AR-Regulated TWEAK-FN14 Pathway Promotes Prostate Cancer Bone Metastasis

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Abstract

The recurrence of prostate cancer metastases to bone after androgen deprivation therapy is a major clinical challenge. We identified FN14 (TNFRSF12A), a TNF receptor family member, as a factor that promotes prostate cancer bone metastasis. In experimental models, depletion of FN14 inhibited bone metastasis, and FN14 could be functionally reconstituted with IKKβ-dependent, NFκB signaling activation. In human prostate cancer, upregulated FN14 expression was observed in more than half of metastatic samples. In addition, FN14 expression was correlated inversely with androgen receptor (AR) signaling output in clinical samples. Consistent with this, AR binding to the FN14 enhancer decreased expression. We show here that FN14 may be a survival factor in low AR output prostate cancer cells. Our results define one upstream mechanism, via FN14 signaling, through which the NFκB pathway contributes to prostate cancer metastasis and suggest FN14 as a candidate therapeutic and imaging target for castrate-resistant prostate cancers. Cancer Res; 74(16); 1–12. ©2014 AACR.

Introduction

Metastasis to the bone is one of the most clinically important features of prostate cancer, although relatively little is known about specific pathways that promote tumor growth in the bone. Metastatic prostate cancer is treated with androgen deprivation therapy, which initially reduces symptoms and tumor cell growth, although recurrence of castration-resistant prostate cancer (CRPC) is almost universal (1). Reconstitution of androgen receptor (AR)-mediated signaling is a central mechanism leading to CRPC (2, 3). Although some level of AR output, measured by expression of AR target gene signatures, is almost always observed in prostate cancer, there is significant intertumoral heterogeneity, especially associated with progression (4–6). Following the development of castration resistance, a decrease in the level of an AR gene signature commonly expressed in prostate cancer indicates either reduced AR transcriptional activity or a shift in AR-dependent transcriptional specificity (6, 7). AR output is influenced by factors such as PTEN levels and the differentiation status of tumors (8–10). In the case of PTEN loss or N-cadherin expression, compensatory non-AR pathways are thought to significantly contribute to tumor growth.

Histopathology of end-stage bone metastases acquired at autopsy or as a result of surgical resections for spinal cord compressions or pathologic fractures (11–13) has shown that bone metastases are heterogeneous, even within a single patient. Importantly, although nuclear AR staining is usually prominent in most cells, non-neuroendocrine AR-negative tumor cells are clearly observed in both CRPC and treatment-naive metastases (11–14). These findings imply that AR-independent survival in the bone microenvironment occurs, and the mechanisms contributing to such survival are of great interest. The heterogeneity of metastatic disease suggests that second-generation AR-directed therapies such as abiraterone and enzalutimide most likely will need to be complemented by therapies directed against non-AR pathways.

We describe here the characterization of Fn14 (TNFRSF12A) as a marker of clinical prostate cancer metastases and a determinant of experimental metastatic capability. Fn14, a TNF receptor family member, is the cognate receptor for TWEAK, a TNF-like cytokine, produced most prominently by infiltrating immune cells (15). Fn14 expression is low or absent in most normal tissues but can be activated by physiologic mediators such as mitogens, hormones, and cytokines in epithelial, mesenchymal, and neuronal cell types (16). Activation of the TWEAK–Fn14 axis leads to context-dependent responses, including a prominent role in normal tissue repair and inflammation (15, 16). TWEAK-stimulated Fn14 signaling occurs through multiple pathways, including NFκB, MAPK, and CDC42/RAC (16, 17).
The overexpression of Fn14 has been reported in various solid tumors, where higher Fn14 expression in some tumor types has been shown to correlate with more advanced grade and poorer prognosis (18, 19), although it has not been previously addressed whether Fn14 is functionally required for an aggressive phenotype in vivo. Fn14-targeted agents based upon antibody binding specificities are being developed and have shown promise in preclinical oncology studies as a new class of therapeutics (20, 21). The study described here uniquely addresses the role of Fn14 in the development of bone metastasis, identifies the p50/p65 NFκB pathway as a sufficient and necessary downstream mediator of Fn14-dependent bone metastasis, and establishes an association between Fn14 expression and a low canonical AR gene signature phenotype.

Materials and Methods

shRNA-mediated gene silencing and expression rescue in DU145/RasB1 cells

All genetically engineered cell lines were established using lentiviral vectors following standard procedures (22). Fn14 gene (TNFRSF12A) was silenced with two Fn14 shRNAs in the pLKO.1 lentiviral vector, designed by The RNAi Consortium (TRC; Open Biosystems) and targeting the following sequences: 5′-ATGAAATGATGATGAGGGCC−3′ (sh1) and, 5′-AGGATGGGCCAAACGACGCG-3′ (sh2). Expression rescued genes included Fn14 (Genecopeia), IKKβS32/36A and NIKAT3 in a pFUGW lentiviral vector with a FerH promoter (23). To achieve controlled expression of target genes, DU145/RasB1 cells stably expressing a Tet-On 3G transactivator were first established and further infected with a pFUGW lentiviral expression clone containing either AR (NM_000044.3) or mutant IκBα super repressor (IκBαS32/36A) under the control of the TRE3G promoter with an IRES-mCherry reporter. AR or IκBαSR expression was induced with 1 μg/mL doxycycline.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ-Magna ChiP A Kit (Millipore) with a modified protocol (24). ChIP reactions were set up using rabbit monoclonal AR antibody [Clone ER179(2), Epitomics] at 4°C overnight. PCR primers are listed in Supplementary Table S1.

In vivo metastasis assay

Metastatic activity was determined using 6- to 7-week-old male athymic nude mice (Ncr nu/nu) as previously described (22).

Tissue microarray analysis

Tissue microarray analyses (TMA) containing primary prostate cancer (UWTM4A48) or metastases (UWTM2A22) were constructed at the University of Washington (25). The NYU Prostate Cancer Biochemical Recurrence array, CAP6 TMA, was acquired from the Prostate Cancer Biorepository Network (PCBN; http://www.prostatebiorepository.org).

Immunohistochemical (IHC) staining conditions are described in Supplementary Table S2. The intensity scoring method for Fn14, TWEAK and p65: 0, none; 1, weak; 2, moderate/intermediate; 3, strong; and 4, strong and obscuring details. The percentages of tumor cells scored for Fn14 and TWEAK were 0, none; 1, up to 25%; 2, up to 50%; 3, up to 75%; and 4, up to 100%. Because a large number of cores contained a low percentage of nuclear p65 staining, an expanded scale was used for scoring: 0, none; 1, up to 10%; 2, up to 25%; 3, up to 50%; 4, up to 75%; and 5, up to 100%. This system yielded a 16 or 20-point combined staining score. For Fn14 and TWEAK, combined scores were <2, negative/weak; 3–6, intermediate; and 8–16, strong. For nuclear p65, combined scores were 0, negative; 1–3, weak; 4–6, intermediate; and 8–20, strong.

Clinical outcome analysis using human tumor datasets

mRNA expression data under the GEO accession numbers GSE21034 (26) and GSE35988 (27) were used. The Taylor dataset represents 151 primary and 19 metastatic tumors from patients treated by radical prostatectomy at Memorial Sloan-Kettering Cancer Center. The Grasso dataset represents a large cohort of heavily pretreated patients with lethal metastatic disease comprising cancer-matched benign prostate tissues, 59 localized prostate cancers, and 35 metastatic CRPCs. The expression data and resulting Z-scores were log2 normalized.

A previously described androgen-responsive gene signature (28) was used to determine the correlation of Fn14 expression level and AR output. This gene set was scored for each tumor by summing the expression Z-scores within the human prostate cancer cohort. Tumors were median-stratified by Fn14 expression and the mean score of androgen response signature was determined in each group.

Statistical methods

In vivo animal results, TMA analysis, and clinical outcome analysis are expressed as plots showing the median and box boundaries extending between 25th to 75th percentiles, with whiskers down to the minimum and up to the maximum value. All in vitro data are expressed as mean ± SE. Data were analyzed using Prism software (GraphPad Software, Inc.) and differences between individual groups were determined by the Student t test or ANOVA followed by Bonferroni post test for comparisons among three or more groups. P < 0.05 was considered statistically significant. Log-rank test was used for survival curve analysis. The association of Fn14 and nuclear p65 was analyzed using contingency tables and the Fisher exact test.

Results

Fn14 is required for experimental prostate cancer bone metastasis

We previously described the development of a metastatic prostate cancer xenograft model, based upon the activation of Ras effector pathways in the DU145 cell line (22). Activation of the Ras pathway is commonly observed associated with prostate cancer progression (26, 29). To enrich for bone metastatic activity, DU145 RasG37 tumor cells were isolated from three independent bone metastases and expanded in culture. Inoculation of the bone-derived clones (DU145/RasB), compared with the parental RasG37 transformed cells, demonstrated higher metastatic capacity as determined by more rapid...
development of metastasis and formation of more numerous and larger metastatic lesions (30). To determine whether changes in secreted factors or surface receptors correlated with bone metastatic capacity, supernatants from the cultured parental and bone-derived clones were assayed using a commercial antibody array designed to detect proteins within this functional class. Two of the most robust differences observed in all three bone-derived clones compared with the non-enriched parental culture were increased levels of the cytokine, TWEAK, and its cognate receptor, Fn14 (Fig. 1A). Western blot analyses performed on cell extracts confirmed high cell-associated Fn14 in bone-derived clones (Fig. 1B). To analyze the functional significance in bone metastasis development of the Fn14–TWEAK pathway, Fn14 was depleted in DU145/RasB1 cells using two different lentivirus-encoded Fn14 shRNAs (Fig. 1C). We investigated Fn14 in the development of metastasis because it is anticipated to act cell autonomously. In contrast, TWEAK, a secreted product of the microenvironment, potentially can be contributed by a nontumor source. Depletion of Fn14 had no effect on the in vitro growth rate (Supplementary Fig. S1A). Following inoculation of Fn14-depleted cells, mice developed significantly less bone (Fig. 1D) and brain metastasis (Supplementary Fig. S1C) compared with controls as measured by bioluminescent imaging at week 4 and by the percentage of mice with bone lesions as determined by histology and/or X-ray at morbidity (Table 1). Mice inoculated with Fn14-depleted cells maintained a normal body weight (Supplementary Fig. S1B) and survived longer (Fig. 1E). Morbidity in these

Figure 1. Fn14 pathway activity is necessary for experimental bone metastasis. A, TWEAK and Fn14 protein levels as determined by the protein array analysis of conditioned media from parental DU145 (P), DU145/RasG37 (G37), and three bone metastatic clones (B1, B2, and B3). B, Western blot analysis of whole-cell lysates measuring Fn14 in parental DU145(P), G37, and three bone metastatic clones (DU145/RasB1, B2, and B3). C, Western blot analysis for Fn14 in DU145/RasB1 cells infected with lentivirus vector alone (EV) or lentivirus encoding silencing shRNA directed toward Fn14 (Fn14shRNA1 or Fn14shRNA2). D, bioluminescence signal of bone metastasis per mouse for mice bearing tumor cells described in C at week 4. EV: n = 9; shRNA1 and shRNA2: n = 10 per group; *P < 0.05 versus control. E, survival curve of tumor-bearing mice in the three groups from D. *P < 0.05 versus EV. F, the incidence of bone metastasis in mice bearing PC3/EV tumors and mice bearing PC3/Fn14 shRNA1 or two tumors. **P < 0.01 versus EV.
mice mainly was due to brain metastasis. The bone metastasis inhibitory effect was rescued by reexpression of Fn14 (Table 1), demonstrating specificity of the phenotype for Fn14 depletion. To address the generality of this finding, we depleted Fn14 in PC3 prostate cancer cells, originally isolated from a patient bone metastasis. PC3 cells express high levels of Fn14, and although depletion did not affect growth \textit{in vitro} (Supplementary Fig. S1D), it significantly inhibited bone metastasis as measured by bone metastasis development (Figure 1F), tumor burden, and survival (Supplementary Fig. S1E and S1F). Taken together, these data support a functional role for Fn14 in bone metastatic capability.

**Fn14 is expressed in a high percentage of clinical prostate cancer metastases**

To analyze the prevalence of Fn14 expression in patient samples relative to prostate cancer progression, we performed IHC using antibodies directed against Fn14 in tissue arrays containing a large number of bone and soft-tissue metastases as well as normal prostate, benign prostatic hyperplasia (BPH), and primary prostate cancer specimens (Fig. 2A). Representative images in bone metastases of either weak or strong staining, composed of membranous and cytoplasmic labeling, are shown in Supplementary Fig. S2A. A semi-quantitative scoring system of 0 to 16, reflecting intensity of staining and percentage of positive tumor cells, was used. The mean total score (Fig. 2B) as well as individual intensity and extent (Supplementary Fig. S2B) scores of metastatic samples were significantly higher than samples in the other categories. The distribution of scores is shown in Fig. 2C, illustrating Fn14 expression in 10% of normal prostate epithelium, 25% of BPH, 40% of primary prostate cancer, and 75% of metastatic samples. The distribution of scores between bone and soft-tissue metastases was similar. We next investigated the correlation of Fn14 expression in primary prostate specimens with tumor progression, measured as increasing PSA levels with time after prostatectomy, usually an indication of recurrence outside the prostate (Fig. 2D). Patients with Fn14-positive specimens (22.5% in this array) had significantly shorter time to biochemical recurrence, consistent with published data from an independent cohort of patients (18). These data from patient populations show that Fn14 expression occurs in a significant proportion of clinical bone metastases and support the correlation in primary prostate cancer of Fn14 expression with increased aggressiveness.

An important question concerns the availability of the TWEAK ligand. TWEAK staining in tissue sections was observed across various cellular components, including the cytoplasm, membrane, and extracellular matrix. A high proportion of epithelial cells was stained in most sections, whereas the staining of stromal and immune components was variable (see Supplementary Fig. S2C for examples of staining). Semi-quantitative analyses demonstrated that relatively similar levels of TWEAK ligand are constitutively present in normal and pathologic prostate as well as metastatic tissues (Fig. 2E).

**The canonical NF\( \kappa \)B pathway reconstitutes Fn14-depleted prostate cancer metastasis**

The identification of signaling pathways that contribute to the establishment of prostate cancer bone metastasis is of therapeutic interest. Fn14 signaling activates canonical and noncanonical NF\( \kappa \)B pathways, mediated by p65/RELA and p52/RELB complexes, respectively (16). Depletion of Fn14 in DU145/RasB1 cells inhibited TWEAK-stimulated p65 and p52 nuclear translocation by more than 50% (Fig. 3A). To determine whether NF\( \kappa \)B pathways are necessary for the development of DU145/RasB1 bone metastases, we made use of a doxycycline-inducible, degradation-resistant 1kBoSR, which prevents the translocation of p65 into the nucleus and indirectly inhibits synthesis of the p52 precursor, p100 (31). Prior doxycycline induction of 1kB\( \kappa \)XSR led to inhibition of both NF\( \kappa \)B pathways following TWEAK treatment \textit{in vitro} (Fig. 3B), whereas Fn14-dependent MAPK pathways were not affected (Supplementary Fig. S3A). Figure 3C shows the experimental design for investigating the influence of NF\( \kappa \)B signaling during metastasis development. 1kB0SR was induced either from the time of tumor cell inoculation into the arterial circulation (day 0) or after micrometastases had been established (day 14). Expression of 1kBoSR beginning at either day 0 or 14 led to significantly less metastases after 4 weeks as compared with control mice (Fig. 3D), although 1kB0SR had a relatively less dramatic effect on \textit{in vitro} cell growth (Supplementary Fig. S3B). The inhibition of metastasis by 1kB0SR also was reflected by the maintenance of body weight (Supplementary Fig. S3C) and increased survival (Fig. 3E). These results indicate that blocking NF\( \kappa \)B even after the establishment of micrometastases inhibits clinically observable metastasis development.

### Table 1. The role of the canonical and noncanonical NF\( \kappa \)B pathway in bone metastasis development

<table>
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<tr>
<th></th>
<th>DU145/RasB1 EV</th>
<th>DU145/RasB1 Fn14KD EV</th>
<th>DU145/RasB1 Fn14KD</th>
<th>DU145/RasB1 FnIKKBA177E/S181E</th>
<th>DU145/RasB1 Fn14KD NIK( \kappa )T3</th>
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<td>Bone metastasis</td>
<td>14/18 (78%)</td>
<td>5/19 (26.3%)</td>
<td>12/19 (63.2%)</td>
<td>17/18 (94.4%)</td>
<td>5/16 (31.2%)</td>
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<td>Vs. Fn14KD</td>
<td>*<strong>, P &lt; 0.01</strong></td>
<td><strong>, P &lt; 0.05</strong></td>
<td><strong>, P &lt; 0.05</strong></td>
<td><strong>, P &lt; 0.05</strong></td>
<td>NS</td>
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NOTE: Shown are the number and percentage of mice that developed bone metastasis. Fn14-depleted DU145/RasB1 (Fn14KD) were super-infected with lentiviruses expressing the indicated genes. Mice were euthanized at morbidity, between 6 to 9 weeks after tumor cell inoculation. Abbreviation: NS, nonsignificant.
To determine whether either NFκB pathway individually is sufficient for replacing the Fn14-dependent signal leading to bone metastasis, we used a rescue approach in Fn14-depleted cells. A constitutively active form of IKKβ, IKKβS177E,S181E, was used to reconstitute the p50/p65 pathway, and a truncated form of NIK lacking the TRAF3 binding site (NIKΔT3) was used to rescue the p52/RELB pathway (Supplementary Fig. S3D; ref. 23). As shown in Fig. 3F, IKKβS177E,S181E expressing Fn14-depleted cells demonstrated elevated levels of nuclear p65 with or without TWEAK treatment, whereas control cells demonstrated nondetectable nuclear p65. Similarly, NIKΔT3-expressing Fn14-depleted cells showed increased p52 nuclear translocation independent of TWEAK treatment (Fig. 3F). Table 1 summarizes the bone metastatic capacity in each group, demonstrating that NFκB activation is sufficient to reconstitute Fn14-dependent metastasis. The bone metastasis phenotype was reconstituted in Fn14-depleted cells by IKKβS177E,S181E expression, whereas NIKΔT3 expression alone appeared to be insufficient, suggesting a greater contribution by the p65 pathway. Taken together, these data show that NFκB activation is necessary and sufficient for the development of Fn14-dependent bone metastasis.

Nuclear NFκB p65 is observed in clinical prostate cancer metastases

To determine the prevalence of the canonical NFκB pathway in prostate cancer primary tumors and metastases, tissue arrays previously characterized for Fn14 labeling were stained with an anti-total p65 antibody. Labeling was observed both in the nuclear and cytoplasmic compartments. Nuclear labeling of prostate epithelial cells marked activation of the NFκB pathway. Representative images of total p65 labeling in normal prostate and benign and malignant prostate pathologies are shown in Fig. 4A. A semi-quantitative scoring system of 0

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**Figure 2.** Expression of Fn14 in normal and pathologic prostate tissues. A, representative histologic images depicting normal prostate, BPH, prostate cancer (PCa), and bone metastasis stained for Fn14. B, plots summarizing the Fn14 scores in different groups of clinical samples quantified for UWTMA22 and UWTMA48. The number of patient tissue samples is indicated. *, P < 0.05; ***, P < 0.001. C, percentage of tissue samples with negative/weak (0–2, open bar), intermediate (3–6, gray bar), or high (≥8, black bar) Fn14 expression from B. The shaded sections of the bars in the metastasis group represent soft-tissue metastasis, whereas the solid sections represent bone metastasis. D, Kaplan-Meier curve of biochemical recurrence rate (BCR) in Fn14-positive (≥3) and Fn14-negative (<2) patient populations from CAP6 TMA. *, P < 0.05 with log-rank test. E, average scores for TWEAK staining in epithelium in UWTMA22 and UWTMA48. n = 27 (normal), 28 (BPH), 51 (PCa), and 75 (metastasis).
(negative) to 20 (extensive, strong labeling), reflecting a combination of staining intensity and the percentage of positive cells, was used to examine nuclear total-p65 expression across cores. Cores were marked as negative if less than 1% of the prostate epithelial cells were positive for nuclear NFκB p65 staining.

Primary prostate tumors and metastases had a statistically significant increase in the combined values of intensity and percentage positivity compared with normal prostate or BPH samples (Fig. 4B & C). The relatively low overall scores are due to the majority of samples demonstrating a low overall frequency of nuclear staining, consistent with another large-scale study (32). This may be the result of transient p65 nuclear localization secondary to robust negative feedback mechanisms, which have been described for the p50/p65 pathway (33). Interestingly, the categorization of staining intensities for samples with 1% to 10% positive cells, which fall into the overall weak/intermediate categories, demonstrated a higher percentage of intense staining in metastatic samples (Fig. 4D), suggesting relatively increased signaling in advanced disease samples. Notably, we also observed a significant association between Fn14 and nuclear p65 staining in metastatic prostate cancer specimens (Fig. 4E), suggesting that Fn14 signaling leads to NFκB activation in clinical disease.

Fn14 expression is inversely correlated with AR status

One clinically important property of tumor cells is AR transcriptional output, which can be heterogeneous within...
and between tumors (4, 14). We observed that Fn14 and TWEAK expression were significantly and highly expressed in AR-negative compared with AR-positive prostate cancer cell lines (Fig. 5A), in agreement with published results (18). To determine whether AR signaling was able to influence Fn14 or TWEAK expression in prostate cancer cells, we overexpressed AR in PC3 and DU145 cells using a doxycycline-regulated system. As shown in Fig. 5B, when AR is induced in DU145/RasB1 cells, Fn14 and TWEAK RNA levels were decreased after DHT treatment, whereas the AR antagonist MDV3100 reduced this effect. Consistent with this finding, Fn14 protein levels were also reduced by the presence of AR (Fig. 5C). In LAPC4 cells, Fn14 protein levels were regulated by endogenous AR in the anticipated pattern, demonstrating decreased and increased levels with DHT and MDV3100 treatment, respectively (Fig. 5D).

To interrogate Fn14 and TWEAK expression in patient populations, we analyzed a microarray dataset from Memorial Sloan-Kettering Cancer Center composed of 131 primary and 19 metastatic prostate cancer samples (26). Because TWEAK and Fn14 expression appeared to be regulated approximately in parallel in cell lines, we analyzed the relative expression of the two genes in individual patient samples. Interestingly, Fn14 and TWEAK RNA levels in individual patients revealed a significant correlation ($R = 0.59; P < 0.0001$; Fig. 6A). TWEAK protein levels tend to be constitutively present, whereas Fn14

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**Figure 4.** NFκB activation in prostate cancer clinical samples. **A**, representative images and (scores) of tissue cores, including normal prostate tissue (0), BPH (8), primary prostate cancer (4: UWTMA 48), and bone metastasis (3: UWTMA22) stained for p65. In the bone metastasis core, the cytoplasmic staining is strong, whereas nuclear staining is <10%. **B**, plots summarizing the p65 scores in different groups of patient samples. The numbers of patient tissue samples in each group are: normal (29), BPH (28), primary prostate cancer (55), and metastasis (115). $^* P < 0.01$ versus normal; $^{**} P < 0.01$; $^{***} P < 0.001$ versus BPH. **C**, percentage of TMA tissue samples with negative, weak (1–3), intermediate (4–6), or high (≥6) nuclear p65 staining from B. **D**, tissue samples from B with 1% to 10% of cell nuclear p65 positive were further analyzed for staining intensity. Shown is the distribution of the selected samples for staining intensity with weak (1), intermediate/moderate (2), and strong (3) intensity. **E**, distribution of Fn14 (positive = ≥3) and nuclear p65 (positive = ≥1) expression in metastatic samples from B ($n = 103$). $P < 0.05$. 

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**Figure 5.** **A**, representative images of AR-negative (AR-N) versus AR-positive (AR-P) prostate cancer cell lines stained for Fn14 and TWEAK. **B**, RNA expression levels of Fn14 in DU145 cells following induction of AR. **C**, protein expression levels of Fn14 in DU145 cells following induction of AR. **D**, RNA expression levels of TWEAK in DU145 cells following induction of AR. **E**, protein expression levels of TWEAK in DU145 cells following induction of AR.

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protein levels increase with progression (Fig. 2), implying that there may be different posttranscriptional regulation of TWEAK and Fn14. We then analyzed AR pathway activation relative to Fn14 mean-stratified expression by using a previously reported mRNA signature of AR target genes (28), which demonstrated an inverse relationship between Fn14 expression and AR transcriptional output (Fig. 6B). This finding was validated in an independent dataset (27) for a cohort of CRPC samples (Supplementary Fig. S4A). The Fn14-high samples demonstrated more variability in the AR activity signature than the Fn14-low samples, suggesting more heterogeneity within Fn14-high prostate cancers.

**AR downregulates the transcription of TWEAK and Fn14**

To analyze whether AR directly regulates the transcription of Fn14 and TWEAK, potential androgen receptor response elements (ARE) were mapped in the TWEAK and Fn14 promoter/enhancer regions (Fig. 6C). LNCaP cells, which express endogenous AR, were used to perform ChIP assays with anti-AR antibodies, which confirmed AR binding to two AREs (A1 and A3) in TWEAK and two AREs (A5 and A7) in Fn14 upstream regions (Fig. 6D). Similar ChIP results were obtained in doxycycline-induced AR-expressing DU145 cells (Supplementary Fig. S4B). In LNCaP, binding sites for FOXA1, an AR complex cofactor, were detected at the same positions as AR

![Figure 5. AR downregulates TWEAK and Fn14 expression.](image)

A. RT-PCR analysis for Fn14 and TWEAK in two AR-positive prostate cancer cell lines, LNCaP and 22RV1, and two AR-negative prostate cancer cell lines, PC3 and DU145. B. RT-PCR analysis for AR, TWEAK, and Fn14 in DU145/RasB1 cells expressing Tet-on TA and TREG3-AR with or without prior doxycycline induction for 2 days and subsequently treated with DHT (5 nmol/L) or MDV3100 (10 μmol/L) for 2 days. C. Western blot analysis of whole-cell lysates for AR and Fn14 protein levels following doxycycline induction of AR in the indicated cells. Cells were treated or untreated with DHT (5 nmol/L) for 2 days. D. Western blot analysis of Fn14 protein level in LAPC4 treated with DHT (5 nmol/L) or MDV3100 (10 μmol/L) in charcoal-stripped serum for 2 days.
To determine whether AR activation was able to directly regulate TWEAK and Fn14 transcription, the TWEAK upstream region between −4,006 and −2,183 and the Fn14 upstream region between −2,434 and +1 were separately cloned into an RFP reporter construct and transfected into LNCaP cells. The two reporters demonstrated approximately similar activity patterns. AR transcriptional activation with DHT suppressed reporter activity, whereas suppression of basal AR transcription with the AR antagonist, MDV3100, induced reporter activity (Fig. 6E). Mutations in the defined AREs, especially TWEAK A1 and Fn14 A7, decreased DHT-dependent suppression and MDV-dependent induction relative to no treatment, demonstrating AR-mediated effects. Consistent with the results in LNCaP, AR-mediated suppression of reporter activity also was observed upon doxycycline induction in DU145 cells (Supplementary Fig. S4C). Taken together, these data suggest that AR binding to Fn14 and TWEAK upstream regions is transcriptionally suppressive.

Figure 6. AR downregulates Fn14 and TWEAK transcription in prostate cancer cells. A, correlation analysis of TWEAK and Fn14 expression levels in the MSKCC prostate cancer dataset. r = 0.63; P < 0.001. B, AR activity signature genes expressed as a summed Z-score for samples separated on the basis of Fn14 expression above the median (Fn14 high) or below the median (Fn14 low) in the MSKCC dataset. *** P < 0.001. C, schematic of predicted AREs in the Fn14 and TWEAK promoter regions. D, qChIP analysis of AR and FOXA1 binding to predicted AREs in the TWEAK and Fn14 promoters/enhancers illustrated in C. LNCaP cells were maintained in DHT-containing media. The binding activity of each protein to each site is given as a percentage of total input then normalized to individual immunoglobulin G (IgG) background. E, TWEAK and Fn14 promoter reporter activity as measured by relative median fluorescence intensity (MFI) in LNCaP cells. Cells were treated with vehicle (Veh) or DHT (5 nmol/L) or MDV3100 (10 μmol/L) for 72 hours. Mutant TWEAK (mutant A1, M1; mutant A3, M3) and Fn14 (mutant A5, M5; mutant A7, M7) promoter reporters were used as controls. *, P < 0.05 versus Veh; #, P < 0.05 versus DHT. Data represent the mean ± SEM.
Because higher Fn14 and TWEAK RNA levels in patient samples correlate with lower AR signature gene expression, Fn14 and TWEAK may represent genes expressed in association with overall lower AR activity or altered AR specificity.

Discussion

Although bone metastasis is a primary reason for morbidity and mortality in patients with progressive prostate cancer, relatively little is known about the mechanisms responsible for establishing and expanding prostate cancer bone metastases (34). Using experimental models in addition to molecular and histologic analyses of clinical samples, we present evidence that Fn14 is one determinant of prostate cancer bone metastatic capacity. Fn14 staining also has been observed in about 50% of clinical breast cancer bone metastases, although the number of samples analyzed was small (35).

Of particular interest, Fn14 expression suggests a mechanism link between prostate cancer bone metastasis and inflammation as the TWEAK–Fn14 axis promotes local inflammation (15). An inflammatory microenvironment in the bone contributes to the so-called “vicious cycle” mediated by interacting stromal, immune, and tumor elements and resulting in bone remodeling and enhanced tumor cell survival and proliferation (36). From the data presented here, it seems that Fn14-expressing prostate cancer cells often produce autocrine TWEAK in addition to TWEAK secretion from infiltrating inflammatory cells. The bone metastasis model used in this study identifies the Fn14 pathway as one upstream regulator of NFκB signaling in prostate cancer, and shows that the canonical NFκB pathway is sufficient to replace Fn14 downstream signaling in this context. Fn14-mediated responses are usually proinflammatory as a result of NFκB induction of inflammatory proteins, including cytokines, chemokines, adhesion molecules, and metalloproteases (37). Thus, Fn14-dependent tumor responses are anticipated to increase inflammatory cell infiltration and activation. In addition, the Fn14–TWEAK axis has been shown to autonomously regulate tumor cells with respect to survival, proliferation, migration, and progenitor expansion (15, 16). Thus, both autonomous and nonautonomous Fn14-mediated functions may contribute to prostate cancer bone metastasis.

Our data are consistent with various preclinical models that support a role for NFκB in prostate cancer metastasis. An alternative mechanism of NFκB activation, loss of the DAB2IP tumor suppressor, leading to experimental prostate cancer soft-tissue metastasis has been described (38). Of interest, coordinated RAS and NFκB activation occur in the DAB2IP model and in the model investigated here. DAB2IP loss regulates both RAS and NFκB activation, whereas the DU145/Ras model expresses constitutive RAS activation in addition to Fn14-regulated NFκB. It remains an open question as to how directly or indirectly RAS signaling is associated with Fn14 and TWEAK expression in prostate cancer. In another study, the manipulation of NFκB pathway components in human prostate cancer cell lines demonstrated that NFκB activation is necessary and sufficient for tumor cell growth following intra-tibial injections (39). Finally, a role for the NFκB pathway has been implicated in models of castration-resistant growth (40, 41); castration resistance strongly correlates clinically with the development of metastasis (12, 13).

The increased presence of NFκB signaling in tumor tissue relative to normal tissue and a positive correlation with Fn14 expression was confirmed with IHC staining for nuclear p65 (Fig. 4). The low frequency of positive cells may reflect the normally transient nature of p50/p65 signaling, and are consistent with a recent study showing a significant association of even low numbers of nuclear p65 positive cells with higher Gleason score, increased biochemical recurrence, and the development of metastases (32, 42, 43).

Fn14 expression is regulated by a variety of extracellular stimuli, including growth factors and hormones, although the molecular mechanisms of such regulation are mostly unknown (16). As shown here, Fn14 expression seems to increase in prostate cancer cells in which AR activity is suppressed, intrinsically low, or shifted in specificity. We describe an AR-dependent, negative regulatory component influencing TWEAK and Fn14 expression in prostate cancer cells. (Fig. 6). The ability to identify prostate cancer cells with a modified AR response, which are anticipated to be less sensitive to androgen deprivation therapy (ADT), is of potential utility considering the inter- and intratumoral heterogeneity of prostate cancer (4–6). Thus, an Fn14-targeted agent may permit the imaging and/or therapeutic treatment of metastatic tumors, especially cells that escape first-line ADT due to modified AR signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Yin, Y.-N. Liu, H. Tillman, B. Barrett, S. Hewitt, L. Fang, E. Corey
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