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Altered Fibroblast Growth Factor Receptor 4 Stability Promotes Prostate Cancer Progression[1]

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Abstract

Fibroblast growth factor receptor 4 (FGFR-4) is expressed at significant levels in almost all human prostate cancers, and expression of its ligands is ubiquitous. A common polymorphism of FGFR-4 in which arginine (Arg^{388}) replaces glycine (Gly^{388}) at amino acid 388 is associated with progression in human prostate cancer. We show that the FGFR-4 Arg^{388} polymorphism, which is present in most prostate cancer patients, results in increased receptor stability and sustained receptor activation. In patients bearing the FGFR-4 Gly^{388} variant, expression of Huntingtin-interacting protein 1 (HIP1), which occurs in more than half of human prostate cancers, also results in FGFR-4 stabilization. This is associated with enhanced proliferation and anchorage-independent growth *in vitro*. Our findings indicate that increased receptor stability and sustained FGFR-4 signaling occur in most human prostate cancers due to either the presence of a common genetic polymorphism or the expression of a protein that stabilizes FGFR-4. Both of these alterations are associated with clinical progression in patients with prostate cancer. Thus, FGFR-4 signaling and receptor turnover are important potential therapeutic targets in prostate cancer.

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Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer deaths in American men [1]. Multiple genetic and epigenetic alterations have been described in prostate cancer, which can promote initiation and progression of this disease. Prominent among these changes are alterations in the fibroblast growth factor (FGF) signaling pathway (for review, see Kwabi-Addo et al. [2]). Fibroblast growth factors have a broad range of biologic activities that can play an important role in tumorigenesis including promotion of proliferation, motility, and angiogenesis and inhibition of cell death [2–5]. It is well established that multiple FGF receptor ligands are increased in prostate cancer [6–12] and, on the basis of correlations with clinical and pathologic parameters, seem to play a role in prostate cancer progression. Fibroblast growth factors interact with a family of four distinct, high-affinity tyrosine kinase receptors, designated FGFR-1–4 (for review, see Powers et al. [3]). Activation of FGF receptors leads to signal transduction through multiple pathways [2,3,13], and all of these pathways have been shown to be up-regulated in prostate cancer, and each contributes to prostate cancer initiation and progression [2,14].

There is now clear-cut evidence for the involvement of FGFR-4 in prostate cancer initiation and progression. All of the FGFs that are increased in human prostate cancer tissues are potent activators of FGFR-4. Our group [15] and others [16,17] have shown increased expression of FGFR-4 in prostate cancer by quantitative reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemistry. Strong expression of FGFR-4 in prostate cancer cells, as assessed by immunohistochemistry, is significantly associated with increased clinical stage and tumor grade and decreased patient survival [16]. On the basis of these studies, it is clear that FGFR-4 is expressed in almost all human prostate cancers.

A germ line polymorphism in the *FGFR-4* gene, resulting in the expression of FGFR-4 containing either glycine (Gly^{388}) or arginine (Arg^{388}) at codon 388 was identified several years ago, and the presence of the FGFR-4 Arg^{388} allele was 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 [18]. Since the initial

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report, further studies in a variety of malignancies, including soft tissue sarcomas [19], melanoma [20], lung adenocarcinoma [21], and head and neck squamous cell carcinoma [22,23] have linked the presence of the FGFR-4 Arg^{388} allele with aggressive disease and adverse clinical outcomes. There have been some discordant reports regarding the role of the FGFR-4 Arg^{388} in breast cancer [24], but this may be explained by differences in treatment regimen, because recent studies indicate that the FGFR-4 Arg^{388} polymorphism modulates response to chemotherapy [25]. We have found that the presence of homozygosity for the FGFR-4 Arg^{388} allele is significantly associated with prostate cancer incidence in white men [15]. Furthermore, the presence of the FGFR-4 Arg^{388} polymorphism is correlated with the occurrence of pelvic lymph node metastasis and biochemical (PSA) recurrence in men undergoing radical prostatectomy [15]. Expression of the FGFR-4 Arg^{388} variant results in increased cell motility and invasion as well as up-regulation of genes such as *uPAR* [15] and *Ehm2* [26], which are known to promote invasion and metastasis. These *in vitro* observations may explain, in part, the increased aggressiveness of prostate cancer in men bearing this polymorphism. This polymorphism is very prevalent in the white population, because approximately 45% of individuals are hetero- or homozygous for this allele in all white populations studied to date.

Although the expression of the FGFR-4 Arg^{388} variant is associated with prostate cancer initiation and aggressive disease, an important question remains: what is the molecular basis for the difference between the two FGFR-4 variants? Achondroplasia is caused by a similar mutation in FGFR-3 (Gly^{380} to Arg^{380}). Increased FGFR-3 signaling due to this mutation inhibits proliferation in chondrocytes [27,28] and hence the phenotype. Elegant studies have shown that this mutation leads to decreased receptor turnover due to increased receptor recycling and decreased targeting of receptors to lysosomes [27,28]. We therefore sought to determine whether a similar phenomenon occurs with the FGFR-4 Arg^{388} variant. In addition, we have begun to investigate other proteins that can potentially modulate FGFR-4 receptor stability. One such protein is Huntingtin-interacting protein 1 (HIP1). It can interact with clathrin as well α-adaptin, which is part of the AP-2 late endocytic complex [29]. It has been shown that HIP1 can stabilize epidermal growth factor (EGF) receptors [29,30], and the primary site of such stabilization seems to be through stabilization in early endosomes [29]. Overexpression of HIP1 leads to transformation of NIH3T3 cells [30]. Of note, it has been shown using immunohistochemistry that there is a moderate to strong expression of HIP1 in approximately 50% of clinically localized prostate cancers and that absence of HIP1 expression is associated with complete absence of biochemical recurrence after radical prostatectomy [31]. We report here that the FGFR-4 Arg^{388} variant has markedly decreased degradation and increased phosphorylation after ligand binding when compared to the Gly^{388} variant. Conversely, the interaction with HIP1 stabilizes the FGFR-4 Gly^{388} variant and results in increased proliferation and soft agar colony formation. Thus, altered FGFR-4 receptor trafficking is a common feature in human prostate cancer, which is associated with changes in cellular behavior *in vitro* and clinical progression.

Materials and Methods

Tissue Samples and Cell Lines

RNA were extracted from snap-frozen cancer tissues (>70% cancer) and benign peripheral zone tissue from men undergoing radical prostatectomy as described previously [32]. PNT1A, a nontumorigenic SV40-immortalized human prostatic epithelial cell line, and the PC3, LNCaP, and DU145 prostate cancer cell lines were all maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). 293T cells were maintained in DMEM with 10% FBS.

Sh-FGFR-4 and Sh-HIP1 Lentivirus

Lentiviruses to knock down FGFR-4 or HIP1 RNA were generated using Block-iT Lentiviral RNAi Expression System (Invitrogen, Carlsbad, CA). Primers used for lentiviral constructs were as follows: Sh R4 F 5′CACCGCATAGGGACCTCTCGAAT ATTCGAAAATATTC-GAGAGGTCCCTATGC-3′, Sh R4 R 5′AAAAGCATAGG-GACCTCTC GAATATTTTCGAATA TTCGAGAGGTCCCT ATGC-3′, Sh-HIP1F 5′- CACCGGACTCAGACT GTCAGCAT-CACGAATGATGCTGACAGTCTGAGTCC -3′, Sh-HIP1R 5′-AAAAGGACTCAGAC TGTCAGCATCATTCGTGATGCTGA-CAGTCTGAGTCC -3′. Lentiviral constructs were generated according to the manufacturer's instruction using 293FT cells. After infection with lentivirus, cells were selected in blasticidin (2 μg/ml)–containing medium. RNA was extracted from pooled cells, and mRNA levels of target genes were quantitated by quantitative RT-PCR.

Expression Vector Cloning, Transfection, and Stable Selection

The full-length FGFR-4 Gly^{388} and Arg^{388} isoforms were obtained from DU145 and PC3 cell line cDNA, respectively, and cloned into Topo-V5 expression vector (Invitrogen) by using primers of FGFR-4 Exp F: 5′- CCT GAGAGCTGTGAGAAG G-3′; and FGFR-4 Exp R: 5′-GGATCCAGCTCCTTCCCC-3′. Annealing temperature was 60°C. HIP1 constructs were cloned into pCMV-Tag2B expression vector (Stratagene, La Jolla, CA), which has a Flag Tag at N terminal in frame. The full-length HIP1 and N-terminal truncated HIP1(–183) sequences were obtained from PNT1A cell line cDNA by RT-PCR using primers with a 5′ *Sal*I cutting site and a 3′ *Xho*I site. Primers were as follows: HIP1 Exp F (*Sal*I): 5′- ACGCGTCGACATGGAT-CGGAT G-3′; HIP1 Exp F2 (*Sal*I): 5′-ACGCGTCGACCAGTTAA CAGTGGAG -3′; HIP1 Exp R (*Xho*I): 5′-CCGCTCGAGTTCTT-TTTCGGTTACCA C-3′. All constructs were sequenced to confirm the absence of mutations due to the PCR reaction and verify the accuracy of the tag sequences in frame. The expression of full-length proteins was confirmed by Western blot using cell lysate from transiently transfected 293T cells overexpressing FGFR-4 or HIP1. Anti-V5 antibody (1:5000) from Invitrogen and anti-Flag antibody (1:2000) from Stratagene were used. To generate stable cell lines, 3×10^5 cells per 60-mm dish were prepared 24 hours before transfection. Approximately 2 μg of plasmid was transfected with 6 μl of Fugene6 (Invitrogen) in a total volume of 4 ml of OPTI-MEM without serum. Five hours after transfection, 1 ml of FBS was then added to each dish to achieve a final serum concentration of 20%. After an additional 18 hours of incubation, cells were refed with complete medium and then split 1:3 after 48 hours. The next day, selection was initiated by the addition of G418 at 200 μg/ml. Selection was carried out for 2 weeks, and long-term cultures were routinely maintained in G418 (100 μg/ml).

Generation of PNT1A Cells Stably Expressing FGFR-4 Gly^{388} and Arg^{388}

FGFR-4 Gly^{388} variant (GI 33873872) transcript was amplified and cloned into pCR2.1-Topo (Invitrogen) using primers GTTC-TAGAGCCATGCGGCTGCTGCTGGCCCTGTTGGG and

CGGGCCCGC TGTCTGCACCCCAGACCCGAAGGGG. The FGFR-4 encoding fragment was released by *Xba*I/*Apa*I and subcloned into pcDNA 3.1/V5-His-Topo vector (Invitrogen) to create a V5-tagged FGFR-4 expression cassette. The V5-tagged FGFR-4 Arg^{388} variant was obtained by site-directed mutagenesis of the above construct using primers CCTGGCCTTGGCTGTGCTCCTGCTG CTGGCCAGGCTGTATCG and GCCGTGGAGCGCCTGCCC-TCGATACAGCCTGGCCAG CAGCAG (codon 388 from GGG to AGG). The above V5-tagged FGFR-4 protein-encoding cassettes were released by *Xba*I/*Pme*I digestion and inserted into a lentiviral vector pCDH-MCS1-EF1-Puro (SBI, Mountain View, CA) digested by *Xba*I/*Swa*I to create pCDH-FGFR4(Gly^{388})-EF1-Puro and pCDH-FGFR4(Arg^{388})-EF1-Puro. By cotransfection of pCDH-FGFR4 (Gly^{388})-EF1-Puro and pCDH-FGFR4(Arg^{388})-EF1-Puro with the necessary packaging plasmids in 293 cells, lentiviruses were generated to transduce PNT1A cells. After transduction, PNT1A cells expressing FGFR-4 Gly^{388} and Arg^{388} were stably selected in puromycin-containing medium.

Western Blot and Immunoprecipitation

Total protein was extracted from cells using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). For Western blots, 40 μg of protein extract/lane were electrophoresed, transferred to nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Piscataway, NJ), and incubated overnight with an anti-V5 monoclonal antibody (at 1:5000 dilution; Invitrogen), anti-Flag M2 monoclonal antibody (at 1:2000 dilution; Stratagene), anti–FGFR-4 (at 1:500 dilution; Santa Cruz Biotechnology), anti-HIP1 (at 1:10,000 dilution; Novus Biologicals, Littleton, CO), or anti–β-actin (at 1:5000 dilution; Sigma, St Louis, MO). Membranes were washed and treated with mouse anti-goat IgG (at 1:5000 dilution; Santa Cruz Biotechnology) or goat anti-rabbit (at 1:50,000 dilution; Santa Cruz Biotechnology) conjugated to horseradish peroxidase. The antigen–antibody reaction was visualized using an enhanced chemiluminescence (ECL) assay (Amersham Biosciences) and was exposed to ECL film (Amersham Biosciences). For immunoprecipitation, 1 mg of protein lysate from each sample was incubated with anti-V5 (1:500) overnight at 4°C. Tyrosine phosphorylation of FGFR-4 $Tyr^{641/642}$ was detected using phospho-FGF receptor-specific mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA) at 1:1000 dilution.

Quantitative Real-Time PCR

Quantitative RT-PCR was carried out using the basic procedure described previously [32]. Primers for HIP1 (GenBank Accession No. NM_005338). HIP1 RT Forward: 5′-TGCTCTGCTGGAA-GTTCTG-3′; HIP1 RT Reverse: 5′-CTGGCGGTCACTCAT CTG -3′. Primers for β-actin were as described previously [32]. All real-time PCR efficiencies were controlled in the range of 100 ± 5%.

Receptor Degradation Assay

293 cells, 2×10^6, were plated into each 10-cm dishes 24 hours before transfection. Cells were transfected with 8 μg of plasmid in total in each dish. At 48 hours after transfection, cells were trypsinized and washed three times with ice-cold PBS. Cell pellets were resuspended in PBS at ~2.5×10^7/ ml. A total of 200 μl of 10 mM biotin was added into each 1-ml PBS cell suspension. After 15 minutes of incubation at 4°C, cells were washed three times using PBS with 100 mM glycine. They were then replated at 37°C with complete DMEM plus 100 ng/ml FGF2 and 20 U/ml heparin for different periods. Cells were collected at 0, 6, 24, and 48 hours and were lysed with 1 ml of RIPA buffer with 1% Triton and then sonicated. For immunoprecipitation, 60 μl of streptavidin–agarose beads were used for 1-mg lysate of each sample, with overnight incubation at 4°C.

In Vitro *Binding Assay*

To investigate which domain of HIP1 protein associates with FGFR-4, we made a series of purified HIP1 proteins with truncated domains by using Variflex Bacterial Protein Expression System (Stratagene). We used N-terminal SBP-SET2c for HIP1 proteins expression due to different enzyme cutting sites. Primers used were as follows: HIP1 Exp F V (*Sma*I): 5′-TCC CCC GGG ATG GAT CGG ATG-3′; HIP1 Exp F2 V (*Sma*I): 5′-TCC CCC GGG CAG TTA ACA GTG G -3′; HIP1 Exp F3 V (*Sma*I): 5′-TCC CCC GGG GAG ATC AGT GGA TTG-3′; HIP1 Exp F4 V (*Sma*I): 5′-TCC CCCGGG ACTCAGCTCAAA C-3′; HIP1 Exp F5 V (*Sma*I): 5′- TCC CCCGGGGCCAGA ATAGAG-3′; HIP1 Exp R V (*Xho*I): 5′-CCGCTCGAGCTA TTCTTTTTC GGTTAC-CAC-3′. Purified proteins, dissolved in streptavidin-binding buffer at 0.2 μg/μl, were verified by electrophoresis and Coomassie Blue staining before being applied to the binding assay. The 293T cell lysate (350 μl) in RIPA buffer from cells with transient overexpression of FGFR-4 was mixed with 50 μl of different purified HIP1 domain proteins. After incubation at 4°C overnight, streptavidin beads were spun down and washed three times with phosphate-buffered saline/ 0.5% Tween 20 followed by standard Western Blot by using anti-V5 antibody.

Proliferation Assays

PNT1A or DU145 cells overexpressing HIP1 protein or vector controls were plated at 1×10^5 per 60-mm dish. Cells were grown in RPMI 1640 with 10% FBS or 0.5% FBS, or 1% insulin–transferrin–selenium (Sigma) plus 20 ng/ml FGF2, and were counted at 2, 4, 6, and 8 days. Cell number was determined in triplicate using a Coulter counter.

Soft Agar Colony Formation Assay

35 mm dishes with 0.5% base agar layer mixed with 1× culture medium plus 10% FBS were prepared before the seeding of cells. Approximately 10^5 PNT1A cells transfected with HIP1 expression vector or control were plated in 0.35% top agar layer over the base agar. Plates were stained with crystal violet, and cell colonies were counted after incubating at 37°C in a humidified incubator for 3 weeks.

Migration Assay

Cells were seeded at 2.5×10^6 in 60-mm diameter culture dishes in complete medium and analyzed using a classic scratch wound method as described previously [15].

Results

The FGFR-4 Arg^{388} Variant Has Decreased Degradation and Sustained Phosphorylation after Ligand Stimulation Compared to the Gly^{388} Variant

To assess differences in receptor degradation between the two FGFR-4 variants after ligand stimulation, we transfected 293T cells

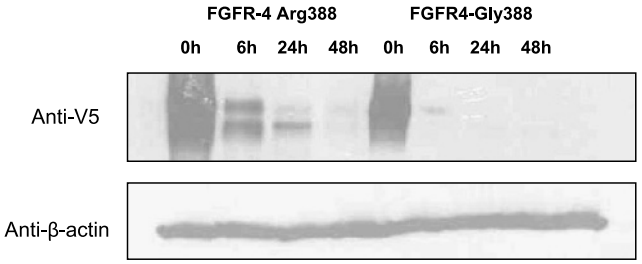

Figure 1. Increased stability of the FGFR-4 Arg^{388} variant after ligand stimulation. 293T cells were transfected with V5-tagged FGFR-4 Arg^{388} or Gly^{388} and cell surface receptors labeled with biotin. Cells were then stimulated with FGF2 and lysed at the indicated time. Labeled receptors were then immunoprecipitated with streptavidin–agarose and FGFR-4 detected by Western blot of the immunoprecipitates with anti-V5 antibody. Western blot of an aliquot of the lysate used for immunoprecipitation with anti–β-actin antibody is shown.

with either the Arg^{388} or Gly^{388} FGFR-4 variant, biotinylated cell surface receptors, and lysed cells at intervals after ligand (FGF2) stimulation. Fibroblast growth factor 2 is a known ligand of FGFR-4 [33] and is present at increased levels in prostate cancer [7]. Biotin-labeled proteins were then precipitated using streptavidin beads, and the precipitates were used for Western blots with anti-V5 antibody recognizing a V-5 tag on the transfected FGFR-4 variants. As can be seen in Figure 1, the FGFR-4 Arg^{388} variant is much more stable than the Gly^{388} variant. We have repeated this experiment multiple times with essentially identical results. Thus, the FGFR-4 Arg^{388} variant is degraded much more slowly than the Gly^{388} variant after ligand binding.

To determine whether there were differences in FGFR-4 receptor phosphorylation between the two variants, we expressed the FGFR-4 Arg^{388} and Gly^{388} variants in the immortalized normal prostatic epithelial cell line PNT1A using a lentivirus engineered to express V5-tagged FGFR-4. This cell line is heterozygous for the FGFR-4 Arg^{388}/Gly^{388} locus, and quantitative RT-PCR reveals that both lentivirus infected cell lines express approximately 90-fold higher levels of FGFR-4 than control PNT1A cells (data not shown). Thus, while some native FGFR-4 Arg^{388} is present in the Gly^{388} overexpressing cell line, it constitutes approximately 1% of total FGFR-4 in that cell line and similar considerations hold for the Arg^{388}-overexpressing cell line. We have shown previously that PNT1A cells expressing the FGFR-4 Arg^{388} variant have significantly increased motility when compared to cells expressing the FGFR-4 Gly^{388} variant. To confirm this finding and verify that the V5-tag does not alter the biologic activity of FGFR-4, we carried out a scratch assay using the two cell lines. As seen in Figure 2*A*, cells expressing the FGFR-4 Arg^{388} variant migrated and filled the wound much more quickly than cells expressing the Gly^{388} variant, consistent with our prior results. We stimulated cells with FGF2 after overnight incubation in medium without FGFs. Cells were harvested at intervals and FGFR-4 receptor phosphorylation assessed using an FGF receptor phosphorylation–specific antibody after immunoprecipitation with anti-V5 antibody. This antibody specifically recognizes a completely conserved tyrosine phosphorylation site present in all FGF receptors [$Tyr^{653/654}$ in FGFR-1; $Tyr^{642/643}$ in FGFR-4 (NP_998812.1)]. This site is phosphorylated in response to ligand binding and is essential for FGF receptor activity [34]. As can be seen in Figure 2*B*, there is a sustained phosphorylation of the FGFR-4 Arg^{388} variant in comparison to the Gly^{388} variant after ligand stimulation. Thus, the FGFR-4 Arg^{388} variant displays increased stability and much higher levels of sustained activation than the Gly^{388} variant.

HIP1 mRNA Is Increased in Prostate Cancer and Increased Expression Is Associated with PSA Recurrence after Radical Prostatectomy

Previous immunohistochemical studies have shown that HIP1 protein is increased in prostate cancer and that absence of HIP1 staining was associated with a favorable prognosis after radical prostatectomy [31]. To confirm these findings using an alternative approach, we

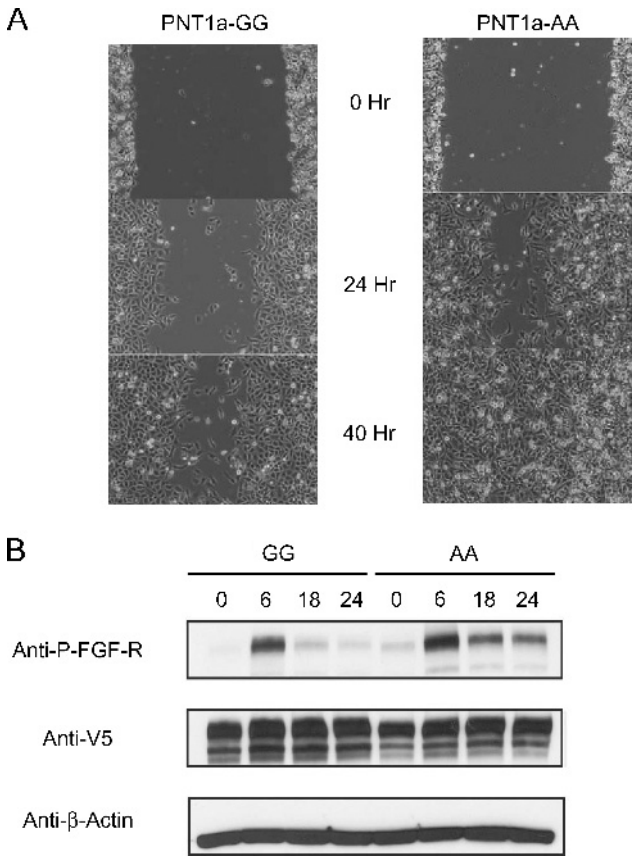

Figure 2. Sustained phosphorylation of the FGFR-4 Arg^{388} variant after ligand stimulation. (A) PNT1A cells expressing V5-tagged FGFR-4 Arg^{388} or Gly^{388} were seeded at 2.5×10^6 in 60-mm diameter culture dishes in complete medium. Cells were gently scraped with a plastic tip. The medium was removed, and cells were washed twice with PBS. Complete medium was added, and cells were allowed to scatter/migrate into the area of clearing for a total of 40 hours, and photomicrographs were taken at 0-, 24-, and 40-hour time points. Scratch assays were performed four times, and representative results are shown. (B) PNT1A cells expressing either V5-tagged FGFR-4 Arg^{388} (AA) or Gly^{388} (GG) were plated. After overnight incubation in medium with insulin as the only growth factor, cells were stimulated with FGF2 and lysates were prepared at the indicated times. Tagged FGFR-4 was immunoprecipitated with an anti-V5 antibody, and the phosphorylated receptor was detected by Western blot of immunoprecipitates with anti–phospho-FGF-R antibody ($Tyr^{641/642}$). Western blot with anti-V5 antibody is shown to confirm equivalent immunoprecipitation.

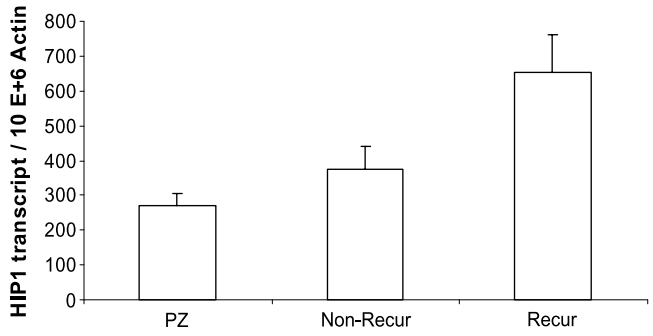

Figure 3. Quantitation of HIP1 transcripts by quantitative RT-PCR in prostate cancer. Quantitative RT-PCR was performed on RNA extracted from benign peripheral zone tissue from radical prostatectomies (PZ) or prostate cancers from patients with no PSA recurrence within 5 years of surgery (Non-recur) or with PSA recurrence (Recur) within 5 years of surgery. β-Actin transcript levels were used for normalization. Mean ± SD is shown.

used quantitative RT-PCR to evaluate HIP1 mRNA expression in normal peripheral zone tissue and cancer tissue from men with no PSA recurrence within 5 years after radical prostatectomy *versus* men with PSA recurrence within 5 years of radical prostatectomy (Figure 3). We found that HIP1 mRNA is significantly increased in prostate cancer ($P = .018$, Mann-Whitney test) and is higher in cancers with biochemical recurrence after radical prostatectomy *versus* those with no recurrence ($P = .05$; Mann-Whitney rank sum test). Thus, in agreement with prior studies, our results indicate that HIP1 may play an important role in promoting prostate cancer aggressiveness.

HIP1 Stabilizes FGFR-4 and Promotes Sustained Activation after Ligand Stimulation

It has been shown previously that HIP1 can increase receptor stability of EGF receptors. To determine the biochemical and biologic effects of HIP1 on FGF signaling and FGFR-4, we established cells lines from the immortalized normal prostate cell line PNT1A and LNCaP prostate cancer cells expressing HIP1 under a constitutive promoter. As can be seen in Figure 4*A*, for both types of cells, expression of HIP1 in a clone was associated with markedly increased FGFR-4 protein levels. Quantitative RT-PCR to determine FGFR-4 receptor mRNA expression levels revealed no increase and, in most cases, slight decreases in FGFR-4 mRNA levels (Figure 4*B*). Thus, HIP1 increases FGFR-4 protein through a posttranscriptional mechanism. To determine whether this was due to altered receptor stability, we cotransfected 293T cells with either the FGFR-4 variant or the HIP1 expression constructs. We evaluated receptor stability of biotin-labeled surface receptors at intervals after ligand stimulation, followed by precipitation of biotin-labeled proteins from cell lysates using streptavidin beads, and followed by Western blot analysis with anti-V5 antibody recognizing a V-5 tag on the transfected FGFR-4 variants. We have found that HIP1 seems to significantly increase the stability of the FGFR-4 Gly^{388} variant, while having, at most, minor effects on FGFR-4 Arg^{388} stability (Figure 5).

HIP1 Interacts Directly with FGFR-4

We next carried out reciprocal immunoprecipitation Western blot studies to determine whether there is a direct interaction between the FGFR-4 variants and HIP1. V5-tagged FGFR-4 and Flag-tagged HIP1 were cotransfected into 293T cells. Immunoprecipitation with

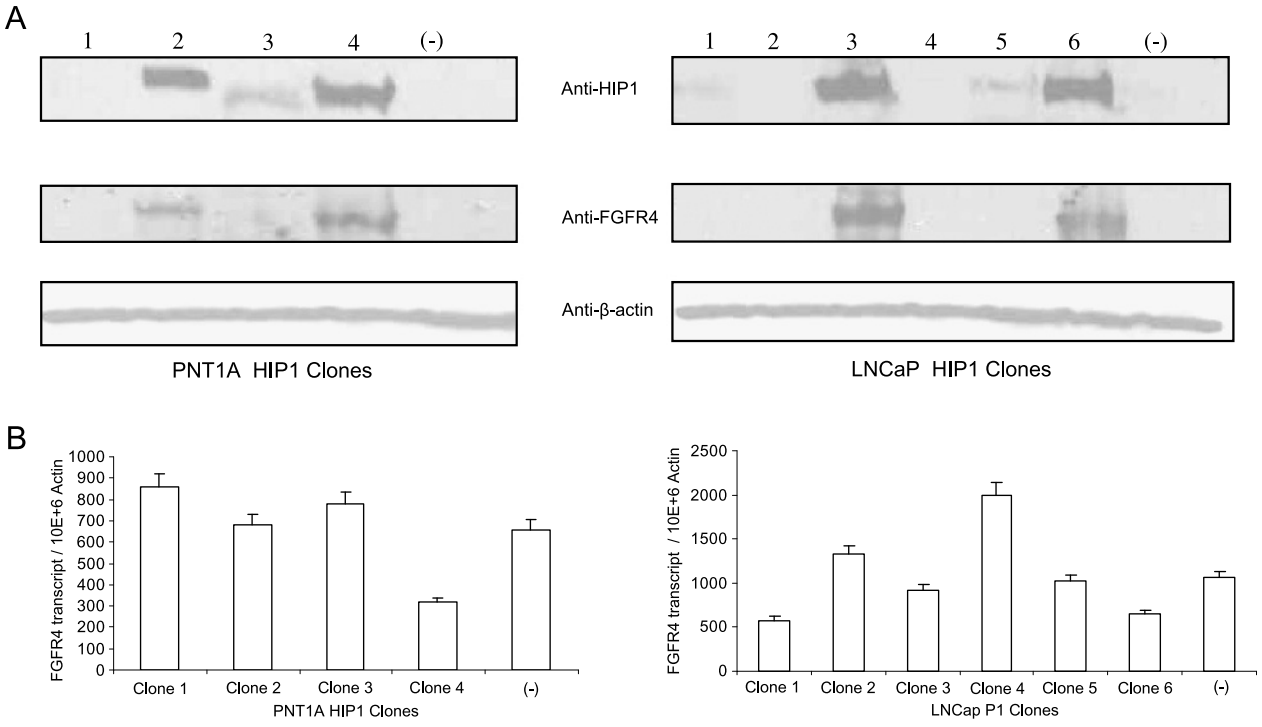

Figure 4. Stabilization of FGFR-4 by HIP1 in prostate epithelial and prostate cancer cells. Immortalized normal prostate epithelial cells (PNT1A) or prostate cancer cells (LNCaP) were transfected with HIP1 in the TOPO-V5 expression vector and stable cells lines selected. (A) Expression of HIP1 and FGFR-4 protein was evaluated by Western blot with β-actin as a loading control. (B) Expression of FGFR-4 mRNA was determined by quantitative RT-PCR with normalization to β-actin transcript levels.

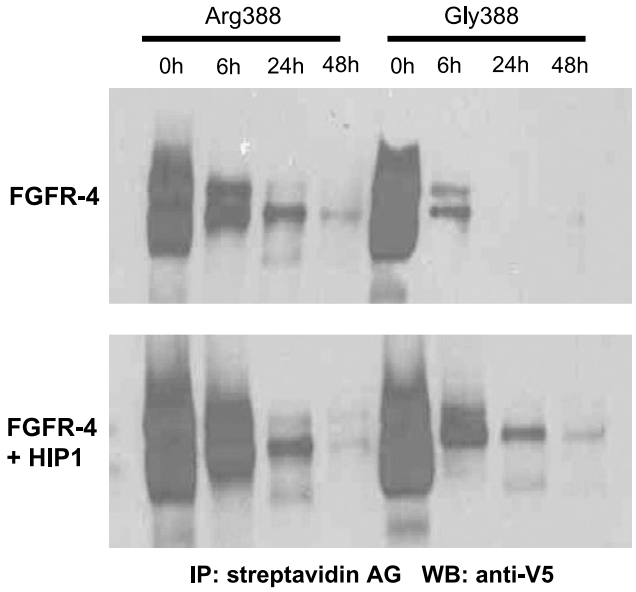

Figure 5. Stabilization of FGFR-4 Gly^{388} by HIP1. 293T cells were transfected with V5-tagged FGFR-4 Arg^{388} or Gly^{388} with or without HIP1, and cell surface receptors were labeled with biotin. Cells were then stimulated with FGF2 and lysed at the indicated time. Labeled receptors were then immunoprecipitated with streptavidin–agarose, and FGFR-4 was detected by Western blot of the immunoprecipitates with anti-V5 antibody.

anti-V5 antibody followed by Western blot with anti-Flag antibody (or the converse) revealed direct interaction between both FGFR-4 variant and HIP1, including an N-terminal truncated HIP1 (Figure 6*A*). Similar results were seen in prostate cancer cell lines (LAPC4 and LNCaP; data not shown). To map the domains of HIP1, which mediate the interaction with FGFR-4, we made a series of HIP1 deletion constructs (Figure 6*B*) that were then expressed in bacteria and proteins purified using streptavidin beads (Figure 6*C*). We then incubated the purified HIP1 proteins with lysates from 293T cells transfected with V5-tagged overnight and immunoprecipitated with streptavidin beads. The beads were washed, and Western blots were performed using an anti-V5 antibody. As shown in Figure 6*D*, the region between amino acids 601 and 798 containing the coiled-coil domain of HIP1 is required for HIP1–FGFR-4 interaction.

HIP1 Potentiates the Biologic Activities of FGFR-4

We next examined the biologic affects of increased HIP1 expression. Cell lines expressing two- to fivefold higher levels of HIP1 were established by stable transfection. In both PNT1A and DU145 prostate cancer cells, increased expression of full-length HIP1 increases proliferation in the medium containing FGF2 and insulin as the only growth factors (Figure 7*A*). This effect was not seen with a truncated receptor that lacks the lipid-binding domain of HIP1, which is required for its membrane localization in cells [29]. It should be noted that DU145 cells are homozygous for the Gly^{388} allele, whereas PNT1A cells are heterozygous Gly^{388}/Arg^{388}. The HIP1-overexpressing PNT1A cells were then infected with a lentivirus expressing ShRNA targeting HIP1 or FGFR-4. As can be seen in Figure 7*B*, knockdown of FGFR-4 decreases growth of HIP1-overexpressing cells below that of control PNT1A in defined medium with FGF2 and insulin as the only growth factors. Thus, FGFR-4 is a major contributor to HIP1-induced growth in response to FGFs in prostate epithelial cells. Similar increases in proliferation in PNT1A cells expressing HIP1 were seen in serum-containing medium that was significantly decreased by the down-regulation of FGFR-4 with ShRNA (Figure 7*B*). These results imply that, even in medium with serum, which contains a variety of growth factors, FGFR-4 is a major effector of HIP1-mediated proliferation. Similar increases in proliferation in HIP1-overexpressing PNT1A cells were seen in the medium containing only 0.5% serum (data not shown). PNT1A cells are immortalized but not fully transformed and do not form colonies in soft agar. Expression of HIP1 leads to colony formation in soft agar (Figure 7*C*). Down-regulation of FGFR-4 by 50% with ShRNA partially abolishes this phenotype (HIP1 *vs* HIP1 ShR4, $P = .03$; Mann-Whitney), again implying that FGFR-4 plays a role in mediating HIP1 effects even in serum-containing medium. It should be noted that down-regulation of HIP-1 by ShRNA resulted in even more marked decreases in proliferation and colony formation (Figure 7, *B* and *C*), implying that other receptors also contribute to the phenotype induced by HIP1 overexpression.

Discussion

We have shown previously in a large study of men with clinically localized prostate cancer that heterozygosity of the FGFR-4 Arg^{388} was associated with increased risk of pelvic lymph node metastasis at the time of radical prostatectomy and increased risk of biochemical recurrence [15]. Heterozygosity of the FGFR-4 Arg^{388} has been associated with aggressive disease and clinical progression in a variety of other malignancies [18–23]. Our prior *in vitro* studies have shown that expression of the FGFR-4 Arg^{388} allele results in increased motility and invasion. Of note, recent studies by Sahadevan et al. [17] have shown that knockdown of FGFR-4 in PC3 cells (homozygous for Arg^{388}) decreases proliferation and invasion *in vitro*. Thus, it is clear that the FGFR-4 Arg^{388} allele can promote prostate cancer progression and is more effective than the Gly^{388} allele in this regard. However, FGFR-4 Gly^{388} may also promote progression because Sahadevan et al. [17] have shown that knockdown of FGFR-4 in DU145, which are homozygous for the Gly^{388} allele, can decrease proliferation and invasion of these cells *in vitro*. We have found that DU145 cells express basal levels of HIP1 (data not shown), which may be enhancing the biologic activity of the FGFR-4 Gly^{388} allele in these cells.

Our current data indicate that the FGFR-4 Arg^{388} variant has increased receptor stability and sustained phosphorylation, which probably explains its ability to promote prostate cancer initiation and progression. Fibroblast growth factor receptor 4 associates with HIP1, and the Gly^{388} variant is significantly stabilized by this interaction and results in phenotypes associated with cancer, including increased proliferation in low growth factor conditions and colony formation in soft agar. The decreased receptor degradation and sustained phosphorylation after ligand stimulation that we observed may substantially amplify receptor signaling by increasing surface receptor concentrations and local FGF ligand (by decreasing degradation) and by increasing the pool of early endosomes with bound ligand, which can continue to transduce signals [29]. Further studies, including direct comparison of the FGFR-4 Arg^{388} and Gly^{388} alleles with other FGF receptors, are needed to understand the molecular basis of the increased receptor stability and sustained phosphorylation after ligand stimulation observed with the FGFR-4 Arg^{388} variant.

We have also demonstrated that the FGFR-4 Gly^{388} variant is stabilized after ligand stimulation by the presence of HIP1. This may be

mediated through a direct interaction through the coiled-coil domain located between amino acids 601 and 798. A recent study by Bradley et al. [35] have shown a similar direct interaction between HIP1 and EGF receptor, which requires a domain between amino acids 381 and 814 of HIP1. It has also been shown that amino acids 690 to 752 of HIP1 are required for the transformation by the HIP1/PDGR beta fusion gene [36]. Thus, the domain we have identified overlaps with the regions identified for direct interaction of HIP1 with EGF receptors, although it is not clear that the interacting domains are the same. HIP1 alters clathrin-mediated membrane trafficking of EGF receptors [29,30], so it is likely that it also has a similar affect on FGFR 4 Gly^{388}, although other mechanisms are possible as well.

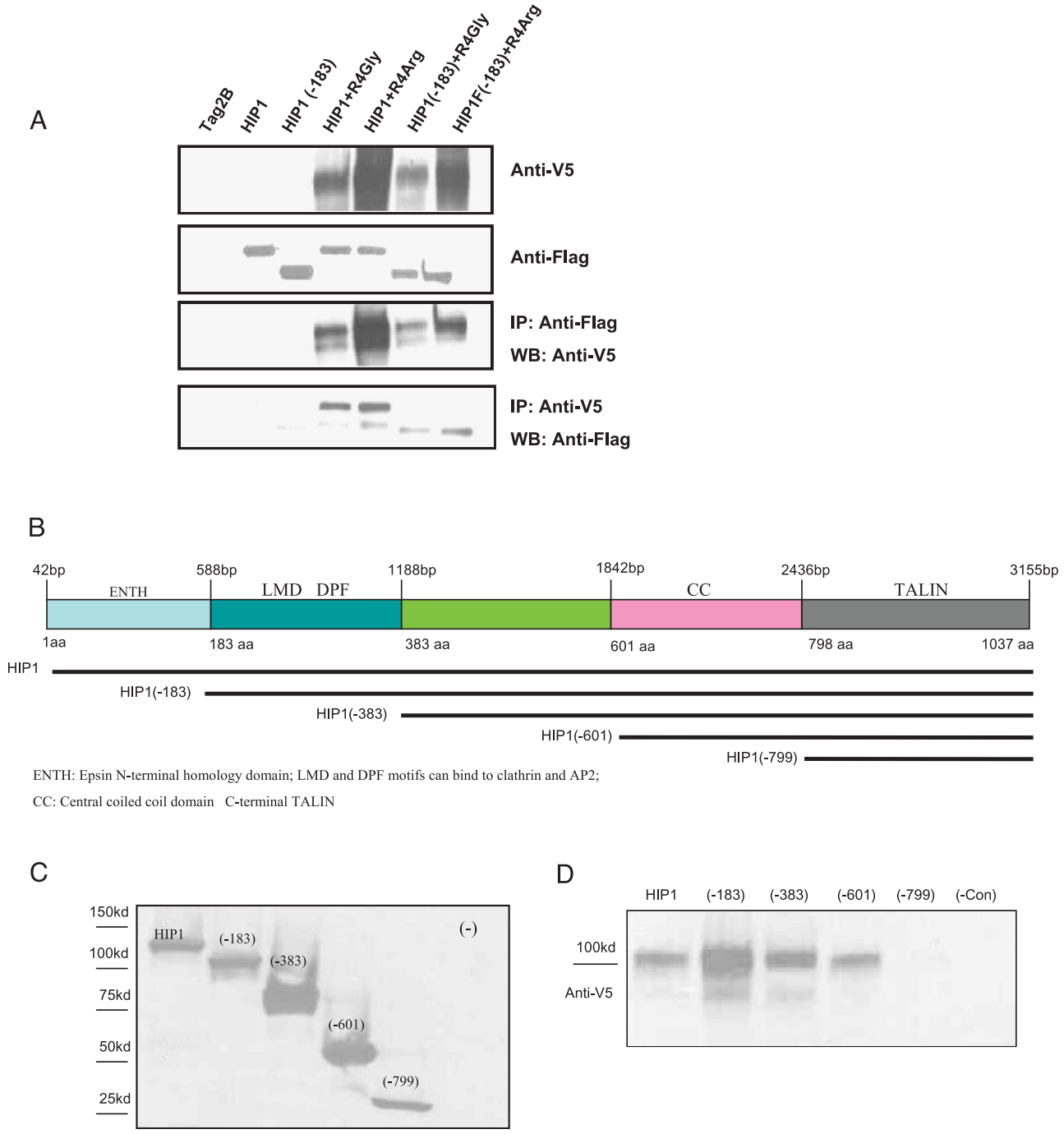

Figure 6. Direct interaction of FGFR-4 and HIP1. (A) 293T cells were transfected with Flag-tagged HIP1 or N-terminally truncated HIP1 (−183) with or without either FGFR-4 Arg^{388} or Gly^{388} (both V5-tagged). Vector control (Tag2B) is also shown. Cell lysates were then analyzed by Western blot using anti-V5 or anti-Flag antibody or were used for reciprocal immunoprecipitation and Western blot analysis with the two antibodies. (B) Map of HIP1 showing major domains and deletion fragments used to prepare bacterial fusion proteins. (C) Purified HIP1 and HIP1 deletion bacterial fusion proteins on Coomassie blue–stained polyacrylamide gel after electrophoreisis. (D) 293T cells were transfected with V5-tagged FGFR-4 and lysates prepared and incubated with purified HIP1 and HIP1 deletion constructs. Complexes were then immunoprecipitated with streptavidin beads, and Western blot was performed with anti-V5 antibody. Control is streptavidin beads but no purified protein.

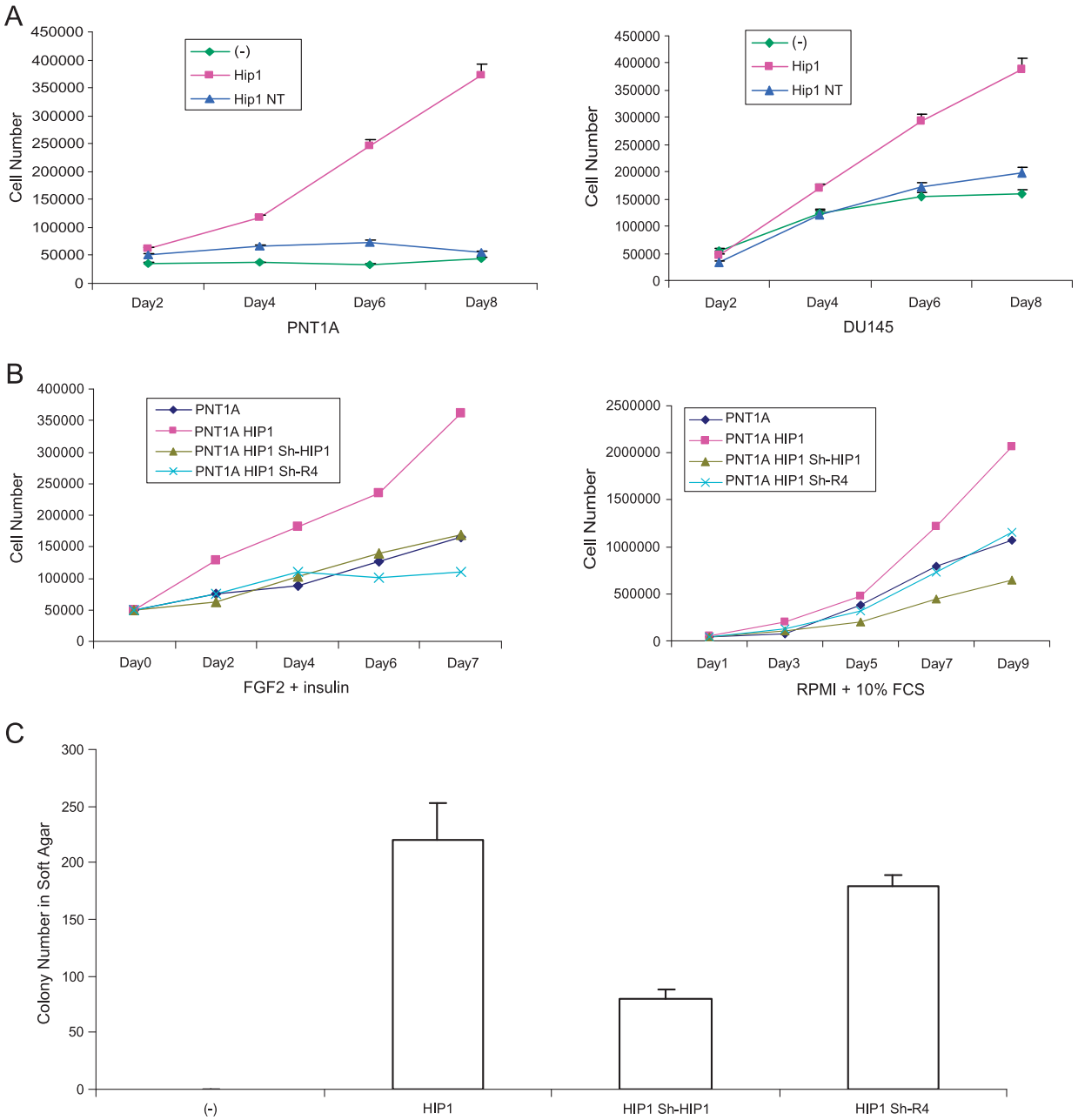

Figure 7. Biologic affects of HIP1 in prostate and prostate cancer cell lines. (A) Prostate (PNT1A) or prostate cancer (DU145) cell lines expressing full-length HIP1, an amino terminal–truncated HIP1 (NT) or vector only were plated, and growth was determined in a defined medium containing FGF2 and insulin as the only growth factors. Cell number was determined at 2-day intervals. Mean ± SD of triplicates is shown. (B) PNT1A cells overexpressing HIP1 were infected with lentivirus expressing ShRNA targeting HIP1 or FGFR-4, and stable expressors were selected and pooled. Quantitative RT-PCR showed 70% and 60% knockdown of HIP1 and FGFR-4 mRNA, respectively (data not shown). Cells were then plated, and grown was determined in defined medium with FGF2 and insulin as the only growth factors or serum-containing medium. Cell number was determined at 2-day intervals by cell counting. Mean ± SD of triplicates is shown. (C) PNT1A cells as described in (B) were plated in soft agar. Colony formation was evaluated by counting. Mean ± SD of triplicates is shown.

Interestingly, HIP1 did not further increase Arg^{388} stability, perhaps because this variant is already extremely stable after ligand stimulation. Further mechanistic studies are needed to determine the molecular basis by which HIP1 stabilizes the FGFR 4 Gly^{388} but not the Arg^{388} variant.

Rao et al. [30] have shown that HIP1 can strongly enhance EGF receptor signaling, with potent biologic effects in NIH3T3 cells. We have demonstrated that HIP1 has similar potent effects in prostate and prostate cancer cell lines that are mediated in part through FGFR-4, even in the culture medium containing serum, which is a poor source of FGFs. Given the high local concentrations of FGF2 in tumors *in vivo* [7] and the prevalent expression of other FGFR-4 binding FGF ligands as autocrine factors [6,9–12], it is likely that FGFR-4 plays an even more important role in mediating the effects of HIP1 in human cancers *in vivo*. Other growth factor receptors, including other FGF receptor family members as well as EGF

receptor family members, may well contribute to the biologic activities of HIP1 in prostate cancer. Rao et al. [30] noted that HIP1 increases the level of FGFR-4 (and FGFR-3) protein in NIH3T3 cells and hypothesized that these growth factor receptors may contribute to the phenotype in HIP1-expressing NIH3T3 cells, although their data indicated that EGF receptors probably played a dominant role in these cells. Thus, the relative importance of different receptors in mediating the phenotypes in cells expressing HIP1 may be cell type–specific. Finally, it has been reported that HIP1 can act as an androgen receptor coactivator [35], which may also promote prostate cancer progression independent of its activities on growth factor receptors. It should be noted that both PNT1A and DU145 cells do not express androgen receptor, so the biologic effects we have observed are not due to androgen receptor activation.

On the basis of the results reported here and by others, altered FGFR-4 stability and/or trafficking is extremely common in prostate cancer. Our prior studies have shown that more than half of all white men with prostate cancer are hetero- or homozygous for the FGFR-4 Arg^{388} allele [15], and immunohistochemical studies have shown that almost all prostate cancers express FGFR-4 [15–17]. Furthermore, approximately 50% of clinically localized prostate cancers express moderate to strong levels of HIP1 [31], and presumably the expression of HIP1 is independent of the FGFR-4 genotype, although there might be a selection for HIP1 expression in prostate cancers homozygous for the Gly^{388} variant. If these two factors are independent, it is likely that at least 75% of prostate cancers have alterations of FGFR-4 signaling through receptor stabilization, and on the basis of our studies and those of others, this altered signaling has important biologic and clinical consequences. Thus, enhanced FGFR-4 signaling is one of the most common alterations in human prostate cancer. Fibroblast growth factor receptor 4 is therefore an important therapeutic target in prostate cancer, and the development of such therapies, as are being developed for other receptor tyrosine kinases [37], should be pursued vigorously.

References

[1] Jemal A, Siegel R, Ward E, Murray T, Xu J, and Thun MJ (2007). Cancer statistics, 2007. *CA Cancer J Clin* **57**, 43–66.

[2] Kwabi-Addo B, Ozen M, and Ittmann M (2004). The role of fibroblast growth factors and their receptors in prostate cancer. *Endocr Relat Cancer* **11**, 709–724.

[3] Powers CJ, McLeskey SW, and Wellstein A (2000). Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer* **7**, 165–197.

[4] Ozen M, Giri D, Ropiquet F, Mansukhani A, and Ittmann M (2001). Role of fibroblast growth factor receptor signaling in prostate cancer cell survival. *J Natl Cancer Inst* **93**, 1783–1790.

[5] Reiland J, Kempf D, Roy M, Denkins Y, and Marchetti D (2006). FGF2 binding, signaling, and angiogenesis are modulated by heparanase in metastatic melanoma cells. *Neoplasia* **8**, 596–606.

[6] Dorkin TJ, Robinson MC, Marsh C, Neal DE, and Leung HY (1999). aFGF immunoreactivity in prostate cancer and its co-localization with bFGF and FGF8. *J Pathol* **189**, 564–569.

[7] Giri D, Ropiquet F, and Ittmann M (1999). Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. *Clin Cancer Res* **5**, 1063–1071.

[8] Gravdal K, Halvorsen OJ, Haukaas SA, and Akslen LA (2006). Expression of bFGF/FGFR-1 and vascular proliferation related to clinicopathologic features and tumor progress in localized prostate cancer. *Virchows Arch* **448**, 68–74.

[9] Ropiquet F, Giri D, Kwabi-Addo B, Mansukhani A, and Ittmann M (2000). Increased expression of fibroblast growth factor 6 in human prostatic intraepithelial neoplasia and prostate cancer. *Cancer Res* **60**, 4245–4250.

[10] Dorkin TJ, Robinson MC, Marsh C, Bjartell A, Neal DE, and Leung HY (1999). FGF8 over-expression in prostate cancer is associated with decreased patient survival and persists in androgen independent disease. *Oncogene* **18**, 2755–2761.

[11] West AF, O'Donnell M, Charlton RG, Neal DE, and Leung HY (2001). Correlation of vascular endothelial growth factor expression with fibroblast growth factor-8 expression and clinico-pathologic parameters in human prostate cancer. *Br J Cancer* **85**, 576–583.

[12] Heer R, Douglas D, Mathers ME, Robson CN, and Leung HY (2004). Fibroblast growth factor 17 is over-expressed in human prostate cancer. *J Pathol* **204**, 578–586.

[13] Eswarakumar VP, Lax I, and Schlessinger J (2005). Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* **16**, 139–149.

[14] Martin-Orozco RM, Almaraz-Pro C, Rodriguez-Ubreva FJ, Cortes MA, Ropero S, Colomer R, Lopez-Ruiz P, and Colas B (2007). EGF prevents the neuroendocrine differentiation of LNCaP cells induced by serum deprivation: the modulator role of PI3K/Akt. *Neoplasia* **9**, 614–624.

[15] Wang J, Stockton DW, and Ittmann M (2004). The fibroblast growth factor receptor-4 Arg^{388} allele is associated with prostate cancer initiation and progression. *Clin Cancer Res* **10**, 6169–6178.

[16] Gowardhan B, Douglas DA, Mathers ME, McKie AB, McCracken SR, Robson CN, and Leung HY (2005). Evaluation of the fibroblast growth factor system as a potential target for therapy in human prostate cancer. *Br J Cancer* **92**, 320–327.

[17] Sahadevan K, Darby S, Leung H, Mathers M, Robson C, and Gnanapragasam V (2007). Selective over-expression of fibroblast growth factor receptors 1 and 4 in clinical prostate cancer. *J Pathol* **213**, 82–90.

[18] Bange J, Prechtl D, Cheburkin Y, Specht K, Harbeck N, Schmitt M, Knyazeva T, Muller S, Gartner S, Sures I, et al. (2002). Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele. *Cancer Res* **62**, 840–847.

[19] Morimoto Y, Ozaki T, Ouchida M, Umehara N, Ohata N, Yoshida A, Shimizu K, and Inoue H (2003). Single nucleotide polymorphism in fibroblast growth factor receptor 4 at codon 388 is associated with prognosis in high-grade soft tissue sarcoma. *Cancer* **98**, 2245–2250.

[20] Streit S, Mestel DS, Schmidt M, Ullrich A, and Berking C (2006). FGFR4 Arg^{388} allele correlates with tumour thickness and FGFR4 protein expression with survival of melanoma patients. *Br J Cancer* **94**, 1879–1886.

[21] Spinola M, Leoni V, Pignatiello C, Conti B, Ravagnani F, Pastorino U, and Dragani TA (2005). Functional FGFR4 $Gly^{388}Arg$ polymorphism predicts prognosis in lung adenocarcinoma patients. *J Clin Oncol* **23**, 7307–7311.

[22] Streit S, Bange J, Fichtner A, Ihrler S, Issing W, and Ullrich A (2004). Involvement of the FGFR4 Arg^{388} allele in head and neck squamous cell carcinoma. *Int J Cancer* **111**, 213–217.

[23] da Costa Andrade VC, Parise O Jr, Hors CP, de Melo Martins PC, Silva AP, and Garicochea B (2007). The fibroblast growth factor receptor 4 (FGFR4) Arg^{388} allele correlates with survival in head and neck squamous cell carcinoma. *Exp Mol Pathol* **82**, 53–57.

[24] Jezequel P, Campion L, Joalland MP, Millour M, Dravet F, Classe JM, Delecroix V, Deporte R, Fumoleau P, and Ricolleau G (2004). G388R mutation of the *FGFR4* gene is not relevant to breast cancer prognosis. *Br J Cancer* **90**, 189–193.

[25] Thussbas C, Nahrig J, Streit S, Bange J, Kriner M, Kates R, Ulm K, Kiechle M, Hoefler H, Ullrich A, et al. (2006). FGFR4 Arg^{388} allele is associated with resistance to adjuvant therapy in primary breast cancer. *J Clin Oncol* **24**, 3747–3755.

[26] Wang J, Cai Y, Penland R, Chauhan S, Miesfeld RL, and Ittmann M (2006). Increased expression of the metastasis-associated gene *Ehm2* in prostate cancer. *Prostate* **66**, 1641–1652.

[27] Monsonego-Ornan E, Adar R, Feferman T, Segev O, and Yayon A (2000). The transmembrane mutation G380R in fibroblast growth factor receptor 3 uncouples ligand-mediated receptor activation from down-regulation. *Mol Cell Biol* **20**, 516–522.

[28] Cho JY, Guo C, Torello M, Lunstrum GP, Iwata T, Deng C, and Horton WA (2004). Defective lysosomal targeting of activated fibroblast growth factor receptor 3 in achondroplasia. *Proc Natl Acad Sci USA* **101**, 609–614.

[29] Hyun TS, Rao DS, Saint-Dic D, Michael LE, Kumar PD, Bradley SV, Mizukami IF, Oravecz-Wilson KI, and Ross TS (2004). HIP1 and HIP1r stabilize receptor tyrosine kinases and bind 3-phosphoinositides via epsin N-terminal homology domains. *J Biol Chem* **279**, 14294–14306.

[30] Rao DS, Bradley SV, Kumar PD, Hyun TS, Saint-Dic D, Oravecz-Wilson K, Kleer CG, and Ross TS (2003). Altered receptor trafficking in Huntingtin interacting protein 1–transformed cells. *Cancer Cell* **3**, 471–482.

[31] Rao DS, Hyun TS, Kumar PD, Mizukami IF, Rubin MA, Lucas PC, Sanda MG, and Ross TS (2002). Huntingtin-interacting protein 1 is overexpressed in prostate and colon cancer and is critical for cellular survival. *J Clin Invest* **110**, 351–360.

[32] Wang J, Cai Y, Ren C, and Ittmann M (2006). Expression of variant TMPRSS2/ERG fusion messenger RNAs is associated with aggressive prostate cancer. *Cancer Res* **66**, 8347–8351.

[33] Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G, and Goldfarb M (1996). Receptor specificity of the fibroblast growth factor family. *J Biol Chem* **271**, 15292–15297.

[34] Mohammadi M, Dikic I, Sorokin A, Burgess WH, Jaye M, and Schlessinger J (1996). Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. *Mol Cell Biol* **16**, 977–989.

[35] Bradley SV, Holland EC, Liu GY, Thomas D, Hyun TS, and Ross TS (2007). Huntingtin interacting protein 1 is a novel brain tumor marker that associates with epidermal growth factor receptor. *Cancer Res* **67**, 3609–3615.

[36] Ross TS and Gilliland DG (1999). Transforming properties of the Huntingtin interacting protein 1/platelet-derived growth factor beta receptor fusion protein. *J Biol Chem* **274**, 22328–22336.

[37] Watson GA, Zhang X, Stang MT, Levy RM, Queiroz de Oliveira PE, Gooding WE, Christensen JG, and Hughes SJ (2006). Inhibition of c-Met as a therapeutic strategy for esophageal adenocarcinoma. *Neoplasia* **8**, 949–955.