Over-expression of cysteine proteinase inhibitor cystatin 6 promotes pancreatic cancer growth

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Pancreatic ductal adenocarcinoma (PDAC) shows the worst mortality among the common malignancies and development of novel therapies for PDAC through identification of good molecular targets is an urgent issue. Among dozens of over-expressing genes identified through our gene-expression profile analysis of PDAC cells, we here report CST6 (Cystatin 6 or E/M) as a candidate of molecular targets for PDAC treatment. Reverse transcriptase–polymerase chain reaction (RT-PCR) and immunohistochemical analysis confirmed over-expression of CST6 in PDAC cells, but no or limited expression of CST6 was observed in normal pancreas and other vital organs. Knock-down of endogenous CST6 expression by small interfering RNA attenuated PDAC cell growth, suggesting its essential role in maintaining viability of PDAC cells. Concordantly, constitutive expression of CST6 in CST6-null cells promoted their growth *in vitro* and *in vivo*. Furthermore, the addition of mature recombinant CST6 in culture medium also promoted cell proliferation in a dose-dependent manner, whereas recombinant CST6 lacking its proteinase-inhibitor domain and its non-glycosylated form did not. Over-expression of CST6 inhibited the intracellular activity of cathepsin B, which is one of the putative substrates of CST6 proteinase inhibitor and can intracellularly function as a pro-apoptotic factor. These findings imply that CST6 is likely to involve in the proliferation and survival of pancreatic cancer probably through its proteinase inhibitory activity, and it is a promising molecular target for development of new therapeutic strategies for PDAC. (*Cancer Sci* 2008; 99: 1626–1632)

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the western world and shows one of the worst mortality rates among the common malignancies, with a 5-year survival rate of only 5%.[1,2] Approximately 37 170 new cases were diagnosed to have a pancreatic cancer in 2007 and nearly 33 370 died of this disease in the United States.[3] Currently, the only curative treatment for PDAC is surgical resection, but the majority of PDAC patients are diagnosed at an advanced stage, and only 15–20% of the patients are candidates for surgical resection at the time of diagnosis. Of those who undergo potentially curative surgery, most patients eventually relapse and die of their disease.[2] Some approaches in the combination of surgery with chemotherapy, including 5-FU or gemcitabine, with or without radiation, can improve patients' quality of life[1,2] but those treatments have a very limited effect on long-term survival of PDAC patients owing to its extremely aggressive and chemo-resistant nature.

To overcome this difficult situation, development of novel molecular therapies for PDAC through identification of molecular targets is an urgent issue now. Previously, we generated gene-expression profiles of PDAC cells using genome-wide cDNA microarrays in combination with laser microdissection to enrich populations of cancer cells for the analysis.[4] Among dozens of genes being over-expressed in PDAC cells, we here investigated cystatin E/M (CST6) as a novel molecular target for this disease. Cystatins are endogenous inhibitors of mammalian lysosomal cysteine proteinases, such as cathepsins B, L, H and S, and the plant cysteine proteinases papain, acinidin and ficin. They function both intracellularly and extracellularly[5–7] and they control the catalytic function of target proteases by forming reversible high-affinity complexes. Among three distinct subfamilies belonging to the cystatin super families, the family 1 cystatins, represented by cystatin A (stefin A) and cystatin B (stefin B), lack disulfite bonds as well as signal peptides and function only intracellularly. Family 2 cystatins, C, D, S, SA, SN, E/M, and F, are secreted proteins of 115–120 amino acids with two interchain disulfide bonds. Family 3 cystatins, L- and H-kininogens, are complex glycosylated cytoplasmic proteins with type-2-like cystatin domains and bradykinin moiety.[5–7] Cystatin E/M (CST6), belonging to family 2, is mainly secreted as a N-glycosylated (17 kDa) and an unglycosylated (14 kDa) form.[8] This cystatin member was identified independently by two research groups;[8,9] one group identified it to be down-regulated in breast cancer by comparing the differential transcripts between primary and metastatic breast cancers and it has been considered to suppress metastasis or invasion of breast cancer by modulating proteolysis of cell matrix or other mechanism.[10,11] However, its detailed function still remains controversial.

In this study, we demonstrate that CST6 was over-expressed specifically in PDAC cells and its over-expression promoted PDAC cell growth. This growth-promoting effect is likely to be dependent on its proteinase-inhibitory domain and intracellular pro-apoptotic cathepsin B can be one of the putative substrates of CST6 in PDAC cells. Our finding of the CST6 role in PDAC cells could shed light on novel understanding of development and progression of pancreatic carcinogenesis and also implicate CST6 to be one of the promising molecular targets for PDAC treatment.

Materials and Methods

Cell lines. PDAC cell lines KLM-1, PK59 and PK-1 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). All cell lines were grown in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO_2. FreeStyle 293F Cell line was purchased from Invitrogen (Carlsbad, CA). This cell line was grown in FreeStyle 293 Expression Medium (Invitrogen) and maintained in a 37°C

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incubator containing a humidified atmosphere of 8% CO_2 in air on an orbital shaker platform rotating at 125 r.p.m.

Semi-quantitative reverse transciptase–polymerase chain reaction (RT-PCR). Purification of PDAC cells and normal ductal epithelial cells from pancreatic cancer tissues was described previously.[4] RNA from the purified PDAC cells and normal pancreatic ductal epithelial cells were subjected to two rounds of RNA amplification using T7-based *in vitro* transcription (Epicentre Technologies, Madison, WI) and synthesized to single-strand cDNA. Total RNA from human pancreatic cancer cell lines was extracted using Trizol reagent (Invitrogen) according to the manufacturer's recommended procedures. Extracted RNA was treated with DNase I (Roche Diagnostic, Basel, Switzerland) and reversely transcribed to single-stranded cDNA using oligo (dT) primer with Superscript II reverse transcriptase (Invitrogen). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification by monitoring *α-tubulin (TUBA)* as a quantitative control. The primer sequences we used were 5′-AAGGATTATGAGGAGGTTGGTGT-3′ and 5′-CTTGGGTCTGTAACAAAGCATTC-3′ for *TUBA*, 5′-GGCAGCAACAGCATCTACTACTT-3′ and 5′-ACAGTTGTGCTTTAGGAGCTGAG-3′ for *CST6*. All reactions involved initial denaturation at 94°C for 2 min followed by 23 cycles (for *TUBA*) or 28 cycles (for *CST6*) at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

Northern blotting analysis. Human multiple-tissue blots (Clontech, Palo Alto, CA) were hybridized for 16 h with ^{32}P-labeled *CST6* cDNA (Mega Label kit, GE Healthcare, Piscataway, NJ). Probe cDNA of *CST6* was prepared as a 255-bp PCR product using primers 5′-GGCAGCAACAGCATCTACTACTT-3′ and 5′-ACAGTTGTGCTTTAGGAGCTGAG-3′. Pre-hybridization, hybridization and washing were performed according to the manufacturer's instruction. The blots were autoradiographed at –80°C for 10 days.

Generation of antibodies specific to CST6 protein. A cDNA fragment encoding the human CST6 protein lacking its signal peptide was amplified by PCR using primers 5′-CGCGGATCCGCCGCAGGAGCGCATGGTCGG-3′ and 5′-CCGGAATTCTCACATCTGCACACAGTTGTG-3′, which contained *BamHI* and *EcoRI* restriction sites indicated by the underlines, respectively. The product was cloned into pET28b vector (Novagen, Madison, WI) to produce a fusion protein bearing an N-terminal 6-His tag, which was purified with TARON Superfluity Metal Affinity Resin (BD Biosciences, Franklin Lakes, NJ) under native conditions according to the supplier's protocol. This recombinant CST6–6His was used to immunize rabbits; the polyclonal antibody was affinity-purified using Affi-gel 10 (Bio-Rad Laboratories, Hercules, CA) conjugated with the 6-histidine fused CST6 protein.

Immunohistochemical staining. Conventional tissue sections from PDAC were obtained from surgical specimens that were resected in Osaka Medical Center for Cancer and Cardiovascular Diseases with the appropriate informed consent and Institutional Review Board (IRB) approval. Tissue microarray samples, where 31 PDAC tissues and two endocrine-tumor tissues were spotted in duplicate, were purchased from ISU ABXIS (Seoul, Korea). The sections were deparaffinized and autoclaved at 108°C in citrate buffer, pH 6.0 for 15 min. Endogenous peroxidase activity was quenched by incubation in Peroxidase Blocking Reagent (Dako Cytomation, Carpinteria, CA) for 30 min. After incubation with FBS for blocking, the sections were further incubated with rabbit anti-CST6 polyclonal antibody (dilution 1:5000) at room temperature for 1 h. After washing with phosphate-buffered saline (PBS), immunodetection was performed with peroxidase labeled antirabbit immunoglobulin (Envision kit, Dako Cytomation). Finally, the reactants were developed with 3, 3′-diaminobenzidine (Dako Cytomation). Counterstaining was performed using hematoxylin.

Small interfering RNA (siRNA)-expressing constructs specific to CST6. To down-regulate endogenous *CST6* expression in PDAC cells, we used psiU6BX3.0 vector for expression of short hairpin RNA against a target gene as described previously.[12] The U6 promoter was cloned upstream of the gene-specific sequence (19-nt sequence from the target transcript, separated from the reverse complement of the same sequence by a short spacer, TTCAAGAGA), with five thymidines as a termination signal and a neo cassette for selection by Geneticin (Invitrogen). The target sequences for *CST6* were 5′-GTGGTTCCCTGGCAGAACT-3′ (*#448*), and 5′-GAAGCAGCACGACTTCTTC-3′ (*#EGFP*) as a negative control. The mismatch sequences from *#448* were 5′-TTGTTTCCCTAGCAGAACT-3′(*#448a*), 5′-ATGTTTCCCTTGCAGAACT-3′ (*#448b*), and 5′-TTGATTCCCTTGCAGAACT-3′ (*#448c*). The human PDAC cell lines, PK-1 and PK-59 cells, were plated on 10-cm dishes and transfected with *#448, #448a-c*, and *#EGFP* siRNA-expression vectors using FuGENE6 (Roche) according to the manufacturer's instruction. Cells were selected by 0.15 mg/mL (for PK-59) or 0.2 mg/mL (for PK-1) Geneticin (Invitrogen). Cells were harvested 7 days after transfection to analyze knock-down effect on CST6 by Western blotting using the above rabbit anti-CST6 polyclonal antibody (dilution 1:1000). After incubation in appropriate medium containing Geneticin for 9 days, the cells were fixed with 100% methanol and stained with 0.1% of crystal violet-H_20 for colony formation assay. In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell viability was measured using Cell-counting kit-8 (DOJINDO, Kumamoto, Japan) at 11 days after transfection. Absorbance was measured at 490 nm and at 630 nm as a reference with a Microplate Reader 550 (Bio-Rad).

Generation of CST6-overexpressing cells and growth assay. The cDNA encoding an open reading frame of *CST6* was amplified by PCR using the primer pair with restriction enzyme sites: 5′-GGGGTACCGAATGGCGCGTTCG AACCTCC-3′ and 5′-CCGGAATTCCATCTGCACACAGTTGTGCT-3′ (*KpnI* and *EcoRI* sites shown by underlines, respectively). The amplified cDNA product was cloned into pcDNA3.1/myc-His A(+) vector (Invitrogen). The plasmids were transfected into the CST6-null PDAC cell line, KLM-1, using FuGENE6 (Roche) according to the manufacturer's recommendation. Transfected cells were selected with 0.5 mg/mL Geneticin (Invitrogen) and clonal KLM-1-derivative cells were subcloned by limiting dilution. Myc-tagged CST6 expression in these clonal cells was examined by Western blotting using anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and antiβ-actin antibody (Sigma), and three of them that expressed CST6 constitutively were used for further experiments (KLM1–CST6). Control KLM-1 cells transfected with pcDNA3.1/myc-His A(+) vector were also established (KLM1–Mock). The growth rates of these established clones were monitored by Cell-counting kit-8 (DOJINDO). 2×10^6 cells of the CST6-overexpressing KLM1-1 clone (CST6-1) and KLM1–Mock clone (Mock-1) were inoculated in the flank of 8-week-old female nude mice, and the long (L) and short diameters (S) of the tumors were measured weekly. The tumor volumes were calculated as $L \times L \times S \times 0.52$ mm^3.

Generation of recombinant CST6 and CST6 autocrine/paracrine assay. We generated three recombinant forms of human CST6: wild-type (wt); a mutant form lacking the protease-inhibitory site (MVG at codon 34–36, Δ1); and a mutant form with replacement of the glycosylated asparagine at codon 137 by asparatic acid (N137D). To create the wild-type form, the entire coding sequence of *CST6* cDNA was amplified by RT-PCR with primers 5′-GGAATTCGATGGCGCGTTCGAACC TCCCGCT-3′ (forward) and 5′-TAAAGCGGCCGCAACATCTGCACACAGTTG TGCT-3′ (reverse) and the product was inserted into the *EcoRI* and *NotI* sites of pCAGGS for vector to express a FLAG-tagged protein. Site-directed mutagenesis was performed to generate mutant CST6-expressing vectors using the QuickChange XL

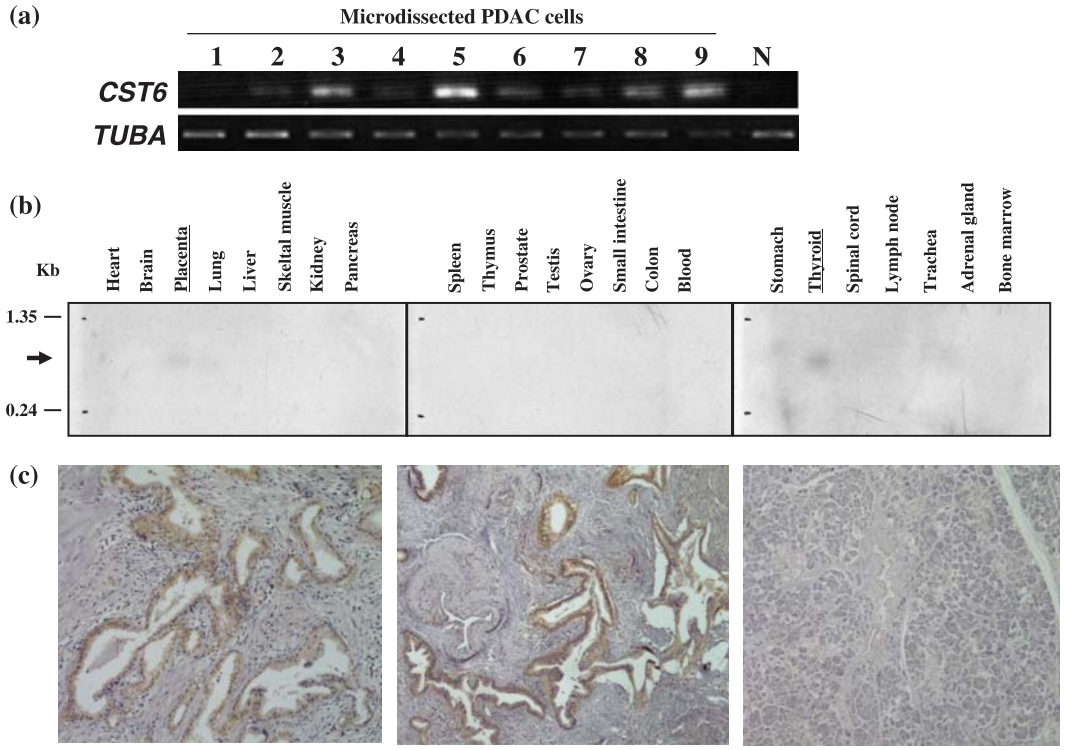

Fig. 1. Cystatin 6 over-expression in pancreatic ductal adenocarcinoma (PDAC) cells. (a) Reverse transcriptase–polymerase chain reaction (RT-PCR) for mRNA expressions of *CST6* and *TUBA* in the microdissected PDAC cells[1–9] compared with normal pancreatic ductal epithelial cells (N), which were also microdissected. (b) Northern blot analysis revealed that *CST6* was expressed restrictively at the placenta and thyroid tissues. (c) In immunohistochemical study using anti-CST6 antibody, intense staining was observed in PDAC cells (left and middle panels, original magnification ×200). Strong positive staining of CST6 was observed at the cytoplasma of PDAC cells. In normal pancreatic tissue, acinar cells and normal ductal epithelium cells showed no staining (right panel, original magnification ×200).

Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's procedure. Complementary primers for the mutants were as follows: for Δ1, 5′-CGGGCCCGGCCGCAGG-AGCGCGAACTCCGGGACCTGTCGCCC-3′ and 5′-GGGCGA-CAGGTCCCGGAGTTCGCGCTCCTGCGGCCGGGCCCG-3′ and for N137D, 5′-GTCCTTGTGGTTCCCTGGCAGGACTCCT-CTCAGCTC CTAAAGC-3′ and 5′-GCTTTAGGAGCTGAGAGG-AGTCCTGCCAGGGAACC ACAAGGAC-3′. These plasmids were transfected into FreeStyle 293-F using FuGENE6 (Roche). After incubation for 48 h, the suspension was centrifuged and the supernatant was purified with anti-FLAG M2 affinity gel (SIGMA). After washing, the recombinant protein was eluted by 0.1 M glycine HCl (pH 2.5) containing 10% ethylene glycol. Fractions were mixed with 1 M tris-HCl (pH 8.8) immediately, and dialyzed in PBS. Recombinant CST6-wt, Δ1, and N137D were added to culture medium of KLM-1 cells at several concentrations (wt: 0, 2, 10, and 50 ng/mL, Δ1 and N137D: 50 ng/mL), supplied with 2% FBS. The growth rates of the cells were measured by Cell-counting kit-8 (DOJINDO).

Cathepsin B activity assay and cathepsin B inhibitors. Intracellular cathepsin B activities of KLM1–CST6 clones (CST6-1, -2, and -3) and KLM-1-mock clones (Mock-1, -2, and -3) were measured by the use of InnoZyme Cathepsin B Activity Assay Kit, Fluorogenic (CALBIOCHEM, San Diego, CA) according to the manufacturer's recommendations. This kit utilizes the ability of cathepsin B to digest the synthetic substrate Z-Arg-Arg AMC (7-amino-4-methylcoumarin). The amount of free AMC is measured fluorometrically at excitation wavelength 360–380 nm and emission wavelength 440–460 nm. The activity of cathepsin B can be quantified using an AMC standard. We used the 50 μg cell lysate of each clone. We treated KLM-1 cells with the specific inhibitors to cathepsin B, CA-074, and its membrane-permeable analog, CA-074Me (Peptides International, Inc., Louisville, KY). Cell viability was evaluated by MTT assay using Cell-counting kit-8 after 4 days exposure to each of the cathepsin B inhibitors.

Results

Over-expression of CST6 in PDAC cells. Among dozens of genes that were over-expressed in PDAC cells in our genome-wide cDNA microarray analysis[4] we focused on *CST6* for this study owing to reasons described below. RT-PCR analysis confirmed *CST6* over-expression in eight of the nine PDAC cells (Fig. 1a). Northern-blot analysis using a *CST6* cDNA fragment as a probe identified a 0.6-kb transcript to be expressed only in the thyroid and also faintly in the placenta; CST6 expression was not detectable in any vital organs including lung, heart, liver and kidney (Fig. 1b). Using a polyclonal antibody specific to the CST6 protein we generated, we performed immunohistochemical analysis on PDAC tissue sections and found its strong staining in PDAC cells (Fig. 1c), but no staining in the normal pancreas (Fig. 1c). Furthermore, we performed immunohistochemical analysis on the tissue microarrays for PDAC and detected its positive staining in 22 (44%) of 50 PDAC tissues examined.

Effect of *CST6*-siRNAs on PDAC cell growth. To further investigate the function of CST6 in PDAC cells, we constructed several siRNA-expression vectors specific to *CST6* and transfected each of them into PDAC cell line, PK-59, which endogenously expressed high levels of CST6. RT-PCR experiments indicated a significant knock-down effect of endogeneous *CST6* when we transfected the *#448* siRNA-expression construct (Fig. 2a).

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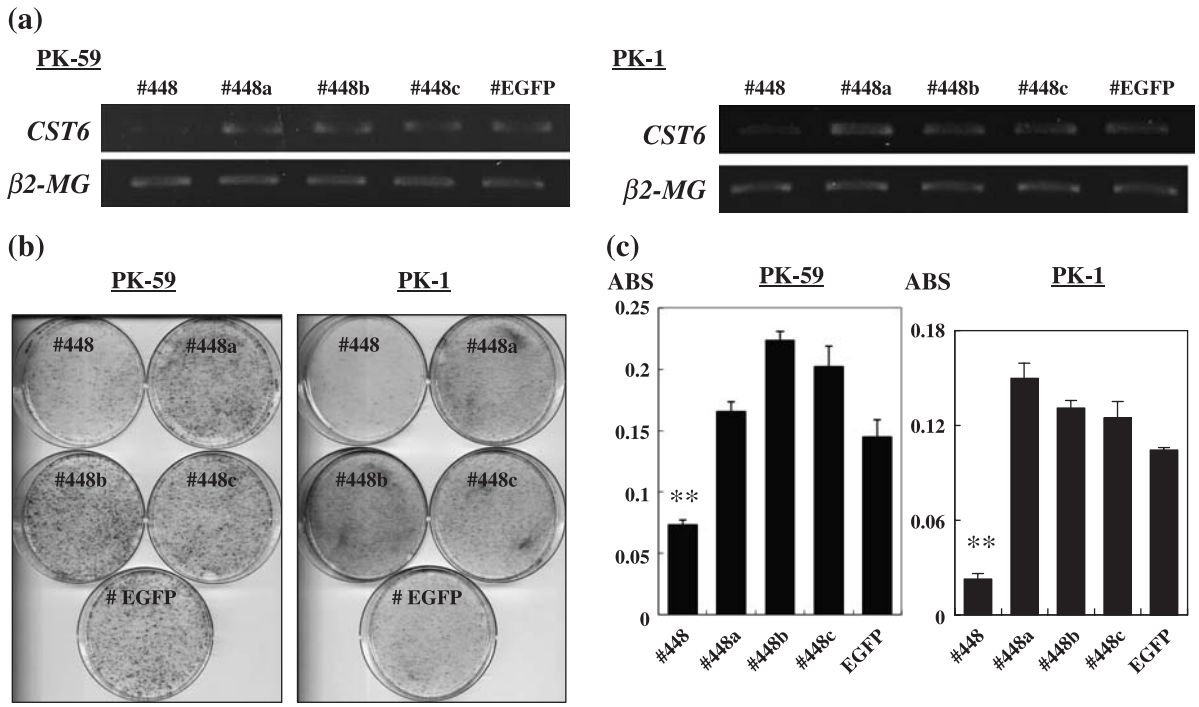

Fig. 2. Effect of CST6–small interfering (si)RNA on the growth of pancreatic ductal adenocarcinoma (PDAC) cells. (a) Knock-down effect of siRNA on *CST6* in PDAC cell lines, PK-1 and PK-59. Semi-quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using cells transfected with each of siRNA-expressing vectors to *CST6* (*#448*) and its mismatch siRNA-expression vectors (*#448a*, *b*, *c*) as well as a negative control vectors (*#EGFP*), which confirmed the knock-down effect by *#448*, but not by the mismatch *#448a-c* vectors. *β2-MG* was used to quantify RNAs. (b) Colony formation assay of PK-1 and PK-59 cells transfected with each of indicated siRNA-expressing vectors to *CST6* (*#448*, *#448*a-c) and a negative control vector (*#EGFP*). Cells were visualized with 0.1% crystal violet staining after 14-day incubation with Geneticin. (c) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of each of PK-1 and PK-59 cells transfected with indicated siRNA-expressing vectors to CST6 (*#448*, *#448*a-c) and a negative control vector (*#EGFP*). Each average is plotted with error bars indicating SD (standard deviation) after 14-day incubation with Geneticin. ABS on *Y*-axis means absorbance at 490 nm, and at 630 nm as reference, measured with a microplate reader. These experiments were carried out in triplicate. **Means *P*-value of <0.01 (Students' *t*-test).

Colony-formation assays (Fig. 2b) and MTT assays (Fig. 2c) using *#448* revealed a drastic reduction in the number of viable cells compared with a negative control *#EGFP* for which no knock-down effect was observed. Subsequently, we evaluated the specificity of *#448* siRNA using several *#448* variant constructs containing three-base mismatch sequences to *CST6* and transfected each of them into PDAC cells. The mismatched siRNA-expression vectors (*#448a*, *b*, and *c*) showed no knock-down effect on *CST6* expression and did not affect the cell viability (Fig. 2b,c). When we used another CST6-positve PDAC cell line, PK-1, we observed similar effects (Fig. 2). On the other hand, the siRNA *#448* did not affect the cell viability of another PDAC cell line KLM-1 that did not express *CST6*, which excluded a possibility of the 'off-targeting' effect (data not shown).

Over-expressed CST6 promoted PDAC cell growth. To examine the oncogenic effect of CST6, we established cell lines that expressed wild-type CST6 constitutively using KLM-1 cells in which CST6 expression was hardly detectable by RT-PCR and Western blot analysis. As shown in Fig. 3(a), Western blot analysis using anti-CST6 antibody confirmed high level of CST6 expression in three KLM-1-derivative clones (CST6-1, -2 and -3), but no expression in three KLM1–Mock clones (Mock-1, -2 and -3). In addition, we confirmed CST6 protein in the culture medium from each of these KLM1–CST6 clones (data not shown). MTT assays demonstrated that these three KLM1–CST6 clones more rapidly grew *in vitro* than KLM1–Mock clones (Fig. 3b), suggesting that over-expression of CST6 in PDAC cells promoted cell proliferation. Furthermore, we evaluated the effect on *in vivo* growth by inoculating KLM1–CST6 clone (CST6-1) or KLM1–Mock clone (Mock-1) into the flank of nude mice and found that KLM1–CST6 cells grew more rapidly than KLM1–Mock cells *in vivo* (Student's *t*-test, $P < 0.05$) as shown in Fig. 3(c).

Secreted CST6 promoted cell growth, dependently in its proteinase inhibitor domain. Although CST6 is a secretory protein, it is likely to function both intracellularly and extracellularly.[5–7] To examine biological effects of CST6 on cell growth, we generated recombinant CST6 protein (wild-type: wt) using the mammalian FreeStyle 293F system (Fig. 4a) and performed a cell growth assay in the presence of recombinant CST6 protein at several concentrations (2–50 ng/mL). Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 4a) identified two bands for recombinant wild-type CST6 protein (CST6-wt), the higher molecular weight of which was reported to be an N-glycosylated form.[8,9] In the presence of wild-type CST6 protein in culture medium, cell proliferation was significantly enhanced in a dose-dependent manner, implicating that secreted CST6 could function as an oncoprotein in an autocrine/paracrine manner (Fig. 4b). Furthermore, to investigate the function of CST6 as a protease inhibitor as reported previously,[9] we generated two recombinant mutant CST6 proteins: a mutant form lacking the protease-inhibitory site (Δ1); and a mutant form in which an N-glycosylation site was replaced (N137D) (Fig. 4a). PDAC cells were treated with 50 ng/mL each of the wild-type or mutant CST6 proteins (wt, Δ1, and N137D) in culture medium. Neither of the mutant CST6 proteins revealed the growth-promoting effect, whereas the wild-type CST6 clearly promoted

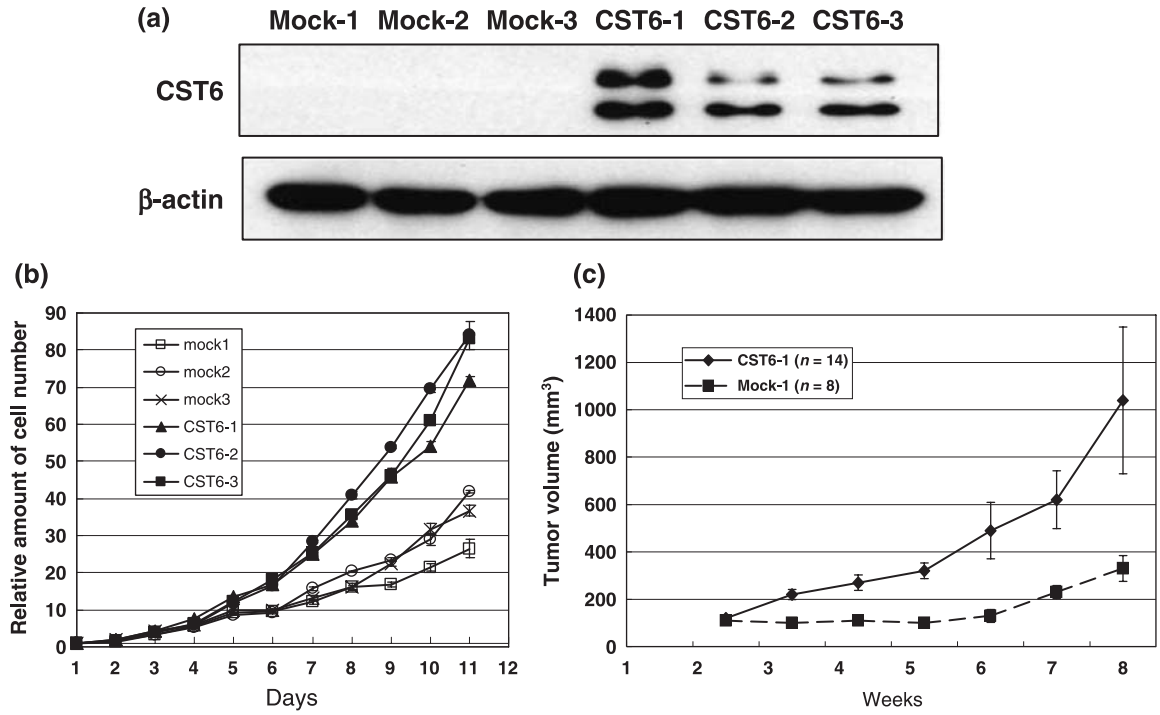

Fig. 3. Over-expression of CST6 promoted cell proliferation. (a) Western blot analysis of KLM-1 cells expressing exogenous CST6 or those transfected with mock vector. Exogenous introduction of CST6 expression was validated with anti-Myc-monoclonal antibody. β-Actin served as a loading control. (b) *In vitro* growth rate of KLM-1 clones expressing high level of exogenous CST6 (CST6-1, -2 and -3) and those transfected with mock vector (Mock-1, -2, and -3). X- and Y-axes represent the day point after seeding and the relative amount of the cell number that was calculated in absorbance of the diameter by comparison with the absorbance value of day 1 as a control. Each average is plotted with error bars representing standard deviation (SD). These experiences were in triplicate altogether. (c) *In-vivo* growth rate of CST6-1 clone expressing exogenous CST6 and those transfected with mock vector (Mock-1). X- and *Y*-axis represent the week point after inoculation and tumor volume (mm^3). Each average is plotted with error bars representing SD.

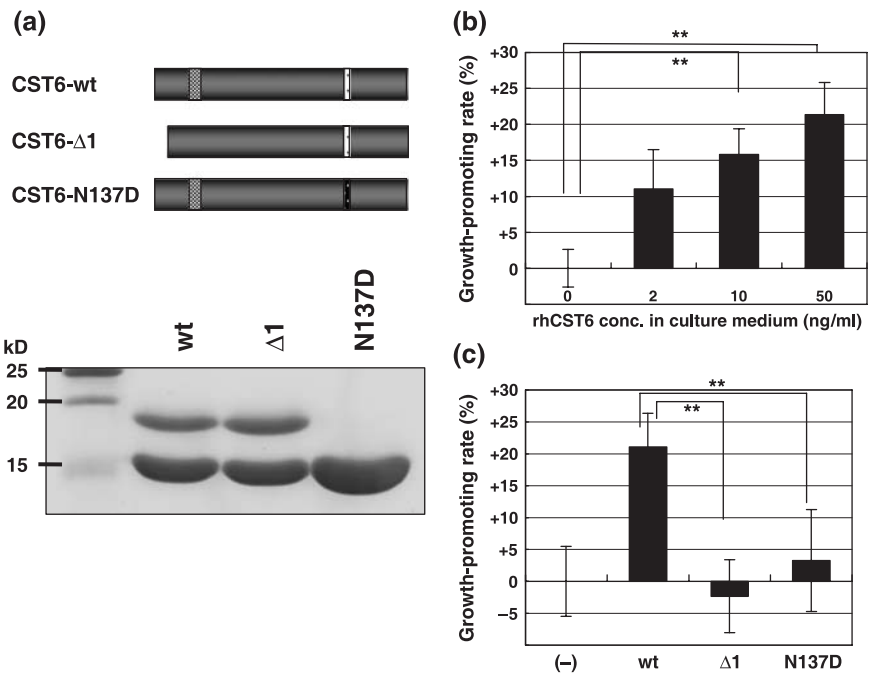

Fig. 4. Recombinant CST6 protein stimulated cancer cell growth and mutant proteins lacked the growth-promoting activity. (a) The wild-type (CST6-wt) and two mutant forms of recombinant CST6 proteins were generated in 293F mammalian cells. CST6-△1 was lacking the protease inhibitory site (MVG at codon 34–36) and CST6-N137D had the N-glycosylated asparagine residue (codon 137) replaced by aspartic acid. Recombinant proteins were purified and analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). (b) KLM-1 cells were incubated with recombinant CST6-wt at serial concentration (0, 2, 10, and 50 ng/mL), supplied with 2% fetal bovine serum (FBS), and the growth-promoting effect by rhCST6 was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (c) KLM-1 cells were incubated with 50 ng/mL recombinant CST6-wt, CST6-△1, or CST6-N137D supplied with 2% FBS. *Y*-axis represents relative growth-promoting rate at day 6, which was calculated by comparison with the absorbance value of 0 ng/mL CST6 as a control. Each average is plotted with error bars representing SD. These experiences were in triplicate altogether. **Means *P*-value < 0.01 (Students' *t*-test).

the cancer cell growth at the same dose of mutant ones (Fig. 4c). This result indicated that the protease inhibitory activity and the N-glycosylation at the 137th asparagine residue were critical for its growth-promoting effect.

Over-expressed CST6 reduced intracellular cathepsin B activity. CST6 is categorized as an inhibitor of cysteine proteinase and can inhibit enzymatic activity of cathepsin B and other proteinases.[9] Intracellular cathepsin B can act as the main

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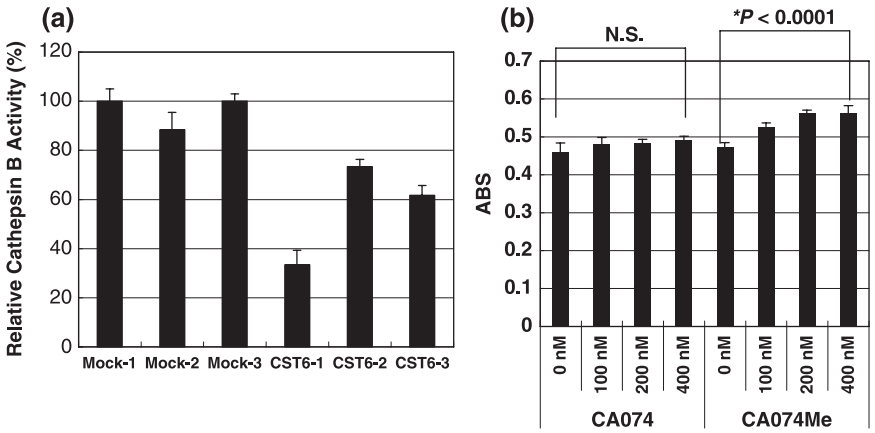

Fig. 5. Over-expression of CST6 reduced cathepsin B activity and intracellular cathepsin B was involved with pancreatic ductal adenocarcinoma (PDAC) growth. (a) Activity of intracellular cathepsin B was measured in KLM-1 clones expressing high level of exogenous CST6 (CST6-1, -2 and -3) and those transfected with mock vector (Mock-1, -2, and -3). *Y*-axis represents the relative activity of cathepsin B that was calculated in fluorescence intensity by comparison with Mock-1 clone as a control. (b) KLM-1 cells were treated with the specific inhibitors to cathepsin B (CA-074 and CA-074 Me), and cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 4 days exposure of each of cathepsin B inhibitors. ABS on *Y*-axis means absorbance at 490 nm, and at 630 nm as reference measured with a microplate reader. These experiments were carried out in triplicate. Membrane-permeable cathepsin B inhibitor CA-074Me enhanced PDAC cell growth significantly ($P < 0.0001$, Students' *t*-test), whereas non-permeable inhibitor CA-074 did not.

executors of caspase-independent cell-death induced by tumor necrosis factor and its intracellular activity is associated with apoptosis.[13–15] Hence, we focused on the intracellular activity of cathepsin B associated with CST6 expression. We measured cathepsin B activity using a fluorogenic proteinase substrate in the lysate from the PDAC cells transfected with CST6. KLM-1-derivative cells over-expressing CST6 (CST6-1, 2 and 3) showed significant reduction of the intracellular cathepsin B activity, compared with KLM1–Mock cells (Fig. 5a). Then, in order to examine the intracellular activity of cathepsin B for its involvement in PDAC cell viability, we treated PDAC cells with the specific inhibitors to cathepsin B (CA-074 and CA-074 Me). Membrane-permeable inhibitor CA-074Me enhanced PDAC cell growth significantly ($P < 0.0001$), whereas non-permeable inhibitor CA-074 did not (Fig. 5b), indicating the significant effect of the intracellular activity of cathepsin B on PDAC cell viability. These findings implicated that CST6 could promote PDAC cell growth, probably through the inhibition of intracellular cathepsin B activity and its pathway.

Discussion

In this report, we investigated a biological effect of CST6 over-expression on PDAC cells. Since it was restrictively expressed in adult normal organs, CST6 could be an appropriate and promising molecular target for a novel therapeutic approach with minimal adverse effect. CST6 was reported its limited expression in human cutaneous epidermis[16] and its physiological role is considered to keep the balance of protease and antiprotease in skin surface and to regulate cornification and keratinocyte differentiation.[16,17] However, its detailed function in the epidermis or cancer cells is still unclear. Previous studies had reported that CST6 was down-regulated in metastatic breast cancers[10,11] and could have a role to suppress the metastatic or invasive phenotype of breast cancer cells by inhibiting extracellular proteinase activity. On the other hand, recent reports indicated that CST6 was up-regulated in head and neck cancers[18] and thyroid cancers[19] and might induce anti-apoptotic property to cancer cells. Furthermore, another study investigating breast cancer failed to confirm CST6 down-regulation in breast cancer cells[20] as similar to our microarray analysis on the microdissected breast cancer cells, which also failed to detect down-regulation of CST6 expression in breast cancer cells.[21] Hence, although down-regulation of CST6 expression in breast cancer may still be the subject of discussion, our functional analysis implied that CST6 is more likely to function as an oncogene, but not as a tumor suppressor gene, at least in pancreatic carcinogenesis.

CST6 has a potential to inhibit intracellular cathepsin B of lysosomal proteinases,[9] which could act as a dominant execution proteinase in the caspase-independent apoptosis pathway.[13,14] Our cathepsin B activity assay clearly demonstrated that CST6 over-expression reduced intracellular cathepsin B activity, leading to growth stimulation. Cathepsin B inhibitors can reduce the response to apoptosis inducers, and cells deficient or down-regulated in cathepsin B are more resistant to tumor necrosis factor-mediated or drug-induced apoptosis.[13–15] And cathepsin B-dependent lysosomal death pathway could play a major role in the immortalization and/or transformation in carcinogenesis.[22] Consistent with these findings, our study showed that membrane-permeable cathepsin B inhibitor CA-074Me promoted PDAC cell growth, whereas non-permeable cathepsin B inhibitor CA-074 did not. Therefore, over-expressed CST6 could promote PDAC cell growth, in part, by inhibiting intracellular activity of cathepsin B and other proteinases associated with pro-apoptosis pathway.

The treatment with recombinant CST6 protein stimulated PDAC cell proliferation dose-dependently, whereas CST6-Δ1 lost its growth-promoting effect on PDAC cells, implicating that the proteinase inhibitory function of secreted CST6 could play a critical role in its growth-promoting effect. How extracellular CST6 could mediate the growth-promoting signal and affect the intracellular activity of cathepsin B or other proteinases is unknown. However, another cystatin family member, cystatin C (CST3), could be endocytosed to reach the lysosomal compartment and function[23,24] and extracellular CST6 could affect intracellular cathepsin B probably after its endocytotic transportation into the cell as well as cystatin C, although the receptor for CST6 and CST3 remains unclear. Furthermore, N-glycosylation of cell-surface or extracellular proteins can regulate their endocytosis and intracellular trafficking in the cell[25] and, in our study, the non-glycosylated form of CST6 (N137D) also lost its growth-promoting effect when we

treated PDAC cells with this recombinant protein and this mutant form could be deficient to be endocytosed to lysosome where cathepsin B and some of apoptosis-related proteinases are located.

In summary, we here demonstrated CST6 over-expression in PDAC cells and this over-expression promoted cancer cell proliferation by its inhibitory activity on intracellular proteinases. Suppression of CST6 function in cancer cell by small molecules inhibiting its proteinase inhibitory activity or neutralizing CST6 by antibodies could provide a promising new approach to molecular therapy for lethal PDAC.

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