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Original Article

FRAS1 knockdown reduces A549 cells migration and invasion through downregulation of FAK signaling

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Abstract: Distal metastasis is the major cause of death for the vast majority of lung cancer patients. Many extracellular matrix (ECM)-related molecules are proposed to be associated with the migration and invasion of cancer cells. FRAS1 encodes an ECM protein, however, little is known about its function on tumorigenesis and metastasis of lung cancer. In this work, FRAS1 was silenced by shRNA in non-small cell lung cancer (NSCLC) A549 cell line. The capacities of A549 cells to migrate and invade were decreased markedly after FRAS1 knockdown. The shRNA knockdown of FRAS1 was found to be specific and had no effect on A549 cells proliferation. Western blot experiments demonstrated that FRAS1 knockdown inhibited FAK signaling but not Src signaling. Overall, we found that FRAS1 knockdown reduces A549 cells migration and invasion ability through downregulation of FAK signaling.

Keywords: Lung cancer metastasis, migration and invasion, FRAS1, FAK signaling

Introduction

FRAS1 encodes an extracellular matrix (ECM) protein that appears to function in the regulation of epidermal-basement membrane adhesion and organogenesis during development. Mutations in this gene cause Fraser syndrome, a multisystem malformation that can include craniofacial, urogenital and respiratory system abnormalities [1, 2]. However, the roles of FRAS1 on tumorigenesis and metastasis remain unknown.

Lung cancer is the leading cause of cancer death in men and women worldwide. Patients with lung adenocarcinoma are often diagnosed with metastasizing symptoms and die of early and distal metastasis [3]. Metastasis is made up of a cascade of interrelated and sequential steps, including cell adhesion, ECM degradation, cell movement, and invasion. ECM-related molecules have been associated with the migration and invasion of lung cancer cells in vitro and in vivo, such as FBLN3 [4], Integrin β1 [5-7], LAMB3 [6], MMP2 [8], CD44s [9], COL6A1 [10], fibronectin [11], and Tissue Factor Pathway Inhibitor-2 (TFPI-2) [12]. Furthermore, FRAS1 was proposed to be a biomarker for human endometrial carcinoma [13]. These reports demonstrated the possibility that FRAS1 is involved in lung cancer metastasis. It is of great interest to address this hypothesis in lung cancer cells.

In present work, FRAS1 was silenced by shRNA in Non-small cell lung cancer (NSCLC) A549 cells, the migration and invasion capacities were investigated by transwell assay. The results demonstrated that FRAS1 actually regulates A549 cells migration and invasion rather than proliferation via mediating the FAK signaling.

Materials and methods

Cell culture

The human NSCLC cell line A549 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM medium (Gibco) supplemented with 10% FBS (Hyclone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) in a humidified atmosphere

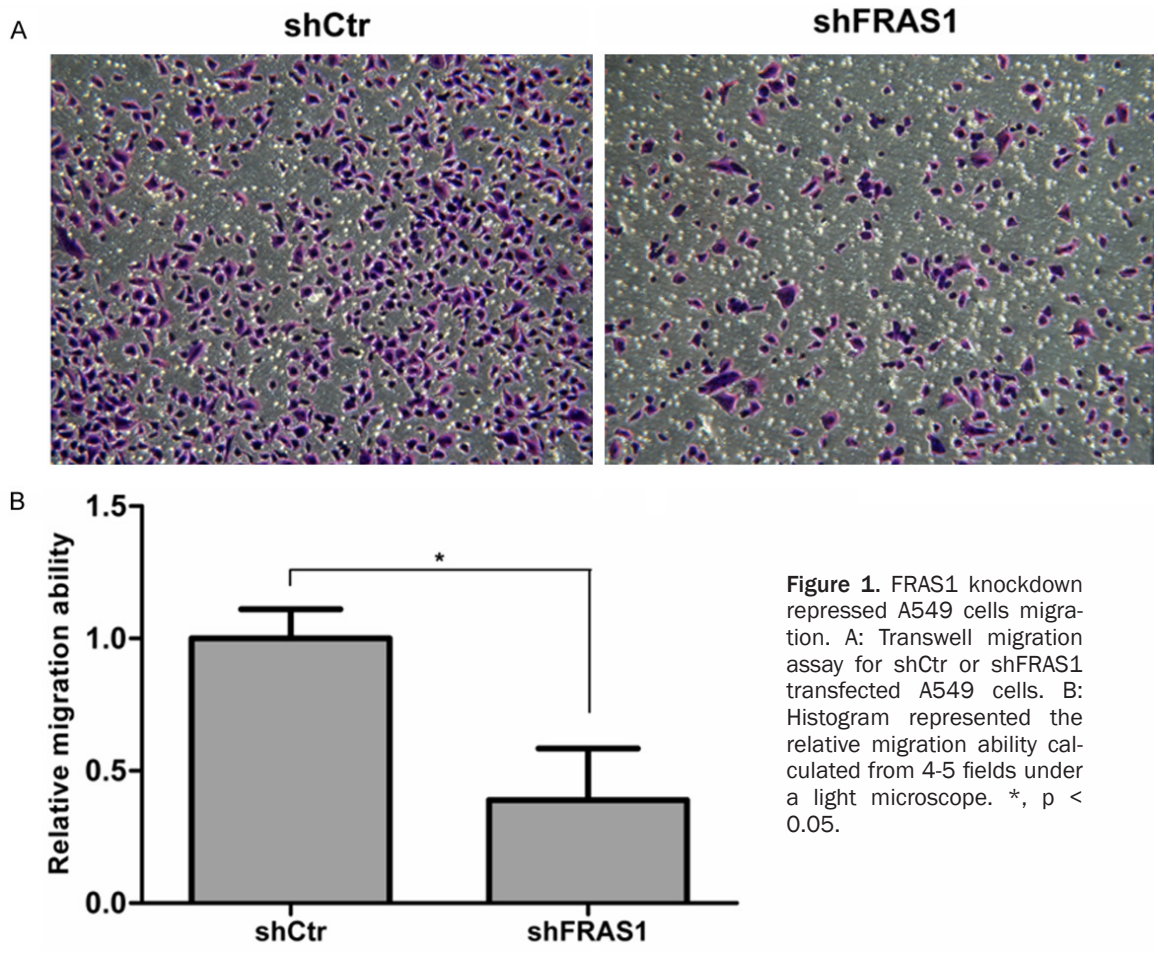

Figure 1. FRAS1 knockdown repressed A549 cells migration. A: Transwell migration assay for shCtr or shFRAS1 transfected A549 cells. B: Histogram represented the relative migration ability calculated from 4-5 fields under a light microscope. *, $p < 0.05$.

containing 5% CO_2 at 37°C. Cells in the exponential growth phase were used for all the experiments.

Construction and infection of shFRAS1 lentivirus

A customized lentiviral shRNA targeting FRAS1 was constructed into lentivirus vector (Invitrogen, BLOCK-iT™ Lentiviral RNAi Expression System, K4944-00), shFRAS1 lentivirus was packaged and titered in HEK293T cells according to the manufacturer's protocol. A549 cells were infected by shRNA lentivirus at MOI of 5 in the presence of polybrene (8 μg/ml).

Cell migration and invasion assay

Migration assays were performed in Transwells (Corning Inc., 8.0-μm pore size). For migration assay, 96 h post transduction of shFRAS1 or shCtr (control shRNA, shRNA with empty vector), 2.5×10^4 cells in serum-free medium were added to the upper wells. Media containing 10% FBS were added to the lower wells. Cells that migrated through the filter after 24 h were stained with 0.2% crystal violet and counted by phase microscopy.

Cells transfected with shFRAS1 or shCtr were cultured separately until 80% confluence. The cells were then washed three times with phosphate-buffered saline (PBS) and cultured in serum-free media overnight before being subjected to an ECM invasion assay in vitro. The invasion assay was conducted using BioCoat Matrigel Invasion Chambers with 8 μm pores (BD Biosciences, Bedford, MA) according to the manufacturer's instructions. Briefly, 2.5×10^4 cells were resuspended in fresh serum-free media and seeded into the upper chamber of a 24-well transwell plate, while the lower chamber contained fresh culture media with 20% FBS. The cells were allowed to invade for 22 hours (37°C, 5% CO_2 atmosphere), and the chambers were then washed with PBS. Those cells that did not invade through the membrane

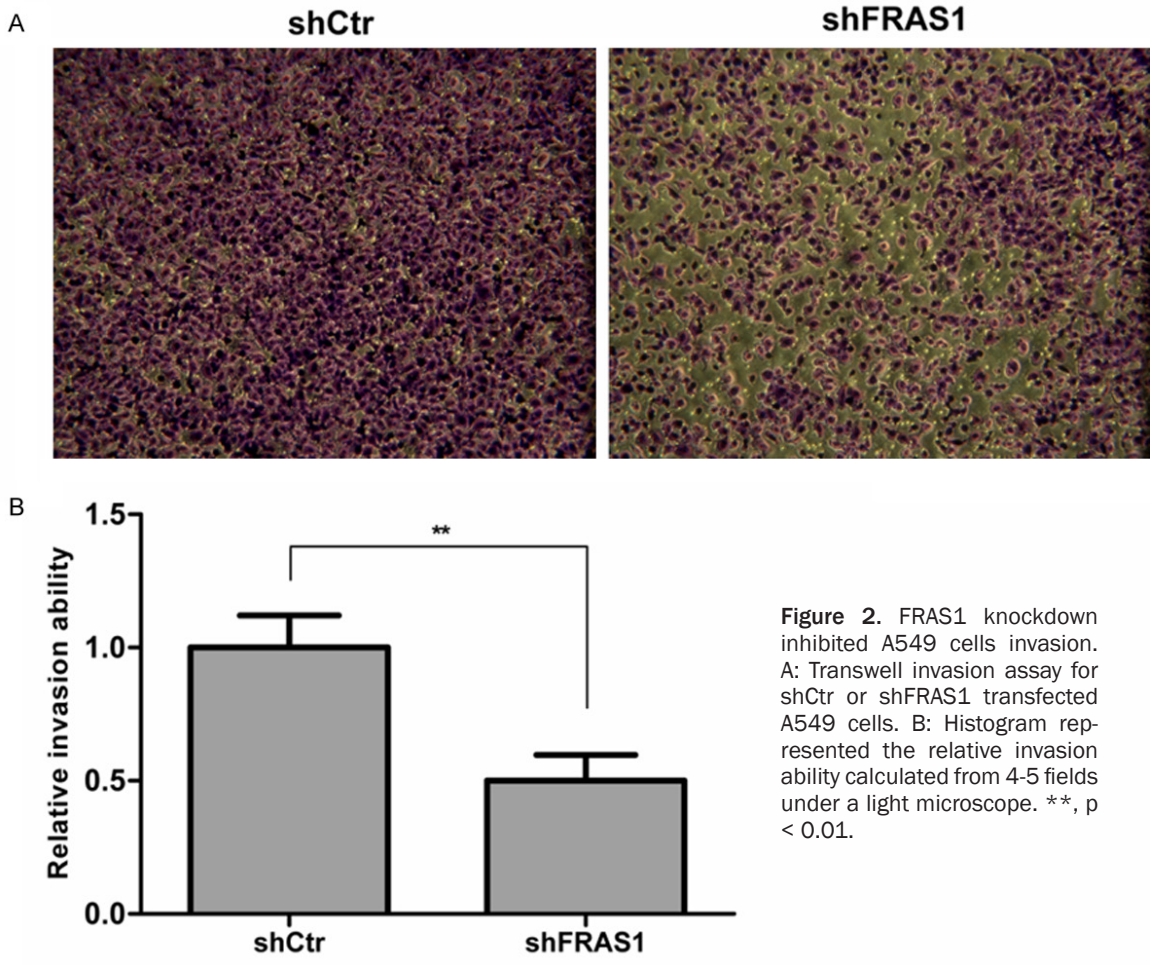

Figure 2. FRAS1 knockdown inhibited A549 cells invasion. A: Transwell invasion assay for shCtr or shFRAS1 transfected A549 cells. B: Histogram represented the relative invasion ability calculated from 4-5 fields under a light microscope. **, $p < 0.01$.

were removed. The invading cells on the lower surface of the membrane were fixed with cold methanol, stained with 0.2% crystal violet and examined. The cells on each membrane were counted in no less than five fields under a light microscope.

Quantitative real-time PCR (qPCR)

Total RNA was synthesized to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with mixture of oligo-dT and Random Primer (9 mer). Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. Gene expression was calculated relative to expression of hActb endogenous control and adjusted relative to expression in A549 transfected with shCtr. The primers for qPCR validation were list as below: hActb: forward: 5'-GCATCCCCCAAAGTTCACAA-3', Reverse: 5'-GGACTTCCTGTAACAAC GCATCT-3'; FRAS1: forward: 5'-AATAGCTGCCAACCAATG-CTG-3', Reverse: 5'-CAAGAGC ACACACTACATGG-AG-3'.

MTS cell proliferation assay

The tetrazolium compound (MTS) cell proliferation assay is a quantitative colorimetric assay for mammalian cell survival and proliferation. A549 cells (4×10^3) were grown in 100 μl of culture medium containing serum per well in a 96-well plate. After 24 h, the cells were treated by shFRAS1 or shCtr lentivirus for 96 h. Every treatment for each cell line was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 2 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol.

Protein isolation and western blotting

Cell pellets were resuspended in 1 × SDS loading buffer (1 mmol·L^{-1} Na_3VO_4, 10 mmol·L^{-1} NaF, 1 mmol·L^{-1} PMSF) containing protease inhibitors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for

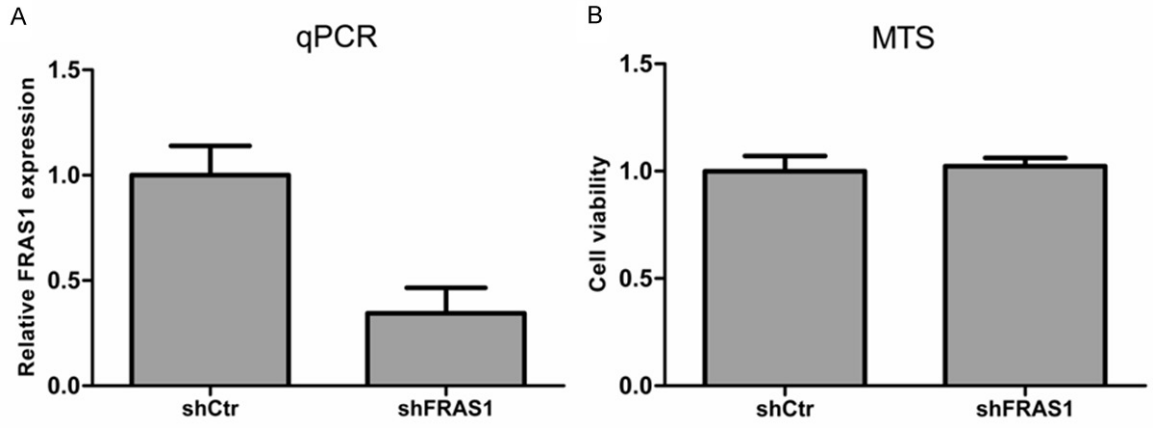

Figure 3. FRAS1 does not influence the proliferation of A549 cells. A: qPCR assay for FRAS1 knockdown efficacy. B: Silencing of FRAS1 had no effect on A549 cell proliferation. The cell viability was examined by MTS assay.

GAPDH (Abmart, 080922), AKT (Santa Cruz, sc-8312), p-AKT (Santa Cruz, SC-7985-R), Src (Epitomics, 3795-S), p-Src (Epitomics, 5595-1, pY418), FAK (Epitomics, 1700S), p-FAK (Epitomics, 2211-1, pY397) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

Results

FRAS1 knockdown inhibits the migration and invasion of A549 cells

To investigate the roles of FRAS1 on the migration and invasion of A549 cells, FRAS1 was silenced by shRNA in A549 cells and the migration and invasion of A549 cells was examined by transwell migration and invasion assay. The results showed that following FRAS1 knockdown the migration ability of A549 cells decreased by 61% (**Figure 1**) and the invasion ability decreased by 50% (**Figure 2**), suggesting that FRAS1 is vital for A549 cells to exert the capacities of migration and invasion and hence might play roles in lung cancer metastasis.

FRAS1 does not influence the proliferation of A549 cells

To rule out the off-target effect and the influence on cell proliferation of shRNA knockdown, the gene-silencing efficacy and the cell viability were detected respectively by qPCR and MTS assay. The knockdown efficacy of FRAS1 by shRNA was 66% (**Figure 3A**), suggesting the shRNA knockdown is specific and the effects on A549 cells migration and invasion after FRAS1 knockdown is not possibly caused by off-target effect. Meanwhile, FRAS1 knockdown did not influence the proliferation of A549 cells (**Figure 3B**), indicating that FRAS1 is really associated with the migration and invasion ability other than proliferation of A549 cells.

FRAS1 regulates the migration and invasion of A549 cells via mediating the FAK signaling

And then the underlying mechanisms by which FRAS1 plays roles on A549 cells migration and invasion were investigated by immunoblotting assay (**Figure 4**). The results demonstrated that the AKT and Src signaling were not altered significantly, while FAK signaling was dramatically downregulated by FRAS1 knockdown, proposing that FRAS1 regulates the migration and invasion of A549 cells via mediating the FAK signaling.

Discussion

Distal metastasis is the major cause of death for the vast majority of lung cancer patients. Many ECM-related molecules are proposed to be associated with the migration and invasion of cancer cells. FRAS1 encodes an ECM protein, however, little is known about its function on tumorigenesis and metastasis of lung cancer.

In this work, FRAS1 was silenced by shRNA in NSCLC A549 cell line. The capacities of A549 cells to migrate and invade were examined by

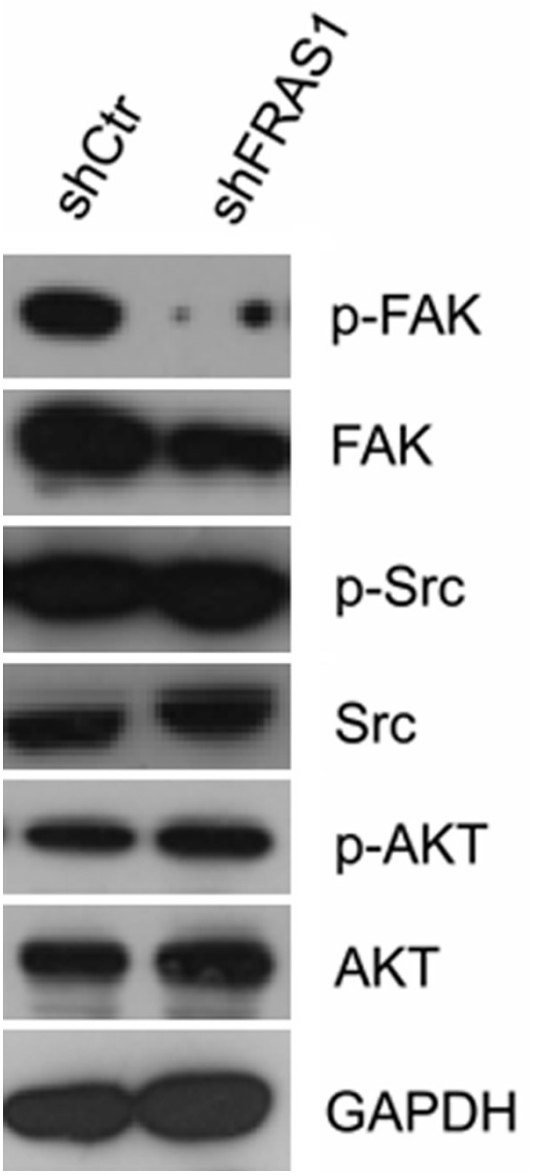

Figure 4. Western blot for several signaling molecules. The total and phosphorylation levels of AKT, Src and FAK proteins were detected for A549 cells infected with shCtr or shFRAS1, GAPDH served as an input control.

transwell assay. The underlying mechanisms were investigated by western blot. We found that FRAS1 knockdown reduces A549 cells migration and invasion through downregulation of FAK signaling.

Previous studies have suggested some possible signaling pathways by which ECM-related molecules regulate migration and invasion of cancer cells, such as IGF1R/PI3K/AKT/GSK3β signaling [4], Src signaling [14], ERK1/2 signaling [5, 14], p38MAPK signaling [15], epithelial-mesenchymal transition [7], NF-kB and STAT3 signaling [16]. Interestingly, our western blot results demonstrated that it is the FAK signaling but not Src and AKT signaling that is repressed following FRAS1 knockdown. Src and FAK proteins are both non-receptor protein-tyrosine kinases implicated in cancer invasion and metastasis [17-21]. Both kinases are involved in focal adhesion and adjacent adhesion to mediate the transmission of extracellular signaling to intracellular downstream molecules. The activation of Src and FAK requires the release of auto-inhibitory protein conformations that result in increased kinase activity. The full catalytic activation of FAK needs the Src-mediated phosphorylation of tyrosine residues in the kinase loop of FAK [22, 23]. Our results demonstrated that FRAS1 knockdown suppressed FAK signaling but not Src signaling, suggesting that FRAS1 is specific and necessary for FAK but not Src activation. Therefore, it deserves further investigation of FRAS1 expression plus FAK activation status in lung cancer metastasis tissue samples. On the other hand, the status of ERK1/2 signaling, p38MAPK signaling, NF-kB and STAT3 signaling after FRAS1 knockdown may also present clues for the underlying mechanisms and hence warrant further examination.

Taken together, FRAS1 knockdown reduces A549 cells migration and invasion through downregulation of FAK signaling.

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Disclosure of conflict of interest

None.

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References

[1] McGregor L, Makela V, Darling SM, Vrontou S, Chalepakis G, Roberts C, Smart N, Rutland P,

Prescott N, Hopkins J, Bentley E, Shaw A, Roberts E, Mueller R, Jadeja S, Philip N, Nelson J, Francannet C, Perez-Aytes A, Megarbane A, Kerr B, Wainwright B, Woolf AS, Winter RM, Scambler PJ. Fraser syndrome and mouse blebbed phenotype caused by mutations in FRAS1/Fras1 encoding a putative extracellular matrix protein. Nat Genet 2003; 34: 203-208.

[2] Vrontou S, Petrou P, Meyer BI, Galanopoulos VK, Imai K, Yanagi M, Chowdhury K, Scambler PJ, Chalepakis G. Fras1 deficiency results in cryptophthalmos, renal agenesis and blebbed phenotype in mice. Nat Genet 2003; 34: 209-214.

[3] Tsai CM, Yen GC, Sun FM, Yang SF, Weng CJ. Assessment of the anti-invasion potential and mechanism of select cinnamic acid derivatives on human lung adenocarcinoma cells. Mol Pharm 2013; 10: 1890-1900.

[4] Kim IG, Kim SY, Choi SI, Lee JH, Kim KC, Cho EW. Fibulin-3-mediated inhibition of epithelial-to-mesenchymal transition and self-renewal of ALDH+ lung cancer stem cells through IGF1R signaling. Oncogene 2013; [Epub ahead of print].

[5] Wang H, Wu C, Wan S, Zhang H, Zhou S, Liu G. Shikonin attenuates lung cancer cell adhesion to extracellular matrix and metastasis by inhibiting integrin beta1 expression and the ERK1/2 signaling pathway. Toxicology 2013; 308: 104-112.

[6] Wang XM, Li J, Yan MX, Liu L, Jia DS, Geng Q, Lin HC, He XH, Li JJ, Yao M. Integrative analyses identify osteopontin, LAMB3 and ITGB1 as critical pro-metastatic genes for lung cancer. PLoS One 2013; 8: e55714.

[7] Gupta SK, Oommen S, Aubry MC, Williams BP, Vlahakis NE. Integrin alpha9beta1 promotes malignant tumor growth and metastasis by potentiating epithelial-mesenchymal transition. Oncogene 2013; 32: 141-150.

[8] Toupance S, Brassart B, Rabenoelina F, Ghoneim C, Vallar L, Polette M, Debelle L, Birembaut P. Elastin-derived peptides increase invasive capacities of lung cancer cells by post-transcriptional regulation of MMP-2 and uPA. Clin Exp Metastasis 2012; 29: 511-522.

[9] Ko YH, Won HS, Jeon EK, Hong SH, Roh SY, Hong YS, Byun JH, Jung CK, Kang JH. Prognostic significance of CD44s expression in resected non-small cell lung cancer. BMC Cancer 2011; 11: 340.

[10] Chiu KH, Chang YH, Wu YS, Lee SH, Liao PC. Quantitative secretome analysis reveals that COL6A1 is a metastasis-associated protein using stacking gel-aided purification combined with iTRAQ labeling. J Proteome Res 2011; 10: 1110-1125.

[11] Jia D, Yan M, Wang X, Hao X, Liang L, Liu L, Kong H, He X, Li J, Yao M. Development of a highly metastatic model that reveals a crucial role of fibronectin in lung cancer cell migration and invasion. BMC Cancer 2010; 10: 364.

[12] Iochmann S, Blechet C, Chabot V, Saulnier A, Amini A, Gaud G, Gruel Y, Reverdiau P. Transient RNA silencing of tissue factor pathway inhibitor-2 modulates lung cancer cell invasion. Clin Exp Metastasis 2009; 26: 457-467.

[13] Xu J, Min W, Liu X, Xie C, Tang J, Yi T, Li Z, Zhao X. Identification of FRAS1 as a human endometrial carcinoma-derived protein in serum of xenograft model. Gynecol Oncol 2012; 127: 406-411.

[14] Luo Y, Liang F, Zhang ZY. PRL1 promotes cell migration and invasion by increasing MMP2 and MMP9 expression through Src and ERK1/2 pathways. Biochemistry 2009; 48: 1838-1846.

[15] Zhu H, Liu XW, Cai TY, Cao J, Tu CX, Lu W, He QJ, Yang B. Celastrol acts as a potent antimetastatic agent targeting beta1 integrin and inhibiting cell-extracellular matrix adhesion, in part via the p38 mitogen-activated protein kinase pathway. J Pharmacol Exp Ther 2010; 334: 489-499.

[16] Yoon S, Woo SU, Kang JH, Kim K, Shin HJ, Gwak HS, Park S, Chwae YJ. NF-kappaB and STAT3 cooperatively induce IL6 in starved cancer cells. Oncogene 2012; 31: 3467-3481.

[17] Canel M, Serrels A, Frame MC, Brunton VG. E-cadherin-integrin crosstalk in cancer invasion and metastasis. J Cell Sci 2013; 126: 393-401.

[18] Hao HF, Naomoto Y, Bao XH, Watanabe N, Sakurama K, Noma K, Tomono Y, Fukazawa T, Shirakawa Y, Yamatsuji T, Matsuoka J, Takaoka M. Progress in researches about focal adhesion kinase in gastrointestinal tract. World J Gastroenterol 2009; 15: 5916-5923.

[19] Zhao J, Guan JL. Signal transduction by focal adhesion kinase in cancer. Cancer Metastasis Rev 2009; 28: 35-49.

[20] Cabodi S, Di Stefano P, Leal Mdel P, Tinnirello A, Bisaro B, Morello V, Damiano L, Aramu S, Repetto D, Tornillo G, Defilippi P. Integrins and signal transduction. Adv Exp Med Biol 2010; 674: 43-54.

[21] Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol 2006; 18: 516-523.

[22] Frame MC, Patel H, Serrels B, Lietha D, Eck MJ. The FERM domain: organizing the structure and function of FAK. Nat Rev Mol Cell Biol 2010; 11: 802-814.

[23] Superti-Furga G, Gonfloni S. A crystal milestone: the structure of regulated Src. Bioessays 1997; 19: 447-450.