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The inhibition of pancreatic cancer invasion-metastasis cascade in both cellular signal and blood coagulation cascade of tissue factor by its neutralisation antibody

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ABSTRACT

Tissue factor (TF), the initiating cell surface receptor for the blood coagulation cascade, plays an important role in malignant transformation of the pancreas, although the precise mechanism remains unresolved. Here, we report that the TF – factor VIIa complex in human pancreatic cancer cells produced a significant amount of MMP-9 and promoted invasion ability *in vitro* and invasion and metastasis *in vivo*. For treatment, we successfully developed an anti-human TF monoclonal antibody that inhibits both cellular signalling and blood coagulation cascade via TF. Invasive capability and MMP-9 expression were significantly reduced by the antibody. The antibody inhibited not only tumour invasion in the orthotopic model, but also haematogenous metastasis in the portal-injection liver metastasis model. In conclusion, the TF-VIIa complex plays an important role in invasion-metastasis by enhancing tumour cell infiltration ability and forming microthrombi. The newly established anti-human TF neutralisation antibody may be useful for the treatment of pancreatic and other invasive cancers.

1. Introduction

In cancer invasion and metastasis, the cancer cells degrade the basement membrane and intravasate into lymphatic or blood microvessels. The cells are then transported to a new location and become clogged within the microvessels, proceeding to grow following extravasation.[1] These steps include cancer cell invasion, degradation of the basement membrane and stromal extracellular matrix (ECM), and formation of microthrombi. The matrix metalloproteinase (MMP) family represents important enzymes that degrade ECM and facilitate tumour invasion.[2] Amongst them, MMP-9 is well-known as one of the most important factors in facilitating invasion and metastasis in pancreatic cancer.[3]

Tissue factor (TF), the initiating cell surface receptor for the coagulation cascade, activates factor VIIa. The TF-VIIa complex activates factor X, and consequently this protease cascade forms fibrin clots.[4,5] The relationship between cancer and blood coagulation was initially described by the French surgeon Trousseau.[6] Cancer patients, especially those with pancreatic, stomach, and glioma cancer, often suffer from a state of hypercoagulation and venous thrombosis, leading to

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patient morbidity and mortality.[7–9] In another study using a fibrinogen-deficient transgenic mouse model, fibrinogen appeared to be an important element of the metastatic potential of circulating tumour cells.[10] Meanwhile, TF plays an important role in not only blood coagulation but also cell signalling in which the TF-VIIa complex phosphorylates extracellular-regulated kinase 1/2 (ERK1/2) via protease-activated receptor-2 (PAR-2).[11] Moreover, its complex promotes the expression of interleukin-8 (IL-8) and invasion in breast cancer cell lines.[12] However, the concrete involvement of TF in tumour invasion-metastasis has not yet been fully evaluated.

Human pancreatic cancer has one of the worst prognoses amongst cancers.[13] Invasion and metastasis advancing beyond the pancreas are typical. Direct invasion to nearby organs, such as the stomach, duodenum, colon, spleen and kidney frequently occurs. Distant metastasis to the liver and peritoneal dissemination are also commonly seen.[14,15] In terms of the relationship between TF and pancreatic cancer, TF expression is an important early event in malignant transformation of the pancreas.[16] TF expression may contribute to the aggressiveness of pancreatic cancer that would stimulate tumour invasiveness, and evaluation of the primary tumour for TF expression may identify patients with a poor prognosis.[17,18]

Therefore, elucidation of the relationship between TF and pancreatic cancer invasion-metastasis may lead to the development of new therapeutic strategies as well as a better understanding of pancreatic cancer biology.

2. Materials and methods

2.1. Cell lines

Human pancreatic cancer cell lines BxPC3, Panc1, Capan1, and MIA PaCa-2 were purchased from the American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% faetal bovine serum (FBS) (Cell Culture Technologies, Gaggenau-Hoerden, Germany), 100 units/mL streptomycin, and 2 mmol/L L-glutamine (Sigma, St. Louis, MO, USA) in an atmosphere of 5% CO_2 at 37 °C.

2.2. Immunocytochemistry

Cells (1×10^5) were seeded on a 4-well culture slide (BD Biosciences, Bedford, MA, USA), which was incubated for 24 h at 37 °C. Then, after removal of the medium, the sections were rinsed with phosphate buffered saline (PBS) and soaked in 4% paraformaldehyde phosphate buffer solution for 15 min. The sections were then rinsed with PBS, and endogenous peroxidase activity was blocked with a 0.3% hydrogen peroxide solution in 100% methanol for 20 min. After the sections were rinsed with PBS three times for 5 min each, non-specific protein binding was blocked with 5% skim milk (BD, Franklin Lakes, NJ, USA) in PBS for 30 min at room temperature, followed by washing three times with PBS for 5 min. A mouse monoclonal antibody against human TF (Calbiochem, La Jolla, CA, USA) or a rat monoclonal antibody against human TF (established by USA) named as 1849 was added, incubated for 1 h, and rinsed three times with PBS for 5 min each. The sections were incubated for 30 min with EnVision™/HRP (Dako, Glostrup, Denmark) directed against each primary antibody. The sections were rinsed three times with PBS and incubated using the DAB+(3,3-diaminobenzidine tetrahydrochloride) Liquid System (Dako, Glostrup, Denmark) for 30 s. Finally, the sections were rinsed with water and counterstained with haematoxylin solution.

2.3. Immunohistochemistry

10^7 cells of each pancreatic cancer cell line were injected subcutaneously in 4-week-old female BALB/c nude mice. When the tumour volume reached 300 mm^3, tumours were excised from the mice under anaesthetic. Immunohistochemical analysis was conducted as described previously.[19] As a primary antibody, we used the rat monoclonal antibody against human TF that we created. In addition, we used goat anti-rat IgG/HRP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as a secondary antibody.

2.4. Transfection of BxPC3 cells with TF short hairpin RNA (TF shRNA), green fluorescent protein (GFP), and luciferase

Lentiviral particles were purchased from Sigma–Aldrich. BxPC3 cell suspension (1000 cells/100 μL) was seeded on a 96-well plate, which was incubated for 24 h at 37 °C. After removal of the medium, 100 μL of medium containing hexadimethrine bromide (final concentration of 8 μg/ml) was added to the cells. Viral particles carrying TF shRNA or non-target shRNA (multiplicity of infection (MOI) = 20) were added to the cells. After selecting the infected cells using 2 μg/ml puromycin, we established a TF-knockdown cell line (BxPC3 TFshRNA) and a control cell line (BxPC3 mock). For the detection of micrometastasis and microinvasion in pancreatic orthotopic tumour xenografts, both BxPC3 mock and BxPC3 TFshRNA were infected with viral particles carrying GFP (MOI = 20). In addition, the BxPC3 cell line stably expressing firefly luciferase and YFP mutant Venus ($BxPC3^{Luc}$) was established. In brief, the coding sequence for firefly luciferase and Venus was subcloned into the pIRES vector (Clontech Laboratories, Mountain View, CA, USA). The fragment consists of Luciferase-IRES-Venus generated from the plasmid with the restriction enzymes Nhe1 and Not1. This fragment was subcloned into the pEF6/V5-His vector (Invitrogen, Carlsbad, CA, USA) to generate plasmids of pEF6-Luciferase IRES Venus. BxPC3 were seeded on 6-well plate 24 h before transfection. The cells were transfected with pEF6-Luciferase IRES Venus using Lipofectamine™ LTX with Plus™ Reagent (Invitrogen) according to the manufacturer's instructions, and then incubated for 48 h at 37 °C. The cells were then passaged in medium containing blasticidin (10 μg/ml; InvivoGen, San Diego, CA, USA) to select the blasticidin resistance gene integrated in the pEF6/V5-His plasmids.

2.5. Real-time PCR analysis for MMPs and TF

Total RNA was extracted from pancreatic cancer cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesised from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in accordance with

the manufacturer's instructions. We analysed the expression of five genes: *matrix metallopeptidase 2 (MMP-2)*, *matrix metallopeptidase 7 (MMP-7)*, *matrix metallopeptidase 9 (MMP-9)*, *TF*, and *GAPDH* as an internal control gene. For all of these genes, we used commercially available TaqMan primers and probe mixture (Applied Biosystems). The reporter dye at the 5′ end of the probe was FAM™, and the quencher dye at the 3′ end was minor groove binder (MGB). Real-time PCR was performed using precycling heat activation at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 3 s, and annealing/extension at 62 °C for 30 s, in the Applied Biosystems 7500 Fast Real-Time PCR System. Relative quantification of the total RNA in each sample was conducted using the comparative Ct (threshold cycle) method. In this analysis, the formulas for the relative quantification of each of the genes were as follows: (dCT of each gene) = (Ct of each gene) – (Ct of *GAPDH*), and (Relative quantification of each gene) = $2^{-(\text{dCt of each gene})}$.

2.6. TF ELISA assay

TF protein was measured by IMUBIND® Tissue Factor ELISA Kit (American Diagnostica, Greenwich, CT, USA) in accordance with the manufacturer's instructions.

2.7. Cell invasion assay

BxPC3 mock and BxPC3 TFshRNA (2.5×10^4 cells diluted in 500 μL of serum-free DMEM) were added to the upper wells of a Transwell® 24-well insert coated with Matrigel™ (BD Biosciences) according to the manufacturer's instructions. DMEM with 10% FBS was added to the lower wells. Cells were incubated for 22 h in an atmosphere of 5% CO_2 at 37 °C. Cells in the top wells were removed using cotton swabs. Invaded cells were stained and counted in three different viewing fields. In addition, to avoid the effect of factor VIIa in FBS, 500 μL of cell suspension in serum-free high-glucose DMEM was added to the upper wells after they were deprived of serum for 2 h, and high-glucose DMEM supplied with 1% BSA and 0.5% FBS was poured into the lower wells. Factor VIIa and anti-TF antibodies were also added to the lower wells. At the end of 48 h incubation at 5% CO_2, 37 °C, the cells in the top wells were removed and invaded cells were counted. Every invasion assay was conducted two times each.

2.8. Gelatin zymography

SDS-gelatin zymography was performed with 10% zymogram gel (Invitrogen). Cells (2×10^5) were seeded on a 6-well plate and incubated for 24 h at 37 °C. The medium was removed and incubated for 24 h in serum-free DMEM, after which the medium was collected. Tris–Glycine SDS Sample Buffer (Invitrogen) was added and incubated for 30 min at 37 °C. After electrophoresis at 4 °C, the gel was shaken with zymogram renaturing buffer for 30 min. Next, the zymogram renaturing buffer was removed and zymogram developing buffer (Invitrogen) was added and shaken for 30 min. The gel was added to new zymogram developing buffer and was incubated for 30 h at 37 °C. The gel was fixed and then stained with Quick CBB (Wako Chemicals). MMPs were quantified using Image J software.

2.9. Effect of anti-TF mAb on invasion and metastasis in nude mice

To assess metastasis and invasion, we established an orthotopic pancreatic cancer mouse model as described previously.[19] Briefly, BxPC3 mock or BxPC3 TFshRNA expressing GFP (5×10^6 cells) was injected into the body of the pancreas of nude mice after laparotomy under anaesthesia. The mice were sacrificed 5 weeks after the injection of cancer cells. An OV110 fluorescence microscope (Olympus, Tokyo, Japan) was used to observe several organs and a dissemination score was calculated to evaluate local tumour invasion and distant metastasis as previously described.[20] Tumour dissemination was quantified as follows: every manifestation of tumour infiltration or metastasis was credited with one point. Additional points were awarded for massive local infiltration, multiple metastatic nodules and metastatic nodules >50 mm^3. In treatment experiments, mice inoculated with $BxPC3^{Luc}$ or BxPC3 mock expressing GFP cells on Day 0 were treated for seven consecutive days with 1849 mAb (400 μg/mouse) as a TF neutralisation antibody, or PBS as a control. The IVIS imaging system (Caliper Life Sciences, Hopkinton, MA, USA) was used to observe tumour invasion of mice inoculated with $BxPC3^{Luc}$. After 40 days, mice inoculated with cells expressing GFP were sacrificed. A dissemination score was calculated. To assess the inhibition of haematogenous metastasis by anti-TF antibody, we established a portal-injection liver metastasis model. BxPC3 cells expressing luciferase (5×10^5 cells) were directly injected into the portal vein after intravenous administration of 1849 mAb (500 μg/mouse) or PBS as a control. The IVIS imaging system was used to detect liver micrometastasis. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of the National Cancer Center, Japan; these guidelines meet the ethical standards required by law for proper conduct of animal experiments in Japan.

2.10. Statistical analysis

Student's *t*-test was used for the statistical analyses unless otherwise mentioned. $P < 0.05$ was considered significant.

3. Results

3.1. TF-positive human pancreatic cancer cells, BxPC3, enhance invasion potential in vitro and in vivo

We used the BxPC3 cell line in a series of our experiments because real-time PCR analysis and immunostaining of TF showed that BxPC3 strongly expressed TF amongst the four pancreatic cancer cell lines (Fig. 1A). Then, we examined the status of TF expression for BxPC3 *in vitro* and *in vivo*. Immunostaining revealed TF expression specifically in cancer cells contacting with stromal tissues, namely the invasive front *in vivo*, although TF expression was uniformly observed in all cells *in vitro* (Fig. 1B). To determine if TF affects pancreatic cancer invasion, we established TF-knockdown BxPC3 cell lines. BxPC3 cells were infected with TF shRNA lentivirus (BxPC3 TFshRNA) and non-target shRNA lentivirus (BxPC3

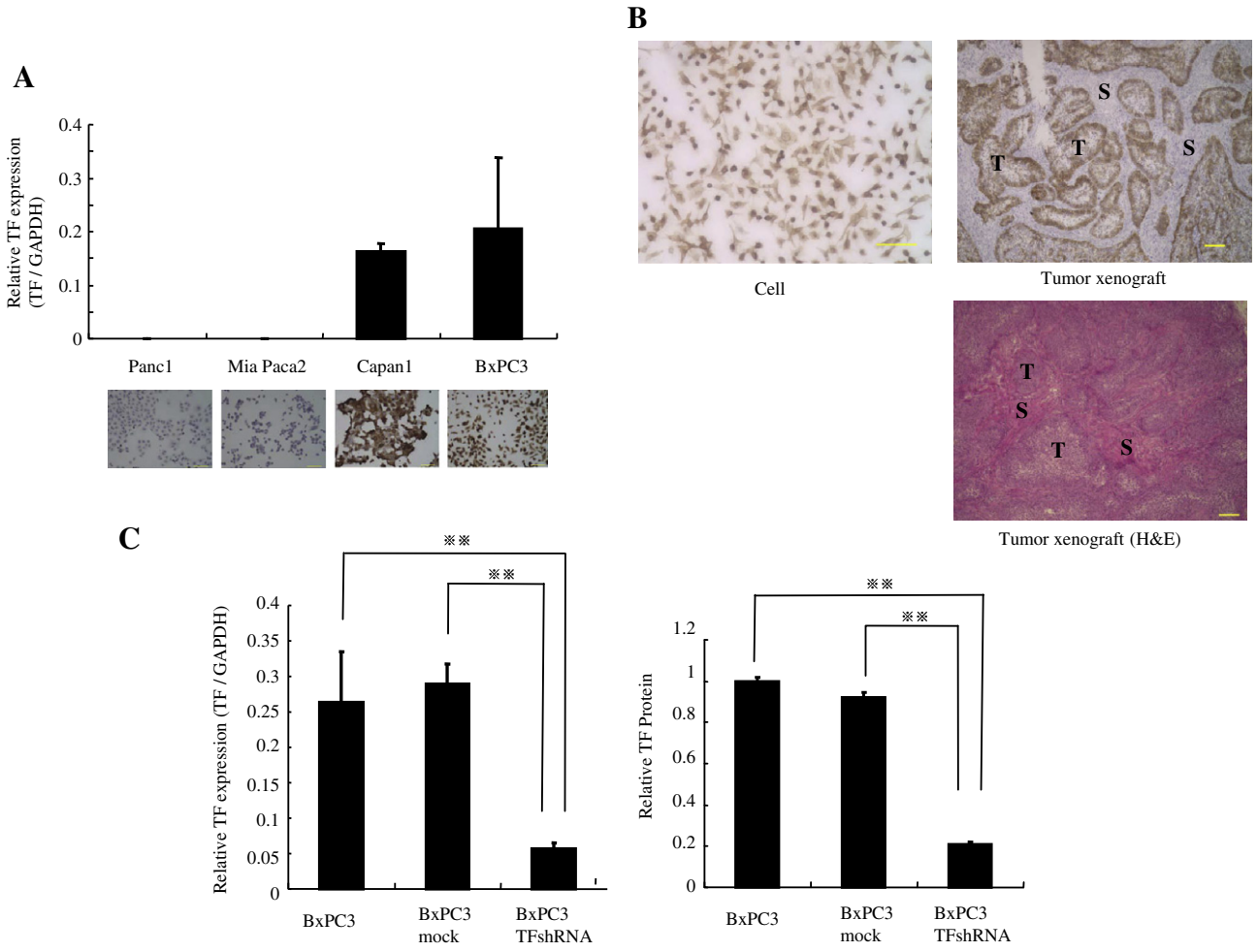

Fig. 1 – TF knockdown cells decrease the invasion ability. (A) Expression of TF amongst the four human pancreatic cancer cell lines *in vitro*. Total RNA was isolated and mRNA was quantitated by real-time PCR. Data are expressed as the mean ± SD. N = 3–4. The images show the staining of TF. Scale bar 100 μm. (B) Pattern of expression of TF in cultured cells and tumour xenografts. Scale bar 100 μm. T and S mean 'tumour' and 'stroma', respectively. (C) mRNA and protein level of TF in BxPC3, BxPC3 mock and BxPC3 TFshRNA. mRNA was quantitated by real-time PCR and TF protein was quantitated by TF ELISA. Data are expressed as the mean ± SD. N = 3–4. ** P < 0.01. (D) Invasion ability in BxPC3 mock and BxPC3 TFshRNA. BxPC3 mock and BxPC3 TFshRNA diluted in 500 μL of serum-free DMEM were added to the upper wells of a Transwell™ 24-well insert coated with Matrigel®. DMEM supplied with 10% FBS was added to the lower wells. Data are expressed as the mean ± SD. * P < 0.05. The images show the invasion cells. Scale bar 100 μm. (E) Representative fluorescence images of liver, mesentery, and stomach bearing BxPC3 mock GFP or BxPC3 TFshRNA GFP orthotopically. GFP signal denotes the presence of tumour cells. (F) A dissemination score was calculated to evaluate local tumour invasion and distant metastasis as previously described. Data are expressed as the mean ± SD. N = 4–6. ** P < 0.01.

mock) as a control. BxPC3 TFshRNA appeared to reduce TF expression by 80% at both the mRNA level and protein level (Fig. 1C). BxPC3 TFshRNA showed significant reduction in invasive ability compared with BxPC3 mock in DMEM medium containing 10% FBS (Fig. 1D). We next examined if TF promotes BxPC3 metastasis and invasion in the pancreatic orthotopic tumour xenografts. In the BxPC3 mock orthotopic tumour model, extensive invasion and metastasis was observed in the liver, mesentery and stomach (Fig. 1E). On the other hand, invasion and metastasis was suppressed in the BxPC3 TFshRNA orthotopic tumour model (Fig. 1E). The progression score reflecting tumour invasion and metastasis was significantly decreased in the BxPC3 TFshRNA tumour compared with the BxPC3 mock tumour (Fig 1F).

3.2. *TF-VIIa complex induces MMP-9 expression and promotes cancer cell invasion*

To determine a possible TF-mediated invasion factor in the BxPC3 cell line, we examined MMP-2, MMP-7 and MMP-9 expression levels by real-time PCR. Expression of MMP-2 and MMP-7 was very low both in BxPC3 mock and BxPC3 TFshRNA. In contrast, MMP-9 was highly expressed in BxPC3 mock and was significantly reduced in BxPC3 TFshRNA (Fig. 2A). We, therefore, clarified that TF induced MMP-9 production and promoted the invasion ability of pancreatic cancer cells under DMEM containing 10% FBS (Fig. 1). To avoid the effect of factor VIIa in FBS on MMP-9 expression and to determine whether or not the TF-VIIa complex induces MMP-9 followed by promoting

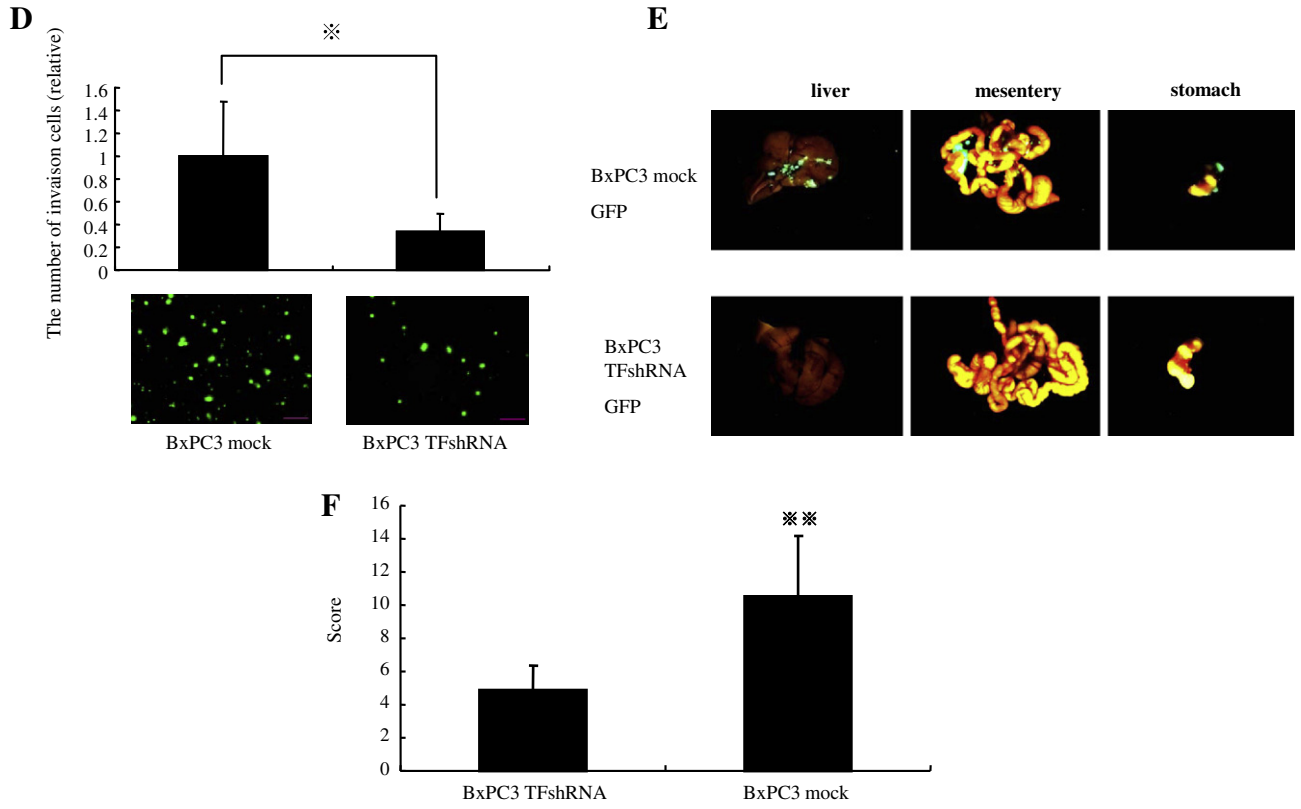

Fig 1. (*continued*)

BxPC3 cell invasion, BxPC3 was cultured under FBS-free DMEM (serum starvation). Gelatin zymography showed that BxPC3 mock with factor VIIa significantly increased MMP-9 production compared with BxPC3 mock without factor VIIa (Fig. 2B). However, MMP-9 was not detected in BxPC3 TFshRNA, with or without factor VIIa (Fig. 2B). Invasion assay showed that factor VIIa significantly enhanced the invasion ability of BxPC3 mock but not BxPC3 TFshRNA. This data strongly suggested that the TF-VIIa complex promotes MMP-9 production followed by enhancing cancer cell invasion (Fig. 2C).

3.3. *Establishment of neutralisation antibody that inhibits both the blood coagulation cascade and cell signalling of TF*

To establish an anti-human TF neutralisation antibody, we screened 14 hybridoma clones producing rat anti-human TF antibody. The supernatant of hybridoma clones 444 and 1849 significantly inhibited factor X activity (Supplemental Fig. A). Next, we purified the antibody from clones 1849, 72 and 130. The inhibition ability of these clones is strong, modest, and poor, respectively. We next observed whether or not these antibodies inhibit human blood clotting. Antibody 1849 strongly inhibited fibrin clotting in a concentration-dependent manner and prolonged the clotting time to the same level as spontaneous clotting without TF. Antibody 72 modestly inhibited fibrin clotting. In contrast, antibody 130 was unable to inhibit fibrin clotting (Supplemental Fig. B). The same results were obtained using mouse plasma (Supplemental Fig. C). These results suggested that rat anti-human TF antibody purified from hybridoma clone 1849 possessed the most potent TF neutralising effect in terms of blood coagulation cascade. We also determined whether or not antibody 1849 inhibits the TF-VIIa-mediated cell signalling pathway. To determine if 1849 inhibits MMP-9, we examined MMP-9 production by gelatin zymography in BxPC3 mock in the presence of factor VIIa. It was observed that factor VIIa promoted MMP-9 production in BxPC3 mock again (Fig. 2B, Supplemental Fig. D). Interestingly, TF neutralisation antibody 1849 suppressed TF-VIIa-mediated MMP-9 production partly, but the non-specific antibody was unable to suppress production (Supplemental Fig. D). This result showed that antibody 1849 could inhibit not only fibrin clotting but also TF-related cell signalling. Immunocytochemistry with 1849 showed TF in TF-positive cells (Capan1 and BxPC3), whereas there was no detectable TF in TF-negative cells (Panc1 and Mia Paca2) (Supplemental Fig. E). In addition, we confirmed that human TF interacts with mouse factor VIIa, and 1849 inhibits the interaction of human TF and mouse factor VIIa (Supplemental Fig. C), but 1849 does not react with mouse TF (data not shown).

3.4. *Inhibitory effect of anti-TF mAb (1849) on cancer cell invasion in vitro*

Since 1849 was able to block both TF-related cell signalling and the blood coagulation function (Supplemental Figures), we predicted that it would inhibit both the promotion of cancer invasion via TF-related cell signalling and haematogenous metastasis via TF-related blood coagulation function. First, we examined the invasion ability in BxPC3 mock in the presence of factor VIIa and/or TF neutralisation antibody

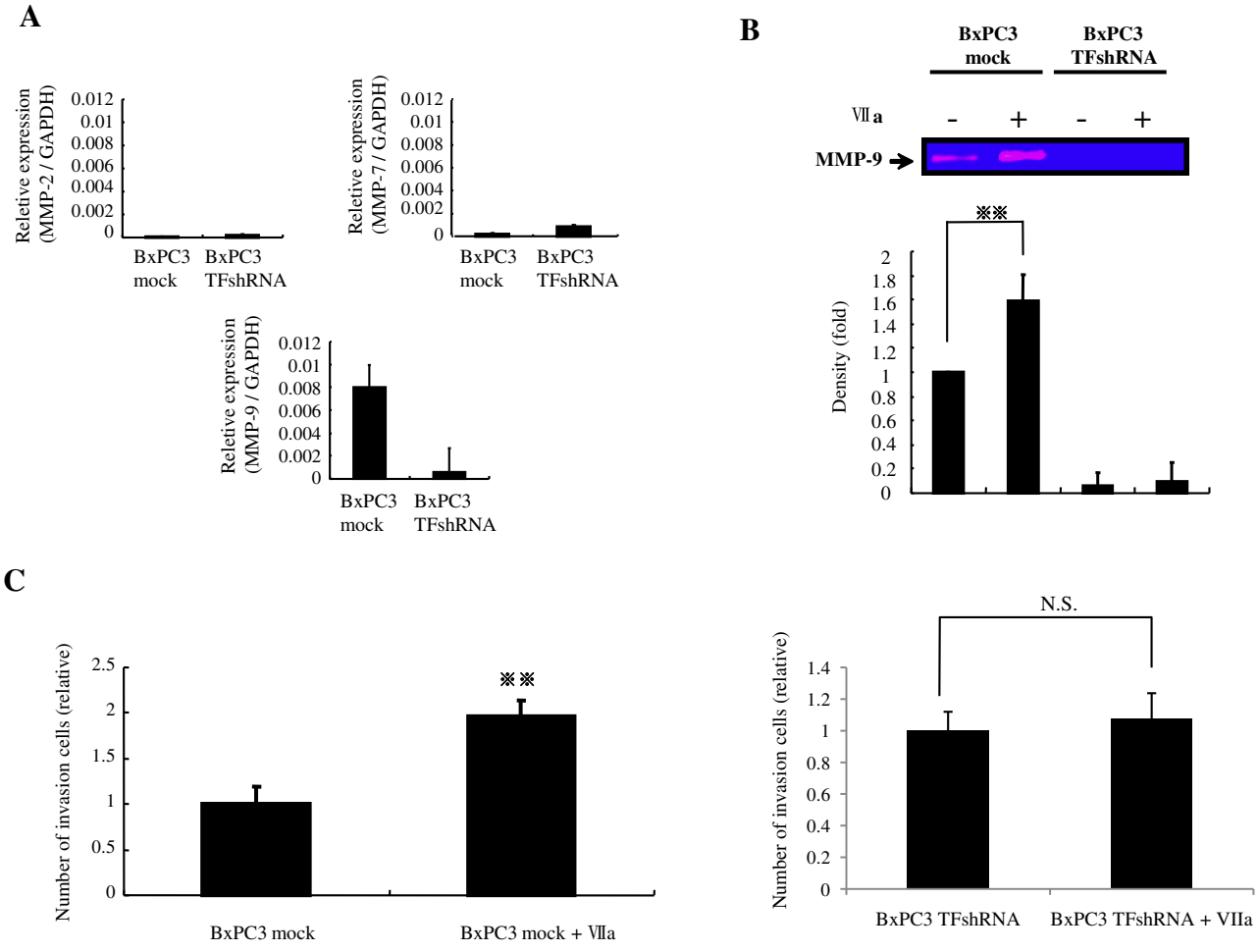

Fig. 2 – TF-VIIa complex promotes the expression of MMP-9 and invasion. (A) Expression of MMP-2, MMP-7 and MMP-9 by real-time PCR in BxPC3 mock and BxPC3 TFshRNA. Data are expressed as the mean ± SD. (B) Gelatin zymography of culture medium in BxPC3 mock and BxPC3 TFshRNA with or without factor VIIa (5 nM) after starvation for 2 h. MMP-9 was quantified using Image J software. Data are expressed as the mean ± SD. ** $P < 0.01$. (C) Invasion ability in BxPC3 mock or BxPC3 TFshRNA treated with factor VIIa (50 nM). Data are expressed as the mean ± SD. ** $P < 0.01$. N.S. means not significant.

1849. *In vitro* invasion study showed that the TF neutralisation antibody inhibited the invasion of BxPC3 mock in the presence of factor VIIa compared with the non-specific antibody (Fig. 3A).

3.5. *Effect of anti-TF mAb (1849) in mouse model*

In the portal-injection liver metastasis model, cancer cells stayed in the liver and luminescence continued to increase in a time-dependent manner in the control group. On the other hand, in the mice receiving 1849 treatment, luminescence began to decrease from 5 to 10 h after the injection of cancer cells (Fig. 3B). 1849 completely inhibited liver metastasis on Day 4, but the control did not (Fig. 3C). In the orthotopic pancreatic tumour xenograft model, remarkably strong invasion and metastasis were manifested in the liver, mesentery and stomach in the control group (Fig. 4A). In contrast, TF neutralisation antibody 1849 was able to suppress invasion (Fig. 4A). Furthermore, the progression score reflecting invasion and metastasis in the pancreatic orthotopic tumour xenograft was significantly lower in the 1849-treatment group compared with that in the control group (Fig. 4B). In addition, cancer spreading beyond the pancreas was observed in the control group. On the other hand, pancreatic cancer cells remained within the pancreas even after 6 weeks of injection of cancer cells in the 1849-treatment group (Fig. 4C). Kaplan–Meier analysis showed a significant improvement in survival rate in the 1849 treatment group compared to the control group (Fig. 4D). A significant difference in body change between the 1849-treatment mice and the control mice was not observed (Fig. 4E).

4. Discussion

Pancreatic cancer is the most refractory neoplasm and possesses several clinicopathological characteristics. First, pancreatic cancer exerts extensive invasion and metastasis to other organs.[14,15] Second, thrombosis occurs most frequently in patients with pancreatic cancer.[7] Third, expression of TF may contribute to the aggressiveness of pancreatic cancer that stimulates tumour invasiveness, and evaluation of the primary tumour for TF expression may identify patients with a poor prognosis.[17,18] We, therefore, hypothesised that TF

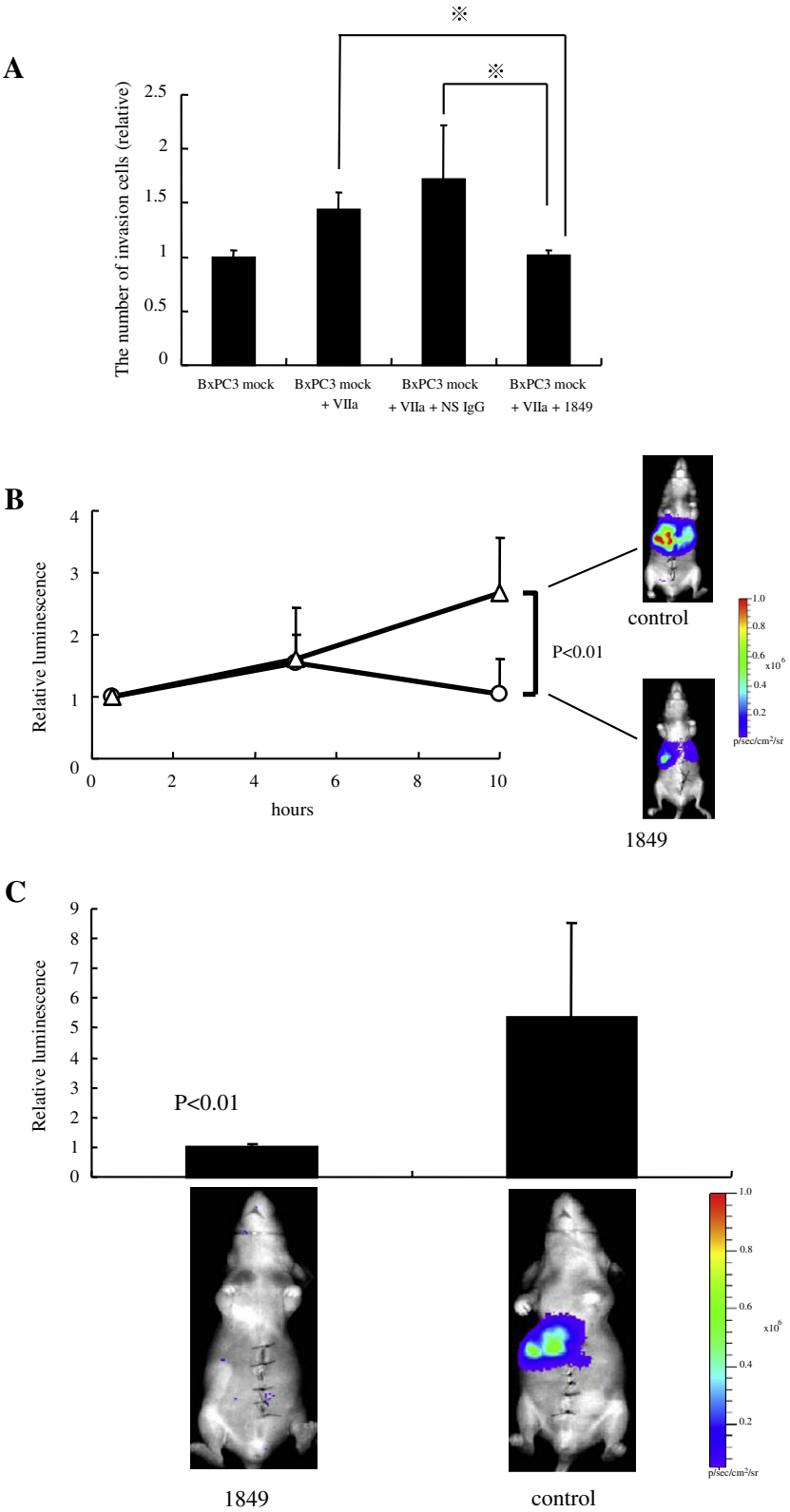

Fig. 3 – Anti-human neutralisation antibody 1849 inhibits cancer cell invasion and haematogenous metastasis. (A) Invasion ability in BxPC3 mock treated with factor VIIa (50 nM) clone 1849 (100 μg/ml), or non-specific antibody (anti-human CD20 antibody, 100 μg/ml). Data are expressed as the mean ± SD. * $P < 0.05$. (B) Inhibition of early-phase metastasis by 1849 in portal-injection liver metastasis model. Luminescence intensity was measured by IVIS imaging system 0, 5, and 10 h after injection of cancer cells. Data are expressed as the mean ± SD. $N = 6$. (C) Inhibition of late-phase metastasis by 1849. Luminescence intensity was measured by IVIS imaging system 4 days after injection of cancer cells. Data are expressed as the mean ± SD. $N = 5$.

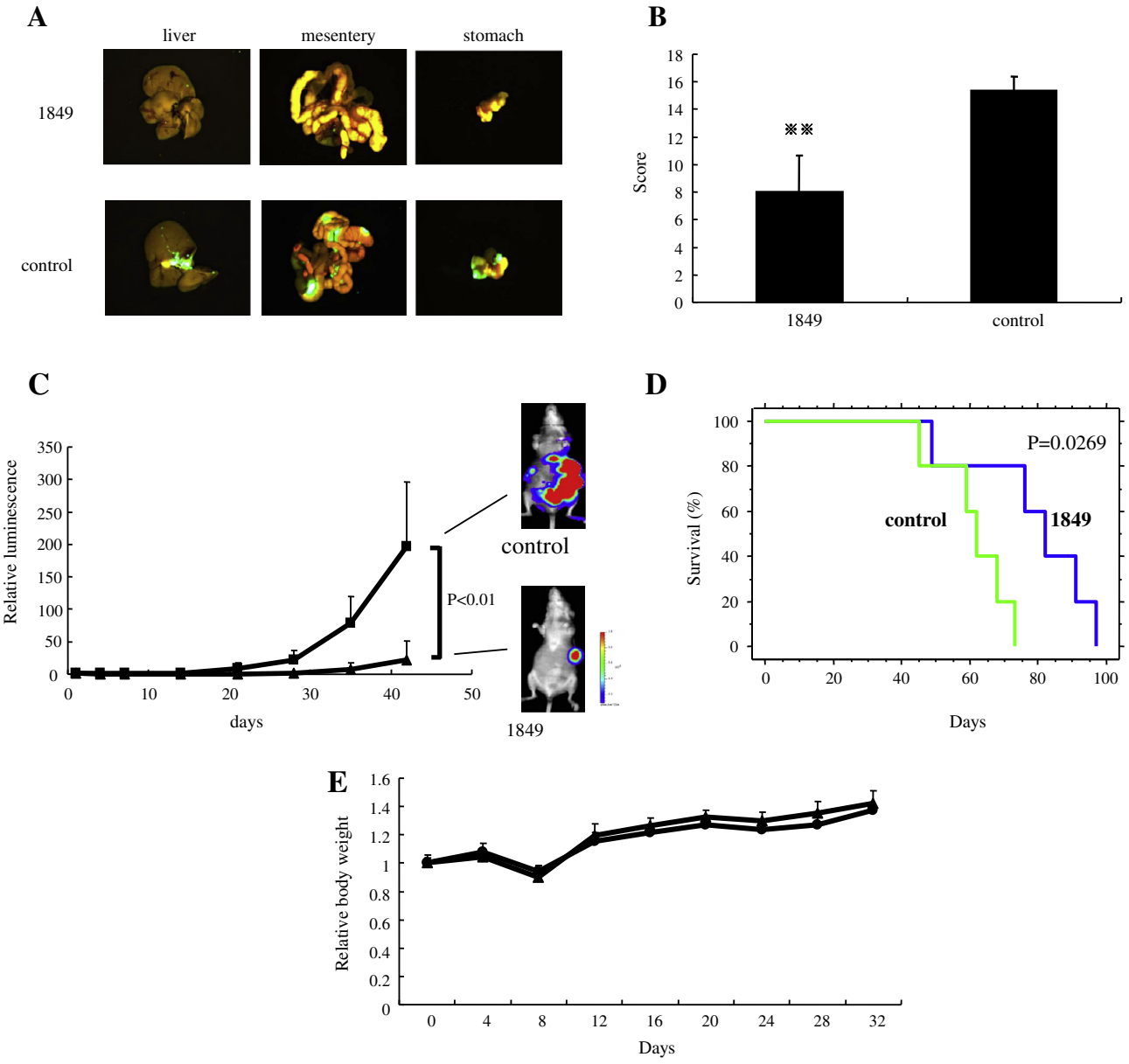

Fig. 4 – The effect of 1849 in the mouse model. (A) Representative fluorescence images of liver, mesentery and stomach bearing BxPC3 mock GFP treated with 1849 or PBS. GFP signal denotes the presence of tumour cells. (B) A dissemination score was calculated to evaluate local tumour invasion and distant metastasis as previously described. Data are expressed as the mean ± SD. N = 5–6. ** P < 0.01. (C) Inhibition of invasion and metastasis by 1849 in pancreatic orthotopic tumour xenografts. Relative luminescence was quantitated by IVIS imaging system. Representative luminescence intensity images were obtained in the individual control and the 1849-treated group. Data are expressed as the mean ± SD. N = 5. D. Effect of 1849 treatment on survival. Survival was assessed by Kaplan–Meier analysis. Blue line represents the 1849 treatment group. Green line indicates the control group. N = 5. E. Body weight change by 1849. Mice inoculated with BxPC3 cells subcutaneously on Day 0 were treated for seven consecutive days with 1849 mAb (400 μg/mouse, ●) as the TF neutralisation antibody, or PBS (▲) as the control. N = 3.

plays an important role in the invasion-metastasis cascade of pancreatic cancer.

In this study, the expression pattern of TF in BxPC3, TF-abundant cancer cells, was different between *in vitro* and *in vivo*. These results corresponded with a previous study on the human squamous cell carcinoma cell line A431.[21] Both A431 and BxPC3 are uniformly expressed *in vitro*. A431 xenografts were heterogeneously stained for TF; however, our study showed BxPC3 xenografts highly expressed in the area of cancer cells contacting with stromal cells, namely the invasion front. In fact, invasion assay showed that BxPC3 TFshRNA cell lines strongly inhibited cell invasion compared with BxPC3 mock cell lines. Real-time PCR analysis showed that the expression of MMP-9 in the BxPC3 TFshRNA cell line decreased compared with BxPC3 mock. To date, many reports have shown that MMP-9 promotes the invasion of pancreatic cancer.[3,22] In BxPC3, the invasion ability was suppressed through the inhibition of MMP-9 expression by SiRNA.[23]

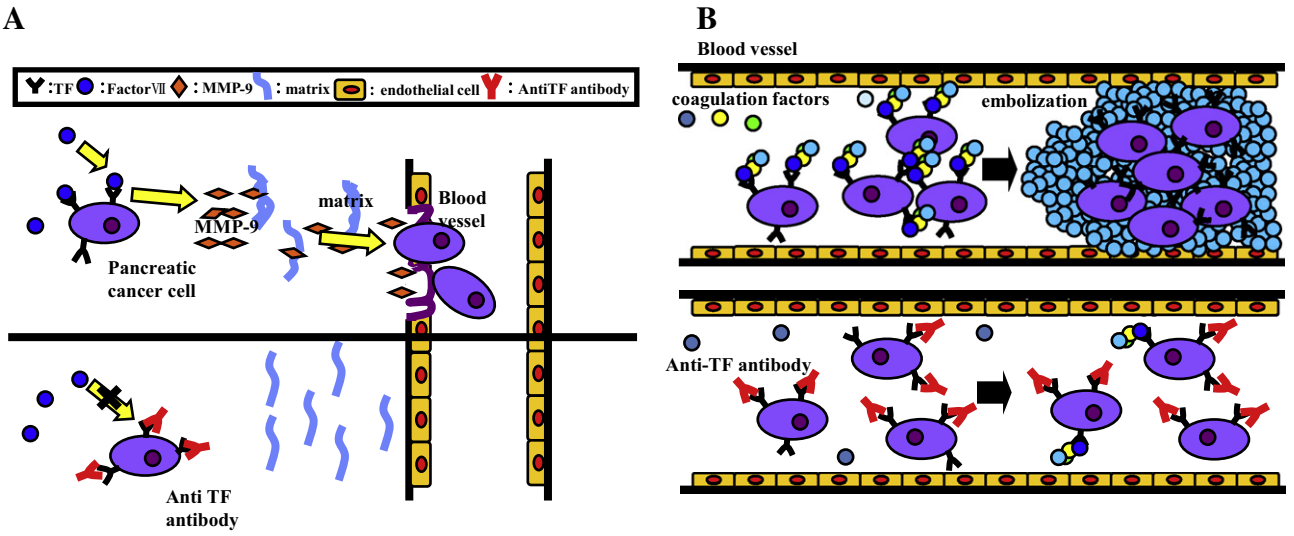

Fig. 5 – Possible role of TF-VIIa complex on invasion and metastasis. And the implication of anti-human TF neutralisation antibody, 1849, against the tumour metastasis. (A) TF-VIIa in pancreatic cancer induces MMP-9 expression and invades stromal tissue and basement membrane. 1849 inhibits invasion by inhibiting TF-related cell signalling. (B) TF-VIIa promotes embolisation in micro blood vessels. 1849 inhibits blood coagulation and traps cancer cells.

Taking our present data and other reports together, TF promotes the invasion ability of BxPC3 by expressing MMP-9.

Previous reports showed the relationship between coagulation factors and MMPs. In normal tissue, PAR-2, which is downstream from TF-VIIa complex signalling, mediates MMP-9 release in airway epithelial cells.[24] Thrombin mediates an increase in MMP-1 and MMP-3 in human endothelial cells.[25] In cancer tissue, PARs mediate MMP-2 and MMP-9 in prostate cancer.[26] Also, in colorectal adenocarcinoma cells, the TF-VIIa complex induces the expression of MMP-7.[27] However, in pancreatic cancer cells, the relationship between coagulation factor(s) and MMP(s) remains unclear. Our study is the first evidence that the TF-VIIa complex induces MMP-9 and promotes invasion in pancreatic cancer cells. Moreover, we also showed that TF promoted invasion *in vivo* using pancreatic orthotopic tumour xenografts.

Next, we newly established a TF neutralisation antibody for the treatment of pancreatic cancer. We found that the anti-TF antibody 1849 decreased the release of MMP-9. Therefore, we propose that 1849 inhibits TF-related cell signalling by blocking the TF-VIIa complex. Also, blood coagulation assay showed that 1849 inhibited TF-induced blood coagulation to the same level as the TF-free sample. We suggest from this result that 1849 can almost completely inhibit TF-related blood coagulation.

We found that 1849 inhibited pancreatic cancer invasion in the *in vitro* invasion assay. We suggest that this anti-invasion effect of 1849 is due to MMP-9 suppression via TF-related cell signalling. Also, 1849 completely blocked haematogenous metastasis in the portal-injection liver metastasis mouse model. This result suggested that the anti-haematogenous metastasis effect of 1849 is due to blocking the TF-related blood coagulation cascade.

Since the metastatic ability of BxPC3 TFshRNA decreased significantly as compared to that of BxPC3 mock *in vivo*, we tested if our newly developed anti- TF mAb suppressed the metastasis of BxPC3. We used the orthotopic pancreatic tumour xenograft model to examine whether or not 1849 could block invasion and metastasis in the mouse model, because the orthotopic model is similar to human pancreatic cancer in terms of progression, invasion, and metastasis.[19] 1849 strongly inhibited invasion and metastasis. Moreover, the survival rate in the 1849 treatment group was significantly improved compared with that of the control group.

The metastasis process includes many steps. Cancer cells invade the basement membrane and the pericytes of blood vessels. Cancer cells survive in the bloodstream and end up in the micro blood vessels of distant organs. MMP-9 plays an important role in invasion. Fibrin facilitates metastasis by enhancing the sustained adherence and survival of tumour cell emboli in the blood vessels of distant organs.[10] Also, a recent study showed that fibrin increases the metastatic potential of circulating tumour cells by impeding natural killer cells.[28] 1849 almost completely inhibited both blood coagulation and TF-VIIa complex mediated MMP-9 release.

In summary, we found that the TF-VIIa complex upregulated MMP-9 and promoted cancer invasion in pancreatic cancer. The cancer cells subsequently degraded the stromal extracellular matrix and basement membrane to intravasate into the blood vessels (Fig. 5A). Next, they formed microthrombi within the vessels (Fig. 5B). The double-blocking effect of our newly developed TF neutralisation antibody 1849 could be a useful tool in the treatment of pancreatic cancer invasion-metastasis.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.04.028.

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