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FOS-Related Antigen 1 (FRA-1) Switches Breast Cancer Cells from a Luminal to a Basal-like Subtype with Spontaneous Metastasis Behavior

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Submitted
Abstract
For tumor cells to escape the primary tumor and invade into surrounding tissue and circulation, the activation of an epithelial-to-mesenchymal (EMT) transition is important to acquire a migratory and invasive behavior. This genetic program is marked by the loss of the expression of epithelial markers such as E-cadherin and gain of expression of mesenchymal markers, for example N-cadherin and vimentin. The transcription factor FOS-related antigen 1 (Fra-1) has increased expression in mesenchymal-like compared to epithelial-like breast cancer cell lines but direct evidence for a role for Fra-1-mediated EMT in association with metastasis formation is lacking. In this study, we demonstrated that ectopic expression of Fra-1 is sufficient to induce a full EMT genetic program in the luminal breast cancer (BC) cell line MCF7. This resulted in cell scattering, anchorage independent growth and increased cell migration with enhanced focal adhesion turnover. Importantly, Fra-1 expression induced tumorigenesis as well as metastasis formation in an in vivo orthotopic BC mouse model. Gene expression profiling followed by unsupervised hierarchical clustering demonstrated a switch towards basal B-like breast cancer cells upon introduction of Fra-1 in the luminal MCF7 cell line. Finally, knockdown of Fra-1 in basal B cell lines reduced their migratory capacity and metastatic potential. Together the results indicate that Fra-1 is a dominant factor in breast cancer progression and metastasis and pin-point Fra-1-based signaling as a valid target for development of breast cancer therapies.

Key words
Fra-1, migration, epithelial-to-mesenchymal transition, breast cancer, basal B subtype, metastasis
Introduction

Breast cancer (BC) is a heterogeneous disease. Genetic profiling of BC samples allowed the identification of five major BC molecular subtypes: luminal A, luminal B, normal breast-like, HER2-enriched and basal-like [1; 2]. Each subtype has unique biological and prognostic features and is treated according to the best fitting therapeutic regime. The basal subtype comprises the most aggressive and invasive tumors. Invasive cancer cells disseminate from the primary tumor site and invade into surrounding stroma and tissue, ultimately ending up in distant organs to form secondary tumors, a process called metastasis. This may be associated with an epithelial-to-mesenchymal transition (EMT) in which cells lose cell-cell contacts and gain a scattered phenotype. In particular downregulation of the cell-cell adhesion molecule E-cadherin is an important feature of this genetic program [3]. Gene expression profiling of a panel of human BC cell lines that harbor different modes of E-cadherin promotor inactivation, identified the transcription factor Fra-1 to be highly upregulated in cell lines with inactive E-cadherin. In addition, Fra-1 expression was associated with a more fibroblastic, scattered phenotype [4]. EMT is regulated by multiple signals including pathways activated by growth factors and extracellular matrix. One of the main effectors of these signals is the small GTPase Ras. Although Ras has many effectors, previous studies have shown that ERK2 is a critical regulator of active Ras-induced EMT via Fra-1/ZEB1/2 pathway in normal breast cancer cells [5]. Yet, a dominant role for Fra-1 in the induction of EMT independent of Ras and in the context of cancer metastasis is unknown.

Fra-1, also called FOS-related antigen 1 (FOSL1), is a member of the Fos family, together with Fos, FosB and Fra2. These transcription factors bind to TPA-responsive elements (TRE) in promotor or enhancer regions [6]. Being part of the activation protein 1 (AP-1) complex, it regulates genes involved in many biological processes such as differentiation, survival and proliferation [7; 8]. Fra-1 activity itself is regulated by post-translational modification. Phosphorylation by different kinases (e.g. mitogen-activated protein kinase (MAPK), protein kinase A (PKA) and C (PKC)) influences Fra-1 stability, DNA binding activity and trans-activating potential of the transcription factor [9; 10]. Inhibition of PKCθ activity resulted in a reduction in Fra-1 abundance in estrogen receptor (ER) negative BC cells [11]. Several studies have reported increased expression of Fra-1 in different cancer cell lines including lung [12], brain [13], bladder [14] and colon [15], but Fra-1 was mostly studied in breast cancer [16]. In a mouse mammary gland adenocarcinoma cell line, Fra-1 was positively correlated with high malignancy and negatively correlated with
E-cadherin expression [17]. In an other study, a cDNA array with four highly invasive and nine weakly invasive human BC cell lines defined 24 genes that are differently expressed, including Fra-1 [18]. In addition to genetic profiling, quantification of Fra-1 protein levels in human breast tumor samples revealed a correlation between Fra-1 expression and increasing tumor grade [19; 20].

Cancer progression is associated with several biological processes, including sustained cell proliferation and enhanced cell migration and invasion [21]. Fra-1 expression has been associated with some of these hallmarks [16; 18; 22]. Ectopic expression of Fra-1 in mouse epithelial adenocarcinoma cells with low intrinsic motility showed an increase in the rate of cell diffusion and mean cell speed. In addition, inhibition of Fra-1 in a highly motile mouse fibroblastoid cell line resulted in a significant reduction of cell speed [16; 23]. A role of Fra-1 in oncogenic Ras-induced tumor motility could be explained by regulation of RhoA GTPase activity through β1-integrin signaling, as was shown in colon carcinoma cells [24]. Such a role is possibly related to the control of the expression of transcriptional E-cadherin repressors by Fra-1 [25; 26]. While these combined results suggested a role of Fra-1 in EMT regulation, so far, no study conclusively supported that Fra-1 by itself is sufficient to induce EMT. Moreover, a direct role for Fra-1 in cancer metastasis is still lacking.

Here our aim was to determine the role and mechanism of Fra-1 in breast cancer progression and metastasis. We demonstrated that ectopic expression of Fra-1 in the luminal BC cell line MCF7 transformed them into highly motile and metastatic cells in an in vivo orthotopic BC mouse model. Gene expression profiling informed that MCF7-Fra-1 cells underwent a full EMT, and clustered with basal B-like BC cell lines. Importantly, stable knockdown of Fra-1 in basal B cell lines decreased cell migration and blocked spontaneous BC metastasis in vivo. Taken together, our data indicates that Fra-1 itself is sufficient to induce a full EMT program thereby driving spontaneous breast cancer metastasis.

**Materials and Methods**

**Cell culture**

MCF7 (ATCC-CRL-5803), MDA-MB-231 (ATCC-HTB-26), BT549 (ATCC-HTB-122) and 4T1 cells (ATCC-CRL-2539), as well as all other human BC cell lines mentioned in this manuscript were cultured in RPMI (GIBCO, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (PAA, Pasching, Austria) and 100 International Units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells
Fra-1 expression induces EMT and increases metastatic potential.

were maintained in a 5% CO₂ humidified chamber at 37°C.

**Generation stable Fra-1 overexpressing and knockdown cell lines**

Stable Fra-1 expressing MCF7 cells were generated using a retroviral vector containing mouse Fra-1 cDNA tagged with 8×myc [27]. A pBabe empty vector was used as control.

Stable Fra-1 knockdown cell lines were generated using lentiviral MISSION shRNA vectors (Sigma Aldrich, St. Louis, MO, USA). The human sequence (TRCN0000019541) used was CCGGCCTGCTGCAAGTAGTAGTCTCGAGTACTCTTTGCGATGCGCTGAGGTTTTT, and the mouse sequence (TRCN0000042686) was CCGGCCTCGCTCAGCGAAGTAGTCTCGAGTACTCTTTTCCGGTACGCGGAGGTTTTTG. A non-targeting vector was used as control. Transduced cell lines were maintained in 2 µg/mL puromycin containing selective medium.

**Antibodies**

Mouse anti-Fra-1 antibody (C-12, imunostaining), rabbit anti-Fra-1 (R-20, Western Blot) and rabbit anti-c-Myc were purchased from Santa Cruz (Santa Crux, CA, USA). Mouse anti-FAK (clone 4.47) and mouse anti-SRC were purchased from Upstate (Millipore, Billerica, MA, USA). Rabbit anti-FAK Y397, Y576, Y861 and rabbit anti-paxillin (PXN) Y118 were purchased from Biosource (Invitrogen, Carlsbad, CA, USA). Mouse anti-PXN, mouse anti-p130Cas, mouse anti-β-catenin, mouse anti-E-cadherin and mouse anti-N-cadherin were purchased from BD Transduction (Franklin Lakes, NJ, USA). Rabbit anti-SRC Y418 was purchased from Invitrogen. Rabbit anti-p130Cas Y246 was purchased from Cell Signaling (Danvers, MA, USA). Goat anti-vimentin and mouse anti-tubulin were purchased from Sigma Aldrich (St. Louis, MO, USA). Rabbit anti-ZO1 was purchased from Zymed (Invitrogen). Rhodamin phalloidin was purchased from Molecular Probes (Invitrogen).

**Soft agar colony assay**

Cells were plated in 1.5 mL top agar (0.34% w/v LMP agarose in complete medium) on top of 2.5 mL bottom agar (0.66% w/v LMP agarose in complete medium) in a 6-well plate (Corning, New York, NY, USA). After 7 days, 150 µL of a 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma Aldrich) was added and incubated overnight at 4 °C. Digital images of the well were taken with a Canon IXUS digital camera. Colonies were counted and size was determined using a particle analysis macro in ImageJ (NIH, Bethesda, MD, USA).
Transient siRNA mediated gene knockdown

siGENOME smartpool against human Fra-1 or GFP as negative control (Dharmacon, Lafayette, CO, USA) was used in a 50 nM reaction. Transfection was done using INTERFERin (Polyplus-transfection, New York, NY, USA) using manufacturers guidelines. Medium was refreshed next day. After 48-72 hr, cells were used in various assays.

Live cell imaging random cell migration assay

Glass bottom 96-well plates (Greiner Bio-one, Monroe, NC, USA) were coated with 20 µg/µL collagen type I (isolated from rat tails) for 1 hr at 37°C. Cells were plated and after 48 hr, cells were pre-exposed to 0.1 µg/uL Hoechst 33342 (Fisher Scientific, Hampton, NH, USA) to visualize nuclei. After refreshing the medium, cells were placed on a Nikon Eclipse TE2000-E microscope fitted with a 37°C incubation chamber, 20x objective (0.75 NA, 1.00 WD) automated stage and perfect focus system. Automatically, 3 positions per well were defined and the Differential Interference Contrast (DIC) and Hoechst signal was acquired every 10 min for a total imaging period of 12 hr. After the imaging period, plates were fixated using 4% paraformaldehyde and stored for later immunostaining. All data was converted and analyzed using custom made ImagePro Plus macros [28]. Cell migration was quantified by tracking of nuclei in time.

Total Internal Reflection Fluorescence (TIRF) imaging of FA dynamics

CELLview glass bottom dishes with four compartments were coated for 1 hour at 37°C with 10 µg/uL fibronectin (Sigma Aldrich) in the case of MCF7 and 20 µg/µL collagen type I (isolated from rat tails) in case of MDA-MB-231. Cells expressing GFP- or mCherry-paxillin were used for this assay and were plated 24 hr prior to imaging. Dishes were mounted on a Nikon Eclipse TE2000-E microscope fitted with a 37°C incubation chamber, 60x oil objective (1.49 NA, 0.12 WD) and perfect focus system. Focal adhesions were visualized using TIRF with the 488 or 561 nm lasers for one hour, with one-min interval. The cytoplasmic GFP/mCherry signal was acquired every 5 min using widefield fluorescence. Data was exported as .tif images and both channels were merged while making .avi files. The cytoplasmic channel was used to manually track the cells and determine cell migration velocity within the same experiment. FA dynamics was analyzed by making time color overlays using a custom made plug-in from Image-Pro Plus (MediaCybernetics, Bethesda, MA, USA).
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**Western Blotting**

Cells were scraped in ice-cold TSE (10 mM Tris-HCL, 250 mM sucrose, 1 mM EGTA pH 7.4) supplemented with inhibitors. After sonication of cell lysates, protein concentration was determined by Bio-Rad protein assay (Hercules, CA, USA) using IgG as internal standard. 30 μg of total cellular protein was separated on 7.5 or 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, USA). Blots were blocked in 5% w/v bovine serum albumin in TBST (0.5 M NaCl, 20 mM Tris-HCl, 0.05% v/v Tween20 pH 7.4) and probed with primary antibody (overnight, 4°C) followed by incubation with secondary antibody either Dylight-649 or Horse radish peroxidase-coupled (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Dylight-649 was directly visualized by scanning on Typhoon imaged 9400 (Amersham Biosciences, Uppsala, Sweden). HRP probes were first activated by incubation with Enhanced Chemiluminescence reagent (Amersham Biosciences) and detected using film.

**Immunofluorescence staining**

Cells were cultured on 12-mm 10 μg/μL fibronectin coated coverslips and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Coverslips were blocked in TBP (0.1% v/v Triton-X and 0.5% w/v bovine serum albumin in PBS) for 1 hour at room temperature and overnight incubated with primary antibody, followed by 1 hour incubation of fluorescently labeled secondary antibody. Next, coverslips were incubated with 2 μg/mL Hoechst 33258 (Sigma Aldrich) to visualize nuclei and mounted in Aqua Polymount (Polysciences, Warrington, PA, USA). Cells were imaged using a Nikon Eclipse TE 2000-E confocal microscope fitted with a 20x objective (0.75 NA, 1.00 WD) and 4 times digital zoom.

**RNA isolation**

Cells were cultured in P60 dish (Corning) and 48 hr after seeding, RNA was isolated using mirVana miRNA isolation kit (Ambion, Life Technologies, Carlsbad, CA, USA), following manufacturers guidelines. RNA quality and concentration was determined using Nanodrop ND-1000 and samples were stored at -80°C.

**FOSL1 mRNA levels in human breast cancer cell lines**

Expression data of 36 BC cell lines was available in GEO (series GSE16795) and levels of FOSL1 (Fra-1) were extracted using probeset 204420_at [29].
Gene expression profiling MCF7-ctrl and MCF7-Fra-1
RNA integrity and quality was assessed using the Agilent bioanalyser (Agilent Technologies, Palo Alto, CA, USA). The synthesis of labeled cRNA and hybridization steps were performed by Service XS (Leiden, The Netherlands) using the Affymetrix 3’ IVT-Express Labeling Kit (#901229) and the Affymetrix Human Genome U133 plus PM arrays. Scanning of the Array Plates was performed using the Affymetrix GeneTitan scanner. BRB Array Tools software (developed by Dr. Richard Simon and BRB-ArrayTools Development Team; http://linus.nci.nih.gov/BRB-ArrayTools.html) was used to normalize the expression data using the Robust Multichip Average (RMA) method. Significantly differentially expressed genes (p-value < 0.001) between the various experimental conditions were identified with an ANOVA test followed by calculation of the false discovery rate according to Benjamini and Hochberg (1995).

Gene expression profiling human breast cancer cell lines
RNA from 52 human BC cell lines, cultured in Rotterdam, and MCF7-ctrl and MCF7-Fra-1 cells, cultured in Leiden, was hybridized in triplicate to Affymetrix HT HG-U133+ PM Array Plate according to manufactures procedure (Affymetrix, Santa Clara, CA, USA). Raw data was processed with Affymetrix Expression console, using RMA parameters. The data of each triplicate was averaged per probeset and the top 10% (n=5472) variable probesets was used to cluster all cell lines. Data was median centered and clustered using average linkage correlation.

RT QPCR
RNA was treated with 1 U of RNase-free DNase RQ1 (Promega cat #M6101) per 1 µg RNA for 30 min at 37°C in appropriate buffer. DNase was inactivated by incubation in Promega’s stop solution for 10 min at 65°C. Bulk Mg2+ was removed by using Amicon ultra 0.5-mI centrifugal filters (Millipore cat #UFC510096) in two consecutive diluting washes. cDNA synthesis was performed with iScript (Bio-Rad cat # 170-8891) following the manufacturer’s instructions. Quantitative PCR was done using the Fast SYBR master mix kit (Applied Biosystems cat # 4385618) for the genes of interest and reference genes. Primers were designed using Primer Express 1.0 Software (Perkin Elmer Applied Biosystems): SNAI1 forward CAGGACTCTAATCCAGAGTTTACCTTC, reverse GGGATGGCTGCCAGCA. SNAI2 forward GCCAAACTACAGCGAACTGGA, reverse TGTGGTATGACAGGCATGGAG. ZEB1 forward TGTTACCAGGAGGAGGAGACGTG, reverse TCTTGCCTTCCTTTTCTGTCA. ZEB2 forward CGAGCGGCATATGAGAC, reverse GCCACACTCTGTGCATTTGAA. Housekeeping
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genes TBP forward CGGCTGTAAAATTCCCTTC, reverse CACACGCAAGAACACGTGA and GAPDH forward TGCACCACCAACTGCTTAGC, reverse GGCATGGACTGTGGTGTAG. Plates were run on the LightCycler 480 (Roche). The average threshold cycle of triplicate reactions was used for all subsequent calculations using the deltaCT method.

Spontaneous and experimental metastasis assays
Experiment with 4T1 was done as previously described [30]. In short, female Rag2⁻/-γc⁻/- mice were orthotopically injected with 100,000 4T1 cells in the fourth mammary gland (6-8 animals per group). Tumor growth was monitored over a 3-weeks period and then mice were sacrificed and tumor and lungs were isolated. Tissue was processed for immunohistochemical analysis. Lungs were injected with Indian ink to count surface metastasis. Experiments with MCF7 were done similarly, though in this case 1,000,000 cells mixed with 1 part 8.4 mg/ml growth factor reduced matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were injected in the tumorigenesis/spontaneous metastasis model. 500,000 cells were injected directly in the tail vein for experimental metastasis. Next to lungs, also liver, reproductive organs, axillary and branchial lymph nodes were isolated and inspected for presence of tumor cells and metastatic nodules.

Statistics
Student’s t test was used to determine significant differences between two means (P<0.05 or P<0.01). Values are represented as mean ± sem. Significant differences are marked in the graphs.

Results
Fra-1 is higher expressed in basal breast cancer cell lines compared to luminal cell lines
To get a detailed overview on the distribution of Fra-1 expression in breast cancer (BC) cell lines, we first determined the expression of FOSL1 mRNA in a large panel of human BC cell lines (n = 36) and classified them as luminal (including luminal A, luminal B and ErbB2 positive cell lines) or basal (including basal A, basal B and triple negative cell lines) [31]. While luminal cell lines had hardly any expression, the basal cell lines showed significant high expression of Fra-1 at mRNA level (Fig 1A). To confirm whether this also corresponded to an increased Fra-1 protein level and localization in the nuclear compartment, we performed an immunofluorescence
staining for Fra-1 on the same cell line panel (Fig 1B). Indeed the basal BC cell lines had an increased Fra-1 protein expression, which was primarily located in the nucleus. In addition, while the luminal cells showed compact cell islands containing cell-cell contacts and an epithelial morphology, the Fra-1 positive basal cells were elongated and more scattered.

**Figure 1:** Fra-1 is higher expressed in basal BC cell lines compared to luminal cell lines. A) Fra-1 mRNA levels were extracted from available gene expression profiling of 36 human BC cell lines. Cell lines were classified luminal or basal and grouped accordingly. B) Immunofluorescence staining of Fra-1 (in green) confirmed the increase of Fra-1 protein level and nuclear localization in basal cell lines. For general cell morphology, actin was visualized using rhodamin phalloidin (in red).
Fra-1 expression induces EMT and increases metastatic potential

We wondered whether Fra-1 expression was sufficient to induce a scattered phenotype in luminal BC cells. We chose the well-studied luminal cell line MCF7 and stably transduced it with a pBabe-control (MCF7-ctrl) or a pBabe-myc-Fra-1 construct (MCF7-Fra-1) (Fig 2Ai). Overexpression of Fra-1 in this cell line clearly induced strong cell scattering (Fig 2Aii). In a soft agar assay MCF7-ctrl cells displayed little colony formation; instead MCF7-Fra-1 cells had six times increased capacity to form colonies indicated by increased average colony size (Fig 2B). Because the switch in morphology after introducing Fra-1 expression in MCF7 was quite dramatic, we anticipated that the MCF7-Fra-1 cells had gained migratory capacities. A random cell migration assay demonstrated that MCF7-Fra-1 cells doubled their migration speed (18.54 μm/hr ± 0.70) compared with MCF7-ctrl cells (8.85 μm/hr ± 0.55) (Fig 2C). Cell migration relies on focal adhesion (FA) turnover. Therefore, we next determined the effect of Fra-1 expression on FA dynamics. Using TIRF, we visualized GFP-paxillin (PXN), a well-known FA protein, in both MCF7-ctrl and -Fra-1 cells and tracked the position of FAs in time. Note that FAs appeared to be much smaller in MCF7-Fra-1 cells compared to control, indicating less stable FA (Fig 2D). A time color overlay demonstrated an increased FA turnover in MCF7-Fra-1 cells, i.e. the absence of ‘white’ FAs in the overlay and an increase in multi-colored FAs. We next used Western blot analysis to investigate the effect of Fra-1 expression on FA signaling. Introduction of Fra-1 in MCF7 cells did not affect total levels of the FA proteins focal adhesion kinase (FAK), paxillin (PXN), Src kinase (SRC) and p130Cas, yet phosphorylation of these proteins was drastically decreased compared to control cells (Fig 2E). In particular FAK at tyrosine 576, which lies within the kinase domain of this protein, was decreased, indicative for reduced FAK kinase activity. This was reflected in the decreased phosphorylation of binding partner SRC and their downstream targets, PXN and p130Cas.

Introduction of Fra-1 in MCF7 makes these cells tumorigenic and metastatic

The above data suggests an increased aggressiveness of MCF7-Fra-1 cells compared to MCF7-control as well as a role for Fra-1 in breast cancer metastasis formation. To test this hypothesis, we injected MCF7-ctrl and MCF7-Fra-1 cells expressing GFP together with matrigel in the fourth mammary gland of 10-11 week old immunodeficient mice and monitored their capacity to form tumors at site of injection. We injected two groups of mice with MCF7-Fra-1 cells. In the first group,
Expression of Fra-1 in the luminal MCF7 induces cell scattering, anchorage independent growth, cell migration and altered focal adhesion signaling. A) MCF7 cells were transduced with either pBabe-control vector (MCF7-ctrl) or pBabe-myc-Fra-1 (MCF7-Fra-1) and expression of Fra-1 and myc was confirmed by Western Blot (i) and immunofluorescence (ii). Expression of Fra-1 induced cell scattering. B) When grown in soft agar, MCF7-Fra-1 cells showed increased survival and growth (P<0.01). C) MCF7-Fra-1 cells have gained migratory capacity compared to MCF7-ctrl cells (P<0.01). D) FA dynamics were studied using TIRF imaging of GFP-paxillin (PXN). MCF7-Fra-1 had decreased FA size and increased FA turnover as indicated by the absence of ‘white’ FAs in the time color overlay. E) Downstream FA signaling was studied using Western Blot. A decrease in phosphorylation of FA proteins was seen after expression of Fra-1 in MCF7.
Figure 3: Fra-1 expression in MCF7 induces tumor growth and metastasis formation. A) MCF7-crtl and MCF7-Fra-1 cells were injected into the fourth mammary fat pad of immunodeficient mice. Tumor growth was monitored in time. MCF7-Fra-1 Tumor + Metastasis (T+M) group was sacrificed when tumor reached maximum volume, whereas group Tumor + Removal + Metastasis (T+R+M) had the first primary tumor removed and sacrificed when the second tumor reached maximum volume (i). Tumors were weighted after isolation (ii, P<0.01) and checked for GFP positive cells by Fluorescence whole organ imaging (FLI) or confocal laser scanning microscopy (GFP signal). After processing, tumor sections were stained with H&E (iii). B) Liver, lung and reproductive organs were examined for the presence of GFP positive cells. C) In an experimental metastasis assay, GFP positive macrometastases were detected in liver and lung after injection of MCF7-Fra-1 cells. D) The distribution of metastasis in both orthotopic and experimental metastasis assay was scored. Scale bar = 100 µm.
tumor growth was monitored and mice were sacrificed when tumors reached an average volume of ~1 cm³ (911 ± 236 mm³). For a second group, tumors were surgically removed when they had an average volume of ~0.1 cm³ (113 ± 57 mm³) to allow disseminated tumor cells to form visible metastatic outgrowth in distant organs. All mice in this group had a second mammary gland tumor growing at site of removal. These mice were sacrificed when the second tumor reached an average volume of ~1 cm³ (1259 ± 688 mm³). No tumors were formed in mice injected with MCF7-ctrl cells, but instead a small clump of injected matrigel was isolated from the mammary gland (Fig 3Ai). In contrast, MCF7-Fra-1 cells formed large tumors (Fig 3Aii). H&E staining showed that the small clump of matrigel isolated from MCF7-ctrl group still contained viable MCF7 cells (closed arrow). The MCF7-Fra-1 tumors had an organized tumor structure (closed arrow) containing large blood vessels (open arrow). The detection of GFP signal shortly after isolation of the tissue indicated the presence of living MCF7 cells within these tumors (Fig 3Aiii).

We anticipated that MCF7-Fra-1 cells would also be able to form metastasis in distant organs. In both MCF7-Fra-1 tumor/metastasis groups, MCF7-Fra-1 cells were detected in liver, lung, ovary, uterus, axillary and brachial lymph nodes and none in organs isolated after injection of MCF7-ctrl cells (n=6; Fig 3B and 3D). Mostly single cells and small micrometastases were detected, possibly due to the fast tumor growth and, thus, relatively short time frame of the experiment, providing insufficient time for complete outgrowth of metastases. To determine whether Fra-1-mediated metastasis of MCF7 cells was directly related to enhanced signaling in the primary tumor microenvironment, we also injected MCF7-ctrl and MCF7-Fra-1 cells directly into the circulation by tail vein injection. Mice were sacrificed 4 weeks after injection and main organs were isolated and inspected for GFP positive cells. This time, macrometastases could be detected by eye and Fluorescence Imaging (FLI) (Fig 3C), but lymph nodes were negative for GFP positive cells. This suggested that these organs are reached prior to intravasation of tumor cells into the blood system. Four out of five mice injected with MCF7-Fra-1 cells showed metastasis, compared to none of the mice injected with MCF7-ctrl cell (Fig 3D). In conclusion, these data indicate that ectopic Fra-1 expression is sufficient to transform the non-tumorigenic MCF7 cell line into a highly invasive tumor cell line.

*Fra-1 expression in the luminal MCF7 is inducing an EMT and basal B phenotype*

Introduction of Fra-1 in MCF7 resulted in a scattered and metastatic phenotype. We first determined if these cells underwent a full EMT genetic program, using
Figure 4: Fra-1 expression induces an EMT switch in MCF7. A) Gene expression profiling was done on MCF7-ctrl and MCF7-Fra-1 cells. A selection of the most strongly affected genes was used for pathway analysis using GeneGO software (MetaCore™). Top 4 affected pathway categories are shown. B) The most strongly affected pathway after introducing Fra-1 into the MCF7 cell-line, was the regulation of epithelial-to-mesenchymal transition (EMT). Genes downregulated in MCF7-Fra-1 compared to -ctrl are circled in green, upregulated genes in red.
Affymetrix gene expression profiling by microarray analysis. After introduction of Fra-1, 9,055 genes were altered in their expression compared to control. We next performed a network analysis for a selection of genes (criteria parametric p-value < 1e-07 and fold discovery rate (FDR) < 1e-07, n= 2,791 genes) using GeneGO software (Metacore™) to identify the pathways associated with the Fra-1-mediated phenotypic switch (Fig 4A). Indeed, the most affected pathway was the regulation of epithelial-to-mesenchymal transition (EMT). In total, the expression of 22 out of 64 genes described in this pathway was affected (Fig 4B), including the upregulation of E-cadherin suppressors SNAI1/2 and ZEB1/2 was determined using RT-qPCR. Except SNAI1, all were upregulated in MCF7-Fra-1 (P<0.01).

**Figure 5:** Introduction of Fra-1 in MCF7 switches them to basal B-like cells. A) Unsupervised hierarchical clustering showed MCF7-Fra-1 clustered with basal B cell lines, whereas MCF7-ctrl clustered with luminal cell lines. B) Western Blot analysis of EMT markers E-cadherin, N-cadherin and vimentin in MCF7-ctrl and -Fra-1 and C) immunofluorescence confirmed the switch in EMT markers. D) Expression of E-cadherin suppressors SNAI1/2 and ZEB1/2 was determined using RT-qPCR. Except SNAI1, all were upregulated in MCF7-Fra-1 (P<0.01).
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Slug and Twist1 and downregulation of E-cadherin and Claudin-1. Other important pathways were related to cell adhesion (e.g. tight junctions) and cytoskeleton regulation (e.g. keratin filaments).

We showed that Fra-1 is not expressed in luminal, and medium to highly expressed in basal A and metastatic basal B human BC cell lines (Fig 1). We wondered whether MCF7-Fra-1 cells had switched into more basal-like BC cells. We used Affymetrix datasets of a panel of 52 human BC cell lines and performed an unsupervised hierarchical clustering, taking the top 10% variable genes as input. The MCF7-ctrl cell line clustered with parental MCF7 and other luminal cells. In contrast, the MCF7-Fra-1 cell line clustered with basal B cells (Fig 5A and SFig 1). Basal B cell lines are characterized by an EMT switch and accompanied with a change in related marker proteins including the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and vimentin [32]. MCF7-Fra-1 cells also underwent a similar full EMT switch, since the expression of E-cadherin was lost and N-cadherin and vimentin increased (Fig 5B and 5C). The downregulation of E-cadherin protein levels in the MCF7-Fra-1 cells could be explained by upregulation of transcriptional E-cadherin repressors. RT-qPCR showed an upregulation of SNAI2, ZEB1 and ZEB2 in these cells (Fig 5D). No difference in SNAI1 expression was found, but parental MCF7 cells do already express SNAI1 [33].

**Fra-1 knockdown in basal B breast cancer cell lines reduced their migratory capacity and metastatic potential**

We showed that expression of Fra-1 in a luminal BC cell line induces a basal B-like BC cell line. We next wanted to test whether knockdown of Fra-1 in basal B cell lines affects their phenotype. We choose two human basal B cell lines, MDA-MB-231 and BT549 that have relative high and moderate Fra-1 levels respectively (Fig 1). We created stable Fra-1 knockdown cell lines using lentiviral shRNA constructs, which was confirmed by immunofluorescence staining. With a random cell migration assay we showed that knockdown of Fra-1 resulted in a decrease of migration velocity (Fig 6A). As expected, actin staining revealed a switch from scattered to a more epithelial phenotype upon Fra-1 knockdown (Fig 6B). We next used a mCherry-PXN MDA-MB-231 cell line to determine the effect of Fra-1 knockdown on FA dynamics. Fra-1 was transiently depleted using siRNA. In agreement with the stable knockdown, migration was also decreased under these conditions. FAs were visualized using TIRF and color overlays were made. Knockdown of Fra-1 resulted in enlargement and stabilization of FAs, indicating decreased aggressiveness of these cells (Fig 6C).
To study the effect of Fra-1 knockdown on the metastatic potential, we used the 4T1, a mouse adenocarcinoma mammary gland tumor cell line that is highly invasive and for which we previously established a reliable spontaneous BC metastasis model [30]. Upon stable knockdown of Fra-1 in 4T1 cells, the cultured cells became more tightly packed (Fig 7A and 7B). When injected in the fourth mammary fat pad of 8-10 week old immunodeficient mice, both control and Fra-1 knockdown 4T1 cells were able to induce tumor formation at site of injection. Tumors formed after injection of Fra-1 knockdown cells were significantly reduced in

**Figure 6: Knockdown of Fra-1 in basal B cell lines reduces cell migration and cell scattering.** A) In a random cell migration assay, MDA-MB-231 and BT549 with a stable knockdown of Fra-1 were studied. In both cell lines, cell migration was decreased upon knockdown (P<0.01). B) Immunofluorescence staining of Fra-1 (green) confirmed the knockdown and actin (red) staining revealed a more epithelial phenotype of the cells upon Fra-1 knockdown. C) MDA-MB-231 cells expressing mCherry-paxillin were used to study the effect of Fra-1 knockdown on focal adhesion (FA) dynamics. Transient knockdown of Fra-1 in this cell line also reduced cell migration (i, P<0.05). FAs were visualized using TIRF and appeared enlarged and more stable after Fra-1 knockdown (ii).
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weight compared to shControl, but no difference was found when compared to the parental cell line (Fig 7C). However, we observed a dramatic effect in the ability to colonize the lungs. After knockdown of Fra-1, the number of surface lung metastases was significantly reduced compared to the parental 4T1 and shControl 4T1 groups. Histological analysis of paraffin sections of the lung confirmed this observation (Fig 7D). These data strongly indicate that Fra-1 is a critical determinant for breast cancer progression and metastasis.

**Figure 7:** Knockdown of Fra-1 in the invasive breast cancer cell line 4T1 prevents metastasis formation. A) Knockdown of Fra-1 in 4T1 cells was confirmed by Western Blot and B) increases the compactness of the cell clusters. C) After injection of 4T1 cells with or without knockdown of Fra-1 in the fourth mammary fat pad of immunodeficient mice, no difference in tumor growth was observed (i); 4T1-shCtrl tumors were increased in tumor weight (ii, P<0.05). D) After isolation, lungs were injected with Indian ink (i) and surface metastases were counted (ii, P<0.01). The small lobe was processed for immunohistological analysis and stained with H&E (iii).
Discussion

In this study we provide strong support for a critical role of Fra-1 to mediate breast cancer metastasis formation. We showed that: 1) Fra-1 can induce a full EMT genetic program in non-invasive and non-metastatic luminal BC MCF7 cells; 2) that this Fra-1-induced EMT switch is associated with a highly migratory phenotype and enhanced metastasis; 3) that Fra-1 induces a transcriptional program in luminal BC cells that ensures a more basal B-like association and 4) that knock down of Fra-1 in basal-like BC cells inhibits spontaneous metastasis formation.

Fra-1 expression caused a complete EMT switch of luminal BC cells. Different transcriptional repressors of E-cadherin were affected by Fra-1 expression, including Slug and Twist1, which was confirmed by RT-qPCR an upregulation of SNAI2 and ZEB1/2 in MCF7-Fra-1. The Fra-1-ZEB1/2 axis has previously been described to induce EMT in normal-like breast cancer cells. While these repressors are typically downstream of TGF-β signaling [34], the expression of TGF-β or its receptor was not affected by Fra-1 and pharmacological inhibition of TGF-β receptor did not inhibit the EMT phenotype (data not shown). Possibly, Fra-1 directly drives the expression of the different E-cadherin repressors leading to a full EMT program. Recent data indeed supported such a role and demonstrated that Fra-1 is essential in ZEB1 and ZEB2 expression, induced by expression of constitutively active ERK2 in MCF10A cells [5]. Interestingly, miR-221/222 was recently demonstrated as a target of Fra-1, which suppresses the expression of the TRPS1, a repressor of ZEB2 [26].

Our transcriptomics data analysis revealed additional receptor signaling routes that could contribute to Fra-1-induced EMT including Wnt-, HGF- and Endothelin-1-mediated signaling. Possibly Fra-1 induces the expression of these ligands with the subsequent activation of their receptors and initiation of the EMT program. Indeed, HGF signaling has been linked to EMT during embryogenesis as well as cancer progression [35] and is a well-studied target for anti-cancer therapy [36; 37]. It has been proposed that Fra-1 is a key player in HGF-induced proliferation in human malignant mesotheliomas [38], but a link between Fra-1 and HGF signaling in the context of EMT has not been established before. HGF signaling is known to have crosstalk with other signaling routes, including Wnt signaling via induction of β-catenin [39]. Our data indicates that Fra-1 expression promotes a Wnt signaling program. Canonical Wnt signaling is linked to EMT via hypoxia-inducible factor 1 alpha (HIF1α) in human prostate cancer cells [40; 41]. Activation of canonical Wnt signaling in periodontal ligament fibroblasts resulted in increased Fra-1 mRNA levels [42]. Our data suggests that Fra-1 also acts upstream of Wnt. Further work is
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required to decipher the role of these different signaling programs in Fra-1-mediated EMT and cancer metastasis.

EMT is generally accompanied by an increase in cell migration. Indeed Fra-1 mediated EMT in MCF7 cells increased the cell motility, while knockdown of Fra-1 in human basal B-like BC cells inhibited cell migration. Such a drastic behavioral switch was supported by our transcriptomics data: cell adhesion signaling and cytoskeleton remodeling were the second most important pathway category affected, including downregulation of E-cadherin and upregulation of N-cadherin. Although enhanced motility is associated with EMT, the enhanced cell migration induced by Fra-1 is not necessarily a direct result of EMT but appears as a separate biological process independently activated by Fra-1, but in parallel with EMT. A well-studied kinase that controls cell adhesion and migration is FAK. In general, enhanced FAK levels are associated with increased tumor grade and metastasis [43; 44]. In this study, we showed that total levels of FA proteins such as FAK and PXN were not changed after expression of Fra-1. This indicates that possibly additional pathways are involved in Fra-1-mediated cell migration and subsequently metastasis, such as integrin β1 signaling. Previously, Fra-1 has been linked to integrin β1 to control RhoA GTPase. We observed a complete loss of integrin β1 protein expression in MCF7-Fra-1 cells and a gain in integrin β3 expression (data not shown). This switch potentially initiates the acquisition of a migratory phenotype. Further studies are required to establish what cell migratory machinery controls enhanced motility caused by Fra-1 expression.

Fra-1 is reported to be upregulated in several human cancers, in particular BC. Targeting the Fra-1 pathway for therapeutic intervention of BC harbors valuable potential. This could be achieved by targeting upstream Fra-1 regulators, such as PKCθ or ERK2 [5; 11], or target downstream Fra-1 signaling, in particular that involved in Fra-1 mediated EMT and migration. To further elucidate additional drugable candidate genes that underlie Fra-1 driven migration/invasion, a RNA-interference knockdown screen for Fra-1 driven tumor cell migration will be valuable. Our MCF7-Fra-1 cells would be a good model system for this. Interesting, a Fra-1 vaccine has already been proven successful in the killing of Fra-1 positive breast cancers in a mouse model [45] supporting Fra-1 as an effective anti-cancer drug target.

Claudin-low breast cancer has been identified as a subgroup within the basal B BC with poor disease outcome. Tumors in this group are characterized by low expression of claudin 3,4 and 7 and E-cadherin. When compared to basal B-like BC, this subgroup is more enriched in EMT features and (mesenchymal) stem cell-associated biological processes [46]. Another feature of claudin-low tumors is the
increased expression of matrix-metalloproteinase (MMP)-9. MMP9 is a well known transcriptional target of Fra-1 and is involved in degradation of extracellular matrix during cancer invasion and other tissue remodeling events [47]. In gene expression profiling of the MCF7-Fra-1 cells, we observed not only the decreased expression of E-cadherin, but also of several claudin family members, including 3, 4 and 7. This indicates that expression of Fra-1 not only induced EMT, but also reduced claudin expression, suggesting enrichment in stem-like properties of our MCF7-Fra-1 cells. Evidence in support of this hypothesis is the dramatic decreased expression of another putative BC stem cell marker, CD24, in MCF7-Fra-1 cells. Also earlier studies demonstrated that mammmary cells undergoing an EMT induced by Snail or Twist increased stem-like capabilities [48].

Cancer invasion and metastasis are initiated and maintained by intrinsic tumor cell signaling, such as the induction of an EMT, but also signaling from surrounding tissue. Fra-1 was previously shown to play a role in cancer progression by remodeling the tumor microenvironment. Fra-1 expression in tumor-associated macrophages (TAMs) is upregulated upon interaction with BC cells. Fra-1 in turn initiates the activation of the IL-6/JAK/Stat3 signaling pathway, which results in a malignant switch. Knockdown of Fra-1 in TAMs reduced tumor cell invasion, angiogenesis and metastasis in a mouse BC model [49]. Here we showed that Fra-1 expression in MCF7 altered expression of endothelin-1, endothelin receptor type A (EDNRA) and platelet derived growth factor 1 (PDGF-1) (Fig 4B). These factors are associated with blood vessel function and have a role in angiogenesis [50]. This observation is supporting the previous link between Fra-1 and angiogenesis via expression of MMP2 [51], though it is suggesting an additional signaling route.

To summarize, we have demonstrated that expression of the transcription factor Fra-1 alone is sufficient to induce a full EMT and is required for breast cancer metastasis. Further exploration of the biological role of (in)direct downstream Fra-1 target genes is likely to lead to the identification of additional relevant and novel anti-metastasis targets.
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Supplemental Figure 1: full heatmap clustering human BC cell lines. Unsupervised hierarchical clustering showed MCF7-Fra-1 clustered with basal B cell lines, whereas MCF7-ctrl clustered with luminal cell lines.
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