Genes Upregulated in Prostate Cancer Reactive Stroma Promote Prostate Cancer Progression In Vivo

Olga Dakhova¹, David Rowley², and Michael Ittmann¹,³

Abstract

Purpose: Marked reactive stroma formation is associated with poor outcome in clinically localized prostate cancer. We have previously identified genes with diverse functions that are upregulated in reactive stroma. This study tests the hypothesis that expression of these genes in stromal cells enhances prostate cancer growth in vivo.

Experimental Design: The expression of reactive stroma genes in prostate stromal cell lines was evaluated by reverse transcriptase (RT)-PCR and qRT-PCR. Genes were knocked down using stable expression of short-hairpin RNAs (shRNA) and the impact on tumorigenesis assessed using the differential reactive stroma (DRS) system, in which prostate stromal cell lines are mixed with LNCaP prostate cancer cells and growth as subcutaneous xenografts assessed.

Results: Nine of 10 reactive stroma genes tested were expressed in one or more prostate stromal cell lines. Gene knockdown of c-Kit, Wnt10B, Bmi1, Gli2, or COMP all resulted in decreased tumorigenesis in the DRS model. In all tumors analyzed, angiogenesis was decreased and there were variable effects on proliferation and apoptosis in the LNCaP cells. Wnt10B has been associated with stem/progenitor cell phenotype in other tissue types. Using a RT-PCR array, we detected downregulation of multiple genes involved in stem/progenitor cell biology such as OCT4 and LIF as well as cytokines such as VEGFA, BDNF, and CSF2 in cells with Wnt10B knockdown.

Conclusions: These findings show that genes upregulated in prostate cancer-reactive stroma promote progression when expressed in prostate stromal cells. Moreover, these data indicate that the DRS model recapitulates key aspects of cancer cell/reactive stroma interactions in prostate cancer.

Introduction

Prostate cancer remains the most common malignancy affecting men and the second leading cause of cancer-related death of men in the United States. It has been appreciated for many years that the tumor microenvironment plays an important role in the initiation and progression of prostate and other cancers (1, 2). The tissues surrounding the cancer cells in prostate cancer are distinct from the normal mesenchymal tissues of the prostate and consist of a mixture of fibroblasts, myofibroblasts, nerves, endothelial cells, immune cells, other cells, and altered extracellular matrix. Men with tumors having the most profound histologic alterations of reactive stroma, which is termed reactive stroma grade 3 (RSG3), have reduced biochemical recurrence-free survival, and/or increased prostate cancer-specific death in studies of tissue microarrays (3), biopsies (4), and radical prostatectomy specimens (5), which was independent of other clinical and pathologic parameters. This finding indicates that reactive stroma plays a critical role in prostate cancer progression and thus is an important therapeutic target. Of note, reactive stroma is relatively genetically stable compared with cancer cells so that it cannot respond as readily to selective pressures induced by therapies via mutation or other genomic alterations and thus represents an excellent therapeutic target.

To understand the mechanisms by which reactive stroma can influence tumor behavior, we have previously examined global changes in gene expression in prostate cancer–reactive stroma grade 3 relative to paired benign prostatic stroma using expression microarray analysis of laser-captured RNAs from these two tissue types (6). By focusing on grade 3 reactive stroma, which is associated with prostate cancer progression, we sought to identify key changes in prostate reactive stroma that are associated with aggressive prostate cancer. A total of 544 unique genes were higher in the reactive stroma and 606 unique genes were lower based on microarray analysis compared with benign stroma. The upregulated genes were associated with a variety of biologic...
The interaction of prostate cancer cells and reactive stroma in DRS model recapitulates key tumor-regulatory aspects of cancer progression. Moreover, these data indicate that the DRS model. These findings further implicate these gene sets expressed in DRS stromal cells and that knockdown using simultaneous in nude mice and tumor formation and/or 10 or 28 days after injection and tumors excised, weighed, and sections prepared for histologic examination and other studies. Tumors were scored at either 10 days (to examine early events) or at 28 days (to stringently compare tumorigenesis). It should be noted that DRS stromal cells can both partially replace Matrigel and when injected in the presence of Matrigel further enhance tumor growth (7).

Knockdown of reactive stroma genes with lentiviral shRNAs
Human GIPZ lentiviral shRNA mirs targeting Wnt10B (RHS4430-98418580), c-Kit (RHS4430-98418428), COMP (RHS4430-200217395), Gli2 (RHS4430-200280814), and Bmi1 (RHS4430-200171042) were obtained from Open Biosystems. HPS-19I stromal cells were infected with virus and stably selected with puromycin. Knockdown was confirmed using qRT-PCR of RNAs extracted after stable selection. Control vector was nonsilencing GIPZ Lentiviral shRNA mir control from Open Biosystems (RHS4346).

Differential reactive stroma tumor studies
Stromal cells with knockdown or vector controls were used to subcutaneously inoculate nude mice as described previously (7). Briefly, each injection consisted of 2 x 10^6 LN CaP prostate cancer cells and/or 2 x 10^6 stromal cells and/or 100 μL of Matrigel. Mice were injected over each flank to yield two tumors per mouse. Mice were euthanized 10 or 28 days after injection and tumors excised, weighed, and sections prepared for histologic examination and other studies. In the two-way DRS model, cells were injected without Matrigel and the presence and size of tumors scored at 10 days. For the three-way DRS model, Matrigel was used in addition to cells. Tumors were scored at either 10 days (to examine early events) or at 28 days (to stringently compare tumorigenesis). It should be noted that DRS stromal cells can both partially replace Matrigel and when injected in the presence of Matrigel further enhance tumor growth (7).

Immunohistochemistry and TUNEL
Immunohistochemistry for Bmi1 was performed as follows. After blocking endogenous peroxidase with hydrogen peroxide in methanol, antigen retrieval was performed in citrate buffer, pH 6.0 (Diagnostic BioSystems) for 20 minutes in a steamer. Nonspecific staining was blocked for 20 minutes with Background Blocker (Biocare Medica). Slides were incubated in primary antibody (Bmi1 rabbit Mab, Abcam, cat #ab126783) at 1:150 overnight at 4°C. Detection was performed using Rabbit on Rodent HRP-Polymer (Biocare Medical) for 30 minutes at room temperature followed by stable DAB Plus (Diagnostic BioSystems) for...
5 minutes at room temperature. Slides were then counterstained using CAT Hematoxylin (Biocare Medical) for 15 to 30 seconds. All rinses made using Tris-Tween Buffer, pH 7.4. Immunohistochemical staining for Ki67 and CD31 and TUNEL were carried out as described previously (10).

Western blot analysis

Whole-cell protein lysates (10–15 μg) were separated in 7.5% SDS/PAGE and transferred on nitrocellulose membrane as described previously (11). Immunoblotting was performed with mouse monoclonal anti-human ErbB2 antibodies (ab8054, Abcam) used at 1:2,000 dilution in 5% nonfat milk in PBS with 0.05% Tween-20 overnight at 4°C. Goat anti-mouse HRP-conjugated antibodies were used as secondary at 1:5,000 in the same buffer for 1 hour at room temperature. Signal was detected using ECL reagent (GE Healthcare).

Image analysis

Tumors from the 10-day DRS experiment were analyzed using InForm image analysis software (10). InForm allows to perform learning-example automated image processing. Eight to 10 images were taken at ×100 magnification with a Nuance camera to cover all tissue area. Training was performed on 10 to 15 representative images. First tissue segmentation was performed using three categories (LNCaP, stroma, Matrigel) with training at fine segmentation resolution level and small pattern scale. It was followed by nuclear cell segmentation in a chosen tissue category and scoring with Double Positivity mode. After that, batch analysis was performed on all available images using training algorithm, and data were merged in one file to analyze. Image analysis of anti-CD31 immunohistochemistry was performed as above except that anti-CD-31 stained area divided by the total area of the stromal compartment was calculated.

PCR arrays

RNAs from 19I cells with knockdown of Wnt10B or controls were used to analyze gene expression using an RT-profiler PCR array for human mesenchymal stem cell–related genes from SABiosciences (SuperArray PAHS-082A) according to the manufacturer’s instructions. Confirmatory qRT-PCR of individual genes was carried out using primers and conditions shown in Supplementary Table S1.

Results

Tumorigenc potential of human prostate stromal cell lines

To evaluate the tumorigenic potential of three human stromal cell lines established from prostates from human organ donors (19I, 19B, and 33B), we examined their ability to support LNCaP tumorigenesis in the differential reactive stroma model (7) using two-way DRS injections. In the two-way DRS model, LNCaP prostate cancer cells and a prostate stromal cell line (DRS stromal cells) are mixed and injected subcutaneously in nude mice alone, with Matrigel, or with the indicated prostate stromal cell line. After 10 days, the number of palpable tumors was scored. The percent tumor formation is shown and the number of injections evaluated is indicated over each bar.

Expression of reactive stromal genes by human prostate stromal cell lines

We next examined the expression of 10 different genes that we had previously demonstrated to be upregulated in human prostate cancer–reactive stroma compared with benign stroma, based on both expression microarray’s and qRT-PCR of laser-captured stroma. We chose a variety of different types of genes to evaluate based on their potential protumorigenic activity, including growth factor receptors (c-Kit, ErbB2, TGFBR2), transcription factors (Gli2, FoxA1, Bmi1), axon guidance genes (Robo3), secreted growth factors (Wnt10b, FGF19) and genes encoding extracellular matrix (COMP). Expression was evaluated using semi-quantitative and/or qRT-PCR as shown in Fig. 2A. In some cases, there was relatively invariant expression of mRNAs between cell lines, for example ErbB2 and Bmi1 (Fig. 2A and B). However, for most genes, there was variable expression between different cell lines that in many cases was statistically significant when evaluated by qRT-PCR (Fig. 2B). Most of the RSG3-induced genes were expressed at easily detectable levels with the exception of FoxA1, which was only weakly detected in a single cell line (19I) and FGF19, which was not expressed (data not shown). Given that ErbB2 is potentially targetable in prostate cancer, we

Figure 1. Differential reactive stroma cell lines variably enhance LNCaP tumorigenesis in a 10-day DRS model. LNCaP cells were injected subcutaneously in nude mice alone, with Matrigel, or with the indicated prostate stromal cell line. After 10 days, the number of palpable tumors was scored. The percent tumor formation is shown and the number of injections evaluated is indicated over each bar.
sought to confirm expression of this protein in DRS stromal cells by Western blotting. As can be seen in Supplementary Fig. S1, ErbB2 protein is expressed in all three DRS stromal cell lines. Thus, all of the human stromal cell lines expressed multiple reactive stroma genes at variable but easily detectable levels.

**Functional analysis of reactive stroma genes**

To evaluate the functional significance of the reactive stroma genes expressed in the human prostate stromal cell lines, we carried out stable knockdowns of five of these genes using lentiviral shRNAs followed by the evaluation of tumor formation in the DRS model. Our initial studies used a 28 day three-way DRS model in 19I cells to evaluate the biologic activities of c-Kit and Wnt10B. As can be seen in Fig. 3, an approximately 80% decrease in Wnt10B mRNA led to a 50% decrease in tumor incidence and more than 80% decrease in tumor weight in tumors that were measurable. Similarly, a 60% knockdown of c-Kit resulted in approximately 40% decrease in tumor incidence and, in the measurable tumors, there was an 80% decrease in tumor weight. Thus, knockdown of either of these genes resulted in significant inhibition of tumorigenesis.

We then extended these studies to a 10 day three-way DRS model using 19I cells after knockdown of gene expression using lentiviral shRNAs. Use of the 10-day model allows evaluation of earlier events involved in tumorigenesis. In
these studies, we evaluated cells with knockdown of Wnt10B, COMP, Gli2, or Bmi1 mRNA. We evaluated target gene expression by qRT-PCR before injection, which showed approximately 70% to 80% knockdown in all cases (Fig. 4A). Tumorigenesis in a 10-day DRS model showed a significant decrease in tumor size in all the knockdown cell lines versus control 19I cells (Fig. 4B). It should be noted that at the 10-day timepoint there is still a significant amount of Matrigel in the tumors (Fig. 4C), as the cellular elements have not had as much time to grow and the Matrigel has not been resorbed to the same degree as in 28-day tumors. Thus, the absolute decrease in tumor weight is constrained by the presence of this injected material. To confirm continued knockdown of the targeted gene, we performed immunohistochemistry to detect Bmi1 protein expression in tumors with knockdown of Bmi1 in the 19I cells and control tumors. As can be seen in Fig. 4D, Bmi1 is expressed in a subset of spindle-shaped cells in the stroma. It should be noted that much of the stroma, even at this early timepoint consists of mouse stromal cells, including endothelial cells, as

Figure 3. Tumorigenesis in a 28-day DRS model is inhibited by knockdown of Wnt10B or c-Kit. LNCaP cells were injected subcutaneously in nude mice with Matrigel and 19I DRS prostate stromal cells with shRNAs targeting Wnt10B (A) or c-Kit (B) or vector controls. Gene knockdown was confirmed by qRT-PCR before injection. After 28 days, the number of palpable tumors was scored and palpable tumors were excised and weighed. All values are relative to vector controls and are expressed as mean ± SEM, n = 18 injections in a single experiment. Significant differences relative to 19I vector control cells are indicated by asterisks. For gene expression, P < 0.01, t test. For tumor weight, P < 0.003, t test. For tumor incidence, P = 0.01, Fisher exact test.

Figure 4. Inhibition of tumorigenesis by reactive stroma gene knockdown in a 10-day DRS model. LNCaP cells were injected subcutaneously in nude mice with Matrigel and 19I DRS prostate stromal cells with shRNAs targeting Wnt10B, COMP, Gli2, Bmi1, or vector controls. A, gene expression before injection by qRT-PCR. B, tumor weight after 10 days. All values are relative to controls and are expressed as mean ± SEM. Results represent two separate experiments with 12 injections in each experiment. Significant differences relative to control cells are indicated by asterisks. For gene expression: P < 0.001, t test. For tumor weight, P < 0.001, t test. C, histology of 10 day DRS tumors from control and shBmi1 tumors. Residual Matrigel is present (arrowheads) as are islands of LNCaP cells (arrows) and stromal cells and blood vessels (double arrows). Note the smaller and more sparse clusters LNCaP cells and decreased blood vessels in the shBmi1 tumors. D, immunohistochemistry with anti-Bmi1 antibody. LNCaP cells (arrows) are positive as are a subset of stromal cells (half shaded arrows). Staining of stromal cells was visibly decreased in tumors with shBmi1 stromal cells and this was confirmed by image analysis. All images ×200 magnification.
demonstrated previously by Alu in situ hybridization (7). In the tumors from mice with Bmi1 knockdown 19I cells, there was a visible decrease in spindle-shaped cells in the stroma expressing Bmi1. The LNCaP cell also express Bmi1, as has been demonstrated previously (13), which acts as an internal positive control. We then carried out Inform image analysis to quantitate Bmi1 staining in the stroma. Using multispectral imaging, the stromal and cancer cell compartments were identified by image analysis. All cell nuclei and Bmi1-stained nuclei were then quantitated in the stromal compartment. In control tumors, 13.6 ± 0.2% of stromal nuclei were positive versus 2.1 ± 0.1% in the knockdown tumors (mean ± SEM). This difference was highly statistically significant (P < 0.005, t test), thus confirming continued knockdown of Bmi1 in the stromal compartment in the tumors with Bmi1 knockdown in 19I cells. Thus, we consistently found decreases in tumor-promoting phenotypes in prostate stromal cells with decreased expression of genes induced in reactive stroma.

To determine the reason for the observed decrease tumor size when the reactive stroma genes were knocked down, we carried out image analysis of tumors using Inform image analysis software. In all cases, the number of LNCaP cells, as determined by image analysis of random fields in the tumors with Inform software, was significantly decreased in tumors with target gene knockdown compared with controls (Fig. 5A), with Bmi1 knockdown cells showing the most significant decrease in cancer cells per tumor. It should be noted that the decrease in LNCaP tumors cells is greater than the decrease in tumor weight (Fig. 4B) since, particularly at this earlier timepoint, Matrigel accounts for a significant fraction of the tumor (Fig. 4C) as described above. We then carried out image analysis to determine the percentage of proliferating LNCaP cells by Ki67 immunohistochemistry (Fig. 5B) as well as the percentage of apoptotic cells by TUNEL analysis (Fig. 5C). In tumors with Wnt10B knockdown stromal cells, there was a significant increase in apoptosis without change in proliferation of LNCaP cells. The tumors from the COMP knockdown stromal cells showed a similar pattern, although the increase in apoptosis was not statistically significant (P =
0.06; \( t \) test). Tumors using stromal cells with knockdown of Gli2 showed a significant decrease in proliferation and a marginal increase in apoptosis (\( P = 0.09; \ t \) test). Tumors using stromal cells with knockdown of Bmi1 showed a significant decrease in the proliferative fraction of LNCaP cells as well as a marked increase in apoptotic LNCaP cells. These observations are consistent with decreased overall number of LNCaP cells in all cases, but the exact cause of the decrease in LNCaP cells is variable based on the target gene that is knocked down. In contrast, in all cases, decreased angiogenesis was observed, as determined by quantitative analysis of anti-CD31 immunohistochemistry (Fig. 5D).

Wnt family members have been implicated in mesenchymal stem cell and/or progenitor cell maintenance and function. Wnt10B is a relatively poorly characterized member of the Wnt family but recent studies have implicated it in human mesenchymal stem cell/progenitor cell function in adipose tissue and bone (14, 15). As described above, Wnt10B is upregulated in prostate cancer reactive stroma, is expressed in the prostate stromal cell lines and knockdown decreases tumorigenesis by increasing apoptosis in LNCaP cells and decreasing angiogenesis. We therefore sought to determine whether knockdown of Wnt10B can impact expression of genes associated with mesenchymal stem/progenitor cell function. RNAs from 19I cells with knockdown of Wnt10B or controls were used to analyze gene expression of using an RT-profiler PCR array for 85 human mesenchymal stem cell–related genes from SABiosciences. All of these genes are known to be involved in mesenchymal stem cell biology, including genes involved in the maintenance of stemness, stem cell markers, differentiation markers, and other stem cell–related genes. A total of 74 genes showed detectable expression. Genes with low expression (\( C_t > 32 \)) were excluded from further analysis. A total of 10 genes showed decreased expression (1.5-fold or more) upon Wnt10B knockdown. These genes are shown in Table 1. No genes showed an increase of 1.5-fold or more. Four of these genes (ABCB1, OCT4, BDNF, THY1) were analyzed by individual qRT-PCR and all showed 54% to 84% decrease in expression compared with controls (Fig. 6), confirming the accuracy of the larger qRT-PCR array. Expression relative to vector controls is shown. All values are means of triplicates. SDs were less than 2% and thus are not shown since they are not visible on the graph. Significant differences relative to control cells by \( t \) test are indicated by asterisks (ABCB1; \( P < 0.001 \); OCT4, \( P = 0.024 \); BDNF, \( P < 0.001 \); THY1, \( P = 0.01 \)).

### Table 1.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Fold change</th>
<th>Unigene ID</th>
<th>NCBI Ref Seq</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>0.4234</td>
<td>Hs.489033</td>
<td>NM_000927</td>
<td>ATP-binding cassette, sub-family B1 (MDR/TAP)</td>
</tr>
<tr>
<td>CSF2</td>
<td>0.4698</td>
<td>Hs.1349</td>
<td>NM_000758</td>
<td>Colony stimulating factor 2 (granulocyte-macrophage)</td>
</tr>
<tr>
<td>OCT4</td>
<td>0.4863</td>
<td>Hs.249184</td>
<td>NM_002701</td>
<td>POU class 5 homeobox 1</td>
</tr>
<tr>
<td>BDNF</td>
<td>0.5586</td>
<td>Hs.502182</td>
<td>NM_001709</td>
<td>BDNF</td>
</tr>
<tr>
<td>VEGFA</td>
<td>0.5586</td>
<td>Hs.73793</td>
<td>NM_003376</td>
<td>VEGF A</td>
</tr>
<tr>
<td>KAT2B</td>
<td>0.6199</td>
<td>Hs.53055</td>
<td>NM_003884</td>
<td>(lysine) acetyltransferase 2B</td>
</tr>
<tr>
<td>THY1</td>
<td>0.6199</td>
<td>Hs.644697</td>
<td>NM_002701</td>
<td>Thy-1 cell surface antigen</td>
</tr>
<tr>
<td>ANPEP</td>
<td>0.6417</td>
<td>Hs.1239</td>
<td>NM_001150</td>
<td>Alanyl (membrane) aminopeptidase</td>
</tr>
<tr>
<td>LIF</td>
<td>0.6417</td>
<td>Hs.2250</td>
<td>NM_002309</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>RUNX2</td>
<td>0.6643</td>
<td>Hs.535845</td>
<td>NM_004348</td>
<td>Runt-related transcription factor 2</td>
</tr>
</tbody>
</table>

NOTE: A total of 10 genes showed decreased expression of 1.5-fold or more. None showed increased expression of a similar magnitude.

Figure 6. Decreased expression of mesenchymal stem cell–associated genes in prostate stromal cells with knockdown of Wnt10B. Individual qRT–PCR reactions were carried out for four mesenchymal stem cell–related genes to confirm the results of the qRT–PCR array. Expression relative to vector controls is shown. All values are means of triplicates. SDs were less than 2% and thus are not shown since they are not visible on the graph. Significant differences relative to control cells by \( t \) test are indicated by asterisks (ABCB1; \( P < 0.001 \); OCT4, \( P = 0.024 \); BDNF, \( P < 0.001 \); THY1, \( P = 0.01 \)).
are relevant to prostate cancer progression, including VEGFA, brain-derived neurotrophic factor (BDNF), and CSF2, were also downregulated by Wnt10B knockdown as well.

Discussion

Formation of prostate cancer reactive stroma is a complex, dynamic process that is characterized by activation of multiple cellular processes. Our current studies show that many genes upregulated in grade 3 reactive stroma can enhance prostate cancer tumor progression in vivo in the DRS xenograft model system. This is a crucial validation of their in vivo significance that strongly supports the relevance of our prior observational studies showing their upregulation in human prostate cancer stroma tissues. Conversely, our studies also indicate that the DRS model accurately mimics tumor–stromal interaction in human prostate cancer and can exploited for understanding the complex interactions of prostate cancer cells and the microenvironment.

Bmi1 is a transcription factor that is involved in maintenance of stem cells (16) and is expressed in all three DRS stromal cell lines studied. When Bmi1 mRNA is knocked down, tumorigenesis is decreased significantly, indicating that Bmi1 can modulate key properties of stromal cells that promote tumorigenesis. The stromal cell lines used in the DRS model have a myofibroblastic phenotype but the expression of Bmi1 indicates they express some genes that are also expressed in mesenchymal stem/progenitor cells. This conclusion is reinforced by our finding that knockdown of Wnt10B, which plays an important role in mesenchymal progenitor function in bone and adipose tissue, decreases expression of stem/progenitor genes such as OCT4, LIF, and CD90 (THY1). It should be noted that immunohistochemistry studies have identified Bmi1-expressing stromal cells in both normal prostate and prostate cancers, although there was no quantitation of the expression in normal versus cancer (17).

Wnt10B is a canonical Wnt and promotes prostate cancer tumorigenesis in the DRS model by decreasing apoptosis in LNCaP cells and increasing angiogenesis. In part, this may be due to the autocrine promotion of expression of stem cell–associated genes as described above. This autocrine stimulation also increases expression of several cytokines. BDNF is a nerve growth factor that can promote axogenesis/neurogenesis, which has been linked to prostate cancer progression by recent clinical and biologic studies (18–20). It also enhances VEGFA expression in an autocrine manner, which may explain in part the observed decrease in angiogenesis when Wnt10B is knocked down given that VEGFA is a potent angiogenic factor. In addition, it is likely that Wnts act in a paracrine manner to directly promote the growth of LNCaP cells by the activation of canonical Wnt signaling, as it has been shown that LNCaP cells can respond to paracrine Wnts (21).

Cartilage oligomeric matrix protein (COMP) is an extracellular matrix protein that is overexpressed in prostate cancer reactive stroma based on our prior studies (6) and those of other groups (22). Our prior bioinformatics analysis showed that COMP is also overexpressed in breast cancer reactive stroma (6), which has been confirmed in an independent analysis (23). A recently published study confirmed the expression of COMP at the protein level in prostate cancer tissues and also showed that COMP was increased in the serum of patients with advanced prostate cancer (24). Our functional studies reveal for the first time that COMP can promote prostate cancer tumor growth. Further studies are needed to determine the mechanism by which COMP enhances tumor growth. Of note, COMP is induced by TGF-β in prostate stromal cells (25). Previous studies from our group have identified connective tissue growth factor (CTGF) as another TGF-β–induced gene that can promote tumor progression in the DRS model system (26). The finding that COMP also promotes tumor progression argues for an important role for TGF-β–induced proteins expressed in stroma in promoting tumor progression in prostate cancer.

c-Kit is a transmembrane tyrosine kinase receptor that is activated by binding to Kit ligand (KL). Our studies indicate that c-Kit is expressed in 2 of 3 DRS stromal cell lines studied and when knocked down tumor growth is decreased. Previous studies by Simak and colleagues (27) have shown that c-Kit is expressed in cultured prostate stromal cells. The same group showed that KL protein was expressed in normal prostate stromal cells as well as approximately 40% of prostate cancers. Thus, there is abundant KL present in prostate cancers from stromal cells or from cancer cells that can activate c-Kit. Our group (6) and others (28) have shown expression of c-Kit is prostate cancer stroma by immunohistochemistry. Of note, some stem/progenitor cells, such as cardiac stem/progenitor cells, express c-Kit (29) but it seems unlikely that c-Kit is linked to expression of stem cell/progenitor genes in the DRS cell lines since it is not expressed in one of the cell lines that expresses Bmi1.

Gli2 is a nuclear transcription factor that is a key downstream target of canonical hedgehog signaling. The Bushman group has shown that hedgehog signaling promotes prostate cancer tumor growth via the activation of hedgehog pathway in myofibroblasts, with no evidence of autocrine stimulation of prostate cancer cells by hedgehog ligands (30, 31). Of note, they show that activation of hedgehog signaling only occurs in prostate cancer tumors with a reactive stroma phenotype (31). Our current studies show directly that Gli2 enhances tumor progression in a tissue recombination model when expressed in the myofibroblastic cells. It should be noted Gli2 transcription can be driven both by hedgehog ligands or noncanonical pathways such as TGF-β (32) and both of these pathways may be potentially active Gli2 transcription in prostate cancer in vivo in different contexts.

An interesting aspect of the DRS stromal cells is their heterogeneous expression of various genes in the different cell lines, even in cell lines isolated from the same organ donor (19A and 19B). Bmi1 is the strongly expressed in all 3 cell lines and knockdown of this gene has the most profound effect on tumorigenesis so it is highly likely that this
gene has an important role in promoting tumorigenesis by stromal cells. However, other genes (c-Kit, Wnt10B, COMP etc) are expressed at variable levels and these genes also impact tumorigenesis. Thus, the pro-tumorigenic factors are variable between different cell lines and multiple factors contribute to the overall tumorigenic activity. Ultimately, it will be useful to examine the impact of gene knockdowns, singly and in combination, in multiple DRS stromal lines other than 19I to define the relative importance of various genes in tumorigenesis.

The reason the various reactive stroma genes are expressed at variable levels in various cell lines is unclear and requires further investigation. However, such heterogeneity of gene expression, in particular TGFBR2, has been documented in carcinoma associated fibroblasts and to a lesser extent in benign stromal tissues by Li et al (21), so the variable expression in cell lines reflects actual in vivo heterogeneity. Work by Franco and colleagues has shown that stromal cell heterogeneity itself may induce paracrine interactions that can promote tumor progression (33). It will be of interest to examine whether mixing various DRS cell lines can promote tumorigenesis in a similar manner.

Our current studies reveal that a key set of genes expressed in reactive stroma can promote tumor progression in vivo. Such activities are potential cancer therapy targets. Targeting the tumor microenvironment is attractive as a therapeutic strategy since the reactive stromal cells are more genomically stable than cancer cells. In addition, when using therapies targeting activation of similar pathways in cancer cells, a portion of therapeutic benefits seen may be due to stromal effects in the tumor microenvironment, which must be accounted for when interpreting results of trials with such agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: O. Dakhova, D.R. Rowley, M.M. Ittmann
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.M. Ittmann
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Dakhova
Writing, review, and/or revision of the manuscript: O. Dakhova, D.R. Rowley, M.M. Ittmann
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Dakhova, D.R. Rowley, M.M. Ittmann
Study supervision: M.M. Ittmann

Acknowledgments
The authors thank Billie Smith of the Human Tissue Acquisition and Pathology Core of the Dan L. Duncan Cancer Center for technical assistance.

Grant Support
This work was supported by grants from National Cancer Institute to the Tumor Microenvironment Network [U54CA126568, DR], the P30 Cancer Center support grant (P30 CA125123) to support the Human Tissue Acquisition and Pathology Core, the Department of Veterans Affairs Merit Review program (to M.M. Ittmann) and by the use of the facilities of the Michael E. DeBakey VAMC.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 30, 2013; revised September 17, 2013; accepted October 15, 2013; published OnlineFirst October 22, 2013.

References


Genes Upregulated in Prostate Cancer Reactive Stroma Promote Prostate Cancer Progression In Vivo

Olga Dakhova, David Rowley and Michael Ittmann