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**ORIGINAL ARTICLE**

# Cloning, expression and functional characterization of the full-length murine ADAMTS-13

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**Summary.** Functional deficiency or absence of the human von Willebrand factor (VWF)-cleaving protease (VWF-cp), recently termed ADAMTS-13, has been shown to cause acquired and congenital thrombotic thrombocytopenic purpura (TTP), respectively. As a first step towards developing a small animal model of TTP, we have cloned the complete (non-truncated) murine *Adamts-13* gene from BALB/c mice liver poly $A^+$ mRNA. Murine ADAMTS-13 is a 1426-amino-acid protein with a high homology and similar structural organization to the human ortholog. Transient expression of the murine *Adamts-13* cDNA in HEK 293 cells yielded a protein with a molecular weight of approximately 180 kDa which degraded recombinant murine VWF (rVWF) in a dose-dependent manner. The cleavage products of murine rVWF had the expected size of 140 and 170 kDa. Murine ADAMTS-13 was inhibited by EDTA and the plasma from a TTP patient.

**Keywords**: ADAMTS-13, thrombotic thrombocytopenic purpura, von Willebrand factor, von Willebrand factor-cleaving protease.

## Introduction

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein with two distinct biological functions. First, it is required for platelet adhesion and platelet aggregation to sites of vascular damage, and second, it acts as a carrier protein for blood-clotting factor VIII in the circulation [1–3]. Only the largest VWF multimers are hemostatically active and can effectively mediate platelet adhesion, particularly under the high shear stress conditions of the arterial capillary system [4–6]. To stop bleeding, secretion of very high-molecular-weight VWF, so-called 'unusually large VWF' (ULVWF) by endothelial cells at the site of vascular injury is therefore of physiological importance. Downstream of the injury site, however, the uncontrolled presence of ULVWF can have severe consequences. If the degradation of VWF is impaired, persistent ULVWF multimers can, by high-affinity interaction with platelet receptors such as GP1b, agglutinate platelets at sites with high levels of shear stress and thereby promote the risk of thrombosis [7]. The disease associated with the formation of platelet and VWF-rich thrombi is life threatening and has been termed thrombotic thrombocytopenic purpura (TTP). Clinical symptoms of TTP include microangiopathic hemolytic anemia, thrombocytopenia, fluctuating neurological impairment, renal dysfunction and fever [8–10].

A plasmatic metalloprotease, originally termed VWF-cleaving protease (VWF-cp) is associated with VWF degradation [11,12], and a deficiency of VWF-cp has been found in plasmas of patients with familial relapsing TTP as well as the presence of an antibody inhibitor to VWF-cp in patients diagnosed with acquired TTP [13–16].

N-terminal sequencing of purified plasmatic VWF-cp [17,18] as well as conventional cDNA cloning [19,20] and a sophisticated positional cloning approach [21] identified the gene encoding for human VWF-cp as a member of a multidomain metalloprotease family called ADAMTS (a disintegrin-like and metalloprotease with thrombospondin type 1 repeat motifs [22,23]). According to the suggested nomenclature of ADAMTS proteins, human VWF-cp was designated ADAMTS-13. Expression studies showed functional activity of recombinant ADAMTS-13 (rADAMTS-13) [24,25] in plasma towards human VWF as a substrate. ADAMTS-13 was identified as the enzyme inhibited by the IgG fraction of TTP patients' plasma, suggesting a causal relation between ADAMTS-13 inhibitory antibodies and TTP. Human rADAMTS-13 was also shown to correct the

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deficiency of VWF-degrading capacity in plasma from patients diagnosed with hereditary TTP [26].

Tremendous progress has been made in explaining the causal relations that ultimately lead to clinically relevant TTP [27]. However, many questions about the cause of TTP are still unanswered and cannot easily be addressed in humans. A small animal model of TTP would therefore be of great clinical interest. Despite the known limitations of animal models, the availability of an animal model faithfully mimicking human TTP would greatly facilitate the development of treatment modalities other than plasma exchange or plasma infusion.

The putative sequence of the murine *Adamts-13* gene has recently been predicted by automated computational analysis (NCBI GenBank: AB_095445) and the full-length cDNA sequence was determined [28], but functional activity of the murine ADAMTS-13 gene product has not yet been verified.

A first step towards development of a murine model for TTP is the cloning and characterization of the murine ortholog to the human ADAMTS-13. In this study we describe the cloning of the complete (non-truncated) murine *Adamts-13* cDNA sequence, an *Adamts-13* mRNA expression analysis, the expression of the murine recombinant ADAMTS-13 (rADAMTS-13) in eukaryotic cells and the initial functional characterization of the activity of the mouse enzyme towards murine recombinant VWF (rVWF).

## Materials and methods

### *Database searches*

Nucleic acid sequence information was obtained by searching the NCBI GenBank (http://www.ncbi.nlm.nih.gov/). The SMART program (http://smart.embl-heidelberg.de) and ClustalW (http://www.ebi.ac.uk/clustalw/) were used for analysing domain structures. Amino acid sequences were compared by use of the EMBL protein database (http://www.ebi.ac.uk/clustalw) and AlignX (Vector NTI Suite 8.0; InforMax, Frederick, MD, USA).

### *Reverse transcription of poly A$^+$ RNA*

To obtain long transcripts, 3 μg of poly A$^+$ RNA from liver of BALB/c mice (Stratagene, San Diego, CA, USA) was heated for 5 min at 64 °C and reverse transcribed for 1.5 h at 42 °C with the Expand RT kit from Roche (Roche Molecular Biochemicals, Mannheim, Germany) in a final volume of 120 μL with concentrations of reagents according to the instructions of the manufacturer using 100 pmol of primer spdT consisting of a poly dT part, a randomly chosen specific sequence (MC 18) and two NN at the 3′ end for specific fixation of the primer (Table 1).

### *Polymerase chain reaction*

All purifications were carried out according to the supplier protocols. Complementary DNA solution (1 μL) was subjected to polymerase chain reaction (PCR) using a thermally activated DNA polymerase (FastStart Taq DNA Polymerase from Roche Molecular Biochemicals, or HotStar Taq polymerase from Qiagen, Hilden, Germany). The PCR was carried out in a total volume of 50 μL containing 1 U of the polymerase in the buffer supplied by the manufacturer (with addition of GC solution as recommended), 200 μM of dNTP and 50 pmol each of forward and reverse primer (Table 1). The samples were overlaid with mineral oil, incubated for 4 min at 95 °C (14 min for HotStar polymerase) and then amplified for 45 cycles in a TRIO-Thermoblock (Biometra, Göttingen, Germany) with the following cycle profile: 50 s at 95 °C, 50 s at 62 °C, and 1–6 min at 72 °C (1 min extension for approx. 1 kb of DNA). The samples were then fractionated on a 1% agarose gel stained with ethidium bromide.

### *Sequencing of PCR products*

For each primer pair multiple independently created PCR products were sequenced on an Applied Biosystems Model 373A Sequencer (Applied Biosystems, Foster City, CA, USA) using the cycle sequencing method with dye terminators (BIG

**Table 1** Sequences of primers used for polymerase chain reaction

| Name | Sequence | Remark |
|---|---|---|
| 6844 | 5′-TTCTGCACCTGGAACTCCTG-3′ | Expr. PCR forward |
| 6956 | 5′-TAGAGGCCAGGCTGTGCCATCAGCTGG-3′ | Expr. PCR reversed |
| 6957 | 5′-ACCTGGGCTGCAGTCAGGACTTA-3′ | Expr. PCR forward |
| 6963 | 5′-GGAGTGCTGCTACGGTACTGGAGTC-3′ | Expr. PCR forward |
| 6964 | 5′-ATGGTCCAATACTCGCCCTGGA-3′ | Expr. PCR reversed |
| 6992 | 5′-ACAAGCCTGCGTGTTGCATC-3′ | Expr. PCR reversed |
| 7577 | 5′-GAGCGAATTCGCCGGCCACCATGAGCCAGCTTTGCCTGTG-3′ | *Eco*RI forward 5′ |
| 7578 | 5′-GAGCGGTACCGTGAGATACTAGTCTTCCCTGC-3′ | *Spe*I reversed 5′ |
| 7589 | 5′-GACAGGTACCACTAGTATCTCACCCAACACC-3′ | *Spe*I forward 3′ |
| 7580 | 5′-GCAGTTCCATGGCTCGAGGCTAAAGTTGCATACTCC | *Xho*I reversed 3′ |
| spdT | 5′-GAGCAAATTCCTGTACTGAC $(T)_{30}$ NN-3′ | Production of cDNA |
| MC18 | 5′-GAGCAAATTCCTGTACTGAC-3′ | Specific part of spdT |
| 7455 | 5′-GCAGCAGGTGCTCTACTGGGAGTC-3′ | Expr. PCR forward |
| 7635 | 5′-CAGCCCTTCTGCACCTGGAAAAGGTCACTA-3′ | Expr. PCR reversed |

Dye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems) and the same primers as used in the PCR. The chromatograms were aligned and assembled to the complete gene sequence using the Sequence Editor (SeqEd; Applied Biosystems).

### *N-terminal amino acid sequence analysis*

The VWF cleavage fragments generated by incubation with murine rADAMTS-13 were separated by reducing SDS–PAGE and were electrotransferred to a Sequi-blot polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA, USA) according to Plaimauer *et al*. [25]. N-terminal amino acid sequence analysis of the excised protein bands was carried out at Toplab (Martinsried, Germany).

### *Construction of a murine Adamts-13 cDNA clone*

The cDNA was first PCR amplified with a variety of primers specific for some of the putative exons. To reduce cloning steps to a minimum number, primer combinations were tested to obtain the longest possible PCR products. Finally, two reverse transcriptase (RT)-PCR products obtained from primers 7577/7578 for the 5′ end and 7580/7589 for the 3′ end (Table 1) were necessary to obtain a cDNA construct containing the whole murine *Adamts-13* sequence. Primer 7577 contains an *Eco*RI restriction enzyme recognition sequence, the Kozak sequence, to enhance translation [29] and an AUG codon, and primer 7580 a *Xho*I site. Primers 7578 and 7589 have a *Spe*I site, which was created internally without altering the amino acid sequence. The 5′-*Adamts-13* PCR fragment obtained from primers 7577/7578 and the 3′ *Adamts-13* fragment from primers 7589/7580 were cloned into the pCR2.1-TOPO vector (TOPO TA cloning kit; Invitrogen, Lofer, Austria). The plasmid containing the 5′ end of *Adamts-13* was digested with *Spe*I, treated with calf intestinal phosphatase (CIP) and the 3′ end of *Adamts-13* cleaved by *Spe*I was ligated into this site. An *Eco*RI/*Xho*I fragment containing the complete murine *Adamts-13* cDNA was then subcloned into pcDNA3.1 (Invitrogen).

### *Multi-tissue Northern blot*

A 768-bp probe (derived from the 5′ end) was excised from the *Adamts-13* cDNA construct by *Eco*RI/*Pml*I digestion, separated on an agarose gel and purified with the Qiagen gel extraction kit (Qiagen). The *Adamts-13* probe and a *β-actin* cDNA control probe were labeled with the random-primed DNA labeling kit (Roche) using $\alpha$-$^{32}$P dCTP (6000 Ci mmol$^{-1}$) and nick columns (Amersham Biosciences, Uppsala, Sweden) for removal of free nucleotides according to the instructions of the suppliers. A commercially available multitissue Northern blot (MTN, poly A$^+$ RNA from BALB/c mice; BD Biosciences, Erembodegem, Belgium) was then hybridized overnight at 60 °C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA, USA), washed twice for 20 min at room temperature with 2 × SSC, 0.05% SDS, once for 20 min at 50 °C with 0.1% SSC, 0.1% SDS and then exposed to a X-ray film. It has to be noted that for the *β-actin* control the presence of a single 2.0-kb band is confirmed for all lanes and a 1.8-kb *actin* isoform may predominate in heart, skeletal muscle and testis lanes.

### *Multi-tissue PCR gene expression panel*

For studying gene expression in different tissues by RT-PCR, a BD MTC™ cDNA panel from BALB/c mice was purchased from BD Biosciences (Palo Alto, CA, USA). The cDNAs were diluted 1 : 3, 1 : 10, 1 : 33, and 1 : 100. Then PCR was performed with 50 pmol each of primer pairs 6844 (forward)/6956 (reversed) and 7455 (forward)/7635 (reversed) (Table 1) or primers specific (supplied by the manufacturer) for the control gene glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) following standard PCR procedures with the following cycle profile: 94 °C for 2 min; five cycles at 94 °C for 30 s and 72 °C for 5 min; five cycles at 94 °C for 30 s and 70 °C for 5 min; 35 cycles at 94 °C for 30 s, 62 °C for 30 s and 70 °C for 5 min.

For embryonic tissues (FVB strain) the mouse Rapid-Scan™ gene expression panel was purchased from Origene Technologies, Inc. (Rockville, MD, USA) and used according to the supplier's instructions. Briefly, each cDNA is diluted into 96 wells to a series of four concentrations (labeled 1000 ×, 100 ×, 10 × and 1 ×), with the lowest concentration (1 ×) being approximately 1 pg cDNA/well. According to the supplier's instructions, 25 μL of PCR premix including 50 pmol of forward primer 7455 and reversed primer 7635 or forward primer 6957 and reversed primer 6992 (Table 1) or primers specific for the control *β-actin* gene (supplied by the manufacturer) were added to each well. The multiwell PCR plate was covered, incubated on ice for 15 min, gently mixed and transferred into the block of a 96-well thermal cycler. PCR was performed as described above. The PCR products were analyzed on a 1% agarose gel.

### *Transfection and cell culture of murine rADAMTS-13*

The murine rADAMTS-13 construct was transiently expressed in HEK 293 (human embryonic kidney fibroblasts; ATCC CRL-1573) cells, routinely grown in DMEM/Ham's F12 (1 : 1) medium (Life Technologies, Lofer, Austria) supplemented with 10% fetal calf serum (full medium). Transient transfection was carried out at 85–95% confluence using lipofectamine 2000 (Life Technologies) according to the supplier's protocol. At 24 h post-transfection medium was changed and serum-free medium was applied to the confluent cells for 24 h. Conditioned medium was collected, clarified by centrifugation and concentrated 20-fold using Amicon Centriprep YM-30 (Millipore, Bedford, MA, USA) at 4 °C. Control cell culture supernatant from cells not transfected was prepared accordingly. The human rADAMTS-13 was expressed as described [25].

### Western blot analysis

Recombinant ADAMTS-13 samples were reduced, denatured, and resolved by SDS–PAGE on 4% stacking/6% separation gels and visualized by Western blotting as described [30]. Recombinant ADAMTS-13 was detected using the monoclonal antibody 242/H2, a subclone of 242/H1 generated as described previously [25], together with alkaline phosphatase-conjugated goat antimouse IgG (Sigma, St Louis, MO, USA) as a detection antibody and 5-bromo-4-chloro-3-indolyl-phosphate/p-nitroblue-tetrazolium (BCIP/NBT) (Promega, Madison, WI, USA) as substrate. Monoclonal antibody 242/H2 is directed against the catalytic domain of human ADAMTS-13.

### Expression of murine rVWF

The murine VWF expression plasmid was a generous gift from D. Ginsburg (University of Michigan). The cDNA encoding murine VWF was amplified by RT-PCR using total lung RNA prepared from CASA/Rk mice. The cDNA was cloned into modified multiple cloning sites of the pcDNA3.1 expression vector (Invitrogen), and is flanked by *Not*I (5′) and *Xba*I (3′) sites. The assembled cDNA contains an optimized Kozak sequence and a 3′ myc epitope tag (EQKLISEEDL), but does not contain the 5′ or 3′ untranslated regions. Fidelity of the PCR was verified by direct DNA sequencing of the entire insert. The rVWF constructs were then transiently expressed in HEK 293 cells as described above for murine rADAMTS-13.

### Assay for VWF-cleaving activity

The assay for VWF-cleaving activity was done as described [31]. Cell cultures were washed twice with 10 mL PBS before adding serum-free medium. Then cell culture supernatant from $1.5 \times 10^6$ HEK 293 cells $mL^{-1}$ containing murine rADAMTS-13 was diluted 1 : 20, 1 : 40, 1 : 80 or 1 : 160 in 50 μL 5 mM Tris–HCl pH 8.0, 12.5 mM $BaCl_2$, 1 mM Pefabloc SC (Roche) followed by 5 min incubation at 37 °C for activation of the protease. $BaCl_2$ was included because it was found to be a strong activator for the proteolysis of VWF multimers by ADAMTS-13 [11]. Twenty-five microliters of 20-fold concentrated conditioned medium (approximately 100 ng) from cells transiently expressing murine rVWF in 5 M urea, 5 mM Tris–HCl pH 8.0 was added and incubated overnight at 37 °C. The reaction was stopped by adding EDTA to a final concentration of 23.5 mM. In the control experiments the cell culture supernatants were incubated with 50 mM EDTA or 0.25 μL of a plasma from a TTP patient with anti-ADAMTS-13 inhibitory antibodies or 9 μg purified IgG from normal human plasma using protein G Sepharose according to Plaimauer *et al.* [25] prior to the addition of rVWF. VWF multimers were analyzed by SDS electrophoresis on 1.4% SeaKem HGT(P)-Agarose (Biozym Diagnostics, Oldenburg, Germany) as described [32], followed by immunoblotting and detection with α-VWF antibody (Dako, Glostrup, Denmark) and goat antirabbit antibody coupled to alkaline phosphatase and BCIP/NBT (Promega).

## Results

### Cloning of the murine ADAMTS-13 and comparison of amino acid sequence

Poly $A^+$ RNA from liver of BALB/c mice was reverse transcribed, the resulting cDNA solution subjected to PCR and the entire murine *Adamts-13* cDNA sequence assembled in a two-step procedure (see Materials and methods). Our RT-PCR procedure yielded a cDNA clone with the deduced murine ADAMTS-13 amino acid sequence identical to the published sequence (NCBI GenBank: AB_095445). The murine ADAMTS-13 precursor polypeptide consists of 1426 amino acid residues and is structurally similarly organized to that of the human counterpart (1427 amino acids, Fig. 1A).

Murine ADAMTS-13 has the typical multidomain structure also found in human ADAMTS-13 [19]. A signal peptide sequence of 37 amino acids (human 33 amino acids) is followed by an exceptionally short propeptide of 39 amino acids (human 41 amino acids), which is terminated by a typical proprotein convertase site [33], RRRR (human RQRR). The metalloprotease domain contains a conserved active site sequence consisting of the HEXXHXXGXXHD motif typical for reprolysine type proteases [34], the conserved M253 residue (human M249) in a proposed Met-turn, and residues E85, D175, C285, and D289, which are predicted to coordinate a structural $Ca^{2+}$ ion (human E83, D173, C281, and D284). The first thrombospondin type 1 region (TSP1-1), with a 50% similarity to murine thrombospondin type 1 repeats (murine TSP1, NCBI GenBank: NP_035710), is found downstream of the disintegrin-like domain. This region is followed by the murine equivalent to the human cysteine-rich/spacer domain containing a RGD sequence putatively mediating integrin interactions [35]. Downstream of the cysteine-rich/spacer domain, seven additional TSP1 repeats (TSP1-2 to TSP1-8) are present. As with human ADAMTS-13, the murine counterpart contains two CUB domains at its C-terminus, a structural feature which has not so far been found in other ADAMTS proteases. Murine and human ADAMTS-13 have an overall similarity of 69% at the amino acid level. Not unexpectedly, high homologies are observed in functional domains such as the metalloprotease- (77%), the disintegrin-like (81%), and the cysteine-rich (80%) domain. The individual domain with the highest degree of conservation is TSP1-1 (88%, Table 2) suggesting an important physiological function. Homologies between the TSP1 regions of human and mice vary between 88% (TSP1-1) and 37% (TSP1-6) (Table 2). ADAMTS-13 has eight asparagines predicted as potential N-linked glycosylation sites at residues 144, 148, 557, 564, 584, 619, 834, 1057 (human ADAMTS-13 has 10 potential N-linked glycosylation sites at residues 142, 146, 552, 579, 614, 667, 707, 828, 1235, 1354). Thus the positions 144 and 148 (metalloprotease domain), position 557 (cysteine-rich domain),

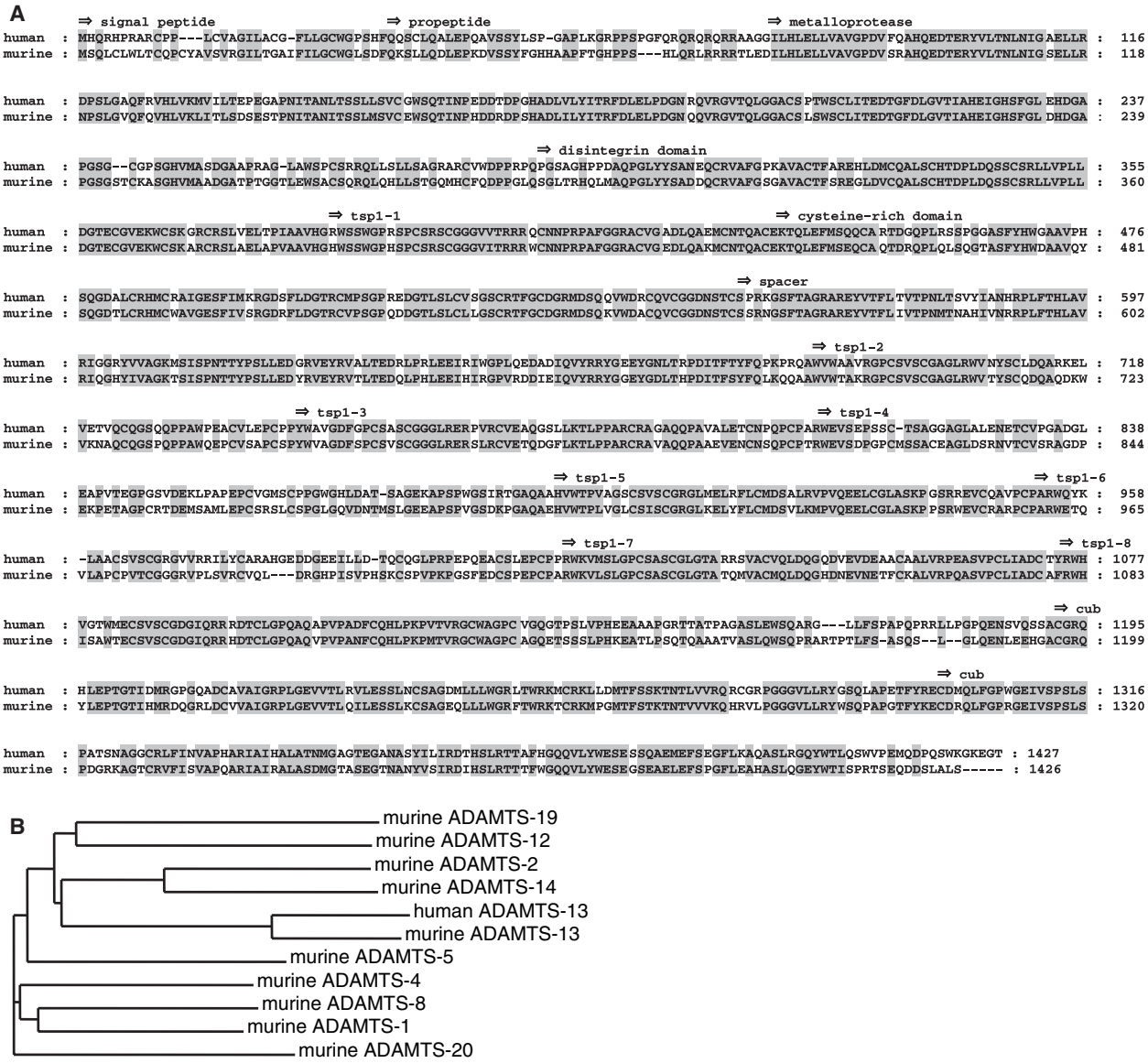


**Fig. 1.** (A) Amino acid sequence comparison of human ADAMTS-13 and murine ADAMTS-13. Start and end of the signal peptide, propeptide, metalloprotease with active site, disintegrin-like domain, thrombospondin type 1 motifs (tsp1), the cysteine-rich domain, spacer, and cub domains are indicated by arrows. (B) Phylogenic tree of the mouse ADAMTS family. The dendogram shows the relation of the complete sequence of murine ADAMTS proteins and human ADAMTS-13.

positions 584 and 619 (spacer domain) and 834 (TSP1-4 domain) are conserved. Both proteins contain the sequence motif WXXW in the TSP1-1 region, which is often modified by C-mannosylation [19,36]. With the exception of TSP1-4 (human and mouse) and TSP1-6 (mouse only) in both proteins, all TSP1 elements contain the conserved sequence motif CS*X*(S/T)CG usually modified by O-glycosylation of the disaccharide Glc-Fuc [19,36].

Interestingly, some amino acid exchanges reported to constitute human ADAMTS-13 single-nucleotide polymorphisms (SNPs) [21,24,26,37] can be found at the equivalent position in the mouse sequence, e.g. residues W7, E448 and V900 in the murine wild-type ADAMTS-13 sequence correspond to the reported human SNPs R7W, Q448E and A900V.

A comparison of the degree of homology between murine ADAMTS-13 and all other murine members of the ADAMTS family described to date as well as with the human ADAMTS-13 sequence is shown in Fig. 1B. The phylogenic tree for the respective human ADAMTS proteins is very similar to the murine counterparts [38–41].

### *Expression pattern of the murine Adamts-13 mRNA*

The expression pattern of the murine *Adamts-13* mRNA was examined in a variety of murine tissues (BALB/c strain) using a

**Table 2** Degree of similarity between human and murine ADAMTS-13 domains

| Domain | Similarity (%) |
|---|---|
| Signal peptide | 39 |
| Propeptide | 43 |
| Metalloprotease | 77 |
| Disintegrin-like domain | 81 |
| TSP1-1 | 88 |
| Cysteine-rich domain | 80 |
| Spacer | 75 |
| TSP1-2 | 71 |
| TSP1-3 | 72 |
| TSP1-4 | 51 |
| TSP1-5 | 77 |
| TSP1-6 | 37 |
| TSP1-7 | 76 |
| TSP1-8 | 57 |
| CUB1 | 74 |
| CUB2 | 64 |

commercially available multitissue Northern filter containing RNA derived from heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis tissue (Fig. 2A). With a probe specific for the 5′ end the filter showed expression of a single mRNA in the liver (lane 5). Probes specific for the 3′ end led to no visible band (data not shown).

In order to verify and to expand this result we used a commercially available cDNA panel of the BALB/c strain to analyze mRNA expression by a more sensitive PCR approach. The PCRs were repeated with two different primer pairs specific for the 5′ part of *Adamts-13* (Table 1), five times each. *Adamts-13* expression was found to be consistently high in liver, medium in lung and spleen, low in skeletal muscle and undetectable in heart, brain, kidney and testis (Table 3). Expression of the control gene *G3PDH* was found to be equally high in all tissues down to the lowest dilutions used in the assay (data not shown).

### *Embryonic-tissue PCR gene expression panel*

To investigate the levels of *Adamts-13*-specific mRNA accumulation in embryonic tissue of mice, 'Mouse rapid-scan gene expression panels' were used (see Materials and methods). Each of these panels contained normalized amounts of cDNA isolated from mouse embryos (FVB strain) at a specific age. The quality of the cDNAs was checked by amplification of the *β-actin* gene as recommended by the manufacturer (Fig. 2E). Serial dilution of the cDNAs and subsequent amplification of *Adamts-13*-specific transcripts by PCR using *Adamts-13*-specific primers gave a good estimate of the relative amount of *Adamts-13*-specific cDNA within the embryonic tissue of a certain age. To select for full-length transcripts the scan was carried out with a forward primer (7455, Table 1) located in the second CUB domain and a reversed primer (7635, Table 1) located between the stop codon and the poly T sequence. The PCR resulted in a 262-bp fragment. To probe additionally for the truncated form of *Adamts-13* [28], primers 6963/6964 (PCR product 383 bp) located in the metalloprotease region were used. ADAMTS-13-specific mRNA increases steadily with the age of the embryo (Fig. 2C,D) without significant differences

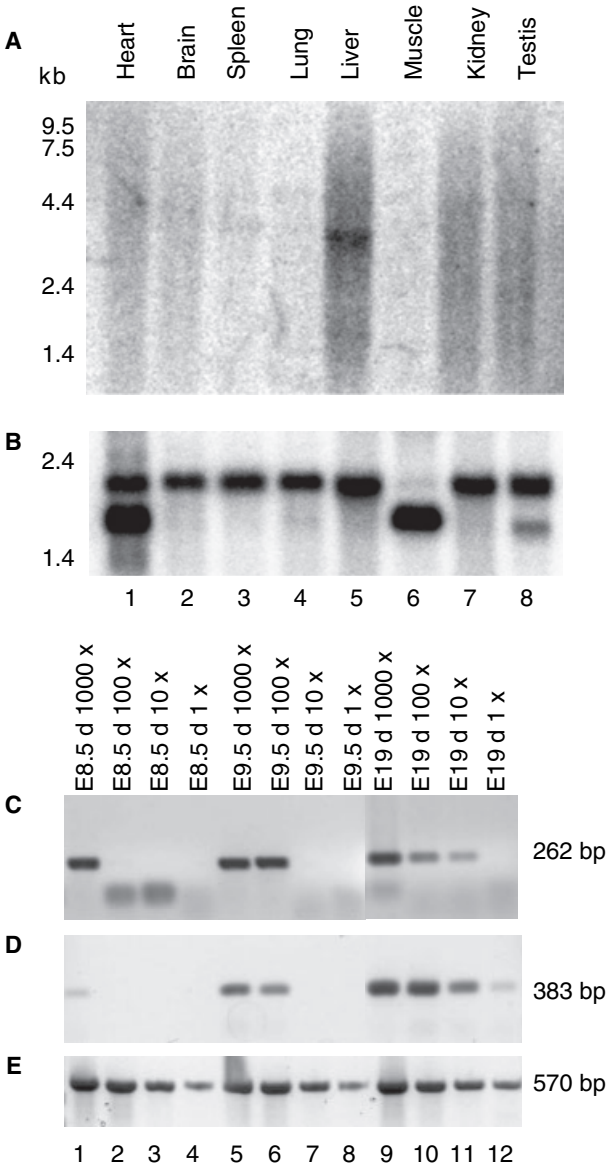


**Fig. 2.** RNA expression pattern of the murine *Adamts-13*. The expression pattern of the murine *Adamts-13* was examined using a multitissue Northern blot. (A) Lane 1, Heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. A $\alpha$-$^{32}$P-labeled 754-bp *EcoR*I/*Pml*I fragment derived from the ADAMTS-13 N-terminus was used as a probe. (B) Multitissue Northern blot analysis with $\alpha$-$^{32}$P-labeled *β-actin* cDNA as control probe. Lane 1, Heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. (C) Agarose gel analysis of polymerase chain reaction (PCR) fragments (specific for the non-truncated form of *Adamts-13*) generated from embryonic tissue derived from the commercially available Mouse Rapid-Scan™ gene expression panel. Four serial dilutions (10-fold) of cDNA prepared from mouse embryonic tissue were PCR amplified. E, Embryo tissue, 8.5 days, 9.5 days and 19 days old, respectively; 1000 ×, highest concentration; 1 ×, lowest concentration of the cDNA. (D) PCR specific for the truncated form of *Adamts-13*. (E) PCR specific for *β-actin* as control for integrity of cDNA.

**Table 3** Semiquantitative analysis of ADAMTS-13 expression in tissues of BALB/c mice

| Tissue | 1 : 3 | 1 : 10 | 1 : 33 | 1 : 100 |
|---|---|---|---|---|
| Heart | – | – | – | – |
| Brain | – | – | – | – |
| Spleen | + | +/– | – | – |
| Lung | + | +/– | – | – |
| Liver | + | + | + | – |
| Muscle | +/– | – | – | – |
| Kidney | – | – | – | – |
| Testis | – | – | – | – |

Normalized first-strand cDNA was diluted as indicated and each dilution was polymerase chain reaction (PCR)-amplified 10 times independently. +, PCRs always positive; +/–, PCRs with mixed results; –, PCRs always negative.

between the two primer pairs. The relative abundance of *Adamts-13* mRNA is highest at an embryo age of 19 days (Fig. 2C, lanes 9, 10, 11, and Fig. 2D, lanes 9–12 are positive) followed by day 9.5 (lanes 5 and 6 are positive) and day 8.5 (just lane 1 is positive). *Adamts-13* mRNA levels were essentially the same for an embryo age of 9.5 and 12 days (data not shown).

### *Expression of murine rADAMTS-13 protein*

The cDNA sequence of *Adamts-13* was cloned in an eukaryotic expression vector and murine ADAMTS-13 was transiently expressed in HEK 293 cells. After 24 h, serum-free conditioned media were analyzed by Western blotting. We detected the expected band reactive with the antihuman ADAMTS-13 monoclonal antibody 242/H2 [25] at a molecular weight of approximately 180 kDa (Fig. 3, lane 3) at the same size as human rADAMTS-13 (Fig. 3, lane 2). In contrast to human ADAMTS-13, a second band with a lower molecular weight [42] putatively derived from a proteolytic processing event was not observed with the murine rADAMTS-13.

### *Degradation of murine rVWF multimers by murine rADAMTS-13*

The biological activity of murine rADAMTS-13 was assayed by its ability to degrade murine VWF multimers *in vitro*. The murine VWF was transiently expressed in HEK 293 cells and

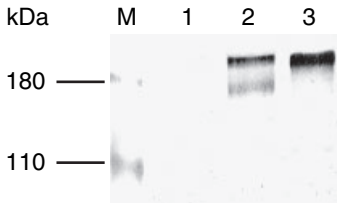


**Fig. 3.** Western blot of transiently expressed murine rADAMTS-13. Conditioned media from HEK 293 cells transiently expressing murine rADAMTS-13 (equivalent to approximately $2.4 \times 10^5$ cells) were loaded on SDS-polyacrylamide gels, blotted to nitrocellulose and immunostained. M, Marker proteins, 1 = vector control, 2 = human rADAMTS-13 from HEK 293 cells, 3 = murine rADAMTS-13 from HEK 293.

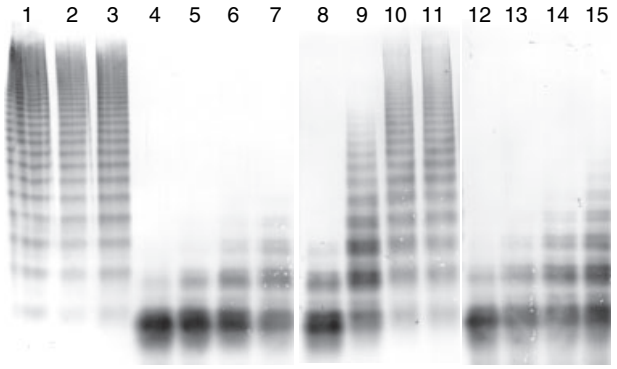


**Fig. 4.** Activity assay for murine rADAMTS-13 by von Willebrand factor (VWF) multimer analysis. HEK 293 cell culture supernatant containing murine rADAMTS-13 diluted 1 : 20 [lane 3 with 50 mM EDTA, lane 4 supernatant alone, lane 8 with thrombotic thrombocytopenic purpura (TTP) plasma, lane 12 with purified IgG from normal human plasma], 1 : 40 (lane 5, lane 9 with TTP plasma, lane 13 with purified IgG from normal human plasma), 1 : 80 (lane 6, lane 10 with TTP plasma, lane 14 with purified IgG from normal human plasma) or 1 : 160 (lane 7, lane 11 with TTP plasma, lane 15 with purified IgG from normal human plasma) and cell culture supernatant of cells not transfected diluted 1 : 20 (lane 2), were incubated with 25 μL (approximately 100 ng) murine rVWF and the cleavage analyzed by VWF-multimer gel electrophoresis. Lane 1, rVWF without any additions.

concentrated conditioned medium containing approximately 100 ng VWF was incubated with diluted cell culture supernatants of HEK 293 cells transfected with the murine *Adamts-13* construct. In contrast to the control cell culture supernatant transfected with the parental construct (Fig. 4, lane 2), the supernatant containing murine rADAMTS-13 degraded the rVWF multimers in a dose-dependent manner from 1 : 20 to 1 : 160 dilutions (Fig. 4, lanes 4–7). Other controls were the addition of the metal ion-chelating agent EDTA (Fig. 4, lane 3) and the addition of plasma from a TTP patient (Fig. 4, lanes 8–11). In both cases ADAMTS-13 activity was strongly inhibited, whereas purified IgG derived from normal human plasma did not inhibit the degradation (Fig. 4, lanes 12–15). Murine rADAMTS-13 also degraded human VWF, although at a much higher protease-to-VWF ratio (data not shown). The specificity of murine rADAMTS-13 was further characterized by N-terminal amino acid sequence analysis of the cleavage fragments of murine rVWF generated by incubation with murine rADAMTS-13 (Fig. 5A,B). As expected, two fragments with the N-terminal sequences SLSCRP and MVTGN corresponding to the 140-kDa and the 170-kDa bands, respectively, were obtained (Fig. 5A,B). The sequence SLSCRP (S764–P770, numbering according to Swiss-Prot: Q8CIZ8) was identical to the region upstream of a putative Furin cleavage site (R763–S764) that separates the murine VWF propeptide from the mature VWF, whereas the sequence MVTGN (M1606–N1610) could be found C-terminal of the predicted ADAMTS-13 cleavage site Y1605–M1606. The murine rVWF preparation contained also free VWF propeptide (Fig. 5A,B). The N-terminal sequence of the approximately 100-kDa fragment was TEKPRD, corresponding to the murine VWF propeptide sequence upstream of the signal peptide cleavage site (C22–T23).

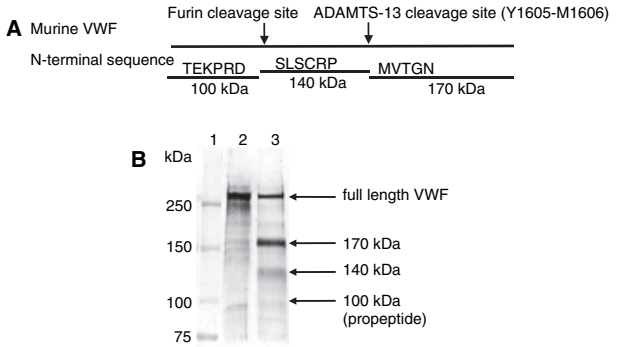


**Fig. 5.** N-terminal amino acid sequence analysis of murine recombinant von Willebrand factor (rVWF) fragments. Murine rVWF was incubated with murine rADAMTS-13, the cleavage fragments were separated by reducing SDS–PAGE electrotransferred polyvinylidene difluoride (PVDF) membrane, and the N-terminal amino acid sequence was determined. To characterize the murine VWF propetide present in the rVWF preparation, the N-terminal sequence of the 100-kDa fragment was also determined. (**A**) Schematic drawing of the expected rVWF-specific bands after processing of propetide containing VWF with Furin and ADAMTS-13. The N-terminal amino acids are given in bold letters. (**B**) Western blot of the three analyzed bands. Lane 1, Molecular weight marker; lane 2, negative control (buffer and rVWF); lane 3, murine rADAMTS-13 and rVWF.

## Discussion

Human ADAMTS-13 plays a pivotal role in the development of acquired as well as in hereditary TTP [19,21,24–26]. Both diseases are associated with the persistence of ULVWF and, after a yet uncharacterized precipitating event, the formation of platelet- and VWF-rich thrombi in the arterial vascular tree. A small animal model for TTP is still lacking. Despite the obvious limitations of animal studies, an animal model would allow us to study the development of the disease and would also be highly beneficial for the development of a treatment strategy other than plasma infusion or plasma exchange. As a first step towards developing a suitable animal model, we were interested in the cloning and biochemically characterizing the murine ADAMTS-13 protein.

From computer-assisted searches of the available mouse genomic sequences, the murine ADAMTS-13 protein sequence and structure was already predicted (NCBI GenBank: AB_095445). We obtained the full-length murine *Adamts-13* cDNA in a two-step procedure from a BALB/c mouse liver mRNA preparation. Our clone matched exactly the sequence predicted by computational analysis. Not unexpectedly, the precursor polypeptide was very similar to the human counterpart in size and structural organization, and had an overall similarity on the amino acid level of 69%. As with human ADAMTS-13 [19,33], compared with other human ADAMTS proteins murine ADAMTS-13 is significantly different from other members of the murine Adamts protease family (e.g. exceptionally short propeptide, two CUB domains at carboxyl terminus), making it the most divergent member of the murine group.

Strain-specific variants of the mouse *Adamts-13* gene were recently identified and an intracisternal A-particle insertion was found in the intron 23 of several mouse strains, including BALB/c mice [28]. This insertion was proposed to lead to a truncated version (1037 amino acids) of ADAMTS-13 because of the introduction of an in frame stop codon by splicing exon 23 to the pseudo-exon 24.

The aim of our study was to clone and express the non-truncated form of the murine ADAMTS-13. By using the appropriate primer combination we have been able to clone the complete murine *Adamts-13* gene from a poly $A^+$ RNA preparation derived from the liver of BALB/c mice. A Northern blot analysis of mRNA derived from various tissues from BALB/c mice with a probe specific for the 5′ end showed a single band around 4 kb in liver tissue as described [28]. Because the same Northern blot treated with a probe specific for the 3′ end gave no signal, this band probably corresponds to the truncated form of murine ADAMTS-13. However, although derived from BALB/c mice, the sources of the mRNA for the Northern blot and for the cDNA cloning were not identical. Our results therefore allow for the interpretation that either our extremely sensitive PCR method might be responsible for successful amplification of residual amounts of full-length *Adamts-13* mRNA, or subpopulations of BALB/c mice might exist that do not harbor the intracisternal A particle (IAP).

Apart from the tissue distribution of ADAMTS-13 expression in adult tissues, we were also interested in the analysis of ADAMTS-13 expression in the developing mouse embryo. Using a semiquantitative PCR-based method, we could show that there is a steady increase of *Adamts-13* mRNA (truncated and non-truncated form, FVB strain) detectable in the growing embryo, reaching highest levels in embryonic tissues of 19 days of gestation. It should be noted that these results do not reveal tissue-specific differential expression during embryonic development.

Transiently transfected HEK 293 cells expressed the murine *Adamts-13* cDNA as a protein of approximately 180 kDa. Murine ADAMTS-13 was detected by Western blot analysis using the antihuman ADAMTS-13 protease domain monoclonal antibody 242/H2 [25] as a detection antibody. Unlike human ADAMTS-13 [42], the murine enzyme does not show any C-terminal proteolytic truncation when secreted in the supernatant of HEK 293 cells. Moreover, the murine rADAMTS-13 is functionally active, as clearly shown by its ability to degrade recombinant murine VWF multimers in a dose-dependent manner. The cation chelator EDTA and plasma from a TTP patient inhibited the degradation of VWF multimers completely. Furthermore, the murine rVWF was cleaved by murine rADAMTS-13 at the appropriate site (amino acid positions Y1605 and M1606), thus generating the expected cleavage fragments.

In summary, we have cloned and functionally characterized the murine ADAMTS-13 protein, which promises to be a helpful tool to explain the physiological role of ADAMTS-13 as well as to gain more insight into the pathopysiological

mechanisms leading to an autoimmune response to ADAMTS-13. Moreover, the availability of recombinant murine ADAMTS-13 will be instrumental for the development of an animal model for TTP, and the explanation of the specific role of VWF in arterial thrombosis.

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## Contribution of authors

K.B. and B.P. expressed and characterized the murine ADAMTS-13. D.V. and S.P. were responsible for cloning and PCR experiments. G.A. was responsible for sequencing. D.G.M. and H.L.L. conducted the work with the murine VWF expression plasmid. F.D. contributed to the design of the study and critically reviewed the study proposal. K.Z. analyzed data and made a major contribution to writing the paper. F.S. conceived the study, was the project leader, and contributed major intellectual input to the design of the study.

## Disclosure of conflicts of interest

D. Motto and H. Lemmerhirt declare that they have no conflicts of interest regarding this study.

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