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Annexin II promotes invasion and migration of human hepatocellular carcinoma cells *in vitro* via its interaction with HAb18G/CD147

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HAb18G/CD147, a member of the immunoglobulin family enriched on the surface of tumor cells, is reported to be correlated with invasion, metastasis, growth, and survival of malignant cells. Here, we found that annexin II, a 36-kDa Ca^{2+}- and phospholipid-binding protein and *in vivo* substrate for tyrosine kinase and PKC, is a new interaction protein of HAb18G/CD147 in human hepatocellular carcinoma (HCC) cells. In the present study, we explored the unclear role of annxin II in HCC invasion and migration and the interaction effects between HAb18G/CD147 and annexin II. Our data show that downregulation of annexin II in HCC cells significantly decreased the secretion of MMP, migration ability, and invasive potential, and affected the cytoskeleton rearrangement of tumor cells. The MMP-2 level and invasive potential of HCC cells were regulated by both annexin II and HAb18G/CD147. Also, interaction effects exist between the two molecules in tumor progression, including MMP-2 production, migration, and invasion. These results suggest that annexin II promotes the invasion and migration of HCC cells *in vitro*, and annexin II and HAb18G/CD147 interact with each other in the same signal transduction pathway working as a functional complex in tumor progression. (*Cancer Sci* 2010; 101: 387–395)

Invasion and migration are two central processes of malignant tumors that usually lead to tumor-associated death. Such migratory and invasive events are regulated by different proteolytic enzymes, including serine protease, cysteine protease, and metalloproteases.[1] These proteolytic enzymes degrade the extracellular matrix (ECM) and basement membrane surrounding blood vessels. During metastasis, cancer cells penetrate through the degraded basement membrane and ECM, become implanted in the underlying tissues, and subsequently form secondary tumors.[2]

Among the proteases, MMP are mainly regulated by tumor–stroma interactions via CD147, a highly glycosylated cell surface transmembrane protein belonging to the immunoglobulin superfamily.[3] CD147 was first identified as a factor shed from the surface of tumor cells responsible for stimulating MMP-1 production by fibroblasts. One of the important and most studied functions of CD147 is its role in induction of MMP production via cell interactions – thus the derivation of its other name: extracellular matrix metalloproteinase inducer (EMMPRIN).[4] Accumulating evidence suggests a prominent role for CD147 in mediating interactions both between tumor cells themselves and between tumor cells and 'hijacked' host stromal cells to promote a number of events during cancer progression.[5] Although the detailed mechanisms through which CD147 regulates the phenomenon are not yet known, it is clear that CD147 is a major mediator of malignant cell behavior. Our team previously developed the anti-hepatocellular carcinoma (HCC) mAb HAb18 by using a cell suspension extracted from fresh human HCC tissues to immunize BALB/c mice and prepare hybridomas.[6–8] Its antigen, HAb18G, was identified by screening the HCC cDNA expression library and was named HAb18G/CD147 for its homology to CD147.[3,9] Studies have demonstrated that HAb18G/CD147 promotes the invasion and migration of HCC cells by stimulating both fibroblast cells and tumor cells themselves to produce MMP. Our previous study also showed that HAb18G/CD147 is involved in tumor metastasis and invasion as a signal transduction molecule by regulating Ca^{2+} inflow.[10,11] All of these findings show that HAb18G/CD147 may play some important roles in HCC progression, including adhesion, migration, and enzyme degradation. In light of these findings, Metuximab, a murine HAb18 F(ab')2 fragment specific for CD147 (also known as LICARTIN) has been developed in the iodine^{131}-labeled form and approved as a new drug for clinical therapy of primary HCC patients by the China State Food and Drug Administration (no. S20050039) in April 2005.[12,13]

In addition to MMP, the serine protease plasminogen–plasmin system also plays an active role in tumor invasion and migration. Plasminogen is an inactive enzyme that can be cleaved by tissue-type plasminogen activators (tPA) through hydrolysis of the Arg561–Val562 peptide bond to yield the active serine protease plasmin. Plasmin is a serine protease that degrades most proteins within the ECM, including fibronectin, laminin, and proteglycans.[14] Emerging reports indicate that under pathological conditions, uncontrolled overproduction of plasmin in the tumor microenvironment can accelerate the localized degradation of ECM, which in turn facilitates tumor invasion and migration.[1,15,16] Annexin II, a 36-kDa Ca^{2+} – and phospholipid-binding protein and a substrate for tyrosine kinase and PKC, was found to be a putative co-receptor for both plasminogen and tPA, and to regulate the generation and expression of plasmin and thus affect the malignant phenotype of various human cancers. Studies show that malignant cells with higher expression of annexin II such as MDA-MB-231 cells (an invasive breast cancer cell line) are able to efficiently catalyze the conversion of plasminogen to plasmin whereas MCF-7 cells (a poorly invasive breast cancer cell line) lacking annexin II expression fail to generate plasmin, which strongly indicates that annexin II is required for plasmin generation.[17,18] Further data show that the annexin II-dependent plasmin generation is essential for tumor invasion and migration. These results suggest that annexin II is a regulatory switch for continuous plasmin generation in the tumor microenvironment.[18] On the other hand, membrane-bound annexin II presenting at the inner surface of the plasma membrane may serve as a platform for actin

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assembly and function in maintaining the plasticity of the dynamic membrane-associated actin cytoskeleton, due to the direct interaction of annexin II with polymerized and monomeric actin, which plays an important role in cancer cell migration and invasion.[19–21] Increased expression of annexin II has been described in several types of tumor and is positively related to tumor invasion and migration, including gastric carcinoma, colorectal cancer, pancreatic cancer, breast cancer, high-grade gliomas, kidney cancer, and vascular tumors.[22] In contrast, there is loss of annexin II expression in prostate cancer, and the role of annexin II in prostate cancer appears contradictory.[23,24] Studies have testified that annexin II is upregulation in HCC, but the function of annexin II in HCC invasion and migration remains unclear.

Annexin II and HAb18G/CD147 play important and similar roles in tumor progression, and it has been speculated that there may exist some interaction between annexin II and CD147,[5] but the assumption has never been proven. In the present study, we first explored and proved the possibility of the above hypothesis. In the following experiments, we studied the role of annexin II in HCC invasion and migration. Then we further investigated the interaction between annexin II and HAb18G/CD147 in tumor invasion and migration.

Materials and Methods

Cell lines. Human FHCC-98 hepatoma cells[25] were originated from the tumor tissues of a 39-year-old Chinese male HCC patient and conserved in our laboratory. Human MHCC97-H hepatoma cells[26] were supplied by the Liver Cancer Institute of Fudan University (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 2% L-glutamine at 37°C in a humidified atmosphere of 5% CO_2.

Co-immunoprecipitation. The interaction of HAb18G/CD147 with annexin II in FHCC-98 and MHCC97-H cells was detected using a ProFound Mammalian Co-Immunoprecipitation Kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. Briefly, cells (1×10^6) were lysed using M-per reagent. The lysate was collected onto a coupling gel that was pre-bound with 200 μg of the mouse anti-human HAb18G/CD147 mAb HAb18 (prepared in our laboratory) or anti-JEV mAb (mouse, IgG, kindly provided by the Department of Microorganism, Fourth Military Medical University; negative control), followed by four washes with the co-immunoprecipitation buffer. The coupling gel was then washed with elution buffer, and aliquots of the eluent were analysed by western blotting using annexin II mAb (Zymed Laboratories, South San Francisco, CA, USA) and HAb18 mAb.

Co-localization of annexin II with HAb18G/CD147 in HCC cells. FHCC-98 and MHCC97-H cells were harvested and allowed to attach for 24 h to precoated glass coverslips. They were then fixed in 3.7% formaldehyde in PBS, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA (Fraction V) in PBS for 1 h. Coverslips were incubated with goat anti-CD147 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution and annexin II mAb at a 1:100 dilution in PBS overnight at 4°C. Later the cells were washed and incubated with Alexa 594-conjugated goat anti-mouse (Invitrogen, Carlsbad, CA, USA) or donkey anti-goat IgG-FITC (Santa Cruz Biotechnology) secondary antibody at a dilution of 1:500 for 1 h at room temperature. Cell nuclei were dyed with DAPI (Vector Laboratories, CA, USA). Following washing, the coverslips were mounted onto glass slides by adding anti-fade to prevent the quenching of flourophores. The proteins were visualized with a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

RNA interference. Chemically synthesized, double-stranded siRNA, with 19-nucleotide duplex RNA and 2-nucleotide 3′ dTdT overhangs was purchased from Shanghai GenePharma (Shanghai, China). The sequences for si-annexin II (si-RNA-1) were 5′-GGGUCUGUCAAAGCCUAUAtt-3′ and 3′-ttACCCAGACAGUUUCGGAUA-5′, and the sequences for si-HAb18G were as described previously.[13] Annexin II siRNA (si-RNA-2) was purchased from Santa Cruz Biotechnology. HCC cells were transfected with siRNA using LipofectAMINE 2000 reagent according to the manufacturer's instructions (Invitrogen). Silencer negative control siRNA (snc-RNA) was used as a negative control under similar conditions.

RT-PCR. Forty-eight hours after siRNA transfection, the total RNA was extracted from the cells with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA with the ReverTra Ace-a kit (Toyobo, Tokyo, Japan). All primers were synthesized by Shanghai Sangon Co. (Sangon, Shanghai, China) as follows: annexin II, forward primer 5′-GAGGATGGCTCTGTCATTGATT-3′, reverse primer 5′-CTGGTAGTCGCCCTTAGTGTCT-3′; GAPDH, forward primer 5′-ACCACAGTCCATGCCATCAC-3′ and reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′. The conditions for PCR were one cycle of 94°C for 4 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR products were electrophoresed on 1% agarose gels. All PCR were done in triplicate.

Western blotting. Forty-eight hours after siRNA transfection, HCC cells were harvested in lysis buffer and 10 μg of cellular protein was subjected to 12% SDS-PAGE separation. Proteins were transferred to PVDF microporous membrane (Millipore, Boston, MA, USA) and blots were probed with annexin II mAb. Tubulin was chosen as an internal control and the blots were probed with mouse anti-tubulin mAb (Santa Cruz Biotechnology).

Cell adhesion assay. A 96-well culture plate was coated with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) and blocked with PBS containing 2% BSA, then washed with PBS. Cells in serum-free medium containing 0.1% BSA were added to the wells (2×10^4 cells/well) and incubated at 37°C and 5% CO_2 for 30–60 min. After removing the medium and non-attached cells, 0.1% crystal violet was added for 10 min. The plate was gently washed with tap water and dried in air for 24 h. Then 100 μL of 5% SDS with 50% ethanol was added for 20 min and the absorbance was read at 540 nm.

***In vitro* invasion/migration assays.** The assay was done using chambers with polycarbonate filters (8-μm pore size; Millipore). The upper side of a polycarbonate filter was either not coated or coated with Matrigel to form a continuous thin layer. Twenty-four hours after siRNA transfection, HCC cells (1×10^5) were resuspended in 300 μL of 0.1% serum medium and added into the upper chamber. The snc-RNA-transfected cells were used as a negative control. The lower chamber was filled with 10% FBS medium (200 μL). After 24-h incubation and removal of the cells on the upper chamber of the filter with a cotton swab, the cells on the underside were stained and counted.

Gelatin zymography. Forty-eight hours after siRNA transfection, conditioned medium was collected and separated by 10% acrylamide gels containing 0.1% gelatin. The gels were incubated in 2.5% Triton X-100 solution at room temperature with gentle agitation and then were 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 for 18 h. After the reaction, the gels were stained for 6 h with Coomassie brilliant blue and then destained for 0.5 h. The zones of gelatinolytic activity were shown by negative staining.

Immunofluorescence detection of microfilaments. Twenty-four hours after siRNA transfection, HCC cells were harvested and allowed to attach for 24 h to precoated glass coverslips. They were then washed twice with PBS (pH 7.4), fixed in 3.7% formaldehyde in PBS, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA in PBS for 1 h. Coverslips were

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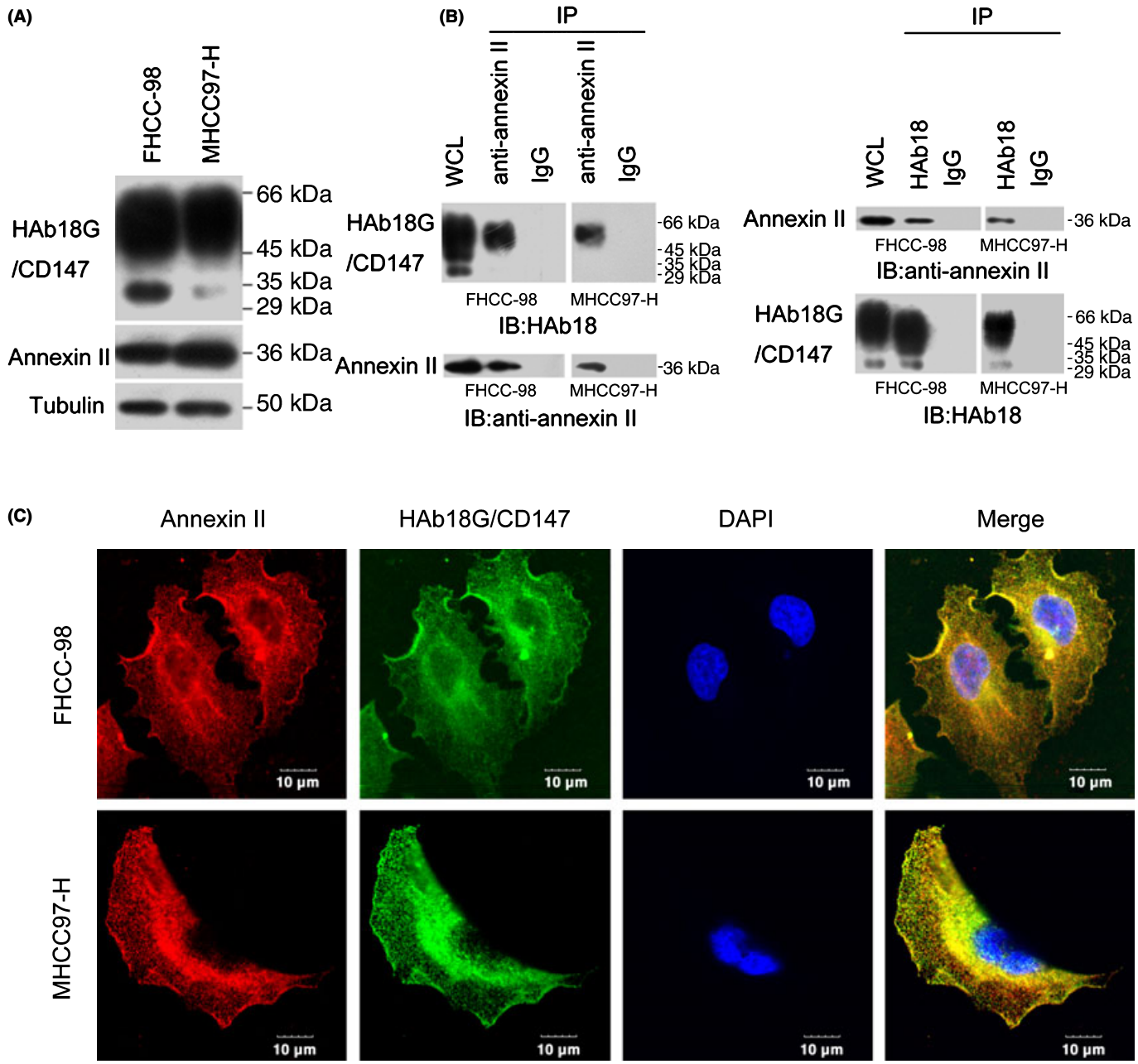

Fig. 1. Co-immunoprecipitation and co-localization of annexin II and HAb18G/CD147 in FHCC-98 and MHCC97-H cells. (A) Expression of HAb18G/CD147 and annexin II in FHCC-98 and MHCC97-H cells. HAb18G/CD147 and annexin II were both highly expressed in the two cell lines. (B) HAb18G/CD147 immunoprecipitated with annexin II. Lysates of FHCC-98 and MHCC97-H cells were subjected to immunoprecipitation with anti-HAb18G/CD147 or anti-annexin II antibody-pre-bound coupling gel. Annexin II and HAb18G/CD147 in the immune complexes were detected by immunoblotting (IB) analysis. Mouse IgG was used as a negative control. (C) Expression and localization of HAb18G/CD147 and annexin II in FHCC-98 and MHCC97-H cells. FHCC-98 and MHCC97-H cells were double-stained for HAb18G/CD147 (green) and annexin II (red). Scale bar = 10 μm. WCL, whole cell lysate.

incubated with annexin II mAb at a 1:100 dilution in PBS for 2 h. Later the cells were washed and incubated with Alexa 594-conjugated goat anti-mouse and FITC–phalloidin solution (1:40) for 40 min in the dark. After each step, the cells were washed twice with PBS, and all of the steps were carried out at room temperature. Finally, the cells were mounted with glycerol, and observed using an Olympus BX60 microscope.

Statistical analysis. All experiments were carried out in triplicate and the results are expressed as the mean ± SD. Statistical significance was determined using the software SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). One-way ANOVA with the Latin Square Design test was carried out for multiple comparisons and two-way ANOVA was used for analysis of interaction effects. Differences were deemed significant if $P < 0.05$.

Results

Co-immunoprecipitation and co-localization of annexin II and HAb18G/CD147 in HCC cells. In order to investigate the interaction between annexin II and HAb18G/CD147, we undertook co-immunoprecipitation and immunofluorescence assays.

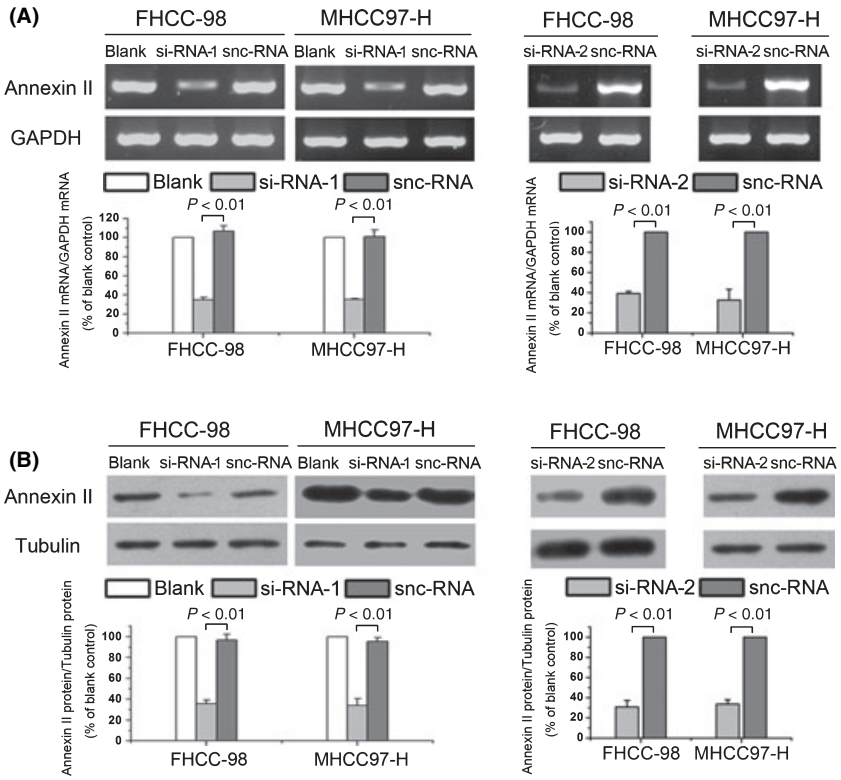

Fig. 2. Annexin II siRNA causes effective and specific downregulation of annexin II expression in hepatocellular carcinoma cells. Forty-eight hours after si-annexin II (si-RNA-1 and si-RNA-2) or silencer negative control siRNA (snc-RNA) transfection, expression of annexin II in both cell lines was examined by (A) RT-PCR and (B) western blotting (top, a representative image; bottom, grayscale analysis of at least three independent experiments). Columns, mean; bars, SD, $P < 0.01$ *versus* corresponding snc-cells (one-way ANOVA).

Protein expression of the two molecules in MHCC97-H and FHCC-98 cells was detected by western blotting. As shown in Figure 1A, HAb18G/CD147 and annexin II were both highly expressed in HCC cell lines. Annexin II was then found to co-immunoprecipitate with HAb18G/CD147 in FHCC-98 and MHCC97-H cell lysates (Fig. 1B), indicating that HAb18G/CD147 and annexin II interact in their native form in HCC cells. To further confirm the result, immunofluorescence double staining was used. The results showed co-localization of annexin II with HAb18G/ CD147 in FHCC-98 and MHCC97-H cells (Fig. 1C).

Silencing annexin II inhibits adhesion and migration of HCC cells, and changes microfilament arrangement. Adhesion and migration are important processes in tumor invasion. To investigate the role of anenxin II in HCC progression, RNA interference was used to downregulate the expression of annexin II in FHCC-98 and MHCC97-H cells. Annexin II siRNA could effectively decrease the mRNA expression of annexin II (Fig. 2A) with inhibitory rates of 66.19 ± 2.87% ($P < 0.01$, si-RNA-1, FHCC-98), 65.22 ± 0.53% ($P < 0.01$, si-RNA-1, MHCC97-H), 60.83 ± 2.24% ($P < 0.01$, si-RNA-2, FHCC-98), and 67.26 ± 10.59% ($P < 0.01$, si-RNA-2, MHCC97-H) compared with that of snc-cells. In addition, the protein expression of annexin II was obviously decreased in annexin II siRNA-transfected cells (si-cells) with inhibitory rates of 61.74 ± 2.84% ($P < 0.01$, si-RNA-1, FHCC-98), 64.83 ± 5.60% ($P < 0.01$, si-RNA-1, MHCC97-H), 69.16 ± 6.34% ($P < 0.01$, si-RNA-2, FHCC-98), and 66.28 ± 4.29% ($P < 0.01$, si-RNA-2, MHCC97-H) compared with that of snc-cells (Fig. 2B).

In the cell adhesion assay, 48 h after transfection, attached cells were significantly decreased in FHCC-98 and MHCC97-H cells (Fig. 3A) with attached rates of 68.05 ± 10.55% ($P < 0.01$, si-RNA-1, FHCC-98), 49.42 ± 8.38% ($P < 0.01$, si-RNA-1, MHCC97-H), 55.27 ± 3.34% ($P < 0.01$, si-RNA-2, FHCC-98), and 50.91 ± 9.16% ($P < 0.01$, si-RNA-2, MHCC97-H) compared with that in snc-cells. In the migration assay, the number of cells migrating through the filter was markedly decreased in the siRNA-transfected FHCC-98 and MHCC97-H cells (Fig. 3C,D) with inhibitory rates of 50.79 ± 4.46% ($P < 0.01$, si-RNA-1, FHCC-98), 40.35 ± 6.43% ($P < 0.01$, si-RNA-1, MHCC97-H), 64.8 ± 7.64% ($P < 0.01$, si-RNA-2, FHCC-98), and 71.08 ± 3.14% ($P < 0.01$, si-RNA-2, MHCC97-H) compared with that of snc-cells.

Actin rearrangement plays a central role in cell motility. To investigate the role of annexin II in actin reorganization involved in the formation of microfilaments, blank control cells, si-cells, and snc-cells were stained with FITC-conjugated phalloidin and anti-annexin II antibody. As shown in Figure 3B, spreading of HCC cells was remarkably inhibited when the expression of annexin II was downregulated. Si-cells presented a longer appearance with a thicker and denser F-actin stress fiber network compared with snc-cells. These data indicate that annexin II plays a role in regulating tumor cell adhesion and migration, as well as actin rearrangement.

Downregulation of annexin II inhibits the invasion of HCC cells. Degradation of the ECM is an important process associated with tumor invasion, in which MMP play a critical role. The results of gelatin zymography showed that MMP secretion was significantly decreased in si-cells with inhibitory rates of 51.91 ± 4.86% ($P < 0.01$, MMP-2, si-RNA-1), 57.91 ± 1.82% ($P < 0.01$, MMP-9, si-RNA-1), 60.78 ± 1.37% ($P < 0.01$, pro-MMP-9, si-RNA-1), 60.40 ± 6.51% ($P < 0.01$, MMP-2, si-RNA-2), 48.96 ± 1.82% ($P < 0.01$, MMP-9, si-RNA-2), and 43.14 ± 0.72% ($P < 0.01$, pro-MMP-9, si-RNA-2) in MHCC97-H cells, and 38.35 ± 5.23% ($P < 0.01$, MMP-2, si-RNA-1), 58.43 ± 0.41% ($P < 0.01$, MMP-9, si-RNA-1), 36.15 ± 2.72% ($P < 0.01$, pro-MMP-9, si-RNA-1), 62.51 ± 3.17% ($P < 0.01$, MMP-2, si-RNA-2), 37.65 ± 0.94% ($P < 0.01$, MMP-9, si-RNA-2), and 39.53 ± 1.63% ($P < 0.01$, pro-MMP-9, si-RNA-2) in FHCC-98 cells compared with that of snc-cells (Fig. 4A). By *in vitro* invasion assay, it was found that the number of cells migrating through the filter was

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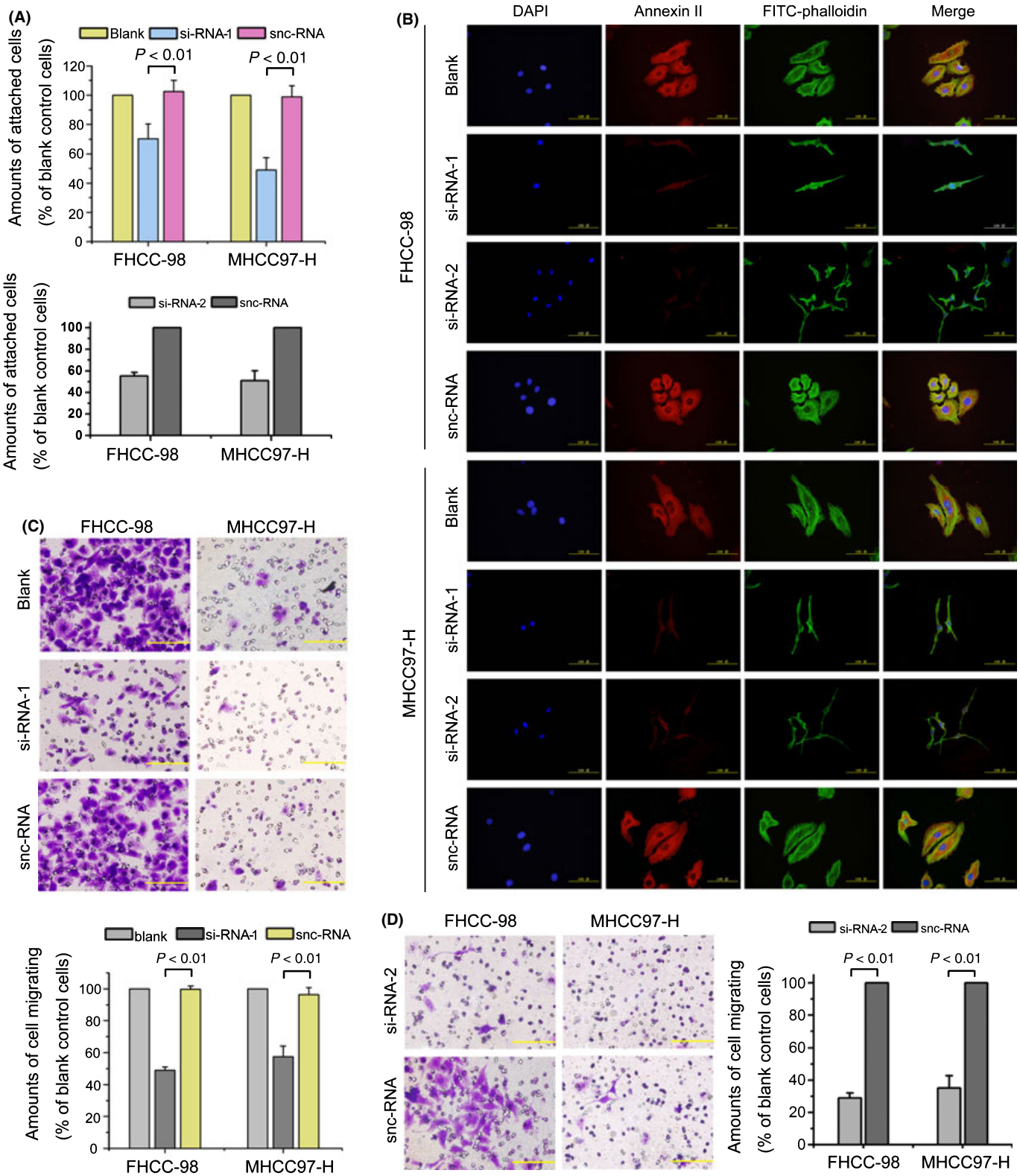
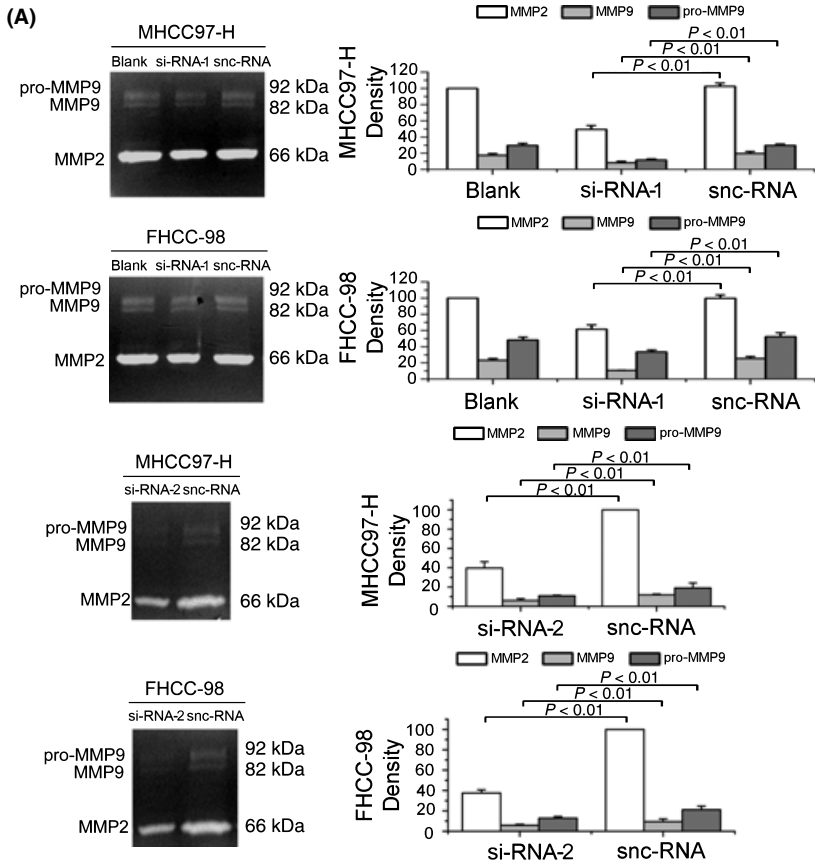


Fig. 3. Silencing annexin II in FHCC-98 and MHCC97-H cells inhibits cell adhesion, cytoskeleton rearrangement, and migration. FHCC-98 and MHCC97-H cells were transfected with annexin II siRNA (si-RNA-1 and si-RNA-2) and silencer negative control siRNA (snc-RNA). (A) Cell adhesion. Forty-eight hours after siRNA transfection, equal numbers of cells were added to the Matrigel-coated wells. One hour later, cells were stained and air dried for another 24 h, then lysed and detected. Top, si-RNA-1; bottom, si-RNA-2. (B) Cytoskeleton rearrangement. Forty-eight hours after siRNA transfection, hepatocellular carcinoma cells were stained with FITC–phalloidin solution and anti-annexin II antibody. The spreading of si-annexin II (si-RNA-1 and si-RNA-2)-transfected cells was remarkably inhibited. (C,D) Migration assay. Twenty-four hours after siRNA (C, Si-RNA-1; D, Si-RNA-2) transfection, equal numbers of cells were added into the upper chamber. Twenty-four hours later, the cells migrating through the filter were stained and counted. (C top, D, left) Representative image. (C bottom, D right) Quantitative analysis for the cells migrating through the filter in three independent experiments. Columns, mean; bars, SD. $P < 0.01$ *versus* corresponding snc-cells (one-way ANOVA). Scale bar = 10 μm.



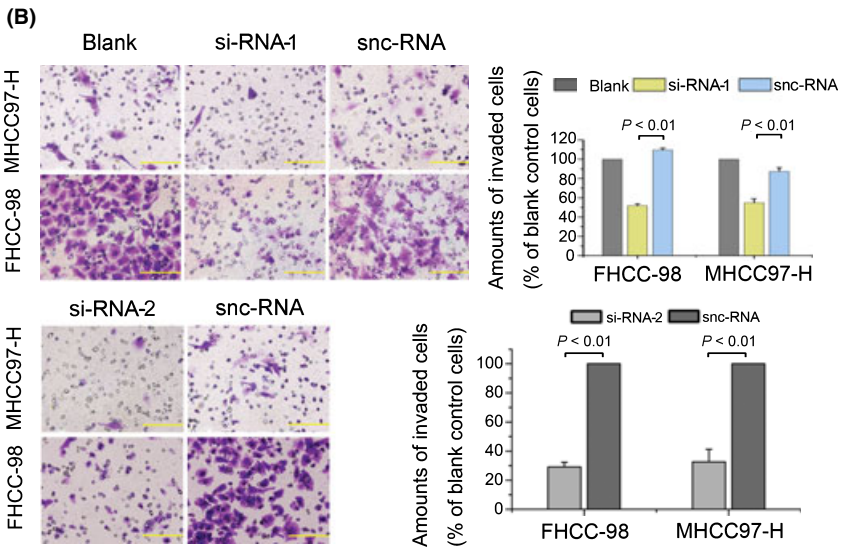


Fig. 4. Silencing annexin II in FHCC-98 and MHCC97-H cells inhibits the invasion potential of tumor cells. FHCC-98 and MHCC97-H cells were transfected with annexin II siRNA (si-RNA-1 and si-RNA-2) and silencer negative control siRNA (snc-RNA). (A) MMP activity analysis. Forty-eight hours after siRNA transfection, MMP activity in the conditioned medium was detected by gelatin zymography (left, representative image; right, grayscale analysis of at least three independent experiments). Columns, mean; bars, SD. (B) Invasion assay. Twenty-four hours after siRNA transfection, equal numbers of cells were added into the Matrigel-coated upper chamber. Twenty-four hours later, the cells migrating through the filter were stained and counted (left, representative image; right, quantitative analysis for the cells migrating through the filter in three independent experiments). Columns, mean; bars, SD. $P < 0.01$ *versus* corresponding snc-cells (one-way ANOVA). Scale bar = 10 μm.

significantly decreased in the siRNA-transfected MHCC97-H and FHCC-98 cells, with inhibitory rates of 37.08 ± 3.95% ($P < 0.01$, si-RNA-1) and 67.38 ± 8.67% ($P < 0.01$, si-RNA-2) in MHCC97-H cells, and 52.52 ± 2.29% ($P < 0.01$, si-RNA-1) and 70.85 ± 3.18% ($P < 0.01$, si-RNA-2) in FHCC-98 cells compared with that of snc-cells (Fig. 4B). These results demonstrate that annexin II enhances the invasion potential of HCC cells through regulating MMP secretion and activation.

Interaction effects between annexin II and HAb18G/CD147 in tumor invasion and migration. In the following experiments, we further investigated the interaction between annexin II and HAb18G/CD147. Data showed that the expression of HAb18G/CD147 in HCC cells was inhibited when HAb18G/CD147-specific siRNA (si-HAb18G) was transfected, with an inhibitory rate of 56.24 ± 3.45% compared with that of snc-cells ($P < 0.01$), but the expression of annexin II was not affected (Fig. 5A, left). The expression of HAb18G/CD147 was not affected by the downregulation of annexin II either (Fig. 5A, right). The result suggested that the function of HAb18G/CD147 in tumor invasion and migration was not mediated by changing the expression of annexin II and vice versa. Gelatin zymography analysis showed that MMP-2 expression was downregulated when HAb18G/CD147

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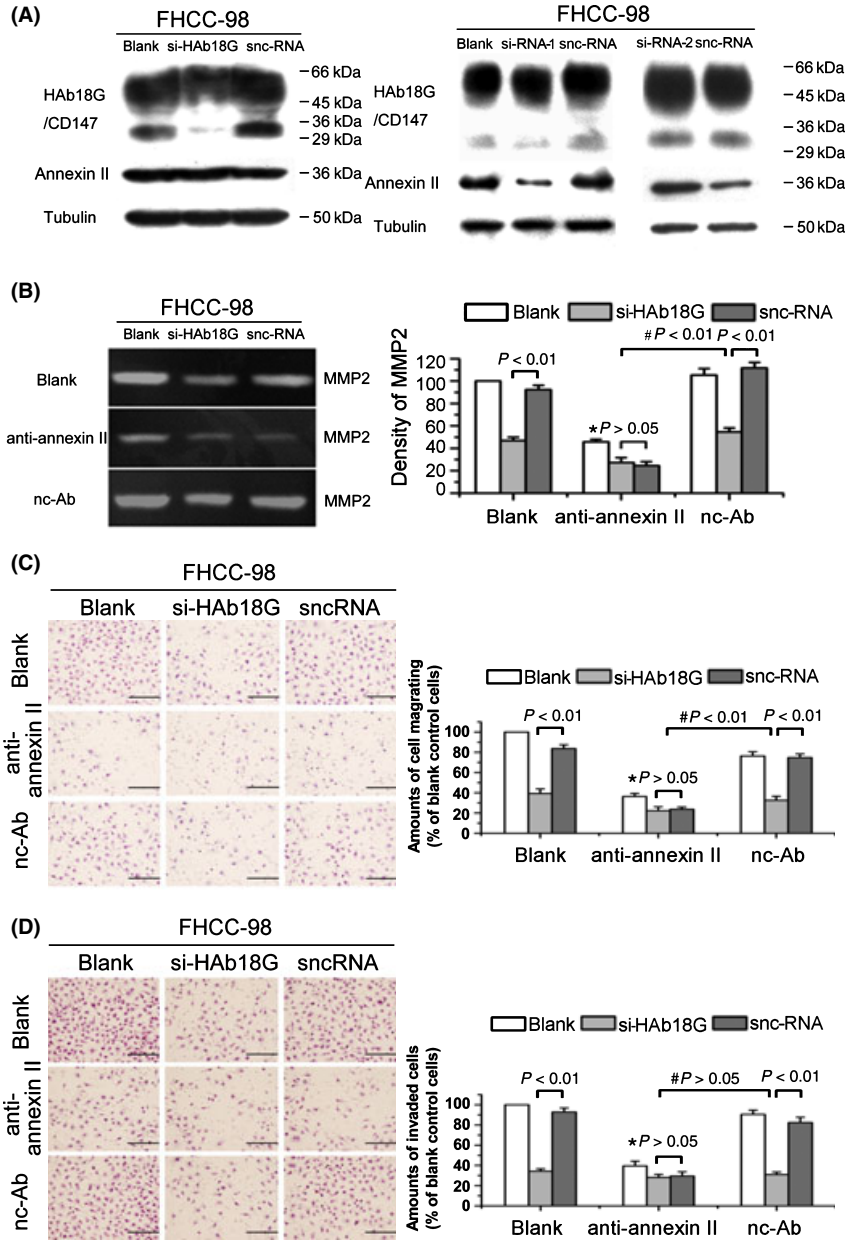

Fig. 5. Interaction effects between annexin II and HAb18G/CD147 in invasion and migration of FHCC-98 cells. (A) Expression of annexin II was not affected by si-HAb18G transfection in FHCC-98 cells (left, representative image). The expression of HAb18G/CD147 also was not affected by the downregulation of annexin II (right, representative image). (B–D) Interaction effects between the two molecules on MMP-2 (B) expression, (C) migration, and (D) invasion of FHCC-98. FHCC-98 cells were transfected with si-HAb18G or silencer negative control siRNA (snc-RNA), and were incubated with annexin II antibody or negative control antibody (nc-Ab) 24 h after transfection. MMP-2 expression and trans-filter cell number under diverse treatment were measured (left, representative image; right, grayscale analysis of at least three independent experiments or quantitative analysis for the cells migrating through the filter in three independent experiments). Columns, mean; bars, SD. *P* and **P versus* corresponding snc-cells, *#P versus* corresponding nc-Ab-cells (one-way ANOVA). Scale bar = 10 μm.

was knocked down or annexin II was blocked ($P < 0.01$), and there existed significant interaction effects in MMP-2 production between the two molecules ($P < 0.01$, two-way ANOVA) (Fig. 5B). Compared with that of snc-cells, the expression of MMP-2 was not affected by si-HAb18G transfection when annexin II was blocked ($P > 0.05$). But the expression of MMP-2 in si-HAb18G-transfected cells was further downregulated when the cells were blocked with anti-annexin II compared with that of nc-Ab blocked cells ($P < 0.01$). Similar results were found in the invasion (Fig. 5C) and migration (Fig. 5D) assays. But a difference in the invasion assay was noticed. That is, the number of si-HAb18G-transfected cells migrating through the filter was not affected by anti-annexin II ($P > 0.05$, one-way ANOVA). These results suggest that annexin II and HAb18G/CD147 have interaction effects in HCC invasion and migration. They may work as a functional complex in tumor invasion and migration in the same signal transduction pathway.

Discussion

Tumor invasion and migration are multistep processes in which many molecules are involved by protein–protein interactions. HAb18G/CD147, also named EMMPRIN, is particularly enriched on the membrane of malignant tumor cells.[27] Previous studies have demonstrated that HAb18G/CD147 promotes the invasion of tumor cells by stimulating MMP production.[28,29] Several molecules have been reported to be associated with HAb18G/CD147 and play important roles in cancer invasion and migration.[30] The precise mechanisms of the HAb18G/CD147-mediated invasion and migration are not yet well known. In the present study, annexin II was found to co-immunoprecipitate and co-localize with HAb18G/CD147 in HCC cells. We discovered that downregulation of annexin II significantly inhibited MMP production and the adhesive and invasive potentials of MHCC97-H and FHCC-98 cells. Also, there were interaction effects between annexin II and HAb18G/CD147 in MMP-2 production,

invasion, and migration of HCC cells. Considering the previous reports and our newly found results, we suggest that annexin II may be involved in HAb18G/CD147-mediated invasion and migration of human hepatoma cells.

HAb18G/CD147 plays a critical role in the invasive and migratory activities of HCC cells by stimulating both tumor cells and paratumoral fibroblasts to express MMP. Xu *et al.* found that si-HAb18G inhibits gelatinase production and actin and focal adhesin kinase (FAK) expression in FHCC-98 via an ERK1/2 signaling pathway.[31] Tang *et al.* suggested that HAb18G/CD147 enhances the invasive and metastatic potentials of human hepatoma cells via integrin $\alpha_3\beta_1$-mediated FAK–paxillin and FAK–PI3K–Ca^{2+} signaling pathways.[32] Qian *et al.* demonstrated that downregulation of CD147 affects the structure and function of HCC cells, and the stress-activated protein kinase (SAPK)–JNK pathway may be involved in the alteration of cell behavior.[33] Although many aspects of HAb18G/CD147 on tumor progression have been studied, the signal transduction pathway and interaction proteins of HAb18G/CD147 remain to be further investigated.

In the present study, we found that MHCC97-H and FHCC-98 cells presented a longer appearance with thicker and denser F-actin stress fiber networks when annexin II was downregulated. These morphological changes can also be observed when HAb18G/CD147 is knocked down, as we previously reported.[32] Annexin II is an F-actin-binding protein enriched at actin assembly sites on both the plasma membrane and endosomal vesicles.[34] It is also found at the membrane–actin cytoskeleton interface and is associated with active actin remodeling sites, like rocketing macropinosomes,[19,35] actin-rich pedestals,[36] and lamellipodia.[37] Besigin, which is another name for HAb18G/CD147, was reported to promote cytoskeleton rearrangement and the formation of lamellipodia.[38] Our results have indicated that annexin II co-immunoprecipitates with HAb18G/CD147 in HCC cells, and the two molecules colocalize in HCC cells, especially in the leading edge of the cells. According to our data, we suggest that annexin II might act as a linker between HAb18G/CD147 and F-actin, and then regulate HAb18G/CD147-induced migration of human hepatoma cells.

Our study also showed that annexin II-specific si-RNA inhibited MMP production of HCC cells, and then inhibited invasion and migration of HCC cells. Evidence has proved that annexin II acts as a high-affinity binding receptor for several proteases and ECM proteins, including plasminogen, tPA, and plasmin[39] and functions in the degradation of ECM. Annexin II exists in two forms: annexin II monomer and annexin II heterotetrameric complex (A2t). The latter consists of two annexin monomers and two copies of S100A10 (p11),[40] a member of the S-100 family. Annexin II monomer localizes to the cytoplasm near the plasma membrane, whereas A2t localizes to the extracellular surface of endothelial and tumor cells and binds to proteases to degrade ECM.[41,42] We found that MMP-2 expression and the invasive ability of HCC cells were not affected by the expression level of HAb18G/CD147 when annexin II was blocked. But MMP-2 expression and the migratory potential of HCC cells can be further downregulated by anti-annexin II, when the expression of HAb18G/CD147 is knocked down first (Fig. 5B,C). Our data illustrated that the invasive ability of HCC cells was not affected by anti-annexin II when HAb18G/CD147 was downregulated first (Fig. 5D). These results suggest that there exist interaction effects between the two molecules. Furthermore, annexin II may function upstream of HAb18G/CD147 in MMP-2 production and tumor migration, and the effect of annexin II is partly, not wholly, mediated by HAb18G/CD147.

We also demonstrated that expression of annexin II is not influenced by the expression of HAb18G/CD147 and vice versa, which suggested that the interaction effects between annexin II and HAb18G/CD147 on HCC invasion and migration are not mediated by regulating the expression mutually. It is known that annexin II plays a central role in the activation of plasmin, which is an activator of MMP. When plasmin is generated at the outer surface of the cellular membrane, it is protected and stabilized by binding to integrins.[18] Our data also showed that downregulating or blocking tumor cell HAb18G/CD147 significantly decreases MMP production, but the exact mechanisms remain obscure. Our previous studies demonstrated that HAb18G/CD147 interacts with $\alpha3\beta1$ integrin.[32] According to these results and the interaction effect between annexin II and HAb18G/CD147 on MMP production, we infer that annexin II, HAb18G/CD147, and integrin may act as a functional complex in plasmin and MMP activation, which facilitates the degradation of ECM and tumor invasion.

In conclusion, our present work shows that annexin II promotes the invasion and migration of HCC cells *in vitro*. Annexin II and HAb18G/CD147 interact with each other in HCC cells and may act as a functional complex, which plays an important role in HCC progression, including adhesion, migration, enzymatic degradation, and invasion.

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