博士論文

論文題目　**Studies on the mechanism of liver fibrosis induced by Oncostatin M**

（オンコスタチン M によるマウス肝線維化促進機構の解析）

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Abbreviations

AST: Aspartate transaminase

ALP: Alkaline phosphatase

ALT: Alanine transaminase

BDL: Bile duct ligation

BMDM: Bone marrow-derived macrophage

CCl_4: Carbon tetrachloride

ECM: Extracellular matrix

FACS: Fluorescence activated cell sorting

GFAP: Glial fibrillary acidic protein

HTVi: Hydrodynamic tail vein injection

HPLC: High performance liquid chromatography

HSC: Hepatic stellate cell

LM: Liver macrophage

LSEC: Liver sinusoidal endothelial cell

MMP: Matrix metalloproteinase

NPC: Non-parenchymal cell

OSM: Oncostatin M

PDGF: Platelet-derived growth factor

TAA: Thioacetamide

TGF-β: Transforming growth factor-β

Timp1: Tissue inhibitor of metalloproteinase 1

Abstract

Oncostatin M (OSM) is a member of IL-6 family cytokines with pleiotropic functions. A previous study showed that OSM contributed to liver regeneration after acute liver injury, partly by regulating the remodeling of extracellular matrix. However, the role of OSM in chronically injured liver accompanying fibrosis is unknown. The current studies show that OSM is continuously expressed during fibrogenesis and responsible for the promotion of fibrosis. OSM-deficient mouse exhibited significant reduction of fibrosis in chronically injured liver compared to wild-type mouse. Conversely, continuous expression of OSM in normal liver by hydrodynamic tail vein injection of OSM cDNA was sufficient to induce remarkable fibrosis with the up-regulation of several fibrogenic genes. Importantly, no sign of hepatic injury was observed in this case, suggesting that OSM-induced fibrosis was not caused by inflammation or hepatic damage but the fibrogenic property of OSM. The OSM receptor was predominantly expressed on hepatic stellate cells (HSCs) and liver macrophages (LMs). OSM directly up-regulated the expression of tissue inhibitor of metalloproteinase-1 gene in HSC *in vitro*, whereas it promoted that of collagen gene via the activation of LMs. In chronically injured liver, infiltrating bone marrow-derived

macrophages were more responsive to OSM for the induction of fibrosis-related genes than liver resident macrophages. In conclusion, OSM is a powerful fibrogenic factor and its persistent expression in chronic liver diseases can be a risk factor for liver fibrosis. OSM is a possible therapeutic target for liver fibrosis.

Chapter I. Introduction

Fibrosis is highly conserved protective response to tissue injuries in the lungs, kidneys, hearts, livers and other tissues and organs. In most situations, fibrosis is preceded by inflammation and complex interaction of components including immune systems, epithelial cells, myofibroblasts and extracellular matrix (ECM) is observed. Of all organs, liver fibrosis has been intensively researched for a few decades. Liver has a remarkable capacity to adapt injury through tissue repair. Robust and self-limiting fibrotic and regenerative response is observed in acute injury. However, fibrosis becomes problematic and clinically relevant when dysregulated and excessive scaring occurs in response to persistent injury and leads to altered tissue function. Liver fibrosis occurs in almost all patients with chronic liver injury. Patients with cirrhosis, i.e. the most advanced stage of liver fibrosis, have poor prognosis because of impaired liver function, adverse symptoms such as gastroesophagial virices and ascites, and hepatocellular carcinoma (1, 2). Cirrhosis is pathologically defined by distorted architecture of the liver with the excess amount of accumulated extracellular matrix (ECM) and subsequent development of nodules of regenerating hepatocytes. At the

clinical level, cirrhosis is accompanied by hepatocellular dysfunction and increased intrahepatic resistance to blood flow, which results in hepatic insufficiency and portal hypertension, respectively. The main causes of liver fibrosis include chronic HCV infection, alcohol abuse, and non-alcoholic steatohepatitis. Regardless of the underlying cause, components of fibrotic liver are similar (described below). Currently, liver fibrosis is considered as wound healing response in solid organ, in which the damaged tissues are encapsulated by ECM. Although the remodeling of ECM is an essential step for restoration of injured liver, chronic liver diseases often accompanies an excessive deposition of ECM. Tremendous efforts have been made to elucidate the general mechanism underling fibrosis progression. Since hepatic stellate cells (HSCs) (formerly known as lipocytes or Ito cells) were identified as the main collagen-producing cell, research focus has been on the mechanism and key regulators of HSCs' activation. Some key fibrogenic mediators and signals that modulate HSCs' activation have been identified (ex. TGF-β, PDGF) (3). Later, even advanced liver fibrosis has been shown to be reversible in some patients (4), motivating researchers to focus on identification of anti-fibrotic therapy. However, the most effective therapy for treating liver fibrosis to

date is eliminating or ameliorating causative agents. Recently, the advent of effective antiviral drugs has been successfully relieving the increasing number of patients from viral hepatitis HCV. It is clear that eliminating virus from liver ameliorate fibrosis spontaneously in some extent. Unfortunately, however, it is generally a slow process and insufficient for most patients (5), suggesting that persistent fibrosis is a major target to treat these patients. Therefore it is increasingly important to unveil the key mechanisms underlying liver fibrosis and establish a novel anti-fibrosis therapy.

Pathogenesis of liver fibrosis

Liver is the central organ for numerous functions such as carbohydrate metabolism, lipid metabolism, drug detoxification and plasma protein production. The minimum functional unit in the liver is called hepatic lobule (Figure I-1 A). Hepatic lobule is hexagonal with the central vein at its center and portal triad, consisting of the portal vein, hepatic artery and bile duct, at each corner. The blood from afferent portal vein flows through sinusoids to efferent central vein and hepatocytes are lined by the sinusoid (Figure I-1 B). Sinusoids are liver specific capillaries consisting of

non-parenchymal cells (NPCs) such as fenestrated liver sinusoidal endothelial cells (LSEC), macrophages, and HSCs (Figure I-1 C). Hepatocytes and NPCs cooperatively functions to maintain liver homeostasis. In addition to these liver-composing cells, the quantity and composition of ECM plays critical role to maintain normal liver function. The balance between ECM production and degradation is important to keep the appropriate amount of ECM in the liver. After acute injury, hepatocytes regenerate to replace the necrotic or apoptotic hepatocytes. In this process, inflammatory response and transient deposition of ECM is accompanied. If the hepatic injury persists, eventually the liver regeneration fails and parenchyma is substituted by abundant ECM, leading to liver fibrosis. The increased ECM in fibrotic liver contains collagens (I, III, and IV), elastin, fibronectin, laminin, hyaluronan, and proteoglycan. As mentioned above, activated HSCs are the main fibrogenic cell type in the injured liver, producing various ECM including type1 collagen (Figure I-2). On the other hand, ECM can be degraded by various proteinases such as matrix metalloproteinases (MMPs) produced by several cell types in the liver (3). Decreased activities of MMP are mainly due to the up-regulation of their specific inhibitor, Tissue inhibitor of metalloproteinases (TIMPs).

In the normal liver, HSC reside in the space of Disse as a quiescent phenotype. Following chronic injury, HSC are activated and transdifferentiated into myofibroblast-like cells, acquiring contractile and fibrogenic properties.

During chronic liver injury, multiple factors from infiltrating immune cells in addition to liver resident cells are implicated in the activation of HSCs. Intensive studies on fibrosis have revealed roles of key cytokines such as TGF-β and PDGF in fibrogenesis (3) and tremendous efforts have been made for treating fibrosis by targeting these cytokines. For example, HSC is considered as a primary target for TGF-β in chronic liver disease. Overexpression of TGF-β by adenovirus-mediated gene transfer promotes fibrosis in mouse liver with elevated collagen1a1 mRNA and TGF-β knockout mice show attenuated liver fibrosis in toxin-induced liver injury (6). Consistently, TGF-β overexpressed transgenic mice progress liver fibrosis with up-regulation of Type1 collagen(7, 8). These fibrogenic effects by TGF-β have been mainly explained by the activation of HSCs. It has been already shown that α1 and α2 Type I pro-Collagen and TIMP-1, -2 are direct target genes of TGF-β in HSC (9, 10). Despite the accumulated evidences about fibrogenic properties of TGF-β in

experimental models, there exists no effective therapy to date for fibrosis through TGF-β. This seems to be partly due to multifaceted roles of the cytokine depending on the cellular context (11). TGF-β has pleiotropic functions and various effect on other type of cells, including anti-mitogenic, pro-apoptotic effect and induction of epithelial-mesenchymal transition of hepatocytes. Though the effects of TGF-β on other cells in the liver have not been intensively investigated, studies on other organs suggest its inhibitory role for liver diseases depending on the stage of liver disease from inflammatory phase to carcinogenesis(11).

Oncostatin M

Oncostatin M (OSM) is a member of IL-6 family cytokines with multiple functions in inflammation, hematopoiesis and development (12). It has been reported previously that OSM contributes to liver regeneration after acute liver injury, partly by inducing tissue inhibitor of metalloproteinase 1 (Timp1) expression and protecting the liver tissue from MMPs-dependent damages. In contrast, OSM receptor knockout (OSMR KO) mice showed enhanced degradation of ECMs due to decreased Timp1 expression and

increased MMP activities in injured liver (13). These findings suggest that transient expression of Timp1 by OSM is beneficial for suppression of extensive tissue damage by MMPs in acute liver injury. Conversely, persistent exposure of OSM in chronically injured liver could be a risk factor for liver fibrosis because of suppression of MMPs activities by induction of Timp1. In the human, there are a few reports showing up-regulation of OSM in patients with liver fibrosis (14, 15). However, the causal relationship and molecular mechanisms linking between OSM signaling and liver fibrogenesis remain largely unknown.

In this study, I have investigated the role of OSM for liver fibrosis in chronic liver injury models using OSM knockout (OSM KO) mouse. In addition, I have developed a novel liver fibrosis model by continuous expression of OSM in normal mouse liver. The results show that OSM elicits a powerful fibrogenic effect on not only chronically injured liver but also even uninjured liver, suggesting that OSM is a powerful inducer of liver fibrosis. Furthermore, I show that that OSM exerts its fibrogenic function via non-parenchymal cells (NPCs) *in vitro* and *in vivo*, demonstrating the novel link

between OSM and liver fibrosis.

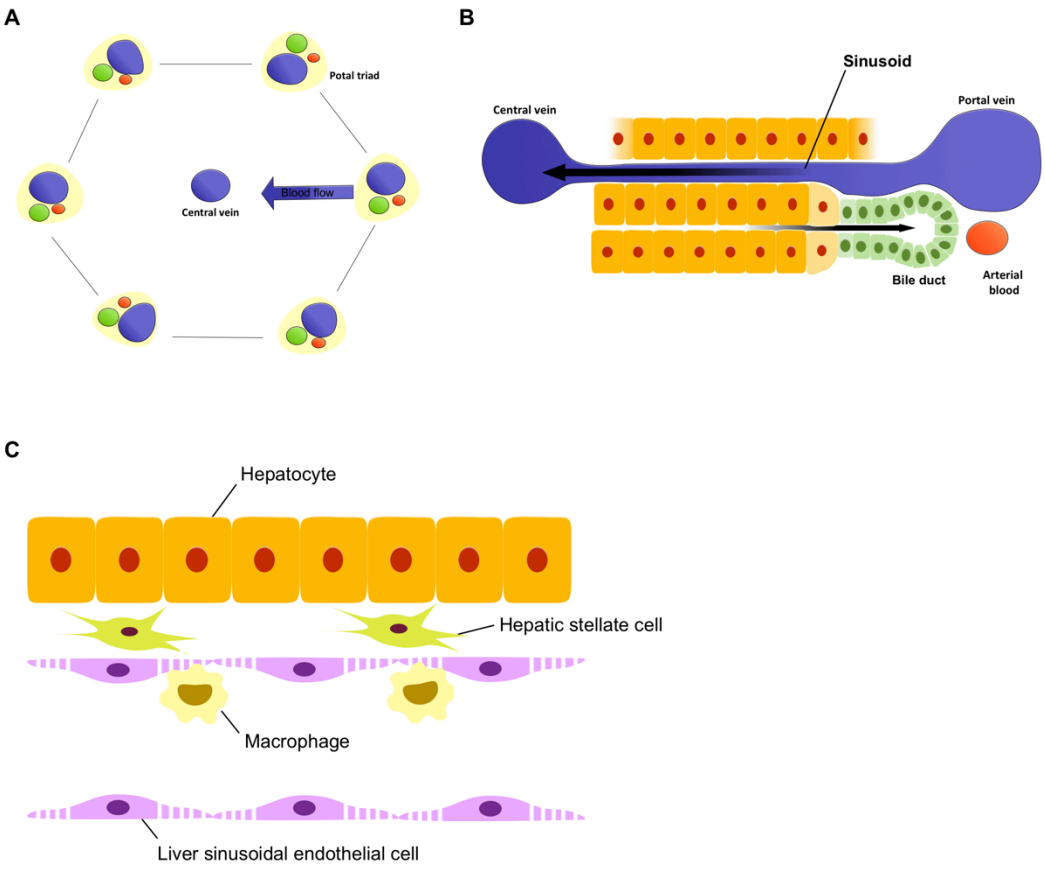

Figure I-1. Schematic diagram of architecture of the liver. (A) Structure of hepatic lobule. (B) Magnified view of the sinusoid lining by hepatocytes. (C) Non-parenchymal cells consisting sinusoid.

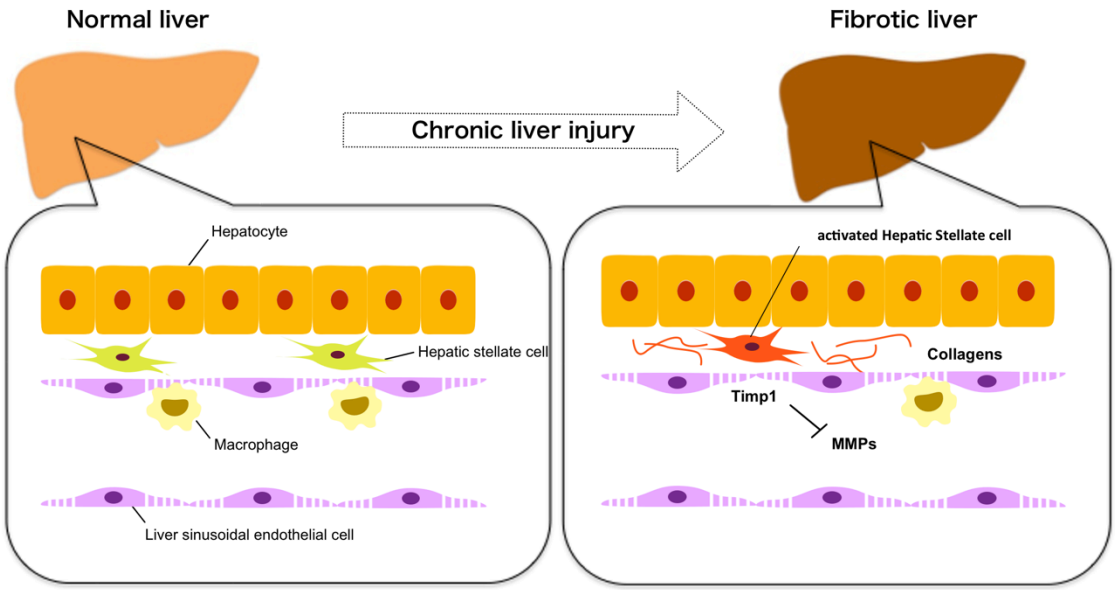

Figure I-2. Schematic model of liver fibrosis. In the chronically injured live, activated HSCs produce collagens. MMPs activity is suppressed by Timp1.

Chapter II. Materials and Methods

II-1. Animals and treatment

In this study, I used 7-9-week-old mice. C57BL/6J mice were purchased from Clea Japan, Inc. (Tokyo, Japan). OSM KO mice were generated as described previously (16). All animals were maintained in a standard Specific-Pathogen Free (SPF) room at our animal facility. I used three liver fibrosis models; thioacetamide (TAA), carbon tetrachloride (CCl_4) and bile duct ligation (BDL) models. TAA was administered with drinking water (0.03% v/v) for 12 weeks. For the CCl_4 model, mice were received biweekly intraperitoneal injection of 10%(v/v) solution of CCl_4 (Wako Pure Chemical, Osaka, Japan) in corn oil (Wako) or vehicle (corn oil) at a dose of 1.0 ml/kg body weight for 6 weeks. Livers were harvested 2 days after the final CCl_4 injection. For the BDL model, common bile duct was ligated in the anesthetized condition. Control mice were sham-operated. The Mice were sacrificed 14 days after the procedure. All animal experiments were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

II-2. Continuous expression of OSM in mouse liver

I used Hydrodynamic tail vein injection method (HTVi) to overexpress OSM continuously in mouse liver as previously described (17). The mouse OSM cDNA was cloned into the vector pLIVE (OSM-pLIVE) with alpha-fetoprotein enhancer and Albumin promoter, which enabled hepatocyte specific expression of the target gene (Fig II-1). OSM-pLIVE was introduced into 8 week-old mice with Trans-EE Hydrodynamic Delivery solution (Mirus Bio, Madison, WI) from the tail vein. pLIVE-LacZ were used as a control.

II-3. Isolation of hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs) and Liver macrophages (LMs)

A cell suspension of NPCs was obtained from mouse liver by the modified collagenase perfusion method, as described previously (17) (Figure II-2). In brief, mouse livers were perfused with basic perfusion solution containing 0.25 g/L of Collagenase-Yakult (Yakult Pharmaceutical Industry Co. Ltd., Tokyo, Japan) and 0.15 g/L of DNAseI (Sigma-Aldrich). HSCs were isolated as described previously (18). The purity was

>90% based on Vitamin A content by UV excitation in flow cytometric analysis. For flow cytometric analysis and fluorescence activated cell sorting (FACS) of NPCs, CantoII (Becton, Dickinson and Company) and Moflo XDP (Beckman-Coulter) were used, respectively. Liver sinusoidal endothelial cells (LSECs) were isolated from NPCs by FACS using anti-mouse Stabillin-2 antibody as previously described (17). For LMs isolation, I skipped the step of percoll centrifugation to prevent the loss of liver resident macrophage population. For primary culture of liver macrophages, the samples were sorted from normal liver by auto-MACS pro (Miltenyi Biotec) using PE-conjugated F4/80 antibody and anti-PE micro-beads. The sorting was performed twice to increase the purity of macrophage. For mRNA analysis, LM was sorted by a combination of rat anti-F4/80 (Clone: BM8), CD11b (Clone; M1/70), Ly6G (Clone: 1A8) and CD45 (Clone: 30-F11) antibodies (BioLegend).

II-4. Primary culture of HSCs, LSECs and LMs

HSCs, LSECs and LMs were seeded in collagen type1-coated 6-well dishes (BD Bioscience) at 5 x 10^5 cells per well with Dulbecco's modified Eagle's medium

(Sigma-Aldrich) containing 2% B-27 and antibiotics. In LSECs culture, VEGF-A was added at a final concentration of 10 ng/mL. After 12 hrs, unattached cells were washed out and mOSM was added at a final concentration of 10 ng/mL. Total RNA was extracted from cultured cell at 48 hrs after OSM administration. In co-culture experiment, half of HSC culture media were exchanged with supernatant of primary LSECs or LMs culture for 3 days, and cultured for 48 hrs until RNA extraction.

II-5. H&E , Picro-sirius red (PSR) staining and Immunohistochemistry

Liver cryosection (8 μm) was mounted on a glass slide and fixed at room temperature for 10 minutes with 4% PFA for H&E staining and immunohistochemistry and over-night with Bouin's solution (Sigma) for Picro-Sirius Red (PSR) staining. PSR staining was performed as described previously (17). For immunohistochemistry, rat anti-mouse stabillin-2 antibody(17), rat anti-mouse F4/80 antibody (Clone: BM8, BioLegend), polyclonal rabbit anti-mouse glial fibrillary acidic protein (GFAP) antibody (Dako), rabbit anti-mouse collagen type I antibody (BIO RAD) were used. All images were captured using All-in-one BZX-700 (Keyence).

II-6. Quantification of liver injury and fibrosis

Hepatic damages were evaluated by serum level of Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline Phosphatase (ALP) (JSCC standardized method), and Total bilirubin (enzyme method) (measured by ORIENTAL YEAST Co.). The accumulation of collagen in the liver was evaluated by PSR staining and hydroxyproline content. PSR positive area was analyzed in consecutive 35 high-power fields (magnification x100) jointed by Keyence BZ-analyser (Total jointed area were 19.6mm^2). For the quantitative analysis of hydroxyproline, harvested livers were lysed by HCl, and the hydoxyproline content was measured by High performance liquid chromatography (HPLC).

II-7. Total RNA extraction and quantitative RT-PCR

Total RNA of liver sample was isolated by using TRIZOL reagent (Invitrogen). Quantitative RT-PCR analysis was performed using Light Cycler 480 (Roche Diagnostics). All the tested genes were normalized by beta-actin.

II-8. Statistical Analysis

All data are shown as mean +/- SEM. For comparison of two groups, a two-side unpaired *t* test was used. For correlation analysis, the Pearson correlation coefficient was calculated. $P<0.05$ was considered statistical significant.

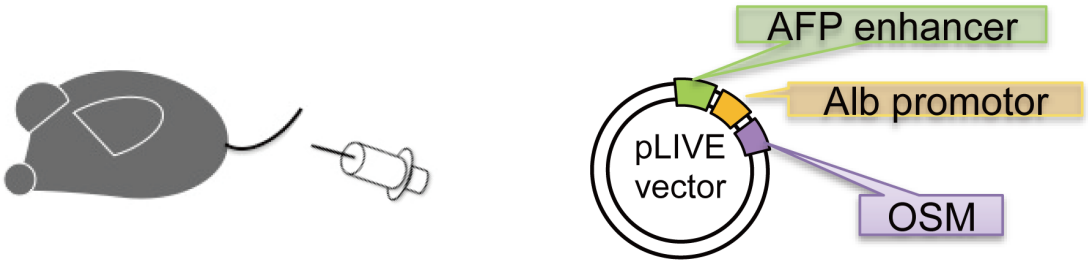

Figure II-1. Schematic view of the hydrodynamic tail vein injection (HTVi) method. The targeted mouse cDNA was cloned into the vector pLIVE with alpha-fetoprotein enhancer and Albumin promoter, which enabled hepatocyte specific expression of the target gene. LacZ was used as control. These vectors were introduced into 8 week-old mice from tail veins with delivery solution.

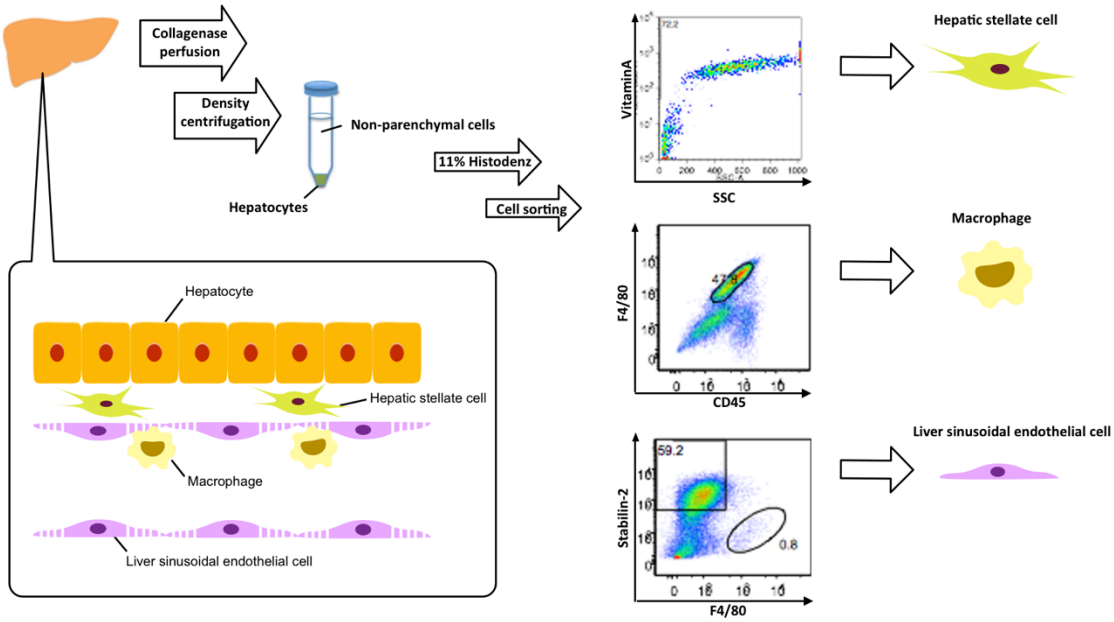

Figure II-2. Isolation methods of liver-composing cells. A cell suspension of Non-parenchymal cells was obtained from mouse liver by the collagenase perfusion method followed by density centrifugation. Hepatic stellate cells were isolated by density gradient using 11% Histodenz. The purity was >90% based on Vitamin A content by UV excitation in flow cytometric analysis. By using FACS, macrophages and liver sinusoidal endothelial cells were isolated as CD45+F4/80+ cells and F4/80-Stabilin-2+ cells, respectively.

Chapter III. Results

III-1. OSM expression is sustained during liver fibrogenesis

It was reported previously that OSM expression is transiently induced in mouse models of acute liver injury (13). To evaluate the relationship between OSM expression level and liver fibrosis, I first examined whether OSM is continuously up-regulated in the chronically injured liver. Three different well-established models of liver fibrosis, i.e. repetitive carbon tetra chloride injection model (CCl_4), thioacetamide administration in drinking water model (TAA) and bile duct ligation model (BDL), were employed in the study. Although these models exhibited distinct degree and location of fibrogenesis, they all showed accumulation of collagens as shown by picro-Sirius Red (PSR) staining (Figure III-1. A-D. Quantitative analysis of fibrotic area (Figure III-2. A, C, E) and quantitative real-time RT-PCR (qRT-PCR) of liver mRNA (Figure III-2. B, D, F) after insult revealed that the gene expression of *Osm* as well as fibrosis-related genes such as *Type1 collagen* and *Timp1* was up-regulated during fibrogenesis. Intriguingly, the degree of OSM expression was gradually increased along the progression of liver fibrosis in the CCl_4 model and TAA model. Because the damage of hepatocyte measured

by serum alanine aminotransferase (ALT) was decreased or unchanged during chronic exposure to hepatotoxin such as CCl_4 and TAA (19), the increased expression of OSM was not due to a simple reflection of the magnitude of hepatitis.

III-2. Lack of OSM alleviates fibrosis in chronic liver injury

To investigate whether OSM signal is responsible for fibrogenesis in chronically injured liver, I evaluated the status of fibrosis in OSM KO mouse liver. I firstly examined the phenotype of OSM KO mice without treatment. H&E staining of the liver of 8-week-old OSM KO mouse showed no phenotype concerning hepatic injury (Figure III-3 A). The analysis of liver-specific serum markers showed no difference between WT and OSM KO mice. (Figure III-3. B). PSR staining of the livers and quantitative analysis of fibrosis-related genes showed no differing phenotype between OSM KO mice and wild type mice (Figure III-4. A and B). Next, I administered chronic liver injury on OSMKO mice. I chose oral administration of TAA as the experimental fibrosis model, because this model showed remarkable fibrosis with relatively mild elevation of serum ALT, a liver injury marker, compared to other models. This resembles the

situation which the patients with chronic hepatitis progress remarkable fibrosis without severe elevation of ALT (20). After 12 weeks of TAA administration, PSR staining revealed that the fibrotic area was remarkably reduced throughout the liver of OSM KO mice compared to wild-type (WT) mice (Figure III-5 A). Quantitative analysis of Sirius red positive area in broad area of liver specimen showed significant reduction of collagen deposition in the liver of OSM KO mouse (Fig III-5 B and C). Consistent with the observation, the hydroxyproline content of the liver, which reflects the amount of collagens, was decreased significantly in OSM KO mice compared to WT mice (Figure III-5 D). The expressions of fibrosis-related genes, collagen 1a1 and Timp1 were also significantly reduced in OSM KO mouse liver (Figure III-5 E), suggesting that sustained expression of OSM in wild-type mice contributes to the progression of fibrosis in chronically injured liver. Importantly, the differential sensitivity of hepatocytes to TAA does not account for the phenotype, because both injured area of liver shown by H&E staining and the serum ALT and AST levels showed no significant difference between the two groups during fibrosis progression (Figure III-6 A and B).

Therefore, I supposed that the amelioration of liver fibrosis in OSM KO mice was due to the absence of direct effect of OSM on NPCs.

III-3. Continuous expression of OSM is sufficient for liver fibrogenesis

Because continuous expression of OSM was implicated in liver fibrogenesis, I next investigated whether OSM itself has fibrogenic effects on the liver. To address the issue, I used hydrodynamic tail vein injection (HTVi) method, which enables continuous expression of exogenous cDNA in the liver (Materials and Methods). After gene delivery of OSM cDNA by HTVi, OSM expression was maintained for at least 4 weeks (Figure III-7 A). Interestingly, the mice expressing OSM by HTVi (OSM-HTVi mouse) showed considerable degree of liver fibrosis after 2 weeks of the injection compared to the control mice (Figure III-7 B), indicating that OSM is a strong inducer of liver fibrosis. Fibrosis area in the broad area of liver specimen was quantitatively analyzed as Sirius red positive area, which showed significant increase of fibrosis in OSM-HTVi livers (Fig III-8 A and B). As expected, the expression level of Timp1 was elevated in OSM-HTVi mice (Figure III-8 C), because Timp1 is a transcriptional target of OSM

signaling (21). In addition, the OSM expression level exhibited a positive correlation with the expression levels of Timp1 and type1 collagen in OSM-HTVi livers (Figure III-8 D). In the fibrotic liver, Timp1 is supposed to contribute to the accumulation of ECM by suppressing MMPs activities. To examine whether the up-regulation of Timp1 by OSM accounts for severe fibrosis in OSM-HTVi treated mice, Timp1 cDNA was delivered into the liver by HTVi method. As shown in Figure III-9 A and B, continuous expression of Timp1 in the liver was not sufficient to induce liver fibrosis. In addition, unlike OSM-HTVi livers, the expression of type1 collagen gene was not induced in Timp1-HTVi livers (Figure III-9 C). These results indicated that continuous expression of OSM elicited the up-regulation of type1 collagen as well as Timp1, of which synergistic effects might result in remarkable liver fibrosis.

III-4. OSM induces liver fibrosis without hepatic injury.

Liver fibrosis develops usually in the background of chronic liver injury and inflammation. To investigate whether continuous expression of OSM caused fibrosis by inducing liver injury or not, hepatic damage in OSM-HTVi mouse was assessed by

measurement of serum liver disease markers (Figure III-10 A). Surprisingly, the levels of ALT, AST were within normal range at any time point during fibrogenesis in OSM-HTVi mice despite severe fibrosis progression. Consistent with the results of serum tests, the histological analysis showed no sign of apparent injury such as necrosis evaluated by H&E staining, while severe hepatic injury was observed around fibrotic area in the liver with continuous TAA administration (Figure III-10 B). These results strongly suggested that liver fibrosis in OSM-HTVi mouse was caused by a direct effect of OSM rather than a consequence of immune response following hepatocyte injury.

To identify the cell type responsible for OSM-induced fibrosis, the expression of OSM receptor (OSMR) was examined in liver-composing cells. OSMR was dominantly expressed in NPCs such as hepatic stellate cell (HSC), liver sinusoidal endothelial cells (LSEC) and liver macrophage, whereas it was marginally expressed in hepatocyte (Figure III-10 C). To examine the relationship between these NPCs and fibrosis, I firstly performed immunohistochemical analysis (IHC) to confirm that these NPCs really exist in the fibrotic area. Control, OSM-HTVi and TAA-injured livers were analyzed by using antibodies against GFAP, Stabillin-2 and F4/80, cell-specific markers for HSCs,

LSECs and macrophage, respectively (22, 23). The results showed that LSECs were close to HSCs throughout the liver irrespective of liver fibrosis (Figure III-11 A and B). By contrast, macrophages were dominantly located around the portal vein rather than the central vein in normal liver (Figure III-12 A and B). Intriguingly, in both models of liver fibrosis induced by OSM and TAA, numerous liver macrophages were observed around the central vein where fibrosis prevailed. These results suggested that OSM acts on these NPCs to induce liver fibrosis.

III-5. OSM induces expression of Timp1 but not collagen in HSCs

Activated HSCs are considered as the main fibrogenic cells that express both Timp1 and type1 collagen during fibrogenesis. To determine whether OSM is able to activate HSCs directly to express these fibrogenic genes, I performed primary culture using freshly isolated HSCs from normal mouse liver (Figure III-13 A and B). In the presence of OSM, cultured HSCs displayed myofibroblast-like morphological change (Figure III-13 C) and dramatically up-regulated Timp1 expression, which were considered signs of HSC activation (Figure III-13 D). However, the expression of type1 collagen gene was

not promoted in HSCs *in vitro* by OSM stimulus (Figure III-13 D), while it was up-regulated in OSM-HTVi liver *in vivo*. This discrepancy between *in vitro* and *in vivo* experiments may be explained by the difference of the microenvironment surrounding HSCs, i.e. the contribution of another OSM-responsive NPCs to HSC activation *in vivo*. Because LSECs highly expressed OSM receptor and resided in the vicinity to HSCs (Figure III-10 C, III-11), I examined whether LSEC is implicated in OSM-induced HSC activation. LSECs isolated from normal liver using anti-Stabilin-2 antibody were cultured with or without OSM for 3 days and then each supernatant was added in the primary culture of HSCs (Figure III-14 A). Although morphological changes were observed in the LSECs stimulated with OSM (Figure III-14 B), I found no significant difference in the expression of Type I collagen gene in HSCs irrespective of OSM stimulation (Figure III-14 C). These results suggested that LSEC did not produce any secretory factors that involved in OSM-induced HSC activation.

III-6. OSM promotes collagen expression in HSCs through the activation of liver macrophages

The role of macrophage for fibrogenesis is of considerable interest and a subject of intensive research in various organs. It was recently reported that OSM plays a role in ameliorating insulin resistance of obese mouse by directly switching the phenotype of adipose tissue macrophages (24). Because liver macrophages also express OSMR (Figure III-10 C) and numerous macrophages were observed in fibrotic area (Figure III-12), I investigated the role of macrophages in fibrogenesis. The F4/80 positive macrophages isolated from normal liver were cultured in the presence or absence of OSM and then each supernatant was added in the primary culture of HSCs (Figure III-15 A). I found that the supernatant from macrophage cultured with OSM promoted the expression of Type1 collagen gene in HSCs compared to that without OSM (Figure III-15 C). These results suggested that some secretory factors from OSM-stimulated macrophage are required for the fibrogenic activation of HSCs. In fact, the expression of fibrogenic cytokines such as TGF-β up-regulated in macrophage cultured with OSM (Figure III-15 B). Next, I analyzed macrophages isolated from OSM-HTVi liver (Fig III-16 A). These macrophages isolated from OSM-HTVi liver showed significantly higher expression of fibrogenic cytokines than those from control-HTVi livers (Figure

III-16 B). F4/80 positive cells isolated from OSM-HTVi liver and control-HTVi liver were cultured for 3 days and each supernatant was added to primary HSC culture. F4/80 positive cells from OSM-HTVi liver has higher ability to induce Type I collagen genes in HSC compared to the F4/80 positive cells form control-HTVi liver (Fig III-16 C). These results suggested OSM-stimulated macrophages contribute HSC activation.

III-7. OSM differentially affects heterogeneous subpopulations of liver macrophages

The F4/80 positive liver macrophages include at least two distinct populations: liver resident macrophages constitute a majority of macrophages in uninjured liver, while the macrophages/monocytes derived from bone marrow comprise a minor population in steady state condition. The latter macrophages remarkably increase after hepatic injury and are further categorized into various subtypes, which exert diverse functions in homeostasis, disease progression and regression from injury (25). Recent studies have demonstrated that liver resident macrophages and bone marrow-derived macrophages (BMDMs) are identified as $F4/80^{hi}$ $CD11b^{mid}$ cells and $F4/80^{lo}CD11b^{hi}$ cells by FACS,

respectively (26, 27). In order to reveal which population of liver macrophage is critical to OSM-induced fibrosis, I examined the composition of hepatic macrophages in the fibrotic liver model by FACS analysis. In the TAA model, the ratio of liver resident macrophages and BMDMs dynamically changed compared to that of intact liver (Figure III-17 A), i.e. the increase of BMDM fraction and the decrease of liver resident macrophage fraction in injured liver. However, no significant difference in cell populations was observed between WT and OSM KO mice (Figure III-17 B), suggesting that the alleviation of liver fibrosis in OSM KO mice depends on the activation status of OSM-responsive macrophage. To reveal which type of macrophage is responsible for liver fibrogenesis by OSM stimulus, I isolated each fraction of liver macrophage from chronically injured WT and OSM KO livers, respectively (Figure III-18 A). Expression analysis of TGF-β and PDGF-B in each fraction revealed that both genes were downregulated in OSM KO BMDMs compared to WT BMDMs, while those were less affected in liver resident macrophages between both genotypes (Figure III-18 B), suggesting that BMDMs may be more responsible for OSM-induced fibrogenic phenotype.

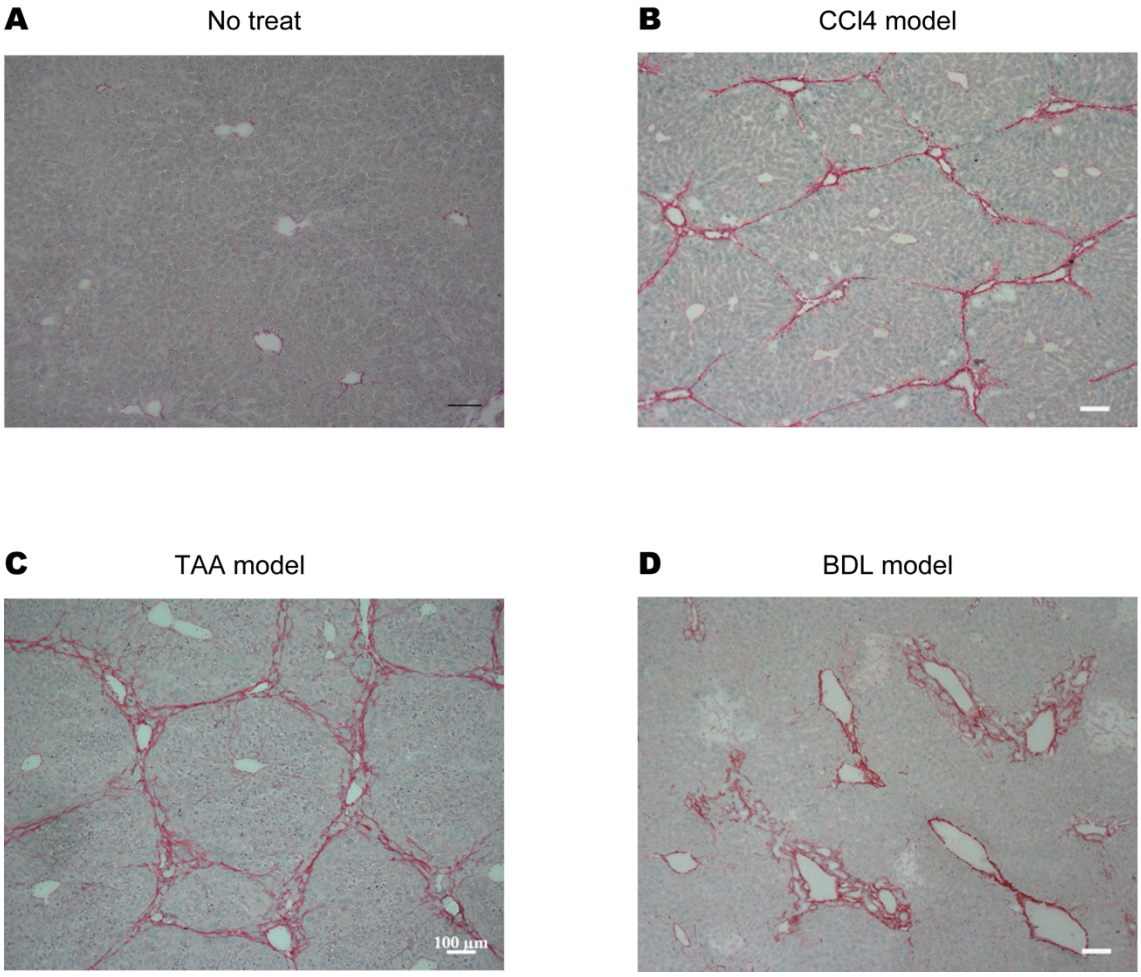

Figure III-1. Liver fibrosis in the three well-established models. Each panel shows representative image of PSR staining of the liver with (A) no treatment (control), (B) repeated CCl_4 injection, (C) TAA administration and (D) bile duct ligation. PSR staining shows collagen fibers as red color. Scale bar = 100μm.

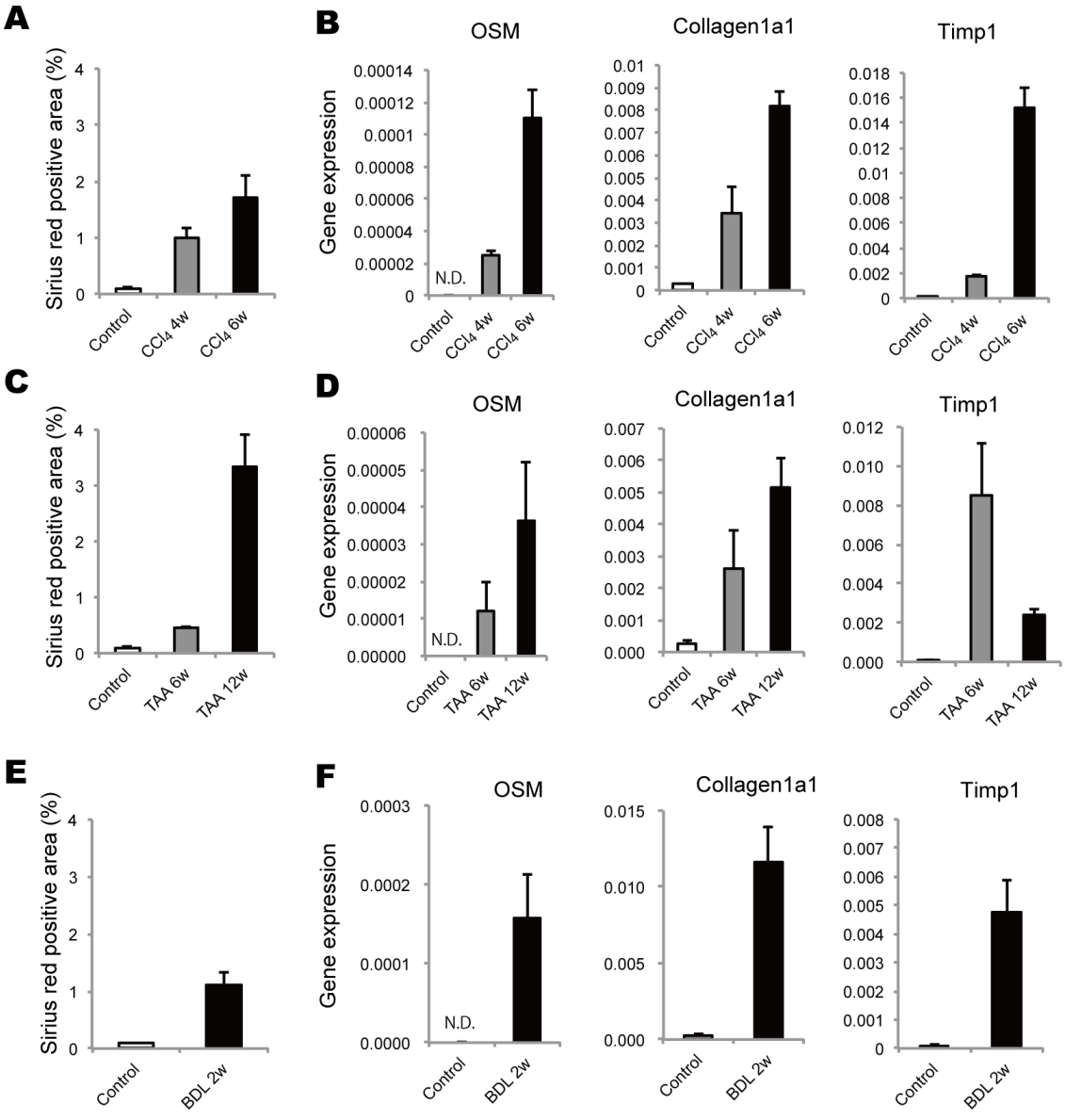

Figure III-2. Expression of OSM and fibrosis-related genes during liver fibrogenesis. Mice were treated with repeated CCl_4 injection, TAA administration and BDL followed by harvest at serial time points during fibrogenesis. (A, C, E) Quantitative analysis of fibrosis area showed progression of fibrosis in time-dependent manner. (B, D, F) Messenger RNA expression levels of *Osm*, *collagen1a1* and *Timp1* in each time point of models. RNA was extracted from whole liver. Data represent means +/-standard error of the mean, n=4-5. N.D., not detected.

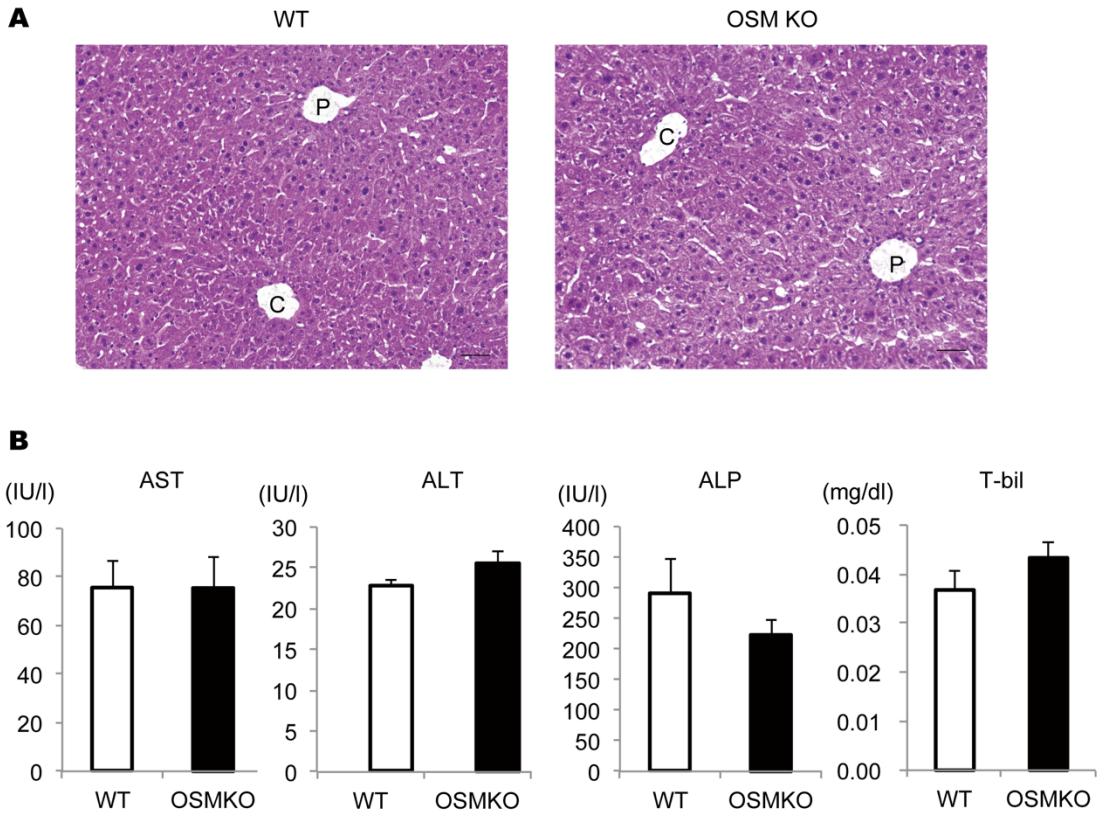

Figure III-3. Liver phenotype of OSM KO mice with no treatment. (A) H&E staining showed no sign of hepatic injury in OSM KO mice. Representative images are shown. Scale bar = 50 μ m. P: portal vein, C: central vein. (B) Serum marker of hepatic injury showed no difference between WT and OSM KO mice. N-=3. Data are shown as mean+/- SEM.

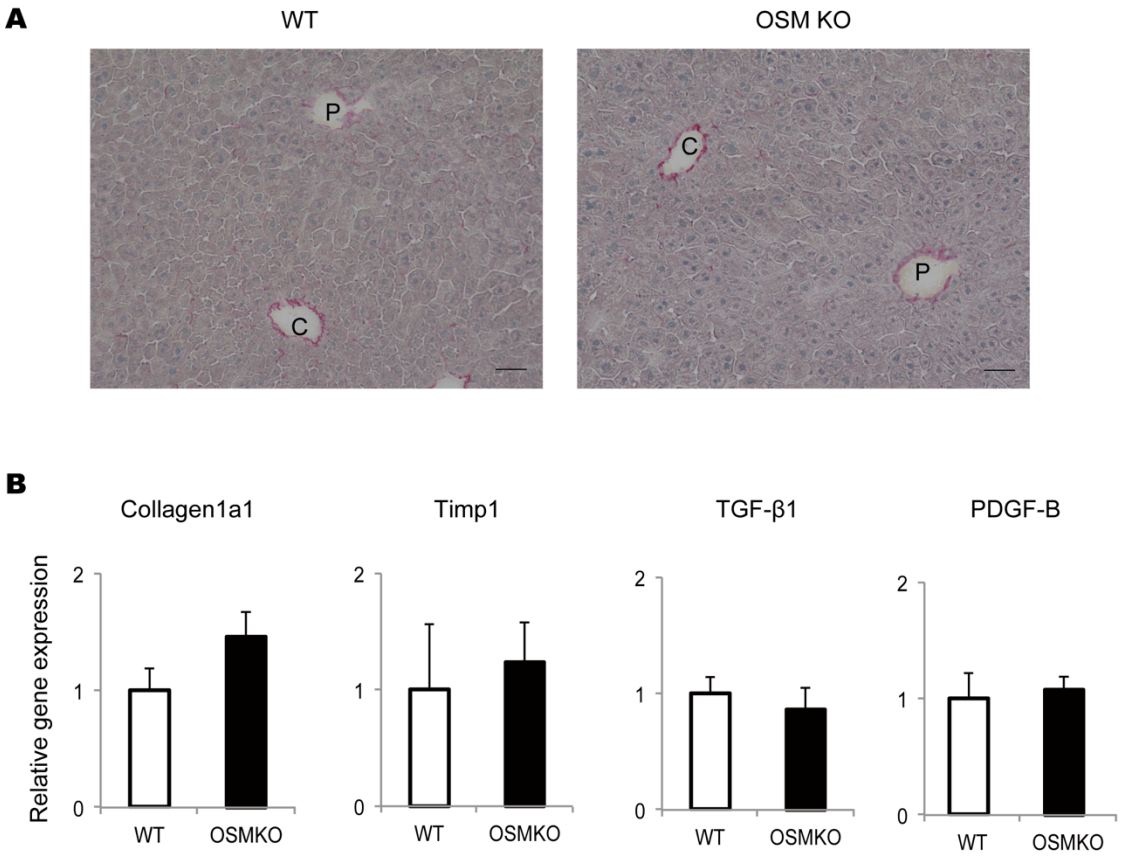

Figure III-4. Fibrosis-related phenotype of the liver from WT and OSM KO mice with no treatment. (A) PSR staining showed no sign of fibrosis in the intact liver of both WT and OSM KO mice. P: portal vein, C: central vein. Representative images are shown. Scale bar = 50 μ m. (B) Messenger RNA expression of fibrosis-related genes of WT and OSM KO mouse livers. N=3. Data are shown as mean+/- SEM.

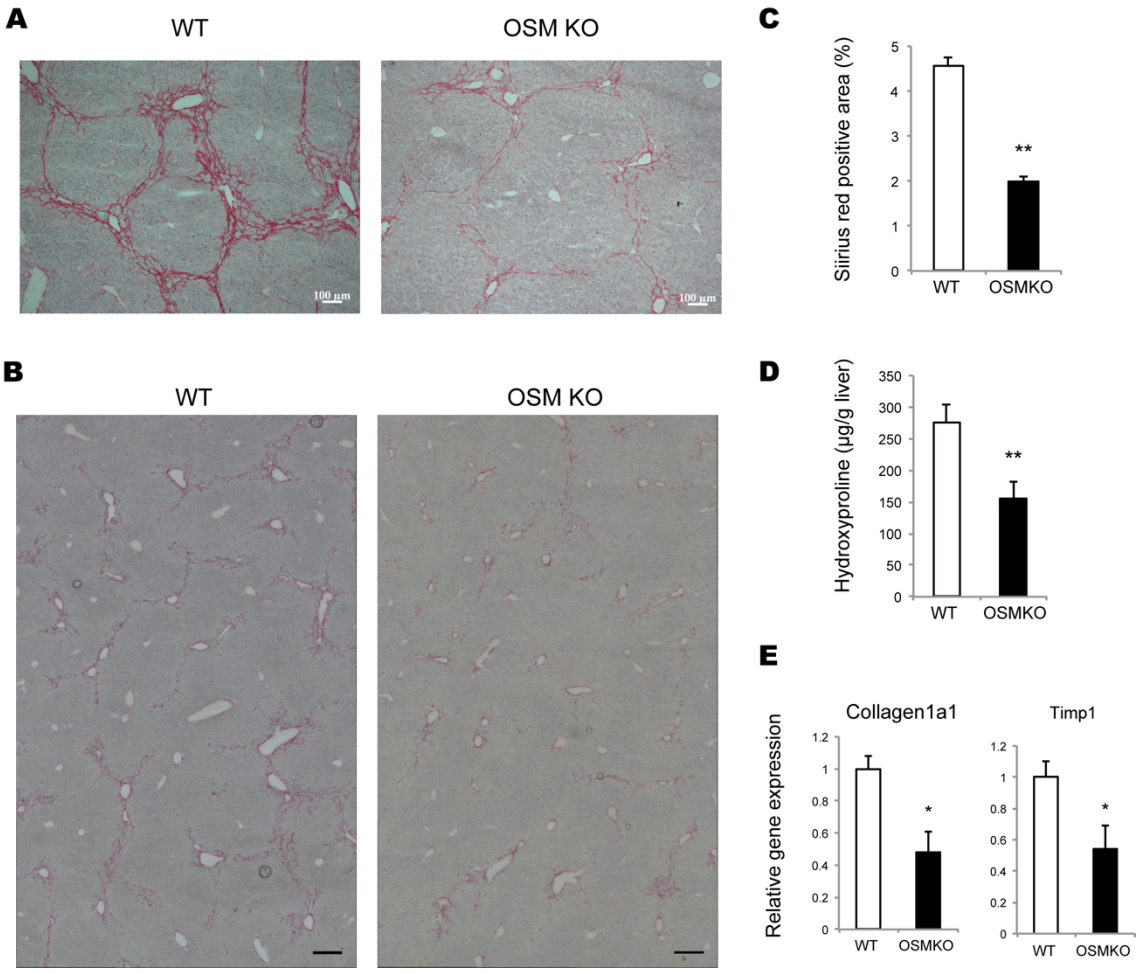

Figure III-5. OSM KO mice show reduced fibrosis after chronic liver injury. WT and OSM KO mice were treated with 0.03% TAA in drinking water and harvested after 12 weeks of administration. (A) Representative PSR staining are shown for WT control and OSM KO mice. Scale bar = 100μm (B) Jointed images of PSR staining for broad area of liver specimen (Representative images are shown, Scale bar = 300μm. (C) Quantitative analysis of fibrotic area by PSR staining and (D) hydroxyproline content of WT and OSM KO mouse livers (N=4). (E) Messenger RNA expression of fibrosis-related genes (n=8-9) of WT and OSM KO mouse livers. All data are shown as mean+/- SEM, **P*<0.05, ***P*<0.01.

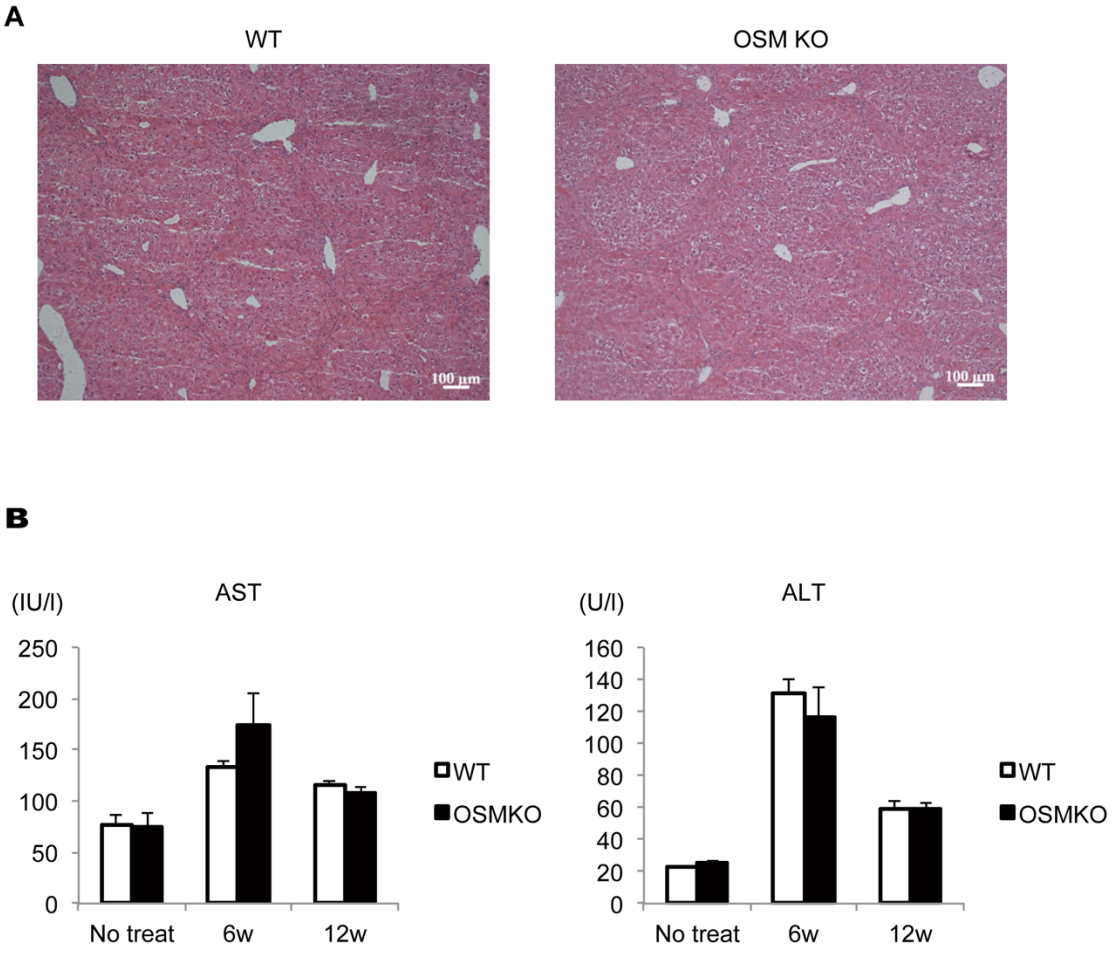

Figure III-6. Hepatic injury of WT and OSM KO mice in the TAA model. (A) H&E staining of chronically injured liver with TAA showed no difference in necrotic area between WT and OSM KO mice. Representative images are shown. Scale bar = 100 μ m. (B) Serum marker of hepatocyte damage during fibrosis progression showed no significant difference between WT and OSM KO mice. N=3. Data are shown as mean+/- SEM.

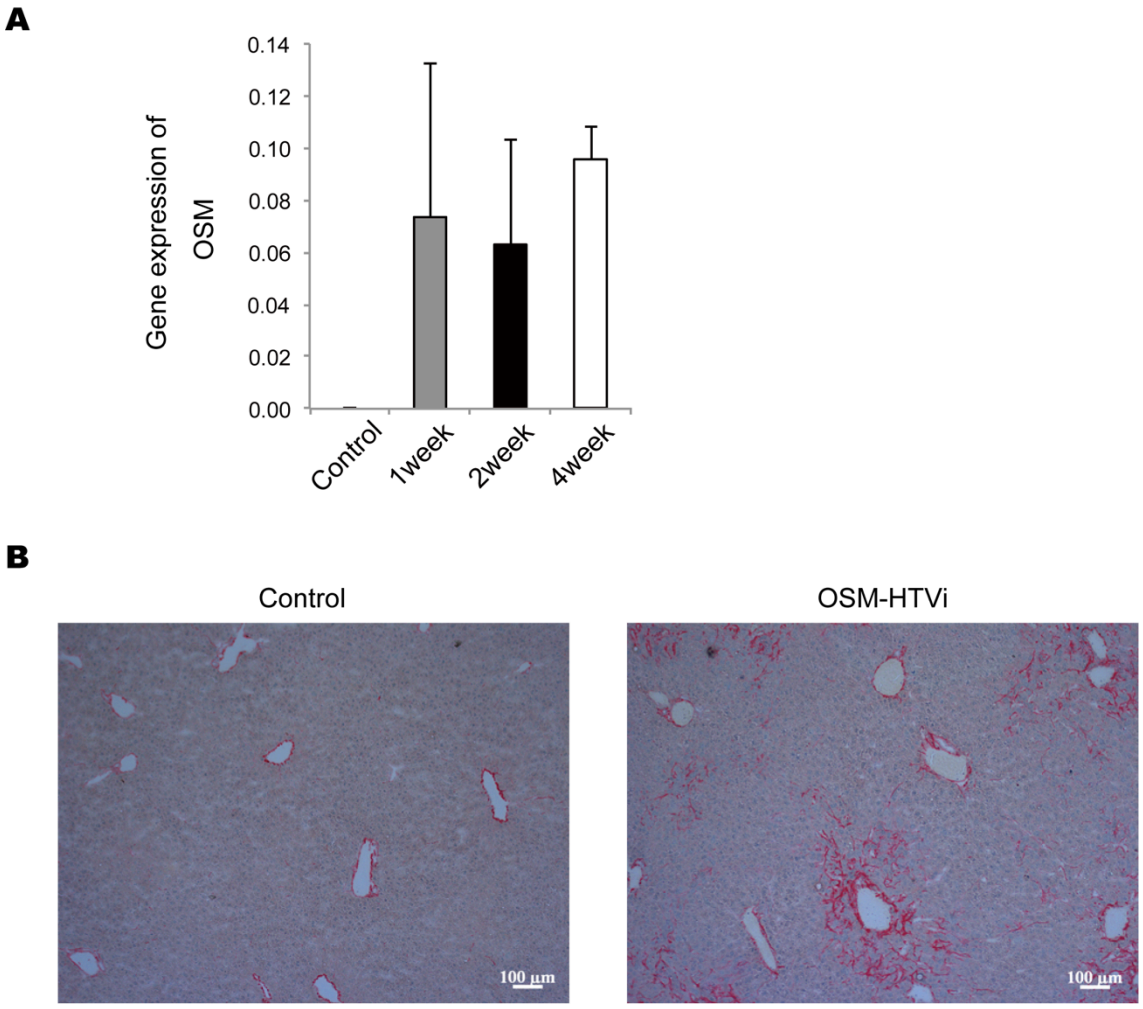

Figure III-7. Continuous expression of OSM induces liver fibrosis. (A) OSM expression in serial time points after OSM-HTVi (n=4 per each time point). (B) Representative images of PSR staining of the liver with LacZ-HTVi (control) and OSM-HTVi mice 2 weeks after injection are shown. Scale bar = 100μm.

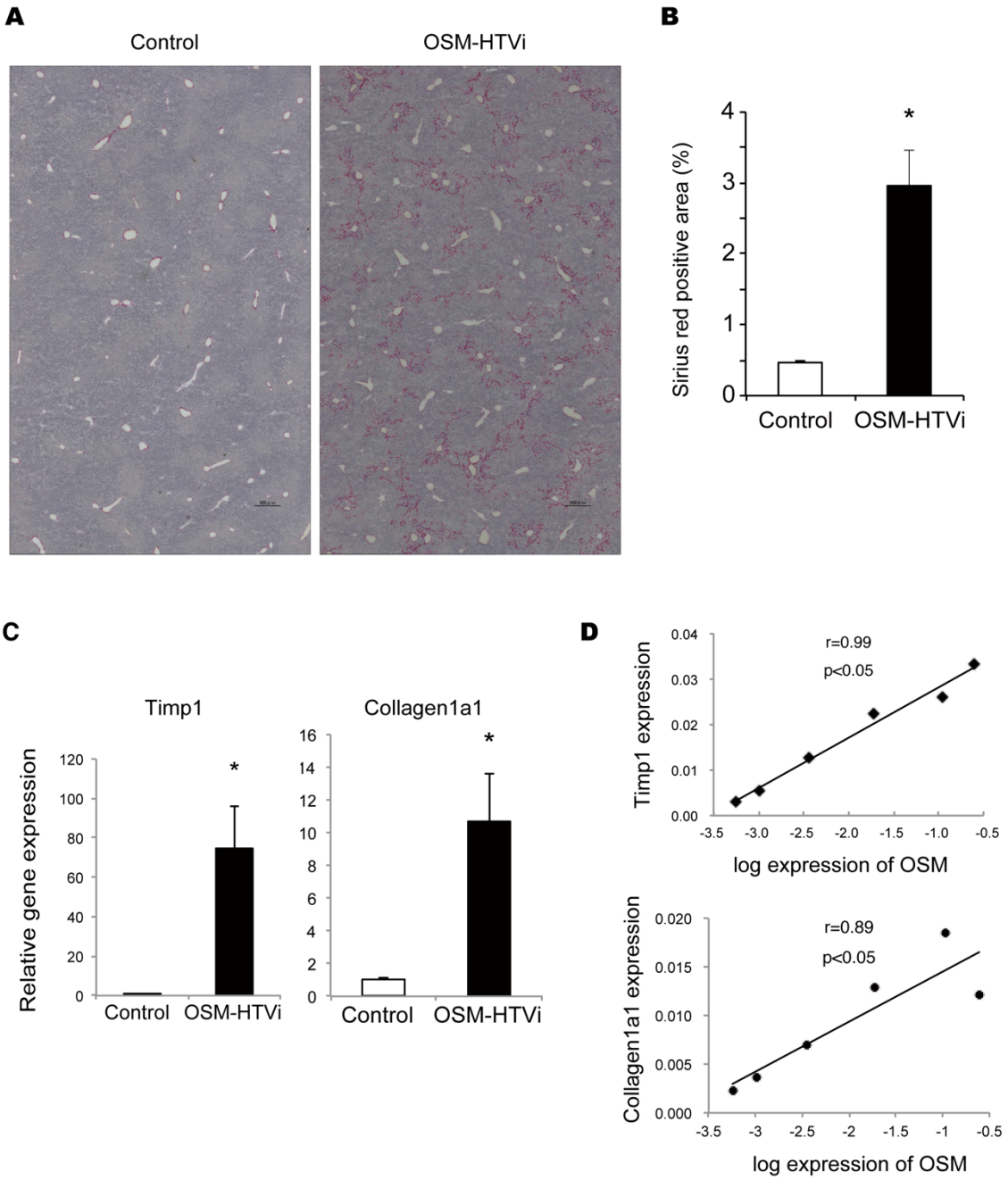

Figure III-8. Analysis of OSM-induced liver fibrosis. (A) Representative jointed images of liver section with PSR staining showed extensive fibrosis in the OSM-HTVi liver. Scale bar = 300μm. (B) Quantitative analysis of fibrotic area (n=6) is shown. (C) Expression analysis (whole liver) for *collagen1a1* and *Timp1* mRNA after 2 weeks of HTVi (n=6). (D) Correlation between the expression level of fibrosis-related genes (*collagen1a1* and *Timp1*) and that of OSM at 2 weeks after HTVi (n=6). Data are shown as mean+/- SEM, **P*<0.05. r: Pearson's correlation coefficient.

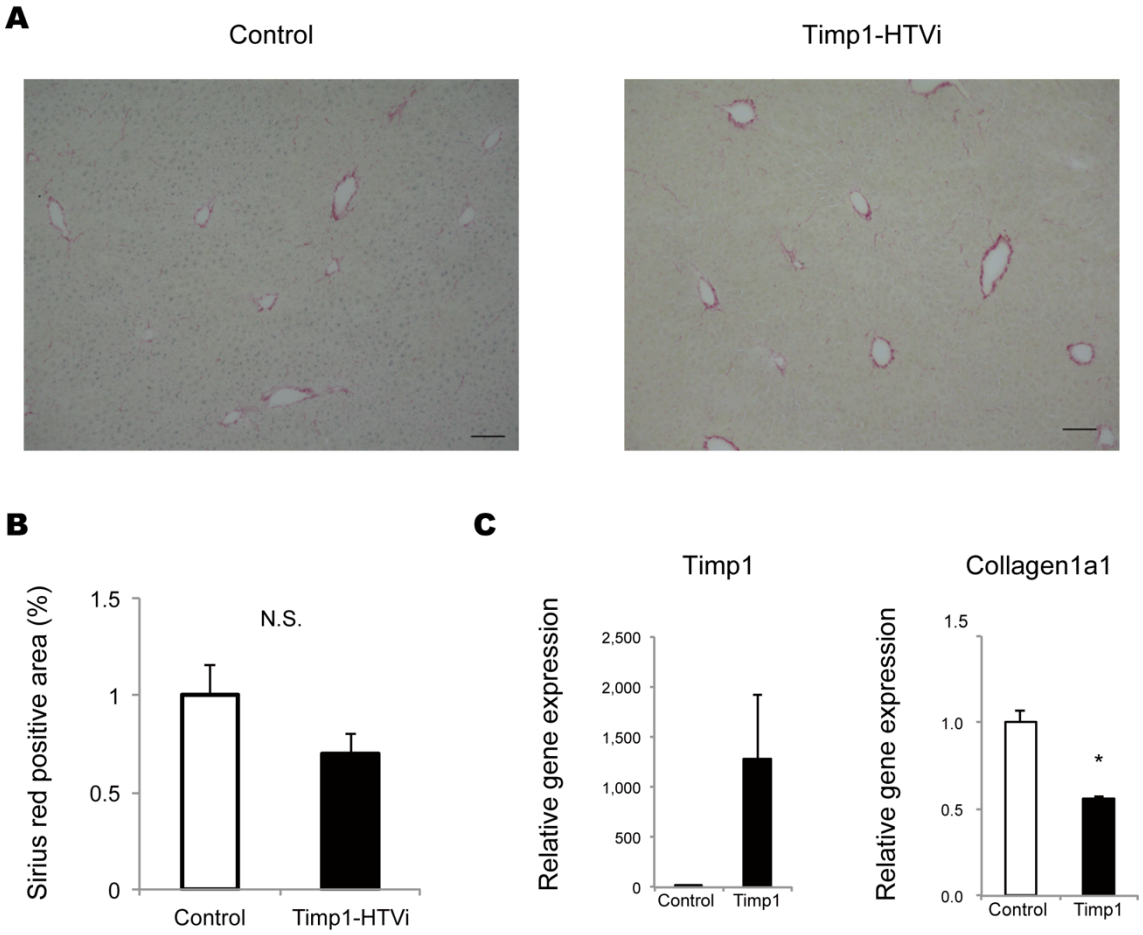

Figure III-9. Analysis of the liver with Timp1-HTVi. (A) Representative images of PSR staining are shown for liver sections of LacZ-HTVi (control) and Timp1-HTVi mice 2weeks after injection. Scale bar = 100 μ m. (B) Quatitative analysis of fibrotic area and (C) expression analysis (whole liver) for *Collagen1a1* and *Timp1* mRNA at 2 weeks after HTVi (n=3-4). Data are shown as mean+/- SEM, **P*<0.05.

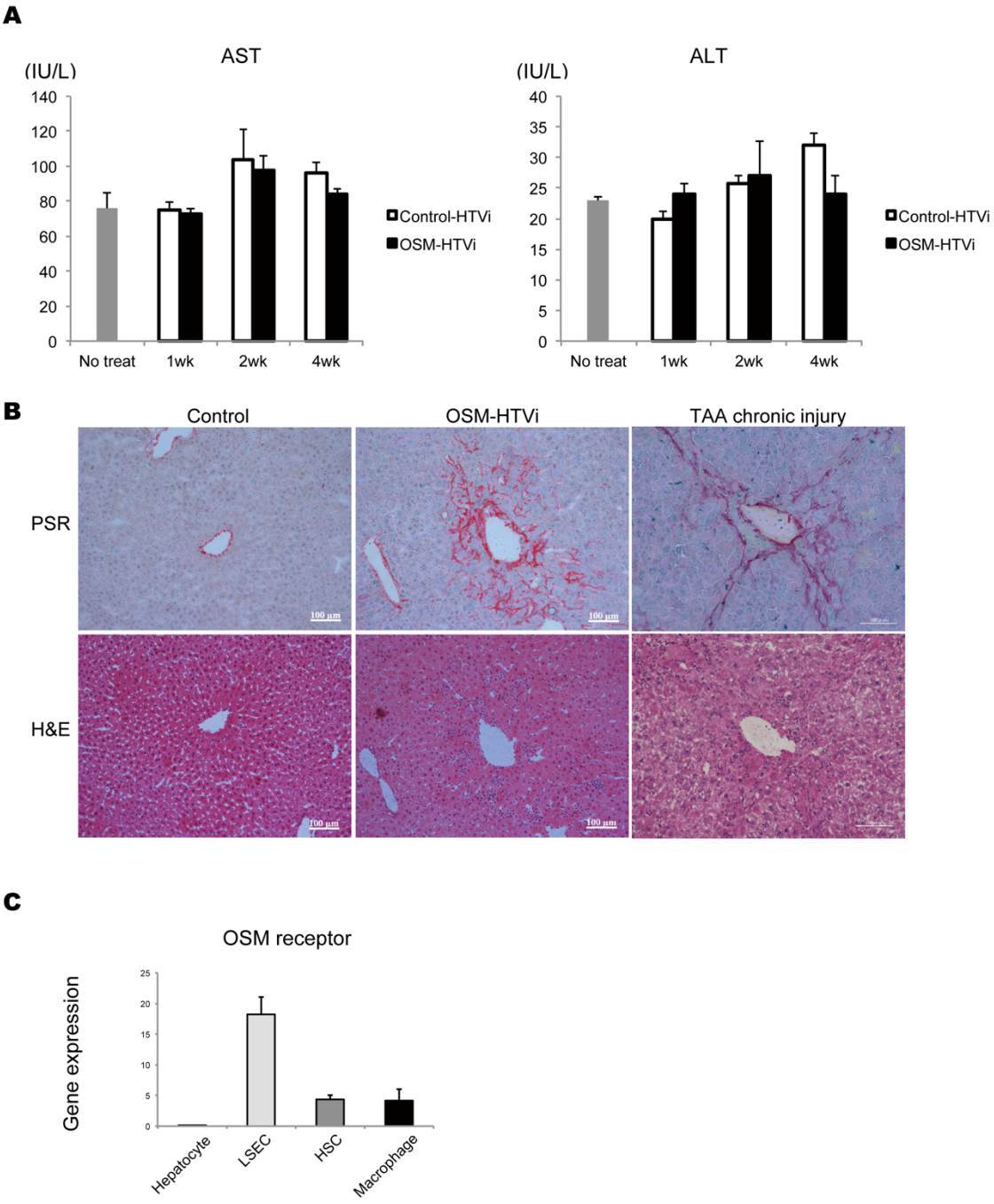

Figure III-10. OSM induces fibrosis without hepatic injury. (A) Serum AST, ALT, total level in control and OSM-HTVi mice in each time points (N=3). (B) PSR staining (upper panels), H&E staining (lower panels) of serial sections of LacZ-HTVi (control), OSM-HTVi and TAA-injured livers. (C) The expression profile of OSMR in hepatocyte, LSEC, HSC and macrophage, isolated from normal liver as described in Methods, measured by quantitative real-time PCR (N=4). All data are shown as mean+/- SEM.

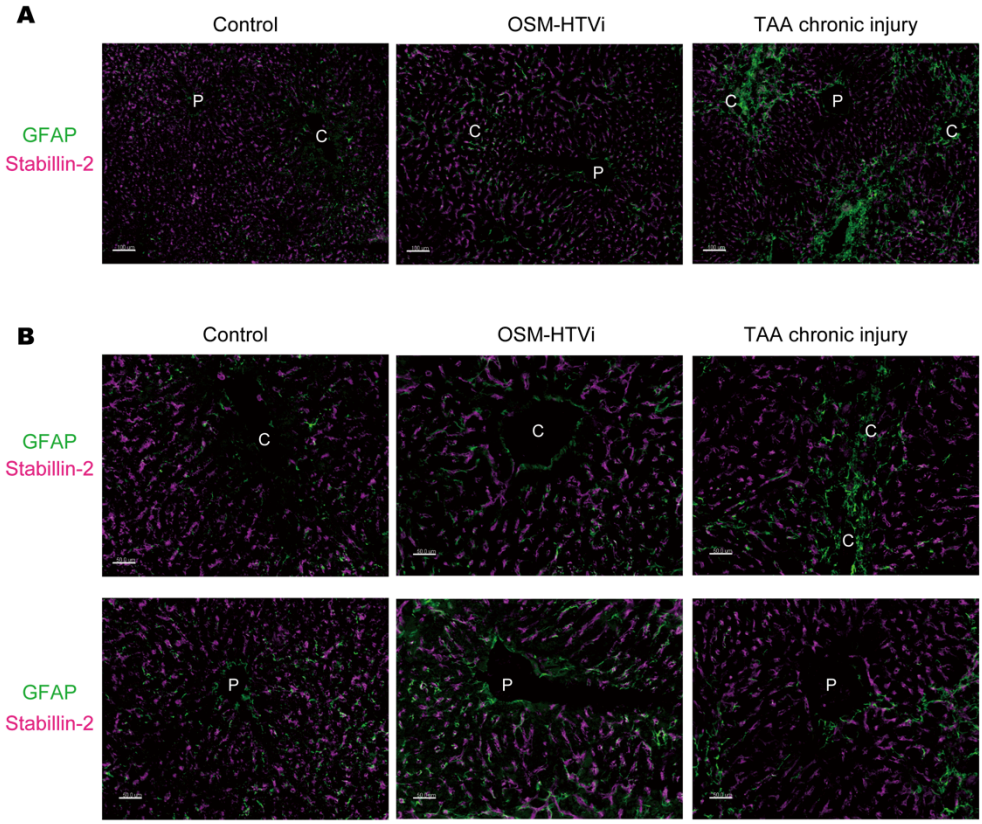

Figure III-11. Immunohistochemical analysis of control, OSM-HTVi and TAA-injured livers using antibodies against GFAP (HSC) and Stabilin-2 (LSEC). LSECs were observed close to HSCs throughout the liver (A) Scale bars = 100μm. Magnified views around central vein (B, upper panel) and portal vein (B, lower panel). Scale bars = 50μm. C: central vein. P: Portal vein.

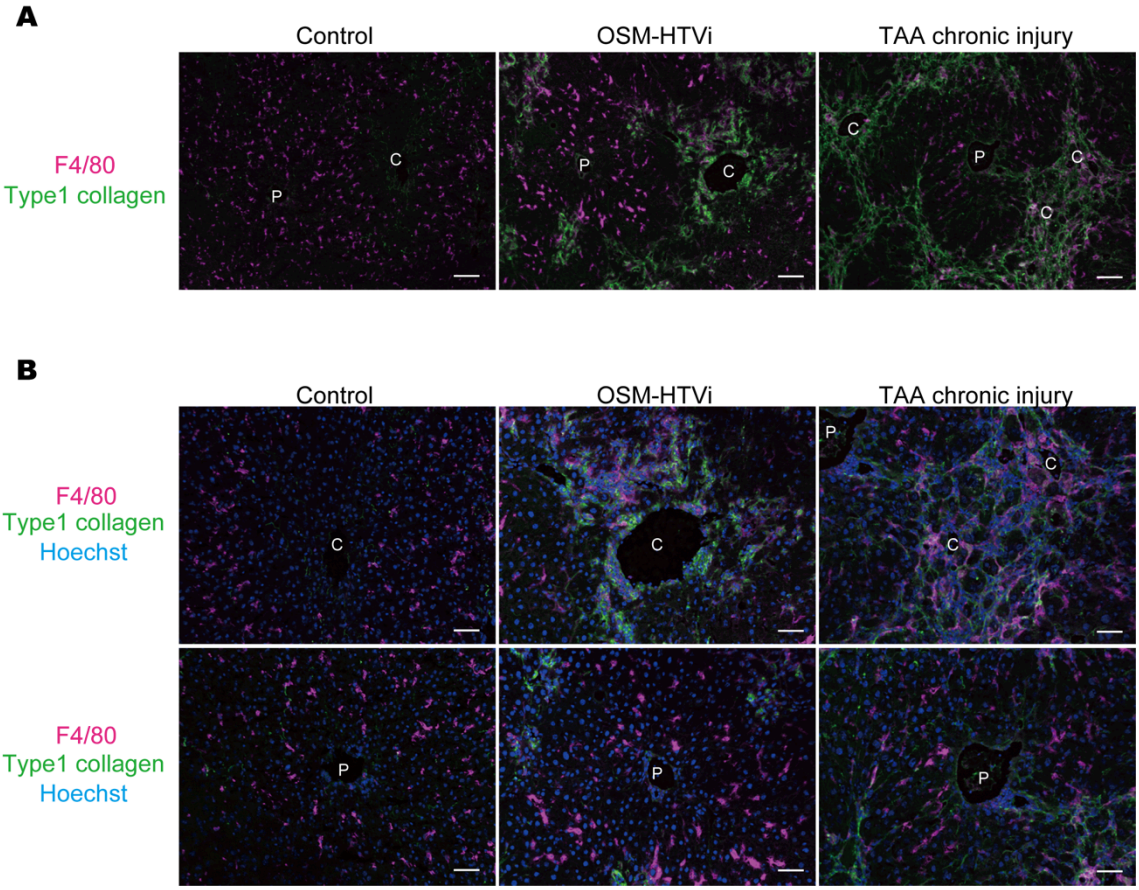

Figure III-12. Immunohistochemical analysis of control, OSM-HTVi and TAA-injured livers using antibodies against F4/80 (macrophage) and Type I collagen. (A) Liver macrophages are dominantly observed around portal vein in normal state. In both OSM-induced and TAA-induced fibrotic livers, macrophage were observed around central vein where fibrosis occurred. Scale bars shown are 100μm in (A) and 50μm in (B). C: central vein. P: Portal vein.

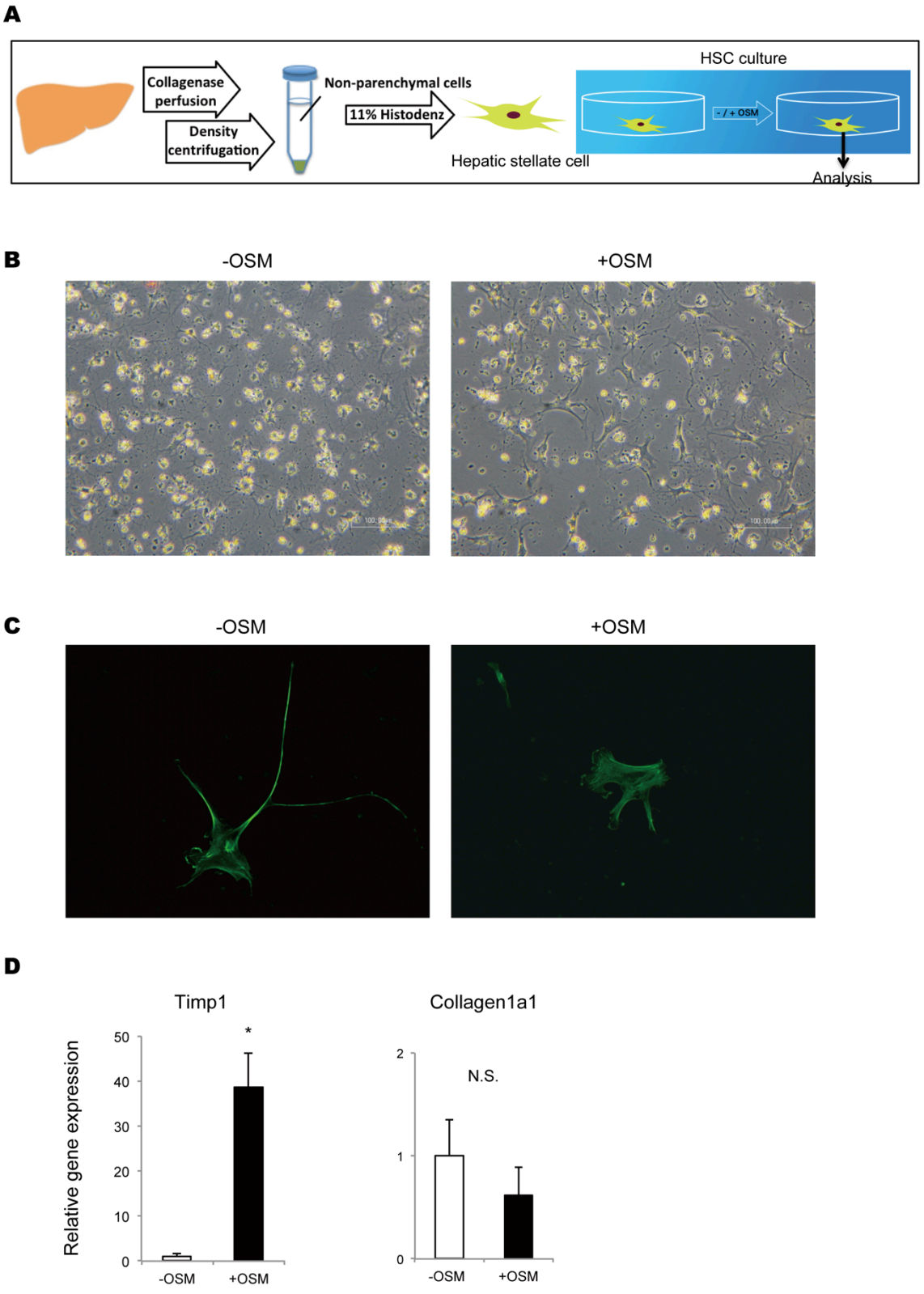

Figure III-13. OSM directly induce Timp1 in HSCs primary culture. (A) Schematic view of the experiments. Freshly isolated HSCs from normal liver were cultured with or

without OSM and analyzed. (B) Representative morphology of HSCs in bright fields and (C) HSCs stained by phalloidin (green) cultured with or without OSM. (D) Expression profiles of *Timp1* and *Collagen1a1* of HSCs after 2 days of culture with or without OSM. Data are shown as mean +/- SEM. *P<0.05. N=4

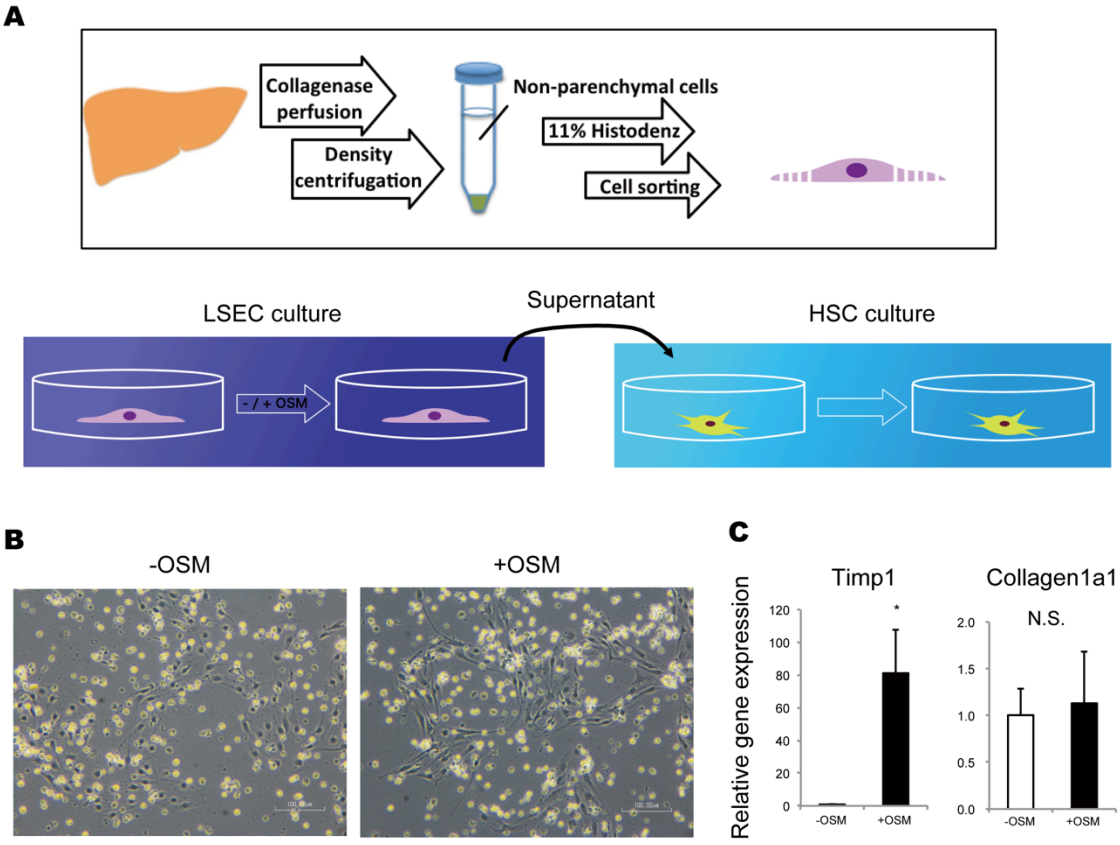

Figure III-14. Analyses of the interactions between HSCs and LSECs *in vitro*. (A) Schematic view of the experiments. Freshly isolated LSEC were cultured with or without OSM and supernatant were obtained. Then, primary cultures of HSCs were stimulated with the supernatant from these LSEC cultures. (B) Morphological changes were observed in OSM-stimulated LSECs. (C) The expression of Timp1 and Collagen1a1 in HSCs were analyzed by qRT-PCR. (N=4) Data are shown as mean +/- SEM. **P*<0.05.

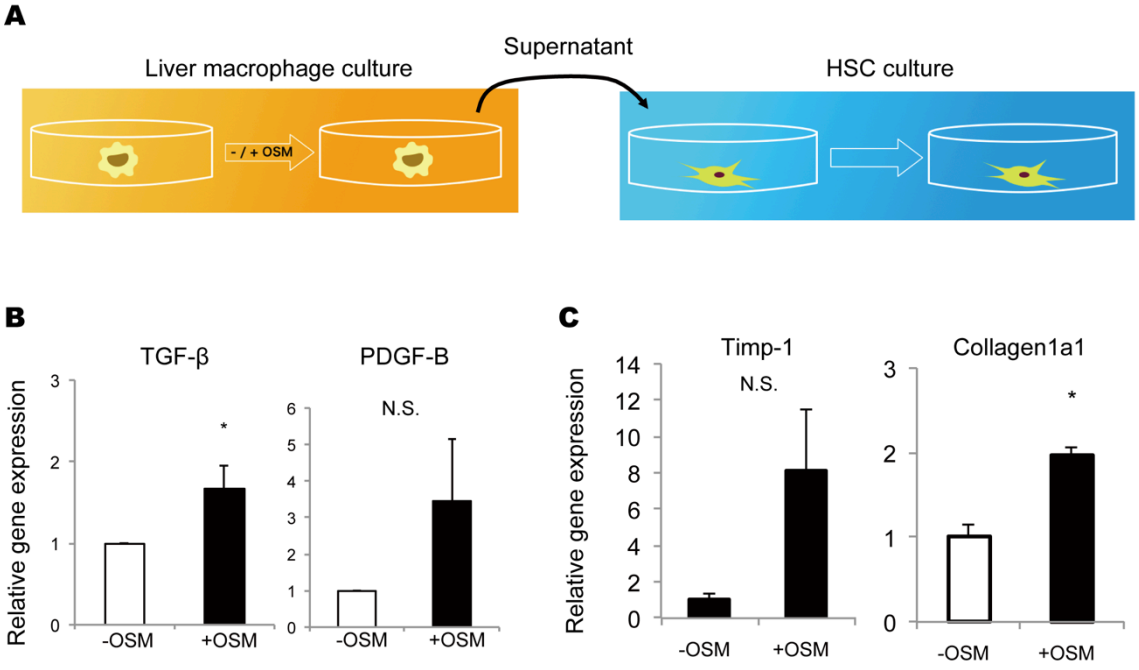

Figure III-15. Analyses of the interactions between HSCs and macrophages. (A) Schematic view of the experiments. Liver macrophages were isolated from normal liver by using magnetic beads-based cell sorting (MACS) and cultured with or without OSM. Supernatants from these macrophage cultures were added to the primary culture of HSCs. (B) Expression profile of *Tgf-β1* and *Pdgf-b* mRNA in liver macrophage cultured with or without OSM (C) QRT-PCR analyses of *Timp1* and *Collagen1a1* expression of HSCs stimulated with the supernatant of liver macrophage culture with or without OSM (N=4). All data are shown as mean +/- SEM. **P*<0.05.

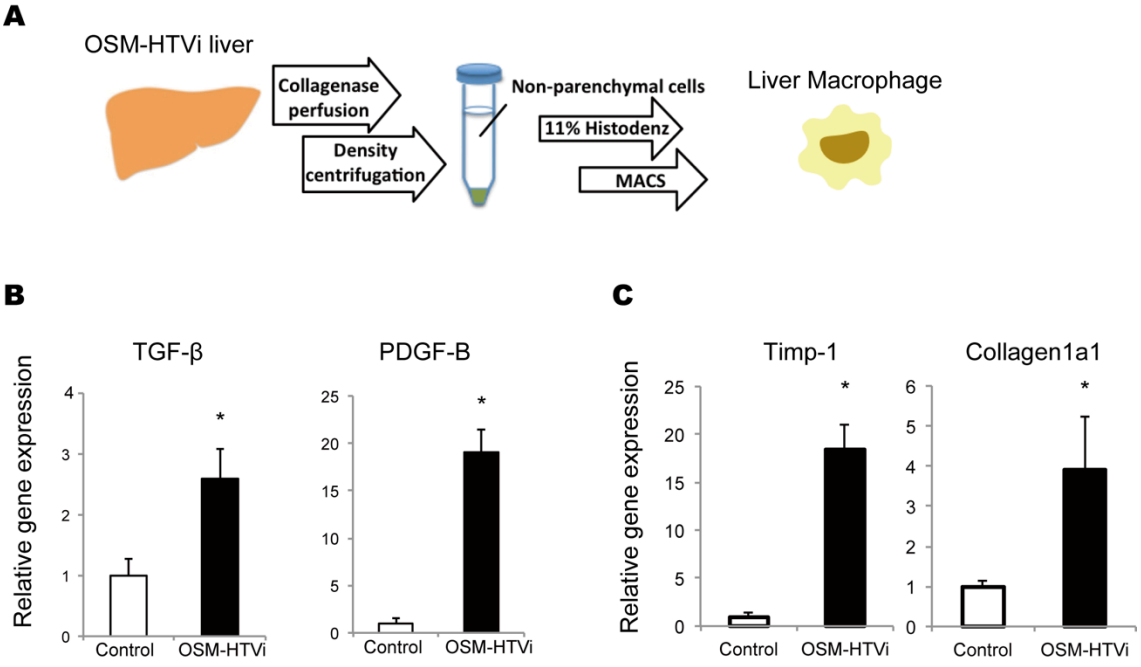

Figure III-16. Analysis of the fibrogenic property of F4/80 positive cells from OSM-HTVi liver. (A) Schematic view of the experiments. F4/80 positive cells were isolated from the liver with OSM-HTVi for 2weeks by using MACS. (B) Expression profile of *Tgf-β1* and *Pdgf-b* mRNA in liver macrophage isolated from the liver with LacZ-HTVi (control) and OSM-HTVi. (C) Isolated F4/80 positive cells from control and OSM-HTVi liver were cultured and each supernatants were added to the primary culture of HSCs. QRT-PCR analyses of *Timp1* and *Collagen1a1* expression of the HSCs are shown (N=4). All data are shown as mean +/- SEM. **P*<0.05

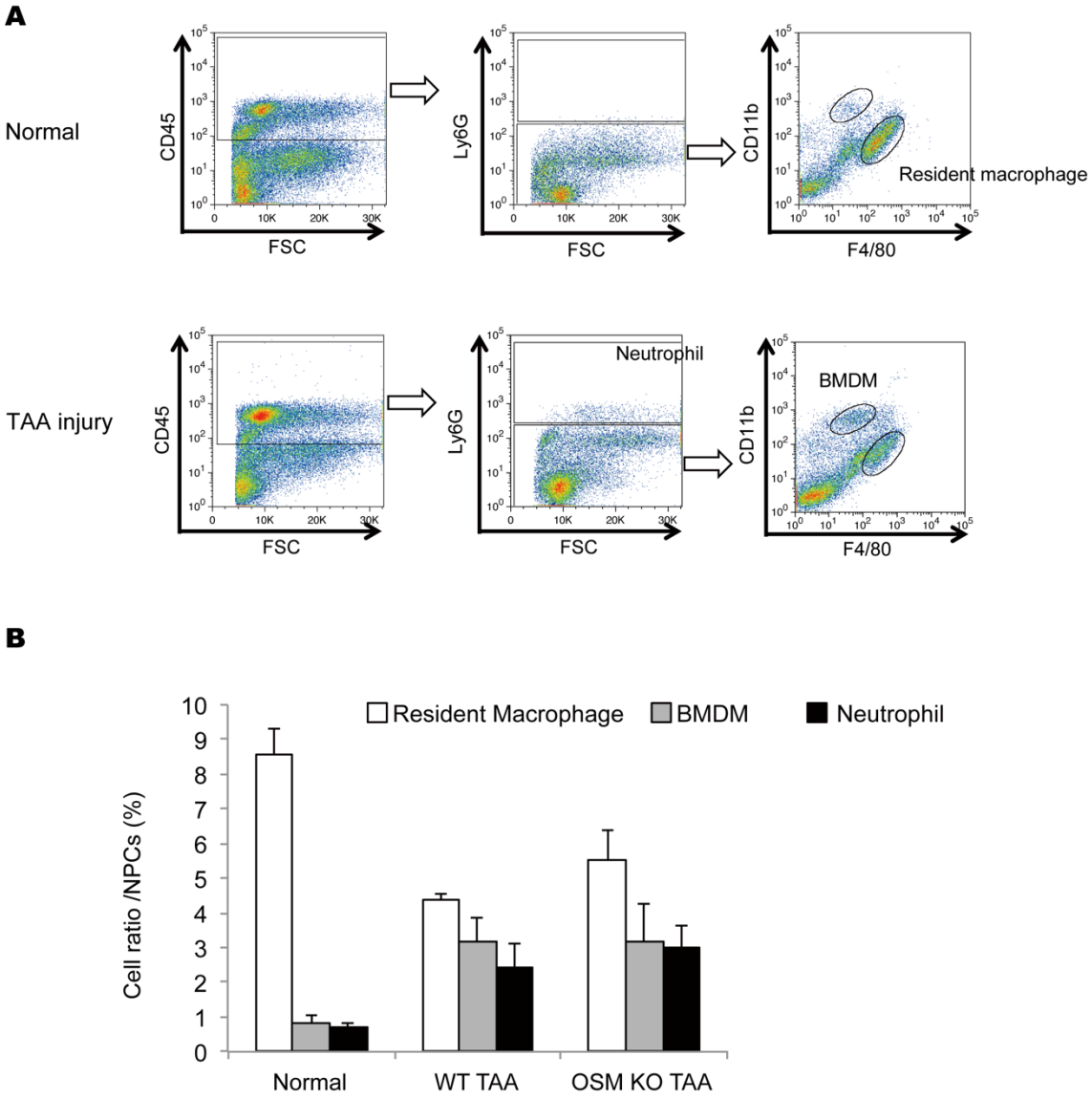

Figure III-17. Analyses of the subpopulations of F4/80 positive cells in TAA-induced chronic liver injury. (A) The subpopulation of F4/80 positive cells was isolated from control liver (uninjured) and TAA-induced fibrotic liver by FACS using anti-CD45, Ly6G, F4/80 and CD11b antibodies. Neutrophil were identified as viable CD45+ Ly6G^{hi} cell and excluded from subsequent macrophage gating. FACS analysis indicates that F4/80^{hi} CD11b^{mid} resident macrophages decrease and F4/80^{lo} CD11b^{hi} BMDMs increase in chronically injured liver compared to uninjured liver (representative plots are shown). (B) The ratios of subpopulation of F4/80 positive cells relative to total non-parenchymal cells in normal livers, injured WT and OSM KO livers by TAA administration. Data are shown as mean +/- SEM. N= 4

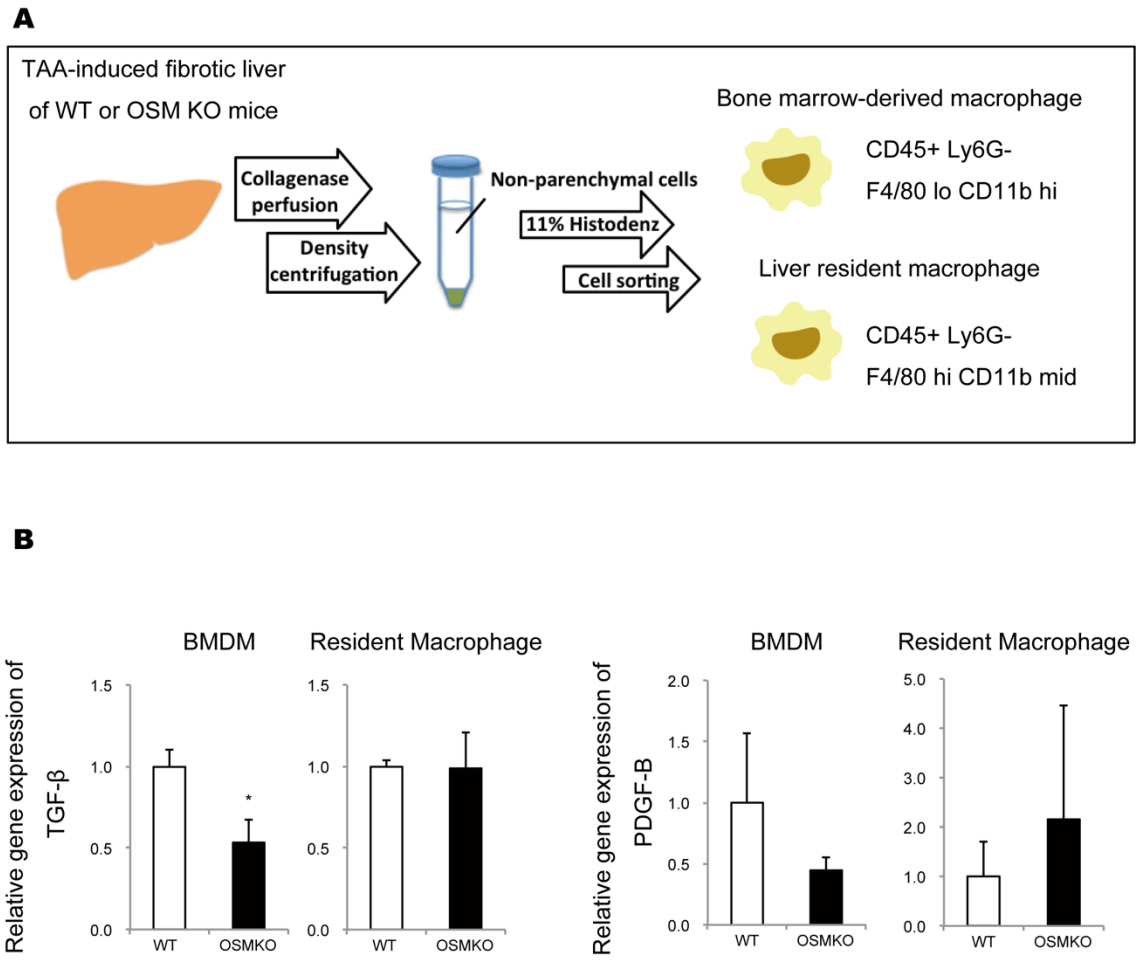

Figure III-18. Analysis of fibrogenic properties of BMDM and liver resident macrophage in chronically injured liver with TAA. (A) Schematic view of the experiments. (B) Quantitative RT-PCR of fibrosis-related genes of each subpopulation of F4/80 positive cells in the liver with chronic injury. Comparison of relative expression of *Tgf-β1* (left) and *Pdgf-b* (right) in BMDMs and resident macrophages between WT and OSM KO mouse livers showed significant difference in TGF-β expression of BMDM between WT and OSM KO mouse. Data are shown as mean +/- SEM. **P*<0.05. N=4.

Chapter IV. Discussion

The aim of this study is to elucidate the role of OSM during fibrosis progression in the chronically injured liver. It was reported previously that transient expression of OSM is beneficial to repair acutely injured liver by protecting tissue damage and that OSM effectively induces Timp1 production in fibroblast (28-30) and lung, muscle and liver (13, 31). Thus ECM remodeling is critical for restoration from injury. However, a factor with beneficial effects on acute injury does not necessarily exhibit the same effect on chronic injury. The current study showed that persistent expression of OSM aggravates liver fibrosis by affecting HSC and liver macrophages differentially (Figure IV-1). This study demonstrated that continuous expression of OSM in the liver is necessary and sufficient to induce remarkable fibrosis and that OSM is a strong inducer of Timp1 in mouse quiescent HSCs. However, continuous expression of Timp1 by HTVi was not sufficient to induce liver fibrosis unlike the case of OSM expression by HTVi (Figure III-9 A). These results are consistent with the reports by Yoshiji et al. (32, 33), showing that Timp1 transgenic mouse exhibited no sign of fibrosis in the intact liver but fibrosis development was promoted when the liver was injured by hepatotoxin.

In the current study, mRNA of Collagen1a1 was significantly reduced in Timp1-HTVi liver compared to control liver with unknown reason (Fig III-9 C). At least, Timp1-HTVi did not induce collagen 1a1 gene as OSM-HTVi did. Therefore, the up-regulation of collagen as well as Timp1 by OSM will account for OSM-induced liver fibrosis. Although the role of OSM on the production of collagens has not been well documented, only one *in vitro* study showed that OSM stimulated collagen production in human HSCs by a post transcriptional mechanism without enhancing gene expression of type 1 collagen (34). Consistently, the results of the current study showed that collagen gene expression was not directly induced in mouse HSC by OSM *in vitro*, whereas continuous expression of OSM induces type1 collagen gene expression *in vivo*. Therefore, I hypothesized that OSM up-regulates type1 collagen gene in HSCs via their surrounding cells.

Recent accumulating evidence has demonstrated that macrophage is a critical regulator of fibrosis in various organs (35-39). In addition, it was reported recently that OSM is able to switch the phenotype of macrophages in adipose tissue (24). In line with these evidences, I demonstrated that liver macrophages resided in the vicinity to HSCs

in fibrotic area (Fig III-12) and that OSM promoted the fibrogenic activity of mouse HSCs indirectly by inducing the expression of fibrogenic factors in liver macrophages (Fig III-15 and 16). However, my results do not exclude the possibility that anti-apoptotic effect by Timp1 in HSCs may contribute to liver fibrosis (40).

In addition to liver resident macrophages, infiltrating BMDMs increase in the liver during chronic injury. These liver macrophages are activated by various stimuli derived from dead cells and immune cells, and constitute the microenvironment for regulating fibrosis (25). Although previous numerous studies have shown the contribution of liver macrophages to fibrosis (41-46), recent studies have focused on the distinct roles of macrophage subsets in the process of liver fibrosis. BMDM or its subset isolated by a cell sorter was reported to contribute to fibrogenesis (45). A more recent study suggested the existence of macrophage subtypes contributing to the resolution of fibrosis via MMPs (27). In this study, I provided evidence that the fibrogenic property of BMDMs was enhanced by OSM. Quantitative real-time PCR showed higher expression of TGF-β in BMDMs from the liver of WT mice compared to OSM KO mouse with chronic liver injury. Although TGF-β is the most well-known fibrogenic

factor, further investigation to confirm that this fibrogenic cytokine contribute to the OSM-stimulated activation of HSC is required. In addition, the results do not exclude the possible involvement of liver resident macrophages in liver fibrogenesis or fibrolysis, because liver resident macrophages also express OSMR. The role of OSM on liver resident macrophages in liver fibrosis needs further investigation.

In the pathogenesis of chronic liver injury, continuous hepatocyte injury produces causative agents to trigger immune response. Multiple effectors such as cytokine and chemokine from infiltrating cells as well as liver resident cells are involved in forming a complicated microenvironment for fibrosis. This complexity makes it difficulty to elucidate the mechanism underlying liver fibrosis even in a well-established model. The OSM-HTVi model I have developed is a simple and useful model to investigate the mechanism of fibrogenesis in the liver. In this method, cytotoxic inflammation is not induced, allowing us to uncover the core roles of OSM in fibrogenesis. In human liver diseases, some patients develop severe fibrosis with only mild elevation of ALT, and some patients suffer from persistent residual fibrosis despite successful removal of causative agents. Therefore, persistent OSM expression may be involved in the

pathogenesis in such patients. In this line, some studies showed increased OSM in patients with chronic liver injury (14, 15). In the current study, I demonstrated that persistent expression of OSM induces liver fibrosis by affecting HSCs and liver macrophages cooperatively, suggesting that OSM signaling will be a possible target for an anti-fibrotic therapy. However, the timing of blocking OSM signal may be critical because OSM has also a beneficial effect on the regeneration in acutely injured liver as shown in the previous study (13).

In conclusion, persistent expression of OSM is a potential risk factor for liver fibrosis. The crosstalk between HSCs and liver macrophages is one of the key pathogenesis underlying OSM-induced liver fibrosis. Similar mechanism could be expected in fibrotic diseases in the other organs.

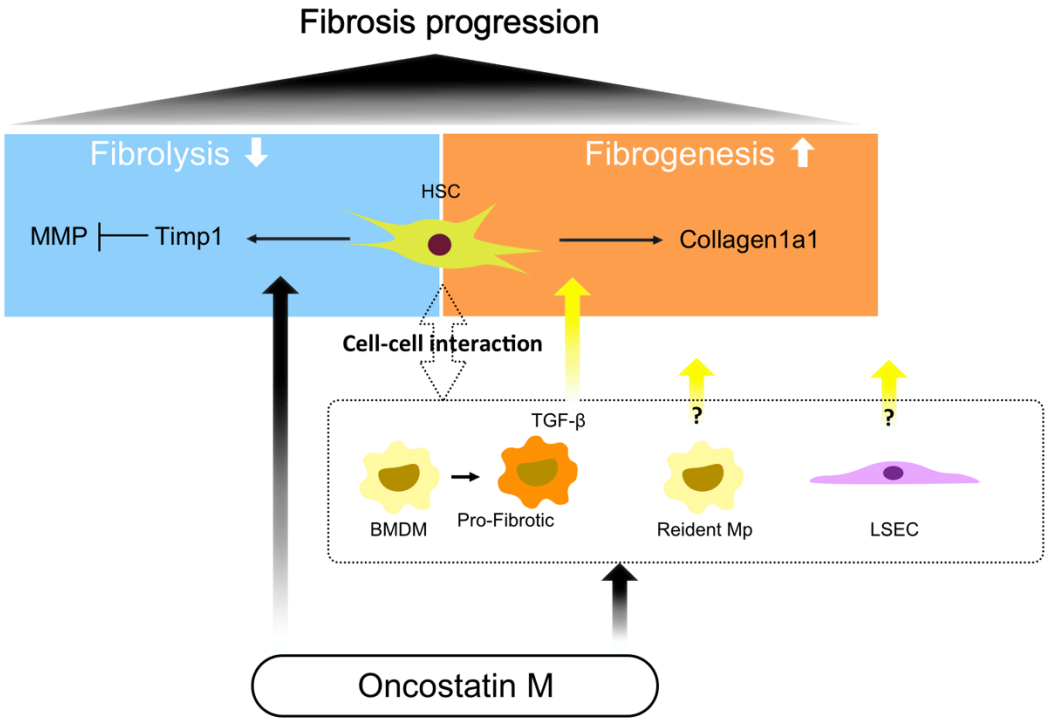

Figure IV-1. Shematic model of the mechanism of fibrosis progression induced by Oncostatin M.

Acknowledgements

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Reference

1. Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. Lancet 2014;383:1749-1761.
2. Schuppan D, Afdhal NH. Liver cirrhosis. Lancet 2008;371:838-851.
3. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209-218.
4. Hammel P, Couvelard A, O'Toole D, Ratouis A, Sauvanet A, Flejou JF, et al. Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. N Engl J Med 2001;344:418-423.
5. Trautwein C, Friedman SL, Schuppan D, Pinzani M. Hepatic fibrosis: Concept to treatment. J Hepatol 2015;62:S15-24.
6. Hellerbrand C, Stefanovic B, Giordano F, Burchardt ER, Brenner DA. The role of TGFbeta1 in initiating hepatic stellate cell activation in vivo. J Hepatol 1999;30:77-87.
7. Kanzler S, Lohse AW, Keil A, Henninger J, Dienes HP, Schirmacher P, et al. TGF-beta1 in liver fibrosis: an inducible transgenic mouse model to study liver fibrogenesis. Am J Physiol 1999;276:G1059-1068.
8. Ueberham E, Low R, Ueberham U, Schonig K, Bujard H, Gebhardt R. Conditional tetracycline-regulated expression of TGF-beta1 in liver of transgenic mice leads to reversible intermediary fibrosis. Hepatology 2003;37:1067-1078.
9. Nieto N, Dominguez-Rosales JA, Fontana L, Salazar A, Armendariz-Borunda J, Greenwel P, et al. Rat hepatic stellate cells contribute to the acute-phase response with increased expression of alpha1(I) and alpha1(IV) collagens, tissue inhibitor of metalloproteinase-1, and matrix-metalloproteinase-2 messenger RNAs. Hepatology 2001;33:597-607.
10. Inagaki Y, Truter S, Greenwel P, Rojkind M, Unoura M, Kobayashi K, et al. Regulation of the alpha 2(I) collagen gene transcription in fat-storing cells derived from a cirrhotic liver. Hepatology 1995;22:573-579.
11. Dooley S, ten Dijke P. TGF-beta in progression of liver disease. Cell Tissue Res 2012;347:245-256.

12. Tanaka M, Miyajima A. Oncostatin M, a multifunctional cytokine. Rev Physiol Biochem Pharmacol 2003;149:39-52.
13. Nakamura K, Nonaka H, Saito H, Tanaka M, Miyajima A. Hepatocyte proliferation and tissue remodeling is impaired after liver injury in oncostatin M receptor knockout mice. Hepatology 2004;39:635-644.
14. Liang H, Block TM, Wang M, Nefsky B, Long R, Hafner J, et al. Interleukin-6 and oncostatin M are elevated in liver disease in conjunction with candidate hepatocellular carcinoma biomarker GP73. Cancer Biomark 2012;11:161-171.
15. Znoyko I, Sohara N, Spicer SS, Trojanowska M, Reuben A. Expression of oncostatin M and its receptors in normal and cirrhotic human liver. J Hepatol 2005;43:893-900.
16. Minehata K, Takeuchi M, Hirabayashi Y, Inoue T, Donovan PJ, Tanaka M, et al. Oncostatin m maintains the hematopoietic microenvironment and retains hematopoietic progenitors in the bone marrow. Int J Hematol 2006;84:319-327.
17. Yagai T, Miyajima A, Tanaka M. Semaphorin 3E secreted by damaged hepatocytes regulates the sinusoidal regeneration and liver fibrosis during liver regeneration. Am J Pathol 2014;184:2250-2259.
18. Suzuki K, Tanaka M, Watanabe N, Saito S, Nonaka H, Miyajima A. p75 Neurotrophin receptor is a marker for precursors of stellate cells and portal fibroblasts in mouse fetal liver. Gastroenterology 2008;135:270-281 e273.
19. Salguero Palacios R, Roderfeld M, Hemmann S, Rath T, Atanasova S, Tschuschner A, et al. Activation of hepatic stellate cells is associated with cytokine expression in thioacetamide-induced hepatic fibrosis in mice. Lab Invest 2008;88:1192-1203.
20. Liedtke C, Luedde T, Sauerbruch T, Scholten D, Streetz K, Tacke F, et al. Experimental liver fibrosis research: update on animal models, legal issues and translational aspects. Fibrogenesis Tissue Repair 2013;6:19.
21. Tong L, Smyth D, Kerr C, Catterall J, Richards CD. Mitogen-activated protein kinases Erk1/2 and p38 are required for maximal regulation of TIMP-1 by oncostatin M in murine fibroblasts. Cell Signal

2004;16:1123-1132.
22. Nonaka H, Sugano S, Miyajima A. Serial analysis of gene expression in sinusoidal endothelial cells from normal and injured mouse liver. Biochem Biophys Res Commun 2004;324:15-24.
23. Nonaka H, Tanaka M, Suzuki K, Miyajima A. Development of murine hepatic sinusoidal endothelial cells characterized by the expression of hyaluronan receptors. Dev Dyn 2007;236:2258-2267.
24. Komori T, Tanaka M, Senba E, Miyajima A, Morikawa Y. Lack of oncostatin M receptor beta leads to adipose tissue inflammation and insulin resistance by switching macrophage phenotype. J Biol Chem 2013;288:21861-21875.
25. Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. J Hepatol 2014;60:1090-1096.
26. Holt MP, Cheng L, Ju C. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. J Leukoc Biol 2008;84:1410-1421.
27. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, et al. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. Proc Natl Acad Sci U S A 2012;109:E3186-3195.
28. Richards CD, Shoyab M, Brown TJ, Gauldie J. Selective regulation of metalloproteinase inhibitor (TIMP-1) by oncostatin M in fibroblasts in culture. J Immunol 1993;150:5596-5603.
29. Richards CD, Kerr C, Tanaka M, Hara T, Miyajima A, Pennica D, et al. Regulation of tissue inhibitor of metalloproteinase-1 in fibroblasts and acute phase proteins in hepatocytes in vitro by mouse oncostatin M, cardiotrophin-1, and IL-6. J Immunol 1997;159:2431-2437.
30. Botelho FM, Edwards DR, Richards CD. Oncostatin M stimulates c-Fos to bind a transcriptionally responsive AP-1 element within the tissue inhibitor of metalloproteinase-1 promoter. J Biol Chem 1998;273:5211-5218.
31. Kerr C, Langdon C, Graham F, Gauldie J, Hara T, Richards CD. Adenovirus vector expressing mouse oncostatin M induces acute-phase

proteins and TIMP-1 expression in vivo in mice. J Interferon Cytokine Res 1999;19:1195-1205.
32. Yoshiji H, Kuriyama S, Miyamoto Y, Thorgeirsson UP, Gomez DE, Kawata M, et al. Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. Hepatology 2000;32:1248-1254.
33. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, et al. Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. Hepatology 2002;36:850-860.
34. Levy MT, Trojanowska M, Reuben A. Oncostatin M: a cytokine upregulated in human cirrhosis, increases collagen production by human hepatic stellate cells. J Hepatol 2000;32:218-226.
35. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008;8:958-969.
36. Vernon MA, Mylonas KJ, Hughes J. Macrophages and renal fibrosis. Semin Nephrol 2010;30:302-317.
37. Hardie WD, Glasser SW, Hagood JS. Emerging concepts in the pathogenesis of lung fibrosis. Am J Pathol 2009;175:3-16.
38. Sica A, Invernizzi P, Mantovani A. Macrophage plasticity and polarization in liver homeostasis and pathology. Hepatology 2014;59:2034-2042.
39. Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. Semin Liver Dis 2010;30:245-257.
40. Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, et al. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis. J Biol Chem 2002;277:11069-11076.
41. Matsuoka M, Tsukamoto H. Stimulation of hepatic lipocyte collagen production by Kupffer cell-derived transforming growth factor beta: implication for a pathogenetic role in alcoholic liver fibrogenesis. Hepatology 1990;11:599-605.

42. Nieto N. Oxidative-stress and IL-6 mediate the fibrogenic effects of [corrected] Kupffer cells on stellate cells. Hepatology 2006;44:1487-1501.
43. De Minicis S, Seki E, Uchinami H, Kluwe J, Zhang Y, Brenner DA, et al. Gene expression profiles during hepatic stellate cell activation in culture and in vivo. Gastroenterology 2007;132:1937-1946.
44. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 2007;13:1324-1332.
45. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, et al. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology 2009;50:261-274.
46. 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.