SHORT COMMUNICATION

# Higher sensitivity of *Adamts12*-deficient mice to tumor growth and angiogenesis

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ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) constitute a family of endopeptidases related to matrix metalloproteinases. These proteases have been largely implicated in tissue remodeling and angiogenesis associated with physiological and pathological processes. To elucidate the *in vivo* functions of ADAMTS-12, we have generated a knockout mouse strain (*Adamts12*$^{-/-}$) in which *Adamts12* gene was deleted. The mutant mice had normal gestations and no apparent defects in growth, life span and fertility. By applying three different *in vivo* models of angiogenesis (malignant keratinocyte transplantation, Matrigel plug and aortic ring assays) to *Adamts12*$^{-/-}$ mice, we provide evidence for a protective effect of this host enzyme toward angiogenesis and cancer progression. In the absence of Adamts-12, both the angiogenic response and tumor invasion into host tissue were increased. Complementing results were obtained by using medium conditioned by cells overexpressing human ADAMTS-12, which inhibited vessel outgrowth in the aortic ring assay. This angio-inhibitory effect of ADAMTS-12 was independent of its enzymatic activity as a mutated inactive form of the enzyme was similarly efficient in inhibiting endothelial cell sprouting in the aortic ring assay than the wild-type form. Altogether, our results show that ADAMTS-12 displays antiangiogenic properties and protect the host toward tumor progression.
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## Introduction

Cancer progression depends not only on the acquisition of new properties by neoplastic cells, but also on a complex cross talk occurring between tumor cells and their microenvironment implicating different types of cells, soluble mediators and cell membrane-associated molecules (Nyberg *et al.*, 2008). The contribution of proteolytic enzymes to cancer progression has long been associated with their ability to degrade extracellular matrix components and has been recently extended to their capacity to control the activity and bioavailability of these mediators (Egeblad and Werb, 2002; Cauwe *et al.*, 2007; Overall and Blobel, 2007). Recently, the generation of animal models involving gain or loss of function of matrix metalloproteinases (MMPs) has led to the surprising discovery of tumor-suppressive function for some proteases (Lopez-Otin and Matrisian, 2007). These host-protective proteases are not produced by tumor cells, but mainly by tumor infiltrating cells including inflammatory cells (MMP-8) (Balbin *et al.*, 2003) and fibroblastic cells (MMP-19) (Jost *et al.*, 2006). These recent findings have broken the dogma of proteases as simple positive regulators of cancer progression and emphasize the urgent need in identifying individual proteases as host-protective partners or tumor-promoting agents.

Among proteases with putative tumor-suppressive functions are the ADAMTS (a disintegrin and metallo-proteinase with thrombospondin motifs), MMP-related enzymes characterized by the presence of at least one thrombospondin type I domain (TSP-1) (Cal *et al.*, 2001; Porter *et al.*, 2004, 2005; Bai *et al.*, 2009). Their multi-domain structure endows these secreted proteins with various functions including the control of cell proliferation, apoptosis, adhesion and migration (Noel *et al.*, 2008; Rocks *et al.*, 2008; Bai *et al.*, 2009). It is worth noting that ADAMTS-1 and ADAMTS-8 display antiangiogenic properties (Vazquez *et al.*, 1999; Liu *et al.*, 2006). Our recent studies have highlighted the antitumor properties exhibited by ADAMTS-12 (Cal *et al.*, 2001; Llamazares *et al.*, 2007). In accordance with this concept of ADAMTS-12 being a host-protective enzyme, *ADAMTS12* is epigenetically silenced in human tumor samples and tumor cell lines (Moncada-Pazos *et al.*, 2009). However, the exact contribution of ADAMTS-12 during the different steps of cancer progression including angiogenesis remains to be elucidated. To address this important issue, we have generated mutant mice lacking the *Adamts12* gene. *Adamts12* deficiency did not cause obvious abnormalities

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during embryonic development or in adult mice. Therefore, mutant mice provide a novel and useful tool to investigate Adamts-12 functions in pathological angiogenesis. Through different complementary approaches, we provide evidence that Adamts-12 protects the host against tumor angiogenesis, growth and invasion.

## Results

### Adamts12-*deficient mice are viable without any obvious phenotype*
The biological functions of ADAMTS-12, a metalloprotease-related enzyme overexpressed in human cancers (Porter *et al.*, 2004), are poorly understood. To establish a mutant mouse strain deficient for *Adamts12* gene (KO, *Adamts12*^{−/−} mice), the targeting vector was designed to replace the exons 6 and 7 (corresponding to the N-terminal part of the catalytic domain) by a neomycin-phosphoglycerate kinase (NEO-PGK) cassette (Figure 1a) and to introduce a frame shift. Embryonic stem clones generated by homologous recombination (Figure 1b) were injected into C57Bl/6J blastocysts to generate chimeric males. Heterozygous mice from the F1 generation were intercrossed to generate *Adamts12*-deficient mice that were obtained in the Mendelian ratio. We checked mice genotypes by PCR (Figure 1c).

The expression of *Adamts12* was determined by reverse transcriptase (RT) PCR in different organs. In WT mice, *Adamts12* was expressed in some organs, including the ovary, mammary gland, uterus, lung, ear cartilage and lymph node (Figure 1d). *Adamts12* was not expressed in the heart, kidney, bone, liver, brain, intestine, testis, muscle, skin, eyes and spleen (data not shown). As expected, tissues from *Adamts12*^{−/−} mice did not produce any Adamts-12 in all organs tested, as assessed by RT–PCR amplification using primers targeting the catalytic domain (Figure 1d) and the pro-domain (data not shown). These RT–PCR analysis show that no residual mRNA was produced in mutant mice because of the frame-shift introduced during exon replacement.

Despite *Adamts12* deficiency, mutant mice developed normally were fertile and had long-term survival rates indistinguishable from those of their wild-type (WT) counterpart. No obvious phenotype was detected. These findings clearly indicate that *Adamts12* is dispensable for embryonic and adult mouse development and growth.

### Adamts12 *deficiency affects tumoral angiogenesis*
In the course of the phenotyping of *Adamts12*^{−/−} mice, we evaluated its expression in pathological conditions. *Adamts12* expression was first investigated during laser-induced choroidal neovascularization by RT–PCR analyses performed at various time points after laser burn (Lambert *et al.*, 2003). *Adamts12* expression was never detected at any stage of choroidal neovascularization (data not shown), excluding the potential implication of Adamts-12 in choroidal angiogenesis.

The overexpression of *ADAMTS12* in various human cancers (Cal *et al.*, 2001) prompted us to explore its putative functions in tissue remodeling associated with cancer development. We applied three models of angiogenesis, which have been previously successfully used to evaluate the contribution of different metalloproteinases during angiogenic processes (Masson *et al.*, 2002; Berndt *et al.*, 2006, 2008). The transplantation system is a highly sensitive tool to inspect the kinetic of early steps of host stromal response to tumor signals (Mueller and Fusenig, 2004; Jost *et al.*, 2007, 2008). Malignant PDVA cells issued from carcinogen treatment of murine keratinocytes (Fusenig *et al.*, 1983) precultured on a type I collagen gel were transplanted onto WT ($n = 14$) and *Adamts12*-deficient mice ($n = 14$). An early endothelial cell migration toward the tumor layer can be visualized through double immunostaining carried out to distinguish tumor cells (keratin positive) and vessels (CD31 positive cells or type IV collagen-positive basement membrane) (Blacher *et al.*, 2008). Two different patterns of invasion were observed 21 days after transplantation (Figure 2). A low vascularized profile scored $+$ was characterized by blood vessel infiltration of the collagen gel without reaching the tumor layer. Then, once blood vessels have reached tumor cell layers, malignant keratinocytes formed tumor sprouts that invaded downward the remodeled host tissue and were intermingled with new vessels (Figure 2). Such highly vascularized pattern was scored $+ + +$. Tumor vascularization and invasion was improved in *Adamts12*^{−/−} mice. Indeed, 86% of transplants were highly vascularized (scored $+ + +$), whereas 14% of samples were scored $+$. In WT mice, the percentage of transplants scored $+ + +$ fell down to 57% and that of low vascularized transplants reached 43% ($P = 0.027$, $\chi^2$-test) (Figure 2a).

To further confirm this distinct invasive profile, an original method of computerized image analysis (Blacher *et al.*, 2008) allowing a concomitant quantification of blood vessel recruitment and tumor cell invasion was applied to all samples. The distribution of tumor cell and endothelial cell densities was determined as a function of distance to the top of the tumor (Figures 2b and c). With this aim, a grid formed from the dilatation of tumor boundaries was superposed onto tumor and vascularization images as previously described (Blacher *et al.*, 2008). The degree of superimposition of curves corresponding to vessel and tumor densities determines tumor vascularization. Yellow spots in Figure 2 delineate areas of tumor in which tumor cells and blood vessels were intermingled, reflecting tumor vascularization. In *Adamts12*^{−/−} mice, invasion and migration process took place to a much larger extent (Figures 2b and c; $P < 0.05$). A stronger invasion characterized by a deeper extension of tumor cells was observed in *Adamts12*^{−/−} mice (migration up to $L_{max} = 3.55 \pm 0.4$ mm in KO mice versus $L_{max} = 2.5 \pm 0.4$ mm in WT mice, $P < 0.05$, Mann–Whitney test). In addition, blood vessels migrated roughly toward tumor cells leading to increased overlapping area of tumor cells and blood vessels (Figures 2b and c, yellow spot). Indeed, the area of
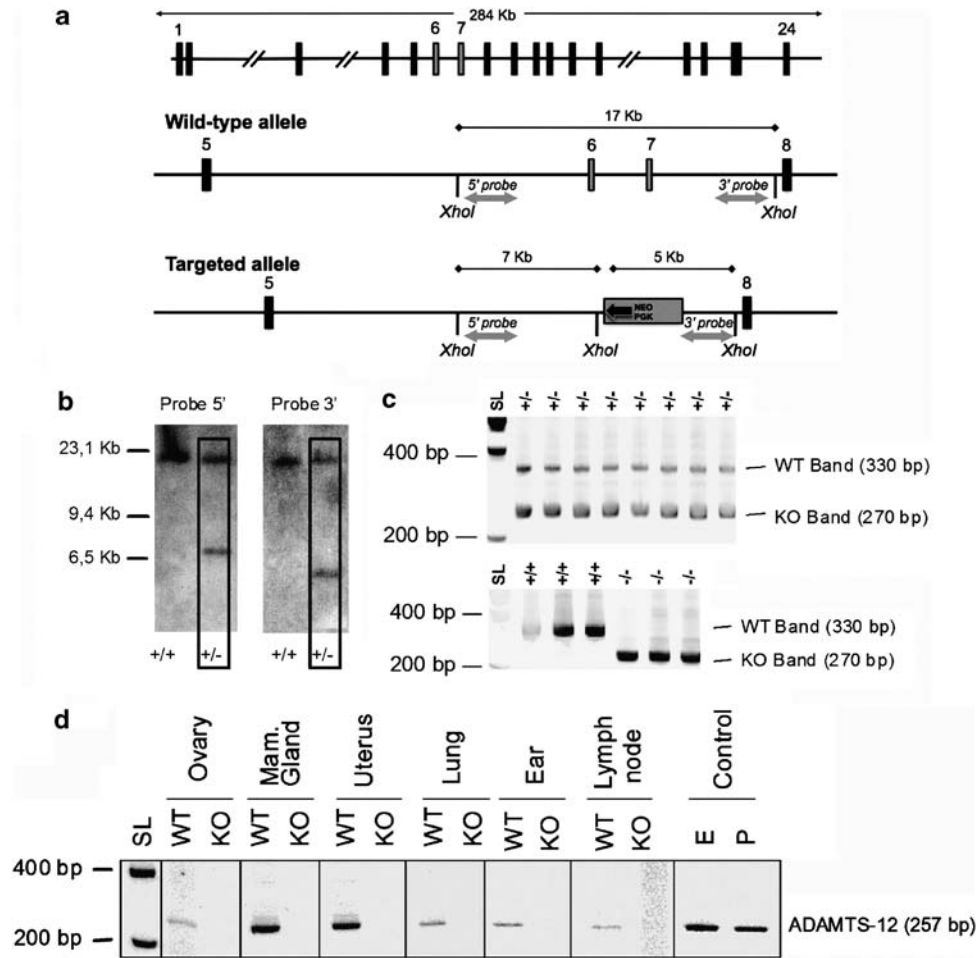
**Figure 1** Targeted disruption of mouse *Adamts12* gene. (**a**) Schematic illustration of the targeting strategy (top, WT allele; bottom: targeted allele). A genomic clone encoding *Adamts12* was obtained from a mice 129/SvJ strain genomic DNA library. The exons 6 and 7 were replaced by a neomycin-phosphoglycerate kinase (NEO-PGK) cassette. Predicted sizes of Xho I fragments are indicated (rectangle, exon; double-headed arrows: probes for detecting the homologous recombination event). (**b**) Xho I Southern blot analysis of +/+ and +/− ES129 cells. The targeted embryonic stem (ES) clones obtained by homologous recombination were then injected into blastocysts (10–15 cells per blastocyst of day 4). (**c**) Example of PCR genotyping from tail biopsies of *Adamts12* WT (+/+), *Adamts12* heterozygous (+/−) and homozygous *Adamts12* (−/−) mice generated in C57BL/6 genetic background. (**d**) RT–PCR analysis of *Adamts12* expression in WT and KO mouse tissues. Positive controls are embryo (E) and placenta (P). Representative gels are displayed with the expected size (bp) of RT–PCR products on the right.

vessels intermingled with tumor cells was twice higher in mutant mice (0.27 versus 0.13 mm² in WT mice).

These data indicate that *Adamts12* deficiency in host tissue is associated with an acceleration of the angiogenic response and increased tumor invasion into the host tissue. It is worth noting that malignant keratinocytes used in this model did not express *Adamts12 in vitro*, whereas human ADAMTS-12 was detected in tumor transplants. The RT–PCR analysis of tumor cell layer and the host compartment separated through laser microdissection revealed that *Adamts12* was expressed by host cells rather than by tumor cells (Figure 2d). These results show an induction of host *Adamts12* expression following tumor cell transplantation *in vivo*. These data are in line with our recent study showing that *ADAMTS12* expression by fibroblasts is enhanced in co-culture with cancer cells (Moncada-Pazos *et al.*, 2009). They are also in full agreement with a previous report

showing that *ADAMTS12* as other family members (*ADAMTS2*, *7*, *8* and *10*) are predominantly expressed in stromal fibroblasts from human mammary tissues, but not in breast cancer cells (Porter *et al.*, 2004). Altogether, these observations suggest that fibroblastic cells are an important source of ADAMTS-12 and respond to the presence of tumor cells by producing this enzyme.

*Adamts-12 affects* in vivo *and* ex vivo *angiogenesis*
The angiogenic response to basic fibroblast growth factor (bFGF) was then investigated in WT and mutant mice. Matrigel (500 μl) containing heparin (10 U/ml) and bFGF (250 ng/ml) were injected subcutaneously into mice (*n* = 16). To quantify functional vessel recruitment, hemoglobin concentrations were measured in the plugs harvested 7 days after injection (Berndt *et al.*, 2006). The angiogenic response induced by bFGF was again higher
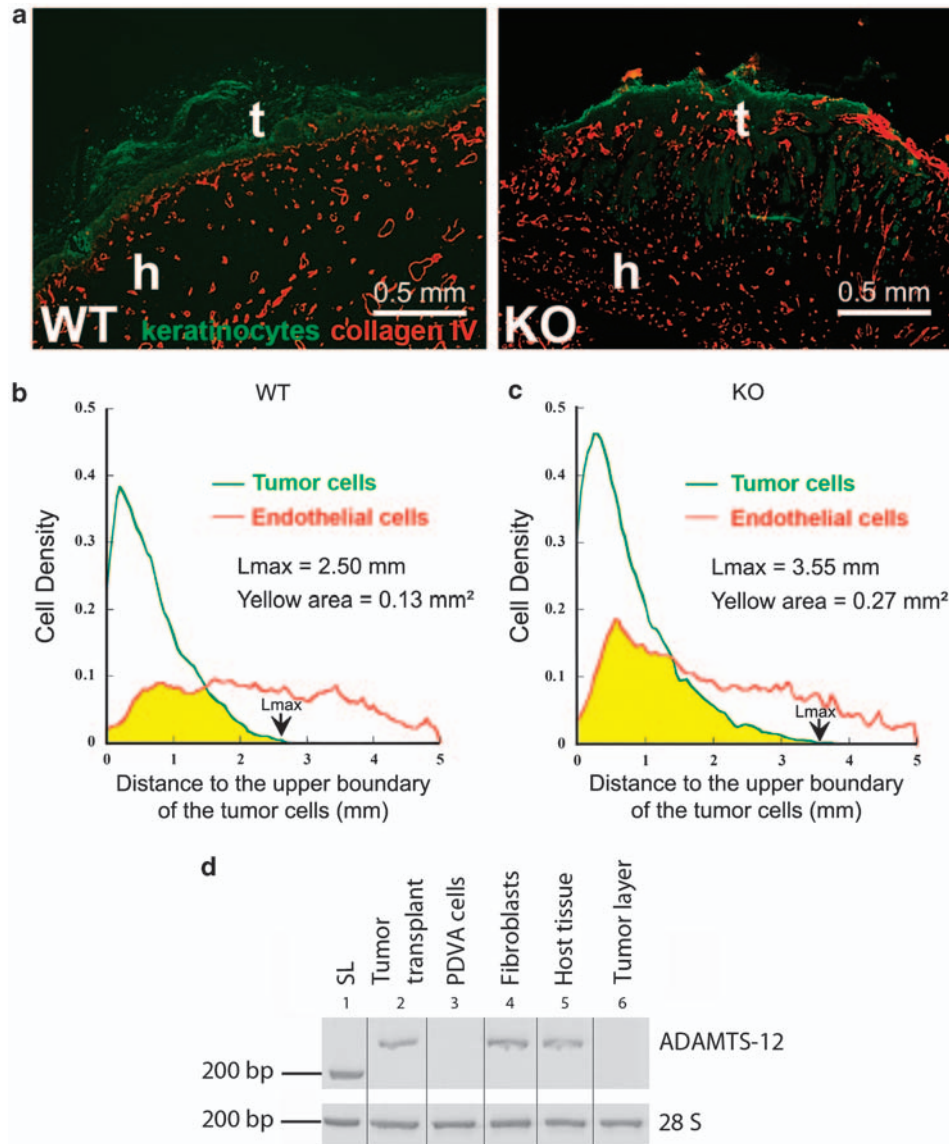
**Figure 2** Histological analysis of tumor transplanted into mice. Malignant murine PDVA keratinocytes precultured on a collagen gel were covered by a silicone chamber and transplanted for 3 weeks on the back of 6- to 8-week-old mice *Adamts12*$^{+/+}$ (WT) or *Adamts12*$^{-/-}$ (KO) mice ($n=14$ per experimental groups). (**a**) Double immunostaining of tumor transplant resected from WT (left panel) and KO mice (right panel) were carried out on cryostat section to identify tumor cells (anti-keratin antibody, green, rabbit polyclonal antibody, Dako, Glostrup, Denmark) and vessels (anti-type IV collagen or anti-CD31, red guinea pig polyclonal antibody, Sigma-Aldrich, St Louis, MO, USA). t, tumor; h, host tissue. Magnification: $\times 40$. Bar, 0.5 mm. (**b, c**) Tumor and endothelial cell density distributions determined by computerized image analysis with the Aphelion 3.2 software from Adsis (Meythet, France). After image binarization/segmentation, the upper boundary of the tumor was automatically detected and a grid was constructed with the successive dilations ($n=1, 2, 3...$) of this upper boundary. Tumor and vessel densities were determined on each interval of the grid. Results were drawn in function of the distance to the upper tumor limit. Intermingling of tumor cells and vessels is shown by superimposition of the respective curves (yellow areas). The maximal distance of tumor cell invasion ($L_{max}$) is indicated by an arrowhead. (**d**) RT–PCR analysis of Adamts-12 expression in the whole tumor transplant (2, tumor transplant), in PDVA cells cultured *in vitro* (3, PDVA cells), in *in vitro* cultures of fibroblasts (4, fibroblasts), in the host tissue compartment of tumor transplant (5, host tissue) and in the tumor layer of tumor transplant (6, tumor layer). The mRNA of the tumor layer and the host compartment were extracted from samples isolated here with laser microdissection performed on tumor transplant cryostat sections. SL (1): molecular weight (smart ladder).

in *Adamts12*$^{-/-}$ than in WT mice. In WT mice, the mean hemoglobin content was two-fold reduced ($0.65 \pm 0.1$ Hb (mg/ml) per mg of plug in WT mice versus $1.65 \pm 0.5$ Hb (mg/ml) per mg of plug in *Adamts12*$^{-/-}$ mice), and the percentage of poorly vascularized plugs (hemoglobin concentration lower than 0.5 Hb (mg/ml) per mg of plug)

reached 56% (9 of 16) in WT mice, whereas it was $<12\%$ (2 of 16) in mutant mice ($P<0.05$).

To further study the contribution of Adamts-12 in neovessel formation, aortic explants issued from mutant or WT mice were embedded into a three-dimensional type I collagen gel in the presence of serum

corresponding to their genotype (Figure 3a). An increased angiogenic response was observed in *Adamts12*^{-/-} mice as evidenced by increased sprout density (Figure 3b, left graph) and vessel length (Figure 3b, right graph). As the formation of capillary-like structures is associated with the spreading out of mural cells, we performed immunohistochemical labeling on whole mount of aortic rings. Interestingly, increased mural cell recruitment detected by NG2 labeling was observed in IB4-positive capillaries issued from *Adamts12*^{-/-} mice (Figure 3c). Computerized quantification revealed that the mean area of colocalization between IB4-positive endothelial cells and NG2-positive mural cells was twice higher in sprouts issued from *Adamts12*^{-/-} mice as compared with those from WT explants (Figure 3d). These data reflect an increased maturation of blood vessels in the absence of Adamts-12. Altogether, these findings indicate that *ADAMTS12* is a negative regulator of angiogenesis.

The finding that *Adamts12* deficiency resulted in an acceleration of the angiogenic response led us to postulate that cells overexpressing ADAMTS-12 would have opposite effect and would inhibit it. To address this

question, we next evaluated the impact of medium conditioned by a population of MCF7 cells overexpressing human FLAG-tagged ADAMTS-12 (MCF7p ADAMTS-12 FLAG) on neovessel formation in another experimental model using aortic rings issued from a rat. The presence of ADAMTS-12 in the conditioned media was assessed by western blot (Figure 4d) using an anti-FLAG antibody allowing the detection of a specific band of molecular mass $\approx 175$ kDa. In accordance with our hypothesis, the medium of cells overexpressing ADAMTS-12 inhibited the angiogenic response as revealed by a reduction vessel length (Figure 4b) and vessel maturation through pericyte coverage (Figure 4c).

We next determined whether the antitumorigenic effect of ADAMTS-12 could be ascribed to its catalytic function. With this aim, new MCF7 transfectants were generated to produce inactive mutants of ADAMTS-12 or intact ADAMTS-12. Point mutations of two key amino acids of the catalytic site (H465Q/E466A) were performed as previously described (Cal *et al.*, 2001). The mutated ADAMTS-12 form was produced at similar levels than the WT form as assessed by western
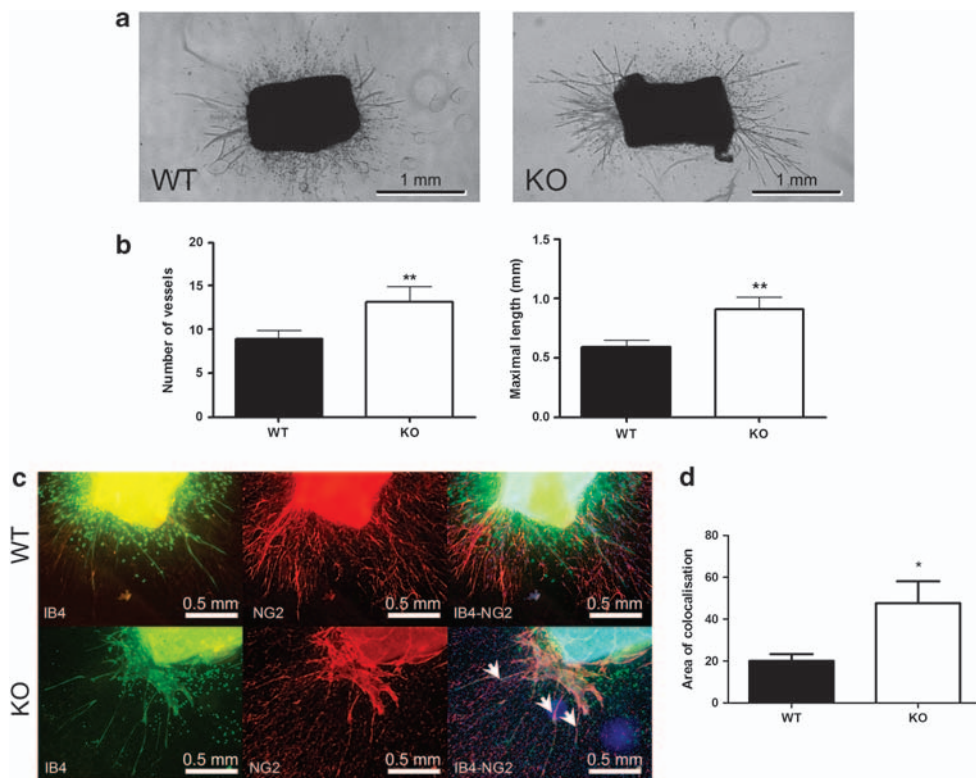


**Figure 3** Capillary outgrowth from mouse aortic rings cultured in a collagen gel. (**a**) Morphological observation of explants of mouse thoracic aorta issued from *Adamts12*^{+/+} (WT) and *Adamts12*^{-/-} mice (KO). Magnification: $\times 25$. Bar, 1 mm. (**b**) Computerized quantification. Two parameters are shown: number (left graph) and maximal length of vessels (right graph). **$P < 0.01$ (Mann–Whitney test). (**c**) Characterization of cells spreading out of aortic rings. Immunostainings were performed on whole-mount rings to identify endothelial cells (Griffonia Simplifolia isolectine B4/Alexa Fluor 488, green, Molecular Probes Inc., Eugene, OR, USA), pericytes (rabbit anti-NG2 chondroitin sulfate proteoglycan antibody, red, Sigma-Aldrich) and pericytes covering endothelial cells (IB4 + NG2, merged in yellow) (white arrowheads). Magnification: $\times 40$. Bar, 0.5 mm. (**d**) The area of colocalization (mm²) between pericytes and endothelial cells was determined by using a computer-assisted method quantification by implementing an algorithm using MATLAB7.1 software. The assay was conducted by using at least triplicate culture per condition and results are those of a representative assay out of three. *$P < 0.05$ (Mann–Whitney test).
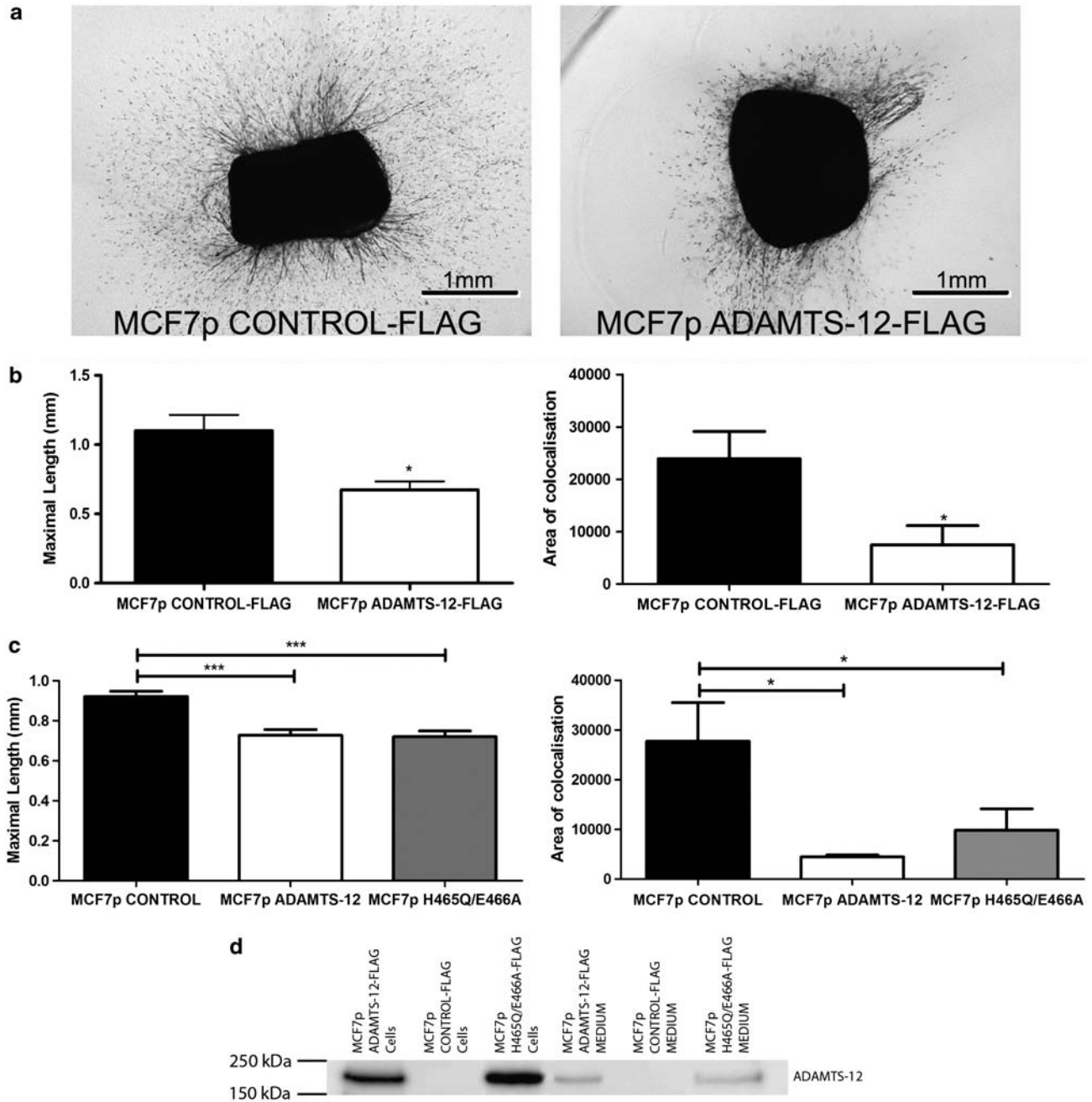
**Figure 4** Capillary outgrowth from rat aortic rings cultured in the presence of ADAMTS-12. Wistar rat aortic explants were cultured in MCDB-131 medium (2.5 ml) supplemented with medium (2.5 ml) conditioned by a population of breast adenocarcinoma MCF7 cells expressing (MCF7pADAMTS12-FLAG) or not (MCF7pCONTROL-FLAG) tagged ADAMTS-12. (**a**) Morphological observation of aortic explants in the absence of ADAMTS-12 (MCF7pCONTROL-FLAG) or in the presence of ADAMTS-12 (MCF7pADAMTS-12-FLAG). Magnification: × 25. Bar, 1 mm. (**b**) Maximal length of vessels was quantified by computerized image analysis (left panel). The area of colocalization (mm²) of pericytes and endothelial cells was determined by using a computer-assisted method applied to immunostainings performed on whole mounts (see legend of Figure 3) (right panel). (**c**) Impact of point mutation of ADAMTS-12 catalytic site. Populations of MCF7 cells expressing WT ADAMTS-12 (MCF7pADAMTS-12), inactive mutant ADAMTS-12 (MCF7pH465Q/E466A) or not expressing ADAMTS-12 (MCF7pCONTROL) were generated by stable transfection, and their conditioned media were tested in the rat aortic ring assay. The double point mutation in the catalytic site did not affect the inhibitory effect of ADAMTS-12 on vessel sprouting (left panel) and on vessel coverage by pericytes (right panel). Similar results were obtained with two clones of each transfectants. The assay was conducted by using at least triplicate culture per condition and results are those of a representative assay out of three. *$P < 0.05$; ***$P < 0.001$ (Mann–Whitney test). (**d**) Western blot analysis of the ADAMTS-12-expressing MCF7 cells (MCF7p ADAMTS-12-FLAG), control cells (MCF7p CONTROL-FLAG) and the mutated ADAMTS-12 (MCF7p H465Q/E466A-FLAG) in cell extracts and conditioned media concentrated 10-fold using Amicon Ultra (Millipore, Billerica, MA, USA). ADAMTS-12 was visualized by using an antibody raised against the Flag epitope with anti-FLAG-M2 antibodies (Sigma-Aldrich).

blotting (Figure 4d). These mutations in the catalytic domain did not impair the antiangiogenic effect induced by medium conditioned by transfectants (Figures 4d and e). Indeed, the medium conditioned by cells overexpressing intact ADAMTS-12 or mutated ADAMTS-12 inhibited the spreading out of endothelial cells as assessed by a reduction of vessel length (Figure 4d) and of vessel maturation through pericyte coverage (Figure 4e). These results suggest that the catalytic activity of ADAMTS-12 is dispensable for its angioinhibitory function.

Our data are in line with our previous report showing that ADAMTS-12 inhibits the formation of vascular endothelial growth factor-induced tubular structures in BAE-1 cells (Llamazares *et al.*, 2007). The finding of antiangiogenic effects for a member of the ADAMTS family is not unprecedented as it has also been described for ADAMTS-1 and ADAMTS-8 (Vazquez *et al.*, 1999; Dunn *et al.*, 2006). However, the originality of our findings relies on the identification of a key host ADAMTS-12 contribution in the host protection toward the angiogenic response induced by tumor cells. This report based on the generation of a novel loss-of-function animal model provides the first demonstration of the antiangiogenic properties of a single host ADAMTS. Moreover, in contrast to ADAMTS-1 whose proteolytic activity is apparently required for its antiangiogenesis property (Iruela-Arispe *et al.*, 2003), the angioinhibitory property of ADAMTS-12 does not depend on its catalytic activity. We cannot ruled out the possibility that ADAMTS-12 can sequester vascular endothelial growth factor through its C-terminal thrombospondin domain(s) and the spacer region as previously described for ADAMTS-1 (Luque *et al.*, 2003). Addressing this issue would require a careful dissection of the complex C-terminal part of ADAMTS-

12 containing seven thrombospondin repeats, two spacers and a PLAC domain, which is out of the scope of this study.

In conclusion, we propose that the ADAMTS-12 production induced in response to the presence of cancer cells could have a beneficial protective effect toward tumor growth and invasion. The new generation of *Adamts12*-deficient mice provides a suitable tool to give new insights into the *in vivo* functions of this enzyme, which are presently unknown. In addition, this new transgenic tool paves the way for further investigations on the biological functions of ADAMTS-12 in various pathological conditions in which the enzyme is putatively involved such as asthma (Kurz *et al.*, 2006) and arthritis (Bai *et al.*, 2009).

## Conflict of interest

The authors declare no conflict of interest.

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