

Anti-angiogenic action of the C-terminal domain of tenomodulin that shares homology with chondromodulin-I

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Summary

Tenomodulin (TeM) is a type II transmembrane glycoprotein that contains a C-terminal domain with homology to the mature, secreted form of chondromodulin-I (ChM-I), a cartilage-derived angiogenesis inhibitor. TeM transcripts have been found in hypovascular tissues such as tendons and ligaments but the biological activity of TeM has not yet been fully explored. Using an adenovirus expression system, we utilized the forced expression and subsequent secretion of the human TeM C-terminal 116 amino acids (Ad-shTeM) in human umbilical vein endothelial cells (HUVECs) to assess the anti-angiogenic properties of TeM. The C-terminal 120 amino acids of the human ChM-I precursor (Ad-shChM-I) was similarly expressed in HUVECs as a comparison study. Transduction of both Ad-shTeM and Ad-shChM-I resulted in significant impairment of the tube-forming activity of HUVECs, when cultured in Matrigel. Similarly, conditioned medium from COS7 cells, transfected with plasmid DNA encoding shTeM or shChM-I, inhibited tube formation of HUVECs when compared to medium derived from either COS7 cells

transfected with control vector or from non-transfected cells.

Upon infection of HUVECs with Ad-shTeM or Ad-shChM-I, DNA synthesis stimulated by vascular endothelial growth factor (VEGF) was reduced to 40-50% of normal levels. Additionally, in a modified Boyden chamber assay, migration of HUVECs in response to VEGF was significantly affected following transduction of either Ad-shTeM or Ad-shChM-I and these transduced HUVECs were found to spread well on type I collagen or fibronectin, but not on vitronectin. Furthermore, the transduction of either Ad-shTeM or Ad-shChM-I in human melanoma cells resulted in suppression of tumor growth in association with decreased vessel density *in vivo*. Hence, we have demonstrated that, similarly to ChM-I, the C-terminal domain of TeM exhibits both anti-angiogenic and anti-tumor activities when expressed in a secreted form.

Key words: Angiogenesis inhibitor, Tenomodulin, Chondromodulin-I, Tendon

Introduction

The balance between stimulators and inhibitors of angiogenesis plays a dynamic role in the regulation of tissue vascularity during development and growth. Under some pathological conditions, however, an imbalance between these factors disturbs the vascular quiescence that needs to be maintained in adult tissues. It has been well documented that vascular endothelial growth factor (VEGF) and angiopoietin, among others, play central roles in the induction of angiogenesis (Carmeliet et al., 1996; Suri et al., 1996). Identification of the tumor-generated angiogenesis inhibitors, angiostatin and endostatin, have also shed light on the importance of such negative regulators during the maintenance of tissue vascularity (O'Reilly et al., 1997; O'Reilly et al., 1994).

In order to identify novel physiological inhibitors of angiogenesis, we previously characterized the growth inhibitory activity of vascular endothelial cells from fetal

cartilage, which is known to be typical avascular tissue (Hiraki et al., 1997). We isolated and purified the factor that promoted this growth inhibition, and subsequent amino acid sequencing identified it as chondromodulin-I (ChM-I) (Hiraki et al., 1991). Transfection of ChM-I precursor cDNAs into either COS7 cells or CHO cells resulted in secretion of mature ChM-I (120 amino acids) as a 25 kDa glycoprotein following processing at the furin cleavage site of the type II membrane ChM-I precursor protein (Azizan et al., 2001; Hiraki et al., 1997; Hiraki et al., 1999). *In situ* hybridization and immunohistochemistry indicated that ChM-I is specifically localized in the avascular zone of cartilage during endochondral bone formation (Hiraki et al., 1997; Shukunami et al., 1999). We demonstrated that purified recombinant human ChM-I inhibited tumor angiogenesis and growth *in vivo* (Hayami et al., 1999) and also stimulated proliferation of chondrocytes and osteoblasts (Hiraki et al., 1991; Mori et al., 1997). Our recent analysis of ChM-I knockout mice revealed an osteopetrotic phenotype,

suggesting that ChM-I acts as a bone remodeling factor following birth (Nakamichi et al., 2003).

Three independent groups have previously reported the identification of a novel gene encoding tenomodulin (TeM), which has homology to ChM-I (Brandau et al., 2001; Shukunami et al., 2001; Yamana et al., 2001). TeM is a putative type II transmembrane protein (317 amino acid residues) that has a domain at the C terminus (Phe²⁵⁵-Val³¹⁷) with homology to the cysteine-rich domain of the ChM-I precursor (Phe²⁷²-Val³³⁴). Within this 63 amino acid region, 42 amino acids are identical between TeM and ChM-1. All eight cysteine residues in the mature ChM-I protein are present in this domain and form four intramolecular disulfide bonds (Hiraki et al., 1991; Neame et al., 1990). These bonds are critical for the bioactivity of ChM-I, which is abrogated by their reduction (Hiraki et al., 1997; Hiraki et al., 1999).

Although the TeM and ChM-I precursor proteins have only limited sequence identity outside their cysteine-rich domains, they show remarkable similarity in their structural motifs in these outside regions, including the presence of a hydrophobic transmembrane domain at the N-terminal side and a BRICHOS domain at their center (Sanchez-Pulido et al., 2002). The TeM protein, however, lacks a complete furin cleavage site (Fig. 1). In the present study, we first confirmed that TeM was expressed as a type II transmembrane protein: transfection of *TeM* cDNA in COS7 cells resulted in the expression of a 40-45 kDa cell surface protein without any appreciable intra-molecular cleavage, as assessed by western blot analysis. Cell-surface TeM was identified as a type II transmembrane protein by a cell-surface biotinylation technique and subsequent confocal immunofluorescence analysis of transfected COS7 cells expressing the entire hTeM coding region. Further western blot analysis also detected endogenous TeM protein in extracts of skeletal muscle as 40-45 kDa double immunoreactive bands.

Materials and Methods

Reagents

Anti-FLAG monoclonal antibody (M2) was purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-TeM polyclonal antibodies against a polypeptide corresponding to the human/mouse TeM protein sequence between amino acids 127-136 were generated by our laboratory. VEGF was purchased from R&D (Minneapolis, MN, USA).

Cell culture

COS7 cells were obtained from the Japanese Collection Research Bioresources Cell Bank and were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO₂ in air. The 293 human embryonic kidney cell line, constitutively producing adenovirus E1 proteins, was purchased from Clonetics and maintained in DMEM containing 10% FBS at 37°C under 5% CO₂ in air. Human umbilical vein endothelial cells (HUVECs) were also obtained commercially (Clonetics) and grown in EGM2 medium (EBM complete medium with supplements; Clonetics) at 37°C under 5% CO₂ in air. Cells growing at between passages 4 and 8 were used in the experiments. The murine melanoma cell line (BL-6) (Nakayama et al., 1994) was kindly donated by the Department of Dermatology, Osaka University Graduate School of Medicine (Suita, Japan) and the cells were grown in DMEM/HAMF-12 containing 10% FBS.

Plasmid construction and transfection

Cloning of the entire coding region of human TeM cDNA (hTeM; GenBank Accession No. AF234259) was performed by 5'- and 3'-RACE (rapid amplification of cDNA ends) using Marathon Ready human fetal cDNA (Clontech, Palo Alto, CA) and the following oligonucleotide primers: forward (5'-GAG ACC ATG GCA AAG AAT CCT CC-3'), reverse (5'-CTC CTA TTA GAC CCT CCC CAG C-3'). The amplified hTeM cDNA was subcloned into the Srf-I site of the pCR-Script vector (Stratagene, La Jolla, CA, USA) and verified by sequencing. N-terminal and C-terminal FLAG-tagged hTeM (FLAG-hTeM and hTeM-FLAG, respectively) were then generated by PCR from the pCR-Script-hTeM construct using the following primers: forward primer 1 (5'-GCG CGA ATT CTC TAC CAT GGA CTA CAA AGA CGA TGA CGA CAA GGC AAA GAA TCC TCC AG-3'), reverse primer 1 (T3 primer: 5'-AAT TAA CCC TCA CTA AAG GG-3') for FLAG-hTeM and forward primer 2 (T7 primer: 5'-TA ATA CGA CTC ACT ATA GGG-3'), reverse primer 2 (5'-GCG CGC GGC CGC TCA CTT GTC GTC ATC GTC TTT GTA GTC GAC CCT CCC CAG CAT GCG GGC-3') for hTeM-FLAG. FLAG-hTeM and hTeM-FLAG amplified cDNAs were then cloned into the *EcoRV* site of pCAGGS, a previously described mammalian expression vector (Niwa et al., 1991), resulting in the pCAGGS-FLAG-hTeM and pCAGGS-hTeM-FLAG vectors, respectively.

To enable forced secretion of the 116-amino acid C terminus of human TeM (shTeM; Glu²⁰²-Val³¹⁷) and the 120-amino acid C terminus of the human ChM-I precursor corresponding to mature ChM-I (shChM-I; Glu²¹⁵-Val³³⁴), the corresponding cDNA fragments were generated by PCR using the following primers: forward primer 3 (5'-GAG GGA GAA GAT CTT CAC TTT CC-3'), reverse primer 3 (5'-AAT TAA CCC TCA CTA AAG GG-3') for shTeM and forward primer 4 (5'-GAA GTG GTA AGA AAA ATT GTT CC-3'), reverse primer 4 (5'-GCG CGG CCG CTC ACT CCA TGC CCA AGA TAC GGG C-3') for shChM-I. The amplified cDNA fragments encoding shTeM and shChM-I were separately cloned into the *EcoRI* site of the pCAGGS vector which contained both the preprotrypsin secretion signal and FLAG epitope upstream of the MCS, resulting in the pCAGGS-FLAG-shTeM and pCAGGS-FLAG-shChM-I vectors, respectively.

Transfection of cloned expression vectors into COS7 cells was performed using Lipofectamine 2000 (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Western blot analysis

Cell culture medium was collected and then eluted through a Butyl-Toyopearl column (Tosoh, Tokyo, Japan). After drying, the sample was dissolved in 1× SDS sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, and 10% glycerol. The corresponding adherent cells were washed twice with PBS and an equivalent amount of 1× SDS sample buffer was then added. Cell lysates were harvested and cleaned by centrifugation. Muscle and liver samples, dissected from 4-week-old DDD mice, were homogenized in buffer containing 8 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA and the resulting tissue extracts were centrifuged to remove cellular debris. Aliquots (~200 μg of total protein) of each supernatant were diluted in 2× SDS sample buffer.

Samples were electrophoresed on 12.5% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). After preincubation with blocking buffer (5% nonfat milk, 0.1% Tween 20, and 0.1% NaN₃ in PBS), the membranes were incubated with either rabbit anti-TeM polyclonal antibody against the synthetic peptide corresponding to the human/mouse TeM sequence from Thr¹²⁴ to Glu¹³⁶, or anti-FLAG monoclonal antibody M2 (Sigma-Aldrich, St Louis, MO, USA) followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody or HRP-conjugated anti-mouse IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA), respectively. Peroxidase activity was detected

and visualized by the enhanced chemiluminescence (ECL) plus system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Cell surface biotinylation and immunoprecipitation

COS7 cells transiently transfected with either the pCAGGS-FLAG-hTeM or the pCAGGS-hTeM-FLAG vector were biotinylated on the cell surface using the ECL protein biotinylation system (Amersham Pharmacia Biotech). Cells were solubilized with lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1% Triton-X100) and cell lysate proteins were immunoprecipitated with anti-FLAG monoclonal antibody M2 (Sigma-Aldrich), resolved by SDS-PAGE and immunoblotted with either streptavidin-DRP or anti-TeM polyclonal antibody. Proteins were visualized by ECL plus (Amersham Pharmacia Biotech).

Cell surface binding

COS7 cells transiently expressing the entire coding region of TeM tagged with FLAG were grown in a Lab-TeK II CC2 chamber slide (NALGE Nunc International Corp, Naperville, IL, USA) in DMEM supplemented with 10% FCS. The cells were then incubated with anti-FLAG M2 antibodies conjugated to FITC (1:100, Sigma Aldrich) for 2 hours at 4°C. Following extensive washing three times with cold DMEM to remove unbound antibody, the cells were fixed in PBS containing 4% paraformaldehyde for 10 minutes and then washed with TBS three times and mounted with Prolong Antifade (Molecular Probes). Cells transfected with empty vector were used as a negative control. Antibody labeling was visualized on a Zeiss LSM510 Laser-scanning microscope and image analysis was performed using the standard system operating software.

Adenovirus construction and production

FLAG-shChM-I, FLAG-shTeM and FLAG-hTeM cDNA fragments were subcloned into the *EcoRI* restriction site of pCAGGS containing an upstream preprotrypsin secretion signal and FLAG epitope (Niwa et al., 1991). These three FLAG-tagged inserts were then subcloned into the pKSCX-EGFP construct (Tashiro et al., 1999). The pKSCX-EGFP expression cassette is flanked by *SwaI* sites and contains a strong CAG promoter, IRES, EGFP and a poly(A) signal, respectively. The expression cassette of FLAG-shTeM (4.4 kb), FLAG-shChM-I (4.4 kb) or FLAG-hTeM (5.1 kb) was excised by digestion with *SwaI*, ligated with *SwaI*-digested pALC3.0 containing the E1-defective adenoviral genome, and introduced into *E. coli* with an in vitro λ phage packaging kit (Stratagene) according to the manufacturer's instructions.

The preparation method for the replication-defective E1-recombinant adenoviral vector has been previously described (Tashiro et al., 1999). Briefly, to generate infectious recombinant adenoviral vectors, 1 μ g of the indicated expression cosmid and 0.1 μ g of pMC1-Cre for the expression of Cre recombinase were cotransfected into 293 cells using Lipofectamine 2000 (Gibco-BRL). Cytopathic effects (CPE) were visible within 10 days and the culture media from cells with CPE were harvested and centrifuged to obtain supernatant fractions containing adenoviral vectors. After addition of the supernatant to 293 cells grown in large scale cultures for several further days, 293 cells with complete CPE were harvested, lysed by six cycles of freezing and thawing, and centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatants, which contained the adenoviral vector, were titrated according to standard procedures described previously (Tashiro et al., 1999). Contamination by replication-competent virus was ascertained by infecting COS7 cells and HUVECs with viral stocks and there was no sign of viral replication in the experimental cells. All manipulations of adenoviral vectors were performed in accordance with both institutional and national biosafety regulations.

Adenoviral gene transduction of HUVECs

HUVECs in culture prior to passage 8 were seeded in 75 cm² flasks and grown with 15 ml EGM2 medium to approximately 80% confluence. The cells were then infected with the indicated adenoviral vectors at a multiplicity of infection (MOI) of 10² to 10³ plaque forming units (pfu) per cell for 6 hours, washed twice with PBS, replenished with the fresh EGM2 medium, and further cultured for subsequent experiments. The transduction efficiency of adenoviral infection was monitored by fluorescent microscopy (Carl Zeiss, Munich, Germany).

Dil-Ac-LDL uptake

To confirm both the presence and maintenance of vascular endothelial characteristics following adenoviral transduction, Ac-LDL uptake assays were performed in uninfected HUVECs (wild type) and in HUVECs infected with the indicated adenoviral vectors. Cells were incubated with 10 μ g/ml Dil-Ac-LDL (Molecular Probes, Leiden, The Netherlands) in EGM2 medium for 6 hours and further incubated with 10 nM Hoechst 33342 (Sigma-Aldrich) for 2 hours. Dil-Ac-LDL uptake in living cells was analyzed using a fluorescence microscope, and merged images were generated with imaging software (Carl Zeiss).

DNA synthesis

HUVECs at early passages (4-8) were harvested with trypsin/EDTA and suspended in EGM2 at a density of 50,000 cells/ml. The cells were seeded into a 96-well collagen I-coated plate (100 μ l per well) and grown to subconfluence. The cells were then starved in 0.5% FBS-containing α MEM for 6 hours and stimulated with 20 ng/ml VEGF (R&D, Minneapolis, MN, USA) for another 12 hours. Cells were labeled with BrdU during the last 4 hours of this incubation. A MAP kinase inhibitor, PD98059 (New England Biolabs, Beverly, MA, USA) was used at a concentration of 50 μ M. Incorporation of BrdU into DNA was determined with a bromo-2'-deoxyuridine labelling kit III according to the manufacturer's instructions (Roche, Basel, Switzerland). Absorbencies at 405 nm were measured using a Model 450 Microplate Reader (Bio-Rad, Hercules, CA, USA).

Matrigel tube formation assay

24-multiwell culture plates (Costar, Corning, NY) were coated with growth factor-reduced Matrigel (0.4 ml; Becton Dickinson Labware, Bedford, MA, USA) and incubated at 37°C for 30 minutes. HUVECs starved for 4 hours were treated with trypsin-EDTA and suspended in the culture medium for 20 minutes. The cells were seeded at a density of 20,000 cells per well on polymerized Matrigel in the presence of VEGF (20 ng/ml). After incubation at 37°C for 6 hours, photographs were taken under a phase contrast light microscope (Carl Zeiss). To quantitatively evaluate the capillary-like morphogenesis of HUVECs, total lengths of tube-like structures per field were measured using image processing and analysis software (NIH image version 1.61, available in the public domain via the National Institute of Health, Bethesda, MD). Each experiment was performed four times.

Adhesion and spreading assays

Adhesion assays were performed essentially as described elsewhere (Tang et al., 1999) with minor modifications. Briefly, both transduced and wild-type early-passage (4-8) HUVECs were cultured for 24 hours, harvested with trypsin/EDTA and suspended in the culture medium for 20 minutes. After re-suspension of cells at a density of 5 \times 10⁵ cells/ml in α MEM containing 0.1% BSA, cells were inoculated in 96-multiwell plates coated with either type I collagen, fibronectin or vitronectin (100 μ l per well), and were incubated for 1 hour at 37°C. Cells were then rinsed with PBS, fixed with 4%

paraformaldehyde in PBS for 30 minutes, rinsed again with PBS, stained with 0.2% crystal violet in 80% methanol for 30 minutes, and finally rinsed 3 times with deionized water. The crystal violet stain was eluted with 0.1 M sodium citrate (pH 4.2) in 50% ethanol and the number of adherent cells in each well was evaluated by the measurement of absorbance at 596 nm using a microplate reader (BioRad). Cell spreading was monitored by crystal violet staining after incubation of the transfected cells in chamber slides coated with the indicated extracellular matrix at 37°C for 1 hour, as described above. Cells were also photographed using a fluorescence microscope (Carl Zeiss).

Cell migration assays

Migration of HUVECs was assessed using modified Boyden chambers (Costar) with the upper chamber containing a polycarbonate membrane filter of 8.0 μ m pore size. Membrane surfaces were coated with vitronectin (5 μ g/ml) for 1 hour at 37°C. Both transfected and non-transfected HUVECs at early passages (4-8) were starved in 0.5% FBS-containing α MEM for 4 hours, and then harvested with trypsin/EDTA. After suspension in the medium for 20 minutes and transfer to the migration buffer (α MEM containing 0.1% BSA), cells were seeded at a density of 5×10^4 cells per well (final volume, 100 μ l) on the filter. Cell migration was induced by addition of 600 μ l migration buffer containing 20 ng/ml VEGF to the lower chamber. After incubation for 4 hours at 37°C, the number of migrating cells was counted under a microscope using a hemocytometer (6 fields per filter) after staining of the cells on the lower surface of the membrane filter with Hematoxylin. Cells on the upper surface of the membrane were removed by mechanical cleaning and extensive washing with deionized water.

In vivo tumor growth model

Anti-tumorigenic and angiogenic activities were evaluated by in vivo tumor growth model of the melanoma cell line BL-6 that forms vascular-rich solid tumors when implanted subcutaneously. Tumors were generated by subcutaneously implanting 1×10^6 BL-6 melanoma cells transfected with either Ad-shChM-I, Ad-shTeM or Ad-EGFP onto the backs of 6- to 8-week-old C57BL/6 mice. Animals were humanely sacrificed at the specific indicated times according to institutional animal care guidelines and tumor weights were

measured. Three independent experiments with 16 mice in each group (shChM-I- or shTeM-transfected cells, mock transfected cells, and wild-type cells) were carried out. Animals were sacrificed on day 21 for immunohistological analysis. Data are given as the mean \pm s.d. (standard deviation) of four mice from each group. For the measurement of vascular density in tumors, micro cryostat sections of each tumor were prepared according to standard procedures. The sections were incubated with 1% BSA in PBS for 4 hours at room temperature, followed by incubation with a monoclonal antibody against PECAM (CD31: Molecular Probes) at a dilution of 1:400 in 1% BSA in PBS overnight. After further incubation with rhodamine-conjugated antibody and 10 nM Hoechst dye 33342 (Sigma-Aldrich) for 2 hours, the sections were photographed and the images merged with a fluorescence microscope coupled to an imaging computer system (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analyses for comparison of data were made with one-way analysis of variance (ANOVA), followed by Scheffé's multicomparison test. A *P* value of <0.05 was considered statistically significant.

Results

Immunodetection of endogenous TeM protein in mice

A Kyte-Doolittle hydrophathy plot revealed that TeM is a type II transmembrane protein with a C-terminal 63 amino acid region (from Phe²⁵⁵ to Val³¹⁷) that shows high homology to a C-terminal domain of mature ChM-I (Fig. 1) (Shukunami et al., 2001). TeM has a hydrophobic transmembrane region in its N terminus, but a classic signal sequence was not found. Additionally, a cleavage site (Arg-Glu-Arg-Arg) present in ChM-I is not present in TeM (Shukunami et al., 2001), although another potential cleavage motif (Arg-X-X-Arg) is present at position 233-236 in both human and mouse TeM (Barr, 1991). To further analyze the biochemical characteristics of TeM proteins, COS7 cells were transiently transfected with cDNAs containing the entire coding region of TeM that had been separately FLAG-tagged at both the N and C terminus.

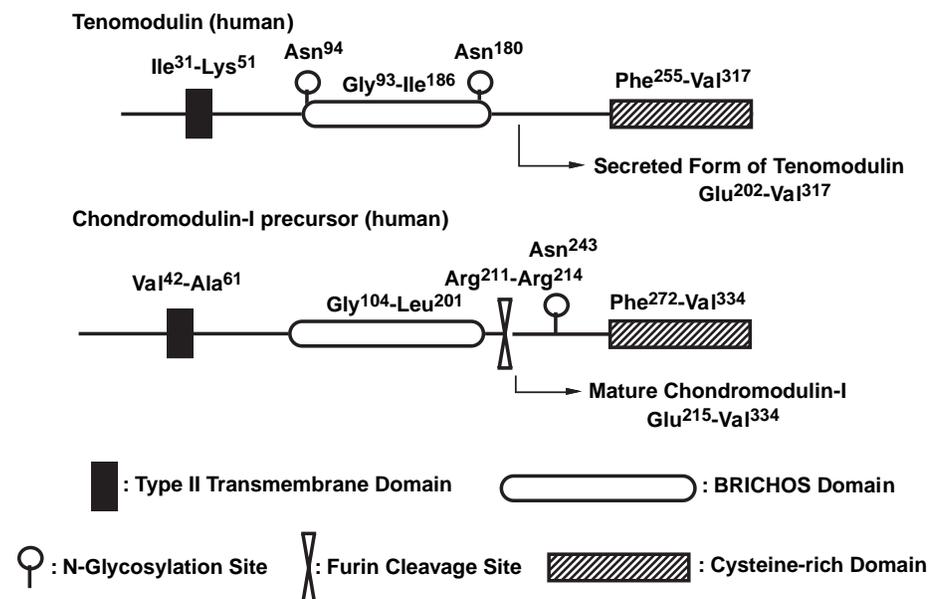


Fig. 1. Structural features of human tenomodulin (TeM) and the human chondromodulin-I (ChM-I) precursor proteins deduced from their cDNA sequences. Analysis of primary amino acid sequences reveals several structural features in the putative TeM protein. These include a type II transmembrane domain at the N terminus, a BRICHOS domain and a cysteine-rich domain (Sanchez-Pulido et al., 2002). The TeM protein contains two N-glycosylation sites within the BRICHOS domain. The highest homology with the ChM-I precursor was scored in the cysteine-rich domain at the C terminus (Sachdev et al., 2001; Shukunami et al., 2001).

As predicted from the deduced amino acid sequence, expressed recombinant TeM protein was detected by western blotting as a double band, with approximate molecular masses of 40 and 45 kDa, that most probably resulted from differential glycosylation (Yamana et al., 2001),

No immunoreactive band was detected in culture medium from COS7 cells that had been transfected with cDNA containing full-length human TeM (Fig. 2A). However, upon transfection of either the FLAG-tagged C-terminal 116 amino acids (Glu²⁰²-Val³¹⁷) of TeM or the FLAG-tagged C-terminal 120 amino acids (Glu²¹⁵-Val³³⁴) of the ChM-I precursor, under the control of the preprotrypsin secretion signal, both FLAG-shTeM or FLAG-shChM-I bands could indeed be detected in the culture media as well as in cell lysates (Fig. 2A). Cell-surface biotinylation experiments indicated that TeM is present as a transmembrane protein with its C terminus exposed to the exterior surface of cells (Fig. 2B). The expression of full-length

C-terminal FLAG-tagged hTeM on the cell surface of transfected COS7 cells was also demonstrated using confocal fluorescence microscopy, which detected the specific interaction of FITC-conjugated anti-FLAG M2 monoclonal antibodies with the FLAG epitope (Fig. 2C). Permeabilized cells transfected with hTeM, FLAG-tagged at either terminus showed diffuse fluorescent signals throughout the cell bodies (Fig. 2D,F). No fluorescent labeling was observed in COS7 cells transfected with empty vector (Fig. 2G,H). These studies indicated that TeM is a type II transmembrane protein with a short cytoplasmic tail and with the C terminus expressed on the exterior cell surface.

We performed a series of biochemical analyses of the translation products of the TeM gene, using a COS7 cell experimental system, as the biochemistry of endogenous TeM proteins that have not been previously reported. Extracts from mouse muscle and liver were analyzed by SDS-PAGE and we

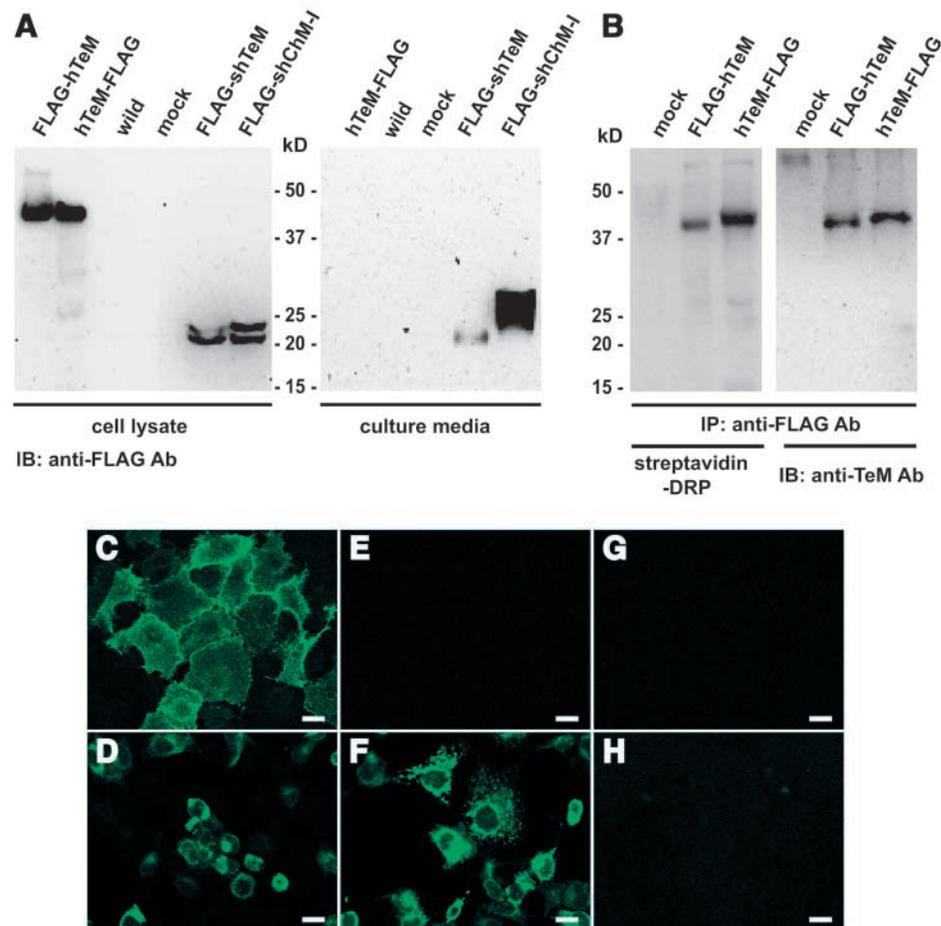


Fig. 2. Characterization of the TeM protein. (A) Detection of human TeM and ChM-I proteins expressed in COS7 cells. COS7 cells were transfected with empty vector (mock), N-terminal FLAG-tagged (FLAG-hTeM) or C-terminal FLAG-tagged (hTeM-FLAG) full-length hTeM (wild), N-terminal FLAG-tagged secreted human TeM (Glu²⁰²-Val³¹⁷) (FLAG-shTeM) and N-terminal FLAG-tagged secreted human ChM-I precursor (Glu²¹⁵-Val³³⁴) (FLAG-shChM-I). Cell lysates and culture media were analyzed by western blotting using anti-FLAG monoclonal antibodies. (B) Identification of human TeM as a transmembrane protein by cell surface biotinylation. COS7 cells transfected with empty vector (mock), FLAG-hTeM cDNA or hTeM-FLAG cDNA were biotinylated on their cell surfaces. The expressed protein was immunoprecipitated with anti-FLAG antibody and detected by streptavidin binding and anti-TeM antibody, respectively. (C,E,G) COS7 cells expressing TeM tagged with either FLAG or empty vector were incubated with FITC-conjugated anti-FLAG M2 antibody for 2 hours at 4°C under nonpermeabilizing conditions and fixed with PBS containing 4% paraformaldehyde. (D,F,H) Permeabilized COS7 cells were incubated with FITC-conjugated anti-FLAG M2 antibody. Cells expressing hTeM-FLAG are shown in C and D, FLAG-hTeM in E and F and empty vector in G and H, respectively. Scale bars: 20 μm.

observed electrophoresis patterns of these extracts that were similar to previously reported observations (Louis and Sekeris, 1976; Scopes and Penny, 1971). We then performed western blot analysis using an antibody raised against synthetic peptides corresponding to the human TeM sequence from Thr¹²⁴ to Glu¹³⁶ (Fig. 3). Endogenous mouse TeM was indicated by the presence of a double band with a molecular mass of approximately 45 kDa in extracts from muscle. By contrast, no immunoreactive band was detected in extracts from liver (Fig. 3). We also performed western blot analysis using antibodies raised against the human TeM sequence Ile²⁴⁵ to Gly²⁵² (Yamana et al., 2001) and obtained similar results (data not shown). Thus, there was no indication of the existence of shorter cleaved forms of TeM in muscle. As revealed by immunohistochemistry, TeM proteins was exclusively present in epimysium and tendon, where TeM transcripts were abundantly expressed (data not shown) (Shukunami et al., 2001). These results indicate that TeM is a type II transmembrane protein and that its C terminus is exposed on the exterior surface of both epimysium and tendon/ligaments during embryonic development.

Inhibition of tube formation of HUVECs following treatment with conditioned medium from hTeM transfected COS7 cells

To test for possible biological activity of TeM proteins in vascular endothelial cells, we prepared conditioned medium from COS7 cells that had been transfected with either the full-length or the secretable C-terminal 116 amino acids of human TeM. For comparison, the C-terminal mature protein derived from the ChM-I precursor was also expressed in COS7 cells in its secretable form. pCAGGS-shChM-I or pCAGGS-shTeM were transiently transfected into COS7 cells by lipofection and the conditioned medium was collected after 48 hours. Production of shChM-I or shTeM was confirmed by western blotting (Fig. 2A). When treated with conditioned medium containing either shTeM (the secreted C-terminal domain of TeM) or shChM-I (the secreted mature ChM-I domain),

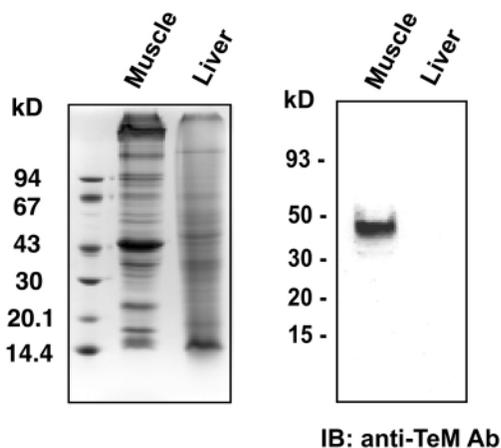


Fig. 3. Detection of TeM protein in mouse skeletal muscle. Tissue extracts were prepared from muscle and liver of 4-week-old DDY mice. Western blotting was carried out with anti-TeM antibodies. Immunoreactive bands were detected in muscle but not in liver extracts.

HUVECs failed to form tubular structures in culture on Matrigel (Fig. 4A,B). Conditioned medium from COS7 cells transfected with the entire extracellular domain of TeM, from His⁵² to Val³¹⁷, also inhibited tube formation of HUVECs (data not shown). By contrast, conditioned medium prepared from mock-transfected COS7 cells did not affect HUVEC tube formation (Fig. 4A,B). In addition, conditioned medium from COS7 cells expressing other regions of TeM (His⁵²-Glu²⁰¹ or His⁵² to Glu²⁵⁴) also did not inhibit tube formation of HUVECs (data not shown).

Recombinant adenoviral expression of the human TeM C-terminal 116-amino acids and the human ChM-I C-terminal 120-amino acids

To examine the angiogenic properties of TeM, we generated adenoviral vectors that expressed either the C-terminal region of human TeM, containing residues Glu²⁰² to Val³¹⁷ (Ad-shTeM), or full-length human TeM (Ad-hTeM) (Fig. 5A). For comparison, we similarly constructed an adenoviral vector (Ad-shChM-I) expressing the 120 amino acids corresponding to mature human ChM-I (Fig. 5A). The adenoviral vectors had been modified to express GFP fusion protein products under the control of an IRES cassette and containing the preprotrypsin secretion signal. For the efficient generation of recombinant adenoviruses, we utilized the *Cre-loxP* recombination system and the circular form of the adenoviral genome cloned into a cosmid for the purposes of constructing E1-substituted adenoviral vectors (Tashiro et al., 1999). In this system, the backbone of the pALC3.0 cosmid vector was modified by replacing the E1 region of the circular form of adenoviral genome with the *loxP*-flanked cosmid cassette.

Cotransfection of a *Cre*-expression plasmid with the indicated pALC3.0 cosmid vector into 293 cells efficiently generates recombinant adenoviral vector that contains the indicated expression cassette, but lacks the cosmid vector backbone. Within 8 days of transfection, cytopathic effects (CPE) was visible in each of the wells containing 293 cells that had been cotransfected with the cosmid vector containing the expression cassette and the *Cre*-expression plasmid. Most HUVECs was deemed to have been infected as determined by the expression of EGFP (Fig. 5B). As shown in Fig. 5C, normal Dil-AcLDL up-take was observed in HUVECs transduced with Ad-EGFP.

Secretion of shTeM or shChM-I into the conditioned medium of HUVECs following adenoviral transduction was confirmed by western blotting with anti-N-terminal FLAG monoclonal antibodies. As shown in Fig. 5D, HUVECs infected with Ad-shTeM synthesized and secreted a ~21 kDa protein which is the predicted molecular mass. Additionally, HUVECs infected with Ad-shChM-I produced a ~25 kDa protein, which was visible as a broad band, probably the result of glycosylation at Asn²⁴³ (Fig. 5D). No immunoreactive bands were detected in conditioned medium from HUVECs transduced with control adenoviral vectors lacking an expression cassette or from uninfected HUVECs.

Inhibition of VEGF-mediated HUVEC tube formation by expression of either shTeM (Glu²⁰²-Val³¹⁷) or shChM-I (Glu²¹⁵-Val³³⁴)

HUVECs assemble into multicellular capillary-like structures

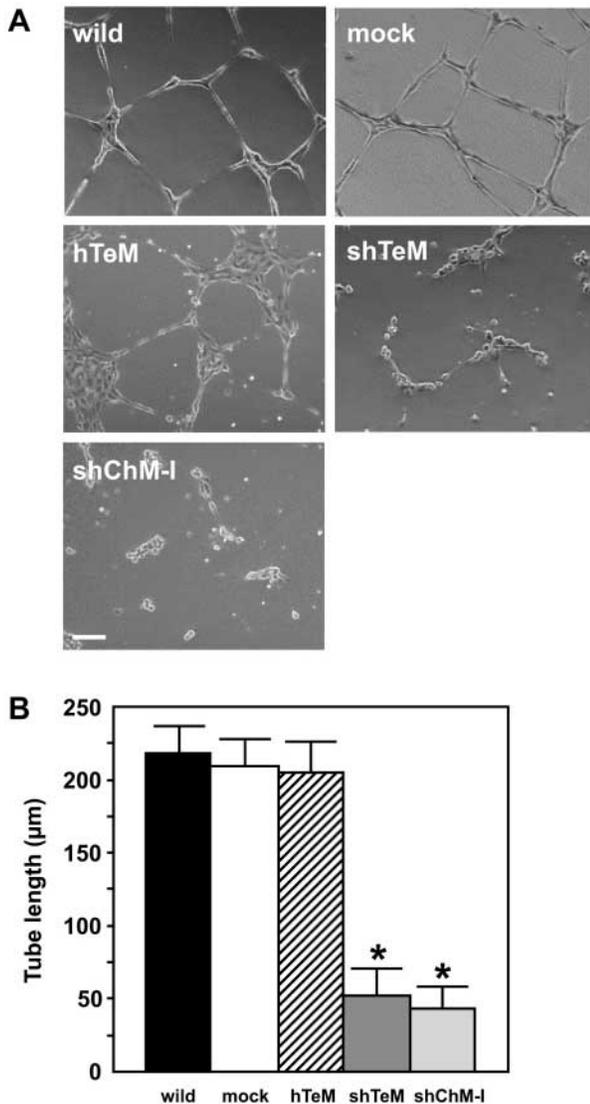


Fig. 4. Effects of conditioned media derived from COS7 cells expressing hTeM, shTeM or shChM-I on HUVEC tube formation. (A) HUVECs were suspended in a mixture of culture media from COS7 cells transfected with the indicated cDNAs and fresh EBM medium at a ratio of 1:1. The cells were then plated on growth factor-reduced Matrigel in the presence of VEGF (20 ng/ml). Representative phase-contrast microscopic images after 6 hours incubation are shown. HUVECs suspended in fresh medium derived from wild-type untransfected COS7 cells and in medium collected from either mock- or hTeM-transfected cultures organized themselves into tubular networks. By contrast, HUVECs suspended in media derived from either shTeM- or shChM-I-transfected COS7 cells did not form tubular structures. Scale bar: 100 μ m. (B) Total tube length per field was measured using image processing and analysis software. The data represent the mean \pm s.d. from three independent experiments. * P < 0.01 versus HUVECs suspended in the EBM medium.

when plated on Matrigel in the presence of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2). This tube formation process recapitulates numerous aspects of angiogenesis including branching, migration and differentiation (Folkman and

Haudenschild, 1980; Grant et al., 1991). When plated on growth factor-reduced Matrigel in the presence of 10 ng/ml VEGF, uninfected HUVECs (wild-type) or HUVECs infected with control adenovirus expressing only EGFP (Ad-EGFP) elongated and assembled themselves into multicellular capillary-like structures within a few hours (Fig. 6A). By contrast, tube formation was significantly inhibited in HUVECs transduced with Ad-shChM-I (Fig. 6A). Similarly there was a significant impairment of the cellular response of capillary formation to VEGF, when HUVECs were infected with Ad-shTeM (Fig. 6A). The total lengths of the capillary-like structures was significantly shorter in adenoviral-transduced HUVECs than in either wild-type cells or Ad-EGFP transfectants (Fig. 6B). We also established stable transfectants from the mouse endothelial cell line MSS31 (Namba et al., 2000) to measure the effects of forced expression of either shTeM or shChM-I under the control of the chicken β -actin promoter, and confirmed that FGF-2-induced tube formation of these cells on Matrigel was also markedly inhibited (data not shown). HUVECs were also transduced with Ad-hTeM to express full-length membrane-tethered TeM and although it cannot be readily evaluated as to what extent insoluble membrane tethered-TeM affects junctional contacts between endothelial cells, apparent tube-like cellular alignments were visible (Fig. 6A,B).

Effects of adenoviral expression of both shTeM and hChM-I on cellular proliferation, migration and attachment of HUVECs

The rate of DNA synthesis in HUVECs after infection with adenovirus was determined by measuring BrdU incorporation. Quiescent cultures were incubated with or without VEGF (20 ng/ml) for 12 hours, and the cells were labeled with BrdU during the last 4 hours of incubation. VEGF markedly stimulated DNA synthesis in non-transfected wild-type HUVECs (Fig. 7). As the mitogenic function of VEGF requires the activation of p42/44 MAP kinase (Hata et al., 1999; Seetharam et al., 1995), the MAP kinase inhibitor PD98059 completely abolished the stimulation of DNA synthesis, in response to VEGF, in both uninfected and adenoviral transduced cells. Moreover, control vector, Ad-EGFP, did not in any way inhibit VEGF stimulation of DNA synthesis (Fig. 7). Hence, adenoviral infection per se does not affect either the basal or the stimulated rate of DNA metabolism in these cells. However, adenoviral expression of shTeM and shChM-I in HUVECs resulted in a 40-50% reduction in VEGF-stimulated DNA synthesis (Fig. 7).

To examine the impact of shTeM and shChM-I on endothelial cell migration, HUVECs were infected with Ad-shTeM or Ad-shChM-I (Fig. 8). Migration was evaluated using a modified Boyden chamber assay with vitronectin-coated filters. As a chemoattractant, VEGF (20 ng/ml) was added to the lower chamber. HUVECs infected with or without recombinant adenoviruses were cultured in α MEM containing 0.5% FBS for 4 hours and then harvested with trypsin/EDTA. After suspending cells in the culture medium for 20 minutes and washing in migration buffer (α MEM containing 0.1% BSA), cells were seeded at a density of 5×10^4 cells per well (final volume, 100 μ l) on the upper surface of filters. After 4 hours of incubation, VEGF clearly induced migration of both

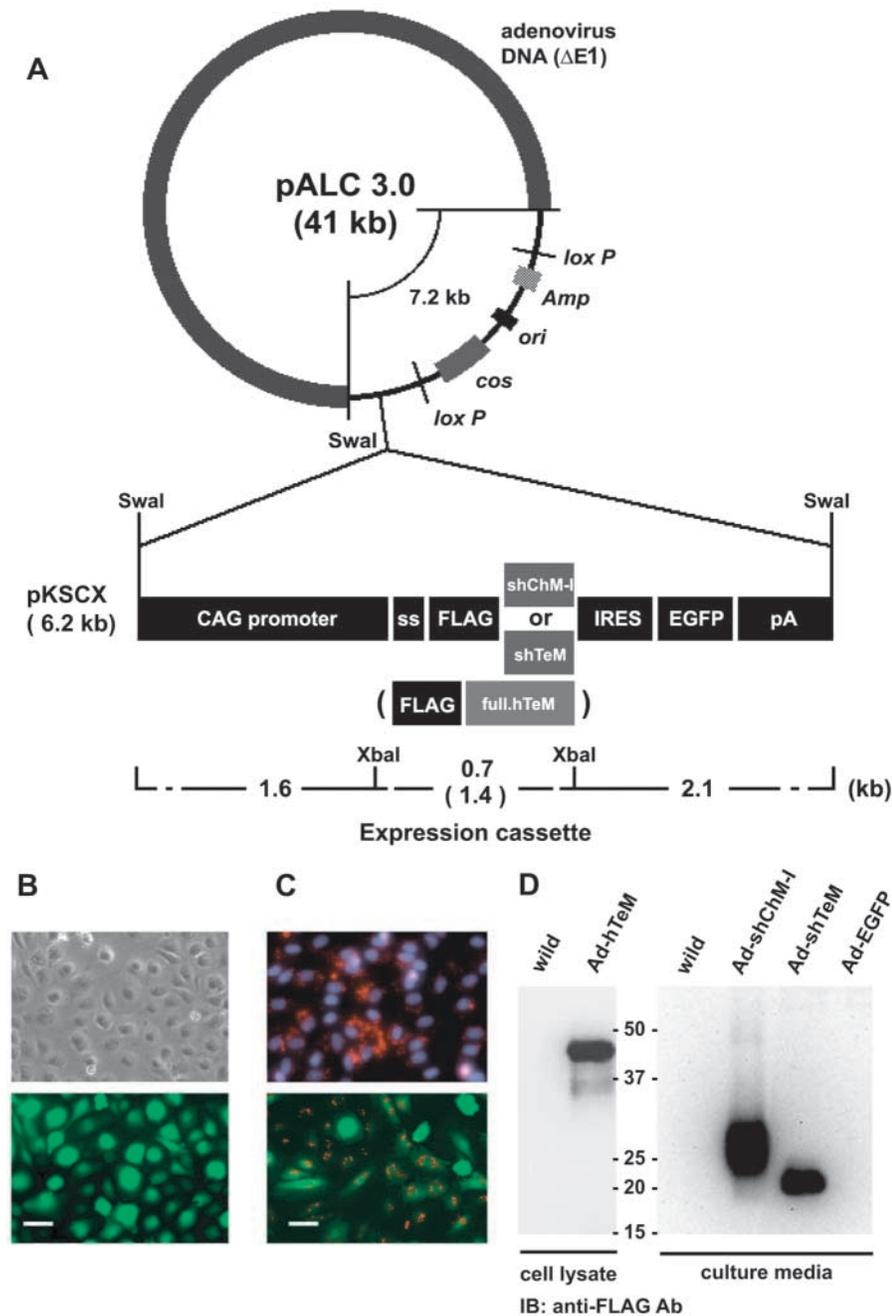


Fig. 5. Construction and expression of Ad-shTeM, Ad-shChM-I and Ad-hTeM in adenoviral vectors. (A) The structure of the pALC3 cosmid vector with an expression cassette containing either a shTeM, shChM-I or hTeM insert. The 34-kb adenoviral genome is indicated as a closed ring. The 7-kb cosmid vector backbone is flanked by *loxP* sites and includes a *cos* site, a bacterial *ori* site and the *ampicillin resistance gene* (*Amp*). The expression cassette consisting of the cytomegalovirus immediate-early enhancer-chicken β -actin hybrid (CAG) promoter, the preprotrypsin secretion signal (ss), the indicated FLAG inserts, an internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) cDNA followed by a polyadenylation signal (pA) was cloned into the *SwaI* site of the pALC3 vector, resulting in the pALC3-shChM-I, pALC3-shTeM or pALC3-hTeM cosmid. Recombinant adenoviruses were generated upon cotransfection of a Cre-expression plasmid with pALC3-shChM-I, pALC3-shTeM or pALC3-hTeM cosmids into 293 cells and are designated as Ad-shChM-I, Ad-shTeM or Ad-hTeM. Empty vector expressing EGFP alone was designated as Ad-EGFP. (B) Phase-contrast microscopic image (upper panel) indicating the cobblestone-like morphology of HUVECs 24 hours after adenoviral infection. The fluorescence microscopic image (lower panel) shows efficient adenoviral gene transduction in HUVECs. Scale bar: 40 μ m. (C) Fluorescence microscope images of uninfected HUVECs (upper panel) or HUVECs transduced with Ad-EGFP. Dil-AcLDL uptake is observed as red spots regardless of adenoviral transduction. Cell nuclei were stained blue-violet with Hoechst dye 33342 (upper panel). Green fluorescence is observed in the HUVECs expressing EGFP (lower panel). Scale bar: 40 μ m. (D) Cell lysates from uninfected HUVECs (wild) or HUVECs infected with Ad-hTeM were directly harvested with 1 \times SDS sample buffer and then subjected to western blotting (left panel). Secreted proteins were concentrated by a Butyl-Toyopearl column from conditioned medium derived from wild type HUVECs (wild) or HUVECs transduced by Ad-EGFP, Ad-shChM-I or Ad-shTeM, and subjected to western blot analysis by anti-FLAG monoclonal antibodies.

uninfected and Ad-EGFP-transduced control HUVECs through a porous filter coated with vitronectin (Fig. 8A). By contrast, the number of migrating cells was markedly reduced when they were transduced with either shTeM or shChM-I (Fig. 8B).

We then evaluated whether either shTeM or shChM-I altered cellular attachment to different extracellular matrices. HUVECs infected with or without adenoviruses were seeded onto plastic dishes coated with extracellular matrix components such as type I collagen, fibronectin and vitronectin. Following 1 hour of incubation at 37°C, unattached cells were removed by a gentle rinse with PBS and plates were evaluated for adhesion and spreading (Fig. 9A). Both

uninfected and Ad-EGFP-transduced control cultures had similar numbers of attached cells and showed a polygonal extension on dishes coated with each of the extracellular matrix proteins (Fig. 9B). However, cells expressing shTeM or shChM-I appeared rounded and failed to spread on vitronectin-coated dishes.

Suppression of both angiogenesis and tumor growth

Both the growth and persistence of solid tumors are dependent on angiogenesis, the inhibition of which has been shown to be an effective strategy for tumor treatment. To test the anti-angiogenic and anti-tumor activities of shTeM or shChM-I in

vivo, BL-6 melanoma cells were transduced with Ad-shTeM, Ad-shChM-I or Ad-EGFP (control) and 1×10^6 of each set of transduced cells were subcutaneously injected into C57BL/6

mice. Both wild-type melanoma cells and Ad-EGFP-transduced melanoma cells formed tumors rapidly. By contrast, however, Ad-shTeM- and Ad-shChM-I-transduced melanoma cells formed tumors that were 46% and 60% smaller, respectively, when compared to Ad-EGFP-transduced melanoma cells (Fig. 10A-C).

Angiogenesis within these melanoma cell tumor tissues was evaluated by counting the number of microvessels in sections stained with antibodies reactive to PECAM-1, a marker for vascular endothelial cells. As shown in Fig. 10D, adenoviral expression of shTeM or shChM-I resulted in visible inhibition of angiogenesis in implanted tumors and microvessel densities were $63 \pm 7\%$ and $71 \pm 5\%$ of Ad-EGFP transduced tumor, respectively (Fig. 10E). By contrast to the inhibitory effect of shTeM or shChM-I on the proliferation of HUVECs (Fig. 7), the growth rate of BL-6 melanoma cells was not affected upon transduction of Ad-shTeM or Ad-shChM-I (data not shown). These data therefore indicate that adenovirus-mediated overexpression of shTeM or shChM-I inhibited tumor growth by counteracting tumor-induced angiogenesis.

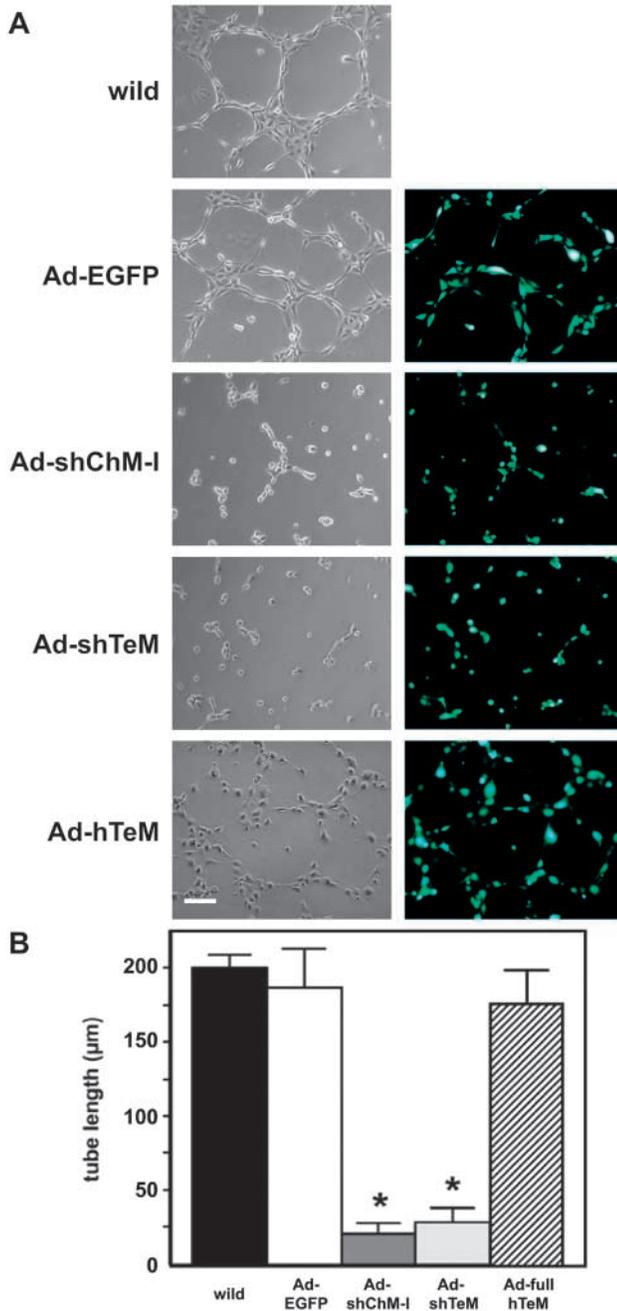


Fig. 6. Inhibition of HUVEC tube formation (A) HUVECs were transduced with Ad-EGFP, Ad-shChM-I or Ad-shTeM and then plated on growth factor-reduced Matrigel in the presence of VEGF (20 ng/ml). Representative phase-contrast micrographs (left panels) and fluorescence micrographs (right panels) after 6 hours incubation at 37°C are shown. Wild-type HUVECs (wild) or HUVECs transduced with either Ad-EGFP or Ad-hTeM organized into tubular networks, whereas HUVECs transduced with either Ad-shChM-I or Ad-shTeM were inhibited from doing so. Scale bar: 100 µm. (B) Total tube length per field was measured by image processing and analysis software. The data represent the means±s.d. from four independent experiments. * $P < 0.01$ versus wild-type HUVECs.

Discussion

TeM protein shows the highest sequence homology to the ChM-I precursor in its C-terminal cysteine-rich domain (Fig. 1) (Brandau et al., 2001; Shukunami et al., 2001; Yamana et al., 2001). In the case of the ChM-I precursor, this domain contains all eight cysteine residues found in the mature ChM-I protein, which form four disulfide bonds with a specific configuration. These disulfide bonds appear to be critical for the anti-angiogenic activity of ChM-I in vitro (Hiraki et al., 1997; Hiraki et al., 1999). However, owing to its hydrophobic nature and the restricted configuration of these disulfide bonds,

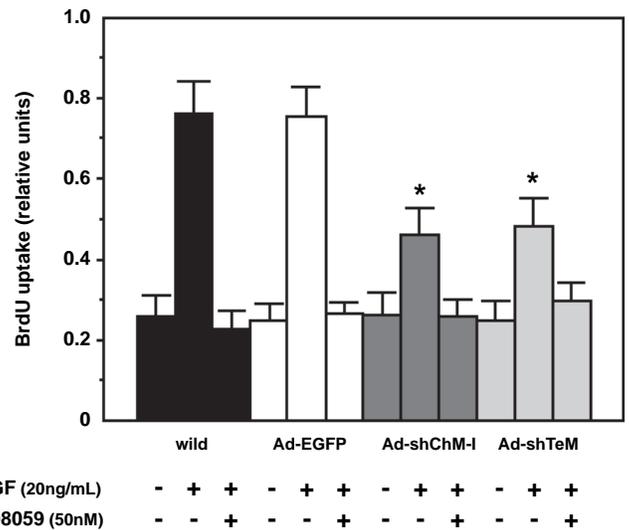


Fig. 7. Inhibition of HUVEC proliferation following transduction with either Ad-shChM-I or Ad-shTeM. HUVECs transduced with either Ad-EGFP, Ad-shChM-I or Ad-shTeM were plated into 96-well plates. The cells were starved in 0.5% FBS-containing αMEM for 6 hours and then incubated with VEGF (20 ng/ml) for 12 hours. The rate of DNA synthesis was evaluated by BrdU incorporation followed by measurement of BrdU incorporation followed by measurement of BrdU ELISA chemiluminescence. A MAP kinase inhibitor, PD98059 (50 µM), was used as a negative control. Data are mean±s.d. of triplicate measurements.

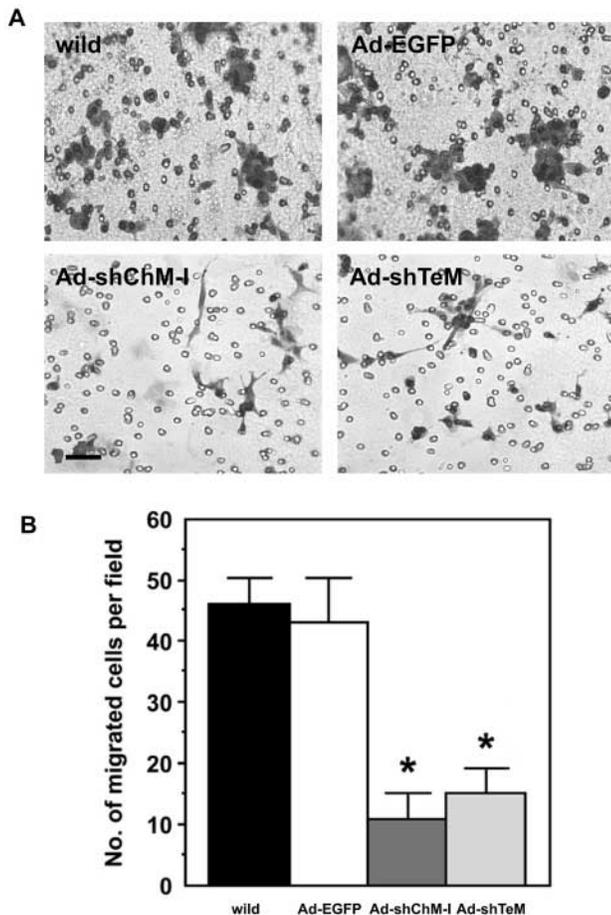


Fig. 8. Inhibition of migration in HUVECs transduced with Ad-shChM-I or Ad-shTeM. (A) Cell migration was evaluated using a modified Boyden chamber assay with vitronectin-coated filters. Representative images of HUVECs migrating to the lower side of the membranes in the presence of VEGF (20 ng/ml) are shown. Bar, 50 μ m. (B) The number of cells migrating to the lower side of the membranes was measured. Data are mean \pm s.d. of triplicate measurements. * P < 0.01 versus wild-type HUVECs.

it has, so far, not been possible to synthesize the ChM-I cysteine-rich domain alone. We therefore decided to carry out experiments utilizing the forced expression and secretion of the C-terminal 116 amino acids of human TeM (shTeM) tagged with a FLAG epitope in HUVECs under the control of the preprotrypsin secretion signal and the CAG promoter (Fig. 5). For comparison, the C-terminal 120 amino acids of the human ChM-I precursor (shChM-I, corresponding to mature ChM-I protein) were similarly expressed using adenovirus vectors.

As predicted, based upon previous observations (Hiraki et al., 1997; Hiraki et al., 1999), autocrine secretion of shChM-I resulted in the marked inhibition of angiogenic responses in HUVECs induced by VEGF, which included DNA synthesis, tube formation and cell migration (Figs 6-8). Thus, the anti-angiogenic properties of ChM-I were evident upon forced expression of the C-terminal portion of the precursor protein. We demonstrate in this study that the forced expression of the TeM C-terminal region, in its secreted form (shTeM), clearly resulted in the inhibition of angiogenic responses in HUVECs

to a similar extent as that of shChM-I expression (Figs 6-8). Sequence similarities between shChM-I and shTeM exist within the C-terminal cysteine-rich domain (Hiraki et al., 1999; Shukunami et al., 2001), of which the amino acid sequence is evolutionally conserved in vertebrates (Sachdev et al., 2001). The reduction-sensitive nature of ChM-I bioactivity indicates a crucial role for this domain which contains four intramolecular disulfide bonds (Hiraki et al., 1997; Hiraki et al., 1999). The kringle domain is a well-known example of an anti-angiogenic sequence motif, and contains the triple-disulfide-linked structure present in angiostatin and other related peptides (Cao et al., 1997; Nakamura et al., 2001). However, no kringle domain exists in either TeM or the ChM-I precursor, which may suggest that the C-terminal cysteine-rich domain constitutes a novel anti-angiogenic sequence motif.

Cell adhesion to extracellular matrices via integrins is one of the principal requirements for angiogenic response of vascular endothelial cells (Brooks et al., 1994). Additionally, vascular endothelial cells have been shown to express the type I collagen receptor integrin $\alpha_2\beta_1$, fibronectin receptor $\alpha_5\beta_1$ and vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Eliceiri and Chersesh, 1999). Expression of shTeM or shChM-I clearly blocked VEGF-induced migration of HUVECs through a porous filter coated with vitronectin (Fig. 8), whereas migration through filters coated with type I collagen or fibronectin was not affected in these transduced cells (data not shown). Cell attachment was similarly modulated by the expression and secretion of shTeM or shChM-I (Fig. 9): adhesion of HUVECs to vitronectin-coated dishes was significantly reduced, whereas adhesion to either type I collagen or fibronectin was not affected. Since transduction of Ad-shTeM or Ad-shChM-I did not influence the surface expression of either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ in HUVECs (data not shown), it is unlikely that the alterations in cell adhesion and migration to vitronectin were mediated by the decreased expression of vitronectin receptors. Recently the $\alpha_v\beta_3$ integrin was found to be a predominant receptor for angiostatin and other anti-angiogenic kringle containing peptides, indicating that the anti-angiogenic action of angiostatin is mediated by the $\alpha_v\beta_3$ signal mechanism (Tarui et al., 2001). In the present study, we demonstrated that the expression of either shTeM or shChM-I also caused vitronectin-specific alterations in cell adhesion and migration in HUVECs (Figs 8, 9). These results suggest the possibility that the anti-angiogenic activity of TeM or ChM-I is partly coordinated by integrin-mediated signals, although the mechanisms underlying ChM-I activity are not fully understood.

In solid tumors, the normal configuration of blood vessels is generally disrupted and the microvessels are primitive and leaky. It also seems to be relatively easier to counteract tumor-induced angiogenesis by administration of a single anti-angiogenic factor, in comparison to the modification of vascular networks during development in which angiogenesis is more precisely controlled by multiple mechanisms. Indeed, clinical and laboratory studies have already reported robust evidence indicating that tumor angiogenesis is inhibited by administration of anti-angiogenic factors (Tonini et al., 2003). We have also reported that recombinant ChM-I protein effectively suppresses the growth of chondrosarcoma and colon adenocarcinoma by inhibiting angiogenesis in vivo (Hayami et

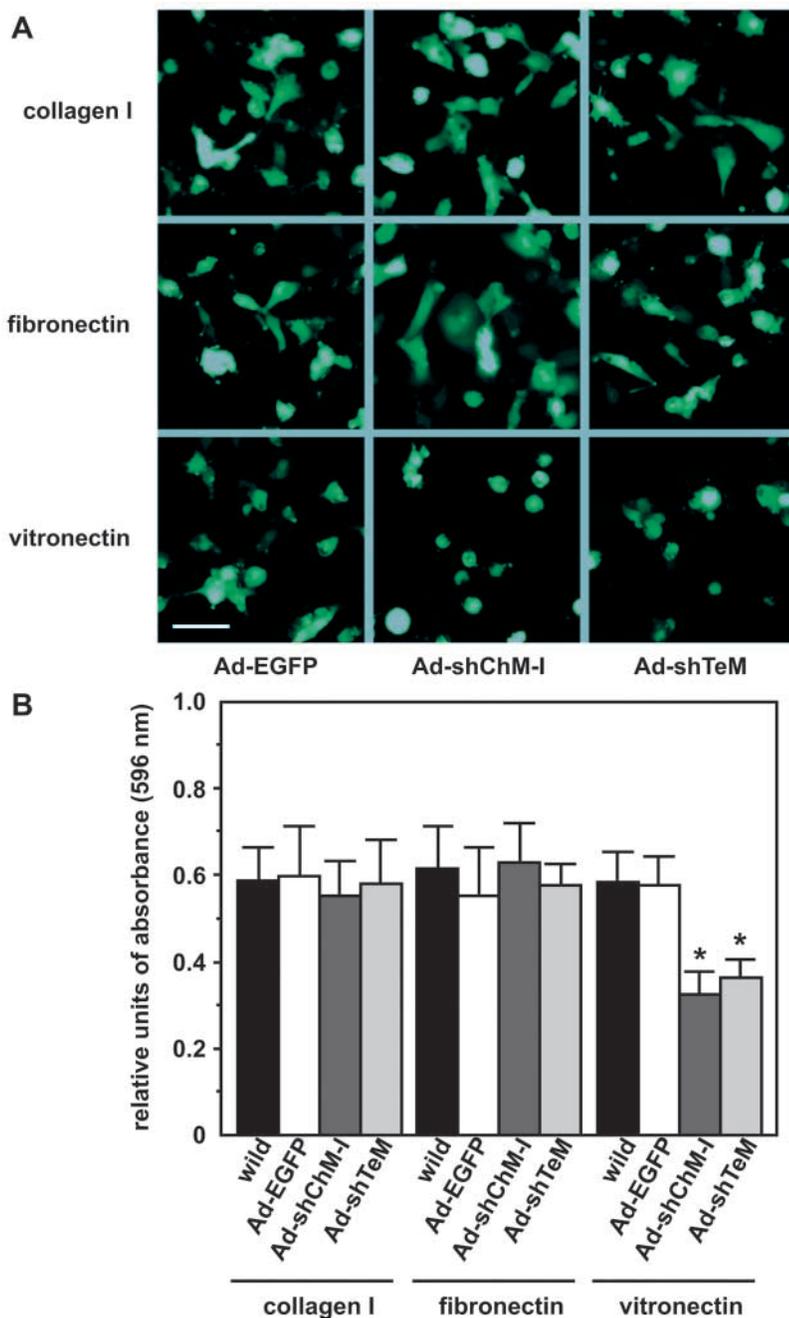


Fig. 9. Inhibition of cell adhesion and spreading via vitronectin in HUVECs transduced with Ad-shChM-I or Ad-shTeM. (A) Both adenovirus-transduced and wild-type HUVECs were harvested with trypsin/EDTA and suspended in culture medium for 20 minutes. The cells were re-suspended in α MEM containing 0.1% BSA at a density of 5×10^5 cells/ml and seeded on 96-well culture plates coated with type I collagen, fibronectin or vitronectin. After incubation for 1 hour at 37°C , unattached cells were removed by gentle washing with PBS. Cellular morphologies of adherent cells were observed and photographed by fluorescent microscopy. Bar, 100 μm . (B) For quantification of adhesion, the adherent cells were stained with crystal violet. The crystal violet stain was eluted with 0.1 M sodium citrate (pH 4.2) in 50% ethanol and the number of adherent cells in each well was evaluated by the measurement of absorbance at 596 nm. Data are means \pm s.d. of triplicate assays.

Many angiogenesis inhibitors identified to date are proteolytic fragments of larger precursor molecules (Tonini et al., 2003). Mature human ChM-I (120 amino acids) is also secreted from chondrocytes as a C-terminal fragment of a type II transmembrane protein (334 amino acids) after cleavage at its furin processing site (Arg-Glu-Arg-Arg) (Hiraki et al., 1997). No processing occurs when this precursor cleavage site is mutated to RERQ-SLVR or when the precursor cDNA is expressed in the furin-deficient CHO cell line (Azizan et al., 2001). Similarly, TeM is a putative type II transmembrane protein containing a BRICHOS domain, the functional role of which has not been fully elucidated (Sanchez-Pulido et al., 2002). However, unlike the ChM-I precursor, the TeM protein does not contain a furin cleavage site (Arg-Glu-Arg-Arg) (Fig. 1), although there is another potential processing site, Arg-Xxx-Xxx-Arg (Barr, 1991), at position 233-236 (Brandau et al., 2001; Shukunami et al., 2001; Yamana et al., 2001). Cell surface biotinylation and cell surface labeling experiments indicate that the TeM protein is indeed expressed as a transmembrane protein in a type II configuration.

In conventional angiogenesis assays, expression of full-length hTeM did not inhibit tube formation in HUVECs (Fig. 6). A novel member of the TNF family VEGI is a type II transmembrane protein predominantly expressed in endothelial cells (Zhai et al., 1999). The anti-angiogenic properties of VEGI were also demonstrated following forced expression of its secreted form via gene transfer, but no anti-angiogenic activity was evident when full length VEGI was expressed. Intracellular signaling pathways, mediating the actions of membrane-tethered ligands, have been described in some instances such as the Notch-Delta or the Ephrin-Eph system (Sullivan and Bicknell, 2003). However, the molecular mechanism by which the C-terminal region of TeM or ChM-I exerts its anti-angiogenic effects has not yet been described. As previously reported (Shukunami et al., 2001), endogenous TeM is specifically expressed in mouse embryos on tenocytes and ligament fibroblasts that give rise to

al., 1999). It is likely, therefore, that the C-terminal ChM-I-like domain of TeM has anti-tumor activity in vivo, and indeed expression of both Ad-shTeM and Ad-shChM-I results in the effective inhibition of angiogenesis, followed by suppression of tumor growth (Fig. 10A-C). The number of vascular vessels was also decreased in tumors transduced with either Ad-shTeM or Ad-shChM-I (Fig. 10D-E). These results strongly suggest that shTeM acts as an angiogenesis inhibitor both in vitro and in vivo. As already demonstrated by several other groups, anti-angiogenic gene therapy, with adenoviral vectors expressing anti-angiogenic proteins, could prove to be effective future treatments for solid tumors. The conserved anti-angiogenic domain present in both TeM and ChM-I might therefore be a useful target for an anti-tumor drug.

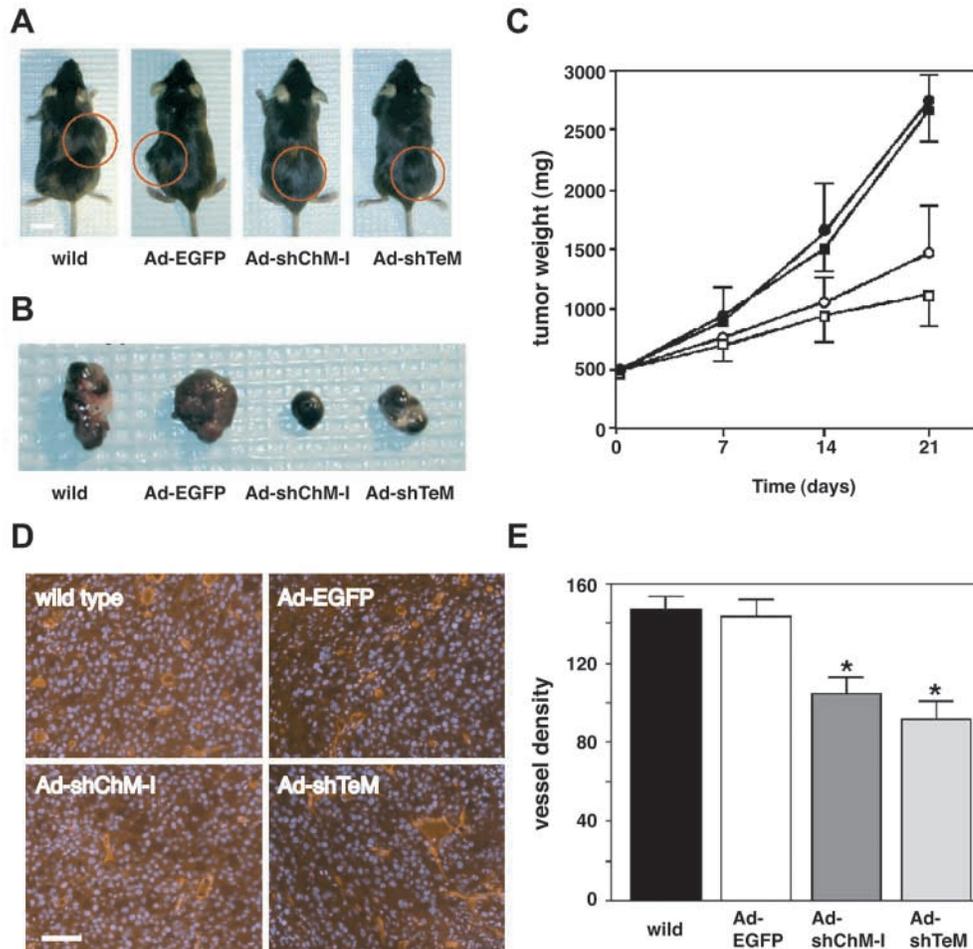


Fig. 10. Suppression of both tumor growth and tumor-induced angiogenesis by transduction of Ad-shChM-I or Ad-shTeM. (A) C57BL/6 mice at 21 days following implantation of melanoma cells (BL-6). Red circles indicate implanted tumors. Scale bar: 1.2 cm. (B) Images of subcutaneously injected melanoma tumor cells, transduced with or without Ad-shChM-I, Ad-shTeM and Ad-EGFP, were photographed also on day 21. (C) Time course of increase in tumor weight following implantation. At 7, 14 and 21 days after subcutaneous implantation of both transduced and non-transduced melanoma cells, the tumor volume was determined in Ad-shChM-I (open boxes), Ad-shTeM (open circles), Ad-EGFP (filled boxes)-transfected groups or in the wild-type group. The plots represent the mean values of tumor volumes from four animals in each group. Bars indicate \pm s.d. (D) Reduced angiogenesis in Ad-shChM-I or Ad-shTeM transduced tumors is apparent. Vascularization in tumors on day 21 following implantation is visualized by staining of PECAM-1 with monoclonal antibodies. Cell nuclei are stained blue-violet with Hoechst dye 33342 as. Scale bar: 400 μ m. (E) Microvessel numbers were counted from four different random fields of both adenovirus transduced or non-transduced melanoma cells. Data are represented as mean values (\pm s.d.) of microvascular densities per field ($\times 40$) from sections of Ad-shChM-I-, Ad-shTeM-, Ad-EGFP-transduced or non-transduced tumors ($P < 0.005$).

hypovascular dense connective tissues during development. When considering the anti-angiogenic potentials of the TeM C-terminal region, therefore, it is reasonable to assume that membrane-tethered TeM protein participates in forming an anti-angiogenic surface on cells of the dense connective tissue. It is possible to speculate also that TeM takes part in the regulation of either angiogenesis or vascular invasion through anti-angiogenic cell surface contacts with adjacent endothelial cells in dense connective tissues, under both normal and pathological conditions. However, because of the current lack of an appropriate assay system, it is still uncertain whether membrane-tethered TeM acts as an angiogenesis inhibitor on such cell surfaces.

Accumulating evidence suggests that the extracellular domains of several integral type I or type II transmembrane proteins are cleaved and released from cells in a soluble form

by ectodomain shedding (Kheradmand and Werb, 2002). Ectodomain shedding has been reported to profoundly affect the biological activity of its target proteins and to play a critical role during development as demonstrated in TACE/ADAM-17 knockout mice which have abnormal lung development and die at birth (Zhao et al., 2001). Several members of the ADAM family and of the metalloproteinases are involved in the release of transmembrane proteins (Black and White, 1998). As demonstrated in this report, endogenous TeM protein was detected by western blotting as a double band of between ~40 and ~45 kDa in tissue extracts (Fig. 2C). No immunoreactivity with bands of smaller molecular mass was detected, indicating the absence of TeM cleavage products. Immunohistochemical analyses of TeM were consistent with in situ hybridization data showing expression of TeM mRNA in the epimysium of skeletal muscle and tendinous dense connective tissue

surrounding the bone precursors (data not shown). Although cleavage products of TeM were not detected in extracts of skeletal muscle from 4-week-old mice by western blot analysis, this does not preclude the possibility that the extracellular domain of TeM might be cleaved by shedding enzymes and released from the cells as a soluble factor to modify angiogenesis during development. Indeed, we found that ChM-I could in fact act as a substrate for MMP-13, which is expressed in hypertrophic chondrocytes (unpublished data). Thus, it is also possible to speculate that some metalloproteinases or ADAMs expressed in developing tendons and ligaments may participate in the processing of the extracellular domain of TeM.

The locomotive organs contains a variety of specialized forms of supporting connective tissues. Among them, cartilage and tendon/ligaments are avascular or hypovascular as characterized by a poor and limited blood supply (Benjamin and Ralphs, 2000; Kuettner and Pauli, 1983; Young and Heath, 2000). By contrast, the adjacent mesenchyme including muscle, bone and synovium are well vascularized. Microcapillaries in these tissues never invade cartilage or tendon/ligaments under physiological conditions and the anti-angiogenic properties of these tissues are regulated by multiple molecular mechanisms. TeM proteins are localized to the sites of attachment of muscle to skeletal tissues that delimit the extension of the vasculature (C.S., unpublished data). Both the bioactivity and localization of ChM-I and TeM indicate that these molecules may play a part in the maintenance of an anti-angiogenic state.

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