Role of Fascin in Xenografted Tumorigenesis in Nude Mice: A Histological Study

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Abstract

Tumorigenesis is a sequential multi-step process of genetic alteration with an effect on cell proliferation, invasion, and metastasis. Fascin is an actin-bundling protein found in invadopodia and is involved in cell invasion and motility. Recently, aberrant expression of fascin in carcinoma cells was reported. The purpose of this study is to investigate the role of fascin in tumor growth and progression. Fascin-depleted cell line was established using lentiviral shRNA silencing and applied in in vivo xenografted tumorigenesis of mouse tongue. Histopathological features in in vivo tumors were investigated.

Tumor growth was significantly decreased in mice inoculated with fascin-depleted cell line compared with wild type Mock. Induced tumors in Mock displayed features of poorly differentiated carcinoma with invasion to the muscle of tongue. On the contrary, limited infiltration and encapsulated tumor masses were observed in mice inoculated with fascin-depleted cell. Invasion to cervical lymph node was identified in 6 out of 10 mice in the Mock cell inoculation group, but there were no tumors in the fascin-depleted cell inoculation group. Cytokeratin was strongly detected in a regional lymph node in Mock cell inoculated group.

These results indicate that fascin improves the productivity of malignant tumors to increase infiltration into the regional stroma and promote tumor progression. Fascin can be employed as a valuable biomarker targeting metastatic oral cancer.

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common malignant epithelial neoplasm affecting the oral cavity, pharyngeal regions, and salivary glands, and is a major cause of cancer morbidity and mortality worldwide[1]. OSCC accounts for more than 90% of all oral malignant lesions. OSCC is caused by multiple cell signal changes due to complex factors such as an individual's genetic predisposition and exposure to environmental carcinogens. However, despite the availability of advanced clinical diagnostic systems and advanced therapeutic options, the overall 5-year survival rate for patients with OSCC has remained poor over the past decades[2]. Therefore, it is necessitated to improve the diagnosis prediction of cancer and develop an effective anti-cancer drug.

Metastatic cancer cells have increased invasiveness and motility. In order to increase the invasive activity, cancer cells form a protrusive cell shape and move through the surrounding extracellular matrix. Cellular protrusions are readily observed in invasive cancer and consist mainly of actin polymers[3]. Invasive cancer cells polymerize G-actin, assemble scaffold components to F-actin, and build protrusive structures known as invadopodia. Invadopodia formation leads to focalized degradation of regional matrix, thereby facilitating invasion and migration of cancer cells. Therefore, invadopodia is considered as an appropriate target for understanding cancer invasion mechanism and for the discovery of anti-cancer drugs.

Fascin is an actin-bundling protein that regulates the dynamics of the cytoskeletal structure of invadopodia and plays an important

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role in cancer invasion and metastasis. Fascin has been suggested as an effective maker of poor prognosis and a potential therapeutic target in human tongue squamous cell carcinoma[4]. In this study, we developed a fascin-depleted cell line using lentiviral shRNA silencing and used this cell line for generating a xenografted tumor model in nude mice to investigate morphological and immunohistochemical changes.

Materials and methods

Material and reagents

All reagents used in cell culture were purchased from Gibco BRL Co. (Rockville, MD, USA). Cholera toxin, hydrocortisone, insulin, apo-transferrin, T3, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All reagents used in this research were of analytical grade. Antibody for Fascin, Cytokeratin AE1/AE3 and beta-actin were purchased from Dako (Dako, Carpinteria, CA, USA).

Cell lines

YD-10B human OSCC cells were obtained from the Department of Oral Pathology, College of Dentistry, Yonsei University (Seoul, Korea). Cells were grown in DMEM/F12 (3:1 ratio) medium supplemented with 10% fetal bovine serum (FBS), $1x10^{-10}$ M cholera toxin, 0.4 mg/ml hydrocortisone, 5 µg/ml insulin, 5 µg/ml apo-transferrin and $2x10^{-11}$ M triiodothronine (T3) in a humidified atmosphere of 5% CO_2 at 37˚C.

Fascin depletion

Fascin 1-specific shRNA (h) lentiviral particles were transduced in cultured cells with 5 µg/ml polybrene according to the manufacturer's protocol (Santa Cruz Biotechnology, CA, USA). Puromycin (10 g/ml) was added to select stably transduced cells. Puromycin-resistance cells were analyzed to verify the fascin depletion by Western blot. Control shRNA (h) lentiviral particles-A (Santa Cruz) were also used as a negative control.

Western Blotting

Total protein was prepared with RIPA buffer containing protease inhibitor cocktail tablets (Merck KGaA, Darmstadt, Germany). Equal quantities of protein were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membrane was blocked with 5% skim milk in PBS and subsequently incubated with primary antibody (1:1,000 dilution) in 5% skim milk overnight at 4°C. Then membrane was incubated with respective horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution) for 2 h at room temperature. Targeted proteins were visualized using Enhanced Chemiluminescence Detection kit (Amersham Life Science, Parsippany, NJ, USA).

Animals and xenograft

Male Balb/c *nu/nu* mice (5 weeks; mean body weight, 18±1.5 g) were purchased from the Nara Bio Inc. (Seoul, Korea) and maintained at 20-22°C on a 12 h light/dark cycle. All animal studies were performed in accordance with experimental protocols that were approved by the animal ethics committee of Eulji University. Cells ($5×10^4$ cells/0.1 ml in media) were injected into tongue of mice with a 0.5-ml insulin syringe under anesthesia (n=10/group) and allowed to establish tumors for 6 weeks. At the end of experiments, the mice were sacrificed by cervical dislocation, and tumor volume was calculated using the formula: Volume=(length/width^2)/2. Subsequently, tongues and regional lymph were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin blocks.

Histological analysis

Deparaffinized tissue were rehydrated and conducted antigen retrieval via autoclave treatment of the sections in 0.01 M citrate buffer (pH 6.0). After blocking with 10% normal goat serum, the sections were incubated with a primary antibody at a 1:100 dilution in background reducing diluent (Dako). The sections were rinsed with PBS and incubated with biotinylated anti-mouse/anti-rabbit IgG (H+L) (1:100 dilution in background reducing diluent), followed exposure to horseradish peroxidase streptavidin (1:200 dilution in background reducing diluent). Staining was performed by incubating with 3,3'-diaminobenzidine (DAB) buffer. The sections were counterstained with hematoxylin, followed by dehydration and mounting. Hematoxylin and eosin (H&E) staining was also performed.

Statistical analysis

The analysis for statistical purposes was conducted using InStat GraphPad Prism ver. 5.01 statistical software (GraphPad Software, Inc., San Diego, CA, USA). The comparison

between tumor sizes was performed using the paired *t* test. A *P* value of <0.05 was considered to be statistically significant.

Results

Fascin protein was strongly overexpressed in human OSCC cells but was not expressed in human normal cells, such as epithelial cells (Figure 1).

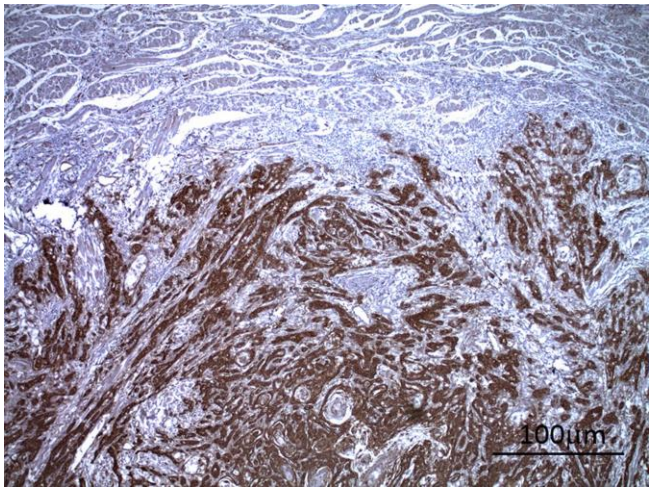

Figure 1. Fascin was expressed in primary oral squamous cell carcinoma (OSCC) cells. Fascin was not expressed in normal epithelial cells and stromal cells (original magnification ×40).

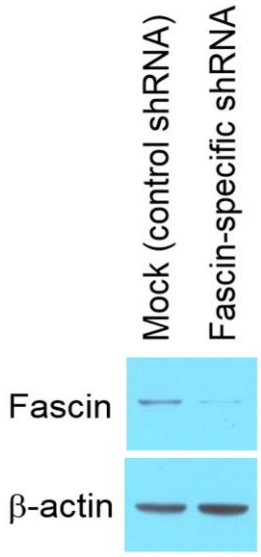

Figure 2. Fascin-depleted cell line was established using lentiviral shRNA silencing. Non-effective shRNA was used as a negative control (Mock). Down-expression of fascin was demonstrated using Western blotting. beta-actin was employed as a loading control.

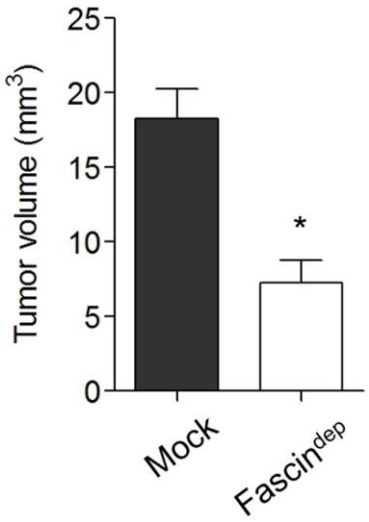

Figure 3A

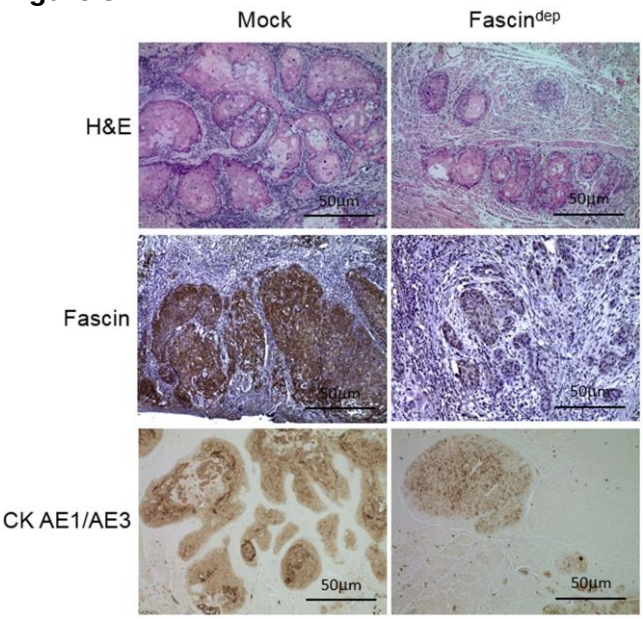

Figure 3B

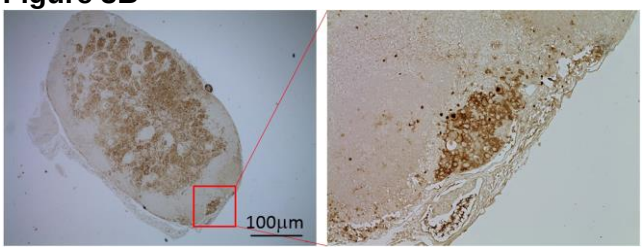

Figure 3C

Figure 3. The role of fascin in tumor growth and metastasis was evaluated in athymic nude mice. (A) Cells were injected into the mouse tongue and tumor volume was calculated at the end of 6 weeks experiment. Mock, wild type cell inoculated group; Fascin^{dep}, Fascin-depleted cell inoculated group. *P<0.05 versus Mock. (B) Histopathological features were observed using hematoxylin and eosin staining (H&E). A poorly

differentiated squamous cell carcinoma and intense infiltration of cancer cells into the nearby stroma were observed in the tongue infiltrated with Mock cells (Mock). Small masses of the tumor and limited invasion were observed in the tongue infiltrated with fascin-depleted cells (Fascin^{dep}). (original magnification ×40). The expression of fascin was identified in xenografted tissue Mock and Fascin^{dep}. Cytokeratin AE1/AE3 expression was investigated to verify the epithelial origin of the tumors. (C) Lymph node from wild type Mock cell inoculated group was immunostained with anti-cytokeratin AE1/AE3. [original magnification ×10 (left), ×40 (right)]

It was mainly expressed in the cytoplasm of the OSCC cells. In order to investigate the effect of fascin on tumor growth and progression, fascin-depleted cell line was established using lentiviral shRNA silencing (Figure 2). The fascin-depleted cell line was applied in xenografted *in vivo* tumorigenesis of mouse tongue and histopathological features of *in vivo* tumors were observed. As shown in Figure 3A, tumor volume in mice inoculated with fascin-depleted cell line was reduced by 2.54-fold at the end of the six weeks compared with wild type Mock ($p<0.05$). Tumor mass was observed in 10 out of 10 mice in the wild type Mock inoculation group (Table 1).

Group (5×10^4 cell/tongue)	Mass formations	Cervical lymph node metastasis
Mock	10 / 10	6 / 10
Fascin^{dep}	8(small mass) / 10	0 / 10

(Mock, wild type cell inoculated group; Fascin^{dep}, Fascin-depleted cell inoculated group)

Table 1. Tumorigenesis and regional lymph node metastasis in mouse tongue xenograft.

Induced tumors in Mock inoculation group displayed features of poorly differentiated carcinoma with keratin pearl and invasion to the regional muscle of tongue (Figure 3B). Whereas small masses of tumors were present in the fascin-depleted cell inoculated group. Limited infiltration and encapsulated tumor masses were observed in mice inoculated with fascin-depleted cell. Fascin expression was reduced in tissue from fascin-depleted cell inoculated group compared with the Mock tumor. The epithelial origin of the tumors was verified based on the detection of cytokeratin AE1/AE3. Table 1 reveals the presence of regional lymph node metastasis in 6 out of 10 mice in the Mock inoculation group whereas no lymph node metastasis was present in fascin-depleted cell inoculated group. Specimen from the lymph node of Mock inoculated mice demonstrated the presence of cytokeratin AE1/AE3 positive cells (Figure 3C).

Discussion

The increased early diagnosis rate of cancer and the development of effective treatment strategies lead to an improvement in cancer treatment and prognosis. However, there has been a constant increase in the incidence of cancer with no improvement in survival rate. Consequently, the development of prognostic biomarkers for malignant cancer is urgently necessitated. Oral cancer accounts for approximately 90% of the squamous cell carcinoma cases[5]. The most common sites of cancers of the mouth and oral cavity are the floor of the mouth, the pharynx including the soft plate, the lips, and the tongue. Salivary glands, the gingiva, the hard palate or roof of the mouth, and the buccal mucosa and soft tissues inside the cheeks are also the sites of cancer. As such, it is difficult to develop accurate biomarkers and anti-cancer drugs since the oral tissue is heterogenous. While early OSCCs, which are poorly differentiated and not metastasized, exhibit relatively good prognosis, most of the OSCCs are diagnosed at a late stage of the disease.

In this study, we investigated the role of fascin in cancer growth and progression and evaluated whether fascin is a useful target for diagnosis of oral cancer. The fascin depleted cell line was established using lentiviral shRNA silencing and applied in *in vivo* xenografted tumorigenesis of mouse tongue. Previously, we have reported that fascin is intimately involved in cancer growth and invasion[6,7]. However, histopathological features in *in vivo* tumors were not observed. Based on the changes in histopathological features as well as cancer growth by fascin depletion, fascin can be verified as a useful diagnostic target for OSCC. Xenograft animal models more precisely reflect the morphology and character of their respective original tumor[8]. Tumorigenesis by YD-10B human OSCC cells, derived from cancerous tissues of patient with tongue cancer is highly reproducible in mouse xenograft thus making it easier to observe cancer growth. In this study, YD-10B

cells with fascin depletion showed inhibited cancer growth in xenograft model compared to wild type Mock. Poorly differentiated carcinoma with invasion was observed in the tumor of wild type Mock. Whereas limited infiltration and encapsulated small tumor masses were observed in the tumor of fascin-depleted cells. The reduction of the invasion activity by fascin depletion is consistent with our previous results of the Matrigel-coated transwell invasion assay and 3-dimensional culture system[6].

The prognosis of OSCC varies based on a number of factors that are related to the tumor, treatment, and patient[9]. The 5-year survival rate of total oral cancer patients after the operation were 75.7%[10]. The 5-year survival rate of patients with recurrence of cervical lymph node was decreased to 46.7%. The 5-year survival rate in the advanced stages does not exceed 12% and most of the patients with advanced OSCC usually die within the first 30 months of their disease[11,12]. The degree of lymph node metastasis caused by fascin depletion was also investigated. All the regional lymph node was extracted when the mouse was sacrificed, and metastasis was verified through histological and immunohistochemical study. Typically, 60% of lymph node metastasis was identified in the Mock inoculation group for 6 weeks experiment period. Cytokeratin (CK) AE1/AE3-positive cells were extensively detected in the lymph nodes. However, lymph node metastasis was not present in any of the fascin-depleted cell inoculated group although small tumor masses were observed at 80% of tongue. No CK AE1/AE3-positive cells were detected in the lymph node in the fascin-depleted cell inoculated group. These results indicate that fascin is a valid biomarker targeting metastatic oral cancer.

Targeted molecular therapy using monoclonal antibodies, has been applied to head and neck cancer patients[13]. Limited or nonexistent side effects are the advantage of targeted molecular therapy. Epidermal growth factor receptor (EGFR), cyclooxygenase-2 (COX-2), peroxisome proliferator-activated receptor γ (PPARγ), and progesterone receptor are the main focused molecules. These molecules are associated with the proliferation and the differentiation of OSCC[14]. EGFR monoclonal antibodies (cetuximab, panitumumab, zalutumumab and nimotuzumab), EGFR tyrosine kinase inhibitors (gefitinib, erlotinib, lapatinib, afatinib and dacomitinib), vascular endothelial growth factor (VEGF) inhibitor (bevacizumab) or vascular endothelial growth factor receptor (VEGFR) inhibitors (sorafenib, sunitinib and vandetanib) and inhibitors of phosphatidylinositol 3-kinase/serine/threonine-specific protein kinase/mammalian target of rapamycin are being applied clinically. Fascin has been suggested a prognostic biomarker and therapeutic target for human tongue squamous cell carcinoma[4]. It is also evident through our *in vivo* study that fascin is intimately involved in tumor growth and metastasis of OSCC. For clinical application of fascin as an effective target molecular therapeutic molecule, further comprehensive clinical studies are necessary.

Conclusions

Fascin is intimately involved in tumor growth and progression in *in vivo* study. Fascin can be served as a valuable biomarker targeting metastatic oral cancer. Further studies are necessary for clinical applications.

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Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

Ethics approval

Approval was received from the Institutional Review Board of Yonsei University College of Dentistry. (2-2015-0058).

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