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The Involvement of HAb18G/CD147 in Regulation of Store-operated Calcium Entry and Metastasis of Human Hepatoma Cells*

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The present study examined the effect of hepatoma-associated antigen HAb18G (homologous to CD147) expression on the NO/cGMP-regulated Ca^{2+} mobilization and metastatic process of human hepatoma cells. HAb18G/CD147 cDNA was transfected into human 7721 hepatoma cells to obtain a cell line stably expressing HAb18G/CD147, T7721, as demonstrated by Northern blot and immunocytochemical studies. 8-Bromo-cGMP (cGMP) inhibited the thapsigargin-induced Ca^{2+} entry in a concentration-dependent manner in 7721 cells. The cGMP-induced inhibition was abolished by an inhibitor of protein kinase G, KT5823 (1 μM). However, expression of HAb18G/CD147 in T7721 cells decreased the inhibitory response to cGMP. A similar concentration-dependent inhibitory effect on the Ca^{2+} entry was observed in 7721 cells in response to a NO donor, (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP). The inhibitory effect of SNAP on the thapsigargin-induced Ca^{2+} entry was significantly reduced in HAb18G/CD147-expressing T7721 cells, indicating a role for HAb18G/CD147 in NO/cGMP-regulated Ca^{2+} entry. Experiments investigating metastatic potentials demonstrated that HAb18G/CD147-expressing T7721 cells attached to the Matrigel-coated culture plates and invaded through Matrigel-coated permeable filters at the rate significantly greater than that observed in 7721 cells. Both the attachment and invasion rates could be suppressed by SNAP, and the inhibitory effect of SNAP could be reversed by NO inhibitor, N^G-nitro-L-arginine methyl ester. The sensitivity of the attachment and invasion rates to cGMP was significantly reduced in T7721 cells as compared with 7721 cells when cells were pretreated with thapsigargin. The difference in the sensitivity between the two cells could be abolished by a Ca^{2+} channel blocker, Ni^{2+} (3 mM). These results suggest that HAb18G/CD147 enhances metastatic potentials in human hepatoma cells by disrupting the regulation of store-operated Ca^{2+} entry by NO/cGMP.

HAb18G is a new hepatoma-associated antigen recently cloned by hepatoma monoclonal antibody HAb18 screening from human hepatocellular carcinoma cDNA library (1). HAb18G is abundantly expressed in human hepatoma tissues and on the cell surface of several highly metastatic hepatoma cell lines as detected by immunohistochemistry using monoclonal antibody against HAb18G (2–4). HAb18G is a highly glycosylated transmembrane protein of 60 kDa with an ectodomain consisting of two regions exhibiting the characteristics of the immunoglobulin superfamily (1). The amino acid sequence of HAb18G is identical to that of CD147, which has recently generated a great deal of interest (5). Other names for CD147 include human basigin, extracellular matrix metalloproteinase inducer (EMMPRIN),[1] and human leukocyte activation-associated M6 antigen (6–9). Homologues in other species include rat OX-47 antigen, mouse basigin or gp42, chicken HT7 molecules, and rabbit homologue (10, 11). A role for CD147 as an adhesion molecule has been proposed, and it has been reported to bind to a variety of cell types including endothelial cells and fibroblasts (5).

Our previous studies have demonstrated that HAb18G stimulates fibroblast cells to produce elevated levels of several matrix metalloproteinases (MMPs) (1), including MMP-1, MMP-2, and MMP-9, which are well known for prompting invasion of hepatoma cells (12–14). Lim *et al.* found that EMMPRIN up-regulated MMP-1 mRNA expression, which was dependent on tyrosine kinase activity (15). However, substrates or ligands for CD147 or enzymes that modify CD147 are still not known. It is not clear whether CD147 (or HAb18G, basigin, EMMPRIN, M6, etc.) is directly involved in cell adhesion or as an adhesion signal transmitting molecule or a regulator of adhesion.

The process of proliferation, invasion, and metastasis is a complex one that involves both the autonomy of the malignant cells and their interaction with the cellular and extracellular environments. The way in which tumor cells respond to cellular and extracellular stimuli is regulated through transduction of those signals and translation into cellular activities. A frequent common denominator of the different signal transduction pathways is cellular Ca^{2+} mobilization. Ca^{2+} has been shown to play a role in the regulation of proliferation, invasion, and metastatic potential (16–18). The motility and adhesion properties of several normal cell types have been shown to work through G protein-sensitive pathways, suggesting that this type of cell signaling may be involved in tumor invasion and metastasis (19–21). Extracellular matrix components, such as

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[1] The abbreviations used are: EMMPRIN, extracellular matrix metalloproteinase inducer; PKG, protein kinase G; Br, bromo; PBS, phosphate-buffered saline; NPBS, normal phosphate-buffered saline; SNAP, *S*-nitroso-*N*-acetylpenicillamine; L-NAME, N^G-nitro-L-arginine methyl ester; MMP, matrix metalloproteinase; AM, acetoxylmethyl ester.

laminin, collagen type IV, fibronectin, and thrombospondin, also have been shown to stimulate tumor cell migration (22–27). Ca^{2+} appears to play a pivotal role in extracellular matrix-induced tumor cell migration as demonstrated in the A2058 human melanoma cells stimulated with type IV collagen and fibronectin (24, 28, 29).

Ca^{2+} release from internal stores by either inositol trisphosphate (IP_3) (*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, often referred to as "store-operated" or "capacitative" Ca^{2+} entry (30). Several factors have been reported to modulate this Ca^{2+} entry, including small molecular weight G protein(s), serine/threonine phosphatase, tyrosine kinase or phosphatase, cAMP, NO, and guanosine 3′,5′-cGMP (31–34). Among these, NO and cGMP have received much attention recently. It has been proposed that NO and cGMP may play a key role in regulating Ca^{2+} entry into pancreas acinar cells (35, 36). It was shown that the Ca^{2+} content of the internal stores apparently regulated NOS activity and subsequently NO and cGMP, which had a biphasic effect on restoring Ca^{2+} entry after it had been blocked by NOS/guanylate cyclase inhibitors. These experiments indicated that store-operated Ca^{2+} entry was regulated tightly by $[cGMP]_i$. Negative regulation of store-operated Ca^{2+} entry by cGMP via a protein kinase G-dependent mechanism has also been observed recently in other cell types including endothelial cells (34). Thus, cGMP appears to play an absolutely crucial role in regulating store-operated Ca^{2+} entry. However, very little is known about the physiological importance of store-operated Ca^{2+} entry in tumor cells, and virtually nothing is known about the intracellular regulation of store-operated Ca^{2+} entry involved in the process of cancer metastasis, although NO has been implicated in cancer metastasis through Ca^{2+}-dependent mechanisms involving different MMPs (37).

We have recently demonstrated the presence of a NO-cGMP-regulated store-operated Ca^{2+} entry pathway in human hepatoma 7721 cells (38). In contrast to other highly metastatic hepatoma cells, 7721 cells have been shown to have lower metastatic activities and do not express HAb18G (2–4). It appears that the expression of HAb18G is associated with high metastatic potencials of human hepatoma cells. The present study further explored the involvement of HAb18G/CD147 in regulating the Ca^{2+} entry signaling pathway and metastatic potentials by stably transfecting HAb18G into 7721 cells. We found that the expression of HAb18G/CD147 reduced the sensitivity of the store-operated Ca^{2+} entry to NO/cGMP and enhanced the metastatic potentials of human hepatoma cells.

EXPERIMENTAL PROCEDURES

Materials—8-Br-cGMP, (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP), N^G-nitro-L-arginine methyl ester (L-NAME) and thapsigargin were obtained from Calbiochem (La Jolla, CA). RPMI 1640, fetal bovine serum, Geneticin (G418 sulfate), and Lipofectin transfection reagents were purchased from Life Technologies, Inc. Fura2/AM was obtained from Molecular Probes, Inc. (Eugene, OR). TRIZOL for total RNA isolation was from Life Technologies, Inc., and ExpressHyb™ hybridization solution for Northern blot was from CLONTECH Laboratories, Inc. (Palo Alto, CA). All restriction endonucleases were purchased from Promega Corporation (Madison, WI), and [^{32}P]dCTP was from Amersham Pharmacia Biotech. Fluorescein isothiocyanate-conjugated rat anti-mouse IgG and Matrigel were obtained from Becton Dickinson Labware (Bedford, MA). Other reagents were from Sigma.

Cell Culture—The human 7721 hepatoma cells (obtained from the Institute of Cell Biology, Academic Sinica) were cultured with RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2% L-glutamin at 37 °C in a humidified atmosphere of 5% CO_2.

Transfection Experiment and Northern Blot Analysis of the Expression—The expression vector pcDNA3-HAb18G was made in our laboratory. The human 7721 hepatoma cells were seeded onto 35-mm plates at a density of 1×10^5/plate. After cells were grown to 60–80% confluency, transfection was performed using Lipofectin transfection reagent according to the manufacturer's instructions. Briefly 2 μg of expression plasmid DNA and 15 μl of Lipofectin transfection reagent in RPMI 1640 serum and antibiotic-free medium were incubated together for 45 min at room temperature to allow liposome-DNA complexes to form. The cells were washed and incubated with serum and antibiotic-free medium after the addition of liposome-DNA complexes for 8 h. The transfection solution was removed, and fresh medium with serum was added and incubated for another 48 h. Medium supplemented with G418 (400 μg/ml) was added to the cells. The G418 selection medium was changed every 2–3 days, until Lipofectin-treated control cells died. After a 3-week culture in selection medium, the cells were cloned by clonal dilution.

Total RNA was isolated from cells using trizol Reagent, and 20 μg was resolved on 1% agarose/formaldehyde gels and transferred to nitrocellulose membranes. Hybridization was done using HAb18G cDNA fragment as the probe in ExpressHyb™ hybridization solution according to the manufacturer's instructions. The membranes were exposed to autoradiographic film for up to 2 days.

Immunocytochemistry—Human hepatoma monoclonal antibody HAb18 was produced and characterized in our laboratory (39). 7721 and HAb18G-transfected T7721 cells were seeded onto sterile glass coverslips in 15-cm cell culture dishes. Following attachment, coverslips were washed in PBS and then held in 100% methanol at −20 °C for 30 min. Methanol was removed, and the cells were immunostained. All further processing was carried out at room temperature. For immunostaining, the cells were rehydrated in PBS for 5 min and then were held in blocking solution (1% bovine serum albumin in PBS) for 1 h. The coverslips were rinsed briefly in PBS, incubated in a solution of the primary antibody HAb18 (1 μg/ml in blocking solution) for 1 h, and subjected to four 5-min PBS washes. The cells were then incubated with rat anti-mouse IgG-fluorescein isothiocyanate at 3 μg/ml in blocking solution for 1 h. Following five 5-min washes in PBS, coverslips were mounted in glycerol:water solution (v/v, 1:1) on glass slides. Fluorescence microscopy was taken on.

Measurement of Intracellular Free Calcium Concentration—$[Ca^{2+}]_i$ was measured using Fura2/AM. The cells were loaded with the dye by incubation with 5 μM Fura2/AM for 45 min in dark at 37 °C in normal PBS (NPBS) containing 2 mM $CaCl_2$, pH 7.4. The cells were then washed and resuspended in NPBS. To start the experiment, the cells were pretreated with 4 μM thapsigargin for 20 min. Then the cells were washed with and maintained briefly in PBS containing no Ca^{2+} and 2 mM EGTA. Unless stated otherwise, the cells were pretreated with or without chemicals (*i.e.* 8-Br-cGMP or $NiCl_2$) for 5 min. The fluorescent signal was monitored and recorded by an LS-50B luminescent spectrometer (Perkin Elmer). The excitation light at 340 and 380 nm was provided by a 150 W Xenon arc lamp (Perkin Elmer) and a filter wheel (Perkin Elmer) containing 340- and 380-nm interference filters (Perkin Elmer). The emitted fluorescence at 510 nm was collected by a photomultiplier tube and recorded.

Cell Adhesion Assay—The wells of the 96-well culture plate were coated with Matrigel at a concentration of 5 μg/ml and incubated at 4 °C overnight. The coated wells were blocked with PBS containing 2% bovine serum albumin for 30 min and then washed with PBS. Cells suspension in serum-free medium containing 0.1% bovine serum albumin was added to the wells (2×10^4/well) and incubated at 37 °C, 5% CO_2 for 30–60 min with or without test agents (*i.e.* 8-Br-cGMP, SNAP, or L-NAME). After removing medium and nonattached 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. 0.1 ml of 5% SDS with 50% ethanol was added for 20 min, and then the plate was read at 540 nm.

Cell Migration Assay—The chemotactic cell migration assay was performed using 24-well transwell units (COSTAR Corning Inc.) with an 8-μm pore size polycarbonate filter according to the method described by Mensing *et al.* (40). Each lower compartment of the transwell contained 600 μl of 0.5% fetal bovine serum as chemoattractant or 0.5% bovine serum albumin as negative control in RPMI 1640. Cells (1×10^5) in 0.1 ml of RPMI 1640 containing 0.1% bovine serum albumin were added into the upper compartment of the transwell unit and incubated for 6 h at 37 °C in a humidified atmosphere containing 5% CO_2. After removing the cells from the upper surface of the membrane with a swap, cell numbers on the underside were determined using colorimetric crystal violet assay.

Invasion Assay—The procedure for the chemotactic cell invasion test was the same as in the chemotactic cell migration assay, except that the upper side of polycarbonate filter was coated with Matrigel (5 μg/ml in

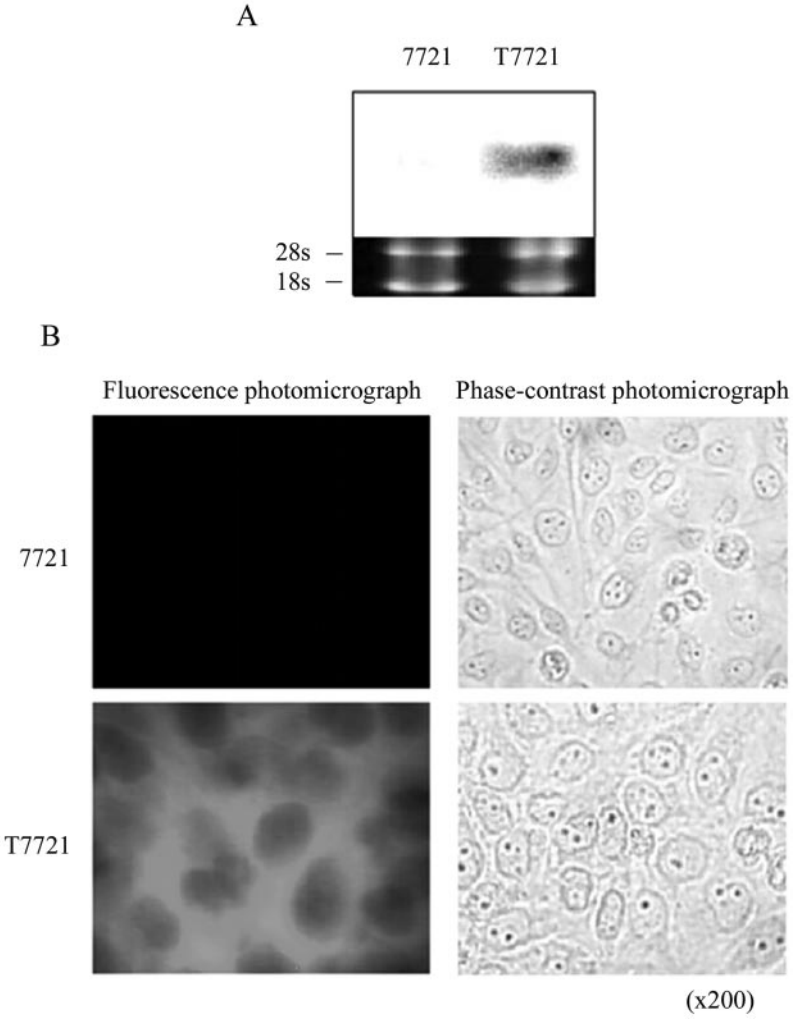

FIG. 1. **Stable transfection of HAb18G into human hepatoma cells.** *A*, Northern blot analysis of HAb18G mRNA lever in 7721 and T7721 cells. Total RNA was isolated and fractionated on a 1% agarose gel containing 0.22 M formaldehyde and transferred by capillary blotting and cross-linked to a nitrocellulose membrane. The blot was then probed with ^{32}P-labeled HAb18G cDNA. The results demonstrate expression of HAb18G mRNA in a transfected T7721 cell line. *B*, immunocytochemical demonstration of expression of HAb18G. 7721 and T7721 cells were fixed and stained with monoclonal antibody HAb18, followed by fluorescein isothiocyanate-conjugated rat anti-mouse IgG antibody. T7721 cells were positive by staining. *Left panels*, fluorescence photomicrograph; *right panels*, phase contrast photomicrograph.

cold medium) to form a continuous thin layer. Prior to the addition of cell suspension, the dried layer of Matrigel matrix was rehydrated with medium without fetal bovine serum for 2 h at room temperature, and the incubating time varied with a maximum of 72 h. Agents such as 8-Br-cGMP or SNAP were added in the upper compartment after the addition of cell suspension. When thapsigargin treatment assay was carried out, the cells were incubated with 4 μM thapsigargin for 20 min prior to addition of cell suspension. The cells remaining in the upper compartment were completely removed with gently swabbing. The number of cells invaded through the filter into the lower compartment was determined using colorimetric crystal violet assay.

RESULTS

Stable Transfection of HAb18G into Human Hepatoma Cells—We have demonstrated in previous studies that store-operated Ca^{2+} entry in human 7721 hepatoma cells was negatively regulated by NO/cGMP (38). To examine the effect of HAb18G, we transfected HAb18G cDNA into human 7721 hepatoma cells and cloned after G418 selection in the present study. A hepatoma cell line stably expressing HAb18G was obtained and named T7721. The expression of HAb18G in T7721 but not 7721 cells was demonstrated by Northern blot (Fig. 1*A*) and immunocytochemistry (Fig. 1*B*).

Thapsigargin-induced Store-operated Ca^{2+} Entry—Thapsigargin, a potent inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, was used to deplete intracellular Ca^{2+} stores and induce Ca^{2+} entry from extracellular space. After treatment with 4 μM thapsigargin in Ca^{2+}-free and 2 mM EGTA-containing medium for 20 min, the addition of 2 mM $CaCl_2$ induced a rise in $[Ca^{2+}]_i$ because of Ca^{2+} entry (Fig. 2). The thapsigargin-induced elevation of $[Ca^{2+}]_i$ was completely blocked by Ni^{2+} (3 mM), a potent blocker of Ca^{2+} entry that competes for Ca^{2+}-binding sites (41), in both 7721 cells and T7721 cells (Fig. 2), confirming the presence of the store-operated Ca^{2+} entry in both cell types.

Effect of HAb18G on NO/cGMP-sensitive Store-operated Ca^{2+} Entry—cGMP, an activator of PKG, has been shown to regulate the store-operated Ca^{2+} entry (34, 36). The present study examined the effect of 8-Br-cGMP on the store-operated Ca^{2+} entry in both human 7721 and T7721 hepatoma cells. Fig. 3 shows that application of 8-Br-cGMP reduced the thapsigargin-induced $[Ca^{2+}]_i$ rise in 7721 cells in a concentration-dependent manner. 2 mM 8-Br-cGMP almost completely inhibited the Ca^{2+} entry with 96.9 ± 1.9% inhibition (Fig. 3, *A* and *C*). The cGMP-induced inhibition was reversed by a specific PKG inhibitor, KT5823 (1 μM) (Fig. 4). However, the inhibitory effect of 8-Br-cGMP on the thapsigargin-induced $[Ca^{2+}]_i$ rise was significantly attenuated in HAb18G-expressing T7721 cells with only 25.3 ± 13.1% inhibition observed at 2 mM 8-Br-cGMP (Fig. 3, *B* and *C*).

NO is an important intracellular signal molecule that activates soluble guanylye cyclase to synthesize cGMP (42). The present study was carried out to investigate the involvement of NO in regulation of the store-operated Ca^{2+} entry using a NO donor, SNAP, to trigger production of endogenous cGMP. Similar to the effect of 8-Br-cGMP, SNAP inhibited the thapsigargin-indued Ca^{2+} influx in a concentration-dependent manner (Fig. 5), and the inhibitory response in 7721 was significantly greater than that observed in HAb18G-expressing T7721 cells,

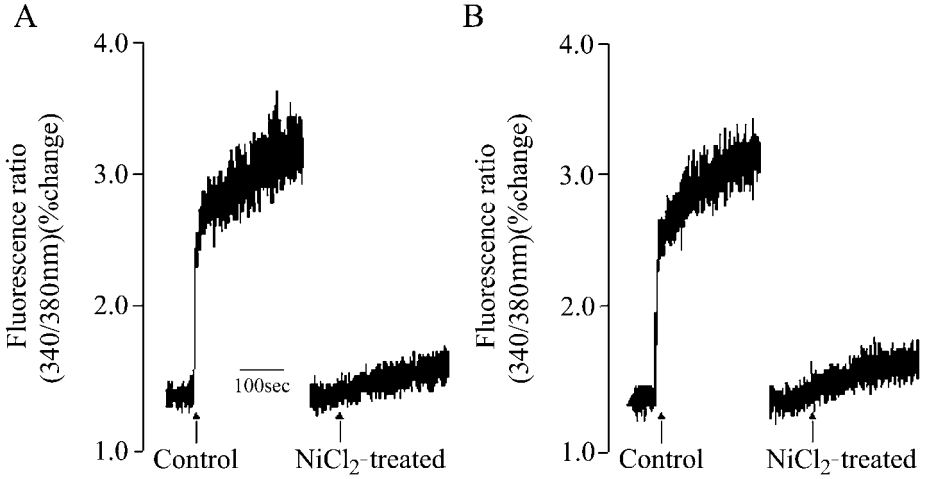

FIG. 2. **Effect of Ni^{2+} on thapsigargin-induced rise in $[Ca^{2+}]_i$.** $[Ca^{2+}]_i$ was monitored in a Fura2/AM-loaded human hepatoma cell line. The cells were incubated in NPBS with 4 μM thapsigargin for 20 min and then resuspended in calcium-free PBS. Ni^{2+} was introduced 5 min prior to the measurement. At the time indicated by the *arrow*, the media were changed to the respective media containing 2 mM $CaCl_2$ without EGTA. *A*, 7721 cells. *B*, T7721 cells.

48.3 ± 12.5% as compared with 19.8 ± 4.1% inhibition observed at 100 μM SNAP, respectively ($p < 0.01$; Fig. 5).

Effect of HAb18G on Metastatic Processes—HAb18G/CD147 is a potential adhesion molecule that has been shown to stimulate expression and release of MMPs through some kinase signaling pathways (1, 15, 43). It is now widely acknowledged that some MMPs (such as MMP-2 and MMP-9) are regulated by NO-cGMP-Ca^{2+} signaling pathways (37, 44, 45). The present study tested the involvement of HAb18G and NO in regulating metastatic potentials of the human hepatoma cells by examining cell adhesion, migration, and invasion processes *in vitro*.

We used Matrigel-coated plates and chambers as models for investigation of possible signaling pathways involved in extracellular matrix-induced metastatic processes. Matrigel is a solubilized basement membrane preparation containing almost all of the extracellular matrix components (46). The following studies were carried out with Matrigel as adhesion substratum. Fig. 6*A* shows that after 30 min of incubation, a significant increase in the amount of cells attached to the Matrigel-coated plates, expressed as fractions of total seeded cells, was observed in HAb18G/CD147-expressing T7721 cells (53.4 ± 1.8%) as compared with 7721 cells (34.6 ± 0.6%, $p < 0.001$).

We used Transwell chambers to investigate cell migration. The ability of HAb18G/CD147-expressing T7721 cells to migrate from one compartment to the other in the Transwell chambers (without Matrigel) was significantly greater than that of 7721cells, 36.4 ± 0.6% as compared with 19.8 ± 1.1% after 36 h, respectively ($p < 0.001$; Fig. 6*B*)

The Transwell chambers were also coated with Matrigel to assay for the ability of cells to invade through the reconstructed basement membrane. As depicted in Fig. 7, at various time intervals, the invasive potential, measured as fractions of total seeded cells invaded through the membrane, of HAb18G/CD147-expressing T7721 cells was significantly greater than that observed in 7721 cells ($p < 0.05$).

Involvement of NO/cGMP in Metastatic Processes—We investigated the possible involvement of NO/cGMP in cell adhesion and invasion processes. Pretreatment of SNAP (200 μM) for 30 min significantly reduces the fraction of attached cells in both 7721 and T7721 cells by 34.6 ± 1.2 and 34.4 ± 5.0%, respectively ($p > 0.05$), and L-NAME (100 μM), a NOS blocker, reversed the effect of SNAP (Fig. 8). Invasion assay also showed that the fraction of cells invaded through Matrigel-coated filter was decreased by treatment with 8-Br-cGMP (2 mM) in both 7721 (31.1 ± 8.2%) and T7721 (17.6 ± 2.1%) ($p < 0.05$). These results indicated that NO/cGMP may be involved in the metastatic processes of hepatoma cells.

The Involvement of NO/cGMP-sensitive Ca^{2+} Entry in Metastatic Processes—As shown above, the store-operated Ca^{2+} entry was negatively regulated by NO/cGMP, and the sensitivity of the store-operated Ca^{2+} entry in T7721 cells to NO/cGMP was significantly reduced as compared with that in 7721 cells. However, although both cell attachment and invasive ability was significantly inhibited by either NO or cGMP, there seemed to be no significant difference between the two cell types in the sensitivity of their metastatic potentials to NO/cGMP treatment. (The inhibition rates on invasion were 13.0 ± 1.7 and 11.0 ± 1.4% in 7721 and T7721, respectively; $p > 0.05$.) Further experiments were performed using thapsigargin to elicit store-operated Ca^{2+} entry. In the presence of thapsigargin (4 μM), the cGMP-induced inhibition of cell invasion observed in 7721 cells was significantly greater than that observed in HAb18G-expressing T7721 cells, 26.2 ± 3.1 and 16.7 ± 3.1%, respectively, $p < 0.05$. This was consistent with the earlier Ca^{2+} measurements showing greater sensitivity of the thapsigargin-induced Ca^{2+} entry in 7721 cells to NO/cGMP as compared with T7721 cells.

The possible involvement of the NO/cGMP-sensitive Ca^{2+} entry in cell attachment and invasion was further demonstrated using Ni^{2+}. After treatment with Ni^{2+} (3 mM), the fraction of cells attached was 58.7 ± 2.6 and 60.9 ± 5.0% in 7721 and T7721 cells, respectively ($p > 0.05$). Similarly, Ni^{2+} treatment resulted in 14.4 ± 1.0 and 13.8 ± 1.1% cells invaded in 7721 and T7721 cells, respectively ($p > 0.05$). Taken together, the difference in metastatic potential (*i.e.* attachment and invasion) between 7721 and T7721 cells was abolished by blocking the Ca^{2+} entry with 3 mM Ni^{2+}. Those results suggest that the HAb18G-induced difference in metastatic potentials involved Ca^{2+} entry.

DISCUSSION

Invasion and metastasis are complicated and fatal cascades in human hepatoma, but the underlying molecular mechanisms involved remain largely unknown. Previous studies have implicated a hepatoma-associated antigen, HAb18G (also named CD147 and EMMPRIN), in metastasis (1, 9, 15). The present study has further demonstrated the involvement of HAb18G in the modulation of NO/cGMP-sensitive store-operated Ca^{2+} entry and metastatic potentials of human hepatoma cells. This has been made possible by using a cell line stably expressing HAb18G obtained by transfection of HAb18G into the human hepatoma 7721 cell line, which has been shown to possess a low metastatic rate (47) and a NO/cGMP-sensitive store-operated Ca^{2+} entry pathway (38).

The present results have clearly demonstrated that the ex-

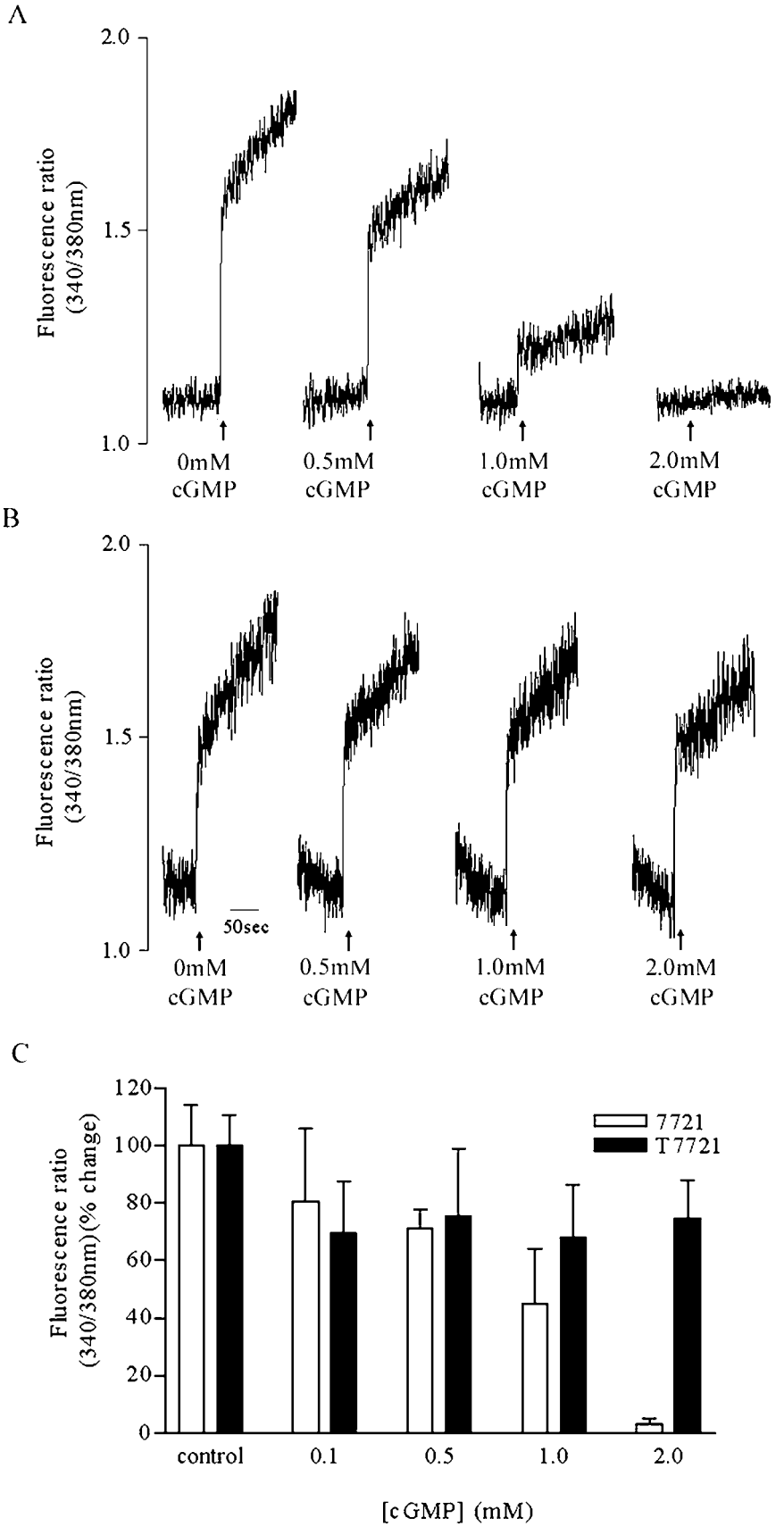

FIG. 3. **A dose-dependent inhibition effect of 8-Br-cGMP on thapsigargin-induced Ca^{2+} influx in hepatoma cells.** The cells were incubated in NPBS with 4 μM thapsigargin for 20 min. 8-Br-cGMP at the concentrations indicated below the record was introduced 5 min prior to the measurement. At the time indicated by the *arrow*, 2 mM $CaCl_2$ was added into the medium. *A*, 7721 cells. *B*, T7721 cells. *C*, concentration-dependent inhibition by 8-Br-cGMP on thapsigargin-induced Ca^{2+} entry in hepatoma cells (7721 and T7721 cells). The values are the percentages of change from base line, and they are expressed as the means ± S.E. (n = 4–6).

pression of HAb18G attenuates the sensitivity of store-operated Ca^{2+} entry to NO/cGMP in the hepatoma cells. Although the thapsigargin-induced store-operated Ca^{2+} entry appears to be the same in both 7721 and HAb18G-expressing T7721 cells, as evidenced by the magnitude of the Ca^{2+} entry and its blockage by Ni^{2+}, the sensitivity of the Ca^{2+} entry to cGMP or the NO donor, SNAP, was significantly different in the two cell types. cGMP or SNAP inhibits the thapsigargin-induced Ca^{2+}

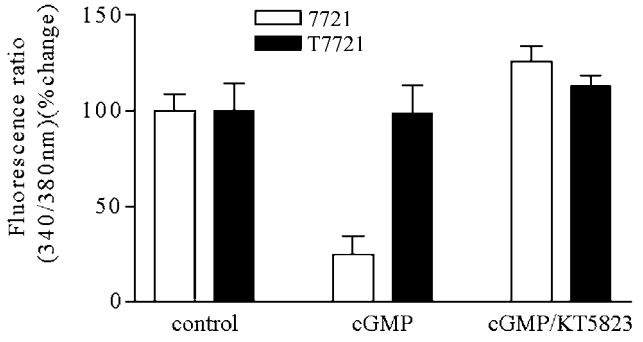

FIG. 4. **Effects of PKG inhibitor on thapsigargin-induced Ca^{2+} influx.** The Ca^{2+} entry fluorescence ratios shown were obtained in cells without chemical treatment (*control*) or treated with 2 mM 8-Br-cGMP (*cGMP*) or both cGMP and 1 μM KT5823 (*cGMP/KT5823*). The cells were incubated in NPBS with 4 μM thapsigargin for 20 min. 2 mM 8-Br-cGMP or plus 1 μM KT5823 was introduced 5 min prior to the measurement. 1 mM $CaCl_2$ was introduced to the medium to obtain the fluorescent signal of Ca^{2+} entry. The values are the means ± S.E. (n = 4–6).

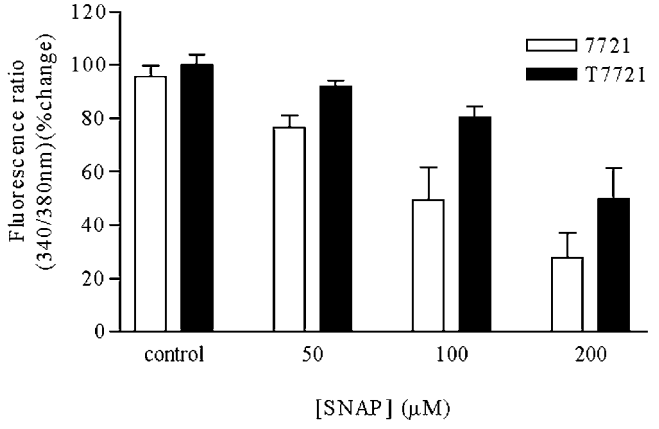

FIG. 5. **Concentration-dependent inhibition by SNAP on the thapsigargin-induced Ca^{2+} entry in hepatoma cells.** Intracellular Ca^{2+} concentration was presented by fluorescence ratio of Fura2. The cells were incubated in NPBS with 4 μM thapsigargin for 20 min and resuspended in calcium-free PBS. 2 mM $CaCl_2$ was introduced to the medium to obtain the fluorescent signal of Ca^{2+} entry. The values are the means ± S.E. (n = 4–6).

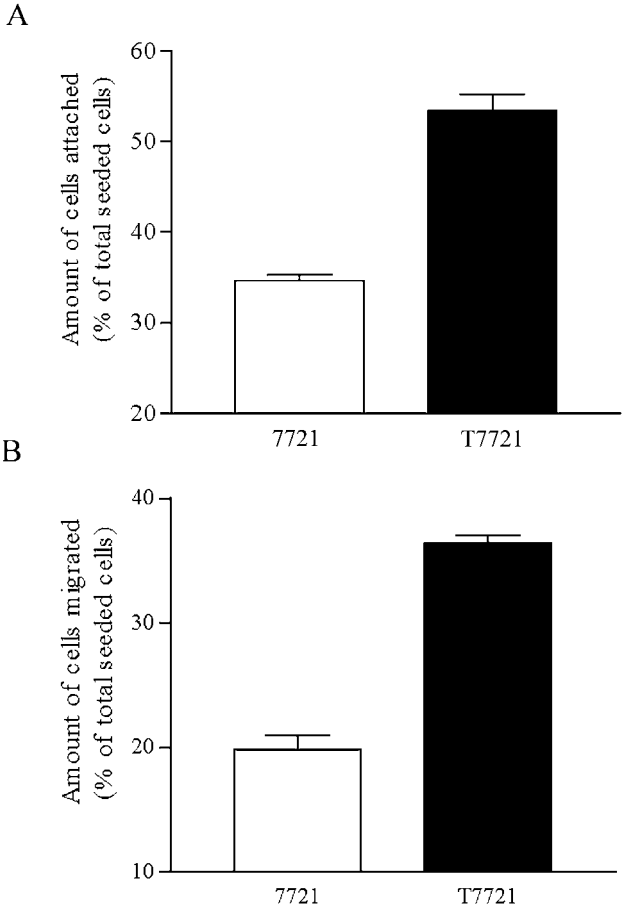

FIG. 6. **Adhesion and migration potentials of human 7721 hepatoma cells and HAb18G-transfected T7721 cells to Matrigel.** *A*, adhesion assay. The cells were suspended in serum-free medium and seeded into the Matrigel (5 μg/ml)-coated wells. After the incubation for 30 min at 37 °C, the percentage of adhered cells was determined by using the colorimetric crystal violet assay. The values are the means ± S.E. (n = 6–8). *B*, migration assay. The amount of migrated cells was determined in Transwell chambers as described under "Experimental Procedures." The values were presented as the means ± S.E. (n = 3–4).

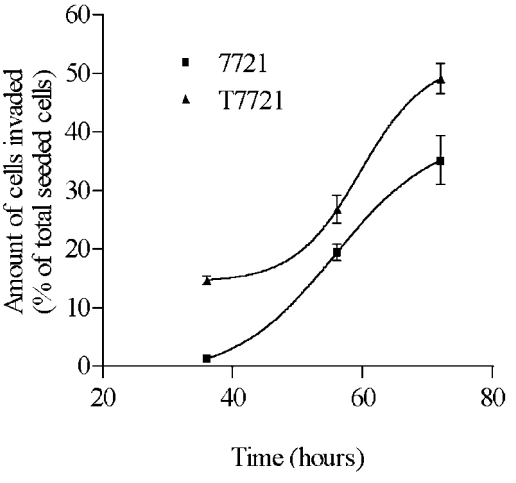

FIG. 7. **The time course change of the invasive potential of human 7721 hepatoma cells and HAb18G-transfected T7721 cells (36–72 h).** The invasive potential was evaluated in Transwell chambers as described under "Experimental Procedures." The values were presented as the means ± S.E. (n = 3–4).

entry in a concentration-dependent manner in 7721 cells, and this effect of cGMP can be reversed by highly specific PKG inhibitor, indicating a negative regulatory mechanism involving NO/cGMP via PKG-dependent pathway. The sensitivity of the thapsigargin-induced Ca^{2+} entry to either cGMP or SNAP was greatly attenuated in the HAb18G-expressing T7721 cells, suggesting that the negative regulation of the Ca^{2+} entry by NO/cGMP could be opposed by the expression of HAb18G.

It is well known that Ca^{2+} plays a role in the regulation of proliferation, invasion, and metastatic potentials (17, 37, 48–50). However, very little is known about the significance of the store-operated Ca^{2+} entry and its regulation in the process of cancer metastasis. The present study has demonstrated the possible involvement of the NO/cGMP-sensitive store-operated Ca^{2+} entry and a possible role of HAb18G in metastatic processes in human hepatoma cells. The present results obtained from assays for cell adhesion, migration, and invasion processes involved in metastasis indicated a greater metastatic potential of the HAb18G-expressing T7721 cells as compared with that of 7721 cells. This was evidenced by the observation that for a given period of time, the fraction of cells attached to or invaded through Matrigel substrate or migrated from one compartment to the other across Transwell filter in the HAb18G-expressing T7721 cells was significantly greater than that in 7721 cells. The fact that cell attachment and invasion could be greatly inhibited by Ni^{2+} suggests that Ca^{2+} entry

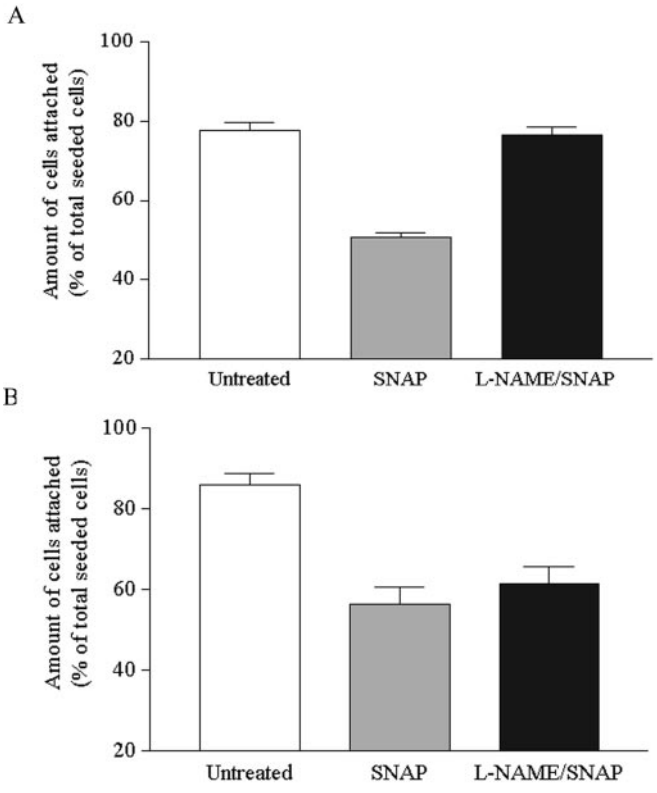

FIG. 8. **Effects of SNAP and L-NAME on cell adhesion to Matrigel.** The cells were suspended in serum-free medium supplemented with 200 μM SNAP or together with 200 μM L-NAME and seeded into the Matrigel (5 μg/ml)-coated wells. After incubation for 30 min at 37 °C, the percentage of adhered cells was determined using colorimetric crystal violet assay. *A*, 7721 cells. *B*, T7721 cells. The values are the means ± S.E. (n = 6–8).

plays a role in these processes. Furthermore, the observed difference in the attachment and invasive ability between the two cell types was also abolished by Ni^{2+} indicates that the greater metastatic potential induced by HAb18G expression may be through a mechanism involving Ca^{2+} entry.

One of the interesting observations is that cell attachment and invasion in both cell types could be inhibited by either cGMP or SNAP, whose effect could be reversed by a NO synthase inhibitor, suggesting the involvement of NO/cGMP in the metastatic processes. However, there was no significant difference between the two cell types in the sensitivity of their metastatic potentials to cGMP or SNAP treatment. This would first tend to suggest that the effect of HAb18G expression exerted directly or indirectly on the metastatic potentials did not involve NO/cGMP. However, when cells were pretreated with thapsigargin to empty the intracellular Ca^{2+} store, which is expected to elicit store-operated Ca^{2+} entry, a significantly greater inhibition of cell invasion by cGMP in 7721 cells was then observed as compared with that in HAb18G-expressing T7721 cells. These results suggest that metastatic potentials in human hepatoma cells depend on the Ca^{2+} homeostasis in the cells and that NO/cGMP and HAb18G may play opposing roles in modulating the cellular Ca^{2+} homeostatic states, especially the store-operated Ca^{2+} entry. The abolishment of observed differences between the two cell types in their metastatic potentials by Ni^{2+} is consistent with the notion that Ca^{2+} entry, which is most likely to be NO/cGMP-sensitive, is involved in the mechanism by which HAb18G influences the metastatic potentials of the hepatoma cells. It should be noted that the present results do not exclude the possible involvement of HAb18G in inducing Ca^{2+} release from the intracellular store, thus contributing to the metastatic potentials of the hepatoma cells. The fact that a significant difference in the cGMP-induced inhibitory effect on cell invasion between 7721 and T7721 cells was only observed after treatment with thapsigargin, which affects intracellular Ca^{2+} store as well as promotes Ca^{2+} entry, suggests that the intracellular Ca^{2+} store or the intracellular Ca^{2+} homeostatic state is critical for the determination of HAb18G-dependent and NO/cGMP-sensitive metastatic potentials. It is not clear from the present study whether HAb18G is directly involved in modulating the intracellular Ca^{2+} homeostatic state. What is clear, however, from the present results obtained from both the Ca^{2+} measurements and the metastatic potential assays is that the negative regulation of Ca^{2+} entry by NO/cGMP can be inhibited by HAb18G expression, which appears to influence the metastatic potentials of the hepatoma cells.

The present finding is consistent with previous results from various laboratories demonstrating a role for HAb18G/CD147 in cancer metastasis (1, 51, 52). The immediate question followed is how HAb18G/CD147 affects the metastatic potentials of tumor cells via NO/cGMP-sensitive Ca^{2+} entry. Many reports have indicated that Ca^{2+} is involved in cancer metastasis by regulating expression and/or release of MMPs (37, 45, 49, 53). MMPs have been implicated in several aspects of tumor progression, including invasion through basement membrane and interstitial matrices, angiogenesis, and tumor cell growth (12–14). The release of MMPs appears to be regulated by intracellular Ca^{2+}, although it is still unresolved whether intracellular Ca^{2+} store or Ca^{2+} entry plays a major role (37, 54, 55). Tumor-derived NO has also been reported to promote the invasiveness of certain human and animal tumors, and the increased invasiveness resulted from alterations in the balance in the production of MMPs and their natural inhibitors (tissue inhibitors of metalloproteinase) (56). Together with our previous observation that HAb18G stimulates production of several MMPs from fibroblasts (1), the present results suggest a possible link between HAb18G and metastatic potentials through NO/cGMP and Ca^{2+} entry and MMPs. Like in many other cells, it appears that the Ca^{2+} entry in the hepatoma cells is normally safeguarded by NO/cGMP. However, the expression of HAb18G/CD147 may disrupt the Ca^{2+} entry regulation by NO/cGMP, thus allowing greater Ca^{2+} influx into the cells, leading to greater production and/or release of MMPs and, therefore, enhanced metastatic potentials. This may explain why HAb18G/CD147 is abundantly expressed in several highly metastatic cancer cells (2–4, 57, 58).

However, it remains to be elucidated how the expression of HAb18G/CD147 leads to attenuation of the response of store-operated Ca^{2+} entry to NO/cGMP and thus enhanced metastatic potentials. It is possible that HAb18G/CD147 may up-regulate or down-regulate the expression of cellular factors that are related to the NO/cGMP-sensitive pathway. For example, down-regulation of PKG may render the store-operated Ca^{2+} entry insensitive to NO/cGMP. On the other hand, overexpression of Ca^{2+} channels that are responsible for store-operated Ca^{2+} entry may also reduce the effectiveness of NO/cGMP to control the Ca^{2+} entry, considering that more NO/cGMP would be required to shut down these overexpressed Ca^{2+} channels. The investigation of these possibilities is currently underway in our laboratory.

In summary, the present studies have provided evidence indicating that store-operated Ca^{2+} entry in human hepatoma cells is negatively regulated by NO/cGMP via PKG-dependent pathway and that this regulation is opposed by the expression of HAb18G/CD147. The present results have also suggested that the expression of HAb18G/CD147 may enhance the met-

astatic potentials in human hepatoma cells by disrupting the regulation of the Ca^{2+} entry by NO/cGMP.

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