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MKK7 mediates miR-493-dependent suppression of liver metastasis of colon cancer cells

Hiroaki Sakai,[1] Ai Sato,[1] Yuki Aihara,[1,6] Yoshinori Ikarashi,[2] Yutaka Midorikawa,[3,7] Michael Kracht,[4] Hitoshi Nakagama[5] and Koji Okamoto[1]

[1]Division of Cancer Development System; [2]Central Animal Division, National Cancer Center Research Institute, Tokyo; [3]Teikyo University School of Medicine University Hospital, Kawasaki, Japan; [4]Rudolf Buchheim Institute for Pharmacology, Biomedical Research Center, Giessen, Germany; [5]Division of Refractory Cancer, National Cancer Center Research Institute, Tokyo, Japan

Key words

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Correspondence

Koji Okamoto, Division of Cancer Development System, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.

Tel: +81-3-3542-2511 (ext. 4550); Fax: +81-3-3542-2530; E-mail: kojokamo@ncc.go.jp

[6]Present address: Sysmex Corporation, Kobe, Japan.
[7]Present address: Department of Digestive Surgery, Nihon University School of Medicine, Tokyo, Japan.

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The prognosis of advanced colon cancer patients is profoundly affected by the presence or absence of liver metastasis. miR-493 functions as a potent suppressor of liver metastasis, and low-level miR-493 expression in human primary colon cancer is associated with an elevated incidence of liver metastasis. We previously showed that *IGF1R* is a target gene of miR-493, and that the inhibition of *IGF1R* partly explains how miR-493 suppresses liver metastasis. However, major functional targets that mediate the antimetastatic activity of miR-493 remain elusive. Here, we extended our search for target genes and identified *MKK7*, a mitogen-activated protein kinase kinase, as a novel target of miR-493. miR-493 inhibits MKK7 expression by targeting the binding site at the 3′-UTR of the *mkk7* gene. MKK7 was expressed in six out of seven colon cancer cell lines examined but not in non-transformed colon epithelial cells, and its expression was required for the activating phosphorylation of JNK. RNA interference-mediated inhibition of MKK7 resulted in marked suppression of liver metastasis of colon cancer cells. A significant decrease of metastasized cells by the MKK7 knockdown was observed, even at early stages of the metastatic settlement, in accordance with a time course of the miR-493-mediated inhibition of the metastasis. Immunohistochemical examination in human primary colon tumors revealed that the occurrence of liver metastasis is associated with elevated levels of MKK7. Thus, *MKK7* is a major functional target of miR-493, and its suppression thwarts liver metastasis of colon cancer cells.

The lethality of colon cancer is mainly caused by distant metastasis, especially in the liver.[1] Overall, chemotherapy or radiotherapy has only limited effects on liver metastasis, and surgical resection, if possible, is still the main option to cope with it.[2] Hence, a novel therapeutic strategy is needed to fight liver metastasis.

MicroRNA (miRNA) is short RNA (~22 nt) that, in association with other associated proteins, inhibits multiple target genes by regulating their mRNA stability and/or translation.[3,4] miRNA is differentially expressed during development of many types of cancer,[5] including colon cancer.[6] miRNAs are shown to regulate many aspects of cancer development, including the metastatic processes,[7,8] and hence regarded as molecules that may be used for innovative cancer therapy as well as for diagnostic tools.[9]

In our previous study, we carried out a lentivirus-based functional screening for antimetastatic miRNAs,[10] and identified miR-493 as an miRNA whose overexpression blocks formation of metastatic foci of colon cancer in the liver.[11] The inhibition of liver metastasis by miR-493 may be clinically relevant, as a high level of miR-493 expression in primary colon cancer is inversely related to the presence of liver metastasis. The inhibitory effect of miR-493 on liver metastasis was clearly observed even at early phases of liver metastasis, indicating that miR-493 blocks initial settlement of cancer cells in liver mesenchyme. Subsequently, insulin-like growth factor 1 receptor (*IGF1R*) was identified as a direct target of miR-493. Although the inhibition of *IGF1R* partly suppressed liver metastasis,[11] the inhibitory effect by *IGF1R* knockdown was modest in comparison to that by miR-493 expression. These suggest that there are unknown target genes of miR-493 that mediate its antimetastatic function.

In this paper, we looked for unknown target genes of miR-493, in order to develop a full picture of miR-493-mediated inhibition of liver metastasis. We looked for direct targets of miR-493, and identified *MKK7* as a novel target of miR-493. In the following experiments, we showed that MKK7 plays an important role in regulation of liver metastasis of colon cancer cells.

Materials and Methods

Cell lines and plasmids. All colon cancer cell lines and their derivatives were cultivated in DMEM supplemented with 10% FBS. A luciferase reporter for the 3′-UTR of MKK7

(MKK7-UTR) was created by inserting a 469-bp 3′-UTR DNA fragment spanning the predicted miR-493 target site into the 3′ end of Renilla luciferase of psiCHECK-2 (Promega, Madison, MA, USA). A luciferase reporter with a mutated 3′-UTR was generated by substituting five nucleotides at the miR-493 target site. A lentiviral vector that expresses MKK7 was generated by inserting *myc*-tagged mouse MKK7-β1[12] into pLenti6/V5-DEST. Lentiviruses were produced in 293T cells as previously described.[11]

Liver metastasis assays. HCT116/GFP or DLD-1/GFP (2×10^5 cells)[11] were transfected with 6 pmol siRNA (Qiagen; Invitrogen, Maryland, MD, USA) or 4 pmol miRNA mimics (pre-miR miRNA precursors; Ambion, Carlsbad, CA, USA) in the presence of 20 μL HiPerFect (Qiagen, Hilden, Germany) for 2 days. Subsequently, the GFP-expressing cells were mixed with HCT116/red fluorescent protein (RFP) or DLD-1/RFP at a 1:1 ratio, suspended in normal growth medium containing 50% Matrigel (BD Biosciences, Bedford, MA, USA), and liver metastasis assays were carried out as previously described.[11] A fraction of the cell mixture before the injection was evaluated by flow cytometry to measure original ratios of GFP-expressing cells/RFP-expressing cells. Two to 10 days after splenic injection, the inhibitory effects of siRNA or miRNA on liver metastasis were evaluated by counting ratios of GFP-positive foci *versus* RFP-positive foci by *in vivo* imaging (OV110; Olympus, Tokyo, Japan), or by measuring the ratios of the number of GFP- or RFP-expressing cells by flow cytometry (FACSCalibur; BD Biosciences). All mouse procedures were carried out according to the Guidelines for Animal Experiments, approved by the committee for ethics of animal experimentation of the National Cancer Center (Tokyo, Japan), and carried out in accordance with institutional policies.

Luciferase reporter assays. A dual-luciferase reporter construct with or without the 3′-UTR of MKK7 was transfected into HCT116/GFP cells in the presence of a miR-493 mimic or its control, and Firefly and Renilla luciferase activities were measured by the Dual-Luciferase Reporter System (Promega) 2 days after transfection.

Immunohistochemical analysis. Tumor tissues were resected from patients with informed consent at the Teikyo University Hospital (Kawasaki, Japan), and all procedures were carried out under the protocol approved by the Ethics Committee of Teikyo University Hospital. Freshly frozen samples of human primary colon tumors were sectioned, fixed in acetone, and immunostained with anti-MKK7 antibody (clone 2G5; Abnova, Taipei City, Taiwan). Staining with the secondary antibody and the detection steps were carried out as previously described.[11] The extent of the staining was visually evaluated on a scale of 1 (no staining) to 4 (strong staining). Approximately 800 cells were evaluated for each sample by two observers, and the mean value for each staining was calculated.

Western analysis. Cells were lysed in RIPA buffer and used for Western blot analyses as previously described,[11] with anti-IGF1R (Cell Signaling, Danvers, MA, USA), anti-actin (Sigma, St. Louis, MO, USA), anti-JNK (clone 56G8; Cell Signaling), anti-phospho-JNK (Thr183/Tyr185) (81E11; Cell Signaling), anti-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-MKK7 (10F7; Santa Cruz Biotechnology) antibody.

Statistical analysis. The other data were analyzed using the two-tailed Student's *t*-test. All data are presented as the mean ± SD.

Results

***MKK7* is a novel target of miR-493.** Our previous study showed that miR-493 directly targets IGF1R, and the inhibition of IGF1R induces cell death of metastatic cells and suppresses liver metastasis of colon cancer cells.[11] Because the extent of the suppression by IGF1R inhibition is modest in comparison to that caused by miR-493, it is likely that an unknown target gene(s) plays important roles on the inhibition of metastasis by miR-493.[11] In order to look for crucial target genes of miR-493, we used a searching criterion that differs from the one used in our previous studies: statistical significance ($P < 0.05$), instead of twofold cut-off, was used to select candidate genes through expression analyses of miR-493-transfected HCT116 colon cancer cells (Fig. 1a). We combined the new criterion from the expression studies with the targets predicted from a combination of *in silico* programs (Targetscan, PITA, miRanda).[11] The examination of overlapped genes between the criterion and the predicted targets led to identification of 16 candidates as potential targets of miR-493 (Fig. 1a), whereas only eight genes were identified if the previous criterion with a twofold cut-off was applied.[11]

A search of published reports for the 16 candidate genes indicated that some are linked to regulation of cell death, suggesting that they may mediate miR-493-dependent inhibition of liver metastasis. Western blot analyses of these genes revealed that introduction of miR-493, but not its star form,[10] into HCT116 colon cancer cells inhibited expression of MKK7 (Fig. 1b). Similarly, induction of miR-493 specifically inhibited MKK7 expression in another colon cancer cell line, DLD-1 (Fig. 1c). A miR-493 target site is located at the 3′-UTR region of the *MKK7* gene (Fig. 1d), and the luciferase reporter assay indicated that the 3′-UTR region is responsible for the miR-493-mediated inhibition (Fig. 1e). Notably, the mutation of the miR-493 target site largely neutralized the inhibition by miR-493 (Fig. 1e). These results indicate that *MKK7* is a novel target gene of miR-493.

MKK7 expressed in colon cancer cells and involved in activating phosphorylation of JNK. Next, we examined MKK7 expression in colon cancer cell lines. Western blot analyses showed that MKK7 was expressed in six out of seven colon cancer cell lines examined (DLD-1, HCT116, HT29, RKO, SW480, and SW620), whereas the level of expression in non-transformed FHC colon epithelial cells was much lower than those in the cancer cells (Fig. 2a).

MKK7 is involved in activating phosphorylation of JNK.[13–15] Because JNK activation affects many aspects of cancer development, including metastatic processes,[16] it is possible that MKK7 regulates liver metastasis by phosphorylating JNK at the regulatory site. Therefore, we were interested in determining whether MKK7 inhibition abolished activating phosphorylation of JNK (Thr183/Tyr185) in our experimental settings. We did not observe significant phosphorylation of JNK under standard culture conditions, however, treatment of HCT116 cells with sorbitol, which can activate the MKK7–JNK pathway,[13] induced phosphorylation of JNK. Notably, siRNA-mediated inhibition of MKK7 markedly inhibited the phosphorylation of a major form of JNK (Fig. 2b). In accordance with this, MKK7 inhibition by miR-493 suppressed the phosphorylation of JNK (Fig. S1). Thus, our data indicate that MKK7 is expressed in most colon cancer cell lines, and that inhibition of MKK7 by miR-493 blocks the activating phosphorylation of JNK.

Inhibition of MKK7 suppresses liver metastasis of colon cancer cells. Identification of *MKK7* as a novel target gene of miR-493

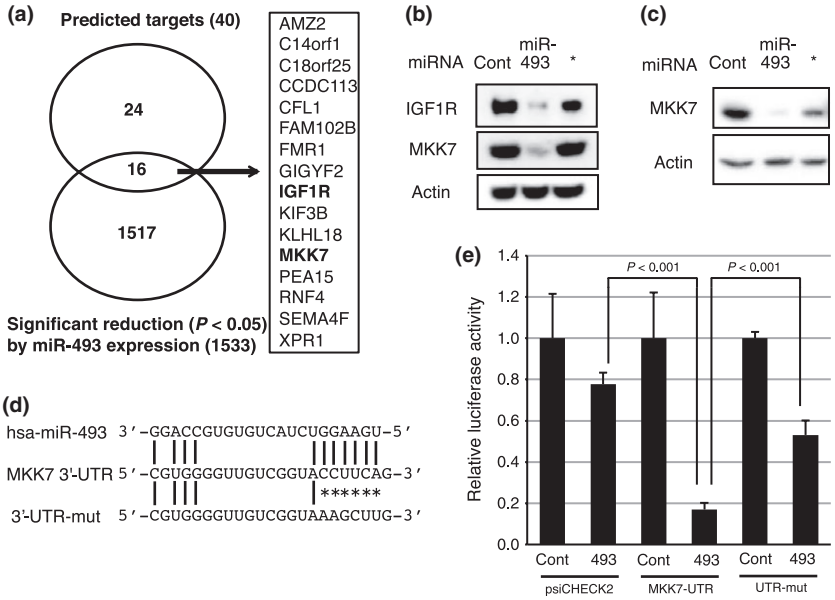

Fig. 1. *MKK7* is a novel target of miR-493. (a) Two-cycle Venn diagram that shows the overlap of predicted target genes of miR-493 (40 genes) and genes inhibited by miR-493 expression (1533 genes). (b, c) Inhibition of *MKK7* by miR-493. HCT116 cells (b) or DLD-1 cells (c) were transfected with the indicated miRNA mimics, and used for Western blot analyses with the indicated antibodies. Asterisk, a star form of miR-493; cont, control. (d) Sequence comparison of MKK7 3′-UTR (positions 2052–2074; mRNA RefSeq, NM_145185.2), mutated (mut) MKK7 3′-UTR, and human (hsa) miR-493. *Mutated nucleotides. (e) Transient luciferase assays. The indicated reporter plasmid was transfected into HCT116 cells together with control oligo (Cont) or miR-493 (493), and luciferase activity was measured 2 days after transfection.

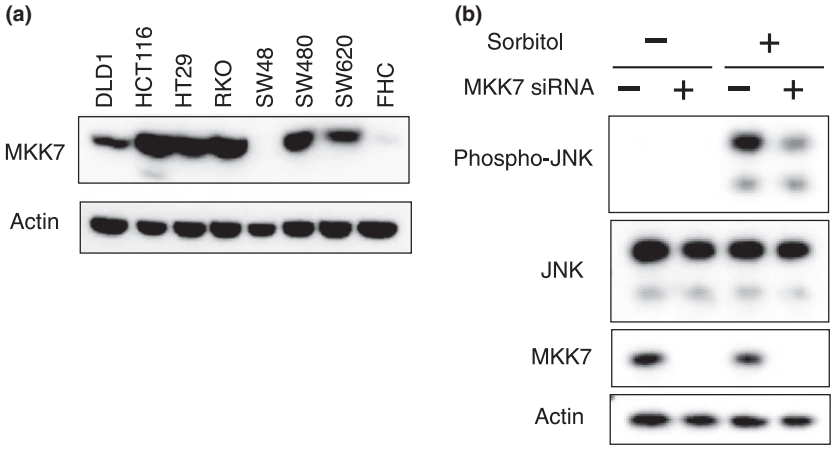

Fig. 2. MKK7 is expressed in colon cancer cells and involved in activating phosphorylation of JNK. (a) Western blots of MKK7 for the indicated colon cells. (b) Inhibition of JNK phosphorylation by MKK7 siRNA. HCT116 cells were transfected with control or MKK7 siRNA. Two days after transfection, the cells were treated with 0.5 M sorbitol or buffer for 30 min, and used for Western blot analyses with the indicated antibodies.

prompted us to examine whether MKK7 is involved in liver metastasis of colon cancer cells. We inhibited MKK7 expression in GFP-expressing HCT116 cells[11] by siRNA-mediated knockdown (Fig. 3a), and after mixing them with an equal number of the RFP-expressing HCT116 cells, injected the mixture of these cells into the spleen of immunocompromized NOG mice. Ten days after the splenic injection, formation of metastatic foci of both GFP-expressing and RFP-expressing cells was confirmed by dual fluorescence imaging (Fig. S2, left panel). Subsequently, the transfected HCT116/GFP and HCT116/RFP cells were recovered from the metastasized liver, and the ratio of the GFP cells *versus* the RFP-positive internal control cells was calculated by flow cytometry to determine the inhibitory effect of each oligonucleotide on liver metastasis.[11] Two different siRNAs that correspond to MKK7 were used (Fig. 3a), and in both cases the knockdown of MKK7 caused significant reduction (~60%) of liver metastasis in comparison to the control siRNAs (Fig. 3b).

The mixture of the siRNA-introduced GFP-expressing cells and the RFP-expressing cells was also used to examine whether the knockdown of MKK7 showed an inhibitory effect under standard growth conditions *in vitro* as well as under metastatic conditions. Under the *in vitro* conditions (cultivated for 10 days), the extent of the growth inhibition by MKK7 knockdown was relatively modest (20–30%) (Fig. S3). Considering that the growth rate under *in vitro* conditions is obviously faster than in metastasized liver, it is likely that the inhibitory effects under the *in vitro* growth conditions are overestimated in comparison to those under the conditions of liver metastasis. These data indicate that the inhibitory effects of the MKK7 knockdown on liver metastasis do not merely reflect the effects on cell growth/survival *in vitro*, and suggest that the inhibitory effects of the MKK7 knockdown is specific to the growth/survival in the liver microenvironment.

In order to determine whether the inhibitory effect of the MKK7 knockdown on liver metastasis of HCT116 cells is also observed in other colon cancer cells, we examined another colon cancer cell line, DLD-1. The siRNA-mediated knockdown of MKK7 was carried out in GFP-expressing DLD-1 cells (Fig. 3c), and the mixture of the GFP-expressing and RFP-expressing cells was injected into the spleen of NOG mice to generate liver metastasis. In contrast to HCT116 cells, whose metastatic foci vigorously proliferate, metastasized DLD-1 cells did not proliferate into large foci (data not shown), and it was technically difficult to recover enough metastasized DLD-1 cells from the liver by flow cytometry.

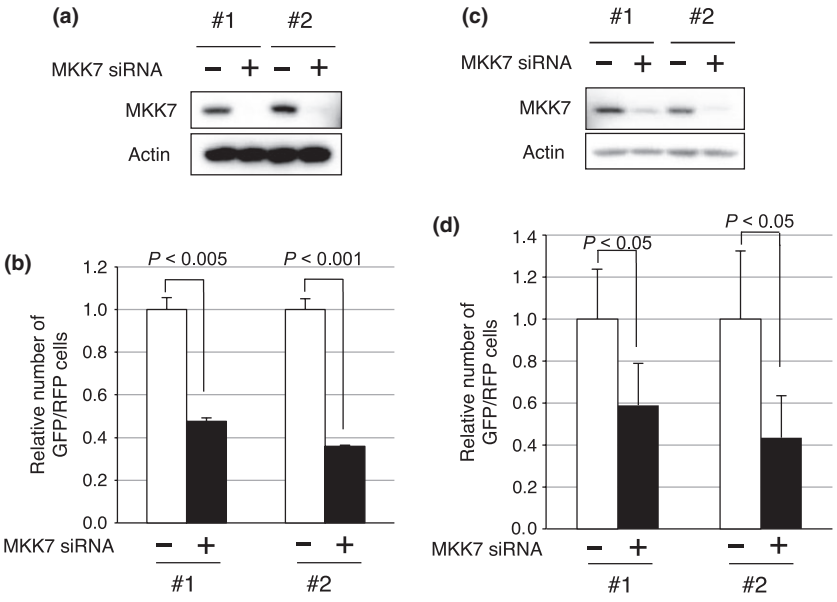

Fig. 3. Inhibition of MKK7 suppresses liver metastasis of colon cancer cells. (a, c) Western blot analyses of siRNA-transfected cells. HCT116/GFP cells (a) or DLD-1/GFP cells (c) were transfected with control or MKK7 siRNA. #1 and #2 designate a set of siRNAs from Invitrogen and Qiagen, respectively. The transfected cells were used for Western blot analyses with anti-MKK7 or anti-actin antibody. (b, d) Inhibition of liver metastasis by MKK7 siRNA. HCT116/GFP cells or DLD-1/GFP cells were transfected with the indicated siRNA, and the mixtures of the transfected HCT116/GFP cells and HCT116/red fluorescent protein (RFP) cells were injected into the spleen. (b) Ten days after the splenic injection of the mixture of the cells, metastasized cells were isolated from the liver and the number of GFP-positive and RFP-positive cells were counted by flow cytometry. The inhibitory effect of each siRNA was evaluated by calculating the GFP/RFP ratio. (d) Ten days after the splenic injection of a mixture of DLD-1/GFP and DLD-1/RFP cells, GFP/RFP dual fluorescence images for metastasized liver were taken. The inhibitory effect was evaluated by counting the number of GFP/RFP fluorescent foci.

Hence, in order to evaluate the effect of the MKK7 knockdown in DLD-1 cells, we instead counted the number of metastasized foci, as carried out in our previous studies.[11] The counting of the GFP- and RFP-positive foci revealed that MKK7 knockdown significantly inhibited the foci formation in the liver (Fig. 3d). Taken together, these data indicate that MKK7 inhibition suppresses liver metastasis in at least two different colon cancer cells.

Previously, we showed that IGF1R suppression mediates miR-493-dependent inhibition of liver metastasis. In order to determine whether inhibition of both IGF1R and MKK7 synergistically suppress liver metastasis, we inhibited these targets alone or in combination in the metastasis assays shown in Figure 3(b). The simultaneous inhibition of both genes did not result in synergistic inhibition of liver metastasis (Fig. S4), suggesting that the metastasis-regulatory functions of these genes are redundant, converging on the same pathway that leads to the blockage of metastatic settlement.

Inhibition of MKK7 blocks liver settlement of metastasized cells. It was shown that miR-493 expression in HCT116 cells blocks settlement of the metastasized cells in the liver, at least in part, by inducing cell death at liver mesenchyme.[11] Hence, we examined whether the inhibition of liver metastasis is observed during the early phase of mesenchymal settlement of metastatic cells by MKK7 inhibition, as well as by miR-493 introduction. The GFP-expressing HCT116 cells were transfected with either the control or MKK7 siRNAs (Fig. 4a, upper panel), mixed with the RFP-positive cells and then used to generate metastatic foci in the liver, as we did in experiments shown in Figure 3. Two or 4 days after the splenic injection, the presence of the metastatic cells in liver mesenchyme was visualized by imaging (Fig. S2, right panel), and the GFP- and RFP-positive cells were counted to calculate the extent of the inhibition of metastasis. In accordance with the time course by miR-493 expression,[11] inhibition of MKK7 suppressed the settlement of metastatic foci by day 4 (Fig. 4a, lower panel). Thus, the inhibition of MKK7 blocks formation of metastatic foci during the early phase of the mesenchymal settlement.

In order to confirm that MKK7 mediates the suppression of liver metastasis by miR-493, HCT116/GFP cells were infected with lentiviruses that express exogenous MKK7 (Fig. 4, left panel). The introduced MKK7 was functionally active, as its overexpression in 293T cells was capable of inducing the activating phosphorylation of JNK (Fig. S5). The lentiviral introduction of MKK7 partially rescued the inhibition of liver metastasis by miR-493 (Fig. 4, right panel). Collectively, our data indicate that MKK7 serves as one of the major downstream targets of miR-493.

High levels of MKK7 in colon cancer are associated with liver metastasis. In light of the functional association of MKK7 to metastasis (Figs 3,4), we examined whether levels of MKK7 are associated with the presence of liver metastasis in clinical specimens. Human primary tumors with or without synchronous liver metastasis were immunostained with MKK7, and the extent of the staining was quantified (Fig. 5a). The comparison of the staining indicated that MKK7 was expressed in specimens with liver metastasis at levels higher than those without metastasis (Fig. 5b). These data are consistent with the role of MKK7 as a regulator of liver metastasis.

Discussion

Development of liver metastasis of colon cancer, like other types of distant metastasis, is a result of a series of complex biological events that cancer cells have successfully gone through.[17,18] Among the series of events, the settlement of metastatic cells into liver mesenchyme is regarded as one of the crucial steps for clinical intervention.[19] However, the detailed mechanisms of the metastatic settlement of cancer cells remain largely unclear.

miR-493 expression in colon cancer blocks formation of metastatic foci and thus prevents liver metastasis of colon cancer cells.[11] Previously, we searched for its target genes to understand the mechanism of how miR-493 prevents liver metastasis, and found that *IGF1R* was a direct target whose expression was suppressed by miR-493.[11] However, the extent of inhibition of liver metastasis caused by IGF1R inhibition was less striking than that by miR-493 expression. Hence, it is likely that there are other target genes that mediate inhibition of liver metastasis by miR-493.

Through the extended screening, we identified *MKK7* as a novel target of miR-493. MKK7 expression was observed in

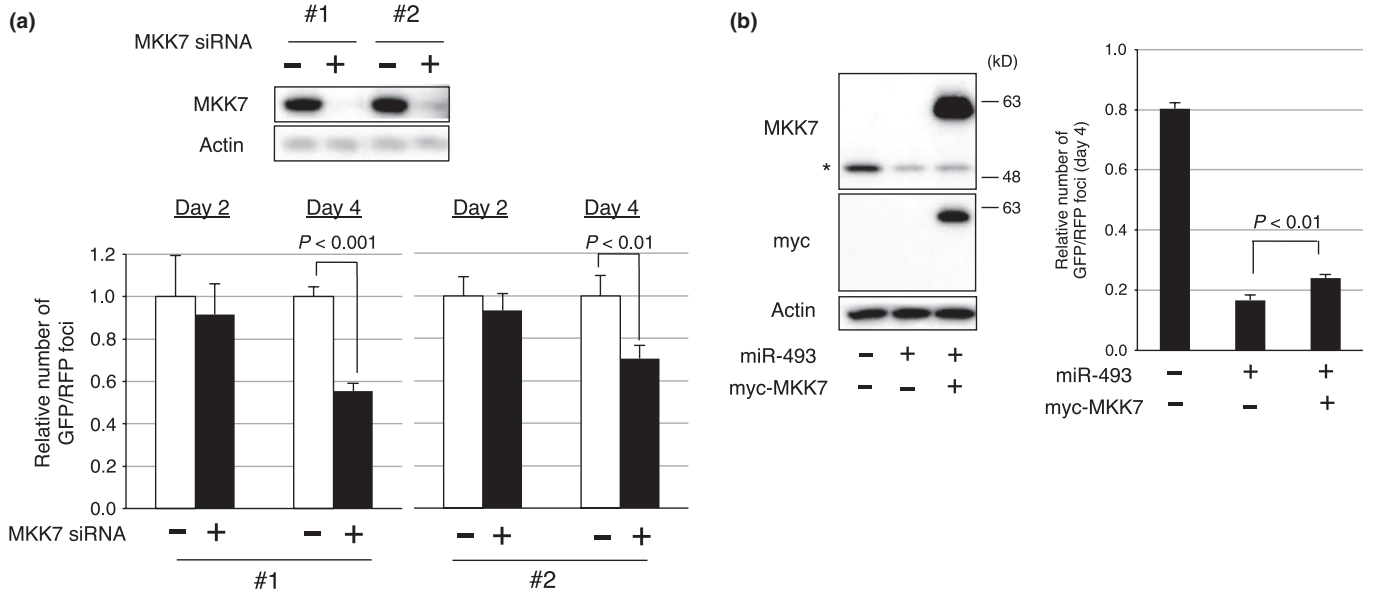

Fig. 4. MKK7 inhibition blocks liver settlement of metastasized cells. (a) Upper panel, Western blot analyses of siRNA-transfected cells: #1, a set of siRNAs from Invitrogen; #2, a set of siRNAs from Qiagen. Bottom panel: Two or 4 days after the splenic injection of a mixture of HCT116/GFP and HCT116/RFP cells, GFP/RFP dual fluorescence images for metastasized liver were taken. The inhibitory effect was evaluated by counting the number of GFP/RFP fluorescent foci. (b) Left panel, Western blot analyses of control or miR-493-transfected HCT116/GFP cells after the lentiviral introduction of *myc*-tagged MKK7-β1. The control or MKK7-introduced cells were transfected with the control oligo or miR-493, and used for Western blot analyses. *A major form of endogenous MKK7, which presumably represents the γ form of MKK7 based on its molecular weight. Right panel, a partial rescue of miR-493-mediated inhibition of liver metastasis by expression of exogenous MKK7. The transfected cells were mixed with HCT116/RFP, and used to generate liver metastasis. The ratio of GFP/RFP-positive foci was calculated at day 4.

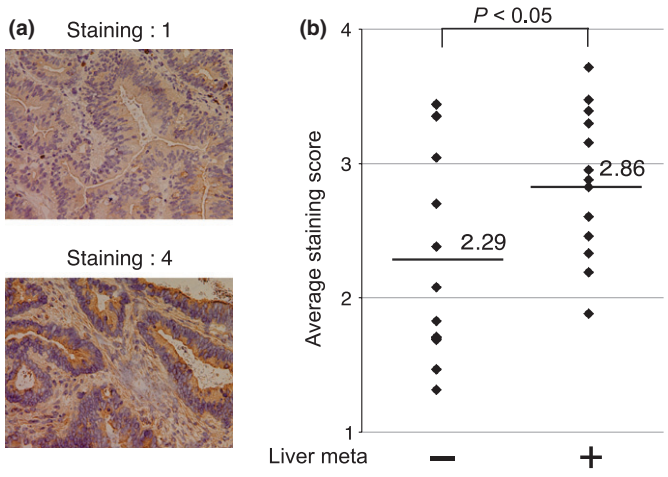

Fig. 5. High levels of MKK7 in colon cancer are associated with liver metastasis. (a) representative immunostaining of MKK7 (grades 1 and 4) (b) Levels of MKK7 immunostaining of primary colon tumor without metastasis (meta) (−) or with synchronous metastasis (+).

most colon cancer cells examined (Fig. 2a), and inhibited by miR-493 in two colon cancer cells, through the binding site located at the 3′-UTR (Fig. 1e). Similar to miR-493 expression, MKK7 inhibition reduced the metastatic settlement at early phases of foci formation (Fig. 4a). These data are in agreement with our observation that *MKK7* is a direct target of miR-493 that mediates the antimetastatic function of the miRNA.

Notably, inhibition of MKK7 inhibited >50% of liver metastasis of HCT116 cells (Fig. 3b), and a significant reduction of liver metastasis was observed in DLD-1 cells (Fig. 3d). Thus, the inhibition of MKK7 plays a major role in miR-493-mediated suppression of liver metastasis. The metastasis-stimulating effect of MKK7 overexpression in miR-493-transfected cells was rather moderate (Fig. 4b), as was the case with IGF1R.[11] Combined with the results presented in Figure S4, our data suggest that major targets of miR-493, such as MKK7 and IGF1R, are redundant in the metastasis-suppressive functions.

MKK7 is a mitogen-activated protein kinase kinase that is activated in response to a variety of cellular stresses.[20] Activated MKK7, together with MKK4, phosphorylates and activates downstream kinase, JNK.[21,22] Both MKK4 and MKK7, by preferentially phosphorylating different residues in the activating loop, synergistically activate JNK.[21,22] Activated JNK in turn phosphorylates a variety of target proteins that include transcription factors (e.g. c-Jun, ATF-2, Elk-1, p53, and c-Myc) and other proteins.[20]

MKK7, as well as its downstream kinase JNK, is reported to play a context-dependent and cell type-specific role in cancer development.[16,20,21] This is likely to be explained by its plethora of downstream targets, and context-dependent phosphorylation and functions of the targets dictate the effect of altered activity of the MKK7–JNK kinase cascade. Our data suggest that MKK7 activation, and presumably the following activation of downstream JNK, are required for metastatic settlement of colon cancer cells in the liver, and inhibition of MKK7 leads to reduced metastatic foci. In future, the potential importance of the MKK7–JNK cascade for prevention of liver metastasis will be evaluated.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Inhibition of the activating phosphorylation of JNK kinase by miR-493.

Fig. S2. Inhibition of liver metastasis by MKK7 siRNA.

Fig. S3. Inhibition of *in vitro* cell growth by MKK7 siRNA.

Fig. S4. Simultaneous inhibition of insulin-like growth factor 1 receptor (IGF1R) and MKK7 by the corresponding siRNAs.

Fig. S5. Induction of activating phosphorylation of JNK kinase by MKK7 overexpression.