Exogenous Fibroblast Growth Factors Maintain Viability, Promote Proliferation, and Suppress GADD45α and GAS6 Transcript Content of Prostate Cancer Cells Genetically Modified to Lack Endogenous FGF-2

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Abstract
Understanding processes regulating prostate cancer cell survival is critical to management of advanced disease. We used prostate cancer cell transfectants genetically modified to be deficient in either endogenous fibroblast growth factor (FGF-1) or endogenous FGF-2 to examine FGF maintenance of transfectant survival and proliferation and FGF-2-regulated expression of transfectant growth arrest DNA damage (GADD) and growth arrest sequences (GAS) family genes (known modulators of cell cycle progression and survival) and the AS3 gene (an androgen-modulated effector of prostate cell proliferation). When propagated in the absence of exogenous FGFs, FGF-2-deficient transfectants undergo exponential death, whereas FGF-1-deficient transfectants proliferate. Exogenous FGF-1, FGF-2, FGF-7, or FGF-8 promote survival and proliferation of FGF-2-deficient transfectants and enhance FGF-1-deficient transfectant proliferation. Transfectants express FGF receptor FGR1, FGR2(IIIb), FGR2(IIIc), and FGR3 transcripts, findings consistent with the effects of exogenous FGFs. FGF-2-deficient transfectants express high levels of AS3, GADD45α, GADD45γ, GAS8, and GAS11 transcripts and moderate levels of GADD153, GAS2, GAS3, and GAS6 transcripts and lack demonstrable GAS1 or GAS5 transcripts. FGF withdrawal-mediated death of FGF-2-deficient transfectants did not significantly affect cell AS3, GADD153, GADD45γ, GAS2, GAS3, GAS7, GAS8, or GAS11 transcript content, whereas GADD45α and GAS6 transcript content was elevated. These studies establish that endogenous FGF-2 dominantly regulates prostate cancer cell survival and proliferation and that exogenous FGFs may assume this function in the absence of endogenous FGF-2. Additionally, we provide the first evidence that FGFs regulate prostate GADD45α and GAS6 transcript content. The latter observations suggest that GADD45α and GAS6 proteins may be effectors of processes that regulate prostate cancer cell survival. Additional studies are required to examine this possibility in detail.


Introduction
Clinical literature establishes that current methods for management of advanced prostate cancer provide palliation of limited duration. In virtually all instances, relapse and disease progression occur (1, 2). An extensive body of studies establishes that fibroblast growth factors (FGFs) prominently modulate normal prostate development and growth and are dominant regulators of prostate cancer cell function (3-10). Our prior studies showed that genetic modification that generates prostate cancer cell transfectants deficient in endogenous FGF-2 promoted cell death when transfectants are cultured in the absence of exogenous FGFs (FGF withdrawal). In contrast, exogenous FGF-2 promoted survival and proliferation of FGF-2-deficient transfectants (11). These findings suggested a potentially novel role for FGFs as mediators of prostate cancer cell survival.

Steroid and mitogen modulation of intracellular signaling pathways that affect prostate cancer cell caspase-mediated apoptosis has been examined intensively. Utility of these studies as a model of prostate cancer cell survival has been limited by the fact that the principal model (LNCaP) is not representative of many other prostate cancer cells that are resistant to effector-induced apoptosis (12-15). Significantly, there has been an absence of studies examining mitogen modulation of growth arrest DNA damage (GADD) and growth arrest sequences (GAS) family gene function in prostate cancer. The GADD and GAS proteins, established regulators of cell cycle progression and survival, were first identified by their rapid induction following genotoxic stress (16-18). Subsequently, it has been shown that activity of the multifunctional GADD and GAS gene family members is modulated by effectors such as interleukin-1, transforming growth factor-β, interleukin-6, colony-stimulating factor (17), interleukin-18 (19), epidermal growth factor (20), and vitamin D (21). Additionally, a significant body of literature establishes that GADD or GAS modulation of cell cycle and survival likely occurs by independent processes that seem to involve independent signaling pathways (17, 22-25). These considerations and the recent identification of the novel AS3 prostate protein that is a mediator of proliferation arrest in prostate cancer cells (26) and normal rat prostate (27) caused us...
to consider that GADD and GAS gene family proteins may affect prostate cancer cell survival. In this report, we document the ability of exogenous FGF-1, FGF-2, FGF-7, and FGF-8 to function as survival factors and/or promote proliferation of prostate cancer cells deficient in endogenous GFGs. Additionally, we detail our findings of the consequences of exogenous FGF withdrawal-mediated cell death on GADD and GAS gene family and AS3 transcript content of FGF-2-deficient transfectants.

Results

Stable Expression of FGF-1 or FGF-2 Antisense RNA Profoundly Down-Regulates Prostate Cancer Cell FGF-1 or FGF-2 Content, Respectively

Western analysis of extracts prepared from stable prostate cancer cell transfectants shows marked down-regulation of FGF-1 content in FGF-1 antisense RNA transfectants and complete loss of detectable 21.5- and 17.0-kDa FGF-2 isoforms and profound down-regulation of the 19.5-kDa FGF-2 isoform of FGF-2 antisense RNA transfectants (Fig. 1). Western analyses also establish that neoplastic transformation is associated with expression of novel 19.5- and 21.5-kDa FGF-2 isoforms that are not expressed in normal rat ventral prostate. The expression of these novel FGF-2 isoforms is characteristic of transformation and is seen in multiple neoplastic human tissues (28-30). Quantitative densitometric analysis of band intensity revealed that the residual FGF-1 content of FGF-1-deficient transfectants (aF1 clones) was 25% to 40% of that characteristic of vector control or FGF-2-deficient transfectants (aF2 clones). Comparable analysis revealed that the 21.5- and 17.0-kDa FGF-2 isoforms were not detectable in FGF-2-deficient transfectants and that content of the 19-kDa FGF-2 isoform of these transfectants was 20% to 40% of that characteristic of vector control or FGF-1-deficient transfectants. Densitometric analysis also showed that FGF-1 content of FGF-2-deficient transfectants

Blot:

![Blot Image]

FIGURE 1. Constitutive expression of FGF antisense RNA profoundly diminishes FGF expression in stable FGF-1 and FGF-2 antisense transfectants. Western analyses of FGF-1 and FGF-2 content of AXC/SSH rat ventral prostate extracts (VP), recombinant rat FGF-1 and rat FGF-2 (Std), or lysates prepared from individual clones of vector control transfectants (VC): FGF-2 antisense RNA transfectants (FGF-2-deficient transfectants, aF2) or FGF-1 antisense RNA transfectants (FGF-1-deficient transfectants, aF1). Aliquots of cell lysates or ventral prostate extract were separately adsorbed to heparin-agarose. Solubilized heparin-agarose–adsorbed transfectant lysates corresponding to 5 × 10⁶ cells/well in 96-well plates on medium either containing or lacking FGF-1 (100 ng/mL) or FGF-2 (10 ng/mL) as indicated. Viable cell content was determined by quantification of formazan production. Lines, obtained by unweighted linear regression analysis. Points, mean of quadruplicate wells. For clarity, the SD error bars (SD was ≤10% of the mean) have been omitted. Values are the half-times for either population doubling (positive values) or viable cell loss (negative values). Half-times were obtained by solution of the regression equations. Data were obtained using FGF-1-deficient transfectant clone 2 (aF1 clone 2) or FGF-2-deficient transfectant clone 2 (aF2 clone 2; Fig. 1). Representative of those obtained using FGF-1-deficient transfectant clone 6 (aF1 clone 6) or FGF-2-deficient transfectant clone 4 (aF2 clone 4; Fig. 1).

and FGF-2 content of FGF-1-deficient transfectants was comparable with that of vector controls. Thus, these analyses establish the specificity of the FGF-1 antisense RNA-mediated reduction in transfectant FGF-1 content and FGF-2 antisense RNA-mediated reduction in transfectant FGF-2 isoform content.

Antisense RNA-Mediated Decrements in Transfectant FGF Content Differentially Affects Transfectant Survival and Proliferation

Representative data show FGF-1-deficient transfectants proliferate exponentially in the absence of exogenous FGF-1 (FGF withdrawal) during 5 days of culture on 5% charcoal-stripped fetal bovine serum (CS-FBS). (CS-FBS was used only to minimize potential contributions of steroids in FBS.) Monolayers were harvested and cells were plated (5 × 10⁵ cells/well) in 96-well plates on medium either containing or lacking FGF-1 (100 ng/mL) or FGF-2 (10 ng/mL) as indicated. Viable cell content was determined by quantification of formazan production. Lines, obtained by unweighted linear regression analysis. Points, mean of quadruplicate wells. For clarity, the SD error bars (SD was ≤10% of the mean) have been omitted. Values are the half-times for either population doubling (positive values) or viable cell loss (negative values). Half-times were obtained by solution of the regression equations. Data were obtained using FGF-1-deficient transfectant clone 2 (aF1 clone 2) or FGF-2-deficient transfectant clone 2 (aF2 clone 2; Fig. 1). Representative of those obtained using FGF-1-deficient transfectant clone 6 (aF1 clone 6) or FGF-2-deficient transfectant clone 4 (aF2 clone 4; Fig. 1).

![Graph Image]
in the absence of exogenous FGF-2 (FGF withdrawal). However, addition of FGF-2 to culture medium shows this mitogen functions both as a survival factor and as a promoter of FGF-2-deficient transfectant exponential proliferation (Fig. 2, bottom). These initial analyses were expanded to include vector controls and analyses of the effects of exogenous FGF-7 and FGF-8. The latter FGFs are prominent products of normal and neoplastic prostate and possess potential to modulate neoplastic prostate cell function (31-33). These analyses showed that exogenous FGF-1, FGF-2, FGF-7, and FGF-8 function both as survival factors and as promoters of FGF-2-deficient transfectant proliferation (Fig. 3, bottom). In contrast, exogenous FGF-7 failed to enhance proliferation of FGF-1-deficient transfectants, whereas exogenous FGF-1, FGF-2, and FGF-8 promoted comparable enhancement of FGF-1-deficient transfectant proliferation (Fig. 3, middle). With the exception that exogenous FGF-7 (100 ng/mL) seemed to decrease vector control proliferation, neither exogenous FGF-1, FGF-2, nor FGF-8 affected vector control proliferation (Fig. 3, top). These replicate analyses also showed that vector control transfectant and FGF-1-deficient transfectant proliferation rates during culture in the absence of exogenous mitogen were identical, having mean ± SD doubling times of 215 ± 8 and 238 ± 20 hours, respectively (Fig. 3). During identical culture, parental cell mean ± SD doubling time was comparable (185 ± 20 hours) with that of vector control and FGF-1-deficient transfectants and was not affected by exogenous FGFs (data not shown). These results show that neither stable transfection nor FGF-1 antisense RNA-mediated reduction in FGF-1-deficient transfectant FGF-1 content significantly alters the basal proliferation rate of these transfectants. Thus, exogenous FGFs only enhance proliferation and/or survival of FGF-deficient prostate cancer cell transfectants, establishing specificity of exogenous FGF effects.

**Cell Count and Viability Analyses Confirm FGF Modulation of FGF-1-Deficient Transfectant Proliferation and FGF-2-Deficient Transfectant Survival and Proliferation**

Cell count and viability analyses showed that FGF-1-deficient transfectants cultured in the absence of exogenous FGF-1 proliferate and maintain a population viability that was indistinguishable from that of cells at the time of plating. Similarly, addition of FGF-1 to culture medium enhanced FGF-1-deficient transfectant proliferation and also maintained population viability that was indistinguishable from that of cells at the time of plating (Fig. 4). Using these limited counting data, the approximated doubling times for FGF-1-deficient transfectants propagated in either the absence or the presence of exogenous FGF-1 were 198 or 89 hours, respectively. These results agree with those of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analyses detailed in Figs. 2 and 3. Comparable analyses of the effect of exogenous FGF-2 on FGF-2-deficient transfectant survival and proliferation showed that 5 days culture in the absence of exogenous FGF-2 resulted in a 30% decline in the number of surviving transfectants. Notably, viability of the surviving FGF-2-deficient transfectant population was indistinguishable from that of cells at the time of plating (Fig. 4). In contrast, culture of FGF-2-deficient transfectants in the presence of exogenous FGF-2 promoted their survival and proliferation. Viability of proliferating FGF-2-deficient transfectants was indistinguishable from that of cells at the time of plating (Fig. 4). Using these limited counting data, the approximated half-life for FGF-2-deficient transfectants propagated in the absence of exogenous FGF-2 was 240 hours, whereas the doubling time for these transfectants during propagation in the presence of exogenous FGF-2 was 116 hours. These results agree with those of the MTT analyses detailed in Figs. 2 and 3.

**FGF Receptors of Prostate Cancer Cells**

The facts that both exogenous FGF-7 and FGF-8 effectively promoted survival and/or proliferation of FGF-deficient transfectants (Fig. 3) and their known differential affinity for FGF receptors (FGFR) relative to FGF-1 and FGF-2 (34) required characterization of prostate cancer cell FGFR content.

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**FIGURE 3.** FGFs do not affect vector control transfectant proliferation, enhance FGF-1-deficient transfectant proliferation, and are required for FGF-2-deficient transfectant survival. Transfectants were propagated and prepared for assay as detailed in the legend to Fig. 2. Results for paired, replicate, independent analyses of transfectants cultured in parallel in either the absence (FGF withdrawal) or the presence of FGF. The interval for a 2-fold change (half-time) in population cell number was determined by solution of the unweighed linear regression equations derived from the viable cell data for each analysis. *, P < 0.05, significantly different from the mean value for cells simultaneously cultured in the absence of FGF by the paired-samples t test. Bars, SE. Data were obtained using vector control transfectant clone 2 (vector control clone 2), FGF-1-deficient transfectant clone 2 (aF1 clone 2), or FGF-2-deficient transfectant clone 2 (aF2 clone 2; Fig. 1). Representative of those obtained using vector control transfectant clone 3 (vector control clone 3), FGF-1-deficient transfectant clone 6 (aF1 clone 6), or FGF-2-deficient transfectant clone 4 (aF2 clone 4; Fig. 1).

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**FGFs in Prostate Cancer Cell Survival**

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prostate cancer cells (Fig. 5). Simultaneous analyses for rat prostate cancer cell FGFR4 failed to generate ds-cDNA. In contrast, as reported by others (35), FGFR4 transcripts were demonstrable in rat kidney RNA preparations (data not shown).

The results of restriction analysis of the ds-cDNAs obtained using these primer pairs are shown in Fig. 5 and summarized in Table 2. These analyses confirmed primer-specific amplification of the FGFR1, FGFR2, and FGFR3 transcripts. Because the FGFR2(IIIb) and FGFR2(IIIc) isoforms are reported to have markedly different specificity for FGF-7 (34), we employed restriction analysis to characterize FGFR2 isoform content of rat prostate cancer cells. The primer pair detailed in Table 1 predict a ds-cDNA for the FGFR(IIIc) isoform that is cleaved by Avai to yield overlapping bands of 239 and 249 bp, whereas this amplification product lacks a HinII cleavage site. In contrast, these primers predict a ds-cDNA for the FGFR(IIIc) isoform that is cleaved by HinII to yield a 246-bp band and overlapping bands of 119 and 113 bp (9). The results of restriction analyses are shown in Fig. 6. Quantification of band intensities (Fig. 6) established that the FGFR2(IIIc) isoform represented 82 ± 3% (mean ± SD, parental cells) or 95 ± 2.7% (mean ± SD, transfectants) of prostate cancer cell FGFR2 content. Predominance of the FGFR2(IIIc) isoform is characteristic of advanced human prostate cancer (36, 37).

GADD and GAS Family Gene Expression in Rat Prostate Cancer Cells

Based on published sequence data, we designed primers for rat GADD and GAS gene family members and the AS3 gene (Table 1). We used these to perform survey analyses of the effect of FGF-2 withdrawal on FGF-2-deficient transfectant GADD and GAS gene family members and AS3 transcript content. The initial analyses were accomplished by performing RT-PCR and obtaining aliquots of the amplification products at PCR cycles 25, 30, 35, and 40 that were subjected to analysis of the FGFR2(IIIc) isoform that is cleaved by HinII to yield a 246-bp band and overlapping bands of 119 and 113 bp (9). The results of restriction analyses are shown in Fig. 6. Quantification of band intensities (Fig. 6) established that the FGFR2(IIIc) isoform represented 82 ± 3% (mean ± SD, parental cells) or 95 ± 2.7% (mean ± SD, transfectants) of prostate cancer cell FGFR2 content. Predominance of the FGFR2(IIIc) isoform is characteristic of advanced human prostate cancer (36, 37).

Table 1. Identity of the Primer Pairs Employed for Rat Prostate Cancer Cell Transcript Amplification

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Sense Primer (5'→3')</th>
<th>Antisense Primer (5'→3')</th>
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<tr>
<td>AS3* U95825</td>
<td>ACACCAAAAAATTATCTGCTC</td>
<td>AAGCCTCCCCCTTATTATCTG</td>
</tr>
<tr>
<td>FGFR1 NM_024146</td>
<td>CGATGATGATGACTCCT</td>
<td>TTCGTTGACAACTCATACAG</td>
</tr>
<tr>
<td>FGFR2 NM_053429</td>
<td>TTGGTGGAATGCATACCC</td>
<td>GCTGTCCTACACCAAGGAC</td>
</tr>
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<td>FGFR3 NM_051599</td>
<td>ACGTGCACAGTTGACTCT</td>
<td>GAAACAAGGAGTAAAGG</td>
</tr>
<tr>
<td>FGFR4 NM_113546</td>
<td>ATCTGGAGTCTCGGAAGTG</td>
<td>CACACCCACATTACAG</td>
</tr>
<tr>
<td>FGFR5 NM_024127</td>
<td>GGAGATCGAAAGGATGAC</td>
<td>GAAGATCAGCTGTCAG</td>
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<tr>
<td>FGFR3 NM_024134</td>
<td>TTTGGACAGTGTTGAC</td>
<td>GCCCCTACCACTGACT</td>
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<td>FGFR4 NM_057100</td>
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<td>FGFR3 NM_024134</td>
<td>TTTGGACAGTGTTGAC</td>
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<td>FGFR4 NM_057100</td>
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<td>FGFR5 NM_058088</td>
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<tr>
<td>FGFR4 NM_119859</td>
<td>ACGACATCATCTCCAAAC</td>
<td>AGGAGGACACCTGCTG</td>
</tr>
</tbody>
</table>

*Primers were designed to amplify rat sequence having high homology to the reported sequence for human AS3 (27).
^Primers were designed to encompass the alternately spliced sequences encoding either FGFR2(IIIb) or FGFR2(IIIc) and have been detailed by others (9).

GADD and GAS Family Gene Expression in Rat Prostate Cancer Cells

Based on published sequence data, we designed primers for rat GADD and GAS gene family members and the AS3 gene (Table 1). We used these to perform survey analyses of the effect of FGF-2 withdrawal on FGF-2-deficient transfectant GADD and GAS gene family members and AS3 transcript content. The initial analyses were accomplished by performing RT-PCR and obtaining aliquots of the amplification products at PCR cycles 25, 30, 35, and 40 that were subjected to analysis of the FGFR2(IIIc) isoform that is cleaved by HinII to yield a 246-bp band and overlapping bands of 119 and 113 bp (9). The results of restriction analyses are shown in Fig. 6. Quantification of band intensities (Fig. 6) established that the FGFR2(IIIc) isoform represented 82 ± 3% (mean ± SD, parental cells) or 95 ± 2.7% (mean ± SD, transfectants) of prostate cancer cell FGFR2 content. Predominance of the FGFR2(IIIc) isoform is characteristic of advanced human prostate cancer (36, 37).

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cell GADD153, GADD45α, GAS2, GAS3, GAS7, GAS8, or GAS11 transcript content. Additionally, neither GAS1 nor GAS5 transcripts were demonstrable in rat prostate cancer cells. However, as reported by others, GAS1 (38) and GAS5 (39) transcripts were demonstrable in NIH 3T3 cell RNA (data not shown). In contrast, these initial analyses indicated that GADD45α transcript content was moderately elevated, GAS6 transcript content was markedly elevated, and AS3 transcript content was unaffected by FGF-2 withdrawal-mediated FGF-2-deficient transfectant death (Fig. 7). Before performing additional detailed studies, we employed restriction analyses to validate the identity of the GADD45α, GAS6, and AS3 amplification products. The predicted restriction fragment yield for the AS3, GADD45α, and GAS6 amplicons are detailed in Table 3 and the results of restriction analyses are shown in Fig. 8. These studies confirmed that the ds-cDNA PCR amplification products generated using AS3, GADD45α, and GAS6 primers are the products of the anticipated amplicons. The absence of detectable PCR product for GAS6 transcripts in RNA prepared from FGF-2-deficient transfectants cultured in the presence of FGF-2 (Fig. 7) cannot be attributed to artifact or absence of primers. Firstly, RNA preparations from FGF-2-deficient cells cultured in either the presence or absence of primers. Secondly, RNA prepared from FGF-2-deficient transfectants cultured in the presence of FGF-2 (Fig. 7) cannot be attributed to artifact or absence of primers. Secondly, RNA preparations from FGF-2-deficient cells cultured in either the presence or absence of primers. Thirdly, RNA preparations from FGF-2-deficient cells cultured in either the presence or absence of primers. Fourthly, RNA preparations from FGF-2-deficient cells cultured in either the presence or absence of primers. In conclusion, these preliminary studies suggest that the 18S primer:18S competimer protocol (detailed by Ambion, Austin, TX) as more appropriate for these initial studies. We first determined that the linear range for PCR amplification of AS3 and GADD45α transcripts comprised cycles 20 to 26 and 28 to 34, respectively. To minimize the effect of 18S transcript amplification on simultaneous amplification of either AS3 or GADD45α transcripts, we did preliminary studies to determine the appropriate 18S primer:18S competimer ratio that yielded comparable simultaneous amplification of 18S and AS3 transcripts or 18S and GADD45α transcripts. The optimal 18S primer:18S competimer ratios for simultaneous PCR amplification of 18S and AS3 transcripts or 18S and GADD45α transcripts were 1.5:9 and 1:14, respectively. Employing these experimental conditions, we found that FGF-2 withdrawal-mediated FGF-2-deficient transfectant death was associated with a >70% mean increase in GADD45α transcript content (Fig. 9). Comparable analyses showed that, within experimental error, FGF-2 withdrawal-mediated FGF-2-deficient transfectant death did not alter transfectant AS3 transcript content (Fig. 9). These results show that observed increases in GADD45α (Fig. 9) and GAS6 (Fig. 7) transcript content following FGF-2 withdrawal-mediated transfectant death is not the consequence of a generalized phenomenon.

**Discussion**

The current studies provide novel data demonstrating differential requirements for endogenous FGF-1 or FGF-2 for maintenance of prostate cancer cell viability. Both kinetic data (Figs. 2 and 3) and cell counting data (Fig. 4) show that, in the absence of exogenous FGFs, endogenous FGF-2 is uniquely essential for prostate cancer cell survival. In contrast, endogenous FGF-1 is not required for cell survival in the absence of exogenous FGFs. These findings suggest that prostate cancer cells have differential requirements for endogenous FGF-1 and FGF-2 for maintenance of cell viability, and that FGF-2 is essential for cell survival in the absence of exogenous FGFs. Additionally, these findings suggest that FGF-2 withdrawal-mediated transfectant death is not the consequence of a generalized phenomenon.

### Table 2. Predicted Restriction Fragment Yield for FGFR1, FGFR2, and FGFR3 Amplicons

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</table>

**Relative Quantitative RT-PCR Analysis of the Effect of FGF-2-Deficient Transfectant Culture in the Absence of FGF-2 (FGF Withdrawal) on Transfectant AS3 and GADD45α Transcript Content**

The 18S rRNA content of rat prostate cancer cells greatly exceeds that of any of the GADD or GAS family genes or the AS3 gene (data not shown). This significantly complicates the design of Taqman protocols (40, 41) for simultaneous, real-time PCR analyses of control (18S) and target (AS3 and GADD45α) transcripts. Our RT-PCR analyses (Fig. 7) failed to show evidence for “nonspecific” amplification of products other than the expected AS3 or GADD45α amplicons. However, the relative insensitivity of ethidium bromide staining would not fully eliminate the possibility of “nonspecific” amplification of minor products other than the desired AS3 or GADD45α amplicons. Such nonspecific amplification of ds-DNAs would confound data interpretation when employing real-time RT-PCR using SYBR Green detection (42). We therefore considered the 18S primer:18S competimer protocol (detailed by Ambion, Austin, TX) as more appropriate for these initial studies. We first determined that the linear range for PCR amplification of AS3 and GADD45α transcripts comprised cycles 20 to 26 and 28 to 34, respectively. To minimize the effect of 18S transcript amplification on simultaneous amplification of either AS3 or GADD45α transcripts, we did preliminary studies to determine the appropriate 18S primer:18S competimer ratio that yielded comparable simultaneous amplification of 18S and AS3 transcripts or 18S and GADD45α transcripts. The optimal 18S primer:18S competimer ratios for simultaneous PCR amplification of 18S and AS3 transcripts or 18S and GADD45α transcripts were 1.5:9 and 1:14, respectively. Employing these experimental conditions, we found that FGF-2 withdrawal-mediated FGF-2-deficient transfectant death was associated with a >70% mean increase in GADD45α transcript content (Fig. 9). Comparable analyses showed that, within experimental error, FGF-2 withdrawal-mediated FGF-2-deficient transfectant death did not alter transfectant AS3 transcript content (Fig. 9). These results show that observed increases in GADD45α (Fig. 9) and GAS6 (Fig. 7) transcript content following FGF-2 withdrawal-mediated transfectant death is not the consequence of a generalized phenomenon.

**FIGURE 5.** Restriction analysis of FGFR1, FGFR2, and FGFR3 PCR amplification products. Restrictions were done using the shown enzymes. Products were separated by electrophoresis on a 2.5% agarose gel containing ethidium bromide. The gel was destained and then photographed.
required for maintenance of prostate cancer cell viability. This requirement is sufficiently dominant that loss of cell viability associated with loss of endogenous FGF-2 isoforms cannot be compensated either by retained full endogenous FGF-1 production or by retained minor production of the 19.5-kDa FGF-2 isoform (Figs. 1–4). Significantly, marked reduction of endogenous FGF-1 in the absence of a change in endogenous FGF-2 (Fig. 1) neither caused death of nor diminished proliferation of FGF-1-deficient transfectants (Figs. 1–4). These results show differential effects of endogenous FGF-2 and FGF-1 as modulators of prostate cancer cell function and identify FGF-2 as the predominant intracellular FGF-regulating prostate cancer cell viability. There is precedence for differential function of endogenous FGF-1 and FGF-2 during their coexpression in target cells. Others have shown that <10% of intracellular FGF-1 is localized in the nucleus (43). In contrast, SK-Hep-1 hepatoma cells produce both low molecular weight (18 kDa) and high molecular weight (22, 22.5, and 24 kDa) FGF-2 isoforms. Notably, >90% of the high molecular weight FGF-2 isoforms are localized in the nucleus, whereas essentially 100% of low molecular weight FGF-2 resides in the cell cytosol (44, 45). Similarly, high molecular weight FGF-2 isoforms promote survival and proliferation of NIH 3T3 cells during culture on 1% serum, whereas low molecular weight FGF-2 does not promote proliferation of identically cultured NIH 3T3 cells (45).

Genetic modification resulting in the loss of endogenous FGFs enhances the effectiveness of exogenous FGFs as mediators of prostate cancer cell survival and/or proliferation (Fig. 3). Neither FGF-1, FGF-2, FGF-7, nor FGF-8 enhances proliferation of vector control transfectants. In contrast, FGF-1, FGF-2, and FGF-8 enhance proliferation of FGF-1-deficient transfectants. In contrast, FGF-1, FGF-2, and FGF-8 enhance proliferation of FGF-1-deficient transfectants, whereas FGF-7 does not (Fig. 3). Notably, all four FGFs readily promote survival and proliferation of FGF-2-deficient transfectants (Fig. 3). The differential effectiveness of exogenous FGF-7 for FGF-1-deficient transfectants as compared with FGF-2-deficient transfectants suggests that, in the presence endogenous FGF-2, potential exogenous FGF-7 effects may be blunted. Our results are consistent with a significant body of data demonstrating that FGF-1 or FGF-2 null mice and FGF-1/FGF-2 double-null mice reproduce normally and lack significant

### Table 3. Predicted Restriction Fragment Yield for AS3, GADD45α, and GAS6 Amplicons

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Restriction Endonuclease</th>
<th>Restriction Fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS3</td>
<td>None</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>DdeI</td>
<td>87, 115</td>
</tr>
<tr>
<td></td>
<td>MwoI</td>
<td>28, 174</td>
</tr>
<tr>
<td>GADD45α</td>
<td>None</td>
<td>291</td>
</tr>
<tr>
<td></td>
<td>AvrII</td>
<td>129, 147</td>
</tr>
<tr>
<td></td>
<td>BstEII</td>
<td>111, 165</td>
</tr>
<tr>
<td>GAS6</td>
<td>None</td>
<td>596</td>
</tr>
<tr>
<td></td>
<td>PstI</td>
<td>150, 445</td>
</tr>
<tr>
<td></td>
<td>BseI</td>
<td>241, 354</td>
</tr>
</tbody>
</table>

FIGURE 6. Restriction analysis for assessment of FGF2[IIIb] and FGF2[IIIc] content of parental prostate cancer cells and stable transfectants. Restrictions were done using the enzymes. Products were separated by electrophoresis on a 2.5% agarose gel containing ethidium bromide. The gel was destained and then photographed.

FIGURE 7. Representative analyses of the effect of FGF-2-deficient transfectant culture in either the presence or the absence of FGF-2 (FGF withdrawal) on apparent AS3, GADD45α, or GAS6 transcript content. Total RNA was prepared from parallel cultures of FGF-2-deficient transfectants propagated in either the presence or the absence of FGF-2. Equal quantities of each RNA preparation were reverse transcribed and equal amounts of the reverse transcribed products were PCR amplified in parallel using the primer pairs validated as shown in Fig. 8. Aliquots of the PCR reactions were withdrawn at cycles 25 (a), 30 (b), 35 (c), and 40 (d) and analyzed by electrophoresis on 2.5% agarose gels containing ethidium bromide. Representative of two replicate independent analyses of FGF-2-deficient transfectant clones 2 and 4 shown in Fig. 1.

FIGURE 8. Restriction analysis of AS3, GADD45α, and GAS6 PCR amplification products. Restrictions were done using the shown enzymes at the indicated incubation temperature. Products were separated by electrophoresis on a 2.5% agarose gel containing ethidium bromide. The gel was destained and then photographed.
their respective FGFRs (34) and may suggest some form of relative mitogenic activity of the individual FGFs on binding to FGF-8 (Fig. 3). This result is not consistent with the predicted cells is comparable with that of either exogenous FGF-1 or we found that exogenous FGF-7 function in prostate cancer cell survival and proliferation (Figs. 3 and 4). However, and their presently demonstrated ability to affect prostate cancer cell survival and/or proliferation.

FGFR3 (Fig. 5; Table 2). These findings are consistent with the known FGFR specificity of FGF-1, FGF-2, and FGF-8 (34) and their presently demonstrated ability to affect prostate cancer cell survival and proliferation (Figs. 3 and 4). However, we found that exogenous FGF-7 function in prostate cancer cells is comparable with that of either exogenous FGF-1 or FGF-8 (Fig. 3). This result is not consistent with the predicted relative mitogenic activity of the individual FGFs on binding to their respective FGFRs (34) and may suggest some form of cooperation among prostate cancer cell FGFRs. We also show that the FGFR2(IIIc) isoform is the predominant FGFR2 of both parental cells and prostate cancer cell transfectants (Fig. 6). As FGFR2(IIIc) isoform predominance is characteristic of advanced human prostate cancer (36, 37), this feature is replicated in these rat prostate cancer cells.

FGF-2-deficient transfectant GADD45α and GAS6 transcript content are increased during FGF withdrawal-mediated transfectant death (Figs. 7 and 9). In contrast, AS3 transcript content of these transfectants was not altered during FGF withdrawal-mediated transfectant death (Fig. 9). These results imply that increases in GADD45α (Fig. 9) and GAS6 transcript content (Fig. 7) occurring during FGF-2 withdrawal-mediated FGF-2-deficient transfectant death are a specific consequence of FGF withdrawal. It is well established that GADD and GAS proteins are potent regulators of cell cycle progression and cell survival (16-18) and that their function is subject to regulation by cytokines (17, 19) and growth factors (20). Thus, our data suggest that GADD45α and GAS6 might be involved in processes that regulate prostate cancer cell survival. This likelihood is the subject of ongoing studies.

FIGURE 9. The effect of FGF-2-deficient transfectant culture in either the presence or the absence of FGF-2 (FGF withdrawal) on GADD45α and AS3 transcript content. FGF-2-deficient transfectants were cultured in the presence of FGF-2 and then harvested in medium lacking FGF-2. The cell preparation was divided, parallel preparations were plated, and cells were cultured for 72 hours in either the presence or the absence of FGF-2 (10 ng/mL). Culture of FGF-2-deficient transfectants in the absence of FGF-2 resulted in a 65% decrease in viable cell content. Total RNA was prepared, reverse transcribed, and amplified employing the relative quantitative PCR conditions detailed in the text. Band intensities for each PCR amplification of a given quantity of RT product (shown as equivalents of RNA PCR amplified) were integrated in quadruplicate and the ratio (–FGF-2)/(+FGF-2) was calculated. Mean ± SD for the 12 individual integrations (four at each of the RNA equivalents). Results are for FGF-2-deficient transfectant clone 2. Representative of those for FGF-2-deficient transfectant clone 4. FGF-2 isoform content of these transfectants is detailed in Fig. 1.

Materials and Methods

Cells and Cell Culture

Detailed characterization of the clonally derived vector control transfectants and the FGF-1-deficient transfectants or FGF-2-deficient transfectants has been described previously (50). Vector controls were generated by CaCl₂ transfection of the pMTneo.1 vector (51) and G418 selection. The FGF-1 antisense RNA vector was prepared by recovering FGF-1 cDNA from vector pFGF-1.8 (52) and directional cloning in antisense orientation in the HindIII/XbaI site of pMTneo.1. The FGF-2 antisense RNA vector was prepared by recovering FGF-2 cDNA from vector pTB784 (52) and directional cloning in antisense orientation in the HindIII/XbaI site of pMTneo.1. Antisense transfectants were generated by CaCl₂ transfection and G418 selection. Clonal selection of stable transfectants was achieved by standard limit dilution in 96-well plates. Culture and subculture of parental cells and transfectants from low-passage stocks on modified MEM containing 0.6 mg/mL Geneticin (G418) and either exogenous FGF-2 (10 ng/mL, FGF-2-deficient transfectants) or exogenous FGF-1 (100 ng/mL, FGF-1-deficient transfectants) were accomplished as detailed previously (50). NIH 3T3 cells (American Type Culture Collection, Manassas, VA) were propagated in the absence of antibiotics on DMEM containing 10% FBS.

Analysis of Ventral Prostate and Transfectant FGF-2 Isoform and FGF-1 Content

Cell culture for extract preparation and cell extract preparation were accomplished as detailed previously (50). Ventral prostate extracts were frozen preparations that had been prepared previously (50). Extract adsorption to heparin-agarose, recovery of adsorbed proteins, and preparation of membranes for antibody probing and membrane development were accomplished as detailed previously (50).
Proliferation Assays

**MTT Analyses.** At 48 to 72 hours prior to initiating the proliferation assay, transfecants maintained on medium containing 10% FBS, 10^{-7} mol/L testosterone, and either 100 ng/mL FGF-1 (FGF-1-deficient transfecants) or 10 ng/mL FGF-2 (FGF-2-deficient transfecants) were passaged to medium lacking testosterone and containing 10% CS-FBS and FGF as detailed in the figure legends. The selection of 10 ng/mL FGF-2 for analyses of FGF-2-deficient transfecant proliferation was based on the results of prior titration studies that showed maximum thymidine incorporation (53, 54) or proliferation (50) was achieved when culture medium contained 1 to 10 ng/mL FGF-2. Comparable analyses showed that maximum thymidine incorporation (53, 54) or proliferation (50) of FGF-1-deficient transfecants was achieved when culture medium contained 50 to 100 ng/mL FGF-1. Proliferation assays were initiated by plating harvested cells (5 \times 10^3 cells/well) on 96-well plates on MEM containing 5% CS-FBS and the requisite FGF. Viable cell content was assessed by quantifying formazan production from tetrazolium salt essentially using methods detailed by Promega (Madison, WI) in the Cell Titer 96 proliferation kit. Plates were read using a microplate reader (Dynatech Laboratories, Inc., Chantilly, VA) programmed to quantify absorbance at 570 nm and background at 630 nm. All assays were done under conditions for which formazan production was directly proportional to the number of viable cells per well (50).

**Cell Counting Analyses.** Cells were prepared for analysis and propagated in 96-well plates as detailed for the MTT analyses. On culture day 5, individual wells were harvested by brief trypsinization and the contents of three wells were combined. Cells were collected by brief centrifugation at \approx 3,000 \times g. After resuspension in PBS, an equal volume of 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO) was added and total and viable cell content was determined by counting in a hemocytometer.

**Reverse Transcription-PCR**

Total RNA was isolated using reagents of the RNAqueous-4PCR kit provided by Ambion. Reverse transcription was done using the SuperScript First-Strand System for RT-PCR (Invitrogen, Carlsbad, CA). PCR amplification of ss-cDNA was done using Platinum PCR SuperMix (Invitrogen) and resultant ds-cDNAs were characterized by electrophoresis on ethidium bromide–containing agarose gels as detailed previously (55). Relative quantitative RT-PCR was done using reagents provided by Ambion and protocols based on those recommended by Ambion. All other PCR primers (Table 1) were prepared by Invitrogen.

**Other Materials**

Reagents for cell culture were obtained and used as described previously (50). Recombinant rat FGF-1 and FGF-2 and CS-FBS were prepared in this laboratory as described previously (50). Aprotinin, NP40, phenylmethylsulfonyl fluoride, leupeptin, hemisulfate, pepstatin A, and heparin-agarose were obtained from Sigma-Aldrich. Primary and secondary antibodies and reagents for enhanced chemiluminescence analysis of membranes were obtained as detailed previously (50). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). All other reagents were reagent grade.

**Other Methods**

Band intensities of Western blots and RT-PCR products were quantified by integration using a PC version of the NIH Image program (Scion Corp., Frederick, MD). Integrations were done in quadruplicate and the mean values were used to calculate relative content. ANOVA and the paired-samples t test were done using programs of the Statistical Package for the Social Sciences (version 11). Doubling times were calculated by solution of the equations derived by unweighed linear regression analysis of semilogarithmic plots of the formazan data.

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**References**

Exogenous Fibroblast Growth Factors Maintain Viability, Promote Proliferation, and Suppress GADD45 α and GAS6 Transcript Content of Prostate Cancer Cells Genetically Modified to Lack Endogenous FGF-2

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