

## Proteomic alterations of fibroblasts induced by ovarian cancer cells reveal potential cancer targets

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The common spread pattern of ovarian cancer is peritoneal implantation. The growth of the shed ovarian cancer cells in the peritoneal cavity is closely related to the tumor microenvironment. Cancer-associated fibroblasts are vital in the tumor microenvironment. It is not clearly defined that the protein expression alters during the activating process of fibroblasts. This study detected the protein alterations in fibroblasts induced by ovarian cancer cells and explored the potential biological relevance through two-dimensional gel electrophoresis and mass spectrometry. Our data showed that the level of CENPE, BAG2, SOD2, GDI2, CORO1C, CFL1, DSTN, CALD1, PHGDH, PDHA1, AKR1B1, TST and TBCA proteins were significantly up-regulated in the fibroblasts co-cultured with ovarian cancer cells, whereas HSPB1, P4HB and VIM were significantly down-regulated. However, only BAG2, SOD2 and CORO1C proteins were confirmed to be significantly increased by western blot analysis. The differentially expressed proteins were mainly involved in metabolic processes, cellular component organization, responses to stimulus, multicellular organismal processes, localization, protein depolymerization, cellular senescence and the mitotic pathway. These data demonstrated that fibroblasts had an altered protein expression pattern after being induced by ovarian cancer cells, and participated in multiple cell processes resulting in tumor progression. The differentially expressed proteins should be considered as targets for cancer treatment.

*Key words: ovarian neoplasms, fibroblast, stroma, biomarker, proteomics*

Epithelial ovarian cancer is the leading cause of gynecologic malignancies. Although patients at early stages have a good prognosis, approximately 75% of patients are diagnosed at late stages and have an approximately 20% ten-year survival rate [1]. As early diagnosis and treatment are particularly important, abundant research efforts have been put into screening the early biomarkers and therapeutic schedules of ovarian cancer.

The common spread pattern of ovarian cancer is peritoneal implantation. Only 15% of the disease is confined to the ovaries [1]. The ovarian cancer cells shed from the primary lesion, and implant onto the peritoneal surface. The malignant growth of these shed cancer cells might depend not only on abnormal genetic changes but also on the microenvironment in which cancer cells exist [2, 3].

At present, an increasing number of studies has suggested that the development and progression of cancer is closely

associated with the tumor microenvironment. Tumor cells exist in a microenvironment composed of fibroblasts, epithelial cells, pericytes, leukocytes, lymphocytes, extracellular matrices, etc. There is a complex interaction network between tumor cells and the stroma. The tumor stroma responds to oncogenic signals from tumor cells and sends signals to tumor cells and to itself [4]. Tumor cells and stromal cells interact and co-evolve on molecular and cellular levels [5]. As a result, stromal cells are reprogrammed and converted into a suitable microenvironment that promotes cancer initiation, progression and metastasis. Moreover, tumor microenvironment heterogeneity is thought to play an important role in poor therapeutic response [2].

Cancer-associated fibroblasts, the activated fibroblasts, play an important role in the interaction between tumor cells and the stroma. They are abundant in the tumor stroma and different from normal fibroblasts. Cancer-associated fibro-

blasts are activated by tumor cells and then contribute to tumor cell proliferation, invasion and progression. It is now evident that cancer-associated fibroblasts secrete signaling factors to promote cancer development and modify the tumor microenvironment [6, 7]. Cancer-associated fibroblasts have been shown to guide prostate cancer cells to invade and promote tumor organoids growth and invasion in a three-dimensional cell culture model [8]. Importantly, the protein alterations that exist within fibroblasts impact tumor progression. However, the alteration of protein expression during the fibroblast activation process is not clearly defined.

Epithelial ovarian cancer contains different histological types, and the serous type accounts for 75% of the total. In this study, the protein expression within fibroblasts during the interaction process between serous ovarian carcinoma cells and fibroblasts was studied in a co-culture system using two-dimensional gel electrophoresis and mass spectrometry. The potential biological relevance of the differentially expressed proteins was analyzed to clarify the impact of these proteins on ovarian cancer development.

## Materials and methods

**Cell culture and protein preparation.** The human ovarian carcinoma cell line Caov-3 was purchased from the China Center for Type Culture Collection (Wuhan, China). The human ovarian carcinoma cell line SKOV-3 and human fetal lung fibroblast cell line MRC-5 were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Caov-3 and SKOV-3 cells were grown in DMEM medium with 10% fetal bovine serum, and MRC-5 cells were grown in MEM medium with 10% fetal bovine serum.

The MRC-5 fibroblasts were seeded in 6-well plates. SKOV-3 and Caov-3 cells were seeded in cell culture inserts (the pore size was 1  $\mu$ m) for 6-well plates. The cells cultured overnight were washed in phosphate-buffered saline (PBS). The co-culture experiment was performed as we described previously. The inserts with ovarian cancer cells (upper chamber) were placed into the 6-well plate with fibroblasts (lower chamber). Inserts without ovarian cancer cells were also placed into plates as a control. After 96 h of incubation in MEM medium with 1% fetal bovine serum, MRC-5 cells were collected and lysed in buffer (8 M urea, 65 mM DTT, 4% CHAPS, 40 mM Tris, and protease inhibitor cocktail). The cell suspension was then sonicated 5 times on ice. After centrifugation for 40 min at  $13\,400 \times g$  at 4 °C, the supernatant was collected, and the protein concentration was measured using a Bio-Rad protein assay kit.

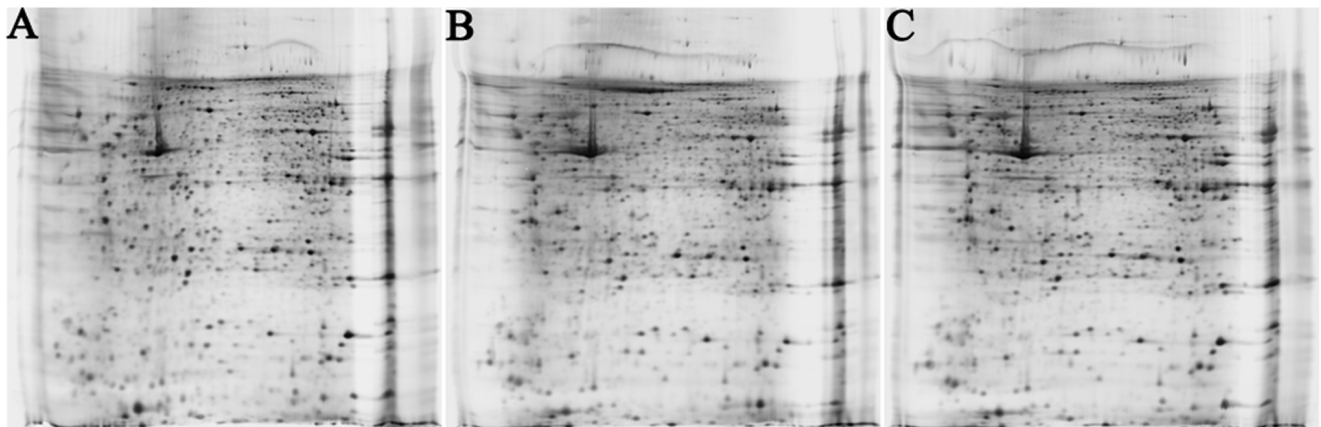
**Two-dimensional gel electrophoresis.** Two-dimensional gel electrophoresis (2-DGE) was used to separate proteins. The first dimension was run on the Ettan IPGphor Isoelectric Focusing System (GE Amersham) with immobilized pH gradient (IPG) strips (nonlinear pH3–10). The IPG strips were rehydrated at 30 V for 12 h in rehydration buffer containing the proteins. Isoelectric focusing (IEF) was

performed at 500 V for 1 h, 1000 V for 1 h, and 8000 V for 8 h. After equilibration, the strips were transferred into 12.5% SDS-polyacrylamide gels for the second dimensional electrophoresis. The electrophoresis was run at 15 mA for 30 min, followed by 30 mA until the bromophenol blue reached the bottom of the gel. After silver staining, the separated protein spots were scanned using UMax Powerlook 2110XL (GE Amersham).

**Mass spectrometry and protein identification.** The differentially stained protein spots were excised from gels, destained and then dried. The gel pieces were rehydrated and incubated with trypsin at 37°C for 24 h. Then, the gel pieces were extracted three times with 60% acrylonitrile (ACN)/0.1% trifluoroacetic acid (TFA) for 15 min with sonication. The resulting peptides were collected and dried. The peptide extracts were spotted onto a MALDI target plate with a saturated matrix solution of CHCA in 50% ACN containing 0.1% TFA. The mass spectra were acquired by a MALDI-TOF/TOF tandem mass spectrometer, 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems). The parameters were: an acceleration voltage of 2 kV, an m/z range of 800–4000, and a signal-to-noise criterion of 50. Acquired mass spectra were searched for sequence matches against the International Protein Index (IPI) human v3.87. The search parameters were a peptide mass tolerance of  $\pm 100$  ppm, a fragment mass tolerance of  $\pm 0.4$  Da, max missed cleavages of 1, and fixed modifications of carbamidomethyl (C).

**Western blotting analysis.** To confirm the MS result, western blotting was used to detect the expression levels of the identified proteins of MRC-5 cells after co-culturing with SKOV-3 or Caov-3 cells. The total protein was separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. After blocking with 5% nonfat milk in PBST (20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h, the membranes were separately incubated with the antihuman antibodies for CENPE and GDI2 (Abcam Ltd., HK), BAG2, SOD2, DSTN, CORO1C, PHGDH, PDHA1, AKR1B1, TST and TBCA (Sigma-Aldrich, Inc.), P4HB (R&D Systems, Inc.), VIM, cofilin, CALD1 and HSP27 (Cell Signaling Technology, Inc.) at 4 °C overnight. Then, the membrane was incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were visualized using an chemiluminescence detection reagent, and imaged by an ImageQuant™ LAS4000 (GE Healthcare LifeSciences).

**Statistical analysis.** Statistical analyses were performed using Student's t-test by SPSS software. The data were expressed as the mean  $\pm$  SD, and a  $p < 0.05$  was considered significant. A Venn diagram of the differentially expressed proteins was performed using the Venny2.1 tool (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>). The functional association network of differentially expressed proteins was built using the STRING search tool (<http://string.embl.de>). A medium confidence of 0.4 was used as the value for edge confidence, and 10 was used as the maximal number



**Figure 1.** Separation of total proteins extracted from MRC-5 cells by two-dimensional gel electrophoresis. (A) MRC-5 cells cultured alone. (B) MRC-5 cells co-cultured with SKOV-3 cells. (C) MRC-5 cells co-cultured with Caov-3 cells.

of predicted functional partners. Gene ontology (GO) was analyzed using the WEB-based GENE SeT AnaLysis Toolkit (WebGestalt, <http://www.webgestalt.org>).

## Results

**Differentially expressed proteins found by 2-DGE.** To investigate the proteome changes of fibroblasts induced by ovarian cancer cells, the proteins extracted from MRC-5 (fibroblast cell line) co-cultured with SKOV-3 or Caov-3 (serous ovarian carcinoma cell line) were analyzed using 2-DGE (Figure 1). The proteins from MRC-5 cultured alone were used as controls. Quantitative analysis showed that a total of 24 protein spots exhibited >1.5-fold change. Eleven protein spots were up-regulated in MRC-5 co-cultured with SKOV-3 cells, whereas one protein spot was down-regulated. Nine protein spots were up-regulated in MRC-5 co-cultured with Caov-3 cells, whereas three protein spots were down-regulated. These proteins were further identified by MALDI-TOF/TOF MS.

**Identification of differentially expressed proteins by MS.** The protein spots with intensity changes >1.5-fold were digested in gels and identified by MALDI-TOF/TOF MS (Figure 2). Sequence matches were performed using the IPI database. As shown in Tables 1 and 2, nine proteins were successfully identified in the MRC-5 co-cultured with SKOV-3 cells group, and ten proteins in MRC-5 co-cultured with Caov-3 cells group were identified. In the MRC-5 co-cultured with SKOV-3 cells group, the up-regulated proteins were identified as centromere protein E (CENPE), BCL2-associated athanogene 2 (BAG2), mitochondrial superoxide dismutase 2 (SOD2), GDP dissociation inhibitor 2 (GDI2), coronin 1C (CORO1C), cofilin 1 (CFL1), destrin (DSTN) and caldesmon 1 (CALD1), and the down-regulated protein was heat shock 27 kDa protein 1 (HSPB1). In the MRC-5 co-cultured with Caov-3 cells group, the up-regulated proteins were identified as CORO1C, SOD2,

phosphoglycerate dehydrogenase (PHGDH), pyruvate dehydrogenase alpha 1 (PDHA1), aldo-keto reductase family 1 member B1 (AKR1B1), thiosulfate sulfurtransferase (TST), BAG2 and tubulin folding cofactor A (TBCA), and the down-regulated proteins were prolyl 4-hydroxylase beta polypeptide (P4HB) and vimentin (VIM).

A comparative analysis between the MRC-5 co-cultured with SKOV-3 cells group and the MRC-5 co-cultured with Caov-3 cells group was performed. As shown in the Venn diagram of Figure 3, a total of 16 differentially expressed proteins were counted. Three of differentially expressed proteins were shared by both groups. The MRC-5 co-cultured with SKOV-3 cells group shared 37.5% of total proteins, and the MRC-5 co-cultured with Caov-3 cells group shared 43.8% of total proteins.

**Potential biological relevance of the differentially expressed proteins.** The differentially expressed proteins identified were further analyzed using GO and the protein-protein interaction network by the WebGestalt and STRING search tools. Most of the differentially expressed proteins were cytoplasmic and mainly related to metabolic processes, cellular component organization, responses to stimulus, multicellular organismal processes, localization, and protein depolymerization. The functional association network of the identified proteins showed direct and indirect links between the identified proteins and other predicted functional partners (Figure 4). Up-regulated protein BAG2 was found to be a partner of HSPA8 and MAPKAPK2, and SOD2 functioned as a partner of AKR1B1 and SOD1, which participated in the cellular response to heat stress and cellular senescence pathways. The up-regulated protein CENPE was found to be a partner of NUF2 and BUB1B in the mitotic pathway, which regulated the cell cycle.

**Identification of differentially expressed proteins by western blotting.** To further confirm the MS result, we used western blotting to detect the expression levels of the identified proteins in MRC-5 cells co-cultured with SKOV-3 or

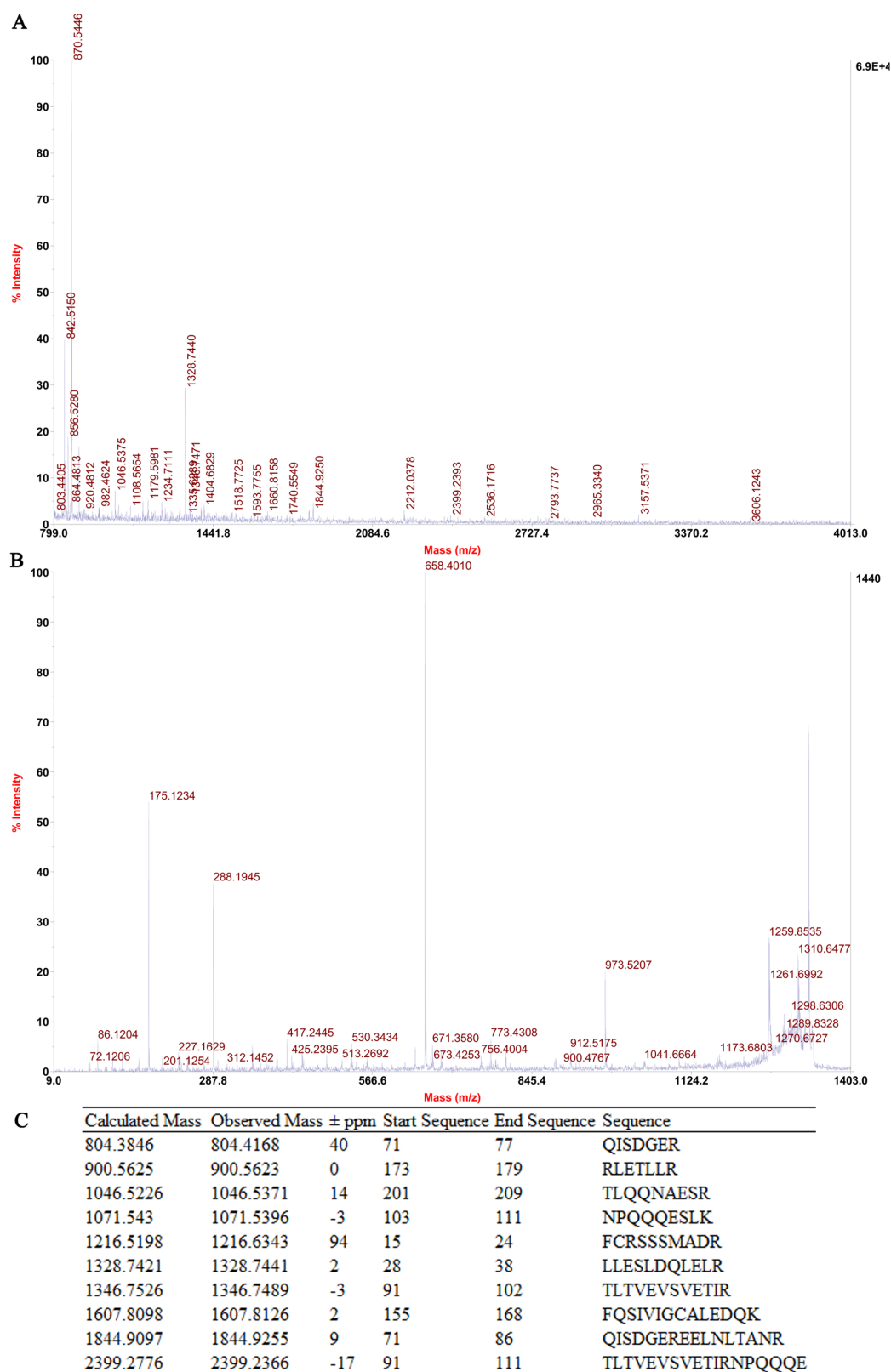


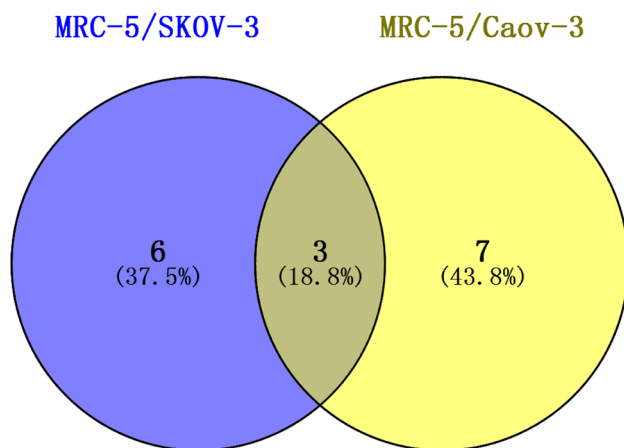
Figure 2. Identification of the representative protein spot by MS. The protein spot was excised from gels, and the digested peptides were analyzed by MALDI TOF/TOF™ Analyzer. (A) MS spectra. (B) MS/MS spectra of ion 1328.74. (C) The identified peptide sequences. The protein was identified as BCL2-associated athanogene 2 (IPI Accession No: IPI00000643).

**Table 1. Differentially expressed proteins identified by MALDI-TOF/TOF after 2-DGE analysis of MRC-5 co-cultured with SKOV-3 cells.**

IPI accession No.	Symbol	Protein name	Protein pI	Protein MW (Da)	MASCOT score	Peptide count	Co-culture/Control ratio
IPI00868715	CENPE	centromere protein E	5.47	313267.6	70	31	3.327
IPI00000643	BAG2	BCL2-associated athanogene 2	6.25	23928.3	257	10	1.995
IPI00847322	SOD2	superoxide dismutase 2, mitochondrial	8.35	24905.7	230	8	1.989
IPI00640006	GDI2	GDP dissociation inhibitor 2	5.91	46046.4	188	14	1.915
IPI01022477	CORO1C	coronin, actin binding protein, 1C	7.71	16867.6	103	4	1.898
IPI00012011	CFL1	cofilin 1 (non-muscle)	8.22	18718.7	304	11	1.859
IPI00473014	DSTN	destrin (actin depolymerizing factor)	8.06	18949.7	164	5	1.788
IPI00926274	CALD1	caldesmon 1	5.66	54183.5	109	13	1.655
IPI00025512	HSPB1	heat shock 27kDa protein 1	5.98	22825.5	627	12	-1.546

**Table 2. Differentially expressed proteins identified by MALDI-TOF/TOF after 2-DGE analysis of MRC-5 co-cultured with Caov-3 cells.**

IPI accession No.	Symbol	Protein name	Protein pI	Protein MW (Da)	MASCOT score	Peptide count	Co-culture/Control ratio
IPI01022477	CORO1C	coronin, actin binding protein, 1C	7.71	16867.6	103	4	2.28643
IPI00847322	SOD2	superoxide dismutase 2, mitochondrial	8.35	24905.7	230	8	2.0935
IPI00642548	PHGDH	phosphoglycerate dehydrogenase	6.47	53678.7	77	4	1.73808
IPI00643575	PDHA1	pyruvate dehydrogenase (lipoamide) alpha 1	8.35	44764.4	79	10	1.65267
IPI00413641	AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	6.51	36229.6	158	11	1.59431
IPI00216293	TST	thiosulfate sulfurtransferase (rhodanese)	6.77	33635.9	164	11	1.56025
IPI00000643	BAG2	BCL2-associated athanogene 2	6.25	23928.3	257	10	1.53444
IPI00973811	TBCA	tubulin folding cofactor A	5.08	14411.2	308	9	1.50953
IPI00878551	P4HB	prolyl 4-hydroxylase, beta polypeptide	4.77	51619.9	166	9	-1.5678
IPI00418471	VIM	vimentin	5.06	53676.1	227	15	-1.77505



**Figure 3. A Venn diagram of differentially expressed proteins. Numbers refer to differentially expressed proteins that belong to each group. MRC-5/SKOV-3 refers to MRC-5 cells co-cultured with SKOV-3 cells. MRC-5/Caov-3 refers to MRC-5 cells co-cultured with Caov-3 cells.**

Caov-3 cells. As shown in Figure 5 and Figure 6, BAG2 and SOD2 proteins were found to be significantly up-regulated when MRC-5 cells were co-cultured with SKOV-3 or Caov-3 cells. The CORO1C protein was found to be up-regulated in MRC-5 cells co-cultured with Caov-3 cells. However, the other differentially expressed proteins found by 2-DGE and MS were not identified by western blotting analysis.

## Discussion

The biological characteristics of tumors are not only connected to tumor cells themselves but also with stroma, which contribute to the proliferation, invasion and metastasis of tumor cells [9, 10]. During cancer progression, the stromal cells can evolve into the activated state, facilitating tumor cell growth through autocrine or paracrine communication and physical effects [11–13]. However, different cancers have different mechanisms. The stroma of epithelial ovarian tumors, Krukenberg tumors and carcinosarcoma

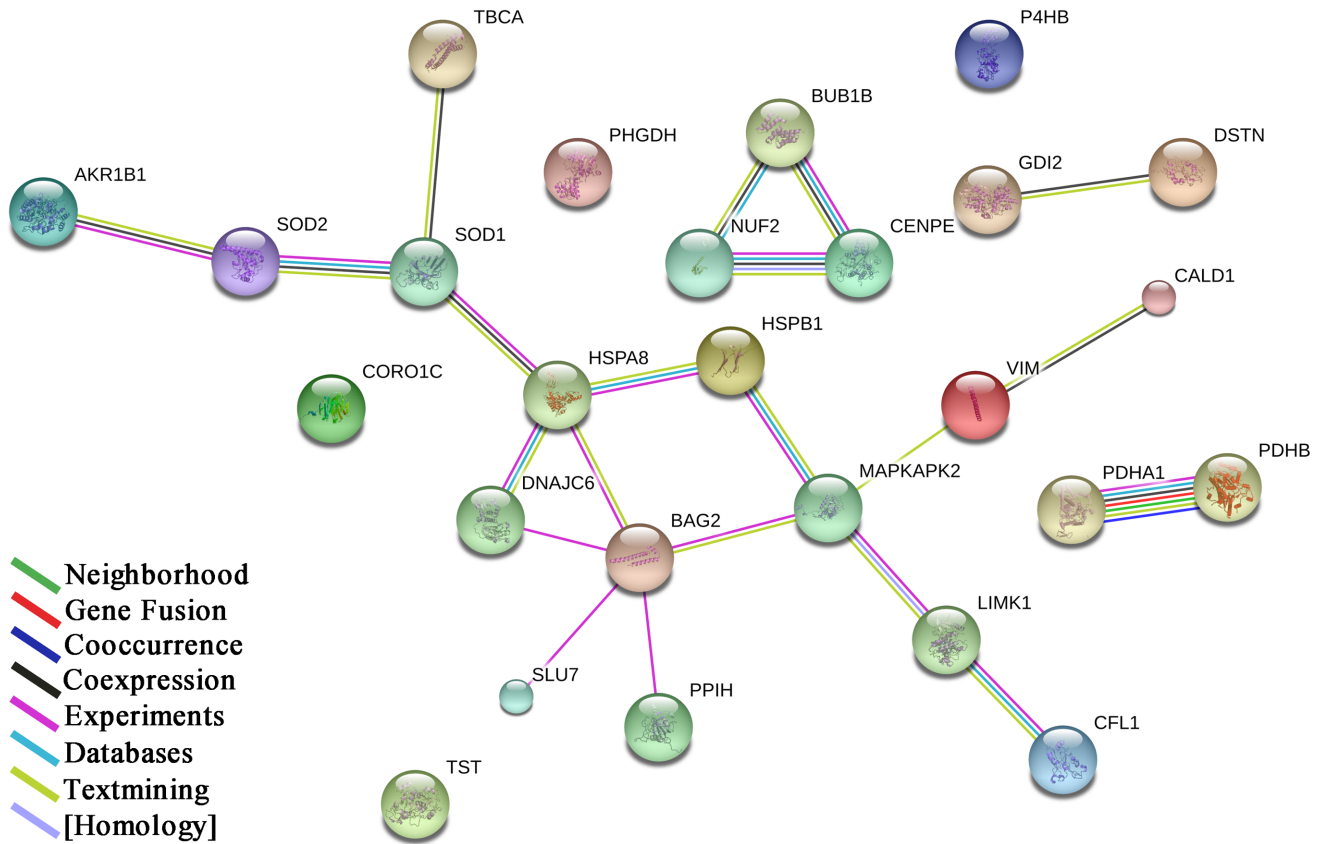


Figure 4. Functional association network of differentially expressed proteins. The network was built using the STRING search tool and showed the direct and indirect links between the identified proteins and other predicted functional partners. Medium confidence of 0.4 was used as the value for edge confidence, and 10 was used as the maximal number of predicted functional partners.

sometimes exhibit endocrine functions, and the functioning stroma can convert androgens to estrogens [14]. Thus, as ovarian cancer is the most malignant gynecological tumor, it is necessary to investigate the special relationships between ovarian cancer and the stroma.

In the present study, we investigated the protein expression alteration of fibroblasts during the interaction between ovarian cancer cells and fibroblasts and analyzed their possible impacts on ovarian cancer development. The data indicated that the level of CENPE, BAG2, SOD2, GDI2, CORO1C, CFL1, DSTN, CALD1, PHGDH, PDHA1, AKR1B1, TST and TBCA were up-regulated in the fibroblasts co-cultured with serous ovarian carcinoma cells, whereas HSPB1, P4HB and VIM were down-regulated. However, only BAG2, SOD2 and CORO1C proteins were confirmed to be significantly increased by western blotting analysis.

BAG2 is a member of the BAG family, which function as co-chaperone proteins of Hsp70/Hsc70 and many other proteins [15]. It has been shown that the BAG family is involved in carcinogenesis. The role of BAG2, one of the six human BAG proteins, in cancer has not been extensively investigated. BAG2 overexpression has been detected in colorectal cancer, lung cancer and breast cancer and is associ-

ated with poor prognosis. BAG2 could be act as a mutant p53 binding protein to facilitate mutant p53 protein accumulation and increase the activity of mutant p53, resulting in the proliferation, migration, metastasis and chemoresistance of

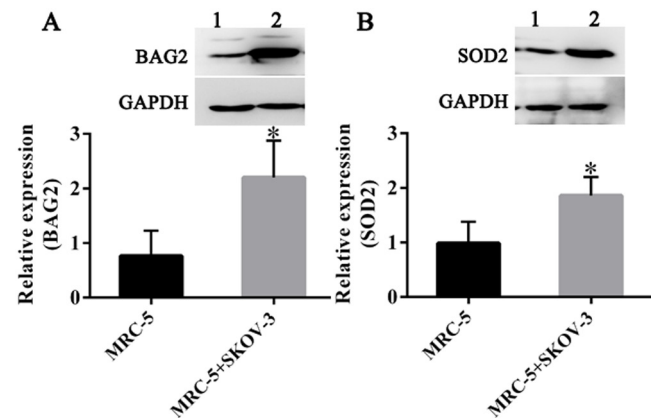
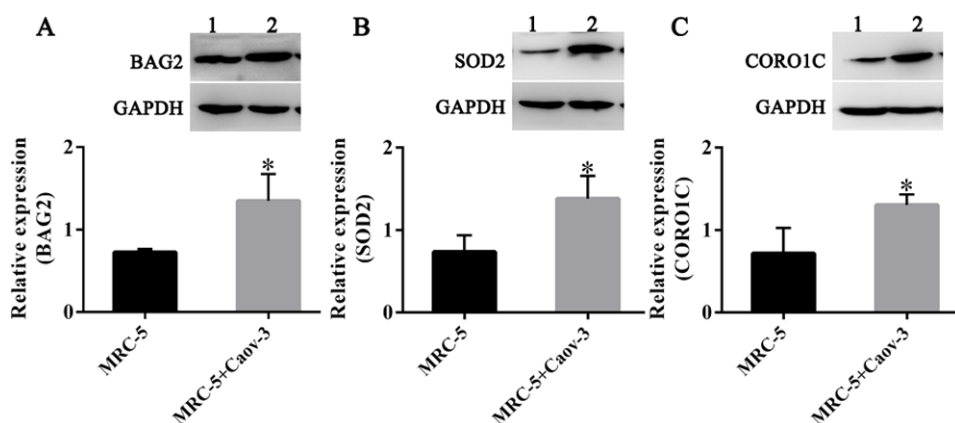


Figure 5. Western blotting validation of differentially expressed proteins in SKOV-3 cells. (A) BAG2 expression. (B) SOD2 expression. Lane 1, MRC-5 cells cultured alone; Lane 2, MRC-5 cells co-cultured with SKOV-3 cells.



**Figure 6.** Western blotting validation of differentially expressed proteins in Caov-3 cells. (A) BAG2 expression. (B) SOD2 expression. (C) CORO1C expression. Lane 1, MRC-5 cells cultured alone; Lane 2, MRC-5 cells co-cultured with Caov-3 cells.

tumor cells [16]. Our study indicated that fibroblasts induced by ovarian cancer cells expressed an elevated level of BAG2, and BAG2 could participate in stress response and cellular senescence pathways. It has also been reported that high levels of BAG2 correlate to cellular senescence as an indirect activator of the p21 pathway [17].

SOD2 is located in the mitochondrial matrix and can catalyze superoxide radical dismutation to  $H_2O_2$  and  $O_2$ . Current studies suggest that SOD2 combines with the UPR<sup>mt</sup> assisting cells to adapt to the high- $H_2O_2$  environment [18]. Elevated steady-state  $H_2O_2$  leads to oxidative inactivation of PTEN and Map kinase phosphatases, which progress the malignant phenotype [19]. SOD2 up-regulation has been observed in advanced breast, prostate and colon cancer tissues and is associated with aggressive types of breast cancer with poorer prognosis. Up-regulated SOD2 is a source of  $H_2O_2$  that activates AMP-activated kinase, thereby sustaining the Warburg effect and promoting cancer cell survival [20, 21]. SOD2 levels increase in cancer-associated fibroblasts of ovarian cancer [22]. Our study indicated that fibroblasts induced by ovarian cancer cells expressed an elevated level of SOD2 that could participate in stress responses and cellular senescence. It has also been reported that cancer-associated fibroblasts of ovarian cancer are mitotically active and resistant to oxidative stress compared with normal fibroblasts. Cancer-associated fibroblasts may avoid  $H_2O_2$ -induced cell death through cellular senescence [22].

CORO1C is a conserved actin binding protein that regulates the actin dependent processes. It is involved not only in cytoskeleton formation and stability, cell motility and membrane trafficking but also in tumor metastasis [23]. CORO1C is more highly expressed in metastatic lymph node tissues than in primary gastric cancer tissues [24]. Elevated levels of CORO1C change the morphology and migratory behavior of cells by promoting more rapid actin dynamics, which contributes to tumor metastasis and may be associated with lower survival in aggressive triple-negative

breast cancer [25, 26]. Knockdown of CORO1C reduces cell polarity and disrupts the cytoskeleton, resulting in the suppression of cell proliferation, migration and invasion in hepatocellular and lung-squamous cell carcinoma [26, 27]. However, the relationship of CORO1C and ovarian cancer is poorly understood. The tumor cell-derived microparticles generated by ovarian adenocarcinoma cells contain several proteins involved in cancer progression, including CORO1C [28]. The fibroblasts induced by ovarian cancer cells overexpressed CORO1C in our study, which might be one of molecular mechanisms of tumor progression via regulating the actin cytoskeleton.

In addition, CENPE was obviously elevated in the fibroblasts induced by ovarian cancer cells in our study. CENPE is one of major spindle checkpoint genes and regulates the cell cycle. Down-regulation of CENPE leads to chromosome misalignment and delayed mitotic progression [29]. However, the clinical correlations and roles of CENPE in cancer are not quite clear at present. In chemoresistant epithelial ovarian cancer, cell cycle regulating genes, including CENPE, are up-regulated [30]. CENPE knockdown induces suppression of neuroblastoma cell proliferation and delays tumor growth via mitotic arrest, which indicates that targeting CENPE may be a novel therapeutic strategy for cancer [31].

In recent years, many of the hallmarks of cancer have been found to be related to the tumor microenvironment, including proliferation, angiogenesis, immune evasion, etc. [32]. The tumor microenvironment should be considered as a target for cancer prevention and treatment, such as the blocking of interactions between cancer cells and cancer-associated fibroblasts [8, 32].

In conclusions, this study indicated that fibroblasts had an altered protein expression pattern after induction by ovarian cancer cells. The differentially expressed proteins might impact metabolic processes, stress responses, cellular senescence, the cytoskeleton and the mitotic pathway, resulting in tumor progression, and should be considered as targets for cancer

treatment. During the interaction process between tumor and fibroblasts, fibroblasts might be activated and then facilitate tumor cell growth through cell-cell or molecular interactions. However, the tumor microenvironment has complex components and dynamic changes in tumor initiation and progression. It is necessary to further elucidate specific molecular mechanisms in the tumor micro environment.

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