FGFR-4 Arg^{388} Enhances Prostate Cancer Progression via Extracellular Signal–Related Kinase and Serum Response Factor Signaling

Abstract

**Purpose:** Increased expression of FGFR-4 and its ligands have been linked to lethal prostate cancer (PCa). Furthermore, a germ line polymorphism in the FGFR-4 gene, resulting in arginine at codon 388 (Arg^{388}) instead of glycine (Gly^{388}), is associated with aggressive disease. The FGFR-4 Arg^{388} variant results in increased receptor stability, sustained receptor activation, and increased motility and invasion compared with Gly^{388}. However, the impact of sustained signaling on cellular signal transduction pathways is unknown.

**Experimental Design:** Expression microarray analysis of immortalized prostatic epithelial cells lines expressing FGFR-4 Arg^{388} or Gly^{388} was used to establish a gene signature associated with FGFR-4 Arg^{388} expression. Transient transfection of reporters and inhibitors was used to establish the pathways activated by FGFR-4 Arg^{388} expression. The impact of pathway knockdown in vitro and in an orthotopic model was assessed using inhibitors and/or short hairpin RNA (shRNA).

**Results:** Expression of the FGFR-4 Arg^{388} protein leads to increased activity of the extracellular signal–related kinase (ERK) pathway, increased activity of serum response factor (SRF) and AP1, and transcription of multiple genes that are correlated with aggressive clinical behavior in PCa. Increased expression of SRF is associated with biochemical recurrence in men undergoing radical prostatectomy. Consistent with these observations, knockdown of FGFR-4 Arg^{388} in PCa cells decreases proliferation and invasion in vitro and primary tumor growth and metastasis in vivo.

**Conclusions:** These studies define a signal transduction pathway downstream of FGFR-4 Arg^{388} that acts via ERK and SRF to promote PCa progression. Clin Cancer Res; 17(13): 4355–66. © 2011 AACR.

Introduction

Prostate cancer (PCa) is the most common visceral malignancy and the second leading cause of cancer deaths in men in the United States. An extensive body of evidence links fibroblast growth factors (FGF) and FGF receptors (FGFR) to PCa initiation and progression (1). FGFs are a family of more than 20 different polypeptide ligands involved in a variety of biological and pathologic processes. FGFRs are transmembrane proteins tyrosine kinase receptors, and there are 4 distinct FGFRs (FGFRs 1–4) that have variable affinities for the different FGFs. Upon binding to FGFRs, various downstream signaling pathways including phospholipase c-γ (PLC-γ), phosphoinositide 3-phosphate (PI3K)/Akt, extracellular signal–related kinase (ERK), and STATs are activated (1, 2). A wide range of cellular phenotypic responses that can be receptor-dependent have been observed following activation of FGFRs, although the basis of these differences is not well understood.

There is strong evidence for the involvement of FGFR-4 in PCa initiation and progression. There is increased expression of several FGFs that preferentially bind to FGFR-4 in PCa, and in many cases this expression is correlated with poor clinical outcome (1, 3–6). There is increased expression of FGFR-4 in PCa (7) by immunohistochemistry (IHC), and strong expression of FGFR-4 is significantly associated with poor clinical outcome (6, 8). Furthermore, our group has shown that a germ line polymorphism in the FGFR-4 gene, resulting in expression of FGFR-4 containing arginine at codon 388 (Arg^{388}), instead of a more common glycine (Gly^{388}), is associated with PCa initiation and progression (7). The Arg^{388} polymorphism is quite frequent in the Caucasian population, as approximately 45% of individuals are hetero- or homozygous for this allele. A recent meta-analysis of multiple studies has confirmed the association of PCa risk and the presence of the FGFR-4 Arg^{388} polymorphism (9). Correlation of the presence of the Arg^{388} allele with poor prognosis has been observed in a...
A germ line polymorphism in the FGFR-4 gene, resulting in arginine at codon 388 (Arg\textsuperscript{388}) instead of glycine (Gly\textsuperscript{388}), is associated with aggressive behavior in a variety of malignancies including prostate cancer (PCa). We have found that expression of FGFR-4 Arg\textsuperscript{388} leads to increased activity of the extracellular signal–related kinase (ERK) pathway, increased transcriptional activity of serum response factor (SRF), and transcription of multiple genes that are correlated with aggressive clinical behavior in PCa when compared with FGFR-4 Gly\textsuperscript{388}. SRF, which is activated by FGFR-4 signaling, is expressed in most PCas, and increased SRF protein is correlated with aggressive clinical behavior. Consistent with these observations, knockdown of FGFR-4 Arg\textsuperscript{388} in PCa cells decreases proliferation and invasion in vitro and primary tumor growth and metastasis in vivo. Thus, ERK and SRF are important therapeutic targets in PCa and potentially other malignancies expressing FGFR-4 Arg\textsuperscript{388}.

**Translational Relevance**

A germ line polymorphism in the FGFR-4 gene, resulting in arginine at codon 388 (Arg\textsuperscript{388}) instead of glycine (Gly\textsuperscript{388}), is associated with aggressive behavior in a variety of malignancies including prostate cancer (PCa). We have found that expression of FGFR-4 Arg\textsuperscript{388} leads to increased activity of the extracellular signal–related kinase (ERK) pathway, increased transcriptional activity of serum response factor (SRF), and transcription of multiple genes that are correlated with aggressive clinical behavior in PCa when compared with FGFR-4 Gly\textsuperscript{388}. SRF, which is activated by FGFR-4 signaling, is expressed in most PCas, and increased SRF protein is correlated with aggressive clinical behavior. Consistent with these observations, knockdown of FGFR-4 Arg\textsuperscript{388} in PCa cells decreases proliferation and invasion in vitro and primary tumor growth and metastasis in vivo. Thus, ERK and SRF are important therapeutic targets in PCa and potentially other malignancies expressing FGFR-4 Arg\textsuperscript{388}.

**Generation of stable cell lines**

PNT1a FGFR-4 Arg\textsuperscript{388} and Gly\textsuperscript{388} cell lines have been described previously (19), and DU145-overexpressing FGFR-4 Arg\textsuperscript{388} and Gly\textsuperscript{388} cells were generated in a similar manner. There was no significant difference in the expression of FGFR-4 in the DU145 cell lines by quantitative reverse transcriptase-PCR (RT-PCR) carried out as described previously (19). The shFGFR-4 lentivirus and stable cell lines were established as described previously (19). A human GIPZ lentiviral shRNAmir individual clone (V2LHS_153469) targeting SRF was obtained from Open Biosystems and used to obtain stable cell lines.

**Expression microarray analysis**

The expression microarray labeling, hybridization, and scanning were conducted as described previously (20). Expression arrays were processed and loess-normalized using BioConductor. Array data have been deposited in the public Gene Expression Omnibus (GEO) database (accession no. GSE20906). To define differentially expressed genes between FGFR-4 Arg\textsuperscript{388} and Gly\textsuperscript{388} samples, we used a criterion of a minimum fold change of 1.4 between each Arg\textsuperscript{388} sample and each Gly\textsuperscript{388} sample. Java TreeView (21) represented expression patterns as color maps. To score each of the prostate tumors of Glinsky and colleagues (22) for similarity to our Arg\textsuperscript{388} gene signature, we derived a "t-score" for each Glinsky tumor in relation to the Arg\textsuperscript{388} signature as previously described (23). The mapping of transcripts or genes between array data sets was made on the Entrez Gene identifier, where multiple human array probe sets referenced the same gene, the probe set with the highest variation represented the gene.

**Quantitative RT-PCR**

RT-PCR assays were conducted as described previously (19). Primers used are summarized in Supplementary Table S1. The PCR conditions were 30-second denaturation, 30-second annealing at the 60°C, and 30-second extension at 72°C for 40 cycles. The relative copy number of transcript for each gene was normalized by transcript level of the β-actin gene. Each experiment was carried out in triplicate.

**Transfection and luciferase reporter assay**

Luciferase reporter assays were carried out as previously described (24). The serum response elements (SRE), AP1, and NFAT reporter constructs were obtained from Stratagene (PathDetect). The androgen responsive ARE reporter construct was obtained from Dr. Carolyn Smith, Baylor College of Medicine, and has been described previously (25). The data presented are the mean of 3 individually transfected wells and the experiments are conducted at least 3 times.

**Matrigel invasion assay**

The Matrigel invasion assays were conducted in triplicate as described previously except that the cells were seeded in
RPMI-1640 medium supplemented with 0.1% FBS (PNT1a) or 0.5% FBS (DU145) and 50 ng/ml FGF2 (7).
For PC3 cells, 5% FBS without added FGF was used. Each experiment was repeated 3 times.

**Immunohistochemistry**

IHC was conducted as described previously (26). Antigen retrieval was conducted using Tris-HCl, pH 9.0. Arrays were incubated for 30 minutes at room temperature with anti-SRF antibody (sc-335; Santa Cruz Biotechnology; 1:500) and staining developed using an Envision kit (Dako). The anti-SRF antibody has been previously validated for IHC (27). Sections incubated with secondary antibody showed no staining.

**Western blotting**

Cells were lysed in modified radioimmunoprecipitation assay buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 2 mmol/L sodium orthovanadate, 1 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 × protease inhibitor cocktail (Roche) and clarified by centrifugation. Protein concentration of the lysates was determined using BCA protein assay kit (Thermo). The extracted proteins (25 μg) were loaded on 10% SDS-PAGE gels. Proteins were transferred onto Immobilon-P polyvinylidene difluoride membranes (Invitrogen), and the membrane was blocked with 5% nonfat milk powder in TBS– Tween 20 (0.1%) for 1 hour at room temperature. The antibodies from Cell Signaling, for example, phospho-p44/42 mitogen-activated protein kinase (MAPK; p-Erk1/2) rabbit monoclonal antibody (Invitrogen R960-25) was used at 1:5,000. After incubation with primary antibodies for overnight at 4°C, horseradish peroxidase–labeled secondary antibodies were then applied to the membranes for 1 hour at room temperature. Signals were visualized using enhanced chemiluminescence Western blotting detection reagents (ThermoScientific).

**PC3 orthotopic mouse model**

PC3 PCa cells expressing a short hairpin RNA (shRNA) targeting FGFR-4 or scrambled shRNA controls, established as described previously (19), were injected orthotopically into the prostates of 8- to 10-week-old nude mice, using 1 × 10⁶ cells in a volume of 20 μL. Mice were sacrificed at 7 weeks after injection, and the primary tumors were harvested and weighed. A full necropsy including pelvic and abdominal lymph nodes was conducted to identify metastasis. All primary tumors and metastases were confirmed by pathologic examination by a pathologist (M.I.). All procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

**Results**

**Expression of FGFR-4 Arg⁸⁸⁸ induces a gene expression signature associated with aggressive disease**

PNT1a are immortalized prostatic epithelial cells, and we have shown that in PNT1a cells expressing exogenous FGFR-4 Arg⁸⁸⁸ or Gly⁸⁸⁸ under the control of the EF1 promoter, almost all FGFR signaling can be attributed to the transfected receptor and the 2 FGFR-4 isofoms are expressed at equivalent levels in the 2 cell lines (19). We have also shown that serum contains FGF ligands capable of activating FGFR-4 (19) and in such conditions the Arg⁸⁸⁸-expressing PNT1a cells display increased invasiveness and motility compared with Gly⁸⁸⁸-expressing cells (7, 19). To further understand the underlying molecular mechanisms of increased cell motility and invasiveness in Arg⁸⁸⁸-expressing cells, microarray studies of biological duplicates were conducted on FGFR-4 Arg⁸⁸⁸- and Gly⁸⁸⁸-expressing PNT1a cells, using Agilent 44k whole genome expression microarrays, to identify the effector genes that may be responsible for phenotypic differences between the 2 variants. A total of 229 genes that were upregulated by at least 1.4-fold in FGFR-4 Arg⁸⁸⁸ cells compared with Gly⁸⁸⁸ cells and 212 genes that showed a similar downregulation (Fig. 1A). Selected genes that were upregulated or downregulated are shown, and the full gene list is shown in Supplementary Tables S2 and S3. Of note was the upregulation of cytokines such as interleukin (IL)-1β, IL-1α, and CXCL1. Other genes included MAIP1, which is associated with invasion; lysyl oxidase (LOX) and several collagen genes, which indicate a more mesenchymal phenotype; transcription factors (ZNFI9 and ZNF22), and genes involved in signaling transduction (GRB1, CRKL, and RelA). IL-18, which is associated with antitumor immunity (28), was downregulated as were several genes known to be downregulated during the transition from prostatic intraepithelial neoplasia and invasive cancer (ITGB2, ref. 29; CD40, ref. 30). We confirmed the upregulation of 7 genes in PNT1a cells expressing Arg⁸⁸⁸ versus Gly⁸⁸⁸ cells, using quantitative RT-PCR (see below). Thus, the expression microarrays accurately reflect changes in gene expression induced by FGFR-4 Arg⁸⁸⁸ and a number of genes with altered expression have relevant biological activities.

Given the association of the FGFR-4 Arg⁸⁸⁸ isoform with aggressive disease, we compared our FGFR-4 Arg⁸⁸⁸ gene signature with the gene expression data of Glinsky and colleagues (22) and examined whether the FGFR-4 Arg⁸⁸⁸ signature was associated with aggressive clinical behavior. As can be seen in Figure 1B and C, patients with the FGFR-4 Arg⁸⁸⁸ gene signature were significantly more likely to recur following radical prostatectomy than men with cancers without the Arg⁸⁸⁸ gene signature (log-rank test, P = 0.003).

**Enhanced induction of SRF and AP1 activities by the FGFR-4 Arg⁸⁸⁸ allele**

To characterize the signaling pathways that account for the observed differences in phenotype and gene expression...
changes between FGFR-4 Arg\textsuperscript{388} and Gly\textsuperscript{388} variants, we transfected a set of luciferase promoter reporter constructs into PNT1a cells expressing either the Arg\textsuperscript{388} or Gly\textsuperscript{388}FGFR-4 allele. We found that luciferase reporter constructs containing SRE, which bind SRF, showed a 13-fold increase in luciferase expression in Arg\textsuperscript{388}-expressing PNT1a cells when compared with the Gly\textsuperscript{388}-expressing cells (Fig. 2A). A 7-fold increase was seen for reporter constructs with AP1 binding sites in the promoter. There were no significant differences in NIFAT or ARE promoter activities (Fig. 2A). It should be noted that PNT1a cells, like all nontransformed prostatic epithelial cells in culture, do not express androgen receptor (AR); so, the ARE is a negative control. To further characterize these differences, we carried out a detailed time course study of these cell lines in both serum-free medium with FGF2 as the only growth factor and serum-containing medium. Cells were plated and the next day transfected with SRE or AP1 reporter constructs and then placed in serum-free media without FGF2 overnight. Cells were then stimulated with FGF2 and luciferase activity was measured in cell extracts collected at intervals over 24 hours. FGF2 was chosen as the ligand, as it stimulates FGFR-4 and has repeatedly been shown to be elevated in PCa (31). SRE expression constructs showed an almost 10-fold increase in luciferase activity in FGFR-4 Arg\textsuperscript{388}-expressing cells, peaking at 3 hours after FGF2 addition (Fig. 2B). The AP1 reporter constructs exhibited an approximately 4-fold increase in activity (Fig. 2B) and had a more sustained increase in activity. The Gly\textsuperscript{388}-expressing cells showed a more modest (4-fold) induction of luciferase activity with SRE constructs and no significant induction with the AP1 constructs. Similar experiments were carried out by serum stimulation and included PNT1a Arg\textsuperscript{388} and Gly\textsuperscript{388} cells lines in which SRF was knocked down using shRNA. As shown in Figure 2C, the FGFR-4 Arg\textsuperscript{388}-expressing cells showed an 8-fold increase in SRE promoter activity by 6 hours as compared with less than 3-fold for the Gly\textsuperscript{388} cells in response to serum stimulation. This response was significantly decreased in shSRF-expressing cells. The AP1 reporter construct showed a 3.5-fold increase in the Arg\textsuperscript{388}-expressing cells by 6 hours after serum stimulation, and the Gly\textsuperscript{388} expression cells showed a 2-fold increase. This response was completely abolished in shSRF-expressing cell lines, indicating that the induction of AP1 is downstream of SRF. Interestingly, the Arg\textsuperscript{388}- and Gly\textsuperscript{388}-expressing cell lines both displayed a transient 2-fold increase in AP1 reporter activity at 15 minutes after stimulation in serum that was not seen in FGF2 serum-free medium. This transient response was not seen in the shSRF-expressing cells and is presumably due to other growth factors in serum that activate SRF via a more transient signal. Thus, both FGFR-4 Arg\textsuperscript{388} and Gly\textsuperscript{388} activate SRF and AP1 signaling but the response in FGFR-4 Arg\textsuperscript{388}-expressing cells is more robust.

**FGFR-4 Arg\textsuperscript{388} activates ERK signaling**

It is known that SRF and AP1 transcription can be activated by ERK signaling in response to growth factor

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**Figure 1.** Gene transcription signature of FGFR-4 Arg\textsuperscript{388} versus FGFR-4 Gly\textsuperscript{388} cells. A, heat map of genes differentially expressed between FGFR-4 Arg\textsuperscript{388} and FGFR-4 Gly\textsuperscript{388} PNT1a cells (yellow, high expression; blue, low expression). B, the expression patterns of the Arg388 gene with patient outcome, treating the coefficient as a continuous variable. C, Kaplan-Meier analysis comparing the differences in risk of disease relapse between human tumors showing activation (yellow line, $t$-score $>0$) of the Arg388 signature and tumors showing deactivation (blue line, $t$-score $<0$) of the signature. Log-rank test evaluates whether there are significant differences between the 2 arms. The univariate Cox test evaluates the association of the Arg388 signature $t$-score with patient outcome, treating the coefficient as a continuous variable.
Figure 2. FGFR-4 Arg388 activates SRE and AP1 transcription. A, PNT1a cells expressing FGFR-4 Arg388 or Gly388 were transfected with luciferase reporter constructs under the control of SRE-, AP1-, NFAT-, and ARE-regulated promoters. Twenty-four hours after transfection protein lysates were prepared and normalized luciferase activity was determined. Mean ± SD of triplicates. B, the cell lines used above were plated and the next day transfected with SRE or AP1 reporter constructs and then placed in serum-free media without FGF2 overnight. Cells were then stimulated with FGF2 and normalized luciferase activity measured in cell extracts collected at intervals over 24 hours. C, PNT1a cells expressing Arg388 or Gly388 with stable expression of shRNA knocking down SRF, or vector controls were transfected and placed in serum-free medium as above. FBS was added (final 10%) after 24 hours and normalized luciferase activity measured in cell extracts collected at intervals over 24 hours.
stimulation (32). To determine whether FGFR-4 Arg$^{388}$ increases ERK signaling when compared with Gly$^{388}$, we serum starved parental PNT1a cells and PNT1a-expressing FGFR-4 Arg$^{388}$ or Gly$^{388}$ overnight and stimulated them with FGF2 or FGF19 or vehicle only. FGF19 activates FGFR-4 in the presence of Klotho coreceptors (33) and PNT1a cells express α-Klotho (unpublished data). FGF2 binds multiple FGFRs, including FGFR-4, with high affinity. Protein lysates were collected at 3 or 6 hours and analyzed by Western blotting. As can be seen in Figure 3A, at 3 hours, the FGFR-4 Arg$^{388}$–expressing cells had significantly increased ERK phosphorylation compared with FGFR-4 Gly$^{388}$–expressing cells and both had higher phosphorylation than in PNT1a cells. At 6 hours, the FGFR-4 Arg$^{388}$–expressing cells maintained high levels of ERK phosphorylation, consistent with the sustained FGFR-4 phosphorylation after FGF stimulation of this isoform as we have shown previously (19). The FGFR-4 Gly$^{388}$ cells had low levels of ERK phosphorylation at this time point, similar to control PNT1a. Thus, FGFR-4 Arg$^{388}$ cells have higher and more sustained ERK phosphorylation than Gly$^{388}$–expressing cells.

To determine whether the increased luciferase activity of SRE and AP1 reporter constructs in FGFR-4 Arg$^{388}$-expressing PNT1a cells was due to enhanced ERK activation, we carried out inhibitor studies with U0126, an inhibitor of mitogen-activated protein kinase kinase, which activates ERK signaling. As shown in Figure 3B, U0126 inhibited luciferase activity, driven by either of these promoters, by approximately 70%. U73122, a PLC-γ inhibitor, decreased activity of the SRE and AP1 reporters by 40% and 25%, respectively. It has been shown previously that PLC-γ can enhance ERK kinase signaling by FGF (34). The c-Jun NH2-kinase (JNK) inhibitor SP600125 did not inhibit reporter activity of either of these promoters. These findings indicate that FGFR-4 Arg$^{388}$ activates SRF and AP1 signaling through increased ERK signaling.

To determine the extent to which ERK can activate the gene expression changes in the FGFR-4 Arg$^{388}$ cells, we examined expression of 7 genes that were identified as being upregulated by FGFR-4 Arg$^{388}$, using microarrays by quantitative RT-PCR with RNAs from Arg$^{388}$ and Gly$^{388}$ PNT1a cells treated with U0126 and control (vehicle only). The results of 4 such studies are shown in Figure 3C. All genes showed increased expression in Arg$^{388}$ cells relative to Gly$^{388}$ cells in the vehicle controls. For 6 of 7 genes tested, U0126 significantly decreased gene expression from 40% to 90% in Arg$^{388}$ cells, with variable decreases in Gly$^{388}$ cells (0% to >90%). One gene (collagen 5a) showed no decrease in response to U0126, although it increased in the Arg$^{388}$–expressing cells. Thus, the majority of genes upregulated in Arg$^{388}$ PNT1a cells are significantly regulated by ERK activity but some are upregulated by other pathways.

We also compared expression in FGFR-4 Arg$^{388}$ and Gly$^{388}$ cells with control PNT1a cells for 4 genes. In all cases, gene expression in the FGFR-4 Arg$^{388}$ PNT1a cells was statistically significantly higher than in PNT1a cells whereas 3 of 4 genes tested had significantly higher expression in the FGFR-4 Gly$^{388}$ cells ($P < 0.01$, t test) than in control PNT1a cells (Supplementary Fig. S1). Interestingly, the only gene that did not show higher expression in the FGFR-4 Gly$^{388}$ was collagen 5a, which is not ERK regulated, and collagen 5a expression was actually lower ($P = 0.02$, t test) in the FGFR-4 Gly$^{388}$ cells than in control PNT1a, suggesting the possibility that FGFR-4 Gly$^{388}$ may negatively regulate activity of a non-ERK pathway. Our data indicate that FGFR-4 Gly$^{388}$ increases ERK activity but to a lesser extent than Arg$^{388}$.

**ERK enhances invasion via upregulation of SRF activity**

We have shown that FGFR-4 Arg$^{388}$ enhances tumor invasion and motility and that it can activate a series of gene expression changes that are associated with aggressive behavior in PCa by ERK and SRF activity. We therefore examined whether inhibition of ERK with U0126 could inhibit invasion in FGFR-4 Arg$^{388}$–expressing PNT1a cells (Fig. 4A). U0126 markedly inhibited invasion of Arg$^{388}$–expressing cells and to a lesser extent that of Gly$^{388}$–expressing PNT1a cells. To determine whether this inhibition involves SRF, we compared invasion of Arg$^{388}$ and Gly$^{388}$ PNT1a expressing a shRNA-targeting SRF. The shSRF-expressing Arg$^{388}$ cells were markedly less invasive and were similar to the Gly$^{388}$ cells (Fig. 4B). To confirm these results in a second system, we expressed FGFR-4 Arg$^{388}$ and Gly$^{388}$ at equal levels (by quantitative RT-PCR) in DU145 PCa cells and evaluated invasiveness in FGF-defined medium with or without U0126. As can be seen in Figure 4C, the FGFR-4 Arg$^{388}$–expressing cells were more highly invasive than the Gly$^{388}$–expressing cells and U0126 markedly inhibited invasion for both cell lines.

**SRF expression in PCa tissues**

The data above implicate SRF as a key target of FGFR-4 Arg$^{388}$. To confirm that SRF is expressed in PCa and determine the clinical relevance of such expression, we analyzed the expression of SRF by IHC using a large PCa tissue microarray with more than 400 PCas from radical prostatectomies. Expression was quantitated as described previously (26, 35), based on a multiplicative index of the average staining intensity (0–3) and extent of staining (1–4) in the cores yielding a 10-point staining index (0–9). Examples of strong (index 9) and weak tumor (index 2) staining are shown in Figure 5A. Staining was exclusively nuclear. Overall, 80% of cancers had detectable SRF expression. Almost half (48%) of all men had moderate to strong expression of SRF (index >4). Strong staining of stromal nuclei was noted (Fig. 5A). This is consistent with ongoing growth factor stimulation of stromal cells by growth factors including FGFs. Expression of SRF was significantly correlated with both extracapsular extension of stromal cells by growth factors including FGFs. Expression of SRF was significantly correlated with both extracapsular extension (0.138, $P = 0.027$) and Gleason score (0.218, $P < 0.0001$). Consistent with this, men with cancers with moderate to strong SRF staining index (>4) had a significantly increased risk of biochemical recurrence following radical prostatectomy (log-rank test = 0.004; Fig. 5B). In addition, SRF expression
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Figure 3. ERK regulation of the FGFR-4 Arg<sup>388</sup> enhanced transcription. A, FGFR-4 Arg<sup>388</sup> (AA)- and Gly<sup>388</sup> (GG)-expressing PNT1a or control PNT1a cells were serum starved overnight were stimulated with FGF2 (25 ng/mL) or FGF19 (50 ng/mL) for 3 and 6 hours. Cell lysates were prepared and Western blot analyses were conducted using antibodies against phospho-p44/42 ERK (p-Erk1/2), p44/42 ERK (Erk1/2), V5-epitope, and β-tubulin. B, FGFR-4 Arg<sup>388</sup>- and Gly<sup>388</sup>-expressing PNT1a cells were treated with U0126 (10 μmol/L) or vehicle only for 24 hours and transfected with SRE or AP1 reporter plasmids and normalized luciferase activity determined at 24 hours. Mean ± SD of triplicates. C, FGFR-4 Arg<sup>388</sup>- and Gly<sup>388</sup>-expressing PNT1a cells were treated with U0126 (10 μmol/L) for 24 hours or treated with vehicle only, RNAs collected, and expression of IL-1ß, MMP1, CXCL1, and collagen 5α determined by quantitative RT-PCR. Values are relative to the lowest expressing sample (Gly<sup>388</sup> treated with U0126 in all cases).

was significantly correlated with proliferation (35), as assessed by Ki67 IHC (r = 0.156, P = 0.03), and negatively correlated with apoptosis (36), as assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (–0.183, P = 0.016). There was also a strong correlation with the percentage reactive
stoma in the tumor ($0.221, P < 0.0001$). Our group has previously shown that cancers with increased reactive stroma are more aggressive than those with only minimal stromal response (37). Finally, we also found a strong correlation with perineural tumor diameter ($0.3, P < 0.0001$). Maru and colleagues (38) have shown that large diameter perineural invasion is a very strong predictor of biochemical recurrence following radical prostatectomy. Thus, SRF is expressed at moderate to high levels in half of PCAs and higher SRF expression is associated with poor outcome following radical prostatectomy, perhaps in part by altering interactions between cancer cells and their microenvironment.

The $\text{FGFR-4 Arg}^{388}$ promotes PCa growth and metastasis

To determine whether $\text{FGFR-4}$ impacts tumor growth and metastasis of PCa cells in vivo, we derived PC3 PCa cells expressing a shRNA targeting $\text{FGFR-4}$ cells using a lentiviral vector. $\text{FGFR-4}$ mRNA was decreased by 72% in these cells compared with PC3 scrambled shRNA control cells. We found a significant inhibition of proliferation (Fig. 6A) and invasion (Fig. 6B) in vitro in the sh$\text{FGFR-4}$ PC3 cells compared with vector controls. We then evaluated primary tumor growth and metastatic potential in an orthotopic model in which sh$\text{FGFR-4}$ or scrambled shRNA control cells are injected directly into the prostates of nude mice for each group. In this model, cancer cells grow initially in a native prostatic environment and metastasize to pelvic and abdominal lymph nodes. We sacrificed mice 7 weeks after injection, weighed all tumors, and submitted primary tumors and lymph nodes for histopathologic analysis. $\text{FGFR-4}$ knockdown significantly inhibited tumorigenicity in this model ($P = 0.01$, Fisher’s exact test). As shown in Figure 6D, in mice with pathologically confirmed primary tumors, primary tumor weight decreased 63% in sh$\text{FGFR-4}$-tumors ($P = 0.02$, t test) and the proportion of mice with lymph node metastasis decreased from 16 of 17 to 5 of 10 (Fig. 6C; $P = 0.02$, Fisher’s exact test). Thus, in PC3 cells, decreased expression of $\text{FGFR-4}$ mRNA is associated with impaired progression at both the primary and metastatic sites.

It should be noted that the mean size of primary tumors in mice without metastasis was smaller than that of primary tumors in mice with metastasis in the group injected with sh$\text{FGFR-4}$ cells (74 vs. 172 mg), but this difference was not statistically significant ($P = 0.08$, t test). Thus, it is difficult to conclusively disentangle effects of $\text{FGFR-4}$ on primary tumor growth and metastasis. This experiment was repeated in a second, independent set of PC3 sh$\text{FGFR-4}$-cells and controls with similar results (data not shown). It should be noted that PC3 cells are homozygous for the $\text{Arg}^{388}$ allele so that this effect is mediated by downregulation of this variant.

Discussion

Increased expression of $\text{FGFR-4}$ and its ligands such as FGF8 and FGF17 in human PCa specimens has been
correlated with aggressive clinical behavior (4–6, 39), including bone metastasis (4) and PCa-specific mortality (6). In addition, the presence of the FGFR-4 Arg388 polymorphism has been associated with more aggressive disease in PCa (7, 40) and other malignancies (10–12, 14, 15).

We have now shown that expression of FGFR-4 Arg388 leads to activation of SRF- and AP1-mediated transcription, primarily via ERK signaling. The transcriptional program induced by FGFR-4 Arg388 signaling is associated with biochemical recurrence after radical prostatectomy. In vitro, SRF and ERK activation by FGFR-4 Arg388 is associated with increased invasion. In vivo, knockdown of FGFR-4 Arg388 in PC3 cells results in decreased tumorigenicity, primary tumor growth, and metastasis. It should be noted that although the FGFR-4 Arg388 variant is more potent at promoting invasion, knockdown of FGFR-4 in DU145 cells (which are homozygous Gly388) markedly inhibits invasion (39) and our data indicate that FGFR-4 Gly388 activates ERK, although to a lesser extent than the Arg388 isoform. Thus, both variants probably contribute to the observed correlation of FGFR-4 expression with aggressive clinical behavior in PCa.

There have been few studies on the role of SRF in PCa. Our data indicate that SRF is expressed in 80% of human PCas. Moderate to high levels are present in about half of human PCas, and elevated expression is associated with aggressive clinical features. It is not clear how SRF protein levels are regulated in PCa cells. It is known that the miR-133 microRNA can target SRF (41), and we have shown that miR-133 is downregulated in human PCa tissues (42) so that it is possible that miR-133 downregulation can increase SRF protein levels in some PCas. Increased levels of SRF protein can potentially enhance cellular response to growth factor activation of the SRF cofactor ELK1 with ERK by increasing total transcription factor complex formation. It is well known that SRF promotes both proliferation and cell migration (reviewed in ref. 43), consistent with our observations that SRF promotes invasion in vitro. The Tindall laboratory has shown that the AR coactivator FHL2 is an SRF target gene (44). Knockdown of SRF inhibits androgen induction of FHL2, transcription of AR target genes, and proliferation in LNCaP cells. Thus, SRF can promote AR activity in PCa, which may be important in its activity in promoting PCa progression.

The role of the ERK pathway in PCa has not been examined to the same extent as other pathways such as the PI3K pathway. Studies in mouse models have provided evidence that the ERK and PI3K pathways can cooperate in prostate carcinogenesis (45). Sprouty proteins, which negatively regulate ERK signaling in response to FGFR activation, are down regulated in PCa (26), suggesting there is pressure to enhance FGFR-mediated ERK activation in PCa.

**Figure 5.** SRF is expressed in PCa and increased expression is associated with biochemical recurrence following radical prostatectomy. Tissue microarrays containing PCa s (n = 387) were analyzed by IHC with anti-SRF antibody and staining was quantitated. A, examples of SRF expression in PCa. Left, strong uniform staining, index 9. Right, weak staining, index 2. Strong staining of stromal cell nuclei was noted (arrow). Bar, 100μm. B, the Kaplan–Meier plot of biochemical recurrence following radical prostatectomy for men with weak staining (< 4; blue) or moderate to strong staining (≥ 4; green).
The extent to which ERK activity in PCa cells is dependent on FGFR signaling, in general, and FGFR-4 signaling, in particular, is unclear. In breast cancer cells, knockdown of FGFR-4 leads to decreased ERK activation (46). Further studies are needed to define the relative importance of FGFRs in comparison to other growth factor receptors in activating ERK in PCa. However, given our finding that knockdown of FGFR-4 in PCa cells significantly decreases proliferation, invasion, tumor growth, and metastasis, it seems likely that this contribution is substantial. It should be noted that ERK signaling can also enhance AR signaling (47), making it a particularly attractive therapeutic target in PCa.

It is established that FGFR signaling leads to activation of PLC-γ signaling. The NFAT transcription factor can potentially be activated via PLC-γ-mediated increase in intracellular calcium. However, we did not observe any difference in NFAT transcriptional activity between FGFR-4 Arg388- and Gly388-expressing PNT1a cells in our luciferase reporter studies. There are 2 potential explanations. One is that there is no difference between the 2 receptor isoforms in PLC-γ signaling. Alternatively, FGFR-4-mediated PLC-γ signaling may not activate NFAT signaling. Studies by Thebault and colleagues (48) in prostate epithelial cells indicate that while different ligands can both activate PLC-γ signaling, some do not activate NFAT signaling because of differences in activation in TRPC calcium channels. Further studies are needed to understand the impact of FGFR-4 signaling on NFAT transcriptional activity.

Immunohistochemical studies in human PCa linking FGFR-4 expression to lethal PCa and the studies reported here indicate that inhibition of FGFR-4 is an important potential therapeutic target in PCa. A number of approaches to inhibiting FGFR activity (49), including FGFR-4-specific approaches (46), are currently under development. Several studies have shown that FGFR-4 activation can enhance resistance to chemotherapy (46, 50), so combination therapeutic approaches with either classic chemotherapy or other targeted agents are most likely to be successful.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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