Involvement of non-triple helical type VI collagen α1 chain, NTH α1(VI), in the proliferation of cancer cells

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Abstract. It has been reported that a polypeptide encoded by collagen type VI alpha 1 chain (*COL6A1*), one of the three α chains of type VI collagen, is strongly associated with the migration and invasion of highly metastatic human pancreatic cancer BxPC-M8 cells and excessive proliferation of LNCaP cells. We previously reported that non-triple helical type VI collagen α1 chain, NTH α1(VI), a non-triple helical polypeptide encoded by *COL6A1*, is not derived from type VI collagen and exists in cancer cell-conditioned media. Therefore, NTH α1(VI) may be involved in cancer cell migration, invasion, and proliferation. The active entity that promotes cellular behaviors in cancer remains unclear. Thus, we predicted that NTH α1(VI) has cancer-promoting activity, such as the ability to induce cell proliferation. This study was conducted to examine whether NTH α1(VI) and/or its derived peptides are involved in cancer cell proliferation. Highly metastatic human pancreatic S2-VP10 cells were used to explore the potential of COL6A1 knockdown in reducing cell proliferation. Moreover, S2-VP10 conditioned medium was assessed after molecular size-fractionation to determine whether the inhibitory effect of COL6A1 knockdown could be rescued by the medium. We showed that S2-VP10-conditioned medium contained COL6A1 polypeptide, but not COL6A2, suggesting that COL6A1 in the conditioned medium of S2-VP10 cells reflects the presence of NTH α1(VI). COL6A1 knockdown repressed S2-VP10 cell proliferation and this repression was rescued using the conditioned medium of S2-VP10 cells. The fraction of conditioned medium containing peptides smaller than 10 kDa rescued the inhibitory effect; however, the fraction containing polypeptides larger than 10 kDa, including NTH α1(VI), did not show rescue activity, indicating that NTH α1(VI) fragmentation is necessary for enhanced cancer cell proliferation. In conclusion, fragmentation of NTH α1(VI) into peptides <10 kDa is required for its cancer cell proliferation-promoting activity.

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Introduction

Type VI collagen secreted from host cells has been shown to influence the proliferation of cancer cells (1-3). High collagen type VI alpha 1 chain (*COL6A1*) mRNA expression was observed in astrocytoma using reverse transcription quantitative PCR (RT-qPCR) analysis (4). Overexpression of *COL6A1* mRNA and the COL6A1 polypeptide was detected in non-small cell lung cancer using RT-qPCR and immunohistochemical (IHC) analyses (5). IHC analysis also indicated that COL6A1 expression is higher in castration-resistant prostate carcinoma than in adjacent normal tissue (6). In addition, gene expression profile analysis showed that *COL6A1* mRNA expression is a predictive marker of poor prognosis in clear cell renal cell carcinoma (7). Proteomic analysis revealed that COL6A1 expression is associated with poor prognosis in patients with glioblastoma (8). Similarly, IHC analysis indicated that COL6A1 expression predicts the prognosis of patients with cervical cancer (9). Thus, previous reports suggest that type VI collagen, which contains the COL6A1 polypeptide as one of the three α chains, is important for cancer cell proliferation.

Zhu *et al* indicated that COL6A1 knockdown (KD) inhibits the proliferation of prostate cancer LNCaP cells. COL6A1 promotes proliferation through the JAK-STAT pathway (6). Owusu-Ansah *et al* also reported that COL6A1 is involved in the migration and invasion of highly metastatic pancreatic cancer BxPC-M8 cells (10). Recently, we reported the existence of a non-triple helical polypeptide encoded by *COL6A1*, NTH α1(VI), in the conditioned media of cancer cell lines (11). NTH α1(VI), rather than the COL6A1 polypeptide of type VI collagen, may be directly involved in cancer cell proliferation, migration, and invasion.

Extracellular matrix (ECM)-derived peptides, known as matrikines or matricryptins (hereafter, matricryptins),

are defined as 'enzymatic fragments of ECM containing exposed matricryptic sites', according to Davis *et al* (12). Arresten, canstatin, and tumstatin are matricryptins derived from the NC1 domains of type IV collagen α1, α2, and α3 chains, respectively; they all possess anti-angiogenic and antitumor activities (13,14). It has been reported that endotrophin, the C5 domain derived from COL6A3, promotes malignant tumor progression (15). As described above, tumors express COL6A1 (5,6), whose expression is associated with the prognosis of patients with certain types of cancer (8,9). Additionally, Willumsen *et al* observed COL6A1- and COL6A3-derived peptides in some tumors (16); as indicated above, NTH α1(VI) is thought to be involved in cancer cell proliferation, migration, and invasion. Therefore, we evaluated whether NTH α1(VI) and/or derived peptide(s) are involved in cancer cell proliferation using highly metastatic human pancreatic cancer S2-VP10 cells, given that S2-VP10 cells constitutively express many matrix metalloproteases (17).

Materials and methods

Antibodies and type VI collagen. A mouse monoclonal antibody (#141) that recognizes COL4A1, NTH α1(IV), COL6A1, and NTH α1(VI) was prepared in our laboratory (Nippon Kayaku, Tokyo, Japan) and characterized as previously described (11,18,19). Rabbit anti-COL6A1 polyclonal antibody (cat. no. NBP1-59126) was purchased from Novus Biologicals. Rabbit anti-COL6A1 (N-term) polyclonal antibody (cat. no. AP6587a) and mouse anti-COL6A2 monoclonal antibody (cat. no. AT1585a) were purchased from Abgent Inc. Mouse anti-HSP90 monoclonal antibody (cat. no. 610418) was purchased from BD Biosciences. Horseradish peroxidase-labeled sheep anti-mouse (cat. no. NA931-1ML) and donkey anti-rabbit (cat. no. NA934-1ML) IgG antibodies were purchased from GE Healthcare. Human type VI collagen was purchased from BD Biosciences.

Small interfering RNAs (siRNAs). Control and COL6A1 siRNAs were purchased from Thermo Fisher Scientific, Inc. The sequences of all siRNAs except for the control siRNA are shown in Table I.

TaqMan probes. Collagen type IV alpha 1 (COL4A1), COL6A1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) TaqMan probes were purchased from Thermo Fisher Scientific, Inc.

Cell lines and cell cultures. The human breast cancer cell line, MDA-MB-436 (20), was purchased from the American Type Culture Collection and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals). The human pancreatic cancer cell line, S2-VP10 (17), was purchased from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan), and cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. Both cell lines were cultured at 37˚C in a 5% CO_2 atmosphere. Notably, we confirmed that both cell lines were mycoplasma-negative.

Transfection of siRNAs and cell proliferation assays. Cells ($1x10^4$/well) were plated into 12-well plates and cultured at 37˚C in a 5% CO_2 atmosphere. After overnight incubation, the cells were transfected with the siRNAs indicated in the figure legends using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 100 μl of OptiMEM (Thermo Fisher Scientific, Inc.) containing 10 pmol of siRNA was mixed with 100 μl of OptiMEM containing 2.0 μl of Lipofectamine RNAiMAX. The mixture was incubated at room temperature for 20 min. After incubation, the mixture was added to the cells, which were maintained at 37˚C in a 5% CO_2 atmosphere. Three hours later, the culture medium was replaced with fresh medium. After siRNA transfection, the indicated amount of type VI collagen, 1 ml of conditioned medium, or 1 ml of DMEM supplemented with 10% FBS was added to the cells. The cells were then cultured for 72 h at 37˚C in a 5% CO_2 atmosphere and subsequently subjected to a cell proliferation assay and cell lysate preparation. In some experiments, the siRNA-treated conditioned medium was collected at the end of the culture period. The conditioned medium was fractionated with Amicon Ultra-4 or Ultra-15 Centrifugal Filter Units (molecular weight cutoff, 10 kDa; Merck KGaA), as indicated in the section 'Conditioned medium for the cell proliferation assay'; the rescue activities of the fractions were assessed in cell proliferation assays.

To evaluate cell proliferation, we calculated proportional proliferation (% of control) assessed by methylene blue staining as described in the section 'Methylene blue staining.'

Methylene blue staining. We assessed cell proliferation by methylene blue staining as previously described with slight modifications (21,22). Briefly, the medium was removed from the wells and the plate was allowed to dry at room temperature. Five hundred microliters of methanol was added to each well, and the plate was kept at room temperature for 2 min to fix the cells. Next, methanol was removed from the wells and the plate was allowed to dry at room temperature. Thereafter, 500 μl of 0.05% methylene blue was added to each well, and the plate was incubated at room temperature for 30 min, after which the methylene blue solution was removed. The plate was washed three times with distilled water; 1 ml of 3% HCl was added to each well to dissolve the methylene blue stain, and the absorbance at 660 nm was measured using a SpectraMAX M3 plate reader (Molecular Devices, LLC) with SoftMAX Pro Software version 6.4.2 (Molecular Devices, LLC). All cell proliferation assays were performed in triplicate and each siRNA, drug treatment, and rescue experiment was repeated at least three times. Data are expressed as the mean ± SD.

Conditioned medium for the cell proliferation assay. A total of $2x10^5$ S2-VP10 cells were plated per well in a 6-well plate and cultured for two days at 37˚C in a 5% CO_2 atmosphere. The conditioned medium obtained after incubation was used to evaluate the ability to rescue the repressed proliferation of KD cells. In some experiments, the conditioned media were fractionated in size using Amicon Ultra-4 or Ultra-15 Centrifugal Filter Units according to the manufacturer's instructions. The conditioned medium was centrifuged at 5,000 x g for 25 min at 4˚C in the filter units, followed by filtration through a 0.45-μm

Table I. siRNA sequences used in this study.

Name	Sequence
COL6A1#1 sense	5'-CCUGUUCUUUGUGCUGGACACCUCU-3'
COL6A1#1 antisense	5'-AGAGGUGUCCAGCACAAAGAACAGG-3'
COL6A1#2 sense	5'-GCAUAGACAAGAAGUGUCCAGAUUA-3'
COL6A1#2 antisense	5'-UAAUCUGGACACUUCUUGUCUAUGC-3'
COL6A1#3 sense	5'-GCCGUCGAUGCCAUGGACUUUAUCA-3'
COL6A1#3 antisense	5'-UGAUAAAGUCCAUGGCAUCGACGGC-3'

polyvinylidene difluoride membrane filter (Merck KGaA); fractions containing molecules larger than 10 kDa (>10 kDa fraction) or smaller than 10 kDa (<10 kDa fraction) were collected separately. Nine volumes of DMEM were added to one volume of the >10 kDa fraction, whereas one volume of FBS was added to nine volumes of the <10 kDa fraction. Each fraction was used in the cell proliferation assay.

Concentration of <10 kDa fraction by acetone precipitation. The proteins were concentrated by acetone precipitation. Briefly, nine volumes of ice-cold acetone were added to one volume of the <10 kDa fraction and the mixture was incubated at -20˚C for more than 2 h. This mixture was centrifuged at 15,000 x g for 10 min at 4˚C, and the pellet was subjected to SDS-PAGE.

Preparation of cell lysates. The cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) and harvested. Lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, and 10 mM phenylmethylsulfonyl fluoride] was added to the cells, which were then incubated on ice for 30 min. After cell lysis, the mixture was centrifuged at 20,000 x g for 15 min at 4˚C, and the supernatants were used as cell lysates. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Ten micrograms of cell lysate were subjected to western blotting.

Western blotting analysis. Western blotting was performed as previously described (11,18). Briefly, an equal amount of 2X concentrated sample buffer containing 2-mercaptoethanol (2-ME) [125 mM Tris-HCl (pH 6.8), 20% glycerol, 10% 2-ME, and 4% SDS] was added to each cell lysate or supernatant and heated at 95˚C for 5 min. Under non-reducing conditions, a 2X concentrated sample buffer without 2-ME was used. The mixture was loaded onto a 7.5% SDS-PAGE gel, 5-20% SuperSep Ace precast gel (Wako Pure Chemical Industries), or 15-20% SuperSep Ace precast gel (Wako Pure Chemical Industries) and electrophoresed. Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Inc.) were used as molecular weight markers. The gel was transferred to a polyvinylidene difluoride membrane (PerkinElmer), which was blocked with Tris-buffered saline-Tween 20 [TBS-T; 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] containing 5% skim milk (BD Biosciences). The membrane was incubated with predetermined antibodies and washed with TBS-T. All primary antibodies, except for the anti-COL6A1 (N-term) and anti-COL6A2 antibodies, were diluted to 1 μg/ml with TBS-T containing 5% skim milk prior to use. The anti-COL6A1 (N-term) and anti-COL6A2 antibodies were diluted to 1 μg/ml with Can Get Signal® (Toyobo). Bound antibodies were detected with horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG secondary antibodies diluted to 0.25 μg/ml in TBS-T containing 5% skim milk. The washed membrane was developed with ECL Western Blotting Detection Reagent (GE Healthcare) and visualized on Hyperfilm ECL (GE Healthcare) according to the manufacturer's instructions. To reuse the membrane for some experiments, it was incubated at 55˚C for 30 min in stripping buffer [62.5 mM Tris-HCl (pH 6.5), 100 mM 2-ME, and 2% SDS] and reprobed with another antibody as previously described (23).

Coomassie Brilliant Blue (CBB) staining. CBB staining was performed using a Rapid Stain CBB Kit (Nacalai Tesque) according to the manufacturer's instructions.

RNA extraction and cDNA synthesis. RNA was extracted and purified using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

cDNA was synthesized using purified RNA and SuperScript III (Thermo Fisher Scientific, Inc.) as follows. Briefly, 500 ng of RNA was mixed with 1 μl of 50 μM oligo $d(T)_{20}$ (Thermo Fisher Scientific, Inc.) and 1 μl of 10 mM dNTP Mix (Thermo Fisher Scientific, Inc.). The mixture was adjusted to 13 μl with sterile distilled water, followed by heating at 65˚C for 5 min. After heat denaturation, the mixture was incubated at 4˚C for 1 min. Next, 4 μl of 5X First-Strand Buffer (Thermo Fisher Scientific, Inc.), 1 μl of 0.1 M DTT (Thermo Fisher Scientific, Inc.), 1 μl of RNaseOUT (Thermo Fisher Scientific, Inc.), and 1 μl of SuperScript III were added to the mixture. For cDNA synthesis, the mixture was incubated at 50˚C for 30 min, followed by denaturation at 75˚C for 15 min. The cDNA was used as a template in RT-qPCR.

Reverse transcription quantitative PCR. The indicated mRNA expression was assessed by RT-qPCR using Rotor Gene Q (Qiagen) according to the manufacturer's instructions. Briefly, a reaction mixture containing 10 μl of 2X TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, Inc.), 1 μl TaqMan probe (Thermo Fisher Scientific, Inc.), 1 μl cDNA, and 8 μl dH_2O was prepared and subjected to qPCR. Cycling conditions were as follows: Pre-denaturation at 95˚C for 10 min; denaturation at 95˚C for 15 sec; annealing and

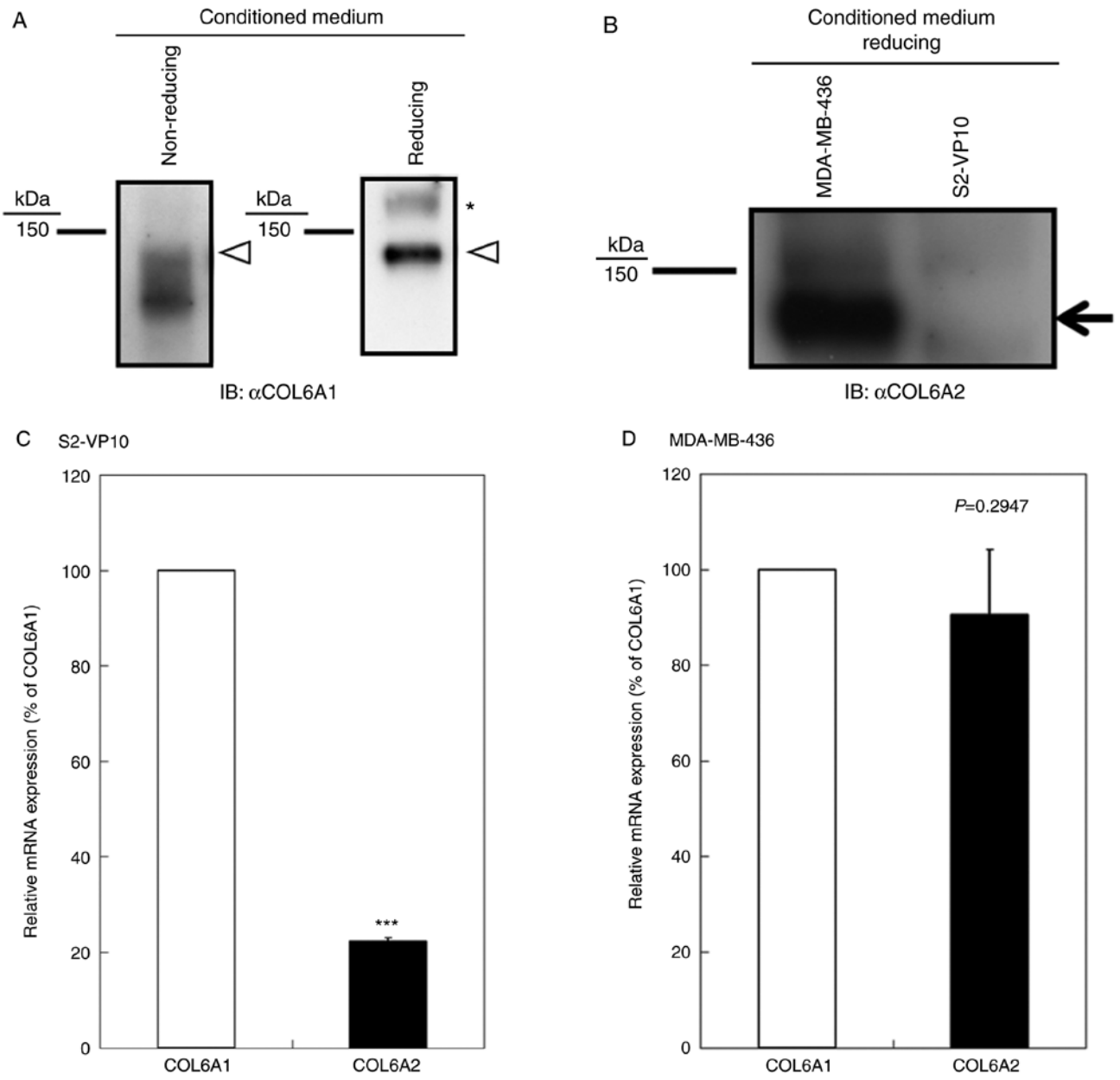

Figure 1. NTH α1(VI) expression in S2-VP10 cells. (A) Representative images of NTH α1(VI) expression in conditioned medium derived from S2-VP10 cells. The conditioned medium was harvested after two days of culture. Western blotting was carried out under non-reducing and reducing conditions, and probing was performed with anti-COL6A1 polyclonal antibody (IB: αCOL6A1); NTH α1(VI) and COL6A1 are indicated by white arrowheads; * indicates non-specific band. (B) A representative immunoblot image of COL6A2 expression in conditioned media derived from S2-VP10 and MDA-MB-436 cells. The conditioned media were harvested after two days of culture. Western blotting was carried out under reducing conditions and probing with anti-COL6A2 monoclonal antibody (IB: αCOL6A2) was performed; COL6A2 bands are indicated by an arrow. (C and D) *COL6A1* mRNA and *COL6A2* mRNA expression in S2-VP10 (C) and MDA-MB-436 cells (D); cells were cultured for 72 h and then *COL6A1* mRNA and *COL6A2* mRNA expression was determined by RT-qPCR. Data are expressed as the mean ± SD (n=3). ***P<0.001 vs. siCont by Student's t test. RT-qPCR, reverse transcription-quantitative PCR; *COL6A1*, collagen type VI alpha 1 chain; NTH α1(VI), non-triple helical type VI collagen α1 chain.

extension at 60˚C for 1 min; number of cycles, 40. After the reaction, the relative ΔCq of the target gene (ΔΔCq) was calculated using *GAPDH* mRNA as an internal standard (24). The expression level of *COL6A2* mRNA was compared to that of *COL6A1* mRNA. All RT-qPCR experiments were performed in duplicate, and each RT-qPCR experiment was repeated at least three times. Data are expressed as the mean ± SD.

Statistical analysis. All statistical analyses were carried out with Exsus version 8.1.0 software (CAC Croit Corp.), and were based on at least three independent experiments; the data are expressed as the mean ± SD. Dunnett's multiple comparison test was used to determine statistical differences among the four groups. Student's t-test was used to determine statistically significant differences between two groups. P<0.05 was considered as statistically significant.

Results

Expression of NTH α1(VI) by S2-VP10 cells: COL6A1 silencing reduces cell proliferation and NTH α1(VI) expression. S2-VP10 cells expressed the NTH α1(VI)/COL6A1 chain

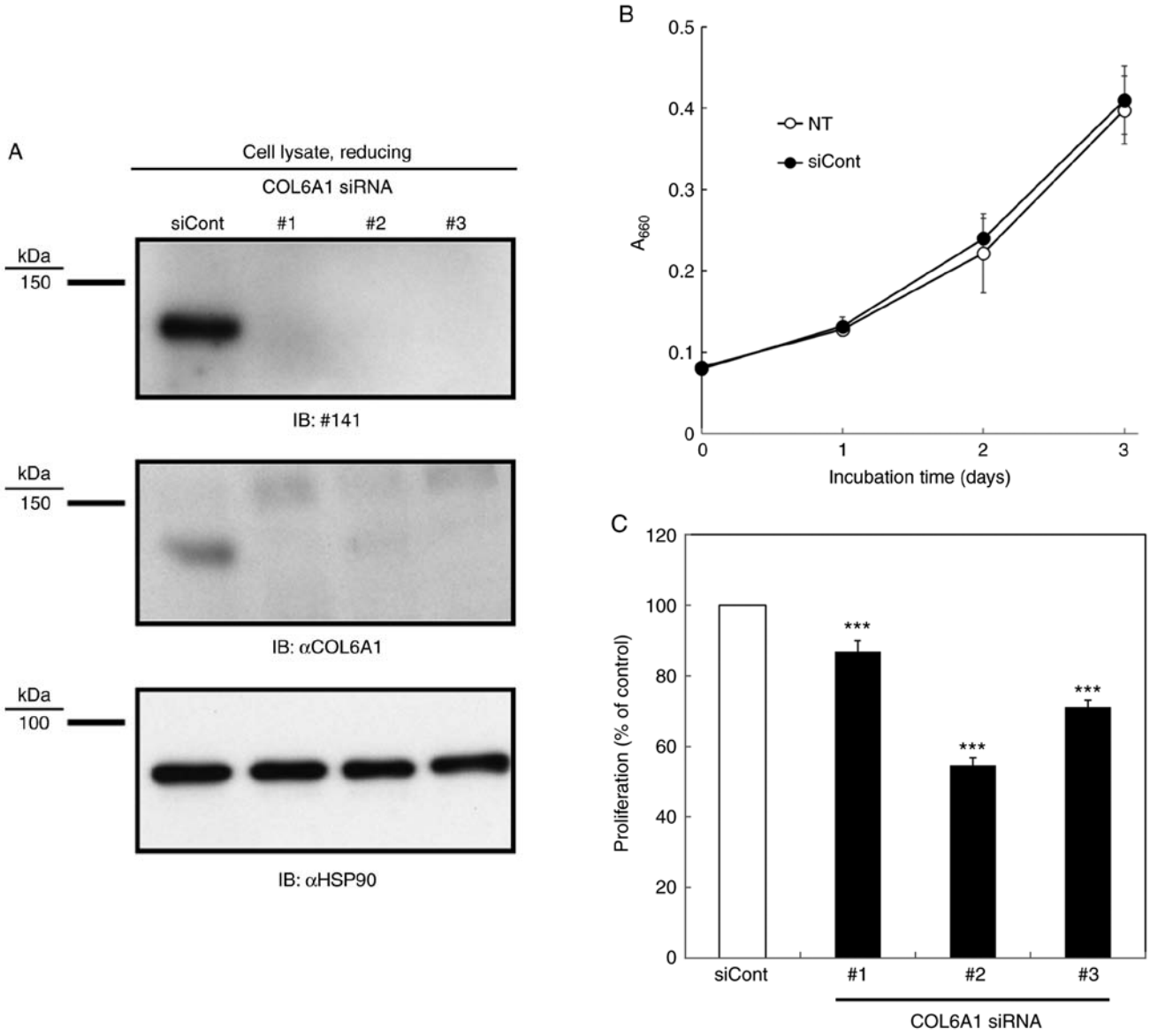

Figure 2. Effect of COL6A1 KD on the proliferation of S2-VP10 cells. (A) Representative images of COL6A1 KD in S2-VP10 cells. The indicated siRNA was transfected into S2-VP10 cells and cell lysates were prepared. Western blotting was carried out under reducing conditions and sequential probing was performed with antibody #141 (IB: #141), anti-COL6A1 polyclonal antibody (IB: αCOL6A1), and anti-HSP90 monoclonal antibody (IB: αHSP90). (B) Typical proliferation curves of S2-VP10 cells. Control siRNA was transfected into S2-VP10 cells, which were cultured for 72 h. Cell proliferation at the indicated culture time determined by methylene blue staining is shown as absorbance at a wavelength of 660 nm. Data are expressed as the mean ± SD (n=4). (C) Effect of COL6A1 KD on the proliferation of S2-VP10 cells. The indicated siRNA was transfected into S2-VP10 cells, which were cultured for 72 h; relative cell numbers without transfection or with siCont transfection were increased by approximately three-fold as shown in (B). Cell proliferation was assessed by methylene blue staining; data are expressed as the mean ± SD (n=3). ***P<0.001 vs. siCont by Dunnett's multiple comparison test. COL6A1, collagen type VI alpha 1 chain; KD, knockdown; IB, immunoblot; siCont, control siRNA; NT, no treatment.

(Fig. 1A), whereas only faint expression of the COL6A2 polypeptide was found (Fig. 1B). Notably, a smear band was observed under non-reducing conditions (Fig. 1A). It is known that COL6A1 contains cysteine residues (1). Therefore, the smear band may have resulted from differences in intramolecular disulfide bonds. In addition, the *COL6A2* mRNA expression level was 22.3±0.7% of the *COL6A1* mRNA expression level (Fig. 1C). We found that MDA-MB-436 cells expressed COL6A1 and COL6A2 during our preliminary experiments (data not shown). Thus, we compared the *COL6A1* mRNA/*COL6A2* mRNA ratio between S2-VP10 and MDA-MB-436 cells. The results revealed that the *COL6A2* mRNA expression level was comparable to that of *COL6A1* mRNA expression (90.5±13.6% of the *COL6A1* mRNA expression level) in the MDA-MB-436 cells (Fig. 1D), indicating that NTH α1(VI) is a major form of the *COL6A1* gene product in S2-VP10 cells, as type VI collagen is composed of α1, α2, and α3 chains (1,25), requiring equal amounts of each chain.

COL6A1 KD repressed COL6A1 expression (Fig. 2A). Next, we confirmed that COL6A1 KD inhibited S2-VP10 cell proliferation as previously reported (6). Representative proliferation curves of S2-VP10 cells transfected with or without control siRNA are shown in Fig. 2B, indicating that the transfection of control siRNA does not affect the proliferation of S2-VP10 cells. Moreover, Fig. 2C indicates that COL6A1 KD inhibited the proliferation of S2-VP10 cells.

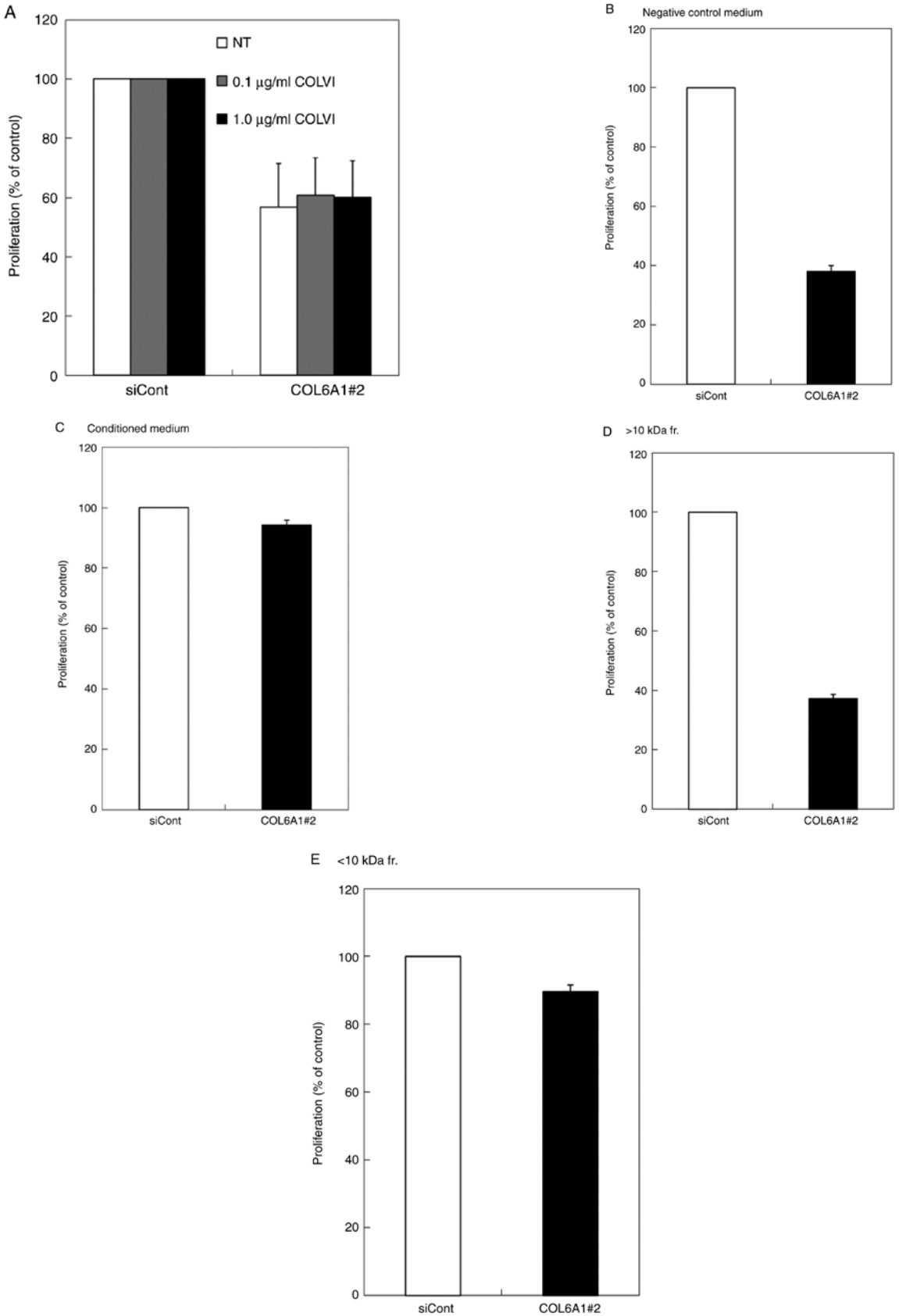

Figure 3. Effect of differently sized fractions from conditioned media on the proliferation of S2-VP10 cells. (A) Effect of type VI collagen on the proliferation of S2-VP10 cells. Transfection with COL6A1 siRNA#2 was the same as that shown in Fig. 2. After siRNA transfection, the indicated amount of type VI collagen (COLVI) was added to the cells, and cell proliferation was determined by methylene blue staining; data are expressed as the mean ± SD (n=3). (B-E) Rescue activities of fractionated S2-VP10 conditioned media on the proliferation of S2-VP10 cells; after siRNA transfection, COL6A1#2 transfected S2-VP10 cells were cultured for 72 h. Then, S2-VP10 cell proliferations using DMEM supplemented with 10% FBS (negative control medium) (A), conditioned medium from S2-VP10 cells (B), >10 kDa fraction (C), and <10 kDa fraction (D) were determined by methylene blue staining. The fractions were prepared using Amicon Ultra Centrifugal Filter Units. Data are expressed as the mean ± SD (n=3). COL6A1, collagen type VI alpha 1 chain; COL6A1#2, COL6A1#2 siRNA; fr., fraction; siCont, control siRNA; NT, no treatment.

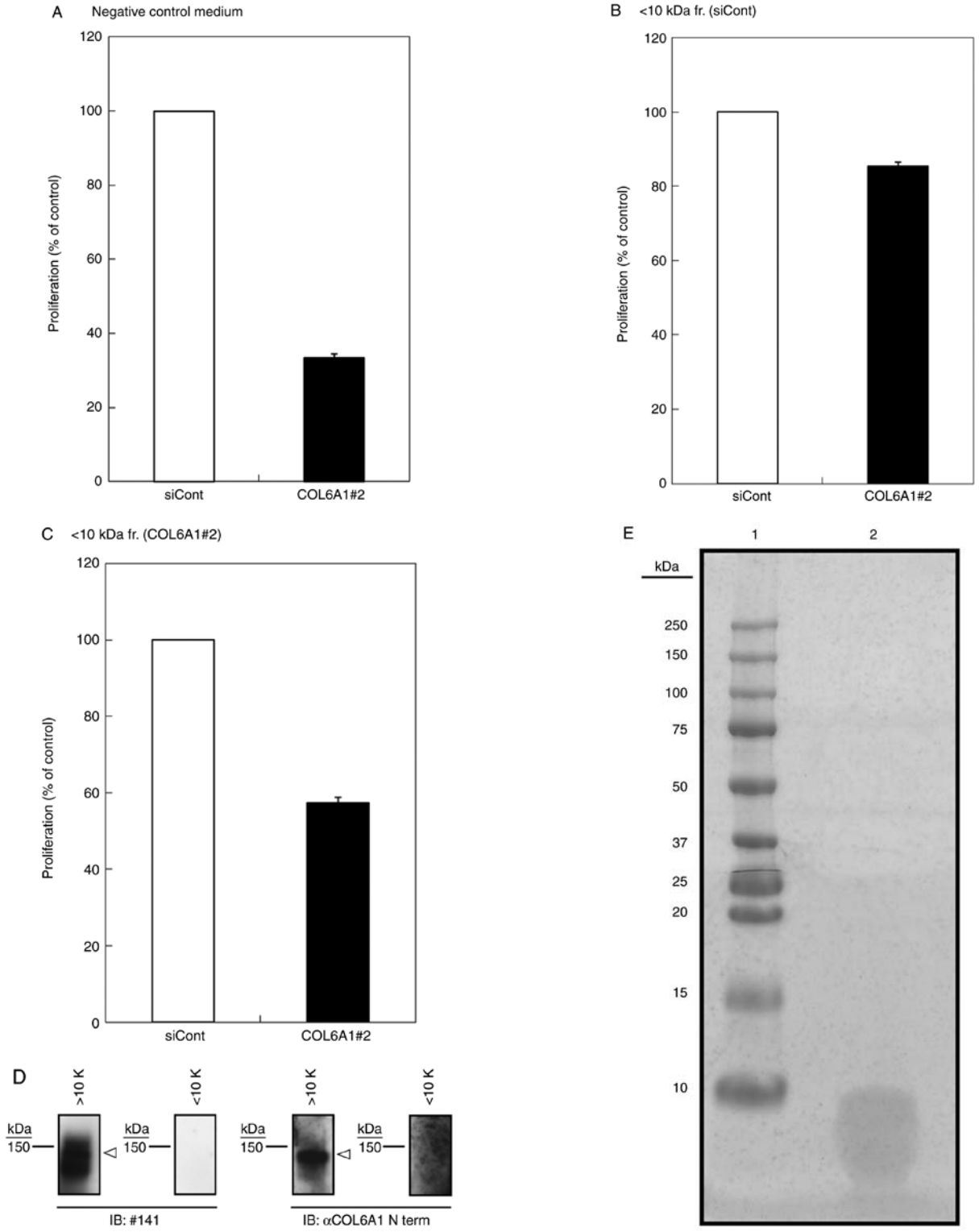

Figure 4. Rescue activities of NTH α1(VI)-derived peptides. (A-C) Rescue activities of siRNA-transfected conditioned media. Transfection with COL6A1 siRNA#2 was the same as that shown in Fig. 2. After siRNA transfection, the cells were cultured for 72 h, after which cell proliferation was determined. The cell proliferations of S2-VP10 cells using DMEM supplemented with 10% FBS as negative control (A), control siRNA-treated <0 kDa fraction (B), and COL6A1 #2 siRNA-treated <10 kDa fraction (C) were determined by methylene blue staining. Data are expressed as the mean ± SD (n=3). (D) Representative immunoblot images of the indicated fraction; the fraction containing polypeptides >10 kDa (>10K) and polypeptides <10 kDa (<10K) were subjected to western blotting under reducing conditions, and probing was performed with antibody #141 (IB: #141) and anti-COL6A1 (N-term) antibody (IB: αCOL6A1 N term). COL6A1 is indicated by white arrowheads. (E) Coomassie Brilliant Blue (CBB) staining of the <10 kDa fraction. Acetone precipitated <10 kDa fraction was subjected to SDS-PAGE and stained with CBB staining. Lane 1, molecular weight marker; lane 2, concentrated <10 kDa fraction. *COL6A1*, collagen type VI alpha 1 chain; NTH α1(VI), non-triple helical type VI collagen α1 chain; COL6A1#2, COL6A1#2 siRNA; fr., fraction; IB, immunoblot; siCont, control siRNA.

Requirement for fragmentation of NTH α1(VI) for enhanced proliferation. Repressed proliferation of S2-VP10 cells by COL6A1 KD suggests that *COL6A1* gene products including type VI collagen, NTH α1(VI), and/or NTH α1(VI)-derived

peptides are involved in cell proliferation. The rescue activity was evaluated by adding type VI collagen or the conditioned medium of S2-VP10 cells to COL6A1-silenced S2-VP10 cells; type VI collagen did not show rescue activity (Fig. 3A), whereas the conditioned medium showed rescue activity compared to DMEM supplemented with 10% FBS (Fig. 3B and C), indicating that NTH α1(VI) and/or derived peptides are responsible for the rescue activity. To test whether NTH α1(VI) or its fragments hold this activity, the conditioned medium was fractionated by size; rescue activity with a strength similar to that of non-fractionated conditioned medium was found in the <10 kDa fraction, whereas the >10 kDa fraction containing NTH α1(VI) showed no rescue activity (Fig. 3C-E), indicating that enhanced proliferation requires NTH α1(VI) fragmentation. To confirm that the active components in the <10 kDa fraction were derived from NTH α1(VI), we assessed the rescue activity of the <10 kDa fraction of COL6A1 KD cells. We observed that the <10 kDa fraction of control siRNA-treated cells was able to rescue cell proliferation (Fig. 4A and B), whereas the <10 kDa fraction of COL6A1 KD cells appeared to exhibit lower rescue activity (Fig. 4C). The slight rescue activity of the <10 kDa fraction of COL6A1 KD cells likely resulted from the small number of active fragments that may have been produced from preexisting NTH α1(VI) prior to KD treatment. Notably, we also confirmed that NTH α1(VI) with a size of 140 kDa was present in the >10 kDa fraction (Fig. 4D) and that the <10 kDa fraction contained only peptides less than 10 kDa in size (Fig. 4E). Therefore, fragmentation of NTH α1(VI) into fragments of less than 10 kDa was responsible for the enhanced proliferation of cancer cells.

Discussion

We showed that peptides of less than 10 kDa, possibly derived from NTH α1(VI) fragmentation, are involved in the proliferation of S2-VP10 cells. Higher expression of collagen type VI alpha 1 chain (COL6A1) is observed in some tumors (4-6), and its expression is associated with poor prognosis (7-9). Zhu *et al* also indicated that COL6A1 overexpression promotes the proliferation of prostate cancer LNCaP cells, and that COL6A1 expression is higher in patients with castration-resistant prostate cancer (6). In addition, secretion of the COL6A1 fragment is upregulated in some types of cancers, including pancreatic cancer (16). This suggests that the peptide fragments described herein are involved in cell proliferation within tumors. Recently, Chen *et al* reported that COL6A1 KD suppresses the proliferation and migration of human aortic vascular smooth muscle cells (26), suggesting that the peptides are also involved in vascular development.

Collagen-derived matricryptins (endostatin, arresten, canstatin, and tumstatin) have anti-angiogenic and antitumor activities (13,14). Hamano *et al* indicated that tumstatin is generated by MMP-9 proteolysis *in vivo* (27). It has also been reported that S2-VP10 cells constitutively express many matrix metalloproteases (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, and MMP-14) and show a highly metastatic phenotype (17). In addition, Willumsen *et al* indicated that the serum level of MMP-generated COL6A1 fragments was upregulated in some types of cancers (16). Similarly, the peptide fragments in the present study may have been generated by one or more of these proteases.

Our results suggest that the growth-promoting mechanism of the peptides is not shared with type VI collagen. As previously indicated, arresten, canstatin, and tumstatin show anti-angiogenic and antitumor activities; these matricryptin sequences are located in non-collagenous NC1 domains. The biological activities of these matricryptins have also been reported to be mediated through integrins (13,14). In addition, endotrophin, the C5 Kunitz-like domain of COL6A3, promotes malignant tumor progression via transforming growth factor β signaling (15). This indicates that non-collagenous regions in collagens have biological functions and that these activities are mediated via specific receptors. COL6A1 has three von Willebrand factor A (vWA) modules, one in its N-terminus and two in its C-terminus (1,28,29). Whittaker and Hynes suggested that the vWA modules in collagen proteins, including COL6A1, are involved in protein-protein interactions with other matrix proteins and possibly with cells (30), suggesting that vWA modules are important for peptide activity.

We showed that the mRNA and protein expression of COL6A2 was low in S2-VP10 cells. This suggests that the chain stoichiometry between COL6A1 and COL6A2 is different. Merl-Pham *et al* also reported that the chain stoichiometry abnormality between COL6A1 and COL6A2 was observed in the extracellular matrix of human lung fibroblasts; they concluded that one of the causes of the stoichiometry abnormality is the existence of NTH α1(VI) (31). This strongly suggests that the origin of the peptides is NTH α1(VI); however, the possibility that the origin of the peptides is a different type VI collagen is not completely excluded. Thus, it is essential to identify the peptide sequences. We are currently investigating this fraction by liquid chromatography-tandem mass spectrometry analysis; the sequence may also provide information about peptide generation as MMPs and other proteases contain consensus sequences for peptide cleavage.

In conclusion, we demonstrated i) that peptides of less than 10 kDa are involved in the proliferation of pancreatic cancer S2-VP10 cells and ii) the peptides are derived from NTH α1(VI) fragmentation.

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Availability of data and materials

The datasets and materials used during the present study are available from the corresponding author upon reasonable request.

Author's contributions

TS designed, conducted and performed the experiments, and wrote the manuscript. KT, KS, HS, TH, YI and MM designed,

reviewed and edited the manuscript. MM designed, conducted and supervised this project. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chen P, Cescon M and Bonaldo P: Collagen VI in cancer and its biological mechanisms. Trends Mol Med 19: 410-417, 2013.
2. Iyengar P, Espina V, Williams TW, Lin Y, Berry D, Jelicks LA, Lee H, Temple K, Graves R, Pollard J, *et al*: Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. J Clin Invest 115: 1163-1176, 2005.
3. You WK, Bonaldo P and Stallcup WB: Collagen VI ablation retards brain tumor progression due to deficits in assembly of the vascular basal lamina. Am J Pathol 180: 1145-1158, 2012.
4. Fujita A, Sato JR, Festa F, Gomes LR, Oba-Shinjo SM, Marie SK, Ferreira CE and Sogayar MC: Identification of COL6A1 as a differentially expressed gene in human astrocytomas. Genet Mol Res 7: 371-378, 2008.
5. Voiles L, Lewis DE, Han L, Lupov IP, Lin TL, Robertson MJ, Petrache I and Chang HC: Overexpression of type VI collagen in neoplastic lung tissues. Oncol Rep 32: 1897-1904, 2014.
6. Zhu YP, Wan FN, Shen YJ, Wang HK, Zhang GM and Ye DW: Reactive stroma component COL6A1 is upregulated in castration-resistant prostate cancer and promotes tumor growth. Oncotarget 6: 14488-14496, 2015.
7. Wan F, Wang H, Shen Y, Zhang H, Shi G, Zhu Y, Dai B and Ye D: Upregulation of COL6A1 is predictive of poor prognosis in clear cell renal cell carcinoma patients. Oncotarget 6: 27378-27387, 2015.
8. Turtoi A, Blomme A, Bianchi E, Maris P, Vannozzi R, Naccarato AG, Delvenne P, De Pauw E, Bevilacqua G and Castronovo V: Accessibilome of human glioblastoma: Collagen-VI-alpha-1 is a new target and a marker of poor outcome. J Proteome Res 13: 5660-5669, 2014.
9. Hou T, Tong C, Kazobinka G, Zhang W, Huang X, Huang Y and Zhang Y: Expression of COL6A1 predicts prognosis in cervical cancer patients. Am J Transl Res 8: 2838-2844, 2016.
10. Owusu-Ansah KG, Song G, Chen R, Edoo MIA, Li J, Chen B, Wu J, Zhou L, Xie H, Jiang D and Zheng S: COL6A1 promotes metastasis and predicts poor prognosis in patients with pancreatic cancer. Int J Oncol 55: 391-404, 2019.
11. Sato T, Takano R, Tokunaka K, Saiga K, Tomura A, Sugihara H, Hayashi T, Imamura Y and Morita M: Type VI collagen α1 chain polypeptide in non-triple helical form is an alternative gene product of COL6A1. J Biochem 164: 173-181, 2018.
12. Davis GE, Bayless KJ, Davis MJ and Meininger GA: Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules. Am J Pathol 156: 1489-1498, 2000.
13. Monboisse JC, Oudart JB, Ramont L, Brassart-Pasco S and Maquart FX: Matrikines from basement membrane collagens: A new anti-cancer strategy. Biochim Biophys Acta 1840: 2589-2598, 2014.
14. Ricard-Blum S and Vallet SD: Matricryptins network with matricellular receptors at the surface of endothelial and tumor cells. Front Pharmacol 7: 11, 2016.
15. Park J and Scherer PE: Adipocyte-derived endotrophin promotes malignant tumor progression. J Clin Invest 122: 4243-4256, 2012.
16. Willumsen N, Bager C and Karsdal MA: Matrix metalloprotease generated fragments of type VI collagen have serum biomarker potential in cancer-a proof of concept study. Transl Oncol 12: 693-698, 2019.
17. Kitamura N, Iwamura T, Taniguchi S, Yamanari H, Kawano MA, Hollingsworth K and Setoguchi T: High collagenolytic activity in spontaneously highly metastatic variants derived from a human pancreatic cancer cell line (SUIT-2) in nude mice. Clin Exp Metastasis 18: 561-571, 2000.
18. Morita M, Sugihara H, Tokunaka K, Tomura A, Saiga K, Sato T, Imamura Y and Hayashi T: Preparation and partial characterization of monoclonal antibodies specific for the nascent non-triple helical form of the type IV collagen alpha 1 chain. Biochem Biophys Rep 9: 128-132, 2016.
19. Sato T, Takano R, Takahara N, Tokunaka K, Saiga K, Tomura A, Sugihara H, Hayashi T, Imamura Y and Morita M: Identification of a common epitope in the sequences of COL4A1 and COL6A1 recognized by monoclonal antibody #141. J Biochem 165: 85-95, 2019.
20. Cailleau R, Olivé M and Cruciger QV: Long-term human breast carcinoma cell lines of metastatic origin: Preliminary characterization. In Vitro 14: 911-915, 1978.
21. Elliott WM and Auersperg N: Comparison of the neutral red and methylene blue assays to study cell growth in culture. Biotech Histochem 68: 29-35, 1993.
22. Dror R, Lederman M, Umezawa K, Barak V, Pe'er J and Chowers I: Characterizing the involvement of the nuclear factor-kappa B (NF kappa B) transcription factor in uveal melanoma. Invest Ophthalmol Vis Sci 51: 1811-1816, 2010.
23. Kaufmann SH, Ewing CM and Shaper JH: The erasable western blot. Anal Biochem 161: 89-95, 1987.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
25. Allamand V, Briñas L, Richard P, Stojkovic T, Quijano-Roy S and Bonne G: ColVI myopathies: Where do we stand, where do we go? Skelet Muscle 1: 30, 2011.
26. Chen Z, Wu Q, Yan C and Du J: COL6A1 knockdown suppresses cell proliferation and migration in human aortic vascular smooth muscle cells. Exp Ther Med 18: 1977-1984, 2019.
27. Hamano Y, Zeisberg M, Sugimoto H, Lively JC, Maeshima Y, Yang C, Hynes RO, Werb Z, Sudhakar A and Kalluri R: Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. Cancer Cell 3: 589-601, 2003.
28. Chu ML, Mann K, Deutzmann R, Pribula-Conway D, Hsu-Chen CC, Bernard MP and Timpl R: Characterization of three constituent chains of collagen type VI by peptide sequences and cDNA clones. Eur J Biochem 168: 309-317, 1987.
29. Cescon M, Gattazzo F, Chen P and Bonaldo P: Collagen VI at a glance. J Cell Sci 128: 3525-3531, 2015.
30. Whittaker CA and Hynes RO: Distribution and evolution of von Willebrand/integrin A domains: Widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13: 3369-3387, 2002.
31. Merl-Pham J, Basak T, Knüppel L, Ramanujam D, Athanason M, Behr J, Engelhardt S, Eickelberg O, Hauck SM, Vanacore R and Staab-Weijnitz CA: Quantitative proteomic profiling of extracellular matrix and site-specific collagen post-translational modifications in an in vitro model of lung fibrosis. Matrix Biol Plus 1: 100005, 2019.