ERK-Dependent Downregulation of Skp2 Reduces Myc Activity with HGF, Leading to Inhibition of Cell Proliferation through a Decrease in Id1 Expression

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Abstract

Hepatocyte growth factor (HGF) has an inhibitory effect on human HepG2 hepatoma cell proliferation. Previously, it was shown that HGF treatment downregulated Id1 and upregulated p16INK4a in an ERK-dependent manner, leading to the inhibition of cellular proliferation. Here, new insight suggests that Skp2, an SCF complex component and potential prognosticator in cancer, is downregulated by injection of HGF into established HepG2 xenograft tumors. The downregulation was evident at both the mRNA and protein level and in an ERK-dependent manner. Critically, high expression of Skp2 restored HGF-inhibited cell proliferation, indicating that the inhibitory effect of HGF required the downregulation of Skp2. However, downregulation was not involved in the HGF-induced upregulation of a CDK inhibitor, p27kip1, a known SCF-Skp2 target. Instead, data revealed that Skp2 regulated Myc activity, which has oncogenic potential in the generation of hepatocellular carcinoma. Elevated expression of Skp2 or a mutant that is unable to associate with the SCF complex was capable of activating Myc, suggesting that Skp2 does not act on Myc as a component of the SCF complex, and thus functions as an activator of Myc independent of its role in ubiquitination. Furthermore, Skp2 regulated Id1 expression by regulating Myc activity, and the regulation of Skp2 is involved in the activity of p16 promoter through regulation of Id1 expression. Overall, these mechanistic findings provide the first evidence that ERK-dependent downregulation of Skp2 reduced Myc activity, leading to HGF-induced inhibition of cell proliferation through decreased Id1 expression.

Implications: This study elucidates the molecular details of HGF-induced inhibition of cellular proliferation in liver cancer cells. Mol Cancer Res; 1–11. ©2013 AACR.

Introduction

Hepatocyte growth factor (HGF) is a pleiotropic glycoprotein produced by stroma cells and associated with heparin in a wide variety of tissues. Its high affinity receptor c-Met is encoded by the c-met protooncogene and is widely expressed in epithelial cells (1). Binding of HGF activates the tyrosine kinase activity of c-Met, leading to cell proliferation, scattering, enhanced motility and so on, and thus HGF plays a key role in tumor–stroma interactions. Ablant activation of c-Met, which can be induced through its mutation and/or overexpression, causes many kinds of tumors (2). Recent efforts to find substances that inhibit the activation of c-Met are thus expected to lead to suppression of the malignant transformation of cells. However, HGF has an opposing effect on the regulation of cell proliferation in accordance with cell type: it promotes the proliferation of some tumor cells, but suppresses that of others (3, 4). This opposing effect is considered to depend on differences in the downstream pathways of c-Met (3–5). Thus, elucidation of the pathways responsible for the effect is expected to lead to new drugs for the suppression of tumor growth.

The binding of HGF to c-Met induces the phosphorylation of several tyrosine residues on c-Met followed by the recruitment of various signal transducers and adaptors such as Grb2 and Gab1, leading to activation of two major signaling pathways, the extracellular signal-regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K) pathways (6, 7). We previously showed that HGF treatment of human HepG2 hepatoma cells inhibited cell proliferation by arresting the cell cycle at G(1) (8). This effect was overcome by partial inhibition of the ERK pathway with a low concentration of the MEK inhibitor PD98059, but not by inhibition of the PI3K pathway (7), showing that strong activation of ERK is essential for the inhibitory effect of HGF, but activation of the PI3K/Akt pathway is not. The strong
activation of ERK by HGF upregulated expression of a Cdk inhibitor, p16<sup>16 Ink4a</sup>, which led to a redistribution of other Cdk inhibitors, p21 and p27, from Cdk4 to Cdk2, resulting in low phosphorylation of pRb and G1 arrest (8, 9). Because the upregulation of p16 expression is crucial to the G1 arrest, we studied the mechanism responsible for it, and found that transcription factor Ets upregulates p16 through downregulation of a repressor protein for Ets, Id1 (10). The expression of Id1 is shown to be regulated at the transcription level (10), but the regulatory mechanism remains to be elucidated.

S-phase kinase–associated protein 2 (Skp2) is an F-box protein in the SCF complex, which consists of Cskp1, Cullin, Rbx1 and an F-box protein (11), and is responsible for the ubiquitination and degradation of many kinds of proteins as an E3 ubiquitin ligase. F-box protein functions as the variable substrate-recognition component of the complex. More than 70 F-box proteins are present in the human genome and each is thought to have specific substrates and functions. Thus, the kinds and doses of F-box proteins define the functions of the SCF complex in cellular processes such as the cell cycle, signal transduction, and transcription. The SCF complex including Skp2 (SCFSkp2) regulates G1–S, the point responsible for defining the progression/arrest of a cell cycle, by regulating the degradation of substrates such as Cdk inhibitors (12). p27<sup>Skp2</sup>, which induces cell-cycle arrest at G1–S with inhibition of Cdk2/4/6, is a representative Cdk inhibitor suppressed by SCFSkp2 (13, 14). Overexpression of Skp2 leads to malignant progression of tumors through acceleration of p27 proteolysis (15, 16), implicating p27 in the function of Skp2 in tumor progression. In contrast, clinical research indicates the absence of an inverse correlation between Skp2 and p27 expression in some human sarcomas (17), suggesting that Skp2 has a p27-independent role in some cancer cells. In fact, a few reports have shown that Skp2 activates the transcription factor Myc through ubiquitination by SCFSkp2, leading to upregulation of target genes involved in cell proliferation such as Cdc2 (18, 19). Also, a recent report showed that Skp2 upregulates expression of the RhoA gene, which has a role in cell invasion, by regulating Myc activity independently of the SCF complex (20). Hence, Skp2 seems to function as a regulator in tumorigenesis and cancer progression through various mechanisms.

We previously showed that HGF treatment of HepG2 hepatoma cells leads to suppression of cell proliferation through a redistribution of the Cdk inhibitors p21 and p27. Also, we showed that HGF treatment induces upregulation of p27 expression (9). In this study, we first examined the expression of Skp2, as the amount of p27 may be regulated via protein degradation with SCFSkp2. We found that Skp2 expression is downregulated by injection of HGF into established tumors from HepG2 cells in mice. We conducted a detailed analysis of the role and mechanism of the downregulation with cultured HepG2 cells, and found that the downregulation occurs in an ERK-dependent manner at the mRNA and protein level, but this downregulation is not involved in the upregulation of p27 expression. We then examined the effect of the downregulation on the transcriptional activity of Myc, which has important roles in hepatocarcinoma (21, 22). We found that the downregulation reduces Myc activity, and Skp2 functions as an activator of Myc independently of its role in ubiquitination. Finally, analysis of Id1, which is involved in the regulation of HepG2 cell proliferation, showed that the reduction in Myc activity caused by the Skp2 downregulation decreases Id1 expression, leading to activation of p16 promoter. We revealed in this study that the downregulation of Skp2 expression, which leads to a reduction in Myc activity, is crucial to the inhibitory effect of HGF on the proliferation of HepG2 hepatoma cells. Another hepatoma cell line, HuH7, proliferation of which is suppressed by HGF in an ERK-dependent manner, also showed ERK-dependent downregulation of Skp2 and Id1 suggesting that some cancer cells, other than HepG2 cells, arrest their proliferation by HGF in the same mechanism as HepG2 cells.

Materials and Methods

Cell culture

HepG2 cells and HuH7 cells were provided by Dr. S. Taketani and Dr. N. Kitamura, respectively, and have been described previously (7–10). Cells were cultured as previously described (9).

Reverse transcriptase-PCR

Total RNA was purified with ISOGEN (Nippon Gene) according to the manufacturer’s instructions. cDNA synthesis was as described previously (10, 23). PCR was conducted with pairs of specific oligonucleotide primers (Skp2: 5′-CTGGTCTGTGCTCCCTCCG-3′ and 5′-CTAGATGTGGAGTGGTTGG-3′, p27<sup>Skp2</sup>: 5′-ATGTCAAACGTGCAGAGTGCAGTGTC-3′). PCR products were resolved with primers as described previously (9). PCR products were resolved on a 1.5% agarose gel and visualized with ethidium bromide staining.

Antibodies and immunoblotting

Detailed antibodies were described in Supplementary Data. Cell lysates were prepared as described previously (10). Equal amounts of protein from the precleared cell lysates (20–70 μg total protein) were resolved by SDS-PAGE on a 12% gel after heat denaturation. Immunoblotting was conducted as described previously (9, 24).

Established tumors originating from HepG2 cells

Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were purchased from Oriental Yeast Co., Ltd. Animal experiments were carried out in accordance with the institutional guidelines of Tokai University (Isehara, Japan). To deplete NK cells in the mice, 200 μL of 1 mg/mL anti-asialo GM1 antiserum (Wako Pure Chemical Industries, Ltd) was administered every 4 days. The mice were inoculated subcutaneously with 5 × 10<sup>6</sup> HepG2 cells into the dorsal flanks. After establishment of tumors, HGF or PBS in a total volume of 200 μL was injected directly into the tumor tissues (100–130 mm<sup>3</sup>) for 7 consecutive days.
Tumor tissues were harvested after sacrifice of the mice and the lysates were prepared as described (10).

Construction of a Skp2 expression plasmid

Skp2 cDNA was amplified to construct a Skp2 expression plasmid with RT-PCR using a pair of oligonucleotide primers specific to the coding sequence of human Skp2 (forward 5'-ACACAAAGCTTACACAGGATGACGACGATAAGATGCACAGGAAGCACCTC-3', including a Flag tag sequence, and reverse 5'-ACACTCGAGTCATAGACAACTGGGCTTTTGACTGTCAG-3'). PCR amplification for constructing an expression plasmid for Skp2-LRR was done using another forward primer, including a Flag tag, and the reverse primer for human Skp2 described above. The PCR products were inserted into pcDNA3.1.

Secreting placentral alkaline phosphatase (SEAP) reporter assay

A reporter construct, pMyc-SEAP (Clontech Laboratories, Inc.), which contains several E-boxes at the 5' of the TATA-like promoter, was used to measure Myc activity, and a control construct containing only the basic TATA-like promoter, pTAL-SEAP, was used as a negative control to measure activity of the basal TATA-like promoter. pMyc-SEAP or pTAL-SEAP was transfected into HepG2 cells with other expression plasmids. After 24 hours, the medium was replaced with fresh medium with or without HGF (50 ng/mL) in the absence or presence of PD98059. The cells were cultured for another 24 hours, and the medium was used for SEAP assays (Clontech Laboratories, Inc.). To determine the net activity of SEAP, the activity in cells with the negative control (pTAL-SEAP) was used as a background value. All values were normalized with activity of β-gal, which was cotransfected with the reporter.

Silencing by siRNA

Synthetic siRNAs for Id1, Myc, and Skp2 were obtained from Integrated DNA Technologies, Inc. Detailed methods were described in Supplementary Data.

Luciferase reporter assays

The p16 promoter construct containing −247 to +1 from the transcription initiation site of the p16 gene (kindly provided by Dr. E. Hara, Japan Foundation for Cancer Research, and described previously in ref. 9) was cotransfected with an expression plasmid for Skp2 and a standard amount of the pSV-β-galactosidase control plasmid (Promega). HGF treatment of the cells and luciferase reporter assays were conducted as previously described (9, 10).

Image manipulation

Adjustments of brightness and contrast with a linear algorithm were applied to the whole image.

Statistical analysis

The paired Student t test was used to test for significance where indicated.

Results

HGF downregulates Skp2 expression at the mRNA and protein level in an ERK-dependent manner

We found that injection of HGF into established tumors originating from HepG2 cells in NOD/SCID mice significantly downregulated Skp2 (Fig. 1A). Skp2 has transformation activity in experimental models, and is sometimes overexpressed in human cancers. To determine the role and mechanism of the downregulation of Skp2, we first conducted a time-course analysis of Skp2 expression in HepG2 cells treated with HGF. Western blot analysis showed that the level decreased for 24 hours, and remained low after 24 hours (Fig. 1B). As HGF treatment suppresses the proliferation of HepG2 cells through strong ERK activity (7, 9, 25), we examined the effect of ERK activity on the expression of Skp2. Treatment with a low concentration of PD98059, which partially inhibits ERK activity and restores cell proliferation suppressed by HGF (7, 9), restored the amount of Skp2 protein (Fig. 1B), indicating that Skp2 expression is regulated in an ERK-dependent manner, and suggesting the relevance of Skp2 to the regulation of cell proliferation. RT-PCR analysis showed that Skp2 mRNA expression was also downregulated by HGF and the amount of Skp2 mRNA was restored by a low concentration of PD98059 (Fig. 1C), showing that Skp2 expression is regulated at the transcription level in an ERK-dependent manner. HGF treatment of another hepatoma cell line, HuH7, proliferation of which is suppressed by HGF in an ERK-dependent manner (Supplementary Fig. S1), also led to ERK-dependent downregulation of Skp2 mRNA (Fig. 1D), suggesting that cancer cells other than HepG2 cells also are responsive to HGF.

To confirm that the strong ERK activity mediates the Skp2 downregulation, we used a HepG2 cell line, in which ERK activity is strongly activated by induction of active Ras (Ras V12) with Isopropyl-β-D-thiogalactopyranoside (IPTG; ref. 7). Induction of the Ras, which suppressed proliferation of HepG2 cells, downregulated Skp2 (Fig. 1E), indicating that strong ERK activation is sufficient for downregulation of Skp2, and supporting that the expression of Skp2 is related to the regulation of proliferation in HepG2 cells.

Downregulation of Skp2 is not involved in the upregulation of p27, while it is involved in the suppression of cell proliferation with HGF

Figure 1B (left) shows that the timing of Skp2 downregulation after HGF treatment coincided with that of p27 upregulation. The expression of p27 mRNA was not altered by HGF (Supplementary Fig. S2), consistent with a report that p27 expression is regulated at the level of protein degradation via Skp2 (26). However, the partial inhibition of ERK activity with the low concentration of PD98059 had no effect on the upregulation of p27 by HGF, and p27 protein expression remained upregulated, even though the level of Skp2 protein was high (Fig. 1B, right). Moreover, with the HepG2 cell line, in which ERK is strongly activated by induction of active Ras with IPTG, we showed that activation of Ras did not alter the level of p27, while it
downregulated Skp2 expression (Fig. 1E). These results suggested that the downregulation of Skp2 is not involved in the upregulation of p27. They also showed that the upregulation of p27 by HGF is not mediated by the ERK activity, and suggested that the upregulation is not involved in the inhibitory effect of HGF on HepG2 cell proliferation.

To directly show that the downregulation of Skp2 is not involved in the upregulation of p27, experiments with high expression of Skp2 were conducted. HepG2 cells were transfected with Skp2, and the protein levels of Skp2 and p27 were examined by Western blot analysis after HGF treatment. High expression of Skp2 did not suppress the upregulation of p27 (Fig. 2A), showing the upregulation to be independent of the downregulation of Skp2. In contrast, cell counts showed that high expression of Skp2 restored the cell proliferation suppressed by HGF (Fig. 2B), indicating that downregulation of Skp2 is essential for the inhibitory effect of HGF on the proliferation of the cells. The data also suggested that the upregulation of p27 is not involved in the inhibitory effect of HGF, as high expression of Skp2 did not suppress the upregulation (Fig. 2A). Although the number of cells was restored with high expression of Skp2 in the presence of HGF, it did not reach the number achieved in the absence of HGF (Fig. 2B). This may simply be due to the transfection efficiency of the construct to express Skp2.

Figure 1. Skp2 is downregulated by HGF in an ERK-dependent manner. A, Western blot analysis to detect Skp2 in established tumors from HepG2 cells in NOD/SCID mice. All bands in analysis with anti-Skp2 and α-tubulin antibodies, respectively, were acquired from a single film exposure. Unprocessed full-length blots are presented in Supplementary Fig. S4. The intensity of the band in the bottom was quantitated using NIH ImageJ software and graphed. Each value represents the mean ± S.D. (n = 3), *P < 0.05; Student t test. B, Western blot analysis to detect Skp2 and p27 in HepG2 cells treated with HGF in the absence (−) or presence (+) of PD98059. Cell lysates were prepared at the indicated times (h) after HGF treatment and subjected to Western blot analysis. Tubulin was used as a loading control. p16 is shown to assess the effect of HGF. The result is representative of three independent experiments. C, RT-PCR analysis of Skp2 in HepG2 cells treated with HGF in the absence (−) or presence (+) of PD98059. Total RNA was purified at the indicated times and subjected to RT-PCR. GAPDH was used as an internal control. p16 is shown to assess the effect of HGF. The result is representative of two independent experiments. D, RT-PCR analysis of Skp2 in HuH7 cells treated with or without HGF for 24 hours in the absence (−) or presence (+) of PD98059. Total RNA was purified and subjected to RT-PCR. GAPDH was used as an internal control. E, time course analysis of cell proliferation (top) and Western blot analysis to detect Skp2 and p27 in cells with forced activation of ERK (bottom). After the active form of Ras was induced with IPTG, cell numbers were counted and cell lysates were prepared at the indicated times after induction. Phospho-ERK and p16 are shown to assess the effect of Ras induction. Experiments were done twice with similar results, and representative data are shown.
Downregulation of Skp2 with HGF reduces Myc activity

A few reports have indicated that Skp2 has another role in activation of the transcription factor Myc (18, 19), and Myc was reported to have important roles in hepatocarcinoma (21, 22). A recent report showed that knockdown of Myc suppressed proliferation of HepG2 cells (27), and we obtained results consistent with this by knockdown of Myc (data not shown), indicating that Myc expression is required for the proliferation of HepG2 cells. Thus, we studied the relevance of Skp2 to Myc in HepG2 hepatoma cells. We first examined Myc activity in HepG2 cells treated with HGF. A promoter-reporter construct, in which the SEAP reporter gene is regulated by binding of Myc on the promoter, was introduced into the cells, and the SEAP activity was analyzed in the absence or presence of HGF. Cells were prepared as described in (A) and cell numbers were counted at the indicated times. Each value represents the mean ± S.D. of triplicate determinants. The results are representative of three independent experiments.

Downregulation of Skp2 with HGF reduces Myc activity

we examined the levels of c-myc, N-myc, and L-myc by RT-PCR. The expression of c-myc was much higher than that of the others (data not shown). Thus, the Myc activity seems to be mostly derived from c-Myc in the cells.

As the Myc protein is known to be unstable (28), it is possible that the inactivation of Myc with HGF simply reflects loss of the protein. However, Western blot analysis showed that the amount of c-Myc is not altered in the presence of HGF (Fig. 3B), suggesting that the reduction in Myc activity with HGF is not caused by loss of the protein. We also analyzed the levels of Max and Mad, because in the Myc/Max/Mad network, the association of Myc with Max leads to transcriptional activation, and that of Mad with Max to repression (29). We did not detect any changes in their expressions in HepG2 cells treated with HGF (Supplementary Fig. S3), suggesting that the reduction in Myc activity by HGF is not regulated by changes in the amounts of Mad and Max.

As the reduction in Myc activity by HGF is mediated through the strong ERK activity, it may be caused by the downregulation of Skp2. We thus examined whether Skp2 regulates endogenous Myc activity. We introduced the
reporter for Myc together with the construct to express Skp2 into the cells. High expression of Skp2 activated Myc activity in the absence or presence of HGF (Fig. 4A), indicating that Skp2 is sufficient to activate endogenous Myc. Next, to directly show that the reduction in Myc activity is caused by the downregulation of Skp2, we introduced Skp2 siRNA into the cells. Knockdown of Skp2 significantly decreased endogenous Myc activity without altering the amount of c-Myc in the absence of HGF (Fig. 4B and C), indicating that Skp2 is essential to activate Myc in proliferating cells. These results showed that Skp2 functions to activate Myc, and thus, downregulation of Skp2 results in the reduction in Myc activity.

Myc activity is regulated by Skp2, but not through SCFSkp2

It was previously shown that Myc is activated via ubiquitination by the SCF complex including Skp2 (SCFSkp2). The ubiquitination results in rapid degradation of Myc (18, 19). However, inhibition of ubiquitination and degradation with knockdown of Skp2 induced no accumulation of Myc activity.
protein (Fig. 4B), suggesting that ubiquitination of Myc by SCFSkp2-LRR is not involved in the regulation of Myc activity. To directly show whether or not the SCF complex is required for the transcriptional activation of Myc by Skp2, we adopted a mutant of Skp2, Skp2-leucine–rich repeats (LRR), which has a deletion in the N-terminus including a part of the F-box domain, resulting in an inability to couple to the SCF complex (18). High expression of not only the wild-type Skp2 but also Skp2-LRR restored the Myc activity reduced by HGF treatment (Fig. 5A), indicating that Skp2 regulates Myc activity, but ubiquitination of Myc by the SCF complex is not involved in the regulation of Myc activity by Skp2. This idea was supported by the finding that high expression of Skp2 and Skp2-LRR did not change the amount of Myc protein (Fig. 5B).

**Figure 5.** High expression of Skp2-LRR restores Myc activity suppressed by HGF. A, detection of endogenous Myc activity in cells with high expression of Skp2 (WT) or Skp2-LRR (LRR). The average fold-decrease in Myc activity compared with activity in the absence of HGF with a Mock transfection is indicated. Each value represents the mean ± S.D. of triplicate determinants from a representative experiment. *P < 0.01; Student’s t test. B, Western blot analysis to detect c-Myc in cells transfected with Skp2 (WT) or Skp2-LRR (LRR). Exogenous Skp2 and Skp2-LRR were detected with anti-Flag and their positions are indicated with * and **, respectively. The signal represented by # is nonspecific. Experiments were carried out twice with similar results and representative data are shown.

**Skp2 is involved in Id1 expression through regulation of Myc activity**

Myc regulates transcription of a wide range of genes responsible for regulation of cell proliferation, transcription, cell motility and so on (29). To uncover the target of Myc activated by Skp2 in the hepatoma cells, and to address the mechanism by which Skp2 affects proliferation through HGF signaling, we focused on a transcriptional regulator, Id1: Id1 seemed to be a target of Myc activated by Skp2, because our previous study showed that Id1, whose the mRNA and protein are downregulated by HGF, is involved in the antiproliferative effect of HGF through regulation of p16 expression (10), and it was recently reported that c-Myc upregulated Id1 expression in human breast cancer cells and prostate cancer cells (30, 31). In addition, Id1 expression in another hepatoma cell line, HuH7, proliferation of which is suppressed by HGF in an ERK-dependent manner (Supplementary Fig. S1), was also regulated in an ERK-dependent manner (Supplementary Fig. S6), suggesting that Id1 is involved in the inhibitory effect of HGF on proliferation of cancer cells other than HepG2. Western blot analysis showed that high expression of Skp2 restored the expression of Id1 suppressed by HGF (Fig. 6A), and knockdown of Skp2 suppressed the expression of Id1 in the absence of HGF (Fig. 6B and Supplementary Fig. S7), showing that Skp2 is involved in the regulation of Id1 expression. In addition, knockdown of Myc suppressed the expression of Id1 in the absence of HGF (Fig. 6C), indicating that Myc regulates Id expression. These results, together with the fact that Skp2 regulates Myc activity, suggest that Skp2 regulates Id1 expression through the regulation of Myc activity. Also, high expression of the Skp2 mutant, Skp2-LRR, restored Id1 expression and cell proliferation suppressed by HGF (Supplementary Fig. S8), suggesting that Id1 expression is regulated by Skp2 in the SCF complex-independent manner. As we previously showed that the downregulation of Id1 results in the activation of the promoter of the p16 gene (10), we next examined the effect of Skp2 on the promoter. A promoter-reporter construct, in which expression of luciferase is regulated by a minimal promoter of the p16 gene (9), was introduced into HepG2 cells together with the construct to express Skp2 in the absence or presence of HGF. While HGF treatment of the cells activated the p16 promoter as previously reported (9), high expression of Skp2 significantly suppressed the activation of the p16 promoter (Fig. 6D). Also, knockdown of Id1 restored the promoter activity suppressed by high expression of Skp2 in the presence of HGF (Fig. 6E, lane 3, 4, and 8). These results supported that Skp2 regulates Id1 expression, leading to regulation of p16 promoter. Higher activity of p16 promoter induced by Id1 siRNA (lane 7 and 8) than that induced by randomized siRNA (lane 3 and 4) in the presence of HGF seems to represent a partial reduction, but not complete elimination, of Id1 by HGF treatment of the cells.

**Discussion**

Recent clinical research suggests that Skp2 has a p27-independent role in some cancer cells (17). In this study, we...
reported that ERK-dependent downregulation of endogenous Skp2 by HGF reduces Myc activity, leading to inhibition of HepG2 hepatoma cell proliferation through a decrease in Id1 expression. The downregulation was also confirmed in established tumors from HepG2 cells in mice. Our data reveal a mechanism by which HGF suppresses cell proliferation through Skp2 downregulation. Our data suggested that Skp2 is physiologically involved in the regulation of Myc activity as its activator independently of its role in ubiquitination, but not in the regulation of p27 degradation. In addition, a hepatoma cell line other than HepG2, HuH7, proliferation of which is also suppressed by HGF in an ERK-dependent manner, also showed ERK-dependent downregulation of Skp2 (Fig. 1D) and Id1 (Supplementary Fig. S6), suggesting that some other cancer cells arrest their proliferation by HGF through Skp2 downregulation in the SCF-independent manner.

Myc regulates the transcription of various genes responsible for controlling cell proliferation, the cell cycle, transcription, cell motility and so on, leading to the initiation, promotion, and progression of a wide range of cancers (32). Among the targets of Myc, we found in this study that expression of Id1 is regulated by Myc through Skp2 (Fig. 6). A comprehensive analysis to find Myc-binding sites in a human genome with ChIP assays showed a binding site in the upstream region of the Id1 gene (33), supporting regulation of Id1 expression by Myc through Skp2. As we previously showed that Id1 is downregulated by HGF (10), which leads to inhibition of HepG2 cell proliferation, we propose the model displayed in Fig. 7 to illustrate the
We showed that Skp2 is downregulated at the mRNA level in an ERK-dependent manner in HepG2 cells treated with HGF (Fig. 1). Previous reports showed that transcription factor E2F regulates Skp2 expression at the mRNA level in an Akt-dependent manner in other types of cells (34). Although it was previously shown that the inhibitory effect of HGF on the proliferation of HepG2 cells is independent of Akt signaling (7), E2F seemed to be a candidate transcription factor responsible for the Skp2 expression in HGF signaling, because we found that HGF treatment of HepG2 cells reduces the levels of E2F1, and E2F activity (data not shown). To address whether E2F regulates Skp2 expression in HGF signaling, we constructed another HepG2 cell line, in which E2F1 is induced by addition of IPTG. Induction of E2F1 was successfully activated an E2F-responsive promoter, but had no effect on Skp2 expression (data not shown). This result indicated that the regulatory mechanism of Skp2 downregulation induced by HGF is different from that in Akt signaling.

It is generally recognized that the amount of p27 protein, which is predominantly regulated by degradation through ubiquitination by SCFSkp2, is responsible for the suppression of cell proliferation (14). The effect of p27 on the proliferation of HepG2 cells was shown in a previous report, in which β1-integrin-mediated downregulation of p27 through upregulation of Skp2 accelerated the proliferation (26). As HGF treatment of HepG2 cells upregulates p27 protein, we had expected the upregulation to contribute to the suppression of cell proliferation by HGF. However, in the present study, our data showed that partial inhibition of the HGF-induced suppression of cell proliferation by HGF, had no effect on the upregulation of p27 induced with HGF (Fig. 1B). Our data also showed that high expression of Skp2 restores the cell proliferation suppressed by HGF without affecting the upregulation of p27 (Fig. 2). These results indicate that the upregulation of p27 does not contribute to the suppression of HepG2 cell proliferation by HGF. Accumulating data suggest that p27 has another role in regulating cell motility and migration independent of its cell-cycle role. p27 increases the stability of actin stress fibers by binding to and inhibiting RhoA in cytoplasm, which results in an increase in cell motility (35). As HGF treatment increases the motility of HepG2 cells, leading to cell scattering, the upregulation of p27 might contribute to the increased cell motility with HGF. The significance of the upregulation of p27 expression in HepG2 cells treated with HGF remains to be elucidated.

It was previously shown that the overexpression of Myc in the liver induces the formation of hepatocellular tumors (36, 37), and its inactivation results in regression of the tumors and differentiation of the tumor cells into normal liver cells such as hepatocytes (21), indicating the importance of Myc activity for hepatocarcinogenesis. Accumulating evidence also indicates an important role for Myc activation in the progression of hepatomas (22, 38, 39). We showed here that endogenous Myc activity is regulated by Skp2 in a hepatoma cell line. Thus, it is likely that Skp2 has an important role in the downregulation of Skp2 in the inhibition of the cell proliferation by HGF. Treatment with HGF downregulates Skp2 expression in an ERK-dependent manner, leading to reduced transcriptional activity of Myc. The reduction in Myc activity decreases Id1 expression, which leads to the activation of a transcription factor, Ets. The activated Ets upregulates p16 expression, which eventually induces inhibition of proliferation in HepG2 hepatoma cells.

Figure 7. Schematic model of the involvement of Skp2 downregulation in the HGF-induced inhibition of cell proliferation. HGF treatment of the cells downregulates Skp2 expression in an ERK-dependent manner, leading to a reduction in Myc activity. The reduction in Myc activity induces a decrease in Id1, which leads to the activation of a transcription factor, Ets. The activated Ets upregulates p16 expression, which eventually induces inhibition of proliferation in HepG2 hepatoma cells.
hepatocarcinoma through the regulation of Myc activity. Another report suggested suppression of Myc-induced hepatocarcinogenesis by HGF: hepatocarcinogenesis was induced in transgenic mice expressing c-Myc, but not in transgenic mice expressing c-Myc in combination with HGF (40). The suppression might be caused by downregulation of Skp2. While we showed that HGF treatments of two hepatoma cell lines, HepG2 and HuH7, downregulate Skp2 mRNA in the presence of HGF, further studies of Skp2 in liver tumors and other hepatoma cells would be required to elucidate the involvement of Skp2 in Myc-induced hepatocarcinogenesis, and its suppression via downregulation of Skp2.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Tanaka
Development of methodology: Y. Inagaki

References

Acknowledgments
The authors thank Dr. Eiji Hata for allowing us to use the -247 promoter. The authors also thank Mr. Takahito Nakayama and Ms. Hiroko Shirai for supporting our experiments and for information on the Id1 promoter, respectively.

Grant Support
This work was supported by Grants-in-aid for Scientific Research (No. 19570124 and No. 22570137; to T. Tanaka, No. 24241044; to T. Ikoma) from the Japan Society for the Promotion of Science.

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Received December 29, 2012; revised May 1, 2013; accepted May 17, 2013; published OnlineFirst October 30, 2013.

OF10 Mol Cancer Res; 2013 Molecular Cancer Research

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Li, Y. Takizawa, T. Hashimoto, Y. Inagaki, M. Komada, T. Tanaka. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Li, Y. Bian, T. Tanaka. Writing, review, and/or revision of the manuscript: M. Komada, T. Tanaka. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Tanaka. Study supervision: T. Ikoma, J. Tanaka, N. Kitamura, M. Komada, T. Tanaka.


Molecular Cancer Research

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Mol Cancer Res  Published OnlineFirst October 31, 2013.

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