

# Fibroblast Growth Factor Receptor 1 Promotes Proliferation and Survival via Activation of the Mitogen-Activated Protein Kinase Pathway in Bladder Cancer

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## Abstract

**Fibroblast growth factor receptors (FGFR) play key roles in proliferation, differentiation, and tumorigenesis. Many urothelial carcinomas contain activating point mutations or increased expression of FGFR3. However, little is known about the role of other FGFRs. We examined FGFR expression in telomerase-immortalized normal human urothelial cells, urothelial carcinoma cell lines, and tumor samples and showed that FGFR1 expression is increased in a high proportion of cell lines and tumors independent of stage and grade. To determine the role of FGFR1 in low-stage bladder cancer, we overexpressed FGFR1 in telomerase-immortalized normal human urothelial cells and examined changes in proliferation and cell survival in response to FGF2. FGFR1 stimulation increased proliferation and reduced apoptosis. To elucidate the mechanistic basis for these alterations, we examined the signaling cascades activated by FGFR1. FRS2 $\alpha$  and PLC $\gamma$  were activated in response to FGF2, leading to activation of the mitogen-activated protein kinase pathway. The level of mitogen-activated protein kinase activation correlated with the level of cyclin D1, MCL1, and phospho-BAD, which also correlated with FGFR-induced proliferation and survival. Knockdown of FGFR1 in urothelial carcinoma cell lines revealed differential FGFR1 dependence. JMSU1 cells were dependent on FGFR1 expression for survival but three other cell lines were not. Two cell lines (JMSU1 and UMUC3) were dependent on FGFR1 for growth in soft agar. Only one of the cell lines tested (UMUC3) was frankly tumorigenic; here, FGFR1 knockdown inhibited tumor growth. Our results indicate that FGFR1 has significant effects on urothelial cell phenotype and may represent a useful therapeutic target in some cases of urothelial carcinoma. [Cancer Res 2009;69(11):4613–20]**

## Introduction

Bladder cancer is the fifth most common cancer in the United Kingdom and the United States (1, 2). Molecular and pathologic studies suggest that bladder cancers comprise at least two major groups (3, 4). The majority of urothelial carcinomas (~70%) are noninvasive papillary tumors (stage T<sub>a</sub>) that commonly recur

but rarely progress and therefore have a good prognosis. Patients with tumors in this group require long-term monitoring, which makes bladder cancer the most expensive cancer to treat (5). Invasive bladder tumors are more aggressive, presenting with penetration of the basement membrane (stage T<sub>1</sub>) or invasion into muscle (stage T<sub>2</sub>). Patients with invasive disease have a much worse prognosis, with a 50% 5-year survival. The high frequency of recurrence of noninvasive urothelial carcinoma and the poor prognosis of invasive urothelial carcinoma highlight the necessity for development of effective targeted therapies.

Fibroblast growth factor receptor (FGFR) 3 was implicated in urothelial carcinoma following the discovery of frequent activating mutations (6) and recent publications show that it may represent a good therapeutic target. Numerous studies have shown significant association of *FGFR3* mutation with noninvasive papillary tumors with good prognosis (7, 8). In addition, a high proportion of tumors, including many invasive nonmutant tumors, show overexpression of FGFR3 (9). Thus, significant numbers of tumors in both major groups of urothelial carcinoma may benefit from FGFR-targeted therapies. Cell culture systems have been used to validate mutant *FGFR3* as a target in bladder cancer (10, 11). These studies showed that the most common *FGFR3* mutations, S249C and Y375C, play a role in regulating proliferation, anchorage-independent growth, and clonogenicity at low density. Examination of the effects of FGFR inhibitors in preclinical urothelial carcinoma models *in vivo* is now required to confirm that dependence on FGFR3 in culture models can be translated into therapeutic efficacy.

In other tumor types, FGFR1 is implicated as an oncogene whose expression is increased compared with normal tissue (12, 13). Constitutive activation of FGFR1 is also associated with the generation of fusion transcripts via chromosomal translocations in myeloproliferative diseases (14). Activation of FGFR1 induces both mitogenic and chemotactic responses in various cell types. In NIH3T3 cells, activated FGFR1 induced a survival response, prevented contact inhibition, and inhibited apoptosis (15). Recently, mouse models of prostate and breast carcinoma have been developed by tissue-specific expression of a conditionally activated, chemically induced dimerization chimeric FGFR1 protein (15, 16). Premalignant prostate cells expressing activated FGFR1 exhibited accelerated progression to malignancy (17). Similar results were observed in a breast model with sustained activation of FGFR1 leading to alveolar hyperplasia and invasive lesions. In addition, a recent report showed increased expression of FGFR1 in a subset of breast tumors and *in vitro* studies showed that FGFR1 signaling contributed to the survival of a breast cancer cell line (18).

Small-molecule inhibitors and antibodies have been used successfully to target FGFR3 in multiple myeloma both *in vitro*

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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and in animal models (19–22). As such inhibitors show activity against other FGFR family members, they could in theory target multiple FGFRs simultaneously in tumors that express more than one family member.

Currently, little is known about the role of other FGFRs in bladder cancer. FGFR1 and FGFR4 transcripts are expressed at low levels in normal urothelium (23) but no information regarding their expression in bladder tumors has been reported. More is known about FGFR2, and evidence suggests that FGFR2b may have tumor suppressor properties (24). However, alternative splicing resulting in expression of FGFR2c has been described and shown to be up-regulated during metastasis in a bladder cancer model (25).

The clear role of FGFR3 in bladder cancer and the possibility that targeted agents may be able to inhibit other FGFR family members prompted us to measure FGFR transcript levels in bladder cancer cell lines. Here, we show that FGFR1 expression is increased in the majority of bladder cancer cell lines and tumors. We examined the effect of increased FGFR1 expression in normal urothelial cells and showed that FGFR1 induces increased proliferation and cell survival. We used short hairpin RNA (shRNA) to knock down FGFR1 in bladder tumor-derived cell lines and showed differential roles of FGFR1 in regulating survival and tumor growth. Our results show that FGFR1 plays a role in several aspects of the urothelial carcinoma transformed phenotype and is implicated in both major groups of urothelial carcinoma.

## Materials and Methods

**Cell lines.** The following cell lines were used: JMSU1, 94-10, 97-7, RT4, RT112, 97-18, BFTC905, SCaBER, DSH1, VMCUB3, SW1710, 96-1, VMCUB2, 97-24, J82, HT1376, 97-1, 647V, 253J, BFTC909, TCCSUP, SD, JO'N, UUMUC3, VMCUB1, 5637, and T24. Cells were grown in standard growth medium at 37°C in 5% CO<sub>2</sub>. Primary normal human urothelial cells (NHUC) or telomerase-immortalized NHUC (TERT-NHUC) were derived from stripped ureteric urothelium (26). NHUC and TERT-NHUC were maintained in KFSM keratinocyte medium (Life Technologies) supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen).

**Quantitative real-time reverse transcription-PCR for FGFRs.** Total RNA was extracted from frozen tumor sections containing >90% tumor cells. RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen) and 1 µg was reverse transcribed in the presence or absence of reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time reverse transcription-PCR analysis was done using SYBR Green I as reporter and ROX as reference dye (Applied Biosystems). FGFR-specific primers and succinate dehydrogenase subunit A control primers were as described (23).

**shRNA constructs.** Oligonucleotides targeting FGFR1 or a nonspecific target (FGFR1 shRNA1 forward oligo 5'-CACAAAGTGTATAAATCTGTTCAAGAGACAGATTATACACTTGTGTTTTGGGCC-3' and reverse oligo 5'-CAAAAAACACAAAGTGTATAAATCTGTCTCTTGAACAGATTTATACACTTTGTG-3', shRNA2 forward oligo 5'-GAAGTGCATACACCGAGACTTCAAGAGAGTCTCGGTGTATGCACTTCTTTTTGGGCC-3' and reverse oligo 5'-CAAAAAAGAAAGTGCATACACCGAGACTCTCTTGAAGTCTCGGTGTATGCACTTCT-3', and nonspecific control forward oligo 5'-CTTCAGCCGTACGCTCGGTTCAAGAGACCGGTAACCGCTGAAGTTTTGGGCC-3' and reverse oligo 5'-CAAAAACTTCAGCCGTTACGCTCGGTTCTCTTGAACCGAGCGTAACGGCTGAAG-3') were cloned into pRetroSuper-puro as described previously (11). pRetroSuper-green fluorescent protein (GFP) was a kind gift from Reuven Agami (27).

**Cloning of FGFR1, production of retroviruses, and transductions.** FGFR1 was amplified by reverse transcription-PCR from cDNA derived from the JMSU1 bladder cancer cell line using FGFR1-specific primers (forward 5'-ctctaactcagaactggg-3' and reverse 5'-tgcgctgttgagtcgcc-3'). The product was cloned into pGEM T-Easy, sequenced, and subcloned into pFB-Hyg. We used site-directed mutagenesis to create FGFR1 Y766F and verified by

sequencing. FGFR1, FGFR1 Y766F, and shRNA constructs were transfected into Phoenix A packaging cells (American Type Culture Collection) using siPORT XP-1 transfection agent (Ambion). After 48 h, viral supernatants were filtered, mixed in equal amounts with medium containing 16 µg/mL polybrene (Sigma), and incubated with cells for 8 h. After 48 h, cells were transferred into medium containing puromycin or hygromycin.

**Western blotting and immunoprecipitation.** Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma) and lysates cleared by centrifugation at 10,000 rpm at 4°C. Antibodies used for Western blotting were anti-phospho-ERK, FRS2α, ERK, actin, FGFR1 (Santa Cruz Biotechnology), phospho-PLCγ, PLCγ, MCL1, phospho-BAD, and 4G10 anti-phosphotyrosine (Cell Signaling). Immunoprecipitation of FGFR1 was carried out at 4°C. Lysates were incubated with rotation overnight with FGFR1 antibody followed by protein A Sepharose beads (Amersham Biosciences) for 2 h. The beads were washed twice in lysis buffer, resuspended in sample buffer (Laemmli, β-mercaptoethanol), and boiled for 3 min and proteins were resolved in 7% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Amersham Biosciences), blocked in 5% bovine serum albumin in PBS 0.1% Tween, and incubated with 4G10 anti-phosphotyrosine antibody or FGFR1 antibody. Bound antibody was detected using anti-mouse horseradish peroxidase-conjugated antibody and chemiluminescence (ECL Plus Kit; Amersham Biosciences).

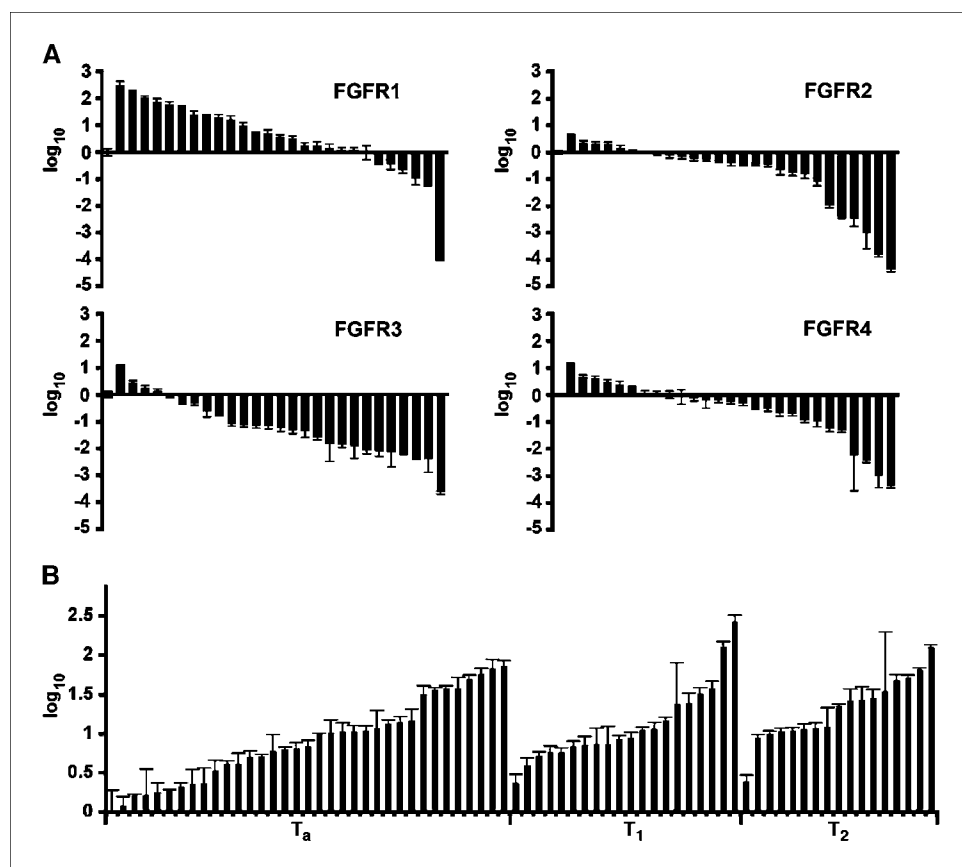
**Phenotypic assays.** For proliferation assays, 5 × 10<sup>4</sup> cells were plated in 6-well dishes. Duplicate wells were counted on day 1, washed in medium without supplements, cultured with heparin and/or FGF2 as described, and counted at relevant time points. Each experiment was done a minimum of three times. Cells were cultured with 10 µmol/L U0126 or 50 µmol/L PD98059 (Calbiochem) for 1 h before the addition of growth factors. Viability and apoptosis assays were done using the Guava EasyCyte System (Guava Technologies) according to the manufacturer's instructions. To determine the proportion of cells undergoing DNA replication, cells were cultured with 10 µmol/L bromodeoxyuridine (BrdUrd) for 1 or 24 h after addition of FGF2. Cells were harvested and fixed in 70% ethanol for 2 h, rehydrated and suspended in 2 mol/L HCl for 20 min, and washed in PBS. Mouse anti-BrdUrd (1:100; DAKO) was added for 1 h, washed in PBS, and incubated with FITC-conjugated rabbit anti-mouse (DAKO) at a 1:50 dilution for 30 min. Cells were washed in PBS and resuspended in propidium iodide/RNase staining buffer (BD Biosciences) for 30 min. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, 3,000 cells per well were plated in a 96-well plate in quadruplicate. PD173074 was added the following day and left for 96 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to the medium for 4 h, the medium was removed, and the precipitate was dissolved in DMSO. For assessment of anchorage-independent growth, 1 × 10<sup>5</sup>, 5 × 10<sup>4</sup>, or 1 × 10<sup>4</sup> cells, in duplicate, were suspended in 0.3% agar in medium and cultured for 21 days. Cultures were fed weekly with 0.3% agar. Viable colonies were stained with *p*-iodonitrotetrazolium violet (Sigma).

**Tumorigenicity testing.** Female BALB/c immunodeficient nude mice (Harlan UK) ages 6 to 8 weeks were used. Mice received CRM diet (SDS) and water *ad libitum*. All procedures were carried out under a project licence issued by the UK Home Office and UK Coordinating Committee on Cancer Research guidelines were followed throughout. Tumor cells in cell culture medium were mixed 50:50 with Matrigel (BD Biosciences) and implanted subcutaneously into each flank. Tumor growth was evaluated as described (28). Briefly, once palpable tumors were evident, daily two-dimensional caliper measurements were taken, and volumes were calculated using the formula: ( $a^2 \times b$ ) / 2, where *a* is the smaller and *b* the larger diameter of the tumor.

## Results

**FGFR1 expression is increased in bladder cancer cell lines and tumor samples.** Recent data have shown that activation and increased expression of FGFR3 is common in bladder tumorigenesis (9). To determine if other members of the FGFR family may

**Figure 1.** Expression levels of FGFRs in bladder cancer cell lines and bladder tumors. Transcript levels were measured by real-time reverse transcription-PCR and normalized to succinate dehydrogenase subunit A. Fold difference compared with NHUC. A, FGFR transcript levels in 27 bladder cancer cell lines; fold change is represented using  $\log_{10}$ . B, FGFR1 transcript levels in 35 T<sub>a</sub>, 20 T<sub>1</sub>, and 18 T<sub>2</sub> bladder tumors.



play a role in bladder cancer, we measured FGFR transcript levels in urothelial carcinoma cell lines and tumors by real-time reverse transcription-PCR (Fig. 1). FGFR1 transcript levels were increased in the majority of urothelial carcinoma cell lines compared with NHUC (Fig. 1A). Transcript levels of FGFR2 to FGFR4 were reduced in the majority of cell lines. To further investigate FGFR1, we measured transcript levels in bladder tumor samples and compared them with uncultured NHUC (Fig. 1B). FGFR1 transcript levels were increased in the majority of tumors. No relationship between expression level and stage or grade was observed.

**FGFR1 regulates proliferation and survival in NHUC.** To model the increased expression measured in low-stage and low-grade tumors and to determine the function of increased expression of FGFR1 in normal urothelium, FGFR1 was cloned and expressed in TERT-NHUC. These cells were designated NHUC-FR1. Numerous FGFR1 isoforms have been identified (29). We amplified and cloned the full-length FGFR1 ( $\alpha$ , IIIC) isoform. Expression was confirmed by Western blotting (data not shown). Immunoprecipitation of FGFR1 and Western blotting with an anti phospho-tyrosine antibody confirmed that FGF2 activated FGFR1 in NHUC-FR1 (Fig. 2A). We also showed that FGF1 activates FGFR1 in NHUC-FR1 (data not shown). However, FGFR3b is expressed in NHUC (23), and as FGF1 but not FGF2 can activate FGFR3b (30), we performed our studies with FGF2 to avoid possible confounding effects of FGFR3 stimulation. NHUC-FR1 and control cells (NHUC-VEC) were cultured in supplement-free medium in the presence of FGF2 for 5 days (Fig. 2B). FGF2 increased the number of NHUC-FR1 cells compared with NHUC-VEC. To confirm that FGFR1 stimulated proliferation, BrdUrd incorporation was measured. NHUC-FR1 cultured with FGF2 showed the highest level of BrdUrd

incorporation ( $24.63 \pm 0.37\%$ ), showing increased proliferation compared with NHUC-VEC ( $0.96 \pm 0.1\%$ ) and cells cultured with heparin alone (NHUC-VEC  $1.57 \pm 0.3\%$  and NHUC-FR1  $1.69 \pm 0.11\%$ ; Fig. 2C). The period for cell viability and apoptosis analysis was derived empirically by analyzing cell survival at different time points (data not shown). Effects were maximal at 96 h post-addition of FGF2 (Fig. 2D). No difference in viability was observed between NHUC-VEC and NHUC-FR1 cultured with heparin alone (NHUC-VEC  $69.5 \pm 3.4\%$  and NHUC-FR1  $73.28 \pm 3.9\%$ ). However, FGF2 significantly ( $P = 0.013$ , Student's *t* test) increased the viability of NHUC-FR1 compared with NHUC-VEC (NHUC-VEC  $69 \pm 3.6\%$  and NHUC-FR1  $84.1 \pm 3\%$ ). Next, we determined the level of apoptosis by Annexin V staining (Fig. 2D). No difference was observed between NHUC-VEC and NHUC-FR1 cultured with heparin (NHUC-VEC  $15.7 \pm 2.2\%$  and NHUC-FR1  $14.9 \pm 1.2\%$ ). In the presence of FGF2, apoptosis decreased significantly in NHUC-FR1 compared with NHUC-VEC ( $5.1 \pm 1.1\%$  compared with  $17.1 \pm 2.8\%$ ;  $P < 0.05$ ).

**Activation and modulation of the mitogen-activated protein kinase pathway.** Both mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase pathways may be activated by FGFR1 (31). In NHUC-FR1, the MAPK pathway was activated (Fig. 3A) and only negligible levels of activated AKT were detected (data not shown). MAPK pathway activation was measured via phosphorylation of ERK1/2, which was maximally activated after 5 min and remained activated up to 60 min post-stimulation. Activation of the MAPK pathway occurs via recruitment of signaling proteins that bind to phosphorylated tyrosine sites on activated FGFR1 and via closely linked docking proteins that become phosphorylated in response to FGF stimulation (31, 32).

FRS2 $\alpha$  and PLC $\gamma$  were phosphorylated in response to activation of FGFR1 (Fig. 3B).

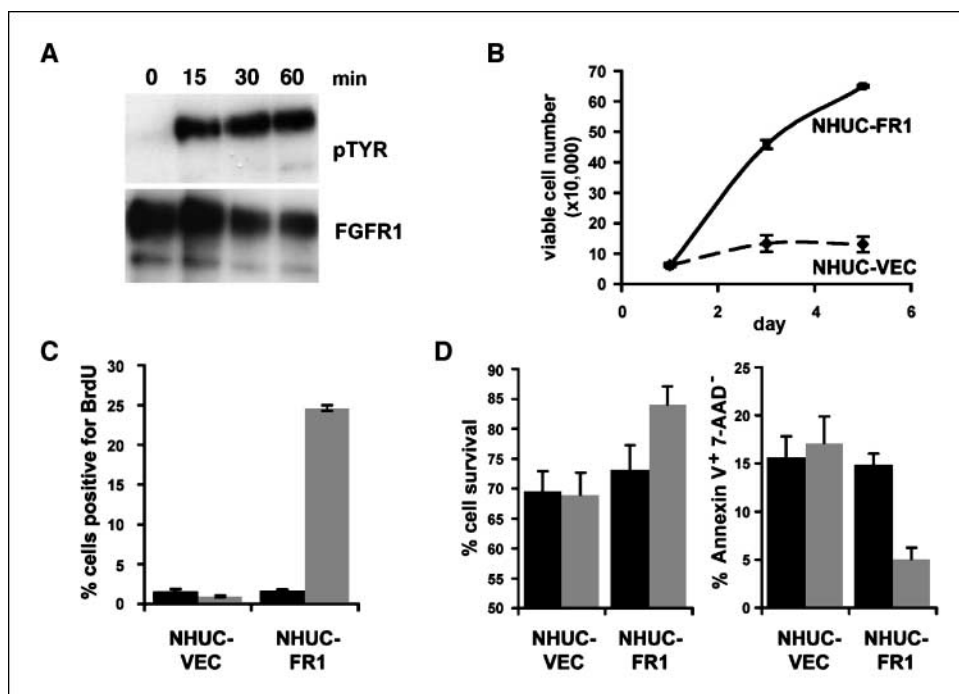
To relate the changes in MAPK activity to the changes in proliferation and survival, we modulated MAPK activation by using MAPK kinase (MEK) 1/2 inhibitors and site-directed mutagenesis of FGFR1. NHUC-VEC and NHUC-FR1 were precultured with MEK1/2 inhibitors, U0126 or PD98059, and then stimulated with heparin and FGF2 for 5 min (Fig. 3C). Both inhibitors prevented FGFR1 activation of ERK1/2. The MEK1/2 inhibitors are reported to inhibit ERK5; hence, previous studies describing phenotypes that were altered with these inhibitors may be a result of inhibiting both ERK5 and ERK1/2 (33). The MEK1/2 inhibitors did not reduce ERK5 activation in our system (Fig. 3C), indicating that the phenotypes identified were the result of inhibiting ERK1/2 activation. It has been reported that inhibition of PLC $\gamma$  activation by mutation of the PLC $\gamma$  binding site on FGFR1 (Y766F) reduces the level of MAPK activation (34). To determine if the level of MAPK activation correlated with the level of proliferation and survival, we used site-directed mutagenesis to destroy the PLC $\gamma$  binding site on FGFR1 (Y766F). Y766F-FGFR1 was transduced into TERT-NHUC (NHUC-Y766F). NHUC-Y766F expressed the same level of FGFR1 as NHUC-FR1 as determined by Western blotting (data not shown). NHUC-Y766F showed no FGF2-induced activation of PLC $\gamma$  and reduced FGFR1 activation of ERK1/2 (Fig. 3D).

**Level of MAPK signaling determines proliferative and apoptotic indices.** NHUC-Y766F and MEK inhibitors were used to determine the effect of modulating MAPK signaling on cell proliferation and survival (Fig. 4). NHUC-Y766F showed reduced levels of DNA replication in the presence of FGF2 compared with NHUC-FR1 (Fig. 4A). In the presence of MEK inhibitors, the levels of DNA replication in NHUC-FR1 and NHUC-Y766F were similar to control cells. FGFR1-induced prosurvival responses were also significantly ( $P < 0.01$ ) reduced in NHUC-Y766F compared with NHUC-FR1 (Fig. 4B). Neither NHUC-FR1 nor NHUC-Y766F showed a difference in FGFR1-induced survival compared with NHUC-VEC when cultured with MEK inhibitors (Fig. 4C), suggesting that the

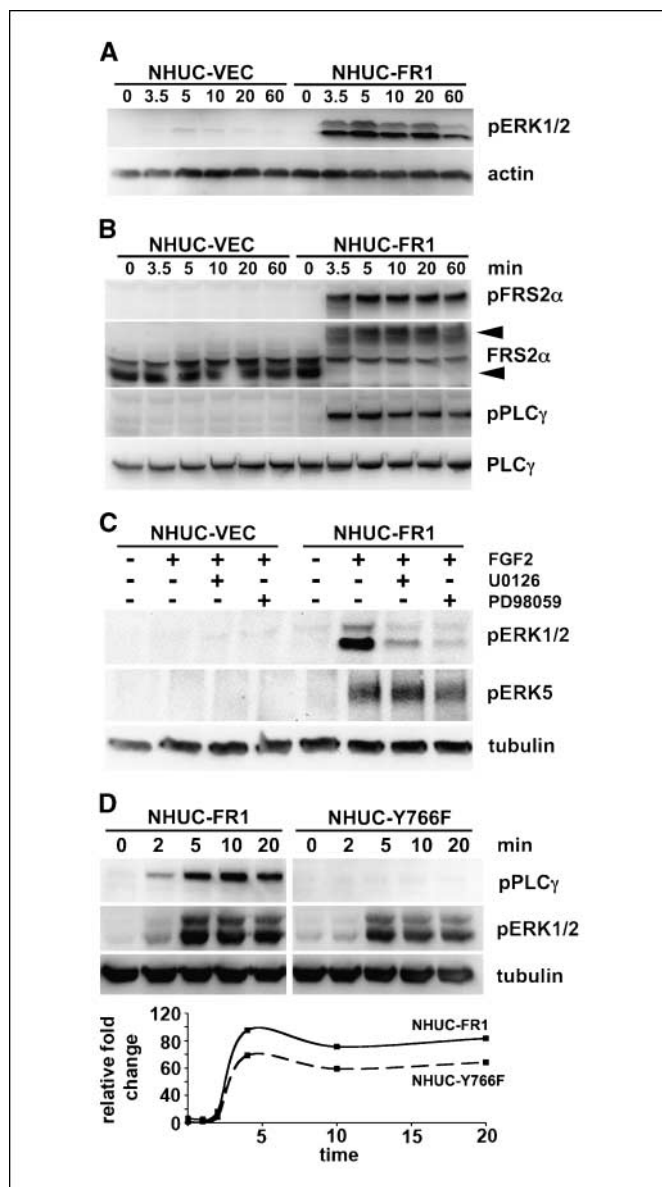
level of MAPK activation determines the observed differences in proliferation and survival.

**MAPK activation regulates prosurvival and proapoptotic factors.** We used Western blotting to identify factors downstream of MAPK signaling that may regulate proliferation and prosurvival responses (Fig. 4D). Cyclin D1 expression was increased in response to FGF2 in a time-dependent manner. Two factors associated with prosurvival and proapoptotic responses are MCL1 and BAD, respectively. Stimulation of FGFR1 increased MCL1 expression and BAD was inactivated via phosphorylation. Increased phosphorylation of BAD occurred at Ser<sup>112</sup> but not Ser<sup>136</sup> (data not shown), indicative of MAPK rather than AKT inactivation. The changes in expression correlated with the level of MAPK activation and observed changes in proliferation and survival (Fig. 2). In addition, MEK inhibitors prevented the increase in cyclin D1 expression, MCL1 expression, and phosphorylation of BAD (Fig. 4E).

**Bladder cancer cell lines show differential dependence on FGFR1 expression.** In an inducible FGFR1-driven model of prostate cancer in mice, early dependence of hyperplasia and prostatic intraepithelial neoplasia on FGFR1 signaling is partially lost in the invasive adenocarcinomas that develop after long-term FGFR1 activation (17). Thus, it has been proposed that a "susceptibility window" may exist for targeting FGFR1 in this tissue. To determine the role of FGFR1 in muscle invasive bladder cancer, we used shRNA to knock down expression in four urothelial carcinoma cell lines, JMSU1, UMUC3, 253J, and HT1197, which are derived from invasive tumors and express high levels of FGFR1. These were transduced with retroviruses containing a U6 promoter (11) driving expression of FGFR1-specific shRNA. Two shRNAs were successful in reducing expression of FGFR1 (Fig. 5A). Interestingly, knockdown of FGFR1 in JMSU1 was consistently less than in the other cell lines. We hypothesized that JMSU1 may be dependent on FGFR1 expression and that only cells with a low level of knockdown could survive. To test this hypothesis, we cloned the shRNAs into a retroviral construct containing GFP, transduced these into UMUC3 and JMSU1, and monitored the number of GFP- and non-GFP-expressing cells by



**Figure 2.** Activation of FGFR1 regulates proliferation and survival. **A**, immunoprecipitation of FGFR1 from NHUC-FR1 cultured with FGF2 for 15, 30, and 60 min. Blots were probed with an anti-phosphotyrosine antibody (*pTYR*) and reprobbed with anti-FGFR1 antibody as loading control. **B**, NHUC-VEC (*dashed line*) and NHUC-FR1 (*solid line*) were grown in supplement-free medium with heparin and FGF2 (10 ng/mL). **C**, NHUC-VEC and NHUC-FR1 were grown in supplement-free medium with heparin (*black column*) or heparin and FGF2 (*gray column*) for 24 h before addition of BrdUrd (*BrdU*). BrdUrd incorporation was measured by fluorescence-activated cell sorting. **D**, NHUC-VEC and NHUC-FR1 were grown in supplement-free medium for 96 h in the presence of heparin (*black column*) or heparin and FGF2 (*gray column*). Viability and apoptosis were analyzed by fluorescence-activated cell sorting.



**Figure 3.** FGFR1 activates the MAPK pathway. Cells were cultured in supplement-free medium for 1 h before culture with heparin and FGF2 for indicated times. **A**, protein lysates were blotted with anti-phospho-ERK (*pERK*) antibodies and then reprobbed with anti-actin. **B**, protein lysates were blotted with phosphospecific antibodies against FRS2 $\alpha$  and PLC $\gamma$ . Blots were reprobbed with anti-FRS2 $\alpha$  or PLC $\gamma$  antibodies. **C**, cells were cultured with DMSO (control), U0126, or PD98059 for 1 h. FGF2 was added for 5 min and lysates were probed with anti-phospho-ERK5 antibody that also cross-reacts with phospho-ERK1/2. Blots were reprobbed with tubulin. **D**, NHUC-FR1 and NHUC-Y766F were cultured with FGF2 and lysates were blotted for phospho-PLC $\gamma$  or phospho-ERK. Loading control, tubulin. Change in phospho-ERK levels in NHUC-FR1 (*solid line*) and NHUC-Y766F (*dashed line*) quantified from a representative experiment.

fluorescence-activated cell sorting (Fig. 5B). The relative proportion of GFP-expressing cells with FGFR1 shRNA expression in NHUC-VEC and UMUC3 cells did not alter compared with the nonspecific control. However, in JMSU1, the proportion of GFP-expressing cells with shRNA targeted against FGFR1 decreased compared with controls, suggesting that these cells are FGFR1 dependent. To test this further, we cultured cells with the FGFR inhibitor PD173074 and measured cell survival by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Compatible with the shRNA data, PD173074 reduced JMSU1 survival but had no effect on TERT-NHUC or UMUC3 (Fig. 5C).

To examine the effects of FGFR1 knockdown on downstream signaling pathways, knockdown cell lines were starved for 24 h and then cultured with FGF2. Similar to NHUC-FR1, the MAPK pathway was activated by FGF2 stimulation in the tumor cell lines without knockdown, increased expression of MCL1 was observed, and both were attenuated by FGFR1 knockdown (Fig. 5D). Phosphorylation of BAD was undetectable in these lines. Cells expressing shRNA2, which was less efficient at knocking down FGFR1 (Fig. 5A), showed less attenuation of signalling than shRNA1 as shown by higher levels of phospho-ERK and MCL1 expression.

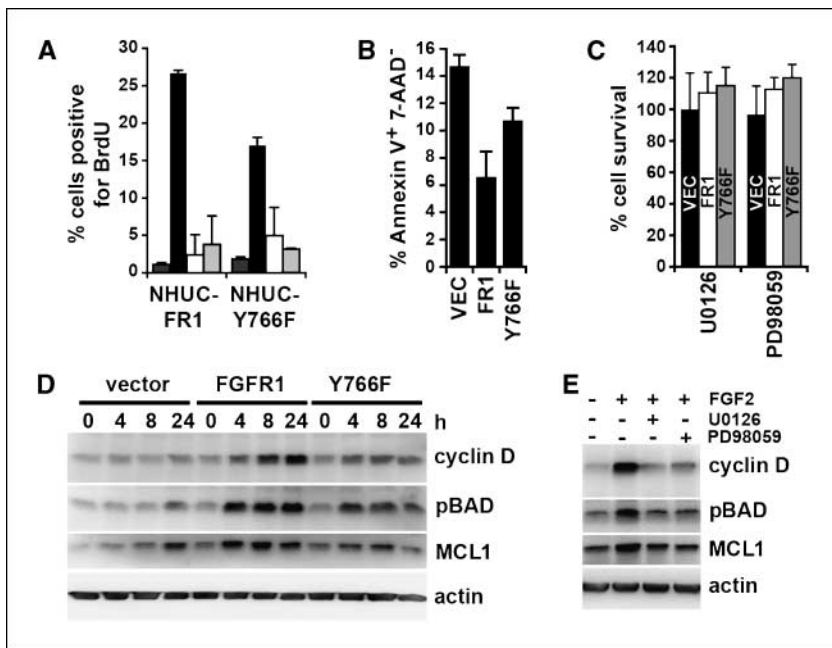
Both UMUC3 and JMSU1 parental lines grow independently of anchorage in soft agar. 253J and HT1197 do not. FGFR1 knockdown reduced the ability of UMUC3 (Fig. 6A) and JMSU1 (Fig. 6B) to form colonies in soft agar, suggesting that FGFR1 plays a role in preventing cell death and promoting anchorage-independent growth.

To determine if knockdown of FGFR1 altered the growth rate or tumorigenicity *in vivo*, cells were injected subcutaneously into nude mice (Fig. 6C and D). FGFR1 shRNAs significantly ( $P < 0.001$ ) reduced the growth of UMUC3 from day 4 *in vivo* compared with the nonspecific control (Fig. 6C). Tumorigenicity tests of JMSU1 were not successful, as only small tumors developed, which inevitably ulcerated independent of cell number or the use of Matrigel. To determine if FGFR1 affected the rate of tumor invasion into surrounding tissue, tumors were harvested at different time points during the experiment (data not shown). No difference in invasion was observed between FGFR1 knockdown cell lines and controls, suggesting that FGFR1 is not a key factor in promoting invasion in these cell lines. 253J and HT1197 formed only transient subcutaneous nodules that regressed within 3 to 4 weeks and this was not affected by FGFR1 knockdown.

## Discussion

Although much recent research has focused on the role of FGFR3 in bladder cancer, little is known about the expression and effects of other FGFR family members in bladder tumorigenesis. We examined the transcript levels of FGFR1 to FGFR4 and showed that FGFR1 expression is increased in a high proportion of bladder cancer cell lines and in the majority of tumors independent of stage and grade. This suggests that up-regulation of FGFR1 expression is an early event in the development of bladder cancer. *FGFR1* is located at 8p12, a region that is amplified in a small proportion of bladder tumors (35, 36), and this may explain some cases of increased expression observed in our study. Amplification of *FGFR1* has also been identified in lobular breast cancer (18). In breast cancer cell lines, amplification of FGFR1 was associated with increased expression and this contributed to cell survival. However, the low proportion of tumors showing amplification in bladder cancer does not account for the high frequency of increased expression, suggesting that this may be due to altered transcriptional regulation or increased transcript stability. Studies of rat and chicken FGFR1 promoters have identified a promoter region and transcription factors that regulate FGFR1 transcription (37, 38). We have identified a similar region in the human FGFR1 promoter that when deleted reduces promoter activity in bladder cancer cell lines,<sup>3</sup> indicating that up-regulation of FGFR1 by transcription factor binding may be causative of high levels of FGFR1 expression in bladder tumors.

<sup>3</sup> Our unpublished data.

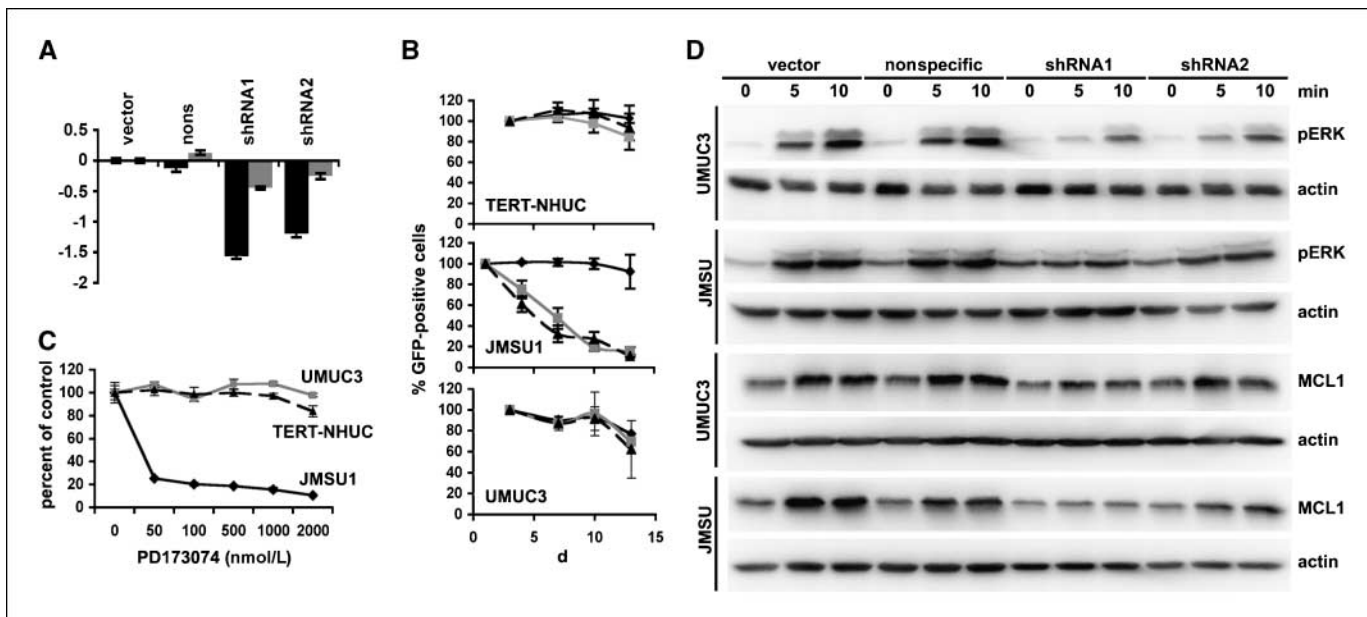


**Figure 4.** MAPK activation regulates proliferation and survival. *A*, NHUC-FR1 and NHUC-Y766F were cultured with heparin (dark gray), FGF2 (black) and U0126 (white), or FGF2 and PD98059 (light gray) for 24 h before addition of BrdUrd. BrdUrd incorporation was analyzed using fluorescence-activated cell sorting. *B*, cells were cultured in supplement-free medium with heparin and FGF2 for 96 h. Apoptosis was analyzed by Annexin V staining. *C*, NHUC-VEC (black column), NHUC-FR1 (white column), and NHUC-Y766F (gray column) were cultured with heparin, FGF2, and U0126 or PD98059 for 96 h. *D*, NHUC-VEC, NHUC-FR1, and NHUC-Y766F were cultured with heparin and FGF2 for indicated times and their lysates were blotted for cyclin D1, MCL1, and phospho-BAD (pBAD). *E*, NHUC-FR1 were cultured with indicated substances for 4 h and their lysates were blotted for cyclin D1, MCL1, and phospho-BAD.

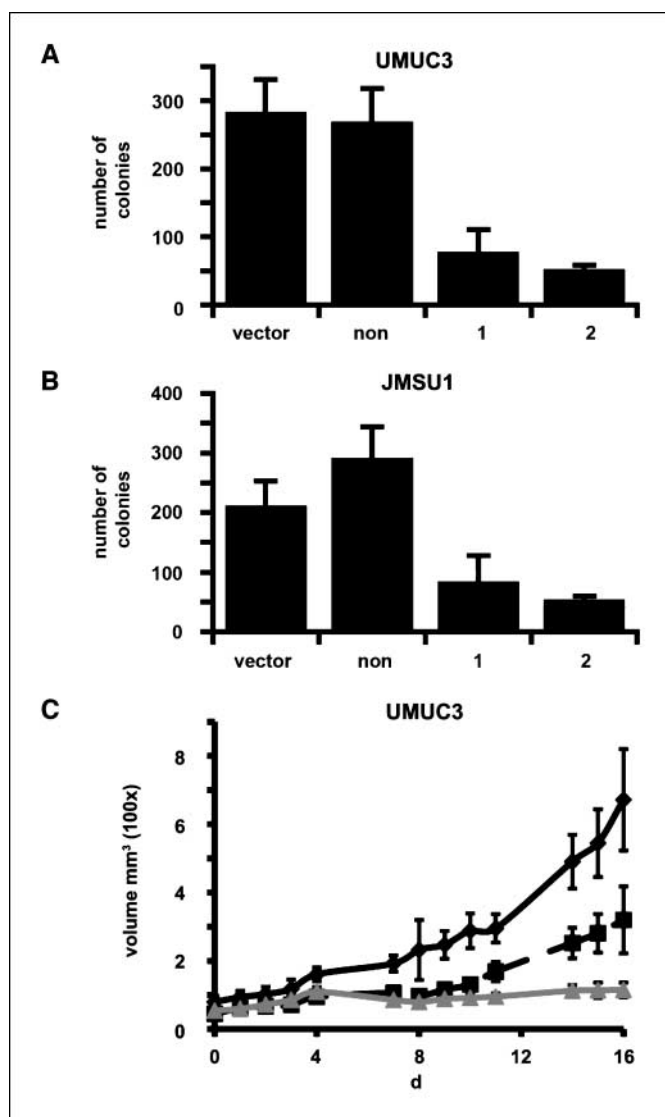
Superficial low-grade bladder cancers have few genetic alterations (3). To recapitulate the increased levels of FGFR1 found in such tumors, we expressed FGFR1 in TERT-NHUC (39) using a retroviral expression system to establish mass populations of cells expressing physiologic levels of receptor. High expression levels of tyrosine kinase receptors can lead to autoactivation as described for ERBB2 (40). However, this did not occur in our system and FGFR1 activation was ligand dependent. Thus, we propose that activation of up-regulated FGFR1 observed in urothelial carcinoma requires ligand. FGF1 and FGF2 levels are increased in the urine of

patients with bladder cancer (41, 42), and increased FGF1 expression has been observed in urothelial carcinoma, with strongest expression in high-grade tumors (43). Furthermore, FGF5 and FGF8 mRNA have been detected in bladder cancer cell lines (44), both of which can stimulate proliferation via FGFR1 (30).

Signaling downstream of FGFRs has been comprehensively studied and reviewed (31). In urothelial cells, ERK1/2 were phosphorylated in response to FGF2 treatment. Much attention has focused on the duration of ERK activation and its role in stimulating proliferation and differentiation (45). In PC12 cells,



**Figure 5.** Bladder cancer cell line dependence on FGFR1. *A*, FGFR1 transcript levels in JMSU1 (gray columns) and UMUC3 (black columns) transduced with vector, nonspecific shRNA (nons), shRNA1, or shRNA2 were measured by real-time reverse transcription-PCR. *B*, TERT-NHUC, JMSU1, and UMUC3 were transduced with nonspecific shRNA (solid black), shRNA1 (dashed black), or shRNA2 (gray) in retroviruses expressing GFP. Percentage of GFP-expressing cells remaining in culture compared with the number at the start of the experiment. *C*, TERT-NHUC (dashed black), UMUC3 (gray), and JMSU1 (solid black) were cultured with FGFR inhibitor PD173074. Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *D*, JMSU1 and UMUC3 were starved in 0.1% FCS for 24 h before culture with heparin and FGF2. Lysates were probed for phospho-ERK, MCL1, and actin.



**Figure 6.** FGFR1 knockdown reduces anchorage-independent growth and growth *in vivo*. UMUC3 (A) or JMSU1 (B) expressing a nonspecific shRNA (*non*), shRNA1, or shRNA2 were cultured in soft agar for 21 d and stained with *p*-iodonitroretrozolium violet. Graph shows viable colonies >1 mm in size per 10 cm<sup>2</sup>. UMUC3 were injected subcutaneously into nude mice and tumor volume measured (C). Solid black line, cells expressing the nonspecific control; dashed black line, cells expressing shRNA1; gray line, cells expressing shRNA2. Bars, SE.

stimulation of ERK1/2 for several hours resulted in differentiation, whereas stimulation that resulted in a relatively short ERK1/2 activation resulted in proliferation. Interestingly, stimulation of FGFR1 in urothelial cells induced sustained activation of ERK1/2, but unlike previously described systems (46, 47), this promoted proliferation rather than differentiation. To determine if FGFR1-induced proliferation and survival were caused by ERK activation, we modulated the level of ERK activation by using either site-directed mutagenesis of FGFR1 or MEK1/2 inhibitors. We created a Y766F mutation in FGFR1, as phosphorylation at Y766 allows binding and activation of PLC $\gamma$ . Inhibition of PLC $\gamma$  activation was shown to reduce the level of MAPK activity in FGF-stimulated L6 myoblasts, indicating that PLC $\gamma$  via PKC modifies the activity of the MAPK pathway (34). In addition, a previous report showed that cells expressing FGFR1 lacking the PLC $\gamma$  binding site still

responded to FGF stimulation although not as efficiently as cells expressing wild-type receptor (48). We also observed decreased MAPK activation and a reduced level of proliferation and survival in Y766F-expressing cells compared with wild-type receptor in response to FGF2. MEK inhibitors prevented FGF2-induced proliferation and survival in NHUC-FR1 and NHUC-Y766F, suggesting that PLC $\gamma$  regulates these phenotypes via activation of the MAPK pathway. This shows that changes in proliferation and survival were a result of ERK activation and that the level of ERK activation is important in regulating the level of response. The level of ERK activation also correlated with changes in downstream signaling proteins, which correlated with the phenotypic changes.

Next, we sought to reduce levels of FGFR1 in bladder cancer cell lines using shRNA to determine the role of FGFR1 in cells derived from invasive carcinoma. Intriguingly, the level of FGFR1 knockdown in JMSU1 was much less than that observed in other cell lines. As we have been able to efficiently knock down other proteins in JMSU1, we hypothesized that JMSU1 are dependent on FGFR1 for survival, that is, they show addiction to this oncogene (49) and our findings supported this. Interestingly, survival or proliferation of UMUC3, 253J, and HT1197 was not dependent on FGFR1 signaling, although these cells express high levels of FGFR1 (data not shown). However, UMUC3 also contain a *KRAS2* mutation and HT1197 contain a *NRAS* mutation (50), which may activate similar signaling pathways to FGFR1 and hence reduce FGFR1 dependence. Although UMUC3 has a *KRAS2* mutation and only a low level of knockdown of FGFR1 was observed in JMSU1, both cell lines showed reduced anchorage-independent growth, a phenotypic marker of transformation. UMUC3 knockdown cells showed decreased tumor volume in nude mice compared with controls. However, the low levels of knockdown of FGFR1 in expanded populations of JMSU1 might not have been sufficient to reduce tumor growth *in vivo*. A major problem in extrapolating results obtained in human bladder tumor cell lines to the situation in bladder tumors in patients is the lack of relevant *in vivo* models. Few urothelial carcinoma cell lines are tumorigenic in immunocompromised mice; thus, we have been unable to test the *in vivo* effects of FGFR1 adequately in other cell lines. It is not clear what the transient subcutaneous growth of 253J and HT1197 represents, but knockdown of FGFR1 had no effect on this phenotype.

A possible explanation for the differences observed between cell lines is suggested by recent work done in a mouse prostate model system in which FGFR1 activation was regulated via an inducible dimerization system and led to stepwise progression to adenocarcinoma (17). Withdrawal of FGFR1 activation before the development of prostate cancer reversed FGFR1-induced prostatic hyperplasia and reduced progression to adenocarcinoma but FGFR1-induced advanced prostate cancer became independent of the inducible system. However, endogenous FGFR1 activity was not inhibited in these experiments, leaving the possibility that endogenous activity compensated for the loss of dependence on the inducible system. Our results suggest that FGFR1-dependent growth may be altered or overcome during bladder tumor development and that invasive tumors may show different degrees of dependence on FGFR1 for growth and survival. This now requires more detailed analysis both in human cancers and in model systems where FGFR1 signaling can be monitored and regulated.

In conclusion, we have shown that FGFR1 expression is increased in bladder cancer and that FGFR1 can drive proliferation and promote survival in normal bladder cells. Proliferation and survival are driven via the MAPK pathway and the level of ERK activation is

directly related to the level of response. We have observed differential FGFR1 dependence in urothelial carcinoma cell lines. Significantly, TERT-NHUC showed no dependence on FGFR1 for either proliferation or survival. This may allow nontoxic therapies targeted to multiple FGFRs to be developed for bladder tumors.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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