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Role of fascin in the proliferation and invasiveness of esophageal carcinoma cells ☆

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Abstract

Fascin, an actin-bundling protein, induces membrane protrusions and increases cell motility in various transformed cells. The overexpression of fascin in esophageal squamous cell carcinoma (ESCC) has been described only recently, but the roles and mechanism still remained unclear. Here, by using RNA interference (RNAi), we have stably silenced the expression of the fascin in EC109 cells, an ESCC cell line. Down-regulation of fascin resulted in a suppression of cell proliferation and as well as a decrease in cell invasiveness. Furthermore, we revealed that fascin might have functions in regulating tumor growth in vivo. The effect of fascin on cell invasiveness correlated with the activation of matrix metalloproteases such as MMP-2 and MMP-9. We examined that fascin down-expression also led to a decrease of c-erbB-2 and β-catenin at the protein level. These results suggested that fascin might play crucial roles in regulating neoplasm progression of ESCC.

Keywords: Fascin; Proliferation; Invasiveness; MMP-9; MMP-2; ESCC; RNAi

Fascins, which belong to a unique family of actin-bundling proteins [1,2], include sea urchin fascin [3–6], HeLa 55-kDa actin-bundling protein [7,8], and the gene products of *Drosophila* singed [9–11]. All of these proteins cause F-actin to aggregate side-by-side into bundles [8] and are localized in structures containing actin bundles including filopodia and stress fibers of cultured cells [1,7,8,12], bristles of Drosophila, actin bundles of *Drosophila* nurse cells [11,13], and microspikes and microvilli of sea urchin eggs and coelomocytes [5,6]. In mammalian cells, fascin is present in membrane ruffles, microspikes, and other motility-associated cell fibers [13,14].

The expression of fascin is highly specific to tissue and cell types. Fascin is abundant in tissues such as brain and spleen and, at cellular level, in specific types of cells such as neuronal and glial cells, microcapillary endothelial cells, and antigen-presenting dendritic cells [15–18]. High-level expression of fascin is observed in many transformed cells and carcinoma cells such as hormone receptor-negative breast carcinomas and ovarian carcinomas [19–21]. Cells expressing high levels of fascin share a common morphologic characteristic of many membrane protrusions in which fascin is predominantly present and show an increased activity of membrane extensions, suggesting that fascin may play a role in extending the membranes either for cell motility or for cell–cell interactions [19,22].

☆ *Abbreviations:* ESCC, esophageal squamous cell carcinoma; RNAi, RNA interference; siRNA, short interfering RNA; FCS, fetal calf serum; TBS, Tris-buffered saline; SEM, scanning electron microscopy; PCNA, proliferating cell nuclear antigen; MMP-2 and MMP-9, matrix metalloproteinases-2 and -9.

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The expression of fascin in human esophageal squamous cell carcinoma (ESCC) has been described only recently. An immunohistochemical study showed that fascin was overexpressed in ESCC and this might play an important role in the progression [23]. Our previous studies also demonstrated that the upregulated expression of fascin might be correlated with the malignant transformation of normal esophageal squamous cells to cancer cells [24]. However, the roles of fascin and the mechanism by which fascin is upregulated in ESCC remain unclear.

To investigate the roles fascin plays in ESCC, we have used the pSUPER RNAi system to stably suppress the expression of the fascin gene in EC109, an ESCC cell line in which fascin is highly expressed and analyzed its effect on the organization of actin cytoskeleton, proliferation, invasive behavior, and explored some involved mechanisms.

Materials and methods

Materials and cell culture. Human ESCC cell line EC109 and uterine cervix cancer cell line HeLa were purchased from the Chinese Academy of Science. They were maintained in 199 medium (Invitrogen) containing 10% fetal calf serum (FCS) in an atmosphere of 5% CO_2 at 37 °C. Monoclonal antibody anti-fascin, anti-PCNA, anti-c-erbB-2 (Dako Corporation), anti-β-catenin (Santa Cruz), anti-β-actin (Sigma), and anti-topoisomerase II (Roche) were used for immunofluorescence and Western blotting. Anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology and Zymed.

RNA interference. The mammalian expression vector, pSUPER.neo.circular (OligoEngine), was used for expression of siRNA in EC109 cells. Briefly, two primer pairs were synthesized, one pair encoding nucleotides 1067–1085 (GGCGTCCAATGGCAAGTTT) followed by a 9 base "loop" (TTCAAGAGA) and the inverted repeat (pSUPER-fascin1), and the second encoding nucleotides 1080–1098 (AAGTTTG TGACCTCCAAGA) again followed by the loop and inverted repeat (pSUPER-fascin2). And pSUPER.neo vector of nonspecific siRNA was taken as native control (pSUPER-con). The primer pairs were annealed and inserted into the *Bgl*II and *Hin*dIII sites of pSUPER.neo and transformed into JM109 competent cells (Promega). Positive clones were identified and verified by using restrictive cleavage and sequenced.

Stable transfection. EC109 cells were plated into six-well plates at 2×10^5 cells per well. After 24 h, cells were transfected with 1 μg RNAi plasmid hybrids using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions. G418 was added into the culture medium (400 μg/ml, Calbiochem) after 24 h. Stable G418-resistant clones were obtained in 7–9 days. The expanded cells were then used for subsequent studies.

Western blotting. Total cell lysates were prepared in a lysis buffer (150 mM NaCl, 1% Triton X-100, and 50 mM Tris–HCl, pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. Lysates were cleared by centrifugation at 14,000*g* for 30 min at 4 °C, separated by SDS–PAGE, and transferred to a PVDF membrane (Millipore). The membrane was incubated in blocking buffer (Tris-buffered saline [TBS], containing 0.1% Tween 20 and 5% nonfat milk) for 1 h at room temperature followed by the addition of the primary antibody for 2 h at room temperature. Then, the membrane was rinsed three times with TBS/Tween 20 and incubated with anti-mouse or anti-rabbit antibody for 2 h at room temperature. Immunoreactive bands were revealed by Western blotting luminol reagent (Santa Cruz). Photographed and quantitative analysis were done by FluorChemTMIS-8900 (Alpha Innotech).

Immunofluorescence microscopy. Cells were seeded on a coverslip and incubated for 24 h. After being washed with cold PBS, cells were fixed with 100% methanol at −10 °C for 15 min and treated with 0.2% Triton X-100 in PBS for 10 min. Cells were subsequently incubated with a blocking solution (10% normal goat serum in PBS) for 20 min, primary antibody overnight at 4 °C and followed by FITC-labeled secondary antibody for 30 min at 37 °C. Finally, the cells were counterstained using propidium iodide (Sigma), mounted in glycerol, and viewed with a fluorescence microscope.

RT-PCR. Total RNA was extracted by using the TRIZOL Reagent (Invitrogen) according to the user's instruction. Purified RNA was reverse-transcribed using Reverse Transcription System (Promega) according to the manufacturer's protocol. PCR was then performed in 22 cycles with 1 μl cDNA as template. The primers were as follows: forward primer 5′CTGGCTACACGCTGGAGTTC3′ and reverse primer 5′CTG AGTCCCCTGCTGTCTCC3′ for fascin gene. Forward primer 5′CCCCTACTGCCTATATCGAC3′ and reverse primer 5′AATG ATACGGGTGCTCTGAG3′ for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene.

Scanning electron microscopy. Cells were cultured on coverslips and harvested in 6, 12, and 24 h, respectively. Cells were then washed with PBS and fixed with 2.5% glutaraldehyde at 4 °C for 12 h. After thoroughly washing with PBS, the fixed cells were dehydrated through a series of ethanol and dried at room temperature. The samples were coated with a thin film of silver (Balzer Union SCD 020 sputter coating unit) and examined under a scanning electron microscope (Philips SEM-501 B).

Cell invasiveness assay. The invasiveness was determined by an invasiveness chamber assay. Cells (1×10^5) were seeded onto the top chamber of a 24-well matrigel-coated micropore membrane filter with 8 μm pores (Millipore), and the bottom chamber was filled with 0.6 ml of 199 medium with 10% FCS as a chemoattractant. The membranes were fixed and stained by Giemsa reagent, and the cells on the upper surface were carefully removed with a cotton swab after 24 h. Invasiveness was quantified by counting 10 random fields under a light microscope (400×). Data obtained from three separate chambers were shown as mean values.

Chamber migration assay. Migration was evaluated using a modified Boyden chamber assay. Cell culture inserts containing polyethylene terephthalate (Millipore) were placed within a 24-well chamber containing 0.6 ml of 199 medium with 10% FCS. 1×10^5 cells were seeded onto the inserts suspended in 0.2 ml of serum-free 199 medium. Non-migratory cells were removed from the upper surface of the filter after incubation for 24 h. Migrated cells were fixed and stained with Giemsa reagent. Migrating cells were quantified based on the procedure as described above.

Gelatin zymography. The transfected cells and untransfected cells were washed and cultured in serum-free 199 medium. Twenty-four hours later, the conditioned medium from 10^7 cells was collected, concentrated 100-fold by using Nanosep 10K centrifugal device (Pall Corporation),and subjected to SDS–PAGE through 10% polyacrylamide gels copolymerized with 1 mg/ml gelatin (Sigma). Gels were rinsed in washing buffer (50 mM Tris–HCl, pH 7.5, 2.5% Triton X-100) at room temperature for 1 h and incubated overnight at 37 °C in incubation buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, and 150 mM NaCl). Gels were fixed and stained with 0.1% Coomassie blue R250. After destaining, gelatinolytic signals were quantified by densitometry (FluorChemTMIS-8900, Alpha Innotech). Gelatinolytic activity was visualized as a clear band against a dark background of stained gelatin. Matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9) are detected by the clear band appearing at 72 and at 92 kDa.

In vivo experiments. To detect the impact of fascin on tumor cell proliferation in vivo, siRNA-treated EC109 cells (1×10^6) were injected into nude mice, with non-transfected EC109 cells as control. These mice were bred under specified pathogen-free conditions (26 °C, 70% relative humidity and a 12-h light/12-h dark cycle). They were fed ad libitum with a breeding/maintenance diet and water containing 1.3 g/L potassium sorbate and 2.0 g/L chloramphenicol (pH 2.5). The use of animals in this study complies with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1985) and the current Chinese law on the protection of animals. Prior to injection, 24 nude mice (4- to 6-week-old) were assigned at random to two groups. Time of the tumorigenesis was recorded and growth curves were made.

Results

Fascin expression in EC109 cells

By using Western blotting, we first examined the level of fascin expression in EC109 cells and HeLa cells. Fascin expression in EC109 cells is almost as high as that in HeLa cells (a putative fascin overexpression tumor cell line), indicating that fascin was also upregulated in EC109 cells (Fig. 1A). Immunofluorescence showed that fascin was localized in the cytoplasm, especially in the membrane protrusions of EC109 cells (Fig. 1B).

Silencing of the fascin gene

To study the function of fascin gene in EC109 cells, pSUPER system was adopted to stably suppress the fascin expression. G418-screened EC109 cells were used for analysis of the silencing effect. Immunofluorescence staining and Western blotting showed that fascin expression decreased markedly in treated cells compared with control. Fascin expression level was efficiently reduced by 92% in PSF1-treated cells and 45% in PSF2-treated cells. Transfection of the vector of nonspecific siRNA (PSC) did not reduce fascin expression level (Figs. 2A–C). Of the two established cell lines, PSF1 showed lower total cellular levels of fascin. Therefore, PSF1 was chosen for further analysis.

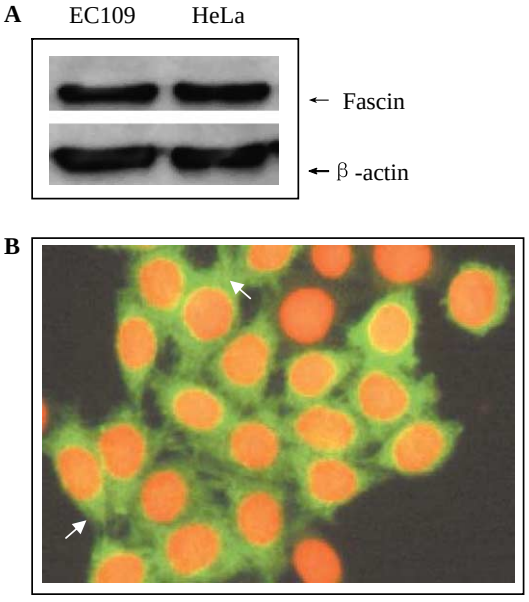

Fig. 1. Expression of fascin in EC109 cells and HeLa cells. (A) Fascin expression was determined by Western blotting analysis. Detection of β-actin was used as a loading control. HeLa cell line served as a positive control of fascin expression. (B) Immunofluorescence distribution of fascin was examined in EC109 cells, the green fluorescence (FITC labeled) corresponded for fascin (shown by arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

After continuing culture and passaging for 2 months, the stable silencing effect of fascin was identified by Western blotting. We found that the three passages maintained a significant reduction in fascin protein levels compared with the pSUPER-con cells and the non-transfected cells. Furthermore, the silencing effect of every passage was almost at the same level (Fig. 2D). To detect the silencing effect on mRNA level, RT-PCR was used and the result showed that mRNA level of the PSF-treated cells was reduced compared to the control or parent cells (Fig. 2E).

Morphological changes in EC109 cells

To explore whether a decreased expression of fascin was involved in the alterations of the cytoskeleton, we studied the morphological changes of the stable transfectants by scanning electron microscopy (SEM). Compared with the pSUPER-con cells, there was a sharp decrease of the membrane protrusions in the pSUPER-fascin cells. The pSUPER-fascin cells showed a smooth edge and fewer projections, displaying the morphology of the typical epithelial cells. In contrast, the pSUPER-con cells showed more elongated membrane projections (Fig. 3).

Proliferation and invasiveness alterations of EC109 cells

An apparent decreased cell proliferation in fascin-silenced EC109 cells was observed during the day-to-day culture. To further explore the underlying mechanism, in vitro detection of proliferating cell nuclear antigen (PCNA) and topoisomerase IIα levels and in vivo experiment on nude mice were performed. Results from Western blotting revealed that the levels of PCNA and topoisomerase IIα were reduced with the fascin knockdown (Fig. 4), and in vivo experiment on nude mice indicated that the tumorigenesis of the siRNA-treated cells decreased significantly compared to the control (Fig. 5).

The motility of pSUPER-fascin cells was examined by determination of their migration through the polyethylene filter in the absence of matrigel. Migration rate of the pSUPER-fascin cells was greatly decreased compared to the control. Fascin down-regulation also dramatically reduced cell invasive properties compared with parental cells. Cell migration was decreased by 62% and cell invasiveness was decreased by 70% (Fig. 6). Zymography analysis of the conditioned media obtained from cells showed two major bands at 92 and 72 kDa that were consistent with the zymographic pattern of MMP-9 and MMP-2 proenzymes, respectively. The activities of both MMP-2 and MMP-9 of pSUPER-fascin cells were significantly lower than those of the control (Fig. 7).

Down-expression of fascin leads to a decrease of c-erbB-2 and β-catenin

At present it has been reported that the upregulation of fascin correlated with altered expression of c-erbB-2/HER-2 and

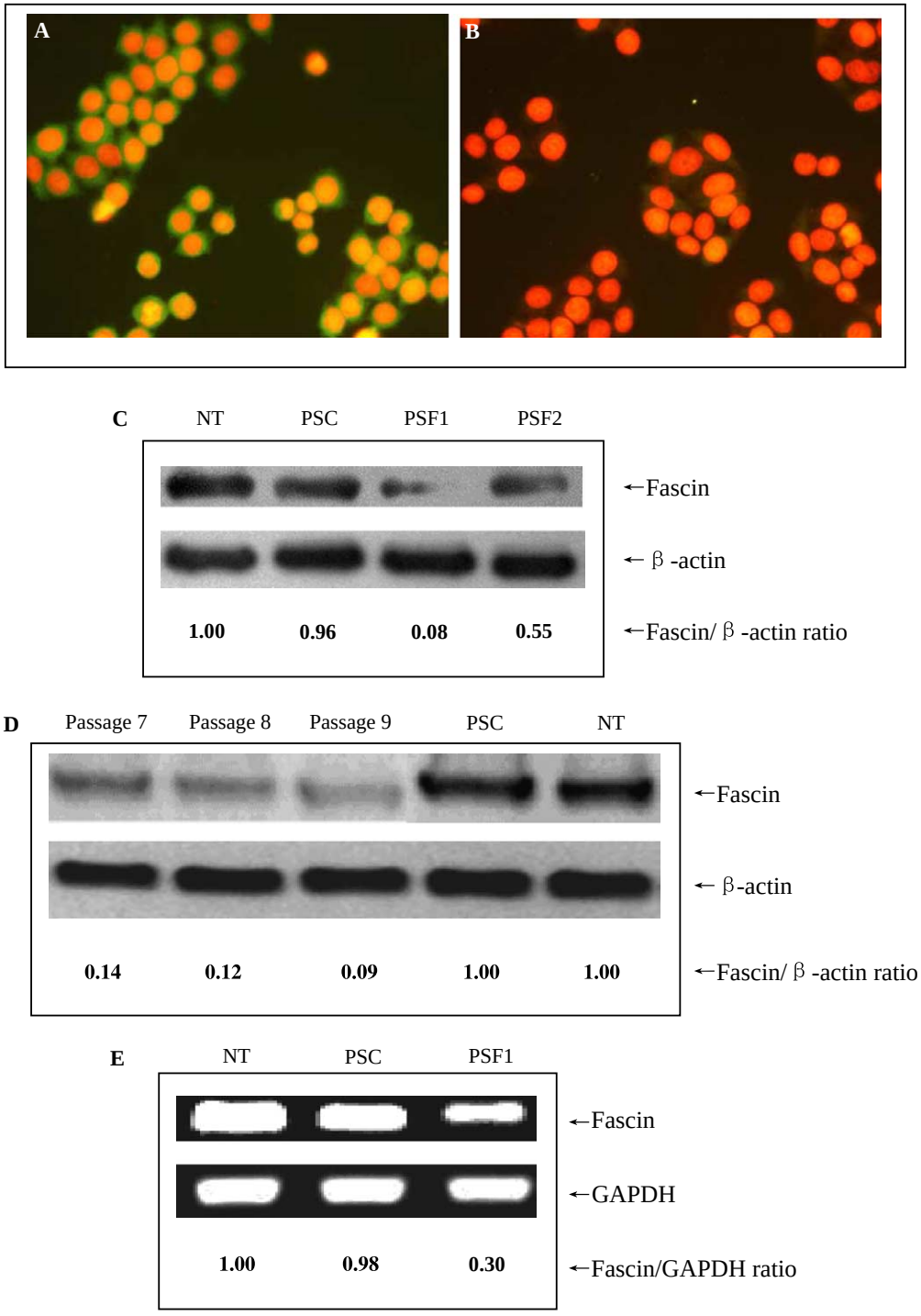

Fig. 2. Fascin gene silencing by siRNA. (A,B) Immunofluorescence analysis for fascin silence, the green fluorescence (FITC labeled) corresponded for fascin. (A) pSUPER-con; (B) pSUPER-fascin. (C) Fascin expression level was determined by Western blotting analysis. (D) Cells from different passages after transfection were obtained and analyzed for the stable silence of fascin level. (E) Fascin mRNA level was examined by RT-PCR. Detections of β-actin and GAPDH were used as loading controls of Western blotting and RT-PCR, respectively. PSC, pSUPER-con; PSF, pSUPER-fascin; NT, non-transfected EC109 cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

β-catenin [25,26]. Meanwhile, the mechanism remained controversial. To address these questions in ESCC, Western blotting was performed. The c-erbB-2 expression was significantly decreased after fascin was suppressed by siRNA while subtle β-catenin down-expression was observed (Fig. 8).

Discussion

Fascin is an actin-bundling protein originally found in the extracts of unfertilized sea urchin eggs [2]. Fascins play roles in the organization of two major forms of actin-based structures: dynamic cortical cell protrusions and

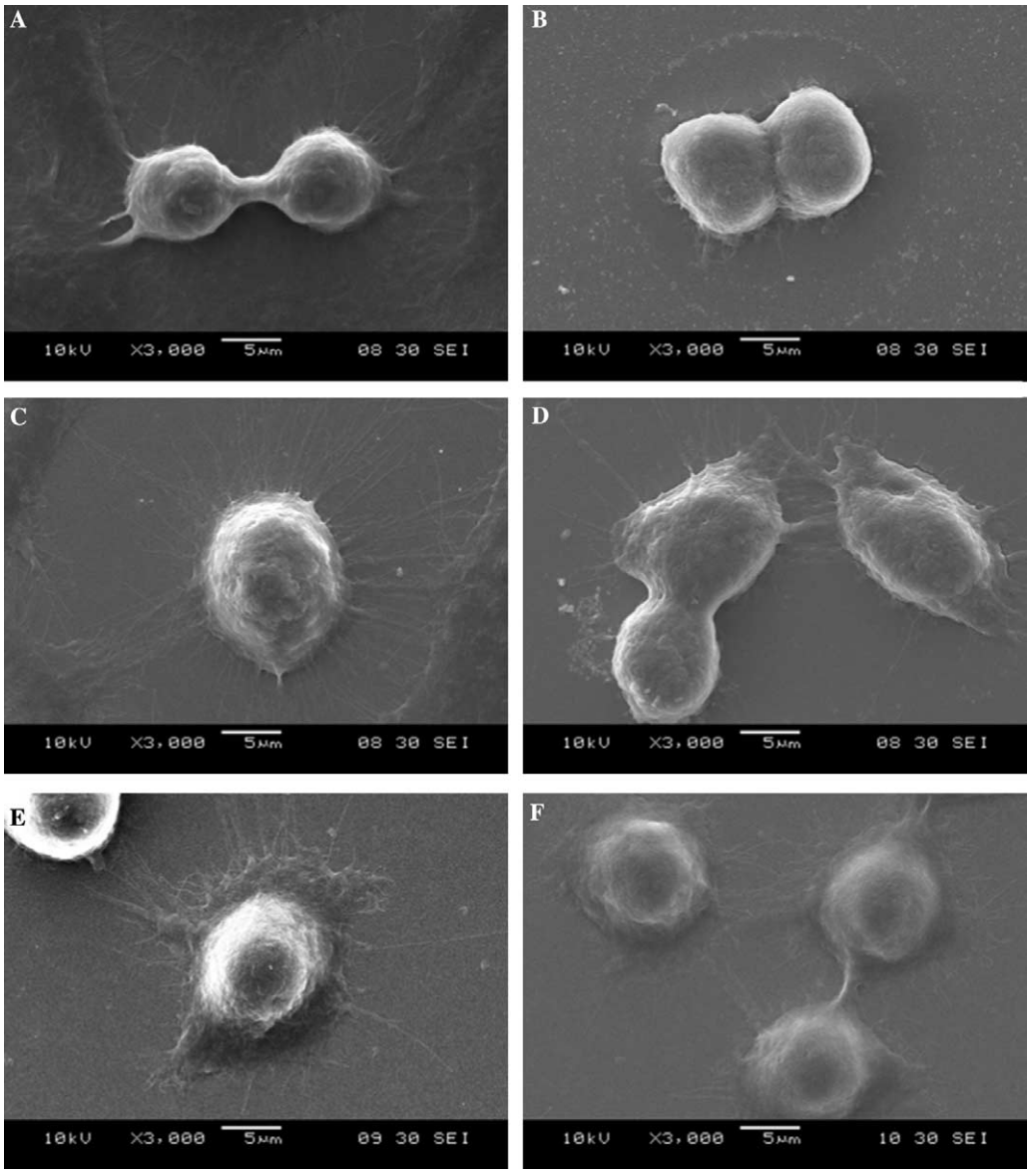

Fig. 3. Decrease formation of membrane protrusions by pSUPER-fascin transfection. (A,C,E) The untreated EC109 cells obtained after different times of culture (6, 12, and 24 h, respectively). (B,D,F) siRNA-treated cells obtained after different times of culture (6, 12, and 24 h, respectively).

cytoplasmic microfilament bundles. High-level expression of fascin is observed in many tumor cell lines such as breast carcinoma and esophageal carcinoma, but the roles and mechanism still remained unclear. For a better understanding of the function of fascin, in the present study we have used the pSUPER system to stably silence fascin gene in EC109 cells.

Our results revealed that fascin was highly expressed in EC109 cells and like many other fascin-high-expression cells, EC109 cells have morphologic characteristics of many membrane protrusions in which fascin is predominantly present. Here we confirmed that expression of fascin was involved in the alterations of the cytoskeleton by using SEM that showed a sharp decrease of the membrane protrusions in the treated cells.

Fascin effects on cell proliferation of colon cancer and nonsmall cell lung cancer have been reported [19,27]. However, these conclusions were mostly based on the morphological postulation rather than molecular study. PCNA functions as a cofactor for DNA polymerase, which is required for both DNA replication and DNA repair [28,29]. DNA topoisomerase IIα has been shown to be required for chromatin condensation, chromosomal segregation during mitosis and linked with active cell proliferation in mammalian cells [30,31], they are usually taken as markers of cell proliferation. Our studies revealed that the protein levels of both PCNA and topoisomerase IIα were decreased after fascin gene silencing. The experiment on nude mice also supported that cell proliferation was suppressed with fascin knockdown. To our knowledge this is the first report on the direct effect of fascin on ESCC cell proliferation. Fascin is an actin-bundling protein and suppression of fascin might affect the assembly of cytoskeletal elements at the cytoplasmic face of the membrane and the

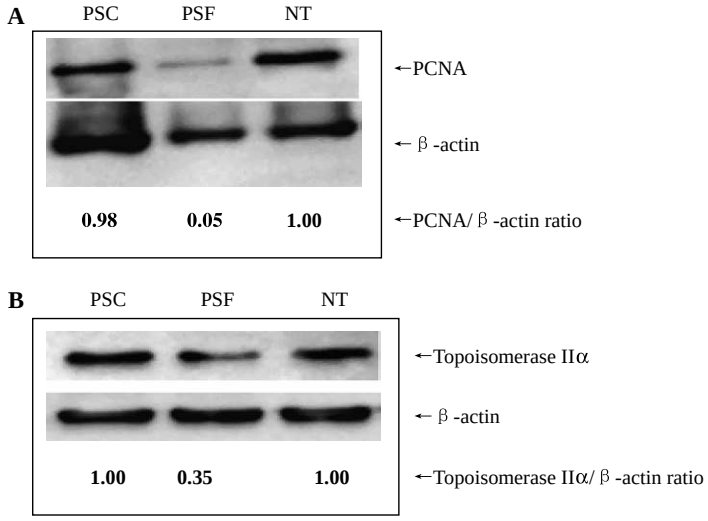

Fig. 4. Expression of PCNA and topoisomerase IIα in siRNA-treated cells. Protein levels of PCNA and topoisomerase IIα were determined by Western blotting analysis. (A) Blotted with anti-PCNA antibody. (B) Blotted with anti-topoisomerase IIα antibody. Detection of β-actin was used as loading control. PSC, pSUPER-con; PSF, pSUPER-fascin; NT, non-transfected EC109 cell.

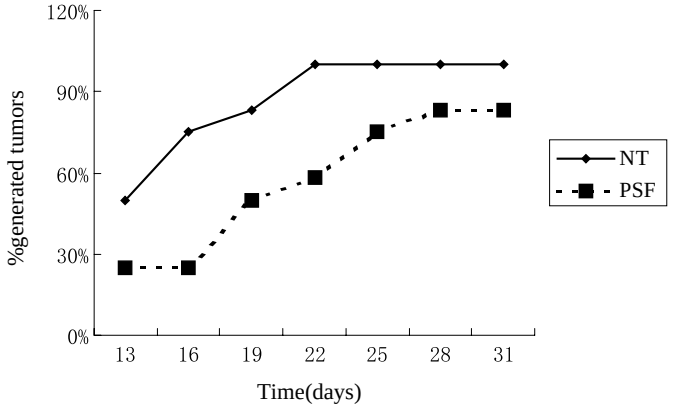

Fig. 5. Tumorigenesis in nude mice. For the tumor cell proliferation experiments, siRNA-treated cells and non-transfected EC109 cells were injected into nude mice. Time of the tumorigenesis was recorded and growth curves were made. Tumor first appeared after 13 days. PSF, pSUPER-fascin cells; NT, non-transfected EC109 cells.

nuclear skeleton and impact the regulation of cell proliferation. Changes in cell proliferation might be a key factor in regulating neoplasm progression and tumor growth [32]. These results are novel and indicate a possible role for fascin in the development of ESCC. Studies to establish more mechanisms involved are underway.

Jawhari et al. [19] and Yamashiro et al. [22] have reported that fascin affected cell migration and invasiveness. More recently, Hashimoto et al. [23] reported that KYSE170, one of the fascin-overexpressed ESCC cell lines, exhibited decreased motility and invasiveness after down-regulation of fascin expression. However, the underlying mechanism needed to be elucidated. Our results of the invasiveness assay and the chamber migration assay confirmed that fascin down-regulation could lead to decreased cell migration and invasiveness. A striking feature of the fascin down-regulation cells was the decreased formation of surface protrusions that play essential roles in cell motility. Besides the decreased formation of protrusions, here we suggested another possible mechanism for fascin effect on cell invasiveness, as shown in the gelatin zymography, was the decreased activity of extracellular matrix proteases such as MMP-2 and MMP-9. MMP-2 and MMP-9 are proteolytic enzymes that digest collagen type IV and other components of the basement membrane. They play a key role in local invasiveness and the formation of distant metastases by malignant tumors [33]. We postulate that the effects of fascin on cell invasiveness involve both changes in cell motility as well as the activity of matrix proteases. However, how fascin affected the activation of MMP-2 and MMP-9 still needs further studies.

C-erbB-2 protein is a 185-kDa transmembrane glycoprotein which is related to the EGF receptors having tyrosine kinase activity [34]. β-catenin, one of the key components of the wnt (wingless-type) pathway and cell–cell adhesion system [35,36], is also a substrate of c-erbB-2 tyrosine phosphorylation [37]. Studies from Tao et al. [25] showed that β-catenin associated with fascin in a non-cadherin complex and discerned a possible involvement of wnt signaling on fascin activity in cancer cells. Grothey et al. [26] reported that a pathway for fascin upregulation was dependent on upregulation of c-erbB-2/HER-2 in breast cancer. It is interesting that our study showed that the protein levels of both c-erbB-2 and β-catenin decreased with fascin knockdown, implicating that fascin might have an effect on the expression of the two proteins by an unknown mechanism. What is the relation among these three molecules and whether c-erbB-2 and β-catenin are involved in the overexpression of fascin in ESCC need further studies.

In conclusion, by using siRNA technology we have successfully silenced fascin gene in EC109 cells and found that decreased level of fascin correlated with decreased formation

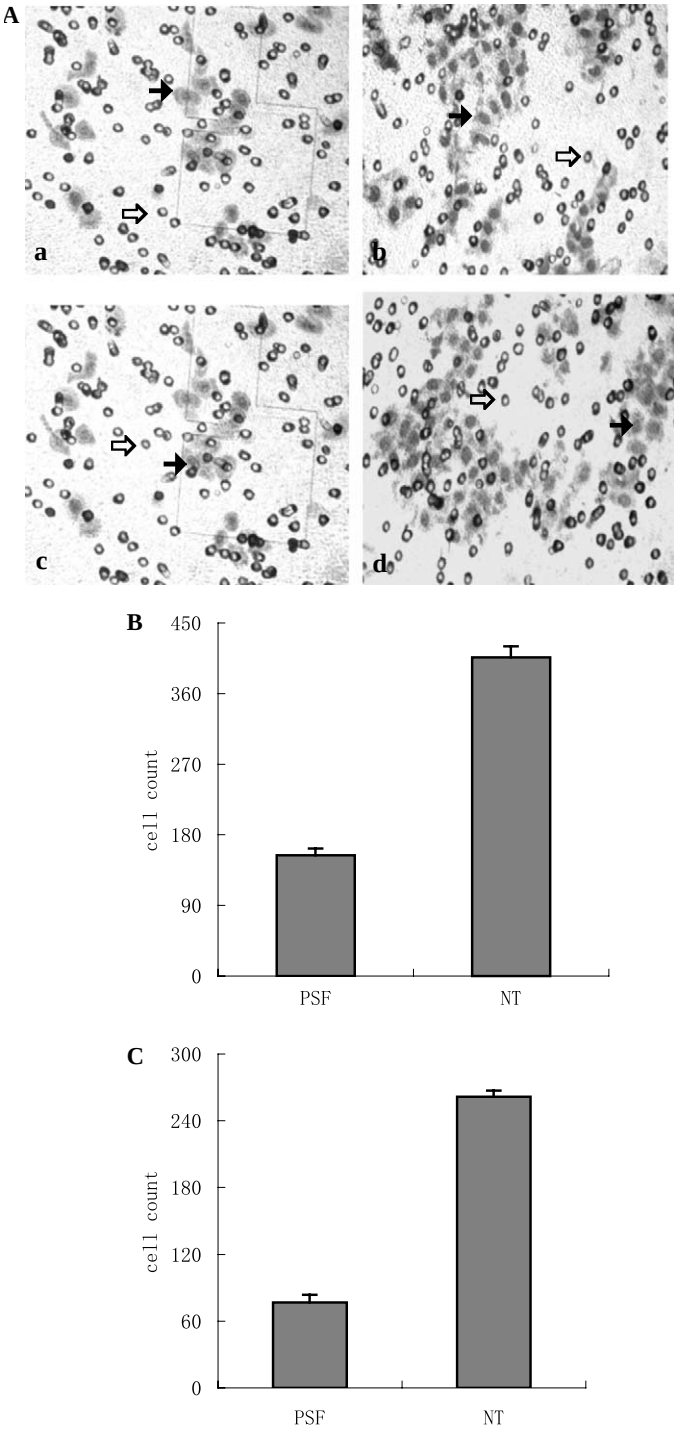

Fig. 6. Role of fascin in cellular invasiveness. (A) Modified Boyden chambers and matrigel-coated invasiveness chambers were used to measure cell migration and cell invasiveness, and representative fields were photographed. Hollow-arrows show the membrane pores, arrows demonstrate cells that have migrated through the membrane. (a,c) pSUPER-fascin cells and (b,d) non-transfected EC109 cells. (B) Cell migration was assessed with modified Boyden chamber inserts. (C) Cell invasiveness was examined with matrigel-coated invasiveness chambers. Migratory cells were fixed and stained with Giemsa reagent. For quantification, the cells were counted in 10 random fields under a light microscope (400×). PSF, pSUPER-fascin cells; NT, non-transfected EC109 cells.

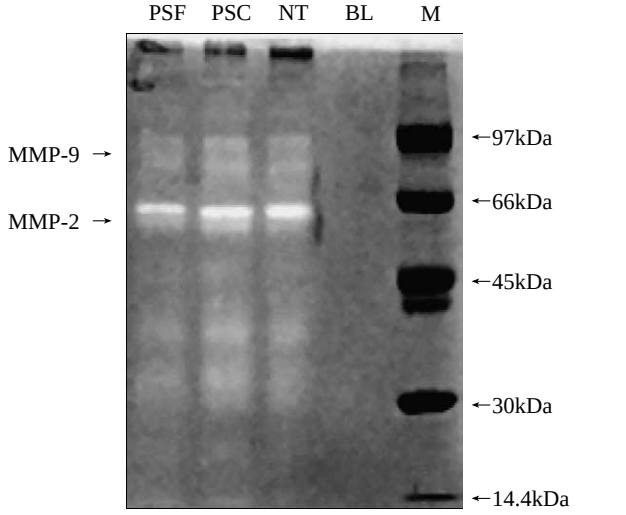

Fig. 7. Gelatin zymography of the conditioned medium. PSF, pSUPER-fascin cells; PSC, pSUPER-con cells; NT, non-transfected cells; BL, blank line; M, protein marker.

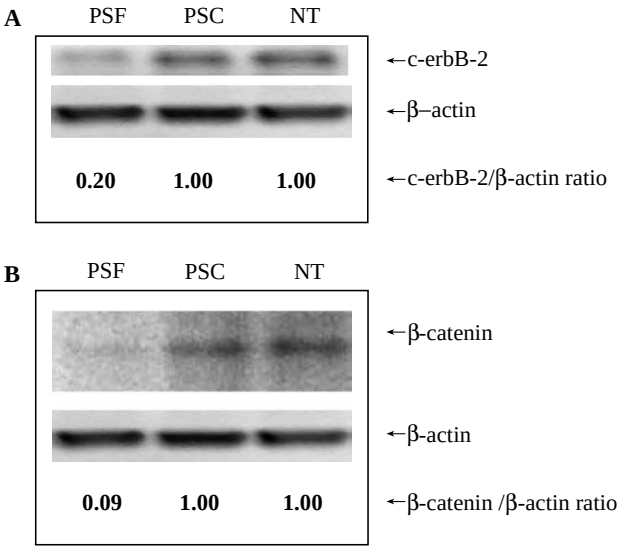

Fig. 8. Expression c-erbB-2 and β-catenin in siRNA-treated cells. Expression levels of c-erbB-2 and β-catenin were determined by Western blotting analysis. (A) Blotted with anti-c-erbB-2 antibody. (B) Blotted with anti-β-catenin antibody. Detection of β-actin was used as a loading control. PSC, pSUPER-con; PSF, pSUPER-fascin; NT, non-transfected EC109 cell.

of dynamic cell protrusions, decreased proliferation, and decreased invasiveness. Furthermore, reduced expression of β-catenin and c-erbB-2 has also been demonstrated. On the basis of these results, we propose that fascin, by participating in the formation of cell protrusions and cell proliferation, may promote invasiveness and metastasis in carcinogenesis.

Acknowledgments

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