Downregulation of caveolin-1 upregulates the expression of growth factors and regulators in co-culture of fibroblasts with cancer cells

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Abstract. Reduced expression levels of caveolin-1 (Cav-1) in tumor stromal fibroblasts influences the occurrence and progression of tumors, particularly in breast cancer, but the relevant molecular mechanism is unclear. The present study aimed to clarify the potential mechanism underlying the promotion of tumor growth by reduced Cav-1 expression levels, by investigating Cav-1-targeted molecules in fibroblasts and breast cancer cells. The expression of growth factors in the ESF fibroblast cell line transfected with Cav-1 small interfering RNA (siRNA) was examined. The expression of apoptotic regulators in the BT474 breast cancer cell line that was co-cultured with the fibroblasts, was also investigated. The transfection of Cav-1-targeting siRNA in ESF cells resulted in efficient and specific inhibition of Cav-1 expression. The downregulation of Cav-1 increased the expression and secretion of stromal cell-derived factor-1 (SDF-1), epidermal growth factor (EGF) and fibroblast-specific protein-1 (FSP-1) in ESF cells. This resulted in the accelerated proliferation of the breast cancer cells. Tumor protein 53-induced glycolysis and apoptosis regulator (TIGAR) was upregulated in the BT474 cells under the condition of co-culture with Cav-1 siRNA fibroblasts, while levels of reactive oxygen species (ROS) were decreased, resulting in apoptosis inhibition in the breast cancer cells. These results demonstrated that the downregulation of Cav-1 promoted the growth of breast cancer cells through increasing SDF-1, EGF and FSP-1 in tumor stromal fibroblasts, and TIGAR levels in breast cancer cells. To the best of our knowledge, the present study supports the hypothesis that Cav-1 possesses tumor-suppressor properties, with the mechanism of Cav-1-dependent signaling involving the regulation of SDF-1, EGF, FSP-1 and TIGAR.

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Introduction

Caveolin-1 (Cav-1) is an important structural and functional component of caveolae, and is known to directly interact via its scaffolding domain with multiple signaling molecules (1). Cav-1 appears to act as a tumor suppressor and an oncogene, depending on the context and type of cancer. Cav-1 reportedly produces inhibitory effects on breast cancer, as it is associated with breast cancer development and progression (2,3). Under normal physiological conditions, Cav-1 is abundantly expressed in breast stromal fibroblasts (4,5). However, Cav-1 expression is reduced in stromal fibroblasts of the breast cancer microenvironment, and negatively correlated with the malignant potential of tumor cells. Breast cancer patients with low or negative Cav-1 expression in stromal fibroblasts often present a low survival rate, whereas the survival rates of those with high stromal Cav-1 expression levels are higher (4,6).

Although the prognostic values of the downregulation of stromal Cav-1 in patients with breast cancer have been reported, the exact mechanism is unclear (7). In order to fully assess the function of Cav-1 as a tumor suppressor, further research into the mechanisms of its expression is required. Additionally, the correlations between Cav-1 expression, tumor stromal fibroblasts and cancer cells must be verified.

Fibroblasts are major stromal cells for cancer and are central to tumorigenesis, tumor growth and metastasis; they secrete multiple factors that may prevent apoptosis, induce proliferation and stimulate tumor angiogenesis (8,9). Thus, a precise understanding of how stromal fibroblasts promote tumor progression is important. Cav-1 downregulation may be a mechanism implicated in the oncogenic transformation of fibroblasts. Decreased expression levels or deleted Cav-1 in fibroblasts can create a tumorigenic microenvironment, but the relevant molecules are not fully clear (10).

Tumor protein 53-induced glycolysis and apoptosis regulator (TIGAR) was discovered in 2005, following p53 activation and detection with microarray analysis (11). The overexpression of TIGAR during cancer development has been noted in various types of tumor. Furthermore, cancer development is often delayed in the case of TIGAR deletion. Recent research has highlighted that the expression and activity of TIGAR can

be disengaged from the p53 response, narrowing the focus of its role in cancer development (12). Nevertheless, the activity of TIGAR and the underlying mechanisms of regulation require further investigation to allow for a more complete understanding of its role in tumor pathology.

The present study aimed to clarify the potential molecular mechanism of decreased Cav-1 in promoting tumor growth through an investigation of Cav-1-targeted molecules in tumor stromal fibroblasts and breast cancer cells. Using siRNA, downregulation of the expression of Cav-1 was performed, and the levels of certain growth factors were assessed, including stromal cell-derived factor-1 (SDF-1), epidermal growth factor (EGF), fibroblast-specific protein-1 (FSP-1) and TIGAR. The current study provides evidence for the role of Cav-1 in tumor suppression.

Materials and methods

Cell culture and co-culture. The human skin fibroblast line CCC-ESF-1 (ESF) and human breast cancer cell line BT474 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). ESF or BT474 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, UT, USA), 10 μg/ml streptomycin and 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. ESF and BT474 cells were co-cultured using polyester Transwell inserts (0.4 μm pore size; Thermo Fisher Scientific, Inc.). Cells cultured on 6-well culture plates were used to detect the expression of proteins. Cells cultured on 24-well culture plates were used to assess levels of reactive oxygen species (ROS), cell proliferation and apoptosis. ESF cells were plated at the bottom of each well of the companion culture plates and allowed to adhere for a minimum of 2 h without apical Transwell inserts. Subsequent to plating, ESF cells were exposed to BT474 cell-conditioned media by placing the BT474 Transwell inserts into the wells previously plated with ESF cells. This method allowed the ESF and BT474 cells to grow in the same medium without direct contact between them. The co-culture models are presented in Fig. 1.

Cav-1 siRNA synthesis and transfection. Cav-1 siRNAs were synthesized by GenePharma Co., Ltd. (Shanghai, China). The following sequences were used: Cav-1 siRNA-1, sense 5'-GCG ACCCUAAACACCUCAATT-3' and antisense 5'-UUGAGG UGUUUAGGGUCGCTT-3'; Cav-1 siRNA-2, sense 5'-CCU UCACUGUGACGAAAUA TT-3' and antisense 5'-UAUUUC GUCACAGUGAAGGTT-3'; Cav-1 siRNA-3, sense 5'-GCC GUGUCUAUUCCAUCUATT-3' and antisense 5'-UAG AUGGAAUAGACACGGCTT-3'; negative control siRNA, sense 5'-GCCGUGUCUAUUCCAUCUATT-3' and antisense 5'-ACGUGACA CGUUCGGAGAATT-3'. ESF-1 cells at 70-80% confluence were transfected with the Cav-1 small interfering RNA or the negative control siRNA by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA and total cellular protein were extracted at 24 and 48 h after transfection, respectively, to verify the effects of the Cav-1 siRNAs.

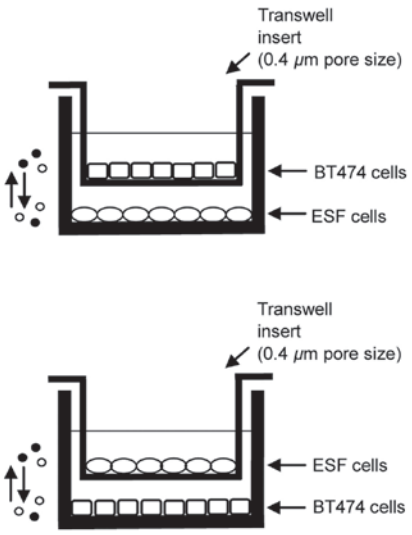

Figure 1. Co-culture models of ESF and BT474 cells. ESF cells were cultured on the bottom of culture plates with BT474 cells cultured on the Transwell inserts, which was placed into the culture plates (top). BT474 cells were cultured on the bottom of culture plates with ESF cells cultured on the Transwell inserts. Experiments were performed on the cells cultured on the bottom of culture plates (bottom).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentrations were measured using an SMA4000 spectrophotometer (Merinton Instrument, Ltd., Beijing, China) at wavelengths of 260 and 280 nm. The extracted RNA was reverse-transcribed into the cDNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). qPCR analysis of the cDNA was performed in triplicate using an IQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and Super Real Premix Plus (Tiangen Biotech Co., Ltd., Beijing, China). Relative changes were calculated using the $2^{-\Delta\Delta Cq}$ method. The primer sequences used were as follows: Cav-1, sense 5'-ACGTAGA CTCGGAGGGACATC-3' and antisense 5'-GCAGACAGC AAGCGGTAAA-3'; FSP-1, sense 5'-CCCCAAGAACAT CCAAAGT-3' and antisense 5'-TTCAGGAACAGCCAC CAGT-3'; SDF-1, sense 5'-CAGGTGGTGGCTTAACAGG-3' and antisense 5'-AAGAGGAGGTGAAGGCAGTG-3'; EGF, sense 5'-CAAAACGCCGAAGACTTACC-3' and antisense 5'-GACCATCCAGAGCCAGACAC-3'; TIGAR, sense 5'-CAGCGGTATTCCAGGATTAG-3' and antisense 5'-ACCTTAGCGAGTTTCAGTCAG-3'. The PCR products were analyzed using CFX Manager 1.6 software (Bio-Rad Laboratories, Inc.).

Western blot analysis. Cells were lysed in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate and protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland). The protein content in each sample was determined using a Bio-Rad Protein assay kit (Bio-Rad Laboratories, Inc.). Equal amounts of protein were separated by electrophoresis on 7.5~10% SDS-polyacrylamide gels (Beyotime Institute of Biotechnology, Haimen, China). The electrophoresed proteins

were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Subsequent to overnight incubation at 4˚C in a blocking buffer [5% non-fat dry milk and 0.05% Tween-20 in Tris-buffered saline (TBST)], the membranes were immunoblotted with antibodies against rabbit anti-human polyclonal Cav-1 and mouse anti-human monoclonal TIGAR (cat. nos. sc-894 and sc-377065; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. Following three washes with TBST for 5 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, sc-2004 and goat anti-mouse IgG, sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Finally, the membranes were developed using Beyotime ECL Plus (Beyotime Institute of Biotechnology).

Flow cytometry analysis. Cells were cultured for 72 h following transfection with Cav-1 siRNA-2, harvested and then fixed with 4% paraformaldehyde. Following a wash with 0.1% Triton X-100, the cells were resuspended in 5% bovine serum albumin (Beyotime Institute of Biotechnology) and incubated with the relevant primary antibody for 1 h at 4˚C. The antibodies against rabbit anti-human polyclonal SDF-1 (cat. no. sc-28876) and rabbit anti-human monoclonal FSP-1 (cat. no. ab124805) were purchased from Santa Cruz Biotechnology, Inc., and Abcam (Cambridge, UK), respectively. The antibody against rabbit anti-human polyclonal EGF (cat. no. BS3549) was procured from Bioworld Technology, Inc. (St. Louis Park, MN, USA). The cells were washed with phosphate-buffered saline (PBS) and then incubated with the appropriate fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit IgG, sc-2012; Santa Cruz Biotechnology, Inc.) for 30 min at 4˚C. The cells were extensively washed with PBS and analyzed on an BDAccuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA). The Cav-1 siRNA-2-transfected cells (ESFsiCav-1 cells) were co-cultured and mono-cultured for 72, 96 or 120 h subsequent to transfection. The culture supernatants were collected and stored at -70˚C. The concentrations of growth factors in the supernatants were determined using a Quantikine ELISA kit and a human SDF-1α Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Standards and sample proteins were added and incubated at room temperature for 2 h. Subsequent to washing, the conjugate was added to the wells for 2 h at room temperature. The reaction was stopped with a stop solution. The optical density (OD) of each well was measured at 450 nm (SMA4000 spectrophotometer) and values were correlated to the standard curve to determine protein concentration.

Measurement of intracellular ROS production. The intracellular generation of ROS in BT474 cells was detected using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA; Santa Cruz Biotechnology, Inc.). BT474 cells were mono- and co-cultured with ESF cells or ESFsiCav-1 cells for 72 h, then incubated with DCFH-DA (20 mM final concentration) at 37˚C for 35 min. The fluorescence was analyzed using an FLx800TBID fluorescence reader (BioTek Instruments, Inc., Winooski, VT, USA) using excitation and emission wavelengths of 475 and 525 nm, respectively. The levels of ROS production were expressed in relative fluorescence units (RFU).

Annexin V binding assay. When BT474 cells reached 80-90% confluence, they were collected by trypsinization (Gibco; Thermo Fisher Scientific, Inc.) and centrifugation at 1,200 x g, and washed once in PBS. An annexin V binding assay was performed with $5x10^5$ BT474 cells for each sample, according to the manufacturer's protocol (Oncogene Science; Nuclea Biotechnologies, Inc., Cambridge, MA, USA), followed by a FACScan cytometry analysis. In brief, cells were incubated in 1.5 μl biotin-conjugated annexin V in 0.5 ml binding buffer for 15 min, and then incubated with phycoerythrin-conjugated streptavidin for 5 min. To distinguish the apoptosis from the other types of cell death, 7-amino-actinomycin (7-AAD) was added prior to FACScan detection on a BDAccuri C6 flow cytometer (BD Biosciences).

Cell counting kit-8 (CCK-8) assay. BT474 cells were seeded in 24-well culture plates and ESF or ESFsiCav-1 cells in Transwell inserts in triplicate. The Transwell inserts plated with ESF or ESFsiCav-1 cells were placed into the wells containing BT474 cells. Fresh medium (100 μl) and CCK-8 solution (40 μl; Wuhan Boster Biological Technology, Ltd., Wuhan, China) were added to each well of the culture plates following culture for 24, 48, 72, 96 and 120 h. The plates were then incubated at 37˚C for 1 h. A 100-μl aliquot of the reactive solution was removed from each sample and then placed in 96-well culture plates. The OD was measured at 450 nm using an ELx800 microplate reader (BioTek Instruments, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was conducted using Student's t-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Interference effects of Cav-1 siRNA on ESF cells. RT-qPCR and western blot analyses were performed to determine the interference effects of Cav-1 siRNA on the ESF fibroblast cell line. The results indicated that the Cav-1 mRNA expression levels in ESF cells were significantly reduced in the siRNA-1, -2 and -3 groups compared with the blank control group at 24 h following transfection with 100 nM Cav-1 siRNA ($P<0.05$; Fig. 2A). Cav-1 mRNA expression levels following siRNA interference were significantly lower in the siRNA-2 group compared with those in siRNA-1 or -3 groups ($P<0.05$; Fig. 2A).

Western blot analysis demonstrated that the Cav-1 protein expression levels were significantly reduced in the siRNA-1, -2 and -3 groups compared with those in the blank control group at 48 h subsequent to transfection with 100 nM Cav-1 siRNA ($P<0.05$; Fig. 2B and C). Cav-1 protein expression in the siRNA-3 group was higher than in the siRNA-1 and -2 groups, however no significant differences were identified. These results indicate the specificity of the siRNA used to target Cav-1. Since the RT-qPCR and western blot results

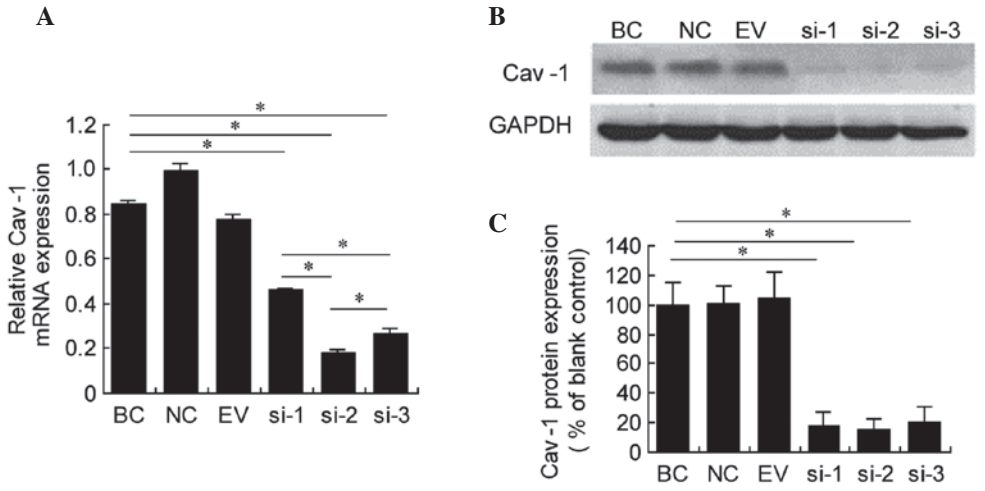

Figure 2. Cav-1 downregulation by siRNA in ESF cells. In order to analyze the interference efficacy of transfection of different sequences of Cav-1 siRNAs into ESF cells, (A) RT-qPCR (at 24 h post-transfection) and (B) western blotting (at 48 h post-transfection) were conducted. (C) Protein expression levels of Cav-1. *P<0.05, comparison shown by brackets. Cav-1, caveolin-1; BC, blank control; NC, negative control siRNA; EV, empty vector; si-1, siRNA-1; si-2, siRNA-2; si-3, siRNA-3.

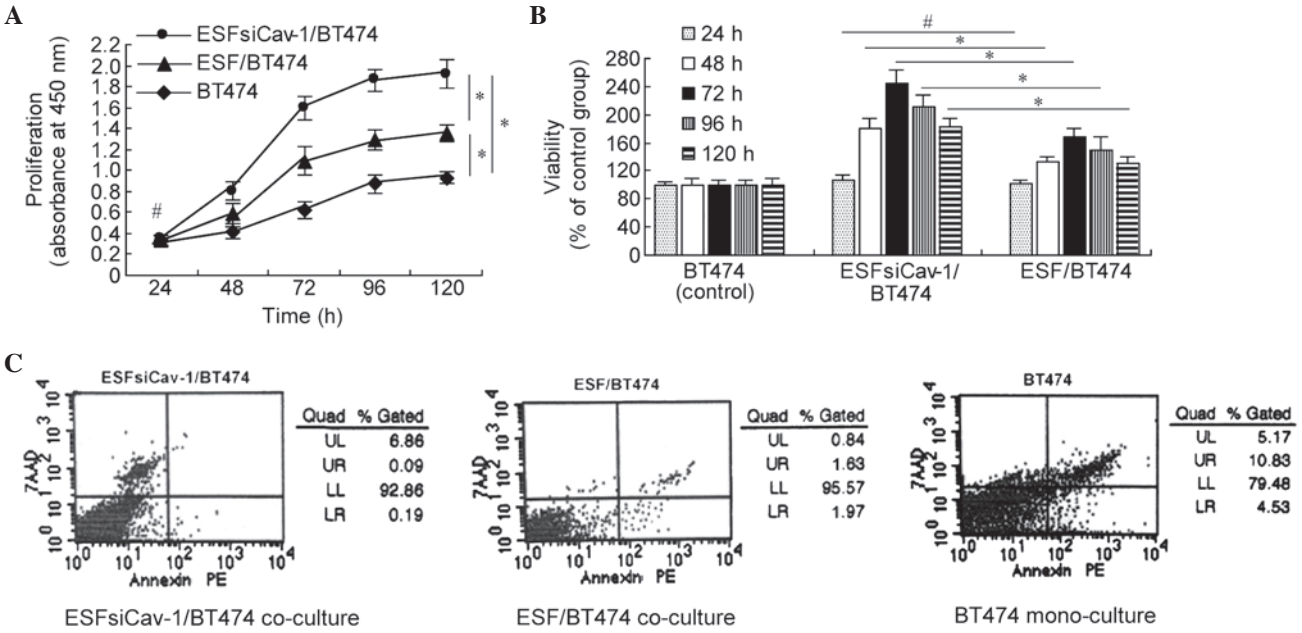

Figure 3. Downregulation of Cav-1 promotes the growth of BT474 cells. (A) BT474 cell proliferation was measured by CCK-8 assay. (B) The viability of BT474 cells was analyzed according to CCK-8 results. (C) Flow cytometry analysis of annexin V-biotin apoptosis detection. Early apoptotic cells are presented in the LR quadrant, late apoptotic cells are presented in the UR. 7-AAD was added prior to FACScan detection to distinguish the apoptosis from the other types of cell death. *P<0.05 and #P>0.05, comparisons shown by brackets. Cav-1, caveolin-1; CCK-8, cell counting kit-8; UL, upper left; UR, upper right; LL, lower left; LR, lower right; 7-AAD, 7-amino-actinomycin.

indicated that the siRNA-2 group was the most successful in reducing Cav-1 expression, it was used as the Cav-1-specific interference sequence for the sequential study.

Downregulation of Cav-1 in ESF cells promotes the growth of BT474 cells. CCK-8 assays from 24 to 120 h following mono- or co-culture were performed in the BT474 breast cancer cell line to determine the effects of Cav-1 downregulation on the proliferation and viability of the BT474 cells. The groups did not significantly differ in the observed levels of cell proliferation at 24 h. However, BT474 cell proliferation was significantly greater in the ESFsiCav-1/BT474 co-culture group than in the ESF/BT474 co-culture or BT474 mono-culture groups at 48, 72, 96 and 120 h (P<0.05; Fig. 3A).

Compared with the BT474 control group, the viability of BT474 cells of the ESFsiCav-1/BT474 co-culture group increased by 80% (48 h), 144% (72 h), 111% (96 h) and 82% (120 h) and those of the ESF/BT474 co-culture group increased by 33% (48 h), 68% (72 h), 49% (96 h) and 31% (120 h). The percentage increases in the ESFsiCav-1/BT747 cells were significantly greater than those in the ESF/BT474 cells (Fig. 3B).

To investigate the effect of Cav-1 downregulation on apoptosis in BT474 cells co-cultured with ESFsiCav-1 cells, an annexin V binding assay was performed as the BT474 cells

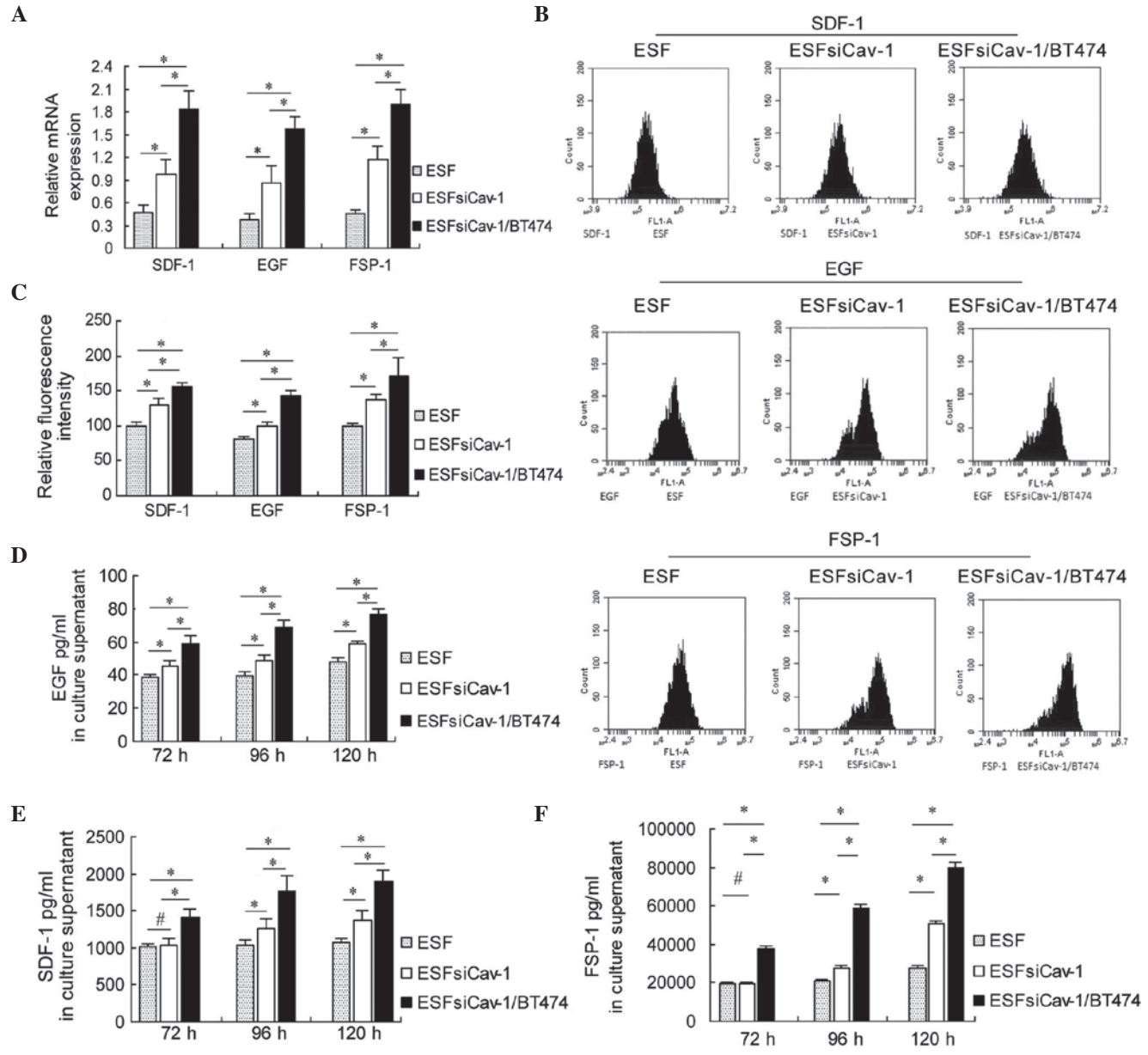

Figure 4. Upregulation of SDF-1, EGF and FSP-1 mRNA and protein levels in ESF cells by downregulation of Cav-1. (A) Reverse transcription-quantitative polymerase chain reaction analysis of the mRNA expression levels of SDF-1, EGF and FSP-1 at 48 h after transfection with Cav-1 siRNA-2. (B) Flow cytometry analysis of the protein expression levels of SDF-1, EGF and FSP-1 at 72 h subsequent to transfection with Cav-1 siRNA-2. (C) Relative fluorescence intensity of SDF-1, EGF and FSP-1 at 72 h after transfection with Cav-1 siRNA-2. (D) EGF (E) SDF-1 and (F) FSP-1 were measured using ELISA at 72, 96 and 120 h after transfection with Cav-1 siRNA-2. *P<0.05 and #P>0.05, comparison shown by brackets. SDF-1, stromal cell-derived factor-1; EGF, epidermal growth factor; FSP-1, fibroblast-specific protein-1; Cav-1, caveolin-1.

reached 80-90% confluence. A 10-fold reduction in the early apoptosis of BT474 cells in the ESFsiCav-1/BT474 co-culture group was observed, compared with the ESF/BT474 cell co-culture group. Furthermore, a 23-fold reduction in the early apoptotic cells in the ESFsiCav-1/BT474 co-culture group was detected, compared with the BT474 cell mono-culture group (Fig. 3C).

Proliferation of BT474 cells was associated with the increase in levels of SDF-1, EGF and FSP-1 in the ESFsiCav-1 cells. The downregulation of Cav-1 in ESF cells promoted the proliferation and viability of BT474 cells. Therefore, the expression of certain proliferation-associated molecules, including SDF-1, EGF and FSP-1, was investigated. ESFsiCav-1 cells were mono- and co-cultured with BT474 cells and the mRNA and protein expression levels of the target molecules were examined by RT-qPCR and flow cytometry. RT-qPCR assay demonstrated that Cav-1 downregulation significantly increased the mRNA expression levels of SDF-1, EGF and FSP-1 in the ESF cells 48 h subsequent to transfection with Cav-1 siRNA-2. Compared with the mono-culture of ESFsiCav-1, the co-culture of ESFsiCav-1 with BT474 exhibited enhanced SDF-1, EGF and FSP-1 mRNA expression, hence exhibiting a synergistic effect (P<0.05; Fig. 4A).

The flow cytometry results were consistent with the RT-qPCR results. SDF-1, EGF and FSP-1 protein expression levels were increased following Cav-1 downregulation, and were significantly higher in the ESFsiCav-1/BT474 co-culture

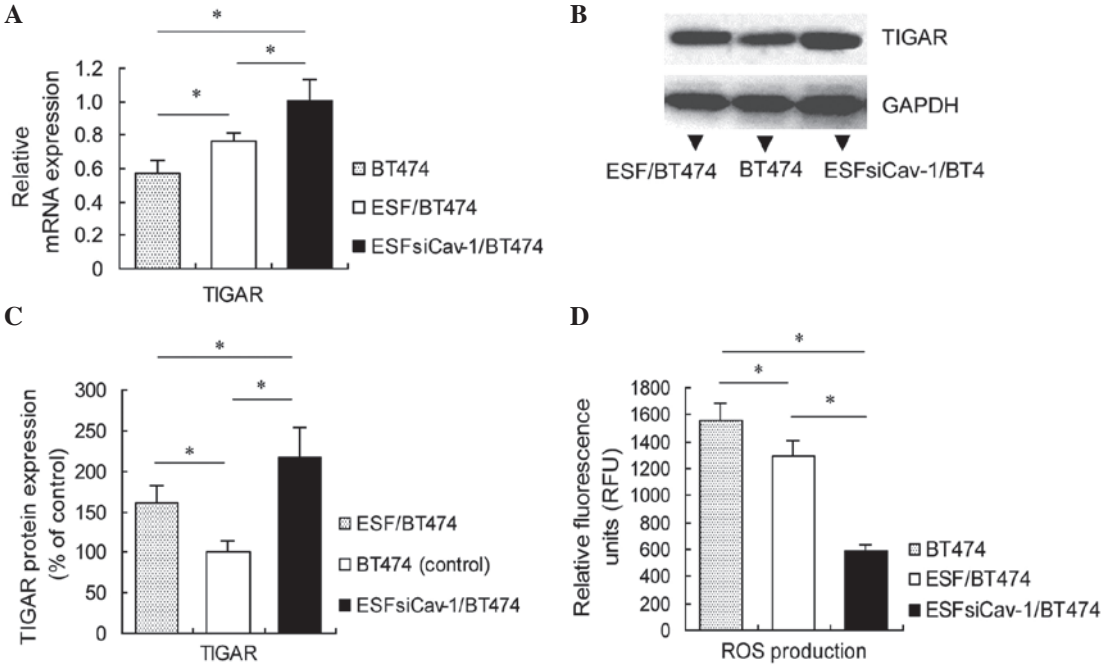

Figure 5. Downregulation of Cav-1 in ESF cells promotes TIGAR expression in BT474 cells. (A) Reverse transcription-quantitative polymerase chain reaction analysis of the mRNA expression and (B) western blot analysis of the protein expression level of TIGAR at 72 h after mono-culture and co-culture. (C) Quantification of TIGAR protein expression levels from western blot analysis. (D) Intracellular ROS analysis using the fluorescent probe DCFH-DA at 72 h after mono-culture and co-culture. *P<0.05, comparisons shown by brackets. Cav-1, caveolin-1; TIGAR, tumor protein 53-induced glycolysis and apoptosis regulator; ROS, reactive oxygen species; RFU, relative fluorescence units; DCFH-DA, 2',7'-dichlorofluorescein-diacetate.

group, compared with the ESF mono-culture group or ESFsiCav-1 mono-culture group at 72 h after transfection with Cav-1 siRNA-2 (P<0.05; Fig. 4B and C).

The concentrations of these molecules in the culture supernatant were determined using ELISA. The results of ELISA indicated that Cav-1 siRNA transfection increased SDF-1, EGF and FSP-1 production in the supernatant of the ESFsiCav-1/BT474 co-culture at 72, 96 and 120 h compared with the control groups (P<0.05; Fig. 4D-F). These data suggest that SDF-1, EGF and FSP-1 are Cav-1-targeted molecules that promote the proliferation of BT474 cells.

Downregulation of Cav-1 promotes TIGAR expression in BT474 cells, alongside inhibition of apoptosis. Apoptosis of BT474 cells was reduced in the co-culture with ESFsiCav-1 cells. Thus, the effects of the downregulation of Cav-1 on the expression of apoptosis regulators in breast cancer cells were investigated. RT-qPCR was used to measure mRNA levels of TIGAR in BT474 cells 48 h after mono-culture and co-culture. The results demonstrated that the BT474 cells from the ESFsiCav-1/BT474 co-culture group expressed significantly higher levels of TIGAR than the cells from the ESF/BT474 co-culture group and those from the BT474 mono-culture group (P<0.05; Fig. 5A). TIGAR protein expression levels were then assessed using western blot analysis 72 h after mono-culture and co-culture, and the results indicated that the TIGAR protein levels were significantly increased in the ESFsiCav-1/BT474 co-culture group compared with the ESF/BT474 co-culture group or BT474 mono-culture group (P<0.05; Fig. 5B and C). The effects of TIGAR expression on ROS regulation can depend, at least in part, on the cell type and context. To elucidate whether the upregulation of TIGAR impacts on ROS production in BT474 cells, the intracellular generation of ROS in BT474 cells was investigated using the fluorescent probe DCFH-DA. As presented in Fig. 5D, co-culture of BT474 and ESFsiCav-1 cells led to a reduction in the fluorescent signal in these cells, compared with the ESF/BT474 co-culture group (589±50 vs. 1298±115; P<0.05) and the BT474 mono-culture group (589±50 vs. 1560±127; P<0.05). Collectively, these results indicate that TIGAR expression is associated with Cav-1 downregulation, and that the upregulation of TIGAR contributes to the inhibition of BT474 cell apoptosis mediated by Cav-1 downregulation.

Discussion

The results from the present study demonstrated that the downregulation of Cav-1 in fibroblasts led to a significant increase in the expression and secretion of the growth factors, SDF-1, EGF and FSP-1. Furthermore, it upregulated the expression of TIGAR, which may accelerate tumor cell proliferation and suppress tumor cell apoptosis.

Fibroblasts from tumor stroma may be more likely to trigger tumor growth compared with normal stroma. These fibroblasts secrete high levels of growth factors, extracellular matrix components and matrix metalloproteinases, but the relevant factors and components are not fully understood (13). The downregulation or loss of Cav-1 expression in stromal fibroblasts is associated with tumor prognosis (14). It has been indicated that Cav-1 loss in stromal fibroblasts of patients with breast cancer may be used as a predictor of the relapse of breast cancer, lymph node metastasis and tamoxifen resistance (15,16). This has not been associated with the expression of the estrogen receptor (ER), progesterone receptor (PR) or

human epidermal growth factor receptor-2 (HER2) (15). In patients with $ER^-/PR^-/HER2^-$ breast cancer or $ER^-/PR^-/HER2^-$ ductal carcinoma, the loss of Cav-1 in stromal fibroblasts has been used as an indicator of unfavorable clinical outcome (4). Cav-1 expression in tumor cells is not correlated with breast cancer prognosis (17). Thus, loss of stromal Cav-1 is a key predictor of a 'lethal' cancer microenvironment.

Loss of stromal Cav-1 is also linked with the poor prognosis of prostate cancer and metastasis of bone and lymph nodes (18); and decreased Cav-1 levels in fibroblasts results in increased levels of myofibroblast markers and extracellular matrix proteins in co-cultured human breast cancer cells with fibroblasts, suggesting that Cav-1 downregulation initiates fibroblast activation in tumorigenesis (19). Myofibroblast markers and glycolytic enzymes were observed to be upregulated in a model of cancer-associated Cav-1-deficient fibroblasts under normoxic conditions (20). However, the mechanisms of phenotype transformation from benign to heterogeneous fibroblasts are unclear. Further investigation is required into the molecules associated with Cav-1 expression and tumor stromal fibroblasts and cancer cells, in order to establish multiple Cav-1-specific therapies and further clarify the mechanisms of Cav-1 in tumor growth.

In the present study, fibroblasts were transfected with synthetic siRNA Cav-1 sequences to determine the effect of the downregulation of Cav-1 on tumor stromal and cancer cells. The results indicated that Cav-1 expression was downregulated in the Cav-1 siRNA-transfected cells (Fig. 2), thus the Cav-1 siRNA sequences effectively interfered with Cav-1 gene expression. The siRNA-2 exhibited a higher interference efficacy than the siRNA-1 or the siRNA-3. Therefore, siRNA-2 was selected as the Cav-1-specific interference sequence for the current study.

Tumor occurrence and development are strongly associated with stromal microenvironment. Additionally, cancer-associated fibroblasts are the major stromal cells in this microenvironment. These fibroblasts are derived from the transdifferentiation of various cells, including quiescent fibroblasts, epithelial cells, endothelial cells, mesenchymal stem cells and pericytes (21,22). Cancer-associated fibroblasts in direct contact with tumor cells secrete various paracrine factors, synthesize oncogenic components and connect oncogenic signal pathways to promote the development and progression of tumor cells (23). In the present study, the co-culture models of fibroblasts with breast cancer cells were established to simulate the breast cancer microenvironment. The reduced levels of Cav-1 in the co-culture were utilized to investigate the association between Cav-1 and fibroblasts and cancer cells through analyzing the expression of cancer-associated molecules in fibroblasts and breast cancer cells.

SDF-1, also termed CXCL12, is a chemotactic cytokine belonging to the large family of CXC chemokines (24-28). SDF-1 induces cell migration, cell adhesion, neutrophil activation and inflammation. Previous studies have reported that SDF-1 is associated with tumor occurrence, metastasis and growth (24,25,29). Stromal cells are key sources of SDF-1, and an increase in SDF-1 expression may be associated with tumor growth. The recruitment of endothelial progenitor cells by SDF-1 and its direct effect on cancer cells may promote tumor angiogenesis (26,30). In the current study, the Cav-1 siRNA fibroblasts/breast cancer cell co-culture group was the most effective in increasing SDF-1 expression amongst the groups investigated. This suggests that downregulated Cav-1 and the co-culture with breast cancer cells synergistically increased SDF-1 expression in fibroblasts, and the tumor inhibition effect of Cav-1 may be associated with the inhibition of the signaling pathways in which SDF-1 participates.

EGF is a peptide consisting 53 amino acids, with a variety of biological functions. It stimulates epithelial cell motility, and is thus required for re-epithelialization. It is also a major stimulator of fibroblast migration and wound contraction, and is hypothesized to affect cell proliferation, embryo development and tumorigenesis (31-33). The effect of Cav-1 downregulation on EGF expression in fibroblasts was investigated in the present study. Downregulation of Cav-1 significantly upregulated EGF expression in the fibroblasts. This indicates the antagonistic relationship between Cav-1 upregulation and EGF expression. The microenvironment of the co-cultured Cav-1 siRNA fibroblasts with breast cancer cells was able to enhance the expression of EGF.

FSP-1 (also termed S100A4) is implicated in numerous stages of tumor progression, including motility, invasion and apoptosis, however, its function remains uncertain (34,35). A previous study demonstrated that the co-injection of $FSP\text{-}1^{+/+}$ fibroblasts with tumor cells restores tumor development and metastasis in $FSP\text{-}1^{-/-}$ animals, whereas co-injection with $FSP\text{-}1^{-/-}$ fibroblasts does not (36). The stromal microenvironment can be altered by FSP-1, in order to favor tumor progression. In the current study, the expression of FSP-1 was significantly higher in the Cav-1 siRNA-transfected fibroblasts than in the control-transfected fibroblasts, which suggests that the downregulation of Cav-1 is an upstream event of FSP-1. The Cav-1 siRNA-induced upregulation of SDF-1, EGF and FSP-1 alters the phenotypes of fibroblasts, causing them to become 'reactive'. The microenvironment of reactive fibroblasts is beneficial to tumor growth. The increased concentrations of SDF-1, EGF and FSP-1 in the culture supernatant of Cav-1 siRNA fibroblasts can accelerate the proliferation of tumor cells. The alterations in proliferation of breast cancer cells were consistent with changes in SDF-1, EGF and FSP-1 expression in the current study, which suggests that high expression levels of SDF-1, EGF and FSP-1 can promote breast cancer cell proliferation.

TIGAR may protect cells from ROS-associated apoptosis, and thus, downregulation of the expression of TIGAR may lead to p53-induced cell death (11,37). It has been determined that p53 is not required for TIGAR expression and activity (12). Therefore, in order to identify the function of TIGAR in cancer development, the factors regulating it require further study. The present study identified that the breast cancer cells from the Cav-1 siRNA fibroblasts/breast cancer cell co-culture group presented the highest increase in the expression levels of TIGAR. Downregulation of Cav-1 in fibroblasts influenced the surrounding tumor cells via SDF-1, EGF, FSP-1 and TIGAR. Initially, downregulation of Cav-1 increased the concentrations of the tumor-associated molecules SDF-1, EGF and FSP-1 in tumor stroma. This triggered the accelerated proliferation of tumor cells, which may synergistically influence the expression of TIGAR in cancer cells, suppressing cancer cell

apoptosis. The downregulation of Cav-1 in fibroblasts may not produce direct effects in tumor cells. However, the resulting altered stromal microenvironment (with increased expression levels of SDF-1, EGF and FSP-1) demonstrates its importance in tumor suppression.

Cancer cells rapidly proliferate, and TIGAR expression levels are upregulated in cancer cells (38). TIGAR functions to limit ROS, thus protecting cells against ROS-induced death. As demonstrated in the current study, the upregulation of TIGAR expression was accompanied by low levels of ROS. The Cav-1-targeted cascade reactions observed in the present study may be the hallmark of a malignant breast tumor.

In summary, the current study highlighted Cav-1-targeted molecules and their regulatory events, including the regulation of SDF-1, EGF and FSP-1 expression and secretion in stromal fibroblasts. Downregulation of Cav-1 promotes the upregulation of TIGAR expression in breast cancer cells, resulting in cancer cell proliferation and the suppression of cancer cell apoptosis. These results provide novel insight into the tumor-suppressor mechanism of Cav-1, indicating that Cav-1-dependent signaling involves SDF-1, EGF, FSP-1 and TIGAR.

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References

1. Qian N, Ueno T, Kawaguchi-Sakita N, Kawashima M, Yoshida N, Mikami Y, Wakasa T, Shintaku M, Tsuyuki S, Inamoto T and Toi M: Prognostic significance of tumor/stromal caveolin-1 expression in breast cancer patients. Cancer Sci 102: 1590-1596, 2011.
2. Chiu WT, Lee HT, Huang FJ, Aldape KD, Tao J, Steeg PS, Chou CY, Lu Z, Xie K and Huang S: Caveolin-1 upregulation mediates suppression of primary breast tumor growth and brain metastases by stat3 inhibition. Cancer Res 71: 4932-4943, 2011.
3. Sotgia F, Martinez-Outschoorn UE, Pavlides S, Howell A, Pestell RG and Lisanti MP: Understanding the Warburg effect and the prognostic value of stromal caveolin-1 as a marker of a lethal tumor microenvironment. Breast Cancer Res 13: 213-225, 2011.
4. Witkiewicz AK, Dasgupta A, Nguyen KH, Liu C, Kovatich AJ, Schwartz GF, Pestell RG, Sotiga F, Rui H and Lisanti MP: Stromal caveolin-1 levels predict early DCIS progression to invasive breast cancer. Cancer Biol Ther 8: 1071-1079, 2009.
5. Patani N, Martin LA, Reis-Filho JS and Dowsett M: The role of caveolin-1 in human breast cancer. Brest Cancer Res Treat 131: 1-15, 2013.
6. Sotgia F, Martinez-Outschoorn UE, Howell A, Prestell RG, Pavlides S and Lisanti MP: Caveolin-1 and cancer metabolism in the tumor microenvironment: Markers, models, and mechanisms. Annu Rev Pathol 7: 423-467, 2012.
7. Du C, Chen L, Zhang HJ, Wang ZC, Liu WC, Xie XD and Xie MJ: Caveolin-1 limits the contribution of BKCa channel to MCF-7 breast cancer cell proliferation and invasion. Int J Mol Sci 15: 20706–20722, 2014.
8. Paola C: Cancer associated fibroblasts: The dark side of the coin. Am J Cancer Res 1: 482-497, 2011.
9. Buckley CD: Why does chronic inflammation persist: An unexpected role for fibroblasts. Immunol Lett 138: 12-14, 2011.
10. Wang Z, Wang N, Li W, Liu P, Chen Q, Situ H, Zhong S, Guo L, Lin Y, Shen J, and Chen J: Caveolin-1 mediates chemoresistance in breast cancer stem cells via β-catenin/ABCG2 signaling pathway. Carcinogenesis 35: 2346-2356, 2014.
11. Jen KY and Cheung VG: Identification of novel p53 target genes in ionizing radiation response. Cancer Res 65: 7666-7673, 2005.
12. Lee P, Vousden KH and Cheung EC: TIGAR, TIGAR, burning bright. Cancer Metab 2: 1, 2014.
13. Yamaguchi H and Sakai R: Direct interaction between carcinoma cells and cancer associated fibroblasts for the regulation of cancer invasion. Cancers 7: 2054-2062, 2015.
14. Witkiewicz AK, Dasgupta A, Sammons S, Er O, Potoczek MB, Guiles F, Sotgia F, Brody JR, Mitchell EP and Lisanti MP: Loss of stromal caveolin-1 expression predicts poor clinical outcome in triple negative and basal-like breast cancers. Cancer Biol Ther 10: 135-43, 2010.
15. Witkiewicz AK, Dasgupta A, Sotgia F, Mercier I, Pestell RG, Sabel M, Kleer CG, Brody JR and Lisanti MP: An absence of stromal caveolin-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancers. Am J Pathol 74: 2023-2034, 2009.
16. EI-Gendi SM, Mostafa MF and EI-Gendi AM: Stromal caveolin-1 expression in breast carcinoma. Correlation with early tumor recurrence and clinical outcome. Pathol Oncol Res 18: 459-469, 2012.
17. Shan-Wei W, Kan-Lun X, Shu-Qin R, Li-Li Z and Li-Rong C: Overexpression of caveolin-1 in cancer-associated fibroblasts predicts good outcome in breast cancer. Breast Care (Basel) 7: 477-483, 2012.
18. Di Vizio D, Morello M, Sotgia F, Pestell RG, Freeman MR and Lisanti MP: An absence of stromal caveolin-1 is associated with advanced prostate cancer, metastatic disease and epithelial Akt activation. Cell Cycle 8: 2420-2424, 2009.
19. Martinez-Outschoorn UE, Pavlides S, Whitaker-Menezes D, Daumer KM, Milliman JN, Chiavarina B, Migneco G, Wiktkiewicz AK, Martinez-Cantarin MP, Flomenberg N, *et al*: Tumor cells induce the cancer associated fibroblast phenotype via Caveolin-1 degradation: Implications for breast cancer and DCIS therapy with autophagy inhibitors. Cell Cycle 9: 2423-2433, 2010.
20. Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, *et al*: The reverse Warburg effect: Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 8: 3984-4001, 2009.
21. Kidd S, Spaeth E, Dembinski JL, Dietrich M, Watson K, Klopp A, Battula VL, Weil M, Andreeff M and Marini FC: Direct evidence of mesenchymal stem cell tropism for tumor and wounding microenvironments using in vivo bioluminescent imaging. Stem Cells 27: 2614-2623, 2009.
22. Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, *et al*: A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3: 301-313, 2008.
23. Jezierska-Drutel A, Rosenzweig SA and Neumann CA: Role of oxidative stress and the microenvironment in breast cancer development and progression. Adv Cancer Res 119: 107-125, 2013.
24. Engl T, Relja B, Marian D, Blumenberg C, Müller I, Beecken WD, Jones J, Ringel EM, Bereiter-Hahn J, Jonas D and Blaheta RA: CXCR4 chemokine receptor mediates prostate tumor cell adhesion through alpha5 and beta3 integrins. Neoplasia 8: 290-301, 2006.
25. Peng SB, Peek V, Zhai Y, Paul DC, Lou Q, Xia X, Eesalu T, Kohn W and Tang S: Akt activation,but not extracellular bignal-reguhted kinase activation,is required for SDF-lalpha/CXCR4-mediated migration of epitheloid carcinoma cells. Mol Cancer Res 3: 227-236, 2005.
26. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL and Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121: 335-348, 2005.
27. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H and Kishimoto T: Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382: 635-638,1996.
28. Lewellis SW and Knaut H: Attractive guidance: how the chemokine SDF1/CXCL12 guides different cells to different locations. Semin Cell Dev Bio 23: 333-340, 2012.
29. Balkwill F: The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol 14: 171-179, 2004.
30. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Juang H, Porter D, Hu M, Chin L, Richardson A, *et al*: Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 6: 17-32, 2004.

31. Li CF, Ma Y, Wei YZ and Xue Y: Relationship between VEGF, EGF and invasion, metastasis of gastric cancer cells. Chin J Cancer Res 21: 122-129, 2009.
32. Baek MK, Kim MH, Jang HJ, Park JS, Chung IJ, Shin BA, Ahn BW and Jung YD: EGF stimulates uPAR expression and cell invasiveness through ERK, AP-l, and NF-kappaB signaling in human gastric carcinoma cells. Oncol Rep 20: 1569-1575, 2008.
33. Hardwicke J, Schmaljohann D, Boyce D and Thomas D: Epidermal growth factor therapy and wound healing-past, present and future perspectives. Surgeon 6: 172-177, 2008.
34. Helfman DM, Kim EJ, Lukanidin E and Grigorian M: The metastasis associated protein S100A4: role in tumour progression and metastasis. Br J Cancer 92: 1955-1958, 2005.
35. Tarabykina S, Griffiths TR, Tulchinsky E, Mellon JK, Bronstein IB and Kriajevska M: Metastasis-associated protein S100A4: spotlight on its role in cell migration. Curr Cancer Drug Targets 7: 217-228, 2007.
36. Grum-Schwensen B, Klingelhofer J, Berg CH, El-Naaman C, Grigorian M, Lukanidin E and Ambartsumian N: Suppression of tumor development and metastasis formation in mice lacking the S100A4(mts1) gene. Cancer Res 65: 3772-3780, 2005.
37. Green DR and Chipuk JE: p53 and metabolism: Inside the TIGAR. Cell 126: 30-32, 2006.
38. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E and Vousden KH: TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 126:107-120, 2006.