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Research Article

Overexpression of HAb18G/CD147 promotes invasion and metastasis *via* α3β1 integrin mediated FAK-paxillin and FAK-PI3K-Ca^{2+} pathways

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Abstract. Mechanism of HAb18G/CD147 underlying the metastasis process of human hepatoma cells has not been determined. In the present study, we found that integrin α3β1 colocalizes with HAb18G/CD147 in human 7721 hepatoma cells. The enhancing effect of HAb18G/CD147 on adhesion, invasion capacities and matrix metalloproteinases (MMPs) secretion was decreased by integrin α3β1 antibodies ($p<0.01$). The expressions of integrin downstream molecules including focal adhesion kinase (FAK), phospho-FAK (p-FAK), paxillin, and phospho-paxillin (p-paxillin) were increased in human hepatoma cells overexpressing HAb18G/CD147. Deletion of HAb18G/CD147 reduces the quantity of focal adhesions and rearranges cytoskeleton. Wortmannin and LY294002, specific phosphatidylinositol kinase (PI3K) inhibitors, reversed the effect of HAb18G/CD147 on the regulation of intracellular Ca^{2+} mobilization, significantly reducing cell adhesion, invasion and MMPs secretion potential ($p<0.01$). Together, these results suggest that HAb18G/CD147 enhances the invasion and metastatic potentials of human hepatoma cells *via* integrin α3β1-mediated FAK-paxillin and FAK-PI3K-Ca^{2+} signal pathways.

Keywords. HAb18G/CD147, hepatoma cells, invasion, metastasis, signal pathways.

Introduction

CD147 is a transmembrane glycoprotein of the immunoglobulin superfamily that is broadly expressed in many cell types, and at high levels in human tumor cells [1]. HAb18G, a hepatoma-associated antigen, cloned by anti-hepatoma monoclonal antibody HAb18 screening of human hepatocellular carcinoma cDNA library, has an identical nucleotide acid and amino acid sequence to CD147. Our previous studies have demonstrated that HAb18G/CD147 promotes the invasion and metastasis of human hepatoma cells by stimulating fibroblast cells to produce elevated levels of metalloproteinases (MMPs) [2].

Although published data support a crucial role for CD147 in migration and metastasis potential of tumor progression, detailed investigation on the precise mechanism is still required. It is becoming increasingly apparent that a full understanding of HAb18G/CD147 functional activities may require understanding of its associations with other molecules and its signal transduction pathway. HAb18G/CD147 has an ectodomain

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consisting of two regions with the characteristics of the immunoglobulin superfamily [3]. The transmembrane domain of HAb18G/CD147 contains a glutamic acid residue and a leucine zipper motif that are implicated in the protein association with the plasma membrane and the dimerization of DNA-binding proteins [4]. These structural characteristics may enable HAb18G/CD147 to interact with other membrane proteins, intracellular enzymes/factors as well as extracellular factors such as βig-h3, as reported previously [5]. Some of these interactions may directly or indirectly regulate integrin-dependent intracellular signal pathways that are involved in cell adhesion, spreading, and migration process [6–8]. In hepatoma cells the molecules that interact with HAb18G/CD147 are still not known, and the signal transduction pathway of HAb18G/CD147 remains to be investigated.

A frequent common denominator of the different signal transduction pathways is cellular Ca^{2+} mobilization. Ca^{2+} has been shown to play roles in the regulation of proliferation, invasion, and metastatic potential by regulating expression and/or release of MMPs [9–11]. Although much is known about the physiological importance of store-operated Ca^{2+} entry in tumor cells, virtually nothing is known about the intracellular regulation of store-operated Ca^{2+} entry involved in the process of cancer metastasis. Our previous data have already demonstrated that elevated expression of HAb18G/CD147 leads to attenuation of the response of store-operated Ca^{2+} entry to NO/cGMP, and enhanced metastatic potentials [3]. However, it remains to be elucidated how the increased metastatic potential induced by HAb18G/CD147 expression is regulated through a mechanism involving Ca^{2+} entry.

The aim of this study is to elucidate the exact signal transduction pathway of HAb18G/CD147 in human hepatoma cells and to identify the mechanism of HAb18G/CD147-regulated Ca^{2+} mobilization that has eluded investigators for many years. We demonstrated that HAb18G/CD147 up-regulates α3β1 integrin activity, and increases the phosphorylation and expression level of FAK and paxillin, leading to cytoskeleton reorganization. HAb18G/CD147 also actives the Ca^{2+} channels through the FAK-PI3K signaling pathway. These activation events may function to enhance the metastatic potentials of human hepatoma cells.

Materials and methods

Cell culture. Human SMMC-7721 and FHCC-98 cells (both obtained from the Institute of Cell Biology, China) as well as T7721 cells stably overexpressing HAb18G/CD147 (developed in our lab) were cultured with RPMI 1640 medium (Gibco, New York, USA) supplemented with 10% FBS, 1% penicillin/streptomycin and 2% L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. G418 was added into the medium for T7721 cells at the final concentration of 400 μg/ml.

Gene silencing. The sense sequence for HAb18G/CD147 siRNAs was 5'-GUUCUUCGUGAGUUCCUCTT-3', 3'-DTDTCAAGAAGCACUCAAGGAG-5' (Ambion). FHCC-98 cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Silencer-negative control siRNA (snc-RNA) (Ambion) was used as negative control under similar conditions.

Reverse transcription-PCR. At 48 h after siRNA transfection, total RNA was extracted from the cells with TRIzol reagents (Invitrogen) and reversely transcribed into cDNA with ReverTra Ace-a kit (Toyobo). All primers and probes were synthesized by Shanghai Sangon Co. as follows: HAb18G/CD147, forward primer 5'-TCGCGCTGCTGGGCACC-3'; reverse primer 5'-TGGCGCTGTCATTCAAGGA-3'; integrin α3, forward primer 5'-CCCCAACTACAGGCGAAAC-3'; reverse primer 5'-CTGCTGCTCTGACACGAAGG-3'; integrin β1, forward primer 5'-ATTTGTCCCGACTTTCTAC-3'; reverse primer 5'-TGCCTACTTCTGCACGAT-3'; MMP9, forward primer 5'-CGCCGCCACGAGGAACAAACT-3'; reverse primer 5'-GGCATTCAGGGAGACGCCCATTT-3'; MMP2, forward primer 5'-TTTGACGGTAAGGACGGACTC-3'; reverse primer 5'-GTGCCCTGGAAGCGGAAT-3'; β-actin, forward primer 5'-CCCAGCCATGTACGTTGCTA-3'; reverse primer 5'-TCACCGGAGT-CCATCACGAT-3'. β-actin mRNA was used as an inner parameter. The PCR conditions for HAb18G/CD147 were 1 cycle of 94 °C for 4 min, 35 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s, finally 72 °C for 10 min. The PCR conditions for integrin α3 were 1 cycle of 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, finally 72 °C for 10 min. The PCR conditions for integrin β1 were 1 cycle of 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, finally 72 °C for 10 min. The PCR conditions for MMP9 were 1 cycle of 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s, finally 72 °C for 10 min. The PCR conditions for MMP2 were 1 cycle of 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, finally 72 °C for 10 min. PCR products were electrophoresed on 1% agarose gels.

Western blot analysis. Cells were lysed in 1 % *n*-octyl *p*-D-glucopyranoside (**OG**) buffer (20 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl, 1 % OG, 1 mmol/l EDTA, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mmol/l PMSF). BCA Protein Assay Kit (Pierce Biotechnology) was employed to determine the total protein density and equal amounts of proteins were separated by SDS-PAGE on a 10 % polyacrylamide gel, and then transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore). After blocked with 5 % non-fat milk, the membrane was incubated for 2 h at room temperature with the designated antibody. Immunodetection was performed using the Western-Light chemiluminescent detection system (Applied Biosystems).

Co-immunoprecipitation. The interaction of HAb18G/CD147 with integrin α3β1 in native cells was detected by ProFound™ Mammalian Co-Immunoprecipitation Kit (Pierce), according to the manufacturer's instructions. Briefly, 7721 cells (1×10^6) were lysed by M-per reagent. The lysate was collected onto Coupling gel that was pre-bound with 200 μg mouse anti-human HAb18G/CD147 monoclonal antibody (mAb) HAb18 [3] (developed in our lab), followed by four washes with the co-immunoprecipitation buffer. Bound proteins were eluted from coupling gel with elution buffer, and aliquots of the eluent were submitted to Western blotting using goat anti-human anti-α3 or anti-β1 antibody (diluted 1:2000; BD). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted 1:5000; Amersham Pharmacia) was used as the negative control.

Immunofluorescence. Cells were allowed to attach for 3 h to precoated glass coverslips. They were then fixed in 3.7 % formaldehyde in PBS, permeabilized with 0.5 % Triton X-100 and blocked with 1 % BSA (Fraction V) in PBS for 1 h. Coverslips were incubated with the indicated antibodies (BD) at a 1:500 dilution, or with rhodamine-phalloidin (Molecular Probes) at a 1:40 dilution in PBS for 20 min. Antibody-treated cells were washed in PBS and incubated with FITC goat anti-mouse or Texas red donkey anti-goat secondary antibodies (Pierce) at a 1:500 dilution in PBS for 1 h. Cell nuclei were dyed with DAPI (Vector Labs). Finally, the cells were mounted using glycerol. Cells probed with rhodamine-phalloidin were washed and immediately mounted and observed by FV1000 laser scanning confocal microscope (Olympus).

Adhesion assay. Wells of a 96-well culture plate were coated with Matrigel (BD) at a concentration of 5 mg/ml and incubated at 4 °C overnight. The coated wells were blocked with PBS containing 2 % BSA for 30 min and then washed with PBS. Cells suspended in serum-free medium containing 0.1 % BSA were added to the wells (2×10^4/well) and incubated at 37 °C, 5 % CO_2 for 30–60 min with or without antibodies (BD) or inhibitors (Cell signaling). After removing medium and non-attached cells, 0.2 % crystal violet was added for 10 min. The plate was gently washed with tap water and dried in air for 24 h. Then 0.1 ml 5 % SDS/50 % ethanol was added for 20 min and then the plate was read at 540 nm.

Invasion assay. Chemotactic cell invasion assay was performed using 24-well Transwell units with an 8-mm pore size polycarbonate filter (Millipore) according to the method described previously [12]. Each lower compartment of the Transwell contained 600 ml 0.5 % FBS as chemoattractant or 0.5 % BSA as negative control in RPMI 1640. The upper side of a polycarbonate filter was coated with Matrigel (5 mg/ml in cold medium) to form a continuous thin layer. Prior to addition of the cell suspension, the dried layer of Matrigel matrix was rehydrated with medium without FBS for 2 h at room temperature. Cells preincubated with antibodies or inhibitors (1×10^5) were suspended in 0.1 ml RPMI 1640 containing 0.1 % BSA and added into the upper compartment of the Transwell unit and incubated for 36 h at 37 °C in a humidified atmosphere containing 5 % CO_2. Cells remaining in the upper compartment were completely removed with gentle swabbing. The number of cells that had invaded through the filter into the lower compartment was determined using a colorimetric crystal violet assay.

Zymography experiments. To detect the expression and activation of MMPs, cells preincubated with antibodies or inhibitors were cultured in serum-free medium and incubated at 37 °C for 5–20 h. The conditioned media was collected and separated by 8 % acrylamide gels containing 0.1 % gelatin. The gels were incubated in 2.5 % Triton X-100 solution at room temperature with gentle agitation and were then soaked in reaction buffer (0.05 mol/l Tris-HCl pH 7.5, 0.2 mol/l NaCl, and 0.01 mol/l $CaCl_2$) at 37 °C overnight. After reaction, the gels were stained for 6 h and were destained for ~0.5 h. The zones of gelatinolytic activity were shown by negative staining.

Measurement of intracellular free calcium concentration. Intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured using Fura2 acetoxymethyl ester (Fura2/AM; Molecular Probes). T7721 cells were preincubated with the PI3K inhibitor wortmannin and LY294002 (Cell signaling) for 40 min before loading with the dye by incubation with 5 μmol/l Fura2/AM for 45 min in the dark at 37 °C in normal

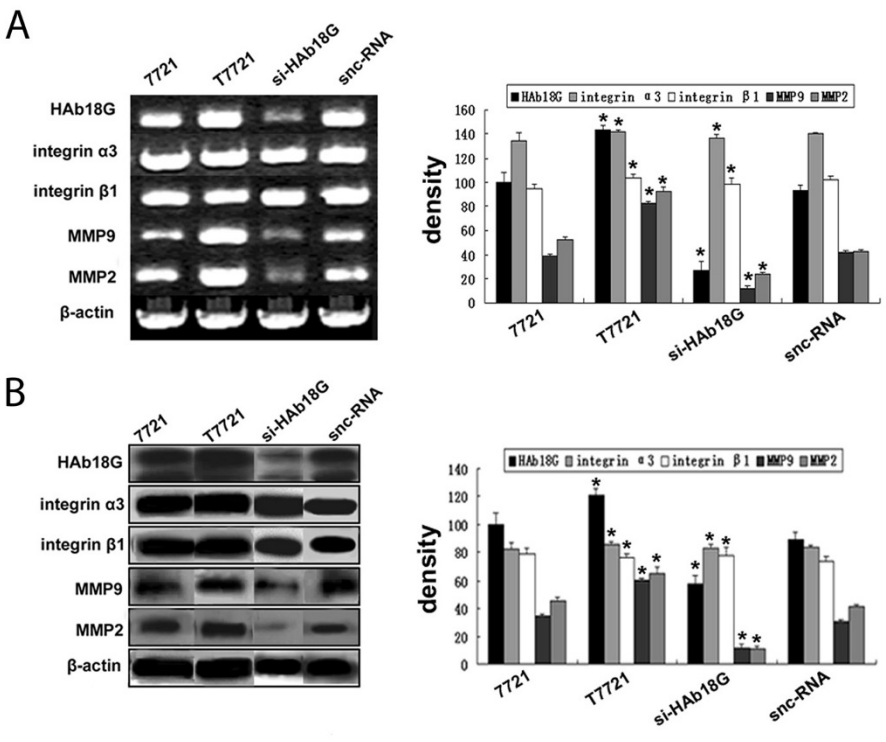

Figure 1. Expressions of HAb18G/CD147, integrin α3, integrin β1, matrix metalloproteinase (MMP) 2 and MMP9 in HAb18G/CD147 overexpressing or silencing hepatoma cells. The mRNA (*A*) and protein (*B*) expressions were tested by RT-PCR and Western blot analyses in 7721 cells, T7721 cells, si-HAb18G-transfected 7721 cells and snc-RNA-transfected 7721 cells. The expression levels were normalized by human β-actin expression. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments. *$p<0.01$ *versus* corresponding controls cells (Student's *t*-test).

PBS (NPBS) containing 2 mmol/l $CaCl_2$, pH 7.4. Cells were then washed and resuspended in NPBS. To start the experiment, cells were pretreated with 4 μmol/l thapsigargin (Calbiochem) for 20 min. After washing off extracellular thapsigargin and Ca^{2+}, cells were pretreated with 2 mmol/l 8-Br-cGMP (Calbiochem) for 5 min. EGTA (2mmol/l) were then added to cells. The fluorescence signal was monitored and recorded by FV300 laser scanning confocal microscope (Olympus).

Results

HAb18G/CD147 co-immunoprecipitates with α3β1 integrin in human hepatoma cells. It has been reported that both α3 and β1 subunits are expressed in human hepatoma cells [13]. To further test the expression levels of α3β1 integrin in cells with different expression levels of HAb18G/CD147, we tested the mRNA and protein expressions of HAb18G/CD147, integrin α3, integrin β1, MMP2 and MMP9 in 7721 cells, T7721 cells, si-HAb18G-transfected 7721 cells and snc-RNA-transfected 7721 cells. As shown in Figure 1, no significant expression modifications were found for integrin α3 and β1 subunits expressions in the four cell lines, but the expressions of MMP2 and MMP9 increased in T7721 cells compared with the expression levels in 7721 cells and they diminished in si-HAb18G-transfected 7721 cells compared with the expression levels in snc-RNA-transfected 7721 cells. α3β1 integrin was then found co-immunoprecipitated with endogenous HAb18G/CD147 in 7721 cell lysates, indicating that HAb18G/CD147 and α3β1 integrin interact in their native conformations (Fig. 2A). To further confirm this result, immunofluorescent double staining was used in human hepatoma FHCC-98 cells. The results showed co-localizations of HAb18G/CD147 with integrin α3 and β1 on the cell membrane (Fig. 2B).

α3β1 integrin mediates HAb18G/CD147-induced metastasis of human hepatoma cells. We then used blocking antibodies to α3 or β1 integrin to examine whether HAb18G/CD147-mediated adhesion, invasion and MMPs secretion are regulated by these integrins. Antibodies to α3 and β1 integrins markedly reduced cell adhesion in 7721 cells and completely abolished the enhanced effect of HAb18G/CD147 in HAb18G/CD147-overespressing T7721 cells ($p<0.01$, Fig. 3A). Further identification of HAb18G/CD147-integrin association was carried out using si-HAb18G-transfected 7721 cells. The addition of α3 and β1 integrins antibodies did not significantly reduce adhesion in si-HAb18G-transfected 7721 cells ($p>0.05$, Fig. 3A). The addition of α3 or β1 integrin antibodies reduced the adhesion rate of 7721 cells and T7721 cells to levels comparable with that in si-HAb18G-transfected 7721 cells. This result demonstrated that HAb18G/CD147 and α3β1 integrin are

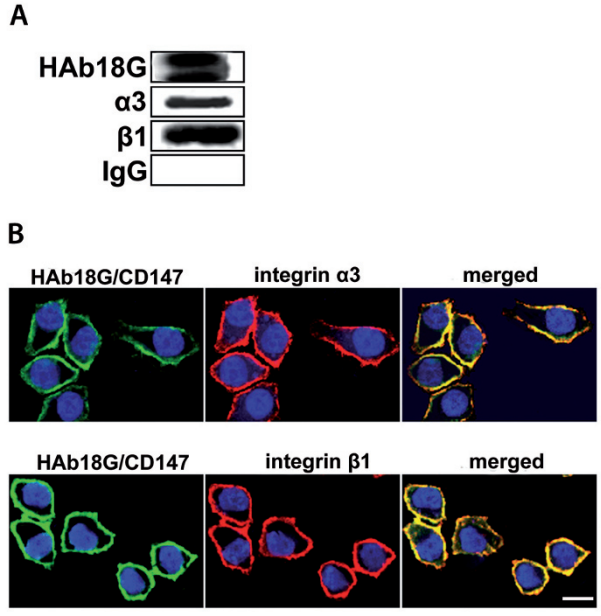

Figure 2. Immunoprecipitation of HAb18G/CD147 with α3β1 integrin in 7721 cells. (*A*) CD147 immunoprecipitation with α3β1 integrin. Lysates of 7721 cells were subjected to immunoprecipitation with anti-HAb18G/CD147 antibody-pre-bound coupling gel, α3β1 integrin in the immune complexes were detected by Western blot analysis. Mouse IgG was used as a negative control. (*B*) Expression and localization of HAb18G/CD147 and α3β1 integrin in FHCC-98 cells. FHCC-98 cells were double-stained for HAb18G/CD147 (green) and integrin α3 and β1 (red). Bar 2 μm.

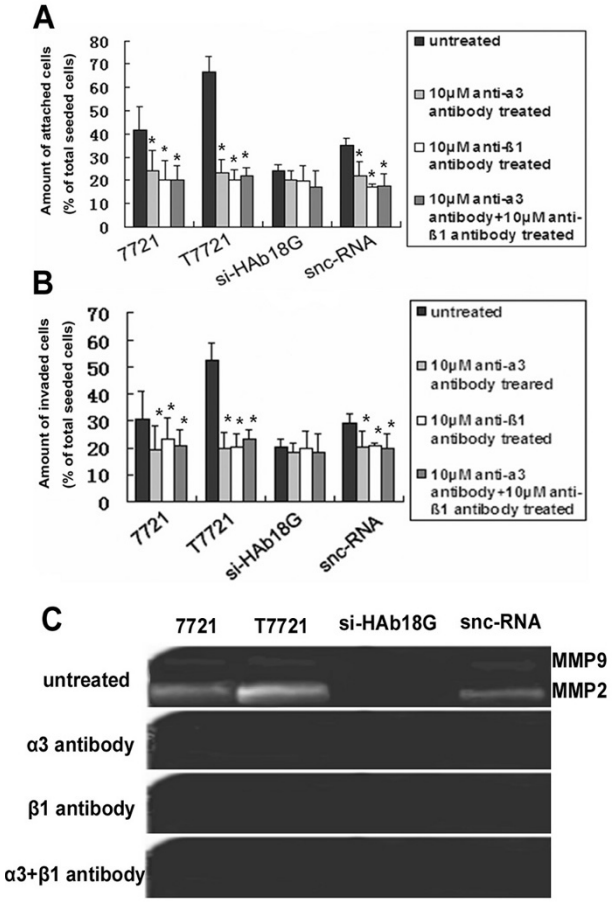

Figure 3. Adhesion and metastasis potential of hepatoma cells with or without α3 or β1 antibody treatment. Adhesion potential (*A*), invasive potential (*B*) and MMP levels (*C*) of 7721 cells, T7721 cells, si-HAb18G-transfected 7721 cells and snc-RNA-transfected 7721 cells incubated with or without α3 or β1 antibody. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments. *$p<0.01$ *versus* corresponding cells with no antibody treatment (one-way ANOVA).

both required and functionally dependent for adhesion. Similar results were also observed in invasion tests (Fig. 3B). In the presence of anti-α3 or -β1 antibody in 7721cells and T7721 cells, MMP2 and MMP9 secretion was reduced to undetectable levels (Fig. 3C).

To explore the nature of the integrins involved in HAb18G/CD147-mediated metastasis and migration, experiments were reproduced with blocking antibodies to α3 associated with antibodies to β1. No significant differences were observed in adhesion potential ($p>0.05$), invasion potential ($p>0.05$) and MMP secretion as compared with those in the groups treated with either α3 antibodies or β1 antibodies.

Expression and phosphorylation levels of FAK and paxillin are enhanced by overexpression of HAb18G/CD147 in human hepatoma cells. FAK, a cytoplasmic tyrosine kinase, plays a major role in integrin signaling by complex formation with paxillin. Paxillin is a key partner protein of FAK that enhances downstream signaling in migration pathways [14, 15]. To identify whether FAK and paxillin are involved in the HAb18G/CD147-mediated and integrin-activated invasion and metastasis processes of human hepatoma cells, the expression levels of FAK, p-FAK (pY397), paxillin and p-paxillin (pY118) were tested by Western blot in cells with different expression levels of HAb18G/CD147. The expression levels of FAK and p-FAK increased 26.7% and 28.6%, respectively, in T7721 cells compared with the expression levels in 7721 cells, and they diminished 32.1% and 44.7%, respectively, in si-HAb18G-transfected 7721 cells compared with the expression levels in snc-RNA-transfected 7721 cells ($p<0.01$, Fig. 4). Paxillin expression and phosphorylation patterns mirrored those of FAK, wth paxillin and p-paxillin expression levels increasing by 28.4% and 65.5%, respectively, in T7721 cells and diminishing by 50.5% and almost 100%, respectively, in si-HAb18G-transfected 7721 cells ($p<0.01$, Fig. 4).

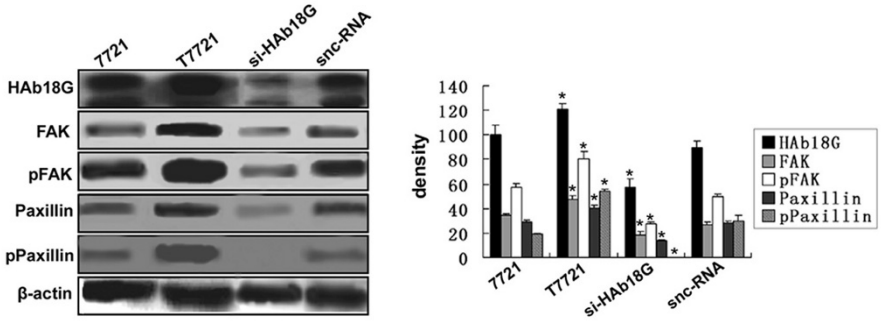

Figure 4. FAK and paxillin expressions in hepatoma cells overexpressing or silencing HAb18G/CD147. FAK, p-FAK(pY397), paxillin and p-paxillin(pY118) expression levels were analyzed in 7721 cells, T7721 cells, si-HAb18G-transfected 7721 cells and snc-RNA-transfected 7721 cells. Protein expressions were normalized by human β-actin expression. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments. *$p<0.01$ *versus* corresponding controls cells (Student's *t*-test).

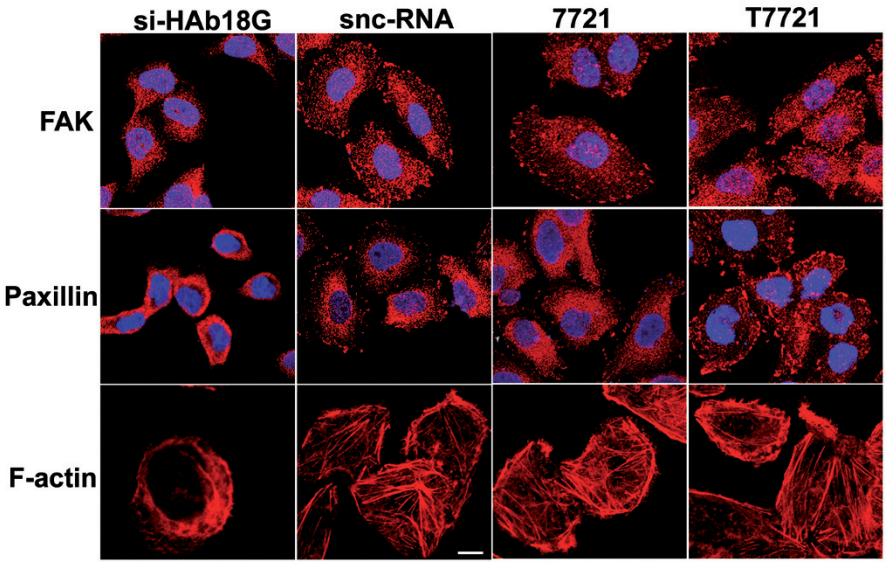

Figure 5. The disposition of focal adhesions and cytoskeleton in HAb18G/CD147-overexpressing or -silencing hepatoma cells. Top and center rows: Cells were probed with either anti-FAK or anti-paxillin antibody. Bottom row: Cells were probed with rhodamine-phalloidin antibody. Shown is a representative of at least three experiments. Bar 2 μm.

Deletion of HAb18G/CD147 reduces the quantity of focal adhesions and rearranges cytoskeleton of human hepatoma cells. Focal adhesions are points of high-strength attachment of the cell to the extracellular matrix (ECM) formed by the leading edge of a migrating or attaching cell in which FAK and paxillin are crucial components [16]. Amount of focal adhesions represents cell adhesion and motion potentials. Cells were stained for FAK and paxillin to assess relative quantity and cellular distribution of focal adhesions. Figure 5 showed a significant decrease of focal adhesions and redistribution of both FAK and paxillin to the periphery of si-HAb18G-transfected 7721 cells compared to those of snc-RNA-transfected 7721 cells, and showed increased focal adhesions in T7721 cells compared to those of 7721 cells. F-actin stress fibers labeled with rhodamine-phalloidin were reduced and disorganized in si-HAb18G-transfected 7721 cells compared with those in snc-RNA-transfected 7721 cells, and there were plentiful and thicker filopodial protrusions in T7721 cells compared to those of 7721 cells (Fig. 5).

HAb18G/CD147 attenuates the negative regulation of the Ca^{2+} entry by PI3K. Our previous data suggested that store-operated Ca^{2+} entry in human hepatoma cells is negatively regulated by NO/cGMP and the negative regulation is opposed by the expression of HAb18G/CD147 [3]. However, how HAb18G/CD147 affects NO/cGMP-sensitive Ca^{2+} entry, and thus promotes the metastatic potentials of tumor cells is not known. It is known that integrin-mediated signals activate the FAK-PI3K pathway and its subsequent signaling functions [17–20]. As inositol trisphosphate (IP3) is the initial trigger for release of intracellular Ca^{2+} stores [17], we speculated that HAb18G/CD147 expression may up-regulate that activity of cellular factors, such as PI3K, and attenuate the negative

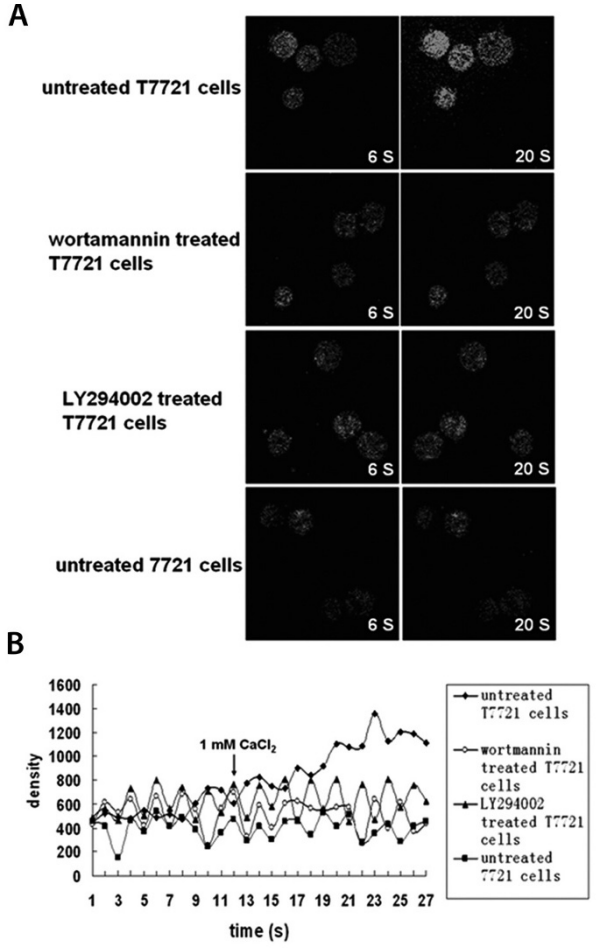

Figure 6. Effects of PI3K inhibitor on the cGMP negative regulation of Ca^{2+} influx in T7721 cells. $[Ca^{2+}]_i$ was monitored in Fura2 acetoxymethyl ester (Fura2/AM)-loaded T7721 cells and 7721 cells. The cells were incubated in NPBS with 4 μmol/l thapsigargin for 20 min and then resuspended in calcium-free PBS; 2 mmol/l 8-Br-cGMP was introduced 5 min prior to the measurement. At the time indicated by the arrow, the media were changed to the respective media containing 2 mmol/l $CaCl_2$ without EGTA. Fluorescence images (*A*) of $[Ca^{2+}]_i$ at 6 s and 12 s. Fluorescence density (*B*) of $[Ca^{2+}]_i$. Data are representative of three independent experiments (univariate ANOVA analysis).

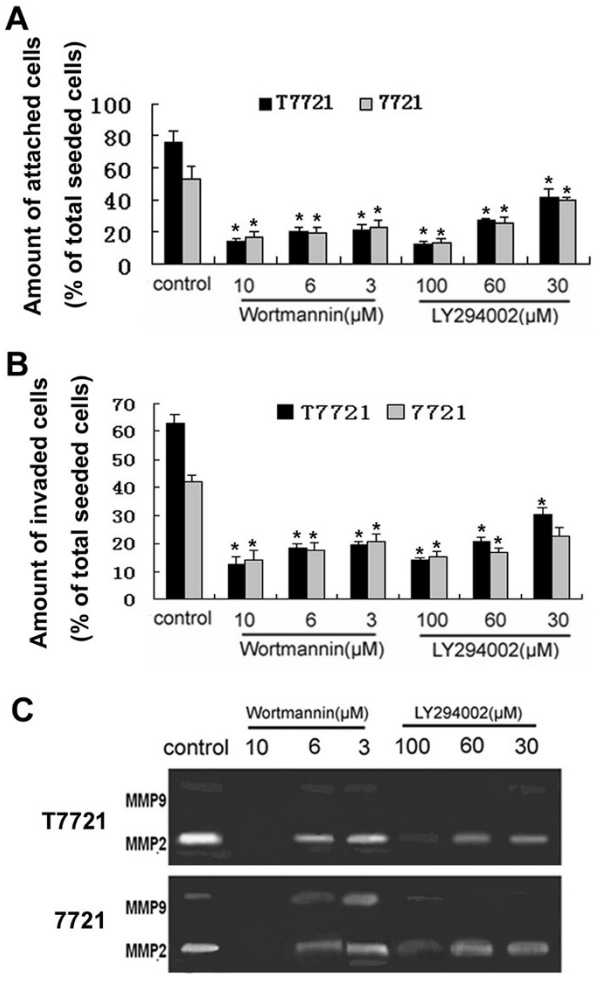

Figure 7. Effects of PI3K inhibitor on adhesion and metastasis potential in T7721 cells and 7721 cells. Adhesion potential (*A*), invasion potential (*B*) and MMP levels (*C*) of T7721 cells and 7721 cells incubated with wortmannin or LY294002 at the indicated concentrations. Bars represent the mean of triplicate samples; error bars represent standard deviation. Data are representative of three independent experiments. *$p<0.01$ *versus* corresponding untreated cells (Student's *t*-test).

regulation of Ca^{2+} entry. To test this hypothesis, we examined the effect of PI3K in NO/cGMP-sensitive store-operated Ca^{2+} entry of T7721 cells. The following inhibitors were employed, wortmannin, an irreversible inhibitor of PI3K pathway and LY294002, a reversible inhibitor of PI3K pathway, which may more mimic the *in vivo* condition. Figure 6 shows that the addition of 10 μmol/l wortmannin or 100 μM LY294002 resulted in 40% decrease of $[Ca^{2+}]_i$ in T7721 cells compared with cells without any inhibitor treatment ($p<0.01$). The previous report showed that 10 μmol/l wortmannin and 100 μmol/l LY294002 effectively blocked chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)-mediated Th2 cell chemotaxis [21], indicating that the effects of wortmannin and LY294002 at the concentration we used are not due to cytotoxicity. Moreover, we checked the concentration of cytosolic Ca^{2+} in 7721 cells and found cytosolic Ca^{2+} in wortmannin- or LY294002-treated T7721 cells were almost the same with that of untreated 7721 cells ($p>0.05$, Fig. 6).

To further confirm the involvement of PI3K in HAb18G/CD147-elevated invasion and metastasis potential, T7721 cells and 7721 cells were exposed to wortmannin and LY294002 for 40 min. As shown in Figure 7, these inhibitors inhibited cells adhesion, invasion and MMPs secretion in a dose-dependent manner in both cell lines.

Discussion

Tumor growth and metastasis are multistep processes involving cell adhesion, proteolytic enzyme degradation of ECM and motility factors, in which the response of tumor cells to cellular and extracellular stimuli is regulated through transduction of signals and translation into cellular activities. It has been demonstrated that elevated HAb18G/CD147 expression is correlated with the progression and invasion potential of human hepatoma cells [13, 22–24]. Previous studies have been focused on the functions of CD147, but the exact molecular mechanism underlying this progression is still largely unknown. In this context, we have investigated the intracellular molecules interacting with HAb18G/CD147 and their signal transduction pathways.

Ligand binding and cell-surface clustering of integrins can lead to the assembly of large multicomponent intracellular signaling complexes in the processes of cells adhesion, proliferation, differentiation, apoptosis and tumor progression. More recently, integrins have been found to associate with many cell-surface molecules [25]. In this study, α3β1 integrin was found to co-immunoprecipitate and colocalize with HAb18G/CD147 in human hepatoma cells. These results demonstrate that α3β1 integrin and HAb18G/CD147 at least are in proximity, if not directly associated in human hepatoma cells. Even though the interaction of α3β1 integrin and HAb18G/CD147 has been largely described in many other cellular lines [8, 26, 27], the exact mechanisms or the molecules that link CD147 to α3β1 integrin have not been reported yet. Our previous study demonstrated that βig-h3, regulated by the expression of HAb18G/CD147, is involved in HAb18G/CD147 signal transduction pathway and mediates HAb18G/CD147-induced invasion and metastasis process of human hepatoma cells [5]. βig-h3, containing an N-terminal signal peptide, four internal FASI repeat domains and an RGD (Arg-Gly-Asp) motif, may interact with α3β1, α1β1, α6β4, αvβ3 or αvβ5 to influence cell attachment and migration in various tumor cells [28–32]. Its highly conserved amino acids (aspartic acid and isoleucine) in the second and fourth FAS1 domains are the binding motifs for α3β1 integrin in human corneal epithelial cells [33]. These results suggested that βig-h3 might act as a bridge between HAb18G/CD147 and α3β1 integrin, and then regulate HAb18G/CD147-induced metastasis of human hepatoma cells. Our results further show that blocking the interaction of HAb18G/CD147 and α3β1 integrin with antibodies specific for α3 and β1 in 7721 cells and T7721 cells reduces cell adhesion, invasion and MMP secretion. However, no significant inhibitory effect is obtained in si-HAb18G-transfected 7721 cells. These results indicate that the enhancing effect of HAb18G/CD147 on cell invasion and metastasis potential is mediated through α3β1 integrin. We also demonstrated that the expression levels of α3 integrin and β1 integrin are not influenced by the expression levels of HAb18G/CD147 in different human hepatoma cell lines. That means the enhancing effect of HAb18G/CD147 is not mediated through the overexpression of α3β1 integrin. It is known that many integrins are expressed with their extracellular domains in a default low-affinity (activity) ligand-binding state (the "off state"); however, cells can change the conformation and affinity of their integrins in response to cellular stimulation in a process often termed "integrin activation". This conformational change results in increased adhesion and subsequent signaling, which mediates events such as cell migration, platelet aggregation, and assembly of ECM [34, 35]. From the above results, we speculate that the positive effect of HAb18G/CD147 is mediated through the up-regulation of α3β1 integrin activity.

Subsequent signaling pathways of integrins consist of many cytoskeleton proteins and enzymes, of which FAK and paxillin are crucial components. The results in our experiments demonstrate that FAK, paxillin and their phosphorylation levels are closely correlated with HAb18G/CD147 expression in human hepatoma cells. As a direct downstream target molecule of integrin activation, FAK connects to integrins through C-terminal domain-mediated interactions. The FAK C-terminal region, which contains the focal adhesion targeting (FAT) domain, also contains binding sites for the integrin-associated proteins talin and paxillin [36]. Even though the initial cloning of FAK has showed that FAK is activated by integrin clustering, the precise mechanism of FAK activation is still not known. A possible explanation for this may be that the cytoplasmic tail of β-integrins (β1, β3 and β5) facilitates FAK activation, potentially involving FAK clustering, autophosphorylation at Y397 and a mechanical linkage of integrins to the actin cytoskeleton [14, 15]. Our results confirm this explanation that HAb18G/CD147, by interaction with α3β1 integrin, facilitates FAK phosphorylation at Y397. Interestingly, in our study we found that not only the phosphorylation levels, but also the expression levels of FAK and paxillin are also elevated. In addition, we found fewer stress fibers, thinner filopodial protrusions and less focal adhesions in si-HAb18G/CD147-transfected 7721 cells. This result directly confirms the interaction of HAb18G/CD147 with FAK and paxillin in adhesion and migration of human hepatoma cells. All the above results suggest that overexpression of HAb18G/CD147 up-regulates α3β1 integrin activity,

and by integrin-FAK signaling linkage increases FAK activity and expression, and subsequently increases paxillin activity and expression.

It is well known that Ca^{2+} plays a role in the regulation of proliferation, invasion, and metastatic potentials. Many reports have indicated that Ca^{2+} is involved in cancer metastasis by regulating expression and/or release of MMPs [37, 38]. Ca^{2+} release from internal stores by either IP3 (*e.g.*, with cholinergic agonists like carbachol) or by inhibition of the Ca^{2+} pump in the internal store (*e.g.*, with thapsigargin or cyclopiazonic acid) stimulates a Ca^{2+} influx in nonexcitable cells [11]. As a strong inducer for intracellular Ca^{2+} store release, IP3-caused intracellular calcium release has been demonstrated in hepatoma cells [17, 39]. The levels of intracellular IP3 mainly depend on the activities of PI3K. PI3K is one of critical downstream signal molecules of FAK pathways [19]. Our previous studies have provided evidence indicating that store-operated Ca^{2+} entry in human hepatoma cells is negatively regulated by NO/cGMP and this regulation is opposed by the expression of HAb18G/CD147 [3]. Both ectodomain and intracellular domains of HAb18G/CD147 are required in this process [4]. Our present results may provide an explanation for this phenomenon. We demonstrate that the inhibitory effect of HAb18G/CD147 on the negative regulation of the Ca^{2+} entry by NO/cGMP is significantly reversed by the specific PI3K inhibitors, wortmannin and LY294002. It is possible that the high level of IP3, stimulated by PI3K, has activated the Ca^{2+} channels, allowing greater Ca^{2+} influx into the cells. The higher Ca^{2+} influx in turn reduces the effectiveness of NO/cGMP on the Ca^{2+} entry, given the fact that more NO/cGMP would be required to shut down these over-activated Ca^{2+} channels. High levels of intracellular Ca^{2+} lead to greater production and/or release of MMPs, thus enhancing metastatic potentials. As shown in this study, the two PI3K inhibitors obviously decrease migration, invasion and MMP secretion of T7721 cells and 7721 cells in a dose-dependent manner.

In conclusion, we report here that HAb18G/CD147, by interacting with $\alpha 3\beta 1$ integrin, activates FAK-paxillin and FAK-PI3K-Ca^{2+} signal pathways, thereby enhancing the metastatic potentials of hepatoma cells. These findings shed new light onto the mechanisms underlying HAb18G/CD147-induced hepatoma cell invasion and migration process and further broaden the investigation of a novel Ca^{2+}-dependent signaling pathway of migration.

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