Cancer cell-induced tissue inhibitor of metalloproteinase-1 secretion by cancer-associated fibroblasts promotes cancer cell migration

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Abstract. Cancer-associated fibroblasts (CAFs) are one of the major components of the cancer stroma in the tumor microenvironment. The interaction between cancer cells and CAFs (cancer-stromal interaction; CSI) promotes tumor progression, including metastasis. Recently, the tissue inhibitor of metalloproteinase-1 (TIMP-1) was reported to promote cancer cell migration and metastasis, which is contrary to its anticancer role as an inhibitor of matrix metalloproteinase. Moreover, CAF-derived TIMP-1 is reported to regulate CAF activity. In the present study, we investigated the effect of TIMP-1 on colon cancer cell migration *in vitro*. The TIMP-1 secretion levels from the CAFs and cancer cell lines were comparatively measured to determine the main source of TIMP-1. Furthermore, the effect of CSI on TIMP-1 secretion was investigated using the Transwell co-culture system. Cancer cell migration was evaluated using the wound-healing assay. The results demonstrated that TIMP-1 promoted the migration of LoVo cells, a colon cancer cell line, whereas TIMP-1 neutralization inhibited the enhanced migration. The TIMP-1 levels secreted from the cancer cells were approximately 10 times less than those secreted from the CAFs. TIMP-1 secretion was higher in CAFs co-cultured with cancer cells than in monocultured CAFs. Furthermore, the migration of LoVo cells increased upon co-culturing with the CAFs. TIMP-1 neutralization partially inhibited this enhanced migration. These results suggest that CAFs are the primary source of TIMP-1 and that the TIMP-1 production is enhanced through CSI in the tumor microenvironment, which promotes cancer cell migration.

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Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase; CAFs, cancer-associated fibroblasts; CSI, cancer-stromal interaction

Key words: cancer-associated fibroblasts, migration, co-culture, colorectal cancer, tissue inhibitor of metalloproteinase 1

Introduction

An important initial process involved in the multistep mechanism of cancer metastasis is the migration of cancer cells. During the process of migration, the cancer cells detach from the primary tumor and enter the lymphatic and blood vessels, which subsequently can result in the formation of tumors at the secondary sites (1,2). Additionally, the stromal extracellular matrix (ECM) undergoes remodeling during cancer cell migration. The cancer cells interact with the ECM and promote ECM remodeling. This interaction affects tissue stiffness and migration of the cancer cells. The matrix metalloproteinases (MMPs), which are released by cancer and stromal cells, play a key role in remodeling the ECM (2-6). The proteolytic activity of MMPs is regulated by the tissue inhibitors of metalloproteinases (TIMPs). The TIMPs are reported to exhibit anticancer activity as they are natural endogenous inhibitors of MMPs. Recently, the function of TIMP-1, which is one of the four identified members of the TIMP family (TIMP-1, -2, -3, and -4), in cancer progression has been gaining attention. Interestingly, recent studies have demonstrated that TIMP-1 may exhibit cancer-promoting effects, such as regulation of cell proliferation, induction of anti-apoptotic signaling, and promotion of angiogenesis, which are independent of MMPs (7-11). Several studies have reported that TIMP-1 promotes cancer cell migration and metastasis (12,13). However, the role of TIMP-1 in cancer cell migration has not been fully elucidated.

An increasing number of studies have focused on the interaction between cancer cells and stromal cancer-associated fibroblasts (CAFs), which is called cancer-stromal interaction (CSI), during the cancer progression (14-17). CAFs maintain an optimal tumor microenvironment for the cancer cells by secreting cytokines and tumor growth and angiogenic

factors (6,14,18-26). Recent studies have suggested that CAFs also play an important role in cancer metastasis through ECM remodeling (6,17,27). Furthermore, several studies suggest that TIMP-1 is derived from the stromal CAFs and that TIMP-1 may regulate the CAF activity during cancer progression (25,28,29). However, the synergistic function of TIMP-1 and CAFs, which is induced through CSI, in cancer cell migration and metastasis is not completely understood.

Hence, in the present study, we focused on the potential role of TIMP-1 in mediating the interaction between cancer cells and CAFs. The aim of the present study was to evaluate the role of CAFs in colon cancer cell migration through CSI via TIMP-1.

Materials and methods

Cell lines. In the present study, we used the human colon cancer cell lines (LoVo, HT29, and HCT116) and CAF cells derived from patients with cancer. All cancer cell lines were purchased from the American Type Culture Collection (ATCC). The cancer cell lines were cultured in DMEM (Merck) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and 1% antibiotic-antimycotic solution (Merck). The CAFs were cultured in DMEM supplemented with 5% FBS and 1% antibiotic-antimycotic solution. All the cells were cultured at 37˚C in a humidified 5% CO_2 atmosphere.

Isolation and culture of human colon fibroblasts. The human colon fibroblast cell lines were established using the resected tumor specimens obtained from patients with colorectal cancer who underwent surgery at the Nagoya City University Hospital (Nagoya, Japan). The study protocol was approved by the Institutional Review Board of Nagoya City University Hospital (Institutional code, 70-00-0071). The technical procedure for establishing the fibroblast cell lines was similar to that described in previous studies (14,22,24). Briefly, the colorectal cancer tissues and nonmalignant tissues were collected from the patients after obtaining written informed consent. The tissues were cut into 2-3 mm^3 cubes using a scalpel. The tissues were cultured in DMEM supplemented with 1,000 U/ml dispase (Godo Shusei) for 2 h. Next, the tissues were cultured in DMEM supplemented with 5% FBS and 1% antibiotic-antimycotic solution at 37˚C in a humidified 5% CO_2 atmosphere. The fibroblasts that were isolated from the cancer tissues were defined as CAFs and those isolated from the nonmalignant tissues were defined as normal fibroblasts (NFs) as described previously (14,22,24). The CAFs and NFs were used for analysis between passages 3 and 6.

Antibodies. The primary mouse anti-vimentin (M0725) and mouse anti-cytokeratin (M3515) antibodies, and the anti-mouse HRP-conjugated secondary antibody (Dako Envision+System, K4001) were purchased from Dako for immunohistochemical staining. The primary mouse anti-α-smooth muscle actin (α-SMA) (ab7817; Abcam) and rabbit anti-fibroblast activation protein α (FAP) (ab28244; Abcam), and the secondary Alexa flour 488-conjugated anti-mouse (ab150113; Abcam) and cyanine (Cy) 3-conjugated anti-rabbit (ab6939; Abcam) antibodies were used for immunofluorescence staining. Primary mouse anti-CD63 (ab59479; Abcam) and rabbit anti-GAPDH (#2118; Cell Signaling, Inc.) antibodies and secondary anti-mouse HRP-conjugated antibody (P0447; Dako) and anti-rabbit HRP-conjugated antibody (P0448; Dako) were purchased for western blotting analysis. The human recombinant TIMP-1 (410-01) was purchased from PeproTech and reconstituted in PBS containing 0.1% BSA to a concentration of 0.2 mg/ml. The human TIMP-1 neutralizing antibody (AF970) was purchased from R&D Systems Inc. and reconstituted in PBS to a concentration of 0.2 mg/ml.

Immunohistochemical and immunofluorescence staining. The isolated fibroblasts were confirmed as CAFs through immunohistochemical and immunofluorescence staining as described previously (14). For immunohistochemical staining, the isolated fibroblasts were fixed with 10% formalin for 10 min. The fixed fibroblasts were blocked with 3% BSA prepared in PBS. Next, the fibroblasts were probed with the primary mouse anti-vimentin (1:80) and mouse anti-cytokeratin (1:80) antibodies for 60 min at 37˚C. The fibroblasts were then probed with the HRP-conjugated secondary antibody for 60 min at 37˚C. The cells were stained with a DAB substrate (Dako) and counterstained with hematoxylin.

For immunofluorescence staining, the fibroblasts were fixed with 10% formalin for 10 min and treated with 0.2% Triton X-100 (MP Biomedicals) for 10 min. The fibroblasts were blocked with 1% BSA prepared in PBS. The fibroblasts were probed with the primary mouse anti-α-SMA (1:200) and rabbit anti-FAP (1:100) antibodies for 60 min at room temperature. Next, the fibroblasts were probed with the Alexa flour 488-conjugated anti-mouse (1:200) and cyanine (Cy) 3-conjugated anti-rabbit (1:1,000) secondary antibodies for 30 min. The fibroblasts were washed with PBS and treated with the ProLong™ Gold Antifade Mountant containing DAPI (P36941; Thermo Fisher Scientific, Inc.) for 10 min at room temperature. The images were captured and analyzed using the KEYENCE BZ-X700 Fluorescence Microscope and BZ-X700 Analyzer (Keyence).

Co-culturing CAFs with the colon cancer cell lines. The CAFs were seeded in 6-well plates at a cell density of $1.0x10^5$ cells/well. The LoVo, HT29, and HCT116 cells were seeded in the Transwell inserts (Falcon Permeable Support for 6-well plate with 0.4 μm Transparent PET Membrane, 353090, Corning Incorporated) at a cell density of $1.0x10^5$ cells/insert. The cells were incubated in DMEM supplemented with 2% FBS for 24 h. Next, each insert was placed in the 6-well plates and co-cultured in DMEM supplemented with 2% FBS. After 48 h co-culture, the conditioned medium was collected. The cancer cell lines and CAFs were monocultured in DMEM supplemented with 2% FBS in the 6-well plates and the respective conditioned medium was collected.

Cytokine antibody array. The secretion of various cytokines by the CAFs was screened through human cytokine antibody array using a commercially available array system for the human MMPs and TIMPs (ab134004; Abcam). The secretion of cytokines in the collected monoculture conditioned medium by the HT29 and CAF cells and the co-culture conditioned medium was analyzed, following the manufacturer's instructions.

Cell survival assay. The effect of TIMP-1 and human TIMP-1 neutralizing antibody on the viability and proliferation of cancer cell lines was evaluated using the Premix WST-1 Cell Proliferation Assay System (Takara Bio), following the manufacturer's instruction. The LoVo, HT29, and HCT116 cells ($3.0x10^4$ cells/well) were placed in 96-well plates and allowed to attach overnight at 37˚C. The growth medium was replaced with a medium containing human recombinant TIMP-1 or human TIMP-1 neutralizing antibody. The number of viable cells was examined at 0, 24, 48 and 72 h by measuring the absorbance at 450 nm with the reference wavelength at 650 nm using a microplate reader (Molecular Devices).

ELISA. The secretion levels of TIMP-1 in the monoculture conditioned medium of cancer cells, CAFs, and the respective co-culture conditioned medium were evaluated by ELISA. The levels of TIMP-1 in the collected conditioned medium were measured using the Human TIMP-1 ELISA Kit (DTM100; R&D Systems Inc.), following the manufacturer's instructions.

Wound-healing assay for colon cancer cell lines in the presence or absence of TIMP-1 and TIMP-1 antibodies. To evaluate cell migration ability, several approaches, such as Transwell assays and wound healing assays, can be used. In this study, we used wound healing assays, which have been used in many other studies, to evaluate the effects of TIMP-1 on the colon cancer cell migration (30-32). The LoVo, HT29, and HCT116 cells were cultured in DMEM supplemented with 2% FBS in 24-well plates until confluency. The wounds were carefully generated by scratching the confluent cells with the 200-μl pipette tips. The cells were washed with PBS. Next, the cells were cultured in DMEM containing 2% FBS and human recombinant TIMP-1 or the control DMEM containing equivalent amount of BSA prepared in PBS. The images from the wound-healing assay were captured and analyzed using the BZ-X700 and BZ-X700 Analyzer (Keyence) at 0, 24, 48, and 72 h.

The effect of TIMP-1 neutralization on cell migration was evaluated by wound-healing assay. The assay was performed in the presence of human recombinant TIMP-1 and in the presence or absence of human TIMP-1 neutralizing antibody or in control DMEM/PBS.

Wound-healing assay for cancer cells co-cultured with CAF. The effect of co-culturing CAFs with cancer cells on promoting cancer cell migration was evaluated by wound-healing assay. The LoVo monoculture was used as the control in the wound-healing assay. The wound-healing assay was performed for the cancers cells co-cultured with CAFs, which were seeded and incubated in the Transwell inserts (Corning Inc.) at the cell density of $1.0x10^5$ cells/well. Furthermore, the role of TIMP-1 secreted by the CAFs in promoting the migration of LoVo cancer cells co-cultured with CAFs was evaluated using the TIMP-1 neutralizing antibody.

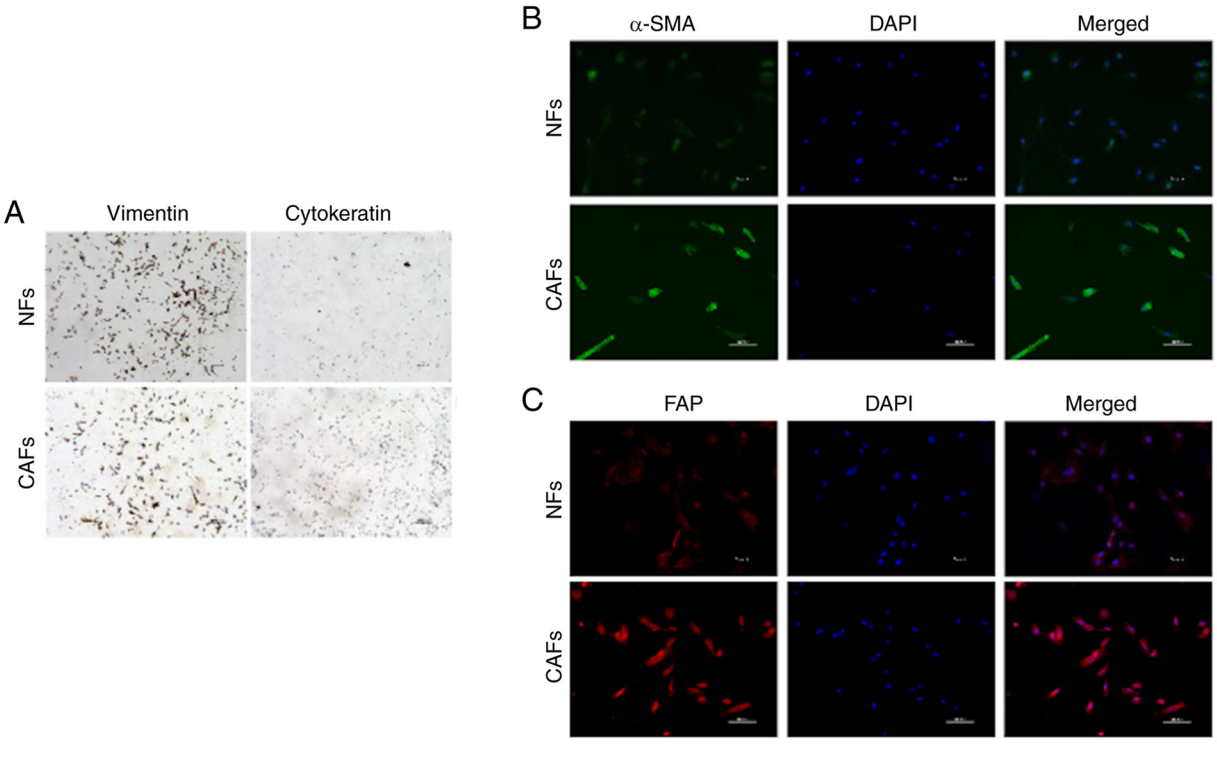
Western blot analysis. Western blot analysis was performed as described previously (14). Protein samples were prepared in RIPA lysis and extraction buffer with Protease Inhibitor Single-Use Cocktail and Phosphatase Inhibitor Cocktail (all from Thermo Fisher Scientific, Inc.). The concentration of each protein was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein were denatured by boiling at 90˚C for 5 min. Proteins (30 μg) were fractionated on 10% Mini-PROTEAN TGX gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Primary and secondary antibody reactions were performed using an iBind Flex Western System (Thermo Fisher Scientific, Inc.). Membranes were incubated with iBind Flex Solution (iBind Flex Buffer, iBind Flex Additive, and distilled water) for 10 min at room temperature to block nonspecific binding. Primary (CD63 and GAPDH) and secondary (polyclonal goat anti-mouse IgGs conjugated to HRP and polyclonal goat anti-rabbit IgGs conjugated to HRP) antibody reactions were performed at room temperature for 2.5 h, following the manufacturer's protocol. Protein-antibody complexes were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). The immunoreactive protein band was detected, and band density was quantified via densitometry using an Amersham Imager 600 (GE Healthcare Life Sciences).

Statistical analysis. Each experiment was repeated at least three times. The data are presented as the mean ± SEM. All statistical analyses were performed using the JMP software version 14 (SAS Institute). The differences between the groups were compared using the Student's t-test or ANOVA followed by post hoc test with Dunnett's test and Tukey's test. The difference was considered statistically significant when the P-value was <0.05.

Results

Isolation and characterization of primary CAFs and NFs. The spindle-shaped cells with large cytoplasm were established from the fresh colorectal cancer tissues and the nonmalignant tissues. The cells were subjected to immunostaining to detect the markers of fibroblasts and CAFs as described previously (14,22). The immunohistochemical staining analysis confirmed that the established cells tested positive for vimentin and tested negative for cytokeratin expression (Fig. 1A). The immunofluorescence staining demonstrated that the cells derived from the cancerous lesions exhibited enhanced expression of α-SMA and FAP, whereas those derived from nonmalignant tissues exhibited weak expression (Fig. 1B and C). These results confirmed that the established cells were fibroblasts. Additionally, the cells derived from cancerous lesions were confirmed as CAFs, while those derived from nonmalignant tissues were confirmed as NFs. The characteristics of patients from whom cancerous lesions and nonmalignant tissues were obtained to isolate the CAFs and NFs are shown in Table I.

Cytokine secretion by the CAFs. The cytokine antibody array was used to analyze the MMPs and TIMPs in the monoculture conditioned medium of HT29 cells and CAFs and the co-culture conditioned medium. The analysis revealed that the levels of TIMP-1 and TIMP-2 in the monoculture conditioned medium of CAFs were higher than those in the monoculture conditioned medium of HT29 cells. Additionally, the co-culture of HT29 cells and CAFs enhanced the secretion



POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	NEG	POS
MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	NEG	POS

Figure 1. Differential expression of MMPs and TIMPs between the established NFs and CAFs evaluated by cytokine antibody array. (A) Representative immunohistochemical staining images showing positive vimentin expression and negative cytokeratin expression in both NFs and CAFs. (B) Representative immunofluorescence staining of α-smooth muscle actin (α-SMA), which was positive in CAFs and negative in NFs. (C) Representative immunofluorescence staining images of fibroblast activation protein α (FAP), which was positive in CAFs and negative in NFs. (D) Cytokine antibody array for MMPs and TIMPs for the monoculture conditioned medium of HT29 cells and CAFs, and the co-culture conditioned medium suggested that CAFs secrete more TIMP-1 and TIMP-2 than the HT29 cells (white arrow heads), and that the co-culture enhanced the secretion of TIMP-1 and TIMP-2 (white arrows). MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; NFs, normal fibroblasts; CAFs, cancer-associated fibroblasts.

of TIMP-1 and TIMP-2 by the CAFs (Fig. 1D). Among these two TIMPs, we focused on TIMP-1 in this study.

TIMP-1 enhances the migration of LoVo cells. The effect of TIMP-1 on colon cancer cell migration was investigated using wound-healing assay, which is an *in vitro* assay for cell migration. We investigated the effect of increasing concentrations of human recombinant TIMP-1 (50, 100, and 250 ng/ml) on the migration of LoVo colon cancer cells. As shown in Fig. 2A and B, TIMP-1 at a concentration of 250 ng/ml significantly enhanced the migration of LoVo cells at 48 h ($P<0.01$) and 72 h ($P<0.05$). The effects of human recombinant TIMP-1 on the survival or proliferation of LoVo cells were evaluated at different concentrations of TIMP-1 using WST-1 assays at 48 and 72 h. The tested concentrations of TIMP-1 did not affect the survival of LoVo cells (Fig. 2C). Next, we assessed the effects of 250 ng/ml TIMP-1 on the migration of HT29 and HCT116 colon cancer cells. The migration of HT29 and HCT116 colon cancer cells was not significantly affected after treatment with 250 ng/ml TIMP-1 (Fig. 2B). As observed in LoVo cells, TIMP-1 did not affect the survival of HT29 and HCT116 cells (Fig. 2C).

To determine whether the differential effect of TIMP-1 on cell migration was due to the differential expression of CD63,

Table I. Characteristics of the patients whose tissues were used to isolate the CAFs and NFs and the figure numbers in which the patient samples were used for experiments.

Patient no.	Age (years)	Sex	Histological type	pTNM[a]	Successfully established fibroblasts	Figure numbers in which the patient samples were used for experiments
#1	68	M	Well-differentiated adenocarcinoma	T3N0M0	CAF#1, NF#1	1B, 1C, 1D, 5A, 5B
#2	61	M	Moderately differentiated adenocarcinoma	T4bN0M0	CAF#2	1A, 5A, 5B
#3	71	F	Moderately differentiated adenocarcinoma	T4bN0M0	CAF#3, NF#3	5A, 5B
#4	50	F	Moderately differentiated adenocarcinoma	T3N1bM0	CAF#4	6B
#5	65	M	Moderately differentiated adenocarcinoma	T4aN0M0	CAF#5, NF#5	5A, 5B, 6A, 6B

[a]According to UICC-TNM classification 8th edition (https://www.uicc.org/resources/tnm). CAF, cancer-associated fibroblasts; NF, normal fibroblast; M, male; F, female.

the expression of CD63 in the cancer cell lines was evaluated via western blotting analysis. The LoVo cancer cells exhibited higher CD63 expression than that found in the HT29 and HCT116 cells (Fig. 3A). The enhanced wound-healing rate between the LoVo cells treated with 250 ng/ml TIMP-1 and untreated LoVo cells at 72 h was approximately twice as that between the 250 ng/ml TIMP-1-treated and untreated HT29 and HCT116 cells (Fig. 3B). This differential effect of TIMP-1 on cell migration may be correlated with the differential CD63 expression in these cancer cell lines.

TIMP-1 neutralization inhibits the TIMP-1-mediated enhanced migration of LoVo cells. The inhibitory effect of TIMP-1 neutralization on TIMP-1-mediated LoVo cell migration was evaluated by wound-healing assay. The migration of LoVo cells treated with 250 ng/ml TIMP-1 was evaluated in the presence or absence of human TIMP-1 neutralizing antibody. TIMP-1-mediated migration of LoVo cells was inhibited by 10 μg/ml of human TIMP-1 neutralizing antibody at 48 h ($P<0.05$), and by 5 μg/ml ($P<0.05$) and 10 μg/ml ($P<0.01$) of TIMP-1 antibody at 72 h (Fig. 4A and B). Next, WST-1 assay was performed to confirm that the inhibition of LoVo migration by different concentrations of TIMP-1 antibody was not due to its inhibitory effect on the LoVo cell survival. The results of WST-1 assay revealed that none of the concentrations of TIMP-1 antibody affected the LoVo cell survival (Fig. 4C).

TIMP-1 secretion levels from cancer cells and CAFs. Previous studies have demonstrated that elevated plasma TIMP-1 levels are associated with adverse long-term outcomes in patients with colorectal cancer (33-35) and that the protein expression of TIMP-1 is upregulated in the stroma of several types of cancer (25,28,36,37). However, the potential source of TIMP-1 in colorectal cancer tissue is poorly elucidated. Hence, we examined whether the source of TIMP-1 secretion is the cancer cells or CAFs *in vitro*. The secreted levels of TIMP-1 in the monoculture conditioned medium of colon cancer cell lines and established CAFs were evaluated using ELISA. As shown in Fig. 5A, the secreted levels of TIMP-1 in the monoculture conditioned medium of the cancer cell lines were too low to measure. However, the secreted levels of TIMP-1 in the monoculture conditioned medium of CAFs were higher than those in the monoculture conditioned medium of cancer cell lines. The secreted levels of TIMP-1 varied depending on the established cell lines. These results suggest that CAFs are the main source of TIMP-1 secretion in the colon cancer tissues.

Co-culturing CAFs with the colon cancer cell lines enhances TIMP-1 secretion. The effect of interaction between cancer cells and CAFs on TIMP-1 secretion was evaluated by measuring the secreted levels of TIMP-1 in the monoculture medium of CAFs and those in the co-culture conditioned medium of CAFs and cancer cell lines. As shown in Fig. 5B, the secretion of TIMP-1 by the CAFs increased when they were co-cultured with the LoVo, HT29, and HCT116 cells. These results indicated that TIMP-1 secretion by the CAFs was enhanced through the interaction between cancer cells and CAFs in the colon cancer tissues.

Co-culturing CAFs with the cancer cells enhances LoVo cell migration and TIMP-1 neutralization inhibits the enhanced cell migration. Although previous studies have demonstrated that the CAFs enhance cancer cell migration in *in vitro* co-culture models in several cancer types (27,38,39), the effects of CAF co-culture and the role of TIMP-1 in colon cancer cell migration have not been examined. Hence, we assessed the effects of co-culturing CAFs with cancer cells and the role of TIMP-1 on LoVo cell migration by neutralizing the TIMP-1 in the co-culture model. As shown in Fig. 6, we observed that co-culturing enhanced LoVo cell migration at all tested time points (24, 48, and 72 h). Furthermore, treatment with 10 μg/ml

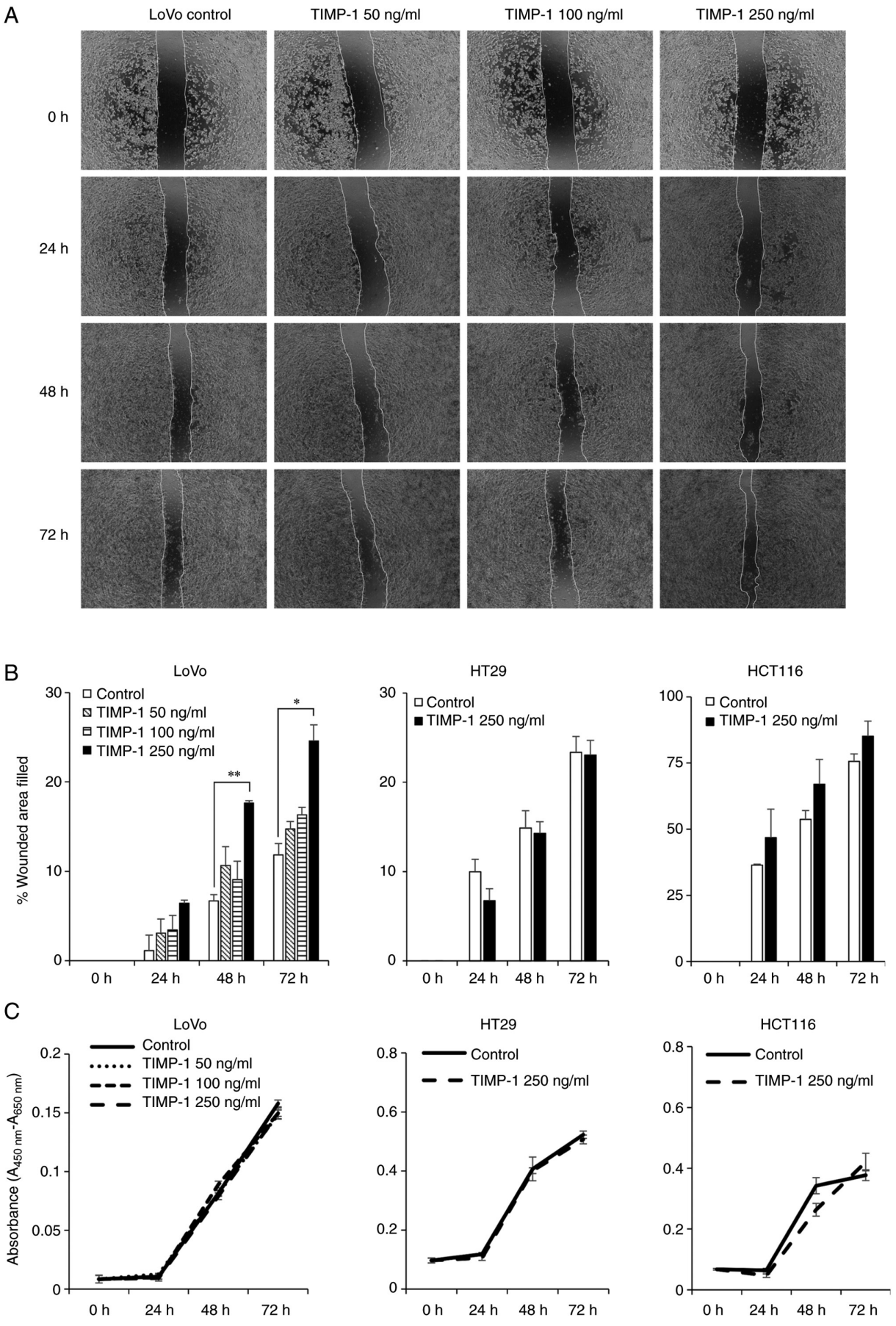


Figure 2. TIMP-1 enhances LoVo cell migration without affecting the cell survival. (A) Representative images of wound-healing assays for LoVo cells treated with increasing concentrations of human recombinant TIMP-1 or control (BSA). (B) TIMP-1 at 250 ng/ml significantly enhanced the migration of LoVo cells at 48 and 72 h but not in the HT29 and HCT116 cells. (C) WST-1 assays demonstrated that the human recombinant TIMP-1 did not affect the survival of colon cancer cell lines. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. TIMP, tissue inhibitor of metalloproteinase.

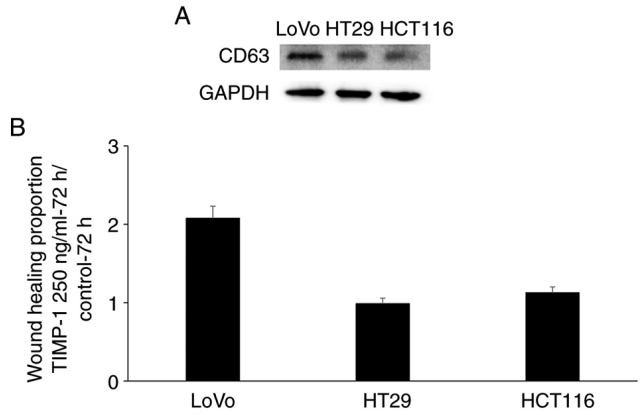


Figure 3. Comparison of CD63 expression in the cancer cell lines and enhanced wound healing by tissue inhibitor of metalloproteinase-1 (TIMP-1). (A) Western blotting analysis suggested that LoVo cells exhibited higher CD63 expression than HT29 and HCT116. (B) The enhanced wound-healing rate between the cells treated with 250 ng/ml TIMP-1 and untreated cells at 72 h. The wound-healing rates in the LoVo cells were approximately twice as high compared to those in the HT29 and HCT116 cells. TIMP, tissue inhibitor of metalloproteinase.

TIMP-1 antibody inhibited the enhanced LoVo cell migration at 48 h ($P<0.05$), and partially inhibited the enhanced migration at 72 h ($P<0.05$, while $P<0.01$ in CAF co-culture vs. control). These observations indicate the secretion of TIMP-1 by the CAFs is enhanced through the interaction with cancer cells, which is one of the important mechanisms underlying colon cancer cell migration.

Discussion

In the last decade, increasing number of studies have demonstrated that the cancer stromal cells promote cancer progression even though they are not malignant cells. Various processes, such as migration, invasion, adhesion, and angiogenesis are necessary for cancer metastasis. The interaction between the cancer cells and stromal cells is reported to be important for progression, angiogenesis, and chemoresistance. This interaction promotes tumor progression more than the cancer cells alone. Among the various stromal cells, cancer-associated fibroblasts (CAFs) are reported to play a potentially important role in tumor metastasis (6,17,36,40). Chemokines and cytokines secreted from the CAFs provide the microenvironment suitable for cancer cell survival, proliferation, invasion, and migration (6,15,16,20,21,23,24,27). However, the role of stromal cells in cancer cell migration and invasion has been poorly studied.

In the present study, we demonstrated that in addition to promoting angiogenesis and cancer progression, CAFs also promoted cancer cell migration. As shown in the present study, co-culturing CAFs with the cancer cells markedly promoted the migration of cancer cells when compared to the monocultured cancer cells. These results concurred with those of a previous study (27). The cell migration rate of cancer cells into the wounded area when co-cultured with CAFs was almost twice as that of the monocultured cancer cells (mean values in 72 h, monocultured cancer cells: 47.6% and cancer cells co-cultured with CAFs: 87.6%).

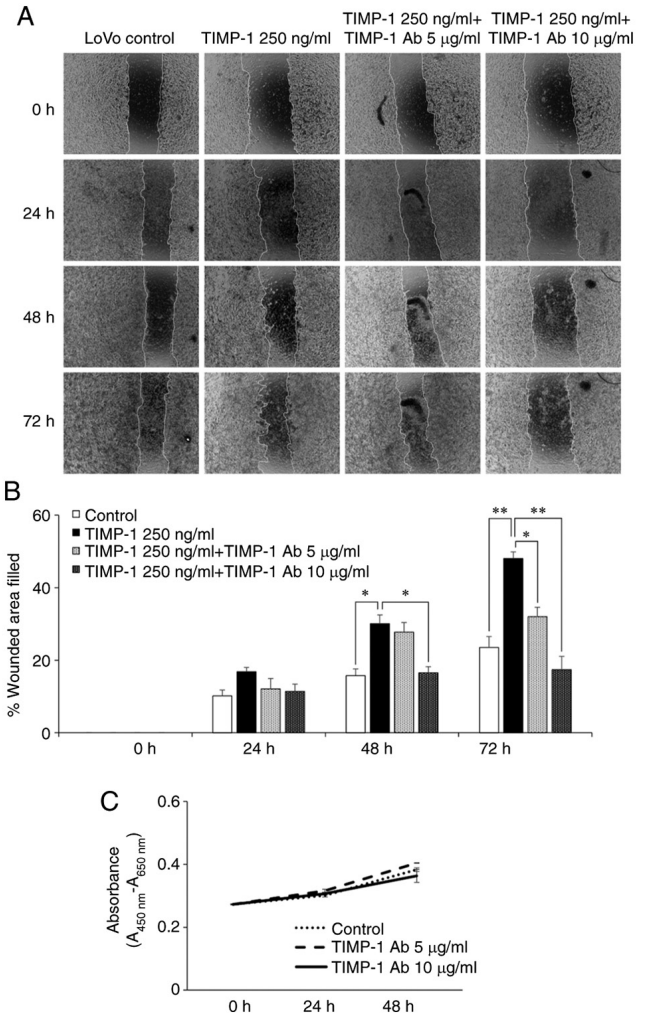


Figure 4. TIMP-1 neutralizing antibody inhibits the TIMP-1-mediated LoVo cell migration without affecting cell survival. (A) Representative images of wound-healing assays for LoVo cells treated with human recombinant TIMP-1 at 250 ng/ml in the presence or absence of human TIMP-1 neutralizing antibody (5 μg/ml or 10 μg/ml), or control (BSA+PBS). (B) The TIMP-1-mediated enhanced migration was inhibited by 10 μg/ml of human TIMP-1 neutralizing antibody at 48 h, and by 5 μg/ml and 10 μg/ml at 72 h. (C) WST-1 assays demonstrated that the TIMP-1 neutralizing antibody did not affect the survival of LoVo cells. Data are presented as the mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$. TIMP, tissue inhibitor of metalloproteinase.

The migration properties of cancer cells enable the metastasis of cancer cells to other organs. Several studies have demonstrated that cancer cell migration involves multistep processes, such as protrusion of the leading edge of the cell, focal contact formation through cell-matrix interaction, focalized proteolysis through the recruitment of surface proteases to extracellular matrix (ECM) contacts, cell contraction by actomyosin, the cell trailing edge detachment, and the proteolytic remodeling of the ECM (2-4). Matrix metalloproteinases (MMPs) play a key role in remodeling the ECM. The proteolytic activity of MMPs is regulated by the tissue inhibitors of metalloproteinase (TIMPs). TIMP-1, a glycoprotein that is detected in various body fluids and the extracellular compartment in various tissues, is a human natural endogenous inhibitor of MMPs. The TIMP-1 forms a noncovalent 1:1 stoichiometric complexes with the MMPs (4). However, increasing evidence indicates that TIMP-1 also exhibits

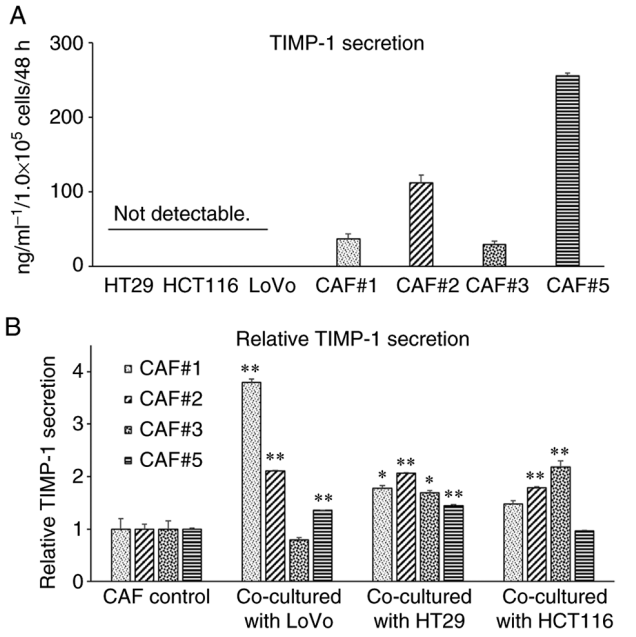


Figure 5. CAFs are the main source of TIMP-1 secretion and the CAFs co-cultured with cancer cell lines exhibit enhanced TIMP-1 secretion. (A) ELISA for measuring the levels of TIMP-1 in the monoculture of colon cancer cell lines and CAFs revealed that the levels of TIMP-1 secreted by the cancer cell lines were too low to measure. The secreted levels of TIMP-1 in the monoculture conditioned medium of CAFs were higher than those in the monoculture conditioned medium of cancer cell lines. The TIMP-1 secretion from CAFs varied depending on the established cell lines. (B) ELISA analysis of the secreted levels of TIMP-1 by the CAFs co-cultured with cancer cell lines revealed that co-culturing CAFs with either of the cancer cell lines enhanced the secretion of TIMP-1. The result of CAF#4 are not indicated because CAF#4 was fully consumed in other experiments. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. CAFs, cancer-associated fibroblasts; TIMP, tissue inhibitor of metalloproteinase.

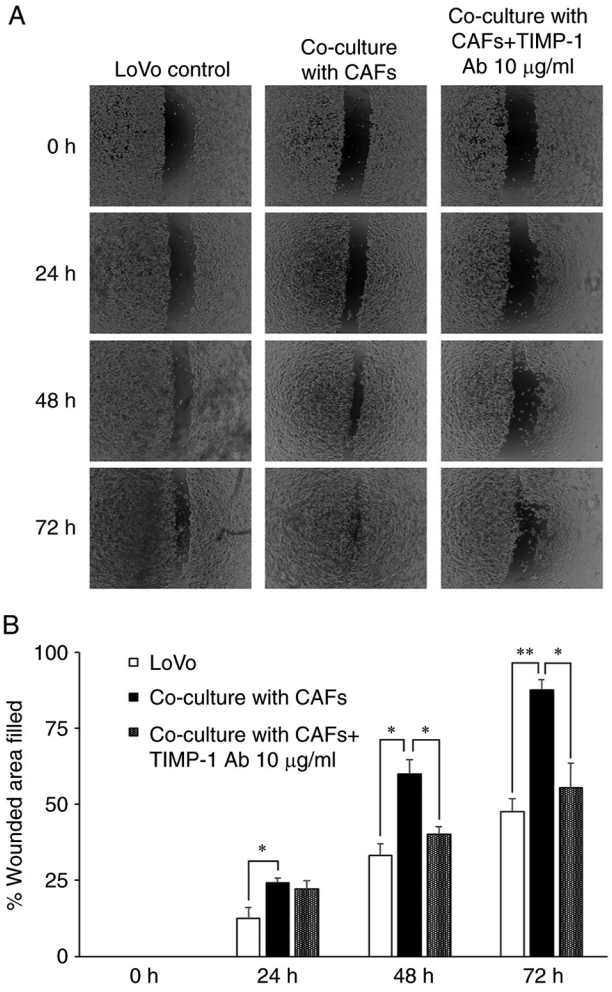


Figure 6. Migration of LoVo cells is enhanced upon co-culturing with CAFs and the neutralization of TIMP-1 partially inhibits the enhanced cell migration. (A) Representative images of wound-healing assays for LoVo cells co-cultured with CAFs in the presence or absence of TIMP-1 neutralizing antibody at 10 μg/ml. (B) Comparison of filled wounded area in the LoVo culture. Co-culturing CAFs with the LoVo cells significantly enhanced the migration of LoVo cells at 24, 48, and 72 h. TIMP-1 neutralizing antibody at 10 μg/ml inhibited the enhanced LoVo cell migration at 48 h, and partially inhibited the migration at 72 h. Data are presented as the mean ± SEM. *P<0.05, **P<0.01. CAFs, cancer-associated fibroblasts; TIMP, tissue inhibitor of metalloproteinase.

tumor-promoting effects, such as regulation of cell proliferation, anti-apoptotic function, cell migration, angiogenesis, and chemoresistance (7,8,12,28,36). The correlation between high plasma TIMP-1 levels and poor prognosis is reported clinically in various malignant neoplasms (8,29,33-35,37).

In the present study, we investigated whether TIMP-1 enhanced colon cancer cell migration. The concentration of TIMP-1 in the conditioned medium of CAF monoculture or CAFs co-cultured with cancer cells at a cell density of 1.0x10^5 cells/48 h was 200 to 300 ng/ml at most. Additionally, previous studies have reported that the plasma TIMP-1 concentration in patients with several malignancies ranged from approximately 100 to 300 ng/ml (8,12,28,33-35). Therefore, we investigated the effect of TIMP-1 on cancer cell migration with a maximum concentration of 250 ng/ml, a value that was also used in a study by Gong *et al* (28). In contrast to the results with LoVo, the same concentration of TIMP-1 did not enhance the migration of HT29 and HCT116 cells. Considering a previous study in which enhanced migration of another colon cancer cell line, DLD-1, was induced by as much as 5 μg/ml of TIMP-1, the reason that TIMP-1 did not enhance HT29 and HCT116 migration may be the use of low concentrations of TIMP-1; however, TIMP-1 concentration above 500 ng/ml appeared to be too high as a component of the tumor microenvironment, considering our previous results and previous studies.

Furthermore, we demonstrated that co-culturing cancer cells with CAFs promoted the migration of cancer cells. The migration of co-cultured cancer cells was similar to that of the cancer cells treated with TIMP-1. Furthermore, treatment with TIMP-1 neutralizing antibody decreased the migration of cancer cells to almost the same level as cancer cells (mean value with antibody: 55.4% and cancer cells alone: 47.6%). These results indicated that TIMP-1 secreted from the CAFs promoted cancer cell migration.

The mechanism underlying the cancer-promoting effect of TIMP-1 involves the conformational activation of integrin β1 and activation of mitogen-activated protein kinase signaling induced by the interaction between TIMP-1 and the TIMP-1 interacting cell surface protein CD63 (11,41). CD63 is a member of the tetraspanins, which are a superfamily of cell surface-associated membrane proteins involved in cell activation, adhesion, differentiation, migration, and invasion. CD63

is detected in the late endosomes, lysosomes, secretory vesicles, and plasma membrane (10). Western blot analysis revealed that the LoVo cells exhibited enhanced expression of CD63, while the HT29 and HCT116 cells exhibited weak CD63 expression; these observations concurred with the results of an earlier study (41). Hence, TIMP-1 may enhance the migration of only LoVo cells. Currently, there are limited data on the expression of CD63 and the effect of TIMP-1 on the migration of various colon cancer cell lines. The results of the present study suggest that the differential effect of TIMP-1 on cell migration can be attributed to differential CD63 expression.

The main source of TIMP-1 secretion in the colorectal cancer tissue has not been well studied. Our study revealed that TIMP-1 was mainly secreted from the CAFs and that the TIMP-1 secretion from the cancer cells was low. Alpizar-Alpizar *et al* and Gong *et al* demonstrated enhanced TIMP-1 expression in the cancer stroma through immunohistochemical staining, which is consistent with our results (25,28). However, Niewiarowska *et al* reported enhanced TIMP-1 expression in cancer cells (29). Moreover, simultaneous TIMP-1 expression in both cancer cells and stromal fibroblasts was also demonstrated by Kahlert *et al* (36). These discordant results may be due to the methodological differences. However, both cancer cells and CAFs may be the potential source of TIMP-1 (25). Although TIMP-1 secretion levels varied between the established CAFs, the correlation between TIMP-1 secretion levels and patient clinicopathological characteristics is unclear. The CAFs are generally a heterogeneous population in each individual. Hence, the secretion of TIMP-1 from the CAFs derived from different individuals may vary (15,16).

Interestingly, the levels of TIMP-1 secreted from the CAFs in the present study were shown to be significantly higher than those secreted from the CAFs co-cultured with the cancer cells. The cytokine antibody array for MMPs and TIMPs revealed that the CAFs secreted various cytokines and that their secretion was influenced by the presence of cancer cells. Enhanced secretion of CAF-derived tumor-promoting factors, such as chemokines, interleukins, growth factors, and transcription factors, is mediated by the interaction between CAFs and cancer cells (14-17,24,27). Our previous studies also demonstrated a similar phenomenon in angiogenesis, where the secretion of VEGFA by the CAFs co-cultured with the cancer cells was significantly higher than that by the monocultured CAFs (14). The results of this study also demonstrated that co-culturing cancer cells potentiates the ability of CAFs to promote cancer cell progression. Additionally, cancer-stromal interaction (CSI) was observed not only during angiogenesis and proliferation but also during cancer cell migration. Consistent with our results, several previous immunohistochemical studies demonstrated the enhanced TIMP-1 secretion from CAFs in the tumor tissues (25,28,36). However, the role of cancer cells in promoting TIMP-1 secretion by the CAFs *in vitro* has never been reported. Our results demonstrated that cancer cells promote enhanced TIMP-1 secretion by the CAFs *in vitro* using simple co-culture models. Further studies are needed to determine the underlying factors and their role in enhancing the TIMP-1 production from CAFs.

In conclusion, this study demonstrated that CAFs exhibit cancer-promoting activity in CD63-positive colon cancer through the secretion of TIMP-1, which can potentially promote colon cancer cell migration. The secretion of TIMP-1 by the CAFs was further enhanced through the interaction with the colon cancer cells. Thus, CAFs and TIMP-1 could be potential novel therapeutic targets for the clinical treatment of patients with colon cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NN, AM, TS, KT and NA performed most of the experiments. NN, TY, YMae, TH and KS collected the cultured fibroblasts. NN, MH and HT designed the study. NN, MH, RO, YMat and HT analyzed the obtained data. ST conducted the entire study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol was approved by Nagoya City University Graduate School of Medical Sciences and Nagoya City University Hospital Institutional Review Board (Institutional code, 70-00-0071). Written informed consent was obtained from the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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