Microenvironmental Regulation of BRCA1 Gene Expression by c-Jun and Fra2 in Premalignant Human Ovarian Surface Epithelial Cells

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Abstract

Reduced BRCA1 gene expression is common in the sporadic form of ovarian carcinoma. The spread of this highly lethal cancer often begins when tumor cell clusters are shed into the fluid of the abdominopelvic cavity such that they can float freely before seeding distant sites on the peritoneal walls and organs. Thus, the microenvironment that tumor cells find themselves in changes dramatically during these early shedding and floating stages of transperitoneal metastasis. To mimic this microenvironmental change in vitro, we released premalignant human ovarian surface epithelial cells from the substratum and forced them to cluster in suspension. Under these conditions, steady state levels of BRCA1 mRNA and protein fell significantly and the transcriptional activation state of the BRCA1 promoter was suppressed. Analysis of the promoter indicated that the previously identified “CRE” element located within the “positive regulatory region” (PRR) contributed to this suppression. More specifically, we show that the suppression was mediated, at least in part, by a suspension culture–driven decrease in the levels of two members of the AP1 transcription factor complex, c-Jun and Fra2, that bind to the CRE element. Therefore, a microenvironmental change that is manifested during the initial stages of ovarian carcinoma dissemination may, potentially, help suppress BRCA1 expression in sporadic tumors and thus promote their progression. Mol Cancer Res; 11(3): 1–10. ©2013 AACR.

Introduction

Epithelial ovarian carcinoma is the leading cause of death due to gynecologic malignancy (1). The great majority of these deaths occur in women who present with high-grade serous subtype tumors that metastasize by spreading transperitoneally through the abdominopelvic cavity. High-grade serous subtype tumors are also notable because they also have the highest rate of BRCA1 abnormalities, a considerable proportion of which occur due to epigenetic silencing rather than mutation (2). Given BRCA1’s myriad roles in acting to maintain genomic stability and differentiation, determining how its expression is silenced during the development of high-grade ovarian tumors is an important goal.

BRCA1 expression is tightly regulated through its promoter, which is located within a 218 bp intragenic region between the bidirectionally controlled BRCA1 and NBR2 genes (3). A strong positive regulatory region (PRR), which consists of 2 subregions, lies within a 56 bp sequence from nucleotide −204 to −148 relative to the BRCA1 start site (4, 5). The first upstream subregion in the PRR is the “RIBS” element (−204 nt to −182 nt), which binds to, and is activated by, a complex of GABPT and GABPβ transcription factors of the ETS family (6). The second subregion is the CRE element located just downstream (−174 nt to −167 nt), which binds to, and is activated by, a complex of c-Jun and Fra2 transcription factors of the AP1 complex (3, 7). To date, the transcriptional regulation of the BRCA1 promoter has been most thoroughly characterized in breast carcinoma cells (8). However, we have shown that the CRE element is active in ovarian carcinoma cells (3).

It has long been assumed that ovarian carcinomas arise within the ovarian surface epithelium (OSE; ref. 9). However, recent findings suggest that some ovarian carcinomas may arise in extraovarian tissues (10). In the case of high-grade serous subtype tumors specifically, there is now considerable evidence that these lesions often arise in lining epithelium of the distal portion of the fallopian tubes, which is very closely related, developmentally, to the OSE (11). The fallopian tube origin of high-grade serous tumors has
many implications for clinical management, including changes to surgical intervention protocols for women who are BRCA1 mutation carriers who choose prophylactic early lesion removal before frank tumor diagnosis (12). In addition, it indicates that the cells of frank high-grade serous tumors have likely already been shed into the fluid of the peritoneal cavity and then later attach to either to the ovarian surface or the abdominopelvic wall where they are most often first discovered (13). Given that altered interactions with extracellular matrix (ECM) substrata have been shown to alter BRCA1 expression in mammary epithelial cells (14, 15), we reasoned that detaching premalignant ovarian cells from the substratum, in an effort to mimic their initial shedding and floating in the peritoneal fluid, might also affect BRCA1 expression.

Premalignant, immortal human OSE cells were cultured either as attached, flat monolayers, or as detached cell clusters in suspension. Under the latter conditions, steady-state BRCA1 mRNA and protein levels were significantly reduced. Furthermore, the transcriptional activity of the BRCA1 promoter was suppressed in suspension and this suppression was mediated, at least in part, by the decreased expression of c-Jun and Fra2, both of which bind to the CRE element within the promoter. This suggests that the microenvironmental loss of cell–substratum adhesion that occurs during the earliest stages of transperitoneal spread may help facilitate the decrease in endogenous BRCA1 expression that has been noted in a significant proportion of high-grade serous ovarian tumors.

Materials and Methods

Cell culture

Extended lifespan human OSE IOSE-80 cells, which have escaped normal growth controls were generated when cells were maintained in 199:105 medium (1:1; National Cancer Institute, Bethesda, MD; ref. 21). The L6 proximal promoter region of BRCA1 was excised from the L6-pRL construct (7) with BgII and HindIII and ligated directly into the pGL4.11 (Luc2P; Promega) basic, minimal promoter vector containing the firefly luciferase reporter. To generate the BRCA1 promoter mutants used here, the respective DNA strands were synthesized, annealed, and ligated into pGL4.11 as previously described (7, 19). For the PRR construct, 2 synthetic primers (5'-GATCTTTCTTCCTCCTGGTCTTCTTCCTTTTA-3' and 5'-AGCTTGT-CTGCCCCGGGGGCAGACA-3') and 5'-AGCTTTGT-CTGCCCCGGGGGCAGACA-3') were designed to include the BRCA1 promoter region from −201 to −155 and to contain adapters of the BgII and HindIII enzymes for annealing and ligation. The "RIBSm" (previously described as "CREs") and "CREm" mutants shown (see Fig. 2) were then generated by site-directed mutagenesis as previously described (6, 7). All promoter/reporter constructs were confirmed by sequencing.

His-tagged c-Jun- and Fra2-containing expression vectors were gifts from Dr. Irina A. Udalova (Imperial College, London; ref. 20). The dominant negative AP-1 mutant "A-fos" expression vector was a gift from Dr. Charles Vinson (National Cancer Institute, Bethesda, MD; ref. 21).

Real-time quantitative PCR

Total RNA was extracted from IOSE-80pc cells maintained in either monolayer or suspension culture. CDNA was then synthesized using a high-capacity cDNA archive kit (Applied Biosystems) and real-time PCR (RT-PCR) analysis was conducted using 20 ng of template in 15 μL reactions containing TaqMan universal PCR master mix as well as BRCA1 primers and probe (HS00173237; Applied Biosystems). 18s rRNA was simultaneously quantified as the internal control. Amplification was conducted in an ABI prism 7500 HT sequence detector using standard settings of 40 cycles with an annealing temperature of 60°C. All samples were assayed in 3 parallel reactions, and 3 independent experiments were carried out with similar results.

Antibodies

The mouse monoclonal anti-BRCA1 antibody (Ab-1) was obtained from Oncogene. Anti-c-Jun (H-79), anti-Fra2 (Q-20), anti-GABPs (H-180), anti-GABPβ (H-265), anti-SP1, and anti-extracellular signal–regulated kinase (ERK)1/2 antibodies were all from Santa Cruz Biotechnology. The anti-His-tag and anti-Flag-tag antibodies were obtained from Applied Biological Materials Inc. and Sigma, respectively. Alexa-Fluor–conjugated secondary antibodies for Western blotting were from Jackson Immuno-Research. Peroxidase-conjugated secondary antibodies for Western blotting were from Molecular Probes (Life Technologies). Real-time quantitative PCR was conducted in an ABI prism 7500 HT sequence detector using standard settings of molecular primers and probes.

Transgenic transfection and luciferase reporter assays

Cells in monolayer culture were transfected using lipofectamine (Life Technologies) with 0.5 μg of BRCA1-
promoter/firefly luciferase reporter constructs together with 0.01 μg of pGL4.73 SV40 promoter/Renilla luciferase reporter construct. The latter reporter served as an internal control for transfection efficiency given that it the SV-40 promoter was not affected by the changes in culture condition. Alternatively, both reporter constructs were cotransfected into cells in monolayer culture with either 0.25 μg each of the expression vectors for wild-type c-Jun and Fra2 together, or 0.50 μg of the A-Fos dominant negative AP1 mutant alone. Twenty-four hours after transfection, the cells were dissociated and cultured for a further 48 hours as either flat monolayers or as cell clusters in suspension. Firefly and Renilla luciferase activities were then determined using a commercial dual luciferase assay kit (Promega). Graphical and multiple t test statistical analyses were conducted using GraphPad Prism6 (GraphPad). A P value of less than 0.05 was considered statistically significant.

c-Jun- and Fra2-specific, as well as random control, siRNAs were purchased from Santa Cruz Biotechnology. Cell monolayers were transfected with siRNAs using lipofectamine RNAiMAX (Life Technologies) with a total of 25 nmol/L siRNA (12.5 nmol/L c-Jun siRNA plus 12.5 nmol/L Fra2 siRNA; or 25 nmol/L total of random siRNAs). After 24 hours, cells were dissociated and were then maintained in either monolayer or suspension culture for a further 48 hours before analysis by Western blotting.

Western blotting

Cells were lysed on ice in radioimmunoprecipitation assay (RIPA) buffer [159 mmol/L NaCl, 50 mmol/L Tris–HCl, pH 7.5, 1% NP-40, 1% deoxycholate, 0.1% SDS, 2 mmol/L EDTA, plus 1 μmol/L/L pepstatin, 10 μg/mL aprotinin, 10 mmol/L phenylmethylsulfonylfluoride (PMSF), and 2 μg/mL leupeptin], sonicated to generate whole-cell lysates, and immunostained with antibodies against the His-tag of the transgenes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) and images were collected using an Olympus FV100 confocal microscope.

EdU proliferation assay

Cells monolayers transfected with His-tagged c-Jun and Fra2 expression vectors were maintained for 24 hours followed by dissociation and maintenance in either monolayer or suspension culture for a further 48 hours. The cells were then incubated for 2 hours with 20 μmol/L 5-ethyl-2'-deoxyuridine (EdU; Invitrogen), which fluorescently labels proliferating cells in S-phase of the cell cycle. Fluorescent, proliferating cells were then quantified using a FACS LSRII flow cytometer (BD Biosciences).

Chromatin immunoprecipitation

Cells were plated at a density of 2 × 10⁵ cells/mL on normal 100-mm tissue culture plates and maintained as flat monolayers for 48 hours. The chromatin immunoprecipitation (ChIP) analysis was conducted according to manufacturer’s instructions for the ChIP-IT Express Kit (Active Motif). This included enzymatic digestion of the chromatin and the magnetic immunoprecipitation of the antibody complexes. Antibodies used were directed against acetylated histone H3 lysine K9 (Abcam) as well as hemagglutinin, GABPα, Fra-2, and c-Jun, all of which were from Santa Cruz.

The PCR reaction was carried out as previously described (22) after adaptation from Bindra and colleagues (23) using the following primers: PF2 5'-GATTGGGACCTCTCTCTCTACREOmut 5'-AGTCTGCCCCGGCAGACT-3'; pR2 5'-TACCCAGAGCAAGGTTGA-3'.

The PCR conditions were 1 × Thermopop buffer, 1 mol/L betaine, 200 μmol/L dNTPs, 250 μg each oligonucleotide in a 25 μL reaction with 0.5 units of Taq polymerase. The reactions conditions were 95°C for 3 minutes, 38 cycles of 95°C, 30 seconds, 60°C, 30 seconds; 72°C, 30 seconds, followed by 72°C for 4 minutes.

Electrophoretic mobility shift assay

Nuclear extracts from cells maintained either as flat monolayers or as clusters in suspension for 48 hours were prepared as previously described (6), with the exception that nuclear proteins were not concentrated by (NH₄)₂SO₄ precipitation, but instead were dialyzed against 10 mmol/L HEPES pH 7.6, 40 mmol/L KCl, 0.1 mmol/L EDTA, and 10% glycerol. Nuclear extracts were then combined with 32P-labeled BRCREO oligonucleotide plus strand 5'-CTTCTCTTCTCTACGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3' or with CREOmut 5'-CTTTCTCTTCTTAAATCAGTCATCCGGGGGCAGACT-3' minus strand 5'-AGTCTGGCCCCGGCAGATGAAAGG-3'.

The binding reactions were conducted for 30 minutes on ice before the addition of 32P-labeled oligonucleotide in binding buffer.

Immunofluorescence

Cell monolayers were transfected with His-tagged c-Jun and Fra2 expression vectors, maintained for 48 hours, fixed in cold methanol, and immunostained with antibodies against the His-tag of the transgenes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) and images were collected using an Olympus FV100 confocal microscope.
BRCA1 expression is suppressed in premalignant OSE cells maintained in suspension culture

Human OSE cells, which are mesodermally derived, do not express E-cadherin. Thus, OSE cells tend to become fibroblastic when they are explanted into monolayer culture (17). Cells of the immortal, premalignant IOSE-80pc line took on this fibroblastic appearance when they were maintained as monolayers on tissue culture plastic (Fig. 1A, Mono). In contrast, when IOSE-80pc cells were maintained in suspension they rounded-up and formed loosely adherent clusters of cells (Fig. 1A, Susp) that are not dissimilar in appearance to the clusters that ovarian carcinoma cells form after they have been shed into the abdominopelvic cavity during the initial stages of transperitoneal spread (24). Interestingly, steady-state BRCA1 mRNA and protein levels were both reduced considerably when the cells were maintained in suspension culture (Fig. 1B and C).

To determine if soluble factors released by IOSE-80pc cells contribute to the differences in BRCA1 expression described earlier, we cultured them in media preconditioned by cells maintained either in suspension or in monolayers (“Media”) and BRCA1 protein levels were assessed by Western blotting. Note that conditioned media did not appreciably alter BRCA1 levels of cells in either monolayer or suspension culture conditions. E, cells were maintained in suspension culture for 48 hours and one half of the cells were then lysed; the remaining cells were replated as monolayers for a further 48 hours and lysed. Both lysates were then subjected to Western blotting. Note that the suppression of BRCA1 was reversed when the cells were replated in monolayer culture.

Figure 1. BRCA1 levels are reduced in suspension culture. A, in monolayer culture, premalignant IOSE-80pc cells were fibroblastic, as expected given their mesodermal derivation. In suspension culture, the cells floated as small, rounded cohesive clusters. B, quantitative RT-PCR indicated that steady-state BRCA1 mRNA levels were reduced in cells maintained suspension culture compared with monolayer culture. C, Western blotting indicated that steady-state BRCA1 protein levels were reduced in cells maintained suspension culture; ERK1/2 levels remained unchanged, and therefore were used as loading controls for all subsequent Western blotting in this study. D, cells maintained in either monolayer or suspension culture conditions (“Culture”) were incubated in media previously conditioned by cells maintained either in suspension or in monolayers (“Media”) and BRCA1 protein levels were assessed by Western blotting. Note that conditioned media did not appreciably alter BRCA1 levels of cells in either monolayer or suspension culture conditions. E, cells were maintained in suspension culture for 48 hours and one half of the cells were then lysed; the remaining cells were replated as monolayers for a further 48 hours and lysed. Both lysates were then subjected to Western blotting. Note that the suppression of BRCA1 was reversed when the cells were replated in monolayer culture.
Transactivation of a PRR within the BRCA1 promoter is suppressed in suspension culture

As the steady state levels of BRCA1 mRNA as well as protein were suppressed in IOSE-80pc cells maintained in suspension, we next assessed the effect of this culture condition on the transactivation state of the BRCA1 promoter.

The BRCA1 promoter, which acts bidirectionally, is located on the long arm of chromosome 17 between the NBR2 and BRCA1 genes (Fig. 2A). Previously, we determined that a 231 bp form of the promoter (i.e., the "L6" promoter, −204nt to +27nt) contains a very strong PRR (i.e., the PRR fragment, −204nt to −155nt) that is maximally active in both ovarian carcinoma and immortalized OSE (IOSE) cells (3). Importantly, the ability of both the L6 promoter and the PRR fragment to transactivate and drive reporter gene expression was reduced when IOSE-80 cells were maintained in suspension culture compared with when they were maintained in monolayer culture (Fig. 2B). While the suspension culture-mediated suppression of the entire L6 promoter was a trend, the decrease in the PRR was larger (i.e., approximately 3-fold) and statistically significant.

**Figure 2.** Transcriptional activation of the BRCA1 promoter is reduced in suspension culture. A, schematic representation and sequence of the BRCA1 promoter constructs. The L6 construct is indicated with a single line, which represents the full-length BRCA1 promoter located between start sites of the NBR2 and BRCA1 genes. The PRR region, located between −201 nt and −155 nt relative to the BRCA1 start site, contains 2 positive-acting elements, RIBS and CRE, which are indicated with open boxes. The "RIBSm" mutant fragment has the same sequence as PRR, but there are 2 previously characterized loss-of-function point mutations, as shown in the sequence of the upstream RIBS element. The "CREm" mutant fragment also has the same sequence as PRR, but there are loss-of-function point mutations in the sequence of the downstream CRE element (3). B, the promoter constructs shown were ligated into the pGL4.11(Luc2P) luciferase reporter vector, and transiently cotransfected into IOSE-80pc cells with the pGL4.73 (hRluc/SV40) vector as an internal transfection efficiency control. Cells were then maintained in either monolayer or suspension culture for 48 hours, lysed, and luciferase activity was assessed. In the left graph, relative L6 promoter activities were compared where the monolayer activity normalized as 100%. Alternatively, in the right graph, relative wild-type PRR, RIBSm, and CREm and promoterless "vector" activities were compared where the wtPRR monolayer activity was normalized as 100% (see Materials and Methods for transfection and assay details; n = 3, ±SD; *, P < 0.05; data shown are from one representative experiment of 3).
The PRR fragment contains 2 positively acting binding elements; the upstream RIBS element and the downstream CRE element (Fig. 2A). When the upstream "RIBS" element was disrupted by mutation (i.e., "RIBSm" in Fig. 2A), the PRR activity fell significantly in monolayer culture. However, the activity of this mutant, which had a wild-type CRE-element, was further reduced in the suspension culture (Fig. 2B). In contrast, when the downstream CRE-binding element was mutated (i.e., "CREm" in Fig. 2A), PRR activity was also greatly decreased in monolayer culture, but there was no further decrease in suspension culture (Fig. 2B). These data suggest that factors binding the positive-acting wild-type CRE element may be reduced in suspension culture.

The binding of c-Jun and Fra2 to the CRE element of the BRCA1 promoter is reduced in suspension culture

Previously, we determined that the RIBS element helps to activate the PRR fragment of the BRCA1 promoter by binding GABPα and GABPβ transcription factor subunits in breast and ovarian carcinoma cells in standard monolayer culture conditions. In contrast, the CRE element contributes to PRR activation by binding c-Jun and Fra2 members of the AP1 transcription factor complex in standard monolayer conditions (3, 6, 7). As such, we carried out ChIP assays and confirmed that GABPα ("Alpha"), c-Jun, and Fra2 all bound PRR-containing chromatin in IOSE-80pc cell monolayers (Fig. 3A). We were unable to assess GABPβ binding in this assay given that, in our hands, the commercially available antibody we used for Western blotting (see later) did not efficiently immunoprecipitate chromatin.

We next assessed the steady state levels of GABPα, GABPβ, c-Jun, and Fra2 proteins in IOSE-80pc cells maintained in the 2 different culture conditions. There was no decrease in the level of either GABPα or GABPβ in suspension culture (Fig. 3B). This observation, and the observation described earlier which indicated that mutating the GABPα/β-binding RIBS element (6) did not abrogate suspension-mediated suppression of the PRR, led us to conclude that changes in the levels of these factors are not likely critical regulators of this phenomenon. In contrast, the steady state levels of c-Jun and Fra2 were both reduced in suspension culture (Fig. 3C). This finding, and the finding described earlier, which indicated that mutating the c-Jun/Fra2–binding CRE element (7) abrogated suspension-mediated suppression of the PRR led us to tentatively conclude that the decrease in these factors may contribute to the suppression of BRCA1. This notion was supported by the data generated by electrophoretic mobility shift assays (EMSA) using the CRE as the probe. Specifically, supershifting with antibodies that recognized c-Jun and Fra2 indicated that complexes formed from nuclear extracts of cells maintained in monolayer culture and that these complexes were reduced when the cells were maintained in suspension (Fig. 3D). Given these findings, and the changes in promoter activity, we decided to next determine if manipulating c-Jun/Fra2 levels affected the suspension culture-mediated suppression of BRCA1 promoter activity.

**Figure 3.** The expression of c-Jun and Fra2, which bind to the CRE element of the BRCA1 promoter, are reduced in suspension culture. A, ChIP indicated that GABPα (Alpha), which interacts with the RIBS element of the PRR, as well as c-Jun and Fra-2, which interact with the CRE element of the PRR, all bind to the BRCA1 promoter under native conditions in IOSE-80pc cell monolayers. Histone H3 (H3) and USF-2 served as positive and negative binding controls, respectively. The absence of antibody (−Ab) and an irrelevant antibody against hemagglutinin (HA) served as technical controls. B, Western blotting of whole-cell lysates indicated that the steady state protein levels of GABPα and GABPβ, both of which bind to the RIBS element, did not decrease in cells maintained suspension culture (Susp) compared with cells maintained in monolayer culture (Mono). C, Western blotting of whole-cell lysates indicated that the levels of c-Jun and Fra2, which bind to the CRE element, both decreased when the cells were maintained in suspension. ERK1/2 served as a protein loading control. D, nuclear extracts from cells maintained in monolayer or suspension culture were used in EMSAs using either a radiolabeled "BRCREO" probe, which contains the entire CRE element with short flanking sequences, or a negative control CRE mutant (CREOmut), which contains the nonfunctional CREm point mutations shown in Fig. 2A. Assays of the wild-type probe were carried out in the absence (−) or the presence of antibodies against Fra2, and c-Jun as well as the Sp1 transcription factors that does not bind the CRE element (Ref. 7). Note that, in monolayer culture, the antibody free-control and the antibody against Sp1 both produced a weak, nonspecific doublet (marked by the asterisks), whereas the Fra2 and c-Jun antibodies both generated a supershift of a single species (arrowheads). In suspension culture only a slight Fra2 supershift remained which suggests that c-Jun and Fra2 binding were both decreased.

**Forced expression of c-Jun and Fra2 activates the BRCA1 promoter in suspension culture**

c-Jun and Fra2 are members of the AP1 family of transcription factors, and AP1 activity can be broadly suppressed by a dominant negative form of Fos that acts to block all AP1 activity (A-Fos mutant; ref. 20). Forced expression of the A-Fos mutant in IOSE-80pc cells decreased the transcriptional activation state of the entire L6 promoter, the PRR and the upstream RIBS mutant, but not the downstream CRE mutant (Supplementary Fig. S1). This finding led us to more specifically investigate the effects of manipulating c-Jun and Fra2 expression on the PRR when the cells were maintained in either monolayer or suspension culture.

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To test the effects of c-Jun and Fra2 specifically, we force expressed both transcription factors together, by dual transfection. In standard monolayer culture conditions, this greatly increased the activation of the L6 promoter and the wild-type form of the PRR fragment (Fig. 4A). Forced expression of c-Jun and Fra2 also increased the activation of the RIBS mutant, but not the CRE mutant, of the PRR (Fig. 4A). The latter result was expected given the fact that the former, but not the latter, still has an intact AP1-binding site for c-Jun and Fra2 (3, 7). Importantly, however, the same pattern of increased activation of the L6 promoter, the wtPRR region and the PRR/RIBS mutant, but not the PRR/CRE mutant, also occurred when the cells were maintained in suspension (Fig. 4B). This further supports the notion that the decrease in endogenous c-Jun and Fra2 levels that occurs in suspension culture may contribute to BRCA1 suppression.

We next assessed the effect of forced c-Jun and Fra2 on IOSE-80pc cell proliferation. In general, the cell spreading that occurs in monolayer culture facilitates proliferative growth factor signaling, a phenomenon that is curtailed as cells round and are released into suspension (25). IOSE-80pc cells fit this paradigm. Specifically, IOSE-80pc proliferation fell significantly when the cells were switched from being flat and spread in monolayer culture to rounded and clustered in suspension culture (Fig. 5). Importantly, while forced c-Jun/Fra2 expression increased proliferation modestly in both the monolayer and suspension culture conditions, the total percentage of proliferating cells remained much lower in the suspension condition. Therefore, the ability of forced c-Jun/Fra2 expression to robustly stimulate BRCA1 promoter transactivation in suspension culture was not likely caused by a general ability to stimulate proliferation back to the levels observed in monolayer culture.

**Figure 4.** Transient transfection of c-Jun and Fra2 increases BRCA1 promoter activity in monolayer and suspension culture. Cells were cotransfected with an empty pcDNA3 expression vector or pcDNA3 vectors containing His-tagged forms of c-Jun and Fra2, together with the promoter/luciferase reporters described in Fig. 2. Cotransfected cells were maintained in monolayer (A) or suspension (B) culture for 48 hours, lysed, and luciferase activity was assessed. The data were then normalized using either L6 promoter activity (left) or wild-type PRR activity (right) in the pcDNA3 expression vector control condition as 100% (see Materials and Methods for details; n = 3, ± SD; **, P < 0.005; data shown are from one representative experiment of 3).
c-Jun and Fra2 regulate endogenous BRCA1 levels in monolayer and suspension culture

When we force-expressed wild-type c-Jun and Fra2 in IOSE-80pc cells by cotransfection, the exogenous forms of the transcription factors were identified by Western blotting for the epitope tag (Fig. 6A, anti-His tag blots), and the overall increase for each could be observed by blotting specifically for either c-Jun or Fra2 in both monolayer and suspension culture (Fig. 6A, anti-c-Jun and Fra2 blots). This forced c-Jun/Fra2 expression increased endogenous BRCA1 protein levels modestly in monolayer cultures and strongly in suspension cultures (Fig. 6A).

To confirm that changes in c-Jun and Fra2 levels alter BRCA1 expression in both monolayer and suspension culture, we also knocked both transcription factors down coordinately using siRNA-mediated cotransfection (Fig. 6B). In response to this coordinated knockdown, BRCA1 protein levels were greatly reduced when the cells were maintained in monolayer culture and there was a further reduction in the already suppressed BRCA1 level when the cells were maintained in suspension culture (Fig. 6B). Therefore, we concluded that the decrease in endogenous c-Jun and Fra2 levels that occurs when IOSE-80pc cells are

Figure 5. Transient cotransfection of c-Jun and Fra2 does not appreciably increase proliferation in monolayer and suspension culture. A, cells transfected with either an empty expression vector or cotransfected with His epitope-tagged versions of c-Jun and Fra2 were immunostained with antibodies against the His tag. Note that approximately 50% of the cells were positive for the epitope-tagged transgenes. B, cells cotransfected with either empty expression vector constructs or with expression constructs containing c-Jun and Fra2 were maintained in monolayer or suspension culture. Proliferative activity was then assessed by the ability of cells to take up fluorescent EdU (see Materials and Methods for details). Note that while maintenance in suspension greatly decreased proliferative rates, there was only a slight increase in the percentage of EdU-positive cells in either monolayer or suspension culture after cotransfection with c-Jun/Fra2 compared with vector control transfectants.

Figure 6. c-Jun and Fra2 regulate endogenous BRCA1 levels in both monolayer and suspension culture. A, cells transfected with an either empty expression vector or cotransfected with His epitope-tagged versions of c-Jun and Fra2 were maintained for 48 hours in either monolayer or suspension culture, lysed, and subjected to Western blotting with the indicated antibodies. Note that cotransfection with c-Jun/Fra2 raised levels of both transcription factors (as is clear from both the anti-His and anti-c-Jun and Fra2 blots), and it specifically increased BRCA1 in both monolayer and suspension culture (i.e., ERK1/2 levels were not affected). B, cells cotransfected with either scrambled (scr) or sequence-specific siRNAs (si) against c-Jun and Fra2 and were maintained for 48 hours in either monolayer or suspension culture. The cells were then lysed, and subjected to Western blotting with the indicated antibodies. Note that cotransfection with siRNAs to c-Jun/Fra2 decreased levels of both transcription factors and specifically decreased BRCA1 in both the monolayer and suspension culture condition.
maintained in suspension culture contributes to the resultant suppression of BRCA1.

**Discussion**

Ovarian carcinoma, which consists of a number of histologically distinct subtypes, is unusual in that it often spreads transperitoneally (26). During this process, tumor cells are first shed from the primary lesion and they float freely, often as small clusters, within the peritoneal fluid of the abdominopelvic cavity (27). It is these floating tumor cell clusters that, if they subsequently adhere to peritoneal surfaces at sites distant from the primary lesion, can expand and become locally invasive. This process is particularly prominent in the high-grade serous tumors, which are shed early in their progression and go on to form the most prominent and lethal ovarian carcinoma subtype by far (28). High-grade serous ovarian carcinoma tumors are also notable in that they often exhibit BRCA1 abnormalities, only a small percentage of which are caused by genetic mutation. Thus, epigenetic suppression is a major factor in the histogenesis of these tumors (2).

The tumor microenvironment is known to regulate BRCA1 expression. This has been best studied in the breast where the ECM, hormones, and hypoxia have all been shown to alter BRCA1 levels by impinging on the gene’s transcriptional and posttranslational regulation (13, 14, 22, 29, 30). Given that release from the ECM is a major microenvironmental change that occurs as ovarian carcinoma begins to disseminate by floating as effusive tumor cell clusters in the fluid of the peritoneal cavity (27) and the fact that BRCA1 levels are reduced in disseminated high-grade serous tumor cells compared with their tissue of origin (31), we released premalignant ovarian cells from the ECM, maintained them as clusters in suspension culture, and determined the effect on BRCA1 levels. Under suspension conditions, BRCA1 levels were decreased considerably and this decrease was mediated, at least in part, transcriptionally. More specifically, we showed that a suspension-mediated decrease in the AP1 transcription factor components c-Jun and Fra2 contributed to inhibition of BRCA1 promoter transactivation and endogenous BRCA1 gene expression. At this point, we cannot state definitively that GABPα and GABPBβ, 2 ETS-like transcription factors that also positively regulate the BRCA1 promoter are not involved in this suppression. However, it was clear that releasing the cells into suspension did not affect the steady state levels of either GABP factor, which is why we focused on c-Jun and Fra2 for the remainder of this initial study. It is also important to note that other, as yet unidentified factors, may be involved in the BRCA1 suppression given the large decrease in wild-type PRR activation we observed in suspension culture that we could not fully recapitulate in either of the RIBS or CRE element mutants.

Cells in monolayer culture have engaged integrins and, because they are spread on a rigid tissue culture substratum, their cytoskeletal tension is high, both of which contribute to the mechanical augmentation of growth factor-mediated signaling that leads to the expression of various factors, including components of the AP1 transcription factor complex (32, 33). At this point, it is not clear if a loss of integrin signaling or a decrease in cytoskeletal tension, or both, contributed to the decrease in c-Jun and Fra2 when IOSE-80pc cells were maintained as rounded cell clusters in suspension. However, this decrease also occurred when the cells were clustered and maintained on compliant reconstituted basement membrane gels where integrins were engaged but the cells remained rounded (data not shown). One way to address this issue definitively would be to alter the rigidity of the substratum after the cells are already attached such that cytoskeleton tension is modulated, whereas the integrins are continuously engaged with the ECM. These are experiments that we are currently attempting by coating silicone rubber ECM substrata that can then be stretched to increase its rigidity after cells are pretreated. It will also be interesting to determine if the suspension-mediated decrease in c-Jun and Fra2 affect other aspects of the ovarian carcinoma phenotype, although at this point, we have not noticed that there is any change in adhesion or invasiveness (e.g., on basement membrane gels).

The BRCA1 promoter constitutes a large CpG island that is hypermethylated in up to 30% of spontaneous ovarian epithelial carcinomas (34, 35). Importantly, this hypermethylation is associated with decreased BRCA1 expression and poor clinical outcome (36). One of the CpG targets for methylation in the BRCA1 promoter is the CRE element (37). In fact, the “CREm” mutation used in this study that eliminated the differential monolayer- and suspension culture-mediated activation and prevented c-Jun/Fra2 binding, removes the major hypermethylation site within this element. Thus, the decreased binding of c-Jun/Fra2 at this site in suspension culture and, presumably, during shedding into the abdominopelvic fluid in vivo, could, potentially, be a form of initial “flexible” transcriptional suppression that subsequently helps to facilitate a “stable” suppression by methylation (38, 39). If this turns out to be the case, it would mean that the process of tumor cell shedding that is an early hallmark of the deadly transperitoneal spread of high-grade serous tumors may help initiate the stable epigenetic suppression of BRCA1 that is prominent in the sporadic form of these tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Received June 29, 2012; revised January 3, 2013; accepted January 3, 2013; published OnlineFirst January 21, 2013.
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Molecular Cancer Research

Microenvironmental Regulation of BRCA1 Gene Expression by c-Jun and Fra2 in Premalignant Human Ovarian Surface Epithelial Cells

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Mol Cancer Res  Published OnlineFirst January 21, 2013.

Updated version  Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-12-0395

Supplementary Material  Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2013/01/21/1541-7786.MCR-12-0395.DC1

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