The ADAMTS12 metalloproteinase exhibits anti-tumorigenic properties through modulation of the Ras-dependent ERK signalling pathway

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Summary

Members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of proteolytic enzymes are implicated in a variety of physiological processes, such as collagen maturation, organogenesis, angiogenesis, reproduction and inflammation. Moreover, deficiency or overexpression of certain ADAMTS proteins is directly involved in serious human diseases, including cancer. However, the functional roles of other family members, such as ADAMTS12, remain unknown. Here, by using different in vitro and in vivo approaches, we have evaluated the possible role of ADAMTS12 in the development and progression of cancer. First, we show that expression of ADAMTS12 in Madin-Darby canine kidney (MDCK) cells prevents the tumorigenic effects of hepatocyte growth factor (HGF) by blocking the activation of the Ras-MAPK signalling pathway and that this regulation involves the thrombospondin domains of the metalloproteinase. We also show that addition of recombinant human ADAMTS12 to bovine aortic endothelial cells (BAE-1 cells) abolishes their ability to form tubules upon stimulation with vascular endothelial growth factor (VEGF). Additionally, tumours induced in immunodeficient SCID mice injected with A549 cells overexpressing ADAMTS12 show a remarkable growth deficiency in comparison with tumours formed in animals injected with parental A549 cells. Overall, our data suggest that ADAMTS12 confers tumour-protective functions upon cells that produce this proteolytic enzyme.

Key words: Cell-cell adhesion, Hepatocyte growth factor, Cell migration, E-cadherin

Introduction

The human ADAMTS family comprises 19 structurally complex metalloproteinases containing a variable number of type-1 thrombospondin (TSP-1) domains in the region of their carboxy-termini (Cal et al., 2002; Llamazares et al., 2003; Porter et al., 2005). Functional studies have demonstrated the participation of these enzymes in a variety of processes, such as collagen maturation, organogenesis, proteoglycan degradation, inhibition of angiogenesis, reproduction and inflammation. Furthermore, deficiency or overexpression of specific ADAMTS proteins is directly involved in serious human diseases. Human ADAMTS proteins can be classified into seven subfamilies in accord with their structural and biochemical features. Notably, ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8 and ADAMTS9 are able to cleave cartilage aggrecan and can contribute to cartilage degradation in osteoarthritic diseases (Porter et al., 2005). In particular, ADAMTS5 is now considered to be the major functional aggrecanase because mice deficient in this enzyme show a marked reduction in the severity of cartilage destruction in comparison with wild-type mice (Glasson et al., 2005; Stanton et al., 2005). ADAMTS2, ADAMTS3 and ADAMTS14 are aminoprocollagen peptidases (Colige et al., 2005; Colige et al., 2002; Wang et al., 2003), and ADAMTS2 deficiency causes Ehlers-Danlos syndrome VIIc in humans and dermatosparaxis in animals, a disease characterized by a decrease in the tensile strength and integrity of the skin, joints and other connective tissues (Colige et al., 1999). ADAMTS13 is the proteinase responsible for cleaving von Willebrand factor, and mutations in the *ADAMTS13* gene cause a severe blood disease named thrombotic thrombocytopenic purpura (Levy et al., 2001). ADAMTS9 (Clark et al., 2000; Somerville et al., 2003) and ADAMTS20 (Llamazares et al., 2003; Somerville et al., 2003) are named the GON-ADAMTS proteins as their gene sequences show a high degree of sequence similarity with the *Caenorhabditis elegans gon-1* gene, which is essential for formation of gonads (Blelloch et al., 1999). Mutations in *ADAMTS10* cause an autosomal recessive disorder of connective tissue called Weill-Marchesani syndrome (Dagoneau et al., 2004). ADAMTS1 and ADAMTS8 are potent angio-inhibitory enzymes (Vazquez et al., 1999), and *Adamts1*-deficient mice display altered morphology and function of organs and reduced fertility (Shindo et al., 2000). It is remarkable that certain members of this metalloproteinase family are dysregulated in tumours of varying origins. These include ADAMTS6 and ADAMTS18 in breast cancer (Porter et al., 2004), ADAMTS19 in osteosarcoma (Cal et al., 2002), ADAMTS20 in breast and colon carcinomas (Llamazares et

al., 2003) and ADAMTS4 and ADAMTS5 in glioblastomas (Held-Feindt et al., 2006).

Despite significant advances in our understanding of the biological functions of some of these enzymes, the roles of other family members, such as ADAMTS12, remain unknown. The ADAMTS12 metalloproteinase was originally identified and cloned in our laboratory by using human foetal lung cDNA samples (Cal et al., 2001) and has been proposed to participate in the initiation and progression of arthritis through its ability to degrade cartilage oligomeric matrix protein (Kevorkian et al., 2004; Liu et al., 2006). However, at present, no data are available concerning a putative role for ADAMTS12 in the development and progression of cancer. To this end, we have analyzed the effects of the exogenous expression of ADAMTS12 in MDCK cells. This cell line constitutes a good model system to study changes in cellular phenotype because it undergoes striking morphological changes, from cobblestone to long-spindle shapes, following HGF treatment, an effect known as 'scattering' (Weidner et al., 1990). Moreover, MDCK cells grown in 3D collagen gels form cyst structures, and HGF stimulation induces tubulogenesis (O'Brien et al., 2002), a phenotype relevant to renal development and carcinogenesis. Early in tubule development, cells form long invasive extensions that represent a partial epithelial-mesenchymal transition (EMT) (O'Brien et al., 2004). The morphological changes that accompany the EMT occur through activation of Met, the HGF receptor, switching on a complex signalling pathway that finally leads to downregulation of E-cadherin, upregulation of vimentin and secretion of several extracellular proteinases that are essential for matrix remodelling (Birchmeier et al., 2003).

Here, we report that expression of ADAMTS12 in MDCK cells prevents the tumorigenic effect caused by HGF stimulation and show that this effect derives from the inhibition of components of the Ras-MAPK signalling pathway. We also show that addition of recombinant human ADAMTS12 to bovine aortic endothelial cells (BAE-1 cells) abolishes their ability to form tubules upon stimulation with VEGF. Finally, we report that tumours induced in immunodeficient mice injected with A549 cells overexpressing ADAMTS12 exhibit a marked growth deficiency in comparison with tumours formed in mice injected with control A549 cells. Overall, our data indicate that ADAMTS12 could act as an angio-inhibitory proteinase with the ability to confer anti-tumorigenic properties to epithelial or endothelial cells.

Results

Generation of stable MDCK clones expressing ADAMTS12

MDCK cells were transfected with the full-length cDNA for ADAMTS12 (Fig. 1A), and the expression of the recombinant protein in the stable clones was examined by both RT-PCR and western blot analysis (Fig. 1B). These analyses allowed us to detect the expression of ADAMTS12 in MDCK clones TS12-4 and TS12-22. A specific band of molecular mass ~175 kDa was visualized by western blot using antibodies against ADAMTS12 and the FLAG epitope (Fig. 1B, and data not shown). Additionally, immunocytochemical analysis of these clones with the antibody against FLAG allowed the ADAMTS12 protein to be localized on the cell surface (Fig. 1C). Likewise, and consistent with previous studies (Cal et al., 2001), the entire protein was not detected in the cell medium. The MDCK clone stably transfected with the empty pCEP vector (cMDCK) did not show immunoreactivity for the antibodies against both ADAMTS12 and FLAG in these assays, and it was subsequently used as a negative control. Clones TS12-4 and TS12-22 were chosen for further evaluation of the putative phenotypic changes accompanying ADAMTS12 expression in MDCK cells.

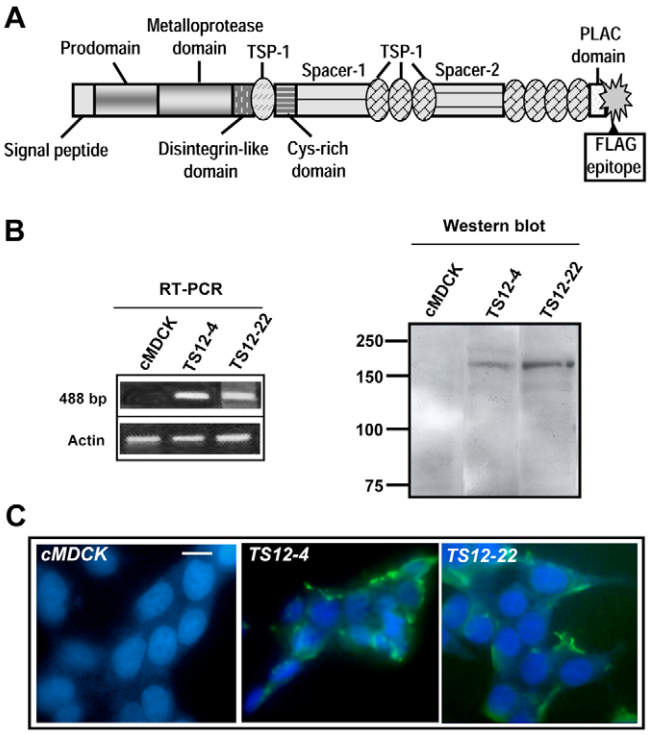

Fig. 1. Characterization of MDCK ADAMTS12 stable transfectants. (A) Domain organization of ADAMTS12 showing the position of the FLAG epitope. (B) RT-PCR and western blot analysis of the ADAMTS12-expressing clones TS12-4 and TS12-22, using the H-142 antibody against ADAMTS12. The cMDCK clone was used as a negative control in these assays. In RT-PCR experiments, amplification of mRNA encoding β-actin was used to ascertain RNA integrity and ensure equal loading. (C) Immunostaining of non-permeabilized cMDCK, TS12-4 and TS12-22 cells using an anti-FLAG antibody. Bar, 10 μm.

MDCK ADAMTS12 clones are refractory to the scattering effect of HGF treatment

Following HGF stimulation, the scattering effect in MDCK cells occurs concurrently with the disassembly of junctional components, which leads to a rapid loss of cell-cell adhesions (Singh et al., 2004). To evaluate whether these effects were modified in the selected ADAMTS12-expressing clones, TS12-4 and TS12-22 clones were cultured in the presence or absence of HGF (50 ng/ml), and morphological changes were visualized 16 hours later. The levels of production of ADAMTS12 and its localization do not seem to be affected by stimulation with the growth factor (data not shown). As can be seen in Fig. 2, cMDCK, TS12-4 and TS12-22 clones formed colonies of similar morphology when cultured in the absence

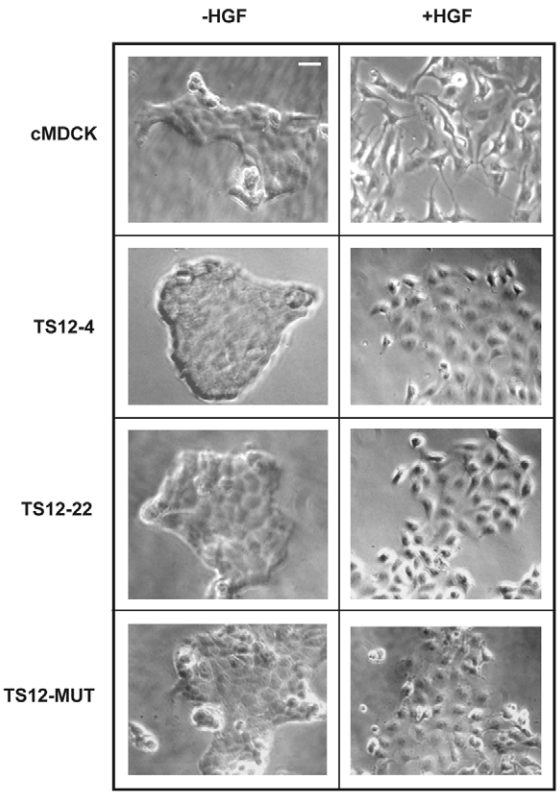

Fig. 2. MDCK ADAMTS12-expressing clones are refractory to the scattering effect of HGF stimulation. The control cMDCK clone shows reduced cell-cell adhesion and has a spindle-shape morphology upon treatment with HGF. By contrast, TS12-4 and TS12-22 still form epithelial-like colonies after 16 hours of incubation with HGF. A MDCK clone that expresses a catalytically inactive mutant version of ADAMTS12 (TS12-MUT) is also refractory to the scattering effect. Bar, 50 μm.

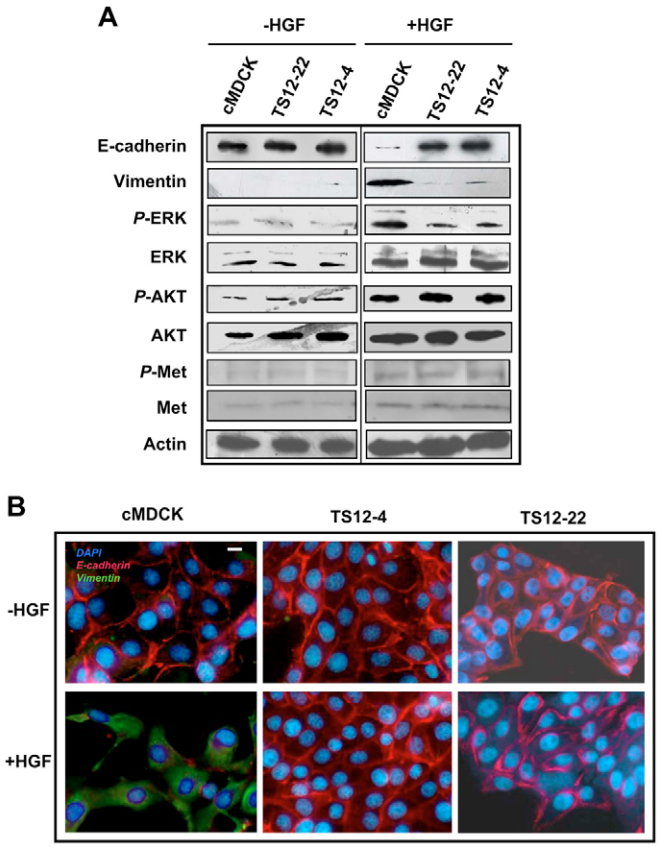

Fig. 3. ADAMTS12 negatively regulates the HGF signalling pathway. (A) Levels of different components of the HGF pathway (E-cadherin, vimentin, ERK, *P*-ERK, AKT, *P*-AKT and *P*-Met) were evaluated by western blot in the selected cMDCK, TS12-4 and TS12-22 clones, in both non-stimulated (– HGF) and stimulated (+ HGF) conditions. Ectodomain shedding of Met was also analyzed in the conditioned medium from these clones (Met). (B) Immunostaining of E-cadherin and vimentin in the selected clones without (– HGF) and with (+ HGF) stimulation by hepatocyte growth factor. Bar, 10 μm.

of HGF. However, following HGF treatment, the cMDCK clone showed the long spindle-shape morphology and reduced cell-cell adhesion characteristic of epithelial cells treated with this growth factor. By contrast, TS12-4 and TS12-22 clones were clearly refractory to HGF stimulation as the cells remained in contact with each other and formed epithelial-like colonies, and the scattering could only be occasionally observed in some cells surrounding the colonies. Likewise, we also assayed a MDCK clone that expresses an ADAMTS12 form containing changes in two key amino acids in the catalytic domain, resulting in an enzymatically inactive enzyme (Cal et al., 2001). Detection of this mutant ADAMTS12 was performed as described for TS12-4 and TS12-22 clones (data not shown). Fig. 2 illustrates that this clone, TS12-MUT, was also refractory to the morphological changes that are expected upon HGF stimulation. This observation, together with the lack of effect of the broad-range metalloproteinase inhibitor ilomastat on the HGF-stimulated TS12-4 and TS12-22 clones (data not shown), suggests that the described effects are not caused by the catalytic domain of the enzyme. Moreover, no changes were observed in the proliferation rates of the selected clones, indicating that ADAMTS12 expression in MDCK cells counteracts the HGF-mediated effects on cell-cell contacts and motility.

ADAMTS12 negatively regulates the HGF signalling pathway

HGF induces cell proliferation and migration through its tyrosine kinase receptor Met (Zhang and Vande Woude, 2003). We asked whether the inhibition of the scattering effect observed in MDCK cells transfected with ADAMTS12 could occur through this metalloproteinase exerting a negative regulatory signal upon the HGF pathway. To address this question, we prepared whole-cell extracts from both growth-factor-treated MDCK clones and untreated control clones, and levels of different components of the HGF pathway were evaluated by western blot. The results shown in Fig. 3A revealed that, in the absence of HGF stimulation, there were no differences in the levels of E-cadherin, vimentin, ERK, phosphorylated ERK (*P*-ERK), AKT, phosphorylated (*P*-AKT) and phosphorylated Met (*P*-Met) among the three clones analyzed, cMDCK, TS12-4 and TS12-22. Likewise, we have found that the expression of the metalloproteinase did not increase the ectodomain shedding of Met. By contrast, clear differences in the levels of some of the analyzed proteins were apparent when cells were stimulated with the growth factor (+

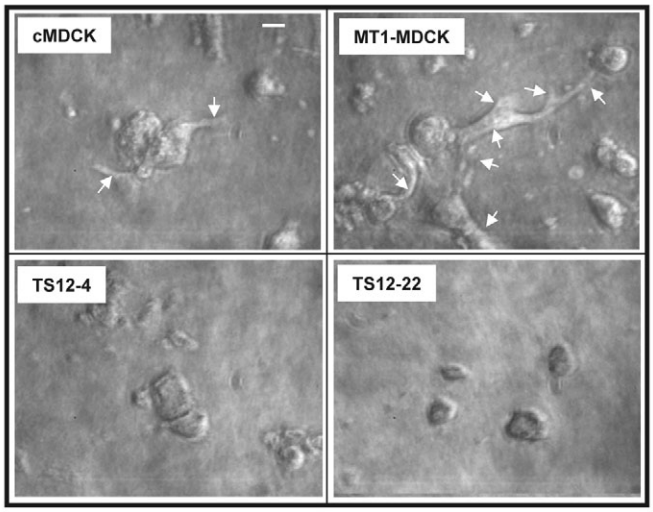

Fig. 4. MDCK cells expressing ADAMTS12 fail to form tubular structures in a 3D collagen matrix. Selected clones expressing ADAMTS12, TS12-4 and TS12-22 were grown within a collagen gel for seven days. Morphological changes such as long extensions (arrows) from the cysts are clearly observed in cMDCK and MT1-MDCK clones. Bar, 50 μm.

HGF, Fig. 3A). Thus, E-cadherin expression was clearly diminished in the cMDCK control clone upon HGF stimulation, whereas, in the ADAMTS12 clones, its expression was similar in both stimulated and non-stimulated cells. In addition, the cMDCK clone produced high levels of vimentin upon HGF stimulation, a change that was not observed in the TS12-4 and TS12-22 clones. Similarly, the levels of the active phosphorylated form of ERK (*P*-ERK) were significantly lower in the clones that expressed ADAMTS12 than in the corresponding controls following HGF treatment. Finally, we also observed that the levels of both AKT and phosphorylated AKT were similar among the analyzed clones in both stimulated and non-stimulated samples.

To further evaluate the observed differences between ADAMTS12-producing clones and control cells, we next performed an immunocytochemical analysis of E-cadherin and vimentin expression in these cells, both in the presence and absence of HGF. As can be seen in Fig. 3B, and in agreement with the results obtained by western blot analysis, cell-surface staining for E-cadherin was clearly detected in TS12-4 and TS12-22 clones, in both stimulated and non-stimulated samples, but negligible or no staining was observed for vimentin. By contrast, the response of cMDCK control cells to HGF stimulation was characteristic of an EMT as vimentin was clearly visible in HGF-treated cells and as E-cadherin levels were virtually undetectable following treatment with the growth factor (Fig. 3B). These results lead us to conclude that expression of ADAMTS12 can negatively regulate the HGF signal-transduction pathway. Moreover, the lowered levels of *P*-ERK detected in the ADAMTS12-expressing MDCK cells when compared with control MDCK cells suggest that the effects mediated by this metalloproteinase arise from an inhibition of the Ras-MAPK cascade and are independent of Met activation.

MDCK clones expressing ADAMTS12 fail to form tubular structures in a 3D collagen lattice

HGF mediates tubular morphogenesis in MDCK cells when grown within 3D collagen networks (Ridley et al., 1995). To evaluate the possible influence of ADAMTS12 expression on MDCK tubulogenic properties, TS12-4, TS12-22 and cMDCK clones were cultured in 3D collagen gels and stimulated with HGF, as described above. As a positive control for this experiment, we used a MDCK clone (MT1-MDCK) that expresses MT1-MMP, a membrane metalloproteinase that induces morphological changes to generate large cavities in the collagen gel (Hotary et al., 2000; Kadono et al., 1998). As can be seen in Fig. 4, MDCK cells that express ADAMTS12 form cysts when cultured in the presence of HGF in 3D collagen gels. However, these clones develop neither any tubular structures nor even any spiking or scattering of the cells that could indicate an early EMT (O'Brien et al., 2004). By contrast, morphological changes were observed in both cMDCK and MT1-MDCK clones. Thus, around 30% of the cysts formed by cMDCK cells, and 41% of those formed by MT1-MDCK cells, showed long extensions from the edge, which were clearly detectable after 7 days of incubation. By contrast, less than 10% of the cysts formed by TS12-4 and TS12-22 clones showed extensions, and such extensions were considerably shorter than those formed by the cMDCK and MT1-MDCK clones. These proportions were maintained when the tubule formation was followed for a longer period (data not shown). These findings indicate that ADAMTS12 expression might affect the ability of MDCK cells to invade a collagen matrix and constitute additional evidence concerning the inhibitory effects that this metalloproteinase can cause on the HGF signal-transduction cascade.

ADAMTS12 lacking thrombospondin domains does not inhibit EMT in HGF-stimulated MDCK cells

To test whether the thrombospondin domains of ADAMTS12 could be involved in the inhibitory effects observed in the TS12-4 and TS12-22 clones, we prepared a new clone, MDCK-ΔTS12, that stably expresses a truncated form of the protein lacking the TSP-1 domains (Fig. 5A,B). Scattering analysis, culture in 3D collagen gels and study of vimentin and E-cadherin expression revealed that this clone does not show any of the inhibitory effects described for those clones that express the full-length protein (Fig. 5C). Similar results were obtained when this assay was performed in the presence of the metalloproteinase inhibitor ilomastat (data not shown), which suggests that these effects are not due to the enzymatic activity of the protein. The two bands detected by western blot analysis of cell extracts could correspond to the entire protein (top band) and the mature ΔTS12 (bottom band), once the prodomain is removed in the trans-Golgi by a furin-like protease. According to these findings, the metalloproteinase and disintegrin domains are not sufficient to trigger the effects observed in the TS12-4 and TS12-22 clones. Hence, the presence of the angio-inhibitory TSP-1 domains as exosites within the metalloproteinase structure seems to be required to induce the negative regulatory effect following HGF stimulation.

Production, purification and enzymatic analysis of recombinant ADAMTS12

To further examine the relevance of ADAMTS12 in EMT

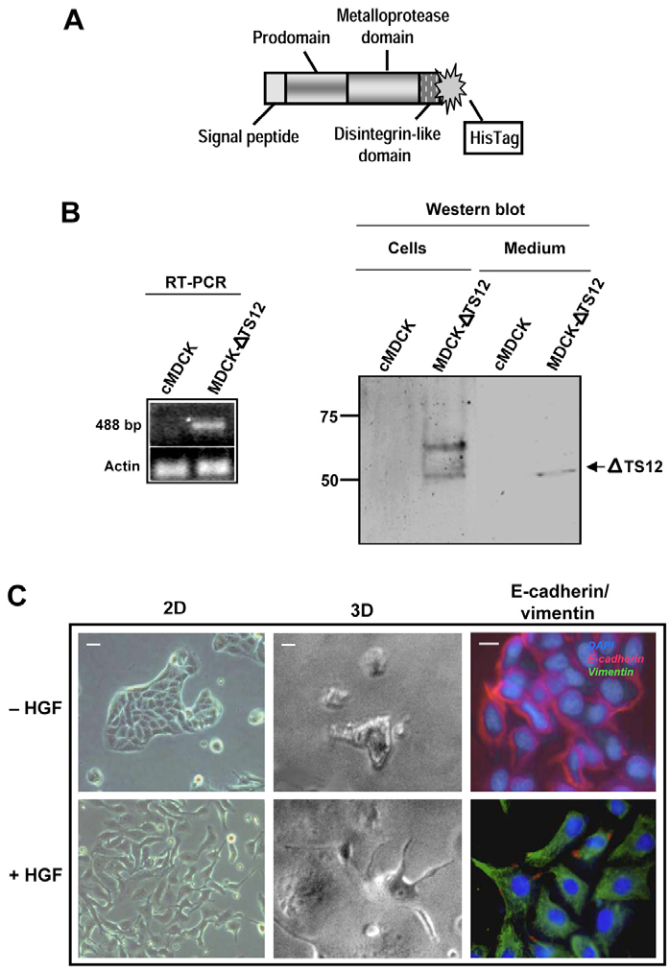

Fig. 5. Cells expressing a truncated ADAMTS12 lacking thrombospondin domains (ADAMTS-ΔTS12) undergo an EMT following HGF stimulation. (A) Domain organization of ADAMTS-ΔTS12. The position of the HisTag epitope is indicated. (B) Expression analysis of ADAMTS-ΔTS12 by RT-PCR and western blot using an antibody against the HisTag. In both cases, the cMDCK clone was used as a control. Truncated ADAMTS12 is located in the conditioned medium of cells that stably express this form of the enzyme. In RT-PCR, amplification of the mRNA encoding β-actin was used to ascertain RNA integrity and ensure equal loading. (C) Scattering assay (2D), tubulogenesis assay (3D) and immunostaining of E-cadherin and vimentin of the ADAMTS-ΔTS12-expressing cells in the absence (– HGF) or presence (+ HGF) of hepatocyte growth factor. Bars, 30 μm (left, centre); 10 μm (right).

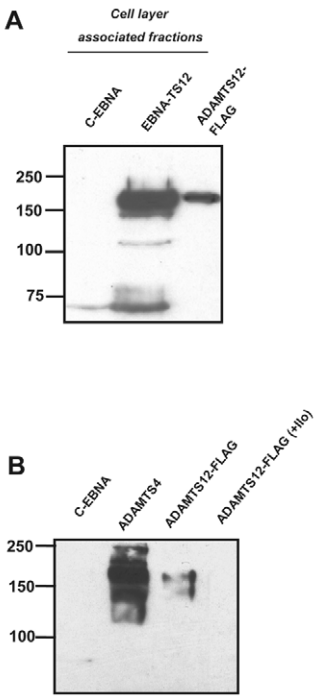

Fig. 6. Purification process and enzymatic activity of ADAMTS12. (A) Western blot analysis of ADAMTS12 during the purification process. c-EBNA and EBNA-TS12 indicate cell-layer-associated fractions from 293-EBNA cells transfected with an empty vector, and the equivalent fraction from 293-EBNA cells expressing ADAMTS12, respectively. Recombinant purified ADAMTS12 is also indicated. (B) Following incubation of aggrecan with recombinant ADAMTS4 (lane 2), purified ADAMTS12 (lane 3) or an equivalent fraction purified from c-EBNA cells (lane 1), samples were deglycosylated and the aggrecan degradation products were detected by using the BC-3 antibody. In lane 4, purified ADAMTS12 was previously incubated with the metalloproteinase inhibitor ilomastat (+Ilo). Molecular mass markers (kDa) in both panels are indicated on the left.

processes, we next generated 293-EBNA cells stably expressing this enzyme (EBNA-TS12), following a strategy similar to that described above for MDCK cells. It was possible to recover the metalloproteinase from the membrane-associated fraction by using a buffer containing 0.5 M NaCl, suggesting that, after secretion, this proteinase is immobilized around the pericellular space (Fig. 6A). After purification of the recombinant ADAMTS12 using an anti-FLAG affinity gel column, we performed a western blot analysis with an antibody against FLAG that allowed the detection of the purified ADAMTS12 (Fig. 6A). The purified enzyme was employed to test its activity by using aggrecan as a potential substrate. An equivalent fraction derived from a 293-EBNA clone stably transfected with an empty vector was used as a negative control (c-EBNA), whereas ADAMTS4, a potent aggrecanase, was used as positive control in this assay. As shown in Fig. 6B, the isolated recombinant ADAMTS12 displayed a clear aggrecanase activity, as assessed by western blot analysis using the BC-3 antibody that detects aggrecan degradation products. Moreover, this activity is abolished when the enzyme is pre-incubated with the metalloproteinase inhibitor ilomastat. These results confirm that recombinant ADAMTS12 produced in 293-EBNA cells is enzymatically active and thereby useful for the analysis reported below.

Effects of ADAMTS12 on VEGF-induced tubulogenesis in BAE-1 cells

To analyze whether the exogenous administration of ADAMTS12 could also affect tubulogenesis in a different system, we chose the endothelial cell line BAE-1, which forms capillary networks in collagen lattices when cultured in the presence of VEGF (Maeshima et al., 2004). In these conditions, purified ADAMTS12 or the corresponding cell-layer-associated

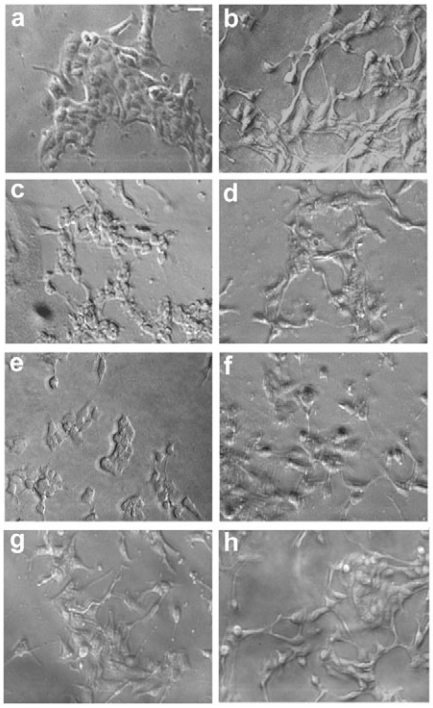

Fig. 7. Effect of ADAMTS12 on tubulogenesis of BAE-1 cells. BAE-1 cells were cultured within a collagen gel for 2 days in the absence (a) or presence (b–f) of 100 ng/ml VEGF. 10 μl of the cell-layer-associated fraction from 293-EBNA cells that express ADAMTS12 (EBNA-TS12) (c), or the same amount of the equivalent fraction from c-EBNA cells (d), were added to the culture. BAE-1 cells were grown in the presence of 10 μl of purified ADAMTS12 (e), or the same amount of the equivalent fraction purified from c-EBNA cells (f). Addition of 50 μl of conditioned medium from cells that express truncated ADAMTS12 (g) or from control cells transfected with an empty vector (h) does not affect the ability of BAE-1 cells to form a capillary network in the presence of VEGF. Bar, 30 μm.

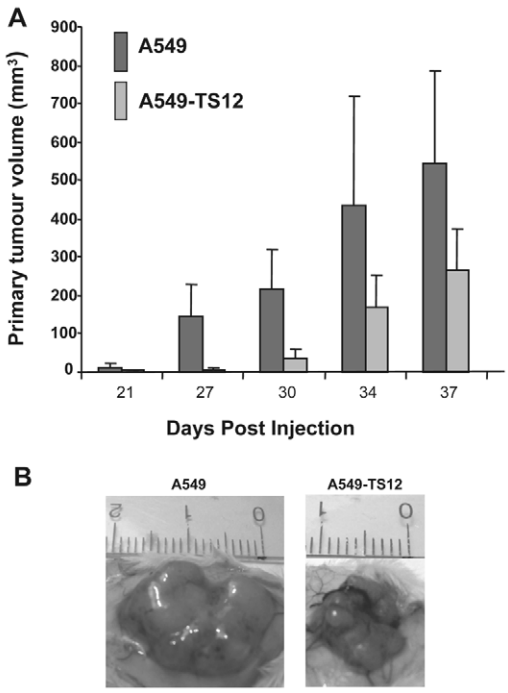

Fig. 8. Inhibition of subcutaneous tumour growth by ADAMTS12. (A) SCID mice were injected with parental A549 cells or with cells from an A549 clone (A549-TS12) expressing ADAMTS12. Tumour growth was followed until day 37, and tumour volumes were calculated after measurements were taken with a calliper. (B) Representative subcutaneous tumours at day 37 following injection are shown for mice that received A549 or A549-TS12 cells

fraction from 293-EBNA cells expressing ADAMTS12 were added to the cell cultures, and tubulogenesis was evaluated 48 hours later. As a negative control, BAE-1 cells under the same culture conditions were incubated with equivalent fractions of 293-EBNA cells stably transfected with the empty vector. As can be seen in Fig. 7, BAE-1 cells cultured in the presence of VEGF formed tubules and ~64% of cell clusters contained capillary structures. By contrast, formation of these capillary networks was drastically reduced in the presence of ADAMTS12, and less than 10% of cell clusters showed any tubular structures. Control cells, grown either without the recombinant ADAMTS12, or in the presence of the cell-layer-associated fraction from control 293-EBNA cells, clearly showed tubular morphogenesis in >40% of the cell clusters. Similar values were obtained when cells were grown in the presence of the protein lacking the thrombospondin domains (ΔTS12), which was carried out by adding to the BAE-1 cells conditioned medium from the MDCK cells that expressed this truncated form of the enzyme. From these assays, we conclude that, in this system, ADAMTS12 inhibits the formation of VEGF-induced tubular structures in BAE-1 cells. It cannot be ruled out that the metalloproteinases can interact with the growth factor. This situation would resemble that found for ADAMTS1, in which this metalloproteinase binds to VEGF through the thrombospondin domain of its C-terminal region (Luque et al., 2003).

ADAMTS12 inhibits tumour growth in vivo

A549 is a human lung adenocarcinoma cell line that forms primary tumours when injected into immunodeficient mice (Rao et al., 2005; Wedge et al., 2002). To evaluate the effect of ADAMTS12 expression in the in vivo tumour formation assay by A549 cells, we first selected a stable clone, A549-TS12, that expresses ADAMTS12. The presence of the metalloproteinase was determined by RT-PCR, western blot and immunocytochemistry (data not shown). A549-TS12 cells were subcutaneously injected in the flank of four SCID mice. Control parental A549 cells were injected in the same conditions in a further four animals, and the evolution of tumour growth was followed for five weeks. As can be seen in Fig. 8A,B, A549-TS12 cells overexpressing the metalloproteinase display a significantly reduced tumour growth rate in comparison with A549-derived tumours. Thus, after 37 days, the average tumour volume in mice that received the A549-TS12 clone was only ~50% of the tumour volume of mice that received the A549 parental cells. Moreover, in the case of the A549-TS12 clone, the tumours were macroscopically visible four weeks after cells injection, whereas the tumours produced by the parental A549 cells could already be detected by three weeks after injection. These results indicate that ADAMTS12 might also confer anti-tumour properties in vivo.

Discussion

ADAMTS12 is a complex metalloproteinase that contains multiple TSP-1 repeats in its C-terminal region and whose expression has been detected mainly in some human foetal tissues as well as in a variety of carcinomas and cancer cell lines (Cal et al., 2001). As a first step to study the putative functional relevance of ADAMTS12 in tumour-associated processes, we have analyzed in this work whether the expression of this proteinase could affect the morphological changes that MDCK cells undergo following HGF treatment. This cellular model has been successfully used to evaluate how different members of the matrix metalloproteinase (MMP) family promote the generation of an invasive phenotype in MDCK cells engineered to overproduce these enzymes (Hotary et al., 2000; Kadono et al., 1998; Kang et al., 2000). Following a similar strategy, and after selection of two MDCK clones that expressed ADAMTS12, we first observed that these clones were very refractory towards undergoing the scattering effect characteristic of MDCK cells upon HGF stimulation. Likewise, within collagen gels, the ADAMTS12-producing cells could form cysts, but neither tubules nor other morphological alterations such as spindle-shaped extensions were observed on the surface of the cysts. Western blot and immunocytochemical analysis showed that HGF stimulation did not downregulate E-cadherin or upregulate vimentin expression in the ADAMTS12-expressing clones, as would otherwise be expected for MDCK cells treated with this growth factor (Bolos et al., 2003).

Analysis of the putative signalling pathways underlying the observed effects induced by ADAMTS12 expression implicated the Ras-MAPK pathway in these events, as deduced from the fact that levels of active phosphorylated ERK were diminished in the ADAMTS12-expressing clones compared with control cells. This signalling cascade is involved in the regulation of epithelial tubule development in MDCK cells (O'Brien et al., 2004) and is necessary and sufficient to initiate tubulogenesis (Hellman et al., 2005). Moreover, sustained activation of ERK seems to be crucial for cell scattering (Liang and Chen, 2001). Our data are consistent with the possibility that ADAMTS12 inhibits the cellular processes that regulate the EMT in HGF-stimulated MDCK cells.

How could ADAMTS12 mediate these effects? The above results were obtained by using MDCK clones that expressed the entire metalloproteinase. However, after performing the same assays with cells producing a mutant ADAMTS12 protein lacking the thrombospondin domains, we observed that this truncated enzyme was unable to inhibit the effects induced by HGF, thereby suggesting that the C-terminal TSP-1 domains of ADAMTS12 might regulate this process. Moreover, MDCK cells that expressed a catalytically inactive ADAMTS12 form were also refractory to the scattering effect. Consistent with this proposal, inhibition of endothelial cell migration by TSP-1 occurs through the interaction with its receptor CD36, and cellular levels of TSP-1 are regulated by tumour-suppressor genes and oncogenes (Ren et al., 2006). It has also been reported that HGF turns off TSP-1 expression in MDCK cells to avoid negative regulatory signals on cell proliferation and migration and that this downregulation depends on the MAPK pathway and operates independently of the phosphoinositide 3-kinase and Stat3 pathways (Zhang and Vande Woude, 2003). Furthermore, and in relation to the possibility that other ADAMTS proteins could play similar roles, it has been reported recently that ADAMTS1 from *Xenopus* also negatively modulates the Ras-MAPK pathway through inhibition of ERK phosphorylation and that the C-terminal region of the protein containing the TSP-1 domains is necessary and sufficient for this function (Suga et al., 2006). Interestingly, both ADAMTS1 and ADAMTS12 are enzymes that undergo several proteolytic processing events to generate several fragments that might play different roles (Cal et al., 2001; Rodriguez-Manzaneque et al., 2000). The balance between the various generated fragments could influence the pro- or anti-metastatic activities of ADAMTS12, as has been shown for ADAMTS1 (Liu et al., 2006; Masui et al., 2001).

To evaluate the potential anti-tumorigenic effects of ADAMTS12 in vivo, we analyzed the growth of the poorly differentiated non-small-cell lung cancer cell line A549, engineered to produce ADAMTS12, in immunodeficient SCID mice. The A549 parental cell line has been used previously to determine that downregulation of the MMP-9 metalloproteinase has a significant inhibitory effect on tumour growth and progression (Rao et al., 2005). Conversely, we observed that A549 cells expressing ADAMTS12 form subcutaneous tumours in SCID mice, but the tumour volumes were considerably smaller than those produced by parental A549 cells. This observation suggests that ADAMTS12 expression might delay tumour growth, which agrees with our in vitro findings on the potential anti-tumour effects of this metalloproteinase. These results, based on using an animal model, open the possibility for future work aimed at evaluating the regulatory roles of ADAMTS12 in other in vivo tumour-associated processes such as angiogenesis or metastasis.

In conclusion, the results presented in this work suggest that ADAMTS12 could confer tumour-protective functions. In agreement with this idea, it has been recently established that Ki-*Ras*-transformed mouse colonocytes expressing high levels of Myc promote the formation of highly vascularized tumours. This effect is due, at least in part, to the downregulation of a series of angio-inhibitory proteins, including ADAMTS12 (Dews et al., 2006). The finding of anti-tumorigenic effects for a metalloproteinase is not unprecedented as it has also been described for other enzymes of this catalytic class, including several MMPs (Acuff et al., 2006; Balbin et al., 2003; Folgueras et al., 2004; Houghton et al., 2006). These findings point to the occurrence of alternative proteinase functions in cancer and also reinforce the need for analyzing the tumour 'degradome' – the entire set of proteinases produced by a specific tumour in a certain stage of development (Lopez-Otin and Overall, 2002). These proteinase-profiling approaches might also contribute to the identification of therapeutic targets for the future design of specific inhibitors that would block only the unwanted activity of these enzymes during tumour progression (Overall and Lopez-Otin, 2002). Generation of mice lacking ADAMTS12, now in progress, will provide an essential tool to understand better the relevance of this enzyme in tumour-associated processes.

Materials and Methods

Cell culture, transfection and cDNA constructs

MDCK, 293-EBNA, A549 and BAE-1 cells were routinely maintained in DMEM medium containing 10% heat-inactivated foetal bovine serum and 100 U/ml penicillin and 50 μg/ml streptomycin. Full-length cDNA encoding ADAMTS12 was amplified by PCR from the vector pcDNA3-ADAMTS12-HA (Cal et al., 2001),

and it was cloned between the *Hin*dIII and *Not*I sites of a modified pCEP4 expression vector (Life Technologies). Additionally, two oligonucleotides (5′-GGCCGACTACAAGGACGACGATGACAAG-3′ and 5′-GGCCCTTGTCATCGTCGTCCTTGTAGTC-3′) were used to introduce a FLAG epitope at the *Not*I site. The generated plasmid, pCEP-TS12, was transfected into MDCK and 293-EBNA cells using the lipofectamine reagent (Life Technologies), as recommended by the manufacturer. MDCK and 293-EBNA clones stably expressing ADAMTS12 or transfected with the empty vector to be used as a negative control were selected in the presence of 2 μg/ml puromycin (Sigma-Aldrich). When indicated, a MDCK clone expressing a mutant ADAMTS12 (TS-12 MUT) was employed. This form of ADAMTS12 contains two mutations in the metalloproteinase domain to produce a catalytically inactive enzyme (Cal et al., 2001). MT1-MMP cDNA was a generous gift from M. Seiki (Institute for Medical Science, University of Tokyo, Japan) and was employed to generate MDCK cells that stably expressed this proteinase. MDCK cells were also transfected with a cDNA encoding a truncated form of ADAMTS12 with a premature stop codon between the Cys-rich and the first TSP-1 domains (MDCK-ΔTS12). A His-tag tail was added to the C-terminus of this protein to facilitate its detection by western blot. To detect this truncated form of ADAMTS12, 300 μl of the conditioned medium was precipitated with an equal volume of chilled acetone.

Western blotting

For western blot analysis, the proteins were resolved by 8 or 12% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and subsequently probed with the indicated antibodies, as recommended by the manufacturers. The primary antibodies used for detection of specific proteins were anti-FLAG-M2 (Sigma-Aldrich), H-142 (anti-ADAMTS12; Santa Cruz Biotech), anti-p44/42 mitogen-activated protein kinase (MAPK), anti-phospho-Akt (Ser473) and anti-Akt (Cell Signalling Technology); anti-ERK, BC-3, anti-VEGF and anti-vimentin (Abcam); anti-E-cadherin (kindly supplied by A. Cano, IIB, Madrid, Spain); anti-MT1-MMP (kindly supplied by A. G. Arroyo, CNIC, Madrid, Spain); and anti-HisTag (GE Healthcare). Anti-*P*-Met antibody was from Biosource International (Camarillo, CA) and anti- Met antibody (clon DL-21) was from Upstate (Charlottesville, VA). Immunoreactive proteins were visualized using HRP-peroxidase-labelled anti-rabbit or anti-mouse secondary antibody and the ECL detection system (Pierce).

Reverse transcription-PCR amplification (RT-PCR)

When indicated, presence of ADAMTS12 in the selected clones was assayed by RT-PCR. Total RNA from these clones was isolated by guanidium thiocyanate-phenol-chloroform extraction, and the cDNA synthesis was carried out using the RNA PCR kit from Perkin-Elmer Life Sciences, as specified by the manufacturer. After reverse transcription using 1 μg of total RNA and random hexamers as primers, PCR was performed with two specific ADAMTS12 oligonucleotides, (5′-TCACGACGTGGCTGTCCTTCT-3′ and 5′-ACCACAG TGTCTGGCAGACGT-3′), corresponding to the metalloproteinase domain. cDNA quality was verified by control RT-PCR reactions using primers derived from the sequence of β-actin.

Immunocytochemical analysis

MDCK cells stably expressing ADAMTS12, or control cells carrying an empty vector, were fixed with 4% paraformaldehyde in phosphate-buffered saline. The non-permeabilized cells were blocked with 15% foetal bovine serum in the same buffer. To detect recombinant ADAMTS12, blocked slides were incubated for 2 hours with the primary antibody against FLAG, followed by 2 hours of incubation with a secondary fluorescein-conjugated sheep anti-mouse antibody (GE Healthcare). Detection of E-cadherin and vimentin was carried out basically as described above except that cells were permeabilized with 0.5% Triton X-100 for 5 minutes before adding the primary antibodies. Secondary antibodies were conjugated with Cy3 and Cy2 (Jackson ImmunoResearch Laboratories), respectively. In all samples, 4′,6′-diamino-2-phenylindole hydrochloride (DAPI) was added at 100 ng/ml to visualize DNA in the cell nucleus. Images were obtained using fluorescence microscopy and a digital camera.

Production and purification of recombinant ADAMTS12

Production and purification of recombinant ADAMTS12 was performed as described by Colige et al. (Colige et al., 2002) for ADAMTS14, with some modifications. In brief, 293-EBNA cells expressing ADAMTS12 (EBNA-TS12) were scraped from the culture plate, suspended in a buffer containing 50 mM Tris-HCl, pH 7.4, and 500 mM NaCl and rotated for 2 hours at 4°C. After centrifugation, the supernatant (cell-layer-associated fraction) was diluted 1:1 (v/v) in the same buffer lacking NaCl, and loaded onto an anti-FLAG affinity gel column (Sigma-Aldrich). Elution was carried out by using a FLAG peptide (Sigma-Aldrich), as recommended by the manufacturer. The purification process was followed by western blot using the anti-FLAG-M2 antibody. The same process was applied to an EBNA clone stably transfected with an empty vector.

Enzyme activity assays

The proteolytic activity of recombinant ADAMTS12 was assessed using aggrecan as a potential substrate. To do this, recombinant purified protein was incubated with 500 nM aggrecan from bovine articular cartilage (Sigma-Aldrich) in a buffer containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl and 10 mM $CaCl_2$ at 37°C for 16 hours. Next, fragments were enzymatically de-glycosylated using aggrecan chondroitinase ABC (0.1 U per 10 μg of aggrecan; Sigma-Aldrich) at 37°C for 1 hour, followed by 2 hours incubation at 37°C with 0.002 U per 10 μg aggrecan of endo-β-galactosidase (Sigma-Aldrich). Reaction products were analyzed by western blot by using the BC-3 antibody. When indicated, the broad-range inhibitor of metalloproteinases ilomastat (Calbiochem) was employed (5 μM final concentration). As a positive control, aggrecan digestion was carried out by ADAMTS4 (Chemicon International) under the same experimental conditions.

Scattering assay

Selected MDCK cell clones were seeded in quadruplicate in a 24-well tissue-culture plate (1×10^4 cells) and were allowed to attach for 6 hours. Next, 50 ng/ml HGF (R&D Systems) was added to two samples of each quadruplicate and pictures were taken after 16 hours of incubation, using a video camera attached to a Zeiss Axiovert 200 M motorized inverted microscope.

Tubulogenesis in 3-D collagen gels

Tubulogenesis assays were carried out using a 3D collagen cell culture system (Chemicon International), following the manufacturer's instructions. Briefly, selected MDCK clones (1.5×10^3 cells) were mixed with 200 μl of chilled collagen and allowed to gel at 37°C in 24-well plates. Next, DMEM medium containing 3% bovine foetal serum and 30 ng/ml HGF was added to the wells. This medium was renewed every 2-3 days and photographs were taken after 7 days, and the percentage of cysts with extensions was quantified using a 40× objective on an inverted microscope (Axiovert 200, Carl Zeiss). In the case of BAE-1 cells, tubule formation was induced by adding 100 ng/ml VEGF to 5×10^3 cells per well growing in the collagen gel, and photographs were taken after 2 days, as described by Maeshima et al. (Maeshima et al., 2004). To semi-quantify tubulogenic activity, the percentage of cell clusters containing tubular structures was determined. When indicated, different amounts of recombinant ADAMTS12 directly extracted from the 293-EBNA cell layer or after purification were added to the wells.

Animals and subcutaneous tumours

Male adult severe combined immunodeficient mice (SCID; C.B-17/IcrCrl-scid-BR) were obtained from Charles River Laboratories (Sulzfeld, Germany), and animal care and experimentation was carried out following institutional guidelines approved by the local animal review board. Mice were kept in sterile cages bedded with sterilized soft wood granulate and fed irradiated rat chow ad libitum with autoclaved water in a 12-hour light-dark cycle. All manipulations were performed in a laminar-flow hood and the mice were intraperitoneally anaesthetized with a mixture of 50 mg/kg ketamine and 10 mg/kg xylazine. For euthanasia, animals were given a lethal dose of ketamine and xylazine. To induce subcutaneous tumours, suspensions of A549 cells or A549 that expressed recombinant ADAMTS12 (A549-TS12) were subcutaneously injected (6×10^6 cells in 0.5 ml PBS) into the left side of four SCID mice. Selection of the clone A549-TS12 was carried out following a procedure similar to that described above for the generation of stable ADAMTS12-expressing MDCK and 293-EBNA cells. The health status of mice and appearance of tumours was monitored routinely and the animals remained healthy throughout the entire experiment. Tumour size was measured with a calliper and tumour volume was determined using the formula: $V=0.4\times A\times B^2$, where A is the largest dimension of the tumour and B is the smallest dimension.

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