Role of Fibroblast Growth Factor Receptor Signaling in Prostate Cancer Cell Survival

Mustafa Ozen, Dipak Giri, Frederic Ropiquet, Alka Mansukhani, Michael Ittmann

Background: **Expression of fibroblast growth factors (FGFs) is increased in a substantial fraction of human prostate cancers *in vivo* and in prostate cancer cell lines. Altered FGF signaling can potentially have a variety of effects, including stimulating cell proliferation and inhibiting cell death. To determine the biologic significance of altered FGF signaling in human prostate cancer, we disrupted signaling by expression of a dominant-negative (DN) FGF receptor in prostate cancer cell lines.** ***Methods:*** **PC-3, LNCaP, and DU145 prostate cancer cells were stably transfected with DN FGFR constructs, and LNCaP and DU145 cells were infected with a recombinant adenovirus expressing DN FGFR-1. The effect of DN FGFR-1 expression was assessed by colony-formation assays, cell proliferation assays, flow cytometry, and cytogenetic analysis. Key regulators involved in the G_2-to-M cell cycle transition were assessed by western blotting to examine cyclin B1 expression and by *in vitro* kinase assay to assess cdc2 kinase activity.** ***Results:*** **Stable transfection of the DN FGFR-1 construct inhibited colony formation by more than 99% in all three cell lines. Infection of LNCaP and DU145 prostate cancer cells with adenovirus expressing DN FGFR-1 led to extensive cell death within 48 hours. Flow cytometry and cytogenetic analysis revealed that the DN FGFR-1 receptor led to arrest in the G_2 phase of the cell cycle before cell death. Cyclin B1 accumulated in DN FGFR-1-infected LNCaP cells, but cdc2 kinase activity was decreased.** ***Conclusions:*** **These findings reveal an unexpected dependence of prostate cancer cells on FGF receptor signal transduction to traverse the G_2/M checkpoint. The mechanism for the G_2 arrest is not clear. Our results raise the possibility that FGF-signaling antagonists might enhance the cell death induced by other prostate cancer therapies. [J Natl Cancer Inst 2001; 93:1783–90]**

Prostate cancer is the most common cancer in U.S. men and the second leading cause of cancer mortality in this group. Alterations in fibroblast growth factor (FGF) signaling have been implicated in the pathogenesis of prostate cancer in animal mod-

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els and by analysis of human prostate cancer tissues and prostate cancer cell lines. For example, Yan et al. *(1)* have shown that progression of prostate cancers in Dunning rats is associated with expression of FGFs not originally present in the tumors and changes in the isoforms of FGF receptors expressed, consistent with autocrine FGF receptor activation. The same group has shown that expression of FGF receptor-1 (FGFR-1) accelerates tumorigenesis in this system *(2)*.

Autocrine expression of FGF6 *(3)* by prostate cancer cells has been identified in 40% of human prostate cancers *in vivo*, and the majority of prostate cancers overexpress FGF8 *(4–6)*. Increased expression of FGFR-1 is present in poorly differentiated human prostate cancers *in vivo (7,8)*. Autocrine expression of FGFs and expression of FGF receptors have been reported in all of the commonly used prostate cancer cell lines—i.e., PC-3, DU145, and LNCaP *(9–11)*. Prostate cancers express appropriate receptors to respond individually to these FGFs *(3,7,8,10, 12)*. However, FGFs have a variety of biologic effects *in vivo* in different contexts, including enhancement of proliferation, motility, and angiogenesis and inhibition of apoptosis, so that the effect of the observed alterations in FGF signaling in prostate cancer may be complex.

To determine the role of FGF signaling in prostate cancer cells, we disrupted FGF receptor signaling by expression of a dominant-negative (DN) FGFR-1 (DN FGFR-1) protein in human prostate cancer cell lines and assessed the effects on cell viability by measuring cell cycle parameters.

Materials and Methods

Transfection of prostate cancer cell lines. PC-3, DU145, and LNCaP human prostate cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The DN FGFR-1 and FGF receptor-2 (FGFR-2) plasmids have been described previously *(13)*. Briefly, stop codons were generated downstream of the transmembrane domain in the complementary DNAs (cDNAs) of each receptor to yield cDNA-encoding proteins containing the extracellular domain and a portion of the intracellular region, 32 amino acids for FGFR-1 and 74 amino acids for FGFR-2. Both truncated cDNAs were cloned into the *Bam*H1 site of pCEP4 (Invitrogen Corp., Carlsbad, CA). Thus, the DN constructs consist of the extracellular and transmembrane domain of the FGF receptor and are truncated so that the kinase domain is not present. The DN protein can dimerize with endogenous receptor following ligand binding but cannot phosphorylate it, so that receptor signaling does not occur; in this manner, it competitively inhibits signaling by the endogenous receptors *(13–15)*. The control was the vector pCEP4 (Invitrogen Corp.). Cells were plated at 3×10^5 cells per 60-mm dish and transfected with 25 μL of Lipofectamine (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) and 3 μg of plasmid for 5 hours in a total volume of 2.4 mL of RPMI-1640 medium without antibiotics. Cells were then refed with RPMI-1640 medium without antibiotics containing 20% FBS. After an additional 18 hours of incubation, cells were refed with growth medium and then split 1 : 3 after 48 hours. The next day, selection was initiated by the addition of 150 μg/mL of hygromycin to select for cells in which plasmids had stably integrated into genomic DNA. In a separate experiment, cotransfection was carried out with 2 μg of PSV2 Neo, which carries a neomycin resistance gene, and 1 μg of FGFR DN plasmid or pCEP4, as described above, and selection was carried out by the addition of 250 μg/mL of G418. This control experiment was carried out to be certain that the reason for the lack of colonies after FGF DN receptor transfection and hygromycin selection was not due to disruption of the hygromycin resistance gene during cloning of the DN cDNAs.

Construction of recombinant adenoviruses. A recombinant adenovirus containing FGFR-1 DN cDNA was constructed by homologous recombination as described previously *(16)*. Briefly, the DN FGFR-1 cDNA was cloned into an adenovirus shuttle vector, pAVS6. The pAVS6 FGFR-1 DN plasmid was then cotransfected with pJM17 plasmid, which carries the adenoviral genome, into the 293 cell line. The E1-defective recombinant adenovirus (FGFR DN adenovirus) was produced by homologous recombination between pAvS6-FGFR-1 DN plasmid and pJM17 in 293 cells. The E1 function is supplied by the 293 cells, which contain an integrated copy of the E1 gene, allowing the cells with E1-defective recombinant adenovirus to express the replication-deficient adenovirus. Adenoviral plaques were screened for the presence of FGFR-1 DN sequences with the use of primers 5′-TTCCAGTACTCTTGGATCGG-3′ and 5′-AGGTACGATGAGACCCGCACC-3′. A positive plaque was amplified and titered with the use of 293 cells. The control adenoviruses were identical, except that they carried either β-galactosidase (LacZ)- or green fluorescent protein (GFP)-coding sequences.

Cell culture and infection of cells. Cell lines were infected with adenovirus in the presence of a minimal volume of infection medium (RPMI-1640 medium supplemented with 2% FBS), incubated for 1 hour at 37 °C in a shaker, and transferred to an incubator (37 °C, 5% CO_2) after the addition of RPMI-1640 medium supplemented with 10% FBS. Preliminary experiments were carried out in all three cell lines with variable concentrations of GFP adenoviruses (10–10^4 virus/cell). The presence of bright green fluorescence was assessed after 24 hours by fluorescence microscopy. To achieve greater than 70% infection by this criterion required 50 plaque-forming units (pfu) per cell for LNCaP and 2×10^3 pfu per cell for DU145; these ratios were, therefore, used in subsequent experiments. PC-3 required greater than 10^4 pfu per cell and thus were not used for subsequent experiments.

Proliferation and colony-formation assays. Cells (5×10^4) were plated in triplicate in 35-mm dishes, allowed to attach to the dish, and infected as described above. Attached cells were then trypsinized and counted with the use of a Coulter counter after 24, 48, and 72 hours for the proliferation assay. For colony-formation assays, 10^3 cells were infected by incubation with adenovirus in 0.3 mL of infection medium and plated on 10-cm dishes. Cells were incubated at 37 °C and supplied with fresh medium every 3 days. After 12 days, they were fixed with 10% formalin and stained with crystal violet, and the colonies visible to the naked eye were counted.

Cell cycle analysis. Cells (2×10^5) were plated in 100-mm dishes and infected with adenoviral vectors as described above, refed, and incubated in growth medium for 24, 48, and 72 hours. Both floating and attached cells were harvested and stained with propidium iodide for DNA cell cycle analysis following a standard protocol as described elsewhere *(17)*. DNA content was measured by use of a flow cytometer (Epics XL-MCI; Beckman Coulter, Miami, FL), and cell cycle analysis was performed with the use of Multi Cycle for Windows version 3.0 software (Phoenix Flow Systems, San Diego, CA). Cell cycle-blocking experiments were carried out by adding 5 μg/mL of aphidocolin (Sigma Chemical Co., St. Louis, MO) to the culture medium, which leads to arrest at the G_1/S checkpoint. After 16 hours, the cells were infected with FGFR DN or GFP adenovirus as described above in the presence of aphidocolin and then maintained for a further 16 hours in the presence of aphidicolin. The medium was removed, and cells were refed with medium without aphidicolin and collected for flow cytometry and determination of cell number at 6, 24, and 48 hours after release. Control cells were maintained in aphidicolin and collected after 48 hours for determination of cell number.

Chromosome preparations and mitotic index. Approximately 80% confluent cell cultures were used for chromosome preparations following standard techniques described previously *(18)*. Briefly, cells were treated with 0.04 μg/mL of Colcemid for 20 minutes, trypsinized, treated with 0.06 *M* potassium chloride for 20 minutes, fixed in 3 : 1 methanol–acetic acid, centrifuged at 1100*g* for 5 minutes at room temperature, and dropped onto wet slides. Slides were stained with Giemsa, and at least 500 cells from each sample were counted for mitotic index and G_2-interphase analysis. G_2/S cells were characterized by a visibly enlarged nucleus, while G_0/G_1 nuclei were of the normal size. Dead cell fragments were identified as having nuclei considerably smaller than normal.

Immunoprecipitation and western blot analysis. Prostate cancer cells were plated at 1×10^6 cells per 10-cm dish and infected with adenoviruses as described above. The cells were collected at each time point and lysed in lysis buffer (i.e., 20 m*M* Tris–HCl [pH 8.0], 2 m*M* EDTA, 1 m*M* dithiothreitol, 0.1% Nonidet P-40, 250 m*M* NaCl, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL benzamidine, and 1 m*M* phenylmethanesulfonyl fluoride) and cleared by centrifugation for 10 minutes in a microcentrifuge at 4 °C at 12 000*g*. For assessment of FGFR-1 phosphorylation, cells were treated with 10 ng/mL of recombinant FGF2 (R&D Systems, Minneapolis, MN) for 10 minutes before cell lysis. Protein concentration was determined by use of a BioRad protein assay (BioRad Laboratories, Hercules, CA). Polyclonal anti-FGFR-1 antibody (sc-15; Santa Cruz Biotechnology, Santa Cruz, CA) (4 μg of antibody to 800 μg of cell lysate) was added to each sample for 2 hours at 4 °C for each immunoprecipitation. The immune complexes were precipitated by incubation with 25 μL of protein A/G

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Sepharose (Pierce Chemical Co., Rockford, IL) for 2 hours at 4 °C. The beads were then washed in buffer containing 10 m*M* HEPES (pH 7.4), 25 m*M* NaCl, and 1 m*M* dithiothreitol (DTT). The washed beads were then boiled in sample buffer and centrifuged at 12 000*g* for 10 minutes at room temperature, and the supernatant was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The resolved proteins were electrotransferred to nitrocellulose membranes and then blocked with a 5% solution of fat-free milk in phosphate-buffered saline containing 0.5% Tween 20 (PBST). The membrane was then incubated with 500 ng/mL of antiphosphotyrosine antibody (PY20; Transduction Laboratories, Lexington, KY) at 4 °C overnight. The membranes then were washed with PBST and were treated with appropriate secondary antibodies. The antigen–antibody reaction was visualized by use of an enhanced chemiluminescence (ECL) assay (Amersham Life Science Inc., Arlington Heights, IL) and exposure to ECL film (Amersham Life Science Inc.). To detect cyclin B1 by western blot analysis, membranes were incubated with 400 ng/mL of mouse monoclonal antibody (sc-245; Santa Cruz Biotechnology) overnight at 4 °C and developed as described above. Control antibody was an anti-β-actin monoclonal antibody (A531; Sigma Chemical Co.) at a 1 : 5000 dilution. Each lane contained 30 μg of cell lysate protein.

cdc2 kinase assay. One milligram of the cell lysate protein was used for each cdc2 kinase assay. One microgram of mouse monoclonal anti-cdc2 antibody (sc-54; Santa Cruz Biotechnology) was added to each sample overnight at 4 °C in a rotator. The immune complexes were precipitated by incubation with 25 μL of protein A/G Sepharose for 1 hour at 4 °C. Sepharose beads were collected by centrifugation at 12 000*g* for 10 minutes at 4 °C and washed four times in wash buffer containing 20 m*M* HEPES, 25 m*M* NaCl, and 1 m*M* DTT. The pellet was resuspended in protein kinase assay buffer (i.e., 50 m*M* Tris–HCl [pH 7.5], 10 m*M* $MgCl_2$, and 1 m*M* DTT). Two micrograms of histone H1 protein (Roche Diagnostics, Indianapolis, IN) and 1 μL of premix containing 3.3 μCi of [^{32}P]adenosine triphosphate (ATP) (4500 Ci/mmol) in protein kinase assay buffer containing 1 m*M* ATP were added to each sample, which was then incubated at 30 °C for 30 minutes. Each sample was boiled in sample buffer and centrifuged for 30 seconds at 12 000*g* at room temperature, and supernatants were analyzed by SDS–PAGE in a 12% gel. The dried gel was then subjected to autoradiography.

RESULTS

Effect of DN FGF Receptor Expression in Human Prostate Cancer Cells

For the determination of the biologic effect of FGF receptor expression in human prostate cancer, DN constructs of FGFR-1 and FGFR-2 were transfected into the three commonly used prostate cancer cell lines (LNCaP, DU145, and PC3) and cells were selected in hygromycin. Both DN constructs consist of the extracellular and transmembrane domains of the corresponding FGF receptor, with a truncation leading to loss of the kinase domain. As described previously, both of these constructs function as DN inhibitors of FGF receptor signaling *(13)*. The DN receptors were both cloned in the pCEP4 vector, which contains a hygromycin resistance gene. Shown in Fig. 1, after selection in hygromycin, only rare colonies were present in any of the three cell lines transfected with either the FGFR-1 or the FGFR-2 DN receptor, whereas numerous colonies were present in control cells transfected with pCEP4 vector control. Inhibition of colony formation by transfection of DN FGF receptor constructs was greater than 99%. To confirm that the effect seen was the result of expression of the DN FGF receptor and not a disruption of expression of the hygromycin resistance gene, we cotransfected the cell lines with the DN receptor constructs and a 2 : 1 excess of plasmid containing a neomycin resistance gene (pSV2Neo). Transfected cells were then selected in G418. Cells transfected with the DN FGF receptors had a 75%–90% reduction in colonies compared with cells transfected with pSV2 Neo and control pCEP4 (data not shown), confirming that the effect was due to the DN receptor expression.

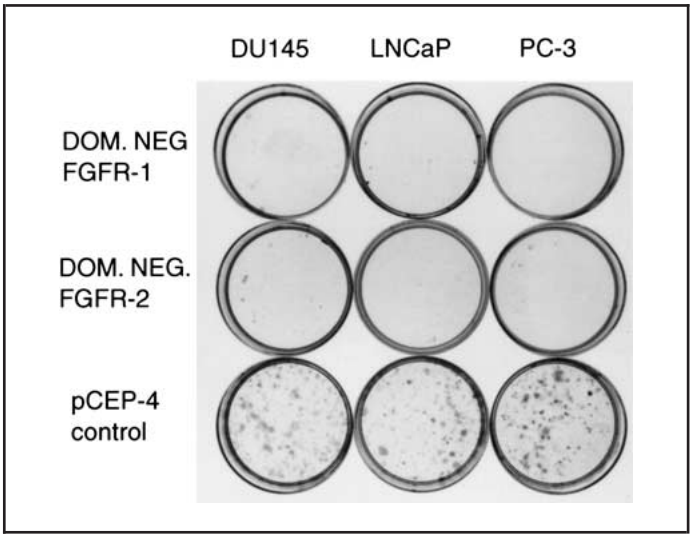


Fig. 1. Stable transfection of fibroblast growth factor receptor (FGFR) dominant-negative (DOM. NEG.) receptors into prostate cancer cells. Prostate cancer cell lines were transfected with DOM. NEG., FGFR-1, or DOM. NEG. FGFR-2 constructs were cloned in the pCEP4 vector, which contains a hygromycin resistance gene, or the pCEP4 vector alone. After 12 days of selection in hygromycin, cells were fixed and stained with crystal violet. Representative plates from each transfection are shown.

To understand the mechanism by which the DN receptor inhibited colony formation, we constructed an adenovirus expressing a DN FGFR-1. To verify that the DN FGFR-1 (FGFR DN) adenovirus was indeed disrupting FGF receptor signaling, we infected LNCaP cells with the FGFR DN adenovirus or LacZ-expressing control virus. Cells were treated with recombinant FGF2 10 minutes before lysis at different intervals after infection. The full-length FGFR-1 was immunoprecipitated, and the immunoprecipitated material was separated on SDS–PAGE gels, and western blot analysis was performed with antiphosphotyrosine antibody. Shown in Fig. 2, there was a marked decrease in phosphorylated FGFR-1 by 24 hours after infection, indicative of decreased transphosphorylation of the full-length receptor and a DN effect on FGF receptor activation. This experiment has been repeated, examining only the 24-hour time point; identical results were obtained. In addition, immunoprecipitation with antiphosphotyrosine antibodies followed by western blotting with anti-FGFR-1 antibody also revealed a marked decrease in phosphorylated FGFR-1 by 24 hours after infection with FGFR-1 DN adenovirus.

The biologic effect of the expression of the DN receptor was profound in both LNCaP and DU145 cells. By 24 hours after infection, many cells were round and refractile and were often

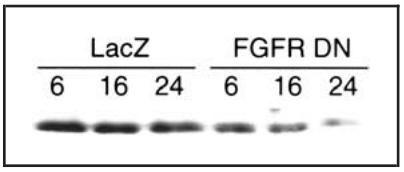


Fig. 2. Inhibition of fibroblast growth factor receptor (FGFR)-1 phosphorylation by infection with FGFR-1 dominant-negative (DN) adenovirus. LNCaP cells were infected with 50 plaque-forming units per cell of FGFR-1 DN (FGFR DN) adenovirus or control (LacZ) adenovirus and, at 6, 16, of 24 hours after infection were stimulated with 10 ng/mL of FGF2. Cells were then lysed, and FGFR-1 protein was immunoprecipitated with anti-FGFR-1 antibody. The immunoprecipitated products were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and western blotting with antiphosphotyrosine antibody was performed.

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detached from the plate. By 72 hours, there was a marked decrease in the number of attached cells. Shown in Fig. 3, infection with the FGFR DN adenovirus led to a 50%–70% decrease in cell number by 3 days after infection, whereas control cells infected with the LacZ control virus were indistinguishable from mock-infected cells and continued to grow exponentially. Similar results were seen in colony-formation assays. LNCaP and DU145 cells were infected with FGFR DN adenovirus and plated at low density. Both cell lines had an approximately 90% reduction in colony number relative to LacZ-infected controls (data not shown). Therefore, expression of DN FGF receptor constructs had a profound inhibitory effect on proliferation and/or viability of all prostate cancer cell lines tested.

Cell Cycle Effects of DN FGFR-1 Expression

For the examination of the effect of the DN FGFR-1 on cell cycle progression, LNCaP and DU145 cells were infected with FGFR DN adenovirus or control LacZ virus and flow cytometry was performed after propidium iodide staining of cells collected 24, 48, or 72 hours after infection. Compared with cells infected with control LacZ virus, both cell types showed a marked increase in the percentage of cells in G_2/M by 24 hours after infection accompanied by a decrease in the fraction of cells in G_1/G_0 (Fig. 4). At 48 hours after infection, there was a continued increase in the percentage of G_2/M cells accompanied by accumulation of dead-cell debris. By 72 hours, there was a marked accumulation of dead-cell fragments in the DN FGFR-1-infected cultures of both cell types (data not shown).

Cells with double the G_1/G_0 DNA content may be in either the G_2 or the M phase of the cell cycle. To determine whether the cells accumulate in G_2 or M, we prepared chromosome spreads of untreated, LacZ, or FGFR DN adenovirus-infected LNCaP cells 72 hours after infection. The percentage of G_0/G_1 cells, cells in mitosis, dead-cell fragments, and G_2/S cells was determined. In addition, the mean chromosome number was determined in the mitotic cells. There was a marked increase in the number of G_2/S cells in FGFR DN adenovirus-infected cells (Fig. 5). Such cells were characterized by a visibly enlarged interphase nucleus. Given that flow cytometry showed an accumulation of cells with 4N DNA content, these cells are G_2- rather than S-phase cells. The percentage of mitotic cells was decreased twofold, consistent with an accumulation of G_2- rather than M-phase cells. There was also a marked increase in the number of dead-cell fragments as well as a corresponding decrease in the number of G_0/G_1 cells. The mean chromosome number in FGFR DN adenovirus-infected cells was the same as in untreated cells (data not shown).

The experiments described above clearly show that the expression of FGFR DN receptor leads to both G_2 arrest and cell death, but it is not clear that the cell death observed is temporally related to the G_2 arrest or whether it can occur throughout the cell cycle. To examine this question, we treated LNCaP cells with aphidicolin for 16 hours at concentrations leading to the arrest at the G_1/S boundary, as determined by preliminary experiments. Cells were then infected with adenovirus-expressing DN FGFR-1 or control GFP-expressing adenovirus and maintained in aphidicolin for an additional 16 hours. Cells were then released from the aphidicolin block, and flow cytometric cell cycle analysis was performed at 6, 24, and 48 hours after release. Cells infected with either FGFR DN adenovirus or control GFP adenovirus were able to traverse the S phase and enter G_2, with more than 75% of the cells having a G_2/M DNA content 6 hours after release in both cases (Fig. 6, A). However, the cell number was equivalent in the FGFR DN expressing and control samples at 6 hours after release (Fig. 6, B), indicating minimal cell death during passage through the S phase of the cell cycle. At 24 hours and, to a lesser extent, 48 hours after release from the G_1 block, there was a higher percentage of FGFR DN expressing cells in G_2 (Fig. 6, A), consistent with inhibition of progression through the G_2 checkpoint. The increase in FGFR DN-expressing cells in G_2 was associated with a marked decrease in the number of FGFR DN-expressing cells at 24 and 48 hours after release (Fig. 6, B), while cells infected with control virus had resumed proliferation and increased in number. However, cells that were maintained for the same period of time in G_1 by the continued presence of aphidicolin did not undergo substantial death, even

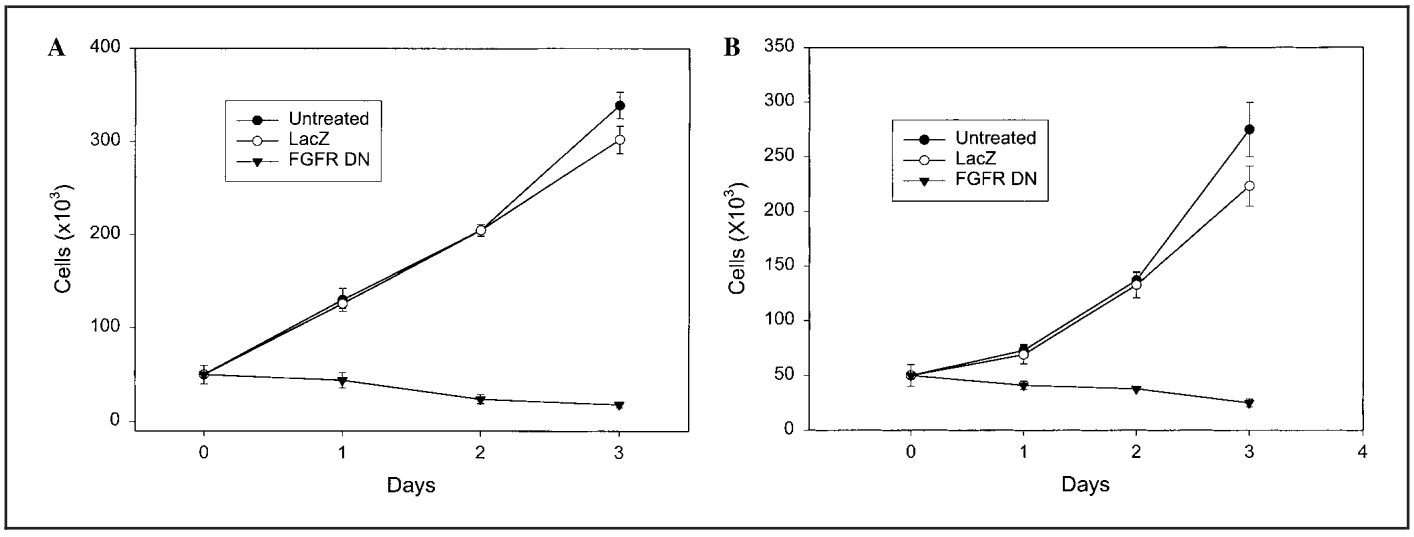


Fig. 3. Effect of fibroblast growth factor receptor (FGFR)-1 dominant-negative (DN) adenovirus on prostate cancer cell proliferation and viability. LNCaP (**A**) or DU145 (**B**) prostate cancer cells were plated at 5×10^4 cells per 35-mm dish and infected with FGFR-1 DN or control (LacZ) adenovirus or mock infected (untreated). The cell number was determined by counting with the use of a Coulter counter at 24, 48, and 72 hours after infection. All values are the mean of triplicate determinations, with the 95% confidence interval as indicated.

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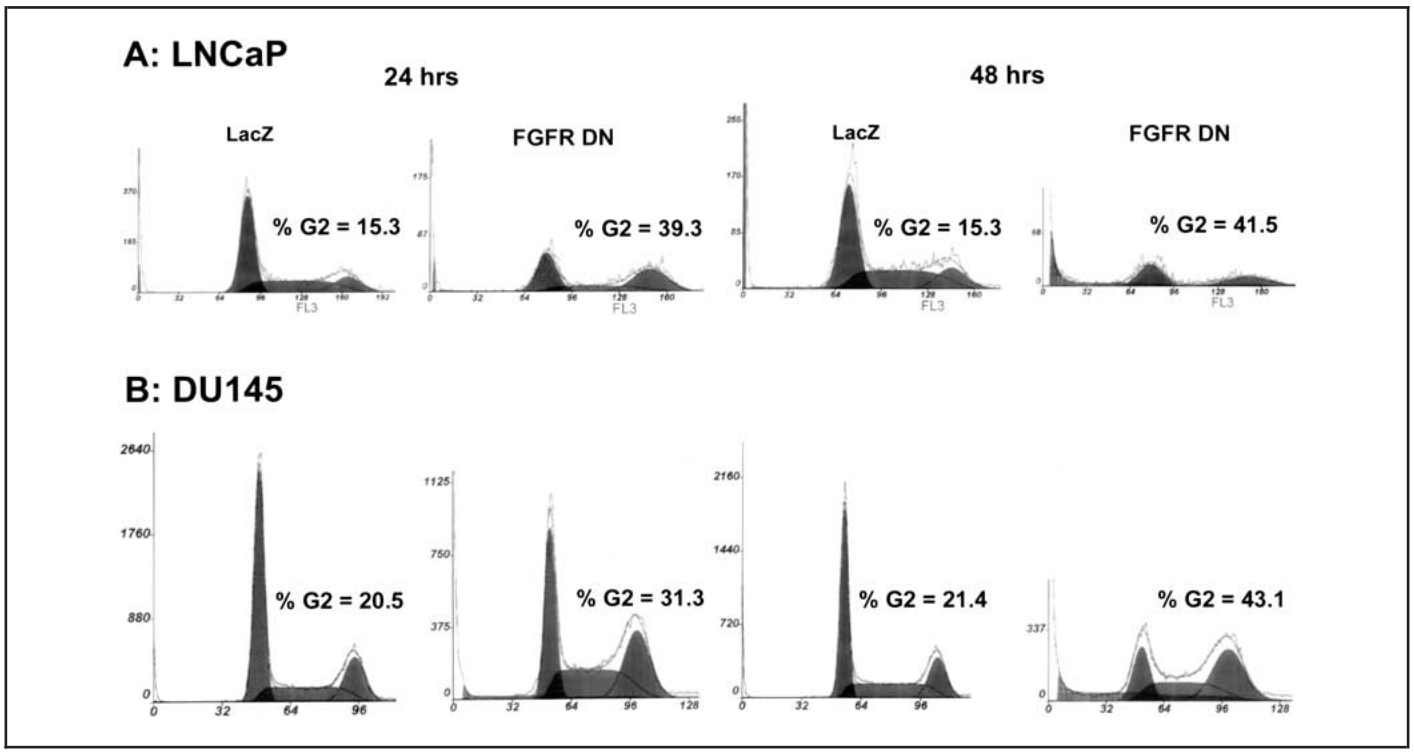


Fig. 4. Cell cycle analysis of fibroblast growth factor receptor (FGFR)-1 dominant-negative (DN)-infected cells. LNCaP (**A**) or DU145 (**B**) prostate cancer cells were plated at 2×10^5 cells per 100-mm dish and were infected with FGFR DN or control (LacZ) adenovirus. At 24 or 48 hours, floating cells were collected, and attached cells were collected after trypsinization, pooled, and stained with propidium iodide, and flow cytometry was performed. In each case, cell number is represented on the ***y*-axis,** with the corresponding fluorescence at 550 nm is shown on the ***x*-axis.** Flow cytometry data are seen as a **line,** and the **filled area** represents the results of cell cycle analysis by the use of Multi Cycle cell cycle analysis software. The percentage of G_2/M as determined with this software is indicated for each analysis. The 95% confidence intervals for the percent G_2/M are as follows: **A**) LNCaP: LacZ, 24 hours (13.8 to 16.9); FGFR DN, 24 hours (38.3 to 40.4); LacZ, 48 hours (12.9 to 17.7); and FGFR DN, 48 hours (33.4 to 49.6); **B**) DU145: LacZ, 24 hours (19.5 to 21.5); FGFR DN, 24 hours (29.9 to 32.6); LacZ, 48 hours (20.2 to 22.5); and FGFR DN, 48 hours (41.0 to 45.2)

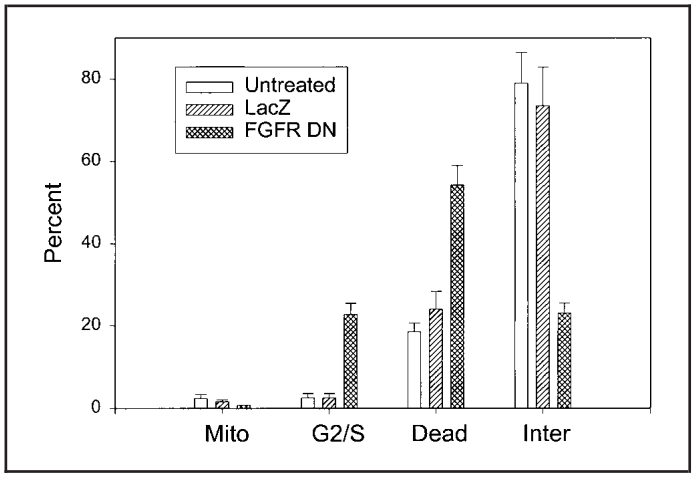


Fig. 5. Cytogenetic analysis of fibroblast growth factor receptor (FGFR) dominant-negative (DN)-infected cells. LNCaP cells were infected with FGFR DN or LacZ adenovirus or mock infected. After 72 hours, chromosome spreads were prepared and 500 cells were evaluated. The percentage of cells in mitosis (Mito), G_2/S (visibly enlarged nuclei), and G_0/G_1 (Inter) or present as dead-cell fragments (Dead) is shown. The mean and 95% confidence intervals are shown.

when expressing the DN FGFR, as can be seen by comparing the number of cells 6 hours after release from aphidicolin and after 48 additional hours of G_1 block (Fig. 6, B). Thus, cell death is associated with an arrest in G_2 and does not occur in cells arrested in G_1 or traversing the S phase. Taken together, these experiments demonstrate that prostate cancer cells infected with FGFR DN-expressing adenovirus arrest in the G_2 phase of the cell cycle and subsequently undergo death.

Effect of FGF DN Receptor Expression on Cyclin B1 and cdc2 Kinase

The key regulator of the G_2 to M transition is the accumulation of cyclin B–cdc2 kinase complexes that are then activated by dephosphorylation by cdc25 during the G_2/M transition *(19)*. We, therefore, evaluated the effect of FGFR-1 DN receptor expression on these key regulatory molecules. Infection of LNCaP cells with FGFR DN-expressing adenovirus led to an accumulation of cyclin B1 by as early as 6 hours after infection (Fig. 7, A). However, as seen in Fig. 7, B, there was a marked decrease in the cdc2 kinase activity (as measured by *in vitro* kinase assay with histone H1 as a substrate) in cells infected with FGFR DN virus when compared with lacZ-infected controls. Thus, although cyclin B1 accumulates in FGFR DN-infected cells, there appears to be a failure to accumulate active cyclin B–cdc2 complexes.

Discussion

Autocrine expression of FGFs by human prostate cancer cells is common both *in vivo (3–6)* and *in vitro (9–11)*, and prostate cancer cells express the appropriate FGF receptors to respond to these FGFs *(3,7,8,10,12)*. Thus, autocrine stimulation of FGF

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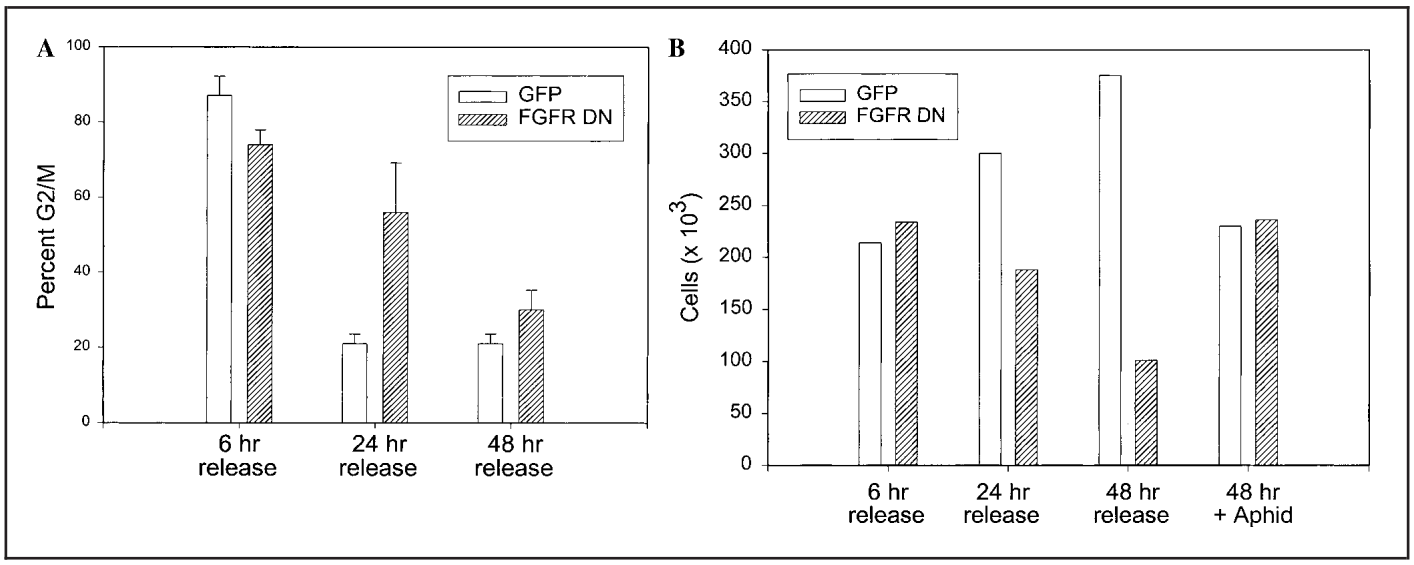


Fig. 6. Analysis of fibroblast growth factor receptor (FGFR)-1 dominant-negative (DN)-infected cells after release from G_1/S block. **A)** LNCaP cells were arrested at the G_1/S checkpoint by incubation with aphidicolin (aphid.) (5 μg/mL) for 16 hours and infected with adenovirus expressing FGFR DN protein or green fluorescent protein (GFP). The cells were maintained in aphid. for an additional 16 hours and then released from the G_1/S block by removal of aphid. Cells were collected at 6, 24, and 48 hours after release, and cell cycle analysis was performed by flow cytometry after propidium iodide staining. The percentage of cells in G_2/M was calculated by use of Multi Cycle cell cycle analysis software. The 95% confidence intervals are as indicated. **B)** Cell number as determined by Coulter counter on LNCaP cells treated as described above. The cell number was also determined on LNCaP cells maintained in aphid. for 48 hours and not released from G_1/S block (48 hours + aphid.). Values shown are for a single determination. Our usual standard deviation for triplicate determinations of cell number is less than 10% of the total cell number.

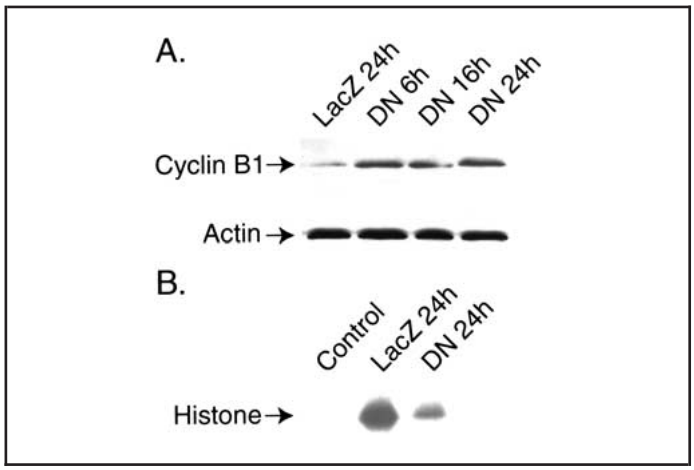


Fig. 7. Analysis of cyclin B1/cdc2, complexes in FGFR-1 dominant-negative (DN)-infected cells. **A)** LNCaP cells were infected with FGFR-1 DN (FGFR DN) or LacZ adenovirus, and cell lysates were collected at the indicated times after infection. Thirty micrograms of protein lysate was then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blotting with anti-cyclin B1 antibody. The blot was then stripped and reprobed with an anti-β-actin antibody as a loading control. **B)** LNCaP cells were infected with FGFR-1 dominant-negative (DN) (FGFR DN) or LacZ adenovirus, and cell lysates were collected after 24 hours. *In vitro,* cdc-2 kinase assays were then performed with 1 mg of cell lysate protein with histone H1 as a substrate for the immunoprecipitated cdc-2 complexes. Control was a lysate from LacZ-infected cells, mock immunoprecipitated without anti-cdc-2 antibody.

receptors appears to be common in human prostate cancer. FGFs can act as growth factors for normal prostatic epithelial cells, so it has been generally believed that one of the major consequences of this autocrine stimulation of FGF receptors in prostate cancer is to enhance cell growth and division. Indeed, FGFs have been shown to enhance net proliferation of prostate cancer cells *in vitro,* both by the addition of exogenous growth factors and by antisense approaches *(3,7,11).* For example, antisense inhibition of FGF8b expression leads to decreased net proliferation, soft-agar colony formation, and *in vivo* tumorigenicity in DU145 cells *(11).* The net increase in cell proliferation in response to FGFs can be due to classic growth factor stimulation of cell growth and division, inhibition of cell death, or a combination of these two mechanisms. Our results indicate that FGF receptor signaling may act to provide an important survival signal in prostate cancer cells. Adenovirus-mediated expression of DN FGF receptor has been shown to induce apoptosis in cultured rat smooth-muscle cells *(20),* in which autocrine FGF2 signaling is thought to occur *(21),* so that there is precedent for our observation that FGF receptor signaling may be an essential survival factor when autocrine FGF signaling is occurring. We have also shown that disruption of FGF signaling leads to an arrest in G_2 in prostate cancer cells. This linkage of FGF receptor activation to progression through the G_2/M checkpoint has not been described previously to our knowledge, and FGFs have generally been thought to be factors promoting exit from G_0 and G_1 progression. Thus, our results indicate that FGFs play a critical role in prostate cancer survival by a novel mechanism that has not—again, to our knowledge—been reported previously.

Many aspects of the underlying mechanisms by which the DN FGF receptor leads to G_2 arrest have yet to be elucidated. We have demonstrated that the DN FGFR-1 receptor decreases FGFR-1 activity. However, there are four types of FGF receptors, and all three prostate cancer cell lines express multiple types of FGF receptor. Transphosphorylation between different forms of FGF receptors has been demonstrated *(22),* so that it is likely that the DN FGFR-1 receptor also inhibits the activity of these other receptors. The observation that stable transfection of the DN FGFR-2 construct also led to a profound inhibition of colony formation is consistent with this idea. However, proof of

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this hypothesis is difficult, given the complex interactions among multiple FGF receptors expressed at variable levels in different cell lines and the variable affinities of currently available antibodies for each receptor. It is also possible that the DN FGF receptors might interact with proteins other than FGF receptors and that some of the observed phenotypes may be the result of such interactions.

The mechanism by which G_2 arrest is induced by FGFR-1 DN receptor expression is unclear. We have demonstrated a decrease in cdc2 kinase activity in FGFR-1 DN-expressing cells, and since cdc2 kinase activity is essential for the G_2/M transition *(19),* this decreased activity is assumed to be critical in the observed G_2 arrest. The exact mechanism by which cdc2 kinase activity is decreased is not known, but it is not the result of decreased expression of cyclin B1, because this critical regulator of cdc2 is increased in treated cells. Further investigations of the multiple factors controlling cdc2 activity are necessary to determine the mechanism leading to G_2 arrest.

We have found that disruption of FGF signaling is lethal for all three prostate cancer cell lines tested. By contrast, infection with similar amounts of FGFR DN adenovirus had only a slight effect on primary prostatic epithelial cells (data not shown). This inability of the FGFR DN adenovirus to induce G_2 arrest and cell death could be a result of differences between primary cells and cancer cell lines in the levels of expression of the different FGF receptor types or could reflect a fundamental difference in the dependence of the cancer cells on autocrine FGF stimulation. However, not all of the cell lines are dependent on FGF receptor activity, because clones expressing FGFR DN receptors were easily established in NIH3T3 cells with the use of these same vectors *(13).* A pancreatic cancer cell line expressing an FGFR-1 DN construct has been established *(23),* and Yayon et al. *(24)* were able to establish a similar melanoma cell line. However, using four other melanoma cell lines, Yayon et al. *(24)* were not able to establish cell lines expressing the DN construct, implying that there may be a strong selection against expressing the FGFR-1 DN construct in these cells. Given the evidence that autocrine stimulation by FGFs may play an important role in melanoma *(25),* we are currently investigating the effect of FGFR-1 DN adenovirus infection in melanoma cell lines. The effect of FGF DN receptors in other malignancies in which FGF receptor activation is thought to play a role will also need to be evaluated.

In summary, there is abundant evidence of increased FGF receptor signaling in prostate cancer based on analysis of animal models, human tissues, and human prostate cancer cell lines. In addition to the known effects of FGFs on cell proliferation, motility, and angiogenesis, which may all promote prostate cancer progression, we now have evidence that FGFs may act as essential survival factors as well. Small molecule inhibitors of FGF receptor signaling are already undergoing phase I clinical trials as cancer therapy *(26),* and our data support the potential for these agents as treatments of prostate cancer. Our finding that loss of FGF signaling leads to G_2 arrest suggests that FGF-receptor signaling antagonists might act synergistically with other cancer therapies leading to G_2 arrest, such as radiotherapy or treatment with alkylating agents. Indeed, it has been shown that FGF2 inhibits radiation-induced apoptosis in some contexts *(27)* so that blocking the FGF signal transduction pathway may enhance radiation-induced cell death. Further work is needed to evaluate these therapeutic possibilities and to understand the mechanism by which loss of FGF receptor signaling leads to G_2 arrest and cell death in prostate cancer.

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NOTES

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