

The Fibroblast Growth Factor–Inducible 14 Receptor Is Highly Expressed in HER2-Positive Breast Tumors and Regulates Breast Cancer Cell Invasive Capacity

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Abstract

Genomic characterization is beginning to define a molecular taxonomy for breast cancer; however, the molecular basis of invasion and metastasis remains poorly understood. We report a pivotal role for the fibroblast growth factor–inducible 14 (Fn14) receptor in this process. We examined whether Fn14 and its ligand tumor necrosis factor–like weak inducer of apoptosis (TWEAK) were expressed in breast tumors and whether deregulation of Fn14 levels affected malignant behavior of breast cancer cell lines. Analysis of TWEAK and Fn14 in publicly available gene expression data indicated that high Fn14 expression levels significantly correlated with several poor prognostic indicators ($P < 0.05$). Fn14 expression was highest in the HER2-positive/estrogen receptor–negative (HER2⁺/ER⁻) intrinsic subtype ($P = 0.0008$). An association between Fn14 and HER2 expression in breast tumors was confirmed by immunohistochemistry. Fn14 levels were elevated in invasive, ER⁻ breast cancer cell lines. Overexpression of Fn14 in weakly invasive MCF7 and T47D cells resulted in a marked induction of invasion and activation of nuclear factor- κ B (NF- κ B) signaling. Ectopic expression of Fn14tCT, a Fn14 deletion mutant that cannot activate NF- κ B signaling, was not able to induce invasion. Moreover, ectopic expression of Fn14tCT in highly invasive MDA-MB-231

cells reduced their invasive capability. RNA interference–mediated inhibition of Fn14 expression in both MDA-MB-231 and MDA-MB-436 cells reduced invasion. Expression profiling of the Fn14-depleted cells revealed deregulation of NF- κ B activity. Our findings support a role for Fn14-mediated NF- κ B pathway activation in breast tumor invasion and metastasis. (Mol Cancer Res 2008;6(5):725–34)

Introduction

Breast cancer is the most commonly diagnosed malignancy in Western women. Metastasis of primary breast tumor cells through the blood or lymphatic system to distant organs is responsible for the majority of breast cancer deaths (1). Breast tumorigenesis and progression involves the breakdown of epithelial cell architecture as the cells transition to poorly differentiated malignant phenotypes (2). Effective tumor cell metastasis requires sequential completion of a complex, multistep process, which includes proteolysis of the extracellular matrix, cell migration and invasion, intravasation, and increased cell survival (3, 4). Although numerous proteins have been identified as regulators of breast tumor invasive and metastatic capacity (5, 6), the identification of additional proinvasive and/or prometastatic molecules, especially in early lesions, is required to further define the malignant cell phenotype.

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a multifunctional cytokine implicated in the pathogenesis of various human diseases, including cancer (7, 8). TWEAK acts on cells via binding to the TNF receptor superfamily member fibroblast growth factor–inducible 14 (Fn14; ref. 9). TWEAK and Fn14 may each play a role in tumor progression. Specifically, TWEAK is a potential tumor angiogenesis factor (10) and may also promote tumor cell migration (11) and survival (12). Additionally, Fn14, which is overexpressed in advanced esophageal (13, 14) and brain (11, 15) tumors, may regulate tumor cell motility (14, 15). The effects of TWEAK-Fn14 signaling in breast cancer have not yet been reported. Fn14 gene expression was shown to be elevated in breast tumor specimens when compared with normal breast tissue (16); however, this study did not assess stratification of Fn14 expression relative to breast tumor biological subtype or

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clinical information. Here, we report for the first time a significant correlation between Fn14 and HER2 overexpression in breast tumors. We also show that Fn14 has proinvasive activity in biologically diverse breast cancer cell lines and that Fn14-mediated invasion requires nuclear factor- κ B (NF- κ B) activation. Our novel findings indicate that the Fn14 receptor is a regulator of breast cancer cell invasive capacity in multiple biological contexts and thus a potential therapeutic target for breast cancer.

Results

High Fn14 mRNA Expression in Patient Tumor Samples Correlates with Clinical Indicators of Poor Prognosis and the HER2-Positive/Estrogen Receptor-Negative Intrinsic Subtype

We conducted an analysis of TWEAK and Fn14 mRNA expression in the Netherlands Cancer Institute microarray data set derived from 295 primary breast tumors (17). Comprehensive clinical diagnostic and prognostic variables are available for the tumors in this data set, as well as intrinsic subtype classification annotation (18). We first examined whether TWEAK or Fn14 mRNA expression levels correlated with clinical prognostic indicators. High TWEAK expression showed a statistically significant association with metastasis and ER⁻ status (Table 1). High Fn14 expression positively correlated with metastasis, more than four positive lymph nodes, and ER⁻ status. Low Fn14 expression correlated with lymph node-negative disease and "well-differentiated" tumors, both indicative of good prognosis. Interestingly, when the same analysis was conducted on patients who did not receive chemotherapy, elevated Fn14 expression correlated more strongly with metastasis.

We then examined TWEAK and Fn14 mRNA expression levels with respect to the five intrinsic subtypes previously described by Sorlie et al. (19), namely, basal-like, HER2-positive/estrogen receptor-negative (HER2⁺/ER⁻), normal breast-like, luminal A, and luminal B. TWEAK expression

levels were not significantly associated with any specific tumor subtypes (data not shown). However, high levels of Fn14 expression were observed in the HER2⁺/ER⁻ subtype and this positive association was highly statistically significant ($P = 0.0008$; Fig. 1A).

The Fn14 and HER2 Receptors Are Frequently Coexpressed in Breast Tumors

To validate our observation that Fn14 expression levels correlated with HER2 and/or ER status, we did an immunohistochemical survey on a breast tissue microarray (TMA). Expression was scorable for both proteins in 259 individual patient samples. A scoring system of 0 to 3 for Fn14 protein expression level was used along with existing immunohistochemical scoring guidelines for HER2 and ER (20-22). Fn14 protein expression was rarely detected in normal breast epithelium. Of the 193 tumors staining positive for Fn14 (score 1-3), 111 of these also stained positive for HER2 (score 1-3), a statistically significant association ($P = 0.0005$). When comparing the 193 Fn14-positive tumors with the 66 tumors with a HER2 score of 2 or 3, the association was still present ($P = 0.018$). In addition, whereas none of the ductal carcinoma *in situ* samples scored 2 or 3 for Fn14, 42% of the invasive ductal carcinoma samples stained 2 or 3 for Fn14. Analysis of samples staining negatively for Fn14 showed that 29% were ductal carcinoma *in situ*, 23% were invasive ductal carcinoma, and 55% were invasive lobular carcinoma samples. Interestingly, four of nine ductal carcinoma *in situ* samples staining strongly for HER2 (2 or 3) were also weakly Fn14 positive (score 1). Representative examples of the staining patterns we observed are shown in Fig. 1B and C.

Fn14 Expression Levels Correlate with Breast Cancer Cell ER Status, Molecular Subtype, and Invasive Potential In vitro

To begin to address whether Fn14 receptor signaling had functional implications for the malignant behavior of breast cancer cells, we first examined TWEAK and Fn14 mRNA expression levels in a selection of human breast cancer cell lines of biologically distinct subtypes and invasion/metastasis potential (23-25). Both TWEAK and Fn14 mRNA levels were relatively high in the ER⁻, highly invasive, basal-like MDA-MB-436 and MDA-MB-231 cell lines (Fig. 2A). Flow cytometric analysis confirmed that the MDA-MB-231 cells coexpress high levels of cell surface-associated TWEAK and Fn14 (Fig. 2B). We also observed that high Fn14 protein expression in breast cancer cell lines correlated with ER status and increased invasive capacity *in vitro* (Fig. 2C).

Ectopic Expression of Fn14 in MCF7 and T47D Cells Increases Invasive Capacity

We next investigated whether Fn14 expression levels could modulate breast cancer cell invasion through a Matrigel barrier *in vitro*. A replication-deficient adenoviral construct expressing myc epitope-tagged Fn14 (AdFn14) was used for high-efficiency gene transfer into the weakly invasive MCF7 and T47D cell lines (which express low endogenous levels of Fn14). Initial dose-response studies using AdFn14 infection of

Table 1. Correlation of Fn14 and TWEAK Expression Levels and Indicators of Good or Poor Prognosis in Breast Tumors

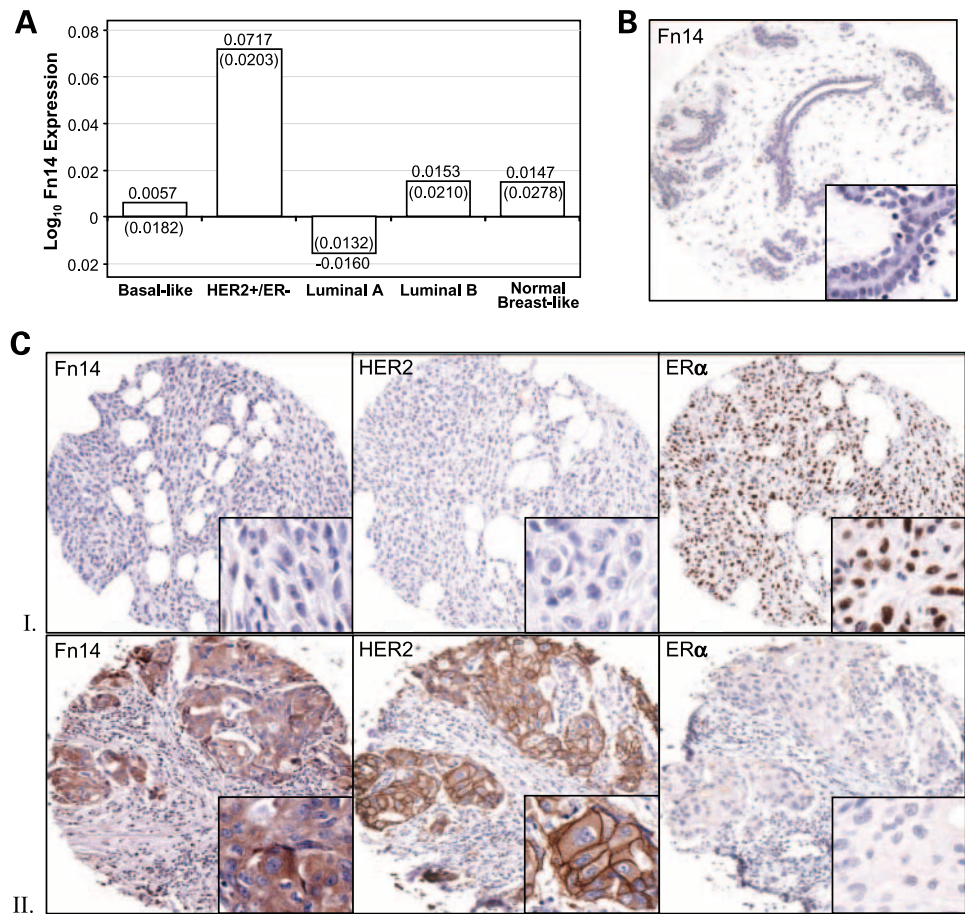
Gene	Prognostic variable	Threshold*	<i>P</i> *	Threshold [†]	<i>P</i> [†]
<i>Fn14</i>	Event metastasis	0.120	0.0058	0.120	0.0013
	Node negative	-0.140	0.0022	0.000	0.0023
	>4 positive nodes	0.100	0.0194	0.059	0.2283
	Well differentiated	-0.180	0.0242	-0.180	0.0811
	ER ⁻ status	0.160	0.0364	0.016	1.0000
	>2 cm diameter	0.120	0.0632	0.016	0.1861
<i>TWEAK</i>	Event metastasis	0.015	0.0288	0.015	0.3266
	Node negative	-0.045	0.9331	-0.066	0.5234
	>4 positive nodes	-0.077	0.2607	0.061	0.9307
	Well differentiated	-0.088	0.5077	-0.066	0.2215
	ER ⁻ status	0.084	0.0352	0.084	0.0472
	>2 cm diameter	-0.019	0.0985	0.007	1.0000

NOTE: Fisher's exact analysis of high and low Fn14 or TWEAK average expression threshold is shown (*P* values of <0.05 are shown in bold). The average expression threshold derived from expression ratios provided by van de Vijver et al. (17) was calculated as described in Materials and Methods.

*All 295 patients.

[†]Patients who did not receive chemotherapy ($n = 185$).

FIGURE 1. Elevated Fn14 expression is associated with the HER2⁺/ER⁻ breast tumor subtype. **A.** *In silico* mining of the breast cancer microarray data set from van de Vijver et al. (17) was done to examine Fn14 mRNA expression levels in different tumor subtypes. Average log₁₀ expression values are graphed with the SE in parentheses. Sample numbers for each subtype are as follows: basal-like (*n* = 53), HER2⁺/ER⁻ (*n* = 35), luminal A (*n* = 123), luminal B (*n* = 55), and normal breast-like (*n* = 29). The only statistically significant correlation detected was between high Fn14 expression and the HER2⁺/ER⁻ subtype (*P* = 0.0008). **B** and **C.** Immunohistochemical analysis of Fn14, HER2, and ER expression was conducted using a breast TMA. Fn14 antibody staining on a normal breast tissue sample is shown in **B**. In panel **C**, series I shows Fn14, HER2, and ER antibody staining on a specimen from a patient with ER⁺ invasive ductal carcinoma breast cancer. Series II shows Fn14, HER2, and ER antibody staining on a specimen from a patient with ER⁻ invasive ductal carcinoma breast cancer.



MCF7 cells indicated that Fn14 expression levels increased with increasing multiplicity of infection (MOI; Fig. 3A). This MOI range did not induce cytotoxicity (data not shown), and we chose a MOI of 20 to 40 for subsequent experiments. MCF7 and T47D cells were infected with either AdFn14 or an adenovirus encoding the bacterial β-galactosidase protein (AdLacZ). Uninfected, vehicle-treated cells were used as a negative control, and 12-*O*-tetradecanoylphorbol-13-acetate-treated cells served as our positive control (26) for induction of invasion. Infection with AdFn14, but not AdLacZ, promoted an ~4-fold increase in MCF7 cell invasive capacity and an ~6-fold increase in T47D cell invasive capacity (Fig. 3B).

The Fn14 TNF Receptor-Associated Factor-Binding Site Is Required for Fn14-Triggered MCF7 Cell Invasion

The TNF receptor-associated factors (TRAF) are a family of adaptor molecules involved in TNF receptor superfamily signaling (27). Four different TRAFs are able to bind the Fn14 cytoplasmic tail, as determined using *in vitro* binding or yeast two-hybrid approaches, and site-specific mutagenesis studies have identified the Fn14 TRAF-binding site (28, 29). Mutated receptors missing this site are unable to elicit a cellular response (12, 15, 28, 30). To investigate whether Fn14-stimulated invasive activity requires TRAF association, MCF7 cells were infected with either AdFn14, AdLacZ, or an adenovirus encoding a Fn14 cytoplasmic tail deletion mutant

missing the TRAF-binding site (AdFn14tCT; ref. 12). Western blot analysis confirmed Fn14 expression in the infected cells (Fig. 3C). Ectopic expression of Fn14 increased MCF7 invasiveness by ~5-fold, whereas Fn14tCT expression had no significant effect on invasive activity (Fig. 3D).

Ectopic Expression of Fn14tCT in MDA-MB-231 Cells Reduces Invasive Capacity

The CD40 receptor, like Fn14, is a member of the TNF receptor superfamily. A previous study showed that ectopic expression of a CD40 mutant receptor lacking TRAF-binding sites can act as a dominant-negative inhibitor of wild-type CD40 function (31). In consideration of these findings, we hypothesized that Fn14tCT overexpression in breast cancer cells expressing high endogenous levels of Fn14 might alter their invasive capacity. To test this hypothesis, highly invasive MDA-MB-231 cells were either left uninfected or infected with AdLacZ or AdFn14tCT and then cellular invasion was measured. Although AdLacZ infection inhibited MDA-MB-231 invasion by ~15%, AdFn14tCT infection resulted in a greater inhibitory effect at the same MOI (48% decrease). Additionally, an ~70% decrease in invasion was observed at an AdFn14tCT infection MOI of 40 (Fig. 4A). These results indicate that overexpression of Fn14tCT can interfere with endogenous Fn14-mediated invasion.

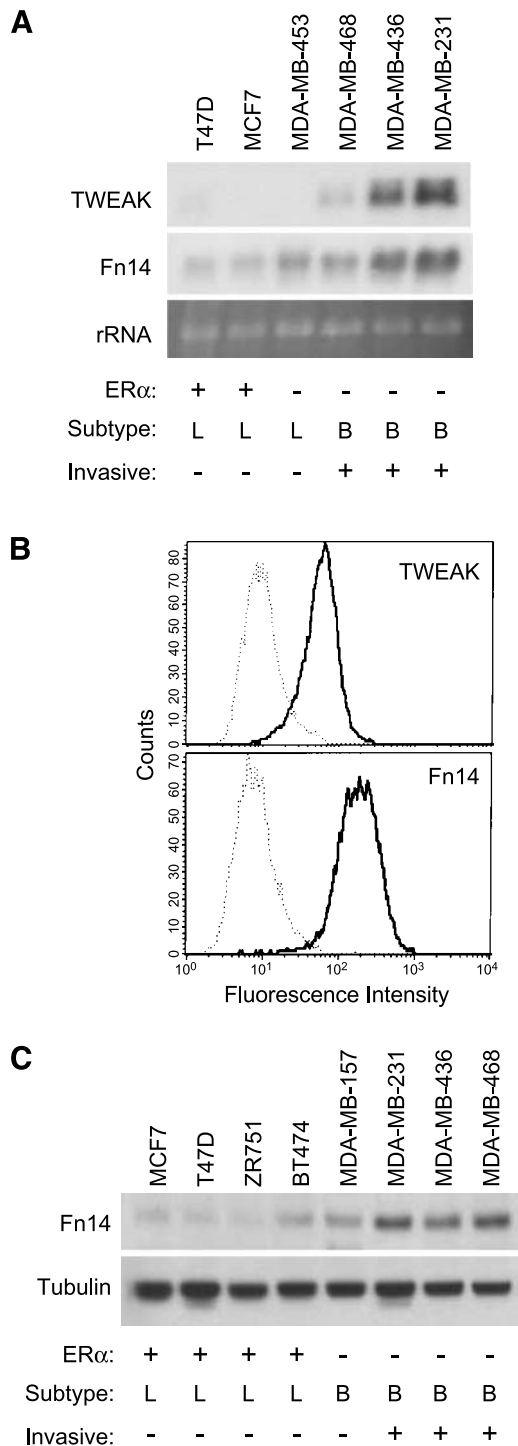


FIGURE 2. Fn14 and TWEAK expression levels correlate with breast cancer cell invasive capacity. **A.** Total RNA was isolated from the indicated cell lines and equal amounts of each sample were analyzed by Northern blot hybridization using radiolabeled TWEAK and Fn14 cDNA probes. A photograph of the 28S rRNA band shows that equal amounts of RNA were present in each gel lane. L, luminal; B, basal. **B.** MDA-MB-231 cells were stained with phycoerythrin-labeled anti-TWEAK antibody (*top, dark line*), anti-Fn14 antibody (*bottom, dark line*), or isotype control IgG (*top and bottom, light line*) and then analyzed by flow cytometry. **C.** Cell lysates were prepared from the indicated cell lines and equal amounts of protein were subjected to SDS-PAGE and Western blot analysis using anti-Fn14 and anti-tubulin antibodies.

Fn14 Depletion by RNA Interference in MDA-MB-231 and MDA-MB-436 Cells Reduces Invasive Capacity

We next examined the effects of either transient or stable Fn14 knockdown in the highly invasive MDA-MB-231 and MDA-MB-436 breast cancer cell lines using either Fn14-targeted small interfering RNA (siRNA) or short hairpin RNA (shRNA) molecules. In the first set of experiments, the MDA-MB-231 and MDA-MB-436 cell lines were transfected with either Fn14 siRNA or firefly luciferase (GL2) control siRNA. Western blot analysis confirmed effective Fn14 protein depletion (Fig. 4B, *top*). Fn14-depleted cells were less invasive than cells transfected with GL2 siRNA; specifically, Fn14 siRNA transfection inhibited MDA-MB-231 cell invasiveness by ~58% and MDA-MB-436 cell invasiveness by ~78% (Fig. 4B, *bottom*). Cell proliferation was not affected by Fn14 knockdown in either cell line (data not shown). In a second set of experiments, we determined whether stably transfected MDA-MB-231 cell lines with reduced endogenous Fn14 expression levels also displayed attenuated invasive potential. Here, MDA-MB-231 cells were transfected with either a Fn14 shRNA-producing plasmid designed to inhibit endogenous Fn14 expression or a nonsilencing shRNA-producing control plasmid and clonal cell lines were isolated. Two control lines (C4 and C12) and two experimental lines exhibiting minimal Fn14 expression (F1 and F4; Fig. 4C, *top*) were used in invasion assays. Although there was some clonal variability between the two control and two experimental lines with respect to invasive capacity, the MDA-MB-231 control lines that expressed endogenous Fn14 were more invasive than the Fn14-deficient lines (Fig. 4C, *bottom*).

Gene Expression Profiling of Breast Cancer Cell Lines following Fn14 Depletion Indicates Disruption of the NF- κ B Signaling Pathway

We did gene expression profiling on the MDA-MB-231 and MDA-MB-436 cell lines following siRNA-mediated inhibition of Fn14 expression as an approach to identify the mechanistic basis for Fn14-mediated invasive capacity. We identified 161 genes that were significantly up-regulated or down-regulated in the Fn14-depleted breast cancer cell lines (Fig. 5A; Supplementary Fig. S1). We used the controlled vocabulary of the Gene Ontology (a curated hierarchy of functionally annotated genes) to categorize the 161 genes into functional groups. Several cell processes were altered with high statistical significance in response to Fn14 depletion, including cytokine- and chemokine-mediated signaling, the immune response, cell cycle control, and the response to extracellular stimuli (see Supplementary Fig. S1). Regulation of the I κ B/NF- κ B cascade was a recurring ontology with high statistical significance in our analysis, implicating the NF- κ B pathway as a likely mediator of Fn14-mediated invasion.

TWEAK Addition or Ectopic Fn14 Expression in Breast Cancer Cell Lines Can Activate the NF- κ B Pathway

TWEAK treatment of various cell types has been shown to induce NF- κ B pathway activation (7) but this has not been examined in breast cancer cell lines. Therefore, TWEAK was

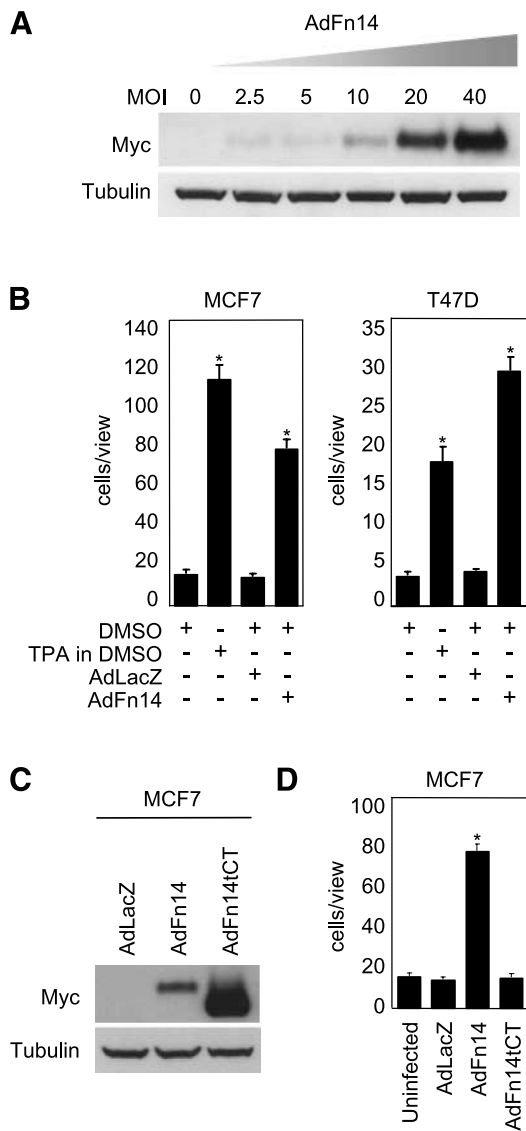


FIGURE 3. Ectopic Fn14 expression in weakly invasive breast cancer cells promotes cellular invasion, an effect requiring the Fn14 TRAF-binding site. **A.** MCF7 cells were either left uninfected or infected at the indicated MOI values with an adenoviral construct encoding myc-tagged Fn14 (AdFn14). Cell lysates were prepared and equal amounts of protein were subjected to SDS-PAGE and Western blot analysis using anti-myc and anti-tubulin antibodies. **B.** MCF7 and T47D cells were infected with AdFn14 or AdLacZ and cellular invasion was quantitated after 16 h of incubation. 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 10 nmol/L) treatment was used as a positive control for cell invasion. *, $P < 0.001$, compared with vehicle (100% DMSO) or AdLacZ control. **C.** MCF7 cells were infected with AdLacZ, AdFn14, or an adenovirus encoding a myc-tagged Fn14 protein with a truncated cytoplasmic tail lacking the Fn14 TRAF-binding site (AdFn14tCT). Equal amounts of protein were subjected to SDS-PAGE and Western blot analysis using anti-myc and anti-tubulin antibodies. **D.** MCF7 cells were either left uninfected or infected with the indicated adenoviruses and invasion assays were conducted as above. *, $P < 0.001$, compared with AdLacZ control.

added to serum-starved MDA-MB-231 and MDA-MB-436 breast cancer cells, the cells were harvested at several time points, and Western blot analysis was conducted to monitor I κ B α phosphorylation, an indicator of NF- κ B pathway activation. We observed transient phosphorylation of I κ B α in

both cell lines (Fig. 5B). Additionally, an increased level of total I κ B α was noted at the 60-min time point, consistent with prior studies indicating that the I κ B α gene is itself a target of NF- κ B (32). Because it has been reported that ectopic Fn14 expression in NIH3T3 cells (28) and HEK293 cells (29) leads to NF- κ B activation, we investigated whether this could also occur in MCF7 cells. The cells were infected with either AdLacZ or AdFn14 and harvested at several time points consistent with early induction of invasion. We detected higher levels of phosphorylated I κ B α in the AdFn14-infected cells in comparison with the AdLacZ-infected cells by Western blot analysis (Fig. 5C), indicating that Fn14 overexpression in breast cancer cells can stimulate NF- κ B activation.

Fn14 Proinvasive Activity Requires NF- κ B

Finally, we sought to functionally validate whether Fn14-mediated invasion required NF- κ B activation. MCF7 cells were infected with either AdLacZ alone or AdFn14 alone or in the presence of either SN50, a synthetic peptide that inhibits NF- κ B-mediated transcription (33), or SN50M, a negative control peptide. Cell invasion assays were then conducted *in vitro*. We found that SN50 completely inhibited Fn14-stimulated invasion (Fig. 5D), whereas SN50M had no effect. These results indicate that NF- κ B activation plays a pivotal role in Fn14-mediated invasion of breast cancer cells.

Discussion

This study is the first to report a significant association between high levels of Fn14 expression in human breast tumors with poor prognostic indicators and the HER2⁺/ER⁻ intrinsic subtype. Additionally, we report for the first time that Fn14 expression levels have a significant effect on breast cancer cell invasive capacity. Specifically, we show that ectopic expression of wild-type Fn14 can stimulate breast cancer cell invasion through Matrigel *in vitro*, whereas ectopic expression of a dominant-negative Fn14 mutant or depletion of endogenous Fn14 by RNA interference inhibits invasion. Additionally, we found that TWEAK addition or ectopic Fn14 expression in breast cancer cells activates NF- κ B signaling and that Fn14-stimulated cell invasion requires NF- κ B function.

TWEAK gene expression has been detected in numerous tumor types, but in some cases, the expression level is similar or even lower than the corresponding nondiseased tissue (7). In contrast, elevated Fn14 expression has been consistently detected in several human tumor types including liver, esophageal, and brain tumors (7). In a previous report, Fn14 overexpression was detected in a panel of breast tumor specimens; however, no stratification of breast tumor subtype or clinical information was provided (16). Here, we conducted *in silico* analysis of TWEAK and Fn14 mRNA expression levels in an annotated microarray data set derived from 295 stage I and II breast cancer patients (17, 18). We found that high TWEAK and Fn14 expression were both associated with metastasis and ER⁻ status, two indicators of poor prognosis. High Fn14 expression was also associated with four or more positive lymph nodes, another indicator of poor prognosis. A trend was also observed for high Fn14 expression and primary

tumor size. It is important to note that no tumor histology is available for this breast tumor data set; thus, the contribution of TWEAK and Fn14 expression from nonmalignant cells in these samples is unknown.

The *HER2/neu* gene, encoding a member of the epidermal growth factor receptor family of tyrosine kinases, is amplified and overexpressed in ~25% of primary breast tumors (34), with ~50% of these tumors being ER⁻ (35). HER2 overexpression, which results in constitutive kinase activation, is associated with increased metastatic potential and poor patient survival (36). HER2⁺/ER⁻ tumors are one of the five intrinsic subtypes of breast cancer identified by gene expression profiling (19). Our *in silico* analysis revealed a significant association between high Fn14 mRNA expression and the HER2⁺/ER⁻ subtype. Immunohistochemical staining of a breast TMA confirmed that the HER2 and Fn14 receptors are frequently coexpressed in breast tumors. It is not yet clear if there is a direct mechanistic link between HER2 and Fn14 gene expression levels; indeed, the HER2⁺ BT474 breast tumor cell line does not express high levels of Fn14 (Fig. 2C). It is, however, of interest to note that ectopic HER2 expression can promote NF- κ B activation (37) and HER2⁺ tumors have constitutive NF- κ B pathway activation (38). *Fn14* is a NF- κ B-inducible gene (15); thus, it is possible that NF- κ B activity may be one mechanism leading to activation of *Fn14* gene expression in HER2⁺ tumors *in vivo*. Furthermore, because ectopic *Fn14* expression can also promote NF- κ B activation (Fig. 5C), both HER2 and Fn14 signaling in breast tumors may contribute to constitutive NF- κ B pathway activation and adverse clinical consequences, such as thera-

peutic resistance (39). Although trastuzumab has proven efficacy for treatment of HER2⁺ metastatic breast cancer (40, 41), the response rate for those patients with relatively high HER2 levels is <40%, with a median duration of response between 9 and 12 months (41, 42). The correlation we have observed between Fn14 and HER2 expression suggests that new therapeutic agents targeting Fn14 or its downstream signaling mediators, either alone or in combination with HER2 or NF- κ B inhibitors, may be effective in improving the response and survival of HER2⁺ breast cancer patients.

Our breast cancer cell line survey of Fn14 expression levels is consistent with an earlier report by Michaelson et al. (16), who examined Fn14 expression in some of these same ER⁺ and ER⁻ lines. The molecular basis for differential Fn14 expression in ER⁺/weakly invasive versus ER⁻/invasive breast cancer cell lines is unknown. Although we did not observe high levels of Fn14 expression in basal-like breast tumors (Fig. 1A), we did observe constitutively high Fn14 expression in the basal-like cell lines (Fig. 2). The reason for this discrepancy is not clear; however, TWEAK can induce Fn14 expression (15), so it is possible that the high levels of TWEAK expressed by the basal-like cell lines may contribute to Fn14 overexpression. Our observations suggest that an *in situ* analysis of both TWEAK and Fn14 expression in a series of invasive breast tumors is warranted.

We found that Fn14 levels can modulate breast cancer cell invasive behavior using several experimental approaches. First, ectopic Fn14 expression in the weakly invasive MCF7 and T47D cell lines stimulates invasive capacity, and this effect only occurs if the receptor contains the Fn14 cytoplasmic tail

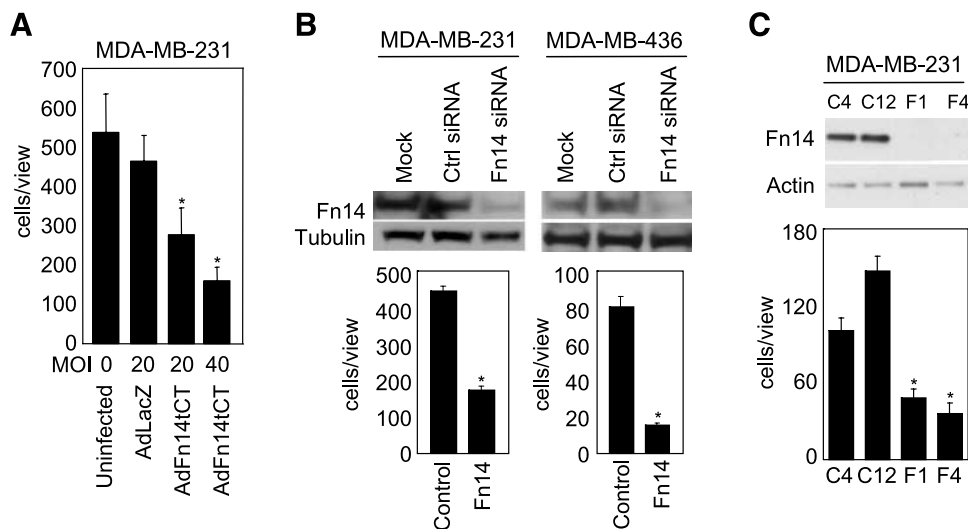


FIGURE 4. Inhibition of Fn14 signaling or expression in highly invasive breast cancer cells reduces cellular invasion. **A**, MDA-MB-231 cells were left uninfected or infected with either AdLacZ or AdFn14tCT at the indicated MOI. At 8 h after infection, the cells were plated into Boyden chambers and invasion was quantitated after 8 h of incubation. *, $P < 0.001$, compared with AdLacZ control. **B**, MDA-MB-231 and MDA-MB-436 cells were transfected with the transfection reagent alone (mock) or this reagent combined with equal concentrations of either GL2 luciferase control siRNA or Fn14-specific siRNA. Top, following a 48-h incubation, cells were harvested for plating into invasion chambers and the remaining cells were collected for Western blot analysis using anti-Fn14 and anti-tubulin antibodies. Bottom, cellular invasion was quantitated after 8 h (MDA-MB-231 cells) or 16 h (MDA-MB-436 cells) of incubation. Two separate Fn14 siRNAs gave very similar results (data for Fn14i-475 are shown). *, $P < 0.001$, compared with control siRNA. **C**, Top, MDA-MB-231 clonal cell lines stably transfected with control (C4 and C12) or Fn14-specific (F1 and F4) shRNA plasmids were harvested and equal amounts of protein were subjected to SDS-PAGE and Western blot analysis using anti-Fn14 and anti-actin antibodies. The invasive capacity of the four stable cell lines was assessed by Boyden chamber invasion assay. Bottom, cellular invasion was quantitated after 4 h of incubation. *, $P < 0.001$, compared with average invasion observed for C4 and C12 cell lines.

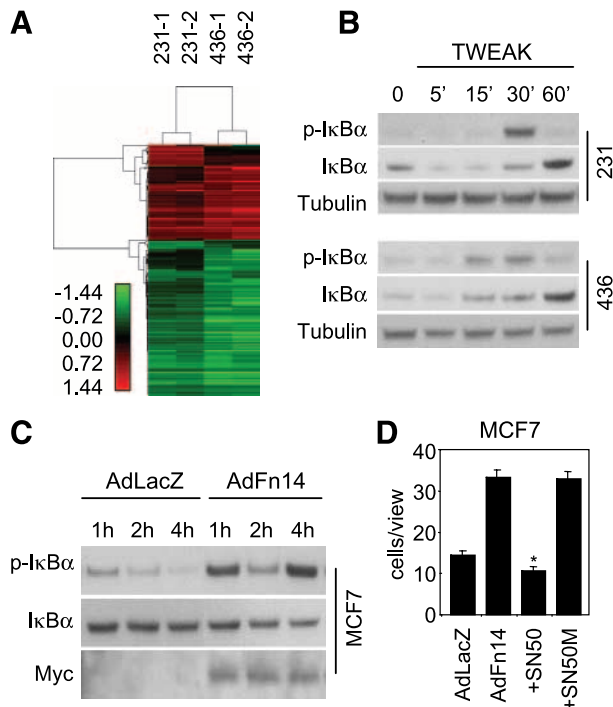


FIGURE 5. TWEAK-Fn14 signaling in breast cancer cells activates the NF- κ B pathway, and this pathway is critical for Fn14-triggered breast cancer cell invasion. **A.** Two-dimensional hierarchical clustering dendrogram of 161 genes significantly regulated following siRNA-mediated Fn14 depletion ($P < 0.001$) in MDA-MB-231 and MDA-MB-436 breast tumor lines. Duplicate arrays are biological replicates. **B.** MDA-MB-231 and MDA-MB-436 cells were incubated in reduced serum conditions for 16 h and then treated with TWEAK (100 nmol/L) for the indicated lengths of time. Cell lysates were prepared and equal amounts of protein were subjected to Western blot analysis using anti-phosphorylated I κ B α (p-I κ B α), anti-I κ B α , and anti-tubulin antibodies. **C.** MCF7 cells were incubated in medium containing 10% charcoal dextran-treated serum for 48 h and then infected with either AdLacZ or AdFn14 for the indicated lengths of time. Cell lysates were prepared and equal amounts of protein were subjected to Western blot analysis using anti-phosphorylated I κ B α , anti-I κ B α , and anti-myc antibodies. **D.** MCF7 cells were infected with either AdLacZ or AdFn14 for 8 h in the presence of either the NF- κ B peptide inhibitor SN50 or the control peptide SN50M as indicated. Cell invasive capacity was then measured. *, $P < 0.001$, compared with AdFn14 infection alone.

residues, which confer signaling competence. This proinvasive effect could be due to ligand-dependent and/or ligand-independent Fn14 signaling. We favor the second mechanism because these two lines express low levels of TWEAK mRNA, the invasion medium contains a small amount of fetal bovine serum (a potential source of TWEAK), and ligand-independent signaling has been documented after overexpression of other TNF receptor superfamily members (43, 44). Second, ectopic expression of a signaling-defective Fn14 mutant in the invasive MDA-MB-231 cell line reduced invasive capacity, presumably by acting as a dominant-negative inhibitor of endogenous Fn14. Third, RNA interference-mediated Fn14 knockdown in MDA-MB-231 and MDA-MB-436 cells also reduces invasive capacity, again showing an important role for endogenous Fn14 in cellular invasive potential.

We did global gene expression profiling to identify genes affected by siRNA treatment of MDA-MB-231 and MDA-MB-436 cells and 161 genes had a statistically significant change in

expression. Gene Ontology analysis indicated several biological processes highly represented by these 161 genes, most notably NF- κ B pathway regulation. NF- κ B signaling complexes can be activated by a large number of stimuli and control the transcription of many target genes encoding a diverse set of proteins involved in inflammation, the adaptive immune response, and cancer (45, 46). The mechanism by which Fn14 signaling through NF- κ B activation contributes to invasive behavior is still poorly understood. Previous studies have indicated that TWEAK treatment of various cell types promotes NF- κ B activation (7) and we observed a similar response in the MDA-MB-231 and MDA-MB-436 breast cancer cell lines. Additionally, ectopic Fn14 expression in MCF7 cells activated the NF- κ B pathway, consistent with earlier Fn14 overexpression studies using either NIH3T3 (28) or HEK293T (29) cells. This previous work showed that Fn14-stimulated NF- κ B activation requires an intact TRAF-binding site (28, 29) and that dominant-negative forms of TRAF2 and TRAF5 can suppress the stimulatory effect (29). Because NF- κ B-regulated genes are implicated in cellular motility, matrix degradation, and invasion (46), we investigated whether Fn14-stimulated MCF7 cell invasion was dependent on NF- κ B function using SN50, a cell-permeable peptide that inhibits NF- κ B activity by preventing p50/RelA (p65) nuclear translocation (33). We found that Fn14 proinvasive activity was completely inhibited by SN50 treatment. Taken together, these findings indicate that Fn14 regulation of breast cancer cell invasive capacity involves the canonical NF- κ B signaling pathway.

In summary, we show here that Fn14 is overexpressed in breast tumors and may contribute to the invasive nature of breast cancer cells. Our findings using breast cancer cells cultured *in vitro* are consistent with previous studies indicating that Fn14 expression levels can influence glioma (15) and esophageal (14) cell invasion capacity *in vitro*. We have expanded on this earlier work by showing that (a) a Fn14 mutant can act as a dominant-negative inhibitor of Fn14-triggered cell invasion, (b) Fn14 overexpression can induce NF- κ B pathway activation and induce changes in the global gene expression pattern, and (c) NF- κ B pathway inhibition can block Fn14-triggered cell invasion. Finally, the data reported here showing HER2 and Fn14 coexpression in some breast tumors are novel and may have potential clinical implications for a subset of breast cancer patients.

Materials and Methods

Cell Lines and Culture Conditions

Cell lines were obtained from the American Type Culture Collection and maintained in either DMEM (BT474 cells), RPMI 1640 (MCF7, ZR751, T47D, MDA-MB-436, and MDA-MB-231 cells), or Leibowitz's (MDA-MB-157, MDA-MB-468, and MDA-MB-453 cells) medium with 10% fetal bovine serum. Cells were grown at 37°C with either 5% CO₂ or no CO₂ (if cultured in Leibowitz medium) at constant humidity. Media and supplements were purchased from Invitrogen.

Northern Blot, Western Blot, and Flow Cytometric Analysis

Total RNA was isolated and Northern blot hybridization was done as previously described (10). Whole-cell protein lysates

were prepared as previously described (47). Western blot analysis was done as previously described (11). The Fn14 polyclonal antibody was previously described (48). The myc epitope monoclonal and the I κ B α and phosphorylated I κ B α polyclonal antibodies were from Cell Signaling Technology. The α -tubulin polyclonal antibody was from MP Biomedicals and the actin monoclonal antibody was from Sigma. Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences and Promega. The Fn14 and phosphorylated I κ B α antibodies were incubated in 5% protease-free and γ -globulin-free bovine serum albumin (Sigma) in TBS with 0.07% Tween 20 at 4°C overnight. All other antibody incubations were done as previously described (11). Flow cytometry was done as previously described (10).

Preparation of Recombinant Adenoviruses and Cell Infection

Isolation and propagation of all recombinant E1-deleted adenoviral constructs have been previously described (12, 15). The two *Fn14* constructs contained an NH₂-terminal myc epitope tag to facilitate immunodetection. Breast cancer cells were infected at matched MOI and cultured for an additional 8 h before harvesting for either Western blot analysis or invasion assays.

siRNA Transfections

siRNA oligonucleotides targeting Fn14 and the firefly luciferase GL2 mRNA were purchased from Qiagen. The siRNA sequences are as follows: Fn14i-1, 5'-catcattctagagcagctct (15); Fn14i-2, 5'-gagggagaattattaataaa; and GL2 luciferase, 5'-aacgtacgcggaaactactcgatt. The siRNAs were transfected at a final concentration of 60 nmol/L using Lipofectamine 2000 (Invitrogen) as previously described (12). No cytotoxicity was observed (data not shown).

Isolation of Stably Transfected MDA-MB-231 Cell Lines

MDA-MB-231 cells were transfected with pSM2-based plasmids encoding either a Fn14 mRNA-targeted shRNA or a nonsilencing shRNA with no homology to known mammalian genes. Both plasmids were obtained from Open Biosystems, Inc. Plasmids were introduced using the Arrest-In Transfection Reagent according to the manufacturer's instructions (Open Biosystems). Cells were grown in complete medium supplemented with 200 ng/mL puromycin (Cellgro). Resistant cell colonies were clonally expanded and screened for Fn14 expression levels by Western blot analysis. Two control MDA-MB-231 cell lines (expressing the nonsilencing shRNA) and two MDA-MB-231 cell lines exhibiting significant Fn14 knockdown were selected for further analysis.

Cell Invasion Assays

Assays were conducted using Boyden chambers precoated with Matrigel (BD Biosciences). Either 1×10^5 cells (for MDA-MB-231) or 2×10^5 cells (all other cell lines) were resuspended in 100 μ L of invasion buffer (medium containing 0.3 g/L L-glutamine, 0.5% fetal bovine serum, and 1% bovine serum albumin) and then seeded into 24-well-size Boyden chambers and incubated for either 4 h (MDA-MB-231 shRNA derivatives), 8 h (MDA-MB-231), or 16 h (MCF7, T47D, and MDA-MB-436).

Lower chambers contained invasion buffer without additional additives. The NF- κ B peptide inhibitor SN50 and the control peptide SN50M (EMD Biosciences, Inc.) were used at 50 μ mol/L. 12-*O*-tetradecanoylphorbol-13-acetate (Sigma) was used at 10 nmol/L. Cells were fixed and stained according to the manufacturer's protocols. Assays were done in triplicate. Statistical analysis was done using the paired Student's *t* test and differences were considered to be statistically significant at $P < 0.05$.

Microarray Analysis of Global Transcriptional Response following siRNA-Mediated Fn14 Depletion

Total RNA was isolated from cell lines as previously described (47). RNA integrity and purity were measured using a 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop ND-1000 Spectrophotometer (NanoDrop). Fn14 mRNA depletion was confirmed using quantitative reverse transcription-PCR. The ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to quantify amplified product using the comparative cycle threshold (C_T) method. Gene-specific primers are as follows: Fn14, 5'-ccaagctctcccaa (forward) and 5'-tgggcctagtgctc (reverse); β -actin, 5'-gcaaacctgtacgccaaca (forward) and 5'-cctcggccacattgtgaac (reverse). Labeled cRNA probes were prepared and hybridized to the manufacturer's protocols and washed in an ozone-controlled environment (<0.1 ppb). Slides were scanned using the Agilent Microarray Scanner (model G2505B). Data were extracted, processed, and normalized using Agilent Feature Extraction software (v8.1.1.1). All arrays were quality controlled for a minimum median intensity of >85 units and a maximum average background level of 50 units in each channel. The variance of the abundance measurements on a single chip was estimated by determining the median SD of the log₂ ratios observed for 100 genes that have 10 identical oligonucleotide detectors each, printed randomly across the chip. In this experimental series, the largest median SD measured was 0.079. This variance is associated with a 99.9% confidence interval for the ratio fold change of 0.85 to 1.18 (Gene Expression Omnibus accession GSE8871).

Microarray Data Filtering and Cluster Analysis

Data were filtered using the *P* value of the ratio as calculated by Agilent Feature Extraction software (v8.1.1.1). Genes selected were significantly up-regulated or down-regulated ($P < 0.001$) in all four Fn14 siRNA treatment arrays and nonsignificant in the GL2 siRNA arrays. Two-dimensional hierarchical clustering was done as previously described (49) using uncentered Pearson correlation and average centroid linkage. Pathway analysis was done using GeneGo.⁴

Analysis of TWEAK and Fn14 mRNA Levels in Publicly Available Expression Array Data

Gene expression data obtained by the Netherlands Cancer Institute from 295 clinically annotated breast tumors were used for this analysis (17, 18). Gene expression levels for TWEAK

⁴ <http://www.genego.com>

and Fn14 were transformed to a binary variable (high or low expression) via thresholding and tested for association with each binary clinical variable using the Fisher's exact test. To avoid the introduction of bias in threshold selection, leave-one-out cross-validation (50) was used to classify TWEAK or Fn14 expression in each sample as being high or low. The strength of this approach was that no sample was involved in selecting the threshold to be used to define its expression level, thus removing selection bias from the analysis. To determine significance of the association between expression level (high or low) and the clinical variables, Fisher's exact tests were done on the contingency tables for each discrete clinical variable.

TMA Immunohistochemistry

Breast cancer TMA slides were obtained from the Translational Genomics Research Institute TMA core facility (Phoenix, AZ). Sections (5 μ m) were sequentially cut and stained for ER α , HER2, and Fn14 using a BondMax TMA autostainer (Vision Biosystems, Inc.). Both the ER (clone 6F11) and HER2 antibodies were obtained from Novocastra Ltd. The Fn14 monoclonal antibody PA48 (used at 2.5 μ g/mL) was provided by Biogen Idec, Inc. Antigen retrieval for Fn14 was done as previously described (15). Endogenous peroxidase activity was quenched by incubating slides in 3% hydrogen peroxide for 10 min at 25°C. Slide blocking was done with background Sniper from Biocare. Slides were counterstained with hematoxylin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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