Acta Pharmaceutica Sinica B 2019;9(6):1163−1173

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Acta Pharmaceutica Sinica B

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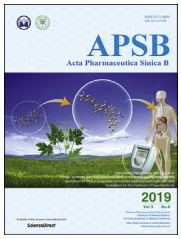

ORIGINAL ARTICLE

PEP06 polypeptide 30 is a novel cluster-dissociating agent inhibiting αv integrin/FAK/Src signaling in oral squamous cell carcinoma cells

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Received 1 July 2019; received in revised form 9 October 2019; accepted 11 October 2019

KEY WORDS

Oral squamous cell carcinoma;
Tumor cell clusters;
Collective migration;
Metastasis;
αv integrin/FAK/RC

Abstract Collectively migrating tumor cells have been recently implicated in enhanced metastasis of epithelial malignancies. In oral squamous cell carcinoma (OSCC), αv integrin is a crucial mediator of multicellular clustering and collective movement *in vitro*; however, its contribution to metastatic spread remains to be addressed. According to the emerging therapeutic concept, dissociation of tumor clusters into single cells could significantly suppress metastasis-seeding ability of carcinomas. This study aimed to investigate the anti-OSCC potential of novel endostatin-derived polypeptide PEP06 as a cluster-dissociating therapeutic agent *in vitro*. Firstly, we found marked enrichment of αv integrin in collectively

Abbreviations: CTC, circulating tumor cell; ECM, extracellular matrix; EMT, epithelial−mesenchymal transition; FAK, focal adhesion kinase; HNSCC, head and neck squamous cell carcinoma; MCA, multicellular aggregates; OSCC, oral squamous cell carcinoma; poly-HEMA, polyhydroxylethyl-methacrylate; RGD, Arg-Gly-Asp.

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Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2019.10.005

signaling;
RGD

invading multicellular clusters in human OSCCs. Our study revealed that metastatic progression of OSCC was associated with augmented immunostaining of αv integrin in cancerous lesions. Following PEP06 treatment, cell clustering on fibronectin, migration, multicellular aggregation, anchorage-independent survival and colony formation of OSCC were significantly inhibited. Moreover, PEP06 suppressed αv integrin/FAK/Src signaling in OSCC cells. PEP06-induced loss of active Src and E-cadherin from cell−cell contacts contributed to diminished collective migration of OSCC *in vitro*. Overall, these results suggest that PEP06 polypeptide 30 inhibiting αv integrin/FAK/Src signaling and disrupting E-cadherin-based intercellular junctions possesses anti-metastatic potential in OSCC by acting as a cluster-dissociating therapeutic agent.

1. Introduction

Oral squamous cell carcinoma (OSCC) originating from the epithelium of oral cavity is the most prominent malignancy of head and neck region[1]. In spite of advances in surgical techniques, radiation and multidrug chemotherapy, OSCC metastasis to regional lymph nodes remains the major clinical challenge correlating with poor prognosis and high mortality[2,3]. Several lines of evidence support the concept that epithelial−mesenchymal transition (EMT) is dispensable for metastasis of carcinomas[4]. Likewise, maintenance of E-cadherin-based adherent junctions is commonly observed in collectively spreading multicellular clusters of lung adenocarcinoma, breast and prostate cancer[5−7]. In OSCC, collective mechanism of cell migration and invasion is highly dependent on the presence of E-cadherin and p-120 catenin for intercellular contacts[8,9]. Importantly, cluster-based dissemination of tumor cells has recently emerged as far more advantageous for establishing metastases in comparison to single cell seeding[10]. Abundance of circulating tumor cell (CTC) clusters in the bloodstream is strongly correlated with adverse clinical outcomes of breast and prostate cancer patients[11]. Another study has demonstrated an association between the presence of CTC clusters in blood of peripheral vein and poor prognosis of patients with lung cancer[12]. It has been revealed that in 80% of solid tumors the spread *via* lymphatic system precedes distant metastasis *via* vascular system. Mohammed and colleagues[13] showed that clusters of lymph-circulating tumor cells constitute extraordinarily efficient metastatic precursors. Recently emerged evidence suggests that lymph node metastases could colonize distant organs by directly invading lymph node blood vessels[14]. Importantly, the prevalence of CTC clusters has been identified in advanced head and neck squamous cell carcinoma (HNSCC) with multiple lymph node metastases and reccurent disease[15]. It is now becoming clear that interference with clustered organization of carcinomas might be a valuable therapeutic tool for inhibiting metastatic potential of cancer cells[16]. Therefore, identification of cancer-specific molecules responsible for collective behavior of OSCC is of paramount importance for discovery of novel anti-metastatic therapies.

Numerous studies have demonstrated that tumorigenesis is orchestrated by integrin receptors, the heterodimeric transmembrane glycoproteins mediating interactions between cancer cells and tumor microenvironment[17]. Ligation of certain integrins to the substrate-specific domain in extracellular matrix (ECM), comprised of Arg-Gly-Asp (RGD) sequence, triggers multiple oncogenic signaling pathways with focal adhesion kinase (FAK) and non-receptor tyrosine kinase Src as the key mediators[18]. Among all RGD-recognizing integrin receptors, αv receptor has a major impact on OSCC aggregation, thereby affecting survival of anchorage-deprived cells[19]. Previous studies have confirmed that overexpression of integrin αv is closely associated with lymph node metastasis of laryngeal and hypopharyngeal carcinomas, pointing towards essential role of integrin αv in lymphovascular spread of head and neck cancer[20]. Furthermore, a recent research has identified that inhibition of αv integrin significantly reduces speed and directionality of collectively migrating HNSCC cell cohorts *in vitro*[21]. Although production of αv heterodimers is known to increase with invasive growth of primary tumors of maxillofacial area[22], the contribution of αv integrin receptors to metastatic spread of OSCC remains yet to be defined.

Blockage of αv integrins by RGD-based compounds represents a promising strategy for targeted therapies in HNSCC[23]. The overall pharmacological effect of RGD-containing peptidic agents is likely to be mediated *via* dual mechanisms, direct antitumor activity as well as interference with endothelial cell functions in cancer vasculature[24]. Given the highly vascularized nature of HNSCC, targeting neoangiogenesis could be a reasonable approach for depriving tumor of adequate nutrition and improving patient outcomes[25]. In such a context, our attention has been paid to a novel RGD-based PEP06 polypeptide, originating from the active fragment of natural inhibitor of angiogenesis endostatin. Terminal fusion of RGDRGD hexapeptide to the 1−24 amino acid residues of NH_2-fragment of endostatin is thought to result in amplified therapeutic potential of PEP06 polypeptide 30 due to direct targeting of cancer-specific cellular processes[26,27]. Recently, our colleagues have demonstrated a strong antitumor effect of PEP06 on colorectal cancer mediated through interactions between RGD motifs and αvβ3 integrins in tumor cells[28]. We speculated that PEP06 polypeptide as RGD-derivative from endostatin might possess direct therapeutic efficacy against OSCC in the context of interference with clustered organization of cancer cells. The present study identified for the first time overexpression of αv integrin in collectively invading multicellular clusters in human tumor specimens, as well as association of this receptor with metastatic progression of primary OSCCs. We showed here that PEP06 polypeptide targeting the αv integrin/FAK/Src signaling pathway significantly suppressed cell clustering on

fibronectin, migration, multicellular aggregation, anchorage-independent survival and colony formation of OSCC *in vitro*. Further analysis revealed that PEP06-mediated perturbation of active Src (Tyr416) and E-cadherin in intercellular contacts contributed to diminished collective movement of OSCC cells. Our results indicate that suppression of αv integrin/FAK/Src signaling is likely the underlying molecular mechanism by which PEP06 elicits cluster-dissociating potential in OSCC cells, suggesting the perspectivity of PEP06-based therapy for eliminating OSCC metastasis.

2. Materials and methods

2.1. OSCC clinical samples and immunohistochemical analysis

Paraffin-embedded specimens of 15 non-metastatic, 15 metastatic primary OSCCs and 5 cervical lymph node metastases were collected from the archives of pathological department. OSCC patients, aged 40−78 years, included 21 males and 14 females. The clinicopathological characteristics of primary OSCC cases are summarized in Supporting Information Table S1. The study was organized in accordance with the Declaration of Helsinki, and the experimental protocol was approved by the Ethics Committee of Harbin Medical University (Harbin, China) on 25 December 2017 (Approval No. 2017115). Immunohistochemical staining of αv integrin was conducted by standardized protocols. Briefly, 5-μm sections were deparaffinized and rehydrated. Antigen retrieval was performed in Tris/EDTA buffer (pH 9.0) according to the manufacturer's instructions. Sections were incubated with primary antibody against αv integrin (ab179475, Abcam, Cambridge, UK; 1:500) at 4 °C overnight. Next day, HPR-conjugated secondary antibody (Zhongshan Goldenbridge Biotechnology, Beijing, China) was applied to the samples at room temperature for 20 min, and 3,3′-diaminobenzidine (Zhongshan Goldenbridge Biotechnology) was used to visualize immunoreactivity. Tissues were counterstained with haematoxylin (Zhongshan Goldenbridge Biotechnology). Immunohistochemical staining was detected under a microscope DP73 (Olympus, Tokyo, Japan). The αv integrin immunoreactivity in 30 primary tumor specimens was analyzed by two board-certified pathologists using the Quickscore method[29].

2.2. Drug treatments

Desiccated PEP06 was kindly provided by the Department of Pharmacology, Harbin Medical University. For *in vitro* studies, PEP06 was prepared as described previously and applied at low, medium and high concentrations (50, 100, and 200 μg/mL)[28]. Glucose solution (5%) was used in the vehicle control. The recombinant human endostatin, ENDOSTAR® (Simcere Pharmaceutical Group, Shandong, China) at a concentration of 100 μg/mL was used as a control drug.

2.3. Cell culture

CAL 27 and SCC-15 cell lines derived from human tongue squamous cell carcinoma were used as *in vitro* model in the present study as these OSCC cells tend to migrate collectively with retain E-cadherin-positive contacts[8]. The cell lines were obtained from American Type Culture Collection and cultured as previously reported[8,21].

CAL 27 cells growing in suspension were used for assessment of cell clustering capacity *in vitro*[30]. To this end, cells were maintained for 72 h with different dilutions of reagents on non-adhesive 6-well plates coated with polyhydroxylethyl−methacrylate (poly-HEMA; Sigma−Aldrich, St. Louis, MO, USA) as described previously[31]. Following incubation, three images were captured for every sample, and the size (area μm^2) of at least 15 clusters per each condition was analyzed in Image J software (v1.52a, National Institutes of Health, Bethesda, MD, USA).

2.4. Cell detachment assay

Ninety-six well culture plates were coated with fibronectin (Sigma−Aldrich) as described previously[32]. CAL 27 cells at a density of 5×10^4 were seeded onto fibronectin-coated wells with serum-free medium in triplicates and incubated at 37 °C for 1 h. The adherent cultures of CAL 27 cells were then exposed to the indicated dilutions of reagents for 4 h. Following incubation, detached cells were removed by gentle washing with PBS. The adherent cells were fixed with 4% paraformaldehyde and stained with crystal violet. Three random fields of each well were captured by a digital camera DP21 (Olympus) at 100× magnification under a microscope CKX41 (Olympus). Subsequent quantification of total number of adherent cells as well as counting of clustered and single cells for each condition was carried out using Image J software.

2.5. Wound healing assay

CAL 27 and SCC-15 cell were seeded onto six-well plates and allowed to form confluent monolayer overnight. Next day, a wound was created by scraping the cell monolayer with 200 μL pipette tip as described previously[33]. The cultures were washed three times with fresh medium to remove debris and treated with varying dilutions of PEP06 (50, 100 and 200 μg/mL) or endostatin (100 μg/mL) for 48 h. The images of wounds were captured for each group at 0 and 48 h, and the gap width was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). The relative migration distance was measured from the edge of the initial wound at 0 h and normalized to the negative control.

2.6. Transwell migration assay

For cell migration assay, 3×10^4 CAL 27 cells that had been starved overnight were seeded in 300 μL of serum-free DMEM with different dilutions of reagents into the upper chamber of Transwell® Polycarbonate Membrane Inserts (Corning, Corelle, NY, USA). The bottom chambers contained 600 μL of medium with 10% FBS that served as a chemoattractant. After 48 h of incubation, non-migratory cells on the upper surface of the membranes were removed with cotton swabs. The Transwell inserts were fixed with 4% paraformaldehyde, stained with crystal violet for 15 min, and washed three times with PBS. The number of migrated cells in every group was determined by photographing the membranes under 200× magnification in three randomly selected fields followed by quantification with Image J software.

2.7. Anchorage-independent growth assay

For cell viability assay in non-adherent conditions, reagents were added simultaneously with 1×10^4 CAL 27 cells in serum-free medium in triplicates, and cell suspensions were maintained for 72 h in 96-well plates coated with poly-HEMA (Sigma−Aldrich)[34]. Following incubation, 20 μL of CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Madison, WI, USA) containing MTS were loaded into culture medium of each well and incubated for 4 h. Optical density was measured at 490 nm with a reference wavelength of 650 nm on a microplate reader (Epoch; BioTek Instruments, Winooski, VT, USA).

2.8. Colony formation

The colony-forming capacity of non-adherent OSCC cells was analyzed as described previously[7]. Briefly, 1×10^4 CAL 27 cells were pretreated on poly-HEMA-coated 96-well plates for 24 h. The cells were then resuspended in Matrigel® Matrix (Corning) with different dilutions of reagents and cultured for another 7 days. The images of samples were captured on day 8, followed by quantification of the colonies.

2.9. Quantitative real-time PCR

After 72 h of treatment, CAL 27 and SCC-15 cells cultured on poly-HEMA were centrifuged at 4 °C for 5 min, rinsed with PBS and harvested for RNA extraction using Trizol reagent (Invitrogen Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. Total RNA was reversely transcribed to cDNA by ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Real-time PCR was conducted on ABI 7500 fast Real Time PCR system (Applied Biosystems, Waltham, MA, USA) using SYBR® Green Realtime PCR Master Mix (Toyobo) as described previously[35]. The sequences of primer pairs were as follows: *ITGAV*, forward 5′-CGCTTCTTCTCTCGGGACTC-3′ and reverse 5′-CTGGGTGGTGTTTGCTTTGG-3′; *GAPDH*, forward 5′-AAGAAGGTGGTGAAGCAGGC-3′ and reverse 5′-TCCAC-CACCCAGTTGCTGTA-3′. The relative levels of mRNAs were determined by the $2^{-\Delta\Delta CT}$ method with GAPDH as an internal control.

2.10. Western blot assay

Following 72 h of treatment, total protein was extracted from CAL 27 cells cultured on poly-HEMA using RIPA Lysis Buffer (Thermo Fisher Scientific, Waltham, MA, USA) with protease and phosphatase inhibitors (Roche, Mannheim, Germany). Equal amounts of protein samples were separated through SDS-PAGE and transferred onto nitrocellulose membrane BioTrace™ NT (Pall Life Sciences, Ann Arbor, MI, USA), followed by blocking. The primary antibodies were used to probe the blots at 4 °C overnight: anti-αv integrin (1:5000; Cat#ab179475, Abcam), anti-phosphorylated FAK (Tyr397, D20B1, 1:1000; Cat#8556, Cell Signaling Technology Inc., Santa Cruz, CA, USA), anti-FAK (1:1000; Cat#3285, Cell Signaling Technology Inc.), anti-phosphorylated Src (Tyr416, D49G4, 1:1000; Cat#6943, Cell Signaling Technology Inc.), anti-Src (36D10, 1:1000; Cat#2109, Cell Signaling Technology Inc.), and anti-β-actin (1:1000; Cat#TA-09, Zhongshan Goldenbridge Biotechnology). Next day, the membranes were washed and probed with fluorophore-conjugated anti-mouse (1:10,000; Cat#P/N 925-32210, LI-COR Biosciences, Lincoln, NE, USA) and anti-rabbit (1:10,000; Cat#P/N 925-32211, LI-COR Biosciences) secondary antibodies at room temperature for 1 h. Blots were scanned with Odyssey® Imaging system (LI-COR Biosciences) and analyzed using LI-COR Image Studio Software 4.0 (LI-COR Biosciences) with β-actin as an internal control.

2.11. Immunofluorescence assay

Double-labeling immunofluorescence assay was applied to study the co-localization of E-cadherin and phosphorylated Src (Tyr416) proteins in CAL 27 cells exposed to different reagents for 48 h. Cellular co-distribution of the proteins in migrating CAL 27 cells was analyzed after 48 h of incubation in wound healing assay as described previously[36]. Briefly, cells cultured on glass coverslips were fixed with ice-cold 4% paraformaldehyde and blocked with goat serum. Subsequently, samples were incubated with primary antibodies against E-cadherin (1:200; Cat#610181, BD Transduction Laboratories, San Diego, CA, USA) and phosphorylated Src (Tyr416, 1:400; Cat#6943, Cell Signaling Technology Inc.) at 4 °C overnight, followed by detection with the corresponding secondary antibodies. Nuclei were stained with DAPI (Beyotime Biotech, Jiangsu, China). The images of samples were captured using confocal microscope LSM 700 (Carl Zeiss, Oberkochen, Germany).

2.12. Statistical analysis

Data are represented as mean ± SD from at least three independent experiments for each group. Statistical analysis between multiple groups was conducted by one-way ANOVA followed by Bonferroni multiple comparison test unless otherwise stated in the legend using GraphPad Prism 6.0c (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Integrin αv is highly expressed in collectively invading multicellular clusters and associated with lymphovascular metastasis of human OSCC

We first sought to explore the involvement of αv integrin receptors in multicellular seeding of human OSCC. As depicted in Fig. 1A, the invasive front of primary tumor exhibited robust enrichment of tumor cell clusters overexpressing αv integrin. Remarkably, accumulation of αv integrin was retained in multicellular tumor nests invaded into adjacent tissues. Perineural spread is a well-recognized route for OSCC dissemination that represents an independent pathological parameter for predicting regional lymph node metastasis and poor survival outcomes[37]. We found here that perineural invasive regions of OSCC displayed as integrin αv+ cohesive multicellular clusters. Furthermore, the maintenance of αv integrin was readily observed in OSCC cells in regional lymph node metastases.

To assess the contribution of αv integrins to metastatic spread of OSCC, the primary tumor specimens were stratified regarding the regional lymph node status (N stage according to TNM classification of American Joint Committee on Cancer and Union for International Cancer Control). We observed that metastatic progression of OSCC to regional lymph nodes was associated with a

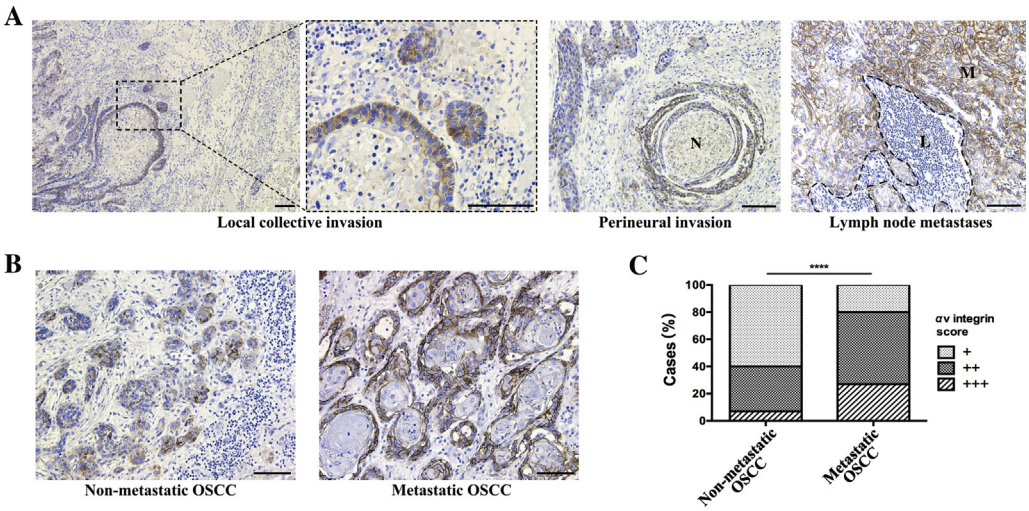

Figure 1 Integrin αv is overexpressed in collectively invading tumor cell clusters and is correlated with metastatic progression of human OSCC. (A) Integrin αv is enriched in multicellular clusters at the invasive front of primary tumor (local collective invasion), along the peripheral nerves (perineural invasion) and retained in regional lymph node metastases. N (nerve), L (lymphocytes), M (metastasis). Hash marks indicate the edge of metastasis in lymph node. (B) Representative images of αv integrin immunohistochemical staining in primary non-metastatic and metastatic OSCC specimens. (C) The frequency of OSCC cases with the indicated scores of αv integrin immunoreactivity stratified by lymph node status (Fisher's exact test, $P < 0.0001$). Scale bars: 100 μm.

higher score of αv integrin immunoreactivity in primary cancerous lesions (Fig. 1B and C). Together, these findings imply the potential role of αv integrin in the lymphovascular dissemination of human OSCC.

3.2. *PEP06 polypeptide diminishes cell clustering on fibronectin and impairs migration pattern of OSCC in vitro*

Malignant progression of OSCC involves series of adhesion-dependent mechanisms that ultimately govern metastatic spread of primary tumor[21]. Aiming to investigate the therapeutic potential of PEP06 polypeptide in OSCC *in vitro*, we first analyzed its impact on adhesive properties of cancer cells. When CAL 27 cells cultured on fibronectin for 1 h were exposed to PEP06 polypeptide for another 4 h, remarkable detachment occurred in a concentration-dependent manner (Fig. 2A and B). Notably, unlike the control and endostatin groups, where adherent cells gathered in multicellular clusters, the majority of PEP06-treated cells existed in a dispersed pattern (Fig. 2C).

We further assessed the effect of PEP06 polypeptide on migratory capacities of OSCC cells. The results of wound healing assay demonstrated a concentration-dependent inhibitory effect of PEP06 on the migration of CAL 27 and SCC-15 cancer cells (Fig. 2D, Supporting Information Figs. S1A and B). Next, we analyzed the ability of OSCC cells to move through the membrane pores in Transwell chambers. As illustrated in Fig. 2E, the numbers of migrated CAL 27 cells in the PEP06 groups were significantly lower as compared to controls. Along with the qualitative changes, the migration behavior of CAL 27 cells has also altered. Control cells migrated through the porous membrane were observed as multicellular cancer groups, whereas CAL 27 cells exposed to PEP06 appeared as smaller strands or individually. Collectively, these results indicate that PEP06 could diminish cancer cell clustering on fibronectin and dramatically suppress OSCC migration *in vitro*.

3.3. *PEP06 produces inhibitory effects on cell aggregation, anchorage-independent survival and colony formation of OSCC cells*

Previous studies have ascertained that metastatic outgrowth of ECM-detached cancer cells relies on their ability to form multicellular clusters[7,11]. Therefore, we next explored whether PEP06 could alter aggregation of OSCC cells. To address this issue, we employed methodology of *in vitro* culturing of cancer cells in suspension, a model mimicking growth pattern of CTC clusters[30]. Unlike control CAL 27 cells that aggregated into multicellular clusters, PEP06-treated samples displayed as loose clumps and single cells under non-adherent conditions (Fig. 3A). As depicted in Fig. 3B, incubation of suspended CAL 27 cells with different concentrations of PEP06 polypeptide for 72 h hindered aggregation and resulted in apparent decrease of tumor cell cluster size. Under such conditions, a concentration-dependent inhibition of anchorage-independent survival was observed in PEP06-treated CAL 27 cells (Fig. 3C).

On the basis of these findings, we hypothesized that PEP06 polypeptide could also affect the colony formation of OSCC cells. To this end, non-adherent CAL 27 cells were first pretreated with PEP06 (50, 100 and 200 μg/mL) and endostatin (100 μg/mL) for 24 h and subsequently cultured in 3D Matrigel with different dilutions of compounds for 7 days according to the established protocol[7]. As it is shown in Fig. 3D, PEP06 treatment led to remarkable suppression of colony formation by CAL 27 cells. We found that the numbers of colonies in PEP06-treated samples were significantly decreased on day 8 in comparison to controls (Fig. 3E). Taken together, the data suggest that PEP06-induced suppression of multicellular aggregation contributes to colony-forming deficiency of OSCC cells.

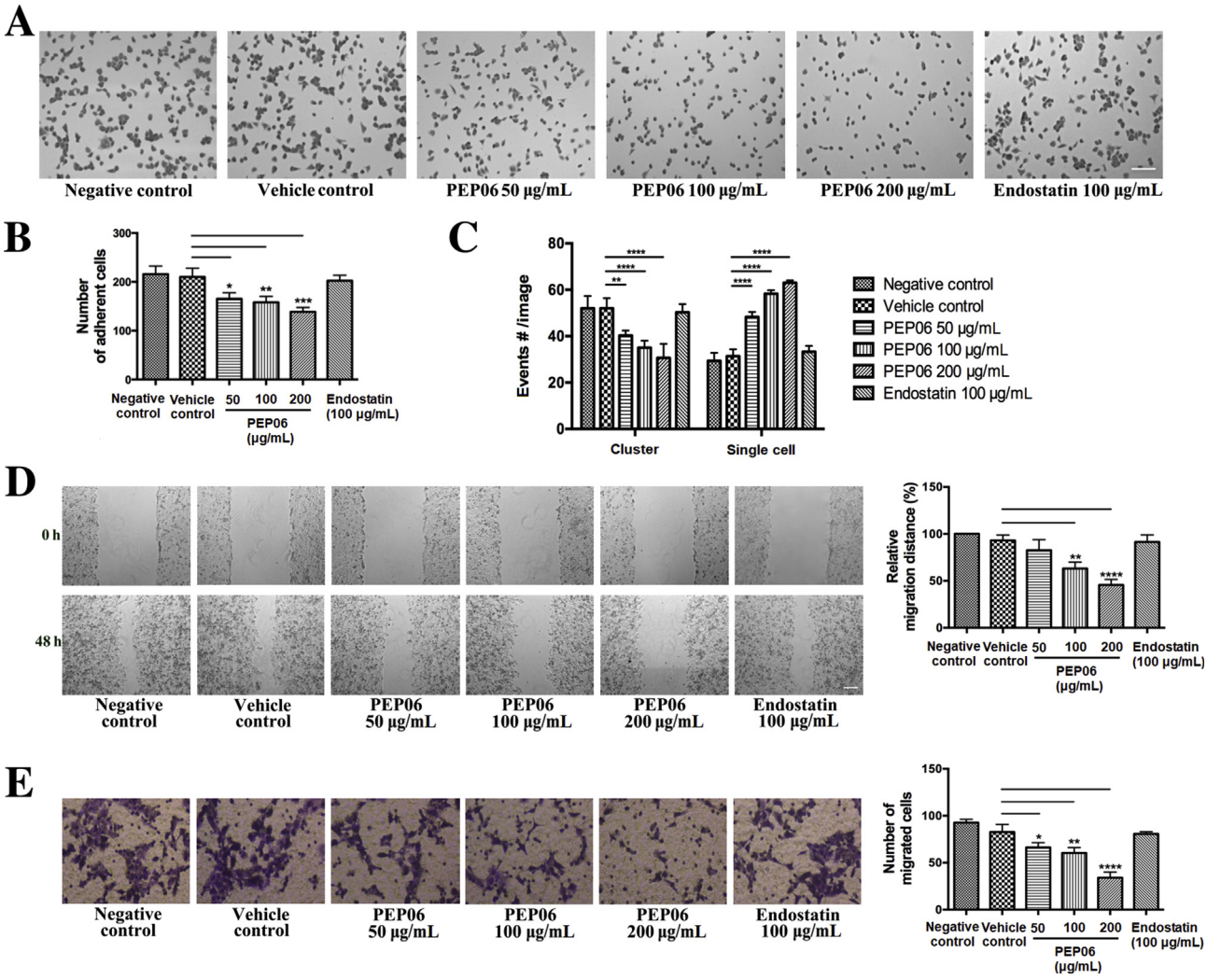

Figure 2 PEP06 polypeptide alters adhesive and migratory capacities of OSCC cells. (A) Representative images showing adherent on fibronectin CAL 27 cells treated with the indicated reagents for 4 h and stained with crystal violet. Scale bar: 50 μm. (B) Number of adherent CAL 27 cells on fibronectin in the detachment assay. (C) Counts of clustered and single CAL 27 cells per image in the detachment assay. (D) Representative phase-contrast images of treated CAL 27 cells captured at 0 and 48 h in the wound healing assay (scale bar: 200 μm) and the averaged cell migration distance in the presence of test drugs relative to the negative control. (E) Representative images showing migrated CAL 27 cells exposed to the indicated compounds for 48 h in Transwell chambers and stained with crystal violet. Scale bar: 50 μm. Number of migrated through the membrane pores CAL 27 cells in the Transwell migration assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data are expressed as mean ± SD from there independent experiments.

3.4. PEP06 polypeptide inhibits the αv integrin/FAK/Src signaling pathway in OSCC cells

It has been demonstrated that blockade of αv integrins by their antagonists leads to inhibition of the associated signaling effectors and, most importantly, alters the expression of αv integrin receptors in cancer cells[38]. Here, quantitative real-time PCR analysis revealed suppression of *ITGAV* gene encoding αv integrin in suspended CAL 27 and SCC-15 cells exposed to PEP06 polypeptide for 72 h (Fig. 4A and Supporting Information Fig. S1C). The results of Western blot assay further confirmed the down-regulation of αv integrin receptor in PEP06-treated cells cultured in non-adherent conditions (Fig. 4B and E).

Upon integrin clustering, FAK is autophosphorylated at Tyr397 site and activates Src kinase through Tyr416 phosphorylation, thereby triggering multiple molecular pathways implicated in oral tumorigenesis[39]. Given the determinant role of FAK signaling in mediating clustered organization of carcinoma cells[40], we next addressed the impact of PEP06 on FAK activity in non-adherent CAL 27 cells. It was observed that PEP06 prevented phosphorylation of FAK and Src at Tyr397 and Tyr416 sites, respectively, without affecting the abundance of total Src. Meanwhile, a remarkable decrease of total FAK protein was detected in CAL 27 cells treated with 100 and 200 μg/mL of PEP06 (Fig. 4C, D and E). Consistent with our findings, it has been previously revealed that depletion of integrin αv contributes to loss of FAK protein expression and significantly attenuates progression of squamous cell carcinoma[41]. Overall, these results demonstrate that PEP06 acts as a potent inhibitor of the αv integrin/FAK/Src signaling pathway in OSCC cells.

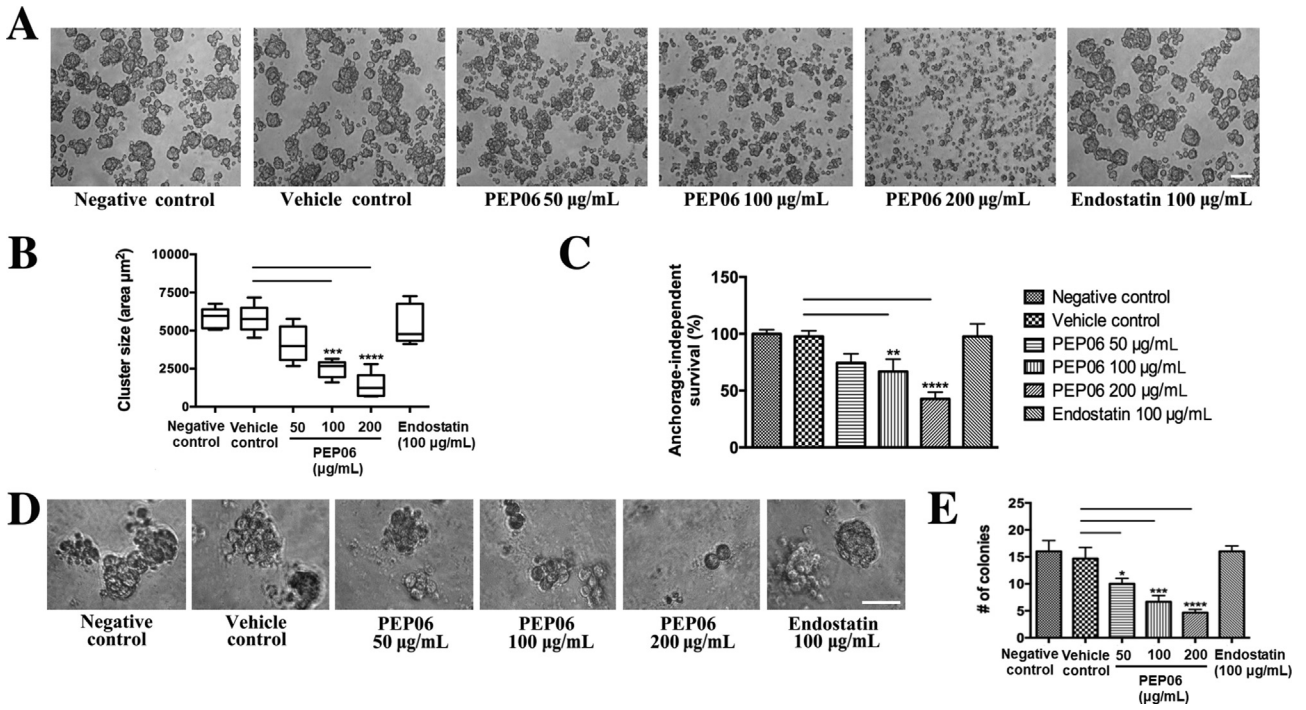

Figure 3 PEP06 polypeptide hampers multicellular aggregation, anchorage-independent growth and colony formation of OSCC cells. (A) Representative phase-contrast images of non-adherent CAL 27 cells on poly-HEMA exposed to the indicated reagents for 72 h. Scale bar: 100 μm. (B) The median size (area, μm^2) of at least 15 clusters of CAL 27 cells calculated for each condition after incubation with the indicated compounds for 72 h in suspension. (C) Anchorage-independent survival of CAL 27 cells treated with the indicated reagents for 72 h by MTS test, calculated relative to the negative control. (D) Representative phase-contrast images of colonies at day 8 formed by pretreated for 24 h non-adherent CAL 27 cells seeded in Matrigel with the indicated reagents. Scale bar: 50 μm. (E) Counts of colonies formed by CAL 27 cells in Matrigel for each condition at day 8. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. For (C) and (E) data are presented as mean ± SD from triplicate experiments.

3.5. PEP06-induced loss of active Src (Tyr416) and E-cadherin from cell−cell contacts contributes to attenuated intercellular cohesion and diminished collective migration of OSCC cells

In order to gain further insight into the inhibitory effects produced by PEP06, we analyzed the cellular distribution of active Src (Tyr416) and E-cadherin in adherent OSCC cells. Consistent with their cohesive phenotype, CAL 27 cells in the vehicle control and endostatin-treated groups exhibited robust coexpression of both phosphorylated Src (Tyr416) and E-cadherin in intercellular contacts (Fig. 5A). In contrast, PEP06-treated cells displayed pronounced perturbation of the proteins critical for cell−cell contacts coincident with delocalization of E-cadherin. Consistently, we observed emergence of separated cells with cytoplasmic E-cadherin immunostaining upon PEP06 treatment.

Overexpression of active Src kinase has been shown to facilitate migration of OSCC cohorts by stabilizing E-cadherin-based junctions[8]. To define the functional consequences of PEP06-induced dissociation of phosphorylated Src (Tyr416) and E-cadherin, we employed confocal microscopy to monitor migrating CAL 27 cells in wound healing assay. As depicted in Fig. 5B, CAL 27 cells in the vehicle control and endostatin groups migrated as multicellular cohorts with retained active Src (Tyr416) and E-cadherin in intercellular junctions of leading edge. Meanwhile, treatment with PEP06 led to loss of phosphorylated Src (Tyr416) and E-cadherin from cell−cell contacts and abrogated collective movement of cancer cells. Together, these results support the notion that PEP06 polypeptide has the potential to inhibit E-cadherin-based collective migration of OSCC cells.

4. Discussion

It has been traditionally assumed that metastases of carcinomas are seeded by individual cells that acquire tumor-initiating capacities through activation of EMT program. However, since the identification of multicellular clusters with epithelial phenotype across the stages of metastatic cascade, collective mechanism has emerged as a driving force in cancer dissemination owing to its remarkable efficiency[42]. It has been demonstrated that OSCC specimens exhibit maintenance of cytokeratins and E-cadherin[43], consistent with the collective invasion pattern of diverse epithelial malignancies[5,6,44]. The present study has identified for the first time overexpression of αv integrin in collectively invading multicellular clusters of human primary OSCC specimens. It is noteworthy that collective spread as the prevalent invasion mode has recently been implicated in distant metastasis of human breast cancer of both invasive ductal and lobular carcinomas[45]. Herein, metastatic progression of OSCC to regional lymph nodes was associated with a higher score of αv integrin immunoreactivity in the primary cancer lesions, thereby further elaborating the pro-metastatic function of αv integrin which has been confirmed for laryngeal and hypopharyngeal carcinomas[20], breast adenocarcinomas and renal clear cell cancer[46].

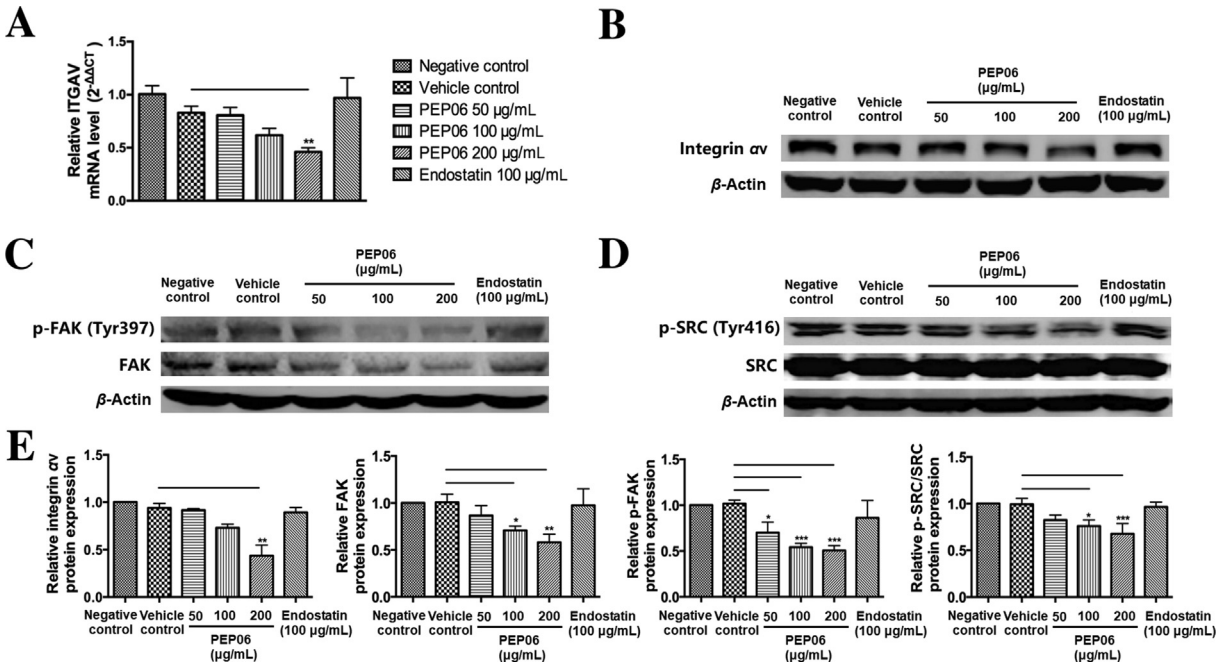

Figure 4 Inhibitory effect of PEP06 polypeptide on αv integrin/FAK/Src signaling pathway in OSCC cells. (A) Expression of ITGAV (αv integrin-encoding gene) in CAL 27 cells maintained with the indicated reagents for 72 h under non-adherent conditions, detected by real-time PCR. GAPDH was used as internal control. Western blot analysis of integrin αv (B), phosphorylated FAK (Tyr397) and total FAK (C), phosphorylated Src (Tyr416) and total Src (D) expression in CAL 27 cells incubated with the indicated compounds for 72 h in suspension. β-Actin served as loading control. (E) Densitometric analysis of integrin αv, total FAK, p-FAK and p-Src/Src levels in CAL 27 cells, calculated relative to the negative control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data are expressed as mean ± SD from there independent experiments.

The revealed contribution of αv integrin to metastatic progression of OSCCs implies the potential susceptibility of oral tumorigenesis to therapeutic agents targeting αv integrins, namely interfering with collective tumor cell dissemination. Importantly, recent uncovering of the biological significance of CTC clusters in metastatic outgrowth of carcinomas has opened a new era of anti-metastatic therapy targeting clustered cancer cells[47]. Aceto and colleagues[11] have shown that knockdown of plakoglobin, a component of adherent junctions and desmosomes, led to disruption of cell−cell contacts in a monolayer of breast cancer cells and failure of cluster formation, thereby suppression of lung metastases *in vivo*[11]. Another group suggested urokinase, a clinical thrombolytic compound, for antimetastatic therapy that dissociates CTC clusters and increases the survival rates of tumor-bearing animals[48]. The present study demonstrates that PEP06 targeting αv integrins disrupts intercellular integrity, indicating this novel polypeptide as a potential therapeutic agent for limiting cluster-based metastasis of OSCC. To our knowledge, this is the first example of small molecule compound that possesses the cluster-dissociating property in OSCC. We showed here that PEP06 treatment diminished αv integrin expression in OSCC cells and led to significant suppression of the FAK/Src signaling axis that is deemed critical for metastatic spread. Previously, maintenance of phosphorylated Src (Tyr416) and E-cadherin proteins in intercellular contacts has been shown to have positive impact on cohesive phenotype and collective movement of HNSCC[8]. We found here that PEP06 polypeptide caused concentration-dependent inhibition of OSCC cell motility with remarkable changes in the migratory pattern. Consistently, PEP06-mediated abrogation of OSCC collective movement *in vitro* was evident by impaired intercellular integrity in migrating cells, paralleled by pronounced loss of phosphorylated Src (Tyr416) and E-cadherin from cell−cell junctions. The inhibitory effects of PEP06 also coincide with a recent report where αv integrin but not α5β1 heterodimer is implicated in the control of HNSCC collective migration[21]. Given the previously confirmed association between endostatin and α5β1 integrins[49], it is not surprising that the migratory pattern of endostatin-treated OSCC cells in this study share the common characteristics of control samples.

One of the most significant findings of the present study is that PEP06 treatment results in striking abrogation of OSCC cell aggregation and colony formation. It has been previously documented that multicellular aggregates, but not single suspended OSCC cells, are capable of anchorage-independent survival, indicating the vital importance of intercellular adhesion for ECM-detached cells[50]. Notably, Cheung et al.[7] have recently revealed ~15-fold greater colony-forming capacity of aggregated tumor cells and ~100-fold increase in metastatic outgrowth of clustered cells *in vivo versus* single cell seeding. Another study has identified that metastatic advantage of tumor cell clusters in experimental breast cancer model is associated with their resistance to apoptosis following seeding in the lungs[11]. Here, we propose that PEP06-induced suppression of the αv integrin/FAK/Src pathway contributes to inhibition of multicellular aggregation and anchorage-independent survival, ultimately leading to diminished colony formation of OSCC cells. Our results are essentially in agreement with the previous studies on that among all RGD-binding integrins, αv subunit exerts critical survival signals transmitted *via* the FAK cascade in non-adherent OSCC cells[19,51]. It has been also demonstrated that upon blockade of FAK signaling in a microfluidic model mimicking microcirculation *in vitro* tumor cell clusters dissociate into single cells and fail to

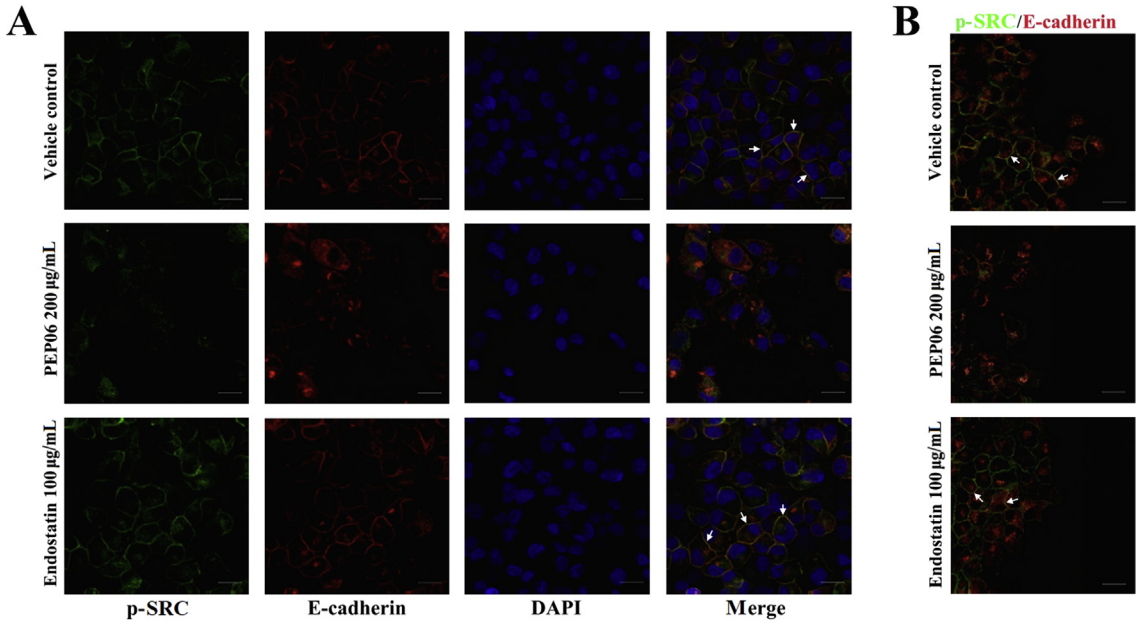

Figure 5 PEP06-mediated perturbation of active Src (Tyr416) and E-cadherin in intercellular contacts contributes to diminished collective movement of OSCC cells. (A) Immunofluorescent analysis of distribution of phosphorylated Src (Tyr416) and E-cadherin proteins in CAL 27 cells exposed to the indicated reagents for 48 h. (B) Immunofluorescent staining of p-Src (Tyr416) and E-cadherin in migrating CAL 27 cells upon treatment with the indicated compounds for 48 h in wound healing assay. Scale bars: 20 μm. The arrows point to the colocalization of active Src (Tyr416) and E-cadherin proteins in CAL 27 cells.

traverse through capillary-sized constrictions, pointing toward FAK as a target for limiting cluster-driven metastasis[52]. Importantly, multicellular clusters migrating through 3-μm constrictions as cohesive strands experience less prevalent nuclear envelope rupture in comparison to single cells[53]. It is noteworthy that beyond affecting phosphorylation of FAK in OSCC cells, PEP06 polypeptide also decreased the level of total FAK. Viewed in the context of established pivotal role of FAK in malignant conversion of HNSCCs[39], our results suggest the perspectivity of PEP06-based therapy for eliminating OSCC metastasis.

The PEP06-mediated interference with αv integrin signaling and loss of E-cadherin from intercellular junctions highlight the importance of both cell−matrix and cell−cell adhesions in collective mechanism of carcinoma cell spread[54]. The ability of E-cadherin-based intercellular contacts to substitute integrin−ECM adhesion and trigger ligand-independent activation of epidermal growth factor receptor in OSCC cells has been coined "synoikis"[55]. Consistent with this scenario, PEP06 polypeptide possesses the exceptional therapeutic potential of limiting OSCC metastatic progression. On contrary, it has been reported that inhibition of β1 integrin in E-cadherin-positive triple-negative breast cancer enhances metastatic colonization in lungs of tumor-bearing animals[56]. The latter example points toward tumor-specific pathophysiological mechanisms; it is thus of pivotal importance to exploit the appropriate targeted therapy based on the cancer origin. In this regard, further *in vivo* experiments are warranted for elucidating the pharmacological effects PEP06 in OSCC with particular focus on its anti-angiogenic potential[28]. Given the crucial role of αv integrin/FAK/Src signaling in the pathogenesis of multiple carcinomas[57], it is likely that PEP06 could also act as αv integrin antagonist and produce beneficial effects on other solid tumors.

In summary, our results demonstrated that PEP06 inhibiting the αv integrin/FAK/Src signaling pathway significantly suppressed cell clustering on fibronectin, migration, multicellular aggregation, anchorage-independent survival and colony formation of OSCC *in vitro*. Meanwhile, PEP06-mediated loss of phosphorylated Src and E-cadherin from intercellular contacts contributed to attenuated intercellular cohesion and diminished collective migration of OSCCs. These findings suggest that PEP06 polypeptide possesses therapeutic potential of eliminating cluster-driven metastasis of OSCC and merits further in-depth investigations.

Acknowledgments

The authors thank Dr. Shuyuan Guo and Dr. Shamil Khusnutdinov for their technical support in immunohistochemical analysis of OSCC clinical samples and also Prof. Yong Zhang for his administrative support. This work was funded by the National Natural Science Foundation of China (grant Nos. 81730012 and 81673426); and the Grant of Republic Bashkortostan for Young Scientists (grant No. 26 GR).

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2019.10.005.

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