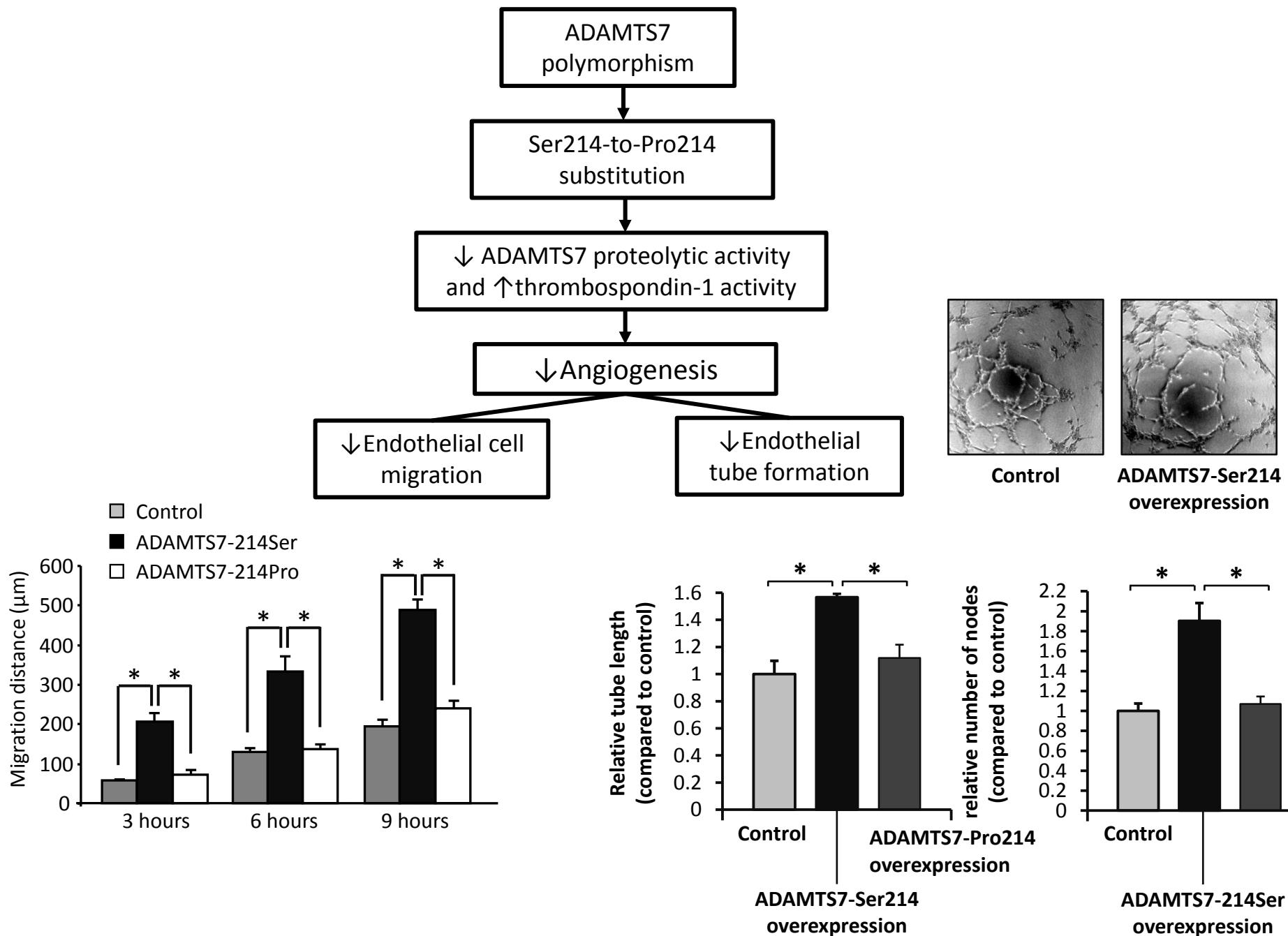


### Highlights

- Serine-to-proline substitution in ADAMTS7 in endothelial cells inhibits angiogenesis
- ADAMTS7 degrades thrombospondin-1, a potent angiogenesis inhibitor
- Augmented expression of ADAMTS7-Ser214 increases endothelial cell migration and neo-vessel tube formation
- The pro-angiogenic effect of ADAMTS7-Ser214 diminishes in the presence of a thrombospondin-1 blocking antibody



# Effect of a coronary-heart-disease-associated variant of ADAMTS7 on endothelial cell angiogenesis

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## Abstract

### *Background and aims:*

Recent studies have unveiled an association between *ADAMTS7* gene variation and coronary artery disease (CAD) caused by atherosclerosis. We investigated if the *ADAMTS7* Serine214-to-Proline substitution arising from a CAD-associated variant affected angiogenesis, since neovascularization plays an important role in atherosclerosis.

### *Methods and results:*

*ADAMTS7* knockdown in vascular endothelial cells (ECs) attenuated their angiogenesis potential, whereas augmented *ADAMTS7*-Ser214 expression had the opposite effect, leading to increased ECs migratory and tube formation ability. Proteomics analysis showed an increase in thrombospondin-1, a reported angiogenesis inhibitor, in culture media conditioned by ECs with *ADAMTS7* knockdown and a decrease of thrombospondin-1 in media conditioned by ECs with *ADAMTS7*-Ser214 overexpression. Cleavage assay indicated that *ADAMTS7* possessed thrombospondin-1 degrading activity, which was reduced by the Ser214-to-Pro substitution. The pro-angiogenic effect of *ADAMTS7*-Ser214 diminished in the presence of a thrombospondin-1 blocking antibody.

### *Conclusions:*

The *ADAMTS7* Ser217-to-Pro substitution as a result of *ADAMTS7* polymorphism affects thrombospondin-1 degradation, thereby promoting atherogenesis through increased EC migration and tube formation.

**Keywords:** *ADAMTS7*; angiogenesis; atherosclerosis; endothelial cell

## 1. Introduction

Genome-wide association studies (GWAS) have revealed an association between genetic variation at the *ADAMTS7* (a disintegrin and metalloprotease with thrombospondin motif, 7) locus on chromosome 15q25 and susceptibility to coronary artery disease (CAD) <sup>1-3</sup>. Furthermore, clinical studies have shown that the CAD-associated variants at this locus increase in coronary atherosclerosis burden in CAD patients, leading to worse clinical outcome <sup>4, 5</sup>.

A lead CAD-associated single nucleotide polymorphism (SNP) at the *ADAMTS7* locus, rs3825807 <sup>1, 2</sup>, deriving from a change from adenine (A) to guanine (G), is a non-synonymous substitution resulting in a serine (Ser) to proline (Pro) replacement at amino acid residue 214 in the pro-domain of the *ADAMTS7* protein. Our previous study shows that this Ser-to-Pro substitution results in lowered *ADAMTS7* proteolytic activity, reduced thrombospondin-5 (TSP-5) cleavage by *ADAMTS7*, and decreased vascular smooth muscle cell (VSMC) migration <sup>6</sup>. This allele-specific effect on VSMC migration likely in part explains the association of the *ADAMTS7*-Ser214 allele with CAD susceptibility and greater coronary atherosclerosis burden <sup>2, 4, 5</sup>, as VSMCs play an important role in the pathogenesis of atherosclerotic plaques, the pathology underlying most CAD cases.

Since vascular endothelial cells (ECs) also produce *ADAMTS7* and since neomicrovessel formation resulting from angiogenesis also contributes to atherosclerotic plaque progression <sup>7-10</sup>, we investigated in the present study a possible role of *ADAMT7* in, and a potential effect of the *ADAMTS7* Ser214-to-Pro substitution, on endothelial cell-derived angiogenesis.

## 2. Materials and methods

### 2.1 Immunohistochemical analysis

Formaldehyde-fixed paraffin-embedded sections of atherosclerotic coronary arteries were deparaffinised, re-hydrated and incubated with sodium citrate for antigen retrieval. The sections were then incubated with a peroxidase blocking solution (3% H<sub>2</sub>O<sub>2</sub>) and then 10% goat serum (Dako). Thereafter, the sections were incubated with a rabbit anti-human ADAMTS7 antibody (Abcam, ab28557) or a rabbit anti-human von Willebrand factor antibody (Abcam, ab9378). Subsequently, the sections were incubated with a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase, followed by incubation with 3, 3'-diaminobenzidine and counterstain with haematoxylin.

### 2.2 Genotyping

Genomic DNA extracted from human umbilical vein endothelial cells (HUVECs) from different individuals was genotyped for rs3825807 with the use of the KASPar (KBiosciences Competitive Allele Specific PCR SNP genotyping system) method. Accuracy of the genotyping results was verified by sequencing of a random selection of the samples.

### 2.3 ADAMTS7 shRNA and endothelial cell infection

A total of 5 clones of ADAMTS7 shRNA plasmids were packaged in lentiviral particles. HUVECs were infected with lentiviral particles of each of these 5 types of ADAMTS7 shRNA, respectively. The ADAMTS7 knockdown efficiencies were determined by immunoblotting analyses of protein extracts of infected cells (Online Supplement Figure S1). The shRNA (ADAMTS7 shRNA1) that gave rise to the highest ADAMTS7 knockdown efficiency (>50%) was used to infect HUVECs in subsequent experiments involving

ADAMTS7 knockdown, in which cultured HUVECs were infected with this ADAMTS7 shRNA or a control shRNA.

#### ***2.4 ADAMTS7 expression plasmids and endothelial cell transfection***

We utilized a previously described plasmid constructed by cloning the full-length *ADAMTS7* cDNA in the pcDNA3.1 vector<sup>11</sup>. The ADAMTS7 sequence in this plasmid corresponded to the A allele for rs3825807, encoding the Ser214 form of the ADAMTS7 protein. We will refer this plasmid as ADAMTS7-Ser214. Using ADAMTS7-Ser214 as a template, we carried out site-directed mutagenesis to generate a new plasmid with the ADAMTS7 sequence corresponding to the G allele for rs3825807 and therefore encoding the Pro214 form of ADAMTS7. We will refer this latter plasmid as ADAMTS7-Pro214. In experiments involving augmented ADAMTS7 expression, cultured HUVECs (heterozygous for rs3825807) were transfected with either ADAMTS7-Ser214 or ADAMTS7-Pro214 or the pcDNA3.1 vector. Immunoblotting analyses confirmed that ADAMTS7 levels were substantially increased in cells transfected with the ADAMTS7-Ser214 plasmid or the ADAMTS7-Pro214 plasmid as compared with cells transfected with the vector plasmid and showed that cells transfected with the ADAMTS7-Ser214 plasmid or the ADAMTS7-Pro214 plasmid had similar levels of increased ADAMTS7 expression.

#### ***2.5 Migration and tube formation assays***

EC migration was measured using the scratch assay technique as described<sup>12</sup>. Matrigel tube formation assays as described<sup>13</sup> were conducted with the use of Matrigel Basement Membrane Matrix (BD Biosciences). The assays were carried out with or without an anti-TSP1 blocking antibody (Santa Cruz, sc-59886) or an isotype control antibody.

## **2.6 Cell proliferation assay**

EC (HUVEC) proliferation was measured using a cell proliferation assay kit (Abcam, ab102501).

## **2.7 Proteomics analysis**

Media conditioned by HUVECs transfected with the ADAMTS7 plasmid, or infected with the ADAMTS7 shRNA, or without transfection or infection, were subjected to mass spectrometry-based quantitative proteomics analysis.

## **2.8 TSP1 in vitro cleavage assay**

Recombinant TSP1 (R&D Systems, 3074-TH-050) was incubated with concentrated media conditioned by HUVECs with or without ADAMTS7 knockdown or transfection with the ADAMTS7-Ser214 plasmid, in the presence or absence of an anti-ADAMTS7 catalytic domain antibody (Biorbyt, orb10042), in a digestion buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl<sub>2</sub>, 2 mM ZnCl<sub>2</sub>, and 0.05% Brij-35, pH 7.5) at 37°C for 8 hours.

## **2.9 Immunoblotting analyses**

Protein extracts from untransfected human coronary artery endothelial cells, untransfected HUVECs, HUVECs transfected with either an ADAMTS7 shRNA or a control shRNA, or HUVECs transfected with either the ADAMTS7-Ser214 plasmid or the ADAMTS7-Pro214 plasmid or the vector, were subjected to immunoblotting with the use of an anti-ADAMTS7 antibody or an antibody against the housekeeping protein GAPDH. Conditioned media or recombinant TSP1 cleavage reaction solutions were subjected to non-denaturing polyacrylamide gel electrophoresis and immunoblotting analyses with the use of an anti-TSP1 antibody.

## 2.10 Statistical analysis

Continuous variables are presented as mean $\pm$ standard deviation. Student's t-test was used to compare between two experimental groups. Analysis of variance was used to compare three or more groups. Categorical variables are presented as counts and percentages. All analyses were performed using SPSS or GraphPad Prism software. All tests were two-sided.

## 3. Results

### 3.1 Vascular endothelial cells express ADAMTS7

Immunohistochemical analysis showed the presence of ADAMTS7 in ECs of neovessels in human atherosclerotic plaques (Figure 1A). Similarly, immunoblotting analysis showed that cultured human ECs produced ADAMTS7 (Figure 1B).

### 3.2 Effect of ADAMTS7 and Ser214-to-Pro on vascular endothelial cell angiogenic ability

To investigate if ADAMTS7 has an effect on angiogenesis, we performed a tube formation assay using ECs (with the Ser214/Ser214 genotype) that have been transfected with either a shRNA to knock down ADAMTS7 or a control shRNA. ADAMTS7 knockdown resulted in a significant decrease in the angiogenic ability of ECs (Figure 2A), suggesting a role of ADAMTS7 in endothelial cell-derived angiogenesis.

We then performed a tube formation assay using ECs (with the Ser214/Pro214 genotype) that had been transfected with either a plasmid (ADAMTS7-Ser214) to augment the expression of the Ser214 version of ADAMTS7, or a plasmid (ADAMTS7-Pro214) to augment the expression of the Pro214 version of ADAMTS7, or the vector plasmid (control). The

experiment showed that augmented expression of ADAMTS7-Ser214 increased EC angiogenic ability, whereas overexpression of ADAMTS7-Pro214 did not exhibit such a pro-angiogenic effect (Figure 2B), indicating that the Ser-to-Pro substitution abolishes pro-angiogenic activity of ADAMTS7. Consistently, non-transfected ECs of the A/A (Ser214/Ser214) genotype for the CAD-associated SNP rs3825807 had higher angiogenic ability than ECs of the G/G (Pro214/Pro214) genotype (Figure 3).

### ***3.3 Effect of ADAMTS7 and Ser214-to-Pro on thrombospondin-1 degradation***

To identify proteins that might mediate the effect of ADAMTS7 on EC angiogenic ability, we performed a proteomics analysis to search for proteins whose levels changed in culture media of ECs that had been transfected either with shRNA to knock down ADAMTS7 or with a plasmid to augment ADAMTS7 (Ser214) expression. The experiment identified several such proteins (Online Supplement Table S1), one of which was thrombospondin-1 (TSP1), a potent angiogenesis inhibitor<sup>14-18</sup>. The assay showed that ADAMTS7 knockdown in ECs resulted in an increase of the concentration of TSP1 in the culture media, whereas augmented ADAMTS7 (Ser214) expression had an opposing effect. Immunoblotting analysis confirmed this finding (Figure 4A).

Subsequently, we investigated whether ADAMTS7 was able to degrade TSP1. We incubated the same amount of recombinant TSP1 protein with media conditioned by ECs, in the presence of a neutralizing antibody against the ADAMTS7 catalytic domain or an isotype control antibody, followed by TSP1 immunoblotting analysis. The experiment showed that after incubating with EC conditioned media, there was significantly less recombinant TSP1 remaining in the assay with the isotype control antibody than in the assay with the ADAMTS7 neutralizing antibody (Figure 4B, comparing lane 1 to lane 5), suggesting that

ADAMTS7 has TSP1 proteolytic activity. Consistently, incubation with media conditioned by ECs that had been transfected with shRNA to knockdown ADAMTS7 led to more recombinant TSP1 remaining undegraded (Figure 4B, comparing lane 2 with lane 1), whereas incubation with media conditioned by ECs that had been transfected with a plasmid to overexpress ADAMTS7 (Ser214) led to less recombinant TSP1 remaining undegraded (Figure 4B, comparing lane 3 with lane 1), further supporting ADAMTS7 having TSP1 proteolytic activity. Furthermore, it was noticeable that media conditioned by ECs transfected with the ADAMTS7-Ser214 expressing plasmid had higher TSP1 degrading activity than media conditioned by ECs transfected with the ADAMTS7-Pro214 expressing plasmid (Figure 4B, comparing band 3 with band 4).

### ***3.4 Effect of the ADAMTS7 Ser214-to-Pro substitution on endothelial cell angiogenic ability is thrombospondin-1 dependent***

To investigate whether the pro-angiogenic effect of ADAMTS7-Ser214 is dependent on the presence of TSP1, we performed tube formation assays of ECs (with the Ser214/Pro214 genotype) that had been transfected with the ADAMTS7-Ser214 plasmid to overexpress the Ser214 version of ADAMTS7 or the ADAMTS7-Pro214 plasmid to overexpress the Pro214 version, in the presence of an anti-TSP1 antibody or an isotype control antibody. The anti-TSP1 antibody used in our experiments recognizes an epitope in the TSP1 stalk region responsible for the anti-angiogenic effect of TSP1 and has previously been shown to be capable of blocking the anti-angiogenic activity of TSP1<sup>14, 17</sup>. Our study showed that ECs overexpressing ADAMTS7-Ser214 had significantly higher angiogenic ability than ECs overexpressing ADAMTS7-Pro214, in the absence of the anti-TSP1 blocking antibody; however, this difference diminished in the presence of the anti-TSP1 blocking antibody

(Figure 5), suggesting that the influence of ADAMTS7 and the Ser214-to-Pro substitution on angiogenesis is related to their effect on TSP1.

### ***3.5 Effect of the ADAMTS7 Ser214-to-Pro substitution on endothelial cell migration***

Having found an effect of ADAMTS7 on endothelial cell angiogenic ability, we investigated its role on endothelial cell migration. Migration assays demonstrated attenuated migratory ability with ADAMTS-214Pro (Figure 6A). This is consistent with slower migration for ECs with G/G genotype (Pro214/Pro214) (Figure 6B). Incubation with TSP1 antibody restore the migratory ability of EC, indicating the influence of ADAMTS7 on EC migration is mediated through TSP1. (Figure 6C)

To investigate whether the ADAMTS7 Ser214-to-Pro substitution had an influence on EC proliferation, we performed a cell proliferation assay on ECs of different genotype. The assay showed a non-significant trend towards lower proliferation of ECs of the G/G genotyped compared with cells of the A/A or A/G genotype. (Figure 7)

## **4. Discussion**

In this study, we found that ADAMTS7 promotes vascular EC angiogenic activity and this pro-angiogenic effect was related to the ability of ADAMTS7 to degrade TSP1, a potent anti-angiogenesis inhibitor<sup>14-18</sup>. Furthermore, our study showed that the TSP1 degrading activity and pro-angiogenic property of ADAMTS7 was affected by the Ser214-to-Pro substitution arising from the CAD-associated, non-synonymous SNP rs3825807.

1 Previous studies have demonstrated that ADAMTS7 inhibits re-endothelialization of injured  
2 arteries and promotes post-injury neointima formation as well as induces de novo  
3 atherosclerosis in hyperlipidemic mice<sup>6, 19-21</sup>. In this study, we focused on the role of  
4 ADAMTS7 in capillary tube formation since atherosclerotic plaques contain neomicrovessels  
5 as a result of angiogenesis from the adventitial vasa vasorum<sup>7</sup>. These fragile vessels provide  
6 an additional passage for infiltration of circulating lipid and leukocytes, leading to  
7 atherosclerotic lesion progression<sup>7-10</sup>. Some other members of the ADAMTS family are  
8 known to play a role in angiogenesis<sup>22-32</sup>, which is another rationale for investigating if  
9 ADAMTS7 also affects angiogenesis.

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24 ADAMTS7 is initially synthesized as a pre-proenzyme which includes a signal peptide, a  
25 pro-domain, a catalytic metalloproteinase domain, a disintegrin-like domain, and a  
26 thrombospondin type-1 motif<sup>33</sup>. The pro-domain is cleaved off by the proprotein convertase  
27 furin during ADAMTS7 maturation/activation<sup>33</sup>. The activated ADAMTS7 possesses  
28 proteolytic activity. The best known proteolytic substrate of ADAMTS7 is thrombospondin-5  
29 (TSP5, also named cartilage oligomeric matrix protein)<sup>34</sup>, an extracellular matrix protein  
30 present in such tissues as vascular walls and cartilages<sup>20, 34</sup>. It has been shown that TSP5 has  
31 no effect on angiogenesis<sup>35</sup>. Therefore, it is unlikely that the angiogenic effect of ADASMT7,  
32 as shown in our study reported here, is related to TSP5. Two other proteins, namely  $\alpha$ 2-  
33 macroglobulin and progranulin (also named granulin-epithelin precursor), respectively, have  
34 also been reported to be proteolytic substrates of ADAMTS7<sup>10, 33</sup>. These two proteins have  
35 both been reported to be able to enhance angiogenesis<sup>36, 37</sup>. Since ADAMTS7 breaks down  
36  $\alpha$ 2-macroglobulin<sup>33</sup> and inactivates progranulin<sup>10</sup>, it is unlikely that the pro-angiogenic  
37 effect of ADAMTS7 is mediated by any of these proteins. On the other hand, our study  
38 indicates that TSP1 is also a proteolytic substrate of ADAMTS7 and that increased

ADAMTS7 production/activity can lead to increased TSP1 degradation. Since TSP1 is a potent angiogenesis inhibitor<sup>14-18</sup>, it is plausible that the pro-angiogenic property of ADAMTS7 is, probably in part, related to its effect on TSP1.

The roles of the different members of the ADAMTS family in angiogenesis appear to be complex, and both pro-angiogenic and anti-angiogenic effects have been reported. For example, full-length ADAMTS1 promotes angiogenesis, whereas certain fragments of ADAMTS1 inhibit the angiogenic process, likely because the anti-angiogenic motif is masked in the full-length ADAMTS1<sup>22, 38</sup>. In our study reported here, we examined the effect of full-length ADAMTS7 and found that it was pro-angiogenic. However, one cannot exclude the possibility that ADAMTS7 might harbor fragments with properties that are different to those of the full-length ADAMTS7.

A previous study showed that adenovirus-mediated overexpression of ADAMTS7 in ECs led to decreased EC migration and proliferation<sup>21</sup>. In contrast, we found that plasmid-mediated overexpression of the Ser214 version of ADAMTS7 (but not the Pro214 version) increased EC migration and did not detect a significant difference between ADAMTS Ser214-to-Pro genotypes in EC proliferation. The reasons for the disparate findings from these two studies are unclear, although it is possible that they might arise from the different experimental approaches and conditions.

Over the last decade, GWAS have identified over 80 CAD-associated genomic loci<sup>1, 2, 39</sup>. However, for many of these loci, the functional mechanisms leading to the genetic effect are still unclear. Functional characterization of these CAD risk variants can aid the understanding of the underlying biological mechanisms and may facilitate the translation of the genetic

discoveries to therapeutic development. As angiogenesis and neovascularization in the  
atherosclerotic plaque plays an important role in plaque growth <sup>7-10</sup>, the finding of our present  
study that ADAMTS7 and the Ser214-to-Pro substitution due to the CAD-associated SNP  
rs3825807 affect vascular EC angiogenic activity provides a new, additional mechanistic  
explanation for the association of genetic variation at the *ADAMTS7* locus with  
atherosclerosis burden <sup>3, 5</sup> and CAD risk <sup>1-3</sup>.

## **Conflict of interests**

The authors declare no competing interests.

## **Financial support**

We thank support of the British Heart Foundation (PG/16/9/31995, RG/16/13/32609, FS/11/28/28758, SP/19/2/344612, RG/19/9/34655), National Natural Science Foundation of China (81370202, 81600339) and Zhejiang Provincial Natural Science Foundation of China (LY19H020009). X.P. was a recipient of a scholarship from the Chinese Scholarship Council. K.C is supported by NIHR Academic Clinical Fellowship. This work falls under the portfolio of research conducted within the NIHR Leicester Biomedical Research Centre.

## **Author Contributions**

X.P., K.C., Q.X., and A.D.M. conducted the experiments; S.Y. and X.P. designed the experiments and wrote the manuscript; S.Y., X.P. K.C., L.Z., C.L, T.R.W., M.J.C., N.J.S., and J.Z. revised the manuscript.

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## Figure legends

### Figure 1. Expression of ADAMTS7 in neovessel endothelial cells in atherosclerotic plaque and cultured vascular endothelial cells

(A). Carotid atherosclerotic plaque histological sections were immunostained for ADAMTS7 (left panel) or the endothelial cell marker vWF (middle panel). Arrowheads highlights the presence of brown colour (DAB) indicating the presence of ADAMTS7 (left panel) and vWF (middle panel) in neovessel endothelial cells inside the plaque. Blue colour (haematoxylin) indicates nuclei. Right panel shows a negative control where the section was incubated with the secondary antibody but no primary antibody. (B). Protein extracts from cultured human coronary artery endothelial cells (HCAEC) or human umbilical vein endothelial cells (HUVEC) were subjected to immunoblotting with an antibody for ADAMTS7 or the housekeeping gene GAPDH.

### Figure 2. Effect of ADAMTS7 on vascular endothelial cell ability

(A). Endothelial cells (of the A/A genotype for rs3825807) were transfected with either an ADAMTS7 shRNA to knockdown ADAMTS7 or a control shRNA, followed by Matrigel tube formation assay. Shown in the figure are representative images of the tube formation assay and in column charts, mean (and SEM, n=6) of relative tube lengths (left) and numbers of nodes (right). (B). Endothelial cells (heterozygous for rs3825807) were transfected with either the ADAMTS7-Ser214 plasmid or the ADAMTS7-Pro214 plasmid or the vector plasmid (control), followed by Matrigel tube formation assay. Shown in the figure are representative images of the tube formation assay and in column charts, mean (and SEM, n=3 different donor cell preparations) of relative tube lengths (left) and numbers of nodes (right).

\* $p < 0.05$ .

### Figure 3. Effect of ADAMTS7 rs3825807 genotype on endothelial cell tube formation

Endothelial cells of the A/A or G/G genotype for rs3825807 were subjected to tube formation assays. Shown in the figure are representative images of the tube formation assay and in column chart, mean (and SEM, n=3 different donor cell preparations) of relative tube lengths (left) and numbers of nodes (right). \* $p<0.05$ .

### Figure 4. Effect of ADAMTS7 on TSP1 degradation

(A). A representative image and quantification data of immunoblotting analyses of media conditioned by endothelial cells (of the A/A genotype for rs3825807) with or without ADAMTS7 knockdown by shRNA or ADAMTS7 overexpression by transfection with the ADAMTS7-Ser214 plasmid. Equal amounts of total proteins were loaded in each lane (as proteins in conditioned media were analyzed, no protein could be used as a loading control). Column chart shows mean (and SEM) of relative band intensities in 4 experiments. (B). A representative image and quantification data of immunoblotting analysis of products from recombinant TSP1 cleavage assays. Recombinant TSP1 (200ng) was incubated without (lane 1) or with 5 $\mu$ l concentrated media conditioned by non-transfected endothelial cells (lanes 2 and 6), endothelial cells with ADAMTS7 knockdown (KD) by shRNA (lanes 3 and 7), endothelial cells transfected with the ADAMTS7-Ser214 plasmid (Ser) (lanes 4 and 8), or endothelial cells transfected with the ADAMTS7-Pro214 plasmid (Pro) (lanes 6-9), in the presence or absence of an ADAMTS7 neutralizing antibody, in a digestion buffer at 37°C for 8 hours. The solutions were subjected to immunoblotting analysis with an anti-TSP1 antibody. Column chart shows mean (and SEM) of relative band intensities in 3 independent experiments. \* $p<0.05$ .

**Figure 5. TSP1 blocking diminishes ADAMTS7-Ser214 effect on vascular endothelial cell angiogenesis activity**

Endothelial cells (heterozygous for rs3825807) were transfected with either the ADAMTS7-Ser214 plasmid or the ADAMTS7-Pro214 plasmid, following by tube formation assay in the presence of an anti-TSP1 antibody or an isotype control antibody. Column chart shows mean (and SEM, n=3 different donor cell preparations) of relative tube lengths and numbers of nodes. \* $p < 0.05$ .

**Figure 6. Effect of ADAMTS7 on endothelial cell migration**

(A). Endothelial cells (heterozygous for rs3825807) were transfected with either the ADAMTS7-Ser214 plasmid or the ADAMTS7-Pro214 plasmid or the vector plasmid (control), followed by migration assay. (B). Migration distance of endothelial cells with AA, AG and GG genotype for rs3825807. (C). Migration distance of endothelial cells (heterozygous for rs3825807) transfected with either the ADAMTS7-Ser214 plasmid or the ADAMTS7-Pro214 plasmid, following by incubation in the presence of an anti-TSP1 antibody or an isotype control antibody. Columns represent mean (and SEM, n=6). \* $p < 0.05$ .

**Figure 7. Endothelial cell proliferation in relation to ADAMTS7 genotype**

Endothelial cells of the A/A, A/G or G/G genotype for rs3825807 were subjected to proliferation assay with the use of a cell proliferation assay kit. Columns represent mean (and SEM, n=4 different donor cell preparations for each genotype).  $p > 0.05$  comparing the different genotypes.

## Response to reviewers' comments

### Reviewer 1:

**Reviewer's Comment:** This study is interesting and well-written. I have no comments.

**Response:** We would like to thank the reviewer for his/her positive comment on our manuscript.

### Reviewer 2:

We would like to thank the reviewer for his/her valuable comments and constructive advice. In the revised manuscript, the reviewer's points have been addressed as follows.

**Reviewer's comment 1:** In the manuscript, the authors state that their studies were the first ones to investigate the possible role of ADAMTS7 in the process of angiogenesis (page 10, lines 46-48). However, in one of the cited publications (36; Kessler T et al. Circulation 2015) it has been shown using in vitro (HUVECs overexpressing ADAMTS7) and in vivo (ADAMTS7-/- mice) models that ADAMTS7 inhibits re-endothelialization of injured arteries and promotes vascular remodelling through cleavage of thrombospondin-1. The only novelty of the manuscript is choice of two mutants to show that ADAMTS7 pro-angiogenic property is Ser214-to-Pro substitution dependent, like seen in CAD. Can please authors comment on it? If it is possible can authors perform experiments with overexpression of full-length ADAMTS7?

**Response:** In response to the reviewer's comment above, we have modified the relevant sentences and they now read as follows:

"Previous studies have demonstrated that ADAMTS7 inhibits re-endothelialization of injured arteries and promotes post-injury neointima formation as well as induces de novo atherosclerosis in hyperlipidemic mice<sup>6, 19-21</sup>. In this study, we focused on the role of ADAMTS7 in capillary tube formation since atherosclerotic plaques contain neomicrovessels as a result of angiogenesis from the adventitial vasa vasorum<sup>7</sup>." (page 11, paragraph 1)

In this study, the experiments with ADAMTS7 overexpression was of full-length ADAMTS7, as described in the Methods. (page 5, paragraph 2, line 1)

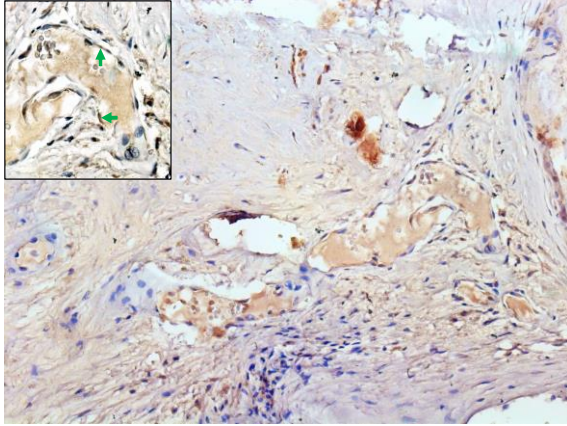
**Reviewer's comment 2:** My biggest concern is that authors are saying that they wanted to investigate the role of ADAMTS7 on endothelial cells angiogenesis because vascular endothelial cells produce ADAMTS7 (page 3, lines 50-58). However, to show the mechanism authors use Human Umbilical Vein Endothelial cells (HUVECs). We all know that, dependent on the source of endothelial cells, they express diverse markers (Kume T; Histol Histopathol. 2010 May;25(5):637-46. doi: 10.14670/HH-25.637 and dela Paz NG et al. Cell Tissue Res. 2009 Jan;335(1):5-16. doi: 10.1007/s00441-008-0706-5. Epub 2008 Oct 30). What is the difference in expression between HUVECs and other endothelial cells (i.e. Human Primary Artery Endothelial cells)? What is the knock-down efficiency of ADMATS7? The authors claim that they used five different shRNA's to knock-down ADAMTS7 but we haven't been presented with any results of the altered ADAMTS7 expression.

**Response:** We thank the reviewer for his/her thoughtful comments. In the revised manuscript, we have added new experimental results showing the primary human coronary artery endothelial cells and human umbilical vein endothelial cells have similar expression levels of ADAMTS7 (Figure 1B). We have now also included data showing the ADAMTS7 levels in cells transfected with the five different ADAMTS7 shRNA (Online Supplement Figure S1) and that among these 5 different

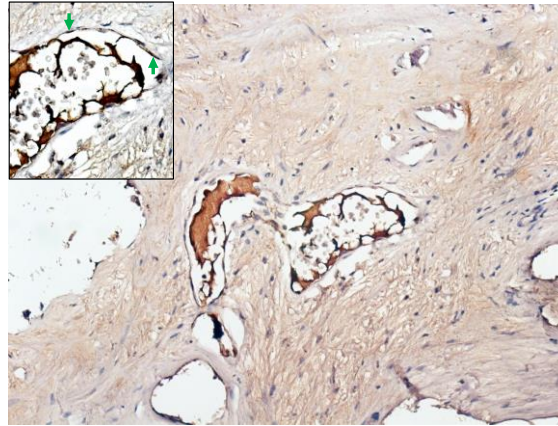
**Figure(s)**  
**Figure 1**

**(A)**

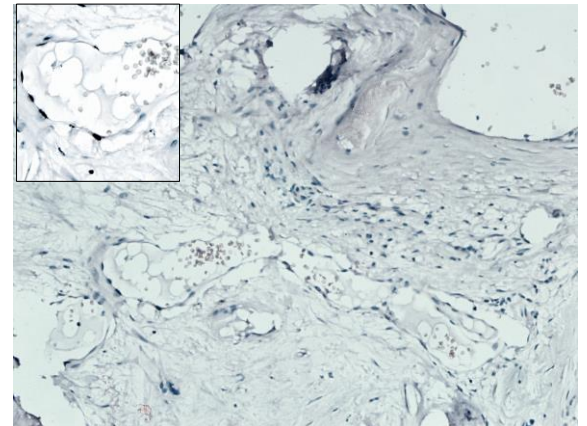
**ADAMTS7**



**vWF**



**Negative control**



**(B)**

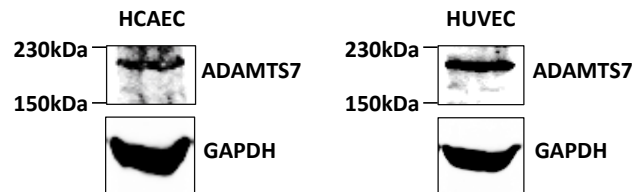
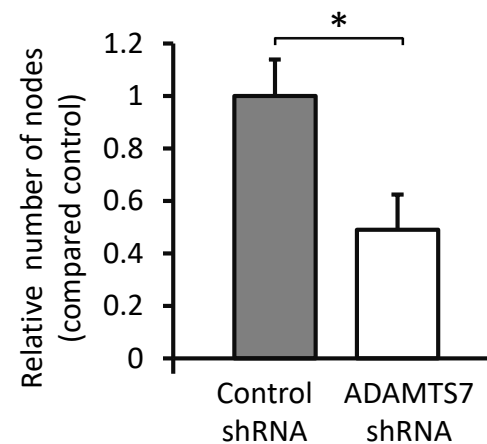
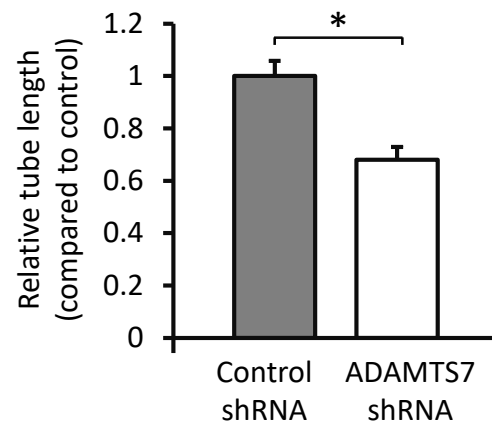
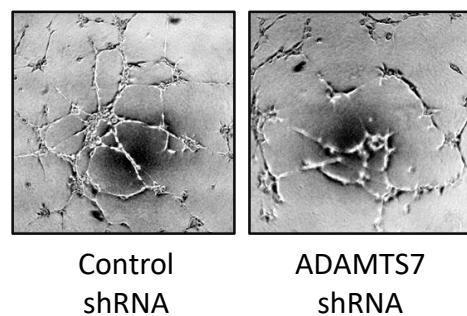


Figure 2

(A)



(B)

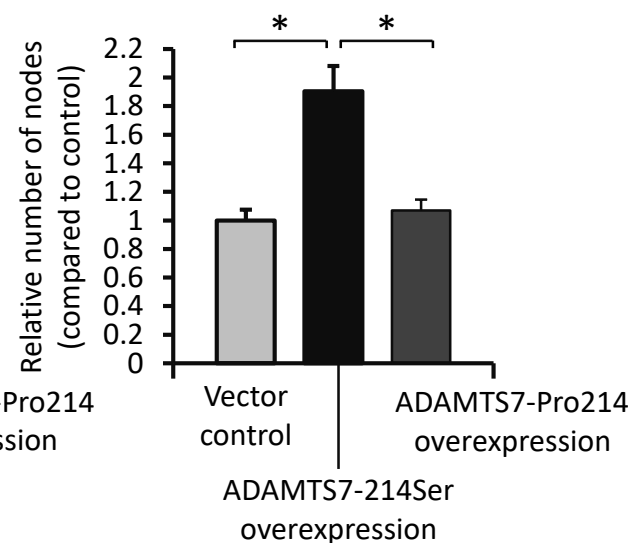
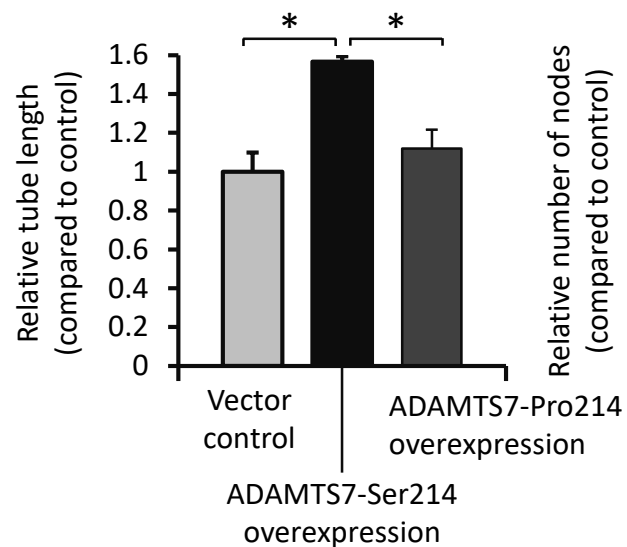
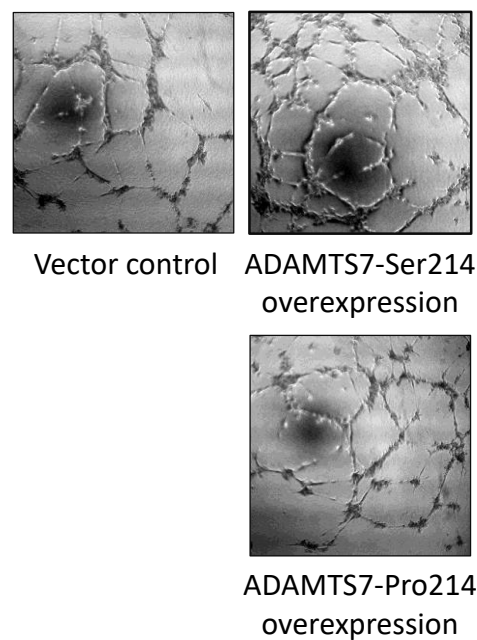


Figure 3

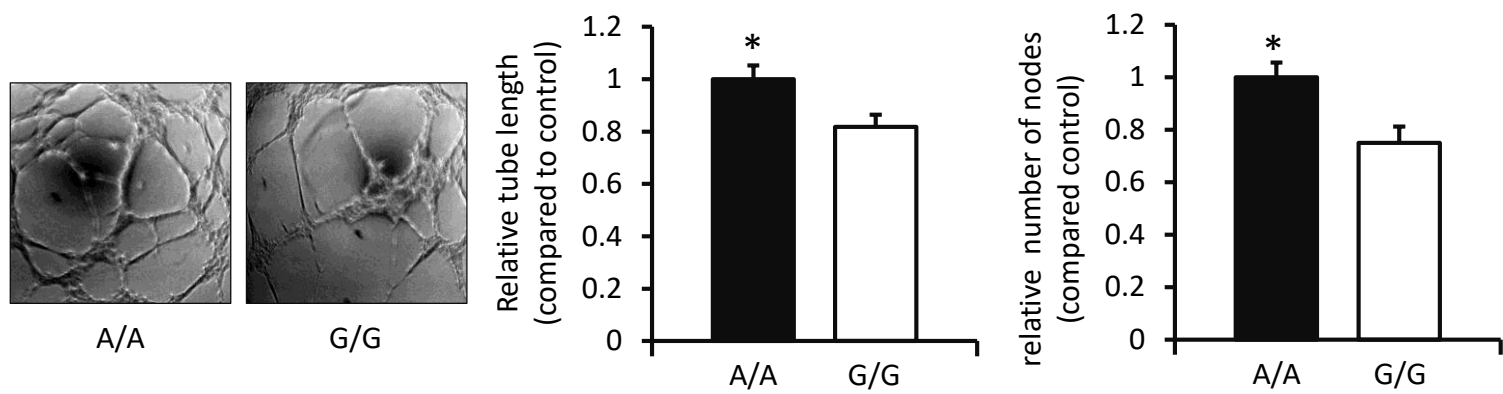
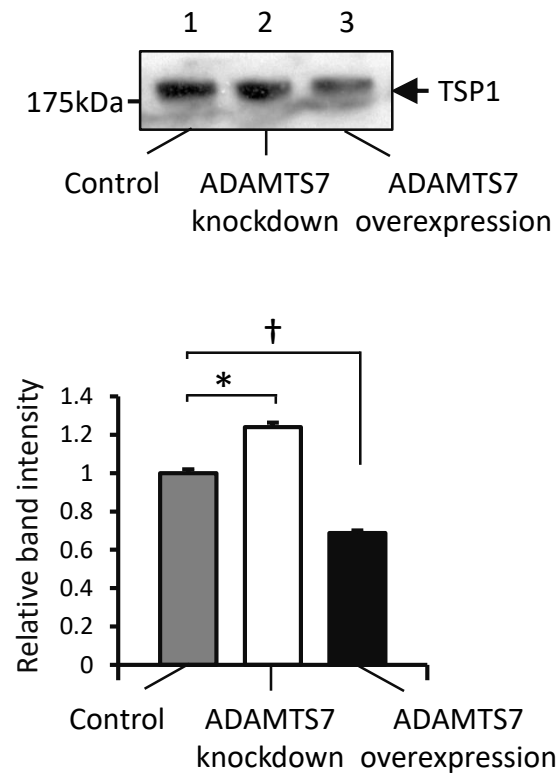


Figure 4

(A)



(B)

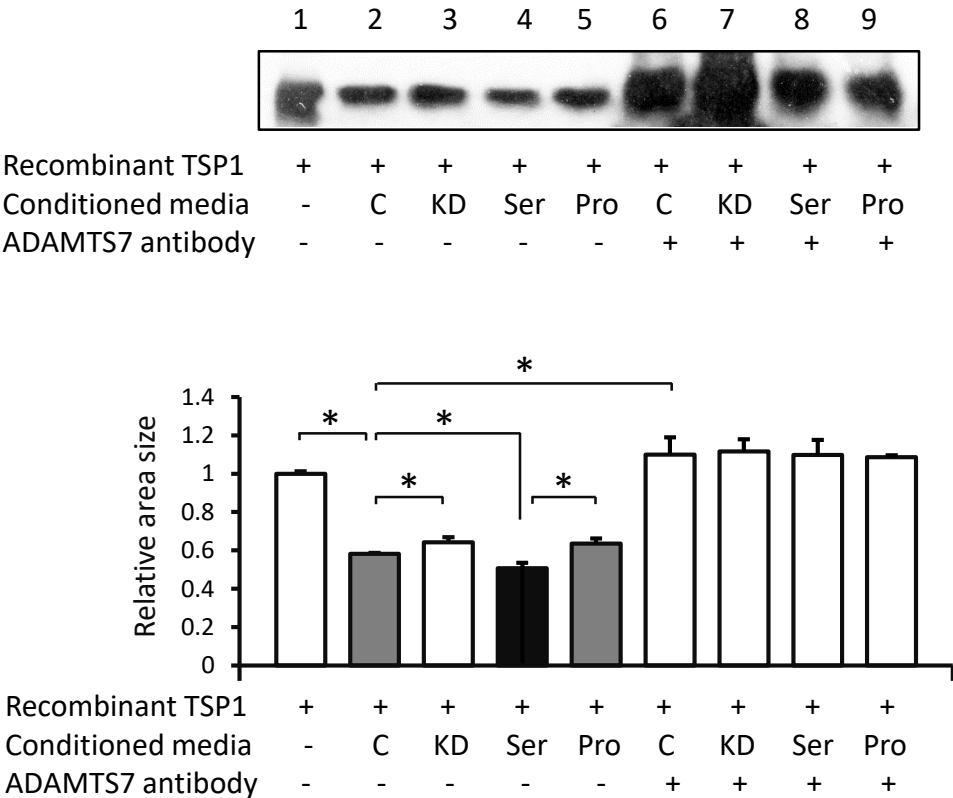


Figure 5

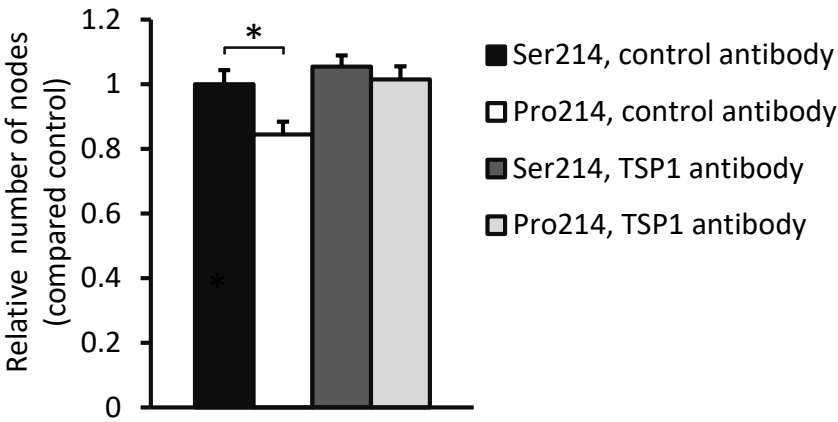
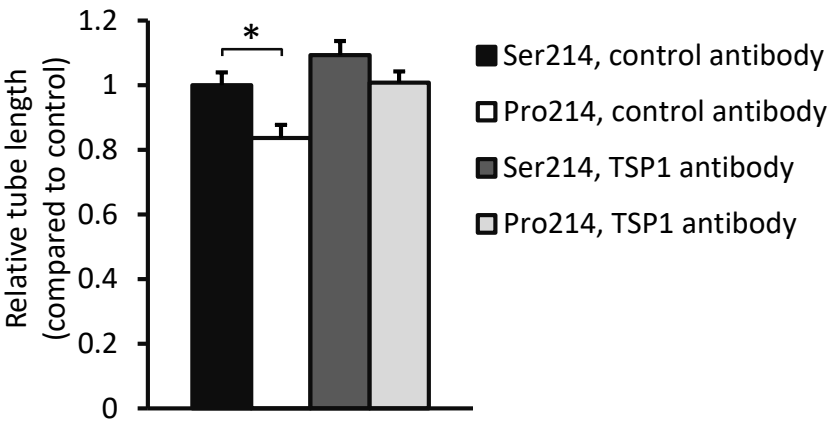
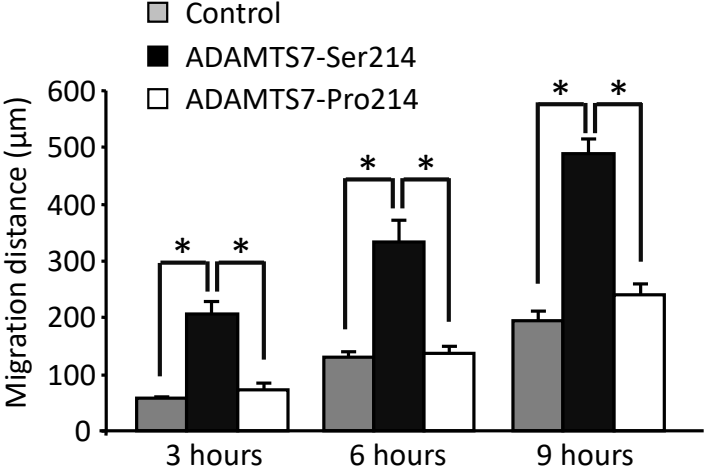
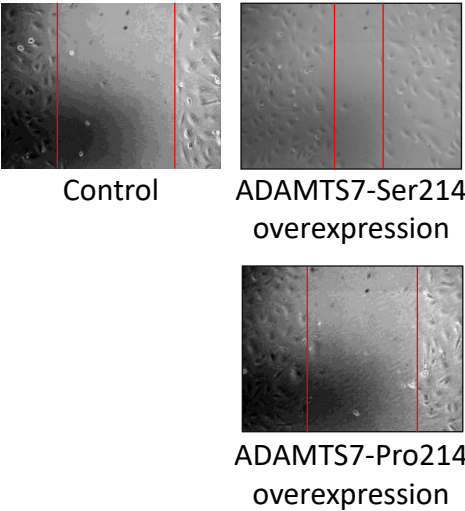
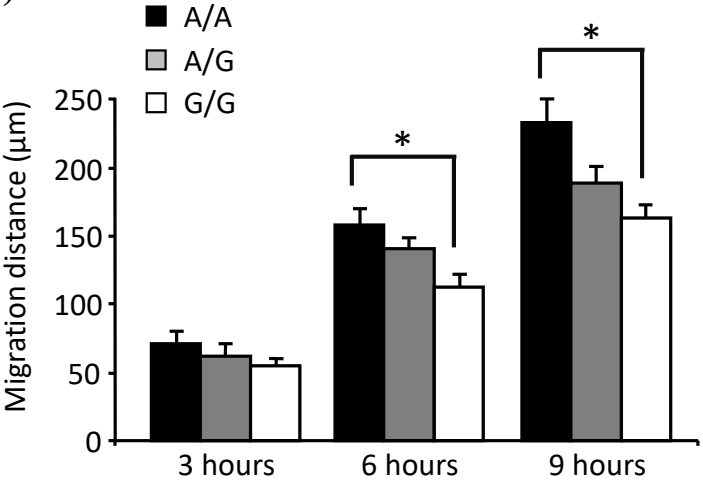


Figure 6

(A)



(B)



(C)

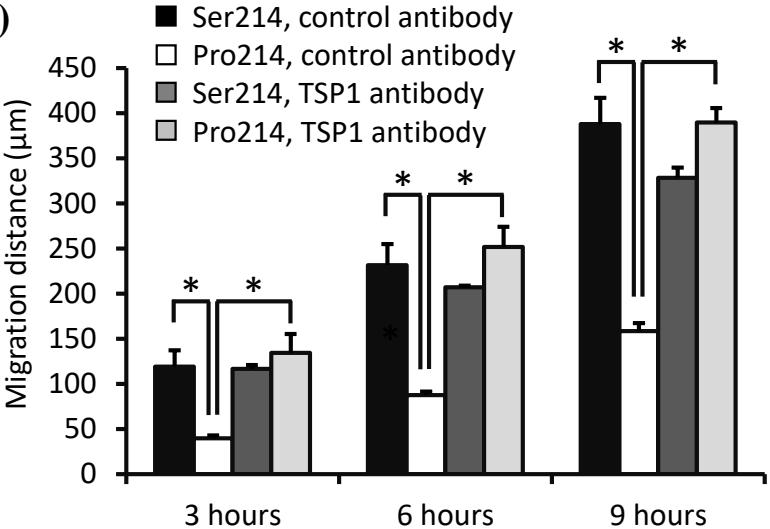


Figure 7

