Inhibition of the proliferation and invasion of hepatocellular carcinoma cells by lipocalin 2 through blockade of JNK and PI3K/Akt signaling

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Abstract. Lipocalin 2 (Lcn2) has been reported to induce cellular proliferation based on its expression in a variety of proliferative cells. Consistent with these findings, the present study demonstrates a significant increase in Lcn2 levels in human hepatocellular carcinoma (HCC) tissues compared with non-tumor liver tissues. However, the role of Lcn2 in hepatocarcinogenesis is far from clear. To investigate the effects of Lcn2 expression on hepatocarcinogenesis, Chang liver and SK-Hep1 HCC cell lines were genetically manipulated to express Lcn2, and the effects on the proliferation and invasion of HCC cells were analyzed. Ectopic expression of Lcn2 in HCC cells significantly inhibited the growth of HCC cells *in vitro* and *in vivo*, reduced the invasive potential of cells, and inhibited the expression of matrix metalloproteinase 2 (MMP-2). Lcn2 may exert its function partly through the inhibition of the c-Jun N-terminal kinase (JNK) and phosphatidyl inositol 3'-kinase (PI3K)/Akt signaling pathways in HCC cells. The selective inhibition of these pathways using pharmacological inhibitors significantly inhibited proliferation, invasion and MMP-2 expression, whereas Lcn2 expression suppressed the JNK and PI3K/Akt pathways. Collectively, these results clearly indicate that Lcn2 may play a protective role against the progression of HCCs by suppressing cell proliferation and invasion. The clinical significance of the present findings should be evaluated further.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; JNK, c-Jun N-terminal kinase; Lcn2, lipocalin 2; MMP-2, matrix metalloproteinase 2; PI3K, phosphatidyl inositol 3'-kinase

Key words: lipocalin 2, hepatocellular carcinoma, MAP kinase signaling, matrix metalloproteinase 2, 1-phosphatidylinositol 3-kinase

Introduction

Lipocalins are a family of proteins that bind to surface receptors and to a variety of lipophilic substances and are characterized by the diversity of their functions. The effect of lipocalins on cell proliferation and differentiation and their potential role in cancer progression have been reported previously (1). One of the members of the lipocalin protein family, lipocalin 2 (Lcn2; also called siderocalin, Ngal, uterocalin, or neu-related lipocalin), is a 25-kDa glycoprotein found in specific granules of human neutrophils (2) and it has been reported to play a role in transporting fatty acids (3) or iron (4), inducing apoptosis in myeloid and lymphoid cells (5), and suppressing bacterial growth through the binding of bacterial catecholate-type ferric siderophores, which leads to iron sequestration (6,7).

Lcn2 is highly expressed in polyoma, SV40 or *neu* transformation (8), growth factor-stimulated fibroblast cells (9), and various malignant tumors (10). Expression of Lcn2 in colon cancer cells suppressed lymph node and liver metastases, which suggests its potential role in enhancing cellular growth but inhibiting metastatic invasion (10,11). The causal relationship between Lcn2 expression and the proliferation and metastasis of tumor cells is not clear and remains to be elucidated.

High expression of Lcn2 in hepatocellular carcinomas (HCC), especially in the advanced components of 'nodule-in-nodule'-type HCC, has been identified by microarray, suggesting a potential role for this protein in the progression of HCC (12,13). In the present study, the effects of Lcn2 expression on the proliferation and invasion of HCC cells was

investigated *in vitro* and *in vivo* in parallel with the expression of MMP-2 due to its important role in cell invasion. The mechanisms and functional pathways mediating Lcn2 signaling were also investigated.

Materials and methods

Reagents and antibodies. Rabbit antibodies against the phosphorylated and non-phosphorylated forms of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK were purchased from Cell Signaling Technology (Beverley, MA). Rabbit antibodies against phospho-Akt and Akt were purchased from BD Biosciences Pharmingen (San Diego, CA). Polyclonal goat antibody against Lcn2 was purchased from R&D Systems (Minneapolis, MN). Peroxidase-conjugated goat anti-rabbit, anti-mouse IgG, and rabbit anti-goat IgG were purchased from KPL (Gaithersburg, MD). Human liver total RNA was purchased from BD Biosciences Clontech (Mountain View, CA). Recombinant human Lcn2 was expressed and purified as described previously (14).

Tissue samples. The comparative expression of Lcn2 in HCC tissues and matched non-tumor surrounding liver tissues was analyzed using the cDNA microarray reported by Lee *et al* (15,16). In addition, fifteen pairs of HCC tissues and corresponding non-neoplastic liver tissues were obtained from patients who underwent surgical resection at the Chonbuk National University Hospital, Jeonju, Korea. The Research Ethics Committee of Chonbuk National University Hospital approved the study. Written informed consent was obtained from each patient. Surgically removed tissues were sampled for histological diagnosis and the remaining tissues were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored until used. The pathology report histologically confirmed HCC and non-tumor tissues. All protocols conformed to the ethical guidelines of the Institutional Review Board.

Cell lines and culture conditions. Chang liver and SK-Hep1 human HCC cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco-BRL) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in 5% CO_2.

Tissue microarray experiment. To analyze the expression profile of Lcn2 in HCC tissues, a tissue microarray experiment was performed using an anti-Lcn2 antibody following the immunohistochemistry method recommended by the manufacturer (AccMax A204, Petagen Inc., Seoul, Korea).

Construction of Lcn2-expressing hepatocellular carcinoma cells. Human Lcn2 cDNA, including its leader sequence, was amplified by polymerase chain reaction using a human adult liver cDNA library (Invitrogen, Carlsbad, CA) as the template with the following oligonucleotide primers: 5'-CACCATGC CCCTAGGTCTCCTGTGGCTG-3' (NGAL5) and 5'-TCCC CGCGGTCAATGGTGATGGTGATGATGGCCGTCGATA CACTG-3' (NGAL3-6H). The amplified cDNA fragment was cloned into the *Spe*I and *Sac*II sites in the pLenti6/V5 vector (Invitrogen) via cloning into a pGEM-T Easy vector (Promega, Madison, WI). The resultant pLenti-L6H plasmid or the parental pLenti6/V5 (mock) were used to transfect the 293FT producer cells together with the packaging mix with the Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). The viral supernatant containing mock or Lenti-L6H lentiviruses was used to transduce Chang liver (Chang-mock and -Lcn2) and SK-Hep1 cells (SK-mock and -Lcn2). After transduction, blasticidin (3 μg/ml)-resistant stable clones were selected and used for further studies.

Preparation of RNA and Northern blot analysis. Total RNA was isolated from cells by using the TRIzol reagent (Gibco-BRL) according to the manufacturer's instructions. Northern blot analysis was performed to detect specific Lcn2 or MMP-2 transcripts. Briefly, equal amounts of total RNA (20 μg for cell lines and 10 μg for HCC liver tissues) were resolved on 1% denaturing formaldehyde-agarose gels and transferred to a Hybond-N membrane (GE Healthcare Life Sciences, Piscataway, NJ). The DNA fragment encoding Lcn2 or human MMP-2 was radiolabeled with the Prime-it II random primer labeling kit (Agilent Technologies Inc., Santa Clara, CA) and used as a probe. Hybridization was performed in ExpressHyb hybridization solution (BD Biosciences Clontech) at 60˚C for 4 h. The filter was washed with 2xSSC (1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.05% SDS at room temperature for 30 min and at 50˚C for 30 min with 0.1xSSC-0.1% SDS. Lcn2 or human MMP-2 mRNA expression was quantified by autoradiography. To ensure equal amounts of RNA were loaded, the same blot was re-probed with a radiolabeled 18S rRNA-specific probe.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA (2 μg) was used as a template to synthesize first-strand cDNA with SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Subsequent amplification was performed for 30 cycles at 95˚C for 1 min, 51˚C for 1 min, and 72˚C for 1 min using the primers: 5'-CACCATGCCCCTAGGTCTCCTGTGG CTG-3' and 5'-TCAGCCGTCGATACACTG-3' for Lcn2; 5'-GCTGGCCCTGGCTCCCACAGG-3' (sense) and 5'-ATA CAAAGCAAACTGCTAATG-3' (anti-sense) for MMP-2; and 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5'-TCC ACCACCCTGTTGCTGTA-3' (anti-sense) for glyceraldehydes 3-phosphate dehydrogenase (GAPDH). The amplified PCR products were resolved by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and quantified.

Cell proliferation assay. Cells were seeded in 96-well culture plates (Thermo Fischer Scientific, Rochester, NY) in DMEM containing 10% FBS in triplicate ($1x10^4$ cells/well), and the number of viable cells was determined by using the CellTiter 96 Aqueous non-radioactive cell proliferation assay kit according to the manufacturer's instructions (Promega) for 7 days.

Colony formation in soft agar. The cell suspension ($1.0x10^4$ cells in 2 ml of DMEM supplemented with 10% FBS and 0.3% agar) was layered onto 60 mm plates containing DMEM

medium with 10% FBS and 0.5% agar. Plating was carried out in triplicate and repeated at least twice. After 17 days of growth, colonies were observed under a phase contrast microscope.

Western blot analysis of Lcn2. Confluent cultures of cells were incubated in DMEM for 72 h in 100 mm culture dishes (Thermo Fischer Scientific). Conditioned media were harvested from each clone and concentrated using centriprep-10 and centricon-10 (Millipore, Billerica, MA). The concentrated proteins were separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad; Hercules, CA). After soaking for 1 h in blocking buffer (5% non-fat dry milk in TBS containing 1% Tween-20), the membrane was incubated for 1 h with polyclonal goat antibodies against Lcn2 followed by HRP-conjugated rabbit anti-goat IgG. Immunoreactive bands were detected by using ECL Western blotting detection reagents (GE Healthcare Life Sciences) according to the manufacturer's instructions.

Western blot analysis of cell signaling molecules in HCC cell lines. The effect of Lcn2 on the activation of the MAPK or PI3K/Akt signaling pathways was analyzed by Western blotting of HCC cell extracts with antibodies to the active phosphorylated forms of these proteins. Lcn2-expressing or control HCC cell lines were cultured in DMEM containing 10% FBS until confluent, washed, and cultured in DMEM supplemented with 0.1% FBS. After 24 h, the medium was replaced with fresh low-serum medium with or without signaling inhibitors such as U0126 (a MEK inhibitor; Promega), SB203580 (a p38 inhibitor; Promega), SP600125 (a JNK inhibitor; A.G. Scientific Inc., San Diego, CA), and LY294002 (a PI3K inhibitor; Biosource, Camarillo, CA). After a 30-min incubation, serum (10% FBS) was added and further incubated for 20 min to determine the levels of phospho-ERK1/2, phospho-p38, phospho-JNK1/2, or phospho-Akt. The cells were washed with PBS and lysed with lysis buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, and 1x protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). The lysates were quantified for protein concentration, separated on 4-20% SDS-PAGE gels, and subjected to Western blot analysis. Equal protein loading was verified using antibodies against the unphosphorylated forms of each molecule.

Matrigel invasion assay. Transwell (6.5 mm diameter) polycarbonate membrane inserts with 8.0 μm pores (Corning Life Sciences, Lowell, MA) were coated with Matrigel (40 μg/each well; BD Biosciences, San Jose, CA). After adding DMEM containing 10% FBS, single-cell suspensions in DMEM were seeded onto the filters ($1x10^5$ cells/100 μl/well) and incubated for 24 h in 5% CO_2 at 37˚C. Filters were then washed and cells on the upper surface were removed with cotton swabs. Cells that had invaded and adhered to the lower surface were fixed with methanol for 15 min and stained with 0.1% (w/v) crystal violet for 15 min. The filters were then extracted with 30% acetic acid. The cells that had invaded were indirectly quantified by determining the absorbance at 595 nm.

In vitro wound migration assay. Cells ($1x10^7$) were plated in 100-mm culture dishes and incubated for 24 h in 5% CO_2 at 37˚C. Confluent cells were wounded by scraping, then washed and incubated for 18 h in DMEM containing 10% FBS. The cells that had migrated into the wounded area were photographed with an Olympus C-3030 digital camera and quantified.

Gelatin zymography. The gelatinolytic activity of MMP-2 was analyzed by zymography, as described by Zhang *et al* (17). Briefly, concentrated conditioned media (3 μg protein per lane) were electrophoresed on 10% Zymogram gels (Invitrogen). The gels were incubated with zymogram renaturing buffer (Invitrogen) for 1 h at room temperature and then with zymogram developing buffer (Invitrogen) for 16 h at 37˚C. The gels were stained with Coomassie Blue R250 and destained with 10% acetic acid-20% methanol until the desired color intensity was obtained.

Subcutaneous implantation of tumors. Male 5-week-old athymic BALB/c nu/nu nude mice (Charles River Laboratories Japan, Yokohama, Japan) were used for the tumor studies. All mice were fed a commercial diet, given water *ad libitum*, and subjected to a 12 h light/12 h dark cycle. Mice (n=5/group) were injected with Lcn2-overexpressing or control HCC cells ($1x10^7$ cells) in the proximal midline of the dorsa. Tumor sizes were measured every 2-3 days and the tumor volumes were determined by using the formula $width^2$ x length x 0.52.

Statistical analysis. Statistical significance was calculated by using Student's t-test. A $p<0.05$ was considered to be statistically significant.

Results

Expression of Lcn2 in HCC tissues and cell lines. To explore the possible role of Lcn2 in hepatocarcinogenesis, the expression of Lcn2 was examined in a panel of HCC cell lines and in human HCC tissues. Lcn2 was overexpressed in two HCC cell lines (Huh7 and Hep3B) out of 6 (Fig. 1A). Northern blot analysis showed that Lcn2 mRNA was expressed at a significantly higher level in 11 out of 15 HCC specimens compared with the adjacent non-tumor liver tissues (Fig. 1B). An expression analysis using gene expression data obtained from the publicly available database Gene Expression Omnibus (accession number GEO1898 and GSE4024) (15,16) revealed that Lcn2 expression in HCC was significantly higher than that of matched non-tumor surrounding liver tissues (Fig. 1C; two sample t-test, p=0.05; n=91). Similar results were observed in the microarray data derived from a cancer profiling database (www.oncomine.org) that showed a significantly higher expression of Lcn2 in HCC tissues (n=103) compared with non-tumor liver tissues (n=76) ($p<0.000005$; data not shown). The protein expression levels of Lcn2 in HCC tissues were determined using a human HCC tissue microarray (Petagen) after staining with anti-Lcn2 antibodies (Fig. 1D). Non-neoplastic liver tissues (n=4) exhibited focal positivity of small intrahepatic bile ducts and the hepatocytes were negative or stained very weakly and diffusely (Fig. 1D; top panel). Of the 35 different liver tumor tissues, 18 tissues showed positive

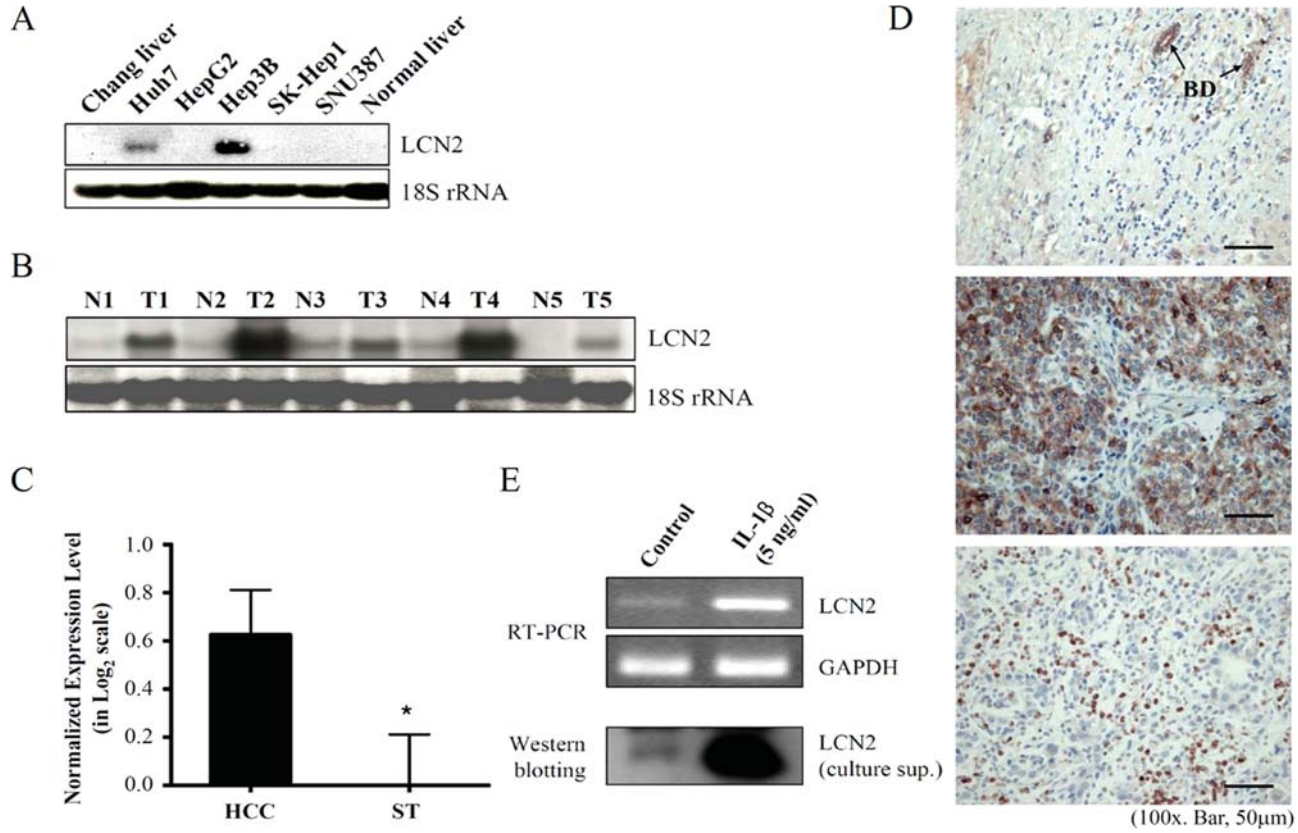

Figure 1. Lcn2 expression in human hepatocellular carcinoma (HCC) and HCC cell lines. (A) Northern blot analysis of Lcn2 expression in HCC cell lines. (B) Northern blot analysis of Lcn2 expression in HCC tissues (T) and matched surrounding non-tumor liver tissues (N). Numbers by N or T represent the tissue samples from each patient. 18S ribosomal RNA is shown as a loading control. (C) Normalized Lcn2 expression level in HCC tissues (n=91) and matched surrounding non-tumor liver tissues (ST) in Log2 scale as determined by using gene expression data (15,16). Values correspond to the mean ± SE. *p=0.05. (D) Immunohistochemical analysis of Lcn2 expression in human HCC tissue microarray. Top panel, non-neoplastic liver tissue; BD, intrahepatic bile duct; middle panel, liver tumor tissue showing strong Lcn2 positivity in both infiltrated cells and liver tumor cells; bottom panel, liver tumor tissue showing infiltrated cells with strong Lcn2 positivity. Magnification, x100. (E) Induction of Lcn2 expression by the treatment of Huh7 cells with 5 ng/ml IL-1ß for 24 h, as assessed by RT-PCR (upper panel) and Western blotting of the culture supernatant (lower panel).

cytoplasmic staining for Lcn2 in HCC cells (Fig. 1D; middle panel). In addition to HCC cells, many granules in the infiltrated cells were also strongly Lcn2-positive in HCC tissues, even in the absence of Lcn2-positivity in tumor cells (Fig. 1D; bottom panel). To investigate whether the expression of Lcn2 in HCC cells is inducible or not, Huh7 HCC cells were treated with 5 ng/ml of inflammatory cytokines including interleukin-1ß (IL-1ß), TGFß, IL-2 and IL-4 for 24 h. Among them, IL-1ß elicited a strong induction of Lcn2 expression as assessed by RT-PCR and Western blot analysis (Fig. 1E).

Construction of Lcn2-expressing HCC cells. To assess the role of Lcn2 in hepatocarcinogenesis, Chang liver and SK-Hep1 cells that do not express Lcn2 were genetically manipulated to overexpress Lcn2 via lentiviral gene transfer. A lentiviral expression vector for Lcn2, pLenti-L6H, was constructed to express Lcn2 constitutively under the control of the cytomegalovirus promoter and, after processing of the leader peptide sequence, to secrete the mature form of the Lcn2 protein into the culture medium (Fig. 2A), as described previously (18). Lcn2 mRNA expression in the transduced Chang liver or SK-Hep1 cells (Chang-Lcn2 and SK-Lcn2, respectively) was determined by Northern blot analysis (Fig. 2B). In addition, the expression of the Lcn2 protein was assessed by Western blot analysis with an anti-Lcn2 antibody. An Lcn2-immunoreactive protein with an apparent molecular mass of ~25 kDa was detected in the culture supernatants of Chang-Lcn2 and SK-Lcn2 cells, respectively (Fig. 2C).

Ectopic expression of Lcn2 inhibits the proliferation, migration, and invasion of HCC cells. To determine whether Lcn2 expression in HCC cells affects their proliferation *in vitro* and *in vivo*, the *in vitro* growth kinetics of Lcn2-expressing and control Chang liver or SK-Hep1 cells was examined by determining the number of viable cells. Ectopic expression of Lcn2 significantly inhibited the *in vitro* proliferation of Chang liver (Fig. 3A) or SK-Hep1 (Fig. 3B) cells by 35.6% ($p<0.00002$) and 12.2% ($p<0.002$), respectively. The role of Lcn2 in the suppression of cell proliferation was confirmed by a similar inhibition of proliferation of Chang liver and SK-Hep1 cells caused by the addition of recombinant Lcn2 protein (data not shown). The effect of Lcn2 expression on anchorage-independent growth was determined in soft agar using SK-Hep1 cells (Fig. 3C). Colony forming efficiency in Lcn2-expressing cells (SK-Lcn2) was markedly reduced relative to parental cells (SK-Hep1) and cells transfected with expression vector (SK-Mock). When those cells were implanted subcutaneously into nude mice, tumor growth was suppressed in Lcn2-overexpressing tumors compared with control tumors and the growth suppression *in vivo* was signifi-

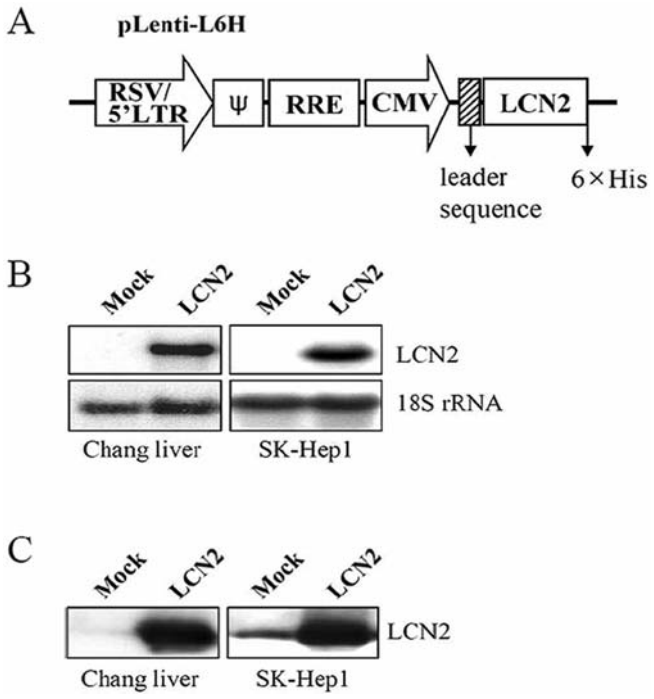

Figure 2. Generation of Lcn2-expressing HCC cells. (A) Schematic representation of the lentiviral expression vector for Lcn2 (pLenti-L6H). The Lcn2 gene including the leader peptide and hexa-histidine tag sequence is depicted. RSV/5' LTR, Rous Sarcoma virus enhancer/promoter and HIV-1 truncated 5'-long terminal repeat (LTR) promoter; Ψ, HIV-1 packaging signal; RRE, HIV-1 Rev response element; CMV, cytomegalovirus promoter. (B) Northern and (C) Western blot analyses of Lcn2 expression in Chang liver and SK-Hep1 cells transduced with either mock lentiviruses prepared using empty expression vector (Mock) or Lcn2-expressing lentiviruses (LCN2), respectively. Cells were transduced with the recombinant Lcn2-expressing lentiviruses, selected with blasticidin for more than 10 days, and analyzed. The culture supernatant was used for Western blot analysis.

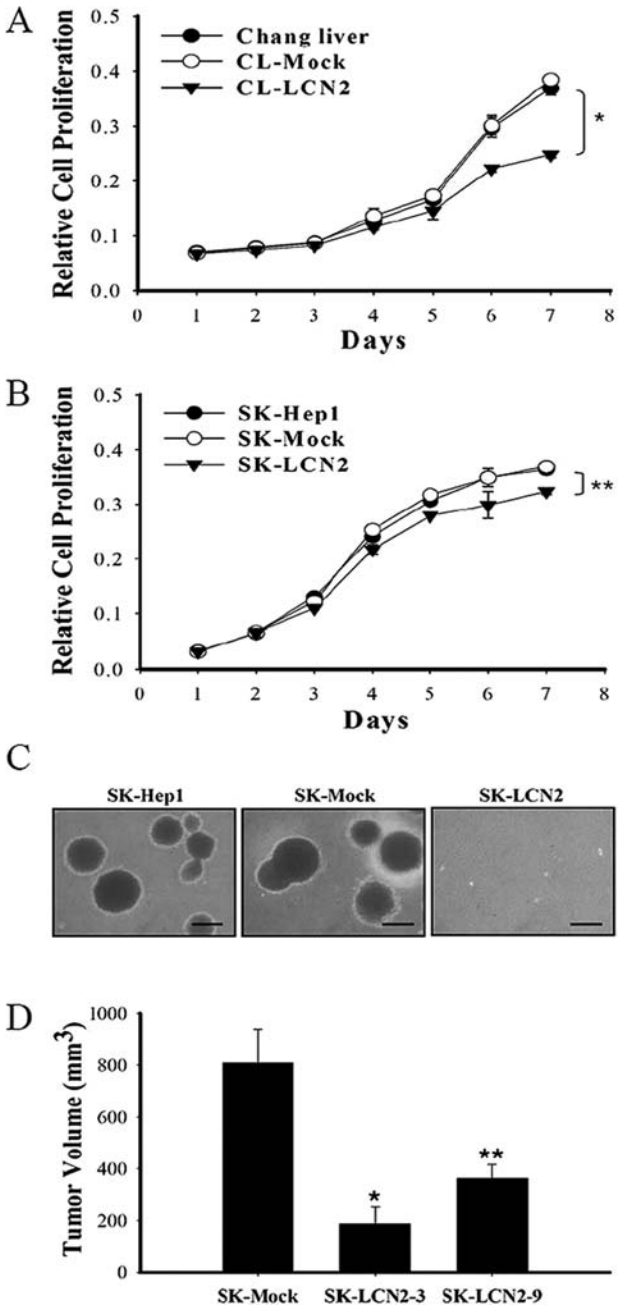

Figure 3. Inhibition of the proliferation of HCC cells *in vitro* and *in vivo* by Lcn2. Effect of Lcn2 expression on the *in vitro* proliferation of (A) Chang liver and (B) SK-Hep1 cells. The number of viable cells was determined at the indicated time points. Each experiment was performed in triplicate. The data shown were obtained from one experiment, which was representative of three separate experiments. Data are presented as mean ± SD. $^{*}p<0.00002$. $^{**}p<0.002$. (C) The colony formation assay in soft agar assessed the anchorage-independent growth of SK-Hep1, SK-Mock, and SK-LCN2 cells. Bar, 0.5 mm. (D) Effect of Lcn2 expression on the growth of subcutaneously implanted SK-Hep1 cells *in vivo*. Tumors were collected 47 days after implantation and the volumes were measured by using the formula width^2 x length x 0.52. SK-LCN2-3 and -9 are individual clones from SK-LCN2 with a high level of Lcn2 expression. Data are presented as mean ± SD. $^{*}p<0.002$. $^{**}p<0.05$.

cantly higher than that *in vitro*. Forty-seven days after implantation, the mean volumes (±SE) of tumors derived from SK-Mock and two different SK-Lcn2 clones (Clone no. 3 and no. 9) were 809±291, 224±143, and 365±105 mm^3, respectively (Fig. 3D).

To evaluate the role of Lcn2 on the migration and invasion of HCC cells, an *in vitro* wound migration assay and invasion assay through Matrigel were employed. The migration of Chang liver and SK-Hep1 cells was significantly inhibited by Lcn2-overexpression for 18 h (Fig. 4A). In addition, ectopic expression of Lcn2 in Chang liver and SK-Hep1 cells suppressed invasion by 27.6% and 34.1%, respectively, when stimulated with 10% serum (Fig. 4B and C), as well as EGF plus TGF-ß1 (data not shown).

Lcn2 overexpression inhibits the expression of MMP-2 in HCC cells. The expression levels of MMPs were assessed to determine whether Lcn2 expression suppresses the invasion of HCC cells by affecting the expression of MMPs. In Chang liver cells, MMP-2 was constitutively expressed while no apparent MMP-9 expression was detected. MMP-2 expression was significantly inhibited in the Lcn2-expressing Chang liver cells (Chang-Lcn2) compared to the control cells (Chang-Mock), as determined by Northern blot analysis. Western blotting and gelatin zymography with the culture supernatants also showed that ectopic expression of Lcn2 significantly reduced the levels of the 72 kDa pro-MMP-2 protein and its gelatinolytic activity in Chang liver cells. Similarly, Lcn2

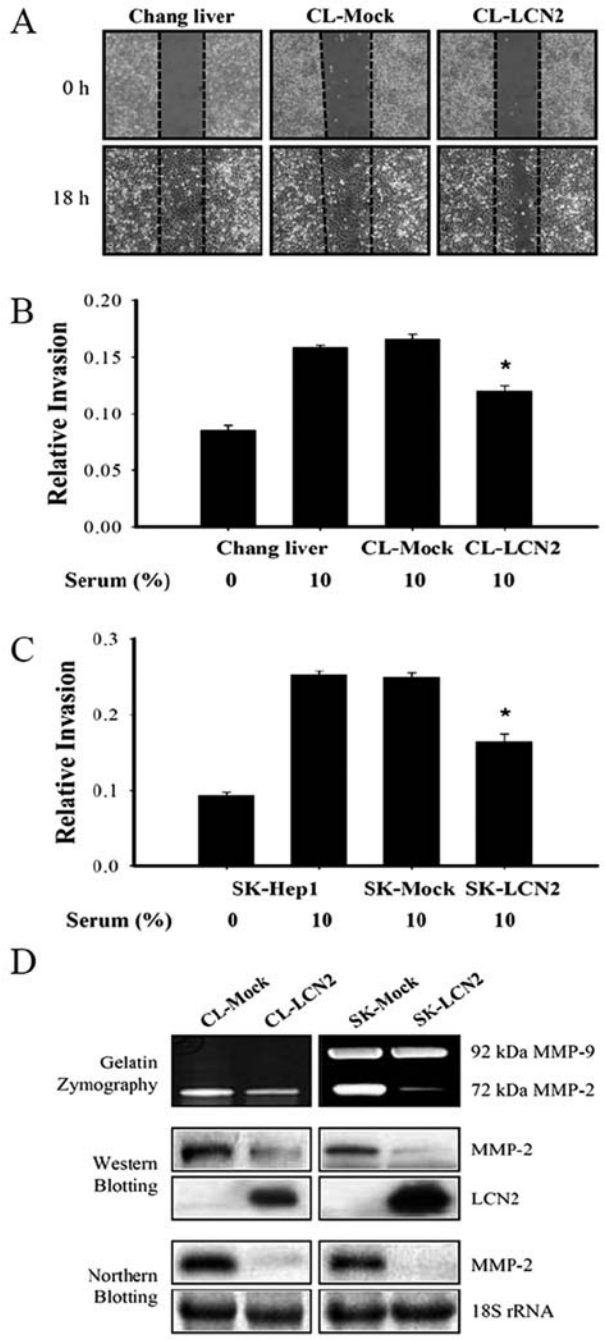

Figure 4. Inhibition of the migration, invasion, and MMP-2 expression of HCC cells by Lcn2. (A) Wound migration assay. Confluent cultures of parental, Mock and Lcn2-expressing Chang liver and SK-Hep1 cells (data not shown) were wounded by scraping, washed, and incubated for 18 h. The cells that migrated into the wounded area were photographed. (B) and (C) Matrigel invasion assay. Lcn2-expressing (B) Chang liver or (C) SK-Hep1 cells were assayed along with their control cells, as described in 'Materials and methods'. Each experiment was performed in triplicate. Data are presented as mean ± SD. $^{*}p<0.0005$ vs. CL-Mock or SK-Mock, respectively. (D) Inhibition of MMP-2 expression in Chang liver (left side) and SK-Hep1 (right side) cells by Lcn2, as assessed by gelatin zymography (top panel), Western blotting against Lcn2 or MMP-2 using the culture supernatant (middle panel), and Northern blotting with total cell RNA (bottom panel). 18S ribosomal RNA is used as a loading control. The data are from one experiment, which was representative of at least three separate experiments.

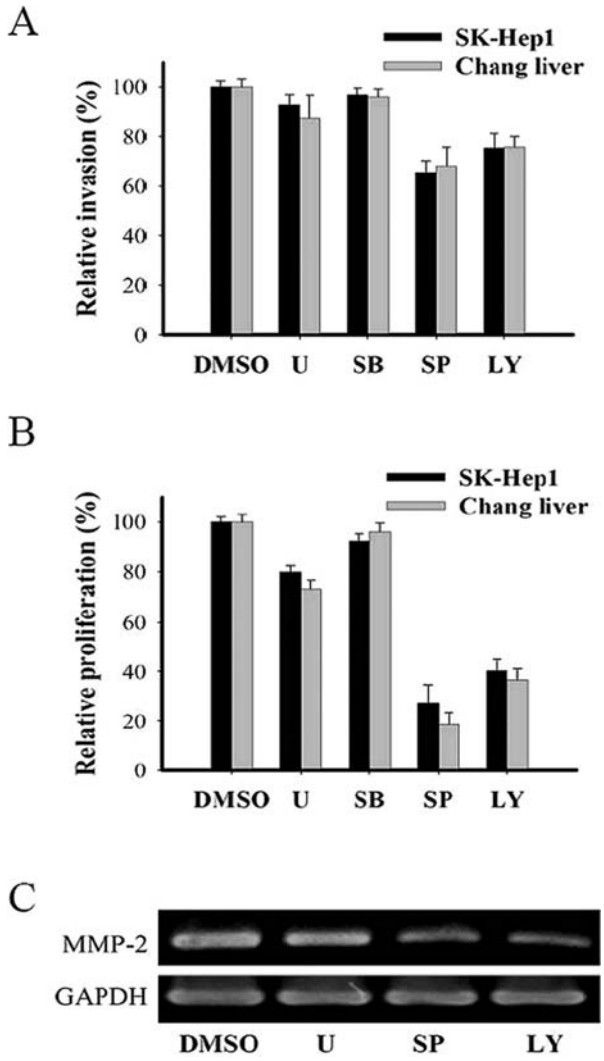

Figure 5. Role of JNK and PI3K/Akt signaling pathways in the proliferation, invasion, and MMP-2 expression in HCC cells. SK-Hep1 (■) and Chang liver (❑) cells were treated with the pharmacological inhibitors indicated and their effects on the (A) proliferation, (B) invasion through Matrigel, and (C) MMP-2 expression were determined as described in the 'Materials and methods' section. Each experiment was performed in triplicate. The data are from one experiment, which was representative of three separate experiments. Data are presented as mean ± SD. DMSO, vehicle control; U: U0126, an inhibitor of ERKs phosphorylation; SB: SB203580, an inhibitor of p38 MAPK; SP: SP600125, an inhibitor of JNK; LY: LY294002, an inhibitor of Akt.

expression in SK-Hep1 cells significantly inhibited the mRNA expression of the MMP-2 gene, MMP-2 protein expression, and MMP-2 activity, without significant effects on the expression of MMP-9 (Fig. 4D). Treatment with recombinant Lcn2 protein showed a similar inhibition of MMP-2 expression in both HCC cell lines (data not shown). Recently, Hanai *et al* (19) have reported that Lcn2 suppresses Ras-transformed 4T1 mouse mammary tumor cell invasiveness *in vitro* and that Lcn2 could facilitate the mesenchymal-epithelial transition by rescuing E-cadherin from proteasomal degradation. However, ectopic expression of Lcn2 in Chang liver and SK-Hep1 cells did not show any significant change in E-cadherin expression (data not shown).

JNK and PI3K/Akt signaling may play a critical role in the proliferation, MMP-2 expression, and invasion of HCC cells.

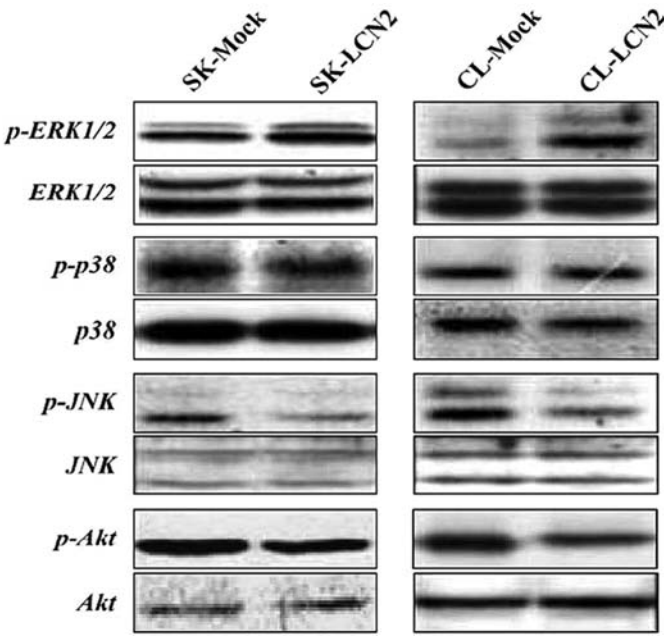

Figure 6. Inhibition of JNK and PI3K/Akt signaling by Lcn2 in HCC cells. Western blot analyses of the cell lysates prepared from Mock and Lcn2-expressing SK-Hep1 (left) or Chang liver cells (right) were performed to determine the effects of Lcn2 expression on the activation of each signaling pathway as indicated in the left side of the figure. Equal protein loading was verified using unphosphorylated forms of each signaling molecule. The data are from one experiment, which was representative of three separate experiments.

To identify the signaling pathway(s) involved in the proliferation and invasion of HCC cells, the effects of the following pharmacological inhibitors were tested on the proliferation and invasion of HCC cells: U0126 for ERKs, SP600125 for SAPK/JNKs, SB203580 for p38 MAPK, and LY294002 for PI3K/Akt, respectively. Inhibition of JNK and/or PI3K/Akt signaling pathways with SP600125 or LY294002, respectively, significantly inhibited the proliferation of HCC cells in a dose-dependent manner. Proliferation was inhibited by 81.3% and 63.5% of the vehicle control, respectively, in Chang liver cells and by 72.5% and 59.7%, respectively, in SK-Hep1 cells. On the other hand, suppression of ERK or p38 MAPK signaling with their respective inhibitors U0126 or SB203580 showed little or no significant difference in cell proliferation (Fig. 5A). Similarly, treatment with SP600125 or LY294002 showed a significant inhibition of invasion through Matrigel in Chang liver (by 31.7% and 24.2%, respectively) or SK-Hep1 cells (by 34.4% and 24.6%, respectively) compared to the vehicle control, whereas U0126 or SB203580 showed little impact on the invasion of HCC cells (Fig. 5B). Moreover, MMP-2 expression was also downregulated by the inhibition of JNK and/or PI3K/Akt, but not ERK signaling pathways (Fig. 5C). Collectively, these results indicate that the proliferation, MMP-2 expression, and invasion of HCC cells are regulated mainly through JNK and/or PI3K/Akt signaling pathways.

Lcn2 inhibits the activation of JNK and/or PI3K/Akt. Next, the effects of Lcn2 on the three subtypes of MAPK and PI3K/Akt signaling pathways were examined. Ectopic expression of Lcn2 in Chang liver and SK-Hep1 cells did not affect the phosphorylation of ERK1/2 and/or p38 MAPK (Fig. 6). Lcn2 functions by inhibiting JNK and/or PI3K/Akt signaling in HCC cells, as seen in the Western blot analysis showing the inhibition of JNK and Akt phosphorylation in Chang-Lcn2 and SK-Lcn2 cells relative to their respective control cells. These results suggest that the above described dependence of proliferation and invasion of HCC cells on the JNK and/or PI3K/Akt signaling pathways is mediated by an Lcn2-induced blockade of the phosphorylation of JNKs and/or PI3K/Akt.

Discussion

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide (20). The incidence is low in the US and high in locations such as Southeast Asia and sub-Saharan Africa. Despite the available treatment options including surgical resection, liver transplantation, and local ablation therapies, the incidence of HCC still nearly equals the mortality rate. This is because the majority of patients present with advanced or unresectable disease, and for those who undergo resection, the recurrence rates can be as high as 50% at 2 years. Although a large number of patients are eligible for systemic therapy, these treatments are minimally effective, can have significant toxicity, and have not been shown to improve patient survival (reviewed in ref. 21). Therefore, new approaches targeting molecular abnormalities specific to HCC are needed to improve patient outcome.

Recently, the number of gene expression profiling studies to define the molecular mechanism of hepatocellular carcinogenesis has increased. These studies have identified genes specifically upregulated or downregulated in HCC tissues and many of them can be used to develop novel diagnostic markers for early detection, prognostic markers for prediction of clinical outcome, and therapeutic targets for the intervention of tumor progression. These studies identified Lcn2 as a gene that is highly upregulated in HCC tissues (12,13). Consistent with prior reports, Lcn2 expression in HCC tissues was significantly upregulated relative to the matched non-tumor liver tissues in the present study. However, Lcn2 upregulation in HCC cell lines was not as significant as that found in HCC tissues, suggesting that the *in vivo* tumor microenvironment may play an important role in the expression of Lcn2 in HCC. Although the mechanism regulating the expression of Lcn2 in HCC cells *in vivo* is not fully understood, one possible explanation is that Lcn2 expression is not a part of the neoplastic process itself, but rather a phenotype induced in neoplastic cells by an accompanying inflammatory reaction. Supporting this hypothesis, the inflammatory cytokine IL-1ß induced Lcn2 expression in HCC cells (Huh7), in primary rat hepatocytes (22), and in human epithelial cells (23). In addition, chronic liver inflammation and hepatic regeneration induced in part by infection with hepatitis B or hepatitis C virus, and the consequent cellular immune responses, may increase the risk of HCC development by favoring the accumulation of genetic alterations in hepatocytes that might trigger specific oncogenic pathways (24). Moreover, Lcn2 has been suggested to function as a modulator of the inflammatory

response, based on findings such as the expression of Lcn2 in tissues exposed to microorganisms (10,25), its induction in murine macrophages by bacterial lipopolysaccharide (26), and the ability of Lcn2 to bind the N-formyl-Met-Leu-Phe peptide and other lipophilic inflam-matory mediators like platelet activating factor and leukotriene B4 (27).

Although several possible mechanisms of Lcn2 expression in HCC have been proposed, the role of Lcn2 on the growth and progression of HCC cells has not yet been elucidated. Lcn2 has been implicated in the induction of cellular proliferation because its expression is associated with a variety of proliferative cells (8-10,28,29). Lcn2 expression promotes breast tumor growth and progression (30-32), and is required for Bcr-Abl-induced tumorigenesis in leukemia cells (33). Lcn2 expression was also found to contribute to thyroid tumor cell survival (34), and to increase colon cancer migration and invasion (35). However, Lcn2 expression suppresses the invasion and liver metastasis of human colon carcinoma cells without affecting cellular proliferation *in vitro* or *in vivo* (14). In the present study, Lcn2 suppressed the proliferation, migration, and invasion of HCC cells *in vitro* and inhibited tumor growth *in vivo*. Similar to our results, Hanai *et al* reported that Lcn2 suppresses Ras-transformed 4T1 mouse mammary tumor cell invasiveness *in vitro* and tumor growth and lung metastases *in vivo* (19). A recent report also suggested that Lcn2 may act as a suppressor of invasion and angiogenesis in advanced pancreatic cancer cells (36). The mechanisms underlying the cell type-specific function of Lcn2 remain to be elucidated. A similar context-specific function has been reported for other genes, especially among the metastasis suppressor genes. For instance, although the mitogen activated protein kinase kinase 4-mediated activation of the JNK/p38 pathway suppresses metastasis in prostate and ovarian carcinomas (37), activation of the same pathway may be associated with malignant transformation of small-cell lung carcinomas (38). Similarly, connective tissue growth factor may have a role in the suppression of lung cancer metastasis (39), while its expression in breast, pancreatic, and skin cancers is associated with decreased disease-free survival (40,41). The findings of the present study will require further work and a more systematic approach to determine the molecular mechanisms that dictate the complexity of the patho-biological roles of Lcn2.

The intracellular signaling mechanism mediated by Lcn2 is not known. In the present study, the Lcn2-induced suppression of proliferation and invasion accompanied by inhibition of MMP-2 expression was mediated by an inhibition of the phosphorylation and activation of JNK and/or PI3K/Akt. Confirming this mechanism, the selective inhibition of either JNK or PI3K/Akt signaling using pharmacological inhibitors significantly prevented the proliferation and invasion through Matrigel *in vitro*, while slight or no significant inhibitory effects were observed in cells treated with ERK or p38 MAPK inhibitors. Consistent with our results, there have been several lines of evidence showing the role of JNK or PI3K/Akt signaling pathways in the proliferation and invasive potential of HCC cells (42-45).

The ratio of matrix metalloproteinase (MMP) to E-cadherin expression has been reported to increase with the pathological stage of disease and to have significant prognostic potential in non-small cell lung carcinoma, renal cell carcinoma, ovarian carcinoma, and prostate carcinoma (46-49). Expression of Lcn2 decreased MMP-2 production in HCC cells without affecting E-cadherin expression, resulting in a decreased MMP/E-cadherin ratio. However, a large clinical study is required to determine whether Lcn2 expression is associated with an improvement in the clinical outcome of HCC patients.

In conclusion, the results of the present study clearly demonstrate that Lcn2 inhibits the *in vitro* and *in vivo* proliferation, migration, invasion, and MMP-2 expression of HCC cells, in part by inhibiting JNK and/or PI3K/Akt signaling. Although the clinical significance of the present findings should be evaluated in the future, these data suggest that Lcn2 may play a protective role in the progression of HCC.

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