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Apoptosis and tumor inhibition by ADAMTS1

The tumor growth inhibitory effect of ADAMTS1 is accompanied by the inhibition of tumor angiogenesis

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Abstract

Angiogenesis plays an important role in tumor progression. Several reports have demonstrated that ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs) inhibited angiogenesis via multiple mechanisms. The aim of this study was to investigate the effect of ADAMTS1 on endothelial cells *in vitro* and on tumor growth with regard to angiogenesis *in vivo*. We examined the effects of the transfection of ADAMTS1 using two constructs, full-length ADAMTS1 (full ADAMTS1) and catalytic domain-deleted ADAMTS1 (delta ADAMTS1). Transfection of both the full ADAMTS1 and delta ADAMTS1 gene constructs demonstrated the secretion of tagged-ADAMTS1 protein into the conditioned medium, so we examined the effects of ADAMTS1-containing conditioned medium on endothelial cells. Both types of conditioned media inhibited endothelial tube formation, and this effect was completely abolished after immunoprecipitation of the secreted protein from the medium. Both types of conditioned media also inhibited endothelial cell migration and proliferation. We then examined the impact of ADAMTS1 on endothelial cell apoptosis. Both conditioned media increased the number of Annexin V-positive endothelial cells and caspase-3 activity and this effect was attenuated when z-vad was added. These results indicated that ADAMTS1

induced endothelial cell apoptosis. We next examined the effects of ADAMTS1 gene transfer into tumor-bearing mice. Both full ADAMTS1 and delta ADAMTS1 significantly inhibited the subcutaneous tumor growth. Collectively, our results demonstrated that ADAMTS1 gene transfer inhibited angiogenesis *in vitro* and *in vivo*, likely as a result of the induction of endothelial cell apoptosis by ADAMTS1 that occurs independent of the protease activity.

Introduction

Angiogenesis, the formation of new blood vessels from preexisting capillaries, is observed under various pathophysiological conditions (e.g., wound healing, diabetic retinopathy and tumor growth)(1). Neovascularization consists of endothelial cell migration, proliferation and new network formation. Tumors require a substantial blood supply, and promote angiogenesis when they grow. Avascular tumors have a severely restricted growth potential. As a result, anti-angiogenic treatments are thus considered to be potentially useful for cancer therapy (2, 3). To date, many anti-angiogenic therapies have been designed to inhibit tumor growth as well as cancer cell dissemination (4, 5).

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) is a newly categorized matrix metalloproteinase (MMP), which has multiple domains, including propeptide, metalloproteinase, disintegrin-like and spacer-region domains, and domains containing thrombospondin (TSP-1) type I motifs (6). ADAMTSs have diverse functions, such as pro-collagen processing (7) and cleavage of Von Willebrand factor (8, 9). One of the best characterized biological functions of ADAMTS is proteolytic activity against extracellular matrix proteins, including proteoglycans (10-13).

ADAMTS1 is the first member of the ADAMTS family that was identified, and has been shown to degrade extracellular matrix proteins such as versican and aggrecan (11, 14). The recombinant ADAMTS1 protein has anti-angiogenic properties(15). These reports supported the observed inhibitory effect of ADAMTS1 on endothelial cell growth and angiogenesis; however, whether ADAMTS1 inhibits tumor progression remains controversial (16-19). Accordingly, we investigated the effect of ADAMTS1 on both endothelial cell apoptosis and tumor growth using two different constructs, a full-length ADAMTS1 and a metalloproteinase-deleted ADAMTS1.

Materials and Methods

ADAMTS1 expression construct and catalytic domain deletion construct

The sequence encoding ADAMTS1 was amplified by polymerase chain reaction (PCR) using the KIAA1344 plasmid (Kazusa DNA Institute, Chiba, Japan) as a template. We designed to fuse ADAMTS1 to the COOH-terminal v5 epitope tag present in the expression vector (pcDNA/v5-D-TOPO; Invitrogen, Carlsbad, CA, USA) and this construct was designated “full ADAMTS1”. The signal peptide and FLAG epitope tag containing a mammalian expression vector with the CMV promoter was a kind gift from Dr. Billy G. Hudson and Dr. Vadim Pedchenko (20). The catalytic

domain deletion construct of ADAMTS1 cDNA with the FLAG epitope tag (delta ADAMTS1) was generated as detailed in Fig. 1. The sequence of each construct was determined to confirm that no amino acid mutations had been caused by the PCR reaction prior to the study.

Cell lines and cultures

Human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo (Osaka, Japan) and cultured in HuMedia-EBM2 (Kurabo) with a kit containing 2% fetal bovine serum (FBS), growth factors, 100 U/mL penicillin, and 100 µg/mL streptomycin, as previously described (5). COS-7 cells were purchased from ECACC (Dainippon Sumitomo Pharmaceutical Co., Osaka, Japan). Rat smooth muscle cells (SMC) and human skin fibroblasts (HSF) were obtained as previously reported (5). Human fibrosarcoma HT1080 cells, human prostate cancer DU145 cells, and Chinese hamster ovarian (CHO)-K1 cells, were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells at passage 3-6 were used for all experiments. The SMC, HSF, HT1080, DU145 and CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37°C under 5% CO₂ and 20% O₂ in a humidified chamber.

Transfection and preparation of conditioned media

Transient transfection was performed as previously described (5, 21) with slight modifications. For the COS-7 cells, lipofectamine 2000 (Invitrogen) was used for transfection according to the manufacturer's protocol. We renewed the DNA-containing medium six hours and twenty-four hours after transfection. After forty-eight hours, the medium was changed, and we collected the conditioned medium after seventy-two hours.

The cells were washed with phosphate-buffered saline (PBS) twice, and then proteins were extracted with cell lysis buffer as previously reported (22, 23). Alternatively, the produced tagged protein was depleted from the conditioned medium by immunoprecipitation of the tagged protein with either an anti-v5 or anti-FLAG M2 affinity gel (Sigma, St. Louis, MO, USA) as previously described (5). Briefly, conditioned media was incubated with affinity gel for overnight at 4°C. Supernatant after centrifugation, in which tagged-protein was no longer detectable, was used as "conditioned medium post-IP" in further experiments. For the transient transfection for the HUVEC, electroporation with microporator (BMS-MP-100; Microporator, Seoul, Korea) was performed as previously described (24). Briefly, cells were removed from the plate with trypsin, centrifuged, and dissolved in serum-free medium. Cell

and constructs were mixed into the buffer and incubated for 5m on ice, then expose to electricity (1000V 30mS, 2-pulse). Cells were plated at a density of 1.0×10^5 cells in 96-well dishes with 100 μ L culture medium without antibiotics. After seeding, cells were allowed to recover for 24h. The medium was then changed and cells were used for the analysis.

Western blot analysis and antibodies

The expression of full ADAMTS1 and delta ADAMTS1 was examined by a Western blot analysis, as described previously.(21, 25) Briefly, 20 μ l of each sample was mixed with 6 \times Laemmli sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, 10% glycerol) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to PVDF membranes (Advantech, Tokyo, Japan) using transfer buffer, and then the membranes were blocked for 1 h in 5% nonfat dried milk in PBS. The membranes were hybridized at room temperature (RT) for 1 h with a mouse anti-v5 antibody (Invitrogen) or an anti-FLAG M2 monoclonal antibody (Sigma). After stringent washing with PBS containing 0.05% Tween 20 (PBS-T) three times for 5 minutes each at RT, the membranes were incubated with the appropriate secondary

antibodies (ICN Pharmaceuticals, Aurora, OH, USA). Following three successive washes with PBS-T, immunoreactive bands were visualized using the enhanced chemiluminescence system (ECL; Amersham Bioscience, Piscataway, NJ, USA).

Endothelial tube formation assay

The endothelial tube formation assay was performed as previously described (5). Briefly, 100 μ L of Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) was applied to 96-well plates, and a suspension of 7.5×10^3 of HUVEC in 50 μ L EBM2 was seeded into each well, and then 50 μ L of each conditioned medium (n=12 each) was added per well. The cells were incubated for 24 h at 37°C and viewed using an IX71 microscope (Olympus). The length of tube formations per well (magnification at 40 \times) was measured using the Scion Image software program by two investigators blinded to the experimental protocols, and the recorded lengths were averaged.

Cell migration assays

The HUVEC migration was assayed as described previously (5, 26). In brief, confluent cells in 48-well plates were scratched with a sterile pipette tip. The cells were washed twice with EBM2 to remove cellular debris. After removal of the

cellular debris, 100 μ L EBM2 with 2% FBS and 100 μ L of each conditioned medium were added to each well. The HUVEC were then incubated at 37°C for 24 h. The cell migration assays were also performed for SMC, HSF and CHO-K1 cells using the same method. After 24 h in culture, the cells were fixed. The cells that had migrated into the denuded area were photographed with a CCD camera and counted with the Scion Image software program.

Cell proliferation assays

Cell proliferation was examined using a CellTiter AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, a suspension of HUVEC, SMC, HSF and CHO-K1 cells (1×10^4 cells/well, passage 3–5) in 50 μ L EBM2 with 2% FBS was added to 96-well plates, and supplemented with 50 μ L of each conditioned medium. After incubation for 24 h or the time indicated, the cell viability was measured as described (27, 28). Z-vad (Peptide Institute Inc, Osaka, Japan) was used to examine whether caspase is involved in this affect. All samples were read in triplicate.

Flow cytometric analysis

An Annexin V Assay Kit (MBL, Nagoya, Japan) was used according to the manufacturer's instructions. Briefly, HUVEC, SMC, HSF and CHO-K1 cells (5×10^5 cells/well) were seeded into 6-well plates in 2 mL EBM2 with 2% FBS. The following day, the medium was changed to 1 mL fresh medium containing 2% FBS plus 1 mL conditioned medium. After 24 h in culture, the attached cells were trypsinized and centrifuged at $3000 \times g$ for 5 min at 4°C . The cells were then washed in PBS and resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2). Five microliters of Annexin V conjugated with biotin and propidium iodide were added, and the cells were incubated in a darkroom for 15 min. The cells were washed again in PBS and then were resuspended in binding buffer. After centrifugation at $3000 \times g$ for 5 min at 4°C , the cells were fixed with 2% formaldehyde and washed with PBS. After centrifugation again, the cells were washed with PBS, and streptavidin-conjugated FITC (DAKO, Glostrup, Denmark) was added, followed by incubation for 15 min at RT. The cells were then washed with PBS again and resuspended in 300 μL PBS. Annexin V-FITC-labeled cells were counted using a FACScaliber flow cytometer (Becton Dickinson). The data were then analyzed using the Cell Quest software program (Becton Dickinson).

Caspase3/7 assay

The caspase 3/7 activity levels were measured using an assay kit (Caspase-Glo3/7 Assay; Promega) according to the manufacturer's instructions. Briefly, HUVEC (1×10^4 /well) were seeded into 96-well plates in 100 μ L EBM2 with 2% FBS. The following day, the medium was removed, and 50 μ L fresh EBM2 containing 2% FBS plus 50 μ L conditioned medium was added. After a 24 h culture, the caspase 3/7 activities were measured using a plate-reading luminometer (Dainippon Sumitomo Pharma. Co., Osaka, Japan).

In vivo tumor studies

All protocols involving experimental animals followed the local institutional guidelines for animal care, which are comparable to those described in the "Guide for the Care and Use of Laboratory Animals" published by the Institute for Laboratory Animal Research (National Institutes of Health Publication No. 85-23, revised 1996). HT1080, DU145 and CHO-K1 cells (1.0×10^6 cells in 50 μ L serum-free DMEM and 50 μ L Matrigel) were inoculated subcutaneously into the right flank of 5- to 7-week-old male BALB/c nude mice (Charles River Japan)(five mice per treatment group) and each tumor growth was monitored with Vernier calipers everyday, as described previously

(29). The volume was calculated according to the formula (volume = length \times width² \times 0.52).(5, 30) When the tumors reached a mean volume of 30–60 mm³, treatment was initiated. The full ADAMTS1 cDNA-containing plasmid (10 μ g) or delta ADAMTS1 cDNA-containing plasmid (10 μ g) was mixed with 10 μ L cationic lipid (DMRIEC; Invitrogen) and then dissolved in 100 μ L OPTIMEM-1 (Gibco). Approximately 20 μ L of the mixture was then directly injected into the tumor every 24 h for 7 days. The CMV/FLAG empty vector (10 μ g) was used as a negative control. Mice were sacrificed by cervical dislocation the day after the final treatment. The tumors were removed, snap frozen in liquid N₂, and embedded in OCT compound (Sakura, Tokyo, Japan).

Immunohistochemical staining

Frozen sections (8 μ m) were placed on silane-coated glass slides (DakoCytomation, Kyoto, Japan) for histological studies, as previously described (31). Blood vessels were detected by immunohistological staining using an anti-CD31 antibody (used at 1:100 dilution) (BD Pharmingen, Franklin Lakes, NJ, USA). Immunoperoxidase staining was carried out using a VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA, USA). The number of the vessels in the tumors was measured as

described previously (32). Anti-Cleaved Caspase-3 (Asp175) antibody (used at 1:100 dilution) (Cell Signaling Technology, Danvers, MA, USA) and anti-CD31 antibody were used for immunofluorescent staining as previously described (33). The tumor section was incubated with two antibodies for overnight at 4°C and then incubated with the secondary antibodies (Alexa Fluor 488-conjugated anti-rabbit IgG and Cy3-conjugated anti-rat IgG, respectively) at appropriate dilution. The immunofluorescent signals were observed with an LSM510 confocal microscope (Carl Zeiss) at the Central Research Laboratory, Okayama University Medical School. All images were processed using Adobe Photoshop CS2. All experiments were repeated using three animals, which we obtained similar results.

Statistical analysis

The statistical analysis was performed using the unpaired Student's *t*-test. $P < 0.05$ was considered to be significant.

Results

Expression of human ADAMTS1 and its fragments

We designed two constructs, full-length ADAMTS1 with a C-terminal v5-tag (full ADAMTS1) and a catalytic domain-deleted construct (delta ADAMTS1) (Fig. 1A).

We first transfected these constructs into COS-7 cells and collected the conditioned medium 72 h after transfection. The fusion proteins with full ADAMTS1 and delta ADAMTS1 were detected by a Western blot analysis (Figs. 1B and C, respectively). Transiently produced proteins from both constructs were detected in the conditioned medium, as well as in cell extracts, in our system. Full ADAMTS1 transfectants showed three major bands in conditioned medium that consisted of the unprocessed form of ADAMTS1 and its processed forms (Fig. 1B). The lowest band (~22 kDa) was considered to be a proteolytic C-terminal fragment based on a previous reported (34). The protein in the delta ADAMTS1-transfected conditioned medium was detected as a single band in conditioned medium and cell extracts (Fig. 1C).

Effect of the conditioned medium on endothelial tube formation

To test the effects of the transfected cell conditioned medium on endothelial cell tube formation, HUVEC were cultured on Matrigel in a mixture of fresh EB2 and conditioned medium, as described in the *Materials and Methods*. Representative results of the endothelial tube formation assay are shown in Fig. 2; both types of conditioned media significantly inhibited the tube formation of HUVEC compared with the control (Figs. 2A, C). These results indicated that both types of conditioned

medium contained factors that effectively inhibited the endothelial cell tube formation. When we depleted v5- or FLAG-tagged fragments from the conditioned medium by immunoprecipitation with an anti-v5 or an anti-FLAG agarose affinity gel, the inhibitory effects on tube formation were abolished (Figs. 2B, D, F). These results indicate that the effects of the conditioned media were due to the tagged proteins.

Inhibition of endothelial cell migration by ADAMTS1

The effects of ADAMTS1-transfected conditioned medium on the migration of HUVEC are shown in Figure 3A. Both types of conditioned media inhibited the migration of HUVEC, while the control (conditioned media from cells transfected with the empty vector) showed no effect on HUVEC migration. This inhibitory effect on migration appeared to be specific to endothelial cells, because neither type of conditioned medium affected the migration of SMC, HSF or CHO-K1 cells compared to the control conditioned medium (Fig. 3B).

Effects of ADAMTS1 on endothelial cell proliferation

We next examined the effects of the conditioned medium from ADAMTS1-transfected cells on endothelial cell proliferation; both types of conditioned

media inhibited HUVEC proliferation. As shown in Figure 4A-D, ADAMTS1 inhibited the cell proliferation of HUVEC, but not SMC, HSF or CHO-K1 cells. This effect was attenuated when Z-VAD (a specific inhibitor of caspases) was added to the conditioned medium, indicating that this effect was mediated by caspases (Fig. 4E). Both types of conditioned media inhibited endothelial cell attachment to collagen I and laminin (supplemental Fig. 1). We further examined the effect of electroporation of ADAMTS1 on HUVEC proliferation. As shown in Fig. 4F, both full and delta ADAMTS1 inhibited endothelial cell proliferation.

Apoptotic effects of ADAMTS1 on endothelial cells

To evaluate the apoptotic effects of ADAMTS1, we examined the Annexin V expression and caspase 3/7 activity. We found that the number of Annexin-positive HUVEC was increased by both types of conditioned media compared to the control (Figs. 5A, B). Interestingly, both the full ADAMTS1 and delta ADAMTS1 constructs caused a distinct shift of the peak of Annexin V fluorescence after 24 h of co-incubation only in endothelial cells, not in other types of cells, such as SMC, HSF and cancer cells (CHO-K1 cells) (Figs. 5A, B). Furthermore, both full ADAMTS1 and delta ADAMTS1 increased the caspase 3/7 activity compared with the control vector-treated

HUVEC (Fig. 5C). We also examined the effect of endothelial cell attachment incubated with each conditioned medium on extracellular matrix-coated plates. As shown in supplemental figure 1A-C, both conditioned media attenuated HUVEC attachment on type I collagen and laminin, but not on fibronectin.

Effects of ADAMTS1 and its deletion construct on tumor growth and angiogenesis

To assess the effects of ADAMTS1 gene therapy on tumor growth, HT1080, DU145 and CHO-K1 cells were inoculated subcutaneously into nude mice. After the establishment of subcutaneous tumors, plasmids in DMRIEC were directly injected into tumors every 24 h for 7 days. An empty vector was used as a negative control. On the 8th day after starting the gene transfer, both the full ADAMTS1 and delta ADAMTS1 constructs significantly had suppressed the growth of subcutaneous tumors compared with the control (Figs. 6A-F). We then examined the vessel distribution in tumors transfected with full ADAMTS1 (Fig. 7A) and delta ADAMTS1 (Fig. 7B), and compared them with cells treated with the empty vector (Fig. 7C) using an anti-CD31 antibody. The empty vector-treated implanted tumors were abundant in vessel formation, but the vessel formation was significantly decreased in tumors treated with the full ADAMTS1 or delta ADAMTS1 construct (Fig. 7D). Next we examined the apoptotic effect on the tumor vasculature by ADAMTS1. As shown in Fig. 7E-G, the

cleaved-caspase signals were observed in the tumor vasculature in the delta ADAMTS1-treated mice. On the other hand, few signals for cleaved-caspase were observed in the control mice (Fig. 7H), indicating that ADAMTS1 gene transfer induced apoptosis in the tumor vasculature *in vivo*. We also examined the transduced protein in tumor tissues. As shown in supplemental figure 2A-C, immunopositive signals for both ADAMTS1 and delta ADAMTS1 were detectable, while there was no signal in tumors treated with the empty vector (supplemental Figure 2D). These results indicated that this tumor does not produce detectable levels of ADAMTS1, and that tumor growth could be reduced by ADAMTS1 gene transduction.

Discussion

In the present study, we demonstrated that ADAMTS1 inhibited angiogenesis *in vitro* and *in vivo*, which suggested that ADAMTS1 induces endothelial cell apoptosis. These inhibitory effects of ADAMTS1 could be achieved independently of its proteolytic activity.

The anti-angiogenic properties of ADAMTS1 have been recently described. One of the most interesting properties of the gene is that it is located on chromosome 21, the locus for Down's syndrome. Reynolds *et al.*, reported that tumor angiogenesis is inhibited in a mouse model of Down's syndrome that has three copies of the ADAMTS1 gene (19). Several reports have suggested that the proteolytic activity of the protein is key to its activity. For instance, ADAMTS1 cleaves large TSP1, and cleaved TSP-1 demonstrates anti-angiogenic activity (13). Iruela-Arispe *et al.* reported that ADAMTS1 binds to the VEGF receptor, which requires protease-mediated activation (35). On the other hand, Fu *et al.*, reported that the proteolytic fragment of versican produced by ADAMTS1 induced pathological angiogenesis (36). In addition, ADAMTS1 null mice demonstrated delayed lymphangiogenesis, thus indicating that ADAMTS1 plays a role in lymphangiogenesis (37). Interestingly, Luque *et al.* reported that the C-terminal half of ADAMTS1 binds to VEGF and affects VEGFR2

phosphorylation and endothelial cell proliferation (38). Rodriguez-Manzaneque et al. reported that deletion of the last two TSP1 motifs reduced the suppression of endothelial cell proliferation (34). Together, these data indicate that the role of ADAMTS1 in angiogenesis is diverse, and that further studies are needed to clarify the numerous roles of the protein. Our present results also demonstrated that the inhibitory effects of ADAMTS1 on endothelial cells is protease activity-independent. We thus confirmed that ADAMTS1 has inhibitory effects on endothelial cells via a proteolytic activity-independent mechanism, and we suggest that the C-terminal half of ADAMTS1 has a crucial role in this activity.

We herein demonstrated that ADAMTS1 plays a role in apoptosis in endothelial cells *in vitro* and in vivo. As reported for other anti-angiogenic molecules, such as TSP-1 (39, 40), ADAMTS1 induced apoptotic changes in endothelial cells, as indicated by the detection of Annexin V expression and increased caspase 3/7 activity (Fig. 5C). Furthermore, z-vad attenuated the effect of ADAMTS1, indicating the involvement of caspase signaling (Fig. 4E). This effect was observed with both constructs, indicating that the proteolytic activity is not required for this effect. It should be noted that ADAMTS1 did not affect the viability of CHO-K1 cells, but did inhibit the growth of tumors resulting from implanted CHO-K1 cells. Interestingly, the inhibition of

proliferation was observed in endothelial cells, but not in other types of cells, including smooth muscle cells, fibroblasts and CHO-K1 cells. ADAMTS1 also induced apoptosis in endothelial cells, but not in CHO-K1 cells, as indicated by the changes in Annexin V expression. We therefore believe that the tumor inhibition, which was accompanied by a decrease in vessel density, that was induced by ADAMTS1 was largely due to the effect of the protein on endothelial cell apoptosis.

There is a limitation of this study. It is not clear whether there are specific receptor(s) for ADAMTS1 on endothelial cells. As shown in this study, ADAMTS1 attenuated endothelial cell attachment (supplemental Fig. 1), suggesting that ADAMTS1 induced anoikis on endothelial cells. Because of multiple functions of ADAMTS1 on inhibiting endothelial cells, apoptotic effect by ADAMTS1 is complex and we suggest that anoikis may be a part of this effect.

In summary, we demonstrated the anti-tumorigenic effect by ADAMTS1 gene transfer that is accompanied by the inhibition of tumor angiogenesis.

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Disclosure Statement

The authors have no conflict of interest.

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Figure legends

Figure 1. (A) Demonstration of the domain structure of full ADAMTS1 and its deletion construct used in the experiments. (B) Cell lysates (lane 1) and conditioned medium (lane 2) derived from full ADAMTS1 transfectants were analyzed. The cell lysates contained pro-ADAMTS1 protein (arrow), and conditioned medium contained two additional proteins (arrowheads). The arrowheads indicate mature ADAMTS1 and C-terminal fragment ADAMTS1 produced by its autocatalytic activity, as previously reported. (C) The cell lysates (lane 1) from delta ADAMTS1 transfectants and conditioned medium (lane 2) derived from delta ADAMTS1 transfectants.

Figure 2. The tube formation assay was carried out with conditioned medium. The conditioned medium obtained from full ADAMTS1-transfected cells before immunoprecipitation (A) and post-immunoprecipitation (B). The conditioned medium obtained from delta ADAMTS1-transfected cells before immunoprecipitation (C). The conditioned medium obtained from delta ADAMTS1-transfected cells post-immunoprecipitation (D) or from the control (E). (F) The length of tube branches in the lower field was measured. * indicates $p < 0.05$. Each column represents the mean \pm SD.

Figure 3. (A) The endothelial cells were incubated with conditioned medium and cell migration was monitored. (B) The inhibitory effect of each conditioned medium on cell migration was compared. Each column represents the mean \pm SD of triplicate samples. * indicates $p < 0.05$ vs. control. NS indicates that two groups were not significantly different.

Figure 4. The inhibition of cell proliferation by transfected-conditioned medium was monitored (A, HUVEC; B, SMC; C, HSF; D, CHO-K1). * indicates $p < 0.05$ vs. control. NS indicates that two groups were not significantly different. (E) The inhibitory effect of HUVEC proliferation by each conditioned medium was compared in the absence or the presence of Z-vad. * indicates $p < 0.05$. (F) The inhibition of endothelial cell proliferation by electroporation with each construct was examined. * indicates $p < 0.05$ vs. control. Each column represents the mean \pm SD of triplicate samples.

Figure 5. (A) The Annexin V expression was examined by FACS analysis. (B) The numbers of Annexin V-positive cells after treatment with the conditioned medium

obtained from both full ADAMTS1 (full) and delta ADAMTS1 (delta)-treated cells were compared with those after treatment with conditioned media obtained from control cells (control). *indicates $p < 0.05$ vs. control. NS indicates that two groups were not significantly different. (C) The caspase 3/7 activities in HUVEC treated with control medium were considered to be 100%. * indicates $p < 0.05$ vs. control.

Figure 6. In the left column (A, C and E), the size of tumors was monitored every day until day 8 (mean \pm SE; $n=5$). In the right column, the size of tumors on day 8 was compared with those in the control group (B, D and F). The size of each tumor on day 1 was considered to be 100% (A and B, HT1080; C and D, DU145; E and F, CHO-K1.). * indicates $p < 0.05$ vs. control.

Figure 7. The vessel formation was examined by immunohistochemistry ((A), full-ADAMTS1; (B), delta ADAMTS1; (C), empty vector). The number of blood vessels was counted (D). * indicates $p < 0.05$ vs. control. The apoptotic signals were observed in the ADAMTS1-treated tumor vasculature (E-G). Sections of the tumor in delta ADAMTS1-injected mice were doubly stained with anti-CD31 antibody (Cy3; E) and anti-Cleaved caspase-3 antibody (FITC; F). (G) Merged images obtained with

anti-CD31 antibody (Cy3) and anti-Cleaved caspase-3 antibody (FITC). Arrows indicate the caspase-3-positive signals observed in the tumor vasculature. In contrast, sections of tumors injected with empty vector showed only negligible FITC signals (green) whereas Cy3 signals (red) were more abundant than in panel G. Scale bar in each panel represents 50 μ m.

Supplemental Figure 1. Inhibition of endothelial attachment by ADAMTS1.

Supplemental figure 2. Distribution of ADAMTS1 gene transfer into tumors.

Fig. 1

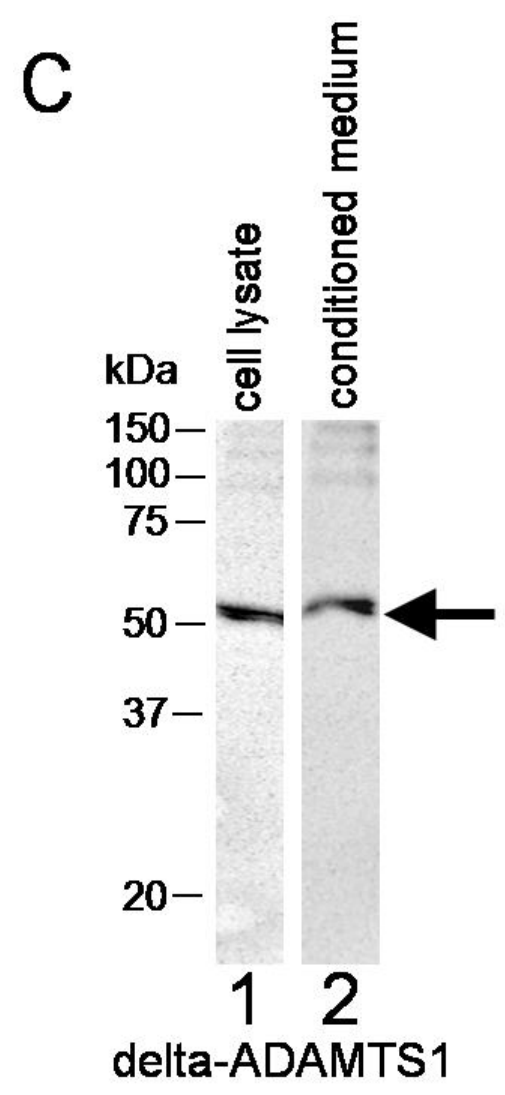
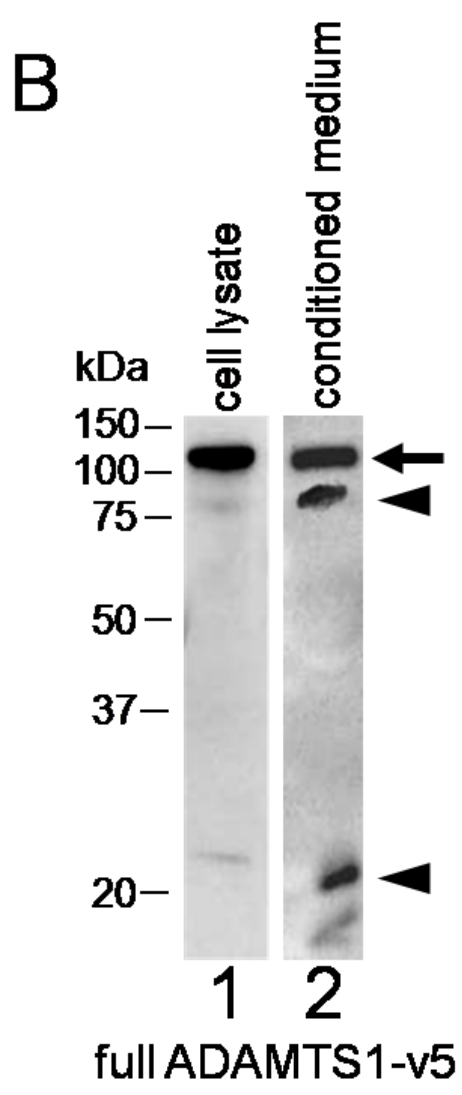
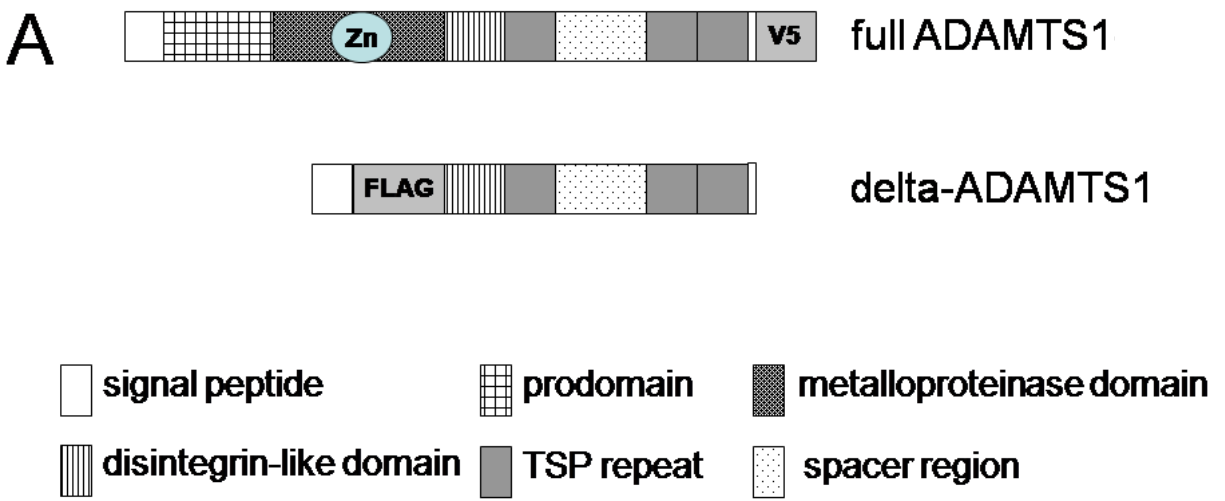


Fig. 2

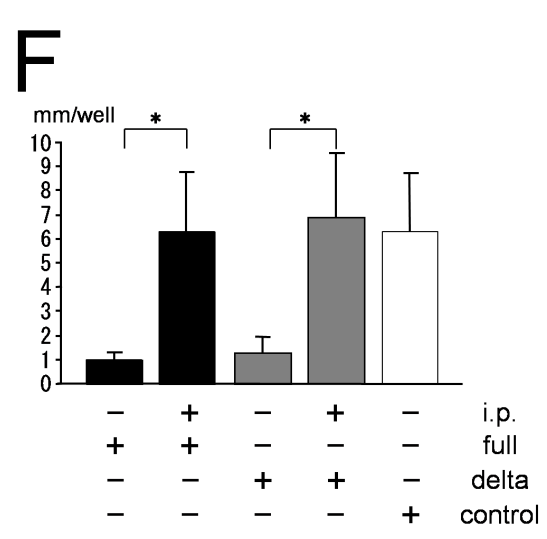
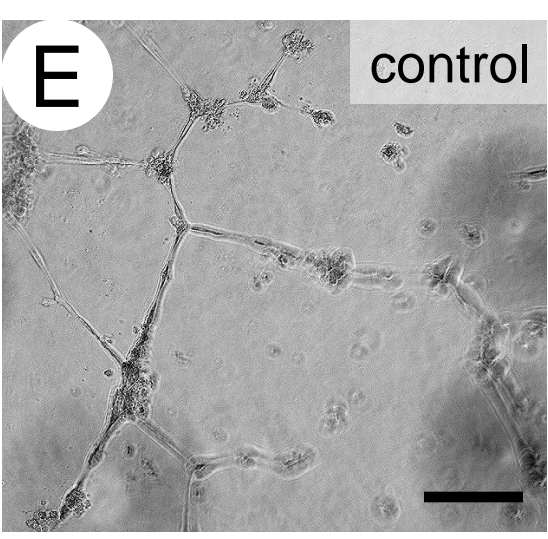
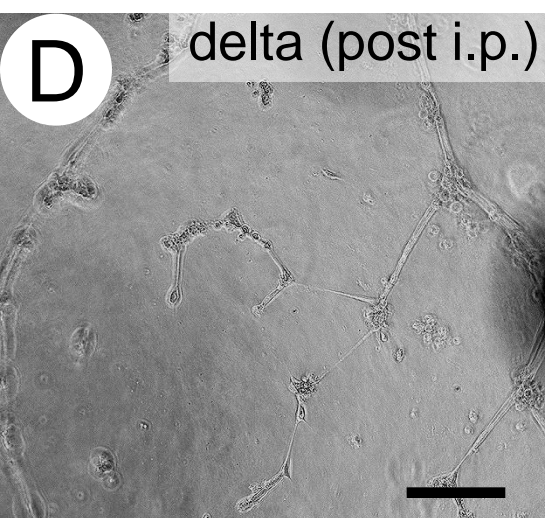
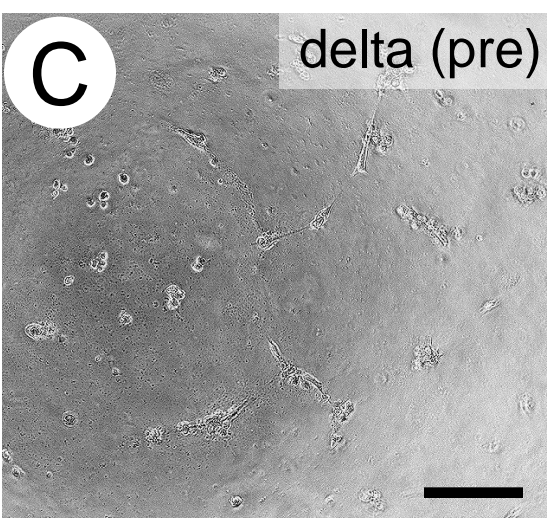
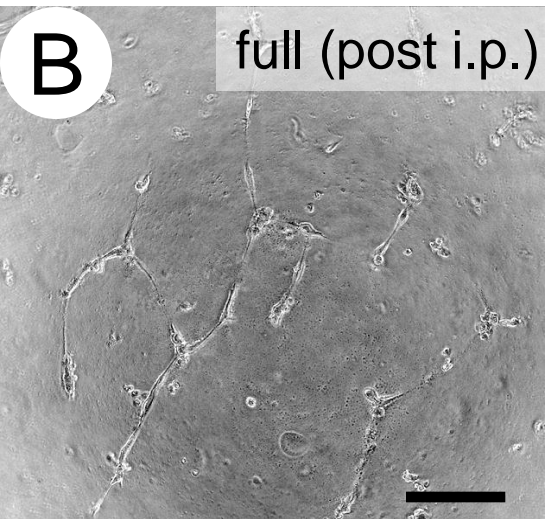
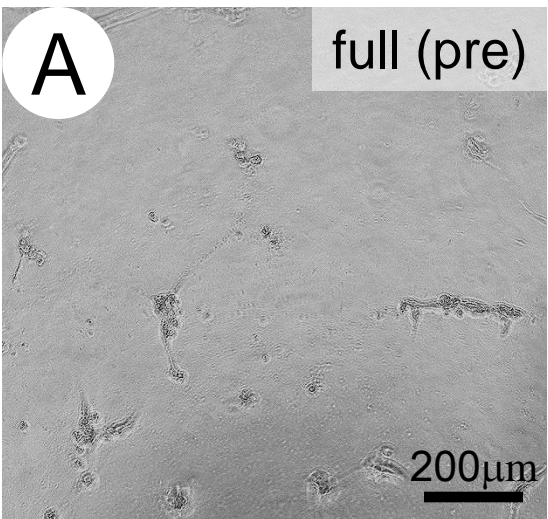
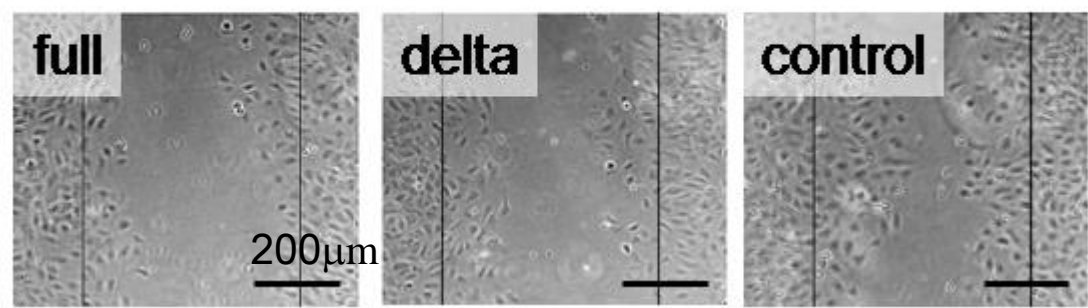


Fig. 3

A



B

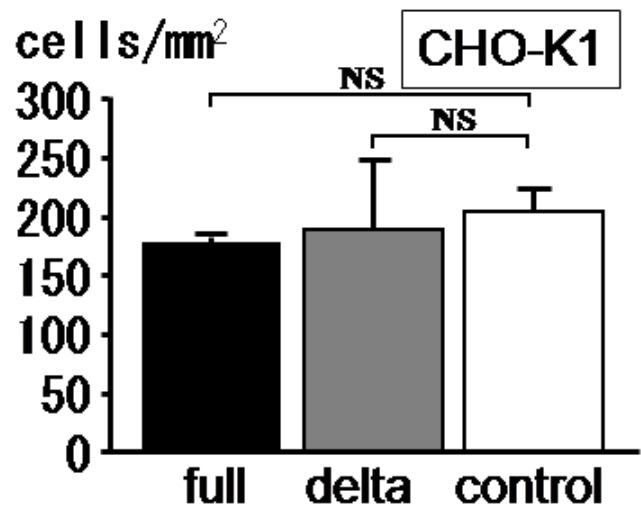
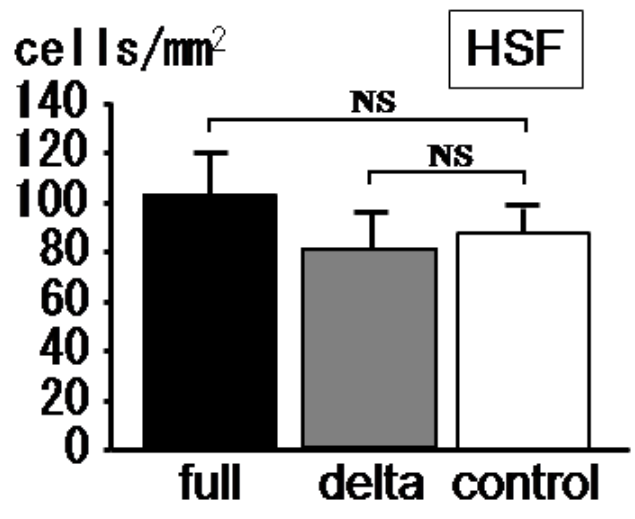
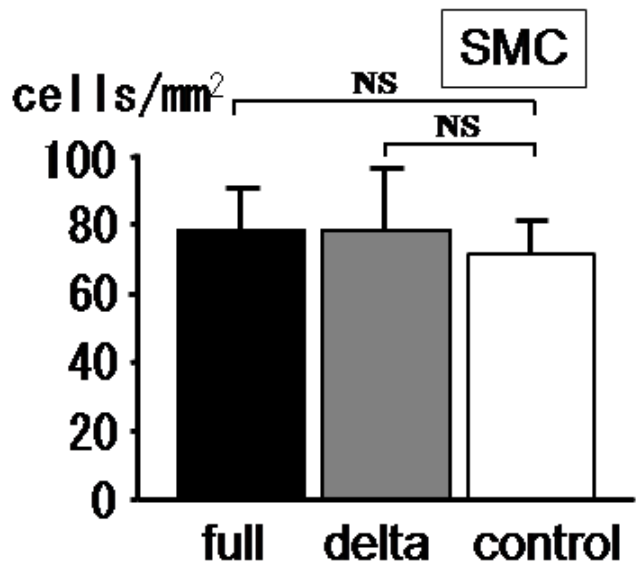
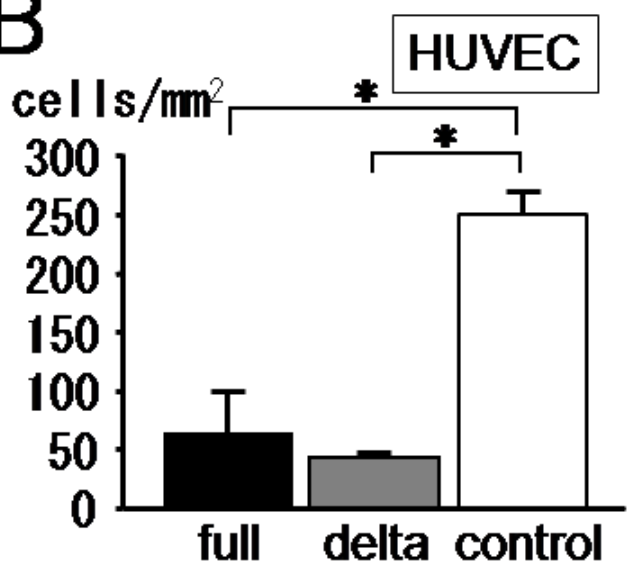


Fig. 4

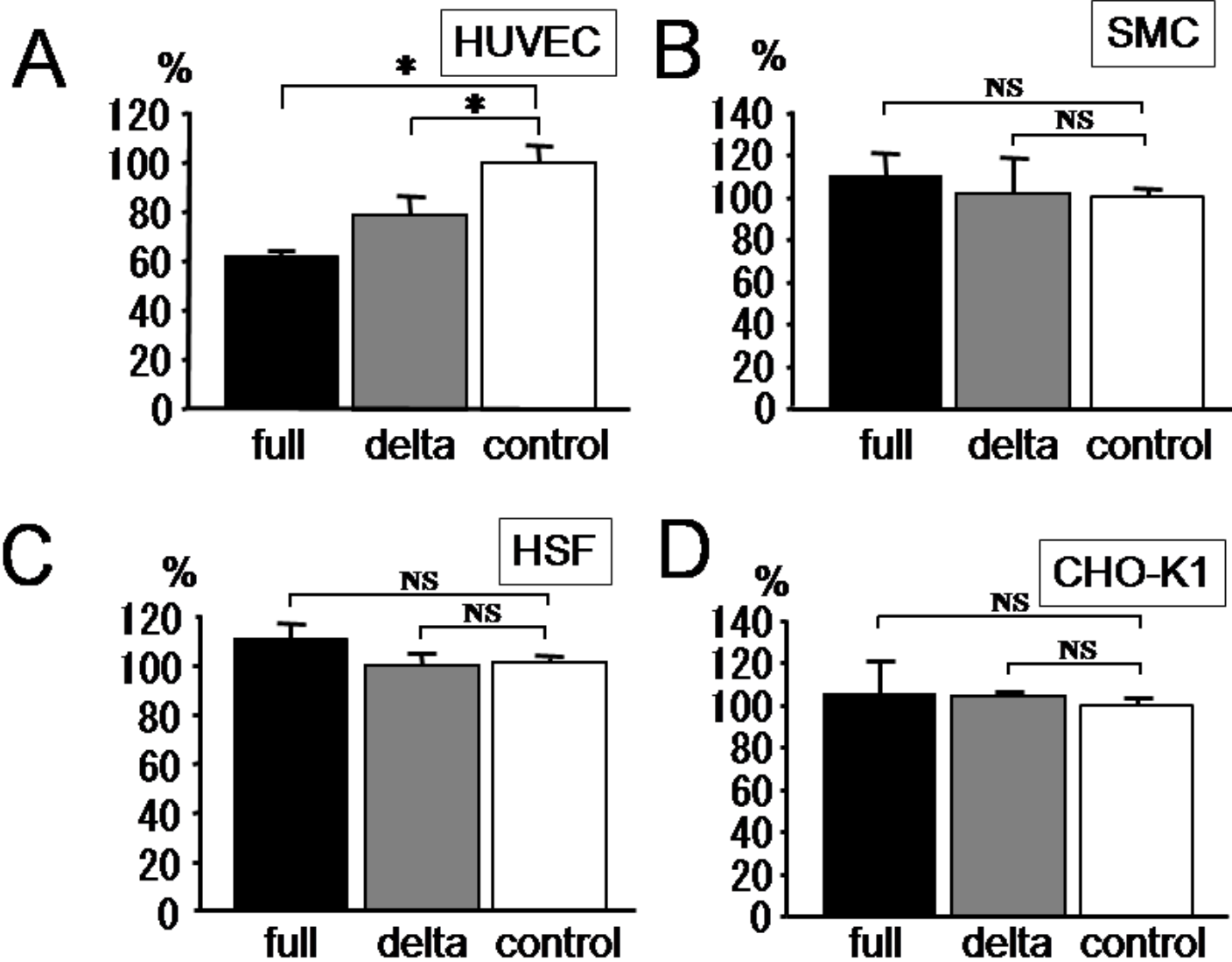


Fig. 4E

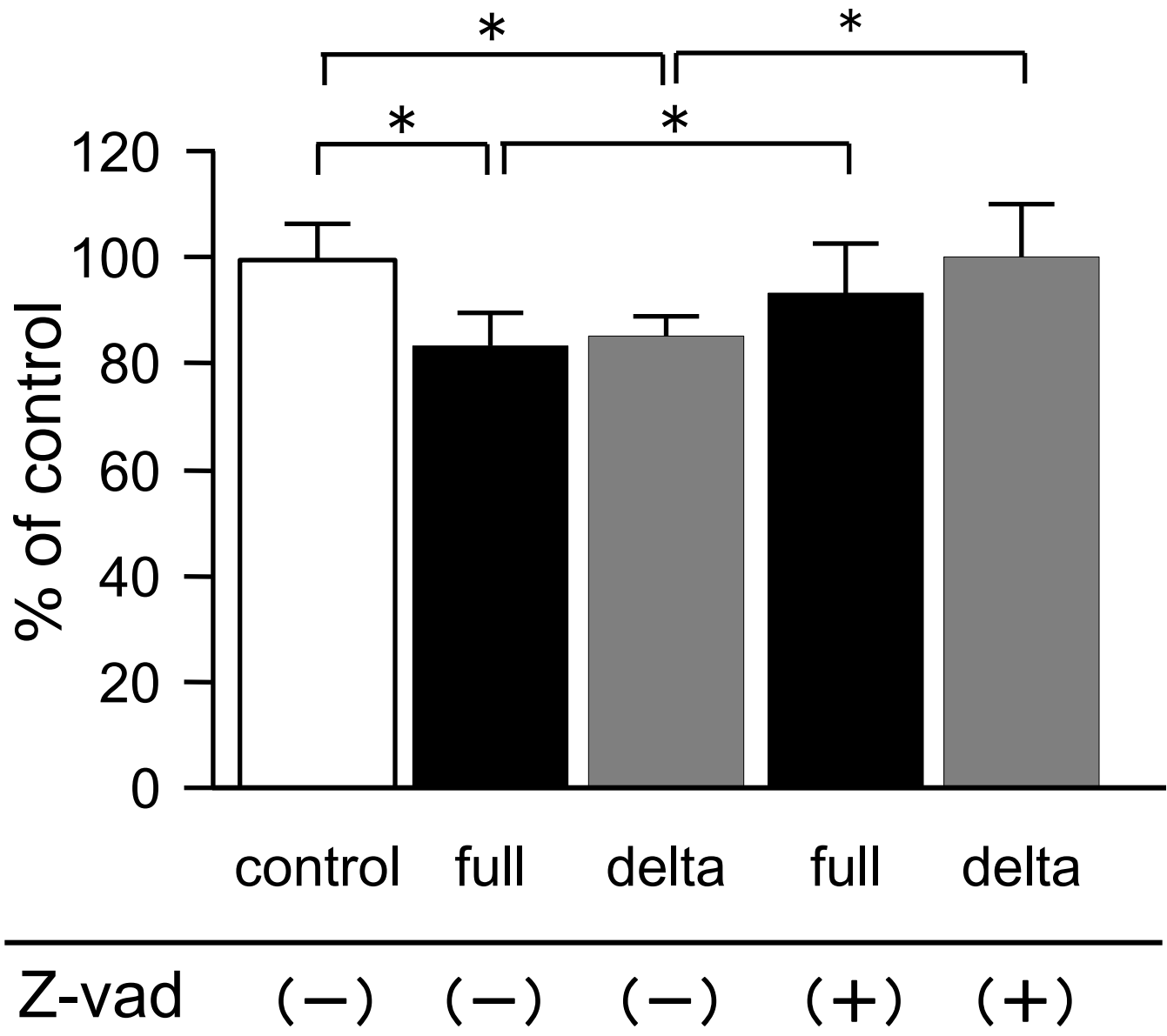


Fig. 4F

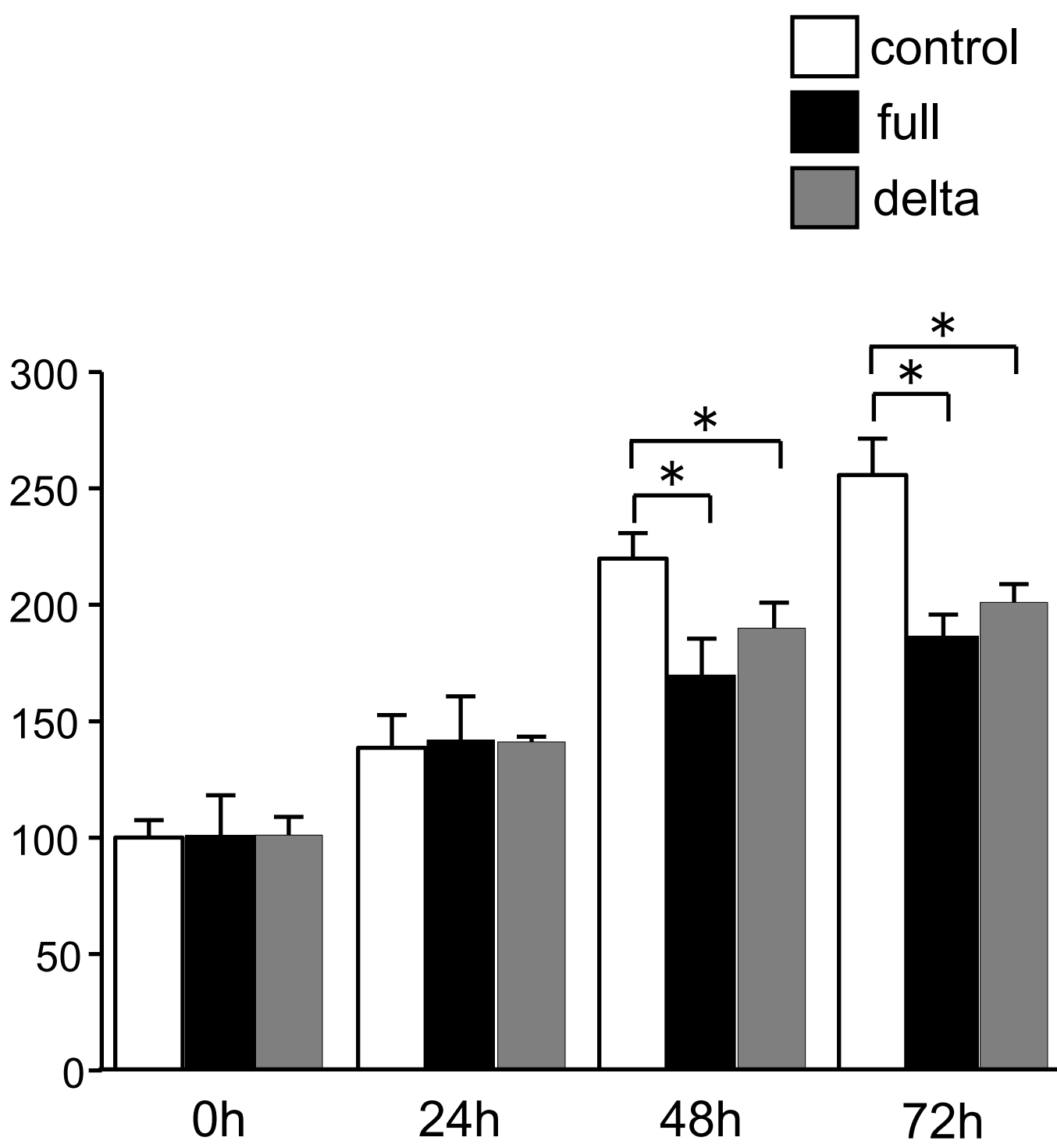
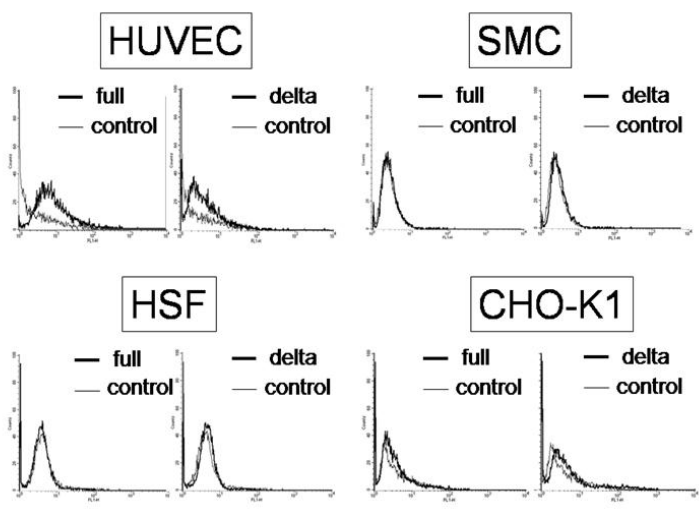
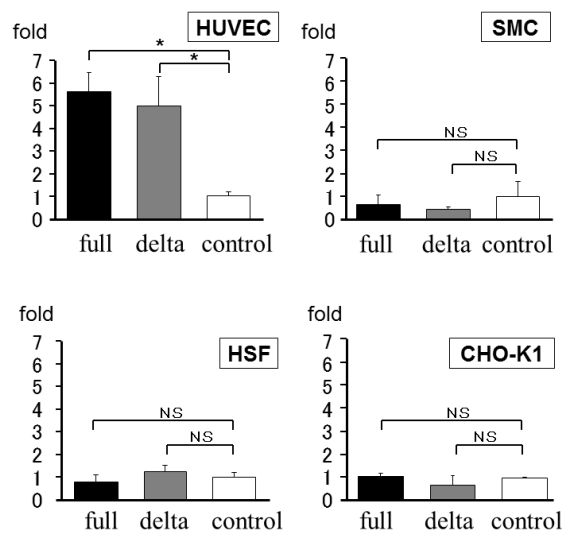


Fig. 5

A



B



C

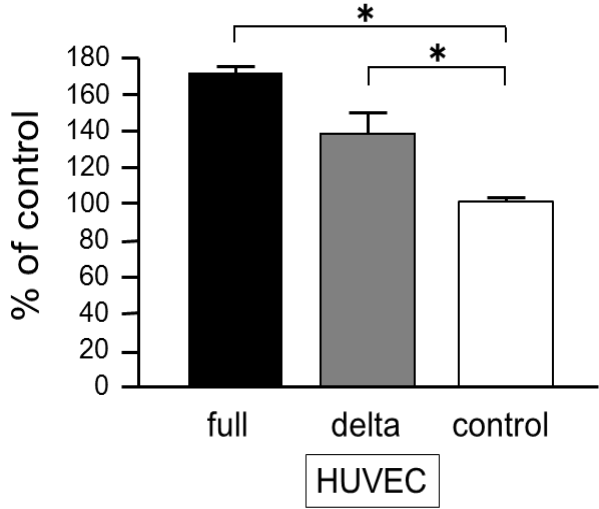


Fig. 6

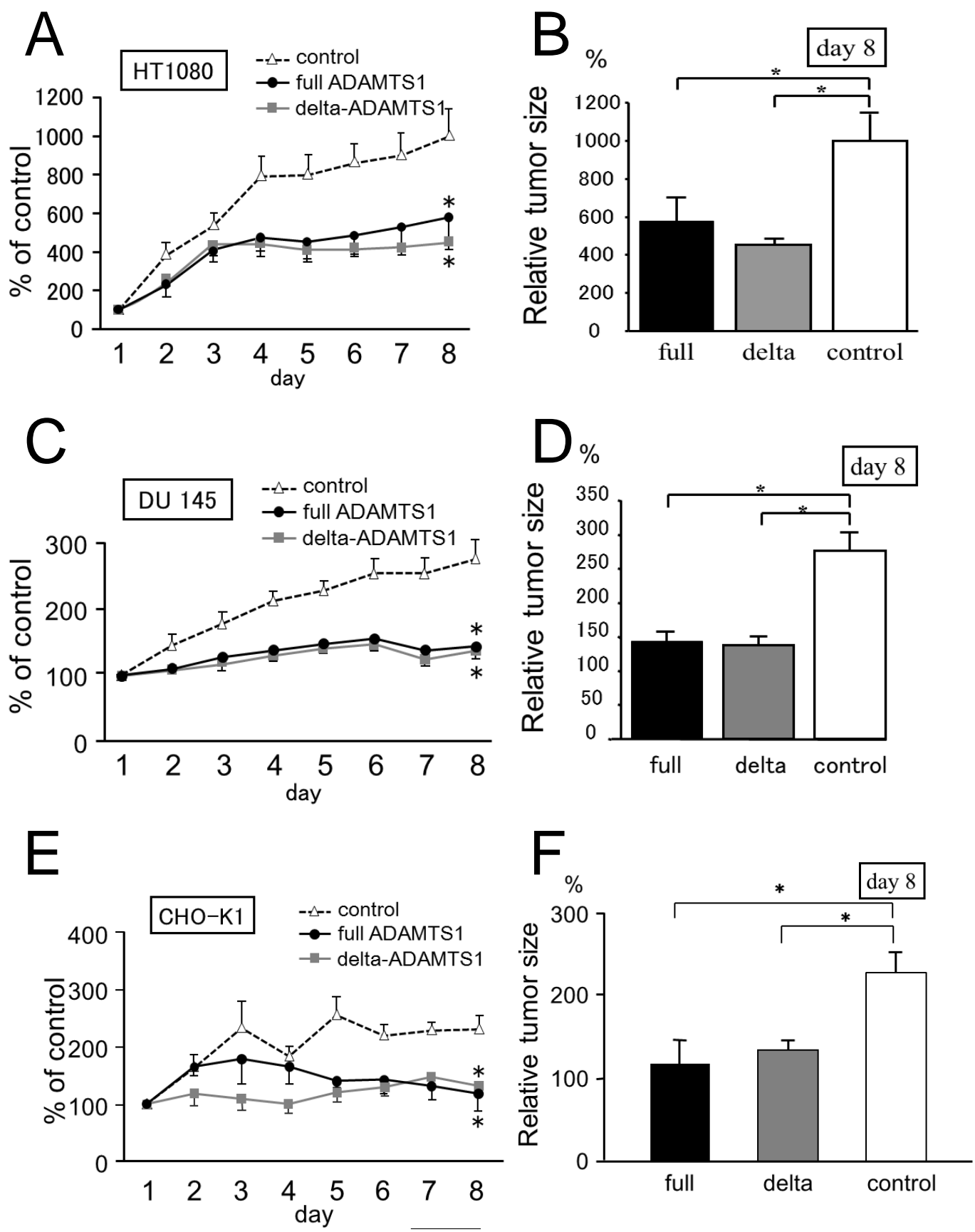


Fig. 7

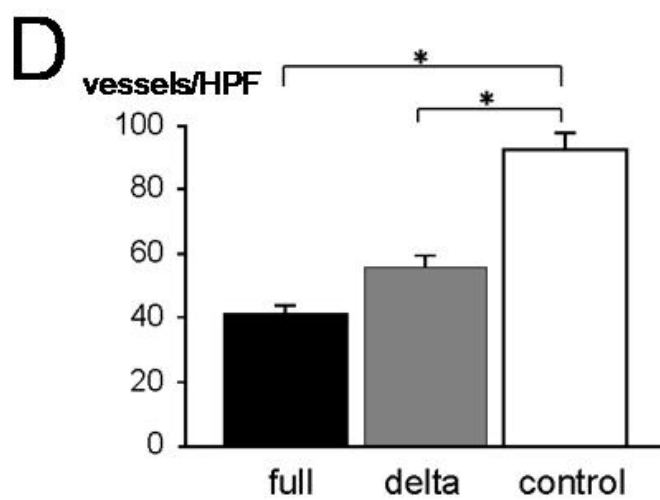
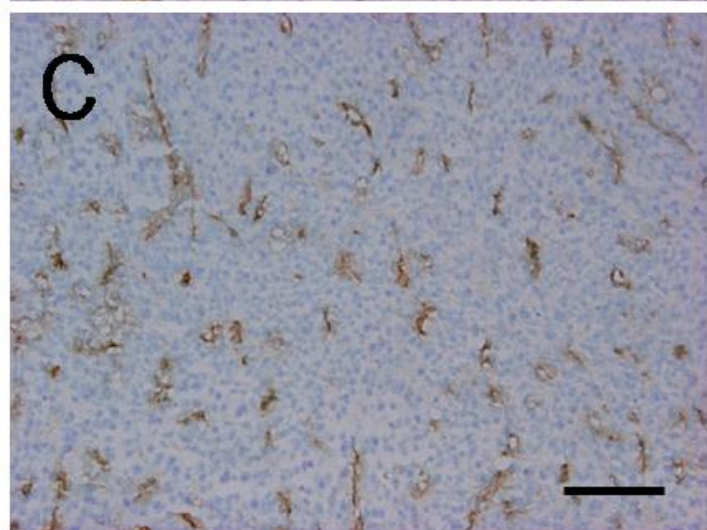
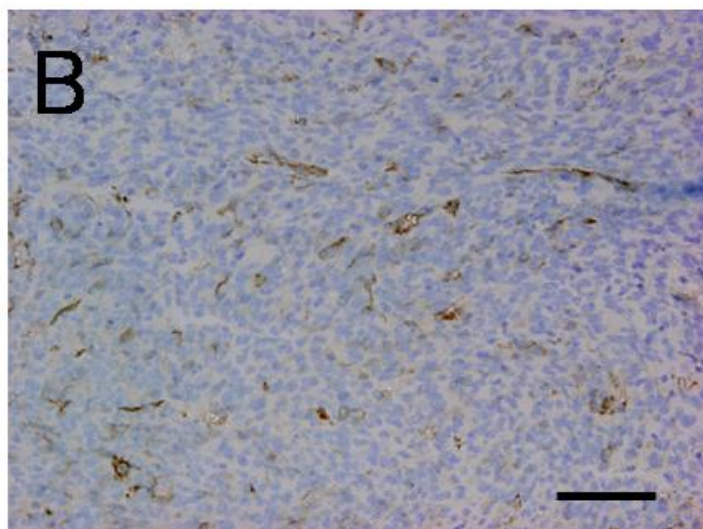
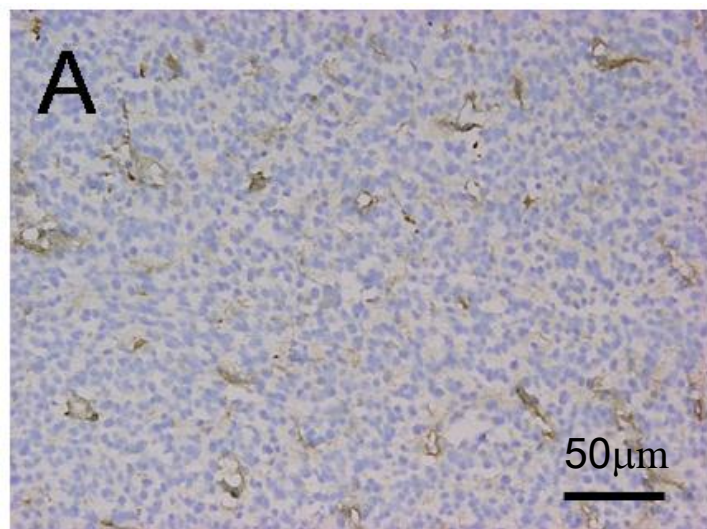
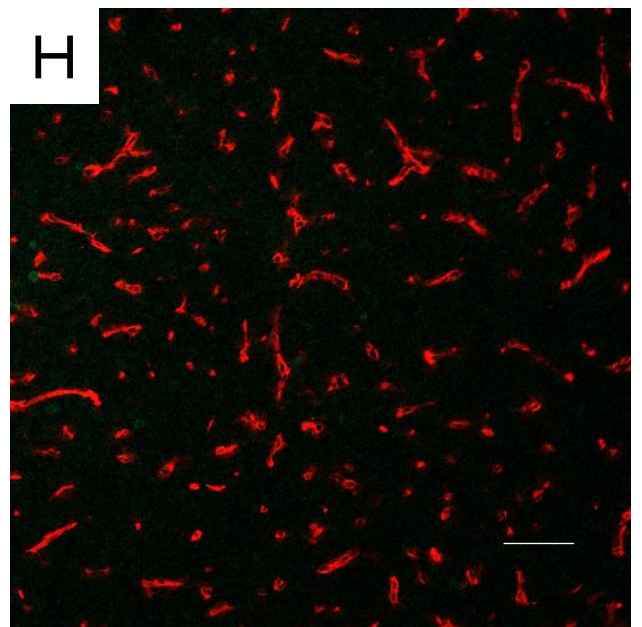
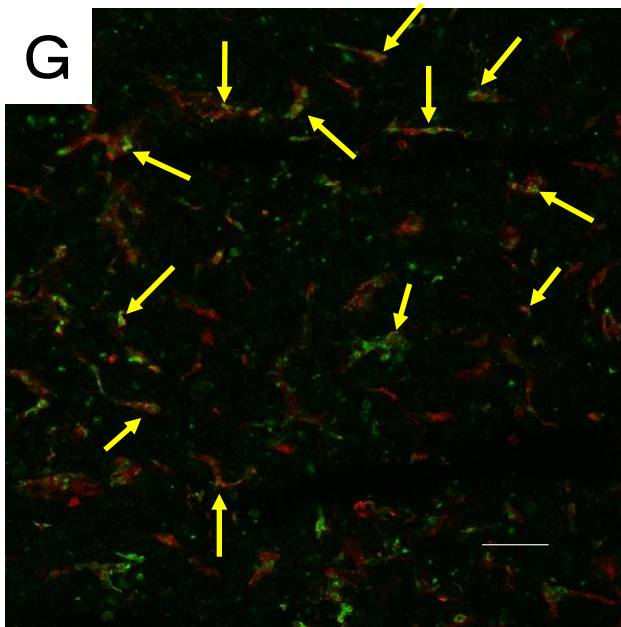
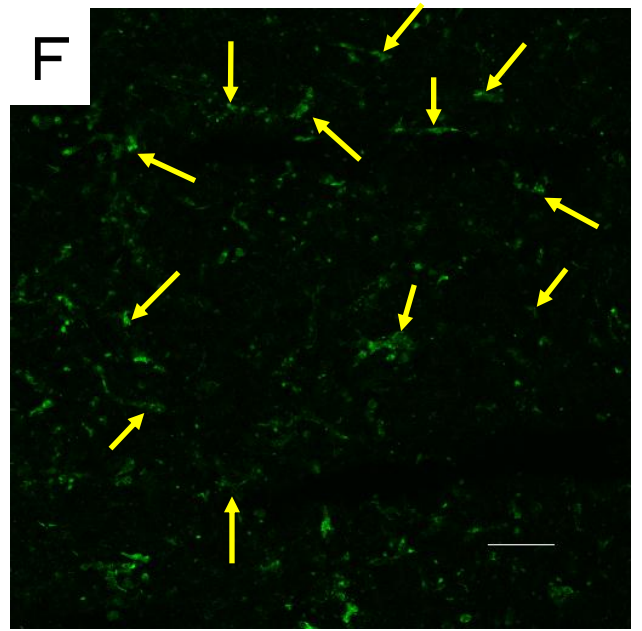
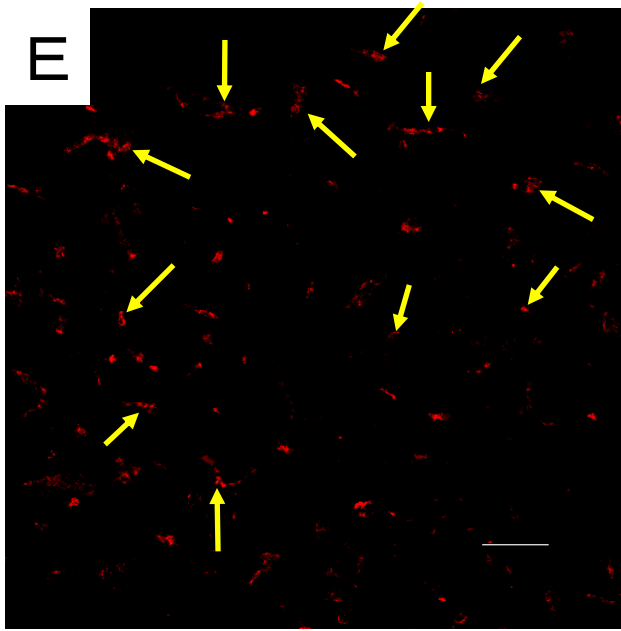


Fig. 7



Supplemental experiments:

Attachment assay

For adhesion assay, extracellular matrix protein (collagen I, laminin, and fibronectin and poly-D-Lysine) coated 96-well plates (BD Pharmingen) were used. HUVEC (1.0×10^4 cells) were suspended in 1:1 mixture of EBM2 and conditioned media, respectively, and incubated on each plate for 30 min. The medium was aspirated and the adherent cells were washed with PBS for three times, and cells were fixed with 2% PFA. Then the cells were washed and stained with 0.2% crystal violet for 5min. Stained cells were dissolved in 500 μ L of 1% SDS and the absorbance at 570nm was measured by microplate reader.

Figure legends

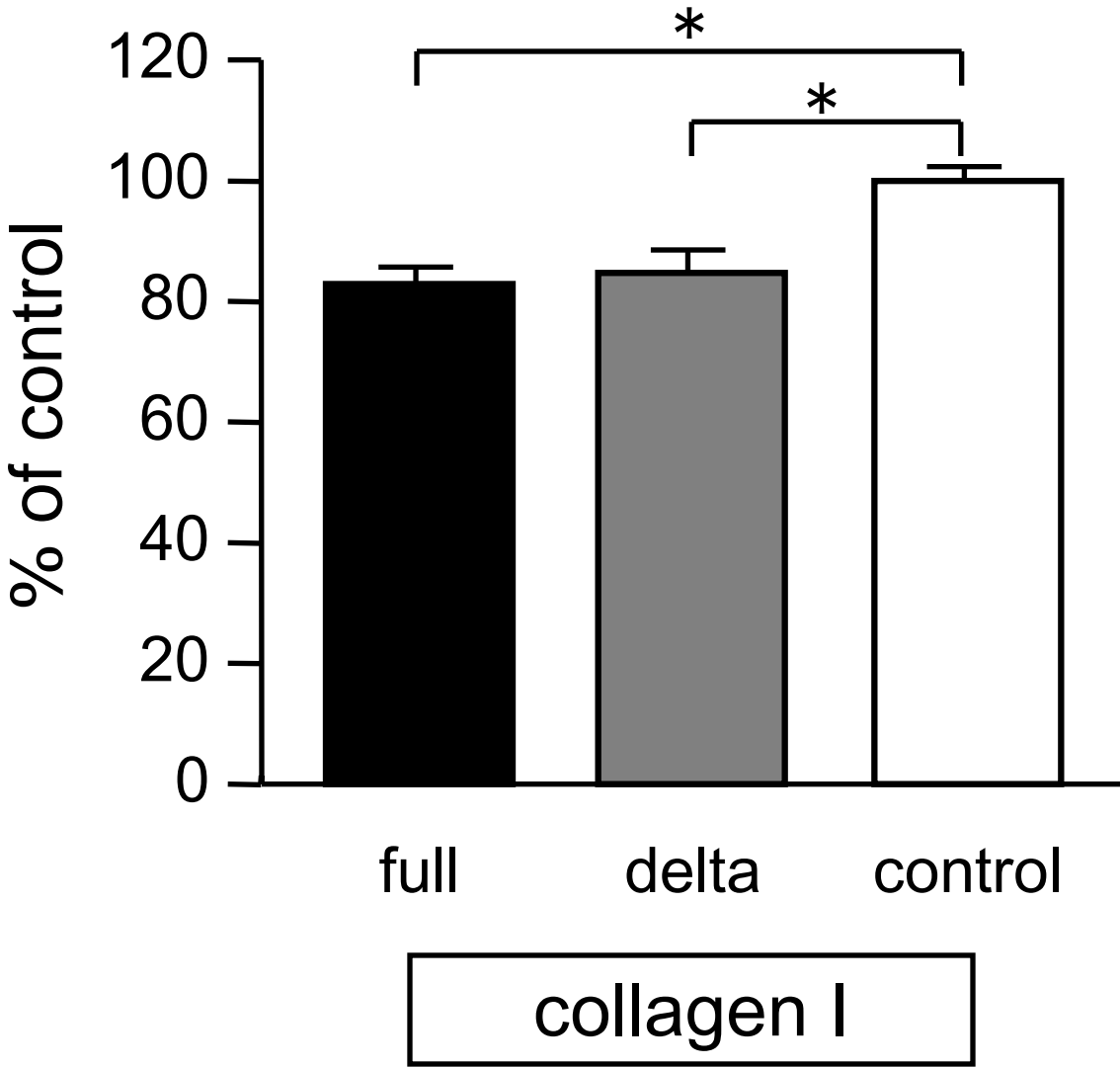
Supplemental Figure 1

Inhibition of endothelial attachment by ADAMTS1. The numbers of attached endothelial cells were measured in each coated plates (**A**: collagen I, **B**: laminin, **C**: fibronectin). The absorbance of attached HUVEC suspended in the empty vector-conditioned media was counted as 100% and the absorbance were shown as relative value. * indicates $p < 0.05$ vs. control. NS indicates that two groups were not significantly different.

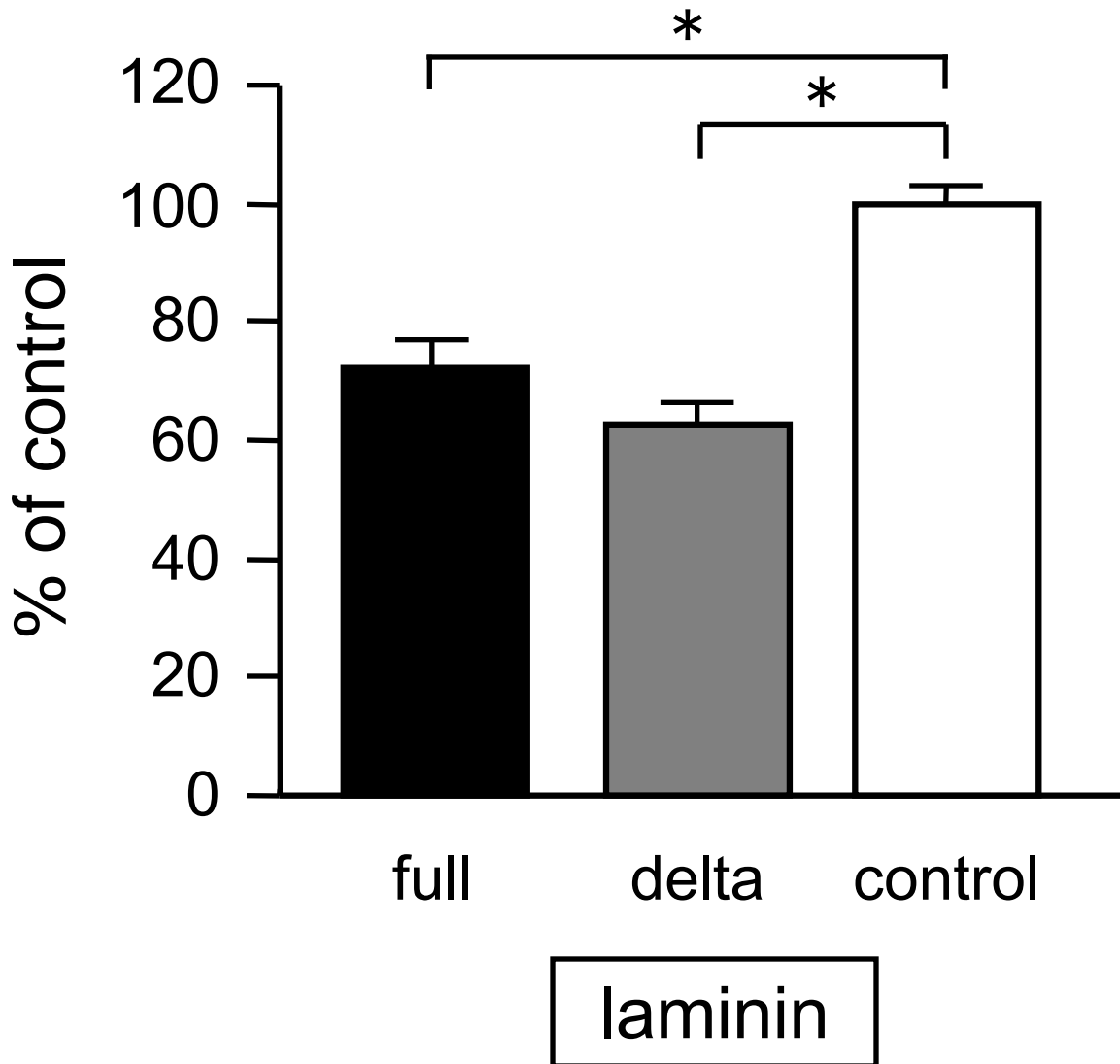
Supplemental Figure 2

Distribution of ADAMTS1 gene transfer into tumors. ADAMTS1 distribution in the tumor tissue after gene transfer was examined by immunohistochemistry. (**A**), ADAMTS1 expression was examined with anti-ADAMTS1 antibody (used at 1:100 dilution)(A-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in tumor tissue transfected with full ADAMTS1. (**B**), Tumor tissue transfected with empty vector and stained with anti-ADAMTS1 antibody. (**C**), Tumor tissue transfected with delta ADAMTS1 and stained with anti-FLAG antibody. (**D**), Tumor tissue transfected with empty vector and stained with anti-FLAG antibody. Scale bar in each panel represents 100 μ m.

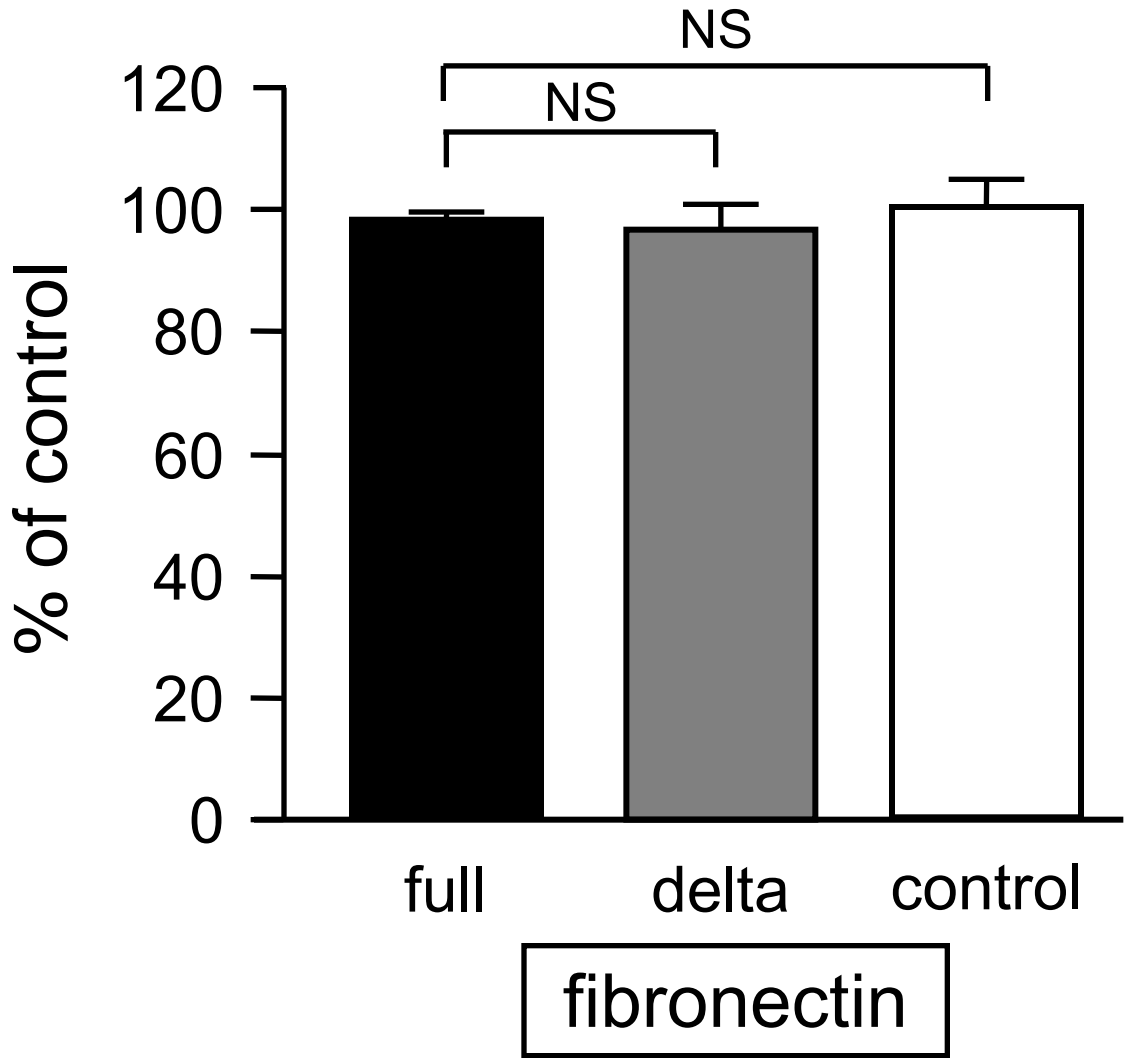
supplemental data: Figure1A



supplemental data: Figure1B



supplemental data: Figure1C



supplemental data: Figure2

