HAb18G/CD147 is involved in TGF-β-induced epithelial-mesenchymal transition and hepatocellular carcinoma invasion

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Abstract

Epithelial-mesenchymal transition (EMT) induced by the transforming growth factor beta (TGF-β) is involved in hepatocarcinogenesis and hepatocellular carcinoma (HCC) metastasis. HAb18G/CD147, a member of the immunoglobulin family, plays an important role in tumor invasion and metastasis. HAb18G/CD147 promotes EMT of hepatocytes through TGF-β signaling and is transcriptionally regulated by Slug. We investigated the role of HAb18G/CD147 in TGF-β-induced EMT in HCC invasion. Two human HCC cell lines, SMMC-7721 and HepG2, were used to determine the role of HAb18G/CD147 in EMT. Upregulation of HAb18G/CD147 induced by the high doses of TGF-β1 in SMMC-7721 (5 ng/mL) and HepG2 cells (10 ng/mL) (P < 0.05). CD147 upregulation was coupled with upregulation of Snail1 and Slug. CD147 knockout significantly decreased the expression of N-cadherin and vimentin, and colony formation ability of SMMC-7721 cells. TGF-β1 enhanced the migration capacity of SMMC-7721 cells, which was markedly attenuated by CD147 knockdown. Thus, HAb18G/CD147 is involved in TGF-β-induced EMT and HCC invasion.

Keywords: CD147; epithelial-mesenchymal transition; hepatocellular carcinoma; TGF-β

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with an annual death rate of about 696,000 cases, among which 80% are in the developing countries (Forner et al., 2012). Invasion and migration are two central processes of HCC that affect the prognosis of the disease and the survival of patient. The 5-year overall survival rate of individuals with HCC is less than 10%.

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine involved in activities, such as differentiation, growth, apoptosis, inflammation, tissue remodeling, and wound healing (Wendt et al., 2012). TGF-β is known to show tumor stage-dependent suppressive and oncogenic properties (Matsuzaki, 2009). The factor inhibits growth of mature epithelial cells and induces apoptosis, thereby acting as a tumor suppressor, whereas during carcinogenesis, from cytostatic effects in hepatocytes to tumor-promoting responses with the development and progression of cancer (Meindl-Beinker et al., 2012).

The epithelial-mesenchymal transition (EMT) is a key developmental program that is often activated during cancer invasion and metastasis (De Craene and Berx, 2013). During EMT, stationary polarized epithelial cells are converted to motile mesenchymal-like cells, accompanied with activation of invasive phenotype and neoplastic cell behaviors, gain of mesenchymal cell markers (e.g. fibronectin, vimentin, α-smooth muscle actin, and N-cadherin), and loss of epithelial markers (e.g. E-cadherin, ZO-1, and α- and γ-catenin) (Kalluri and Weinberg, 2009).

Members of the TGF-β family of growth factors can initiate and maintain EMT in a variety of biological systems and pathophysiological context. TGF-β-derived signals can coordinate the expression and function of Snail, ZEB, and bHLH factors, and promote their interplay in EMT and malignant tumor migration (Peinado et al., 2007). Snail superfamily, the master transcription factor involved in the
EMT, is divided into the Snail and Scratch families, with three members of the Snail family having been described in vertebrates to date: Snail1, Snail2 (Slug), and Snail3 (Peinado et al., 2007). Snail1 could be implicated in the initial migratory phenotype and considered as an early marker of EMT that sometimes contributes to the induction of other factors. By contrast, Slug, Zeb1, Zeb1, and bHLH could be responsible for the maintenance of migratory cell behavior, malignancy, and other tumorigenic properties (Montserrat et al., 2011). Despite all these evidences, the molecular mechanisms of EMT in liver carcinogenesis and tumor metastasis remain unclear.

HAb18G/CD147, a highly glycosylated cell surface transmembrane protein, belongs to immunoglobulin superfamily (Li et al., 2013). CD147 has been reported to be closely related with tumor invasion, metastasis, tumor cell growth, and survival. CD147 induces tumor cell invasion by stimulating production of matrix metalloproteinases (MMPs) by fibroblasts (Weidleet al., 2010). Overexpression of CD147 in tumor cells was significantly associated with poor prognosis of liver cancer and other tumors (Zhang et al., 2007; Chen and Zhu, 2010). However, the potential hepatic oncogenic function of CD147 under inflammatory stimuli has not been yet explored. In our previous study, we found that HAb18G/CD147 is involved in TGF-β-induced EMT in normal hepatocytes, and is directly regulated by the transcription factor Slug (Wu et al., 2011). However, the role of HAb18G/CD147 in the EMT progress of cancer cells is not yet understood.

In this study, we investigated the role of HAb18G/CD147 in EMT of HCC cells under inflammatory stimuli. Our study showed that HAb18G/CD147 is upregulated during TGF-β-induced EMT in hepatocellular carcinoma cell lines and participates the process of TGF-β1-induced invasion.

Materials and methods

Cell culture

Two human HCC cell lines, SMMC-7721 and HepG2, were obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SMMC-K7721 cells (CD147 stably knocked out) was developed and preserved in our laboratory with Zinc finger nuclease technology. SMMC-7721 and SMMC-K7721 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, USA), and HepG2 cells were cultured in DMEM medium. Both types of media were supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin and streptomycin.

Western blot

Cell samples were lysed with RIPA buffer (Beyotime, Jiangsu, China). BCA Protein Assay Kit (Pierce, Rockford, USA) was employed to determine the total protein density. Equal amounts of proteins (10 μg) were subjected to 12% SDS–PAGE separation and then transferred to a polyvinylidene fluoride microporous membrane (Merck Millipore, Billerica, USA). After being blocked with 5% non-fat milk, the membrane was incubated for 1 h at room temperature in PBS buffer. Membranes were then incubated overnight at 4°C with primary antibody in blocking buffer containing 5% non-fat milk at the dilution specified by the manufacturer. Primary antibodies included HAb18 (against HAb18G/CD147, prepared by our laboratory), Snail1 (Santa Cruz, Texas, USA), Slug (Santa Cruz, USA), N-cadherin (Invitrogen, Carlsbad, USA), vimentin (Invitrogen), and GAPDH (Kangchen Bio-tech, Shanghai, China) antibodies. Second antibodies included horseradish peroxidase-conjugated goat anti-mouse IgG(H+L) and rabbit anti-goat IgG(H+L) (Pierce). The proteins were detected using the Amersham enhanced chemiluminescence system (Image Station 4000 MM Pro, XLS180, Kodak, Japan) according to the manufacturer’s instructions.

Immunofluorescence

Expression of E-cadherin, vimentin, and HAb18G/CD147 in SMMC-7721 and SMMC-K7721 cells was detected by immunofluorescence staining. Anti-E-cadherin (BD Biosciences, Franklin Lakes, USA), anti-vimentin (Invitrogen), and HAb18 antibodies were used as primary antibodies at a dilution of 1:500. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Pierce) or with Alexa 594-conjugated goat anti-mouse (Invitrogen) were used as secondary antibody. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Images were obtained with an FV1000 laser scanning confocal microscope (Nikon, Japan).

Real-time RT–PCR

RNA was extracted with the Total RNA Kit I (Omega, Riverside, USA) and reverse transcribed into cDNA using a PrimeScriptTM RT reagent kit (TaKaraBio, Otsu, Japan). Single-stranded cDNA was amplified by quantitative RT–PCR using a SYBR Premix ExTaqTM kit (TaKaraBio) on the Stratagene Mx3005P™ Real-time PCR System (Agilent Technologies, Germany). GAPDH mRNA was used to normalize RNA inputs. All primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China) and are listed in Table 1.

Colony formation assay

The cultured SMMC-7721 and SMMC-K7721 cells were plated on a 0.6% agarose base in 24-well plate (1 × 10² cells
per well) in 1 mL of RPMI-1640 containing 10% FBS and 0.3% agarose (Sigma). At day 15 after plating, colonies with >50 cells were counted. Colony formation rate was calculated as the following formula: colony formation rate = number of colonies/10^3.

**In vitro invasion assay**

Invasion assay was done using chambers with polycarbonate filters (8 μm pore size; Millipore). The upper side of a polycarbonate filter was 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 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 with crystal violet, visualized, and counted under a microscope. Transfection of small interfering RNAs was performed using Lipofectamine 2000 reagent (Invitrogen). si-CD147 (5'-GTTCCTCGT-GAGTTCTC-3') was synthesized by Shanghai GenePharma Co., Ltd. Silencer-negative control siRNA (snc-RNA) was used as negative control under the similar conditions.

**Statistical analysis**

Student’s t-test was applied for comparison of two individual data. One-way analysis of variance (ANOVA) was used to determine the significance of differences among the various groups. The P values and represented data obtained from three independent experiments, with the error bars representing mean ± standard deviation. Statistical significance was set at P < 0.05.

**Results**

**TGF-β1 induces SMMC-7721 cells to undergo EMT**

TGF-β1 is a multifunctional cytokine involved in the regulation of growth and differentiation of both normal and transformed cells. Our previous study has shown that normal liver cell lines undergo EMT after treatment of 2.5 ng/mL TGF-β1, but the same dose of TGF-β1 could not induce the similar responses in HCC cell lines (Wu et al., 2011). After stimulation with TGF-β1, we found that the morphology of SMMC-7721 cells exerted no obvious change at a low dose of TGF-β1 (2.5 ng/mL) (Figure 1A). However, after treatment with TGF-β1 more than 5 ng/mL for 48 h, SMMC-7721 cells acquired a spindle-shaped morphology. Meanwhile, we observed that TGF-β1 induced the loss of cell junctions. Several epithelial and mesenchymal markers are used for detection of EMT, such as E-cadherin, ZO-1, vimentin, and fibronectin (Kalluri et al., 2009). We performed an immunofluorescence using E-cadherin and vimentin as epithelial and mesenchymal markers, respectively. As shown in Figure 1B, the expression of E-cadherin was downregulated, and the mesenchymal marker vimentin was upregulated after stimulation of TGF-β1 (5 ng/mL) compared with that of control.

**TGF-β1 induces upregulation of HAb18G/CD147 and EMT-related transcription factors Snail1 and Slug**

We tested the effects of TGF-β1 stimulation on HAb18G/CD147 expression in SMMC-7721 and HepG2 cells. As shown in Figure 2, CD147 expression increased in response to high doses of TGF-β1 and reached a maximum at the concentration of 5 ng/mL in SMMC-7721 cells (P < 0.001). Meanwhile, CD147 expression reached a maximum at the concentration of 10 ng/mL in HepG2 cells (P < 0.05).

Snail1 and Snail2 (also known as Slug) belong to the Snail superfamily of zinc finger transcriptional repressors that participate in developmental EMT. Snail1 and Slug have now been firmly established as repressors of E-cadherin during EMT (Molina-Ortiz et al., 2012). Our previous studies have shown that the expression of HAb18G/CD147 is directly regulated by Slug during TGF-β1-induced EMT in normal hepatic cells (Wu et al., 2011). To investigate the role of the Snail family in regulation of HAb18G/CD147 in HCC cells, we evaluated mRNA levels of Snail1 and Slug under the stimulation of TGF-β1. Snail1 and Slug expression increased at each concentration of TGF-β1 in SMMC-7721 cells.

### Table 1 Sequences of real-time PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>CD147</td>
<td>5'-ACTCTCACACCCAGCCACCA-3'</td>
<td>5'-GGCTCCAATGTCAGGTCTC-3'</td>
</tr>
<tr>
<td>Snail1</td>
<td>5'-CACATCGGAAAGCACA-3'</td>
<td>5'-AGAAGTGCGGACACA-3'</td>
</tr>
<tr>
<td>Slug</td>
<td>5'-TCCTGTCAGGAGGATT-3'</td>
<td>5'-GAGGAGGTGTCAGATGGA-3'</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>5'-ACAGTGGCACCTACAAAGG-3'</td>
<td>5'-CCGAAGTGCCAGTGAATG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGATGACATCAGAAGGTTGGAAG-3'</td>
<td>5'-TCTTGGAGCCACTGTTGGCCAT-3'</td>
</tr>
</tbody>
</table>
Snail1 expression increased under high doses of TGF-β1 (10 ng/mL) in HepG2 cells \((P < 0.05)\). However, Slug expression was not increased significantly by TGF-β1 stimulation in HepG2 cells (Figure 2).

After treatment with TGF-β1 for 48 h, the protein levels of HAb18G/CD147, Snail1, and Slug were examined by western blotting in HCC cells. HAb18G/CD147 and Snail1 were upregulated in both SMMC-7721 and HepG2 cells. High doses of TGF-β1 (10 ng/mL and 20 ng/mL) significantly increased the level of Slug in SMMC-7721 cells, whereas Slug had remained unchanged in HepG2 cells (Figure 3).

Knockout of CD147 gene decreases expression of mesenchymal markers N-cadherin and vimentin

SMMC-K7721 was derived from SMMC-7721 by knockout of CD147 gene (Basigin) with zinc finger nucleases (Huang et al., 2014). CD147 expression was then detected by western blot analysis and immunofluorescences in both SMMC-7721 and SMMC-K7721 cell lines. As shown in Figure 4A and B, the expression of CD147 in SMMC-K7721 cells decreased to undetectable levels. To determine the role of HAb18G/CD147 in EMT, we tested two mesenchymal markers, N-cadherin and vimentin, in SMMC-7721 and SMMC-K7721 cells. N-cadherin and vimentin expression were markedly reduced in SMMC-K7721 cells compared with that in SMMC-7721 cells (Figure 4A and C), demonstrating the important role of CD147 in maintenance of mesenchymal phenotype of HCC cells. The expression of Snail1 and Slug had remained unchanged in SMMC-K7721 cells, indicating that CD147 did not affect Snail1 and Slug expression and probably CD147 was a downstream factor of Snail signaling in TGF-β1-induced CD147 expression. Colony formation assay was performed to investigate the biological function of CD147. The colony formation ability was weakened 2.5-fold in SMMC-K7721 cells compared with that in SMMC-7721 cells \((P < 0.01)\) (Figure 4D).

Downregulation of CD147 inhibits the TGF-β1-induced HCC cell invasion

Degradation of ECM is an important process associated with tumor invasion, in which MMPs play a critical role (Kessenbrock et al., 2010). CD147 can promote MMP production from fibroblasts and tumor cells. Invasion potential of HCC cells with different treatment was examined by transwell invasion assay to determine the role of CD147. Small interfering RNAs were designed against CD147 (si-CD147) to silence gene expression. As
shown in Figure 5A, TGF-β1 significantly enhanced the invasion potential of SMMC-7721 cells \( (P < 0.001) \), which was attenuated by si-CD147 \( (P < 0.05) \). Furthermore, si-CD147 inhibited TGF-β1-induced invasion in SMMC-7721 cells \( (P < 0.001) \). In the invasion assay, the number of cells migrating through the filter was markedly decreased in CD147-knockout SMMC-K7721 cells compared with SMMC-7721 cells (Figure 5B).

Figure 2  TGF-β1 upregulates Snail1, Slug, and CD147 expression in SMMC-7721 and HepG2 cells. CD147 mRNA level was determined in SMMC-7721 and HepG2 cells in the absence or presence of various concentrations of TGF-β1 for 48 h by real-time PCR. GAPDH was used as control. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).

Figure 3  Upregulation of Snail1, Slug, and HAb18G/CD147 by TGF-β1 stimulation. SMMC-7721 and HepG2 cells were treated with various concentrations of TGF-β1 for 48 h. Snail1, Slug, and HAb18G/CD147 protein levels were detected by western blot. GAPDH was used as control for equivalent loading. The relative amounts of proteins were quantified by scanning densitometry and presented under the corresponding blots.
In this study, we revealed that HAb18G/CD147 was upregulated during TGF-β-induced EMT in HCC cell lines. Knockout of CD147 suppressed the TGF-β-induced invasive in HCC cells.

TGF-β is considered as a crucial molecule in hepatocarcinogenesis (Wu et al., 2012). TGF-β has been found to regulate and induce transcription via transcription factors Snail and Slug by downregulating E-cadherin expression. Accumulating evidence indicates that TGF-β-mediated EMT plays a pivotal role in the dissemination of malignant hepatocytes during HCC progression (Nagaraj and Datta, 2010). In our previous study, CD147 expression by normal hepatic cell line QZG and mouse primary hepatocytes was demonstrated to be upregulated by TGF-β1 and this upregulation was directly regulated by the transcription factor Slug. We concluded that CD147 transcription was activated by Slug through direct interaction with the E-box2 located at the proximal promoter of the CD147 gene as demonstrated by reporter gene assay and chromatin immunoprecipitation. We further found that CD147 is involved in EMT in hepatocarcinogenesis (Wu et al., 2011).

In this study, HAb18G/CD147 expression by HCC cells was demonstrated to be upregulated by TGF-β1. HAb18G/CD147 expression in HCC cells was also upregulated spontaneously in a dose-dependent manner. We observed that Snail1 and Slug expression was both upregulated in SMMC-7721. However, the expression levels for CD147 and Snail1 reached their peaks at 5 ng/mL of TGF-β, and dropped down at >10 ng/mL in SMMC-7721 cells (Figures 2 and 3). The reason for this phenomenon was due to an appearance of apoptosis induced with high concentrations of TGF-β1 in a small subpopulation (data not shown). The surviving cells...
exerted mesenchymal morphology as shown in Figure 1A. A similar phenomenon was observed previously by us in a normal hepatic cell line with lower dose of TGF-β1 (Wu et al., 2011). In Figures 2 and 3, the gene expression levels in SMMC-7721 and HepG2 cells were largely different, reflecting a different response upon the stimulation of TGF-β1. As we know, SMMC-7721 is a highly malignant cell line with characteristics of poor differentiation. On the contrary, HepG2 is a well-differentiated cell line (Yin et al., 2008; Wang et al., 2011). The both well- and poorly-differentiated hepatic tumors can be ordinarily detected with histology in clinical diagnosis. The results suggest that the poorly-differentiated HCC cells are more sensitive to TGF-β1 than the well-differentiated ones on EMT phenotype.

In addition, TGF-β1 significantly enhanced the invasion potential of SMMC-7721 cells, which was attenuated by si-CD147. A large number of studies have shown that CD147 is overexpressed in various cancer tissues, which is capable of inducing the expression of several MMPs, including MMP-1, MMP-2, MMP-3, MMP-9, and MMP-11 (Weidle et al., 2010). MMPs as modulators of tumor microenvironment play crucial roles in extracellular matrix turnover, cell growth, inflammation, angiogenesis, and tumor cell migration by both proteolytic and non-proteolytic methods.

Figure 5  Downregulation of CD147 inhibits the TGF-β1-induced HCC cell invasion. (A) SMMC-7721 cells were transfected with CD147 siRNA (si-CD147) and silencer negative control siRNA (snc-RNA). SMMC-7721 cells were followed by treatment with or without TGF-β1 for 24 h. Twenty-four hours after siRNA transfection and TGF-β1 stimulation, equal numbers of cells were added into the Matrigel-coated upper chamber. The cells migrating through the filter were stained and quantified (scale bar = 100 μm). (B) SMMC-7721 and SMMC-K7721 cells were added into the Matrigel-coated upper chamber. Twenty-four hours later, the cells migrating through the filter were stained and counted. Data were calculated from three independent experiments and presented as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001.
(Kessenbrock et al., 2010). Tumor-associated MMPs can also stimulate processes associated with EMT (Orlichenko and Radisky, 2008). For example, targeting of E-cadherin by MMP-3 or MMP-7 generates a bioactive fragment that promotes invasion and contributes to a cascade of molecular alterations leading to EMT in mammary epithelial cells (Radisky and Radisky, 2010). We have shown in other studies that CD147 promotes MMP-2 and MMP-9 upregulation in HCC cells (Xu et al., 2007). Therefore, the role of CD147-mediated EMT in HCC cells probably was completed by stimulation of MMPs.

**Conflict of interest**

None.

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**References**


