ADAMTS16 Activates Latent TGF-beta, Accentuating Fibrosis and Dysfunction of the Pressure-overloaded Heart

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Short title: ADAMTS16 motif RRFR regulates TGF-β signaling

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Abstract

Aims: Cardiac fibrosis is a major cause of heart failure, and mediated by the differentiation of cardiac fibroblasts into myofibroblasts. However, limited tools are available to block cardiac fibrosis. ADAMTS16 is a member of the ADAMTS superfamily of extracellular protease enzymes involved in extracellular matrix degradation and remodeling. In this study, we aimed to establish ADAMTS16 as a key regulator of cardiac fibrosis.

Methods and Results: Western blot and qRT-PCR analyses demonstrated that ADAMTS16 was significantly up-regulated in mice with transverse aortic constriction (TAC) associated with left ventricular hypertrophy and heart failure, which was correlated with increased expression of *Mmp2*, *Mmp9*, *Col1a1* and *Col3a1*. Overexpression of ADAMTS16 accelerated the AngII-induced activation of cardiac fibroblasts into myofibroblasts. Protein structural analysis and co-immunoprecipitation revealed that ADAMTS16 interacted with the latency-associated peptide (LAP)-TGF-β via a RRFR motif. Overexpression of ADAMTS16 induced the activation of TGF-β in cardiac fibroblasts, however, the effects were blocked by a mutation of the RRFR motif to IIFI, knockdown of *Adamts16* expression or a TGF-β-neutralizing antibody (NAb). The RRFR tetrapeptide, but not control IIFI peptide, blocked the interaction between ADAMTS16 and LAP-TGF-β, and accelerated the activation of TGF-β in cardiac fibroblasts. In TAC mice, the RRFR tetrapeptide aggravated cardiac fibrosis and hypertrophy by upregulation of extracellular matrix proteins, activation of TGF-β, and increased SMAD2/SMAD3 signaling, however, the effects were blocked by TGF-β-NAb.

Conclusions: ADAMTS16 promotes cardiac fibrosis, cardiac hypertrophy and heart failure by facilitating cardiac fibroblasts activation via interacting with and activating LAP-TGF-β signaling. The RRFR motif of ADAMTS16 disrupts the interaction between ADAMTS16 and LAP-TGF-β, activates TGF-β, and aggravated cardiac fibrosis and hypertrophy. This study identifies a novel regulator of TGF-β signaling and cardiac fibrosis, and provides a new target for the development of therapeutic treatment of cardiac fibrosis and heart failure.

Keywords: Cardiac fibrosis, heart failure, ADAMTS16, TGF-β, SMAD2/SMAD3.

1. Introduction

Transforming growth factor (TGF)-β is a master cytokine/growth factor crucially involved in many biological processes such as embryogenesis, angiogenesis, and immune modulation and multiple fibrotic human diseases.[1, 2] TGF-β mediates its biological effects by binding to its receptors TGFβRI/TGFβRII, and phosphorylating SMAD2 and SMAD3 transcription factors, which are then translocated into the nucleus, and activate transcription of target genes.[3] However, the most important checkpoint for TGF-β signaling is at the stage of the activation of TGF-β, a process converting the latent precursor (latency associated peptide (LAP)-TGF-β) to the mature, biologically active TGF-β. The LAP domain is cleaved first from the mature TGF-β, however, the two regions remain associated with each other, preventing receptor binding and signaling and leaving only a small fraction of TGF-β biologically active. The molecular mechanisms for activation of latent TGF-β are not well understood. Proteolysis by plasmin, binding to some integrins, oxidative modifications, and interaction with the extracellular matrix (ECM) protein thrombospondin 1 (TSP-1) were reported to activate latent TGF-β.[2] Blockade of disease-associated TGF-β activation by targeting the latent TGF-β activation mechanism represents a selective approach to develop novel therapeutic strategies to treat related diseases.

Heart failure (HF) is a major public health problem, affecting 26 million people worldwide.[4] However, the 5-year survival rate for HF is 25%-52%, which is worse than most cancers.[5] Cardiac fibrosis is the major factor in the development of HF. Anti-fibrotic therapy is considered to be beneficial in alleviating HF.[6, 7] Excessive accumulation of ECM components leads to fibrosis.[8, 9] Cardiac fibrosis is predominantly mediated by the activation of cardiac fibroblasts, which is a process of conversion from the tissue-resident fibroblasts to proliferating, fibrogenic, and contractile myofibroblasts with increased expression of genes such as *ACTA2* (encoding α-smooth muscle actin, αSMA).[8, 10] Moreover, myofibroblasts also drive ECM remodeling by producing and secreting matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs).[8] The TGF-β expression level is elevated in response to injury[11-13], and TGF-β signaling is associated with repair and fibrosis of different tissues.[11-13] In particular, TGF-β plays a pivotal role in fibroblast activation and ECM production.[9, 11] To date, no clinical therapies are available to effectively block cardiac fibrosis without side effects. Therefore, additional new regulators of cardiac fibrosis need to be identified to facilitate development of novel therapeutic strategies targeting cardiac fibrosis.

ADAMTS16 is one of 19 members of the ADAMTS family of metalloproteinases involved in diverse biological processes, including spermatogenesis/fertilization, neurogenesis, inflammatory responses and cancer.[14, 15] ADAMTS16 was shown to be involved in premature ovarian failure, male genitourinary system dysfunction, esophageal squamous cell carcinoma and blood pressure regulation.[16-21] However, the role of

ADAMTS16 in cardiac fibrosis and heart failure is unknown.

In this study, we identify ADAMTS16 as a novel regulator of cardiac fibrosis. ADAMTS16 is upregulated in a mouse model for cardiac hypertrophy and heart failure, and its expression is strongly correlated with markers for cardiac fibrosis and the activation of cardiac fibroblasts. We show that ADAMTS16 promotes cardiac fibrosis by interaction with the latent form of TGF-β, which leads to TGF-β activation. Furthermore, we show that cardiac fibrosis in TAC mice is accelerated by treatment with a tetrapeptide RRFR peptide derived from ADAMTS16. The RRFR peptide activates TGF-β, promotes the activation of cardiac fibroblasts and cardiac fibrosis, and exacerbates cardiac hypertrophy and heart failure. The effect of *ADAMTS16* overexpression on activation of TGF-β and the effect of the RRFR peptide on cardiac fibrosis and hypertrophy were all blocked by a TGF-β-neutralizing antibody. Taken together, these data suggest that modulating ADAMTS16, in particular, the RRFR motif, may become a powerful new strategy to attenuate cardiac fibrosis and treat patients with HF.

2. Methods

2.1 Plasmids antibodies and peptides

The expression plasmids for *ADAMTS16*, pcDNA-4 ADAMTS16-FLAG-C14 or pCDNA4-ADAMTS16-FLAG-C2, were described previously.[22] The coding region of *ADAMTS16* was PCR-amplified from pcDNA-4 ADAMTS16-FLAG-C14 and subcloned into pcDNA3.1, resulting in another expression plasmid for *ADAMTS16*, pcDNA3.1-ADAMTS16. A mutation of the RRFR motif to IIFI was created in pcDNA3.1-ADAMTS16 using the PCR-based mutagenesis, resulting in an expression plasmid for pcDNA3.1-ADAMTS16-IIFI.

An expression plasmid for *TGFB1* was constructed by amplification of the coding by PCR using human cDNA samples and subcloning into the N-terminal p3*flag-cmv vector (pcDNA3.1-Flag-LAP-TGF-β).

A rabbit anti-ADAMTS16 antibody was purchased from Abcam (Cambridge, MA, USA) (ab45048, 1:1000 dilution for Western blotting). A rabbit anti-β-Tubulin antibody was purchased from Proteintech (Wuhan, Hubei, China) (10068-1-AP, 1:1000 dilution for Western blotting). A rabbit anti-phosphorylated SMAD3 (Ser423+Ser425) antibody was from Bioss (Beijing, China) (bs-3425R, 1:500 dilution for Western blotting). An antibody for phosphorylated SMAD2 (Ser465/467) was from Cell Signaling Technology (Boston, MA, USA) (#3101, 1:1000 dilution for Western blotting). A rabbit anti-Flag antibody was from MBL life science (PM020, 1:1000 dilution for Western blotting). A TGF-β–neutralizing antibody (TGF-β NAb) and control IgG were from R&D Systems (Minneapolis, MN, USA).

The RRFR tetrapeptide from ADAMTS16 and negative control peptide IIFI were synthesized by GENEWIZB (Wuhan, China).

2.2 Isolation of mouse cardiac fibroblasts (MCFs), cardiomyocytes culture and transfection

Neonatal mouse cardiac fibroblasts (MCFs) were prepared from hearts of 1- to 2-day-old C57BL/6 mice by digestion with 0.1% (w/v) collagenase type II (Worthington) for 30 min at 37°C as described previously.[23] The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin. After 4 hours of culture, the non-adherent cells were washed off. The isolated MCFs were cultured in the Dulbecco's Modified Eagle's medium (DMEM) with high-glucose supplemented with 10% (v/v) FBS (Gibco Life Technologies, Gaitherburg, MD, USA) in a humidified incubator with 5% (v/v) CO2 at 37°C. Cardiomyocytes were isolated from mice as previously described.[24]

MCFs were transfected with plasmid DNA (1-2 μg) and siRNA (100 nM) using the FuGENE HD Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instruction. The siRNAs targeting the *Adamts16* mRNA sequence and negative control siRNA (siNC) were chemically synthesized by RiboBio (Guangzhou, Guangdong, China). The siNC does not have homology to any known gene sequences from humans and mice. The sequences of si*Adamts16* duplexes were 5'-GAAGACGCAAGAAAUACAUTT-3' (sense).

2.3 Luciferase assays

HeLa cells were plated in a 24-well plate and transfected with a 3TP-Luc reporter construct and pRL-TK with or without an *ADAMTS16* expression plasmid using Fugene HD as described.[25-28] 30 hours later, cells were used for luciferase assays using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) as described by us previously.[25-28] Luciferase activities were normalized to Renilla luciferase activity.

2.4 ELISA for quantification of TGF-β

To measure the amount of TGF-β in the supernatant of cell culture of MCFs, an ELISA was performed using the mouse TGF-β1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations. Standard curves were created using the CurveExpert 1.3 software program.

2.5 Animal procedures

C57BL/6 mice (12-week-old) were used for all animal studies. Animal care and experimental procedures were approved by the Ethics Committee on Animal Research of Huazhong University of Science and Technology. All animal experiments conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Pressure overload of the heart was induced in 12-week-old male mice (20–25 g) by transverse aortic constriction (TAC) as described.[7] The mice were injected intraperitoneally with the RRFR peptide (0.25 mg/kg body weight) or the same dose of negative IIFI peptide (0.25 mg/kg body weight) twice a week for 8 weeks. TGF-β NAb and control IgG were injected intraperitoneally into mice at the dose of 10 mg/kg body weight twice a week for 8 weeks. Echocardiography was performed by an operator who was blinded to treatments as described.[29-31] After study, all animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and then euthanized by cervical dislocation.

2.6 Quantitative Real-time RT-PCR analysis

Total RNA was extracted from cultured cells or mouse hearts using Trizol (Invitrogen), and 2 μg of RNA samples were used for quantitative real-time PCR analysis with the FastStart Universal SYBR Green Master (Roche, Basel, BS, Switzerland) as described previously.[32, 33] Experiments were performed in triplicate and repeated at least three times.

2.7 Western blot analysis and Co-immunoprecipitation (Co-IP)

Western blot analysis was carried out using protein extracts from transfected cells and mouse hearts as described. Western blot images were captured and quantified using 1-D Analysis Software and Quantity One (Bio-Rad, Hercules, CA, USA).[7, 29, 31]

For Co-IP, MCFs were transfected with pcDNA3.1-ADAMTS16 and pcDNA3.1-Flag-LAP-TGF-β for 48 hours, and lysed with pre-cooled lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Nonidet P-40, proteinase inhibitor cocktail). The cell extracts were pre-adsorbed with 30 μL of Protein A/G PLUS-agarose (SantaCruz, TX, USA) for 4 hours at 4^0C and used for Co-IP with anti-FLAG recognizing LAP-TGF-β and anti-ADAMTS16 antibodies as described.[34]

2.8 Immunohistological analysis and immunostaining

Eight weeks after TAC, mice were euthanized, the hearts were excised, fixed overnight,

paraffin-embedded, and sectioned. Heart sections were stained with Masson staining and Typical Sirius Red staining as described.[29, 30] Immunostaining was performed with MCFs for α-SMA staining as described.[29, 30]

2.9 Cell migration assays

Migration assays with cardiac fibroblasts were carried out as previously described.[24] In brief, cardiac fibroblasts were plated in 6-well plates for 24 h and transfected with pcDNA3.1-ADAMTS16 or pcDNA3.1, and then a wound was made by mechanical scratch with a pipette tip. Cells were incubated with angiotensin II (Ang II, 1 μM) or vehicle for 24 h, and cell migration was quantified according to the ratio of cell coverage to the acellular area.

2.10 Collagen contraction assay

Cardiac fibroblasts were transfected with pcDNA3.1-ADAMTS16 or pcDNA3.1 for 24 h. Cells were then trypsinized, counted and added to a collagen solution (Shengyou Biotechnology, Hangzhou, China). The cell collagen mixture was grown in 96 well culture plates, and then incubated for 1 h under standard conditions for polymerization of collagen cell lattices. The culture medium containing Ang II (1 μM) or vehicle were added. After 8, 16 or 24 hours, the collagen lattice area was measured to calculate the relative contraction ratio of gel contraction.[35]

2.11 Statistical analysis

All quantitative data were shown as mean±s.d. The difference between two groups of variables was compared by the two-tailed, paired or unpaired Student's t-test. For comparisons of more than two groups, one-way analysis of variance was employed for normal distributions and the Kruskal-Wallis test for non-normal or small samples. A P value of < 0.05 was considered as significant.

3. Results

3.1 ADAMTS16 expression is up-regulated in a TAC model for cardiac hypertrophy and heart failure

To characterize the role of ADAMTS16 in cardiac fibrosis and heart failure, we created the transverse aortic constriction (TAC) model in 12-week-old C57BL/6 mice to induce LV hypertrophy, which can progress to heart failure.[7] Eight weeks after TAC, the mice were

used for Western blotting and real-time RT-PCR analyses. The expression levels of both the ADAMTS16 protein and the *Adamts16* mRNA were significantly higher in the hearts from TAC mice than in control sham hearts (***Figure 1A-C***). To identify cell type–specific expression changes of *Adamts16* in normal and pressure-overload hearts, we isolated cardiomyocytes and cardiac fibroblasts from TAC and sham mice. Real-time RT-PCR analysis showed a significantly increased *Adamts16* mRNA level in cardiac fibroblasts but not in cardiomyocytes from TAC mice compared to sham mice (**Supplementary material online, *Figure S1***).

3.2 Up-regulation of ADAMTS16 is associated with cardiac fibrosis and activation of cardiac fibroblasts

Mmp2 and *Mmp9* are involved in pathogenesis of cardiac fibrosis and hypertrophy by regulating ECM homeostasis.[36-38] Real-time RT-PCR analysis with cardiac RNA samples showed that up-regulation of *Adamts16* was strongly correlated with the increased expression levels of *Mmp2* (r=0.8355, P<0.05) and *Mmp9* (r=0.7817, P<0.05) in TAC mice (***Figure 1D***). MMP2 and MMP9 are involved in processing several different collagen types.[39] Similarly, up-regulation of *Adamts16* was significantly correlated with the increased expression levels of *Col1a1* (r=0.7541, P<0.05) and *Col3a1* (r=0.7945, P<0.05) in TAC mice (***Figure 1D***). One key molecular mechanism for cardiac fibrosis is the phenotypic differentiation of fibroblasts into myofibroblasts, which is marked with increased expression of α-SMA encoded by the *Acta2* gene.[9] We overexpressed *ADAMTS16* in isolated primary neonatal mice cardiac fibroblasts (MCFs) treated with Angiotensin II (Ang II). Consistent with the pro-fibrotic effects of AngII, real-time RT-PCR analysis showed that the expression level of the myofibroblast marker *Acta2* was significantly increased by AngII (***Figure 2A***), and interestingly, overexpression of *ADAMTS16* further increased *Acta2* expression significantly at the mRNA level (***Figure 2A***). The interesting finding of upregulation of the myofibroblast marker by overexpression of *ADAMTS16* was confirmed at the protein level of α-SMA using immunostaining and Western blot analysis (***Figure 2B-E***). We also characterized the functional effect of *ADAMTS16* on contractile capability of MCFs. Overexpression of *ADAMTS16* significantly increased the contraction of collagen matrices, an indicator for contraction capacity of MCFs (***Figure 2F*** **and** ***G***). A wound healing assay showed that overexpression of *ADAMTS16* significantly increased the migration of MCFs (***Figure 2H*** **and** ***I***). Together, these data suggest that *ADAMTS16* overexpression induces the activation of MCFs.

3.3 ADAMTS16 interacts with the latent form of TGFβ (LAP-TGF-β)

To identify a molecular mechanism by which ADAMTS16 induces fibroblast activation, we

carefully analyzed the protein structure of ADAMTS16, and found that it has multiple thrombospondin type 1 (TSP1) motifs (***Figure 3A***). Structural modeling analysis showed that the first TSP1 motif spanning amino acid residues 587-667 of ADAMTS16 (SWISS-MODEL: https://swissmodel.expasy.org/) resembles the TSP1 motif of TSP1 (***Figure 3B* and *C***). TSP1 was shown to interact with LAP-TGF-β through a KRFK motif and a WxxW motif (WSPW, WSHW, WGPW).[40] A similar, although not identical, WxxW motif was identified in the TSP1 motif of ADAMTS16 (***Figure 3C***). We did not identify the **KRFK** motif in ADAMTS16, but found an **RRFR** motif which shares similar charged amino acid residues with KRFK (***Figure 3C***). We hypothesized that ADAMTS16 interacts with LAP-TGF-β through the WxxW and RRFR motifs (***Figure 3D***). We co-expressed ADAMTS16 and FLAG-tagged LAP-TGFβ in HeLa cells for Co-IP analysis. An anti-ADAMTS16 antibody successfully precipitated FLAG-tagged LAP-TGF-β from HeLa cell extracts (***Figure 3E***), whereas an anti-FLAG antibody precipitated ADAMTS16 (***Figure 3F***). These data demonstrate the interaction between ADAMTS16 and LAP-TGF-β.

3.4 ADAMTS16 induces the activation of TGF-β

TGF-β activation is indicated by cleavage of the LAP domain from LAP-TGF-β and the release of mature TGF-β. To determine the effect of ADAMTS16 on the activation of LAP-TGF-β, MCFs were co-transfected with an expression plasmid for LAP-TGF-β with or without an ADAMTS16 expression plasmid and used for Western blot analysis. Moreover, we tested the effect of the RRFR motif on ADAMTS16 activation of TGF-β because the KRFK motif, but not the W*XX*W motif, of TSP1 was found to activate LAP-TGF-β.[41] Overexpression of ADAMTS16 decreased the level of LAP-TGF-β (***Figure 4A***), however, the effect was abolished by mutating the RRFR motif to IIFI motif (***Figure 4B* and *C***). Co-IP showed that the mutant ADAMTS16 with IIFI reduced the interaction between ADATS16 and LAP-TGF-β (***Figure 4D***). These data suggest that the RRFR motif can mediate the interaction between ADAMTS16 and LAP-TGF-β and their functional effects.

The activation of TGF-β was directly measured by an ELISA in the supernatant of MCF culture. Overexpression of WT ADAMTS16 markedly increased the amount of active TGF-β released in the culture, however, the effect was abolished by the mutant ADAMTS16 with the IIFI mutation (***Figure 4E***). No significant difference was observed for total TGF-β1 (acid treated) (***Figure 4F***). A sensitive luciferase assay was also used to measure the level of TGF-β activation by measuring TGF-β-mediated promoter activation. A TGF-β-responsive TPE-luciferase reporter gene was co-transfected in HeLa cells with WT-ADAMTS16 or Mut-ADAMTS16, and used for luciferase assays. TGF-β1 treatment (positive control) and overexpression of WT *ADAMTS16* significantly increased TGF-β-dependent transcription activation (***Figure 4G* and Supplementary material online, *Figure S2***), however, the effect was abolished by overexpression of mutant *ADAMTS16* or

TGF-β NAb (***Figure 4G, H*** and **Supplementary material online, *Figure S2***).

To further confirm the important role of ADAMTS16 in TGF-β activation, we knocked *Adamts16* expression down in MCFs using siRNA (***Figure 4I* and Supplementary material online, *Figure S3***). Knockdown of *Adamts16* increased the level of LAP-TGFβ (***Figure 4I***), dramatically decreased the level of active and secreted TGF-β (***Figure 4J***) and significantly reduced TGF-β-dependent transcription activation (***Figure 4L***). No significant difference was observed for total TGF-β1 (acid treated) (***Figure 4K***).

3.5 An RRFR tetrapeptide induces TGF-β activation

Because the RRFR motif of ADAMTS16 is required for binding to and activation of TGF-β in MCFs, we hypothesized that a RRFR tetrapeptide mimicked the function of ADAMTS16 and plays a critical role in TGF-β activation. MCFs were co-expressed with ADAMTS16 and LAP-TGF-β and incubated with different doses of the RRFR peptide. Co-IP showed that the RRFR peptide reduced the interaction between ADAMTS16 and LAP-TGF -β (***Figure 5A***), which further suggests that the RRFR motif of ADAMTS16 is responsible for the interaction between ADAMTS16 and LAP-TGF-β. Western blot analysis showed that the RRFR peptide significantly reduced the level of LAP-TGF-β, an indication for the activation of TGF-β (***Figure 5B***). An ELISA showed that the RRFR peptide, but not the IIFI peptide, significantly increased the release of mature TGF-β in MCF culture media (***Figure 5C***), and induced TGF-β-dependent transcription activation (***Figure 5D***). In addition, the effect of the RRFR peptide was independent of ADAMTS16 as similar results were obtained in MCFs treated with *Adamts16* specific siRNA or negative control siRNA (siNC) (***Figure 5C* and *D*).**

3.6 The RRFR tetrapeptide accelerates cardiac hypertrophy

As the RRFR peptide significantly increased the activation of TGF-β in MCFs (***Figure 5***), we hypothesized that the RRFR peptide would promote cardiac hypertrophy in mice. 12-week-old male TAC mice were treated with intraperitoneal injection of the RRFR peptide (0.25 mg/kg; PBS as negative control) twice a week for 8 weeks. Treatment with the RRFR peptide, aggravated cardiac hypertrophy in TAC mice compared with PBS control, however, the effect was significantly attenuated by TGF-β1 NAb (***Figure 6A***). Echocardiography showed that the RRFR peptide aggravated cardiac dysfunction in TAC mice by decreasing LVEF compared with PBS control, however, the effect was significantly attenuated by TGF-β1 NAb (***Figure 6B***). Similarly, the RRFR peptide significantly increased the ratio of HW/HL compared with PBS control, however, the effect was significantly attenuated by TGF-β1 NAb **(*Figure 6C*).** On the other hand, RRFR treatment did not have significant effect on blood pressure (***Figure 6D***). The plasma level of ANF was also significantly higher with

the RRFR peptide treatment than PBS control, however, the effect was significantly attenuated by TGF-β1 NAb (***Figure 6E***). To determine whether there is a sex difference, similar studies as above were performed for female mice. Interestingly, similar results as found in male mice were obtained in female mice (**Supplementary material online, *Figure S4 A-E***). All together, these data suggest that the RRFR peptide derived from ADAMTS16 promotes cardiac hypertrophy and heart failure, but the effects were abolished by TGF-β1 NAb, suggesting that the RRFR peptide promotes cardiac hypertrophy and heart failure by activating the TGF-β signaling pathway.

3.7 The RRFR tetrapeptide accelerates cardiac fibrosis

To identify the molecular mechanism by which the RRFR tetrapeptide promotes cardiac hypertrophy and heart failure, we analyzed its effects on cardiac fibrosis because TGF-β plays a key role in cardiac fibrosis. In TAC mice 8 weeks after the TAC surgery, Masson staining showed that the RRFR peptide significantly aggravated TAC-induced myocardial fibrosis (collagen content) compared with PBS control, however, the effect was significantly attenuated by TGF-β1 NAb (***Figure 7A-C***). Similar findings were obtained with Sirius red staining (***Figure 7A-C***). Moreover, the RRFR peptide significantly increased the expression levels of *Col1a1*, *Col1a3*, *Mmp2* and *Mmp9* mRNA after TAC compared with PBS control, however, the effect was significantly attenuated by TGF-β1 NAb (***Figure 7D-G***).

TGF-β activation was shown to activate SMAD2/3 (increased phosphorylation), which accelerates cardiac fibrosis, and causes cardiac hypertrophy.[42] Interestingly, we found that the RRFR peptide increased SMAD2/3 phosphorylation in hearts from male TAC mice compared with the PBS control, however, the effect was significantly attenuated by TGF-β1 NAb (***Figure 7H-J***). Similar results as found in male mice were obtained in female mice (**Supplementary material online, *Figure S5 A-G***). Taken together, these results support the notion that the RRFR motif of ADAMTS16 plays a pivotal role in cardiac fibrosis by activating TGF-β signaling (***Figure 7K***).

3.8 TAC affects the expression levels of other members of the ADAMTS family

There are 19 members in the ADAMTS family. We hypothesized that as with *Adamts16*, other *Adamts* genes may show expression differences between TAC mice and sham mice. Real-time RT-PCR analysis showed that the expression level of *Adamts7* was significantly up-regulated in TAC hearts as compared to control sham hearts to the same level as *Adamts16* (**Supplementary material online, *Figure S6***). *Adamts13* was also significantly up-regulated in TAC hearts compared with sham hearts, but not to the scale of *Adamts7* or *Adamts16* (**Supplementary material online, *Figure S6***). On the other hands, the expression levels of *Adamts2, Adamts6, Adamts15* and *Adamts18* were significantly

down-regulated in TAC hearts as compared to control sham hearts (**Supplementary material online, *Figure S6***). *Adamts18* showed the highest down-regulation in TAC mice.

4. Discussion

Cardiac fibrosis contributes critically to cardiac dysfunction in patients with a hypertensive heart and heart failure by causing structural and functional remodeling.[43] Therefore, the reduction of cardiac fibrosis by targeting novel regulators of cardiac fibrosis is considered to be an effective strategy for clinical treatment of heart failure.[9] In this study, we showed that ADAMTS16 is a novel regulator of activation of cardiac fibroblast, TGF-β signaling, and cardiac fibrosis. The expression level of ADAMTS16 was significantly up-regulated in mouse hearts with pressure-overload-induced cardiac hypertrophy and HF associated with cardiac fibrosis (***Figure 1A* and *B***). In addition, we found significant and strong correlation of the expression level of *Adamts16* with that of *Mmp2, Mmp9, Col1a1, and Col3a1* in the hearts of TAC mice (***Figure 1C***). Overexpression of ADAMTS16 triggered MCF activation, proliferation, contraction and migration induced by AngII (***Figure 2***). Under pressure overload, TGF-β is the central player in cardiac fibrosis accompanied by the activation of fibroblasts, leading to the remodeling and deposits of ECM proteins, such as different types of collagen and fibronectin[9, 44]. Recent clinical studies have demonstrated an increased myocardial TGF-β expression level during cardiac hypertrophy and fibrosis.[45] Our results demonstrate that ADAMTS16 interacts with LAP-TGF-β via an interesting RRFR motif, which promotes the release of the LAP from LAP-TGF-β, converting the latent form of LAP-TGF-β to the active form of TGF-β (***Figure 3***). Moreover, increased ADAMTS16 expression contributes to TGF-β-dependent transcriptional activities (***Figure 4***). The effect was inhibited by TGF-β-NAb (***Figure 4H*** and **Supplementary material online, *Figure S2***), indicating an important regulatory role of TGF-β signaling in functions of ADAMTS16. We showed that intraperitoneal injection of the RRFR peptide derived from ADAMTS16 aggravated cardiac fibrosis, hypertrophy and dysfunction in TAC mice, however, the effects were inhibited by TGF-β-NAb (***Figures 6* and *7***). The *in vivo* data again suggest that TGF-β signaling mediates functions of ADAMTS16. A schematic diagram showing the regulation and function of ADMTS16 in cardiac fibrosis/hypertrophy and heart failure is shown in ***Figure 7E***. TGF-β was previously found to stimulate the expression of endogenous *ADAMTS16*,[22] whereas we found here that ADAMTS16 activates TGF-β. The two proteins may form an interesting feed forward loop which exacerbates cardiac hypertrophy and heart failure.

ADAMTS16 was previously shown to control blood pressure by regulating the aortic pulse wave velocity, vascular media thickness, glomerular filtration rate, renal hemodynamics and renal handling of sodium.[20, 21] However, the detailed molecular mechanism is not clear.

Our finding that ADATMTS16 activates TGF-β provides a novel potential molecular mechanism for the regulation of blood pressure by ADAMTS16. Zacchigna et al found that knockout of *Emilin1* increased blood pressure and peripheral vascular resistance, and reduced vessel size in mice.[46] Emilin1 was found to inhibit TGF-β signaling, and inactivation of a single *TGFB1* allele rescued the high blood pressure phenotype in *Emilin1* knockout mice.[46] Therefore, it appears that *Emilin1* knockout mice developed hypertension because of activated TGF-β. Similarly, knockout of *Adamts16* inhibits TGF-β activation, thereby reducing blood pressure in mutant rats with a 17 bp deletion in exon 1 of *Adamts16*.[21] ADAMTS16 was also shown to be involved in the development of the testis, premature ovarian failure, male genitourinary system dysfunction, esophageal squamous cell carcinoma, and optic fissure (OF) closure.[16-21, 47] The results in this study identify a novel function for ADMTS16 in cardiac hypertrophy and HF.

ADAMTS16 is one of 19 members of the ADAMTS family of metalloproteinases[21]. Typical functions of the ADAMTS proteases include processing of procollagens, von Willebrand factor, aggrecan, versican, brevican and neurocan, resulting in the turnover and remodeling of ECM.[48, 49] The data in this study indicate a non-canonical function of ADAMTS16 in promoting the activation of TGF-β and related activities, resulting in the differentiation of cardiac fibroblasts to myofibroblasts and increased proliferation. Several ADAMTS metalloproteinases have been shown to play an important role in cardiovascular diseases. We found that the expression levels of *Adamts7, Adamts13* and *Adamts16* were significantly up-regulated in TAC hearts, whereas *Adamts2, Adamts6, Adamts15* and *Adamts18* were down-regulated by TAC (**Supplementary material online, *Figure S6***). Interestingly, *Adamts7* and *Adamts16* were up-regulated by TAC to the similar level (**Supplementary material online, *Figure S6***), and only the ADAMTS7 and ADAMTS16 proteins share the homologous amino acid sequences at the WxxW and KRFK/RRFR motifs. Genomic variants in ADAMTS7 were significantly associated with risk of coronary artery disease (CAD) and acute myocardial infarction (AMI) in humans, and knockout of *Admts7* reduces atherosclerosis in mice.[50] Wang et al recently showed that knockout of *Adamts2* exacerbated cardiac hypertrophy in TAC mice, and cardiac specific overexpression of *Adamts2* in mice attenuated cardiac hypertrophy.[51] The proposed mechanism is the inhibition of PI3K/AKT signaling in cardiomyocytes by ADAMTS2. Although both ADAMTS16 and ADAMTS2 are involved in regulation of cardiac hypertrophy and HF, they act in completely different manners. First, the two metalloproteinases confer opposite effects on cardiac hypertrophy, with ADAMTS16 as a risk factor and ADAMTS2 as a protective factor. This is consistent with our expression data showing that *Adamts16* was up-regulated in TAC hearts, whereas *Adamts2* was down-regulated (**Supplementary material online, *Figure S6***). Second, ADAMTS2 regulates cardiac hypertrophy by inhibiting the PI3K/AKT signaling in cardiomyocytes, whereas ADAMTS16 activates TGF-β signaling in cardiac fibroblasts and promotes cardiac fibroblast differentiation into myofibroblasts and proliferation. It should be

interesting to investigate the role of other members of the *Adamts* gene family showing either up-regulation or down-regulation in TAC hearts in cardiac fibrosis, hypertrophy and heart failure in the future. It is particularly interesting to characterize *Adamts18* for its role in cardiac fibrosis, hypertrophy and heart failure as it showed the highest down-regulation in TAC mice.

Gunes et al studied the expression levels of ADAMTS16 in thoracic aorta tissue samples from human patients with thoracic aortic aneurysms (TAA) and thoracic aortic dissection (TAD) versus age-matched controls using Western blot analysis.[19] Mutations in multiple gene/protein components of the TGF-β signaling pathway were found to cause TAA and TAD.[52] In this study, we found that ADAMTS16 interacts with LAP-TGF-β, which leads to the activation of TGF-β (***Figure 3***), increased TGF-β signaling, and increased TGF-β-dependent transcriptional activities (***Figure 4***). Therefore, it should be interesting to further investigate whether upregulation of ADAMTS16 in TAA and TAD aortic tissue samples may cause TAA and TAD by regulating TGF-β signaling.

TGF-β is a strong inducer of cardiac hypertrophy.[53] Some studies suggested that TGF-β was secreted from cardiac fibroblasts, and induces the secretion of connecting tissue growth factor (CTGF) from cardiomyocytes; CTGF from cardiomyocytes induces cardiomyocyte hypertrophy following pressure overload via the AKT pathway in a cell-autonomous manner.[53, 54] Another study showed that beta-adrenergic signaling was activated in TGF-β-transgenic mice, leading to increased cardiac hypertrophy.[55] Therefore, ADAMTS16 may also regulate cardiac hypertrophy and heart failure by regulating TGF-β, CTGF, and AKT functions in cardiomyocytes.

TSP1 was found to interact with and activates LAP-TGF-β.[2] Subsequent studies showed that the KRFK motif of TSP1 was critical for interaction with the LSKL motif of the LAP-TGF-β, disrupting LAP-mature domain interactions so that the receptor binding sequences are exposed for TGF-β signaling.[2] ADAMTS16 does not have the exact KRFK motif of TSP1, however, a motif of RRFR was found and shown to mediate the interaction between ADAMTS16 and LAP-TGF-β (***Figures 3* and *4***). *In vivo* mouse studies showed that intraperitoneal injection of the RRFR peptide promoted cardiac fibrosis and exacerbates cardiac hypertrophy in TAC mice (***Figures 6* and *7***). These data suggest that the ADAMTS16 RRFR motif is a new therapeutic target for treatment of cardiac fibrosis, cardiac hypertrophy and heart failure. Future studies may develop therapeutic monoclonal antibodies against the RRFR motif of ADAMTS16 to block the interaction between ADAMTS16 and LAP-TGF-β, which may inhibit the activation of TGF-β signaling and block cardiac fibrosis. Other types of inhibitors against ADAMTS16 function, for example, small chemical inhibitors, may also inhibit cardiac fibrosis and treat cardiac hypertrophy and HF. In addition, TGF-β is known as the most potent pro-fibrogenic cytokine and involved in many other fibrotic diseases, including renal fibrosis, pulmonary fibrosis, liver fibrosis and subepithelial fibrosis[56-59]. Therefore, the ADAMTS16 or its RRFR motif therapeutics may

become a novel treatment of many other fibrotic diseases.

Our study has several limitations. First, although studies in cultured cardiac fibroblasts suggest an important role of ADAMTS16 in activation of cardiac fibroblasts and TGF-β activation, and exogenous infusion of the RRFR peptide from ADAMTS16 enhanced cardiac hypertrophy and fibrosis, the *in vivo* role of endogenous *Adamts16* remains to be established. Global knockout (KO) mice deficient in *Adamts16*, and cardiomyocyte-specific or cardiac fibroblasts-specific KO mice can be developed to demonstrate the critical role of *Adamts16* in cardiac fibrosis and hypertrophy, and distinguish the specific cell type(s) involved. Second, the interaction between endogenous ADAMTS16 and LAP-TGF-β in primary cardiac fibroblasts cells needs to be further analyzed. Third, it may be interesting to determine whether the expression level of ADAMTS16 is affected in human hearts with heat failure.

In conclusion, we show that ADAMTS16 is a central activator of TGF-β to accelerate activation of cardiac fibroblasts. Furthermore, the RRFR motif in ADAMTS16 plays a key role in the activation of TGF-β signaling. Our data in mouse models showed that the RRFR peptide from ADAMTS16 treatment aggravated TAC-induced cardiac fibrosis, hypertrophy and heart failure, also by regulating TGF-β signaling. Together, we demonstrated that the RRFR motif of ADAMTS16 is a key player in the dynamic interplay that regulates TGF-β activation. As TGF-β signaling is involved in numerous fibrotic diseases, the development of inhibitors or neutralizing antibodies of ADAMTS16 or the RRFR motif may serve as a new strategy to treat not only cardiovascular diseases, but also other diseases caused by abnormal TGF-β signaling.

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Figure Legends

Figure 1

The expression level of *Adamts16* is up-regulated and correlated with the levels of *Mmp2*, *Mmp9, Col1a1* and *Col3a1* in the hearts from TAC mice with pressure overload-induced cardiac hypertrophy and heart failure. (***A***) Western blot analysis for ADAMTS16 using heart protein extracts from TAC mice and control Sham mice. Tubulin was used as loading control. Data are shown as mean ± SD. ***P*<0.01 (n=6). (***B***) Real-time RT-PCR analysis for *Adamts16* using total cardiac RNA samples from 16 week old TAC mice and control Sham mice. Data are shown as mean ± SD. ***P*<0.01 (n=6). (***C***) Real-time RT-PCR analysis showed that the expression levels of *Adamts16* significantly correlated with the levels of *Mmp2* (r=0.84), *Mmp9* (r=0.78), *Col1a1* (*r*=0.75) and *Col3a1* (*r*=0.79) in TAC mice. *P*<0.05 (n=12). Statistical analysis was carried out by a Student's *t*-test.

Figure 2

Overexpression of *ADAMTS16* accelerates the activation of cardiac fibroblasts (differentiation of cardiac fibroblasts into myofibroblasts with increased α-SMA). (***A***) Real-time RT-PCR analysis for *Acta2* (encoding α-SMA) in mouse cardiac fibroblasts transfected with pcDNA3.1 or a mammalian expression plasmid for human *ADAMTS16* and treated with Ang II (48 hours) or negative control buffer. (***B***) Immunostaining analysis of α-SMA for mouse primary cardiac fibroblasts treated as in (***A***). Scale bar =12.5 μm. (***C***) Quantification of immunostaining images as in (***B***). (***D***) Western blot analysis for α-SMA using protein extracts from mouse cardiac fibroblasts treated as in (***A***). (***E***) Quantification of Western blotting data as in (***D***). (***F***) Collagen lattice contraction analysis of mouse cardiac fibroblasts treated as in (***A***). (***G***) Quantification of collagen lattice contraction data as in (***F***). (***H***) Migration of cardiac fibroblasts treated as in (A) using scratch wound assays. (***I***) Quantification of scratch wound data as in (***H***). Data are shown as mean ± SD. **P* < 0.05, ***P*<0.01 (n=6/group). Statistical analysis was carried out by a one-way ANOVA test.

Figure 3

ADAMTS16 interacts with LAP-TGF-β1. (***A***) Schematic diagram showing the major structural domains of ADATS16. (***B***) Structural modeling showing overall similarities

between TSP1 and the TSP1-domain of ADAMTS16. (***C***) Alignment of sequences of the proximal TSP1-domain of ADAMTS16 and thrombospondin-1 (TSP1). The homologous amino acid sequences at the WxxW and KRFK/RRFR motifs responsible for binding to the LAP are marked. (***D***) Schematic diagram showing the predicted interaction between ADAMTS16 and LAP-TGF-β via the WxxW and RRFR motifs. (***E***) Co-IP analysis using cellular protein extracts from mouse primary cardiac fibroblasts with co-expression of ADAMTS16 and LAP-TGFβ. An anti-ADAMTS16 antibody (Ab-Adam) was used for immunoprecipitation and an anti-FLAG antibody recognizing LAP-TGFβ was used for immunoblotting. (***F***) Reciprocal Co-IP analysis. An anti-FLAG antibody recognizing LAP-TGFβ (Ab-FLAG) was used for immunoprecipitation and an anti-ADAMTS16 antibody was used for immunoblotting.

Figure 4

ADAMTS16 induces the activation of TGF-β. (***A***) Western blot analysis showed that overexpression of ADAMTS16 decreased the level of LAP-TGF-β in mouse cardiac fibroblasts transfected with LAP-TGF-β. The experiment was independently repeated for three times (n=6). (***B***) Schematic diagram showing wild type (WT) ADAMTS16 and mutant ADAMTS16 with the RRFR motif mutated into IIFI. (***C***) Western blot analysis showed that overexpression of mutant ADAMTS16 did not affect the level of LAP-TGF-β in mouse cardiac fibroblasts transfected with LAP-TGF-β (n=6). (***D***) Co-IP analysis showed that the interaction between LAP-TGF-β and ADAMTS16 was reduced in mouse cardiac fibroblasts by mutation IIFI (n=6). (***E***) ELISA for measurements of active TGF-β (pg/ml) in the supernatant of mouse cardiac fibroblasts co-transfected with LAP-TGF-β and WT-ADAMTS16 or Mut-ADAMTS16. Data are shown as mean ± SD. ***P*<0.01 (n=4). Statistical analysis was carried out by a one-way ANOVA test. (***F***) ELISA for measurements of total TGF-β (acid treated) in the supernatant of mouse cardiac fibroblasts treated as in (***E***). (***G***) Luciferase assays for TGF-β-mediated transcription activation in HeLa cells. Data are shown as mean ± SD. ***P*<0.01 (n=4/group). Statistical analysis was carried out by a one-way ANOVA test. (***H***) Effects of TGF-β NAb on TGF-β-mediated transcription activation in HeLa cells. Data are shown as mean ± SD. ***P*<0.01, NS, not significant (n=4/group). Statistical analysis was carried out by a one-way ANOVA test. (***I***) Western blot analysis showed that knockdown of *Adamts16* expression by siRNA significantly increased the level of LAP-TGF-β in mouse cardiac fibroblast (n=6/group). (***J***) ELISA for measurements of the level of active TGF-β in the supernatant of mouse cardiac fibroblasts transfected with *Adamts16* siRNA or siNC. Data are shown as mean ± SD. ***P*<0.01 (n=4). Statistical analysis was carried out by a Student's *t*-test. (***K***) ELISA for measurements of the level of total TGF-β (acid treated) treated as in (***J***). (***L***) Luciferase assays showing reduced

TGF-β-mediated transcriptional activation by *Adamts16* siRNA. Data are shown as mean ± SD. $^{**}P<0.01$ (n=4). Statistical analysis was carried out by a Student's *t*-test.

Figure 5

The RRFR peptide inhibits the interaction between ADAMTS16 and LAP-TGF-β and induces the activation of TGF-β. (***A***) Co-IP analysis for the interaction between Adamts16 and LAP-TGF-β in mouse cardiac fibroblasts treated with different concentrations of the RRFR peptide. An anti-FLAG antibody was used for immunoprecipitation and an anti-ADAMTS16 antibody was used for immunoblotting (IB). (***B***) Western blot analysis showing the effect of the RRFR peptide on the level of LAP-TGF-β in mouse cardiac fibroblasts. Data are shown as mean ± SD. $^{**}P<0.01$ (n=6). Statistical analysis was carried out by a one-way ANOVA test. (***C***) ELISA showing the effect of the RRFR peptide on the level of active TGF-β in the supernatant of mouse cardiac fibroblasts. Data are shown as mean ± SD. $^{**}P<0.01$ (n=5). Statistical analysis was carried out by a two-way ANOVA analysis. NS indicates not significant. (***D***) Luciferase assays for TGF-β-mediated transcriptional activation in HeLa cells transfected with the TGF-β-responsive 3TPE luciferase reporter gene and treated with *Adamts16* siRNA or negative control siRNA (siNC) for 18 hours. Cells were then incubated with the RRFR peptide or IIFI peptide for 18 hours, and sued for luciferase assays. Data are shown as mean ± SD. $^{**}P<0.01$ (n=5). Statistical analysis was carried out by a two-way ANOVA analysis. NS indicates not significant.

Figure 6

The RRFR peptide accelerates cardiac hypertrophy. (***A***) Morphology of the hearts from Sham mice and TAC mice injected with the RRFR peptide in combination with or without TGF-β1 NAb for 8 weeks. Scale bar = 1 mm. (***B***) Echocardiographic data showing left ventricular ejection fractions (LVEF, n=8/group). (***C***) Ratio of heart weight to tibia length (HW/TL, n=8/group). (***D***) Systolic blood pressure (mmHg, n=8/group). (***E***) Plasma ANF levels (n=8/group). Data are shown as mean ± SD. $^{**}P<0.01$ vs. Sham+vehicle+IgG group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. TAC+vehicle+IgG group; $^{\S}P<0.05$, vs. TAC+vehicle+IgG group; $^{\dagger\dagger}P<0.01$ vs. TAC+RRFR+IgG group. NS indicates not significant. Statistical analysis was carried out by a one-way ANOVA test.

Figure 7

The RRFR peptide accelerates cardiac fibrosis. (***A***) Masson staining (upper panel) and typical Sirius Red staining (lower panel) of the hearts in Sham mice and TAC mice with pressure-overloading for 8 weeks. The collagenous area and myocardial fibrosis of the hearts are graphed for different groups of mice (n=8). Scale bar = 100 μm, and the quantified data are shown at the right (***B***) and (***C***). (***D***), (***E***), (***F***) and (G) represent the effects of the RRFR peptide treatment combined with TGF-β1 NAb or IgG on the mRNA expression levels for *Col1a1*, *Col3a1*, *Mmp2* and *Mmp9* in mice after 8 weeks of TAC (n=8). (***H***) Western blot analysis showing the effects of the combined treatment of the RRFR peptide with TGF-β1 NAb or IgG on levels of SMAD2/3 phosphorylation in mice after 8 weeks of TAC operation (n=8). The quantified data are shown at the right (***I***) and (***J***). (***K***) Diagram showing the molecular signaling pathway for ADAMTS16 as a central activator of TGF-β to accelerate cardiac fibrosis, cardiac hypertrophy and heart failure. Data are shown as mean ± SD. $^{**}P < 0.01$ vs. Sham+vehicle+IgG group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. TAC+vehicle+IgG group; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, vs. TAC+vehicle+IgG group; $^{\dagger\dagger}P<0.01$ vs. TAC+RRFR+IgG group. NS indicates not significant. Statistical analysis was carried out by a one-way ANOVA test.

Figure 1

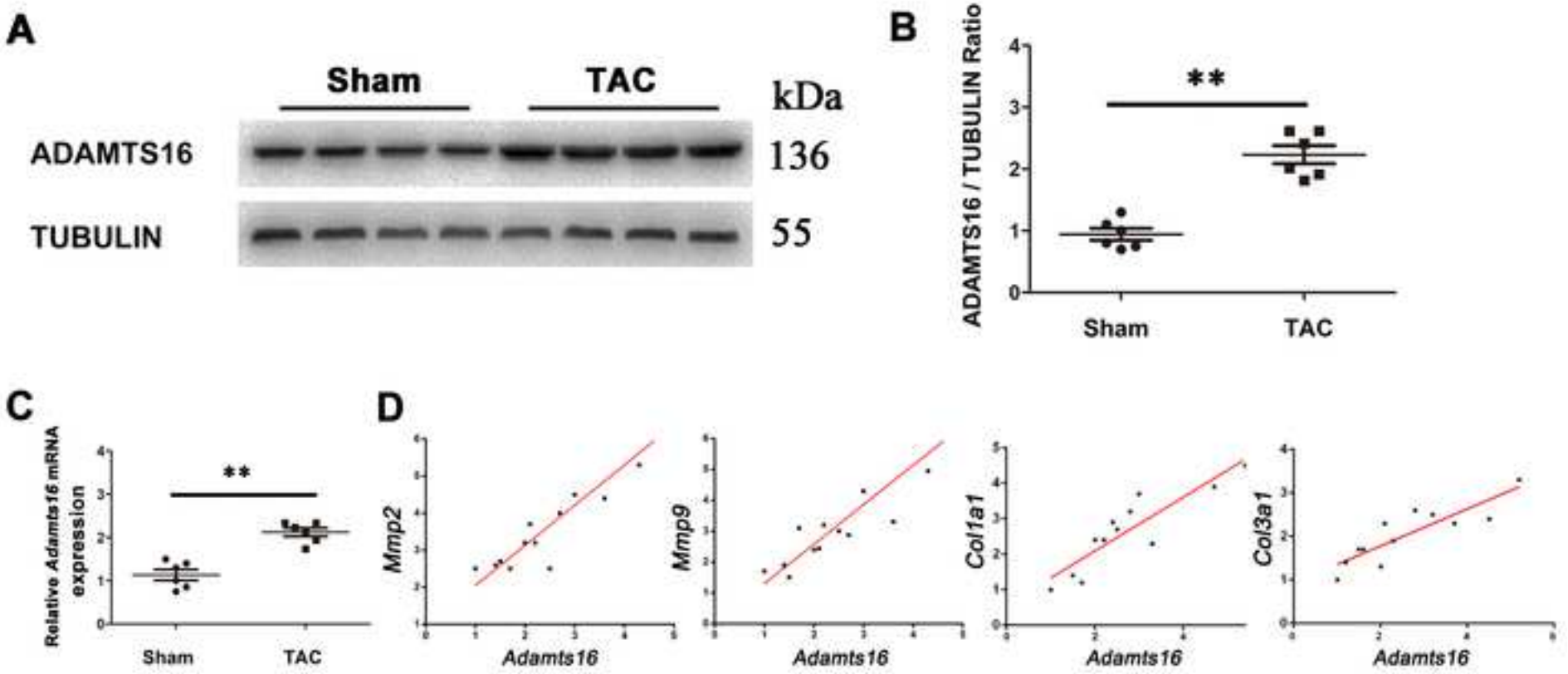
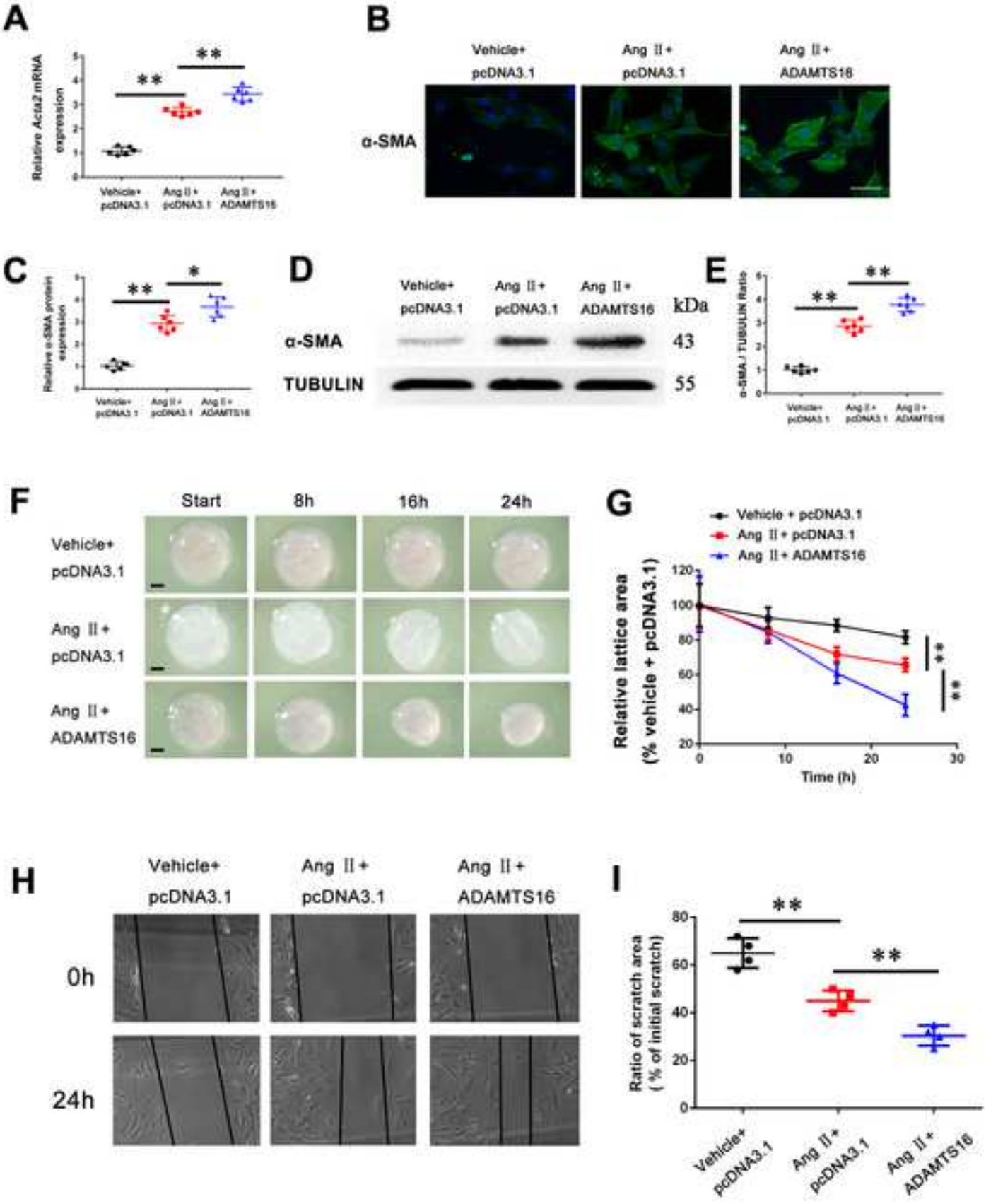


Figure 2



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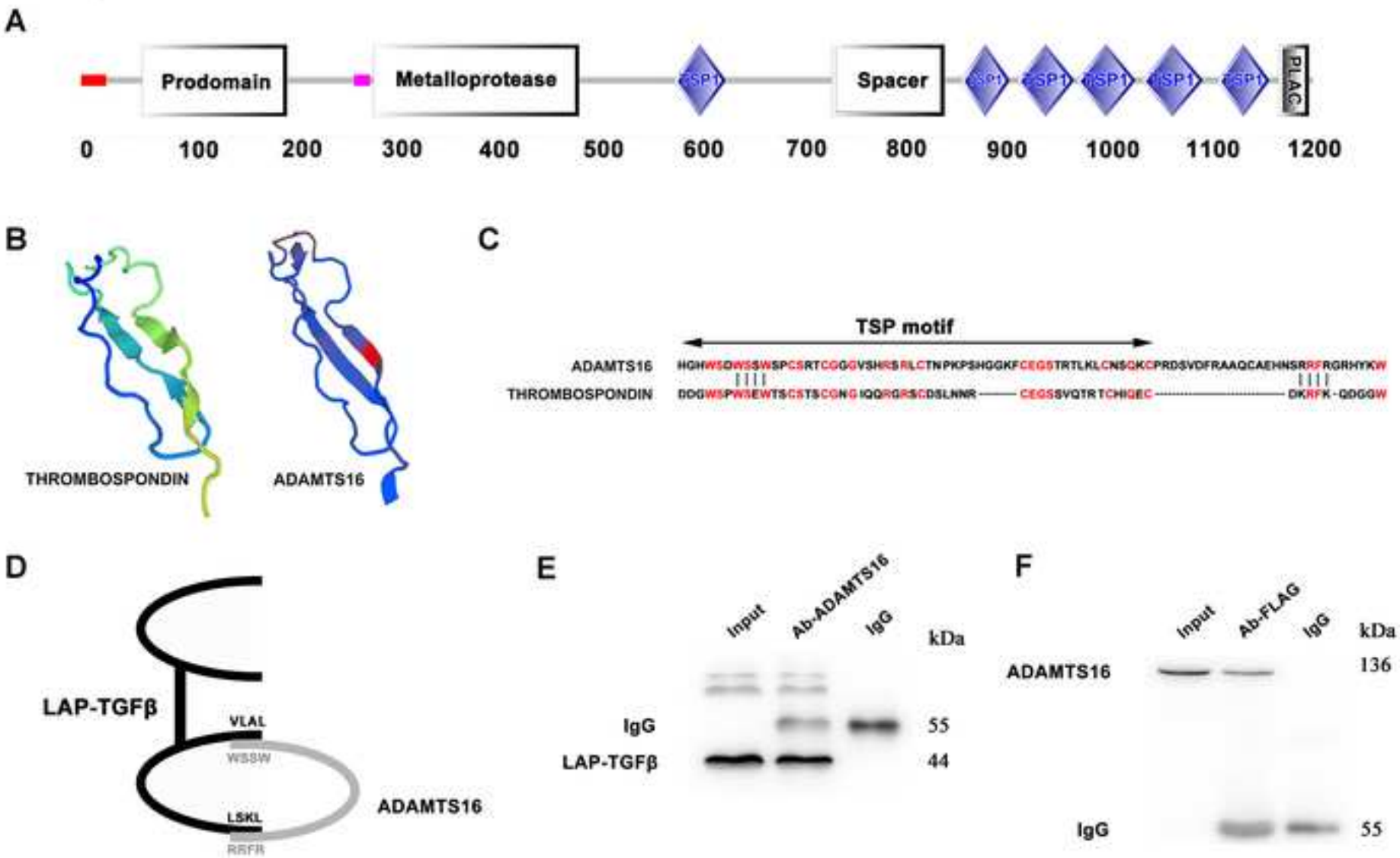

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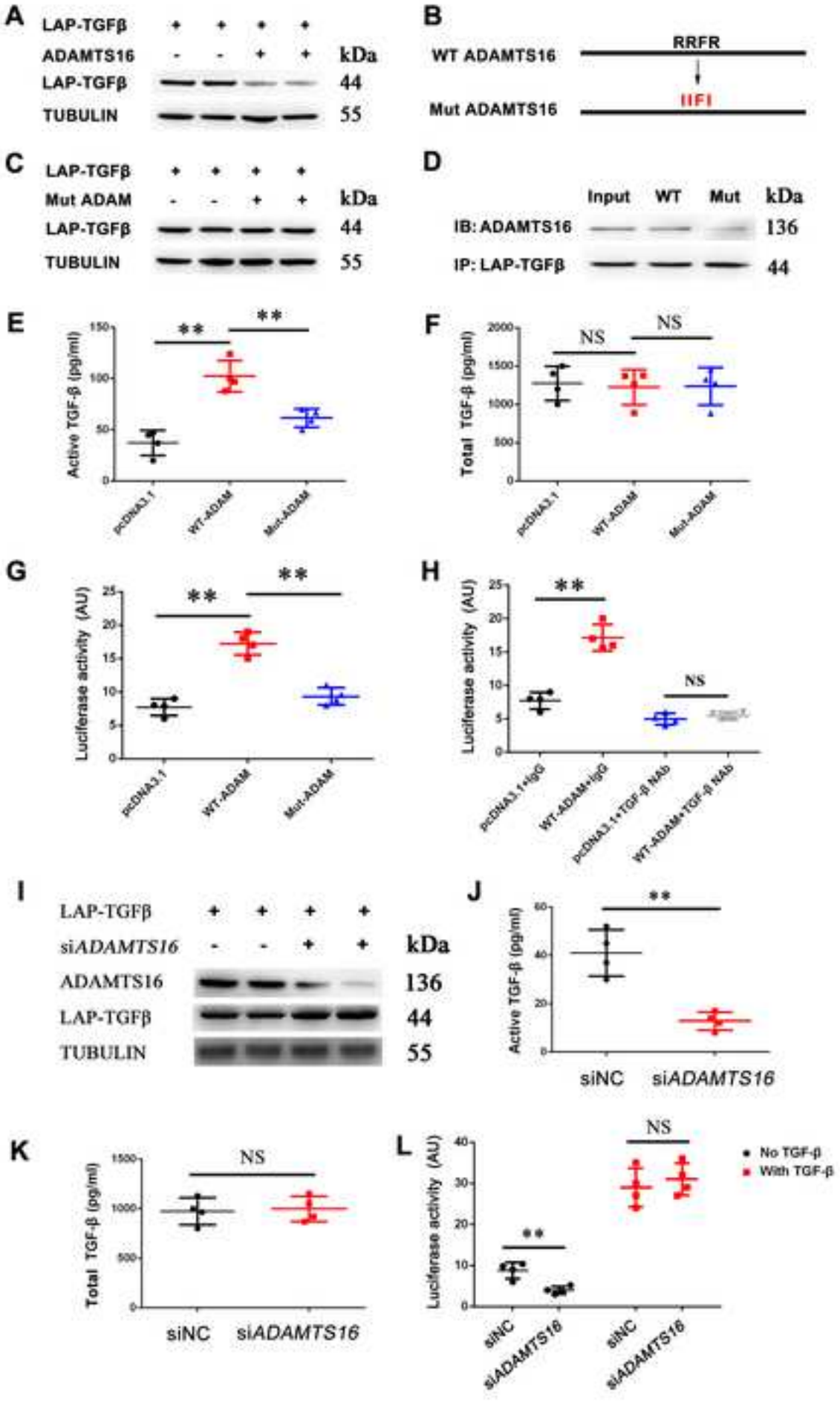


Figure 5

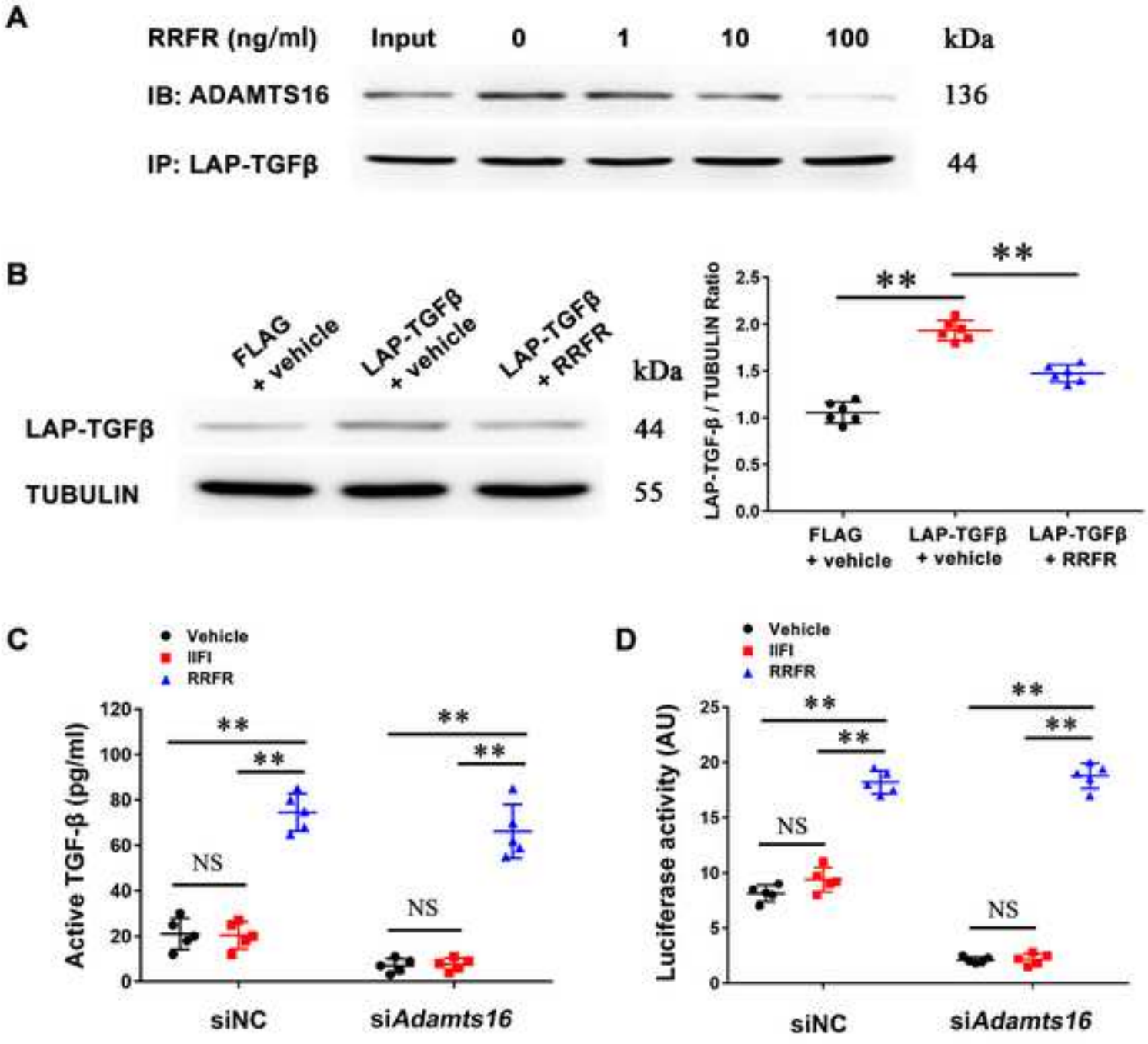


Figure 6

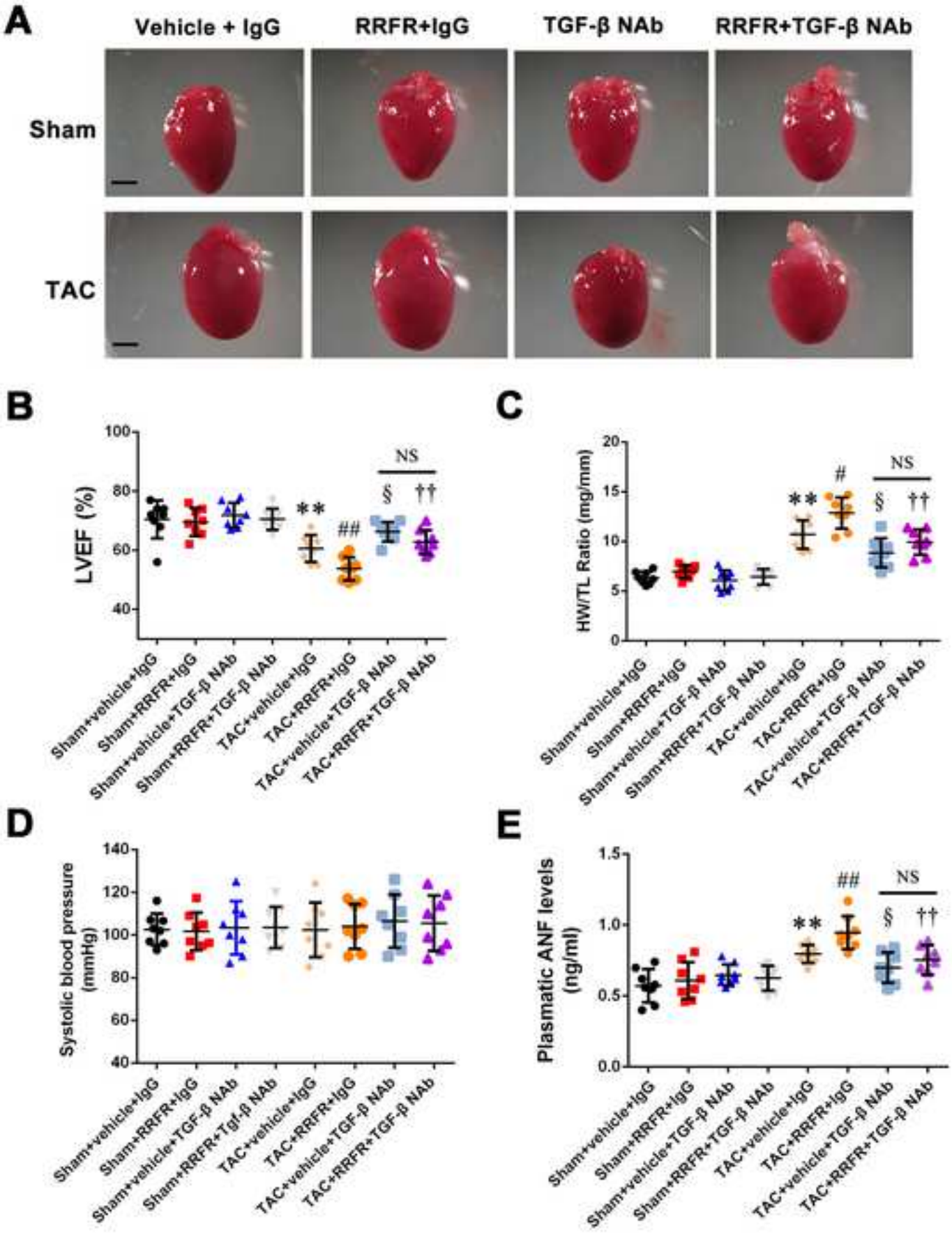
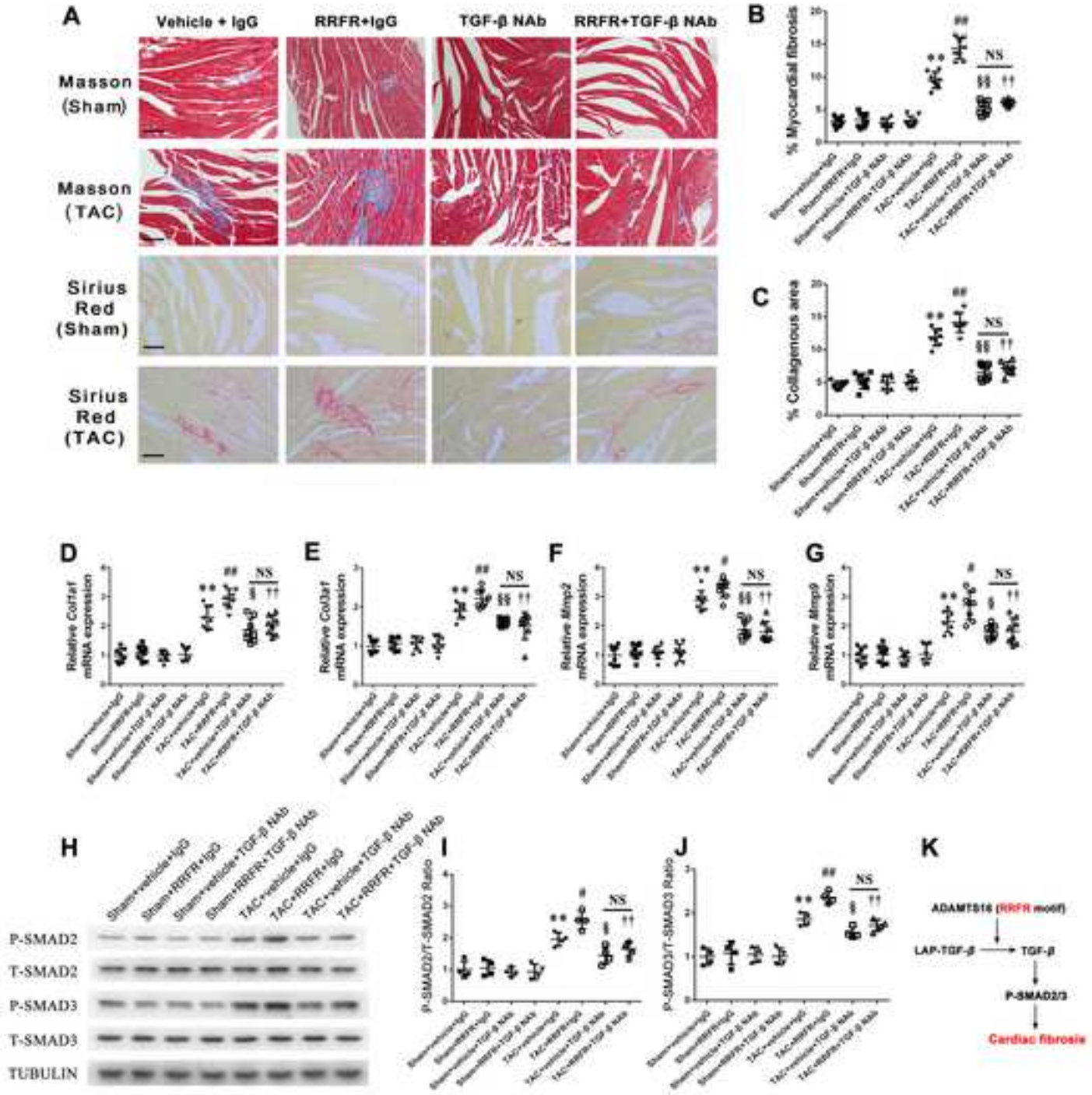


Figure 7



Graphical Abstract