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Cell adhesion and invasion inhibitory effect of an ovarian cancer targeting peptide selected via phage display *in vivo*

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ABSTRACT

Organ-specific metastasis is of great importance since most of the cancer deaths are caused by spread of the primary cancer to distant sites. Therefore, targeted anti-metastases therapies are needed to prevent cancer cells from metastasizing to different organs. The phage clone pc3-1 displaying peptide WSGPGVWGASVK selected by phage display had been identified which have high binding efficiency and remarkable cell specificity to SK-OV-3 cells. In the present work, the effects of selected cell-binding phage and cognate peptide on the cell adhesion and invasion of targeted cells were investigated. Results showed that the adhesive ability of SK-OV-3 to extracellular matrix was inhibited by pc3-1 and peptide WSGPGVWGASVK, and pc3-1 blocked SK-OV-3 cells attachment more effective than the cognate peptide. The peptide WSGPGVWGASVK suppressed the cell number of SK-OV-3 that attached to HUVECs monolayer up to 24% and could block the spreading of the attaching cells. Forthermore, the cognate peptide could inhibit the invasion of SK-OV-3 significantly. The number of invaded SK-OV-3 cells and invaded SK-OV-3-activated HUVECs pretreated with peptide WSGPGVWGASVK was decreased by 24.3% and 36.6%, respectively. All these results suggested that peptide WSGPGVWGASVK might possess anti-metastasis against SK-OV-3 cells.

1. Introduction

Ovarian cancer is usually associated with widespread intra-abdominal metastasis [1–4], and it is the most lethal gynecologic malignancy which leads to approximately 15,460 deaths in the United States in 2011 [5]. Due to its vague symptoms and the lack of effective screening methods, more than 70% of patients with ovarian cancer are diagnosed at advanced stage (stage III or IV) when cancer cells tend to be metastatic [6]. Tumor metastasis is the major cause of death from ovarian cancer, which is usually resistant to conventional treatment. Moreover, metastasis is unpredictable in onset and will increase the clinical impact to the host in exponential manner [7]. Therefore, novel targeted therapies are needed to prevent the ability of cells to metastasize to different organs.

Considering that metastasis is regulated by a series of interaction of adhesion receptors and proteases [8], together with the fact that metastasis process consists of sequential steps and each step can be rate limiting [9], identification of these tumor-specific molecular ligands (several tumor-related peptides and proteins) that target key molecules in the cascade of metastasis would be expected to create a more effective and selective choice to suppress metastasis. Small peptides are pursued as attractive targeting ligands because of their small size, tumorpenetrating ability, low immunogenicity and high affinity [8,10]. Also, the peptides can recognize the cell surface protein of target cells which may be required for proliferation, adhesion, migration, and invasion, so they could modulate the biological behavior of target cells [10]. For example, peptide YIGSR [11] and poly-RGD [12] were reported to inhibit cancer metastasis by preventing cell adhesion. Fused polypeptide Tat-EL P [3] that can inhibit adhesion, spreading, invasion of ovarian cancer cells in cell culture was confirmed with anti-metastasis potential in an experimental model *in vivo*. Thus, the selection of peptides specific for the cells of interest would be very useful to provide a novel strategy for inhibiting metastasis of ovarian cancer.

Phage displayed library is a laboratory technique that uses standard recombinant DNA technology to associate peptides with replicating viral DNAs which include the peptides' coding sequences [13–15]. It is a powerful method that allows direct identification of cancer-specific ligands and their epitopes [16]. Bai et al. [17] isolated a specific peptide that selectively bound to GC9811-P cells which are proved to be a high potential peritoneal metastasis of gastric cancer derived from its parental cell line using a phage display library. The isolated phage-displaying peptide SMSIASPYIALE was a biologically active peptide and could effectively inhibit peritoneal dissemination of gastric cancer. In our previous research, a

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12-mer phage display library was adapted for *in vivo* selection to isolate ovarian cancer-targeting peptides, and one ovarian cancer targeting peptide WSGPGVWGASVK was obtained (the technical details are shown in online Supplementary material) [18]. The peptide was further observed to inhibit the adhesion of SK-OV-3 cells to extracellular matrix (ECM) proteins. Several ECM proteins, such as collagen type I and IV, laminin and fibronectin, have recently been identified to regulate the adhesion and invasion of ovarian cancer cells to peritoneal cells [19–21]. For ovarian cancer metastasis, the initial step is that cancer cells detach from the ovary, move towards the peritoneal mesothelium, and then adhere to the peritoneum [19]. Once the ovarian cancer cells adhere to the peritoneum, they can invade through the peritoneal cell layer and gain access to local organs and form secondary tumors, namely form metastases [22]. For these reasons, it is hypothesized that the inhibition of adhesion and invasion of ovarian cancer cells may be a critical aspect for anti-metastases. In the present study, the effects of selected cell-binding phage and cognate peptide on the biological behavior (especially cell adhesion and invasion) of targeted cells were further investigated.

2. Materials and methods

2.1. Cells and phage libraries

Human ovarian cancer cells SK-OV-3 and human umbilical vein endothelial cells (HUVECs) were purchased from the Replanted Immune Laboratory of Sichuan University in China, and were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 °C. Ph.D.-12 phage display peptide library and M13KE phage were purchased from New England Biolabs. The selected cell-binding phage clon (named pc3-1) was amplified according to the manufacturer's instruction. The selected phage was sequenced following the screening to identify peptides that bind with high affinity to desired target[18]. The cognate peptide WSGPGVWGASVK (Purity > 90%) was commercially synthesized by Shanghai Bootech Co., (Shanghai, China).

2.2. Cell-extracellular matrix adhesion assays

Ability of selected phage pc3-1 or cognate peptide WSGPGVWGASVK to interfere with attachment of cells to different ECM substrates was examined by a cell adhesion assay [23]. Briefly, SK-OV-3 cells suspension were preincubated with phages at 10^5–10^9 TU/well for 30 min at room temperature before plating. The cells were seeded into the 96-well plates which were previously coated with Matrigel (BD Biosciences, USA), fibronectin (FN) and type I collagen (0.5 μg/well) (Sigma, USA) and incubated for 1 h at 37 °C. Subsequently, the unattached cells were removed gently by washing with phosphate buffered saline (PBS), and the number of attached cells was estimated by CCK-8 assay. The absorbance of each well was quantified with a microplate reader (Molecular Devices, USA) at a wavelength of 450 nm. The experiment was performed in triplicate.

2.3. Preparation of tumor conditioned medium

SK-OV-3 cells were seeded in 75 cm^2 flasks and cultured in complete culture medium. When the cells were confluent, the medium was removed. Cells were washed with serum-free medium to remove serum components before exposed to serum-free medium. After culturing for 24 h, the medium was collected, centrifuged at 2000 rpm for 10 min and passed through a 0.22 μm filter to remove cells and cell debris. This supernatant was used as tumor conditioned medium (TCM). The prepared TCM with the addition of 10% FBS was 1:1 (v/v) diluted with complete culture medium was used to stimulate the HUVECs monolayer for 24 h prior to the addition and invasion assay.

2.4. Endothelium adhesion assay

Cancer metastasis is constituted by a series of sequential steps which consisted of proliferation at the primary site, neovascularization, invasion into stroma, entrance to the vessels, survival in the circulation, adhesion to endothelial cells and extravasation, and regrowth at the secondary site [23]. On one hand, it is proved that the endothelium is actively involved in the formation of blood-borne metastases of malignant tumors and the direct adhesive interaction between tumor cells and endothelial cells was the critical step in this process [24–26]. On the other hand, epithelial ovarian cancer metastases occur via the transcoelomic, haematogeneous, or lymphatic route [27]. To make overall plans and take as much as possible factors into account, HUVECs was used to perform the cell adhesion assay.

To investigate the influence of pc3-1/WSGPGVWGASVK on the tumor cell-vascular endothelial cell adhesion, the SK-OV-3 cells in suspension was labeled with a fluorescent dye CFSE and then incubated with confluent monolayer of HUVECs that was stimulated with SK-OV-3-derived TCM. The ability of SK-OV-3 cells to adhere to HUVEC monolayer was determined as follows. HUVECs were seeded on the chamber slides and grew until there is a confluent layer. SK-OV-3 cells were harvested and fluorescent labeled by a live cell labeling kit-CFSE (Dojindo Lab, Kumamoto, Japan) at the concentration of 20 μM according to the manufacturer's instruction. Then the labeled SK-OV-3 cells were layered over monolayers of HUVEC for 1 h in a CO_2 incubator. After 1 h of attachment, the cells were rinsed carefully with PBS and photographed using a fluorescent microscope (Olympus IX-71, Japan). Inhibition of SK-OV-3 cell adhesion to HUVEC was assayed by incubation of SK-OV-3 cells with selected phage, M13KE phage (1.5×10^9 TU) and peptide (100 μM) for 20 min at room temperature prior to the addition to the HUVEC monolayer, respectively. The attached cells were counted using the IPP 6.0 (Image-Pro Plus). Results were represented as the mean number of attached SK-OV-3 cells from ten fields (200×).

In order to observe the spread of SK-OV-3 cells on HUVECs monolayer, the medium of the adherent cells was removed and complete culture medium was added. Then the HUVECs monolayer and attached SK-OV-3 cells were co-cultured for another 48 h. After rinsing carefully with PBS, the cells were photographed using a fluorescent microscope in both phase bright and fluorescent dark fields.

2.5. Cell invasion assay

24-well transwell inserts (8 μm pore size) pre-coated with Matrigel were used to assess cell invasion. In the SK-OV-3 invasion assay, SK-OV-3-cell-suspensions in serum-free medium with/without a 20 min pretreatment of peptide (100 μM) were seeded in triplicate in the upper chamber, and 600 μL of complete culture medium as chemoattractant was added in the lower wells. The chambers were incubated for 24 h at 37 °C.

In the HUVECs invasion assay, SK-OV-3 cells were seeded in the lower wells and grown to 80% confluence. Subsequently, HUVECs suspensions in serum-free medium with/without a 20 min pretreatment of peptide (100 μM) were added to the upper chambers. HUVECs were allowed to invade in a SK-OV-3-associated environment for 24 h.

The inserts were then washed with PBS and the remaining cells on the upper surface of membrane were wiped away with cotton swabs. After fixing with cooled 95% ethanol and washing with

PBS twice, the invaded cells were stained with 4 g/L Trypan Blue and photographed with a microscope (200×). Results were represented as the mean number of invaded cells on each insert in ten random fields. Finally, the inserts were stained with Hochest 33258 (Beyotime Co., Jiangsu, China) and were observed with a fluorescent microscope.

2.6. Statistical analysis

All experiments were done repeatedly three or more times, and the experimental data were presented as means ± standard derivation (SD). Statistical analysis was carried out using Student's *t*-test. Differences at $p < 0.05$ were considered statistically significant.

Table 1
Influence of selected phage on adhesive ability of SK-OV-3 to ECM.

Phage amount (TU)	Relative adhesion (%)		
	FN	Collagen I	Matrigel
10^5	92.63 ± 8.95	91.83 ± 8.07	76.95 ± 8.12[a]
10^6	72.32 ± 13.26[a]	74.50 ± 10.40[a]	67.01 ± 7.10[a]
10^7	59.71 ± 10.32[a]	78.47 ± 14.35[b]	57.19 ± 4.61[a]
10^8	56.81 ± 11.50[a]	69.52 ± 12.01[a]	56.40 ± 5.79[a]
10^9	39.15 ± 4.04[a]	47.05 ± 0.92[a]	47.91 ± 7.82[a]

[a] $p < 0.01$ SK-OV-3+ phage pc3-1 vs. SK-OV-3 without phage.
[b] $p < 0.05$ SK-OV-3+ phage pc3-1 vs. SK-OV-3 without phage.

3. Results

3.1. Effect of phage pc3-1/peptide WSGPGVWGASVK on adhesion ability of SK-OV-3

The effects of phage pc3-1 and cognate peptide WSGPGVWGASVK on the adhesive ability of SK-OV-3 cells to ECM were investigated and the effects of peptide WSGPGVWGASVK on cell-ECM adhesion were performed in our previous research [18](the detailed experimental data are shown in online supplementary material, Fig. S1A–C). The components of ECM are mainly Matrigel, FN and type I collagen.

As can be seen in Table 1, after incubation for 1 h, the number of cells adhered to the ECM in the phage or peptide incubation group were significantly lower than that in the group without preincubation ($p < 0.05$). Results showed that pc3-1 at the concentration of 10^9 TU/well blocked 60% of the SK-OV-3 cells attachment to FN ($p < 0.01$). The cell attachments to collagen I and Matrigel were inhibited by pc3-1 at concentrations as low as 10^6 TU/well and the inhibition was in a concentration-dependent manner as shown in Fig. S2A–C ($p < 0.05$). In addition, it is found that pc3-1 blocked SK-OV-3 cells attachment more effective than the cognate peptide (Fig. S3).

Based on the results above, further confirmation of the ability of pc3-1 and cognate peptide to interfere with the adhesive interaction of cancer cells to endothelium was carried out via cancer cell-endothelial cell adhesion assay, and the results showed that

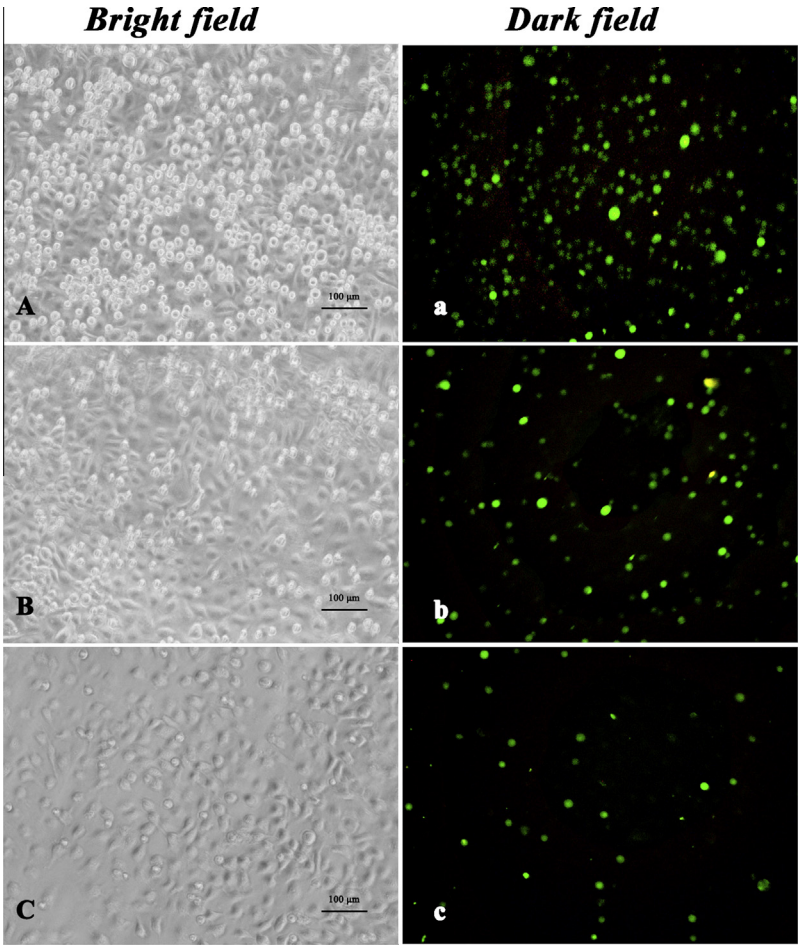

Fig. 1. Influence of selected phage on the adhesion of SK-OV-3 cells on HUVEC cells. SK-OV-3 cells were incubated for 1 h at 37 °C without phage (A, a), with the M13KE phage (B, b) or the pc3-1 (C, c) (Bar = 100 μm).

both phage itself and cognate peptide could inhibit the SK-OV-3 cells-HUVECs adhesion. Briefly, the addition of pc3-1/WSGPGVWGASVK decreased the cell number of SK-OV-3 that attached to HUVECs monolayer after 1 h of incubation, which can be observed in the fluorescent photos (Figs. 1 and 2B). The peptide WSGPGVWGASVK suppressed the cell number of SK-OV-3 that attached to HUVECs monolayer up to 24% (Fig. 2A). After co-culture for another 48 h, the cell area of newly attached SK-OV-3 cells with the pretreatment of peptide WSGPGVWGASVK was decreased by 62% relative to that without the pretreatment and the presence of peptide in cell culture (as shown in Fig. 3A). Moreover, a clear effect of the peptide on cell morphology was observed (Fig. 3B) that most attached cells exposure to the peptide at 100 μM induced a rounded morphology but remained attached to the HUVECs monolayer (Fig. 3B, lower) compared with the well-spread morphology of SK-OV-3 without peptide WSGPGVWGASVK (Fig. 3B, upper). The decreased cell number of initial attached SK-OV-3 (Fig. 2) and the blocked spreading of those cells (Fig. 3B) both contributed to the decreased overall cell area showed in Fig. 3A.

3.2. Effect of peptide WSGPGVWGASVK on cell invasion

Angiogenesis played an important role in tumor growth and metastasis, and tumor vessels were especially different from normal vessels [28,29]. In the previous work, it was confirmed that pc3-1 could target to both tumor cells and tumor vascular endothelial cells. Hence, an *in vitro* remodeling of tumor vascular endothelial cells, namely SK-OV-3-activated HUVECs, was used in this study [30] to investigate the anti-invasion effect of the peptide WSGPGVWGASVK displayed on pc3-1. To examine the effect of peptide WSGPGVWGASVK on the invasiveness of ovarian cancer cells, *in vitro* Matrigel invasion assay was performed (Fig. 4A). As shown in Fig. 4B, the number of invaded SK-OV-3 cells pretreated with peptide WSGPGVWGASVK was decreased by 24.3% compared with that without peptide. Moreover, the effect of peptide WSGPGVWGASVK on HUVECs invasion was further investigated (Fig. 4C). In the assay, HUVECs cells in upper inserts that co-culture with SK-OV-3 in lower wells were allowed to invade toward 10% FBS for 24 h. As a result, the addition of peptide WSGPGVWGASVK decreased the number of HUVECs cells traversed membrane by

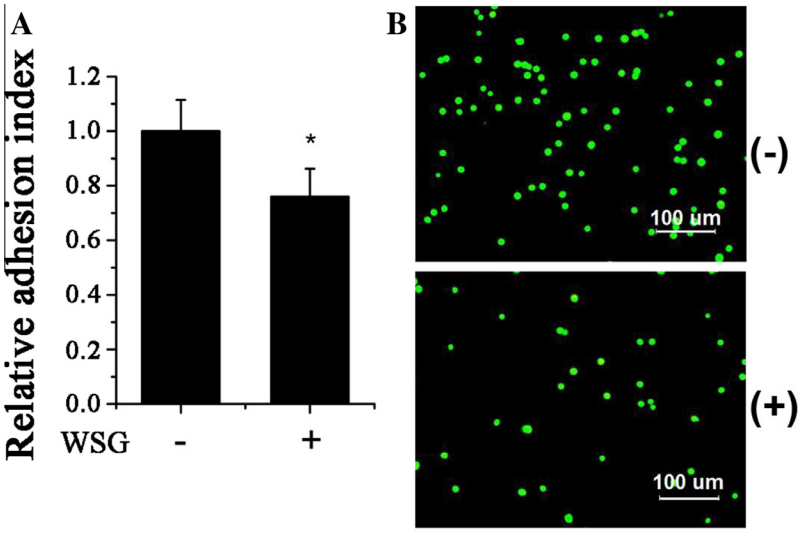

Fig. 2. Inhibition effect of selected peptide on the adhesion of SK-OV-3 cells on HUVEC cells. An asterisk (*) represents a significant difference: p value < 0.05 (Bar = 100 μm).

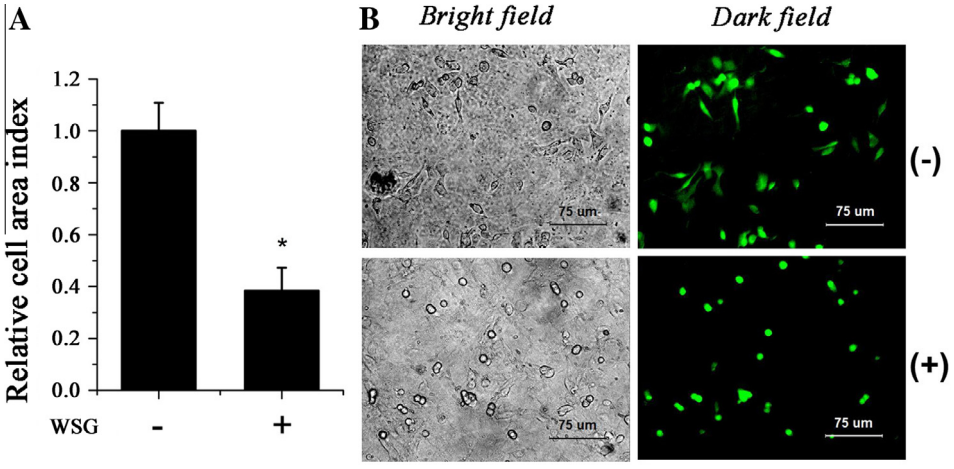

Fig. 3. Inhibition of cell spreading of SK-OV-3 cells on HUVEC cells. (A) Inhibition of cell spreading by the selected peptide. (B) SK-OV-3 cells spread on HUVEC cells $^{*}p$ < 0.05 (Bar = 75 μm).

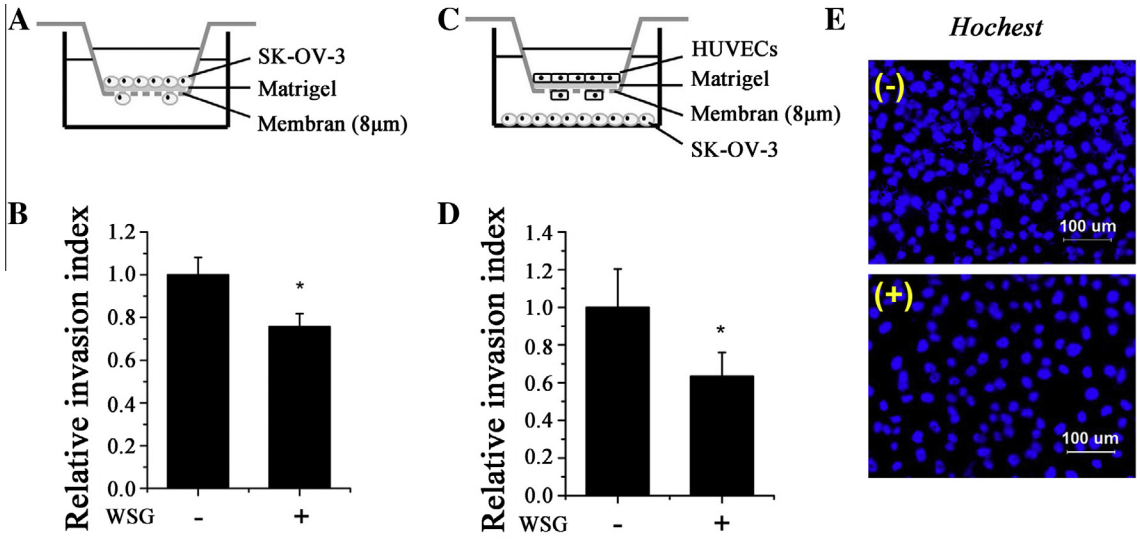

Fig. 4. (A) Schematic drawing of transwell culture of invasion of SK-OV-3 cells. (B) Invasiveness of SK-OV-3 cells preincubated with/without peptide. (C) Schematic drawing of transwell culture of invasion of HUVEC cells. (D) Invasiveness of HUVEC cells preincubated with/without peptide. (E) Invaded HUVEC cells pretreated with 100 μM of peptide. Numbers: cells that invaded through the Matrigel-coated filters (in one microscopic field). Cells in ten random fields on each insert were counted $^{*}p < 0.05$.

36.6% (Fig. 4D). It can be further confirmed by staining the HUVECs cells that traversed across the membrane with Hochest dye (Fig. 4E). These results indicated that invasion of SK-OV-3 cells through Matrigel-coated filters was decreased substantially when cells were preincubated with peptide WSGPGVWGASVK.

4. Discussion

4.1. Effect of phage pc3-1/peptide WSGPGVWGASVK on adhesion ability of SK-OV-3

Phage pc3-1 was identified as an ovarian cancer cell specific ligand. To further elucidate its properties and functions, cognate peptide WSGPGVWGASVK was synthesized. Since inhibition was observed when cells were plated on plastic without substrate (data not shown), an additional possibility is that the pc3-1/peptide binding could inhibit the production of ECM proteins by the cells. The interaction of the tumor cell with various components of the ECM such as integrin, laminin, and FN plays an important role in tumor cell metastasis and invasion [3].The results of cell-ECM adhesion assay showed that pc3-1/peptide could block SK-OV-3 cells attach to ECM. Thus, it was hypothesized that cells treated in suspension with pc3-1/peptide would be coated with the pc3-1/peptide and may be inhibited from attaching to the substrate.

Several proteins as important mediators were involved in tumor cell/endothelium association, including cadherins, selectins, and integrins [31]. In the previous work, it was proved that the peptide WSGPGVWGASVK was a polydom (also named SVEP1) mimic peptide [18]. Polydom/*SVEP1* is a ligand for integrin α9β1 which functioned as a cell surface protein and played a role in mediating cell adhesion in an integrin α9β1-dependent manner [32,33]. Those data provide circumstantial evidence suggesting that peptide WSGPGVWGASVK would be a potential ligand for integrin α9β1 which possessed inhibitory effect on modulating cellular attachment.

4.2. Effect of peptide WSGPGVWGASVK on cell invasion

Cell invasion was key step in the multistage process of pathogenesis of metastasis [34], during which malignant cells spread from the primary tumor to discontiguous organs [7]. Since invasion was shown to be directly depending on motility [31], and cell spreading is a commonly used model system for motility [35], it is expected that blockade of spreading is related to the cell movement and invasiveness.

In this study, a clear effect of the peptide on cell morphology was observed which might further restrain the SK-OV-3 cells spreading on HUVECs. Furthermore, the effect of peptide WSGPGVWGASVK on cell invasion was examined due to the mentioned spreading inhibition activity. As can be seen from the results shown in Fig. 4, invasion of SK-OV-3 cells through matrigel-coated filters was decreased substantially when cells were preincubated with peptide. It is known that increased expression of matrix metalloproteinases (MMP) is linked in cancer cells to facilitate invasiveness [21,23]. The decreased activity of MMP-2 and MMP-9 in cells treated with peptide WSGPGVWGASVK for 24 h (data not shown) indirectly suggested that the inhibition of SK-OV-3 cells invasion caused by preincubation with peptide may be associated with the inactivation of MMP or down-regulate its production.

In summary, the role of peptide WSGPGVWGASVK on the adhesion and invasion ability of SK-OV-3 cells were investigated *in vitro*, and the results demonstrated that the ability of SK-OV-3 cells to bind or invade the defined matrix after peptide WSGPGVWGASVK treatment was significantly impaired. These results suggested that peptide WSGPGVWGASVK might inhibit the metastatic ability of SK-OV-3 cells. Elucidating the mechanism will be very useful to provide a novel strategy for the metastasis of ovarian cancer and peptide WSGPGVWGASVK would be a candidate for anti-metastasis therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.058.

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