RESEARCH ARTICLE

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Fibronectin Regulates the Dynamic Formation of Ovarian Cancer Multicellular Aggregates and the Expression of Integrin Receptors

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Abstract

Objective: To investigate the regulatory role of fibronectin (FN) in the formation of multicellular aggregate (MCA) in ovarian cancer SKOV3 and OVCAR-3 cells and integrin expression. **Methods:** The dynamic formation of MCA in SKOV3 and OVCAR-3 was determined using the liquid overlay technique in the presence or absence of FN, anti-FN, RGD peptide, control RGE. The expression of α3β1, α4β1 and α5β1 integrin in monolayer cells, MCA and FN-treated MCA were determined by flow cytometry and quantitative RT-PCR. **Results:** OVCAR-3 and SKOV3 MCA were formed on the 4th and 8th day and peaked on the 6th and 9th day, respectively. Treatment with different concentrations of FN, LN, type IV collagen and control RGE peptide promoted MCA growth, which was mitigated by anti-FN and RGD peptide. In comparison with monolayer cells, up-regulated α3β1, α4β1 and α5β1 expression were detected in MCA while treatment with FN in both cells. **Conclusions:** OVCAR-3 and SKOV3 cells had varying dynamic formation of MCA in our experimental system. FN enhanced MCA formation in both cells, which was associated with increased expression of 3β1, α4β1 and α5β1 in the MCA. Therefore, FN and these integrins may be new therapeutic targets for intervention of ovarian cancer metastasis.

Keywords: Ovarian cancer- multicellular aggregate- fibronectin- integrin

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Introduction

Ovarian cancer is the third common gynecological malignancy with a high mortality rate. Approximately, 75% of patients with ovarian cancer are diagnosed at an advanced stage, leading to a poor 5-year survival rate (Torre et al., 2016, Gerry and Shih, 2016). During the pathogenic process, epithelial ovarian cancers usually shed their cells into the peritoneal cavity, which promotes diffusion of metastatic seeds and formation of multicellular aggregates (MCA), an anchorage-independent proliferation of cancer cells (Dong et al., 2014). More importantly, accumulating evidence has indicated that the formation of MCA in ovarian cancers is associated with resistance to chemotherapies (Kenny et al., 2014). Previous studies have shown that the diameter of most MCA is between 50-750 μm, and a MCA over 250 μm is more resistant to chemotherapy (Shield et al., 2007, Xing et al., 2007). However, factors that regulate the formation of ovarian cancer MCA have not been clarified.

During the process of MCA formation, ovarian cancer cells have to adhere peritoneal mesothelial cells, invade through the mesothelial monolayer and proliferate in an anchorage-independent manner to form MCA. Previous studies have shown that the extracellular matrix (ECM) molecules are crucial for the formation of glioma and thyroid cancer MCA (Nederman et al., 1984). Laminin can stimulate migration of glioma MCA (Tysnes et al., 1997) and fibronectin (FN) can interact with β1 integrins, particularly for α5β1, to regulate the formation of ovarian cancer MCA (Casey et al., 2001). Another study indicates that α5β1 and other β1 integrins are crucial for the formation of ovarian cancer MCA and chemotherapy resistance (Dong et al., 2010). Recent studies have revealed that the arginine-glycine-aspartic acid (RGD) sequence in FN is responsible for binding to integrin receptors, such as α3β1, α4β1, α5β1, α7β1 and αVβ3, to regulate cell adhesion and MCA formation (Wang et al., 2015, Labat-Robert, 2012, Kumar et al., 2012). On the other hand, laminin interacts with α3β1 to disrupt intercellular adhesion (Kawano et al., 2001). Hence, the role of ligands for integrins in regulating the formation of ovarian cancer MCA remains to be controversial. It is still unclear the dynamic process of ovarian cancer MCA and how FN affects the dynamic formation of ovarian cancer MCA. Furthermore, there is no information on whether

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FN can regulate the expression of integrins during the formation of ovarian cancer MCA.

In the present study, we employed a liquid overlay technique to determine the effect of FN on the dynamic process of MCA formation in ovarian cancer SKOV3 and OVCAR-3 cells and their expression of integrin α3β1, α4β1 and α5β1 in vitro.

Materials and Methods

Cell lines

Ovarian cancer SKOV3 cells were maintained in the Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, China. OVCAR-3 cells were purchased from American Type Culture Collection (ATCC). SKOV3 and OVCAR-3 cells were maintained in DMEM and RPMI 1640 medium (Gibco) supplemented with fetal bovine serum (FBS, Shang Roselle) at 37 ºC in 5% CO_2, respectively, as described previously (Sodek et al., 2009).

MCA formation: liquid overlay technique

SKOV3 and OVCAR-3 cells (1.5×10^4 cells/well) were cultured in triplicate in 24-well plates that had been coated with 1.5% agarose in FBS-free DMEM and RPMI-1640 medium for 10 days, respectively. The cells were exposed to fresh medium every other day and the formation of MCA in individual wells was monitored longitudinally under a microscope. The MCA with a diameter of ≥ 250 μm for SKOV3 or ≥ 50 μm for OVCAR-3 cells was counted because the OVCAR-3 cells grew slowly (Roggiani et al., 2016, Xing et al., 2007).

In addition, the cells were tested in triplicate for the formation of MCA in the presence or absence of different concentrations (10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml) of FN (RD System), 8 μg/ml Laminin (LN), or 8 μg/ml type IV collagen (Sigma).

Moreover, the cells were tested in triplicate for the formation of MCA in the presence or absence of FN (10 μg/ml for SKOV3, 20 μg/ml for OVCAR-3), together with 5 μg/ml RGD, control RGE (arginine-glycine-glutamic acid (Zhai et al., 2016), Ansp), anti-FN IgG or control IgG (Epitomics).

Flow cytometry

The cultured monolayer of SKOV3 and OVCAR-3 cells and MCAs were harvested. The different groups of cells were stained in duplicate with FITC-anti-α3β1, APC-anti-α3 and PE-α4 or control FITC-IgG1, APC-IgG1 or PE-IgG1 (eBioscience). In addition, some cells were stained with FITC-anti-β1 and PE-anti-α5 or control IgG. After being washed, the cells were analyzed by flow cytometry.

Quantitative RT-PCR

The cultured monolayer of SKOV3 and OVCAR-3 cells and MCAs were harvested. Total RNA was extracted from each sample using Ultra-pure RNA Extraction Kit (Cat#CW0581, Cwbio, China). After being qualification and quantification, individual RNA samples were reversely transcribed into cDNA using the HiFi-MMLV cDNA first chain synthesis Kit (Cat#CW0744, CWbio), according to the manufacturers' instruction. The relative levels of a target gene to the control GAPDH mRNA transcripts were determined by quantitative RT-PCR using the Ultra SYBR Mixture (Rox) and specific primers in AB 7500 Real-Time PCR System. The primers were designed according to the gene sequence in the Genebank library using the Oligo 6 software. The relative levels of target gene mRNA transcripts to the control GAPDH were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Statistical method

Data are expressed as the mean ± standard deviation (SD). The difference between groups was determined by Student T test using the SPSS 19.0 statistical software. A P-value of <0.05 was considered statistically significant.

Results

The dynamic formation of MCA in SKOV3 and OVCAR-3

To determine the dynamic formation of MCA, SKOV3 and OVCAR-3 cells were grown a liquid overlay system and the formation of MCA was observed longitudinally. We observed different dynamics of MCA formation between SKOV3 and OVCAR-3 cells. According to the criteria for evaluation of MCA in these two cell lines, OVCAR-3 cells developed a MCA (> 50 μm) on the 4th day post culture and the numbers of MCA increased between the 5-7th (Table 1). The sizes of MCA measured by their diameters increased with time. Notably, we observed that some cells in the MCA began to die on the 7th day. Apparently, the best observation time was on the 6th day post culture as the MCA had a mean diameter of 67.86±14.84 μm (Figure 1). The logarithmic growth time was between 4-6 days post culture. In contrast, SKOV3 cells developed the MCA (a diameter of >250 μm) on the 8th day post culture and the numbers of MCA increased next two days, accompanied by slightly increased sizes. Subsequently, the SKOV3 MCA began to die on the 10th day post culture so that the best observation time was on the 9th day as the MCA had a mean diameter of 263.32±13.23 μm.

FN promotes the formation of MCA in SKOV3 and OVCAR-3

Single cell suspension of SKOV3 and OVCAR-3 cells were grown in the presence or absence of different concentrations (10-80 μg/ml) of FN, 8 μg/ml Laminin (LN), or 8 μg/ml type IV collagen. The MCA formation was observed (Figure 2). First, all groups of SKOV3 cells began to form MCA on the 2nd day in all groups. Compared with the blank control group (absence of exogenous factor), treatment with FN promoted the formation of MCA. Evidentially, SKOV3 cells developed obvious MCA on 2nd day and the MCA had no obvious boundary in the ≥20 μg/ml of FN-treated cells on the 3rd day. However, the MCA was bordered and stacked into a whole, and some cells began to die on the 4th day post culture. Treatment with LN and type IV collagen also enhanced the formation of MCA, but their effects were less than FN because the MCA in the LN and type IV collagen

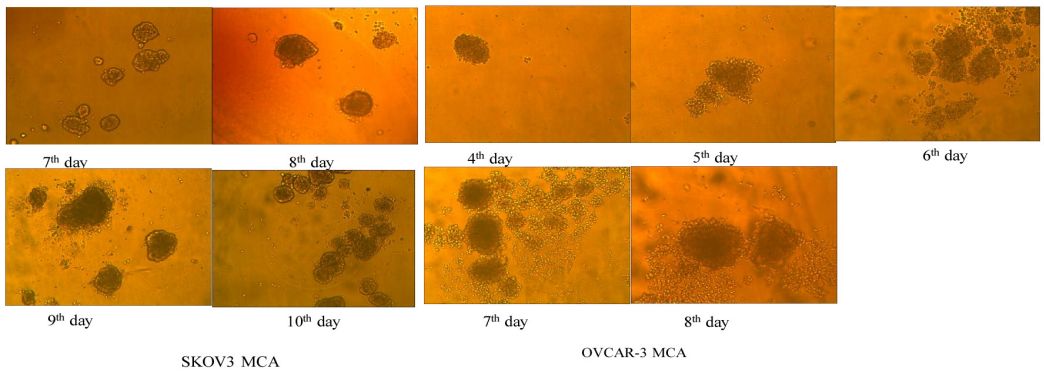

Figure 1. The Dynamic Formation of SKOV3 and OVCAR-3 MCA. SKOV3 and OVCAR-3 cells were cultured in the liquid overlay system in 24-well plates and the formation of SKOV3 MCA with a diameter of ≥250 μm or OVCAR-3 MCA (≥50 μm) was monitored longitudinally. Data are representative images (magnification x 100) of each group of MCA.

groups grew slower and was smaller. Similarly, single cell suspensions of OVCAR-3 cells also began to form MCA on the 2nd day. Compared with the blank control group, treatment with FN also promoted the formation of MCA in OVCAR-3 cells. They began to form distinct MCA on the 2nd day and the MCA had no obvious boundary in the ≥ 40 μg/ml FN groups on the 3rd day. The MCA was bordered, and fused into one, and some cells began to die on the 4th day. In addition, treatment with LN or type IV collagen also promoted the formation of MCA in OVCAR-3 cells, but their efficacy was less than FN. Therefore, we chose to use 10 or 20 μg/ml FN for SKOV3 or OVCAR-3 cells in the following experiments.

To further determine the importance and specificity of FN, SKOV3 and OVCAR-3 cells were tested in triplicate for the formation of MCA in the presence or absence of FN (10 μg/ml for SKOV3, 20 μg/ml for OVCAR-3), together with 5 μg/ml RGD, control RGE, anti-FN IgG or control IgG. The formation of MCA was monitored longitudinally (Figure 3). Treatment with FN promoted the formation of MCA, which was mitigated by treatment with anti-FN antibody, but not obviously with control IgG in SKOV3

Table 1. The Dynamic Growth of MCA in SKOV3 and OVCAR-3

MCA	4th day	5th day	6th day	7th day	8th day		9th day	10th day
OVCAR-3					SKOV3			
Numbers	1	3	6	8	8	2	5	6
Diameter (μm)	52.19	60.03±2.19	67.86±14.84	71.65±17.25	79.35±21.32	254.72±3.67	263.32±13.23	271.82±14.95

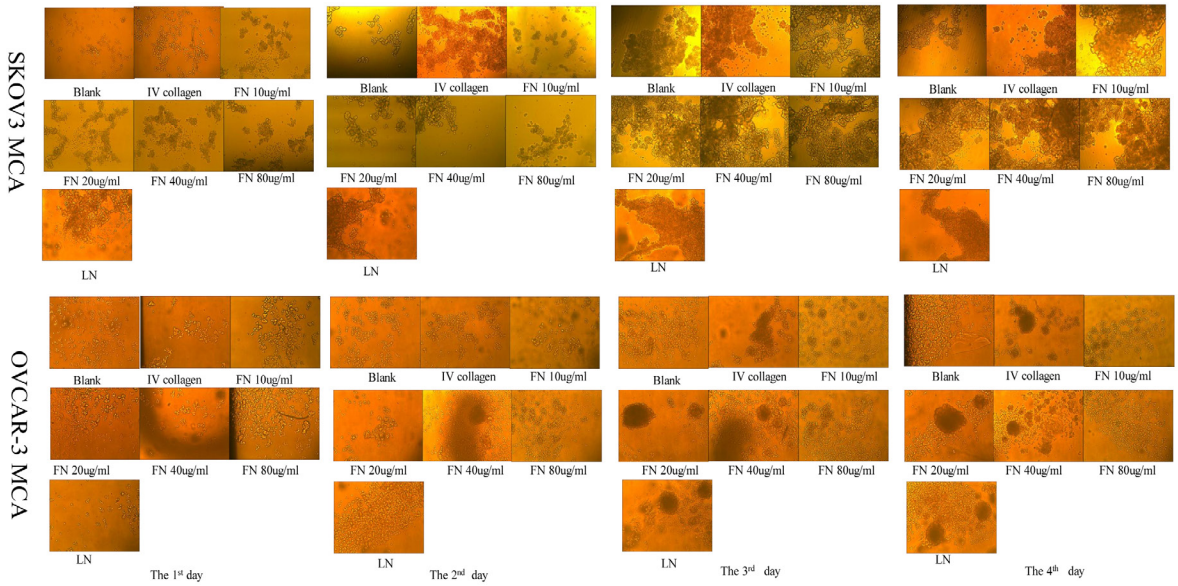

Figure 2. Treatment with FN Promotes the Formation of SKOV3 and OVCAR-3 MCA in Vitro. SKOV3 and OVCAR-3 Cells were Cultured in the Liquid Overlay System in 24-well Plates in the Presence or Absence of Different Concentrations (10 - 80 μg/ml) of FN, 8 μg/ml Laminin (LN), or 8 μg/ml Type IV Collagen for Four Days and the Formation of SKOV3 and OVCAR-3 MCA was Monitored Longitudinally. Data are representative images (magnification x 100) of each group of MCA.

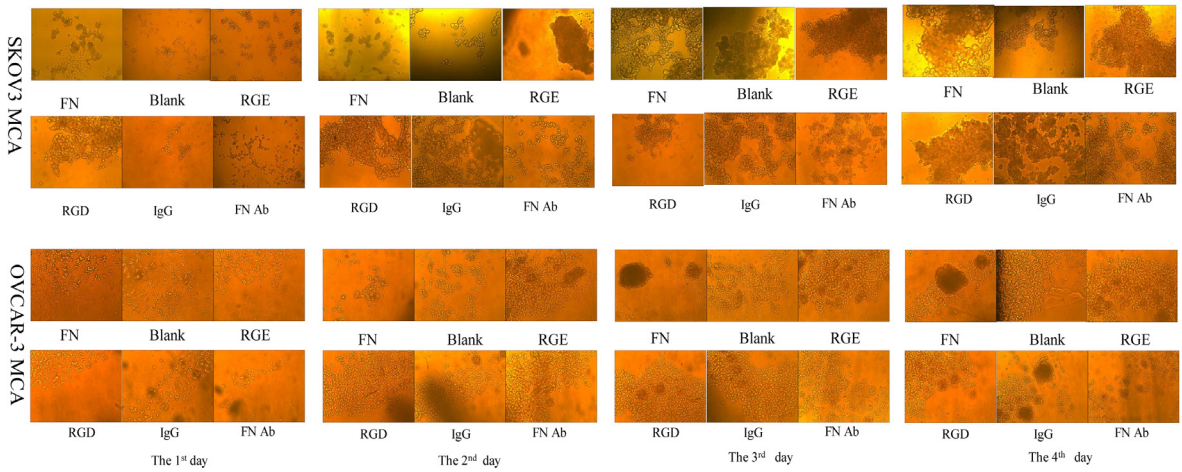

Figure 3. The Formation of SKOV3 and OVCAR-3 MCA Enhanced by FN Depends on Its RGD Sequence. SKOV3 and OVCAR-3 cells were treated with, or without, FN (10 μg/ml for SKOV3, 20 μg/ml for OVCAR-3), together with 5 μg/ml RGD, control RGE, anti-FN IgG or control IgG for testing the formation of MCA for four days. The formation of SKOV3 and OVCAR-3 MCA was monitored longitudinally. Data are representative images (magnification x 100) of each group of MCA.

Table 2. The Relative Levels of α3, α4, α5 and β1 mRNA Transcripts in SKOV3 and OVCAR-3 Cells

Groups	Monolayer		MCA		MCA+FN	
	SKOV3	OVCAR-3	SKOV3	OVCAR-3	SKOV3	OVCAR-3
α3 gene	1	1	9.3635	4	2.7166	12
α4 gene	1	1	13.4309	0.908	49.1207	1.063
α5 gene	1	1	4.6082	0.422	1.0825	1.178
β1 gene	1	1	1.0672	3.918	0.7832	10.918

Data are expressed as the relative mean value of each integrin mRNA transcript from three separate experiments. The relative levels of each integrin mRNA transcripts were designated as 1.

cells. Similarly, treatment with FN, together with RGD, but not with RGE, obviously mitigated the FN-enhanced formation of MCA in SKOV3 cells. Similar patterns of effect of combined treatments on the formation of MCA were observed in OVCAR-3 cells. Clearly, treatment with FN enhanced the formation of MCA in ovarian cancer cells, which was likely dependent on the RGD sequence of FN.

FN modulates the levels of α3β1, α4β1 and α5β1 integrin expression in the MCA of SKOV3 and OVCAR-3 cells

Integrins are transmembrane glycoprotein receptors on the cell surface, and α3β1, α4β1 and α5β1 can also express in the cytoplasm of cells (Kato et al., 2012, Kato et al., 2016). To understand the mechanisms underlying the action of FN, SKOV3 and OVCAR-3 cells were gown in triplicate for the formation of MCA in the presence or absence of FN and the relative levels of α3, α4, α5, and β1 integrin subunit mRNA transcripts to GAPDH were determined by quantitative RT-PCR (Table 2). In comparison with the monolayer of cells, the relative levels of α3, α4, α5 and β1 mRNA transcripts increased in the MCA of both SKOV3 and OVCAR-3 cells, except for α4 and α5 mRNA transcripts in OVCAR-3 cells. Treatment with FN increased the levels of α4 mRNA transcripts, but reduced the levels of α3, α5 and β1 mRNA transcripts in SKOV3 MCAs. Furthermore, treatment with FN also increased the levels of α3, α4, α5 and β1 mRNA transcripts in OVCAR-3 MCAs.

Table 3. Flow Cytometric Analysis of Integrin Expression in SKOV3 and OVCAR-3 Cells

SKOV3	α3β1 ($\bar{X}$ ±s)	α4β1 ($\bar{X}$ ±s)	α5β1($\bar{X}$ ±s)	P
Monolayer cell	0.000±0.000	0.000±0.000	0.013±0.023	0.422
MCA	0.503±0.346	12.030±0.149	0.233±0.047	<0.001
MCA+FN	0.843±0.143	13.187±0.221	0.650±0.147	<0.001
P	0.009	<0.001	<0.001	-
OVCAR-3				P
Monolayer cell	0.003±0.006	0.020±0.000	0.000±0.000	0.001
MCA	1.790±0.499	12.280±0.229	2.090±0.841	<0.001
MCA+FN	2.377±0.502	12.823±0.415	2.090±0.135	<0.001
P	0.001	<0.001	0.003	

Flow cytometry analysis revealed that the levels of α4β1 expression were higher than α3β1 and α5β1 and higher levels of α3β1, α4β1 and α5β1 integrin expression were detected in the MCA of SKOV3 and OVCAR-3 cells, relative to the monolayer of cells (Table 3). Treatment with FN further increased the levels of α3β1, α4β1 and α5β1 expression in SKOV3, but only α3β1 in OVCAR-3 MCA. Collectively, treatment with FN increased the levels of α3β1, α4β1 and α5β1 expression in the MCA of ovarian cancer cells.

Discussion

Ovarian cancer is prone to peritoneal metastasis, even at the early stage, which is difficult for diagnosis. Approximately, 70% of patients with ovarian cancer are diagnosed at late stage, making treatment extreme difficulty. Ovarian cancer cells dropping from the primary site can suspend in the ascites as a single cell or to form MCA (Sodek et al., 2012, Weidle et al., 2016). In this study, we employed a liquid overlap culture technique, an ideal model for the study of ovarian cancer in vitro (Matte et al., 2016, Kato et al., 2016) and found that SKOV3 and OVCAR-3 cells had varying dynamics in the formation of MCA. SKOV3 cells began to form MCA with a diameter > 250 μm on the 8th day and the best observation time was on the 9th day while OVCAR-3 started to have a MCA with a diameter of 50 μm on the 4th day and the best observation time was on the 6th day. Given that MCA can adhere, depolymerize, diffuse and invade the peritoneal mesothelium to form solid tumor rapidly, it promotes ovarian cancer invasion and metastasis (Torchiaro et al., 2016, Weidle et al., 2016). In addition, MCA is more susceptible to chemotherapy and radiation resistance than monolayer cells. Therefore, the MCA cells are responsible for ovarian cancer metastasis and recurrence (Desjardins et al., 2014). The varying dynamics in the formation of MCA in different ovarian cancer cells may provide new insights into understanding the recurrence and metastasis of ovarian cancer.

FN is a kind of non-collagen glycoprotein with high adhesion activity in the extracellular matrix (ECM). We found that treatment with FN enhanced the formation of MCA in both SKOV3 and OVCAR-3 cells and its enhanced effect was more potent than that of type IV collagen and LN. Furthermore, the enhanced effect of FN on the formation of MCA in SKOV3 and OVCAR-3 cells was mitigated by treatment with anti-FN, or RGD, but not control RGE peptide in our experimental system. These suggest that FN may through its RGD motif interact with its receptors of integrins to promote the formation of MCA in ovarian cancers (Widhe et al., 2016, Shi et al., 2016, Chakravarty et al., 2015). Hence, FN promotes the formation of MCA in ovarian cancer cells, dependent on its receptor integrins.

Ovarian cancer cells can invade ECM through the interaction of their cell surface integrins with FN, type IV collagen and LN and secrete catabolic enzymes to digest ECM, leading to local metastasis (Serrano et al., 2013, Micalizzi et al., 2010). FN can bind to α3β1, α4β1, α5β1, α7β1, αIIbβ3 and αVβ3; type IV collagen to α1β1, α3β1, and α8β1; and LN to αLβ2, α2β1, α3β1, α6β1, α7β1, and α6β4 (Kawaguchi, 2016). We found that in comparison with monolayer of cells, up-regulated levels of α3β1, α4β1 and α5β1, particular for α4β1 expression in the formed MCA in both SKOV3 and OVCAR-3 cells while treatment with FN further increased the levels of α3β1, α4β1 and α5β1 expression in the formed MCA. It is possible that the FN-enhanced integrin expression in the formed MCA may promote the formation of focal adhesion complex, through its cytoplasmic region, to activate intracellular focal adhesion kinase (FAK) activation, leading to tumor cell invasion and metastasis (Sun et al., 2014; Li et al., 2017; Kukkurainen et al., 2014; Wu and Reddy, 2012). Actually, the integrin-FAK system has been thought to be one of the important ways to regulate the growth, invasion and metastasis of ovarian tumor cells (Zhang and Zou, 2015; Canel et al., 2013). In addition, the increased integrin expression by FN may enhance the angiogenesis and vascularization in the MCA to promote metastasis and growth of ovarian cancer (Zanella et al., 2015; Ehling et al., 2016; Fraccaroli et al., 2015). Conceivably, FN and its receptor integrins may be new therapeutic targets for intervention of metastatic ovarian cancer.

In summary, our data indicated varying dynamics in the formation of MCA in different ovarian cancer cells. Treatment with FN promoted the formation of MCA in ovarian cancer cells, dependent on its specific motif for binding to its receptor integrins. Treatment with FN increased the levels of α3β1, α4β1 and α5β1 expression in the formed MCA of SKOV3 and OVCAR-3 cells. Therefore, our findings may provide new insights into understand the metastasis of ovarian cancer and aid in design of new therapies for intervention of ovarian cancer.

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