Curr. Res. in Cancer 5, 2011 (11-25)

The role of hepatoma-derived growth factor (HDGF) in cancer development and progression

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Abstract

HDGF (hepatoma-derived growth factor) is a novel growth factor that belongs to a new gene family. Although HDGF was originally identified from the conditioned medium of a hepatoma cell line, HDGF is suggested to promote the proliferation of various kinds of cell via two different mechanisms: a receptor-mediated pathway followed by the activation of MAP kinase (MAPK) signaling, and direct action through its DNA binding after nuclear translocation. HDGF is a unique factor which has multiple functional characteristics, such as anti-apoptotic activity and angiogenic activity, as well its growth stimulating activities. Recent studies have shown that HDGF is considered

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to play significant roles in the development and progression of malignant diseases through these various activities. Furthermore, the expression level of HDGF is an independent prognostic factor for the disease-free and overall survival of various kinds of malignant disease, including hepatocellular carcinoma, pancreatic cancer, cholangiocarcinoma, esophageal cancer, gastric cancer, colorectal cancer, gastrointestinal stromal tumor, and some non-gastroenterological cancers. Therefore, HDGF is suggested to be involved in the development and progression of cancer in various organs.

Introduction

Cancer development and progression consist of many complicated processes (1, 2). One major event is an unlimited cellular proliferation associated with the escape of malignant cells from apoptotic cellular death. In addition, mutual reactions between cancer cells and surrounding stromal cells are also important, and tumor angiogenesis is considered to be essential for the progression and invasion of solid tumors. Therefore, clarifying the molecular systems involved in the anti-apoptotic/ unregulated cancer cell proliferation and tumor neovascularization processes would provide clinically important knowledge for the treatment of malignant diseases.

Hepatoma-derived growth factor (HDGF) is a novel factor that was purified from the hepatoma-derived cell line, Huh-7 (3, 4). Although HDGF was originally identified as a growth factor, HDGF has been also reported to be an anti-apoptotic factor and an angiogenic factor (5, 6), suggesting that it is involved in the development and progression of cancers through several potential mechanisms, including the stimulation of the cellular proliferation, the inhibition of the apoptosis of cancer cells and neovascularization. This article describes the characteristics of this novel factor and its possible roles in cancer development and progression, mainly focusing on hepatocelluar carcinoma (HCC).

Characteristics of HDGF

Molecular characteristics and possible signal transduction of HDGF

HDGF is a 26kDa heparin-binding acidic glycoprotein consisting of 240 amino acids. This growth factor was initially identified from the conditioned media of Huh-7 hepatoma cells. In addition, four novel genes have been identified and reported as HDGF- related proteins (HRPs) (7-9). These proteins share a highly homologous N-terminal region consisting of about 100 amino acids, which is called the HATH (homologous to the amino terminus of HDGF) region. Lens epithelium-derived growth factor (LEDGF),

which was originally reported as a survival factor for the lens epithelium, contains a HATH region, and is considered to be a member of the HDGF family (10).

Although the primary sequence of the HDGF protein does not contain the N-terminal hydrophobic sequence which is characteristic of signal peptides, it is detected in the conditioned media of various types of cells. HDGF is therefore thought to be secreted through a process which differs from the classical Golgi secretion system (5, 6). Recently, Thakar et al. (11) have reported that the N-terminal 10 amino acids of HDGF are required for the secretion of this growth factor. They also showed that phosphorylation of serine 165 in the C-terminal region of HDGF has a significant role in its secretion process.

Although the mechanism(s) in the secretion of HDGF remains unclear, the exogenous administration of HDGF significantly stimulates the proliferation of cells, including fibroblasts, endothelial cells, and fetal hepatocytes, as well as malignant cells (4, 6, 12-14). Furthermore, exogenously supplied HDGF stimulates the phosphorylation of MAP kinase (MAPK) in endothelial cells and gastric epithelial cells (15, 16). These findings strongly suggest the possibility of receptor-mediated signal transduction by HDGF. Recently, part of the HATH region (amino acids 81-100) was reported to be a possible receptor-binding site (17), and we have identified the putative receptor for HDGF (Liu et al, in submission). Based on these findings, the growth promoting effects of HDGF should occur via a receptor-mediated signal transduction pathway, and are followed by the intracellular activation of MAPK.

On the other hand, HDGF has two putative nuclear localization signals (NLSs); the first NLS (NLS1) resides in the HATH region and the second NLS (NLS2) is located in the gene-specific region. HDGF can be transported to the nucleus of cells, as demonstrated by its immunohistological detection, thus suggesting that HDGF has the characteristics of a nuclear factor (18, 19). We have shown that the nuclear translocation of HDGF is essential for the mitogenic activity of HDGF-overexpressing cells. We also found that the NLS2 is especially important for the growth stimulating effects of HDGF (18).

Although it has not been fully clarified how HDGF stimulates cellular growth after nuclear translocation, recent studies have suggested functional roles of the HATH region. The HATH regions of the HDGF family contain a PWWP motif (20, 21), which was originally reported in a candidate gene, WHSC1, for Wolf-Hirschhorn syndrome. An NMR study revealed that the PWWP domain of HDGF has a

characteristic hydrophobic cavity, thus indicating that HDGF likely binds to some component of chromatin through this cavity (22). In addition, HDGF has been suggested to bind to a conserved DNA sequence in the promoter region and suppress the transcription of its target genes, and the presumed DNA binding site is thought to reside in the PWWP domain (23). These findings suggest that the PWWP motif in the HATH region serves as a DNA binding domain for the HDGF protein.

In light of these findings, we hypothesized that HDGF would have two different mechanisms of stimulating cell growth: via a receptor-mediated pathway followed by the activation of MAPK signaling, and direct action through the DNA binding after nuclear translocation. Therefore, HDGF is a unique growth factor which has dual mechanisms for stimulating cellular proliferation.

HDGF functions as both a growth stimulating factor and an angiogenic factor

Although HDGF was originally isolated from the conditioned media of cultured hepatoma-derived cells, several studies have shown that HDGF plays important roles in organ development in the fetus and tissue repair in adults, including the liver, kidneys, lungs, and gut (5, 6, 16, 24, 25). In addition, Everett et al. (12) demonstrated that HDGF is highly expressed in proliferating fetal vascular smooth muscle cells (SMCs) and endothelial cells. They also demonstrated that HDGF expression is induced in vascular SMCs proximal to abdominal aorta constriction, and in neointimal cells after endothelial injury, suggesting functional roles for HDGF in the development and tissue repair of the cardiovascular system. Therefore, HDGF is suggested to function as an angiogenic factor, as well as a growth stimulating factor.

HDGF as a growth stimulating factor

HDGF and non-transformed hepatocyte proliferation

HDGF is highly expressed in immature fetal hepatocytes, especially in the mid-gestation stage of the liver, and its expression was dramatically decreased near birth (13). HDGF expression in hepatocytes decreases with cellular maturation, thereby suggesting that HDGF expression is related to the proliferative activity of hepatocytes. Furthermore, the exogenous administration of recombinant HDGF stimulates the proliferation of cultured fetal hepatocytes, whereas a reduction of HDGF expression severely suppresses the proliferation of these cells. These findings strongly suggest that HDGF plays a significant role in the proliferation of fetal hepatocytes during liver development (13, 14).

In the normal state, the liver is a quiescent tissue and most of mature hepatocytes are out of the replicating phase. However, the liver has the capacity to regenerate in response to cell loss, such as after hepatectomy or drug-induced hepatic injury (26, 27). In both hepatectomized and CCl_4-treated livers, the expression of HDGF was induced in hepatocytes prior to the peak of DNA synthesis. These findings indicate that the HDGF expression increases in parenchymal hepatocytes before DNA synthesis in the regenerating liver, thus suggesting that HDGF is involved in the proliferation of adult hepatocytes, as well as fetal hepatocytes (28).

Involvement of HDGF in the proliferation of various types of non-transformed cells

In the fetus, HDGF is abundantly expressed not only in the liver, but also in various other tissues, including the kidneys, heart, lungs and gut. Oliver et al. purified an endothelial growth factor from the conditioned media of a rat metanephrogenic mesenchymal cell line, and demonstrated that this purified growth factor was identical to HDGF. They have reported that HDGF should have an important role on glomerular capillary formation during nephron morphogenesis (24). HDGF was also expressed abundantly in fetal cardiovascular systems, including the heart and aorta. The HDGF protein is first detected in atrial myocytes, then its expression expands to the ventricular myocytes, endothelial and ventricular outflow cells. In addition, HDGF is strongly expressed in the proliferating vascular SMCs and endothelial cells in fetus (12). Furthermore, exogenous HDGF and endogenous overexpression of HDGF stimulated the growth of vascular SMCs. These findings suggest that HDGF can regulate vascular SMC proliferation during cardiovascular development and neointimal formation in response to vascular injury.

HDGF is highly expressed in the endothelial cells of developing blood vessels in the fetal lungs (16, 29). In a bleomycin-induced lung damage model, HDGF expression is dominantly induced in the bronchial and alveolar epithelial cells, including type II alveolar cells (30), thus suggesting that HDGF is related to the development and tissue repair of the respiratory system. With regard to the proliferation of the gut system, HDGF expression is suggested to have a suppressive role in the maturation of fetal intestinal cells and to be associated with the proliferation of these cells (25).

These research results suggest that HDGF functions as a growth stimulating factor for non-transformed cells and is involved in the development of various organs and in tissue repair processes.

HDGF as an angiogenic factor

HDGF and angiogenesis

Everett et al. (12) demonstrated that HDGF is highly expressed in the fetal cardiovascular system, and is induced during the regeneration of vascular vessels. Transplanted HDGF-overexpressing NIH3T3 cells develop large tumors in nude mice, and these tumors are histologically abundant in vasculature (31). HDGF also stimulates the proliferation and migration of human pulmonary microvascular endothelial cells *in vitro*. In addition, administration of recombinant HDGF significantly promoted blood vessel formation in the chick chorioallantoic membrane assay (16). HDGF has been demonstrated to stimulate the proliferation of human umbilical vein endothelial cells, and recombinant HDGF induces vessel formation *in vitro* (31). Interestingly, the introduction of HDGF in NIH3T3 cells induces the expression of VEGF (vascular endothelial growth factor), which is regarded as the most important angiogenic factor. The overexpression of HDGF significantly upregulates the promoter activity of VEGF, thus suggesting that HDGF promotes the transcription of the VEGF gene. Indeed, VEGF is highly expressed in the tumors developed from HDGF-overexpressing NIH3T3 cells, and growth of the HDGF-overexpressing tumors was partially suppressed by treatment with an anti-VEGF neutralizing antibody (31). Furthermore, HDGF was also reported to induce VEGF in a gastric cancer cell line (32). Therefore, the angiogenic activity of HDGF appears to occur via two mechanisms; one is its direct effect on the proliferation of endothelial cells, and the other is its induction of VEGF.

The introduction of HDGF cDNA into HepG2 hepatoma cells resulted in the formation of larger tumors in comparison to the tumors developed from the control cells (33). Tumors derived from HDGF-overexpressing HepG2 cells rapidly increased in size, although their proliferative activity *in vitro* only moderately increased. In addition, HDGF-overexpressing NIH3T3 cells show only a slight transformation capacity in soft agar, while these cells develop large tumors in nude mice, thus indicating that cells expressing high levels of HDGF had a more prominent growth activity *in vivo* than that expected from the *in vitro* studies (31). Several DNA-chip analyses demonstrated that the overexpression of HDGF upregulated several genes involved in neovascularization, including PDGF-A and Tie-1 (33). Since HDGF-overexpressing tumors are rich in vasculature and several different angiogenic growth factors can be induced by HDGF, the higher growth stimulating effects of HDGF *in vivo* may result from its angiogenic activity, in addition to its growth stimulating effects on cells.

HDGF in apoptosis

Malignant cells are sometimes able to suppress or avoid apoptotic signals, and their unregulated proliferative capacity and expression of many growth factors are suggested to contribute the tumor progression through their anti-apoptotic effects (34). However, the role of HDGF in the apoptotic pathway is still controversial.

HDGF expression has been reported to decrease in radio-resistant cells, and high HDGF expression is related to the sensitivity to irradiation in esophageal cancer cells (35). During the process of TNF/cycloheximide-induced apoptosis of endothelial cells, dephosphorylation of HDGF was shown to be an essential process for the initiation of caspase-dependent apoptosis (36), and knock-down of HDGF inhibits the apoptosis in TNF/cycloheximide-treated HeLa cells (37). In contrast, recent studies have shown that HDGF was involved in the resistance to apoptosis (REF). HDGF has also been reported to be a survival factor for CNS neurons, motor neurons and olfactory epithelium (38-40). The downregulation of HDGF induces the expression and dephosphorylation of the pro-apoptotic protein Bad, and suppresses the Erk-Akt signaling of MAPK, thus leading to the activation of an apoptotic pathway (41). In colorectal cancer cells, knockdown of HDGF induced apoptosis through the mitochondrial pathway, whereas overexpression of HDGF inhibited drug-induced apoptosis, suggesting that HDGF is associated with the resistance of these cells to chemotherapy (42, 43). Furthermore, the blockage of HDGF activated both the Fas-mediated extrinsic and Bad-mediated intrinsic apoptotic pathways in hepatoma cells (41, 44). Therefore, HDGF is also thought to function as a survival factor in hepatoma cells by exerting multiple anti-apoptotic effects.

Based on these recent reports, HDGF can be regarded as an anti-apoptotic survival factor in various cancer cells, although additional studies are required to elucidate the precise roles of HDGF in apoptosis.

HDGF in cancer

HDGF in hepatocellular carcinoma

HDGF is expressed in various hepatoma cell lines, including Huh-7, HepG2, Hep3B, PLC/PLF/5, SK-Hep1, and Mahlavu (5, 6, 18, 33). In addition, the endogenous overexpression of HDGF significantly increases the proliferation and DNA synthesis in hepatoma cells (18), whereas antisense treatment targeting HDGF reduces the cellular proliferation (45). Moreover, HDGF-overexpressing HepG2 hepatoma cells developed larger tumors in a xenograft model using nude mice in comparison to the control tumors (33).

These *in vitro* and *in vivo* experimental studies strongly suggest that HDGF acts as a growth factor for hepatoma cells.

We examined the HDGF expression in the livers of two rodent HCC models. Fisher F344 rats fed with a choline-deficient amino acid (CDAA) diet develop steatohepatitis with the progression to liver fibrosis, and HCC is observed beginning after 52 weeks of age. The Fatty Liver Shionogi (FLS) mouse is an inbred mouse strain that spontaneously develops fatty changes, and at 52 weeks, 90% of male FLS mice develop liver tumors that are histologically confirmed to be hepatocellular adenoma and carcinoma (46). HDGF was expressed more highly in HCC than in the adjacent cirrhotic liver in CDAA-fed rats, and it was also expressed more strongly in HCC than in the adjacent liver with steatohepatitis in the FLS mice. Of note, the HDGF expression increased in the liver of FLS mice before the development of visible solid tumors, suggesting a growth stimulating function of HDGF during the early stage of hepatocarcinogenesis as well as during the progression of HCC (47).

In the previous reports, the protein expression of HDGF in human HCC tissue samples was evaluated by immunostaining, and the levels of the HDGF protein were found to be higher in human HCC tissues than in the adjacent tissues (47). Moreover, the expression level of HDGF is strongly associated with the prognosis of HCC after surgery, and a higher expression of HDGF was found to be related to poorer prognosis (48). In fact, three independent groups (including our group) have demonstrated that HCC patients with a higher HDGF expression level showed an earlier recurrence and an unfavorable overall survival rate compared to those with lower expression levels of HDGF (48-50), and that the HDGF expression was found to be an independent prognostic factor for the disease-free and overall survival in patients after curative resection of HCC. These findings suggest that HDGF plays a significant role in the progression of human HCC.

HDGF in pancreatic cancer

Pancreatic ductal carcinoma is one of the most fatal cancers, and shows a high proliferative and invasive activity, with a poor prognosis. HDGF is strongly expressed in pancreatic cancer cells, as well as hepatoma cells, including various pancreatic ductal carcinoma cell lines such as MIA PaCa-2, PANC-1, PL45 and KP-4 (51). We examined the HDGF expression by immunohistochemistry for 50 patients with primary ductal pancreatic carcinoma who received surgical treatment, and reported that the univariate and multivariate analyses showed nuclear HDGF expression to be an independent prognostic factor for pancreatic

ductal carcinoma after curative resection (51). Our findings suggest that HDGF expression, as evaluated by immunohistochemistry, can be used as a new prognostic factor for pancreatic cancer.

HDGF in cholangiocarcinoma

The expression levels of HDGF and VEGF were examined in patients with human hilar cholangiocarcinoma (52). HDGF and VEGF had a positive correlation, and patients with positive HDGF expression had a significantly poorer overall survival rate than those with negative HDGF expression. A multivariate analysis showed that HDGF expression was an independent prognostic factor. These findings suggested that a high expression of HDGF plays an important role in the development and progression of human hilar cholangiocarcinoma and that HDGF expression can be a valuable prognostic factor for this cancer.

HDGF in esophageal cancer

As described in the "HDGF in apoptosis" section, a previous study suggested that there is a relationship between the expression of HDGF and the radiosensitivity of esophageal cancer cells. HDGF is highly expressed in radiosensitive esophageal cancer cells, and radiotherapy is more effective in patients with a high expression of HDGF than in those with low expression (35). In addition, the induction of apoptosis in cancer cells is thought to be one of the important mechanisms involved in the anti-tumor effects of radiotherapy, and HDGF expression is suggested to be involved in the resistance to apoptotic signals (38-44). Therefore, the mechanism(s) underlying how HDGF expression is associated with the sensitivity to radiation still remains to be clarified.

Although an increased expression of HDGF leads to a higher effect of radiotherapy, HDGF expression is associated with the recurrence and prognosis of cancer in patients with esophageal cancer (53). Patients with a higher expression of HDGF had a poorer disease-free and overall survival compared with those with a lower expression. Further studies are therefore necessary to determine the functional role of HDGF in esophageal cancer.

HDGF in gastric cancer

HDGF is expressed in gastric cancer cells (15, 32). Transfection of HDGF stimulated cellular proliferation via the activation of Erk1/2 signaling, and promoted the anchorage-independent growth of gastric cancer cells (15). Furthermore, a reduction of HDGF in gastric cancer cells induced Bad-mediated apoptotic signaling, and decreased the invasive activity of the cells (32). Interestingly, HGF (hepatocyte growth factor) induced HDGF in a dose-dependent manner, and HDGF induced VEGF expression, thus suggesting that HDGF may be involved in tumor growth by means of its cooperation with these growth factors. In

patients with gastric cancer, higher expression levels of HDGF are significantly associated with higher rates of infiltrative tumor growth, as well as vascular and lymphatic invasion (54). Thus, HDGF is suggested to have a significant role in the development and progression of gastric cancer.

HDGF in colorectal cancer

HDGF has been reported to be involved in the development and progression of colorectal cancer cells (42, 43). HDGF expression is remarkably high in human colorectal cancers, especially in tumors proficient in DNA mismatch repair (25). We have documented that recombinant HDGF stimulated the proliferation of colonic HT-29 cells, whereas a polyclonal antibody against recombinant HDGF significantly suppressed their proliferation (55). Knockdown of HDGF induced apoptosis through the activation of the mitochondrial pathway (42), whereas the overexpression of HDGF resulted in the resistance of colorectal cancer cells to drug-induced apoptosis (43). These findings suggest that HDGF plays an important role in gut epithelial cell proliferation, including that of colorectal cancer cells.

HDGF in gastrointestinal stromal tumors (GIST)

The HDGF protein was detected in GIST tissues (56, 57). An immunohistochemical evaluation suggested that there is a significant relationship between HDGF expression and tumor growth. HDGF expression correlates with tumor mitosis and tumor size. A high expression of HDGF in patients with GIST was related to an early recurrence and poor prognosis, and HDGF expression was reported to be an independent prognostic factor for the disease-free and overall survival of patients after surgical resection (56). Furthermore, with regard to the surgically resected colorectal stromal tumors, it was reported that patients with high HDGF expression had shorter disease-free survival than patients with low HDGF levels, and that HDGF was an independent prognostic factor for patients with colorectal stromal tumors (57).

HDGF in malignant diseases of non-gastroenterological tissues

In addition to the gastroenterological cancers, HDGF is thought to be associated with the development and progression of various malignant diseases in non-gastroenterological tissues. HDGF has been shown to function as a mitogenic factor for lung epithelial cells both *in vitro* and *in vivo*, and previous studies have shown the high HDGF expression correlates with the aggressive biological behavior and poor clinical outcomes reported for non-small cell lung cancer (NSCLC) (5, 16, 58-60). Patients with a high expression level of HDGF show a poorer overall and disease-free survival than those with a low HDGF expression level, thus indicating that HDGF

is a significant independent prognostic factor in NSCLC (58, 59). Furthermore, HDGF has been also reported to be involved in the unfavorable clinical features of many other malignant diseases, including malignant melanoma, nasopharyngeal carcinoma, breast cancer and prostate cancer (61-63). Although HDGF was originally identified as a growth factor for hepatoma cells, HDGF also appears to have significant roles in many kinds of malignant disease, including non-gastroenterological cancers.

Conclusion

HDGF is a novel growth factor belonging to a new gene family. It is a unique molecule with multiple malignant characteristics, such as its ability to act as a growth stimulating factor, an angiogenic factor and a possible anti-apoptotic factor. Numerous studies have demonstrated that the overexpression of HDGF correlates with poor outcomes in various types of malignant diseases. HDGF is considered to play significant roles in the proliferation and survival of cancer cells and also in the induction of angiogenesis. HDGF is therefore suggested to be a potential therapeutic target for many malignant diseases.

Acknowledgements

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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DOI:10.1158/1535-7163.MCT-10-0544

Preclinical Development

Molecular Cancer Therapeutics

Sorafenib Inhibits the Hepatocyte Growth Factor–Mediated Epithelial Mesenchymal Transition in Hepatocellular Carcinoma

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Abstract

The epithelial mesenchymal transition (EMT) has emerged as a pivotal event in the development of the invasive and metastatic potentials of cancer progression. Sorafenib, a VEGFR inhibitor with activity against RAF kinase, is active against hepatocellular carcinoma (HCC); however, the possible involvement of sorafenib in the EMT remains unclear. Here, we examined the effect of sorafenib on the EMT. Hepatocyte growth factor (HGF) induced EMT-like morphologic changes and the upregulation of SNAI1 and N-cadherin expression. The downregulation of E-cadherin expression in HepG2 and Huh7 HCC cell lines shows that HGF mediates the EMT in HCC. The knockdown of SNAI1 using siRNA canceled the HGF-mediated morphologic changes and cadherin switching, indicating that SNAI1 is required for the HGF-mediated EMT in HCC. Interestingly, sorafenib and the MEK inhibitor U0126 markedly inhibited the HGF-induced morphologic changes, SNAI1 upregulation, and cadherin switching, whereas the PI3 kinase inhibitor wortmannin did not. Collectively, these findings indicate that sorafenib downregulates SNAI1 expression by inhibiting mitogen-activated protein kinase (MAPK) signaling, thereby inhibiting the EMT in HCC cells. In fact, a wound healing and migration assay revealed that sorafenib completely canceled the HGF-mediated cellular migration in HCC cells. In conclusion, we found that sorafenib exerts a potent inhibitory activity against the EMT by inhibiting MAPK signaling and SNAI1 expression in HCC. Our findings may provide a novel insight into the anti-EMT effect of tyrosine kinase inhibitors in cancer cells. *Mol Cancer Ther; 10(1); 169–77. ©2011 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third largest cause of cancer-related death in the world annually (1). Recurrence, metastasis, and the development of new primary tumors are the most common causes of mortality among patients with HCC (2). Sorafenib (Nexavar; Bayer HealthCare Pharmaceuticals Inc.) is a small molecule that inhibits the kinase activities of Raf-1 and B-Raf in addition to VEGFRs, PDGFR-β (platelet-derived growth factor receptor β), Flt-3, and c-KIT (3). Two recent randomized controlled trials reported a clinical benefit of single-agent sorafenib in extending overall survival in both Western and Asian patients with advanced unresectable HCC (4, 5). The potential action mechanisms that lead to these clinical benefits are thought to include antiangiogenic effects and sorafenib's characteristic inhibitory effect on Raf-1 and B-Raf signaling.

Meanwhile, growing evidence indicates that the epithelial mesenchymal transition (EMT), a developmental process by which epithelial cells reduce intercellular adhesions and acquire fibroblastoid properties, has important roles in the development of the invasive and metastatic potentials of cancer progression (6–8). To date, numerous clinicopathologic studies have shown positive correlations between the expressions of the transcription factors SNAI1 (snail homologue 1/SNAIL) and SNAI2 (snail homologue 2/Slug), which are key inducible factors of the EMT, and poor clinical outcomes in breast, ovary, colorectal, and lung cancer; squamous cell carcinoma; melanoma, and HCC (reviewed in ref. 6).

Generally, the activation of a wide variety of ligands including FGF (fibroblast growth factor), TGF-β–BMPs (bone morphogenetic protein), Wnt, EGF (epidermal growth factor), VEGF, and HGF (hepatocyte growth factor) and its receptor can upregulate the expression of EMT-regulating transcription factors, including SNAI1, SNAI2, ZEB1, ZEB2, and TWIST (6). Among them, HGF (also known as scattering factor) activates

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-10-0544

DOI:10.1158/1535-7163.MCT-10-0544

the Met signaling pathway, thereby increasing the invasive and metastatic potentials of the cells and allowing the survival of cancer cells in the bloodstream in the absence of anchorage (9). In addition, HGF is well known as a potent angiogenic cytokine, and Met signal activation can modify the microenvironment to facilitate cancer progression (9). Therefore, the HGF-Met signaling pathway is regarded as a promising therapeutic target, and many molecular targeted drugs are under clinical development (10). In HCC, the mRNA levels of HGF and Met receptor are markedly increased compared with those in normal liver (11). A high serum HGF concentration is associated with a poor prognosis for overall survival after hepatic resection, and the serum level of HGF represents the degree of the carcinogenic state in the livers of patients with C-viral chronic hepatitis and cirrhosis (12–14). Thus, we examined the effect of sorafenib on the HGF-Met–mediated EMT in HCC.

Materials and Methods

Reagents

Sorafenib was provided by Bayer HealthCare Pharmaceuticals Inc. U0126, wortmannin (Cell Signaling Technology), and human HGF (R&D Systems) were purchased from the indicated companies. The structures of compounds are shown in Supplementary Figure 1.

Cell culture

The human HCC cell lines HepG2 and Huh7 were maintained in Dulbecco's modified Eagle's (DMEM) medium (Sigma) supplemented with 10% FBS, penicillin, and streptomycin (Sigma) in a humidified atmosphere of 5% CO_2 at 37°C. The cell lines were obtained from the Japanese Collection of Research Bioresources and were grown in culture for less than 6 months.

Scratch assay

The method used for the scratches assay has been previously described (15). Briefly, the cells were plated onto 24-well plates and incubated in DMEM containing 10% FBS until they reached subconfluence. Scratches were introduced to the subconfluent cell monolayer, using a plastic pipette tip. The cells were then cultured with DMEM containing 10% FBS at 37°C. After 24 hours, the scratch area was photographed using a light microscope (IX71; Olympus). The wound distance between edge to edge were measured and averaged from 5 points per 1 wound area, using DP manager software (Olympus). The 2 wound areas were evaluated in an experiment and the experiment was done in triplicate.

Migration assay

The migration assays were done using the Boyden chamber methods and polycarbonate membranes with an 8-μm pore size (Chemotaxicell), as previously described (15). The membranes were coated with fibronectin on the outer side and dried for 2 hours at room temperature. The cells to be analyzed (2×10^4 cells/well) were then seeded onto the upper chambers with 200 μL of migrating medium (DMEM containing 0.5% FBS), and the upper chambers were placed into the lower chambers of 24-well culture dishes containing 600 μL of DMEM containing 10% FBS or with 10 ng/mL of HGF or with HGF and 10 μmol/L of sorafenib. After incubation for 36 hours (HepG2) and 24 hours (Huh7), the media in the upper chambers were aspirated and the nonmigrated cells on the inner sides of the membranes were removed using a cotton swab. The cells that had migrated to the outer side of the membranes were fixed with 4% paraformaldehyde for 10 minutes, stained with 0.1% Giemsa stain solution for 15 minutes, and then counted using a light microscope. Migrated cells were averaged from 5 fields per 1 chamber and 3 chambers were used on 1 experiment. The experiment was done in triplicate.

Morphologic analysis

HepG2 and Huh7 cells (2×10^4 and 1×10^4 cells/well, respectively) were seeded in 6-well tissue culture dishes. After 24 hours of incubation, the cells were stimulated with 10 ng/mL of HGF or control PBS. When the inhibitors were used, the cells were exposed to each inhibitor for 3 hours before the addition of HGF. After 48 hours, the cells were analyzed using a light microscope. The experiment was done in triplicate.

Western blot analysis

The following antibodies were used in this study: phospho-Met (Y1349), Met, phospho-AKT (S473), AKT, phospho-p44/42 mitogen-activated protein kinase (MAPK), SNAI1/Snail, E-cadherin, N-cadherin, vimentin, β-actin antibody horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology), and fibronectin (Santa Cruz Biotechnology). All the experiments were done at least in duplicate. The Western blot analysis was done as described previously (16). The data were quantified by automated densitometry using Multigauge Ver. 3.0 (Fujifilm). Densitometric data were normalized by β-actin in triplicate and the average was shown above the Western blot as a ratio of control sample.

Real-time reverse transcription PCR

The real-time reverse transcription PCR (RT-PCR) method has been previously described (17). Briefly, 1 μg of total RNA from the cultured cells was converted to cDNA using a GeneAmp RNA-PCR kit (Applied Biosystems). Real-time RT-PCR amplification was done using a Thermal Cycler Dice (Takara) in accordance with the manufacturer's instructions under the following conditions: 95°C for 6 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Glyceraldehyde 3-phosphate dehydrogenase (GAPD) was used to normalize the expression levels in the subsequent quantitative analyses. To amplify the target genes, the following primers were purchased from TaKaRa: *CDH1*, forward 5′-TTA AAC

DOI:10.1158/1535-7163.MCT-10-0544

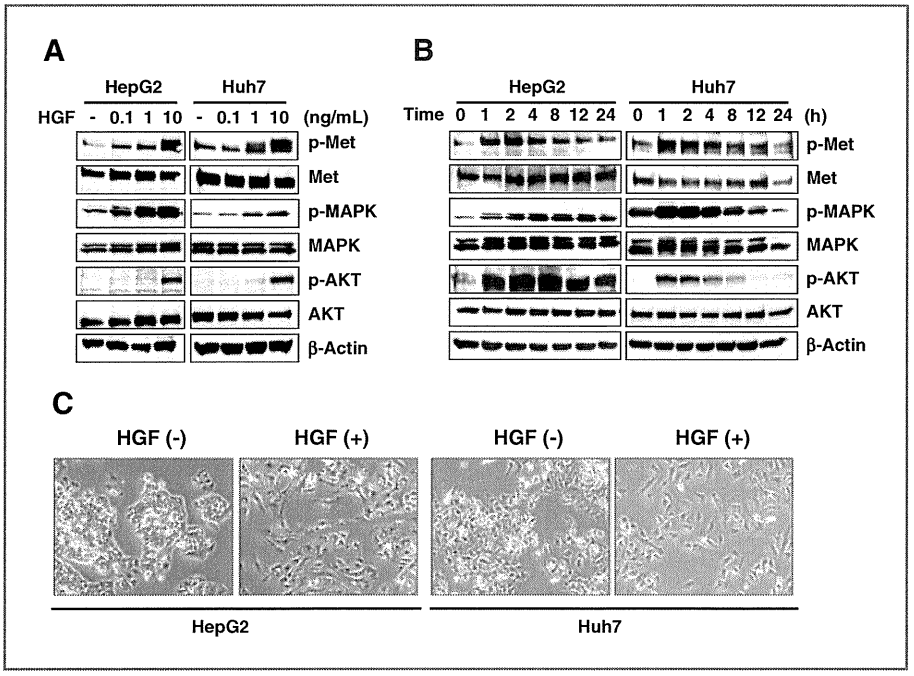

Figure 1. HGF stimulates the Met signaling pathway and induces morphologic changes in HCC. A, HGF stimulation (0, 0.1, 1, and 10 ng/mL) dose-dependently increased the phosphorylation of Met, MAPK, and AKT in the HCC cell lines HepG2 and Huh7. The results of a Western blot analysis are shown. β-Actin was used as a loading control. The serum-starved cells were stimulated with HGF for 60 minutes and then collected for analysis. B, time-course analysis of HGF stimulation. The HCC cells were stimulated with 10 ng/mL of HGF for 0, 1, 2, 4, 8, 12, and 24 hours. The results of a Western blot analysis are shown. C, HGF-mediated morphologic changes included cell scattering and the elongation of the cell shape that are characteristic of the EMT. The HepG2 and Huh7 cells were stimulated with or without 10 ng/mL of HGF for 48 hours and then photographed (magnification × 200).

TCC TGG CCT CAA GCA ATC-3′ and reverse 5′-TCC TAT CTT GGG CAA AGC AAC TG-3′; *CDH2*, forward 5′-CGA ATG GAT GAA AGA CCC ATC C-3′ and reverse 5′-GGA GCC ACT GCC TTC ATA GTC AA-3′; *SNAI1*, forward 5′-TCT AGG CCC TGG CTG CTA CAA-3′ and reverse 5′-ACA TCT GAG TGG GTC TGG AGG TG-3′; *SNAI2*, forward 5′-ATG CAT ATT CGG ACC CAC ACA TTA C-3′ and reverse 5′-AGA TTT GAC CTG TCT GCA AAT GCT C-3′; *VIM*, forward 5′-TGA GTA CCG GAG ACA GGT GCA G-3′ and reverse 5′-TAG CAG CTT CAA CGG CAA AGT TC-3′; *FN1*, forward 5′-GGA GCA AAT GGC ACC GAG ATA-3′ and reverse 5′-GAG CTG CAC ATG TCT TGG GAA C-3′; and *GAPD*, forward 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse 5′-ATG GTG GTG AAG ACG CCA GT-3′.

Small interfering RNA transfection

Three different sequences of small interfering RNA (siRNA) targeting human SNAI1 (Hs_SNAI1_9785, 9786, and 9787) and those of 2 scramble control siRNAs were purchased from Sigma Aldrich Japan. The transfection methods have been previously described (17).

Statistical analysis

The statistical analyses were done using Microsoft Excel (Microsoft) both to calculate the SD and to test for statistically significant differences between the samples using a Student t test. A value $P < 0.05$ was considered statistically significant.

Results

To examine the activity of HGF-Met signaling in HCC cells, we examined the expressions of phospho-Met, Met, phospho-AKT, AKT, phospho-MAPK, and MAPK in the HepG2 and Huh7 cell lines, using Western blotting. The phosphorylation levels of Met, AKT, and MAPK were dose-dependently increased by HGF stimulation (Fig. 1A). A time-course analysis showed that the phosphorylation levels of Met, AKT, and MAPK peaked at 1 to 2 hours after HGF stimulation and gradually recovered to the baseline values at 4 hours later (Fig. 1B). These results indicated that Met signaling is actually capable of being activated in response to HGF in HCC cells.

From a morphologic aspect, the EMT is characterized by an increase in cell scattering and an elongation of the cell shape (18). To evaluate whether HGF mediates the morphologic change that is characteristic of the EMT in HCC cells, cellular morphology was examined after HGF stimulation. HGF clearly mediated both cell scattering and the elongation of the cell shape in HepG2 and Huh7 cell lines (Fig. 1C). These data indicate that HGF mediates

DOI:10.1158/1535-7163.MCT-10-0544

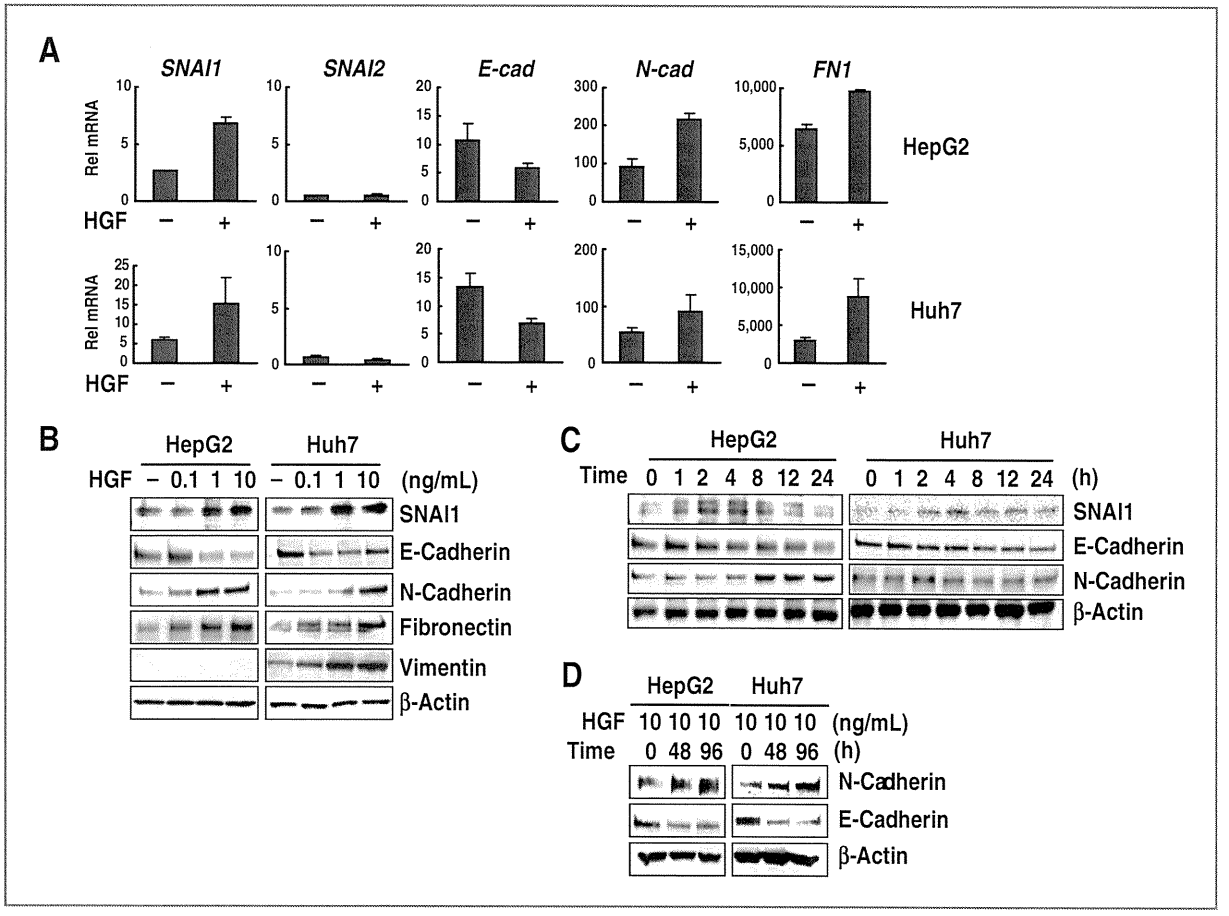

Figure 2. HGF upregulates SNAI1 expression and induces cadherin switching in HCC. A, changes in the mRNA expressions of the EMT-related genes *SNAI1/Snail*, *SNAI2/Slug*, *E-cadherin/CDH1*, *N-cadherin/CDH2*, and *fibronectin/FN1* were determined using real-time RT-PCR. The HepG2 and Huh7 cells were stimulated with or without 10 ng/mL of HGF for 2 hours (SNAI1 and SNAI2) or 48 hours (*E-cad*, *N-cad*, and *FN1*). Rel mRNA, normalized mRNA expression levels (target genes/GAPD $\times 10^4$); E-cad, E-cadherin; N-cad, N-cadherin. B, the HGF-mediated protein expression changes in SNAI1, E-cadherin, N-cadherin, fibronectin, and vimentin were determined using a Western blot analysis. The HepG2 and Huh7 cells were stimulated with HGF at the indicated dose (0, 0.1, 1, or 10 ng/mL) and collected for analysis after 4-hour stimulation for SNAI1 and 72 hours for the others. C, the cells were stimulated with 10 ng/mL of HGF for the indicated time course (0, 1, 2, 4, 8, 12, or 24 hours) and used for analysis. β-Actin was used as a loading control. D, Western blot analysis of E-cadherin and N-cadherin. The cells were stimulated with 10 ng/mL of HGF for 0, 48, and 96 hours and then analyzed.

the morphologic changes that are compatible with the induction of the EMT in HCC cell lines.

Because SNAI1 and SNAI2 are considered to be master regulators of the EMT, changes in the mRNA expression levels of EMT-related genes in response to HGF stimulation were evaluated using real-time RT-PCR (Fig. 2A). HGF stimulation upregulated SNAI1 mRNA expression by more than 2-fold, whereas the baseline expression of SNAI2 was very low compared with that of SNAI1 and did not respond to HGF in either of the HCC cell lines that were examined. Cadherin switching, which is characterized by the downregulation of E-cadherin and the upregulation of N-cadherin, is known as one of the most pivotal cellular events in the EMT (19). Cadherin switching was clearly observed on the basis of mRNA levels after HGF stimulation. The mesenchymal marker fibronectin was also upregulated (Fig. 2A).

Consistent with the mRNA changes, HGF stimulation dose-dependently upregulated the protein expression of SNAI1, N-cadherin, fibronectin, and vimentin and downregulated the expression of E-cadherin in both cell lines (Fig. 2B). Vimentin expression of HepG2 was not detected (baseline vimentin mRNA was also extremely low; data not shown). A time-course analysis showed that HGF upregulated the SNAI1 expression at 2 hours after stimulation and that the expression level recovered to the baseline value at 24 hours thereafter (Fig. 2C). Cadherin switching after HGF stimulation was observed at 8 hours later in HepG2 cells and 48 hours later in Huh7 cells (Fig. 2C and D). Generally, upregulation of SNAI1 is

DOI:10.1158/1535-7163.MCT-10-0544

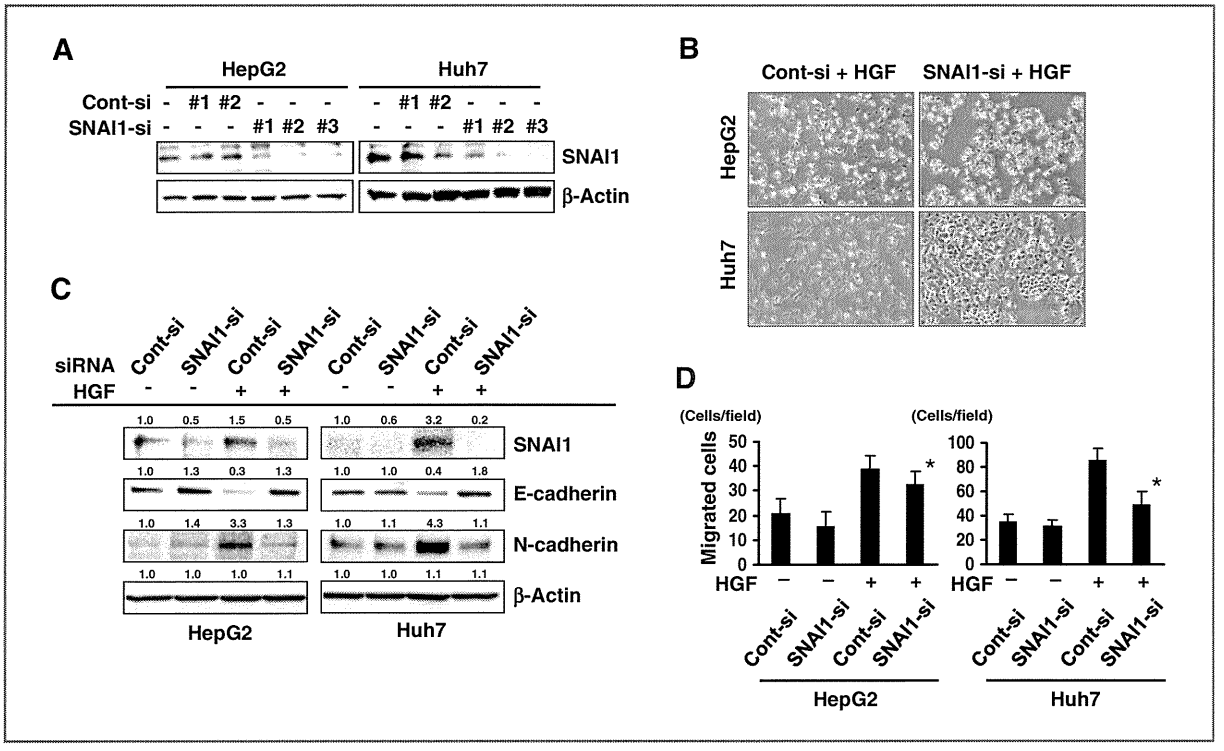

Figure 3. SNAI1 is required to induce the HGF-mediated EMT in HCC cells. A, knockdown of HGF-mediated SNAI1 expression using siRNA. Three sequences of SNAI1-siRNA (1, 2, and 3) were used. The HepG2 and Huh7 cells were treated with or without 50 nmol/L of each siRNA for 48 hours and then were stimulated with 10 ng/mL of HGF. SNAI1-siRNA #2 was effective and was used in subsequent experiments. B, SNAI1 knockdown canceled the HGF-mediated morphologic changes. The HepG2 and Huh7 cells were treated with 50 nmol/L of siRNA for 48 hours and were then stimulated with 10 ng/mL of HGF in all 4 panels. C, SNAI1 suppression by siRNA strongly canceled the HGF-mediated downregulation of E-cadherin and the upregulation of N-cadherin in both HepG2 and Huh7 cells. The cells were treated with 50 nmol/L of siRNA for 48 hours and were analyzed using a Western blot analysis. Densitometric data are shown above the Western blot. D, the siRNA knockdown of SNAI1 inhibited the HGF-mediated cellular migration. The siRNA-transfected HepG2 and Huh7 cells were evaluated using migration assay. The migration assays were conducted using the Boyden chamber methods as described in Materials and Methods. *, $P < 0.05$ (Cont-si vs. SNAI1-si with HGF); Cont-si, control-siRNA; SNAI1-si, SNAI1-targeting siRNA.

observed within few hours, but cadherin switching occurs around 24 hours later after stimulation (20, 21), consistent with our result. These results indicate that HGF meditates the induction of SNAI1, cadherin switching, and the EMT in HCC cells.

Besides SNAI1 and SNAI2, other transcription factors of several genes also have the potential to repress E-cadherin and to induce the EMT; these factors include ZEB1/TCF8, ZEB2/SMAD interacting protein 1, TWIST, E47/TCF3, and TCF4/E2-2 (6). Therefore, we examined whether SNAI1, among several EMT-inducible genes, has a central role in the HGF-mediated EMT in HCC cells. Three sequences of SNAI1-siRNA (1, 2, and 3) were used. A Western blot showed that both sequences 2 and 3 of SNAI1-siRNA completely suppressed the HGF-mediated upregulation of SNAI1 in the HepG2 and Huh7 cells (Fig. 3A); thus, the #2 SNAI1-siRNA was used in the following experiments: The siRNA knockdown of SNAI1 canceled the morphologic changes observed in HepG2 cells undergoing HGF-mediated EMT, whereas the control-siRNA did not (Fig. 3B). Similar results were obtained in Huh7 cells, indicating that SNAI1 is required for the morphologic changes observed in HGF-mediated EMT. Similarly, the siRNA knockdown of SNAI1 strongly canceled the HGF-mediated downregulation of E-cadherin and the upregulation of N-cadherin in both HepG2 and Huh7 cells (Fig. 3C). Those of mRNA expression changes were relatively correlated with the results of Western blot, except for N-cadherin in Huh7 cells (Supplementary Fig. 2A). Regarding the cellular migration, the siRNA knockdown of SNAI1 inhibited the HGF-mediated cellular migration (Fig. 3D). Collectively, these results indicate that SNAI1 is required to induce the HGF-mediated EMT in HCC cells.

In general, SNAI1 expression is regulated by ligand-receptor signal transduction through a downstream signal pathway that includes the Smad, MAPK, AKT, and GSK3 pathways (6, 22, 23). Sorafenib has been shown to inhibit RAF-MAPK signaling in HCC cells (24). Accordingly, we hypothesized that sorafenib might downregulate SNAI1 expression by inhibiting RAF-MAPK signaling, which is a unique activity of sorafenib. As