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ORIGINAL ARTICLE – CANCER RESEARCH

Prognostic impact of transcription factor Fra-1 in ER-positive breast cancer: contribution to a metastatic phenotype through modulation of tumor cell adhesive properties

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Abstract

Purpose The transcription factor Fos-related antigen-1 (Fra-1) has been described to affect the morphology, motility and invasive potential of breast cancer cells. Since tumor cell adhesion plays an essential role in the metastatic process, especially for extravasation from blood vessels, we investigated the influence of Fra-1 on breast cancer cell interactions with the endothelium.

Methods Using Fra-1-overexpressing MCF7 [weakly invasive, estrogen receptor (ER)-positive] and MDA MB231 (strongly invasive, ER-negative) cells, we performed dynamic cell flow adhesion assays on surfaces coated with E-selectin or with human pulmonary microvascular endothelial cells.

Results We found a significant increased adhesion of Fra-1-overexpressing MCF7 cells to E-selectin but also to activate endothelial cells, whereas the MDA MB231 cell line showed moderate enhanced cell rolling and tethering on both coated surfaces. These different adhesion behaviors corresponded to an up-regulation of various adhesion-related proteins such as CD44 and integrin α5 in Fra-1-overexpressing MCF7 cells measured by microarray analysis and flow cytometry in comparison with no deregulation of key adhesion molecules observed in Fra-1-overexpressing MDA MB231 cells. In line with these results and based on cDNA microarray data of breast cancer patients ($n = 197$), high Fra-1 expression significantly correlates with shorter overall survival and higher rate of lung metastasis in ER-positive breast cancer patients ($n = 130$), but has no impact on the prognosis of patients with ER-negative tumors.

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Conclusion Thus, in addition to its pro-invasive and pro-migratory effect, Fra-1 might influence the metastatic potential of breast cancer cells by changing the expression of adhesion molecules, resulting in increased adherence to endothelial cells under flow conditions.

Keywords Fra-1 · Breast cancer · Adhesion · Metastasis

Introduction

One of the leading causes of death in women is breast cancer. It is a heterogeneous disease, and the most important behavior of breast cancer cells is the early lymphogenous and hematogenous metastasis. On the one hand, metastatic dissemination is an inefficient process as only a small subset of cancer cells in a primary tumor has the potential to form metastases; on the other hand, metastasis is responsible for around 90 % of cancer patient mortality. The process of metastasis includes many steps including invasion of the basal membrane and neighboring tissues by cancer cells, intravasation into the vasculature or infiltration of lymphatic vessels, survival in the circulation, extravasation, distant organ infiltration and colonization. These mechanisms require a precise coordination of various signaling pathways and strategies used by the cancer cells to acquire the ability to change their adhesive properties. Specially, the exit of tumor cells from capillary beds into the parenchyma of an organ is strongly affected by their interactions with vascular endothelial cells. Cancer cells have to slow down and then form stable attachment. These processes are mediated by a wide range of ligands and receptors, including selectins, integrins, cadherins, CD44 and immunoglobulin (Ig) superfamily receptors.

The activator protein-1 complex, AP-1, is a key regulator of transcriptional responses induced by various cancer-associated signaling pathways. It consists either of homodimers of Jun family members (c-Jun, JunB and JunD) or heterodimers of Jun proteins with Fos family members (c-Fos, FosB, Fra-1 and Fra-2) or members of the ATF and MAF family (Zhao et al. 2014). Unlike the members of the Jun family, the Fos family proteins need to heterodimerize with members of the Jun family to form transcriptionally active complexes. After dimerization, AP-1 complexes bind to specific DNA consensus sequences as TRE (TPA responsive elements) or CRE (cAMP responsive elements) in the promoter and enhancer regions of several target genes (Angel and Karin 1991; Milde-Langosch et al. 2004). In vitro studies have shown that Jun–Fos heterodimers generate more stable complexes than Jun–Jun homodimers and therefore display a stronger DNA-binding activity and subsequent are more efficient in transcriptional control (Bamberger et al. 1999; Milde-Langosch et al. 2004). Accumulating evidence has implicated AP-1 in the regulation of a variety of cellular processes, including proliferation, differentiation, apoptosis, cell migration, invasion, transformation and adhesion (Hess et al. 2004; Milde-Langosch 2005; Shaulian 2010; Shaulian and Karin 2002; Wagner and Eferl 2005).

In this study, we focused on the transcription factor Fos-related antigen-1 (Fra-1), which is, beside c-Fos, the best studied member of the Fos family and encoded by the fos-like-1 gene (*fosl1*). The *c-fos* and *fra-1* genes are best characterized as immediate early genes, and the transcriptional activity of Fra-1 is regulated both transcriptionally (Casalino et al. 2003; Young and Colburn 2006; Young et al. 2002) and posttranslationally (Smith et al. 1999). The expression of Fra-1 is deregulated in many tumors (Milde-Langosch 2005): its overexpression has been reported in proliferative disorders such as breast, brain, lung, colon, esophageal and thyroid cancer (Belguise et al. 2005; Chiappetta et al. 2000, 2007; Debinski and Gibo 2005; Kustikova et al. 1998; Logullo et al. 2011; Nakajima et al. 2007; Song et al. 2006; Usui et al. 2012; Young and Colburn 2006; Zajchowski et al. 2001). In addition, Fra-1 expression has been shown as a feature of hyperplastic and neoplastic breast epithelium (Chiappetta et al. 2007; Nakajima et al. 2007; Song et al. 2006) with capacity to influence proliferation, migration and invasiveness of breast cancer cells (Belguise et al. 2005; Kustikova et al. 1998). These findings implicate that Fra-1 activity might be functionally involved in breast cancer metastasis. In an immunohistochemical study, Logullo et al. (2011) found a positive correlation between Fra-1 expression and an aggressive phenotype in invasive breast ductal carcinoma.

Since tumor cell adhesion to endothelial cells has been shown to be crucial for hematogenic metastasis of various tumor types, we were interested in the potential influence of Fra-1 on the adhesive properties of breast cancer cells. Therefore, we performed dynamic adhesion assays using two breast cancer cell lines [ER(−) and ER(+)] with stable Fra-1 overexpression. Our results point to a strong influence of Fra-1 on cell adhesion to endothelia in ER-positive tumor cells and provide an explanation for the unfavorable prognostic impact of this protein in this molecular breast cancer subtype.

Materials and methods

Breast cancer cell lines and generation of stable transfectants

MDA MB231 and MCF7 breast cancer cells were cultivated as described before (Bamberger et al. 2001; Milde-Langosch et al. 2001, 2004). MDA MB231 cells express

Fra-1 inherently high, while MCF7 cells do not show any detectable Fra-1 protein expression without transfection. To generate stable clones with increased Fra-1 expression, the full Fra-1 cDNA that had been cloned before in pcDNA3.1+ plasmids was supplemented with a C-terminal FLAG epitope by PCR and subcloned in the bicistronic vector pIRES-P (Genbank no. Z75185) using the NheI and EcoR1 restriction enzymes (Nandy et al. 2003). Transfection of MCF7 and MDA MB231 cells with this plasmid and the empty vector was performed with LipofectAMINE PLUS reagent (Life Technologies, Karlsruhe, Germany). After selection with puromycin (1 μg/ml), a single-cell Fra-1 clone (MCF7 Fra-1#8 and MDA MB231 Fra-1#2) and negative control clones (MCF7 NC and MDA MB231 NC) were chosen for further analysis. The differences in Fra-1 expression were repeatedly confirmed by Western blots during the experiments.

Cultured endothelial cells

Human pulmonary microvascular endothelial cells (HPMEC, Promocell, Heidelberg, Germany) were cultured in endothelial cell growth medium MV (PromoCell), supplemented with Supplement Mix (PromoCell) in vitro under standard culture conditions (37 °C, 100 % relative humidity, 5 % CO_2). For the cell flow assay, HPMEC cells from passage one to twelve were stimulated with 10 ng/ml TNFα for 4 h.

Western blots

Western blot analyses were used to confirm Fra-1 overexpression in both cell lines as described (Milde-Langosch et al. 2004). Total protein extraction was performed using RIPA lysis buffer. For protein detection, the following antibodies were used: rabbit anti-Fra-1 antibody R20 (1:200; Santa Cruz Biotechnologies, Heidelberg, Germany) and goat anti-actin I19 (1:4000, Santa Cruz).

Cell flow assay

To study the influence of Fra-1 on adhesion of breast cancer cells to the endothelium during extravasation, we performed cell flow assays as described before (Dippel et al. 2013; Richter et al. 2011).

Flow cytometry

For assessment of certain adhesion molecules or selectin glycoligand structures on the surface of MCF7 and MDA MB231 transfectants, tumor cells were incubated with the following conjugated antibodies: anti-integrin α5 AF488 clone eBioSAM-1 (eBioscience, Frankfurt, Germany), anti-CD44v6 FITC clone VFF-7 (Invitrogen, Karlsruhe, Germany) and anti-CD 66c APC (R&D Systems, Minneapolis, MN 55413). Respective isotype controls, IgG1 AF488 (eBioscience), IgG2a APC (Dako, Hamburg, Germany) and IgG1 FITC (Miltenyi Biotec, Bergisch-Gladbach, Germany), were used. The stained cells were analyzed in the FACS Calibur (Becton/Dickinson, Heidelberg, Germany) or the CUBE 8 (Partec, Muenster, Germany) and visualized by FCS Express.

Patients cohort

We analyzed the mRNA expression data obtained from primary breast cancer tissue samples from our hospital ($n = 194$). Patients were treated at the University Medical Center Hamburg-Eppendorf, Germany, Department of Gynecology between 1991 and 2002 and selected on the basis of tissue availability. One hundred six patients received anthracycline-based adjuvant chemotherapy regimens [mainly epirubicin/cyclophosphamide (EC) or cyclophosphamide/methotrexate/fluorouracil (CMF)]. Seventy-four patients received endocrine therapy only, eight patients were treated by radiation without any systemic therapy and four patients remained untreated after surgery (no information: two patients). The median follow-up time was 132 months. Informed consent for the scientific use of tissue materials, which was approved by the local ethics committees (HH 05/04/2004), was obtained from all patients. The study was performed in accordance to the principles of the Declaration of Helsinki and REMARK criteria (Altman et al. 2012). No radiotherapy, neoadjuvant chemotherapy or endocrine therapy had been administered before surgery. Adjuvant therapy was applied according to international recommendations.

RNA isolation and microarray analysis

The Affymetrix (Santa Clara, CA, USA) HG-U133A array and GeneChip System™ were used to quantify the relative transcript abundance in the breast cancer tissues ($n = 197$) as described (Ihnen et al. 2008). Starting from 5 μg total RNA, labeled cRNA was prepared using the Roche Microarray cDNA Synthesis, Microarray RNA Target synthesis (T7) and Microarray Target Purification Kit, according to the manufacturer's instructions. In the analysis settings, the global scaling procedure was chosen, which multiplied the output signal intensities of each array to a mean target intensity of 500. Samples with suboptimal average signal intensities (i.e., scaling factors >25) or GAPDH 3′/5′ ratios >5 were relabeled and rehybridized on new arrays.

Total RNA from MCF7 cells which were cultured to 70 % confluence was extracted by lysing the cells in Trizol reagent (Invitrogen) according to the manufacturer's

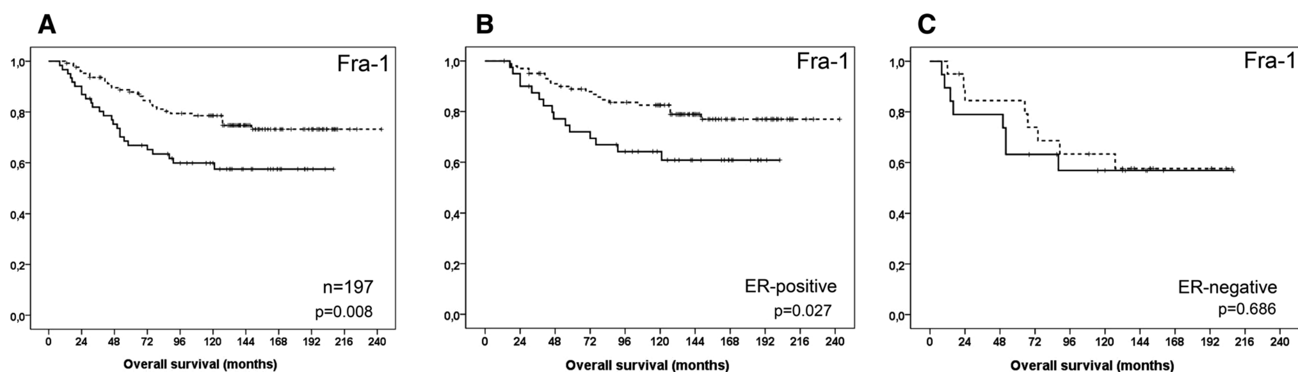

Fig. 1 Prognostic impact of Fra-1 expression in breast cancer patients. Kaplan–Meier curves showing the prognostic value of Fra-1 in **a** 197 patients, **b** 148 ER(+) patients and **c** 41 ER(−) patients included in our collective. *Broken line* low and moderate Fra-1 expression; *solid line* high Fra-1 expression

instructions and further purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Procedures for cDNA synthesis, labeling and hybridization were carried out according to the manufacturer's protocol (Affymetrix). The experiments were performed using Affymetrix Human Genome GeneChip U133 Plus 2.0 as described (Schroder et al. 2010). To compare samples and experiments, the trimmed mean signal of each array was scaled to a target intensity of 100. Absolute and comparison analyses were performed with Affymetrix GCOS (version 1.4, Affymetrix) software using default parameters. To assist in the identification of genes that were positively or negatively regulated in the experiment, we selected genes that were increased or decreased at least 1.9-fold compared to the control.

Statistical analysis

For evaluation of the prognostic significance of Fra-1 mRNA expression in breast cancer patients ($n = 197$), the cohort was first divided into three groups of similar size ($n = 65$–66) according to their expression values representing weak, moderate and high expression, and Kaplan–Meier analyses and log-rank tests were performed. Since cases with weak or moderate expression behaved similarly in survival analysis (Fig. 1a), both groups were combined for further stratified analyses in ER-positive and ER-negative groups. Associations of Fra-1 expression with clinical and histological variables were calculated by Chi-square tests using the following groups: nodal involvement (positive vs. negative), tumor stage (T1, T2, T3, T4), histological grading (G1/G2 vs. G3), estrogen receptor (ER) status and progesterone receptor (PR) status (positive vs. negative), histological type (ductal vs. lobular), differences in adherent cell numbers were calculated by two-sided T tests. All analyses were performed using the SPSS 21 software, and p values of <0.05 were considered to indicate a significant result.

Results

Fra-1 mRNA expression significantly correlates with shorter overall survival and lung metastasis in ER-positive breast cancer patients

Fra-1 mRNA expression was evaluated in 197 tumor samples of breast cancer patients using microarray data. Characteristics of patients included in this collective are shown in Table 1. In general, mRNA expression data for the Affymetrix probeset 204420_at (fosl1) in the tumors were low, ranging from 6.5 to 558.8 (mean 78.5 and median 62.2). According to these values, the cohort was first divided into three groups of similar size displaying low, moderate or high Fra-1 expression. High Fra-1 expression was associated with significantly shorter overall survival by Kaplan–Meier analysis ($p = 0.027$), and the same trend could be observed for progression-free survival ($p = 0.227$). Groups containing patients with low and moderate Fra-1 expression levels showed similar overall and progression-free survival times and were combined in a unique group for further analysis. As expected, Fra-1 retained its negative prognostic significance using these two groups as shown in Fig. 1a (OAS: $p = 0.008$; DFS: $p = 0.085$).

Since Fra-1 expression has been found to be mainly overexpressed in carcinomas with high metastatic potential such as in receptor-negative breast cancer, we were interested in the prognostic value of this transcription factor within the subgroups of ER-positive and ER-negative patients. Interestingly, high Fra-1 expression significantly correlates with shorter overall survival in ER-positive breast cancer patients ($n = 150$; $p = 0.023$; DFS: $p = 0.149$), but has no impact on the prognosis of patients with ER-negative tumors ($n = 41$; Fig. 1b–c). By Chi-square tests, we found significant associations of high Fra-1 expression levels with nodal involvement ($p = 0.019$) and with ER- and

Table 1 Patient characteristics ($n = 197$)

	n (%)
Age (years)	
Median	56.6
Range	29–94
Histological type	
Ductal	140 (71)
Lobular	31 (16)
Others	22 (11)
Unknown	4 (2)
Tumor size (stage)	
<2 cm (pT1)	51 (26)
2–5 cm (pT2)	121 (61)
>5 cm (pT3–4)	20 (10)
Unknown	5 (3)
Grade	
I	20 (10)
II	80 (41)
III-Undifferentiated	92 (47)
Unknown	5 (3)
Lymph nodes	
Positive nodes	136 (69)
Negative nodes	60 (31)
Unknown	1 (0.5)
ER status	
Positive	148 (75)
Negative	41 (21)
Unknown	8 (4)
PR status	
Positive	124 (63)
Negative	65 (33)
Unknown	8 (4)
Distant metastases	
All distant metastases	55 (28)
No distant metastases	120 (61)
Unknown	22 (11)
Bone metastases[a]	34 (17)
Lung metastases[a]	28 (14)
Visceral/hepatic metastases[a]	30 (15)
Brain metastases[a]	15 (8)
Follow-up	
Recurrence	72 (37)
No recurrence	105 (53)
Recurrence unknown	20 (10)
Died of disease	56 (28)
Died of other course	8 (4)
Alive	126 (64)
Unknown	7 (4)
Median follow-up period (months)	133

[a] Multiples included

PR-negative status ($p = 0.006$ and $p = 0.040$), whereas no correlation with age, histological type, grading or stage could be found (Table 1). We further investigated whether the expression of Fra-1 correlated with distant metastasis in general or with metastasis to specific organ sites during recurrence. Thus, we found no association of Fra-1 expression with distant metastasis in general, but a significant correlation with metastasis to the lung ($p = 0.021$) and a weak association of high Fra-1 expression with brain metastasis ($p = 0.056$; Table 2).

Fra-1 regulates tumor cell adhesion to E-selectin and endothelial cells

Tumor cell interactions with the vascular endothelium are crucial for cell extravasation and therefore play an essential role in tumor progression and metastasis. Particularly, E-selectin, which is expressed in activated endothelial cells, has been implicated in metastatic spread in different tumor entities (Barthel et al. 2007). In order to evaluate the influence of Fra-1 on breast cancer cell adhesion, we established stable transfectants with forced Fra-1 overexpression in two human breast cancer cell lines: MCF7 (weakly invasive, ER-positive) and MDA MB231 (strongly invasive, ER-negative). High Fra-1 expression levels compared to the negative control cells (NC), which were generated after transfection with the empty vector, could be confirmed in monoclonal cell populations of either MCF7 (Fra-1#8) or MDA MB231 (Fra-1#2) cells by Western blot analysis (Fig. 2a).

We further analyzed the effect of Fra-1 overexpression on tumor cell adhesion to endothelial cells and to E-selectin under dynamic conditions. Using a laminar flow chamber assay with E-selectin- or HPMEC-coated surfaces, firm adhesion events as well as rolling behavior were evaluated. Cell adhesion or tethering was not observed when capillaries were coated with fetal calf serum, used as negative control for each experiment (data no shown). Interestingly, we observed a different adhesive behavior of MCF7 and MDA MB231 cells in these experiments: MCF7 exhibited firm, irreversible adhesion to endothelial monolayers or E-selectin-coated surfaces, whereas MDA MB231 cells rather demonstrated rolling behavior.

According to current knowledge, tumor cell interactions with endothelial E-selectin consist of a transient binding that facilitates cell tethering and rolling. Once tumor cells slowed down, firm adherence to different adhesion molecules of the endothelium and extravasation take place. In order to mimic the physiological situation, we measured adhesive events of tumor cells on E-selectin-coated capillaries at a shear stress rate of 0.5 dyn/cm^2 and firm adhesion to endothelial cells at a lower rate of 0.25 dyn/cm^2.

Table 2 Fra1 mRNA expression in breast cancer patients ($n = 197$): correlations with clinical and histological tumor characteristics

	n^a	Fra-1 low (Tertial 1–2)	Fra-1 high (Tertial 3)	p
n (%)		52 (50)	52 (50)	
ER status				
Negative	41	20	21	0.006
Positive	148	106	42	
PR status				
Negative	65	37	28	0.040
Positive	124	89	35	
Grading				
G1–2	100	69	31	0.690
G3–4	92	61	31	
Stage				
T1	51	37	14	0.523
T2	121	79	42	
T3–4	20	23	8	
Lymph node involvement				
Negative	136	98	38	0.019
Positive	60	33	27	
Histological type				
Ductal	140	93	47	0.653
Lobular	31	23	8	
Others	22	14	8	
Lung metastasis				
No	147	106	41	0.021
Yes	28	14	14	
Brain metastasis				
No	160	113	47	0.056
Yes	15	7	8	

[a] Missing values to 197: no information

We observed significantly more adhesion events on E-selectin- and HPMEC-coated surfaces for Fra-1-overexpressing MCF7 cells (Fig. 2b; upper left: $p = 0.012$; lower left: $p = 0.002$) compared to NC cells, whereas the influence of Fra-1 on MDA MB231 tethering on E-selectin- ($p = 0.067$) or endothelial-coated surfaces ($p = 0.557$) was less prominent (Fig. 2b, right).

Results of microarray analysis and validation of candidate genes using flow cytometry

Since our analysis showed a prognostic impact of Fra-1 only for ER-positive breast cancer patients, and in vitro dynamic adhesion assays corroborated a strong influence of Fra-1 on the adhesive behavior of MCF7 (ER+) but not in MDA MB231 cells (ER−), we have chosen the breast cancer cell line MCF7 ($MCF7^{Fra-1}$ versus $MCF7^{NC}$) for further microarray analysis. In order to identify Fra-1 target genes that could be involved in metastatic properties of breast cancer cells, cDNA arrays were performed with mRNA isolated from the stably transfected MCF7 Fra-1# 8 and control transfectants (MCF7 NC). For evaluation of gene expression data, a threshold expression value of 50 and a signal log ratio (SLR) of ≥0.9 or ≤−0.9 compared to the control were used. Using these criteria, 1,041 probesets (664 genes) with known function (i.e., signal transduction, immune response, proteolysis or metabolism) were up-regulated, and 535 genes (390 probesets) were down-regulated in MCF7 pIRES Fra-1 cells. Our special attention in this study prevailed in genes, which might influence the adhesive properties of the Fra-1-overexpressing cells (selected genes are shown in Table 3).

Interestingly, overexpression of Fra-1 in MCF7 cells results in up-regulation of several adhesion molecules, notably CD44, CEACAM6 and integrin α5. In addition, Fra-1 overexpression correlates with higher levels of some components of the ECM, i.e., fibronectin (FN1), radixin (RDX) and different collagen isoforms. Furthermore, we found a couple of up-regulated genes participating in O- and *N*-glycosylation processes, namely UDP-GlcNac:betaGal beta-1,3-*N*-acetylglucosaminyltransferase3 (B3GNT3), heparan sulfate 6-O-sulfotransferase2 (HS6ST2) and ST6alpha-*N*-acetyl-neuraminyl-2,3-beta-galactosyl-1,3-*N*-acetylgalactosaminide-alpha-2,6-sialyl-transferase (ST6GALNAC4). On the other hand, diverse cell adhesion molecules or glycosylation enzymes, such as L1CAM, claudin 1 or NCAM1/2, FUCA1 and MAN1A1 (Table 3), were found to be down-regulated in Fra-1-overexpressing MCF7 cells in comparison with MCF7 NC cells.

The regulation of some Fra-1 target genes was validated on protein level by FACS analysis. In comparison with the original cell line MCF7 and the control transfectants MCF7 NC, we could show an obvious up-regulation of integrin α5 [arith. mean 37.55 (NC) to 131.35 (Fra-1#8)] and CD44v6 [arith. mean 34.10 (NC) to 144.59 (Fra-1#8)] on the cell surface of stably transfected MCF7 Fra-1#8 (Fig. 3) and a weak increase in CEACAM6 (arith. mean 9.01) in MCF7 Fra-1 cells compared to control MCF7 cells (arith. mean 5.0) (data not shown). The expression level of further cell adhesion-related proteins such as integrins α1, α2, α4, α6, αV, β1, β3, β4 and β7, ICAM, ALCAM, PSGL-1 and L1CAM was analyzed by FACS. Here, we did not detect deregulation of the mentioned adhesion proteins on MCF7 or MDA MB231 Fra-1-overexpressing cells in comparison with their respective controls.

Discussion

Several studies have described the effect of increased Fra-1 expression in several tumor entities (Chiappetta et al. 2000, 2007; Debinski and Gibo 2005; Kustikova et al. 1998;

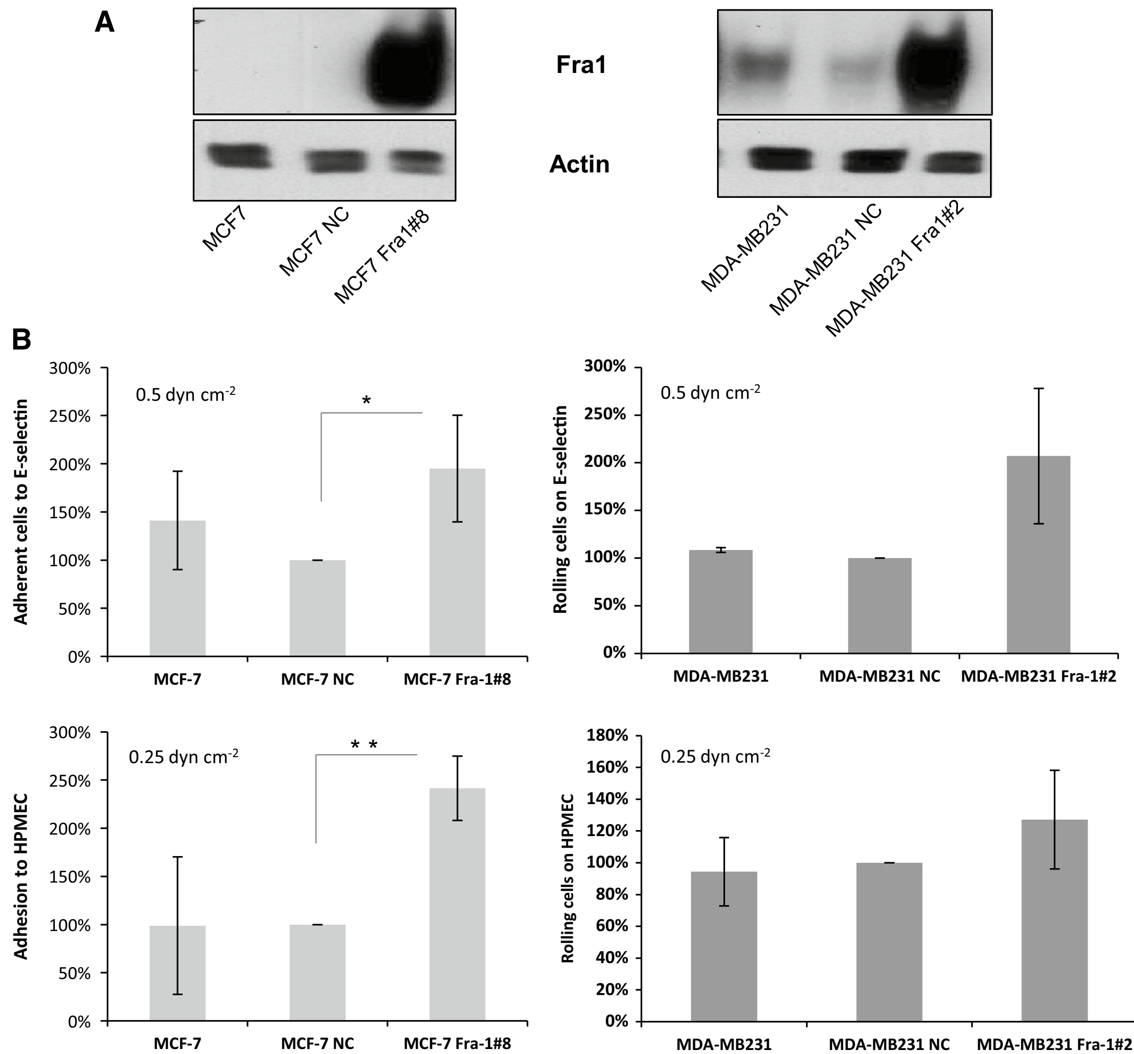

Fig. 2 Dynamic adhesion assays with Fra-1-overexpressing MCF7 and MDA MB231 cells. **a** Western blot analysis showing Fra-1 expression in a stably transfected clone derived from either MCF7 or MDA MB231 cells compared with untransfected cells and cells transfected with empty vector (NC). **b** Percent of adherent or rolling cells on E-selectin (*upper side*) and HPMECs (*lower side*) relative to control ± SEM are shown. *$p < 0.05$, **$p < 0.005$

Logullo et al. 2011; Nakajima et al. 2007; Song et al. 2006; Usui et al. 2012; Zajchowski et al. 2001). In breast cancer cell lines, overexpression of this transcription factor leads to morphological changes, increased motility and invasive behavior in vitro (Belguise et al. 2005; Milde-Langosch 2005; Milde-Langosch et al. 2004). Here, we described for the first time the effect of Fra-1 expression on the adhesive properties of breast cancer cells in vitro and further showed, using microarray data from 194 breast cancer tumor samples, the prognostic relevance of this factor, particularly in the subgroup of ER-positive breast cancer patients.

The association between Fra-1 expression and a more malignant and aggressive phenotype in breast cancer has been evidenced by a variety of descriptive and functional studies (Belguise et al. 2005; Milde-Langosch et al. 2004; Zajchowski et al. 2001), whereas only few reports on the prognostic role of Fra-1 in this entity are known (Logullo et al. 2011). Logullo et al. (2011) investigated Fra-1 expression by immunohistochemistry in ductal carcinomas in situ (DCIS) and invasive ductal carcinomas (IDC) and could corroborate a clear correlation between Fra-1 expression and a more aggressive phenotype in IDC, but no association with overall survival. Our results, based on microarray data of 197 breast tumor samples, showed in contrast to a significant association of high Fra-1 mRNA expression with shorter patient outcome. Interestingly, when analyzing the ER-positive and ER-negative populations separately, we found that the prognostic impact of Fra-1 is restricted to the ER(+) population. This was an unexpected result, since the biological relevance of Fra-1 has been demonstrated in

Table 3 Selection of differentially regulated genes which are involved in adhesion, proteolytic cleavage, glycosylation or ECM by Fra-1 overexpression in MCF7 cells

Probe set ID	Gene symbol	Gene title	Function	f.c.
MCF7 Fra-1# 8 versus MCF7 NC (control)				
209030_s_at	CADM1	Cell adhesion molecule 1	Adhesion	1.9
212063_at	**CD44**	**CD44 molecule (Indian blood group)**	**Adhesion**	**5.3**
201884_at	CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5	Adhesion	4
203757_s_at	**CEACAM6**	**Carcinoembryonic antigen-related cell adhesion molecule 6**	**Adhesion**	**4**
227209_at	CNTN1	Contactin 1	Adhesion	64
214073_at	CTTN	Cortactin	Adhesion	2.1
212977_at	CXCR7	Chemokine (C-X-C motif) receptor 7	Adhesion	8
204750_s_at	DSC2	Desmocollin 2	Adhesion	3.2
201667_at	GJA1	Gap junction protein, alpha 1, 43 kDa	Adhesion	4
201389_at	**ITGA5**	**Integrin, alpha 5**	**Adhesion**	**3**
226534_at	KITLG	KIT ligand	Adhesion	2.5
226622_at	MUC20	Mucin 20, cell surface associated	Adhesion	51.9
221933_at	NLGN4X	Neuroligin 4, X-linked	Adhesion	19.7
216959_x_at	NRCAM	Neuronal cell adhesion molecule	Adhesion	64
212298_at	NRP1	Neuropilin 1	Adhesion	3
239443_at	PCDHB6	Protocadherin beta 6	Adhesion	3.7
221319_at	PCDHB8	Protocadherin beta 8	Adhesion	3
207717_s_at	PKP2	Plakophilin 2	Adhesion	6.9
232317_at	PLXNA4	Plexin A4	Adhesion	45.2
211421_s_at	RET	Ret proto-oncogene	Adhesion	8.5
204268_at	S100A2	S100 calcium-binding protein A2	Adhesion	7.4
206805_at	SEMA3A	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	Adhesion	14.9
46665_at	SEMA4C	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4C	Adhesion	2
225660_at	SEMA6A	Sema domain, transmembrane domain (TM) and cytoplasmic domain, (semaphorin) 6A	Adhesion	26
215856_at	SIGLEC15	Sialic acid-binding Ig-like lectin 15	Adhesion	5.6
209114_at	TSPAN1	Tetraspanin 1	Adhesion	2.6
200973_s_at	TSPAN3	Tetraspanin 3	Adhesion	2.5
212097_at	CAV1	Caveolin 1, caveolae protein, 22 kDa	ECM	13.9
203323_at	CAV2	Caveolin 2	ECM	4.3
209082_s_at	COL18A1	Collagen, type XVIII, alpha 1	ECM	2.6
208096_s_at	COL21A1	Collagen, type XXI, alpha 1	ECM	5.3
213110_s_at	COL4A5	Collagen, type IV, alpha 5 (Alport syndrome)	ECM	22.6
211719_x_at	FN1	Fibronectin 1	ECM	157.5
204969_s_at	RDX	Radixin	ECM	10.5
200838_at	CTSB	Cathepsin B	Proteolysis	2.1
227863_at	CTSD	Cathepsin D	Proteolysis	9.8
202295_s_at	CTSH	Cathepsin H	Proteolysis	3.7
208926_at	NEU1	Sialidase 1 (lysosomal sialidase)	Proteolysis	2.6
229441_at	PRSS23	Protease, serine, 23	Proteolysis	588.1
201666_at	TIMP1	TIMP metallopeptidase inhibitor 1	Proteolysis	14.9
204856_at	B3GNT3	UDP-GlcNAc:betaGal beta-1,3-*N*-acetylglucosaminyltransferase 3	Glycosylation	3.2
MCF7 Fra-1 # 8 versus MCF7 NC (control)				
216627_s_at	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	Glycosylation	2.3
219439_at	C1GALT1	Core 1 synthase, glycoprotein-*N*-acetylgalactosamine 3-beta-galactosyltransferase, 1	Glycosylation	5.6
204417_at	GALC	Galactosylceramidase	Glycosylation	6.1

Table 3 continued

Probe set ID	Gene symbol	Gene title	Function	f.c.
212256_at	GALNT10	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 10 (GalNAc-T10)	Glycosylation	2.1
222773_s_at	GALNT12	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 12 (GalNAc-T12)	Glycosylation	13.9
1552766_at	HS6ST2	Heparan sulfate 6-O-sulfotransferase 2	Glycosylation	3
221551_x_at	ST6GAL-NAC4	ST6 (alpha-*N*-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 4	Glycosylation	3
222549_at	CLDN1	CLAUDIN 1	Adhesion	−9.2
231802_at	CLEC3A	C-type lectin domain family 3, member A	Adhesion	−22.8
202468_s_at	CTNNAL1	Catenin (cadherin-associated protein), alpha-like 1	Adhesion	−2.2
206758_at	EDN2	Endothelin 2	Adhesion	−2.6
204584_at	L1CAM	L1 cell adhesion molecule	Adhesion	−2.1
201105_at	LGALS1	Lectin, galactoside-binding, soluble, 1 (galectin 1)	Adhesion	−12.9
204885_s_at	MSLN	Mesothelin	Adhesion	−8
212843_at	NCAM1	Neural cell adhesion molecule 1	Adhesion	−9.8
205669_at	NCAM2	Neural cell adhesion molecule 2	Adhesion	−3.7
227289_at	PCDH17	Protocadherin 17	Adhesion	−12.1
218677_at	S100A14	S100 calcium-binding protein A14	Adhesion	−3.2
212158_at	SDC2	Syndecan 2	Adhesion	−2.1
209264_s_at	TSPAN4	Tetraspanin 4	Adhesion	−2
205665_at	TSPAN9	Tetraspanin 9	Adhesion	−1.9
1556499_s_at	COL1A1	Collagen, type I, alpha 1	ECM	−4.6
201852_x_at	COL3A1	Collagen, type III, alpha 1	ECM	−14.9
201842_s_at	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	ECM	−3
204688_at	SGCE	Sarcoglycan, epsilon	ECM	−11.3
225776_at	FUT1	Fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase)	Glycosylation	−2.5
214046_at	FUT9	Fucosyltransferase 9 (alpha (1,3) fucosyltransferase)	Glycosylation	−4.3
228303_at	GALNT6	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-Acetylgalactosaminyltransferase 6 (GalNAc-T6)	Glycosylation	−2.6
208116_s_at	MAN1A1	Mannosidase, alpha, class 1A, member 1	Glycosylation	−12.1
222162_s_at	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	Proteolysis	−84.4
1553179_at	ADAMTS19	ADAM metallopeptidase with thrombospondin type 1 motif, 19	Proteolysis	−48.5
207012_at	MMP16	Matrix metallopeptidase 16 (membrane-inserted)	Proteolysis	−25.9

Selected genes for further validation on protein level are indicated in bold

f. c.: Fold change

vitro for ER(+) as well as for ER(−) breast cancer cells, and enhanced proliferation rates, invasivity and motility correlate with high Fra-1 expression levels regardless of ER status (Belguise et al. 2005). Our statistical data, however, suggest a different role for Fra-1 in ER(+) versus ER(−) tumors. In line with our observations, Philips et al. demonstrated an inverse Fra-1-dependent regulation of AP-1 activity by estradiol in ER(+) versus ER(−) breast cancer cell lines and proposed that expression of AP-1-controlled genes might differ in ER(+) and ER(−) breast cancer cells (Philips et al. 1998).

The implication of AP-1 proteins in carcinogenesis has been described shortly after their discovery (Lamph et al. 1988). AP-1-regulated genes include important regulators of invasion, proliferation, differentiation, survival and genes associated with hypoxia and angiogenesis (Milde-Langosch 2005). Moreover, we have recently shown the effect of two AP-1 components, Fra-2 and c-Fos, on the adhesive properties and the metastatic potential of breast and ovarian cancer cells, respectively (Schroder et al. 2010; Oliveira-Ferrer et al. 2014). Regarding the functional role of Fra-1 in breast cancer, there are several experimental studies indicating an association between enhanced Fra-1 expression and proliferation, invasion and motility (Belguise et al. 2005). In addition, Fra-1 has been suggested to participate in the molecular switch during

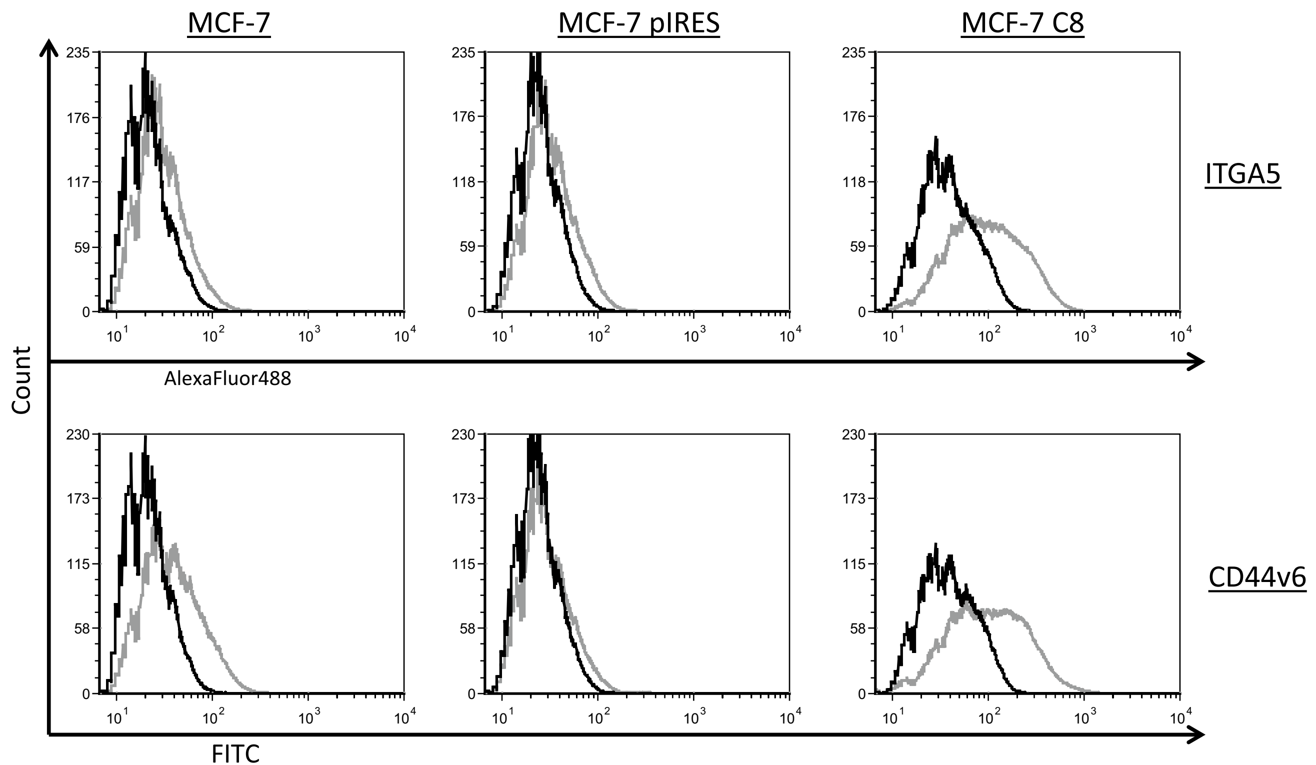

Fig. 3 FACS analysis. Results of flow cytometry showing increased ITGA5 and CD44v6 binding in MCF7 Fra-1#8 cells in comparison with MCF7 and MCF7 NC control cells. Black curves displayed isotype control, and gray curves show specific mAb binding, respectively. Each experiment repeated three times, and histograms of one representative experiment are displayed

epithelial–mesenchymal transition (EMT) (Stinson et al. 2011). Nevertheless, nothing is known about Fra-1-mediated regulation of breast cancer cell adhesion features. Tumor cell adhesion and particularly interactions with the endothelium are critical for tissue colonization and metastasis development. The attachment of cancer cells to the endothelial layer is the first step of the extravasation process and requires the expression of adequate cell adhesion molecules. Mimicking the in vivo situation, we analyzed the effect of Fra-1 on the adhesive behavior of two breast cancer cell lines (MCF7 and MDA MB231) under dynamic conditions. MCF7 showed strong adhesion to endothelial monolayers or E-selectin-coated surfaces, whereas MDA MB231 cells rather demonstrated rolling behavior. By coating the flow channel surface with E-selectin or endothelial cells, we were able to discriminate between two different steps in the adhesion cascade: initial rolling or tethering events mediated by selectins (Tremblay et al. 2008) and firm cell adhesion on stimulated endothelial cells. Fra-1 led to a significant increase in MCF7 cell adhesion events on E-selectin as well as on stimulated endothelial cells under these conditions, whereas the effect on MDA MB231 rolling on both surfaces was less prominent and not significant. Since these two cell lines differ in their ER status [MCF7 is ER(+) and MDA MB231 is ER(−)], one could postulate, in line with our previous results from patient tumor samples, an ER-dependent influence of Fra-1 on the tumor cell adhesion features.

Our present data show that E-selectin binding is significantly increased in Fra-1-overexpressing MCF7 cells under flow conditions. Although the microarray data revealed up-regulation of some enzymes involved in O-glycosylation and the biosynthesis of Lewis antigens (B3GNT3 and B4GALT1), others such as FUT1 and FUT9 (Potapenko et al. 2010) were down-regulated in $MCF7^{Fra-1}$. In fact, we could not detect higher expression levels of the minimal selectin-binding epitopes sialyl $Lewis^{X}$ (CD15 s) and sialyl $Lewis^{A}$ (CA19-9) by FACS analysis (data not shown), indicating that for MCF7 cells alternative E-selectin ligands have to be still postulated.

Enhanced adhesion of MCF7 cells to endothelial cells after Fra-1 overexpression might not only be explained to increased binding to E-selectin but also to other receptors contributing to stable cancer cell adhesion to ECs, i.e., integrins, CD44 and MUC1 (Miles et al. 2008; Kobayashi et al. 2007; Petruzzelli et al. 1999). In $MCF7^{Fra-1}$ cells, up-regulation of integrin alpha5 and CD44 was detected at mRNA as well as at protein level using microarray and

FACS analysis, respectively. This is particularly interesting since integrins follow selectins in the adhesion cascade of leukocytes, and cancer cells follow similar adhesion mechanisms (Strell and Entschladen 2008). CD44 expression on cancer cells strongly correlates with tumor cell adhesion to endothelial cells and metastasis particularly in prostate cancer and breast cancer cells (Draffin et al. 2004; Orian-Rousseau 2010). Interestingly, specific glycosylated forms of CD44 can interact with selectins (Zen et al. 2008). In addition, we detected higher mRNA expression levels of the cell adhesion molecule CEACAM6, which has been previously described to modulate tumor cell adhesion to endothelial cells (Blumenthal et al. 2005).

In contrast to these findings on MCF7 cells, in Fra-1-overexpressing MDA MB231 cells, we could not detect deregulation neither of those CAMs mentioned before (CD44, integrin alpha5 and CEACAM6) nor of other classical mediators of tumor cell adhesion such as integrins alpha 1/2/4/6 and V, integrins beta 1/3/4/7, ICAM-1, ALCAM, PSGL-1 or L1CAM. This underlines the hypothesis of a different regulative function of Fra-1 in ER(+) and ER(−) breast cancer cells.

Conclusion

In conclusion, our data show the prognostic relevance of the transcription factor Fra-1 for patients with ER(+) breast cancer and suggest different roles of Fra-1 in breast tumor cells depending on the ER status. In addition to its known pro-proliferative and pro-invasive effect, Fra-1 might influence breast cancer progression by modulating the adhesion of tumor cells to endothelial surfaces. Single Fra-1 deregulated genes and their relevance in mediating cell adhesion to endothelial cells should be further analyzed in order to identify new target molecules for therapeutic purposes.

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Conflict of interest All authors declare that they have no conflict of interest.

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