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Author manuscript
Oncogene. Author manuscript; available in PMC 2012 December 28.

Published in final edited form as:
Oncogene. 2012 June 28; 31(26): 3111–3123. doi:10.1038/onc.2011.498.

Chitinase 3-like 1 promotes macrophage recruitment and angiogenesis in colorectal cancer

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Abstract

Chitinase 3-like 1 (CHI3L1), one of mammalian members of the chitinase family, is expressed in several types of human cancer, and elevated serum level of CHI3L1 is suggested to be a biomarker of poor prognosis in advanced cancer patients. However, the overall biological function of CHI3L1 in human cancers still remains unknown. Studies were performed to characterize the role of CHI3L1 in cancer pathophysiology utilizing human colorectal cancer samples and human cell lines. Plasma protein and tissue mRNA expression levels of CHI3L1 in colorectal cancer were strongly upregulated. Immunohistochemical analysis showed that CHI3L1 was expressed in cancer cells and CHI3L1 expression had a significant association with the number of infiltrated macrophages and microvessel density. By utilizing trans-well migration and tube formation assays, overexpression of CHI3L1 in SW480 cells (human colon cancer cells) enhanced the migration of THP-1 cells (human macrophage cells) and HUVECs (human endothelial cells), and the tube formation of HUVECs. The knockdown of CHI3L1 by RNA interference or the neutralization of CHI3L1 by anti-CHI3L1 antibody displayed strong suppression of CHI3L1-induced migration and tube formation. Cell proliferation assay showed that CHI3L1 overexpression significantly enhanced the proliferation of SW480 cells. ELISA analysis showed that CHI3L1 increased the secretion of inflammatory chemokines, IL-8 and MCP-1, from SW480 cells through mitogen-activated protein kinase (MAPK) signaling pathway. Both neutralization of

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Conflicts of interest
The authors declare no conflict of interest.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

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IL-8 or MCP-1 and inhibition or knockdown of MAPK in SW480 cells significantly inhibited CHI3L1-induced migration and tube formation. In a xenograft mouse model, overexpression of CHI3L1 in HCT116 cells (human colon cancer cells) enhanced the tumor growth as well as macrophage infiltration and microvessel density. In conclusion, CHI3L1 expressed in colon cancer cells promotes cancer cell proliferation, macrophage recruitment and angiogenesis. Thus, the inhibition of CHI3L1 activity may be a novel therapeutic strategy for human colorectal cancer.

Keywords

chitinase; tumor; microenvironment; chemokine; MAPK

Introduction

Tumors consist of both cancer cells and stromal cells that are recruited to or activated in the local microenvironment. Recent studies indicated that tumor growth and dissemination are the result of dynamic interactions between cancer cells and stromal cells of the tumor microenvironment (Mantovani *et al*., 2008). Macrophages are one of the major populations of infiltrating cells in cancer stroma, and recent reports showed that recruited macrophages within cancers lack the ability to present antigen and contribute to cancer progression by the release of growth and angiogenic factors (Mantovani *et al*., 2002; Pollard, 2004). Tumor angiogenesis is also an essential part of cancer development and involves a multistep process of endothelial cell migration and tube formation (Hanahan and Folkman, 1996). Angiogenesis and macrophage infiltration often share common pathways, and these two biological processes are closely coupled with cancer progression (Ono, 2008).

Chitinases are enzymes that degrade chitin and belong to the family 18 of glycosyl hydrolases (Henrissat and Davies, 1997). Because chitin composes the cell wall of fungi and exoskeletal elements of crustaceans and insects, chitinases are found in organisms that need to protect themselves from fugal infection or to reshape their own chitin (Herrera-Estrella and Chet, 1999). Although chitinases had been considered to be lacking in mammalian bodies due to the absence of chitin, recent studies identified chitinases and chitinase-like proteins in mammals (Rejman and Hurley, 1998; Hakala *et al*., 1993; Shackelton *et al*., 1995). Chitinase 3-like 1 (CHI3L1), also known as human cartilage-glycoprotein 39 (HC-gp39) or YKL-40, is a member of the mammalian chitinase family, but lacks enzymatic activity (Henrissat and Bairoch, 1993). Although CHI3L1 is not synthesized under physiological conditions, the expression of CHI3L1 is observed in patients with several types of cancers and inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and bronchial asthma (Johansen, 2006; Mizoguchi, 2006; Chupp *et al*., 2007: Eurich *et al*., 2009). We recently reported that CHI3L1 is highly induced in colonic epithelial cells under inflammatory conditions and enhances pathogenic bacterial adhesion and invasion on/into colonic epithelial cells (Mizoguchi, 2006; Kawada *et al*., 2007; Kawada *et al*., 2008). It has been shown that CHI3L1 stimulates proliferation of connective tissue cells, including fibroblasts, chondrocytes and synovial cells and modulates expression levels of chemokines and metalloproteases in inflammatory fibroblasts, and also enhances chemotaxis of endothelial cells (Recklies *et al*., 2002; Ling and Recklies, 2004, Malinda *et*

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al., 1999). These data suggest that CHI3L1 may have an important role on stromal cells not only in inflammatory conditions but also in cancer development.

In this study, we focused on analyzing the biological role of CHI3L1 in the tumor microenvironment. For this purpose, we investigated whether CHI3L1 expressed in cancer cells promotes chemotaxis of macrophage and angiogenesis for the progression of colorectal cancer by utilizing human colorectal cancer samples as well as human cell lines.

Results

Expression of CHI3L1 in colorectal cancer

We initially examined the plasma levels of CHI3L1 by ELISA. The plasma CHI3L1 concentration of colorectal cancer patients was significantly higher than that of controls, and the increase was positively associated with TNM stages (Figure 1A). To test whether the increased expression of CHI3L1 can be detected in the colonic tissue of human colorectal cancer, surgically resected samples were subjected to quantitative PCR and immunohistochemical analysis. As shown in Figure 1B, the mRNA levels of CHI3L1/GAPDH in cancer tissues were significantly higher than the adjacent normal tissues of the same patients. Immunohistochemical analysis showed that CHI3L1 was mainly expressed in colorectal cancer cells rather than stromal cells (Figure 1C). Based on the expression levels of CHI3L1, we divided the 61 cases of colorectal cancer into two groups (Figure 1C). Out of 61 cases, 60.7% showed strong expression of CHI3L1 and 39.3% displayed weak expression. Interestingly, strong CHI3L1 expression was significantly correlated with T stage, lymphatic invasion, vascular invasion, and lymph node metastasis (Supplementary Table 1). To further explore a possible role of CHI3L1 in tumor microenvironment, we stained the serial sections with anti-CD68 and -vWF antibodies. Quantitative analysis indicated that the number of infiltrating CD68+ macrophages was significantly higher in cancers than in normal samples. Moreover, the number of infiltrating CD68+ macrophages was significantly higher in cancers with strong CHI3L1 expression than those with weak expression, the mean number of infiltrating macrophages being 121.1 and 95.7, respectively (Figure 1D). Staining of vWF showed a markedly higher microvessel density (MVD) in cancers than in normal samples. Moreover, MVD was higher in cancers with strong CHI3L1 expression than those with weak CHI3L1 expression, the mean number of MVD being 60.0 and 46.7, respectively (Figure 1E). Also, strong expression of CHI3L1 was associated with the concomitant increase of the number of infiltrating macrophages and the MVD (Supplementary Table 2). CHI3L1 expression was thus closely associated with the number of infiltrating macrophages and also with the degree of MVD in human colorectal cancer.

Effect of CHI3L1 on proliferation of colon cancer cells and on migration of macrophages

We next explored whether CHI3L1 expressed in cancer cells influences the cancer progression *in vitro*. We generated a CHI3L1 expression vector and two different kinds of CHI3L1 specific miRNAs as described in Materials and Methods. Western blotting confirmed that the protein level of CHI3L1 was up-regulated by CHI3L1 overexpression in SW480 cells, whereas CHI3L1 specific miRNAs suppressed the protein expression of endogenous CHI3L1 in SW480 cells compared with scrambled negative control miRNA

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(Figure 2A). Cell proliferation assay showed that CHI3L1 overexpression significantly enhanced the proliferation of SW480 cells as compared with empty vector. CHI3L1 miRNAs significantly suppressed the proliferation of SW480 cells (Figure 2B).

Macrophages are a major component of infiltrating cells in cancer stroma and have served as a promoter for cancer progression (Mantovani *et al*., 2008). To investigate the effect of CHI3L1 on macrophage infiltration into cancer stroma, THP-1 cells (human macrophage cells) and SW480 cells (human colon cancer cells) were co-cultured using the double-chamber. We observed increased migration of THP-1 cells when co-cultured with SW480 cells that endogenously expressed CHI3L1 as compared with no cells in the lower chamber (Figure 2C). Moreover, stimulation of SW480 cells with purified CHI3L1 protein further enhanced the migration of THP-1 cells. CHI3L1 overexpression in SW480 cells also increased the migration of THP-1 cells by 2.2 fold as compared with mock transfection. To confirm that the enhanced migration of THP-1 cells is mediated by CHI3L1, studies to inhibit CHI3L1 activity were performed. Anti-CHI3L1 neutralizing Ab treatment significantly inhibited the CHI3L1-enhanced migration of THP-1 cells, although control IgG did not have any inhibitory action. CHI3L1 miRNAs transfection in SW480 cells also significantly inhibited the migration of THP-1 cells (Figure 2D). These results suggest that CHI3L1 expression in colon cancer cells promotes the proliferation of cancer cells and the chemotaxis of macrophages.

Effect of CHI3L1 on migration and tube formation of endothelial cells

Angiogenesis is a key mechanism that supports cancer development by providing nutrients and oxygen (Hanahan and Weinberg, 2000). To examine the influence of CHI3L1 on angiogenesis, SW480 cells and HUVECs (human endothelial cells) were co-cultured using the double-chamber. Under the co-culture with SW480 cells, HUVECs significantly enhanced their migration as compared with no cells in the lower chamber (Figure 3A). Moreover, stimulation of SW480 cells with purified CHI3L1 protein further enhanced the migration of HUVECs. CHI3L1 overexpression in SW480 cells also increased the migration of HUVECs by 1.8 fold as compared with mock transfection. The CHI3L1-mediated HUVECs migration was significantly inhibited by anti-CHI3L1 neutralizing Ab, although control IgG did not have any inhibitory effects. Furthermore, CHI3L1 miRNAs transfection in SW480 cells significantly suppressed the migration of HUVECs (Figure 3B).

Since CHI3L1 stimulated the migration of endothelial cells, we next examined the effect of CHI3L1 on the tube formation of HUVECs. As expected, HUVECs tube formation was significantly enhanced by conditioned medium harvested from SW480 cells (Figure 3C) as compared with basal medium. Moreover, stimulation with purified CHI3L1 protein enhanced the tube formation. We found that the conditioned medium harvested from the CHI3L1-overexpressed SW480 cells further enhanced the tube formation, compared with the conditioned medium harvested from the control vector-transfected SW480 cells. The enhancement of HUVEC tube formation by the conditioned medium derived from the CHI3L1-overexpressed SW480 cells was inhibited efficiently by anti-CHI3L1 neutralizing Ab, although control IgG did not show the inhibitory effect. The transfection of CHI3L1 miRNAs in SW480 cells also significantly suppressed the HUVEC tube formation (Figure

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3D). Taken together, these findings suggest that CHI3L1 expressed in colon cancer cells plays a pivotal role in promoting the angiogenesis.

Enhanced chemotaxis of macrophages and angiogenesis by CHI3L1-induced IL-8 and MCP-1 protein secretions

Next, we further explored the ability of CHI3L1 to promote the release of inflammatory chemokines for migration and tube formation. IL-8 (CXCL8) is one of the extensively studied chemokines as a mitogenic and angiogenic factor for cancer progression. In addition, monocyte chemoattractant protein-1 (MCP-1, CCL2) is also known to be important in tumorigenesis as a macrophage chemoattractant and angiogenic modulator (Singh *et al*., 2007; Lazennec and Richmond, 2010). ELISA showed that CHI3L1 overexpression in SW480 cells significantly and dose-dependently increased the protein secretion of IL-8 and MCP-1 as compared with the control vector-transfected SW480 cells (Figure 4A). Quantitative RT-PCR also showed that CHI3L1 overexpression in SW480 cells significantly increased the mRNA levels of IL-8 and MCP-1 (Figure 4B). We further found that the CHI3L1 miRNAs transfection significantly decreased the release of IL-8 and MCP-1 from SW480 cells (Figure 4C). These results indicate that CHI3L1 enhances the secretion of inflammatory chemokines including IL-8 and MCP-1 from human colon cancer cells.

As shown in Figures 2 and 3, we observed increased migration of THP-1 cells and HUVECs when incubated with CHI3L1-overexpressed SW480 cells in trans-well migration assay. We found that incubation with IL-8 neutralizing Ab significantly diminished the enhanced migration of THP-1 cells and HUVECs induced by CHI3L1 in SW480 cells. Incubation with MCP-1 neutralizing Ab also significantly inhibited the enhanced migration of THP-1 cells and HUVECs by CHI3L1. In contrast, incubation with control goat IgG did not affect the enhanced capability of CHI3L1 (Figure 4D, E). Neutralization with anti-IL-8 or -MCP-1 antibody significantly inhibited the tube formation induced by empty vector-transfected or CHI3L1-treated conditioned medium as compared with control goat IgG (Figure 4F). Taken together, these results suggest that CHI3L1-induced IL-8 and MCP-1 protein secretions from colon cancer cells enhance chemotaxis of macrophages and angiogenesis.

Enhanced chemotaxis of macrophages and angiogenesis through ERK1/2 and JNK signaling in colon cancer cells

The MAPK cascade is an evolutionarily conserved pathway playing an important role in cancer development (Sebolt-Leopold and Herrera, 2004). We, therefore, examined the role of MAPK in CHI3L1-mediated macrophage chemotaxis and angiogenesis. SW480 cells were transfected with CHI3L1 expression- or miRNA- vector, and MAPK phosphorylation was determined using phospho-specific antibodies. CHI3L1 overexpression up-regulated ERK and JNK phosphorylation in SW480 cells (Figure 5A). On the other hand, CHI3L1 knockdown by CHI3L1 miRNAs abolished ERK and JNK phosphorylation induced by CHI3L1 expression in SW480 cells. In contrast, phosphorylation of p38 was unaffected by CHI3L1 overexpression or knockdown.

We explored involvement of MAPK cascade in CHI3L1-induced IL-8 and MCP-1 secretion from colon cancer cells. ELISA showed that ERK inhibitor (PD98059) and JNK inhibitor

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(SP600125) significantly diminished CHI3L1-induced IL-8 and MCP-1 secretion from SW480 cells, but p38 inhibitor (SB203580) had no effect (Figure 5B). Transfection of siRNA for ERK or JNK also significantly diminished the protein secretions of IL-8 and MCP-1 from SW480 cells, but siRNA for p38 had no effect (Figure 5B). Together, these results indicate that CHI3L1 induces IL-8 and MCP-1 secretion from colon cancer cells via ERK and JNK, but not p38 pathway.

We further investigated the involvement of MAPK cascade in CHI3L1-induced macrophage chemotaxis and angiogenesis. Incubation with inhibitor for ERK or JNK (PD98059 or SP600125) as well as transfection of siRNA for ERK or JNK significantly diminished the enhanced migration of THP-1 cells and HUVECs induced by CHI3L1 overexpression in SW480 cells in trans-well migration assay (Figure 5C, D). Furthermore, both addition of inhibitor for ERK or JNK (PD98059 or SP600125) and transfection of siRNA for ERK or JNK during preparation of conditioned medium significantly inhibited tube formation of HUVECs induced by conditioned medium from CHI3L1-overexpressed SW480 cells (Figure 5E). In contrast, p38 inhibitor (SB203580) or p38 siRNA did not affect those effects of CHI3L1.

Effect of CHI3L1 in a xenograft mouse model

We further assessed the effect of CHI3L1 on cancer cell proliferation and in tumor microenvironment *in vivo*. We generated stably transfected HCT116 cells with overexpression of CHI3L1 or control vector using retroviral transduction system. Western blotting showed that protein level of CHI3L1 was up-regulated by CHI3L1 overexpression (Figure 6A). Mice were inoculated subcutaneously with HCT116 cells expressing a control vector in the left flank and a CHI3L1-overexpressed vector in the right flank. Overexpression of CHI3L1 enhanced the tumor growth by 3.5 fold as compared with control during 4 weeks (Figure 6B). To further explore a possible role of CHI3L1 in tumor microenvironment, we stained the serial sections of the implanted tumor with anti-CHI3L1, -F4/80, and -vWF antibodies (Figure 6C). The number of infiltrating F4/80+ macrophages was significantly greater in CHI3L1-overexpressed tumors (Figure 6D). Staining of vWF showed a markedly higher microvessel density (MVD) in CHI3L1-overexpressed tumors (Figure 6E). Thus, we confirmed that CHI3L1 expression was significantly associated with the number of infiltrating macrophages and also with the degree of MVD in a xenograft mouse model.

Discussion

In this study, we showed that CHI3L1 expression was upregulated in cancer cells and has a significant correlation with macrophage infiltration and microvessel density (MVD) in the tumors of human colorectal cancer patients and in a xenograft mouse model. CHI3L1 expression in colon cancer cells significantly promoted the chemotaxis of macrophages and the angiogenesis accompanied by the increased secretion of IL-8 and MCP-1. We further verified that CHI3L1 acted through signal mediators including ERK and JNK to enhance IL-8 and MCP-1 secretions in colon cancer cells.

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High serum level of CHI3L1 is detected in patients with several types of cancer and associated with poor prognosis (Cintin *et al*., 1999; Johansen *et al*., 2006). In agreement with these early reports, CHI3L1 in the plasma was increased with advancing stage of colorectal cancer. In this study, we confirmed that CHI3L1 was predominantly produced by human colorectal cancer cells rather than infiltrating inflammatory cells by immunohistochemical analysis. CHI3L1 expression in cancer cells was significantly associated with the accumulation of CD68-expressing macrophages and MVD. In addition, we showed a significant correlation between CHI3L1 expression in cancer cells and TN stages as well as lymphatic invasion and vascular invasion. Thus, high expression of CHI3L1 in cancer cells was associated with a more aggressive phenotype with a high metastatic potential.

To clarify the role of CHI3L1 in tumor microenvironment, we utilized human colon cancer cells with macrophages or endothelial cells in trans-well migration and tube formation assays. Interestingly, we observed that up-regulation of CHI3L1 in SW480 cells significantly enhanced migration of THP-1 cells and HUVECs and tube formation of HUVECs. The neutralization of CHI3L1 activity by anti-CHI3L1 Ab and inhibition of endogenously expressed CHI3L1 by CHI3L1-specific miRNAs reduced these chemoattractic effects. These results are consistent with the analysis of clinical samples and strongly suggest that CHI3L1 in cancer cells promotes chemotaxis of macrophages and tumor angiogenesis for the progression of colorectal cancer.

What are the mediators for macrophage chemotaxis and angiogenesis induced by CHI3L1 in cancer cells? The interactions between cancer cells and stromal microenvironments are mainly mediated by inflammatory chemokines (Strieter, 2001; Ben-Baruch, 2006). A recent study showed that MCP-1 plays an important role in angiogenesis as well as chemotaxis of macrophages (Salcedo *et al*., 2000). IL-8 is also thought to play a crucial role in tumor angiogenesis (Belperio *et al*., 2000). Therefore, we next examined the possibility that CHI3L1 enhances the release of IL-8 and/or MCP-1 from SW480 cells. As shown in Figure 4, CHI3L1-overexpressed SW480 cells significantly increased the secretion of IL-8 and MCP-1 in a dose-dependent manner as compared with the mock-transfected cells. We further demonstrated that anti-IL-8- or anti-MCP-1- neutralizing antibody significantly suppressed both the macrophage chemotaxis and angiogenesis induced by CHI3L1 overexpression in SW480 cells, suggesting that IL-8 and MCP-1 at least in part mediate the chemotactic and angiogenic actions of CHI3L1 in cancer cells. VEGF is one of the most potent angiogenic factors by inducing endothelial cell migration and tube formation. However, we could not find significant up-regulation of the gene expression of VEGF by quantitative RT-PCR (data not shown). A previous report showed that an inclusion of the anti-VEGF Ab failed to inhibit the endothelial cell angiogenesis induced by CHI3L1 (Shao *et al*., 2009). Therefore, CHI3L1 seems to have another angiogenic activity independent of VEGF. Our study indicated a novel mechanism that inflammatory chemokines induced by CHI3L1 promote not only angiogenesis but also macrophage recruitment, while a previous report showed recombinant CHI3L1 has a direct effect on angiogenesis (Shao *et al*., 2009).

In this study, we also showed that the CHI3L1-induced secretions of IL-8 and MCP-1 were mediated by the activation of MAPK signaling pathway, in particular ERK and JNK but not

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p38. The inhibition or knockdown of ERK and JNK abrogated migration and tube formation enhanced by CHI3L1, supporting ERK and JNK pathways are predominant downstream effectors in the present study. The NF-κB pathway is also activated by CHI3L1 as our group recently demonstrated (Chen *et al*., 2011). However, this effect is observed only in the first few hours after stimulation with purified CHI3L1 protein. We could not find significant decrease of the macrophage chemotaxis and angiogenesis by NF-κB inhibitors (BAY11-7082 and BMS-345541) after transfection of CHI3L1 in the present experimental condition (data not shown). As our previous studies suggested the possibility that CHI3L1 can act as a receptor for bacteria on colonic epithelial cells (Mizoguchi, 2006; Kawada *et al*., 2008), it is interesting to speculate bacteria may activate ERK and JNK signals via CHI3L1.

We further assessed the effect of CHI3L1 on cancer cell proliferation and in tumor microenvironment in *in vivo* experiment. Consistent with the analysis of clinical samples and *in vitro* experiments, CHI3L1 expressed in cancer cells significantly enhanced the xenograft tumor growth as well as the recruitment of macrophages and angiogenesis. Our results from both *in vitro* and *in vivo* studies strongly supported our hypothesis that CHI3L1 promotes tumor growth not only by the proliferation of cancer cells themselves but also by macrophage recruitment and angiogenesis.

In summary, here we demonstrated that the number of infiltrating macrophages and MVD were significantly associated with CHI3L1 expression both in patients with colorectal cancer and in a xenograft mouse model. We also revealed that CHI3L1 induced IL-8 and MCP-1 secretion through ERK and JNK signal pathways. These inflammatory chemokines appear to mediate macrophage recruitment and tumor angiogenesis to promote colorectal cancer development. Thus, the present study highlighted important roles of CHI3L1 in macrophage recruitment and angiogenesis during cancer development and suggests that targeting of CHI3L1 could be of value for the treatment of human colorectal cancer.

Materials and Methods

Patients

Human samples were obtained from patients with colorectal cancer who admitted at Kyoto University Hospital. Surgically resected colorectal cancer specimens were obtained from 61 patients with an average age of 66.8 ± 10.7 (mean ± SD) years. Plasma was obtained from 15 patients with localized colorectal cancers, 16 patients with advanced colorectal cancers, and 12 controls, with an average age of 65.1 ± 12.1, 61.8 ± 14.7, and 54.5 ± 17.4 (mean ± SD) years, respectively. Written informed consents were obtained from all patients in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Kyoto University.

Cell culture and transfection

SW480, HCT116, HUVEC and THP-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SW480 and HCT116 were cultured in DMEM (Gibco-BRL, Rockville, MD) and THP-1 was cultured in RPMI 1640 supplemented with 10% fetal calf serum. HUVEC was maintained using the EGM-2 bullet kit (Lonza, Basel,

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Switzerland). Transient transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Plasmids and reagents

The CHI3L1 expression vector and affinity-purified anti-mouse/human CHI3L1 antibody was described previously (Mizoguchi, 2006). Purified CHI3L1 protein was obtained from Quidel Corporation (San Diego, CA). Recombinant human IL-8, MCP-1 neutralizing antibodies, and normal goat IgG were obtained from R&D Systems (Minneapolis, MN). Rabbit antibodies against phospho-ERK1/2, -p38, and -c-Jun N-terminal kinase (JNK), and total-ERK1/2, -JNK, and -p38 were purchased from Cell Signaling Technology (Denvers, MA). PD98059, SP600125, SB203580, and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO).

RNA interference

Two different kinds of miRNA oligos corresponding to CHI3L1 and the scrambled negative control miRNA were purchased from Invitrogen and cloned into the pcDNA™6.2-GW/EmGFP-miR vector (Invitrogen) with the BLOCK-iT™Pol II miR RNAi Expression Vector Kit according to the manufacturer's instructions. The targeted miRNAs of CHI3L1 were as follows: #1: GGATGGAACTTTGGGTCTCAA, #2: ATGTAAGACTCGGGATTAGTA. Validated siRNAs for ERK1/2, JNK, and p38, and the control siRNA were purchased from Cell Signaling Technology.

Quantitative real time-PCR

Total RNA was extracted from the tissues or cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). Extracted RNA was reverse-transcribed using the first-stand reagent kit (Roche, Mannheim, Germany) and PCR-amplified using FastStart Universal SYBR Green Master (Roche). Primer sequences were as follows: CHI3L1, 5'-GTACAAGCTGGTCTGCTACTTC-3' and 5'-ATGTGCTAAGCATGTTGTCGC-3'; IL-8, 5'-ACTGAGAGTGATTGAGAGTGGAC-3' and 5'-AACCCTCTGCACCCAGTTTTC-3'; MCP-1, 5'-CAGCCAGATGCAATCAATGCC-3' and 5'-TGGAATCCTGAACCCACTTCT-3'; and GAPDH, 5'-ATGGGGAAGGTGAAGGTCG-3' and 5'-GGGGTCATTGATGGCAACAATA-3'.

Cell proliferation assays

SW480 cells (5×10^3) were seeded in 96-well plates. After 36 hrs of transfection, 20 μl of CellTier 96 Aqueous One Solution Reagent (Promega, Madison, WT) was added and absorbance was measured at 490 nm.

Migration assays

Migration assays were performed using 8 μm pore size cell culture inserts within a 24-well plate (BD falcon, Franklin Lakes, NJ), as described previously (Kawada *et al.*, 2009). HUVECs (1×10^4) or THP-1 cells (5×10^5) were seeded into the top of the trans-well chambers precoated with fibronectin, and SW480 cells (2×10^5) were seeded in the lower well. After 4 hrs of incubation, cells in the upper membrane surface were removed with

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cotton swab. Cells on the lower membrane surface were fixed with 4% formaldehyde and stained with propidium iodide (Dojindo, Kumamoto, Japan).

Tube formation assays

HUVECs (5×10^4) were suspended in conditioned medium and seeded on growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) in a 48-well plate, as described previously (Guo *et al*., 2008). After 5 hrs of incubation, tube-forming structures were analyzed by counting the number of connecting branches.

Western blotting

The protein concentration of cell lysates was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and equal amount of total protein were separated by SDS-PAGE and transferred onto nitrocellulose transfer membrane (Whatman, Dassel, Germany), and immunoblotted with various antibodies.

Enzyme-linked immunosorbent assay (ELISA)

Microvue YKL-40 EIA Kit (Quidel) was used for the quantitation of CHI3L1 in human plasma in accordance with the manufacturer's instructions. ELISA kits for the detection of IL-8 and MCP-1 were purchased from R&D systems and eBiosciences (San Diego, CA), respectively.

Xenograft tumor growth assays

HCT116 cells that do not express CHI3L1 endogenously were infected with retrovirus containing CHI3L1 or pMXs-IRES-Puro vector control (Cell Biolabs, San Diego, CA). Five-week-old athymic BALB/c nude mice (CLEA Japan, Tokyo, Japan) were inoculated subcutaneously with 5×10^6 HCT116 cells in the left (vector control) and right (CHI3L1) flank. Animal experiments were performed in accordance with the protocols approved by the Animal Research Committee of Kyoto University.

Immunohistochemistry

Paraffin embedded sections were stained with anti-CHI3L1, -CD68 (Dako, Carpinteria, CA), -von Willebrand factor (vWF) (Dako), and -F4/80 (Abcam, Cambridge, UK) antibodies. The first antibody was incubated at 4C° overnight, and the secondary antibody conjugated with horseradish peroxidase was added. DAB (3,3-diami-nopdbenzidine) substrate (Dako) was introduced and hematoxylin was used for counterstaining. CHI3L1 staining was evaluated as a score of percentage of positive staining cells as follows: weak; < 50% of cells stained, strong; 50% of cells stained.

Statistical analysis

Statistical significance was evaluated by the Mann-Whitney *U*-test for nonparametric data or the Student's *t*-test for parametric data. Clinicopathologic factors were analyzed by Fisher's exact test. P values < 0.05 were considered to be statistically significant.

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Supplementary Material

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Acknowledgments

Financial Support

This work was supported by Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (20790493, 21-40033, 23590937, 21229009), and Health and Labor Science Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labor, and Welfare, Japan, and Research Foundation of Translational Research Center, Kyoto University and by a research grant (R01DK80070) from the National Institutes of Health in USA.

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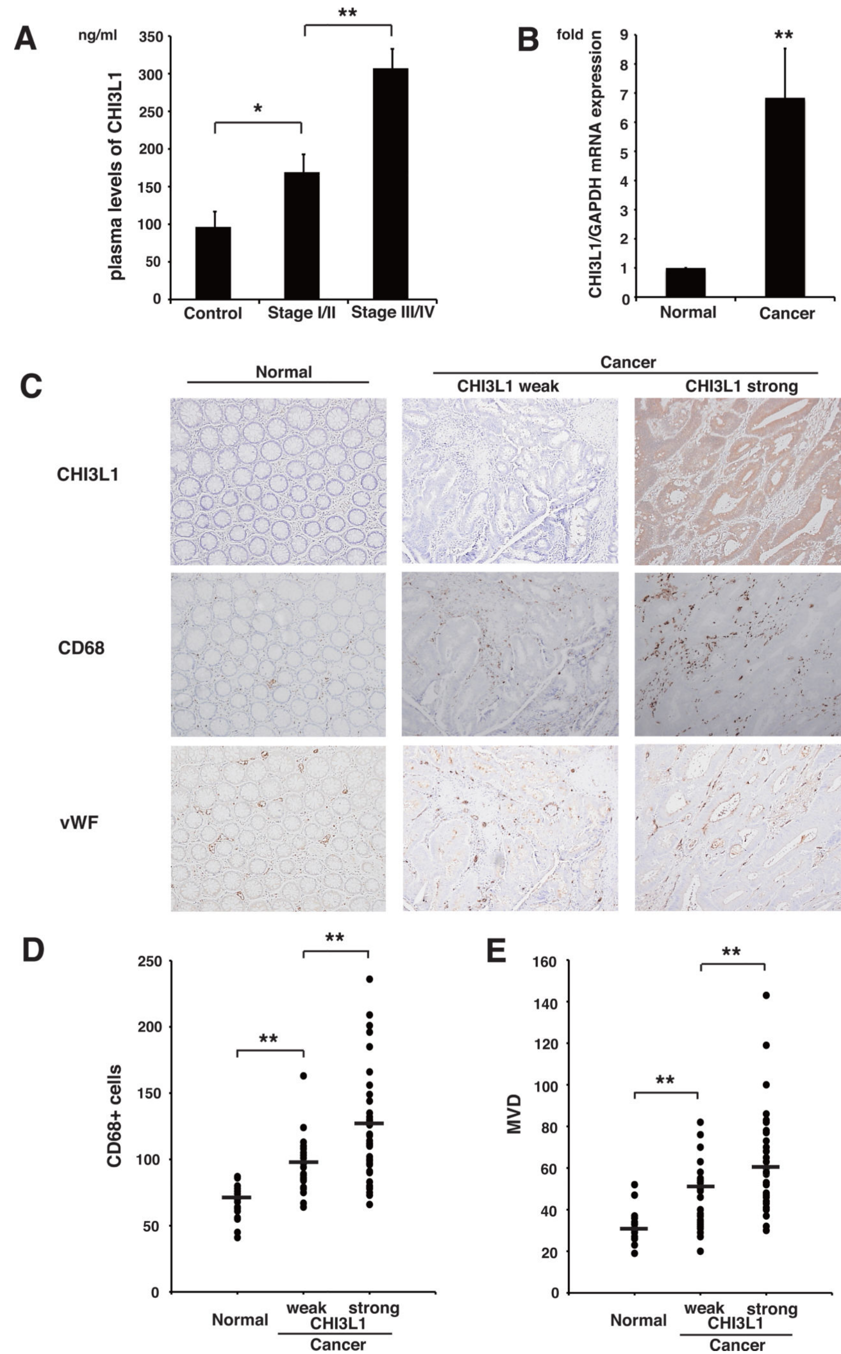

Figure 1. Expression of CHI3L1 in colorectal cancer

(A) Plasma CHI3L1 protein concentrations assessed by ELISA. Plasma CHI3L1 protein was significantly correlated with TNM stage. * $P < 0.05$, ** $P < 0.01$. Normal control; n = 12, Stage I/II; n = 15, and Stage III/IV; n = 16.

(B) CHI3L1 mRNA expression determined by quantitative RT-PCR. The mRNA levels of CHI3L1/GAPDH in cancer tissues were significantly increased as compared with their adjacent normal tissues. ** $P < 0.01$. n = 16

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(C) Immunohistochemical analysis of 61 human colorectal cancer and 18 normal colonic sections stained with anti-CHI3L1, -CD68, and -vWF antibodies. Representative photographs of normal samples (n = 18) and cancers based on the expression level of CHI3L1 in two groups are shown (weak; n = 24, strong; n = 37). Original magnification, x100.

(D) Mean numbers of CD68+ macrophages at x200 magnification. The number of CD68+ macrophages was significantly higher in cancers than in normal samples, and significantly higher in cancers with strong CHI3L1 expression than those with weak expression. Bars indicate the mean number of infiltrating macrophages. ** $P < 0.01$.

(E) Mean MVD at x100 magnification. MVD was significantly higher in cancers than in normal samples, and significantly higher in cancers with strong CHI3L1 expression than those with weak expression. Bars indicate the mean number of MVD. ** $P < 0.01$.

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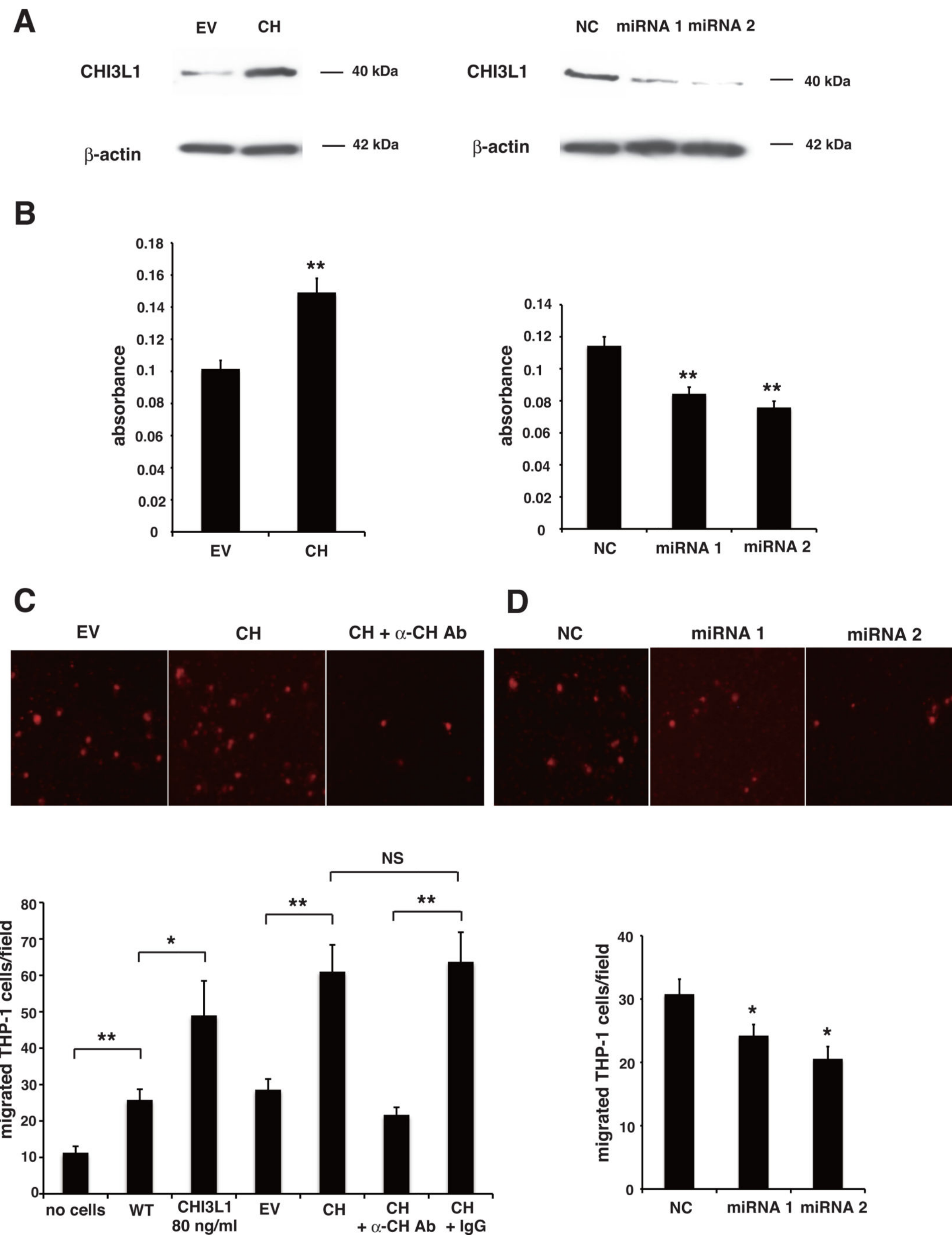

Figure 2. Effect of CHI3L1 on proliferation of colon cancer cells and on migration of macrophages

(A) Western blot analysis of CHI3L1 and β-actin expression. Protein level of CHI3L1 was up-regulated by CHI3L1 overexpression (CH) in SW480 cells as compared with empty vector (EV) transfection (left panel). Two different CHI3L1 specific miRNAs (miRNA 1, miRNA 2) suppressed the protein expression of endogenous CHI3L1 as compared with negative control miRNA (NC) (right panel).

(B) Cell proliferation was assessed by MTS assay. CHI3L1 overexpression (CH) significantly enhanced the proliferation of SW480 cells as compared with empty vector

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(EV) (left panel). Two kinds of CHI3L1 specific miRNAs (miRNA 1, miRNA 2) significantly suppressed the proliferation of SW480 cells as compared with control miRNA (NC) (right panel).

(C) Representative photographs (upper panel) and mean numbers of migrated THP-1 cells (lower panel) at x200 magnification. SW480 cells (2×10^5) and THP-1 cells (5×10^4) were co-cultured for 4 hrs. Migration of THP-1 cells was significantly enhanced by co-culturing with SW480 cells (WT) than without SW480 cells (no cells). CHI3L1 stimulation (80 ng/ml) significantly enhanced the migration of THP-1 cells. Overexpression of CHI3L1 (CH) in SW480 cells significantly enhanced the migration of THP-1 cells as compared with empty vector (EV) transfection. The enhanced migration of THP-1 cells by CHI3L1 was significantly inhibited by anti-CHI3L1 neutralizing Ab (CH + α-CH Ab) as compared with control rabbit IgG (CH + IgG).

(D) Representative photographs (upper panel) and mean numbers of migrated THP-1 cells (lower panel) at x200 magnification. Two kinds of CHI3L1 miRNAs (miRNA 1, miRNA 2) significantly suppressed the migration of THP-1 cells as compared with negative control miRNA (NC).

All results (B-D) are the mean (± SEM) of more than three separate experiments. NS, no significant difference, * $P < 0.05$, ** $P < 0.01$.

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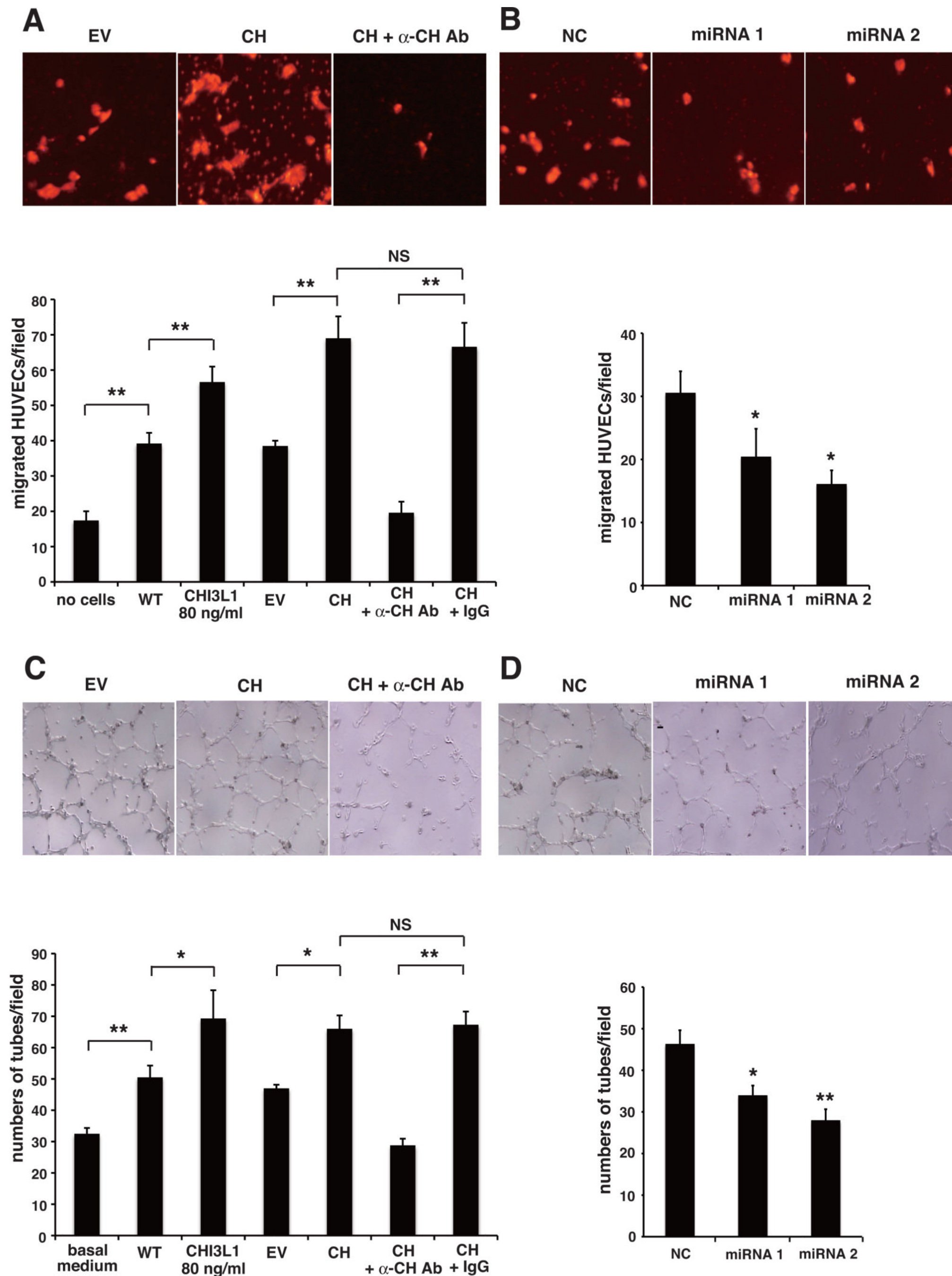

Figure 3. Effect of CHI3L1 on migration and tube formation of endothelial cells

(A) Representative photographs (upper panel) and mean numbers of migrated HUVECs (lower panel) at x200 magnification. SW480 cells (2×10^5) and HUVECs (1×10^4) were co-cultured for 4 hrs. Migration of HUVECs was significantly enhanced by co-culturing with SW480 cells (WT) than without SW480 cells (no cells). CHI3L1 stimulation (80 ng/ml) significantly enhanced the migration of HUVECs. Overexpression of CHI3L1 (CH) in SW480 cells significantly enhanced the migration of HUVECs as compared with empty vector (EV) transfection. The enhanced migration of HUVECs by CHI3L1 was significantly

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inhibited by anti-CHI3L1 neutralizing Ab (CH + α-CH Ab) as compared with control rabbit IgG (CH + IgG).

(B) Representative photographs (upper panel) and mean numbers of migrated HUVECs (lower panel) at x200 magnification. Two kinds of CHI3L1 miRNAs (miRNA 1 and miRNA 2) significantly suppressed the migration of HUVECs as compared with negative control miRNA (NC).

(C) Representative photographs (upper panel) and mean numbers of tube-forming structures (lower panel) at x100 magnification. HUVECs (5×10^4) were incubated in conditioned medium for 5 hrs. Tube formation of HUVECs was significantly enhanced by conditioned medium from SW480 cells (WT) as compared with basal medium. CHI3L1 stimulation (80 ng/ml) significantly enhanced the tube formation of HUVECs. Overexpression of CHI3L1 (CH) in SW480 cells significantly enhanced the tube formation as compared with empty vector (EV) transfection. The enhanced tube formation of HUVECs by CHI3L1 was significantly inhibited by treatment with anti-CHI3L1 neutralizing Ab (CH + α-CH Ab) as compared with control rabbit IgG (CH + IgG).

(D) Representative photographs (upper panel) and mean numbers of tube-forming structures (lower panel) at x100 magnification. Two kinds of CHI3L1 miRNAs (miRNA 1, miRNA 2) significantly suppressed the tube formation of HUVECs as compared with negative control miRNA (NC).

All results (A-D) are the mean (± SEM) of more than three separate experiments. NS, no significant difference, * $P < 0.05$, ** $P < 0.01$.

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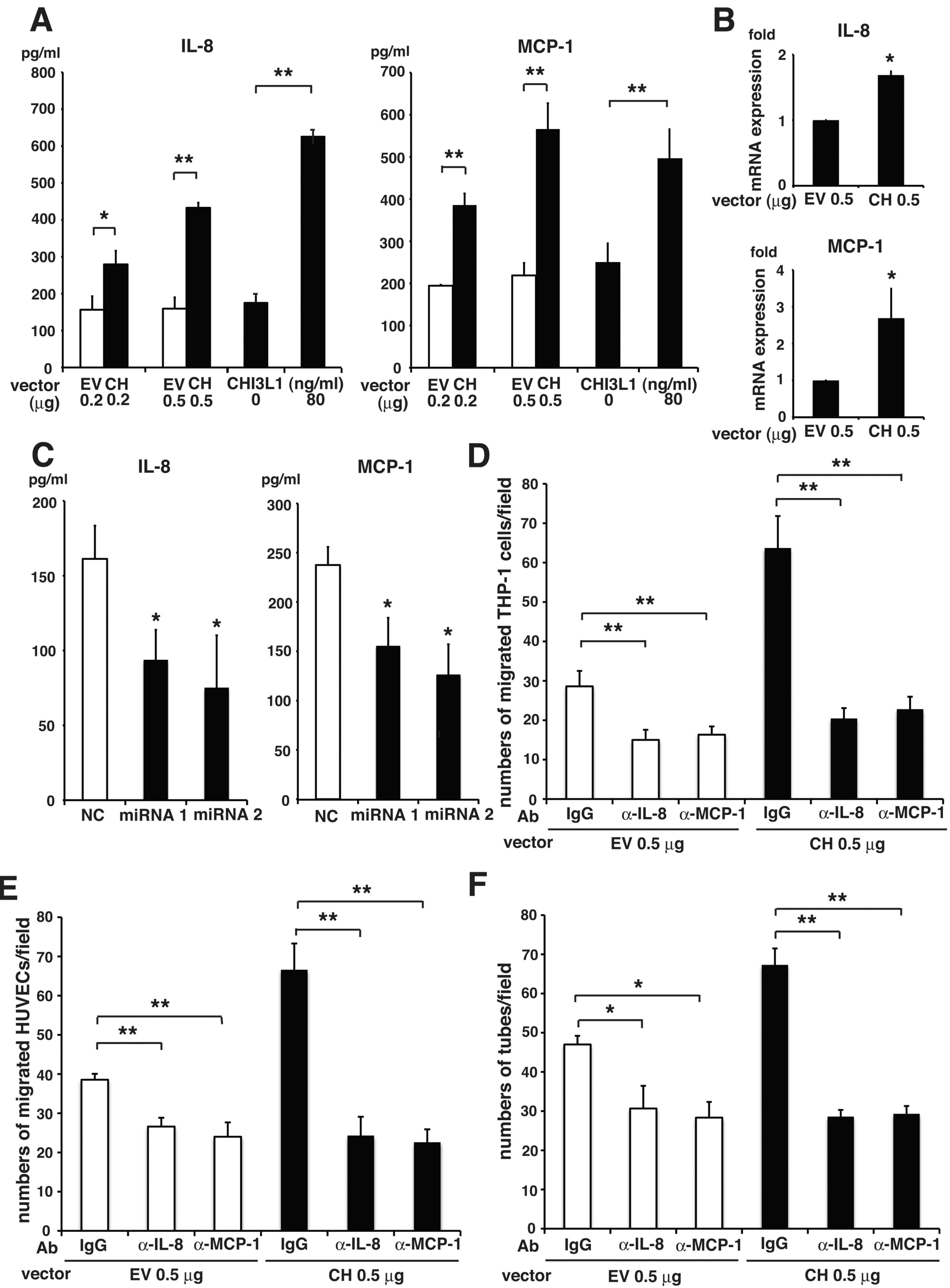

Figure 4. Enhanced chemotaxis of macrophages and angiogenesis by CHI3L1-induced IL-8 and MCP-1 protein secretions

(A) The protein secretions of IL-8 and MCP-1 in the supernatant assessed by ELISA. CHI3L1 transfection (CH) in SW480 cells for 48 hrs significantly and dose-dependently increased the protein secretion of IL-8 and MCP-1 as compared with empty vector (EV) transfection. Stimulation with CHI3L1 (80 ng/ml) in SW480 cells significantly increased the protein secretion of IL-8 and MCP-1.

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(B) The mRNA expressions of IL-8 and MCP-1 determined by quantitative RT-PCR. The mRNA levels of IL-8/GAPDH and MCP-1/GAPDH were significantly increased in CHI3L1 overexpression (CH) as compared with those in empty vector (EV) transfection.

(C) The protein secretions of IL-8 and MCP-1 in the supernatant assessed by ELISA. Two kinds of CHI3L1 miRNAs (miRNA 1, miRNA 2) transfection in SW480 cells for 48 hrs significantly decreased the protein secretions of IL-8 and MCP-1 as compared with negative control miRNA (NC) transfection.

(D) Mean numbers of migrated THP-1 cells at x200 magnification. Incubation with IL-8 or MCP-1 neutralizing Ab significantly diminished the migration of THP-1 cells induced by CHI3L1 in SW480 cells as compared with control goat IgG.

(E) Mean numbers of migrated HUVECs at x200 magnification. Incubation with IL-8 or MCP-1 neutralizing Ab significantly diminished the migration of HUVECs induced by CHI3L1 in SW480 cells as compared with control goat IgG.

(F) Mean numbers of tube-forming structures at x100 magnification. Addition of IL-8 or MCP-1neutralizing Ab during preparation of conditioned medium from empty vector-transfected or CHI3L1-overexpressed SW480 cells significantly inhibited the tube formation of HUVECs as compared with control goat IgG.

All results (A-F) are the mean (± SEM) of more than three separate experiments. * $P < 0.05$, ** $P < 0.01$.

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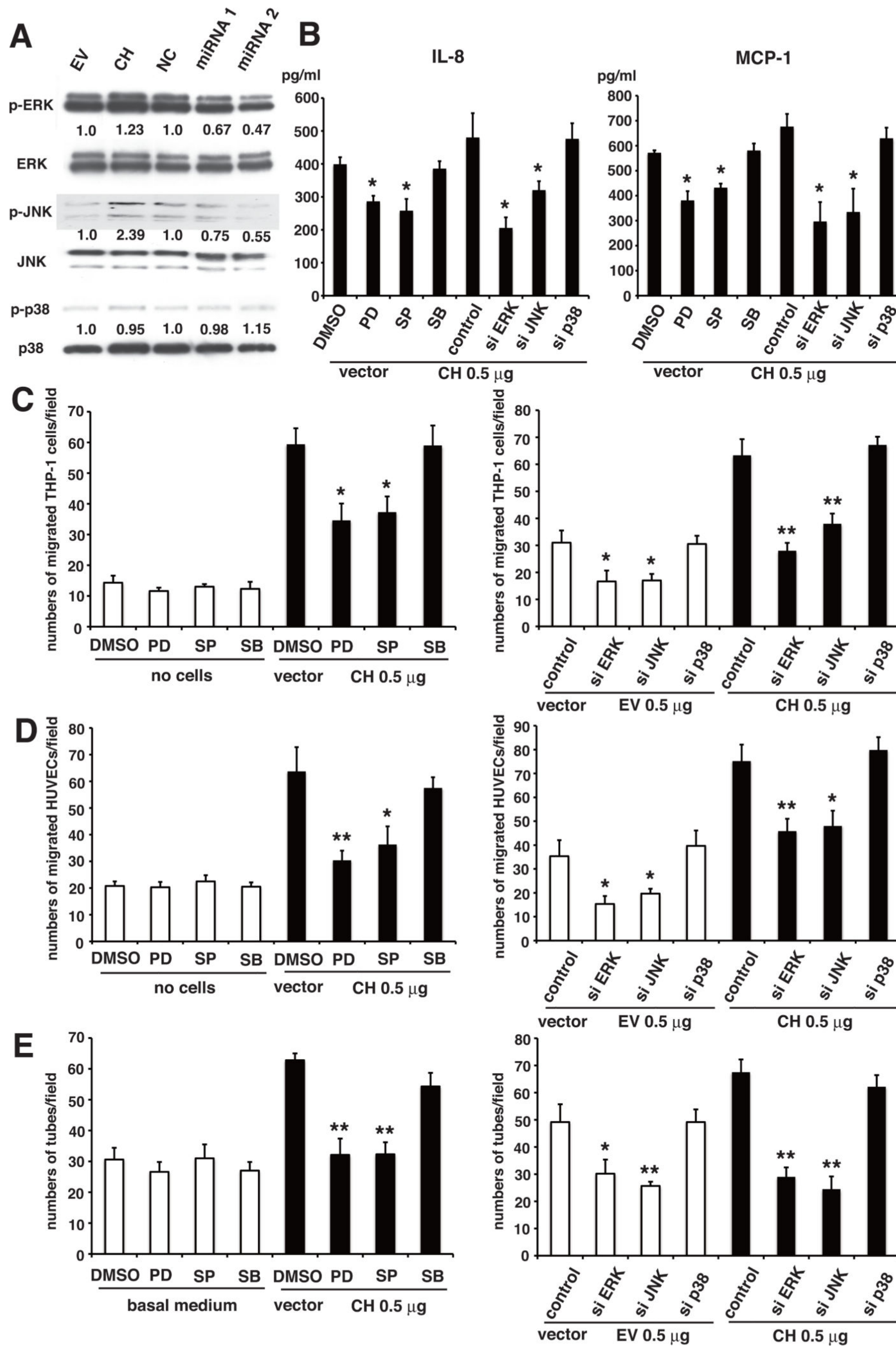

Figure 5. Enhanced chemotaxis of macrophages and angiogenesis through ERK1/2 and JNK signaling but not p38 in colon cancer cells

(A) Western blot analysis of phospho-ERK, -JNK, -p38, and total-ERK, -JNK, -p38 expression in SW480 cells. SW480 cells were transfected with empty- (EV), CHI3L1 expression- (CH), negative control miRNA- (NC), or two kinds of CHI3L1 specific miRNAs (miRNA 1, miRNA 2)- vector for 48 hrs. CHI3L1 overexpression in SW480 enhanced ERK and JNK phosphorylation but not p38. CHI3L1 knockdown diminished ERK and JNK phosphorylation but not p38. The fold expression of phospho-ERK/JNK/p38 normalized by total ERK/JNK/p38 was shown relative to the control (EV or NC).

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(B) The protein secretions of IL-8 and MCP-1 from SW480 cells assessed by ELISA. Incubation with ERK inhibitor (PD98059) or JNK inhibitor (SP600125) significantly diminished the protein secretions of IL-8 and MCP-1 from SW480 cells, but not with p38 inhibitor (SB203580). Transfection of siRNA for ERK or JNK significantly diminished the protein secretions of IL-8 and MCP-1 from SW480 cells, but not for p38.

(C) Mean numbers of migrated THP-1 cells at x200 magnification. Migration of THP-1 cells was not inhibited by MAPK inhibitors when cultured without SW480 cells (no cells). Incubation with ERK inhibitor (PD98059) or JNK inhibitor (SP600125) significantly diminished the migration of THP-1 cells. In contrast, p38 inhibitor (SB203580) did not affect the chemotaxis enhanced by CHI3L1 (left panel). Transfection of siRNA for ERK or JNK in SW480 cells significantly diminished the migration of THP-1 cells, but siRNA for p38 had no effect (right panel).

(D) Mean numbers of migrated HUVECs at x200 magnification. Migration of HUVECs was not inhibited by MAPK inhibitors when cultured without SW480 cells (no cells). Incubation with ERK inhibitor (PD98059) or JNK inhibitor (SP600125) significantly diminished the migration of HUVECs. In contrast, p38 inhibitor (SB203580) did not affect the chemotaxis enhanced by CHI3L1 (left panel). Transfection of siRNA for ERK or JNK in SW480 cells significantly diminished the migration of HUVECs, but siRNA for p38 had no effect (right panel).

(E) Mean numbers of tube-forming structures at x100 magnification. Tube formation was not inhibited by MAPK inhibitors when cultured in basal medium. Addition of ERK inhibitor (PD98059) or JNK inhibitor (SP600125) during preparation of conditioned medium significantly inhibited the tube formation. In contrast, p38 inhibitor (SB203580) did not affect the tube formation enhanced by CHI3L1 (left panel). Transfection of siRNA for ERK or JNK in SW480 cells significantly diminished the tube formation, but siRNA for p38 had no effect (right panel).

All results (B-E) are the mean (± SEM) of more than three separate experiments. * $P < 0.05$, ** $P < 0.01$.

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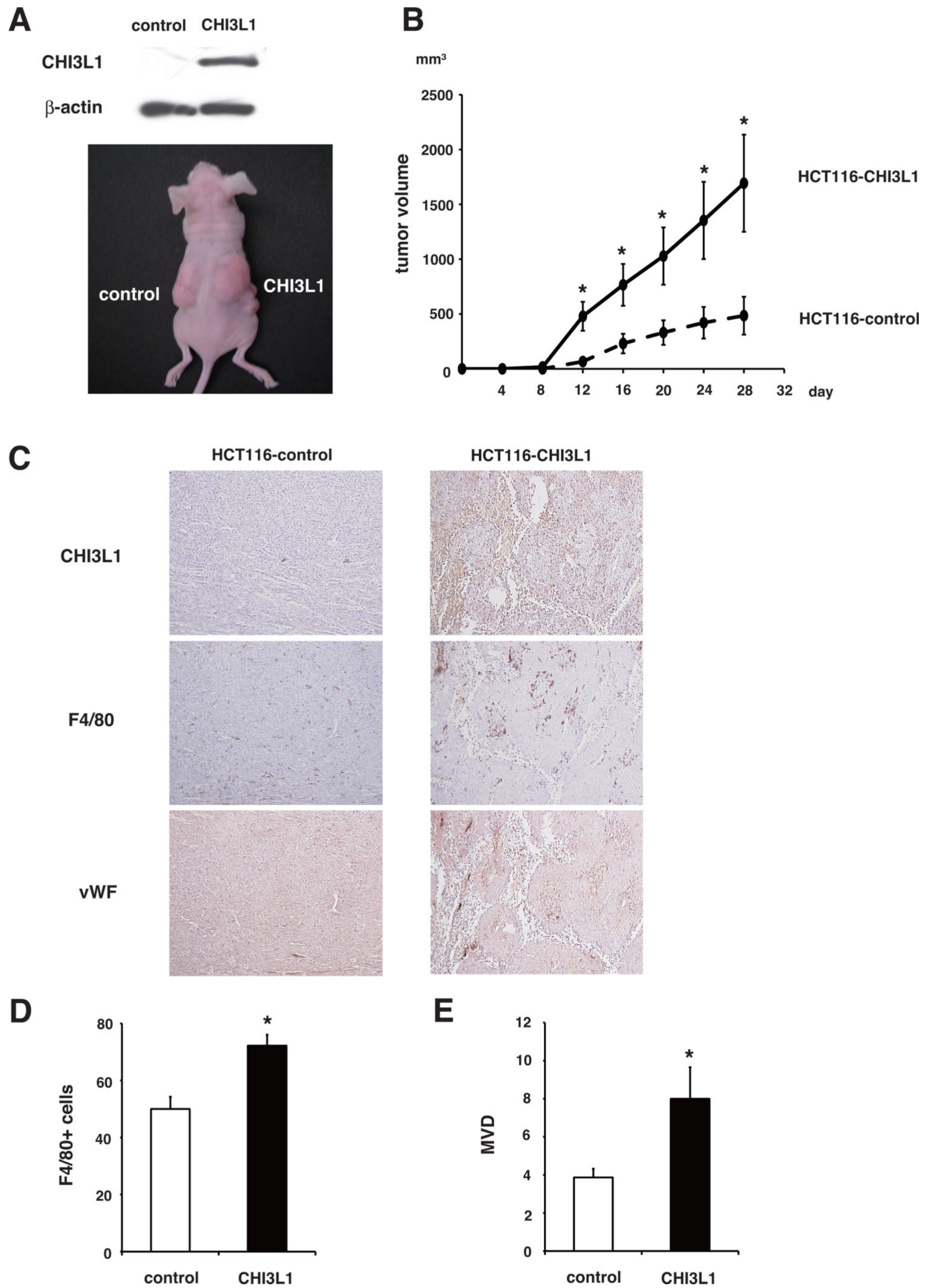

Figure 6. Effect of CHI3L1 in a xenograft mouse model

(A) Western blot analysis of CHI3L1 and β-actin expression (upper panel) and a representative photograph of xenograft-bearing nude mice (lower panel). HCT116 cells were infected with retrovirus containing CHI3L1 or pMXs-IRES-Puro vector control. Protein level of CHI3L1 was up-regulated by CHI3L1 overexpression as compared with control vector. Mice were inoculated subcutaneously with 5×10^6 HCT116 cells in the left (vector control) and right (CHI3L1) flank. Overexpression of CHI3L1 caused 3.5 fold larger tumors than control.

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(B) Tumor growth of HCT116 cells in nude mice. Overexpression of CHI3L1 significantly enhanced xenograft tumor growth as compared with control vector. Tumor size was measured during 4 weeks and calculated as follows: volume = length $\times$ width^2 $\times$ 1/2. n = 6. * $P < 0.05$.

(C) Representative photographs of CHI3L1-overexpressed and control sections stained with anti-CHI3L1, -F4/80, and -vWF antibodies. Original magnification, x100.

(D) Mean numbers of F4/80+ macrophages at x200 magnification. The number of F4/80+ macrophage was significantly higher in CHI3L1-overexpressed tumors than in control tumors. * $P < 0.05$.

(E) Mean MVD at x100 magnification. MVD was significantly higher in CHI3L1-overexpressed tumors than in control tumors. * $P < 0.05$.