Caveolin-1 Upregulation Mediates Suppression of Primary Breast Tumor Growth and Brain Metastases by Stat3 Inhibition

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Abstract
Stat3 activation has been implicated as an important driver of brain metastasis in breast cancer, but the critical targets of Stat3 in this process are yet to be fully defined. In this study, we identified the lipid raft organizing protein Caveolin-1 (Cav-1) as a critical genetic target of Stat3 in this process. In human breast cancers, we found that activated Stat3 correlated with attenuation of Cav-1 in brain metastases relative to primary tumors. Cav-1 promoter activity and gene expression were increased by overexpressing an activated form of Stat3 but decreased by attenuation of Stat3 activity or expression. We identified putative Stat3-binding elements in the Cav-1 promoter and showed a direct repression of Cav-1 transcription by Stat3. Reciprocally, we showed that strategies to increase or decrease Cav-1 expression were sufficient to attenuate or promote breast cancer cell invasion. Furthermore, increased expression of Cav-1 phenocopied the effects of Stat3 activation in blocking primary tumor growth and abrogating formation of brain metastases. Collectively, our findings provide clinical and mechanistic evidence that Cav-1 is a critical target for suppression by Stat3 in driving invasion and metastasis of breast cancer cells. Cancer Res; 71(14); 4932–43. ©2011 AACR.

Introduction
Breast cancer is the most common cancer in women. The 5-year relative survival rate in patients with local breast cancer is 98%. However, the rate is only about 27% in patients with distant metastases (1). Breast cancer frequently metastasizes to the brain. Specifically, breast cancer is responsible for 16% of all cases of brain metastases (2). Furthermore, clinically overt brain metastases occur in 10% to 15% of patients with breast cancer, and 20% of patients have brain metastases at autopsy (3, 4). When metastases are present in the brain, the prognosis for breast cancer is consistently poor, with median survival durations less than 1 year in most cases (2, 3). Despite advances in understanding the causes of and treating primary breast cancer, the biological and molecular mechanisms of brain metastases of this malignancy remain understudied.

The oncoprotein Stat3 is constitutively activated in cancer cells in various types of human cancers (5–12). The Stat3 signaling pathway may impact tumor metastases via regulation of several steps in this process (8, 11, 13). Stat3 is activated by many cytokines and growth factors, including epidermal growth factor and interleukin-6, as well as by oncogenic proteins, such as Src and Ras (14). Conversely, Stat3 activation is negatively regulated by several proteins, including suppressor of cytokine signaling (SOCS)-1 (15, 16). By using our established brain metastases models, we recently described the role and positive regulation of Stat3 activation in melanoma brain metastases (11, 17). However, the underlying mechanisms by which Stat3 promote brain metastases remain unclear.

Caveolin-1 is the principal structural component of caveolar membrane domains in nonmuscle cells, including mammary epithelial cells (18, 19). Caveolae are multifunctional organelles in which caveolin-1 plays direct roles in various events, such as membrane trafficking and various cellular signal transduction (20). Interestingly, researchers have observed reduced caveolin-1 expression levels in various cancers.
number of human cancers (21, 22), suggesting a negative regulatory role for caveolin-1 in tumor development. Although caveolin-1 seems to have both tumor suppressive and oncogenic activity in different types of cancer, recent studies suggested that caveolin-1 inhibits breast cancer cell migration and metastases (21–26). Specifically, in caveolin-1 null mice carrying the MMTV-PyMT transgene, multifocal dysplastic lesions developed throughout the entire mammary tree, and mammary tumorigenesis and lung metastases were enhanced (27, 28). Furthermore, caveolin-1 expression levels are significantly lower in human breast cancer cells than in their normal mammary epithelial counterparts (24). All of this experimental and clinical evidence supports that caveolin-1 is a tumor suppressor in patients with breast cancer.

These findings underscore the importance of determining the roles and molecular mechanisms by which activated Stat3 and caveolin-1 regulates brain metastases. Therefore, we sought to determine the effect of activated Stat3 on caveolin-1 expression, breast cancer invasion, and brain metastases.

Materials and Methods

Cell lines and culture conditions
The NMuMG, MDA-MB-231, BT-474, MCF7, and T-47D cells were obtained from the American Type Culture Collection (ATCC). The brain-metastatic variant MDA-MB-231-BR3 (referred to herein as 231-BR), BT-474-BR, and A375BR were generated from their parental cell lines (11, 29). All of the cell lines including the brain-metastatic variants were authenticated by the Authorization for Characterized Cell Line Core of MD Anderson Cancer Center by using the AmpF/STR Identifiler Kit (Applied Biosystems) and the ATCC fingerprint database. The cell lines were cultured in Eagle’s minimal essential medium supplemented with 10% FBS and were not passaged for more than 6 months before bringing new cells out of freeze or purchasing a new cell aliquot from the ATCC. The last cell authentication was tested in February 2011.

Western blot analysis
Western blotting was carried out by using whole-cell protein lysates; primary antibodies against phosphorylated Stat3 (pStat3; Tyr 705), Stat3, phosphorylated extracellular signal-regulated kinase1/2 (pERK1/2), and ERK1/2 (Cell Signaling Technology). The resulting precipitated DNA samples were analyzed by using PCR to amplify a 212-bp region of the caveolin-1 promoter with the primers 5'-GGGTGCCTGTGGT-3' (sense) and 5'-TGGCA-3' (antisense; ref. 36).

Plasmids and siRNAs
The plasmids pcDNA3.1-SOCS-1, pcDNA3.1-caveolin-1, Stat3C (a constitutively activated mutant of Stat3), Stat3DN (a dominant-negative mutant of Stat3), and control vectors were described previously (14, 16, 17, 30, 31). siRNA sequences targeting SOCS-1, Stat3, and caveolin-1 were as follows: SOCS-1, 5'-AACAUCCUGCUAGACGGCUAt-3' (33); and caveolin-1, 5'-AGACGAGCGAGCGAGACTT-3' (34).

Transient and stable transfection
Transfection of plasmids and siRNAs into breast cancer cells was done by using Lipofectamine 2000 (Invitrogen). For transient transfection, cells were transfected with siRNA or plasmids at different doses as indicated for 48 hours before functional assays were carried out. Stably transfected cell lines were isolated from 231-BR transfected with pcDNA3.1-SOCS-1 plasmid via selection with hygromycin (150 μg/mL).

Pharmacologic agents
JSI-124 was purchased from Indofine Chemicals, Inc. STS-21 was obtained from Biomol. All agents were dissolved in dimethylsulfoxide (DMSO). During the experiment, they were thawed and cultured with culture medium to the appropriate final concentrations.

Promoter reporter, site-specific mutagenesis, and dual luciferase assay
The caveolin-1 promoter luciferase reporter pA3Luc-caveolin-1 (pLuc-Cav) was described previously (31). Mutated pLuc-Cav was generated by site-specific mutagenesis by using the QuikChange site-directed mutagenesis kit (Stratagene). Cells were transfected with the indicated wild-type or mutated pLuc-Cav, siRNAs, or gene-specific expression plasmids. The transfection efficiency was normalized via cotransfection with a β-actin-RL reporter (35). Luciferase activity in the cells was quantified by using a dual luciferase assay system (Promega Corp.).

Electrophoretic mobility shift assay
Electrophoretic mobility shift assay (EMSA) was carried out as described previously (35). Double-stranded oligonucleotides of the putative Stat3-binding sites in the caveolin-1 promoter were used as probes. For supershift analysis of EMSA, extracts of tumor cells were preincubated with anti-Stat3 antibodies (Cell Signaling Technology).

Chromatin immunoprecipitation assay
Tumor cells (2 × 10⁶) were prepared for chromatin immunoprecipitation (ChIP) assay with the ChIP assay kit (Cell Signaling Technology). The resulting precipitated DNA samples were analyzed by using PCR to amplify a 212-bp region of the caveolin-1 promoter with the primers 5'-GGGTGCCTGTGGT-3' (sense) and 5'-GGGTGCCTGTGGT-3' (antisense; ref. 36).

Invasion assay
An invasion assay by using growth factor–reduced Matrigel-coated Invasion Chambers (BD Biosciences) was carried out as described previously (11). FBS (10%) containing medium was placed in the lower chambers to act as a chemoattractant. The cells that penetrated through filter were counted at a magnification of ×200 in 15 randomly selected fields, and the mean number of cells per field was recorded.

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Human tissue samples and immunohistochemical analysis

Human breast ductal carcinoma in situ (DCIS), breast invasive ductal carcinoma (IDC), and breast cancer brain metastases tissue samples were coded without any patient identifiers. The use of the tissue samples was approved by the Institutional Review Board. Immunohistochemical (IHC) analysis was done by using paraffin-embedded sections of the human tissue samples with anti–caveolin-1 or anti–pStat3 antibodies. Staining was scored by 2 investigators blinded to the clinical data by using a 4-tier system that incorporated the percentage of cells positive and intensity for staining: negative, weak, moderate positive, and strongly positive.

MTT assays

Breast cancer cells were seeded at a density of $3 \times 10^5$ cells per well in 96-well plates. At the end of culture for each indicated time points, 20 μL of MTT (5 mg/mL; Sigma-Aldrich) was added to each well, and plates were placed at 37°C for 4 hours. DMSO (100 μL) was added to each well to lyse the cells. Absorbance was measured at a wavelength of 570 nm.

Colony formation in soft-agar assay

Cells ($5 \times 10^2$ cells per well) were mixed with 0.3% agar solution in MEM containing 10% FBS, and the solution was poured on top of a 0.60% agar layer containing MEM and 10% FBS in 12-well tissue culture plates. After 21 days in culture, colonies were then stained with 0.005% crystal violet and examined microscopically.

Animals

Pathogen-free female athymic BALB/c nude mice were purchased from the National Cancer Institute. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with the current regulations and standards of the U.S. Department of Agriculture and Department of Health and Human Services.

Human tumor xenografts

Tumor cells ($10^6$) in 0.1 mL of HBSS were injected into the mammary fat pad of nude mice. The length and width of the resulting tumors were measured by using calipers, and the mean tumor diameter was calculated by using the equation $(L + W)/2$, in which $L$ is the tumor length and $W$ is the tumor width.

Brain metastases

Breast tumor cells ($3 \times 10^5$) were slowly injected into the intracarotid artery of nude mice. The mice were killed 90 days after tumor cell inoculation or when they seemed to be moribund or had clinical symptoms of brain metastases. Their brains were then removed, and 10 serial brain sections were cut every 300 μm. Mean number of large metastases and micro metastases was determined by counting the number of metastases in the 10 sections from 1 hemisphere of each brain as described previously (29, 37).

Statistics

The significance of the data from patient specimens was determined by $\chi^2$ test and Spearman rank correlation test. The significance of the in vitro data and in vivo data was determined by Student’s $t$ test (2-tailed) and Mann-Whitney test (2-tailed), respectively. Two-sided $P$ values less than 0.05 were considered statistically significant.

Results

Altered Stat3 signaling impacts the expression of caveolin-1

First, the expression of caveolin-1, pStat3, and Stat3 protein was determined in the normal mammary gland epithelial line NMuMG and various breast cancer cell lines. Substantially higher level of caveolin-1 but lower level of pStat3 was founded in NMuMG line than in the breast cancer lines (Fig. 1A). Moreover, the decreased level of caveolin-1 protein in brain-metastatic cell lines 231-BR and BT-474-BR was directly correlated with increased level of activated Stat3 (Fig. 1B). Transfection of a constitutively activated mutant of Stat3 (Stat3C) plasmid led to decreased caveolin-1 expression in MDA-MB-231 cells, whereas knockdown of Stat3 expression in 231-BR cells by using Stat3 siRNA did the opposite at both levels of protein (Fig. 1C) and mRNA expression (Supplementary Fig. S1). Altered caveolin-1 expression also affected the levels of matrix metalloproteinase (MMP)-9, a known downstream target gene of caveolin-1 (Fig. 1D; ref. 38). Treatment of 231-BR and BT-474-BR cells with STA-21 (inhibiting DNA-binding activity of Stat3; ref. 39) or JSI-124 (Jak2 inhibitor; ref. 40) led to increased caveolin-1 protein expression (Fig. 1E). These data strongly suggested that activated Stat3 represses caveolin-1 expression in brain-metastatic breast cancer cells.

To assess the mechanism of regulation of caveolin-1 by Stat3, we examined whether inhibition of Stat3 activation suppress caveolin-1 promoter by using the caveolin-1 promoter luciferase reporter pLuc-Cav with a dominant-negative Stat3 (Stat3DN; refs. 41, 42) or the control vector pcDNA3.1 at increasing concentrations. Stat3DN upregulated the activities of pLuc-Cav in 231-BR and BT-474-BR cells (Fig. 2A and B). Also, the Stat3 inhibitors STA-21 or JSI-124 increased the activities of pLuc-Cav in 231-BR cells (Fig. 2C and E) and BT-474-BR cells (Fig. 2D and F). The inhibition of promoter activities was not because of cytotoxicity caused by the inhibitors (Supplementary Fig. S2). These results suggest that Stat3 activation negatively regulates caveolin-1 transcription.

Stat3 binds directly to the caveolin-1 promoter

To determine whether caveolin-1 is a direct transcriptional target of Stat3, we analyzed the caveolin-1 promoter sequence by using the Stat3-binding consensus sequences TT (N4) AA and TT (N5) AA (43). We identified 7 putative Stat3-binding sites in the promoter region of caveolin-1.
elements in the caveolin-1 promoter (Fig. 3A). To functionally characterize these elements, we initially carried out EMSA competition experiments by using a Stat3 consensus binding oligonucleotide as a probe. The competitive probes were 7 oligonucleotides corresponding to the elements. We found that #2 and #6 probes significantly competed with the Stat3 consensus probe for binding to Stat3 (Fig. 3B).

To confirm that Stat3 binds to the caveolin-1 promoter, we used the element #2 as a probe for EMSA. As shown in Figure 3C, incubation of protein extracts from 231-BR cells with the #2 probe resulted in the formation of a major shifted band (DNA–protein complex; lane 2). Moreover, the shifted band was competed out by an unlabeled consensus Stat3 probe (lane 3) and super shifted by an anti-Stat3 antibody (lane 4), indicating that the shifted band represented a Stat3 DNA–protein complex. These in vitro binding experiments indicated that Stat3-binding elements are present in the caveolin-1 promoter and that #2 sequence in the promoter is a high-affinity Stat3-binding element.
Next, we carried out ChIP assays to provide direct evidence that Stat3 is recruited to the endogenous caveolin-1 promoter during transcription \emph{in vivo}. We used 2 pairs of primers designed to amplify segments of caveolin-1 promoter encompassing the #2 or #6 elements for the ChIP assays with 231-BR and A375Br cells. Next, we generated 231-BR and A375Br cells with reduced Stat3 activity by transfecting these cells with the SOCS-1 expression vector, as a previous study showed that activation of Stat3 in brain-metastatic cancer cells was caused by decreased SOCS-1 expression and restoration of SOCS-1 activity resulting in inhibition of Stat3 activation (17). ChIP assays were also carried out with these cells. We found that the region of the caveolin-1 promoter containing Stat3-binding site #2 bound to endogenous Stat3 protein in above cell lines (Fig. 3D). Moreover, both 231-BR and A375Br exhibited higher levels of binding of Stat3 to the region than did 231-BR-SOCS-1 and A375Br-SOCS-1 cells. However, we did not detect binding of Stat3 to the region of the caveolin-1 promoter containing Stat3-binding site #6 in any of the cell lines tested (data not shown). Collectively, these results showed that Stat3 bound specifically to the caveolin-1 promoter and that TTGGGCATAA at positions −571 to −563 in the promoter is a high-affinity Stat3-binding element.

A Stat3-binding site is critical for deactivation of the caveolin-1 promoter in brain-metastatic cancer cells

To assess the functional role of the Stat3-binding site in caveolin-1 gene regulation, we carried out site-directed mutagenesis of Stat3-binding site #2 in the caveolin-1 promoter with a base pair change from TT to CG. We then measured the promoter activity of both wild-type and mutant forms of caveolin-1 promoters in 231-BR and A375Br cells. Mutation of Stat3-binding site #2 significantly increased the promoter activity of caveolin-1, suggesting that this Stat3-binding site acted as a negative regulator of caveolin-1 transcription in these brain-metastatic cancer cells (Fig. 3E).

Alteration of caveolin-1 expression affects breast cancer cell invasion

We examined the invasive ability of breast cancer cells and found that knockdown of Stat3 expression by Stat3 siRNA attenuated the invasiveness of 231-BR cells (Fig. 4A), whereas Stat3C overexpression promoted the invasiveness of MDA-MB-231 cells (Fig. 4C). We also genetically altered caveolin-1 expression in these 2 cell types and found that overexpression of caveolin-1 attenuated the invasiveness of 231-BR cells (Fig. 4B), whereas knockdown of expression of caveolin-1 promoted the invasiveness of MDA-MB-231 cells (Fig. 4D). To assess the inhibitory effect of caveolin-1 on Stat3-induced invasion, we cotransfected both Stat3C and caveolin-1 into MDA-MB-231 cells and found that overexpression of caveolin-1 attenuated the invasiveness of Stat3C-overexpressing MDA-MB-231 cells (Fig. 4E).

Inhibition of Stat3 suppressed the growth and brain metastases of breast cancer cells

Because invasion is a critical step for brain metastasis, we next determined the impact of inhibited Stat3 activation on
breast tumor growth and brain metastases and its relationship with caveolin-1 expression. We used the stable SOCS-1–transfected 231-BR cells, because the cells have lower levels of binding of Stat3 to the caveolin-1 promoter than did 231-BR cells (Fig. 3D). SOCS-1 transfection significantly inhibited breast tumor growth (Fig. 5A) and completely abrogated brain metastases of human breast cancer (Fig. 5B) of 231-BR cells in mice. Furthermore, immunohistochemical studies showed that the expression of activated Stat3 decreased, whereas that of caveolin-1 increased in tumors formed by 231-BR-SOCS-1 cells (Fig. 5C). These data indicated that Stat3 inhibition by SOCS-1 suppressed breast cancer metastases, at least in part, via negative regulation of caveolin-1 expression.

Inhibition of Stat3 by SOCS-1 upregulates the expression of caveolin-1

To provide direct evidence that SOCS-1 inhibited brain metastasis by caveolin-1 upregulation, we determined whether SOCS-1 upregulates caveolin-1 protein expression. We found that the expression level of pStat3, but not phospho-p44/42, was significantly decreased in the 231-BR-SOCS-1 cells (Fig. 5D). Moreover, 231-BR-SOCS-1 cells exhibited a higher level of caveolin-1 than did parental or vector-transfected cells (Fig. 5D). Similar results were found in SOCS-1–transfected A375Br cells (Fig. 5D). In contrast, knockdown of endogenous SOCS-1 expression increased the expression of activated Stat3 and decreased the expression of caveolin-1 protein in MDA-MB-231 cells (Fig. 5E).

Inhibition of Stat3 by SOCS-1 suppressed breast cancer cell invasion and growth

We next determined whether SOCS-1 expression would affect the cell invasion and whether the effect of SOCS-1 on cell invasion is via caveolin-1. First, we found that down-regulation of SOCS-1 by SOCS-1 siRNA enhanced the invasive ability of MDA-MB-231 cells (Fig. 6A and Supplementary Table 1).
Figure 4. Alteration of caveolin-1 expression affects breast cancer cell invasion. A, transfection of siStat3 or siControl in 231-BR cells for 48 hours; B, transfection of pCav1 or control pcDNA3.1 in 231-BR cells for 48 hours; C, transfection of Stat3C or control pcDNA3.1 in MDA-MB-231 cells for 48 hours; D, transfection of siCav1 or siControl in MDA-MB-231 cells for 48 hours; E, MDA-MB-231 cells were transfected with pcDNA3.1, Stat3C, and pCav1 at the indicated amounts for 48 hours. Then, the invasiveness of above cells (in A–E) after the transfection was determined by using the invasion assay in 16 hours. Representative photomicrographs of tumor cells that invaded through a Matrigel-coated filter were taken and shown. The invasive cells were counted in 15 random fields and expressed as the percentage of the control cells. Values are mean (± SD) results of 2 independent experiments (left). *, P < 0.001.
Figure 5. Inhibition of the growth and brain metastases of 231-BR cells by SOCS-1. 231-BR cells were stable transfected with pcDNA3.1 (vector) or pSOCS-1 plasmids. To avoid clonal selection, we carried out 3 independent transfections of pSOCS-1 in the cells and pooled hygromycin-resistant colonies to establish stable transfectants, designated as 231-BR-SOCS-1 #1, #2, and #3. A, 231-BR, 231-BR-Vector, and 231-BR-SOCS-1 (SOCS-1 #1 and #2) cells (10^6 per mouse) were injected into the mammary fat pad of nude mice (n = 5). The resulting tumors were measured at the indicated time intervals. The data are the mean tumor diameters observed in 5 mice per group. B, the above tumor cells (3 × 10^5 per mouse) also were injected into the intracarotid arteries of nude mice. The mice brains were harvested when they were killed, and metastatic tumor formation was assessed histologically (*, P < 0.01). Incidence indicates the number of mice with brain metastasis divided by the total number of mice injected with tumor cells. Micro, mean number of micro metastases. Large, mean number of large metastases. C, expression of pStat3 and caveolin-1 in tumors from experiments of (A) was analyzed by using IHC staining. Representative photos of the IHC staining are shown. D, SOCS-1 upregulated the expression of caveolin-1. 231-BR or A375Br, vector, or SOCS-1 transfected cells were used for analysis of SOCS-1, pStat3, Stat3, caveolin-1, phosphorylated ERK1/2, ERK1/2, MMP-9, and β-actin protein expression by immunoblotting. E, MDA-MB-231 cells transfected with 100 nmol/L siControl or siSOCS-1 were used for analyses of SOCS-1, pStat3, Stat3, caveolin-1, and β-actin protein expression by using immunoblotting.
Conversely, overexpression of SOCS-1 downregulated the invasive ability of 231-BR cells (Fig. 6B). Overexpression of SOCS-1 also downregulated the levels of MMP-9 (Fig. 5D), which is consistent with previous studies indicating that MMP-9 is one of the main mechanisms by which caveolin-1 operates in reducing invasion (28, 38). Furthermore, the inhibitory effect of SOCS-1 on cell invasion was rescued by caveolin-1 siRNA (Supplementary Fig. S4 and Fig. 6C). These data indicated that SOCS-1 inhibition of cell invasion is largely dependent on caveolin-1.

In addition, we investigated whether SOCS-1–transfected 231-BR cells also exhibit abnormalities in proliferation. We found the cell viability has no significant difference among 231-BR vector and 231-BR SOCS-1 cells within the 5 days in MTT assay (Fig. 6C). However, 231-BR SOCS-1 #1 and #2 cells, which have relative higher levels SOCS-1, exhibited reduced proliferation in longer time MTT and colony formation in soft agar assays compared with 231-BR vector cell (Fig. 6D and E). Furthermore, the inhibitory effect of SOCS-1 on the cell proliferation was rescued by Stat3C or A. MB-213 cells were transfected with siControl or siSOCS-1 (100 nmol/L) for 48 hours. Then the invasive ability of the cells was determined by the invasion assay as described in Figure 4. *, P < 0.01. B, the invasive ability of 231-BR, vector, and SOCS-1 cells was determined by the invasion assay. *, P < 0.01. C, 231-BR-SOCS-1 (#1, #2, and #3) cells were transfected with siControl or siCav-1 (100 nmol/L) for 48 hours. Then the invasive ability of the cells was determined by the invasion assay. *, P < 0.01. D, cell viability of 231-BR vector and SOCS-1 cells was determined by MTT assay. Values are mean ± S.D. of triplicate samples of 2 experiments. *, P < 0.05. E, colony-forming capacity of 231-BR vector and 231-BR-SOCS-1 (#1, #2, and #3) cells. F and G, 231-BR-SOCS-1 (#1 and #2) cells were transfected with Stat3CA (0.4 μg), pcDNA3.1 vector (0.4 μg), or siRNAs (siControl or siCav-1, 100 nmol/L) on day 0 and day 3. Then the cell viability at the indicated time points was determined by MTT assay. *, P < 0.05; **, P < 0.01.
caveolin-1 siRNA (Fig. 6F and G and Supplementary Fig. S3). Consistently, Stat3C transfection prevented the increase of Cav-1 expression in SOCS-1–transfected 231-BR cells (Supplementary Fig. S4). These data further support that Stat3 critically regulates breast cancer metastases, at least in part, via negative regulation of caveolin-1 expression and function.

**Increased expression of pStat3 correlates with decreased expression of caveolin-1 in human breast cancer brain metastases**

To determine the relationship between Stat3 and caveolin-1 and the clinical significance of this relationship, we carried out IHC staining for pStat3 and caveolin-1 in 50 human breast DCIS, 50 human breast IDC, and 50 human breast cancer brain metastases samples. We analyzed the data of moderate positive (++) and strongly positive (++++) staining of pStat3 or caveolin-1 by χ² test. The expression levels of pStat3 were significantly increasing in brain metastases compared with DCIS or IDC (P < 0.001), whereas the expression levels of caveolin-1 were significantly decreasing in brain metastases as compared with DCIS or IDC (P < 0.001; Table 1 and Supplementary Fig. S5). Moreover, this inverse correlation between pStat3 and caveolin-1 in these 3 types of samples was statistically significant as determined by Spearman rank correlation test (r = −0.941; P < 0.001; Table 1). These data further support the critical role of pStat3 in caveolin-1 expression and brain metastasis in human breast cancer.

**Discussion**

In this study, we sought to determine the role of interaction between Stat3 activation and caveolin-1 expression in breast cancer brain metastases. Our novel clinical and mechanistic evidence strongly suggested that activated Stat3 binds directly to the caveolin-1 promoter and inhibits its transcription. Conversely, caveolin-1 negatively regulates activation of Stat3 and invasion of brain-metastatic cancer cells. Moreover, suppression of Stat3 activation inhibited the invasion and brain metastases of breast cancer cells in our animal model.

Brain metastasis is one of the major causes of morbidity and mortality in patients with breast cancer. The underlying mechanisms of breast cancer brain metastases remain unclear; however, a full understanding of these mechanisms is essential to controlling brain metastases. In this study, we found that activated Stat3 expression is higher in breast cancer brain metastases than in breast DCIS and IDC tumors. Consistently, several studies have linked activated Stat3 protein expression with metastases of various tumor types (12), and activated Stat3 promotes liver and lung metastases in mouse models (8, 44). Also, recent studies by our group indicated that alteration of Stat3 activation directly impacts melanoma brain metastases (11, 17). These previous findings are substantially extended by those of our present study showing that Stat3 regulates the expression and function of caveolin-1 and the invasive and metastatic properties of breast cancer. Mechanistically, activated Stat3 binds to the promoter of and inactivates the transcription of caveolin-1.

| Table 1. The expression levels of pStat3 and caveolin-1 in human DCIS, IDC, and brain metastasis specimens |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| **Group**                                      | **DCIS**                                       | **Brain mets.**                                 | **IDC**                                        | **Brain mets.**                                 |
| **pStat3 expression level**                     |                                                |                                                |                                                |                                                |
| Case                                           | 50                                             | 50                                             | 50                                             | 50                                             |
| Negative/+                                     | 78%                                            | 32%                                            | 68%                                            | 32%                                            |
| +/+ +++                                       | 22%                                            | 68%                                            | 32%                                            | 68%                                            |
| Caveolin-1 expression level                     |                                                |                                                |                                                |                                                |
| Case                                           | 50                                             | 50                                             | 50                                             | 50                                             |
| Negative/+                                     | 10%                                            | 80%                                            | 42%                                            | 80%                                            |
| +/+ +++                                       | 90%                                            | 20%                                            | 58%                                            | 20%                                            |
| Relationship between pStat3 and Caveolin-1 expression levels |
| Groups                                         | **Case**                                       | **Negative/+**                                 | **+++/+**                                      | **Negative/+**                                 |
| **DCIS**                                       | 50                                             | 78%                                            | 22%                                            | 10%                                            |
| **IDC**                                        | 50                                             | 68%                                            | 32%                                            | 42%                                            |
| **Brain mets.**                                | 50                                             | 32%                                            | 68%                                            | 80%                                            |

P = 0.005, r = −0.941.
NOTE: A Spearman rank correlation coefficient was used to perform overall relationship in expression level of pStat3 and caveolin-1 among DCIS, IDC, and brain metastasis groups, the reported P values are 2-tailed.
Abbreviation: Brain Mets, brain metastasis.
*The significance of the data was determined using the χ² test.
In this study, we also observed that SOCS-1, a negative regulator of Stat3, enhances caveolin-1 expression in brain-metastatic breast cancer cells and inhibits breast cancer invasion and metastases. Interestingly, authors have reported that the caveolin-1 scaffolding domain shares primary sequence similarities with the SOCS-1 pseudosubstrate domain (45); thus, caveolin-1 may also negatively regulate the activation of Stat3 via interactions with members of the Janus kinase family (46). Thus, activation of Stat3 in brain-metastatic cancer cells probably shut down the negative feedback of caveolin-1 by downregulating its expression. This may be one of the mechanisms responsible for sustained Stat3 activation in brain metastases.

Studies have shown that caveolin-1 acts as a tumor suppressor protein in human breast cancer (17–28, 46–48). The mechanisms by which caveolin-1 exerts its inhibitory role in transformation, tumor growth, and metastases include cell proliferation and invasion. Caveolin-1 functions as an endogenous inhibitor in the p42/44 mitogen-activated protein kinase cascade and as a transcriptional repressor of cyclin D1 (46). Caveolin-1 also inhibits secretion and expression of MMP-9 and MMP-2 (28, 38). Consistently, in this study, we found that elevation of caveolin-1 expression by SOCS-1 suppressed tumor growth in vitro and in nude mice and that increased caveolin-1 expression attenuated the invasiveness of breast cancer, whereas decreased caveolin-1 expression promoted the cell growth and invasiveness of breast cancer. Moreover, we observed that caveolin-1 overexpression inhibited Stat3 activation, an important pathway in breast tumor growth and metastases. Thus, caveolin-1 expression may affect brain metastases of breast cancer via multiple mechanisms.

Prior studies have suggested that activated Stat3 promotes tumor growth and metastases, presumably through its critical role in the expression of many genes key to regulation of multiple aspects of tumor cell survival, growth, angiogenesis, and evasion of immune surveillance (7, 49), such as cyclin D1, MMP-2, VEGF, and 10-kDa IFN-γ-induced protein (7, 14, 49). In this study, we obtained evidence that Stat3 activation transcriptionally represses caveolin-1 expression and promotes breast cancer invasion and brain metastases. Our results strongly suggest that Stat3 has an important role in brain metastases of breast cancer through the regulation of the expression of caveolin-1 and could be a potential therapeutic target for brain metastases of breast cancer. It would be of great interest to test the efficacy of Stat3 inhibitors on brain metastases of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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