Hepatic stellate cells secrete *WFA*$^{+}$-M2BP; its role in biological interactions with Kupffer cells

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Abstract

Background and aims

Hepatic stellate cells (HSCs) play a central role in hepatic fibrosis, and are regulated by Kupffer cells (KCs). *Wisteria floribunda agglutinin*-positive Mac-2 binding protein (WFA^+-M2BP) was recently identified as a serum marker for hepatic fibrosis. Although WFA^+-M2BP was identified as a ligand of Mac-2, the function of WFA^+-M2BP in hepatic fibrosis remains unclear.

Methods

Liver specimens were obtained from five patients with cirrhosis, five with chronic hepatitis, and five without hepatic fibrosis. WFA^+-M2BP kinetics were evaluated histologically and in subpopulations of liver cells such as HSCs, Kupffer cells, endothelial cells, biliary epithelial cells, and hepatocytes in *in vitro culture*. The function of WFA^+-M2BP in activated HSCs was evaluated using immunoblot analysis.

Results

Numbers of WFA^+-M2BP-positive cells in liver tissues increased with fibrosis stage. There were significant differences in WFA^+-M2BP levels between fibrosis stages F0 and F1–2 ($P = 0.012$), and between fibrosis stages F1–2 and F3–4 ($P < 0.001$). HSCs were the source of WFA^+-M2BP secretion in *in vitro* cultures of liver cells, as determined by sandwich ELISA. Cells of the human HSC line LX-2 also secreted WFA^+-M2BP. Histologically, tissue sections showed that WFA^+-M2BP was located in Mac-2 expressing KCs. *In vitro* assays showed exogenous WFA^+-M2BP stimulation enhanced Mac-2 expression in KCs, and that HSCs co-cultured with KCs increased α-SMA expression. Finally, Mac-2-depleted KCs with siRNA had reduced α-SMA expression following coculture with HSCs.

Conclusions

WFA^+-M2BP from HSCs induces Mac-2 expression in KCs, which in turn activates HSCs to be fibrogenic.

Keywords: WFA^+-M2BP; Mac-2; hepatic stellate cells; Kupffer cells; M2BPGi

Introduction

Hepatic fibrosis can progress to liver cirrhosis (LC), which is frequently accompanied by complications such as gastrointestinal bleeding, portosystemic encephalopathy, ascites and hepatocellular carcinoma. An improved understanding of the molecular mechanisms involved in hepatic fibrosis may facilitate the development of targeted antifibrotic therapies before hepatic fibrosis becomes severe.

Activation of HSCs is the central event in hepatic fibrogenesis. HSCs are activated in response to liver injury, whereby they assume a more contractile, myofibroblast-like phenotype that is characterized by excessive production of α-smooth muscle actin (α-SMA) and other extracellular matrix (ECM) proteins(1, 2). HSCs also produce cytokines that affect the differentiation and phenotype of immune cells such as Kupffer cells (KCs), B cells, T cells, and natural killer cells (3, 4).

In hepatic fibrosis, Kupffer cells (KCs)—which are resident liver macrophages—localize in close proximity to HSCs to regulate their activation (5). During fibrogenesis in mice, KCs express profibrotic cytokines such as transforming growth factor-β, platelet-derived growth factor-BB and Mac-2 (3, 5). Of these, Mac-2 is a key factor driving HSC activation and ECM production during hepatic fibrosis(6). A clinical trial centered on neutralizing Mac-2 for treatment of hepatic fibrosis is currently underway (7). However, while Mac-2 binding protein was first identified as a ligand of Mac-2, the impact on hepatic fibrosis of the interaction between these two proteins remain unclear.

Wisteria floribunda agglutinin-positive Mac-2 binding protein (WFA^+-M2BP) is a serum marker for hepatic fibrosis that indicates the extent of hepatic fibrosis in patients with CLD (8, 9) resulting from HCV infection (10, 11), NAFLD (12), primary

biliary cholangitis (13), and autoimmune hepatitis (14). WFA^+-M2BP comprises the glycoprotein M2BP in conjunction with *N*-glycan residues that are bound by WFA. However, the exact mechanism underlying the correlation between increasing serum WFA^+-M2BP and progression of hepatic fibrosis remains unknown.

Our current work takes advantage of our ability to obtain primary human liver composite cells—including HSCs and KCs—and use these cells to examine the cellular source and function of WFA^+-M2BP. We report that WFA^+-M2BP is secreted by HSCs and activates KCs with enhanced Mac-2 expression. In turn, these KCs activate HSCs to be fibrogenic.

Methods

Patients and protocol

Between 2014 and 2015, liver tissue specimens were obtained from ten patients at the time of liver resection and from five patients at the time of liver transplantation. The study protocol was approved by the institutional review board of Kyushu University (study ID: 26-210), in compliance with the Declaration of Helsinki, and written informed consent was obtained from each patient. Patient descriptions are provided in Supporting Table 1.

Measurement of WFA^+-M2BP

Concentrations of WFA^+-M2BP in commercially supplied M2BPGi (Sysmex Co.) were quantified by sandwich immunoassay using a fully automatic immunoanalyzer (HISCL-5000 and HISCL M2BPGi Assay kit, Sysmex Co.) (9). All counts were standardized and presented as fold-increases over controls (8, 12).

For methods in more detail, see the Supporting Information.

Results

Correlation between the number of WFA^+-M2BP-positive cells and hepatic fibrosis.

Correlations between hepatic fibrosis and numbers of WFA^+-M2BP cells were investigated using immunohistochemical studies. WFA^+-M2BP cells were defined as those positive for both the WFA epitope and M2BP. Negligible WFA^+-M2BP cells were present in normal livers. As hepatic fibrosis progressed, the number of WFA^+-M2BP cells increased (Fig. 1A). The number of WFA^+-M2BP cells present in a high-power field (HPF) in patients with fibrosis grades F0 (n = 5), F1–2 (n = 5) and F3–4 (n = 5) were 0.7 ± 0.7, 2.6 ± 1.1, and 8.5 ± 1.9, respectively (Fig. 1B). There were significant differences between the number of WFA^+-M2BP cells per HPF in samples from fibrosis stages F0 and F1–2 ($P = 0.012$), and between fibrosis stages F1–2 and F3–4 ($P < 0.001$).

Production of WFA^+-M2BP by liver cell subpopulations.

Concentrations of WFA^+-M2BP in the supernatants of cultured HSCs were significantly higher than those of other liver cells, as determined by sandwich immunoassay ($P < 0.001$; Fig. 2A). Supernatants obtained from cultures of the human HSC line LX-2 also contained WFA^+-M2BP, supporting HSCs as the source of WFA^+-M2BP (Fig. 2B). PCR analysis also confirmed that gene expression of M2BP was significantly higher in HSCs compared with other liver cells ($P < 0.001$; Fig. 2C). Immunocytochemical staining of HSCs showed that WFA epitopes and M2BP were not localized intracellularly (Fig. 3A). However, treatment of HSCs with the protein transport inhibitor brefeldin A (GolgiPlug, 1 μL/mL; BD Biosciences, San Jose, CA) led to intracellular signals from WFA epitopes and M2BP (Fig. 3A). Western blotting revealed that M2BP levels were increased in the supernatants, but not the lysates, of cultured primary HSCs and LX-2 cells (Fig. 3B, Supporting Fig. 3, respectively).

Western blotting also confirmed that M2BP remained intracellular and did not increase in culture supernatants following treatment of HSCs with brefeldin A (Fig. 3C). These indicate HSCs produce WFA^+-M2BP as a secretory protein.

WFA^+-M2BP is present in KCs of patients with hepatic fibrosis in *in vivo*.

Immunohistochemical analysis of KCs—defined as CD68-positive cells present in liver tissue—expressed WFA epitopes and M2BP (Fig. 4A, B). HSCs—defined as α-SMA-positive cells—did not exhibit positive staining for WFA epitopes and M2BP (Supporting Fig. 4A, B).

KCs express Mac-2 in the presence of WFA^+-M2BP from HSCs.

The distributions of Mac-2 were evaluated by immunohistochemical staining. Dual staining with antibodies to Mac-2 and CD68 showed that both were present in KCs (Fig. 5A). Western blotting revealed that Mac-2 expression was enhanced by culturing KCs in HSC-conditioned medium or by the addition of exogenous recombinant human WFA^+-M2BP (Fig. 5B, C). Next, KCs were cultured in conditioned medium from HSCs treated with siRNA targeting M2BP mRNA to evaluate the effect of WFA^+-M2BP on Mac-2 expression in KCs. Inhibition of M2BP expression significantly reduced the ability of conditioned medium to induce Mac-2 expression (Fig. 5B, C).

Mac-2 induces the profibrotic properties of KCs.

Regulation of HSC activation by KCs was evaluated by co-culture of HSCs and KCs. Co-culturing HSCs and KCs increased α-SMA expression, as shown by western blotting, indicating that KCs induce profibrotic properties (Fig. 6A, B). KCs

were then treated with siRNA targeting Mac-2 mRNA to determine whether Mac-2 contributes to these profibrotic effects of KCs. Inhibition of Mac-2 expression significantly impaired the ability of KCs to enhance α-SMA expression (Fig. 6A, B).

Discussion

This study investigated the distribution and role of WFA^+-M2BP in hepatic fibrosis *in vitro* by assessing human primary hepatic constitutive cells. HSCs secreted WFA^+-M2BP, which may be then moved to KCs as shown by immunohistochemistry. WFA^+-M2BP enhanced Mac-2 expression by KCs, with Mac-2 markedly activating HSCs. These findings suggest that WFA^+-M2BP secreted by HSCs may induce and promote hepatic fibrosis in conjunction with KCs.

Activated HSCs secrete microenvironmental signals that promote hepatic fibrosis (4). In this study, we found that HSCs are a source of WFA^+-M2BP as determined by both PCR and sandwich immunoassay using pure populations of multiple intrahepatic cell types. The use of protein secretion inhibitors enables intracellular staining of secretory proteins. For example, intracellular evaluation of IFN-γ and TNF-α requires that cells to be treated with protein secretion inhibitors (15), because the cytokines are readily secreted after the production. We showed that untreated HSCs did not possess intracellular WFA^+-M2BP, but inhibition of protein secretion facilitated intracellular localization of WFA^+-M2BP. Our results of WFA^+-M2BP dynamics may contribute to the fact that WFA^+-M2BP has been reported as a good serum marker reflecting hepatic fibrosis (8, 9).

Consistent with our in vitro study showing that WFA^+-M2BP did not remain intracellularly in HSCs, immunohistochemical staining of liver tissues also identified WFA^+-M2BP outside of HSCs. Immunohistochemistry does not conclusively identify

the cellular source of secreted proteins. However, immunohistochemical analysis may demonstrate the presence of chemokines and cytokines outside of the cells in which they were produced because they are bound by receptors or phagocytosed (16, 17). Feig et al. reported that CXCL12 is secreted by fibroblasts but can localize to cancer cells in tissue sections because cancer cells express receptors that capture CXCL12 (16). Following addition of M2BP to the culture medium, M2BP is present both at the cell membrane and in the cytoplasm, while the expression of Mac-2 also increases in bone marrow stromal cells (18). M2BP induces Akt, JNK, and the Ras signaling via the Raf-ERK axis (19), with PI3K/Akt signaling increasing Mac-2 expression in macrophages (20). Our immunohistochemical study of liver tissues revealed both WFA^+-M2BP and its receptor Mac-2 localized in KCs, suggesting the possibility that WFA^+-M2BP secreted from HSCs induces Mac-2 expression in KCs via PI3K/Akt pathways and colocalizes with Mac-2. Therefore, WFA^+-M2BP and Mac-2 may be important mediators in the close interaction between HSCs and KCs during fibrogenesis.

KCs have been proposed as the master regulator of hepatic fibrosis by controlling activation of HSCs. Profibrotic functions of KCs have been demonstrated using *in vivo* models of hepatic injury induced by carbon tetrachloride, thioacetamide or bile duct ligation in CD11b-DTR transgenic mice. Conditional depletion of KCs using liposomal clodronate resulted in decreased numbers of activated HSCs and attenuated hepatic fibrosis (5). We found that co-culture of KCs isolated from cirrhotic patients with HSCs increased α-SMA expression by HSCs. Connective tissue growth factor expression is induced in activated HSCs—which also express α-SMA— to promote increased production of ECM components including collagens (21, 22). Previous studies using Mac-2 knockout mice exhibit impaired HSC activation and

collagen production, suggesting Mac-2 as a therapeutic target in hepatic fibrosis (6, 7). Our present study found that the profibrotic activity of KCs was blocked by knockdown of Mac-2 expression, providing further evidence for the critical role of KC-derived Mac-2 in activating HSCs. The close relationship between WFA^+-M2BP and Mac-2 that we have identified suggests that neutralizing WFA^+-M2BP may represent another therapeutic approach in hepatic fibrosis, though further studies including *in vivo* assays are required.

In conclusion, we have shown that HSCs are the source of WFA^+-M2BP secretion. HSC-derived WFA^+-M2BP induces Mac-2 expression in KCs, which in turn activates HSCs.

References

1. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, et al. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. Hepatology 2013;58:1461-1473.

2. Kong D, Zhang F, Wei D, Zhu X, Zhang X, Chen L, et al. Paeonol inhibits hepatic fibrogenesis via disrupting nuclear factor-kappaB pathway in activated stellate cells: in vivo and in vitro studies. J Gastroenterol Hepatol 2013;28:1223-1233.

3. Preisser L, Miot C, Le Guillou-Guillemette H, Beaumont E, Foucher ED, Garo E, et al. IL-34 and macrophage colony-stimulating factor are overexpressed in hepatitis C virus fibrosis and induce profibrotic macrophages that promote collagen synthesis by hepatic stellate cells. Hepatology 2014;60:1879-1890.

4. Yi HS, Jeong WI. Interaction of hepatic stellate cells with diverse types of immune cells: foe or friend? J Gastroenterol Hepatol 2013;28 Suppl 1:99-104.

5. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. Nat Rev Immunol 2014;14:181-194.

6. Henderson NC, Mackinnon AC, Farnworth SL, Poirier F, Russo FP, Iredale JP, et al. Galectin-3 regulates myofibroblast activation and hepatic fibrosis. Proc Natl Acad Sci U S A 2006;103:5060-5065.

7. Londono MC, Abraldes JG, Altamirano J, Decaens T, Forns X. Clinical trial watch: reports from the AASLD Liver Meeting(R), Boston, November 2014. J Hepatol 2015;62:1196-1203.

8. Toshima T, Shirabe K, Ikegami T, Yoshizumi T, Kuno A, Togayachi A, et al. A novel serum marker, glycosylated Wisteria floribunda agglutinin-positive Mac-2

binding protein (WFA(+)-M2BP), for assessing liver fibrosis. J Gastroenterol 2015;50:76-84.

9. Kuno A, Ikehara Y, Tanaka Y, Ito K, Matsuda A, Sekiya S, et al. A serum "sweet-doughnut" protein facilitates fibrosis evaluation and therapy assessment in patients with viral hepatitis. Sci Rep 2013;3:1065.

10. Yamasaki K, Tateyama M, Abiru S, Komori A, Nagaoka S, Saeki A, et al. Elevated serum levels of Wisteria floribunda agglutinin-positive human Mac-2 binding protein predict the development of hepatocellular carcinoma in hepatitis C patients. Hepatology 2014;60:1563-1570.

11. Fujiyoshi M, Kuno A, Gotoh M, Fukai M, Yokoo H, Kamachi H, et al. Clinicopathological characteristics and diagnostic performance of Wisteria floribunda agglutinin positive Mac-2-binding protein as a preoperative serum marker of liver fibrosis in hepatocellular carcinoma. J Gastroenterol 2015;50:1134-1144.

12. Abe M, Miyake T, Kuno A, Imai Y, Sawai Y, Hino K, et al. Association between Wisteria floribunda agglutinin-positive Mac-2 binding protein and the fibrosis stage of non-alcoholic fatty liver disease. J Gastroenterol 2015;50:776-784.

13. Umemura T, Joshita S, Sekiguchi T, Usami Y, Shibata S, Kimura T, et al. Serum Wisteria floribunda Agglutinin-Positive Mac-2-Binding Protein Level Predicts Liver Fibrosis and Prognosis in Primary Biliary Cirrhosis. Am J Gastroenterol 2015;110:857-864.

14. Nishikawa H, Enomoto H, Iwata Y, Hasegawa K, Nakano C, Takata R, et al. Clinical significance of serum Wisteria floribunda agglutinin positive Mac-2-binding protein level and high-sensitivity C-reactive protein concentration in autoimmune hepatitis. Hepatol Res 2015;46:613-621.

15. Freer G, Rindi L. Intracellular cytokine detection by fluorescence-activated

flow cytometry: basic principles and recent advances. Methods 2013;61:30-38.

16. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, et al. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A 2013;110:20212-20217.

17. Zweifel M, Mueller C, Schaffner T, Dahinden C, Matozan K, Driscoll R, et al. Eotaxin/CCL11 expression by infiltrating macrophages in rat heart transplants during ongoing acute rejection. Exp Mol Pathol 2009;87:127-132.

18. Silverman AM, Nakata R, Shimada H, Sposto R, DeClerck YA. A galectin-3-dependent pathway upregulates interleukin-6 in the microenvironment of human neuroblastoma. Cancer Res 2012;72:2228-2238.

19. Stampolidis P, Ullrich A, Iacobelli S. LGALS3BP, lectin galactoside-binding soluble 3 binding protein, promotes oncogenic cellular events impeded by antibody intervention. Oncogene 2015;34:39-52.

20. Lin YH, Chou CH, Wu XM, Chang YY, Hung CS, Chen YH, et al. Aldosterone induced galectin-3 secretion in vitro and in vivo: from cells to humans. PLoS One 2014;9:e95254.

21. Liu Y, Liu H, Meyer C, Li J, Nadalin S, Konigsrainer A, et al. Transforming growth factor-beta (TGF-beta)-mediated connective tissue growth factor (CTGF) expression in hepatic stellate cells requires Stat3 signaling activation. J Biol Chem 2013;288:30708-30719.

22. Zheng S, Chen A. Curcumin suppresses the expression of extracellular matrix genes in activated hepatic stellate cells by inhibiting gene expression of connective tissue growth factor. Am J Physiol Gastrointest Liver Physiol 2006;290:G883-893.

Fig. 1

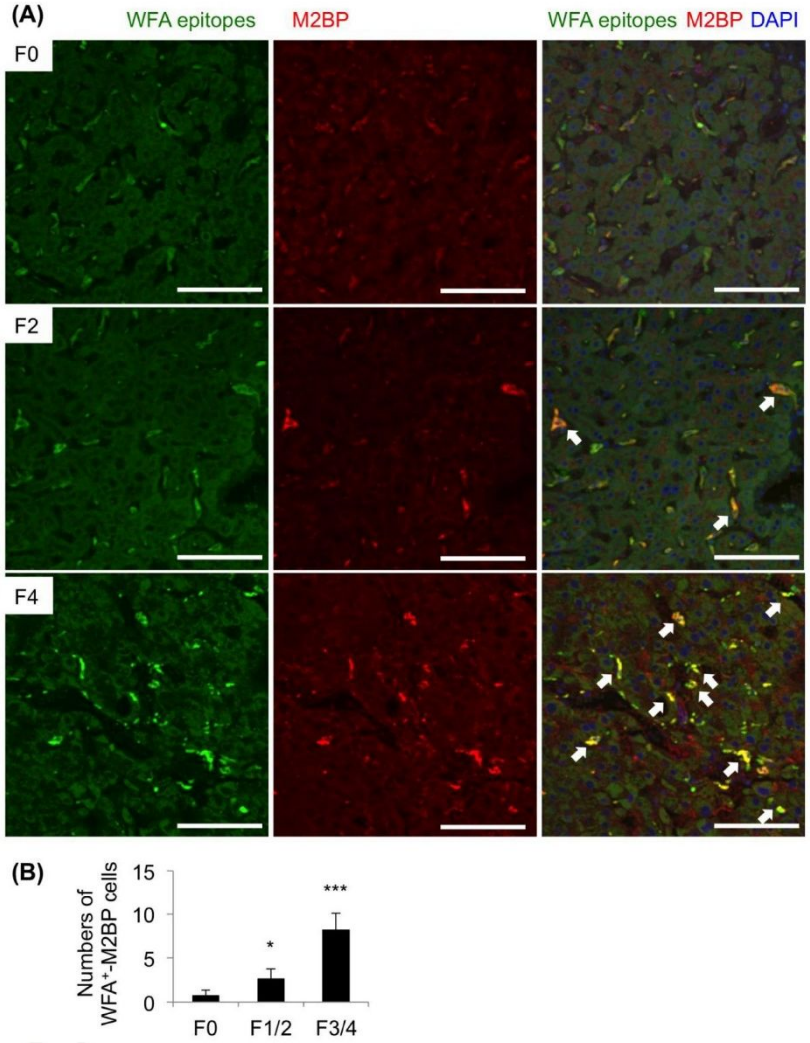

Fig. 1 Numbers of *Wisteria floribunda agglutinin*-positive Mac-2 binding protein (WFA^+-M2BP) cells increase with hepatic fibrosis.

(A) Immunohistochemical staining of tissue sections from resected or explanted livers at different fibrosis stages for WFA epitopes (green) and M2BP (red). Nuclei were

stained with 4′,6-diamidin-2-phenylindol (DAPI; blue). Micrographs were obtained by immunofluorescence microscopy. Arrows indicate WFA^+-M2BP cells defined as WFA epitopes and M2BP double-positive cells. Scale bar, 100 μm. (B) Average number of WFA^+-M2BP cells per high power field in sections from each stage of fibrosis. (F0, n = 5; F1–2, n = 5; F3–4, n = 5) * $P < 0.05$, *** $P < 0.001$ compared with F0.

Fig. 2

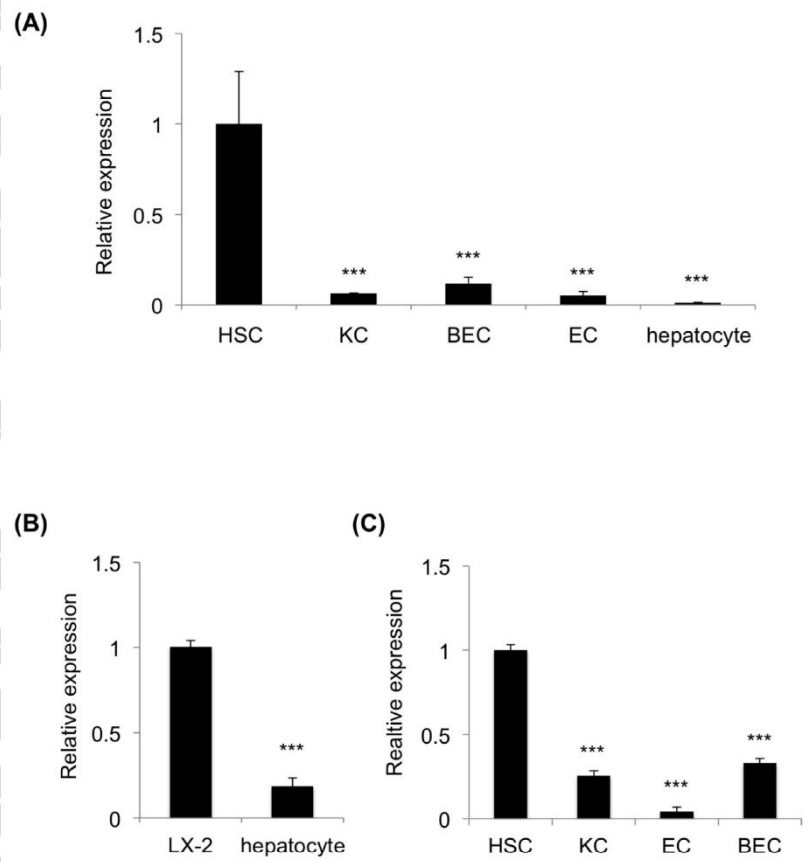

Fig. 2 Secretion of *Wisteria floribunda agglutinin*-positive Mac-2 binding protein (WFA^+-M2BP) by hepatic stellate cells (HSCs).

(A) WFA^+-M2BP concentrations in the culture supernatants of HSCs, Kupffer cells (KC), biliary epithelial cells (BEC), endothelial cells (EC) and hepatocytes were measured by sandwich immunoassay and reported as fold induction over HSCs. *** $P < 0.001$ compared with HSCs. (B) WFA^+-M2BP concentrations in the culture

supernatants of LX-2 cells were measured by sandwich immunoassay and reported as fold induction over hepatocytes. *** $P < 0.001$ compared with LX-2. (C) Realtime quantitative PCR analysis of M2BP mRNA levels in HSCs, KCs, BECs, and ECs. The level in HSCs was set at 1. *** $P < 0.001$ compared with HSCs. Results are presented as the means ± SD of triplicate determinations.

Fig. 3

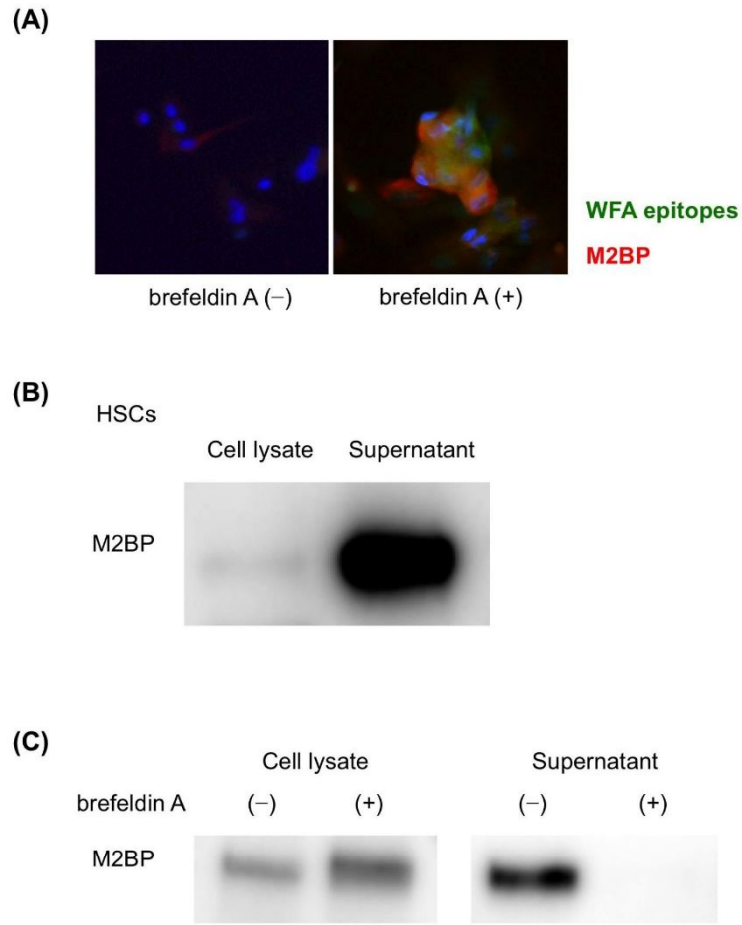

Fig. 3 *Wisteria floribunda agglutinin*-positive Mac-2 binding protein (WFA^+-M2BP) is secreted from hepatic stellate cells (HSCs) and does not remain intracellularly. (A) Immunofluorescent analysis of WFA epitopes (green) and M2BP (red) in primary cultured HSCs treated with a protein transport inhibitor, brefeldin A. Nuclei are indicated by DAPI staining (blue). (B) Western blot analysis of M2BP protein levels in

whole cell lysates and supernatants of HSCs. (C) Western blot analysis of M2BP protein levels in cell lysates and supernatants of HSCs treated with brefeldin A.

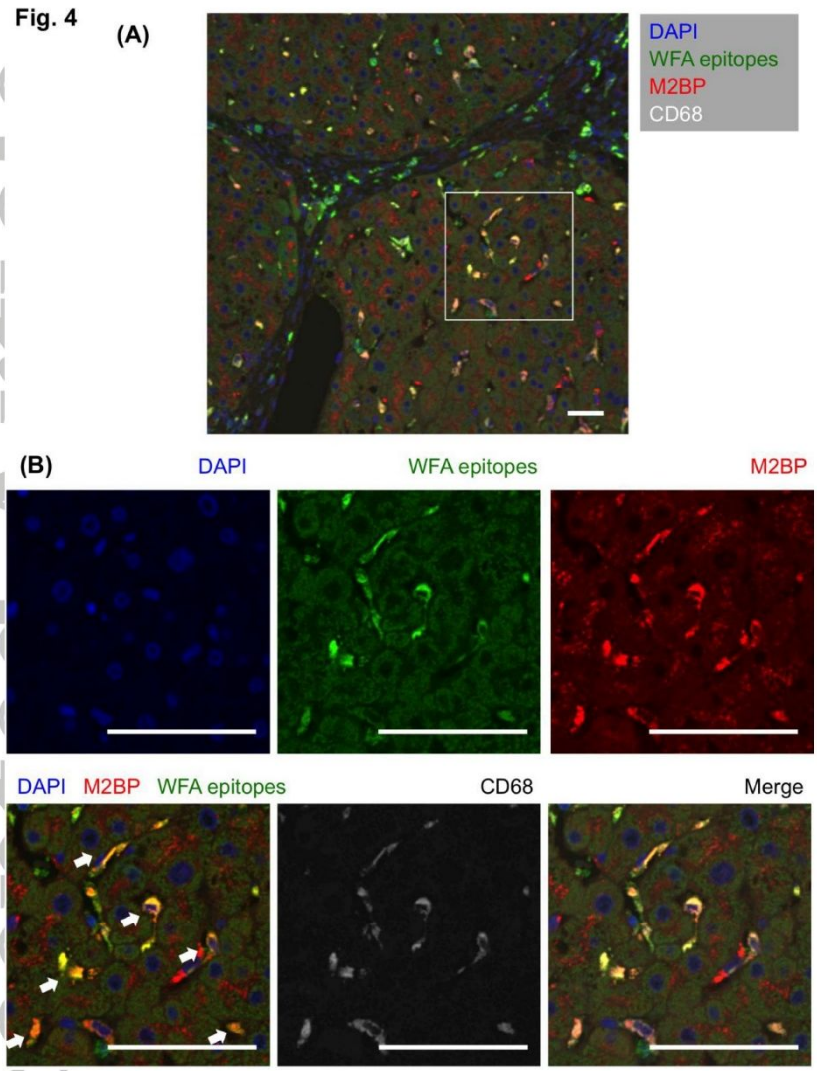

Fig. 4 *Wisteria floribunda agglutinin*-positive Mac-2 binding protein (WFA^+-M2BP) is located in Kupffer cells (KCs) in liver tissues.

(A) Liver samples were stained with antibodies to CD68 (white), WFA epitopes (green), M2BP (red) and with DAPI (blue) and analyzed using immunofluorescence

microscopy with enlarged images indicated in (B). Scale bar, 100 μm. Arrows show WFA^{+}-M2BP cells defined as those staining double-positive for WFA epitopes and M2BP.

Fig. 5

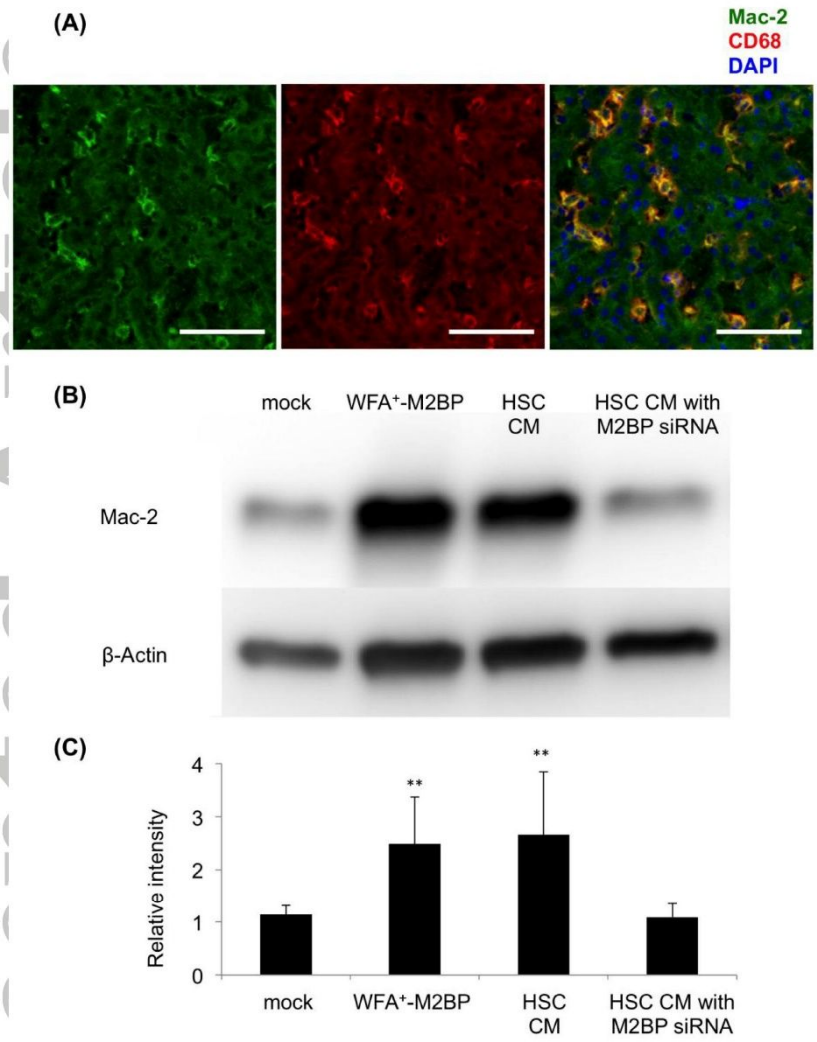

Fig. 5 KCs express Mac-2 in response to HSC-derived *Wisteria floribunda agglutinin*-positive Mac-2 binding protein (WFA^+-M2BP).

(A) Tissue sections of livers were stained with antibodies to Mac-2 (green) and CD68 (red) and with DAPI (blue) and analyzed using immunofluorescence microscopy.

Scale bar, 100 μm. (B) Western blot analysis of Mac-2 protein levels in KCs treated with PBS (mock), WFA^{+}-M2BP (recombinant human M2BP 1 μg/mL), HSC-conditioned medium (HSC-CM), or HSC-CM treated with M2BP siRNA. (C) Bar graph of (B). The band intensities of Mac-2 were quantified and normalized relative to those of β-actin. Results represent the means ± SD of values obtained from five separate experiments. The value of negative control cells was set at 1. ** $P < 0.01$ compared with control.

Fig. 6

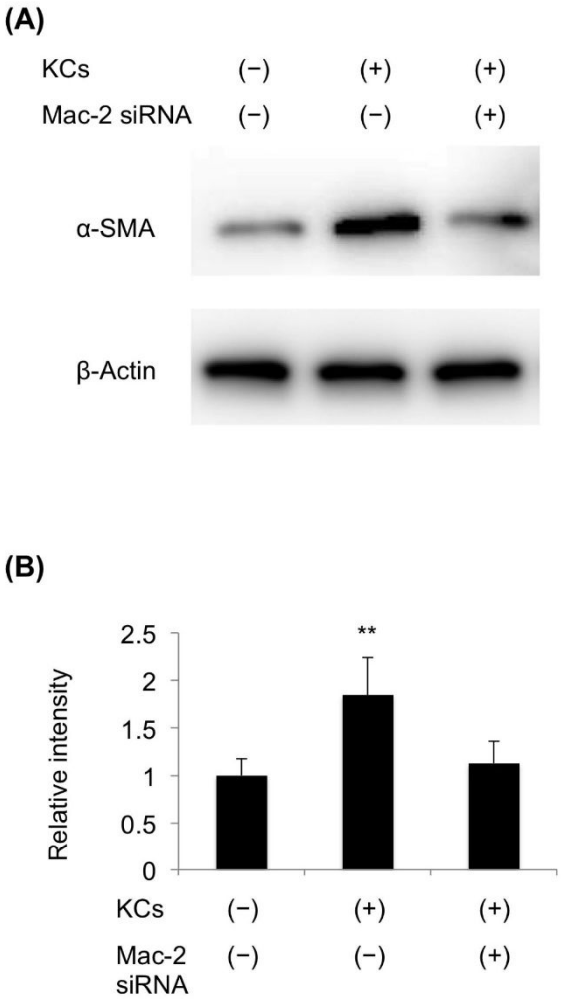

Fig. 6 KCs promote α-SMA expression by HSCs via WFA^{+}-M2BP/Mac-2 interactions. (A) Western blot analysis of α-SMA protein levels in HSCs cultured in the presence or absence of KCs that had been treated with negative control siRNA, or Mac-2 siRNA.

(B) Bar graph of (A). Band intensities of α-SMA were quantified and normalized to those of β-actin. Results represent the means ± SD of the values obtained from five separate experiments. The value obtained for the level in negative control cells was set at 1. ** $P < 0.01$ compared with control.