Integrin-uPAR signaling leads to FRA-1 phosphorylation and enhanced breast cancer invasion

Matthew G. Annis 1,3, Veronique Ouellet 5, Jonathan P. Rennhack 6, Sylvain L’Esperance 7, Claudine Rancourt 7, Anne-Marie Mes-Masson 5, Eran R. Andreche 6 and Peter M. Siegel 1,2,3,4*

Abstract

Background: The Fos-related antigen 1 (FRA-1) transcription factor promotes tumor cell growth, invasion and metastasis. Phosphorylation of FRA-1 increases protein stability and function. We identify a novel signaling axis that leads to increased phosphorylation of FRA-1, increased extracellular matrix (ECM)-induced breast cancer cell invasion and is prognostic of poor outcome in patients with breast cancer.

Methods: While characterizing five breast cancer cell lines derived from primary human breast tumors, we identified BRC-31 as a novel basal-like cell model that expresses elevated FRA-1 levels. We interrogated the functional contribution of FRA-1 and an upstream signaling axis in breast cancer cell invasion. We extended this analysis to determine the prognostic significance of this signaling axis in samples derived from patients with breast cancer.

Results: BRC-31 cells display elevated focal adhesion kinase (FAK), SRC and extracellular signal-regulated (ERK2) phosphorylation relative to luminal breast cancer models. Inhibition of this signaling axis, with pharmacological inhibitors, reduces the phosphorylation and stabilization of FRA-1. Elevated integrin αVβ3 and uPAR expression in these cells suggested that integrin receptors might activate this FAK-SRC-ERK2 signaling. Transient knockdown of urokinase/plasminogen activator urokinase receptor (uPAR) in basal-like breast cancer cells grown on vitronectin reduces FRA-1 phosphorylation and stabilization; and uPAR and FRA-1 are required for vitronectin-induced cell invasion. In clinical samples, a molecular component signature consisting of vitronectin-uPAR-uPA-FRA-1 predicts poor overall survival in patients with breast cancer and correlates with an FRA-1 transcriptional signature.

Conclusions: We have identified a novel signaling axis that leads to phosphorylation and enhanced activity of FRA-1, a transcription factor that is emerging as an important modulator of breast cancer progression and metastasis.

Keywords: Breast cancer, Integrins, uPAR, FRA-1, Invasion

Background

The transcription factor Fos-related antigen 1 (FRA-1) influences tumor heterogeneity [1] and is an important driver of cancer cell stemness and resistance in breast cancer [2]. FRA-1 is a member of the AP-1 family of transcription factors that regulate cell proliferation, differentiation, apoptosis and other biological functions and is encoded by the fosl1 gene (reviewed in [3, 4]). They function as heterodimers composed of one Fos (c-FOS, FOSB, FRA-1 or FRA-2) and one JUN (c-JUN, JUNB or JUND) family member. FRA-1 was originally shown to transform Rat1 fibroblasts [5] and has since been implicated in the invasiveness and progression of several cancers [6–8], with a prominent role in enhancing the malignant phenotypes of breast cancer cells [9–12]. FRA-1 is also a target of the microRNA miR34, which is frequently downregulated in metastatic breast cancer cell
lines and primary breast tumors with lymph node metastases. Forced expression of miR34 impairs cellular invasion and the ability of breast cancer cells to metastasize [13].

In breast cancer, FRA-1 expression is associated with the transition from normal epithelium to hyperplasia/ductal carcinoma in situ (DCIS) [14–16] and elevated FRA-1 correlates with increasing grade in invasive ductal carcinoma [2, 16]. Correlation between FRA-1 expression and clinical outcomes is more controversial. One study failed to detect an association between FRA-1 protein expression and overall survival [16], while others identified positive correlation between FRA-1 gene expression and overall survival [16], while others study failed to detect an association between FRA-1 protein expression and overall survival and higher rates of lung metastases in patients with estrogen receptor (ER)-positive disease but not ER-negative cancers [19].

FRA-1 exerts pro-tumor functions through the numerous transcriptional targets it regulates [10, 20]. FRA-1 targets influence tumor cell proliferation, invasion and metastasis including: plasminogen activator, urokinase/plasminogen activator urokinase receptor (plau/plaur) [10, 21], matrix metalloproteinase-1 (mmp-1) [22], matrix-metalloproteinase-9 (mmp-9) [12], chloride channel accessory 2 (clca2) [18], adenosine receptor A2B (ador2b) [10], AXL tyrosine kinase receptor (axl) [23] and microRNAs, such as miR-221/222 [24]. FRA-1-regulated genes have demonstrated potential therapeutic targets in breast cancer, including AXL [25] and adenosine receptor A2B [10].

Similar to other members in the Fos family of transcription factors [26, 27], FRA-1 is phosphorylated. Two serine residues, S252 and S265, in the c-terminal DEST domain are phosphorylated, leading to increased protein stability by protecting FRA-1 from proteosomal degradation [23, 28–31]. FRA-1 transcriptional activity is correlated with protein stability and phosphorylation status [32] and the c-terminal region of FRA-1 is required for its transforming activity [33]. Receptor tyrosine kinase signaling (including epidermal growth factor receptor (EGFR) and MET), via the ERK pathway, has been shown to mediate FRA-1 phosphorylation in numerous cancers [34, 35]. FRA-1 can also be phosphorylated by protein kinase C (PKC)θ and PKCα on additional serine residues in the c-terminal DEST domain, which is thought to synergize with ERK-mediated phosphorylation to stabilize FRA-1 [2, 11, 36]. AKT signaling has also been shown to regulate the activity of AP-1 complexes, including FRA-1/c-JUN heterodimers [35].

Here, we demonstrate that engagement of the extracellular matrix protein vitronectin (VN), via the integrin and urokinase/plasminogen activator urokinase receptors (uPARs), leads to activation of SRC and mitogen-activated protein (MAP) kinase (MAPK) signaling and ultimately enhanced FRA-1 phosphorylation and the induction of breast cancer invasion.

Methods

Cell lines and culture conditions

The BRC-17, BRC-31, BRC-32, BRC-36 and BRC-196 cell lines were cultured as previously described [37]. All other breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) and cultured as previously described [38]. Where indicated, cells were grown on fibronectin (2 μg/cm²; Millipore, Billerica, MA, USA), vitronectin (40 or 400 ng/cm² as indicated; Peprotech, QC, Canada) or laminin (2 μg/cm²; Trevigen, Gaithersburg, MD, USA).

Reagents and DNA constructs

Dasatinib (LC Laboratories, Woburn, MA, USA), trametinib/dabrafenib/selumetinib/sorafenib (Selleckchem, Houston, TX, USA) and PP2 (Calbiochem, Gibbstown, NJ, USA) were dissolved in dimethylsulfoxide (DMSO) (Bioshop Canada, Burlington, ON, Canada) and added to fresh medium at the indicated concentrations.

Ten nanomoles of siRNA duplex (fosl1 Smart pool: L-004341-00 (GE Healthcare Dharmacon Inc, Lafayette, CO, USA), plaur [29] or Scrambled (sequences listed in Additional file 1: Table S1) was transfected into cells using RNAiMax according to the manufacturer’s protocol (Life Technologies Inc., Burlington, On, Canada). For the rescue of FRA-1 expression, two fosl1 small interfering RNAs (siRNAs) that target the 3’ UTR were used (Additional file 1: Table S1).

The cDNA for fosl1 was purchased from GE Healthcare Bio-Sciences Company (Lafayette, CO, USA) and cloned into an expression vector to add an HA-tag to the N-terminus. Phospho-deficient and phosphomimetic versions were created using Quick-change mutagenesis (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer’s directions. Sequences for the oligonucleotides used to make these mutants are listed in Additional file 1: Table S1.

Immunoblotting

Thirty micrograms of protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), where it was subsequently immunoblotted using the following antibodies: p44/42 MAPK, phospho-p44/p42 MAPK T202/Y204, phospho-FRA-1 S265, phospho-SFK Y416, Phospho-FAK Y925, Phospho-FAK Y576, Phospho-FAK...
Extracellular matrix (ECM) stimulation and gene expression analysis

For the siRNA-mediated knockdown of uPAR, 48 hours post-transfection with siRNA, cells were harvested with 2 mM NaEDTA in PBS, washed with serum-free medium and plated for 30 minutes on culture dishes that were left uncoated or coated with the appropriate ECM. For gene expression analysis, cells were grown on the indicated ECM-coated or uncoated dishes for 18 hours prior to RNA extraction. For the rescue of plaur/fosl1 knockdowns, cells were first transfected with siRNA then, 24 hours later, transfected with the indicated expression plasmid: 24 hours later, these cells were plated for 18 hours on VN-coated dishes prior to RNA extraction. RNA was extracted using RNeasy kits (Qiagen Inc, Toronto, ON Canada) according to the manufacturer's protocol. The quality of RNA was assessed using a 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies, Mississauga, ON, Canada) according to the manufacturer's protocol.

Microarray hybridization experiments were performed at McGill University and the Genome Quebec Innovation Center (Montreal, QC, Canada) using the HG-U133A GeneChip arrays. This chip allows the analysis of approximately 18,400 transcripts and variants, including 14,500 well-characterized human genes, composed of more than 22,000 probe sets. Protocols are available at the Affymetrix Web site (http://www.affymetrix.com/; Affymetrix, Santa Clara, CA, USA). Methods for labeling and hybridization of RNA were previously described [40].

Gene expression statistical analysis

Raw microarray expression data from Neve et al., 2006 was downloaded from (http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-157/) and combined with the data from the five BRC cell lines and together they were pre-processed and clustered using GeneSpring software (V7.3, Agilent Technologies). Pre-processing included first robust multiarray averaging (RMA) normalization, then genes with expression below 0.01 were forced to meet this threshold, per-chip normalization was performed to the 50th percentile, and per-gene normalization to the median. Data are presented as a log ratio, log2. Clustering was performed using the 305 gene classifier as previously described [38] using average linkage with similar branches merged and

In vivo tumor growth and establishment of explant cultures

The fourth mammary fat pad of ten athymic nude mice was injected with 1 × 10^6 BRC cells (n = 10 mice/cohort). Tumor growth was monitored by weekly caliper measurements and volume calculated according to the formula:

\[
\frac{(\pi L \times W^2)}{6}
\]

where L refers to the length and W to the width of the tumor. After 9 weeks of growth, tumors were removed, digested with collagenase B and Dispase I (Roche Diagnostics, Laval, Quebec, Canada) and then incubated with cell growth medium to establish tumor explants.

RNA preparation and microarray analysis

Total RNA was extracted using TRIzol reagent (Gibco/BRL, Life Technologies, Inc, Grand Island, NY, USA) according to the manufacturer's protocol. The quality of RNA was assessed using a 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies, Mississauga, ON, Canada) according to the manufacturer's protocol.

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Cell invasion assays were performed as previously described [39] with the addition, where indicated, of vitronectin (24 ng/ml) within the Matrigel matrix alone or within both the Matrigel matrix and coated on the bottom surface of the Boyden chamber at 400 ng/cm^2. In the latter assay, cells invade towards serum-free medium.

397, N-Cadherin, AKT, phospho-AKT S473 (Cell signaling, Whithby, ON, Canada); Integrins α5, αv, β1, β2, ErbB-2, FRA-1 (Santa Cruz Biotechnology, Dallas, TX, USA); α-Tubulin (Sigma, Oakville, ON, Canada), E-Cadherin (BD Biosciences, Mississauga, ON, Canada), uPAR (R&D Systems, Minneapolis, MN, USA), vimentin (Dako Canada Inc, Burlington, ON Canada), ER (Santa Cruz Biotechnology, Dallas, TX, USA), PR (Santa Cruz Biotechnology, Dallas, TX, USA) and cytokeratin-8 (a kind gift from Dr. Normand Marceau, Université Laval). Blots were incubated with either horseradish-peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Bar Harbour, ME, USA), developed with chemiluminescent HRP substrate (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Bar Harbour, ME, USA), or IR dye secondary antibodies (Licor Inc, Lincoln, NE, USA) and exposed to autoradiography film (Harvard Apparatus, Saint-Laurent, QC, Canada) or IR dye secondary antibodies (Licor Inc, Lincoln, NE, USA) and developed with the Odyssey Imager (Licor Inc, Lincoln, NE, USA). Quantification was performed using the ImageLite Studio software (Licor Inc, Lincoln, NE, USA).
The number of available patient samples for ana
genes within this signature were used for survival analysis
3955, 1747 and 1402, respectively (Fig. 7f-h).

Breast cancer dataset analysis

To assess potential clinical associations between BRA-A levels,
BRA-1 transcriptional activity, and molecular sig
aling components capable of activating BRA-1, the
kmplot.com dataset [42] was used. Patients were split into
high and low expression groups based upon the top quart
tile of patients versus the remainder of patients. Biased ar
rays were excluded from the analysis. No other filters
were applied to the patient population before Kaplan-
Meier analysis.

Statistical analysis

GraphPad Prism 7.0 was used for statistical analysis. For
sample sizes of three, the Shapiro-Wilk test was used to
determine the normality of the data. For samples of eight
or more the D'Agostino test was used to determine the
normality of the data. Unless otherwise stated in the figure
legends Student’s t test was used determine statistical sig
ificance. Supplemental methods and figure legends can
be found in Additional file 2: Document 1: Supplemental
Figure legends and Methods.

Results

Novel breast cancer lines derived from primary human
breast tumors are representative of the intrinsic subtypes

We have examined a set of breast cancer cell lines that
have been isolated directly from primary tumors of
breast cancer patients [37]. We first characterized these
explants by gene expression analysis and performed un
supervised clustering using a 305 gene signature [38] to
classify human breast cancer cell lines as either luminal,
basal A or basal B. Four of the five primary breast cancer
explants (BRC-17, 32, 36 and 196) clustered with other
luminal cell populations, whereas one explant (BRC-31)
clustered closely with basal B cell lines (Fig. 1a).

Generation of the FRA-1 transcriptional activity signature

A publically available dataset ([GSE:46440] [18]) was used
to generate an FRA-1 transcriptional activity signature.
Significance analysis of microarrays (SAM) [41] was used
to identify genes with 1.24-fold increase in control
transfected BT549 cells relative to BT549 cells transfected
with an siRNA pool targeting fos/l1. To apply the gene sig
nature, the average of the signature genes was first calcu
lated from RMA-normalized gene expression data and
subsequently mean-normalized to obtain a value between
0 (low) and 1 (high). The signature was then validated on
human breast cancer cell lines with either high or low
expression groups based upon the top quartile of the gene
expression data. The signature was then used for survival
analysis (Fig. 7c). The number of available patient samples for
analysis of overall survival (OS), relapse-free survival (RFS)
expressed higher levels of the esr1 and erbB2 relative to
the BRC-31 cell population (Fig. 1b). Conversely, the
BRC-31 explant exhibited high expression of egrf relative to
the other explant populations, which is characteristic of
50% of basal breast tumors [43] (Fig. 1b). To further
validate the basal nature of BRC-31 cells, we examined
the expression of epithelial (E-cadherin (cdh1),
cytokeratin-8 (krt8)) and mesenchymal (N-cadherin
(cdhl2), fibronectin (fn1) and vimentin (vim)) markers.
BRC-31 breast cancer cells expressed high levels of
cdh2, fn1 and vim and low levels of cdh1 and krt-8 com
pared to BRC-17, 32, 36 and 196 cells (Fig. 1c).

When injected into the mammary fat pads of athymic
mice, all five human explant cell lines were tumorigenic;
however, only two cell lines (BRC-31 and BRC-36)
demonstrated a reproducible ability to establish primary
tumors (>60% incidence) and maintain sustained growth
(Fig. 1d, e). No metastatic lesions were observed in lung
tissue collected from tumor-bearing mice (data not
shown). To verify phenotypic stability of these tumors
following growth in the mammary fat pad of mice, we
established explants from tumor-bearing mice (explant
A or B). Immunoblot analyses revealed that BRC-31
breast cancer cells retained expression of EGFR, N-
cadherin, fibronectin (FN) and vimentin at higher levels
relative to BRC-32, 36 and 196 breast cancer cells
(Fig. 1f). Conversely, BRC-31 cells exhibited low levels
of Her2, estrogen receptor-α (ERa), E-cadherin and
cytokeratin-8 relative to BRC-32, 36 and 196 cells
(Fig. 1f). These data confirm the basal (BRC-31) and
luminal nature (BRC-17, 32, 36 and 196) of these model
systems and validate the Affymetrix gene expression
profiles (Fig. 1a-c). While the incidence of tumor forma
tion was similar, the BRC-36 cell line, but not the BRC-
31 cell line, exhibited enhanced primary tumor growth
in mice implanted with estrogen pellets (data not
shown). Thus, we have characterized five novel breast
cancer cell lines, four that represent ER-positive luminal
breast tumors and one that represents basal breast tumors.

**Signaling via ERK2 leads to constitutive FRA-1 phosphorylation in basal B breast tumor cell lines**

We next characterized the signaling pathways that were active in the BRC series of cell lines and derived tumor explants. We assessed the activation of the PI3-kinase pathway by examining AKT phosphorylation and the MAPK pathway by detecting ERK1/ERK2 phosphorylation. While the degree of AKT expression and phosphorylation was not noticeably different in the BRC cell lines and explants, we noted that the pattern of ERK1/2 phosphorylation, and to some extent expression of total ERK1/2, was clearly divergent between basal and luminal BRC cell lines. Specifically, we observed that the BRC-31 cell line and both tumor explants exhibited prominent p42 ERK2 expression and phosphorylation relative to the luminal BRC breast cancer cells (Fig. 2a). Previous observations have linked ERK2-dependent signaling to an epithelial-to-mesenchymal transition (EMT) that relied on phosphorylation and stabilization of FRA-1, a component of the AP-1 transcription factor family [31, 44]. Interestingly, the Fos-like antigen 1 (*fosl1*) gene, which encodes FRA-1, is overexpressed in basal breast cancer cell lines when compared to luminal breast cancer cells, with an overabundance specifically in basal B breast cancer cells (Additional file 3: Figure S1A).

The observation that BRC-31 breast cancer cells exhibit preferential ERK2 activation and express markers of an EMT transition (E-cadherin and cytokeratin-8 low; N-cadherin, FN and vimentin high) prompted us to examine the phosphorylation status of FRA-1. In the BRC panel, FRA-1 expression was uniquely elevated in the BRC-31 cells (Fig. 2b). FRA-1 is phosphorylated on serine residues 252 and 265, which leads to protein stabilization [23, 28–31]. In a panel of established human breast cancer cell lines (Fig. 2b) representing the luminal-like and basal-like subtypes we observed that, similar to previous reports [18], FRA-1 expression and phosphorylation of serine-265 is elevated in basal breast cancer cells (Fig. 2b). While ERK1 and ERK2 expression (protein, Fig. 2c; RNA, Additional file 3: Figure S1B) is variable across this panel of cell lines.
lines with no positive correlation with FRA-1 phosphorylation, the ratio of ERK2/ERK1 expression is associated with phosphorylation of FRA-1 (pFRA-1) (Fig. 2c, Spearman correlation value of 0.675 \( P = 0.01 \); RNA, mapk1/mapk3 vs fosl1, Additional file 3: Figure S1A, Spearman correlation value of 0.451 \( P = 0.0004 \)). These observations demonstrate that basal-like breast cancer cell lines possess elevated ERK2 expression relative to ERK1 and exhibit increased FRA-1 phosphorylation.
A signaling axis involving FAK and SRC leads to FRA-1 phosphorylation in breast cancer cells

Given the elevated expression of EGFR in these cells (Fig. 1f), we anticipated that ERK activation occurred downstream of EGFR. While stimulation of BRC-31 cells with EGF led to an increase in pFRA-1/FRA-1 levels, inhibition of the EGFR with small molecule kinase inhibitors (AG1478 or gefitinib) did not alter ERK phosphorylation or basal pFRA1 status in BRC-31 cells (Additional file 4: Figure S2A, B). To identify the signaling pathways responsible for basal pFRA-1 levels in BRC-31 cells, we extended our characterization of the BRC panel of cell lines and examined FAK and SRC phosphorylation. We observed elevated phosphorylation on the FAK auto-phosphorylation site Y397 and on sites that are phosphorylated by SRC (Y576 and Y925) specifically in the basal BRC-31 cell line (Fig. 2d). Consistent with increased SRC activity we also observed increased SRC phosphorylation on Y416 in the BRC-31 cell line. This observation is of interest considering recent data implicating SRC in the phosphorylation of FRA-1 [45]. To determine if the SRC family kinase (SFK)-rapidly accelerated fibrosarcoma (RAF)-MEK pathway led to FRA-1 phosphorylation we treated cells with multiple small molecule kinase inhibitors that individually target each component of this pathway to identify optimal inhibitor concentrations, thus reducing the possibility of off-target effects. Inhibiting the activity of each protein kinase in this pathway reduced the level of FRA-1 phosphorylation relative to vehicle (Fig. 2e, f). These observations link SFK-RAF-MEK signaling activity to S265 phosphorylation on FRA-1.

**Extracellular matrix components engage integrin receptors for FRA-1 activation**

Given that EGFR inhibitors failed to diminish pFRA-1 levels, we reasoned that additional upstream receptors were responsible for SFK activation and ultimately FRA-1 phosphorylation. SFKs can be activated downstream of integrin engagement; thus, we assessed the expression levels of several integrin members. Interestingly, αv, α5, β1, and β3 integrin subunits were uniquely upregulated in BRC-31 breast cancer cells relative to the luminal BRC cell lines (Fig. 3a). These observations argue that integrin receptor-mediated FAK and SRC activation may represent a new mechanism leading to FRA-1 phosphorylation. Distinct integrin receptors bind to specific components of the ECM. For example, αvβ1 receptors bind FN, αvβ3 integrin receptors bind VN and α5β1 integrin receptors bind to laminin (LN). To determine which of these integrin heterodimers was responsible for increased FRA-1 phosphorylation, BRC-31 basal breast cancer cells were plated on VN, FN or LN. Only VN led to a significant increase in FRA-1 phosphorylation in BRC-31 breast cancer cells (Fig. 3b, c), which occurred prior to significant cell spreading on the ECM (Additional file 5: Figure S3A, B). To determine if VN-induced FRA-1 phosphorylation was mediated through ERK1/2, cells were transfected with siRNAs directed against either mapk3 (ERK1), mapk1 (ERK2) or scrambled control (Scrambled) and plated on VN-coated dishes (Fig. 3d). Only knockdown of ERK2, but not ERK1, diminished VN-induced FRA-1 phosphorylation (Fig. 3d).

Using available gene expression data from BT549 breast cancer cells transfected with siRNAs targeting fosli [18] we generated an FRA-1 transcriptional signature composed of genes that are positively regulated by FRA-1 (Table 1). Using four of the top regulated genes from this list, we assessed whether activation of FRA-1 phosphorylation correlated with increased FRA-1 transcriptional activity. Consistent with previous reports linking FRA-1 transcriptional activity with phosphorylation [32], only cells plated on VN, but not FN or LN, exhibited a significant increase in FRA-1 regulated transcriptional targets (Fig. 3e).

Urokinase plasminogen activator receptor (uPAR) is a known regulator of VN signaling in conjunction with integrin receptors [46, 47] and is a known transcriptional target of FRA-1 [48]. We speculated that uPAR could engage with specific integrin receptors to induce FRA-1 phosphorylation, which in turn maintains uPAR expression. Of the integrin receptors expressed in BRC-31 cells, αv, β1, and β3 were also elevated in established basal breast cancer cells (Fig. 4a). There was also strong correlation between high levels of plaur (uPAR) expression and the basal subtype (Fig. 4a, b and Additional file 6: Figure S4). We asked whether reduction of uPAR expression in basal-like breast cancer cells would affect ECM-induced signaling and FRA-1 phosphorylation. When BT549, BRC-31 or HCC1143 cells were plated on plastic, knockdown of uPAR expression using siRNA-mediated approaches did not alter FRA-1 phosphorylation when compared with control-transfected cells (Fig. 4c, d). When plated on VN, BT549, BRC-31 and HCC1143 cells harboring control siRNAs, displayed an increase in FRA-1 phosphorylation. In contrast, when breast cancer cells with diminished uPAR expression were plated on increasing concentrations of VN, we observed a decrease in FRA-1 phosphorylation (Fig. 4c, d). FAK activation, measured by Y925 phosphorylation, was diminished with uPAR knockdown (Fig. 4c); however, we only detected a modest decrease in ERK phosphorylation despite the requirement for ERK2 expression for the phosphorylation of FRA-1 in these cells (Fig. 3c). Taken together, the data argue that VN engages an integrin/uPAR complex to induce downstream SRC/FAK signaling that ultimately leads to FRA-1 phosphorylation.

FRA-1 increases the invasive properties of breast cancer cells [12]. We postulated that mixing VN with Matrigel would further enhance BRC-31 invasion, as this would lead to increased integrin engagement and enhanced
To test this, we coated the bottom surface of the Boyden chamber with VN, mixed VN together with Matrigel in the chamber, and allowed the cells to invade towards serum-free medium. In the absence of VN, BRC-31 and HCC1143 cells did not invade through the Matrigel (data not shown), consistent with our previous observation that LN (the major component of Matrigel) failed to stimulate FRA-1 phosphorylation. In the presence of VN, BRC-31 and HCC1143 cells invaded through the Matrigel/VN mix (Fig. 5a, b, Scr); however, siRNA-mediated knockdown (KD) of FRA-1 (Fig. 5c, d) suppressed VN-induced BRC-31 and HCC1143 cellular invasion (Fig. 5a, b, fosf1 KD). Consistent with a role for uPAR in stimulating the engagement of VN with integrins, siRNA-mediated knockdown of uPAR (Fig. 5c, d) reduced the invasive properties of BRC-31 and HCC1143 cells (Fig. 5a, b, plaur KD). Knockdown of FRA-1 or uPAR had no effect on cell proliferation over the duration of this assay (Additional file 7: Figure S5A-D). These data demonstrate that uPAR and FRA-1 are required for VN-induced cellular invasion.

Phosphorylation of FRA-1 is required for transcriptional activity
To determine if phosphorylation was required for FRA-1 activity, we knocked down the expression of both uPAR and FRA-1 with siRNA in BRC-31 cells (Fig. 6a) resulting in reduced expression of FRA-1 transcriptional targets relative to a non-targeting control (Fig. 6b), confirming the dependence, in part, on FRA-1 for the transcription of these targets. To rescue knockdown of endogenous FRA-1, we overexpressed either wild-type FRA-1, a phospho-deficient FRA-1 mutant (S252AS265A) or a phospho-mimetic FRA-1 mutant (S252DS265D) (Fig. 6a). Expression of wild-type FRA-1 or the phospho-mimetic mutant of FRA-1 restored expression of FRA-1 regulated transcriptional targets (Fig. 6b). In contrast, BRC-31 cells expressing the phospho-deficient mutant of FRA-1 displayed similar levels of these FRA-1 transcriptional targets to BRC-31 cells harboring the vector control (Fig. 6b).
uPA, uPAR and FRA-1 are frequently co-expressed in human breast cancers

We analyzed representative breast cancer cell lines for expression of uPA, the ligand for uPAR, and observed that uPA mRNA expression and secretion was highest in basal breast cancer cells (Additional file 8: Figure S6A, B). We next wished to determine if this signaling axis was also upregulated in patients with breast cancer. As a first step, we examined tumor lysates from five breast cancer patient-derived xenografts (PDX) for the expression of uPAR, uPA and phosphorylated FRA-1 [49, 50]. Two of five PDX samples had elevated uPAR expression...
and detectable FRA-1 phosphorylation (Additional file 8: Figure S6C). The PDX sample showing the highest phospho-FRA-1 levels exhibited the highest uPA expression in tumor lysates (Additional file 8: Figure S6D).

We next assessed clinical correlation between fosl1 expression, FRA-1 activity and breast cancer patient outcomes. Kaplan-Meier analysis of a human breast cancer dataset revealed that fosl1 gene expression alone did not was not significantly prognostic of overall patient survival (Fig. 7a). To validate the robustness of the FRA-1 transcriptional signature (generated from available gene expression data [18] (Table 1)) as a surrogate readout of FRA-1 activity, we used it to segregate breast cancer cells in which FRA-1 phosphorylation status was previously established (Fig. 2b). Importantly, breast cancer cells characterized as high for the FRA-1 transcriptional signature were the same ones that displayed high FRA-1 phosphorylation (Fig. 7b) and the signature was prognostic of poorer overall survival in patients with breast cancer (Fig. 7c). Interestingly, we noted that the correlation between fosl1 mRNA expression and presence of the FRA-1 expression transcriptional signature, while significant, was not very strong (Fig. 7d) consistent with fosl1 expression alone not being prognostic in this dataset (Fig. 7a). This suggests FRA-1 expression alone does not translate to FRA-1 transcriptional activity. We speculated that breast tumors characterized by elevated expression for molecular components of the uPA/uPAR/VN/FRA-1 signaling axis might possess elevated FRA-1 transcriptional activity. Indeed, a molecular components signature (plau/plaur/vtn/fosl1) correlated well with FRA-1 transcriptional activity (Fig. 7e). Kaplan-Meier analysis of the same breast cancer dataset revealed that this molecular component signature was also prognostic in predicting poorer overall survival, recurrence-free survival and distant metastasis-free survival (Fig. 7f, g, h, respectively).

**Discussion**

Phosphorylation provides a rapid mechanism to regulate FRA-1 transcriptional activity depending on the micro-environment encountered by the cell. Here we have described a vitronectin-stimulated pathway that leads to increased FRA-1 phosphorylation, transcriptional activity and invasion in basal-like breast cancer cells. Vitronectin is a soluble ECM protein that is found in the circulation...
and may provide a link between the components of the ECM, via collagen and heparin binding domains within vitronectin, and integrin binding-domain-containing proteins expressed on cells [51]. Vitronectin connects with assembled matrix proteins around wounds and thus may act as a bridge between the circulating tumor cells and areas of vascular damage. This leads to the intriguing possibility that cells engaging vitronectin may activate FRA-1 through increased phosphorylation and result in increased tumor cell extravasation. Indeed, vitronectin can stimulate increased tumor cell invasion [52, 53] and promote tumor growth [53] of breast cancer cells. Vitronectin may also play a role during intravasation as it can be detected in subendothelial regions and

![Fig. 5](image)

**Fig. 5** Vitronectin-induced breast cancer invasion requires urokinase/plasminogen activator urokinase receptor (uPAR) and Fos-related antigen 1 (FRA-1) expression. BRC-31 (a) or HCC1143 (b) breast cancer cells were plated in Boyden chambers in which the bottom surface was coated with vitronectin and the upper surface of the chamber coated with Matrigel mixed with vitronectin. Cells were allowed to invade towards serum-free medium for 24 hours. Quantification of breast cancer cell invasion and representative images are shown. Data from nine independent experiments plotted with the error bars representing the standard deviation. P values are as indicated. Immunoblot analyses of protein lysates from BRC-31 (c) or HCC1143 (d) cells transfected with scrambled (Scr), fos1 (fos1 knockdown (KD)) or plaur (plaur KD) siRNAs. α-Tubulin served as a loading control and representative blots from one of nine independent sets of lysates are shown.

![Fig. 6](image)

**Fig. 6** Phosphorylation of Fos-related antigen 1 (FRA-1) is required for transcriptional activity. a) Immunoblots from BRC-31 cells transfected with siRNA to plaur and fos1 (UPAR KD/FRA-1 KD) or scrambled control were subsequently transfected with either empty vector (VC), FRA-1 wild-type (WT), FRA-1-S252A/S265A (S252A/S265A) or FRA-1-S252D/S265D (S252D/S265D) expression vectors. b) Gene expression analysis of FRA-1 regulated transcriptional targets, chloride channel accessory 2 (clca2), adhesion G protein-coupled receptor G6 (adgrg6), C-X-C motif chemokine ligand 8 (cxcl8) and C-C motif chemokine ligand 5 (ccl5). Expression values are normalized to the scrambled control. Error bars represent the standard error of the mean. *P < 0.02
in small vessels surrounding tumor cells [54]. FRA-1 regulates the expression of multiple proteins involved in cell migration and invasion and it can also promote cell migration by suppressing RhoA activity; however, the exact mechanism has not yet been identified [55, 56].

The glycosyl phosphatidylinositol anchored membrane protein uPAR has been implicated in vitronectin-stimulated tumor cell migration and invasion [46, 47, 57]. As a membrane-associated protein, uPAR requires other membrane receptors including G protein-coupled receptors (GPCRs), certain growth factor receptors and integrin complexes to facilitate intracellular signaling (recently reviewed [58]). The β1-integrin and β3-integrin subunits are frequently reported as important signaling partners for uPAR [59]. Both uPAR and β3-containing integrin receptors can bind vitronectin, with uPAR recognizing the SMB domain and the β3 integrin subunit the Arg-Gly-Asp sequence of vitronectin; however, it is unclear whether a ternary complex indeed forms between uPAR-VN-β3-integrin [60]. Integrin signaling may be enhanced by uPAR through a direct conformational change in the β3-integrin subunit [61] or uPAR may alter the membrane surface leading to integrin activation [62]. It was recently reported that cell spreading induced by uPA-vitronectin is not dependent on β3-integrin signaling, but requires non-ligand dependent activation of β1-containing integrin receptors, which is mediated through changes in membrane tension [62]. It is noteworthy that only vitronectin, and not fibronectin or laminin, was able to strongly stimulate FRA-1 phosphorylation in BRC-31 cells. It remains to be determined what additional cellular components are required to initiate this signaling axis.

binding of urokinase plasminogen activator (uPA) to uPAR can enhance the binding of vitronectin to uPAR due to the fact that uPA and vitronectin utilize mutually exclusive binding sites to simultaneously bind uPAR [63, 64]. Upon uPAR binding, uPA is activated and cleaves the zymogen plasminogen into the active protease, plasmin, ultimately leading to the degradation of ECM components [65]. uPAR is also a substrate for uPA and the presence of soluble uPAR (suPAR) fragments in the circulation of preoperative patients with breast cancer is indicative of poor prognosis [66]. As our experimental system removes any secreted protein, we suspect that uPA is not required for

**Fig. 7** A molecular components signature of plau/plaur/vtn/fosl1 is prognostic in human breast cancer. **a** Kaplan meier analysis of overall survival in patients with breast cancer separated by high or low fosl1 expression. **b** Activity analysis of a Fos-related antigen 1 (FRA-1) transcriptional signature in breast cells with elevated phosphorylated FRA-1 (High pFRA-1 cell lines) or low levels of FRA-1 phosphorylation (Low pFRA-1 Cell lines). The activity index indicates the probability that a given breast cancer cell line will be positive for the FRA-1 signature. **c** Kaplan-Meier analysis in breast cancer patients using an FRA-1 transcriptional activity signature and molecular component signature composed of the top 65 genes regulated by FRA-1 (see Table 1). Patients are divided into high (red) or low (black) transcriptional activity. **d** Correlation between fosl1 expression and FRA-1 transcriptional activity signature. **e** Correlation between the FRA-1 transcriptional activity signature and molecular component signature composed of genes encoding FRA-1 (fosl1) and upstream signaling proteins (plau/plaur/vtn). **f** Kaplan-Meier analysis of breast cancer patients divided into high (red) and low (black) reveals that expression of a molecular component signature is prognostic for decreased overall survival (g), recurrence-free survival (RFS) and (h) distant metastasis-free survival (DMFS). KMplot.com dataset was used in all analysis apart from b where the Neve et al. dataset was used.
vitronectin-stimulated FRA-1 phosphorylation; however, it remains to be determined whether uPA is required for the invasive phenotype.

The controversial relationship between FRA-1 expression and clinical outcome in patients with breast cancer may be, in part, due to the fact that expression levels of FRA-1 may not correlate with phosphorylation status and transcriptional activity. To address this possibility, an “FRA-1 classifier” was constructed by identifying genes from an “FRA-1 transcriptome” (differentially expressed genes in MDA-MB-231 LM2 cells harboring short hairpin RNAs (shRNAs) to FRA-1 versus vector controls cells) that also had prognostic significance in publicly available datasets [10]. The Desmet et al. FRA-1 classifier showed significant prognostic ability to identify distant metastasis across all subtypes with the exception of Her2+/ER- breast cancers. This curated FRA-1 classifier provides a useful readout of FRA-1 activity; however, the subset of genes regulated by FRA-1 likely differ significantly depending cellular context, epigenetic variation and microenvironment. Similar to the datasets examined by Desmet et al., our Kaplan-Meier analysis using fosl1 expression alone did not have prognostic significance in the independent breast cancer dataset utilized in this study; however, a gene signature containing the molecular components of the novel signaling pathway we have delineated in the present study (plau/plaur/vtn/fosl1) was able to identify patients with breast cancer with poor overall survival, recurrence-free survival and distant metastasis-free survival. We speculate that tumors with these signaling components would possess elevated FRA-1 activity and therefore be more aggressive in nature. Until now, FRA-1 phosphorylation has been described downstream of receptor tyrosine kinase signaling, via ERK2 [23, 29–31], and via members of the PKC family [2, 11]. Our data uncover a new signaling pathway, downstream of vitronectin engagement of integrin complexes that is augmented through the uPA/uPAR axis, which ultimately engages SRC/RAF/MEK to mediate FRA-1 phosphorylation.

Many components of the vitronectin-uPAR-integrin signaling axis are transcriptionally regulated by FRA-1 [10, 21], suggesting the existence of a positive feedback loop that further enhances FRA-1 activity. Strategies to suppress the pro-metastatic effects of FRA-1 include targeting downstream transcriptional targets and suppressing their activity [9, 10, 25]. However, given that numerous transcriptional targets likely contribute to the observed FRA-1 effects on breast cancer invasion and metastasis, such an approach may prove ineffective. Here we have demonstrated that vitronectin, and not laminin or fibronectin, stimulates FRA-1 phosphorylation via an uPAR-dependent process, suggesting that targeting this specific upstream axis could prove efficacious [67]. Application of the recently characterized small molecule inhibitors of uPAR-integrin association [68] and antibodies [69] may be beneficial in suppressing this signaling axis and reducing tumor cell invasion and metastases.

Conclusions
We have identified a vitronectin stimulated signaling axis that leads to phosphorylation and stabilization of FRA-1, which is associated with increased transcriptional activity and breast cancer invasion. Notably, components of this signaling axis, along with a transcriptional signature of FRA-1 activity, are associated with poor clinical outcomes in patients with breast cancer. These data highlight FRA-1 as a transcription factor important for promoting breast cancer progression and metastasis.

Additional files

Additional file 1: Oligonucleotides utilized in this manuscript. (XLSX 47 kb)
Additional file 2: Document 1: Supplemental Figure legends and Methods. (DOCX 86 kb)
Additional file 3: Figure S1. Gene Expression of mapk1, mapk3 and fosl1 in human Breast Cancer Cell lines. (PTX1 1395 kb)
Additional file 4: Figure S2. EGFR inhibition is not sufficient to decrease phosphorylation on FRA-1. (PTX1 4508 kb)
Additional file 5: Figure S3. FRA-1 phosphorylation occurs prior to cell spreading. (PTX1 4024 kb)
Additional file 6: Figure S4. Gene Expression of plaur in human Breast Cancer Cell lines. (PTX1 1129 kb)
Additional file 7: Figure S5. Knockdown of plaur or fosl1 does not affect cell proliferation. (PTX1 515 kb)
Additional file 8: Figure S6. Basal-like breast cancer cell lines and patient-derived xenografts (PDXs) that possess elevated FRA-1 phosphorylation display high uPAR and uPA expression. (PTX1 1129 kb)

Abbreviations
ADOR2A2: Adenosine receptor A2b; AXL: Axl tyrosine kinase receptor; CDH1: E-Cadherin; CDH2: N-Cadherin; CLCA2: Chloride channel accessory 2; DM50: Dimethylsulfoxide; EGFR: Epidermal growth factor receptor; EMT: Epithelial-to-mesenchymal transition; ERK: Extracellular signal-regulated kinase; FOS: Fos-related antigen 1; HER2: Human epidermal growth factor receptor 2; KD: Knockdown; KRT: Cytokeratin-8; LN: Laminin; MAP: Mitogen-activated protein; MPP: Matrix metalloproteinase-9; MMP: Matrix metalloproteinase-1; NF: Nerve growth factor receptor 2; PSR: Progesterone receptor; RAF: Rapidly accelerated fibrosarcoma; SCR: Scrambled; SFK: SRC family kinase; siRNA: small interfering RNA; UPA: Urokinase plasminogen activator; UPAR: Urokinase /plasminogen activator urokinase receptor; UTR: Untranslated region; VIM: Vimentin; VN: Vitronectin

Acknowledgements
We thank members of the Siegel laboratory for their thoughtful discussions regarding the current work. We also thank Dr. Harvey Smith for helpful discussions/technical advice and Paul Savage for his help with the patient-derived xenograft samples.

Funding
This research was supported by a grant from the CIHR (MOP-119401) held by PMS. A-MM-M and VO are researchers of the Centre de Recherche du Centre...
Hospitalet de l’Université de Montréal (CRCHUM) and receive support from the FRQS. PMS is currently a McGill University William Dawson Scholar.

Availability of data and materials
Data generated or analyzed during this study are included in this published article and its supplementary information files. The microarray data for the BRC cell lines [GEO:GSE69915] and FRA-1 transcriptional signature [GEO:GSE4644] can be accessed through the GEO repository (https://www.ncbi.nlm.nih.gov/geo). The Kaplan-Meier dataset comprises several gene expression datasets referenced in Gyorffy et al. [42].

Authors’ contributions
MGA and PMS designed all experiments. MGA performed the in vitro and in vivo experiments. VO and A-MM-M performed microarray gene expression analysis of the BRC cell lines. JPR and ERA performed gene expression analysis in the human datasets. SLE and CR provided the BRC cell lines. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The McGill University Facility Animal Care Committee Downtown Campus A approved all animal studies. No additional ethical approvals or consents were required.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Author details
1Goodman Cancer Research Centre, McGill University, Montréal, Québec, Canada. 2Departments of Biochemistry, McGill University, Montréal, Québec, Canada. 3Departments of Medicine, McGill University, Montréal, Québec, Canada. 4Departments of Anatomy and Cell Biology, McGill University, Montréal, Québec, Canada. 5Centre de Recherche du Centre Hospitalier de l’Université de Montréal (CRCHUM) and Institut du cancer de Montréal, Montréal, Canada. 6Department of Physiology, Michigan State University, East Lansing, Michigan, USA. 7Département de Microbiologie et Infectiologie, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Canada.

Received: 3 August 2017 Accepted: 15 January 2018
Published online: 30 January 2018

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