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Original Article

Mucinous and serous ovarian carcinoma cells interact differently with stromal fibroblasts

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Abstract: The interactions between cancer cells and fibroblasts in the stroma are essential for cancer development and progression. Epithelial ovarian cancer is a heterogeneous disease. Each histological type has different clinical characteristics and molecular mechanisms. A better understanding of the different molecular mechanisms may provide novel approaches for the treatment of ovarian cancer. We analyzed the cytokine profiles found in the interaction between ovarian cancer cells and fibroblasts and compared the results of serous and mucinous ovarian carcinoma. The levels of the epithelial neutrophil-activating protein 78 (ENA-78) wassignificantly up-regulated in the conditioned media of fibroblasts co-cultured with serous ovarian carcinoma cells. TIMP metallopeptidase inhibitor 1 (TIMP1), TIMP2 and fibroblast growth factor 2 (basic) (FGF2) were significantly up-regulated, and ten other cytokines were down-regulated when fibroblasts were co-cultured with mucinous ovarian carcinoma cells. The differential expression of these proteins was mainly related to cell locomotion, migration, communication and stimuli response. These data suggested that the cross-talk between serous ovarian cancer and its stroma might be distinct from that of mucinous ovarian cancer and that the interactions between ovarian cancer cells and fibroblasts caused changes in the expression of autocrine or paracrine signals in these cells, which might in turn contribute to cancer development and progression.

Keywords: Ovarian neoplasms, fibroblast, stroma, serous ovarian carcinoma, mucinous ovarian carcinoma

Introduction

Most patients with epithelial ovarian cancer are diagnosed at an advanced stage because the early disease causes few symptoms and early detection is difficult. Despite extensive cytoreductive surgery, chemotherapies and molecular targeted therapy, the majority of patients will experience recurrence and die from refractory disease. Thus, epithelial ovarian cancer is still the most lethal gynecologic malignancy. Although some abnormal changes of oncogenes and tumor suppressor genes have been found to be involved in the etiology of ovarian cancer [1], the development and progression of the disease have not been clearly elucidated.

Previous studies of ovarian cancer have focusedprimarily on the ovarian cancer cells individually rather than the tumor tissue as a whole. However, tumorsdo not consist of a cluster of cancer cells but ratherof a mixture of tumor cells and the surrounding stroma.Recent studies have shown that the surrounding stroma exerts a crucialfunction for cancer cells and is not simply a passive bystander [2, 3]. In the development and progression of cancer, cancer cells continuously recruit and reprogram normal stromal cells to create a suitable microenvironment for themselves. In addition, the stroma surrounding cancer cells promotes the initiation, progression and metastasis of cancer [4-6].

In the complex microenvironment of tumor cells, fibroblasts can promote the malignant transformation of epithelial cells, tumor growth, invasion and metastasis [7, 8]. Cancer-

Table 1. Protein analysis in cytokine antibody array 6

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	NEG	NEG	Blank	Angiogenin	BDNF	BLC	BMP4	BMP6	CK β 8-1	CNTF	EGF	Eotaxin
2	POS	POS	NEG	NEG	Blank	Angiogenin	BDNF	BLC	BMP4	BMP6	CK β 8-1	CNTF	EGF	Eotaxin
3	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Fit-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-γ	IGFBP1	IGFBP2	IGFBP4
4	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Fit-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-γ	IGFBP1	IGFBP2	IGFBP4
5	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
9	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGFB1	TGFB3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS
10	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGFB1	TGFB3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS

associated fibroblasts are different from those in the normal stroma with regard to morphology and function. They exhibit the biological characteristics of myofibroblasts [9]. Cancer-associated fibroblasts are stimulated and activated by cancer cellsand produce paracrine factors, such as stromal cell-derived factor 1, that influence cancer cells [9, 10]. Consequently, the interactions between cancer cells and fibroblasts lead to their activation and promote tumor growth. However, the cross-talk network between ovarian cancer cells and fibroblasts is not clearly defined.

Epithelial ovarian cancer is a heterogeneous disease. Each histological type has different clinical characteristics and molecular mechanisms. A better understanding of the different molecular mechanisms may provide novel approaches for the treatment of ovarian cancer. Therefore, in this study, the cytokine profiles for the interaction between serous or mucinous ovarian carcinoma cells and fibroblasts were detected usingcytokine antibody arrays containing 120 cytokines. Moreover, the potential biological relevance of the differentially expressed cytokines was analyzed using bioinformatics tools.

Materials and methods

Cell culture

The human serous ovarian carcinoma cell line Caov-3 was purchased from the China Center for Type Culture Collection (Wuhan, China). The humanmucinous ovarian carcinoma cell line OMC685 was obtained from Zunyi Medical College (Zunyi, China). The human fetal lung fibroblast cell line MRC-5 was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Caov-3 cells were grown in DMEM medium, OMC685 cells in RPMI 1640 medium, and MRC-5 cells in MEM medium. The mediawere supplemented with 10% fetal bovine serum, and the cells wereculture at 37°C in a 5% CO_2 environment.

Conditioned media collection

The MRC-5 fibroblasts were seeded in 6-well plates at a density of 1×10^5 cells per well and cultured to 60% confluence. The ovarian cancer cells Caov-3 and OMC685 were seeded in cell culture inserts for 6-well plates at a density of 1×10^5 cells per well and cultured overnight. The cells were then washed twice in phosphate-buffered saline (PBS) and incubated in serum-free fibroblast medium. For the co-culture experiments, the inserts with ovarian cancer cells (upper chamber) were transferred to the wells with fibroblasts (lower chamber). Inserts without ovarian cancer cells were also placed in the wells with fibroblasts as a control. The pore size of the cell culture insert was 1 μm, which is sufficient for basolateral diffusion to allow communication between two cell types. After 48 h of incubation, the conditioned media of MRC-5 co-cultured with Caov-3 or OMC685, MRC-5 cultured alone, and Caov-3 or OMC685 cultured alone were collected, centrifuged and frozen at -80°C.

Cytokine array analysis

To evaluate the levels of cytokines in the different conditioned media, the RayBio Human Cytokine Antibody Arrays 6 and 7 (RayBiotech Inc., Norcross, USA) were used. As shown in **Tables 1** and **2**, Cytokine Antibody Arrays 6 and 7 allow the expression levels of 120 different cytokines to be determined. The co-culture

Table 2. Protein analysis in cytokine antibody array 7

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	NEG	NEG	Blank	Acrp30	AgRP	Angiopoietin-2	Amphiregulin	Axl	bFGF	b-NGF	BTC	CCL-28
2	POS	POS	NEG	NEG	Blank	Acrp30	AgRP	Angiopoietin-2	Amphiregulin	Axl	bFGF	b-NGF	BTC	CCL-28
3	CTACK	Dtk	EGF-R	ENA-78	Fas/TNFRSF6	FGF-4	FGF-9	GCSF	GITR-Ligand	GITR	GRO	GRO-α	HCC-4	HGF
4	CTACK	Dtk	EGF-R	ENA-78	Fas/TNFRSF6	FGF-4	FGF-9	GCSF	GITR-Ligand	GITR	GRO	GRO-α	HCC-4	HGF
5	ICAM-1	ICAM-3	IGFBP3	IGFBP6	IGF-I SR	IL-1 R4/ST2	IL-1 RI	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R alpha	IL-6 R	IL-8
6	ICAM-1	ICAM-3	IGFBP3	IGFBP6	IGF-I SR	IL-1 R4/ST2	IL-1 RI	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R alpha	IL-6 R	IL-8
7	I-TAC	Lymphotactin	MIF	MIP-1α	MIP-1β	MIP-3β	MSP-α	NT-4	Osteoprotegerin	Oncostatin M	PIGF	sgp130	sTNF RII	sTNF-RI
8	I-TAC	Lymphotactin	MIF	MIP-1α	MIP-1β	MIP-3β	MSP-α	NT-4	Osteoprotegerin	Oncostatin M	PIGF	sgp130	sTNF RII	sTNF-RI
9	TECK	TIMP1	TIMP2	Thrombopoietin	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	Blank	Blank	Blank	Blank	POS
10	TECK	TIMP1	TIMP2	Thrombopoietin	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	Blank	Blank	Blank	Blank	POS

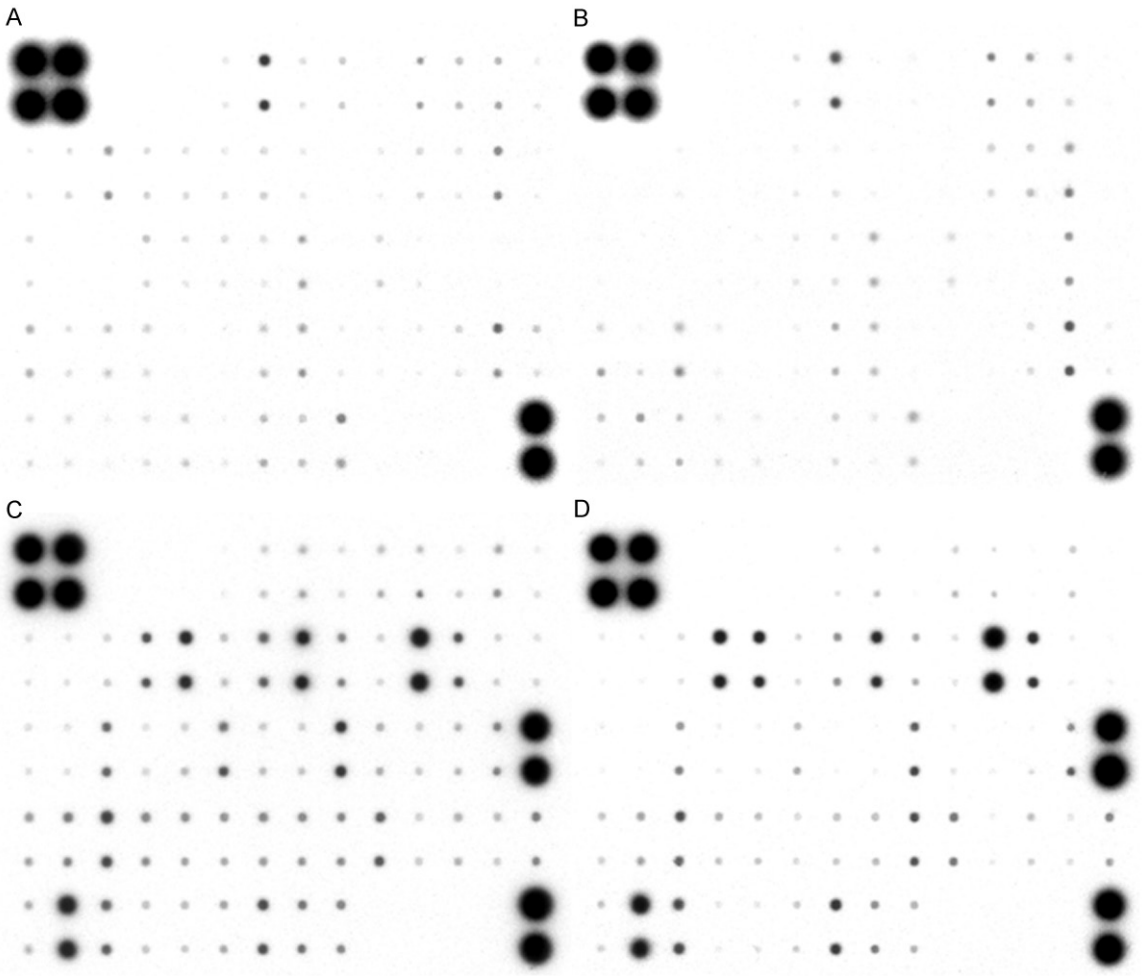

Figure 1. Differential expression of cytokines in the conditioned medium of MRC-5 co-cultured with Caov-3 cells. A. Array 6 incubated with the mixture of the medium of MRC-5 or Caov-3 cells cultured separately. B. Array 6 incubated with the conditioned medium of MRC-5 cells co-cultured with Caov-3 cells. C. Array 7 incubated with the mixture of the medium of MRC-5 or Caov-3 cells cultured separately. D. Array 7 incubated with the conditioned medium of MRC-5 cells co-cultured with Caov-3 cells.

conditioned media of MRC-5 with Caov-3 or OMC685 were analyzed according to the manufacturer's instructions. The mixtures of the conditioned medium of MRC-5 cultured alone and of Caov-3 or OMC685 cultured alone were analyzed as controls. In brief, the array membranes were blocked with blocking buffer for 30 min and then incubated with different conditioned media at room temperature for 2 h. After washing, the array membranes were incubated with diluted biotin-conjugated anti-cytokine antibodies at 4°C overnight. After washing, horseradish peroxidase-conjugated streptavidin was added to the membranes. After incubation and washing, the array membranes were incubated with the detectionbuffers. The chemiluminescence signal was then measuredon the array membranes. For all membranes, the biotin-conjugated IgG was used as a positive control to normalize the expression levels among the different membranes.

Statistical analysis

Statistical analyses were performed using Student's t test, and a P value <0.05 was considered significant. The interacting network of differentially expressed proteins was built using the STRING search tool (version 9.1, http://string.embl.de). The highlevel of confidence of 0.7 was used as the value for edge confidence, and 20 was used as the maximal number of predicted interactions. Gene ontology (GO) was analyzed using the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt, http://bioinfo.vanderbilt.edu/webgestalt/).

Table 3. Differentially expressed proteins in the conditioned media of MRC-5 co-cultured with Caov-3 or OMC685 cells

Symbol	Full name	Co-culture/ Control ratio	*P*-value	Cells co-cultured
CXCL5 (ENA-78)	Chemokine (C-X-C motif) ligand 5 (Epithelial neutrophil-activating protein 78)	2.111	0.049	Caov-3
TIMP2	TIMP metallopeptidase inhibitor 2	2.753	0.021	OMC685
TIMP1	TIMP metallopeptidase inhibitor 1	1.459	0.009	OMC685
FGF2 (bFGF)	Fibroblast growth factor 2 (basic)	1.334	0.035	OMC685
CCL16 (HCC-4)	Chemokine (C-C motif) ligand 16 (Hemofiltrate CC Chemokine 4)	0.765	0.005	OMC685
CSF1 (M-CSF)	Colony stimulating factor 1 (macrophage)	0.617	0.042	OMC685
BMP4	Bone morphogenetic protein 4	0.593	0.044	OMC685
TGFB3	Transforming growth factor beta-3	0.514	0.017	OMC685
CCL22 (MDC)	Chemokine (C-C motif) ligand 22	0.465	0.012	OMC685
ANG	Angiogenin	0.423	0.022	OMC685
IGFBP1	Insulin-like growth factor binding protein 1	0.418	0.011	OMC685
GDNF	Glial cell-derived neurotrophic factor	0.388	0.041	OMC685
CXCL6 (GCP-2)	Chemokine (C-X-C motif) ligand 6 (Granulocyte chemotactic protein 2)	0.372	0.017	OMC685
CXCL13 (BLC)	Chemokine (C-X-C motif) ligand 13 (B-lymphocyte chemoattractant)	0.336	0.030	OMC685

Results

Differentially expressed cytokines identified in the conditioned media of fibroblasts co-cultured with serous ovarian carcinoma cells

To identify the cytokine profileof the interaction between serous ovarian carcinoma cells and their stroma, the co-culture conditioned medium of MRC-5 (fibroblast cell line) with Caov-3 (serous ovarian carcinoma cell line) was analyzed using cytokine antibody arrays for 120 cytokines. The mixtures of the conditioned medium of MRC-5 cultured alone and of Caov-3 cultured alone were used as controls.The cytokineexpression levels were compared between the co-culture group and the control (**Figure 1**). The level of only one cytokine, epithelial neutrophil-activating protein 78 (ENA-78), was significantly up-regulated with a ratio of 2.11 in the co-culture group (**Table 3**), which suggested that ENA-78 could beinvolved in the interaction between MRC-5 and Caov-3 cells.Although the expression changes of 112 cytokines were ≥1.3-fold or ≤0.77-fold, the changes didnot reach statistical significance with *P* values >0.05.

Differentially expressed cytokines identified in the conditioned media of fibroblasts co-cultured with mucinous ovarian carcinoma cells

The clinicopathological features of mucinous ovarian carcinoma differ from those of serous ovarian carcinoma. Therefore, the co-culture conditioned medium of MRC-5 with OMC685 (mucinous ovarian carcinoma cell line) was also analyzed using cytokine antibody arrays in this study (**Figure 2**). The mixtures of the conditioned medium of MRC-5 cultured alone and of OMC685 cultured alone were used as controls. Overall, changes in the expression of 93 cytokines were ≥1.3-fold or ≤0.77-fold compared with the control. However, only 13 cytokines were shown to be significantly up-regulated or down-regulated. TIMP metallopeptidase inhibitor 1 (TIMP1), TIMP2 and fibroblast growth factor 2 (basic) (FGF2) were significantly increased, whereas chemokine (C-C motif) ligand 16 (CCL16), colony stimulating factor 1 (macrophage) (CSF1), bone morphogenetic protein 4 (BMP4), transforming growth factor beta-3 (TGFB3), chemokine (C-C motif) ligand 22 (CCL22), angiogenin (ANG), insulin-like growth factor binding protein 1 (IGFBP1), glial cell-derived neurotrophic factor (GDNF), chemokine (C-X-C motif) ligand 6 (CXCL6) and chemokine (C-X-C motif) ligand 13 (CXCL13) were significantly decreased in the co-culture group (**Table 3**). These results suggested that these 13 cytokines could be involved in the interaction between MRC-5 and OMC685 cells.

Pathways and biological interaction networks involved in the interaction of fibroblasts and ovarian carcinoma cells

To further investigate the potential biological relevance of the differentially expressed cyto-

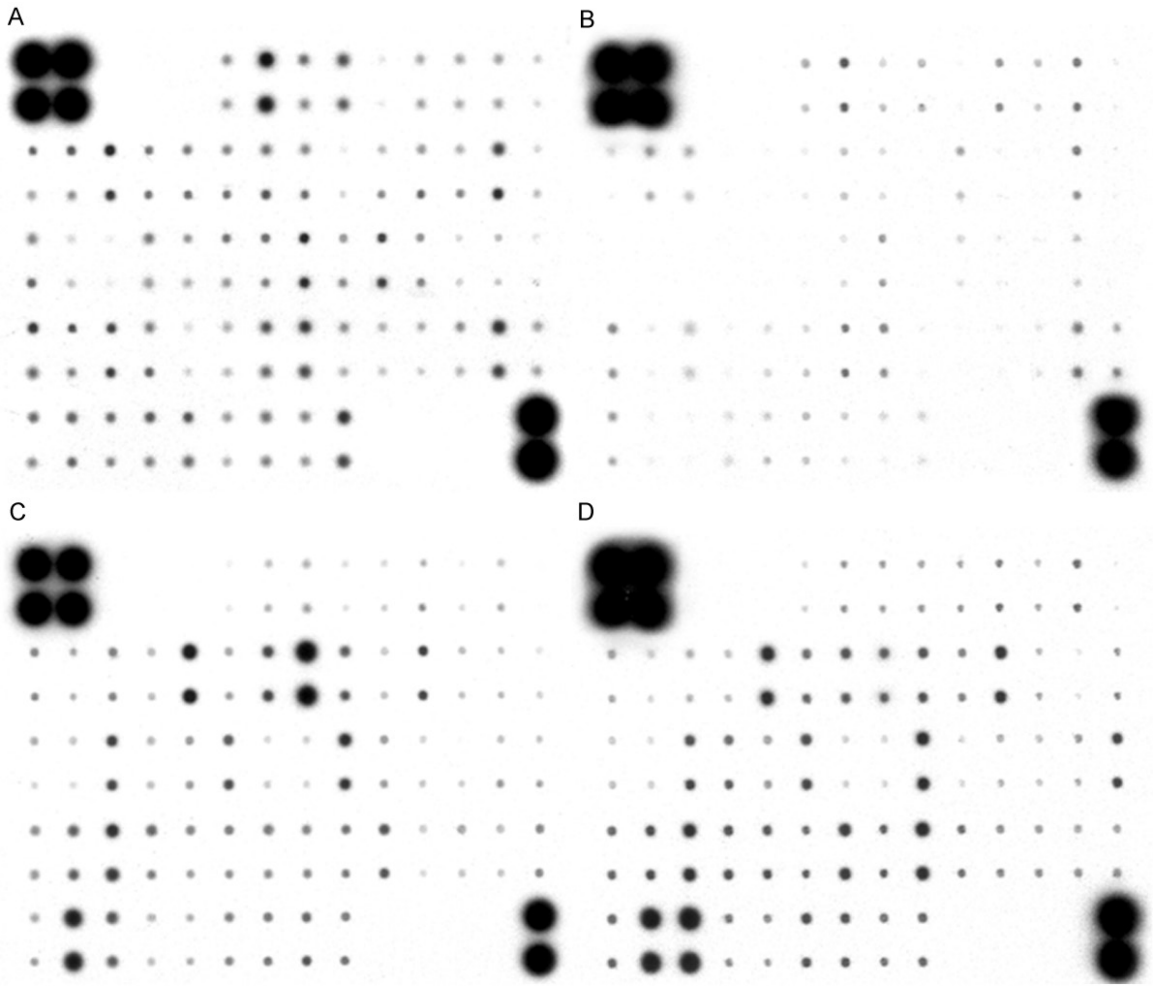

Figure 2. Differential expression of cytokines in the conditioned medium of MRC-5 co-cultured with OMC685 cells. A. Array 6 incubated with the mixture of the medium of MRC-5 or OMC685 cells cultured separately. B. Array 6 incubated with the conditioned medium of MRC-5 cells co-cultured with OMC685 cells. C. Array 7 incubated with the mixture of the medium of MRC-5 or OMC685 cells cultured separately. D. Array 7 incubated with the conditioned medium of MRC-5 cells co-cultured with OMC685 cells.

kines involved in the interaction between ovarian carcinoma cells and their stroma, GO and interacting network analyses were performed using the WebGestalt and STRING search tools. The analysis was limited to the differentially expressed cytokines identified in the MRC-5 and OMC685 co-culture groupbecause only one differentially expressed cytokine was identified in the MRC-5 and Caov-3 co-culture group.

These proteins were found to be associatedwith biological process categories related to cell locomotion, stimuli response, cell communication and biological regulation. The main biological processes were as follows: locomotion and migration (CCL16, CXCL13, TIMP1, ANG, FGF2, CSF1, CXCL6, CCL22, GDNF and BMP4), chemotaxis (CCL22, GDNF, BMP4, CCL16, CXCL13, FGF2 and CXCL6), response to chemical stimulus (TIMP2, CCL16, CXCL13, TIMP1, ANG, FGF2, IGFBP1, TGFB3, CXCL6, CCL22, GDNF and BMP4), response to wounding (CCL16, CXCL13, TIMP1, FGF2, IGFBP1, CSF1, TGFB3, CXCL6 and CCL22), positive regulation of catalytic activity (CXCL13, ANG, FGF2, IGFBP1, CSF1, TGFB3, GDNF and BMP4), positive regulation of protein kinase and transferase activity (BMP4, ANG, FGF2, IGFBP1, CSF1 and TGFB3), regulation of multicellular organismal development (TIMP2, CXCL13, TIMP1, FGF2, CSF1, TGFB3, GDNF and BMP4), cell communication and cell-cell signaling (TIMP2, CCL16, CXCL13, ANG, FGF2, IGFBP1,

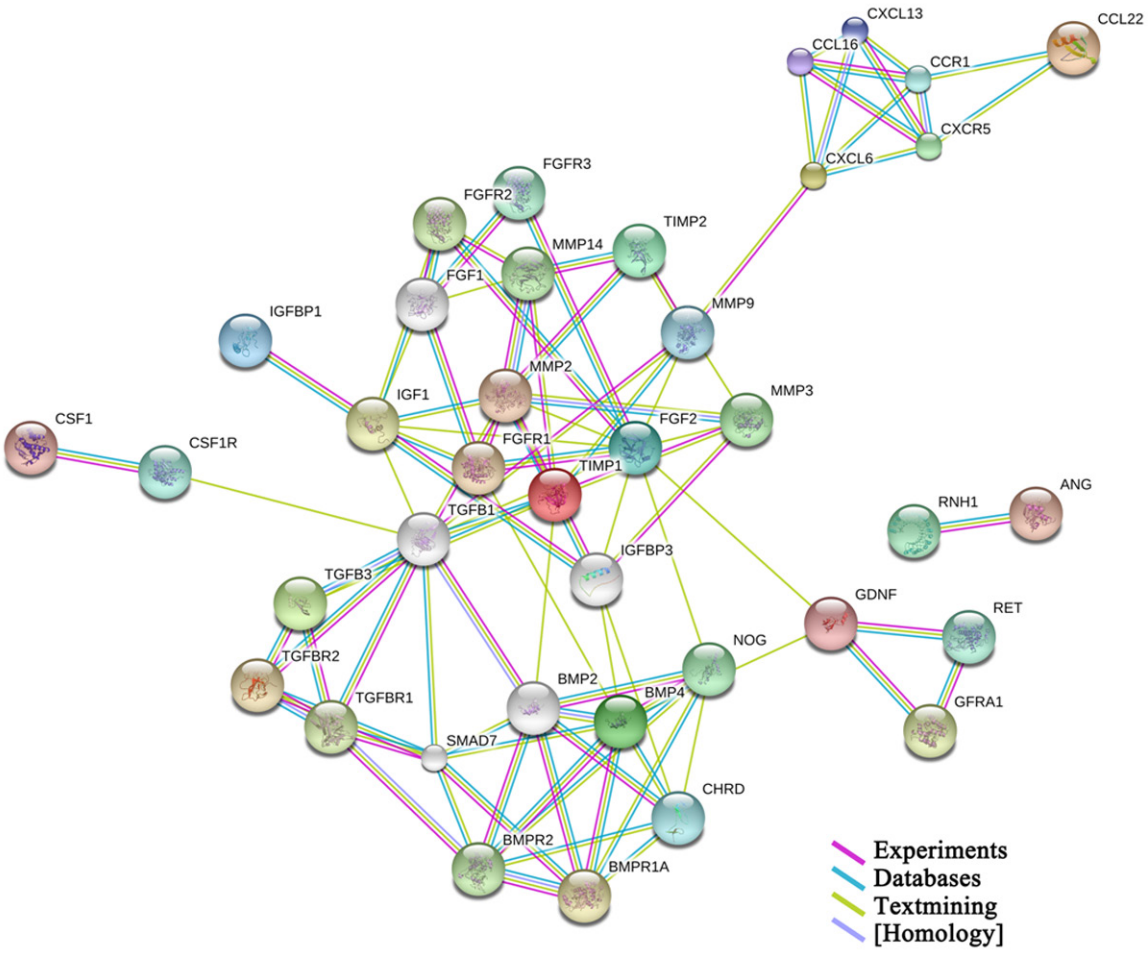

Figure 3. Network of differentially expressed proteins in the conditioned media of MRC-5 co-cultured with OMC685 cells.

CSF1, TGFB3, CXCL6, CCL22, GDNF and BMP4) and negative regulation of cell death (GDNF, BMP4, CXCL13, TIMP1, FGF2 and TGFB3). The interacting network between ovarian carcinoma cells and their stroma was then constructedusing the STRING search tool (**Figure 3**). The network used the differentially expressed proteins identified in the MRC-5 and OMC685 coculture groupas seed nodes and also included the predicted functional partners.

Discussion

In the present study, we investigated the cytokine profiles involved in the interaction between serous or mucinous ovarian carcinoma cells and fibroblasts using cytokine antibody arrays for 120 cytokines. Statistical analyses indicated that the level of ENA-78 was significantly increased in the conditioned media ofthe fibroblasts co-cultured with serous ovarian carcinoma cells. However, the differentiated proteins identified in the conditioned media ofthe fibroblasts co-cultured with mucinous ovarian carcinoma cells were different than those of serous ovarian cancer. TIMP1, TIMP2 and FGF2 were significantly up-regulated, whereas CCL16, CSF1, BMP4, TGFB3, CCL22, ANG, IGFBP1, GDNF, CXCL6 and CXCL13 were significantly down-regulated.

ENA-78, also known as chemokine (C-X-C Motif) ligand 5, can recruit neutrophils and promote angiogenesis. ENA-78 has been reported to beover-expressed and to promote the proliferation, migration, invasion and angiogenesis of cancer cells [11-14]. The expression of ENA-78 by cancer cells could be enhanced by the interaction between the tumor and its stroma [15]. In addition, fibroblasts activated by platelet

derived growth factor B secreted ENA-78 at anenhanced level [16]. In our study, the amount of ENA-78 that was secreted was up-regulated in the co-cultures of serous ovarian carcinoma cells with fibroblasts. This elevated level of ENA-78 could be derived from ovarian cancer cells and/or the activated fibroblasts. ENA-78 has also been found to be up-regulated in ovarian cancer and endometriosis tissues, suggesting that ENA-78 is involved in inflammation and carcinogenesis of theovary [17].

TIMPs are the natural inhibitors of matrix metalloproteinases (MMPs), which participate in the degradation of the extracellular matrix (ECM). In addition to regulating ECM homeostasis, MMPs and TIMPs are also involved in tumor development and progression. Elevatedlevels of TIMP1 have been found in ovarian cancer tissues, plasma and serum and are associated with the aggressive behavior and poor prognosis of ovarian cancer [18-21]. However, conflicting results have been reported regardingthe down-regulation TIMP1 in ovarian cancer tissues [22, 23]. Here, we showed that the secretion of TIMP1 was significantly up-regulated in co-cultures of fibroblasts with mucinous ovarian carcinoma cells. TIMP1 has also been reported to be up-regulated inthe cystic fluids of mucinous ovarian carcinoma, prostate cancer stroma, and lung cancer-associated fibroblasts and in co-cultures of oral squamous carcinoma cells and fibroblasts [24-27]. The high level of TIMP1 promotes the accumulation, proliferation and migration of cancer-associated fibroblasts [25].

In addition to inhibiting the activation of MMPs, TIMP2 is associated with tumor development and progression by MMP-dependent and -independent mechanisms [28]. However, paradoxical results have been reported, such as decreased levels of TIMP2 expression and the antitumor and antiangiogenic roles of TIMP2 in cancer [28-30]. High levels of TIMP2 have been detected in the plasma and tissues of patients with ovarian cancer, whichmay favor tumorigenesis [20, 31]. Conflicting results showed that the levels of TIMP2 were decreased in ovarian cancer tissues, except for clear cell carcinoma, and less abundant in mucinous carcinoma/borderline cystic fluids than in adenoma fluids [24, 32]. TIMP2 is known to be localized to both epithelial tumor cells and stromal cells [31, 33]. The expression of stromal and epithelial TIMP2 ishigher in malignant ovarian tumors than in borderline tumors, either serous or mucinous tumors [23]. The overexpression of TIMP2 in the stromal compartment and simultaneous overexpression in the stromal and tumor compartments are associated with a favorable prognosis of ovarian cancer [34]. In this study, the level of secreted TIMP2 was significantly increased in co-cultures of fibroblasts with mucinous ovarian carcinoma cells. Consistently, TIMP2 was also actively secreted by cancer-associated fibroblasts or myofibroblasts in lung and gastric cancer [27, 35]. The overexpression might be stimulated by tumor-host crosstalk between cancer cells and stroma, which contributes to ECM degradation and cancer dissemination and metastasis [35].

FGF2 has mitogenic and proangiogenic roles and is reported to be involved in tumor growth, angiogenesis and metastasis [36]. The role of FGF2 in ovarian cancer remains controversial. High levels of FGF2 in the tissues, ascites and serum of malignant tumors, including ovarian cancer,have been reported and contribute to cancer development [37-39]. In contrast, Szubert et al. indicated that the expression of FGF2 is lower in ovarian cancer than benign ovarian tumors and normal ovaries [40]. In our study, the expression of FGF2 was significantly elevated in co-cultures of fibroblasts with mucinous ovarian carcinoma cells. Recent studies have consistently shown that hepatocellular carcinoma cells stimulate cancer-associated fibroblasts to produce FGF2 [41]. In turn, FGF2 enables normal fibroblasts to acquire the capacitiesof cancer-associated fibroblasts, which are vital for cancer development [42].

In addition, this study showed the down-regulation of such cytokines as CCL16, CSF1, BMP4, TGFB3, CCL22, ANG, IGFBP1, GDNF, CXCL6 and CXCL13. These cytokines are mainly related to cell locomotion, migration, motility and chemotaxis. The decrease in the levels of these cytokines might contribute to degradation or ligand-receptor binding and internalization.

Conclusions

Taken together, our data suggested that the interaction between ovarian cancer cells and fibroblasts caused changes in the expression of autocrine or paracrine signals in these cells.

These changes might contribute to cancer development and progression by regulating ECM degradation, angiogenesis, cell proliferation and migration. Additionally, our data indicated that the cross-talk between serous ovarian cancer cells and their stroma might be distinct from that of mucinous ovarian cancer cells. However, the details of these differences need to be studied. This example of the molecular mechanisms involved in different histological types of ovarian cancer may promote the development of targeted therapeutics and the application of individualized treatment in ovarian cancer patients.

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Disclosure of conflict of interest

None.

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