**Fra-1 Promotes Breast Cancer Chemosensitivity by Driving Cancer Stem Cells from Dormancy**

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**Abstract**

Fra-1 is a member of the Fos transcription factor family that is highly expressed in multiple cancers, playing important roles in transformation, proliferation, and metastasis. In this study, we observed an inverse correlation between the expression of Fra-1 in human stage II breast cancer tissues and the corresponding level of clinical chemoresistance. Extending these findings *in vitro*, we found that knockdown of Fra-1 in breast tumor cells was sufficient to confer resistance to doxorubicin and cyclophosphamide, whereas enhanced Fra-1 expression could render these cells chemosensitive. The tumor cell side population, which is enriched for cancer stem cells, was found to be associated with chemoresistance. Increased side population fractions were detected among tumor cell lines subjected to Fra-1 knockdown. In contrast, enhanced expression of Fra-1 was correlated with a decreased side population fraction, and significantly, this finding was recapitulated *in vivo*, where tumors with enhanced expression of Fra-1 were found to have blunted growth. Tumor cells subjected to Fra-1 knockdown grew faster and were larger in size. Taken together, our findings suggest that Fra-1 may be an important prognostic marker for breast cancer therapy.

**Introduction**

Chemotherapy has become a routine therapeutic approach for the treatment of cancer, with significant impact on patient survival. Nonetheless, many unsolved issues remain that lead to dose-limiting toxicities of chemotherapy. Among these, cancer relapse and emerging drug resistance remain key factors in determining chemotherapy efficacy.

The cancer stem cell hypothesis suggests that a small population of cells within a tumor will tend to share some common features with stem or progenitor cells, including self-renewal and differentiation. These cancer stem cells (CSC) are thought to be responsible not only for primary tumorigenesis, but also for resistance to chemotherapy and subsequent cancer recurrence. A variety of mechanisms have been proposed to contribute to CSC chemoresistance, including relative quiescence, expression of ATP-binding cassette (ABC) transporters and/or multidrug resistance transporter 1 (MDR1), a more robust DNA repair capability, and the elevated expression of antiapoptotic proteins (1). All of these natural characteristics combine to make CSCs a particularly challenging target for chemotherapy (2, 3).

The Fos family transcription factor Fra-1 has weak transforming activity, due largely to its lack of potent transactivation domains. Fra-1 typically heterodimerizes with Jun family members (c-Jun, JunB, or JunD) to form the activator protein (AP-1) transcription factor complex. Initially identified as an immediate early transcriptional response element following exposure to serum (4), Fra-1 was later found to exhibit transforming activity in rat fibroblasts (5) and thyroid cells (6). Recent studies suggest Fra-1 to be involved in tumorigenesis and cancer progression, with elevated Fra-1 expression detected in breast (7), lung (8), brain (9), colon (10) and prostate cancers (11). Functionally, Fra-1 expression promoted tumor cell proliferation, inhibited apoptosis, and increased cell invasion (12).

Here, we examined Fra-1 for its role in breast cancer progression via its potential effect on CSCs. Unexpectedly, we found that immunohistochemical staining of human stage II breast cancer tissues supported a significant correlation between the expression of Fra-1 and patients’ responses to chemotherapy and outcomes. When we directly tested the function of Fra-1 in breast CSCs, we found that suppression of Fra-1 expression correlated with both an increase in tumor CSCs and a concurrent increase in chemoresistance, whereas ectopic Fra-1 expression correlated with decreased incidence of CSCs and increased chemosensitivity of murine breast cancer cells. Together, these results suggest a novel role for Fra-1 in cancer biology, and raise the possibility that Fra-1 may be a significant prognostic response marker for tumor therapy.
Materials and Methods

Animals, cell lines, and tissue samples
Female BALB/c mice, 6 to 8 weeks of age, were purchased from The Scripps Research Institute Rodent Breeding Facility, La Jolla, CA. All animal experiments and protocols were carried out according to the NIH Guide for the Care and Use of Laboratory Animals and approved by The Scripps Research Institute Animal Care Committee. Murine breast cancer cell line 4TO7 was kindly provided by Suzanne Ostrand-Rosenberg (University of Maryland, College Park, MD). Human 293T cells were a gift from Wen-yuan Hu (Biosettia). A total of 63 stage II breast cancer samples were collected at the Cancer Institute and Hospital of Tianjin Medical University after informed consents had been obtained from all patients.

Lentiviral transduction systems
Murine Fra-1 cDNA was generated by PCR-based amplification with the following primer set: forward primer: 5′-GCCAGGAGTGTAGGAGAGCCCAG-3′; reverse primer: 5′-GGGCCGGATCCTCACAAA-GCCGAGGATGTAAGAGGACC-3′, reverse primer: 5′-GGGC GGATCCTCACAAA-GCCGAGGATGTAAGAGGACC-3′, and cloned into XbaI and BamHI restriction sites of pLV-H1-EF1-MCS-IRES-Bsd expression vector (Biosettia). Three helper plasmids pMDLg/pRRE, pRSV-REV, and pCMV-VSV-G were kindly provided by Wen-yuan Hu (Biosettia). Three helper plasmids pMDLg/pRRE, pRSV-REV, and pCMV-VSV-G were kindly provided by Wen-yuan Hu (Biosettia). Short hairpin RNAs (shRNA) of Fra-1 were inserted into pLV-H1-EF1-puro vector (Biosettia). Sequences of the hairpins and the scramble control vector are the following:

shRNA1:
AAAAGTTCCACCTTGCCAAGCATTTTGGATCCAAAATTGCCACAGGGTGAAC

shRNA2:
AAAAGAAAGGAGCTGACAGACTTCTTGGATCCAAGAAGTTGCT

Scr-shRNA:
AAAAGCTACACTATCGAGCAATTTTGGATCCAAAATTGCCACAGGGTGAAC

Lentiviruses were generated from 293T cells according to the protocol of Single Oligonucleotide RNAi Technology for Gene Silencing (Biosettia). Stable 4TO7 cells were selected by blasticidin (Invitrogen) or puromycin (Sigma-Aldrich) 48 hours after lentiviral transduction.

HOECHST 33342 dye exclusion assay
Cells (1 × 10⁶/mL) were stained with 10 μg/mL HOECHST 33342 (Sigma-Aldrich) with or without verapamil hydrochloride (50 μmol/L, Sigma-Aldrich). Cells were incubated at 37°C for 1 hour as described previously (13).

Cell-cycle analysis
Cells (1 × 10⁶/mL) were fixed with 70% cold ethanol for 1 hour, then incubated in ice-cold PBS containing 50 μg RNase A (QIAGEN) at 37°C for 1 hour. Cells were treated with 10 μg of propidium iodide (PI; 10 μg/mL, Molecular Probes), incubated at 4°C overnight, and data collected on the next day.

Protein expression analysis
Protein expression of Fra-1 was showed by immunoblotting using antibodies (Abs) from Santa Cruz (anti-murine Fra-1, anti-murine β-actin, goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP). To detect expression of Sca-1, 10⁶ cells were harvested and incubated for 1 hour at 4°C in 100 μL of ice-cold fluorescence-activated cell-sorting (FACS) buffer with fluorescein isothiocyanate (FITC)-conjugated anti-Sca-1 Ab (BD Pharmingen). FITC conjugated anti-rat IgG (eBioscience) was used as an isotype control. Intracellular expression of Ki-67 was measured by FACS: 10⁶ cells were fixed and permeabilized following manufacturer’s instructions (eBioscience) and anti-murine Fra-1 Ab (Santa Cruz), FITC-conjugated goat anti-rabbit IgG Ab (Southern Biotech) and FITC-conjugated anti-Ki-67 Ab (Abcam) were used for staining. For histology, paraffin-embedded human tissue samples were stained with Fra-1 Ab (Santa Cruz).

Doxorubicin extinction assay
Cells were seeded at 1.5 × 10⁵/well in a 12-well plate 1 day before doxorubicin treatment (Sigma-Aldrich). Doxorubicin (1.5 μg/mL) was added to each well at different time points (0.5, 1, 2, 4, 6, 8, 20, and 24 hours). The pumping abilities of the cells were measured by FACS.

Analysis of apoptosis
4TO7 tumor cells (3 × 10⁵) were seeded in a 6-well plate and cultured for 24 hours before drug treatment. Cyclophosphamide (500 μmol/L, Sigma-Aldrich) was added into each well on the following day and maintained for 24 hours before Annexin V and PI double staining (BD Pharmingen).

Tumor cell challenge and cyclophosphamide treatment
BALB/c mice (n = 4/group) were divided into 4 experimental groups. A total of 5 × 10⁶ 4TO7 cells with Fra-1 extinction, Fra-1 overexpression, or control vectors were injected s.c. to the left front flank of mice on day 0. From day 3 to day 11, cyclophosphamide was administered to all groups of mice by i.v. injection (30 mg/kg) for a total of 5 times at 2 day intervals. Tumor dimensions were measured in 2 dimensions with microcalipers every other day and tumor volume was calculated as previously described (14).

Statistics
A statistical comparison of experimental groups and controls was determined by Student t test. Findings were regarding as significant if 2-tailed P value were less than 0.05.

Results and Discussion
We evaluated the expression of transcription factor Fra-1 within a panel of 63 paraffin-embedded tissue samples from patients with stage II breast cancer who received chemotherapy following tumor resection by immunohistochemistry. Results from immunohistochemical staining were scored as negative (no Fra-1 expression), low (<75% Fra-1-positive cells,
weak staining), and high (>75% Fra-1-positive cells, strong staining; Fig. 1A–C). We expected to find a correlation between increased Fra-1 expression and disease progression (7). Surprisingly, among the cohort of patients with rapid cancer recurrence (<3 years), there was little staining of Fra-1, with 12 of 20 patients exhibiting no detectable expression, and 8 revealing weak expression. In contrast, each of the tissues derived from patients who failed to recur for 5 or more years, expressed Fra-1, with 9 of 22 patients showing strong expression. Patients whose disease recurred between 3 and 5 years exhibited an intermediate phenotype: 1 of 21 tissues had no detectable Fra-1, whereas the remaining 20 exhibited weak Fra-1 expression (Fig. 1D). This trend was highly significant ($P < 0.001$), suggesting that high Fra-1 expression may indicate a delayed tumor relapse after chemotherapy. Conversely, the complete absence of Fra-1 would seem prognostic for rapid relapse. This correlation between chemosensitivity and Fra-1 expression was further confirmed in a panel of human breast cancer cell lines (Supplementary Fig. S1A and S1B).

To examine the impact of Fra-1 on breast cancer growth and cell-cycle regulation, we generated stable murine breast tumor sister cell lines with either enhanced or suppressed expression of Fra-1. Fra-1 expression was significantly increased in 4TO7-Fra-1 cells, whereas Fra-1 expression was decreased by 2 independent shRNAs, which created the 4TO7-sh1 and 4TO7-sh2 cell lines. An examination of growth and proliferation of these cells via Ki-67 staining (15), indicated that 4TO7-sh1 and particularly 4TO7-sh2 cells revealed a significantly decreased expression of Ki-67 relative to scrambled controls (Supplementary Fig. S2A; $P = 0.12; { }^{*}, P<0.05$). However, Ki-67 expression was increased in 4TO7-Fra-1 cells, which exhibited enhanced Fra-1 expression (Supplementary Fig. S2B; *, $P<0.05$). During further evaluation, we assessed cell-cycle progression as a function of Fra-1 expression by FACS analysis.

Staining of nuclear DNA with PI showed that suppression of Fra-1 resulted in an increased fraction of cells in the G0–G1 population, with a concurrent decrease in incidence of cells in S- and G2–M phases (Supplementary Fig. S3A). Conversely, ectopic expression of Fra-1 correlated with a decrease in the G0–G1 population and increase in S- and G2–M fractions relative to controls (Supplementary Fig. S3B). Together, these data support prior conclusions by others (6) that Fra-1 plays a positive role in regulating breast cancer proliferation in vitro, yet was paradoxically associated with better clinical prognosis in our study.

This paradox might, however, be resolved when one considers both the relative susceptibility of highly proliferative populations to chemotherapeutic therapies currently in clinical use. For example, slowly proliferating tumor cell populations may represent a somewhat enhanced significant risk for more rapid breast cancer relapse. Accordingly, resistance to chemotherapeutic drugs is a characteristic associated with among other factors, an incidence of CSCs.

To explore this, we further conducted Hoechst 33342 exclusion technique to identify populations enriched in CSCs in different 4TO7 cell lines via side population gating protocols (16). Our findings showed a dramatic increase in the CSC-enriched population following suppression of Fra-1 expression, which was dose-dependent according to relative Fra-1 depletion (Fig. 2A and B), whereas increased expression of Fra-1 depleted these CSCs (Fig. 2C and D). To confirm that gating of our side population indeed identified a CSC population, we costained for expression of Sca-1, a CSCs marker (17–19), and found it highly expressed in the gated cells (Fig. 3A). Accordingly, increased staining of Sca-1 was observed in cells lacking Fra-1 expression, whereas decreased expression of Sca-1 was found in cells with enhanced Fra-1 expression (Fig. 3B). Together, these data revealed a strong negative correlation between the expression of Fra-1 and the relative proportion of CSCs, raising the interesting notion that Fra-1 may promote loss of CSC dormancy, and thereby enhance chemosensitivity.

To verify this contention, we first tested doxorubicin uptake as a function of Fra-1 expression. To this end, cells were treated with doxorubicin (1.5 $\mu$g/ml) at different time points (0.5, 1, 2, 4, 6, 8, 20, and 24 hours) and the retention of doxorubicin assessed by quantitation of fluorescence.

Figure 1. Immunohistochemical staining of Fra-1 was carried out in paraffin-embedded tissues from human patients with stage II breast cancer. Fra-1 staining score was defined as negative expression (A), no detectable Fra-1; low expression (B), <75% Fra-1–positive cells and weak staining; high expression (C), >75% Fra-1–positive cells and strong staining. Scale bar, 200 $\mu$m. D, a summary of all immunohistochemical results, indicating a significant association of Fra-1 expression with chemosensitivity ($P < 0.001$).

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Between 8 and 24 hours, a dramatic reduction in retention of doxorubicin was observed in 4TO7-sh2 cells with suppressed Fra-1 expression relative to controls (Fig. 4A, left; **, \( P < 0.01 \)). In contrast, at 20 and 24 hours, increased doxorubicin was evident within 4TO7 cells with enhanced Fra-1 expression (Fig. 4A, right; **, \( P < 0.01 \)). Together, these results indicate that Fra-1 expression may be associated with different sensitivities to chemotherapeutic agents.

Figure 2. Fra-1 expression modulates the proportion of side population cells by Hoechst 33342 dye exclusion assay. A and C, FACS analysis of side population cells stained with Hoechst 33342 dye in the absence (top) or presence (bottom) of the Ca\(^{2+}\) channel blocker verapamil to confirm specificity. B and D, percentage of side population cells was evaluated by 3 separate measurements. Error bars represent mean ± SD, #, \( P = 0.082 \); *, \( P < 0.05 \), versus control group. SP, side population.

Figure 3. Fra-1 expression modulates the proportion of side population cells by Sca-1 staining. A, Sca-1 was highly expressed in side population (SP) cells compared with nonside population (NSP) cells. B, expression of Sca-1 was detected by FACS in 4TO7 cells with varying Fra-1 expression. A typical experiment (of 3) is depicted.
To further extend this result, we evaluated a second commonly used breast cancer therapeutic drug, cyclophosphamide, in our breast tumor sister cell lines. As might be predicted from prior studies of doxorubicin, a remarkably decreased number of cell deaths was observed in 4TO7 cells with Fra-1 suppression (A, left) or ectopic expression (A, right). Apoptosis of 4TO7 cells with Fra-1 extinction (B, left) and ectopic expression (B, right) was evaluated by Annexin V and PI double staining. Cells were treated with cyclophosphamide (500 μmol/L) for 24 hours before FACS analysis. Three independent results were calculated and shown as bar graphs. C, scheme depicts the in vivo experimental approach: 5 × 10^5 4TO7 cells, with varying Fra-1 status, were injected s.c. in the left front flank of female BALB/c mice (n = 4/group) on day 0. All mice were treated with CTX (30 mg/kg) by i.v. injection (day 3–11). D, when 4TO7 tumor growth became visible, tumor volume was monitored and results were graphically displayed. Scale bar, 100 μm. Error bars represent mean ± SD. *, P < 0.05; **, P < 0.01, versus control group.

To determine whether the effect of Fra-1 on chemosensitivity in vitro also extended to tumors growing in vivo, we injected syngeneic female BALB/c mice with 5 × 10^5 4TO7 cells and then evaluated their response to cyclophosphamide treatment. Starting on day 3, and every 2 days thereafter, mice were treated i.v. with cyclophosphamide (30 mg/kg) and tumor sizes tracked. Mice were sacrificed on day 23 and tumors were resected and weighted (Fig. 4C). Following termination of chemotherapy, the 4TO7-sh2 group of mice exhibited enhanced tumor growth relative to control group (Fig. 4D, left; *, P < 0.05). In fact, among 4TO7 cells with enhanced Fra-1 expression, chemotherapy had a significant impact on early tumor development. While these tumors did continue to grow, they propagated at a markedly reduced rate (Fig. 4D, right; ***, P < 0.01). These results also correlated with significant differences in final tumor burden (Supplementary Fig. S4A and S4B) of different Fra-1 expression mice (Supplementary Fig. S4C). Importantly, these in vivo data not only confirmed our in vitro observations of the impact of Fra-1 on chemosensitivity, but also suggested that Fra-1 could become a valuable clinical marker of chemosensitivity.
Together, our analysis of human clinical breast cancer tissues and our preclinical investigations show an unexpected but important finding indicating that patients with high Fra-1 expression are more likely to present with a delay in tumor relapse following chemotherapy. This is surprising because initial studies attributed an oncogenic function of Fra-1 in multiple solid tumors including breast tumor (7–11). We also reported that Fra-1 played a crucial role in modification and maintenance of the malignant phenotype of tumor-associated macrophages, which promoted breast cancer cell survival and invasiveness (20). Thus immune therapies were targeted to Fra-1 in mice (21, 22). In contrast, our current findings reveal that patients with breast cancer lacking Fra-1 are more likely to undergo a rapid tumor relapse after chemotherapy, which may make Fra-1 a valuable prognostic marker for sustained tumor chemosensitivity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Lu, X. Tan, Z. Liu, R. Xiang
Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): D. Lu, Z. Li, D.G. Stupack, R. Xiang
Writing, review, and/or revision of the manuscript: D. Lu, D.G. Stupack, R.A. Reisfeld, R. Xiang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Lu, Z. Liu, R. Xiang
Study supervision: N. Li, R. Xiang

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