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The transcription factor Fra-2 promotes mammary tumour progression by changing the adhesive properties of breast cancer cells

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ABSTRACT

The transcription factor Fra-2 (Fos-related antigen-2) has been implicated in invasion of breast cancer cells, but there is only sparse information about its role in clinical tumours. In the present study, we analysed Fra-2 mRNA expression in a cohort of 167 patients, and found significant correlations between high Fra-2 expression and nodal involvement or reduced disease-free survival. To get more information about the underlying mechanisms, we generated stably transfected MDA-MB231 breast cancer cells with increased Fra-2 expression. Compared with the controls, these clones did not differ in proliferation and motility, but had higher invasive potential. By global gene expression analysis and subsequent validation of selected genes, we identified a number of proteins involved in cell–cell or cell–matrix interactions that were up- or down-regulated in Fra-2 overexpressing cells, e.g. connexin 43, ICAM-1, L1-CAM, integrin beta 2, integrin beta 4, and integrin alpha 6. The association of Fra-2 overexpression and high ICAM-1 or L1-CAM levels could also be demonstrated in our clinical cohort of mammary tumours. In both MDA-MB231 and MCF7 cells, we found an increased attachment of Fra-2 transfectants to components of the extracellular matrix. In addition, we could show a striking increase in the number of rolling cells in flow-through assays using E-selectin coated capillaries, which might indicate a higher capacity of extravasation. In conclusion, our data obtained on breast cancer cell lines and clinical tissue samples suggest that overexpression of Fra-2 promotes breast cancer progression and metastasis by deregulation of genes involved in cell–cell and cell–ECM contacts.

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1. Introduction

Activating protein-1 (AP-1) is a transcription factor complex composed of members of the Jun, Fos, and activating transcription factor (ATF) family that bind as hetero or homodimers to the regulatory sequences of various target genes. Inhibition of AP-1 activity results in blocked proliferation, migration, invasion and experimental metastasis of tumour cells in various systems.[1]

The transcription factor Fra-2 (Fos-related antigen 2) was identified as a serum-inducible gene with homologies to c-Fos, FosB and Fra-1, but with significantly lower transforming activity compared with c-Fos.[2,3] Like the other members of this Fos family, Fra-2 forms heterodimers with Jun family members. In contrast to c-Fos and FosB, but similar to Fra-1, Fra-2 proteins lack a C-terminal transactivating domain and do not stimulate artificial AP-1-responsive promoters *in vitro*.[4,5] Animal models have shown that Fra-2 is required for proper bone and cartilage development, and Fra-2 –/– pups exhibit growth retardation and die within one week after birth.[6] On the other hand, ectopic expression of Fra-2 in transgenic mice results in extensive fibrosis, predominantly in the lung, leading to premature mortality.[7] Investigations on human clinical tissues indicate that Fra-2 might play an important role in the progression of various human tumour types *in vivo*.[8] Fra-2 overexpression was found in salivary gland tumours[9], colorectal cancer[10] and adult T-cell leukaemia[11], and in anaplastic large cell lymphomas. In the latter case, Fra-2 is amongst the genes which are affected by the characteristic t2;5 translocation leading to enhanced Fra-2 expression in the tumour cells.[12]

In breast cancer cell lines and tumour tissues, Fra-2 is detectable in variable amounts and phosphorylation states.[13] In a Western blot study with 75 breast cancer samples, we found a significant association of strong Fra-2 expression with a high frequency of recurrence.[14] In experimental models, Fra-2 expression is associated with an increased invasive potential and motility, whereas proliferation was hardly influenced at all.[14,15] In mouse mammary adenocarcinoma cell lines (CSMLO) with different metastatic potential, strong Fra-1 and Fra-2 expression was present in the metastatic cells, whereas c-Fos and FosB were undetectable.[16] In transfection experiments with these cell lines, overexpression of Fra-2 resulted in activation of osteopontin, thrombospondin and CD44 which are all known to be involved in metastasis.[17]

In order to further investigate the role of Fra-2 in breast cancer progression, Fra-2 mRNA expression was analysed in a cohort of 164 mammary carcinomas with long-term follow-up data. In addition, we performed stable transfection leading to Fra-2 overexpression in the ER-negative MDA-MB231 cells. Amongst the genes which are differentially regulated in these cells, we identified a number of genes involved in cell–cell or cell–ECM interaction which suggested that the effect of Fra-2 is at least partly due to its influence on cell adhesion, especially during extravasation from the bloodstream. This hypothesis was further confirmed by ECM adhesion assays and analysis of attachment to E-selectin-coated surfaces in a laminar flow system. The correlation of Fra-2 expression with two relevant Fra-2 target genes, the cell adhesion molecules ICAM-1 and L1-CAM, was further analysed in our cohort of clinical breast cancer samples.

2. Materials and methods

2.1. Patients

For array-based Fra-2 mRNA detection, samples from 167 patients (mean age 56.2 years; range 29–85 years) were analysed. All patients were treated for breast cancer at the University Medical Center Hamburg-Eppendorf, Germany, Department of Gynecology, between the years 1991 and 2002. Patient selection was based upon availability of tumour tissue. All patients gave written informed consent to access their tissues and review their medical records in accordance with the principles of the declaration of Helsinki after review and approval of the consent form by the local ethics committee (Ethikkommission der Ärztekammer Hamburg). Breast conserving surgery was performed in 53% of patients, and 47% were treated by mastectomy. One-hundred four patients received adjuvant chemotherapy, 106 radiation therapy, and in 91 cases, an endocrine therapy was applied. The median follow-up time was 84 months (range 8–169 months).

Histologically, 116 tumours were diagnosed as ductal carcinomas, 27 as lobular carcinomas, and 24 were of other histological types. Twelve tumours were well-differentiated, 66 moderately differentiated and 83 were poorly differentiated (unknown: 6 cases). According to TMN classification, 39 tumours were T1 (<2cm), 11 cases were T2 (2–5 cm) and 13 tumours T3–4 (>5 cm). Lymph node involvement was detected in 47 cases (28.5%), and 118 tumours were nodal-negative (unknown: 2). One-hundred seventeen tumours (74%) were oestrogen-receptor (ER) positive, and 41 cases (26%) ER-negative. Progesterone receptors (PR) were detectable in 100 cases (63%), whereas 58 tumours (37%) were PR-negative (unknown: 9).

2.2. RNA isolation and microarray analysis

Approximately 50 mg of frozen breast tumour tissue was crushed in liquid nitrogen. RLT-Buffer (Qiagen, Hilden, Germany) was added and the homogenate was centrifuged through a QIAshredder column (Qiagen). The total RNA was isolated from the eluate by the RNeasy Kit (Qiagen) according to the manufacturer's instructions. RNA yield was determined by UV absorbance and RNA quality was assessed by analysis of ribosomal RNA band integrity on an Agilent 2100 Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA).

The Affymetrix (Santa Clara, CA) HG-U133A array and GeneChip System™ was used to quantify the relative transcript abundance in the breast cancer tissues. Starting from 5 μg total RNA, labelled 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 relabelled and rehybridised on new arrays.

Total RNA from MDA-MB231 cells which were cultured to 70% confluence was extracted by lysing the cells in Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions and further purified using the RNeasy Mini Kit (Qiagen). Procedures for cDNA synthesis, labelling and hybridisation 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.[15] 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.

2.3. Cells and transfection

The human mammary carcinoma cell line MDA-MB 231 was cultivated as described.[18] To generate stable clones with increased Fra-2 expression, transfections with the plasmid Fra2–pIRES-P which contains the full Fra-2 cDNA cloned in the bicistronic vector pIRES-P (Genbank no. Z75185) were performed.[15] The correct Fra-2 cDNA sequence in the resulting plasmid Fra2–pIRES-P had been confirmed by sequencing. Transfections with this vector and the empty control plasmid pIRES-P were performed with LipofectAMINE PLUS reagent (Life Technologies, Karlsruhe, Germany). Selection of stably transfected clones started by addition of 1 μg/ml puromycin 48 h after transfection. As negative control, puromycin-resistant cells harbouring the empty vector were cultivated as a batch (MDA-NC). For further analysis, MDA-NC and the MDA–pFra2 clones 2 and 8 were used. Binding of Fra-2, Fra-1, FosB and c-Fos to AP-1 consensus sequences in the generated clones were determined using the TransAm™ AP-1 assay (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions.

2.4. Proliferation, invasion and motility assays

Cellular proliferation was monitored using the Cell Proliferation Kit I (MTT; Roche Diagnostics, Mannheim, Germany) as described.[15] The invasive potential of the cells was tested with Matrigel™ Invasion Chambers (24-well plates; BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions with 25,000 cells per insert as described previously.[15] The bottom of these inserts consists of a porous membrane (8 μm pores), coated with matrigel which resembles human basal membranes in composition. After 48 h incubation, the cells which had passed the membranes were counted under a microscope in 7 identical square fields which together included most of the membrane area without the periphery. For comparison and estimation of cell motility, the same number of control inserts without matrigel membrane (BD Biosciences, Heidelberg, Germany) was filled in the same way.

2.5. Adhesion assays

Adhesion to five substances of the extracellular matrix was assayed using the CytoMatrix™ cell adhesion assay (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions, using 10^4 cells per well which were coated with either fibronectin, vitronectin, laminin, collagen I, collagen IV or, as control, bovine serum albumin (BSA). After 1h incubation at 37 °C, the supernatant was removed, and the adherent cells were stained and quantified by ELISA test.

Adhesion to the endothelial marker E-selectin was analysed in a flow-through adhesion assay using the IV-ibidi treat flow chambers (IBIDI, Munich, Germany) coated with 5 μg/ml E-selectin. The transfected cells were drawn through the flow chamber by a perfusion pump (Perfuser IV, B.Braun Melsungen AG, Melsungen, Germany) with a constant flow of 1704 ml/h, which resulted in a shear stress of 5 dyn/cm^2 and represented physiological blood flow conditions in small vessels. The cell number was adjusted to 100,000 cells/ ml. The suspension flow was digitally recorded by a video camera mounted on the microscope. The number of rolling cells was counted as an absolute number during the entire recording time of 1.5 min. In similar experiments, adhesion to hyaluronic acid was analysed using the IBIDI-μ-slides (poly-L-lysine) (IBIDI) coated with 1 mg/ml hyaluronic acid.

2.6. Western blots

Western blot conditions for AP-1 proteins have been described.[13] For validation of differentially expressed genes, the following antibodies were used: ALCAM Mab NCL-CD166 (1:200; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), rabbit anti-connexin 43 (1:2000; Sigma–Aldrich, Taufkirchen, Germany), mouse anti-CD44 (1:300; Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-ICAM-1 (1:300, Santa Cruz), mouse anti-NCAM-L1 (1:200, Santa Cruz), and goat anti-actin (1:4000, Santa Cruz).

As secondary antibodies, peroxidase-conjugated anti-mouse-IgG (1:2000), anti-rabbit-IgG (1:4000) or anti-goat-IgG (1:4000; all from Santa Cruz), respectively, were used, which were visualised by chemiluminescence reagents (Super Signal West Pico kit, Pierce, Rockfort, Ill.) with Hyperfilm ECL films (Amersham, Braunschweig, Germany). Band intensities were quantified by densitometry (GS-700 Imaging Densitometer, BioRad, München, Germany). The intensities of the specific protein bands were calculated as percentage intensity of the control sample and corrected for equal actin loading.

2.7. FACS analysis

For FACS analysis, the transfected cells were stained with the following mAB for 30 min at 4 °C: PE-conjugated anti-human L1-CAM (CD 171), PE-conjugated anti-human ICAM-1(CD 54); PE-conjugated anti-human CD24 (all from eBiosciences Inc., San Diego, USA) and FITC-conjugated anti-human CD44 (Diaclone, Tepnel Research, Bescancon Cedex, France). Stained cells were analysed with a FACScalibur (BD Biosciences) using the Cellquest software (BD Biosciences). The presence of each adhesion molecule was analysed in three independent experiments.

2.8. RT^2 Profiler PCR array

RNA expression of 84 genes important for cell–cell and cell–matrix interactions was also studied using the Human Extracellular Matrix and Adhesion Molecules RT^2 Profiler PCR Array (SABiosciences Corporation, Frederick, MD, USA). For MDA-NC and MDA–Fra-2 clone 2 RNA, the cDNA was obtained using the Super Array RT^2 First Strand Kit according to the manufacturer's protocol. After genomic DNA elimination, the reverse transcription reaction was performed at 42 °C for 15 min, followed by inactivation of the enzyme at 95 °C for 5 min. The cDNA was mixed with RT^2 SYBR Green/ROX qPCR master mix (SABiosciences Corporation) and 25 μl of the experimental cocktail were loaded into each well of the RT^2 Profiler PCR Array. PCR array experiments were performed on an ABI7500 instrument (Applied Biosystems, Foster City, CA, USA), with the following conditions for amplification: 1 cycle of 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR array data were analysed by the ΔΔCt method. Genes with Ct values greater than 35 cycles were considered as N/A (non-detectable) and assigned a value of 35. Average of five housekeeping genes B2M (Beta-2-microglobulin), HRPT1 (Hypoxanthine phosphoribosyltransferase (Lesch–Nyhan-syndrome)), RPL13A (ribosomal protein 13A), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTB (β-actin) was used to obtain the ΔCt value for each gene of interest. The ΔΔCt value for each gene was calculated by the difference between the ΔCt of the control cells (MDA-NC) and the ΔCt of the transfected cells (MDA–Fra-2). The fold-change for each gene was calculated by $2^{-\Delta\Delta Ct}$.

2.9. Statistical analysis

Correlations between Fra-2 mRNA expression and histological or clinical tumour characteristics were calculated by Chi-square tests using the SPSS 15.0 software. For prognostic parameters, the following groups were compared: histological grade: G1/G2 versus G3; staging: pT1 versus pT2 versus pT3/pT4; nodal involvement versus nodal-negative tumours; ER/PR positive cases versus ER/PR negative tumours; ductal versus lobular versus other carcinomas; age <median versus> median. Pearson correlation was used as a measure of association between variables using the continuous expression values. Kaplan–Meier analysis was also performed using SPSS 15.0 software. OAS was computed from the date of diagnosis to the date of death due to distant metastasis. Survival curves were compared with the logrank test. Univariate as well as multivariate p values for the respective risk factors in the survival model were obtained by a Cox proportional hazards model as implemented in SPSS. All tests were performed at a significant level of $p = 0.05$. All p values are two-sided, and no corrections for multiple testing were applied.

3. Results

3.1. Fra-2 expression in clinical tumour tissues

Fra-2 (FOSL2) mRNA expression in 167 breast cancer samples was evaluated by microarray analysis. Since the Affymetrix genchips harboured three probesets for FOSL2 (205409_at, 218880_at and 218881_s_at), we first compared the expression data of these sets and found highly significant correlations between all of them ($p < 0.001$; not shown). Since similar correlations with established prognostic markers were found for all probesets, we chose one of them (218881_s_at) for further analysis. Expression values for this probeset varied from 12.8 to 447.6 with a median expression of 168.2.

According to their FOSL2 mRNA expression data, all samples were divided into three groups of similar size with low, moderate and high FOSL2 expression. By Chi-square tests, we found highly significant associations of high Fra-2 expression with younger age and nodal involvement (Table 1). In addition, correlations with high grading and a negative ER status were observed. Yet, the latter associations were not linear, and the most favourable prognostic characteristics, i.e. high numbers of well/moderately differentiated, ER-positive and T1 tumours, were found in cases with moderate FOSL2 expression levels (Table 1).

By Kaplan–Meier analysis, high FOSL2 expression levels were associated with a significantly shorter disease-free survival, whereas no significant difference in overall survival was observed (Fig. 1).

RNA expression values for the other AP-1 proteins (c-Jun, JunB, JunD, c-Fos, FosB and Fra-1) are given in the supplemental Table S1. By Kaplan-Meier analysis comparing three groups with weak, moderate and strong expression, we only found significant differences in overall survival if patients were stratified according to c-Fos expression, with a significantly better prognosis in cases showing strong c-Fos expression (Fig. S1). None of the Jun proteins or the remaining Fos family members (FosB, Fra-1) had a prognostic impact in our cohort.

3.2. Characterisation of stably transfected MDA-MB231 cells

In order to further characterise the role of Fra-2 in mammary carcinomas, we established stable transfectants of MDA-MB231 breast cancer cells with the previously described pIRES-P–Fra2 vectors[15] resulting in clones with strongly enhanced Fra-2 expression (Fig. 2B). As control, stable transfectants with the empty vector were generated. By TransAm test we could show that overexpression of Fra-2 resulted in a 1.6–1.7-fold increase in Fra-2 protein binding to AP-1 consensus sequences (Fig. 2A). In contrast, binding of Fra-1 which was the major Fos family member in untransfected cells decreased by 1.7-fold. C-Fos binding increased and FosB binding decreased on a very low level (Fig. 2A). Similar to previous results obtained with MCF7 cells[14,15], Fra-2 overexpression did not result in changes in cell proliferation (Fig. 2C) and in motility (Fig. 2E), whereas the invasive potential of the cells, as measured by matrigel invasion assays, was increased significantly (Fig. 2D). The morphology of the MDA-MB231 was not altered by Fra-2 overexpression (data not shown).

3.3. Results of microarray analysis in Fra-2 transfectants

In order to identify Fra-2 target genes, cDNA arrays were performed with mRNA isolated from the stably transfected MDA-MB231 clones 2 and 8 and control transfectants (MDA-MB231

Table 1 – Correlations of Fra-2 expression and clinical or histological tumour parameters in breast cancer samples.

Parameter	Group	*n*	Fra-2 expression			*p*
			low	moderate	high	
Age						
	</= Median	84	20	28	36	
	>Median	83	36	28	19	0.007
Grading						
	G1-2	78	27	34	17	
	G3	83	28	20	35	0.008*
Tumour size						
	T1	39	9	17	13	
	T2	111	46	33	32	
	T3–4	13	1	4	8	0.023*
Nodal involvement						
	N0	118	49	38	31	
	N1	47	6	18	23	0.001
ER status						
	Negative	41	12	9	20	
	Positive	117	40	46	31	0.023*
PR status						
	Negative	58	19	16	23	
	Positive	100	33	39	28	0.232

* No linear association.

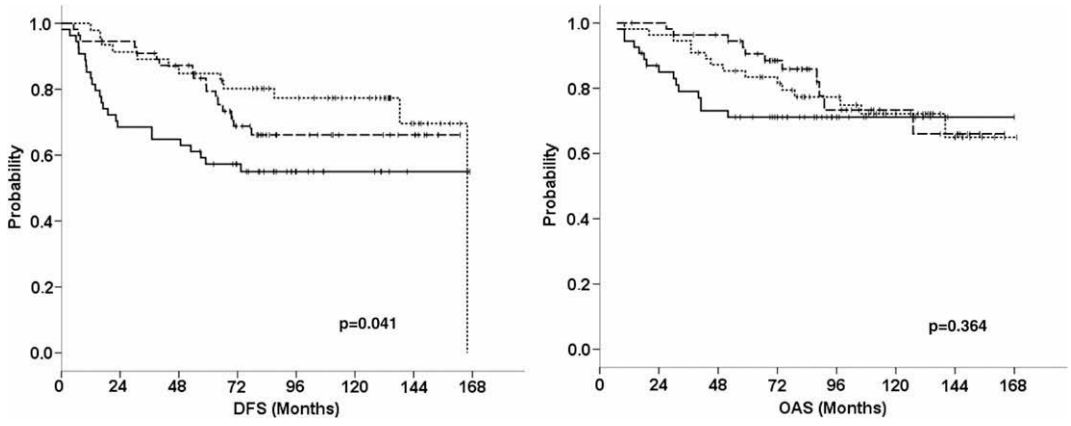

Fig. 1 – Prognostic impact of Fra-2 expression in breast cancer patients (*n* = 167). The differences in disease-free survival (A) and overall survival (B) are shown. Solid line, strong Fra-2 expression; dashed line, moderate Fra-2 expression; dotted line, weak Fra-2 expression.

nc). Genes that were increased or decreased at least 1.9-fold (mean signal log ratio >/= 0.9) compared to the control in both clones were identified. Using this definition, 245 genes were up-regulated, and 263 genes were down-regulated in Fra-2-overexpressing cells which could be attributed to a wide variety of functional groups (Fig. 3A and B).

Since we were mostly interested in genes which might influence the invasive and/or metastatic properties of breast cancer cells, we further concentrated on deregulated genes involved in these processes. In Table 2, a number of differentially regulated genes involved in proteolytic cleavage (carboxypeptidases E and M, cathepsins C and D, matrix metalloproteinase 1, SERPINs B7 and I1, cystatins E/M and F, etc.), adhesion (some integrins, ICAM-1, L1-CAM, CD44, connexin 43, etc.) and metastasis (KISS-1) is shown.

3.4. *Validation of differentially regulated genes*

Since proteins involved in cell–cell and cell–ECM contacts are crucial for the metastatic potential of breast cancer cells, we validated several of those factors on a protein and/or mRNA level. By western blot analysis, we could show highly decreased expression of the gap junction protein connexin 43 (GJA1) and strong overexpression of intercellular adhesion molecule-1 (ICAM-1) in Fra-2 transfectants (Fig. 3C). By FACS analysis, up-regulation of ICAM-1 (38-fold), L1-CAM (3-fold)

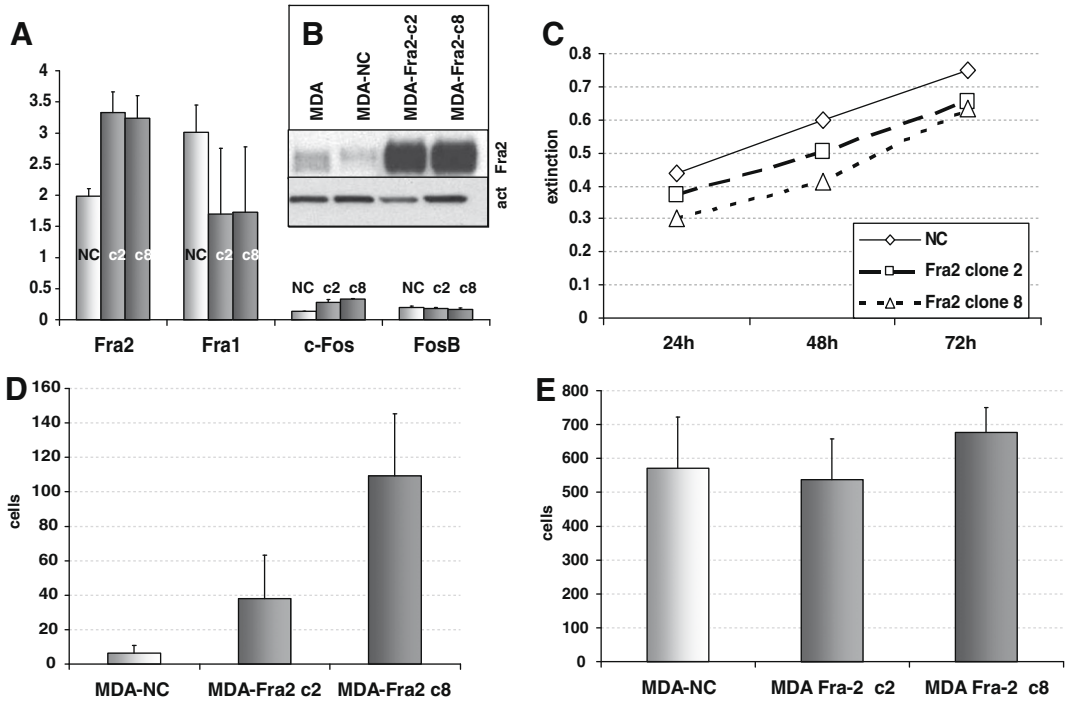

Fig. 2 – Characterisation of stably transfected MDA-MB231 clones 2 and 8. (A) Results of TransAm tests showing changed Fra-2, Fra-1, c-Fos and FosB binding to AP-1 consensus sequences in Fra-2 transfectants. (B) Western blot analysis showing Fra-2 overexpression in clones 2 and 8 relative to the original MDA-MB231 cells and stably transfected cells harbouring the empty pIRES-P vector (MDA-NC). As loading control, actin expression is shown. (C) Proliferation curves (MTT test), results of a representative experiment. (D) Results of invasion assays and (E) migration through control inserts. Mean values of three independent experiments are given.

and, to a weaker extent, CD44 (1.2-fold) on the cell surface could be shown (Fig. 3D). Using the RT^2 Profiler PCR array, downregulation of MMP1 which had been shown in cDNA arrays of Fra-2 transfected cells could be confirmed (Table 2). In addition, up-regulation of ICAM-1, integrin alpha 6, integrin beta 2 and integrin beta 4 was corroborated. In contrast, CD44 results could not be validated by real-time PCR. Using this test, more than 2-fold up-regulated expression was also found for the following genes not included in our list obtained by microarray analysis: catenin alpha 1, integrin alpha 5, integrin alpha V, integrin beta 3 and MMP 11, whereas reduced expression was observed for MMP 14 and MMP 16 (not shown).

3.5. *Influence of Fra-2 overexpression on cell adhesion to ECM components and E-selectin*

Since Fra-2 overexpression resulted in altered expression of proteins involved in cell–cell or cell–matrix interaction, we investigated the adhesive potential of transfected cells. For these experiments, we also included the MCF7–pIRES–Fra2 cells and the corresponding negative control cells (MCF7–pIRES) which were described in a previous publication.[15] In an adhesion assay including 5 ECM substances, Fra-2 overexpression resulted in increased adhesion to collagen I, collagen IV, vitronectin and fibronectin in MCF7 cells (for collagen IV and vitronectin, this difference reached statistical significance). In MDA-MB231 cells, increased Fra-2 expression was associated with significantly increased adhesion to collagen I and weakly increased binding to fibronectin (Fig. 4A). Since adhesion molecules are also involved in binding to endothelial surfaces, we also analysed the transient attachment of the cells ("rolling") to E-selectin in a laminar flow assay, using physiological shear forces (5 dyn/cm^2). E-selectin is expressed by activated endothelial cells and mediates the first, transient steps of adhesion of circulating cancer cells which is then followed by firm binding which is mainly mediated by integrins and adhesion molecules of the immunoglobulin superfamily.[19] Therefore, it seemed feasable to examine the binding of tumour cells to E-selectin under near-physiological conditions. On E-selectin-coated surfaces, there was a strong increase in the number of transiently adherent (rolling) cells relative to the control in MCF7 and MDA-MB231 cells showing Fra-2 overexpression (Fig. 4B and C). In MCF7, Fra-2 transfectants showed a 3–4-fold elevated number of rolling cells, whereas in MDA-MB231 cells, where hardly any adhesion was observed in the control cells, the number of rolling cells was increased more than 100-fold. No firm, irreversible adhesion was observed under our experimental conditions. In a similar experiment with capillaries coated with hyaluronic acid, there was no difference in transient adhesion in MDA-MB231 cells with and without Fra-2 overexpression (not shown).

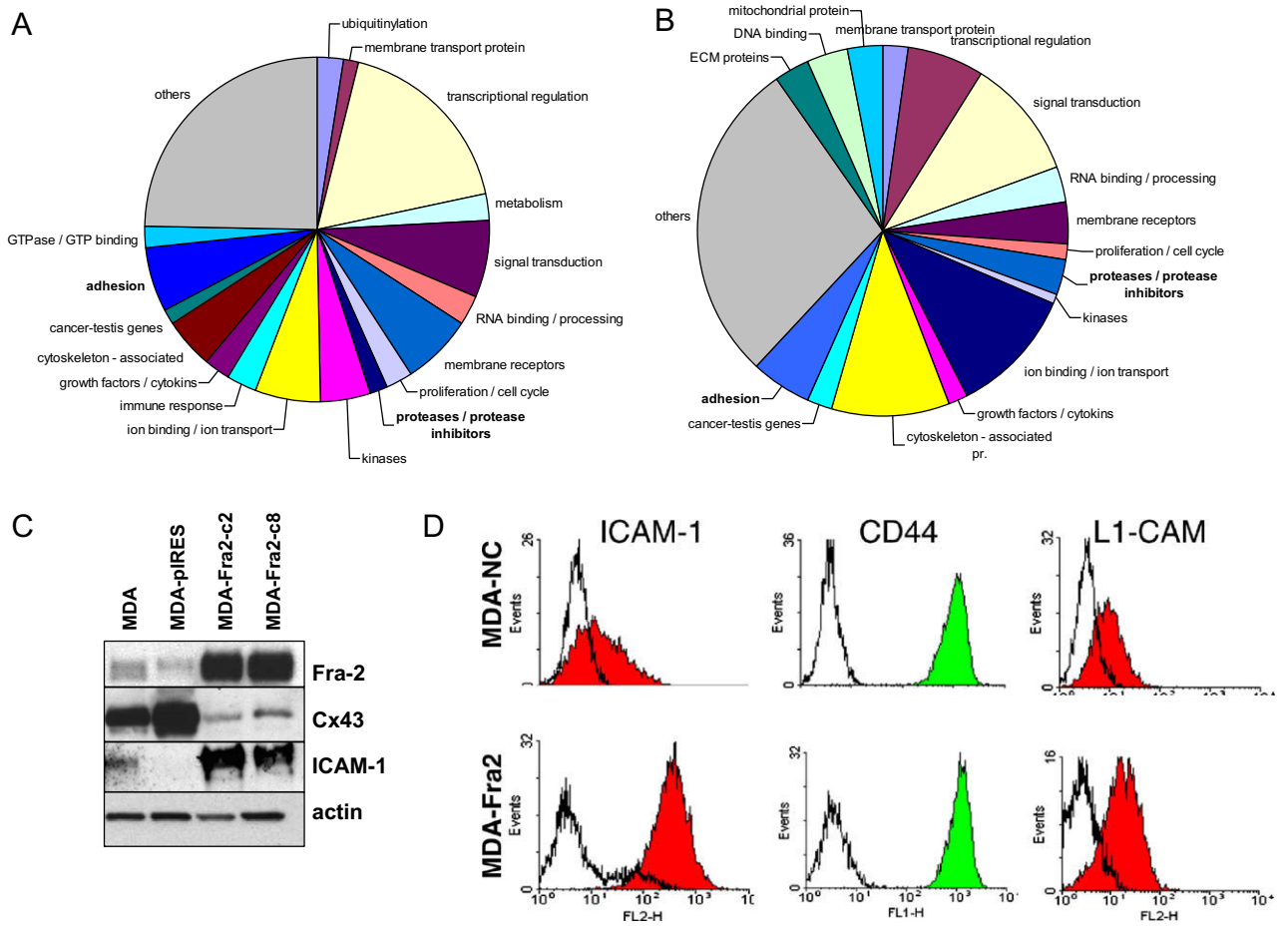

Fig. 3 – Genes with overexpression or downregulation in Fra-2 transfectants. (A) distribution of genes showing down-regulation by >/= 1.9-fold in functional groups. (B) distribution of genes showing up-regulation by >/= 1.9-fold in functional groups. (C) validation of connexin 43 (Cx43) down-regulation and ICAM-1 upregulation in Fra-2 transfectants by western blot analysis. (D) FACS analysis showing strong ICAM-1 overexpression, moderate L1-CAM overexpression and minimally changed CD44 expression in Fra-2 transfectants (bottom, clone 2) relative to the negative control (top).

3.6. *Correlation of Fra-2 expression with expression of adhesion molecules in clinical tumour tissues*

Since Fra-2 influenced the expression of various molecules involved in cell adhesion in breast cancer cell lines, we asked if this regulation would also take place in clinical tumour tissues. Therefore, we correlated expression data obtained by microarray analysis in 167 breast cancer samples. Interestingly, we found a significant correlation of microarray results for Fra-2 (affymetrix probeset 218881_s_at) with ICAM-1 (all 3 probesets) and L1-CAM (1 probeset; Table 3). Similar correlations were found for the two additional Fra-2 probesets (not shown).

4. Discussion

This study was undertaken to examine the role of Fra-2 expression in malignant progression of breast cancer in an experimental system and clinical tumours. Similar to our prior western blot study with a smaller cohort[14], the present analysis of RNA expression data of 167 breast cancer samples has shown that high Fra-2 expression is associated with nodal involvement and early relapse, suggesting that Fra-2 might regulate target genes which are involved in the metastatic cascade in breast cancer. This involvement of Fra-2 during metastatic progression in breast cancer patients is consistent with experimental results obtained with mouse adenocarcinoma cell lines of different metastatic potential which also differ in Fra-1 and Fra-2 expression[16].

Fra-2 is not the only Fos family member present in human mammary tumours (Table S1;[13]). Yet, expression of none of the other Fos proteins (c-Fos, FosB and Fra-1) was associated with poor prognosis in our patients (not shown). High c-Fos expression is even associated with a favourable outcome in our cohort (Fig. S1) which indicates that c-Fos might be replaced by Fra-2 in highly invasive tumours. For FosB and Fra-1, no significant prognostic impact was found. Although Fra-1 is structurally similar to Fra-2, both proteins behave differently *in vivo*: Fra-1 which is strongly expressed in highly invasive breast cancer cell lines[20] is hardly detectable in clinical tumour tissues, whilst Fra-2 expression is abundant in breast cancer samples[8]. As shown in prior studies, FosB

Table 2 – Differentially regulated genes in MDA-MB231 Fra-2 cell lines[a].

Probeset ID	Gene symbol	Gene title	Fold change		
			Clone 2	Clone 8	Validation by RT-PCR array (clone 2)
Decrease:					
201667_at	GJA1	Connexin 43, gap junction protein	−32	−15	n.a.
213865_at	DCBLD2	Discoidin, CUB and LCCL domain containing 2	−2.0	−2.3	n.a.
204751_x_at	DSC2	Desmocollin 2 (demosomal cadherin)	−9.8	−4.9	n.a.
201117_s_at	CPE	Carboxypeptidase E	−3.5	−5.3	n.a.
235019_at	CPM	Carboxypeptidase M	−2.5	−3.2	n.a.
225646_at	CTSC	Cathepsin C	−3.7	−4.9	n.a.
231234_at	CTSC	Cathepsin C	−3.2	−3.7	n.a.
225647_s_at	CTSC	Cathepsin C	−3.0	−4.3	n.a.
201487_at	CTSC	Cathepsin C	−2.1	−3.0	n.a.
201185_at	HTRA1	HtrA serine peptidase 1	−2.8	−6.1	n.a.
205563_at	KISS1	KiSS-1 metastasis-suppressor	−2.1	−11.3	n.a.
204475_at	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	−22.6	−73.5	−157.1
212217_at	PREPL	Prolyl endopeptidase-like	−1.9	−1.9	n.a.
206421_s_at	SERPINB7	Serpin peptidase inhibitor, clade B (ovalbumin), member 7	−22.6	−2.1	n.a.
205352_at	SERPINI1	Serpin peptidase inhibitor, clade I (neuroserpin), member 1	−8.0	−5.7	n.a.
Increase:					
216056_at	CD44	CD44 molecule (Indian blood group)	2.1	2.5	−1.2
1565868_at	CD44	CD44 molecule (Indian blood group)	2.0	2.3	−1.2
217523_at	CD44	CD44 molecule (Indian blood group)	2.0	2.1	−1.2
225369_at	ESAM	Endothelial cell adhesion molecule	2.3	2.1	n.a.
1555480_a_at	FBLIM1	Filamin binding LIM protein 1	4.9	2.6	n.a.
202638_s_at	ICAM1	Intercellular adhesion molecule 1 (CD54)	7.5	3.7	393.5
202637_s_at	ICAM1	Intercellular adhesion molecule 1 (CD54)	5.7	2.8	393.5
201656_at	ITGA6	Integrin, alpha 6	1.7	1.9	131.4
215177_s_at	ITGA6	Integrin, alpha 6	1.7	1.9	131.4
202803_s_at	ITGB2	Integrin, beta 2	1.9	3.7	2.9
204989_s_at	ITGB4	Integrin, beta 4	2.5	2.8	8.6
204990_s_at	ITGB4	Integrin, beta 4	1.9	2.6	8.6
1557080_s_at	ITGBL1	Integrin, beta-like 1 (with EGF-like repeat domains)	2.1	2.0	n.a.
204584_at	L1CAM	L1 cell adhesion molecule	2.0	2.6	n.a.
215418_at	PARVA	Parvin, alpha	2.5	2.3	n.a.
210368_at	PCDHGA8 /// PCDHGA9 /// PCDHGB4	Protocadherin gamma subfamily B, 4 /// A, 8 /// A, 9	2.3	3.2	n.a.
209493_at	PDZD2	PDZ domain containing 2	3.7	3.7	n.a.
226071_at	ADAMTSL4	ADAMTS-like 4	3.2	2.3	n.a.
222446_s_at	BACE2	Beta-site APP-cleaving enzyme 2	3.7	2.1	n.a.
206595_at	CST6	Cystatin E/M	2.3	9.8	n.a.
210140_at	CST7	Cystatin F (leucocystatin)	3.5	3.5	n.a.
200766_at	CTSD	Cathepsin D	2.1	3.5	n.a.

[a] Genes involved in proteolytic cleavage, adhesion or metastasis with at least 1.9-fold change in both clones are presented. n.a.: not analysed.

expression correlates with a well-differentiated, oestrogen receptor-positive phenotype in mammary carcinomas.[21] The RNA expression data shown in Table S1 do not necessarily reflect protein amounts in the tumour cells, since AP-1 proteins have a short, but different half-life and are partly regulated post-transcriptionally by modifications leading to changed protein stability.[22] Since Fos proteins cannot form homodimers, they require the presence of one of the Jun proteins as dimerisation partner. In most tumours, all Jun proteins (c-Jun, JunB and JunD) are expressed (Table S1), and no correlation with clinical outcome was observed for these transcription factors (not shown).

Metastasis is a highly complex process which involves escape of tumour cells from the primary tumour mass, migration and invasion through basal membranes and connective tissues, entry into the vascular or lymphatic system, survival in the circulation, extravasation at different organ sites (including attachment to endothelial cells and diapedesis

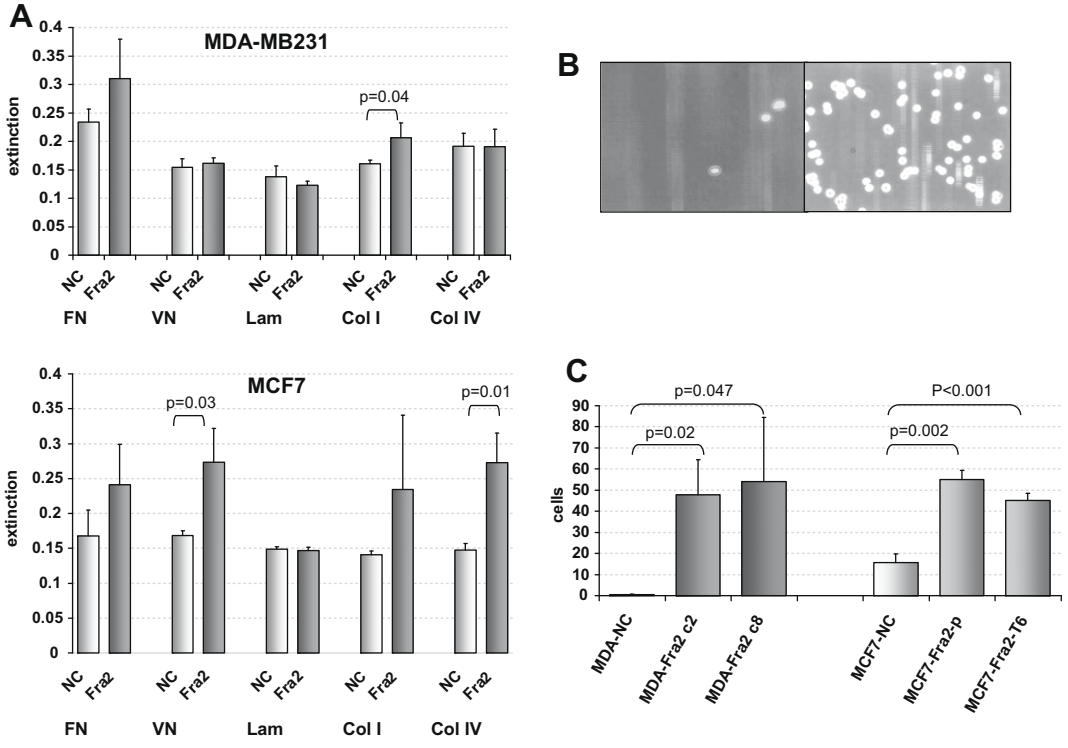

Fig. 4 – Characterisation of adhesive properties of MCF7 and MDA-MB231 Fra-2 transfectants. (A) Results of CytoMatrix™ cell adhesion assay. Adhesion to the ECM components fibronectin (FN), vitronectin (VN), laminin (Lam), collagen I (Col I) and collagen IV (Col IV) in MCF7 (bottom) and MDA-MB231 transfectants (top) is shown. (B) Representative figures showing rolling cells in flow-through adhesion assays. (C) Results of the flow-through adhesion assay showing the number of rolling cells per visual field.

Table 3 – Correlation of Fra-2 expression with potential target genes in clinical tumour samples (*n* = 167).

Gene (probeset ID)	Fra-2 (218881_s_at)	
	r	*p*
ICAM1 (215485_s_at)	0.358	<0.001
ICAM1 (202638_s_at)	0.303	<0.001
ICAM1 (202637_s_at)	0.238	0.002
L1Cam (204584_at)	0.256	0.001

across the endothelium), and proliferation to form a distant metastasis. Our prior and present data have shown that Fra-2 overexpression leads to an increased invasive potential in breast cancer cells.[14,15] In MCF7 and MDA-MB231 cells, Fra-2 does not correlate with enhanced expression of matrix metalloproteinases like MMP9 or MMP2 which are known to be involved in tumour invasion. On the contrary, Fra-2 leads to a strong repression of MMP1 expression. In an earlier study, MMP1 correlated with a negative nodal status and high FosB expression which are favourable prognostic indicators.[14] Thus, instead of matrix metalloproteinases, other proteolytic enzymes are probably causative for the invasive potential of Fra-2 transfectants, i.e. cathepsin D which is associated with metastasis in various tumour types.[23–26] Interestingly, down-regulation by Fra-2 was found for the metastasis suppressor KISS-1 which is involved in the regulation of invasion in breast cancer cells and might further contribute to tumour progression.

The most interesting aspect regarding metastasis is the changed expression of proteins involved in cell–cell and cell–ECM interactions which might have an impact on the metastatic potential of the breast cancer cells. Down-regulation of the gap junction protein connexin 43 (GJA1) and the desmosomal component desmocollin 2 (DSC2) by Fra-2 was found in MCF7 cells in a prior study[15] and now in MDA-MB231 cells. The loss of cell–cell contacts mediated by these proteins might facilitate escape of tumour cells from the primary tumour mass in early steps of metastasis.

In contrast, up-regulation was observed for the stem cell marker CD44 and some integrins which are receptors for components of the extracellular matrix. The up-regulation of CD44 by Fra-2 could not be validated by a RT-PCR array and was only minimal in FACS analysis, which corresponds to the unchanged adhesion of Fra-2 transfectants to capillaries coated with the main CD44 ligand hyaluronic acid. The up-regulation of integrins might, on the one hand, lead to enhanced migration of tumour cells into surrounding tissues. On the other hand, beta-4 integrin transactivates EGFR/Her2 signalling and promotes lung metastasis in breast cancer cells.[27,28]

Up-regulation on both RNA and protein levels was found for two members of the immunoglobulin superfamily of adhesion molecules, ICAM-1 and L1-CAM. L1-CAM is implicated with poor prognosis in various tumour types.[29–31] In a

recent study we could show that high L1-CAM expression which is present in a subset of breast cancers is associated with a significantly shorter overall survival and disease-free interval.[32] ICAM-1 is a key player involved in the adhesion of lymphocytes to endothelial cells[33] and in experimental models it also mediates the interaction of breast cancer cells to endothelia[34] and cell invasion.[35] These properties might indeed contribute to the increased metastatic potential of tumours with high Fra-2 expression *in vivo*. In our mRNA expression data from 167 breast cancer patients, we found significant correlations of Fra-2 mRNA levels with ICAM-1 and L1-CAM expression, respectively, which indicates that the results obtained with the MDA-MB231 cell line also hold true for breast cancer cells in clinical tumours.

As several adhesion molecules were differently expressed in Fra-2 transfectants, we assayed the adhesive properties of the Fra-2 overexpressing cells on ECM substances under static conditions and on E-selectin and hyaluronic acid under flow conditions. In both breast cancer cell lines, an increase of adhesion to collagens and, partly, vitronectin and fibronectin was observed under static conditions, probably due to the overexpressed integrins, which recognise RGD binding domains present in vitronectin and fibronectin.

Concerning flow conditions, the first transient adhesion of leucocytes to endothelial cells during inflammation, the so-called rolling, is mainly mediated by E-selectin.[36] According to the current view, similar mechanisms take place during extravasation of tumour cells in the course of metastasis.[19] We therefore analysed the adhesion of Fra-2 transfected cells in a laminar flow adhesion assay using E-selectin coated capillaries and physiological shear forces. In mock-transfected controls, rolling was only observed in MCF7, but not in MDA-MB231 cells, which corresponds to prior results of Tözeren et al. [37] who studied rolling on activated endothelial cell layers. In their investigation, rolling of MCF7 cells could be blocked by anti-E-selectin antibodies which provide evidence that E-selectin is indispensible for adhesion to activated endothelia. In our study, the number of rolling cells on E-selectin coated surfaces was strongly increased in Fra-2 transfectants both in MCF7 and, especially, MDA-MB231 cells, which probably reflects a higher capacity of extravasation and metastasis *in vivo*.

The question which of the adhesion molecules causes this adhesive effect cannot be answered to date. The problem concerning the interpretation of the results is that E-selectin binds to carbohydrate structures on glycoproteins. Thus, oligosaccharide residues on the overexpressed cell adhesion molecules must be the mediators of this effect. One obvious candiate would be CD44, which has been shown to bind to E-selectin in its variant 4[38] indicating that carbohydrate residues covalently attached to it mediate this interaction. However, this molecule was only minimally or not up-regulated in Fra-2 transfectants which argues against its involvement in our system. Another candidate, ICAM-1, which is strongly up-regulated by Fra-2 was not involved in E-selectin-dependent diapedesis of colon cancer cells in another experimental system.[19] Interestingly, L1-CAM was recently implicated in trans-endothelial migration of dendritic cells, although probably through homophilic interactions.[39] As the natural E-selectin ligands are still not fully understood, further experiments are needed to elucidate this enigma.

In conclusion, our data obtained on breast cancer cell lines and clinical tissue samples suggest that overexpression of the transcription factor Fra-2 promotes breast cancer progression by deregulation of genes involved in cell–cell and cell–ECM contacts. Down-regulation of molecules which mediate cell-cell contacts within gap junctions or desmosomes and up-regulation of adhesion molecules involved in extravasation of tumour cells might facilitate tumour spread and metastasis *in vivo*, leading to a significantly shorter survival of the patients.

Conflict of interest statement

Ralph M. Wirtz is employed at Siemens Healthcare Diagnostics Products GmbH, Cologne, Germany. For the other authors, there are no conflicting interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.02.008.

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