Nuclear Janus-Activated Kinase 2/Nuclear Factor 1-C2 Suppresses Tumorigenesis and Epithelial-to-Mesenchymal Transition by Repressing Forkhead Box F1

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Abstract

Progression to metastasis is the proximal cause of most cancer-related mortality. Yet much remains to be understood about what determines the spread of tumor cells. This paper describes a novel pathway in breast cancer that regulates epithelial-to-mesenchymal transition (EMT), motility, and invasiveness. We identify two transcription factors, nuclear factor 1-C2 (NF1-C2) and Forkhead box F1 (FoxF1), downstream of prolactin/nuclear Janus-activated kinase 2, with opposite effects on these processes. We show that NF1-C2 is lost during mammary tumor progression and is almost invariably absent from lymph node metastases. NF1-C2 levels in primary tumors correlate with better patient survival. Manipulation of NF1-C2 levels by expression of a stabilized version or using small interfering RNA showed that NF1-C2 counteracts EMT, motility, invasiveness, and tumor growth. FoxF1 was found to be a direct repressed target of NF1-C2. We provide the first evidence for a role of FoxF1 in cancer and in the regulation of EMT in cells of epithelial origin. Overexpression of FoxF1 was associated with a mesenchymal phenotype, increased invasiveness in vitro, and enhanced growth of breast carcinoma xenografts in nude mice. The relevance of these findings is strengthened by the correlation between FoxF1 expression and a mesenchymal phenotype in breast cancer cell isolates, consistent with the interpretation that FoxF1 promotes invasion and metastasis. Cancer Res; 70(5); 2020–9. ©2010 AACR.

Introduction

Prolactin, a polypeptide hormone mainly produced by the anterior pituitary, is best known for its effects on the mammary glands, where it regulates glandular growth and development, as well as milk production and secretion (1). The role of prolactin in mammary gland development makes this hormone a candidate factor in the control of initiation and progression of breast cancer. However, it remains controversial whether prolactin promotes tumor growth (2–5) or suppresses invasiveness by upholding a differentiated state (6–8). The issue is complicated by the presence of multiple signaling pathways downstream of the prolactin receptor.

Studies using mammary epithelial cells and tissue from heterozygous prolactin receptor knockout mice showed that prolactin is required for maintenance of the transcription factor nuclear factor 1-C2 (NF1-C2) in the mammary epithelium (9). The NF1 family consists of four genes (NF1-A, NF1-B, NF1-C, and NF1-X), each producing different splice variants, thereby generating a large number of protein isoforms. The expression levels of the different NF1 genes and the ratio between alternatively spliced transcripts vary between cell types and differentiation states (10–13). In the mouse mammary gland, the specific isoform NF1-C2 is the dominating NF1-C protein and exhibits a characteristic temporal pattern during the adult developmental cycle; the mRNA is constitutively present, but the protein decreases during lactation to re-emerge again at involution (9). We previously described a novel pathway through which prolactin maintains NF1-C2 via activated nuclear Janus-activated kinase 2 (Jak2; ref. 14). The mechanism by which Jak2 increases the amount of active NF1-C2 is independent of the signal transducers and activators of transcription (Stat) pathway, relies on direct interaction between Jak2 and NF1-C2, and involves tyrosine phosphorylation of NF1-C2 and protection against proteasomal degradation (14). The known function for NF1-C2 is to activate p53 and participate in the establishment of expression of milk protein genes during pregnancy (15, 16).

Here, we show a novel role for NF1-C2 in tumor development and epithelial-to-mesenchymal transition (EMT) of breast cancer cells. We show (a) that the level of NF1-C2 is higher in normal glandular cells compared with tumor cells and that the NF1-C2 protein is virtually absent from lymph node metastases, (b) that patients with nuclear NF1-C2 in...
their breast cancer cells have better prognosis, (c) that forced expression of NF1-C2 abolished tumorigenicity in nude mice, (d) that NF1-C2 is a suppressor of EMT, (e) that the Forkhead box F1 gene (FoxF1) is a direct target of transcriptional repression by NF1-C2, (f) that FoxF1 is a potent inducer of EMT and invasiveness, and (g) that forced expression of FoxF1 enhanced tumorigenicity in nude mice.

Materials and Methods

**Antibodies.** NF1-C2 antibody (9), HDAC-1 (Santa Cruz), Jak2 (Upstate), Vimentin (Daco), α-tubulin (Sigma), β-catenin (Santa Cruz), E-cadherin (Calbiochem), N-cadherin (Zymed Laboratories), and fibronectin (Sigma) were used.

**Cell cultures.** The mouse mammary epithelial cell line HC11, kindly provided by Dr. R. Ball (Friedrich Miescher Institute), was grown as described previously (16). The human breast cancer cell lines T47D, ZR75, BT474, MDA-MB415, BT20, Hs578T, MDA-MB157, and MDA-MB436 (obtained from American Type Culture Collection) was grown at 37°C in a 5% CO₂/95% air atmosphere in RPMI 1640 supplemented with 10% FCS, 1% PEST, and 5 μg/mL insulin.

**Cell transfections and plasmids.** The NF1-C2 K407E construct was made by the Stratagene Quickchange site-directed mutagenesis kit, and the mutated NF1-C2 open reading frame was cloned into the pcDNA3.1 (+) (Invitrogen) creating the NF1-C2 K407E construct (called NF1-C2S in the text). The open reading frame of FoxF1 was cloned into pcDNA3.1 (+) (Invitrogen) creating the FoxF1 construct.

The different constructs were stably transfected in HC11 cells and MDA-MB436 cells using lipofectin as described (16). The Hs578T cells were plated on glass slides and transfected (Lipofectin, Invitrogen) with a green fluorescent protein (GFP) plasmid encoding a dominant-negative FoxF1 protein (GFP–DNA-binding domain only) fused to GFP (17) or an empty GFP vector.

**Cell cycle analysis.** The proportion of cells in the G1, S, and G2 phases was quantified flow cytometrically following propidium iodide staining. Labeled S-phase cell proliferation was monitored using dual staining techniques as previously described (18).

**Reverse transcription–PCR analysis.** Total RNA was extracted from cells using Sigma-Aldrich GenElute Mammalian Total RNA Miniprep kit. Reverse transcription–PCR (RT-PCR) was performed with Titan One Tube RT-PCR System kit from Roche Applied Science.

**RNA interference.** A 21-nucleotide small interfering RNA (siRNA) duplex targeting mouse NF1-C was custom synthesized by Dharmacon. The sequence used was AUAUUGGCGUUGCC-GCUAA with 3′/5′ overhangs. The control, nontargeting siRNA UAGCGACUAAACACAUCAA with 3′/5′ overhangs was obtained from Dharmacon. Transfection of siRNA duplexes into HC11 cells was carried out using oligofectamine (Invitrogen) according to the manufacturer’s instructions.

**Protein preparation and Western blot analysis.** Whole-cell and nuclear extract preparation, protein concentration determination, and Western blot analysis were carried out as described previously (14).

**Immunofluorescent analysis of cultured cells and breast cancer.** Cells were fixed in 4% formaldehyde/PBS, permeabilized using 0.2% Triton X100 in PBS, and blocked in 3% bovine serum albumin/PBS. After incubation with primary antibodies, the cells were incubated with species-appropriate biotinylated secondary antibodies followed by FITC- or TRITC-conjugated streptavidin (Vector Laboratories). FITC-phalloidin or TRITC-phalloidin [filamentous actin (F-actin); Sigma] staining was achieved following the manufacturer’s protocol. VectaShield/VectaShield-Dapi (3:2) was used for mounting, and the cells were viewed under a fluorescence equipped Zeiss Axioplan2 Imaging microscope.

**Gelatin zymography.** Conditioned media were obtained after a 30-h incubation period of the different MDA-MB436 cells. Secreted matrix metalloproteinase (MMP) activities were detected and characterized by zymography according to the manufacturer’s protocol (Invitrogen).

**Invasion assay.** Invasion assays were performed using BD BioCoat Matrigel Invasion chambers with 8-mm pore size according to the manufacturer’s instructions (VWR International). After 24 h incubation, top cells were removed and bottom cells were counted.

**Anchorage-independent growth assay.** Cells were seeded into six-well plates in 0.4% agar, supplemented with 10% FCS, 1% PEST, and 5 μg/mL insulin on top of a 0.8% agar bed in similar medium according to the manufacturer’s protocol (Millipore). The cultures were incubated for 21 d, and the colonies were measured and photographed.

**Mouse xenografts.** Eight female BALB/c nu/nu mice (Charles River Laboratories) were s.c. injected in the left flank with 1 × 10⁷ MDA-MB436 cells stably transfected with vector control or NF1-C2S and 1 × 10⁶ HC11 cells or HC11 cells overexpressing FoxF1. After injection the general condition of the animals was followed daily. Tumor growth was monitored weekly, and animals were allowed to form tumors of up to 1 cm in diameter. Each tumor was dissected and fixed in formaldehyde. The experiments were approved by the Gothenburg animal ethics committee.

**Patient characteristics and tumor-related factors.** We studied a cohort of 292 patients with stage II invasive breast cancer diagnosed in the South Sweden Health Care Region (1983–1994). All patients were treated with tamoxifen for 2 y, irrespective of estrogen receptor status, and have previously been selected from two randomized trials (19). Effects on distant disease-free survival (DDFS) during a maximum follow-up time of 5 y were studied. Information of clinical outcome, patient, and tumor-related factors were already available (19). In the present study tissue microarrays from paraffin-embedded tumor samples were used with approval by Lund University Medical Ethics Committee. The tissue microarrays were constructed using 3 × 0.6 mm diameter tissue core biopsies as previously described (19).

**Immunohistochemical analysis of breast cancer tissue sections.** Tissue microarrays were incubated with the affinity purified rabbit polyclonal pNF1-C2 antiserum (1:1,000 dilution) according to the protocol described in ref. 9.

**Statistical methods.** Time from diagnosis to first distant metastasis in the two NF1-C2 groups was analysed using
Kaplan-Meier estimates and a log-rank test. The test is two sided, and a \( P \) value of <0.05 was considered significant. Stata 10.1 (StataCorp LP) was used for statistical analysis.

**Results**

The presence of nuclear Jak2/NF1-C2 correlates with improved patient survival in breast cancer. To assess whether the presence of NF1-C2 in breast tumors has any physiologic and clinical relevance, we examined the NF1-C2 protein status in tissue microarrays containing 292 samples from patients with stage II invasive breast cancer. As seen in the representative sections (Fig. 1A), tumor cells stained significantly weaker for NF1-C2 than did normal glandular cells, and virtually no NF1-C2 was detected in lymph node metastases. Of the 292 specimens examined, 25% (74 of 292) stained positive for NF1-C2. Importantly, patients with primary tumors expressing NF1-C2 were found to have better prognosis compared with those without detectable NF1-C2 in the nucleus (Fig. 1B). The difference in DDFS between these two groups is statistically significant (\( P = 0.03 \); log-rank test). This is the first evidence of a prognostic significance of NF1-C2 status in primary breast cancer. Only 1 of 159 lymph node metastases stained positive, consistent with the notion that NF1-C2 is lost during tumor progression and rising the interesting possibility that this loss facilitates metastasis.

Activated Jak2 in the nucleus stabilizes NF1-C2 (14), and we therefore asked if reduced levels of nuclear NF1-C2 in breast cancer cells are associated with a decrease in nuclear Jak2. Western blot of nuclear extracts from eight tumor biopsies, representing the whole range of NF1-C2 levels, showed a clear correlation between nuclear Jak2 and NF1-C2 (Fig. 1C). This suggests that loss of NF1-C2 in the tumors is a consequence of reduced signaling by the prolactin/nuclear Jak2/NF1-C2 pathway and, thus, that this pathway may act to slow down tumor progression.

Modulation of NF1-C2 levels affects proliferation, cell morphology, motility, and cell-cell adhesion. To isolate the effects of NF1-C2 from other pathways and targets downstream of prolactin, we wanted to manipulate the NF1-C2 level, independent of prolactin and Jak2. Overexpression of native NF1-C2 is inefficient, because the protein is rapidly degraded in the absence of nuclear Jak2; a process mediated by interaction with the \( \alpha_7/C8 \)-subunit of the 20S proteasome (14). Deletion analysis identified a small region in the COOH terminus of NF1-C2 required for interaction with \( \alpha_7/C8 \) and for proteasomal degradation. Point mutations in this region were screened by yeast two-hybrid assay, which identified K407E as a substitution that abolished the interaction with \( \alpha_7/C8 \) (Fig. 2A). In cycling cells, NF1-C2 levels drop sharply during S phase (14). To investigate if the K407E substitution affects NF1-C2 stability in vivo, HC11 mammary epithelial cells expressing the mutant protein were synchronized at the G1-S border by a double thymidine block, released and assayed for NF1-C2 content after 4 hours. Consistent with previous results (14), the native NF1-C2 protein in the parental HC11 cells was diminished 4 hours after release from G1-S arrest. However, the NF1-C2(K407E) level remained unaffected, which suggests that K407E substitution protects from proteasomal degradation (Fig. 2A). Taken together, these findings indicate that K407E substitution results in a more stable NF1-C2 protein (hereafter called NF1-C2S).

To assess whether an increased level of NF1-C2 affects the growth characteristics of a mammary epithelial cell line, we compared HC11 cells with and without expression of NF1-C2S by flow cytometry of exponentially growing cells. NF1-C2S-expressing cells showed a markedly decreased proportion of S-G2 compared with control cells (Fig. 2B). No difference was observed in the sub-G1 fraction, suggesting that apoptosis does not differ significantly between the two populations. Expression of NF1-C2S induced striking alterations in morphology and intercellular adhesion. The parental HC11 cells grow as dispersed cells and rarely form clusters (Fig. 2C). In contrast, HC11 expressing NF1-C2S formed...
tightly packed patches of epithelial sheets (Fig. 2C), with increased amounts of membrane-associated E-cadherin and β-catenin—key components of epithelial adherence junctions (Fig. 2C). The cytoskeleton was rearranged, with weaker microtubular outgrowth and less well-developed F-actin-rich filopodia (Fig. 2C). The altered morphology and cytoskeletal modifications were reversible; knockdown of NF1-C2 by siRNA in HC11 cells expressing NF1-C2 restored filopodia formation and increased actin filaments in cells with reduced NF1-C2 nuclear staining (Fig. 2D). Time-lapse imaging confirmed that the morphologic changes had consequences for cell motility, which was impeded by expression of NF1-C2S (Supplementary Movies HC11wt and HC11NF1-C2S).

NF1-C2 suppresses the invasive phenotype of aggressive breast cancer cells. During tumor progression, carcinoma cells undergo EMT, a process in which polarized epithelial cells transition into a mesenchymal phenotype. The suppression of EMT by NF1-C2 suggests a potential mechanism for its antitumor effects.

Figure 2. Modulating NF1-C2 expression affects growth, cell-cell adhesion, and cellular morphology. A, yeast two-hybrid assay of interaction between the α7/C8-binding domain of the 20S proteasome and either wild-type NF1-C2 or NF1-C2(K407E; called NF1-C2S), as indicated (left). HC11 cells and HC11 expressing NF1-C2S were arrested at the G1-S border by a double thymidine block. Nuclear extracts were prepared from cells taken 0 and 4 h after release in fresh medium, analyzed by Western blot with the NF1-C2–specific antibody, stripped, and reincubated with anti-HDAC-1 (right). B, fluorescence-activated cell sorting (FACS) analysis of parental and NF1-C2S–expressing HC11 cells. Data are shown as mean ± SEM. C, phase-contrast microscopic images of parental and NF1-C2S–expressing HC11 cells. Immunofluorescence of parental and NF1-C2S–expressing HC11 cells with antibodies against E-cadherin and β-catenin (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Microtubules and F-actin in parental and NF1-C2S–expressing HC11 cells visualized by anti-β-tubulin FITC-conjugated (red) and phalloidin (green) as indicated. D, NF1-C2S–expressing HC11 cells transiently transfected with siRNA against NF1-C2 and analyzed with the NF1-C2–specific antibody (red) and FITC-conjugated phalloidin (green).
cells undergo dynamic changes to lose their epithelial characteristics and obtain a more motile fibroblastic phenotype, which enables them to proceed with invasion and metastasis (20). Based on the alterations in cellular morphology and adhesion induced by NF1-C2S in the comparatively epithelial-like HC11, we next asked whether NF1-C2S can reverse EMT in a more mesenchymal breast cancer cell line, MDA-MB436. Expression of NF1-C2S in MDA-MB436 cells resulted in an increase in NF1-C2 level compared with the parental cells or cells stably transfected with an empty expression vector or a construct expressing NF1-C2S, with antibodies against NF1-C2 and HDAC-1 (left). Western blot analysis of whole-cell extracts from the three MDA-MB436 lines with antibodies against a mesenchymal marker (vimentin), an epithelial marker (β-catenin), and a loading control (α-tubulin; right). B, MMP-2 activity in conditioned medium from the three MDA-MB436 lines assayed by zymography (top left). Quantification of migration of MDA-MB436 cells expressing NF1-C2S compared with vector-only control cells. Data are presented as the average number of migrated cells per field in three independent experiments (right). Qualitative analysis of soft agar colony formation by MDA-MB436 cells expressing NF1-C2S and vector control cells (bottom left). C, FACS analysis of MDA-MB436 cells expressing NF1-C2S and vector control cells. D, tumors excised from BALB/c nude mice s.c. grafted with vector control MDA-MB436 cells. No tumors developed on mice grafted with MDA-MB436 cells expressing NF1-C2S. Data in B and C are shown as mean ± SEM.
of NF1-C2S completely abolished this ability; five of eight mice grafted with control cells (MDA-MB436 cells stably transfected with empty expression vector) developed massive tumors, whereas no tumor growth could be detected (zero of eight) in mice grafted with cells expressing NF1-C2S (Fig. 3D).

Altogether, these results indicate that NF1-C2 suppresses cell motility and invasion and implicate NF1-C2 as a negative regulator of tumor progression and EMT. The relevance of these observations for the clinic is underscored by the findings that NF1-C2 status of primary breast cancers correlates with survival and that NF1-C2 immunoreactivity is almost invariably lost in lymph node metastases.

**NF1-C2 binds the promoter of the FoxF1 gene and represses its expression.** To identify downstream targets of NF1-C2, we used Affymetrix microarrays to compare the transcriptome of NF1-C2S-expressing MDA-MB436 with that of vector control cells. (The hundred most upregulated and downregulated genes can be found in Supplementary Table S1.) One of the most strongly downregulated genes, i.e., negatively affected by NF1-C2S, was FoxF1—encoding a forkhead transcription factor normally expressed in mesenchymal cells (22). FoxF1 is important for mesoderm differentiation (22), vasculogenesis (23), and organogenesis (24, 25). It also promotes mesenchymal migration by increasing cellular motility (26). Metastatic spread of epithelial cancer cells from the primary tumor to distant organs shares mechanisms with cell migration that occur during embryogenesis. FoxF1 is therefore an interesting candidate target, which could account for the inhibitory effects of NF1-C2 on motility and invasiveness.

Using RT-PCR, we confirmed that expression of NF1-C2S diminishes FoxF1 mRNA in both cell lines (MDA-MB436 and HC11; Supplementary Fig. S1A). To rule out that repression of FoxF1 is a peculiarity of the mutant NF1-C2S protein, we used siRNA to knockdown the endogenous NF1-C2 in

![Figure 4](image_url)

**Figure 4.** Forced expression of FoxF1 is associated with changes in cellular morphology and a shift from epithelial to mesenchymal gene expression. A, phase-contrast micrographs of HC11 cells or HC11 cells overexpressing FoxF1. F-actin in FoxF1-expressing and parental HC11 cell lines visualized by TRITC-conjugated phalloidin (red). Immunofluorescence (green) of FoxF1-expressing and parental HC11 cell lines with anti-β-catenin and anti-fibronectin with nuclei stained by DAPI (blue). B, Western blot analysis of whole-cell extracts from FoxF1-expressing, vector control, and parental HC11 cell lines with anti-β-catenin, anti-E-cadherin (epithelial), anti-N-cadherin, anti-fibronectin (mesenchymal), and anti-α-tubulin (loading control). C, mRNA for E-cadherin, claudin-1, ocludin, desmoglein-1β, desmoglein-2, desmocollin-2, desmoplakin, and GAPDH in FoxF1-expressing and control HC11 cell lines measured by RT-PCR. Primers used can be found in Supplementary Materials and Methods. D, anti-fibronectin immunofluorescence (red) of HS578T cells transfected with a GFP plasmid or a plasmid encoding a dominant-negative FoxF protein (DNA-binding domain only) fused to GFP (green; ref. 17). Nuclei stained with DAPI (blue).
HC11 cells. Knockdown resulted in a modest reduction in NF1-C2 protein level but a dramatic increase in FoxF1 mRNA (Supplementary Fig. S1B), fully consistent with the decrease in FoxF1 obtained by moderate overexpression of NF1-C2S (Supplementary Fig. S1A). To investigate if FoxF1 is a direct target of NF1-C2, we searched conserved regions of the human and murine FoxF1 promoters for potential NF1-binding sites. An excellent match to the NF1 consensus occurs between positions −212 and −199 in the human promoter and at the corresponding location at −1,211 to −1,198 in mouse (Supplementary Fig. S1C). This site binds NF1-C2 from nuclear extract in a gel shift assay, as confirmed by competition with a distinct NF1 site and by supershift with an NF1-C–specific antibody (Supplementary Fig. S1D). Chromatin immunoprecipitation analysis, with MDA-MB436 cells expressing NF1-C2S and two independent antisera (NF1-C– and NF1-C2–specific, respectively), verified that NF1-C2 occupies the FoxF1 promoter in vivo (Supplementary Fig. S1E). These results show that binding of NF1-C2 to the promoter of FoxF1 represses transcription of this gene.

**FoxF1 promotes EMT and is overexpressed in highly aggressive breast cancer cells.** To investigate whether increased expression of FoxF1, on its own, is sufficient to induce EMT, we overexpressed FoxF1 in the epithelial-like HC11 cells. As shown in Fig. 4A, forced expression of FoxF1 was sufficient to induce features of EMT, including a fibroblastic morphology, a significant decrease of epithelial— and a corresponding increase in mesenchymal—protein levels (Fig. 4B). Furthermore, FoxF1 is a potent repressor of *E-cadherin* expression (Fig. 4C), consistent with the behavior of other known EMT inducers, such as Snail, Zeb, and Twist (27–29). In addition to E-cadherin, all other epithelial genes tested were downregulated by FoxF1, including components of tight junctions (*occludin* and *claudin-1*), and desmosomes (*desmocollin, desmoplakin, and desmoglein*), all known to be required for the fully polarized epithelial phenotype (Fig. 4C). Moreover, inhibition of FoxF activity in HS578T cells, normally expressing high levels of FoxF1, reduced the expression of the mesenchymal marker fibronectin (Fig. 4D). These findings support the notion that FoxF1 is capable of orchestrating

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**Figure 5.** FoxF1 promotes tumorigenesis in breast cancer cells. A, RT-PCR analysis of FoxF1 mRNA in different HC11-derived cell lines, as indicated. B, relative migration of HC11 cells expressing NF1-C2S or FoxF1 compared with unmodified cells. C, qualitative analysis of soft agar colony formation by FoxF1-expressing and control HC11 cell lines (left) and quantification of soft agar colony formation (right). D, tumors recovered from BALB/c nude mice s.c. grafted with HC11 cells (wt; four of eight) or HC11 cells expressing FoxF1 (seven of eight; top left). Xenograft tumor sizes from nude mice grafted with FoxF1-expressing and control HC11 cell lines (middle). Histology (right) and anti-fibronectin immunofluorescence (bottom left) of tumors generated by FoxF1-expressing and control HC11 cell lines. Nuclei stained with DAPI (blue). Data in B and C are shown as mean ± SEM.
a complete EMT reprogramming of epithelial cells and provides a likely mechanism for the EMT observed in response to reduced NF1-C2 protein levels.

As illustrated in Fig. 5A and B, there is a strong correlation between the expression level of FoxF1 and invasiveness of HC11 cells. In addition, the FoxF1 overexpressing cells exhibited hallmarks of malignant transformation, such as growth in soft agar (Fig. 5C) and aggressive tumor growth after s.c. grafting on nude mice (Fig. 5D). Tumors derived from FoxF1-expressing cells produced increased amounts of fibronectin compared with control tumors consistent with the in vitro results and a hallmark of EMT (Fig. 5D).

Breast cancer cell lines range from epithelial-like, with low invasiveness, to mesenchymal-like, exhibiting high invasive capacity. Because forced expression of FoxF1 induced EMT, we wanted to see if FoxF1 expression levels correlate with cellular phenotype also in natural isolates. Eight breast cancer cell lines were selected: four weakly invasive and epithelial-like (T47D, ZR75, BT474, and MDA-MB415) and four highly invasive and mesenchymal-like (BT20, HS578T, MDA-MB157, and MDA-MB436; Fig. 6A). FoxF1 expression correlated perfectly with cellular phenotype; all four invasive cell lines had high to moderate levels of FoxF1 mRNA, whereas in the noninvasive, it ranged from low to undetectable (Fig. 6B).

In conclusion, FoxF1 is a repressed target of the prolactin/nuclear Jak2/NF1-C2 pathway and plays a critical role in the induction of EMT. As one of the driving forces behind progression to a mesenchymal phenotype of breast cancer cells, it is likely to facilitate metastatic dissemination.

Discussion

Insights into the mechanisms leading to and protecting against malignancy is a prerequisite for identifying new prognostic markers and therapeutic targets. Understanding the role of prolactin in breast cancer has been complicated by the pleiotropic effects of this hormone on downstream signaling pathways and the existence of isoforms and modifications of both receptor and ligand. We set out to isolate the effects of a recently described and comparatively obscure prolactin response: the nuclear Jak2/NF1-C2 signaling pathway. By modulating the levels of NF1-C2—down by siRNA and up by expression of a stabilized version of the protein—we provide evidence that this pathway suppresses breast cancer growth and progression. The in vitro results were corroborated by the ability of NF1-C2 to inhibit tumor growth in nude mice and the clinical relevance by the observation that NF1-C2 status is prognostic for patient survival. The correlation between NF1-C2 and nuclear Jak2 in breast cancer biopsies supports the notion that the prolactin/nuclear Jak2 pathway is a major factor determining NF1-C2 levels also in cancer. The mechanism by which prolactin induces nuclear translocation of Jak2 is yet unknown. However, reports of opposing effects on growth by different forms of prolactin suggest a ligand selectivity in triggering specific pathways. For example, did phosphorylated prolactin antagonize growth in contrast to the unphosphorylated hormone (30)? A 16-kDa NH2 terminal fragment of prolactin significantly delayed tumor development, reduced the establishment of lung metastases (31), and inhibited angiogenesis (32). It will be important to clarify how the nuclear Jak2/NF1-C2 pathway responds to different versions and modifications of prolactin, for example, the 16-kDa fragment and phosphorylation, and compare this with alternative pathways, such as Jak2/Stat5.

A number of reports have implicated dysregulation of members of the NF1 family in tumor development. c-myc and ras decrease the expression of NF1 genes (33, 34). Identification, by retroviral insertion, of loci that cause cancer in combination with overexpressed platelet-derived growth factor-B tagged the entire NF1 family (35), which supports the idea that loss of NF1 activity facilitates tumor formation. Lack of specific antibodies has hampered identification of the relevant NF1 family members and isoforms involved in different processes. Hence, in most studies of NF1 target genes, the responsible NF1 protein remains unknown, and isoform-specific effects may have gone undetected (36). To our knowledge, the present work represents the first example...
of a specific NF1 isoform being identified as a tumor suppressor or regulator of EMT. The fact that carcinoma dissemination follows from EMT suggests that loss of NF1-C2 may be one event leading to metastasis, further supported by the observation that NF1-C2 immunoreactivity dropped from 25% of primary tumors to <1% of lymph node metastases.

Regarding the mechanism through which NF1-C2 inhibits EMT, the present study identifies transcriptional repression of FoxF1 as a crucial step. FoxF1 encodes a forkhead transcription factor active in mesenchymal cells during embryonic development, and we provide the first evidence for a role of FoxF1 in cancer and in the regulation of EMT in cells of epithelial origin. Genes that induce EMT during tumor progression are frequently involved in the mobilization of cells at events in embryonic development that require cell migration, such as gastrulation and neural crest formation (37, 38). There are no reports of FoxF1 being involved in EMT during development. However, FoxF1 is expressed in nascent mesoderm passing through the primitive streak during gastrulation and is turned on in the sclerotome at the time when somite cells undergo EMT to migrate toward the notochord (22). The expression pattern is, thus, fully compatible with a role for FoxF1 in developmental EMT.

Like most EMT inducers (27–29), FoxF1 is a potent repressor of E-cadherin expression. Another member of the Forkhead family, FoxC2, induces alterations in cellular morphology consistent with EMT. However, in contrast to FoxF1, FoxC2 overexpression did not affect the level of E-cadherin mRNA but instead redirected E-cadherin from the plasma membrane to the cytoplasm (39). Several lines of evidence indicate that different E-cadherin repressors may operate at different steps of the metastatic cascade. Snail has been implicated in the initial migratory phenotype of primary tumors, whereas others, Zeb1, Zeb2, and Twist, may be responsible for maintenance of cell motility, malignancy, and angiogenesis (40, 41). Repression of E-cadherin expression suggests an earlier role for FoxF1, than FoxC2, in EMT. Whereas FoxF1 both inhibits epithelial gene expression and induces the mesenchymal component of the program, other EMT regulators induce mesenchymal genes via activation of FoxC2 expression (39). Future research will reveal how FoxF1 relates to other EMT modulators and establish whether it contributes to metastasis.

Several prognostic gene sets for breast cancer have been reported in the literature but may not necessarily contain genes that are functionally important for tumor progression. In this paper, we show that patients with nuclear NF1-C2 staining in primary tumor cells have better prognosis, and we describe a novel mechanism by which NF1-C2, through direct repression of FoxF1, control tumor progression. We also add new insights into the mechanism underlying EMT and show that NF1-C2 and FoxF1 are two novel potential therapeutic targets against breast cancer invasion and metastasis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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