Activated hepatic stellate cells promote hepatocellular carcinoma cell migration and invasion via the activation of FAK-MMP9 signaling

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Abstract. Activated hepatic stellate cells (HSCs) are the major subtype of stromal cells in the liver tumor microenvironment which can promote the growth and migration of hepatocellular carcinoma (HCC) cells. However, the underlying mechanisms by which activated HSCs exert their oncogenic effects are not fully understood to date. In the present study, we investigated the number of activated HSCs and its clinicopathological significance in HCC and uncovered its correlation with focal adhesion kinase (FAK)-MMP9 signaling. A higher number of activated HSCs was associated with tumor invasion of the portal vein, advanced TNM stage and poorer tumor differentiation. The number of activated HSCs was positively correlated with the expression levels of p-FAK and MMP9 in HCC. Furthermore, we studied the effects of activated HSCs on the migration and invasion of HCC cells *in vitro*. Conditioned medium (CM) from activated HSCs or co-culture with activated HSCs significantly induced the migration and invasion of HCC cells. In addition, activation of FAK-MMP9 signaling in HCC was demonstrated in the presence of activated HSC-CM and of co-culture. Inhibition of FAK-MMP9 signaling in HCC cells with FAK short hairpin RNA (shRNA) abrogated the effects of activated HSCs on HCC cells. Taken together, our data suggest that activated HSCs in the tumor microenvironment promote HCC cell migration and invasion via activation of FAK-MMP9 signaling.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths and the fifth most common cancer worldwide, particularly in countries with a high prevalence of chronic hepatitis virus infections (1). Despite recent advances in the diagnosis and treatment of HCC, it remains a highly lethal disease. The main reason for mortality in HCC patients is tumor progression with metastasis (2). Liver fibrosis is strongly associated with HCC, with 90% of HCC cases arising in cirrhotic livers, and the presence of liver cirrhosis is the main risk factor for the development of HCC (3,4).

The liver tumor microenvironment is a complex mixture of tumoral cells within the extracellular matrix (ECM), combined with stromal cells and the proteins they secrete. Together, these elements contribute to the carcinogenic process (5). Hepatic stellate cells (HSCs), which were previously known as Ito cells, are pericytes found within the perisinusoidal space of the liver. During liver injury due to viral infection or long-term insult of hepatotoxins, HSCs become activated to myofibroblast-like cells, leading to the development of hepatic fibrosis and finally, cirrhosis (6,7). Activated HSCs, belonging to one of the most important stromal cell types in the liver tumor environment, are infiltrated in the stroma of HCC and are localized around tumor sinusoids, fibrous septa and capsules (8,9). They are responsible for the remodeling and deposition of tumor-associated ECM and altered expression of growth factors in the tumor environment (10). Several studies have demonstrated that activated HSCs promote HCC growth and invasiveness. Moreover, the bidirectional interactions between tumors and HSCs further enhance metastatic growth in the liver (11,12). However, the underlying mechanisms by which activated HSCs exert their oncogenic effects are still not fully understood.

Focal adhesion kinase (FAK), a non-receptor cytoplasmic protein tyrosine kinase, plays a central role in a number of cell events including cell proliferation, survival, migration and invasion (13,14). Multiple signals, including integrins clustering in response to ECM and engagement of various growth factor receptors, initiate autophosphorylation of FAK at tyrosine (Tyr) 397 leading to FAK activation. Activation

of FAK further mediates the induction of invasive pathways involving signaling to RAC1, JUN N-terminal kinase (JNK), and matrix metalloproteinases (MMPs) (14,15). Consistent with the biological functions of FAK, studies have shown that elevated FAK expression and activity are associated with HCC metastasis and poor patient prognosis (16,17). Upregulation of FAK-MMP9 signaling is considered to be one of the main pathways that promotes HCC cell invasion and metastasis (18,19).

In the present study, we investigated whether activated HSCs promote HCC cell migration and invasion via activating FAK-MMP9 signaling.

Materials and methods

Patients and specimens. Fifty-two fresh tumor samples were collected from HCC patients who underwent curative resection between 2008 and 2010 at the First Affiliated Hospital of the Medical College, Xi'an Jiaotong University (Xi'an, China). Resected tumors and corresponding non-tumor tissue specimens (at least 2 cm away from the tumors) were immediately cut from the resected liver and fixed in buffered paraformaldehyde for immunohistochemical study. None of the patients had received prior radiotherapy or chemotherapy before the sampling. The clinical data of these patients were obtained from the medical records and are listed in Table I. The tumor-node-metastasis (TNM) staging (6th edition), histopathologic Edmonson's classification, vascular invasion and the normal tumor-adjacent tissues were all confirmed by an experienced pathologist who was blinded to the clinical information. Informed consents were obtained from all patients recruited into this study. This study was approved by the Ethics Committee of the First Affiliated Hospital of the College of Medicine, Xi'an Jiaotong University (Xi'an, China), according to the Helsinki Declaration of 1975.

Immunohistochemical staining. The sections were dewaxed, rehydrated. Antigen retrieval was carried out in citrate buffer and the sections were washed. After neutralization of endogenous peroxidase and blocking of nonspecific binding, sections were separately incubated overnight at 4˚C with the following primary antibodies: rabbit anti-phospho-FAK Tyr 397 (p-FAK) (1:100; Abcam, Cambridge, MA, USA), rabbit anti-MMP9 antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse anti-α-smooth muscle actin (α-SMA) antibody (1:100; Dako, Carpinteria, CA, USA). Subsequently, the sections were serially rinsed and incubated with biotinylated secondary antibodies (Golden Bridge Biotechnology, Beijing, China) according to the manufacturer's instructions. The sections were visualized with diaminobenzidine and counterstained with hematoxylin. For the negative controls, the primary antibody was replaced using phosphate-buffered saline (PBS). The degree of immunohistochemical staining was evaluated independently by 2 observers. The results for p-FAK and MMP9 were semiquantitatively expressed using an immunohistochemical score combined with the percentage of liver cells showing specific immunoreactivity (20). Activated HSCs were identified by their locations, morphologic features and cytoplasmic expression of α-SMA. Areas of vessels, Glisson's capsules, fibrous septa, and collapsed parenchyma were not assessed (21). The number of activated HSCs was evaluated semiquantitatively as follows: grade 0, no positive cells; +, rare positive cells that require careful searching at high power; ++, scattered positive cells easily identified at medium power; +++, scattered or clustered positive cells apparent at low power; and ++++, wide spread positive cells apparent at low power (22).

Cells and cell culture. The human liver cancer cell lines, Hep3B and HepG2, were obtained from the American Type Culture Collection (Manassas, VA, USA). Both cell lines were cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS). Human primary HSCs were isolated from human resection specimens (ScienCell, San Diego, CA, USA). The HSCs were cultured on uncoated plastic plates in DMEM supplemented with 10% FBS and incubated at 37˚C in 5% CO_2. *In vitro* activation of HSCs was gradually achieved during passages. Activated HSCs were used at passages 6-8 for the following experiments. For preparation of conditioned medium, HSCs were grown to 60-70% confluency in 100-mm flasks. Cells were washed 3 times with PBS and then incubated in 10 ml of DMEM containing 0.2% FBS for 24 h. Conditioned media were harvested, centrifuged at 1,000 rpm for 10 min to remove cellular debris, filtered through a 0.22-μm filter and stored at 4˚C.

FAK short hairpin RNA (shRNA) vector and gene transfection. The recombinant plasmid vectors expressing the double-stranded shRNA targeting FAK or the non-target shRNA control (NT shRNA) were purchased from Wolsen Biotechnology (Xi'an, China). Two sets of FAK shRNA vectors, designated as FAK shRNA-1 and FAK shRNA-2, were tested and proven to be efficient in suppressing FAK expression in HCC cells. The target sequences used for FAK shRNA constructs were shRNA-1 (GCCACCTGGGCCAGTATTA) and shRNA-2 (GCGGCCCAGGTTTACTGAA). HCC cells were grown to 60-80% confluence and transfected with FAK shRNA vectors or the NT shRNA vector using FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) for 24, 48 or 72 h, in accordance with the manufacturer's protocol. The transfection rate was assessed by counting the number of positive cells under a fluorescence microscope, which could reach up to 90%.

Co-culture experiments. *In vitro* activated HSCs were cultured in 6-well dishes in medium containing 10% FBS for 12 h at $2x10^5$ cells/well and then maintained in serum-free medium for 18 h prior to the co-culture experiment. HCC cells transfected with the FAK shRNA or NT shRNA were cultured on 0.4-μm pore size cell culture inserts (BD Biosciences, San Jose, CA, USA) for 12 h at $1x10^5$ cells/well and then maintained in serum-free medium for 18 h prior to the co-culture experiment. Inserts were placed in the companion plate for 48 h for the co-culture experiments (Fig. 2B), which allowed diffusion of the medium components but prevent cell migration. Independent culture experiments were carried out at least in triplicate.

Immunocytochemistry and confocal microscopy. Cells were grown on glass coverslips for 48 h, rinsed with Dulbecco's

Table I. Correlation between α-SMA-positive HSCs and clinicopathologic characteristics in the 52 HCC patients.

Clinicopathologic parameters	N	α-SMA-positive HSCs				R-value	P-value
		+	++	+++	++++		
Age (years)							
≤55	25	1	6	10	8	-0.117	0.410
>55	27	4	7	8	8		
Gender							
Male	43	3	13	13	14	0.021	0.881
Female	9	2	0	5	2		
Tumor size (cm)							
<5	16	1	5	4	6	-0.046	0.744
≥5	36	4	8	14	10		
No. of tumors							
Solitary	47	4	13	16	14	0.066	0.642
Multiple	5	1	0	2	2		
Edmonson's staging							
I-II	36	5	12	11	8	0.397	0.004[a]
III-IV	16	0	1	7	8		
TNM staging							
I	35	3	13	12	7	0.346	0.012[a]
II-III	17	2	0	6	9		
Vascular invasion							
Absent	40	5	13	15	7	0.525	<0.001[a]
Present	12	0	0	3	9		
AFP (ng/ml)							
≤20	21	1	4	9	7	-0.146	0.301
>20	31	4	9	9	9		
HBsAg							
Positive	40	4	10	14	12	-0.029	0.840
Negative	12	1	3	4	4		

AFP, α-fetoprotein; TNM, tumor-node-metastasis; HBsAg, hepatitis B surface antigen; α-SMA, α-smooth muscle actin; HSCs, hepatic stellate cells. [a]P<0.05 was considered significant.

PBS (D-PBS) at room temperature, and fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. Goat serum (10%) was applied to prevent nonspecific binding. Subsequently, HCC cells were incubated with the anti-p-FAK antibody (1:100 for cells) overnight at 4˚C. After washing with PBS, the primary antibodies were labeled by Alexa 594 goat anti-mouse/rabbit IgG (1:1000; Invitrogen Life Technologies) for 1 h at room temperature. Then cells were mounted with DAPI and examined by confocal microscopy. For the negative control, the primary antibody was replaced using PBS.

Western blot analysis. Total protein was extracted from the HCC cells exposed to conditioned medium from the activated HSCs or co-cultured with activated HSCs. The protein concentration was quantified using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of proteins (20 μg/lane) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking for 1 h with 5% BSA and washed with TBST, the membranes were incubated overnight at 4˚C with a primary antibody against p-FAK (1:500), FAK (1:1,000; Cell Signalling Technology, Inc., Danvers, MA, USA), MMP9 (1:1,000) and β-actin (1:1,000; Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were incubated with goat anti-mouse or goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10,000) for 1 h at room temperature. The blots were then detected by SuperSignal West Pico chemiluminescent substrate kit (Millipore, Billerica, MA, USA) and exposed to X-ray film. β-actin was measured to control for equal loading.

Cell migration and invasion assay. The migratory ability of HCC cells exposed to conditioned medium harvested from

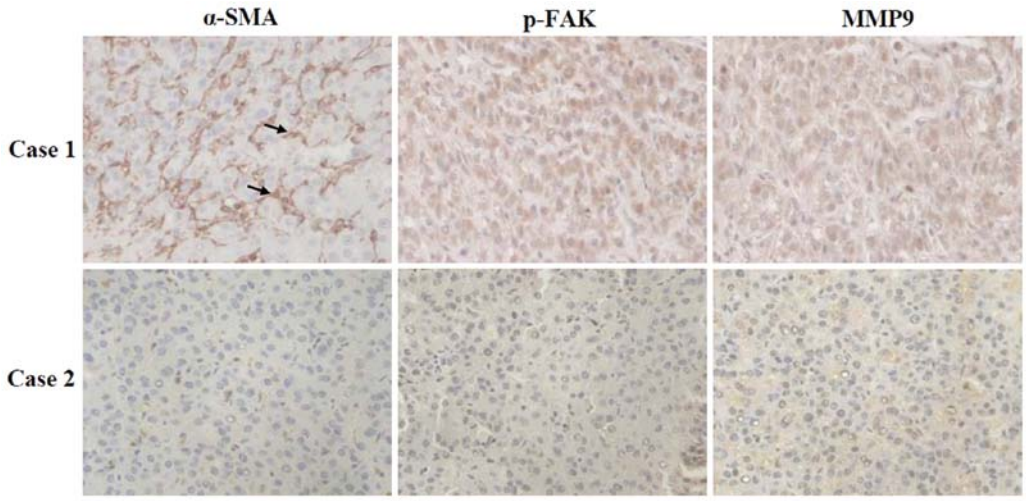

Figure 1. Immunohistochemical staining of α-SMA-positive activated HSCs, p-FAK, MMP9 in human hepatocellular carcinoma (HCC) (x400). In case 1 with a high number of α-SMA-positive activated HSCs (arrows indicate representative cells), there was strong p-FAK and MMP9 protein expression in the same tissue section. In contrast, in case 2 with an extremely low number of α-SMA-positive activated HSCs, there was negative p-FAK expression and weak MMP9 expression. α-SMA, α-smooth muscle actin; HSCs, hepatic stellate cells; p-FAK, phospho-focal adhesion kinase.

activated HSCs was measured by wound healing assay. HCC cells transfected with FAK shRNA or NT shRNA were grown to 90-100% confluence in 6-well plates. Cell monolayers were wounded with a sterile 200-μl pipette tip and then rinsed with PBS to remove cellular debris. The wounded monolayers were cultured in conditioned medium or in control medium and photographed with a phase-contrast microscope at 0, 24 and 48 h. Cell migration was quantitated by measuring the width of the wounds. The invasive activity of HCC cells was assessed using 24-well Transwell inserts with a 8-μm pore size coated with Matrigel (1 mg/ml; BD Biosciences). Forty-eight hours after shRNA transfection, $2.5x10^4$ HCC cells in serum-free medium were added into the upper chamber. Conditioned medium from activated HSCs supplemented with 1% FBS was placed in the lower chambers as chemoattractants. After a 24-h incubation at 37˚C, the cells remaining on the upper surface of the filter were wiped away with a cotton swab. The migrated cells, adhering to the lower surface of the membrane, were fixed with 100% methanol, stained with crystal violet and counted under a light microscope by randomly selecting 5 fields/filter. Each experiment was carried out in triplicate wells and repeated at least 3 times.

Statistical analysis. Statistical analyses were performed with the SPSS 16.0 software. For continuous variables, data are expressed as the means ± SD. Comparison between groups was carried out using the Student's unpaired t-test. The correlations between the number of activated HSCs and clinicopathological features or p-FAK and MMP9 protein expression were analyzed using Spearman's rank correlation coefficient test. Values of $P<0.05$ were considered to be statistically significant.

Results

The number of activated HSCs in HCC and its clinicopathologic significance. The number and distribution of α-SMA-positive activated HSCs in 52 pairs of HCC tumor and adjacent non-tumorous tissues were detected by immunohistochemical staining. The α-SMA-positive activated HSCs were spindle-shaped and were present within the perisinusoidal space and carcinomatous nodules (Fig. 1, left panels). The number of activated HSCs identified within hepatocellular carcinoma was more than that noted in the adjacent non-tumorous tissues. To assess the significance of activated HSCs in HCC, the correlation between activated HSCs and the clinicopathological characteristics of the 52 HCC patients was statistically analyzed and the results are listed in Table I. Patients with a higher number of activated HSCs in HCC tissues were prone to have high rates of vascular invasion, advanced TNM staging and poorer tumor differentiation ($P<0.05$). However, no significant correlation was found between the number of activated HSCs and age, gender, HBV infection, α-fetoprotein (AFP), tumor number and tumor size.

The number of activated HSCs is positively correlated with FAK-MMP9 signaling in HCC. To investigate whether the number of activated HSCs is associated with FAK-MMP9 signaling in HCC, we further determined the expression of p-FAK and MMP9 by immunohistochemical staining. The grading of the extent of activated HSCs, p-FAK and MMP9 are summarized in Table II. Results showed that p-FAK protein was positively stained in the cytoplasm of carcinoma cells (Fig. 1, middle panels), and 53.8% (n=28) of the HCC tumor samples showed positive staining of p-FAK protein. The number of activated HSCs was positively correlated with the p-FAK protein expression level in the HCC tissues ($r=0.475$, $P<0.001$), as assessed by Spearman's rank correlation coefficient test (Table II). MMP9 protein was diffuse with moderate or strong staining in the cytoplasm of tumor cells (Fig. 1, right panels). Positive staining of MMP9 protein was found in 69.2% (n=36) of the HCC tissues. The number of activated HSCs was also positively correlated with MMP9 protein expression level in the HCC tissues ($r=0.446$, $P<0.001$) (Table II).

Activated HSCs induce activation of FAK-MMP9 signaling in HCC cells. In order to study the interaction between acti-

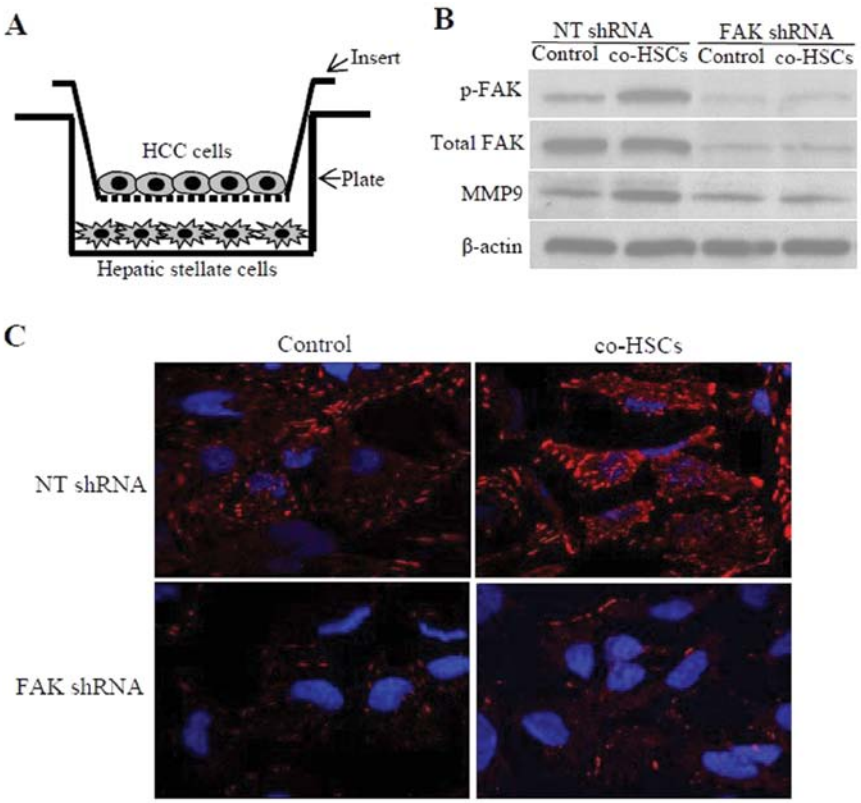

Figure 2. Activated HSCs induce activation of FAK-MMP9 signaling in HCC cells. (A) Schematic representation of the co-culture system of HCC cells and activated HSCs. (B) At 48 h after co-culturing with activated HSCs (co-HSC group), total protein of Hep3B cells transfected with FAK shRNA or NT shRNA was extracted. Western blot analysis was performed using an antibody against p-FAK, FAK, MMP9 and β-actin. (C) Immunofluorescence staining of p-FAK (red) in Hep3B cells (FAK shRNA or NT shRNA group) co-cultured with activated HSCs or control medium. HSCs, hepatic stellate cells; FAK, focal adhesion kinase; HCC, hepatocellular carcinoma; shRNA, short hairpin RNA; NT, non-target.

Table II. Correlation between α-SMA-positive HSCs and p-FAK and MMP9 protein expression in 52 HCC patients.

α-SMA-positive HSCs	p-FAK protein				MMP9 protein			
	-	+	++	+++	-	+	++	+++
+	4	0	1	0	4	1	0	0
++	10	2	0	1	6	7	0	0
+++	8	5	3	2	2	5	8	3
++++	2	6	7	1	4	3	7	2
R-value	0.475				0.446			
P-value	<0.001				0.001			

α-SMA, α-smooth muscle actin; HSCs, hepatic stellate cells; p-FAK, phospho-focal adhesion kinase; HCC, hepatocellular carcinoma.

vated HSCs and HCC cells, a co-culture system was used. Cells were cultured using 0.4-μm pore size cell culture inserts that separate both cell populations, which allow diffusion of medium components but prevent cell migration. Activated HSCs were plated at the bottom of the well plate, and HCC cells transfected with FAK shRNA or NT shRNA were plated on the insert (Fig. 2A). Optimal co-culture conditions were identified in preliminary studies. The most reproducible and consistent results were obtained at the HCC cells:HSCs ratio of 1:2. HCC cells were transfected with FAK shRNA or NT shRNA, and FAK mRNA expression levels were measured by real-time PCR at 48 h after transfection. Cells transfected with FAK shRNA had a >90% reduction in FAK mRNA levels compared to cells transfected with NT shRNA (data not shown). Western blotting showed that at 48 h after co-culture, HCC cells (NT shRNA group) co-cultured with the activated HSCs exhibited significantly higher levels of p-FAK and MMP9 expression when compared to these levels in cells cultured in control medium. However, inhibition of FAK in HCC cells with FAK shRNA dramatically decreased levels of p-FAK and MMP9 expression, and abrogated the differences caused by coculture (Fig. 2B). Furthermore, these results were confirmed by immunocytochemistry performed on HCC cells co-cultured with activated HSCs (Fig. 2C). Consistent with the co-culture experiments, conditioned medium from activated HSCs also significantly increased the expression of p-FAK and MMP9 in HCC cells.

Activated HSCs promote HCC cell migration and invasion dependent on FAK-MMP9 signaling. Since the activation of FAK-MMP9 signaling was significantly correlated with the migratory and invasive potential of HCC cells and is further involved in tumor metastasis (18,19), we investigated whether activated HSCs promote HCC cell migration and invasion depending on FAK-MMP9 signaling. The migratory and invasive abilities of HCC cells exposed to conditioned medium

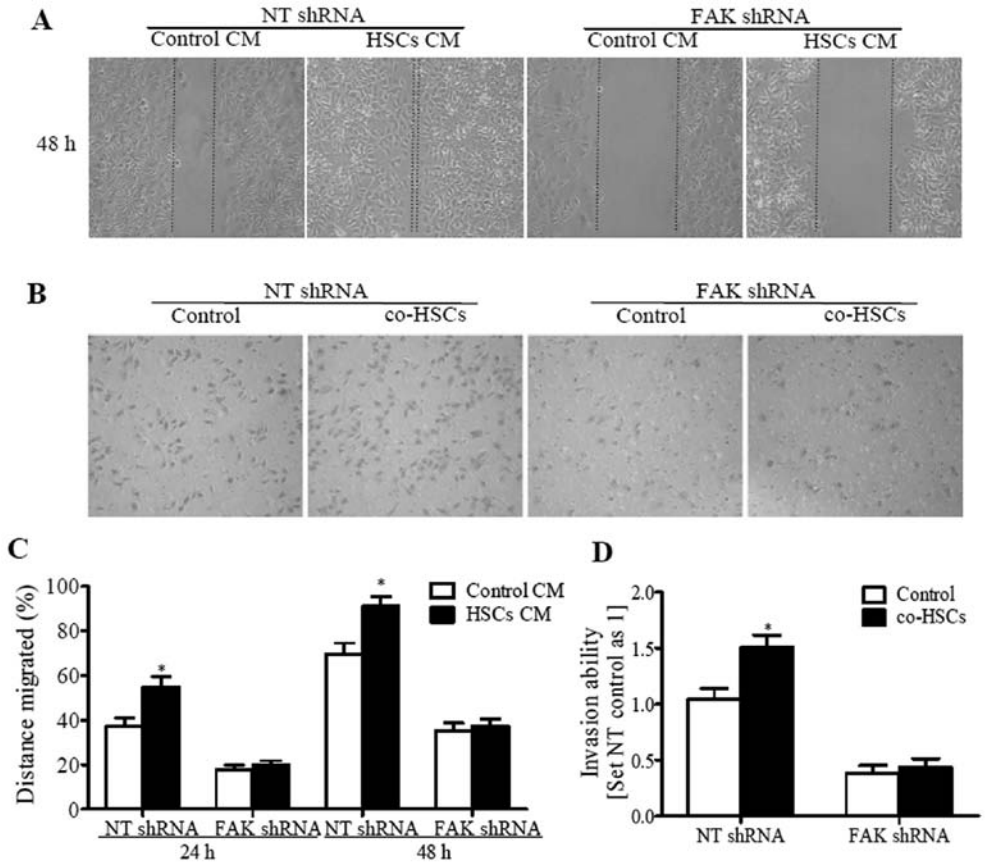

Figure 3. The migration and invasion-promoting effects of HSCs on HCC cells depend on FAK-MMP9 signaling. (A) The migratory ability of Hep3B cells (FAK shRNA or NT shRNA group) exposed to conditioned medium from activated HSCs (HSC-CM) or control medium (control CM) was measured using a wound healing assay. Representative images of the areas between scratch fronts were captured at 48 h after wounds were made. (B) The invasive ability of Hep3B cells (FAK shRNA or NT shRNA group) co-cultured with activated HSCs was measured using the Transwell assay. Transfected cells were allowed to invade through Matrigel-coated Transwell inserts for 24 h. The cells that had migrated to the lower surface of the membrane were visualized by crystal violet staining under microscopic observation. (C) The histogram shows the migratory rate of Hep3B cells (FAK shRNA or NT shRNA group) at 24 and 48 h after scratch wounds were made. (D) The histogram shows the Transwell assay results. The number of cells that had migrated to the lower surface in the NT shRNA control group was set to 1. *P<0.05, HSC-CM vs. control CM, or co-HSCs vs. control. HSCs, hepatic stellate cells; HCC, hepatocellular carcinoma; FAK, focal adhesion kinase; shRNA, short hairpin RNA.

from activated HSCs were measured by wound healing and Transwell assays. The results showed that, in comparison with the control medium, activated HSC-conditioned medium (HSC-CM) significantly enhanced the migratory and invasive potential of HCC cells (NT shRNA group), while knockdown of FAK expression in HCC cells with shRNA diminished the migration and invasion-promoting effect of HSC-CM on HCC cells. Even stronger migration and invasion was observed when HCC cells (NT shRNA group) were seeded in the upper part of the Transwell chamber and activated HSCs were seeded in the bottom part of the chamber (Fig. 3). Similarly, suppression of FAK with shRNA in HCC cells decreased cell migration and invasion, and diminished the difference caused by HSCs. These results indicate that the migration and invasion-promoting effects of HSCs on HCC cells depend on FAK-MMP9 signaling.

Discussion

The interaction of tumor cells with the microenvironment has been recognized to be central for cancer progression and metastatic colonization (23). Stromal components of the microenvironment can contribute to several hallmarks of cancer:

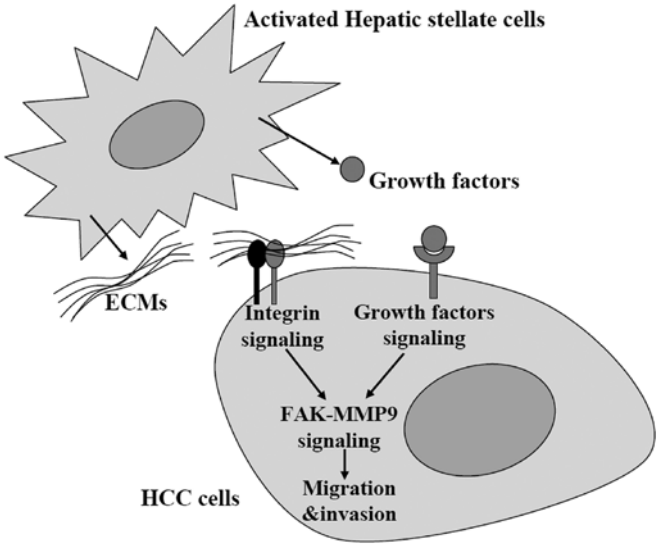

Figure 4. Schematic representation of the possible mechanism involved in the promotion of HCC cell migration and invasion by activated HSCs via activation of FAK-MMP9 signaling. The extracellular matrices (ECMs) and growth factors shown here are secreted by activated HSCs and then bind to their receptors on HCC cells. HSCs, hepatic stellate cells; HCC, hepatocellular carcinoma; FAK, focal adhesion kinase.

sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, and evading immune destruction (5). HSCs are pericytes found within the perisinusoidal space of the liver. During chronic liver injury, HSCs undergo phenotypic transformation from the 'quiescent' to the 'activated' state, and eventually develop into myofibroblasts (24). These changes in cell fate of HSCs towards myofibroblasts provide the cellular basis for the establishment of hepatic fibrosis and cirrhosis, which are characterized by the vast remodeling of the ECM and altered expression of growth factors (25). HSCs have been reported to play a role in the tumoral progression of HCC, since the high density of peritumoral activated HSCs was found to contribute to early recurrences and predict poor clinical outcome in HCC after curative resection (21). In the present study, we demonstrated a similar finding as previously reported (26), that α-SMA-positive activated HSCs are frequently present in carcinomatous nodules, and the number of activated HSCs in HCC is greater than that in adjacent non-tumorous tissues. Moreover, a higher number of activated HSCs in HCC tissues was associated with high rates of vascular invasion, advanced TNM stage, and poorer tumor differentiation. These findings, derived from clinic samples, confirmed the tumor-promoting role of activated HSCs in the microenvironment and further indicated that activated HSCs may be related to HCC metastasis. Indeed, studies have shown that activated HSCs promote HCC migration and invasion, and the bidirectional interactions between tumors and HSCs enhance metastatic growth in the liver (11,12).

The underlying mechanisms by which activated HSCs promote HCC migration and invasion may largely depend on their ECM remodeling and growth factor regulating function. For example, previous studies have shown that hepatocyte growth factor (HGF) and laminin-5 secreted by activated HSCs promote the growth and invasiveness of HCC cell lines through activating the MEK/ERK pathway *in vitro* (11,27,28). Nevertheless, it is reasonable to suppose that more than one mechanism occurs. FAK, a widely expressed non-receptor protein tyrosine kinase has been suggested to play an essential role in metastasis. FAK is phosphorylated at tyrosine (Tyr) 397 and is subsequently activated upon both ECM/integrin and growth factor signaling (14,15,29). Activation of FAK upregulates MMP9 expression through ERK or PI3K, thus promoting cell migration and invasion (19,30). Notably, in the present study we found that the number of activated HSCs was positively correlated with p-FAK and MMP9 expression levels in the HCC tissues. This finding indicates that activated HSCs are associated with FAK-MMP9 signaling in HCC. In order to study the effects of activated HSCs on HCC cells directly, we isolated HSCs from resected normal liver tissues, and activated them *in vitro* by culture. Consistent with previous studies (11,27), conditioned medium from activated HSCs or co-culture with activated HSCs significantly induced the migration and invasion of HCC cells. Furthermore, HCC cells co-cultured with activated HSCs or incubated in conditioned medium exhibited significantly higher levels of p-FAK and MMP9 expression when compared to cells cultured in control medium, while, inhibition of FAK in HCC cells with shRNA dramatically decreased p-FAK and MMP9 expression, and abrogated the differences caused by co-culture or conditioned medium.

In summary, our study revealed that activated HSCs promoted HCC cell migration and invasion via activating FAK-MMP9 signaling (Fig. 4). However, we did not identify which specific ECM components or growth factors secreted by activated HSCs were responsible for the activation of FAK-MMP9 signaling in HCC cells. Based on previous studies (6,11,27), we speculate that HGF, PDGF, collagen and laminin-5 may be possible candidates. Their roles in the entire process will be elucidated in future studies.

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