Fibroblast Growth Factor 2 Promotes Tumor Progression in an Autochthonous Mouse Model of Prostate Cancer

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ABSTRACT

Fibroblast growth factor (FGF) 2 (or basic FGF) is expressed at increased levels in human prostate cancer. FGF2 can promote cell motility and proliferation, increase tumor angiogenesis, and inhibit apoptosis, all of which play an important role in tumor progression. To determine whether FGF2 plays a critical role in prostate cancer progression, we have used the transgenic adenocarcinoma of the mouse prostate (TRAMP) model system. A high percentage of TRAMP mice develop metastatic prostate cancer, and thus the TRAMP model is useful for evaluating cancer progression. TRAMP mice were crossed with FGF2 knockout (FGF2−/−) mice, and tumor progression in TRAMP mice that were either hemi- or homozygous for inactivation of the FGF2 allele was compared with progression in wild-type TRAMP mice. Inactivation of even one FGF2 allele resulted in increased survival, a decrease in metastasis, and inhibition of progression to the poorly differentiated phenotype in primary prostatic tumors. When compared with wild-type mice, poorly differentiated tumors arising in FGF2+/− and FGF2−/− mice expressed higher levels of vascular endothelial growth factor and, in some cases, increased levels of acidic FGF intracellular binding protein, a nuclear FGF1-binding protein. These findings suggest that both FGF2-mediated angiogenesis and intranuclear FGF2 activities may promote tumor progression and support the hypothesis that FGF2 plays a significant role in prostate cancer progression in vivo.

INTRODUCTION

Prostate cancer is the second most frequent cause of cancer deaths in men in the United States. The biology of human prostate cancer is complex, and although many genetic and epigenetic alterations have been detected in human prostate cancer, the role of many of these changes in prostate cancer initiation and progression remains unclear. FGF2, also known as basic FGF, is a member of the FGF family, a group of more than 20 structurally related proteins that control a multitude of cellular processes in different contexts, including proliferation, differentiation, survival, and motility (1). FGF2 also plays a critical role in cancer development due to its role in angiogenesis (2).

FGF2 is expressed in human prostate cancer, as well as in many other malignant neoplasms including melanomas (3), astrocytomas (4), and carcinomas of the breast (5), pancreas (6), lung (7), bladder (8), and head and neck (9). By ELISA, we have shown that there are very substantial quantities of FGF2 in human prostate cancer tissue, and it is present at significantly higher concentrations in cancer tissue compared with normal prostate (10). Immunohistochemical analysis revealed that increased FGF2 was present in stromal cells within the prostate cancer, consistent with a paracrine effect of FGF2 in localized prostate cancer. In contrast, two European groups (11, 12) have examined expression of FGF2 in prostate cancer by immunohistochemistry and detected expression of FGF2 in prostate cancer epithelial cells in the majority of these cases. However, most of the prostate cancers in these studies were locally advanced or metastatic and/or poorly differentiated and thus are much more aggressive than the cancers from radical prostatectomy specimens studied by our group. High levels of expression of FGF2 are present in two of the commonly used prostate cancer cell lines (PC-3 and DU-145), and both of these cell lines are derived from metastatic prostate cancers (2, 13). These observations are consistent with the idea that in advanced and/or poorly differentiated prostate cancers, FGF2 is expressed by the cancer cells and may potentially stimulate growth in an autocrine manner. Thus FGF2 is expressed at high levels in human prostate cancer and can function as either as an autocrine or a paracrine growth factor.

FGFs bind to a family of four distinct transmembrane tyrosine kinase receptors (FGFRs 1–4), and there is strong evidence that these receptors play a role in prostate cancer progression. FGFR-1 and FGFR-4 are potent receptors for FGF2 (14), and these receptors are both expressed by prostate cancer cells (10, 15). Feng et al. (16) have shown that expression of FGFR-1 accelerates tumorigenesis in the Dunning rat prostate cancer model. In agreement with these findings in animal models, there is increased expression of FGFR-1 in poorly differentiated human prostate cancers (10, 17). Thus increased expression of at least one FGF2 receptor is associated with prostate cancer progression.

Whereas it is clear that FGF2 is present at increased levels in prostate cancer and that appropriate FGF2 receptors are expressed by the cancer cells, it has not been established that FGF2 plays an essential role in the progression of cancers arising within the prostate in vivo. To determine whether this is the case, we used the TRAMP model of prostate cancer, an autochthonous transgenic model of prostate cancer that has been used by many groups. TRAMP mice were originally generated by microinjection of a construct harboring a probasin regulatory element to direct expression of the SV40 early genes to prostatic epithelium. The earliest pathology is prostatic intraepithelial neoplasia, and the mice can display well-differentiated adenocarcinoma as early as 12 weeks of age. Ultimately, mice develop poorly differentiated carcinoma by 24–30 weeks of age, and metastatic disease is observed in a high percentage of TRAMP mice by 28 weeks of age (18). It has been demonstrated previously that FGF2 is expressed in prostate cancers arising in TRAMP mice and that there is increased expression of FGFR-1 in poorly differentiated prostate carcinomas in these mice (19). To determine whether FGF2 plays a critical role in cancer progression in this model system, we have crossed TRAMP mice with FGF2 KO (FGF2−/−) mice. FGF2−/− mice are healthy and have subtle phenotypes, such as a decreased wound healing and focal changes in the number of neurons when compared with normal prostate (10).
within the central nervous system (20). Tumor progression in TRAMP mice that were either HT or homozygous for the presence of the null FG2 allele was compared with progression in WT TRAMP mice. We have found that inactivation of even one FG2 allele leads to increased survival, a significant decrease in metastasis, and inhibition of progression to the poorly differentiated phenotype in primary prostatic tumors in TRAMP mice. These findings support the hypothesis that FG2 plays a significant role in prostate cancer progression in vivo.

MATERIALS AND METHODS

Breedings of Mice and Necropsy. FG22/− KO mice (SV129 background) and TRAMP transgenic mice (C57Bl/6 background) have been described previously (18). FG22/−/2/− TRAMP mice were mated, and progeny were screened for the presence of the SV40 large T antigen by PCR as described at the TRAMP model website.4 TRAMP FG22/−/− mice were then mated with nontransgenic FG22/− mice to generate FG22/−−/− littermates from this cross. The strain background of this cross is 50% C57Bl/6 and 50% SV129. The typical background for experiments in TRAMP mice is 50% C57Bl/6 and 50% FVB, and tumor progression in this background is more rapid than that in the C57Bl/6 × SV129 background, based on the results of prior studies (21), when compared with the reported rate of progression in 50% C57Bl/6/50% FVB TRAMP mice. Transgenic male mice bearing the SV40 T antigen from this second cross were followed until they met criteria for euthanasia, including large palpable tumor, huddled posture, immobility, or an obviously moribund appearance. A full necropsy was performed on all animals including microscopic examination of all thoracic, abdominal, and pelvic organs. The primary tumor was excised and weighed. Portions were fixed in formalin for histopathological analysis, and the remainder was snap-frozen in liquid nitrogen. Frozen tumors from euthanized animals were used for RNA and protein extraction. The FG2 genotype was not determined until after euthanasia and necropsy, including microscopic examination, to preclude bias in selection for euthanasia or necropsy interpretation. Tumors were graded as well, moderately, or poorly differentiated as described at the TRAMP model website.4 If some residual well- or moderately differentiated tumor was present in cases with areas of poorly differentiated carcinoma, these were considered to be poorly differentiated carcinomas for the purpose of classification.

Southern Blot Analysis. DNA was extracted as described previously (20). Ten μg of total tissue DNA were digested with 50 units of EcoRI restriction endonuclease (Life Technologies, Inc., Rockville, MD) in a 100-μl reaction volume containing 50 μl of reaction buffer at 37°C for 16 h. The digested DNA was fractionated on a 0.7% agarose gel using electrophoresis and transferred to a positively charged nylon membrane (Roche, Indianapolis, IN). Southern hybridization was performed at 68°C in 10 ml of PerfectHyb Plus hybridization solution (Sigma, St. Louis, MO). The nylon membrane was prehybridized in the above buffer for 15 min. Hybridization was done for 3 h by adding 50 ng of a FG2 genomic fragment (20) that was radioactively labeled with [32P]dCTP (3000 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) using RadPrime Labeling Kit (Life Technologies, Inc.) and included at a concentration of 1 × 106 cpm/μg. Blots were washed according to the manufacturer’s protocol, and signals were visualized by autoradiography.

Preparation of Tissue Protein Extracts. Prostatic tissue samples were pulverized in liquid nitrogen and then homogenized by three strokes, each for 10 s, on ice, in a lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 250 mM NaCl, 0.1% NP40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride using 0.5 ml lysis buffer/200 mg tissue. The homogenate was then incubated for 30 min on ice, and insoluble material was removed by centrifugation for 1 min in a microcentrifuge at 4°C. The protein content of the supernatant was determined as described previously (10).

Heparin Affinity Purification and Western Blot Analysis. For heparin affinity purification of FGFI or FG2 proteins, 250 μg of protein extract were incubated with 50 μl of heparin-agarose overnight at 4°C with agitation. The beads were then washed in buffer containing 10 mM HEPES (pH 7.4), 25 mM NaCl, and 1 mM DTT. The washed beads were then boiled in sample buffer and centrifuged, and supernatant was subjected to SDS-PAGE using a 15% gel. The resolved proteins were electrotransferred to nitrocellulose membranes and then blocked with PBST and 5% fat-free milk. The membrane was then incubated with either 300 μl goat polyclonal anti-FGFI polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 700 ng/rabbit polyclonal anti-FG2 antibody (Santa Cruz Biotechnology) at 4°C. After overnight incubation, membranes were washed with PBST and treated with an appropriate secondary antibody conjugated to horseradish peroxidase at a concentration of 80 ng/ml (Santa Cruz Biotechnology). The antigen-antibody reaction was visualized using an enhanced chemiluminescence assay (Amersham, Arlington Heights, IL) and exposure to enhanced chemiluminesence film (Amersham).

VEGF ELISA. Each well of a 96-well plate was coated with 100 μl of a solution of polyclonal goat anti-VEGF antibody (AF-493-NA; R&D Systems, Minneapolis, MN) at a concentration of 0.5 μg/ml overnight at room temperature in a sealed bag. The next day, wells were washed three times with PBST and incubated for 1 h at room temperature with 300 μl of a blocking solution consisting of PBS containing 1% BSA, 5% sucrose, and 0.05% NaN3. The plate was washed as described above, and standards and samples were added (100 μl/well). Samples were 50 μg of tissue protein diluted to a final volume of 100 μl. Wells were then incubated for 2 h at room temperature. After washing as described above, biotinylated polyclonal goat anti-VEGF antibody (BAF 493; R&D Systems) was added at a concentration of 400 ng/ml for 2 h at room temperature. After washing as described above, detection was carried out by addition of 100 μl/well of a 1:4000 dilution of streptavidin/horseradish peroxidase (Zymed, San Francisco, CA) and incubation for 20 min at room temperature. Wells were washed and incubated with substrate consisting of a 1:1 solution of H2O2 and tetramethylbenzidine (Sigma) at a concentration of 0.1 ng/ml. Stop solution (H2SO4) was added within 30 min, and absorbance at 450 nm was determined using an ELISA plate reader. The sensitivity of this ELISA was found to be <15 pg/ml.

Primer Design and Synthesis. Oligonucleotide primers for FIBP were designed using Molecular Beacon program (PREMIER Biosoft International, Palo Alto, CA). Primers were 5′-AACATCCAGCAGCCTTCC-3′ (sense) and 5′-TCCCTTGTCGACGGCAAGA-3′ (antisense). The nucleotide position for the amplification product as given by the GenBank accession number (AK000893) is 550–742. Oligonucleotide primers for β-actin were designed using Baylor College of Medicine Primer Selection program.5 Primers were 5′-AGACCGGATCTGCAAACTC-3′ (sense) and 5′-TGTTCCGGTGTGTTAAGGTCT-3′ (antisense). The nucleotide position for the amplification product as given by the GenBank accession number (X00351) is 256–435. Primers were carefully designed to cross exon/intron regions and avoid the formation of primer-dimer, hair pin, and self complementarity. Synthetic oligonucleotide primers were obtained from Sigma Genosys (The Woodlands, TX).

cDNA Synthesis and Quantitative Real-Time PCR. Total RNA was extracted from tissues using Trizol reagent according to manufacturer’s protocol (Invitrogen, Carlsbad, CA) and used in first-strand cDNA synthesis. Total RNA (1 μg) was used to synthesize cDNA using a Script cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol. Quantitative PCR was carried out by adding 5 μl of template cDNA to a final 25-μl reaction volume containing 3 mM MgCl2, 0.4 μM each forward and reverse primers, and 2.5 μl of LC-FastStart DNA Master SYBR GREEN 1 (Roche). Real-time PCR was done using the iCycler instrument (Bio-Rad) with optimized PCR reaction conditions. The amplification of FIBP was carried out as follows: a 3-min hot start at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 20 s, and a 72°C extension for 30 s. The amplification protocol for β-actin was carried out as follows: a 3-min hot start at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 20 s, and a 72°C extension for 30 s. Each assay included a negative control, and the experiment was done in duplicate. The fluorescence emitted by the reporter (SYBR GREEN) dye was detected online in real time, and the threshold cycle (Ct) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The Ct value is the fractional cycle number at which the fluorescence generated by the reporter dye exceeds a fixed level above baseline. The FIBP signal was normalized against the relative quantity of β-actin and expressed as ΔCt = (Ctcontrol − Ct sample). The change in FIBP signal relative to the reference signal (control sample) was expressed as ∆ΔCt = (ΔCtcontrol − ΔCtsample). Relative changes in expression were then calculated as 2−ΔΔCt.

http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html.

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Labeling and Hybridization of cDNA for Microarray Analysis. Microarray analysis was performed using 30 µg of total RNA. The cDNA reverse transcription and fluorescent labeling reactions were carried out using Cy3-labeled nucleotides for control and Cy5-labeled nucleotides for experimental samples. Briefly, cDNA synthesis was initiated with incubating RNA with non-labeled 1.5µg oligo dT primer (Gibco BRL) in a 39 µl total volume at 70°C for 10 min and chilled on ice. Then, Cy3- and Cy5-labeled cDTPs (Amersham Life Science, Arlington Heights, IL) were added to the appropriate reactions. To each reaction, we added 5× first-strand buffer (12 µl; 250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM MgCl2), unlabeled nucleotide mix (3 µl; 1 mM dATP, dGTP, and dTTP and 0.1 mM dCTP), 0.1 M DTT (6 µl), 3 µl of Superscript II reverse transcriptase (200 U/µl), and RNase Inhibitor (6 µl; 20 U/µl). After incubation at 42°C for 2 h, cDNA was denatured and neutralized by adding 3 µl of 5 M NaOH and incubating at 37°C for 10 min followed by addition of 15 µl of Tris-HCl (pH 7.5) and 3 µl of 5 M HCl. The mixture of Cy3 and Cy5 reactions was used as probe and purified using Qiagen PCR purification kit (Qiagen, Valencia, CA). The probe was then mixed with an equal amount of UltraHyb hybridization buffer (Ambion, Austin, TX), denatured by a 2-min incubation in boiling water, and hybridized in a Genomic Systems Hybridization station for 4 h at 42°C against a microarray chip carrying 9000 cDNAs obtained from Baylor Microarray Core Facility. After hybridization and posthybridization washes, the slide was scanned immediately in Axon 4000A dual channel scanner (Axon Instruments, Foster City, CA), and the data were analyzed using the Gene Pix version 3.0 software package (Axon Instruments). Genes were considered up- or down-regulated if the expression was enhanced or suppressed expression values over the range of expression. Unsupervised cluster analysis was run on genes identified as the best class predictors using the CLUSFAVOR algorithm based on Euclidean distances and raw expression values (23).

Differences in the proportion of animals with poorly differentiated and metastatic tumors between WT and KO mice were evaluated by Fisher’s exact test. The significance of differences in survival and tumor weight between these groups was determined by t test (two sided).

RESULTS

Progression of Prostate Cancer in TRAMP Mice with Hemi- or Homozygous Inactivation of the FGF2 Allele. To determine whether FGF2 plays an important role in prostate cancer progression in a mouse model of prostate cancer, FGF2−/− and TRAMP mice were mated, and progeny were screened for the presence of the SV40 large T antigen by PCR. TRAMP FGF−/− mice were then mated with nontransgenic male FGF−/− littermates from this initial cross. Transgenic male mice bearing the SV40 T antigen from this second cross were followed until they met criteria for euthanasia, including large palpable tumor, huddled posture, immobility, or an obviously moribund appearance. A full necropsy, including microscopic examination, was performed. One mouse had disseminated lymphoma at necropsy and was excluded from further analysis. A total of 44 TRAMP animals were included in the final analysis, including 10 FGF2−/− WT, 24 FGF2−/− HT, and 10 FGF−/− KO mice. Animals were sacrificed between 25 and 49 weeks of age. WT mice survived an average of 30.7±5.4 (SD) weeks. This survival is very similar to that seen previously in other studies of WT TRAMP mice in this genetic background carried out in this laboratory (21). Both the HT and KO TRAMP mice survived longer than the WT mice, with a mean survival of 36.0±5.4 and 35.1±7.6 weeks, respectively (Fig. 1A). The difference in survival between the WT and HT and KO mice taken together was statistically significant (P<0.03, t test), as was the difference between WT and HT mice (P<0.02). The difference between the WT and KO mice approached statistical significance (P<0.16, t test), and it should be noted that these two groups of animals both had smaller numbers of mice than the HT group, which impacts on the statistical power to detect differences between groups. There was no statistically significant difference between the HT and KO mice (P>0.69, t test). The reason for the difference in survival became apparent upon pathological examination of the necropsy tissues of these mice. The histopathology of the prostate carcinomas in this study was similar to that of TRAMP mice in the C57Bl/6 x FVB background (24), and we have classified the tumors in our study using the criteria described previously for TRAMP tumors in that background. In the WT mice, 70% of the primary tumors were poorly differentiated. In contrast, both the HT and KO mice had a much lower proportion of poorly differentiated primary tumors (Fig. 1B). When WT mice were compared with HT and KO mice taken together, the difference between the proportions of mice with poorly differentiated primary tumors between these two groups was statistically significant (P<0.01, Fisher’s exact test). The average weight of the primary tumor in HT/KO mice was 3.9±3.2 g (mean ± SD versus 7.0±5.3 g in WT mice, and this difference is statistically significant (P<0.05, t test). The fact that the tumors from WT animals were larger than those in HT/KO mice, despite the fact that WT mice were euthanized on average 5 weeks before the HT/KO animals, is consistent with the very high proliferation rate in poorly differentiated tumors and the higher proportion of WT TRAMP mice with such tumors. Prior experience with the TRAMP model has shown that the vast majority of mice with distant metastasis have poorly differentiated primary tumors (24). Concordant with this observation, both HT and KO mice had a significantly lower incidence of metastatic disease than WT mice (Fig. 1C). This difference was again statistically significant (P<0.03, Fisher’s exact test). In summary, inactivation of one or both FGF2 allele(s) is associated with increased survival, a decreased percentage of poorly differentiated primary tumors, and decreased metastasis in TRAMP mice.

FGF2 Is Expressed by Prostate Cancers in WT TRAMP Mice. Huss et al. (19) have demonstrated previously that FGF2 is expressed in prostate cancers in TRAMP mice. Given that FGF2 appears to play an important role in progression to the poorly differentiated phenotype, we sought to confirm this observation, particularly in the poorly differentiated tumors. We analyzed a total of seven cancers from WT animals including five poorly differentiated tumors, one moderately differentiated tumor, and one well-differentiated tumor using partial...
FGF2 promotes tumor progression in prostate cancer.

VEGF, but not FGF1, is up-regulated in poorly differentiated tumors from HT and KO TRAMP mice. Because FGF2 appears to play an important role in progression to the poorly differentiated phenotype, we sought to determine how poorly differentiated tumors can arise in HT or KO mice to gain insight into the specific biological activities of FGF2 that are important in tumor progression. It has been demonstrated previously by RT-PCR that FGF1 is expressed in TRAMP tumors, but the level of FGF1 protein expression was not determined (25). Given that FGF1 and FGF2 share a number of biological properties, including lack of a traditional signal sequence, we sought to determine whether increased expression of FGF1 compensates for the loss of FGF2 in poorly differentiated TRAMP tumors arising in HT or KO TRAMP mice. We therefore analyzed a total of 12 poorly differentiated prostate cancers from animals of all three genotypes for expression of FGF1 by heparin-agarose affinity partial purification followed by Western blotting with anti-FGF1 antibodies. Approximately 40% of TRAMP tumors expressed detectable FGF1 (Fig. 2B). Overall, three of six WT, two of four HT and zero of two KO mice expressed detectable FGF1. Thus, we were unable to detect a significant compensatory increase in FGF1 expression in mice bearing inactivated FGF2 alleles.

FGF2 is well known as an angiogenic factor in vivo (2). Furthermore, it has been demonstrated previously that emergence of the poorly differentiated phenotype in TRAMP prostate cancers is associated with a significant increase in angiogenesis (21). VEGF is also a potent angiogenic factor, and it is expressed in poorly differentiated TRAMP prostate cancers (26). However, there is evidence that FGF2 may directly induce VEGF expression in some systems (27, 28). Thus, it is possible that in the poorly differentiated tumors from HT and KO mice, VEGF might be present at increased levels to compensate for loss of FGF2 in these highly vascularized tumors or might be decreased due to loss of induction by FGF2. We therefore sought to determine whether VEGF is expressed at higher levels in poorly differentiated prostate cancers to compensate for loss of FGF2 in HT and KO TRAMP mice. VEGF protein levels were measured in protein extracts from eight poorly differentiated cancers from mice with different genotypes using ELISA. As shown in Fig. 3, poorly differentiated tumors from both HT and KO mice were found to contain more VEGF than tumors from WT mice. There is a clear trend for increased expression of VEGF in mice with inactivation of one or both FGF2 alleles.

cDNA Microarray Expression Analysis in Poorly Differentiated TRAMP Tumors. We used cDNA microarray expression analysis to gain additional insights into the mechanism by which tumors in mice with inactivation of one or both FGF2 alleles progress to poorly differentiated prostate cancers to compensate for loss of FGF2 in poorly differentiated TRAMP tumors.

purification with heparin-agarose followed by Western blotting with anti-FGF2 antibodies. All cancers expressed FGF2 protein. In general agreement with Huss et al. (19), the poorly differentiated tumors expressed the 25-, 22-, and 18-kDa forms of FGF2 (Fig. 2A). The larger forms arise from translation initiation from CUG codons and have different biological properties than the 18-kDa form, which initiates from an AUG codon. As noted by Huss et al. (19), better differentiated tumors may not express lower molecular mass forms of FGF2.

This is supported by the presence of the SV40 large T antigen by PCR. TRAMP mice with nontransgenic littermates from this initial cross, and male transgenic mice on July 13, 2017. © 2003 American Association for Cancer Research.

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either one or both alleles of FGF2 leads to significantly increased expression of FIBP when compared with similar tumors from WT differentiated tumors from KO and HT mice, there is increased expression of FIBP mRNA than the highest WT tumor. Overall, the average expression of FIBP was more than 6-fold higher in the HT and KO mice when compared with the WT mice. Although the number of samples is limited, taken together, these data indicate that in a subset of poorly differentiated tumors from KO and HT mice, there is increased expression of FIBP when compared with similar tumors from WT mice.

### DISCUSSION

FGF2 is expressed in all stages of human prostate cancer. To determine whether FGF2 plays a critical role in prostate cancer progression, we have compared prostate cancer progression in TRAMP mice with hemi- or homozygous inactivation of FGF2 with that of WT littermate controls. Our results show that inactivation of either one or both alleles of FGF2 leads to significantly increased survival by inhibiting progression to the poorly differentiated phenotype and metastatic disease. It is noteworthy that inactivation of even one FGF2 allele has a strong influence on tumor progression. Analysis of mouse models of cancer has revealed that profound phenotypic consequence can result from haploinsufficiency of tumor suppressor gene function. For example, we have demonstrated previously that loss of even one PTEN allele is associated with increased rates of tumor progression in TRAMP mice (21). Similarly, Goss et al. (30) have recently demonstrated that mice carrying only one allele of the Bloom syndrome gene have increased tumor rates when challenged with murine leukemia virus or crossed with mice bearing a mutated APC tumor suppressor gene. Inactivation of one allele of either the NKK3.1 tumor suppressor gene (31) or retinoid X receptor α gene...
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(32) is associated with development of prostatic intraepithelial neoplasia in mice, although with delayed onset in comparison to mice with homozygous inactivation of these genes. Our results indicate that haploinsufficiency of a gene associated with tumor progression leads to a decreased rate of progression in vivo. Thus, mouse models have revealed that loss of even one allele of a gene that plays a critical role in tumorigenesis can impact tumor progression over the course of the many months required for tumorigenesis in vivo.

There are multiple mechanisms by which loss of FGF2 could inhibit tumorigenesis. FGF2 is a potent growth factor for both normal and neoplastic prostatic epithelial cells (10). Ropiquet et al. (33) have studied the effects of FGF2 expression under the control of a strong promoter on immortalized but nontumorigenic human prostatic epithelial cells. They found that these cells had an increased proliferation rate and had acquired the ability to form colonies in soft agar. They also found that the FGF2-transfected cell lines had increased invasion through Matrigel relative to controls. Greene et al. (34) have shown that expression of FGF2 was increased in highly metastatic sublines of the PC3 cell line when compared with less metastatic sublines. Expression of dominant negative FGFRs, which block FGFR signaling, leads to G2 arrest and cell death in prostate cancer cell lines (35). Finally, FGF2 is well known as a promoter of angiogenesis (2). Thus, FGF2 signaling may potentially promote proliferation, survival, invasion, metastasis, and angiogenesis in prostate cancer, and loss of these activities could all inhibit cancer progression.

Loss of FGF2 inhibits but does not completely prevent progression to the poorly differentiated, metastatic phenotype. To understand which biological activities of FGF2 are important in promoting this phenotype, we investigated the molecular alterations in poorly differentiated tumors arising in HT and KO mice in comparison with WT mice. We found that the tumors in HT/KO mice contained more VEGF compared with WT tumors, implying that the angiogenesis induced by FGF2 is critical in emergence of the poorly differentiated phenotype. This is consistent with the very high microvessel density observed in poorly differentiated TRAMP tumors (21). Although FGF2 can directly induce VEGF in a number of cell types (25, 26), in the poorly differentiated tumors loss of FGF2 was associated with increased VEGF, implying that other factors that can induce VEGF, such as hypoxia, were able to overcome the loss of FGF2 stimulation.

Another potentially interesting difference between poorly differentiated tumors arising in WT and HT/KO mice was the increased expression of FIBP in some of the tumors in HT/KO mice. Although based on a small number of samples, this observation is intriguing because it focuses attention on the intranuclear activities of FGF2 in promoting prostate cancer progression. The high molecular mass forms of FGF2 (22 and 25 kDa) that arise from alternative translation initiation from CUG codons preferentially localize directly to the nucleus and can promote growth in low serum in some cell types (36). The 18-kDa form of FGF2 is released from the cell by mechanisms not involving a signal peptide [reviewed in Dow and deVere White (2)]. It can then interact with cell surface receptors such as FGFR-1 to promote activation of multiple signal transduction cascades. There is also evidence, at least in some cell types, that FGFR-1 can be located in the nucleus and can have direct intranuclear activities by interacting with FGF2 in that location (37). FGF1 also has both cell surface receptor-mediated and intranuclear activities. FGF1 does not have high molecular mass forms but can be translocated to the nucleus after binding cell surface receptors (38). FIBP was identified as a protein that binds to FGF1, but not to a mutant FGF1 that can activate cell surface FGFRs but is not mitogenic except at high concentrations. FIBP is located primarily in the nucleus. Thus FIBP may promote the mitogenic intranuclear activities of FGF1, although this has not been proven to date. The observation that FIBP is up-regulated in tumors from HT/KO mice suggests that it might compensate for loss of the intranuclear activities of FGF2 in some tumors. Although FGF1 was not up-regulated in the tumors from the HT or KO mice, FIBP may interact with FGF1 that is expressed at low levels in all TRAMP tumors (25) or might potentially interact with other FGF family members.

Additional experiments will be needed to determine conclusively whether increased FIBP can compensate for loss of FGF2 in during progression in TRAMP prostate cancers. Whether FGFR-1-mediated activities at the cell membrane also play a role in FGF2-regulated progression will also need to be confirmed, but given the increased expression of FGFR-1 during prostate cancer progression in both animal models and human prostate cancer, this seems likely.

The presence of a neuroendocrine phenotype in poorly differentiated carcinomas in TRAMP mice, as determined by the expression of synaptophysin, has recently been described (24). Focal neuroendocrine differentiation, as assessed by markers such as chromogranin A, occurs in the majority of clinical localized human cancers. Furthermore, neuropeptides, such as bombesin, are expressed by the majority of prostate cancers (39), and prostate cancer cells can respond to exogenous neuropeptides with both increased proliferation and invasion, indicating that the neuroendocrine differentiation may promote progression (40). In fact, increased serum levels of chromogranin A have been associated with advanced clinical stage, poor prognosis, and androgen independence and correlate with cancer tissue levels as determined by immunohistochemistry [for review, see Berruti et al. (41)]. Whereas the prognostic significance of neuroendocrine differentiation in clinical disease is controversial, there is abundant evidence that neuroendocrine differentiation is common in prostate cancer and, based on both clinical and biological observations, that it is associated with disease progression in a significant fraction of cases. Thus the presence of neuroendocrine differentiation in the poorly differentiated carcinomas arising during tumor progression in TRAMP mice is consistent with observations in human prostate cancer. Additional studies of the biology of prostate cancer in the TRAMP model and human prostate cancer, including comprehensive transcriptional profiling, are needed to assess the similarities and differences between progression in the TRAMP model and human prostate cancer.

Our results show that decreased FGF2 can delay progression in the TRAMP model of prostate cancer. However, the fact that FGF2 is decreased in all tissues and cell types in the KO mice leaves ambiguity as to the exact contribution of FGF2 expressed in different cell types to prostate cancer progression and the mode of activity of FGF2 in the various cell types in vivo. For example, FGF2 expressed by the prostate cancer cells can potentially act as an autocrine factor, either by release to the extracellular compartment and interaction with cell surface receptors or by direct translocation to the nucleus. FGF2 released by epithelial cells could also act as a paracrine factor on endothelial and fibroblastic cells to promote angiogenesis or to stimulate secretion of other tumor-promoting factors by these mesenchymal cells. At the same time, FGF2 released by fibroblastic and endothelial cells could act as a paracrine factor on the epithelial cells or as an autocrine factor promoting angiogenesis. Such effects could occur either in the primary site or at sites of distant metastasis. Furthermore, given that loss of even one FGF2 allele can affect tumor progression, it may be that even relatively subtle alterations of FGF2 expression can have important effects on either autocrine or paracrine actions of FGF2 in vivo. To determine the relative importance of these various mechanisms, tissue-specific KOs of the various isoforms of FGF2 would need to be created, and the effect of each KO on progression would need to be assessed. Despite these ambiguities, this study provides further support to the hypothesis that therapies target-
ing FGF signal transduction in general and FGF2 in particular may be clinically useful for treating human prostate cancer.

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