of FGF signaling is an attractive therapeutic target in prostate cancer.

FGFs have significant biological function as positive regulators of angiogenesis. FGF1 and FGF2 were among the first angiogenic factors to be identified (Folkman & Shing 1992, Powers et al. 2000) and other FGFs can also have angiogenic activity. When FGF2 is expressed in prostate cancer cells and cancer stroma it can induce the formation of tumor vasculature. PC3M cells that express high levels of secreted FGF2 were more angiogenic when growing as solid tumors in nude mice in contrast to DU145 cells (Connolly & Rose 1998). In high grade prostate cancers, production of thrombospondin-1, a major inhibitor of angiogenesis, is downregulated while that of stimulatory FGF2 and/or vascular endothelial growth factor (VEGF) rise, and this is associated with increased microvessel density (Doll et al. 2001). Sugamoto et al. (2001) reported similar correlations. It should be noted that the angiogenic factors VEGF and IL-8 are increased along with FGF2 in prostate cancer and can act synergistically to promote angiogenesis. Hypoxia can increase expression of all of these factors by prostatic stromal cells (Berger et al. 2003) and presumably in cancer cells as well, and hypoxia may be responsible, at least in part, for the increased expression of these three angiogenic factors in prostate cancer. Huss et al. (2003) studied the process of angiogenesis and the temporal and spatial expression of the FGF axis during prostate tumor progression in the TRAMP model. They have demonstrated that FGFR-1 IIIb is specifically expressed in new vasculature associated with prostate cancer but not in the vessels of normal mouse prostate. Such increased expression of FGFR-1 could potentiate the effect of increased FGF2 on the tumor vasculature.

Growth factors are key regulators of proliferative and migratory events and FGFs play a role in wound repair (Ortega et al. 1998), which is characterized by both cellular proliferation and migration. In response to FGF2, endothelial cells increase the activation and ligation of integrin αvβ3 to facilitate cellular migration process through the ERK pathway (Elciceri et al. 1998). MMPs are elevated in many types of cancer including prostate cancer (Basset et al. 1990, Matrisian et al. 1991, Pajouh et al. 1991) and are involved in the invasion and metastasis of prostate cancer. MMPs are a family of endopeptidases that require zinc for catalytic activity and are capable of digesting ECM and basement membrane components (Newell et al. 1994). Increased expression of MMPs in prostate cancer leads to proteolytic breakdown of the basement membrane and ECM structures leading to release of FGFs (Mack et al. 1993). Matrilysin, a matrix metalloproteinase, can degrade the extracellular proteins, including proteoglycans, fibronectin, entactin, laminin, gelatin and elastin (Wilson & Matrisian 1996). Its expression has been shown in prostate cancer (Pajouh et al. 1991) and can enhance the invasiveness of the DU145 prostate cancer cell line (Knox et al. 1996, Powell et al. 1993). It has been demonstrated that overexpression of matrilysin in prostate cancer is partially due to the paracrine factors secreted from the surrounding stroma (Klein et al. 1999) and inhibition of FGF receptor signaling can inhibit promatrixin expression along with PSA expression and tumor growth in LNCaP prostate cancer cells (Udayakumar et al. 2003). Thus it is possible that a positive feedback loop could be established in which FGFs promote activity of MMPs which, in turn, leads to increased availability of FGFs by release from the ECM. We have recently demonstrated that immortalized prostatic epithelial cells expressing the Arg388 form of FGFR-4 have enhanced migration, invasiveness and expression of urokinase-type plasminogen activator receptor (Wang et al. 2004b), which plays a key role in cell motility and invasiveness. Thus FGFs can play an important role in invasion and migration by enhancing the activity of multiple proteolytic systems.

Given the biological effects described above it is not surprising that FGFs can enhance metastasis in vivo. Highly metastatic variants of PC3 express higher levels of FGF2 (Greene et al. 1997). Our laboratory has recently demonstrated, by crossing TRAMP mice with FGF2 knockout mice, that inactivation of even one FGF2 allele is associated with decreased metastasis (Polnaszek et al. 2003). Other FGFs probably have a similar effect on metastasis although this has not been established experimentally to date.

FGF2 could affect the cytotoxicity of chemical and other non-physiological stresses inflicted upon the cell. This is particularly important for the interaction of cancer cells with chemotherapy drugs and other DNA-damaging agents, including radiation. Depending on the type of cells studied, the chemotherapeutic or DNA-damaging agent used and the method of exposure to FGF2, such treatment can be either protective or sensitizing. Song et al. (2000b) have shown that the chemoresistance in lung metastases is caused by acidic and basic FGFs (aFGF and bFGF) expressed in solid tumors. Subsequently the same group investigated the effect of FGF inhibitors on doxorubicin activity in human prostate PC3 tumors (Zhang et al. 2001). Addition of suramin (which can inhibit FGF receptor activation) to doxorubicin therapy significantly enhanced the anti-tumor effect, resulting in complete inhibition of tumor growth. The protective effects of FGFs can also involve endothelial cells. For example, FGF2 can increase survival of radiated endothelial cells, which could limit the effectiveness of radiation
therapy in inhibiting tumor angiogenesis (Abdollahi et al. 2003). While further studies are needed, the evidence to date indicates that increased FGF expression could contribute to the resistance of many prostate cancers to radiation and/or chemotherapy.

Androgen receptor activity is critical for prostate cancer cell survival and androgen ablation plays an important role in the treatment of prostate cancer, particularly in men with advanced disease. Unfortunately androgen-independent prostate cancer almost always emerges following androgen ablation, limiting the effectiveness of this therapy. The vast majority of androgen-independent prostate cancers continue to express androgen receptor and androgen receptor target genes such as PSA, indicating that the emergence of androgen independence is usually due to genetic or epigenetic changes that allow for activation of androgen receptor at extremely low androgen levels. Such changes include amplification and overexpression of androgen receptor, mutations of androgen receptor, increased expression of androgen receptor coactivators and activation of androgen receptor transcription by growth factor signaling. Culig et al. (1994) originally demonstrated that FGF7, insulin-like growth factor-I and EGF activate androgen receptor-dependent transcription in prostate cancer cells. It is now clear that multiple growth factors and cytokines can activate androgen receptor-dependent transcription, but the mechanism by which this occurs is still unclear. One potential mechanism is through activation of androgen receptor coactivators by the MAPK pathway (Debes et al. 2004) and has anti-angiogenic activities that inhibit FGF receptors and FGF receptor signaling can affect both the tumor cells directly and tumor angiogenesis. There are numerous approaches that could target FGF receptors and/or FGF receptor signaling in prostate cancer. One approach is to target cancer cells by conjugating FGF ligands to toxins (Davol & Frackelton 1999) or adenoviruses carrying toxic genes (Lanciotti et al. 2003). Another approach would be to develop antibodies targeting FGF receptors that could either directly inhibit their activity or be used to target therapeutic molecules to the cancer cells and tumor vasculature. He et al. (2003) have used an interesting approach in which FGF receptor from a non-mammalian species (Xenopus) was used as a vaccine in mice and which led to inhibition of tumor growth. Finally, small molecule inhibitors of FGF signaling are under active development. SU5402 is a specific inhibitor of FGFR activity (Mohammadi et al. 1997). It has been shown to specifically inhibit the growth of chronic myeloid leukemia cell lines bearing an unusual translocation that results in the production of a BCR–FGFR1 fusion protein (Demiroglu et al. 2001) as well as multiple myeloma cells bearing a rearrangement of FGFR-3 (Grand et al. 2004). In LNCaP cells, low doses of SU5402 have been shown to inhibit secretion of promatrilysin (Klein et al. 1999). Another FGF receptor inhibitor, PD173074, shows activity against breast cancer cell lines (Koziczak et al. 2004) and multiple myeloma cells expressing FGFR-3 fusion protein (Grand et al. 2004) and has anti-angiogenic activities in vivo (Dimitroff et al. 1999). SU5416 and SU668 are broad-spectrum anti-angiogenic tyrosine kinase inhibitors that inhibit FGF receptors as well as VEGF and platelet-derived growth factor receptors and have anti-tumor effects in vivo (Griffin et al. 2002). These two agents are currently undergoing clinical trials for treatment of a variety of malignancies, although SU5416 has recently been reported not to be effective in advanced prostate cancer (Stadler et al. 2004). Finally, it should be noted that a number of chemotherapeutic agents such pegylated interferon-α-2b (Huang et al. 2002) and taxanes (Cassinel et al. 2002) downregulate expression of FGF2 and this may be part of their therapeutic effectiveness. While this effort is only beginning, agents targeting the FGF signaling system in prostate cancer may have therapeutic effectiveness and may well be integrated into patient treatment in the future.

**FGF receptors and FGF receptor signaling as therapeutic targets in prostate cancer**

Tyrosine kinases have emerged as a major potential therapeutic target in cancer therapy. Imatinib, which inhibits the BCR-abl kinase and c-kit, is highly effective in the treatment of chronic myelogenous leukemia and malignant gastrointestinal stromal tumors. Similarly, Her2/neu is an important target in breast cancer therapy. As described above, expression of FGF receptors and increased FGF receptor signaling are ubiquitous in human prostate cancer. Furthermore, since FGF signaling enhances multiple biological processes that promote tumor progression, most critically cell survival, it is an attractive therapeutic target, particularly since therapies targeting FGF receptors and/or FGF signaling can affect both the tumor cells directly and tumor angiogenesis. There are numerous approaches that could target FGF receptors and/or FGF receptor signaling in prostate cancer. One approach is to target cancer cells by conjugating FGF ligands to toxins (Davol & Frackelton 1999) or adenoviruses carrying toxic genes (Lanciotti et al. 2003). Another approach would be to develop antibodies targeting FGF receptors that could either directly inhibit their activity or be used to target therapeutic molecules to the cancer cells and tumor vasculature. He et al. (2003) have used an interesting approach in which FGF receptor from a non-mammalian species (Xenopus) was used as a vaccine in mice and which led to inhibition of tumor growth. Finally, small molecule inhibitors of FGF signaling are under active development. SU5402 is a specific inhibitor of FGFR activity (Mohammadi et al. 1997). It has been shown to specifically inhibit the growth of chronic myeloid leukemia cell lines bearing an unusual translocation that results in the production of a BCR–FGFR1 fusion protein (Demiroglu et al. 2001) as well as multiple myeloma cells bearing a rearrangement of FGFR-3 (Grand et al. 2004). In LNCaP cells, low doses of SU5402 have been shown to inhibit secretion of promatrilysin (Klein et al. 1999). Another FGF receptor inhibitor, PD173074, shows activity against breast cancer cell lines (Koziczak et al. 2004) and multiple myeloma cells expressing FGFR-3 fusion protein (Grand et al. 2004) and has anti-angiogenic activities in vivo (Dimitroff et al. 1999). SU5416 and SU668 are broad-spectrum anti-angiogenic tyrosine kinase inhibitors that inhibit FGF receptors as well as VEGF and platelet-derived growth factor receptors and have anti-tumor effects in vivo (Griffin et al. 2002). These two agents are currently undergoing clinical trials for treatment of a variety of malignancies, although SU5416 has recently been reported not to be effective in advanced prostate cancer (Stadler et al. 2004). Finally, it should be noted that a number of chemotherapeutic agents such pegylated interferon-α-2b (Huang et al. 2002) and taxanes (Cassinel et al. 2002) downregulate expression of FGF2 and this may be part of their therapeutic effectiveness. While this effort is only beginning, agents targeting the FGF signaling system in prostate cancer may have therapeutic effectiveness and may well be integrated into patient treatment in the future.

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