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The role of vascular smooth muscle cell membrane-bound thrombomodulin in neointima formation

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HIGHLIGHTS

- Smooth muscle cells play important roles in injury-induced neointima.
- Thrombomodulin is expressed on smooth muscle cells after vascular injury.
- Thrombomodulin participated in smooth muscle cell phenotype change.
- Thrombomodulin influenced smooth muscle cell proliferation and inflammation.
- Deletion of thrombomodulin on smooth muscle cells caused less neointima.

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ABSTRACT

Background and aims: Thrombomodulin (TM) is an endothelial cell membrane-bound anticoagulant protein expressed in normal arteries. After vascular injury, medial and neointimal smooth muscle cells (SMCs) exhibit large amounts of TM. The purpose of this study was to investigate the physiological significance of vascular SMC-bound TM.

Methods: The morphology, expression of phenotype markers and cell behaviors of cultured aortic SMCs after knockdown of TM were observed. Transgenic mice with SMC-specific TM deletion were generated, and carotid neointima formation was induced by carotid ligation.

Results: Cultured human aortic SMCs displayed a synthetic phenotype with a rhomboid-shaped morphology and expressed TM. TM knockdown induced a spindle-shaped change in morphology with an increased expression of contractile phenotype marker and decreased expression of synthetic phenotype marker. TM knockdown not only attenuated the proliferation of SMCs but also reduced tumor necrosis factor-α-induced nuclear factor-κB activation and interlukin-6 production. In a carotid artery ligation model, transgenic mice with SMC-specific TM deletion (SM22-cre^{tg}/TM$^{flox/flox}$) had significantly less cellular proliferation in arterial walls compared with wild type mice (SM22-cre^{tg}/TM$^{+/+}$). The neointima area and neointima/media area ratio were smaller in SM22-cre^{tg}/TM$^{flox/flox}$ mice at 4 weeks after ligation.

Conclusions: Our results indicate that vascular SMC-bound TM plays a role in changes of the SMC phenotype. It also influences SMC cell behavior and injury-induced neointima formation.

1. Introduction

The pathophysiology of atherosclerosis and vascular injury-induced neointima involves endothelial injury as the initiating event followed by complex inflammatory and immune responses [1,2]. Vascular smooth muscle cells (SMCs) are intimately involved during this process

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[3]. In the media of normal arteries, vascular SMCs assume a contractile phenotype and express a variety of SMC-specific contractile markers, such as α-smooth muscle actin (α-SMA) and calponin. Upon stimulation, the medial SMCs transform into a synthetic, proliferative and proinflammatory phenotype, which is associated with decreased expression of contractile markers and increased expression of synthetic markers, such as vimentin and non-muscle myosin heavy chain (NM-MHC). Activated vascular SMCs are capable of robust proinflammatory responses by producing multiple cytokines, such as interleukin (IL)-6, which is the major regulator of downstream inflammatory cascades [4,5]. The switch of vascular SMC phenotype can be induced by a wide range of factors [6], however, the definite molecular mechanisms underpinning vascular SMC phenotype transition remain to be fully elucidated.

Thrombomodulin (TM) is a cell membrane-bound glycoprotein only expressed on the vascular endothelium in normal arteries. TM binds to thrombin, and the TM-thrombin complex accelerates the rate of protein C activation, which acts as a physiological anticoagulant [7]. Binding of TM to thrombin also prevents thrombin-mediated protease-activated receptor-1 (PAR-1) activation and inhibits its downstream proinflammatory effects on the endothelium [8]. In normal arterial walls, TM is only expressed on endothelial cells, but not medial vascular SMCs. Medial vascular SMCs have been shown to express large amounts of TM during atherosclerosis and neointima formation after vascular injury [9–11]. Proinflammatory cytokines and growth factors such as thrombin, cyclooxygenase-2-derived prostaglandin and platelet-derived growth factor (PDGF)-BB have been shown to stimulate the expression of TM on cultured vascular SMCs [12–14]. Although the presence of TM on vascular SMCs in diseased arteries has been known for years, the physiological and pathological significance of SMC-bound TM remains largely undetermined. The expression of TM has been shown to be decreased on the endothelium in atherosclerotic lesions and in vascular injury-induced neointima [11,15]. Many researchers have speculated that the expression of TM on vascular SMCs is only a compensatory mechanism to balance endothelial TM downregulation, thereby maintaining vascular wall homeostasis. In recent years, TM has been shown to possess multiple direct cellular effects beyond its anticoagulant effects [8,16–18]. We hypothesized that the presence of TM on activated SMCs may alter vascular SMC behaviors and influence vascular injury-induced neointima formation. In the current study, we observed the differences in cell morphology and behaviors between cultured aortic SMCs with and without TM expression. A complete loss of TM in TM knockout transgenic mice causes embryonic lethality [19]. Therefore, we generated vascular SMC-specific TM-deficient mice using the Cre-loxP system to explore the role of vascular SMC membrane-bound TM *in vivo*.

2. Materials and methods

2.1. TM knockdown

Human aortic SMCs (Invitrogen, Carlsbad, CA) were maintained in medium 231 (Invitrogen) containing 5% smooth muscle growth supplement (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cultured human aortic SMCs were grown to 60–70% confluence and transfected with TM siRNA or control siRNA (25 nM) using DharmaFECT transfection reagents (Thermo Scientific, Lafayette, CO) according to the manufacturer's instructions. Cells were treated with siRNA for 48 h before the following experiments. After transfection, the cells were harvested and the knockdown efficiency of TM was evaluated using immunoblotting.

2.2. Microarray analysis

Agilent microarray analysis was carried out according to Agilent Technologies' guidelines. Briefly, RNA samples from cells treated with TM siRNA or control siRNA were collected, amplified and labeled using an Agilent Quick Amp Labeling Kit. The samples were hybridized to an Agilent whole genome oligo microarray and then scanned using an Agilent DNA microarray scanner. The results were imported into Agilent GeneSpring GX software (version 11.0) for further analysis. Differentially expressed genes were identified and the p values were calculated using the t-test.

2.3. TM overexpression

The pEGFPN1 vector was obtained from BD Biosciences Clontech (Palo Alto, CA) and the fluorescent protein-tagged TM expression vector, pEGFPN1-TM, was constructed as previously described [16]. In brief, the human *TM* gene encoding TM amino acid residues 1–575 in chromosomal DNA was amplified by polymerase chain reaction (PCR) using a BamHI forward primer, TM719 (5′-CGGGATCCCGGAATGCTT GGGGTCCTGGTCCTTG-3′) and an EcoRI reverse primer (5′-GGAATTC GGAGTCTCTGCGGCGTCCGCT-3′). The 1.7-kb PCR product was digested with BamHI and EcoRI. The fragment was ligated to the expression vector pEGFPN1 and named pEGFPN1-TM. The pEGFPN1-TM vector was transfected into aortic SMCs using Polyjet transfection reagent (SignaGen Laboratories, Rockville, MD) for 24 h and the transfection efficiency was analyzed by immunoblotting.

2.4. Immunoblotting

Nuclear and cytoplasmic fractions of aortic SMCs were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) following the manufacturer's instructions. Cell lysates were extracted from aortic SMCs and subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting. They were then probed with specific antibodies against human TM, IκB-α, p50, p65, α-tubulin, GAPDH (all from Santa Cruz Biotechnology) and SMC markers, including α-SMA, calponin, NM-MHC and vimentin (all from Abcam).

2.5. Proliferation assay

Cell proliferation in 96-well plates (3×10^3/well) was assessed using a WST-1 assay according to the manufacturer's instructions (Roche, Madison, WI). The medium was replaced every 2 days. Ten μL of WST-1 was added to the wells followed by incubation for 1 h. The absorbance was directly recorded at a wavelength of 450 nm in a microplate reader.

2.6. Quantification of IL-6 and MMP-2

IL-6 mRNA was evaluated by reverse transcription (RT)-PCR, and release of IL-6 into culture supernatants was analyzed using an enzyme-linked immunosorbent assay (ELISA) kit. In brief, total RNA was extracted and reverse transcribed using TaqMan Reverse Transcription Reagents. The gene specific primers were as follows: IL-6 (sense) 5'-AGTTGTGCAATGGCAATTCTG-3'; IL-6 (antisense) 5′-GGAAATTGGGG TAGGAAGGAC-3'; TM (sense) 5′- TACGGGAGACAACAACACCA-3'; TM (antisense) 5′- AAGTGGAACTCGCAGAGGAA-3'. β-actin (sense) 5′-TGTTACCAACTGGGACGACA-3'; β-actin (antisense) 5′-GGGGTGTT GAAGGTCTCAAA-3'. Quantitative RT–PCR was performed and analyzed as previously described [20]. ELISA was used to measure the concentrations of IL-6 (R&D Systems, Minneapolis, MN) in the supernatant of cultured aortic SMCs treated with TM siRNA or control siRNA based on the manufacturers' instructions as previously described [21]. Matrix metalloproteinase-2 (MMP-2) activity in the condensed supernatants was examined by gelatin zymography. Protein (0.5 μg) was subject to electrophoresis with 10% SDS-PAGE containing 0.1% gelatin. The gels were stained with Coomassie blue, and the band intensity was quantitatively determined using Gel-Pro Analyzer software.

2.7. Migration assay

We evaluated the migration ability of aortic SMCs with and without TM knockdown using a 48-well Boyden chamber assay. In the Boyden chamber (Neuro Probe, Bethesda, MD), 50 μL SMC suspension (5×10^4/mL) was added to the upper chamber, and PDGF (20 ng/mL) was added to the lower chamber. The chambers were incubated at 37 °C for 6 h with 5% CO_2. SMCs that did not migrate were scraped off the membrane, and the cells that migrated were fixed and visualized by Liu's staining. The migrated cells were counted using an optical microscope (Leica) at 100X magnification with MetaMorph imaging software (Universal Imaging Corp). Five random fields in each well were counted.

2.8. Vascular SMC-specific TM-deficient mice

Vascular SMC-specific TM-deletion mice were generated using the Cre-loxP system as previously described [20]. In brief, the whole TM gene was designed to flank with two loxP sites, and $TM^{flox/+}$ chimera mice were generated from the Transgenic Mouse Models Core, National Research Program for Genome Medicine, Taiwan (Supplemental Fig. 1A). The $TM^{flox/+}$ mice were bred with wild type C57BL/6 mice for at least 5 generations. We used SM22-cre transgenic mice in which cre recombinase was driven by the Tagln promoter and mainly expressed in vascular SMCs [22]. To generate vascular SMC-specific TM-deficient mice, $TM^{flox/flox}$ mice were crossbred to transgenic mice carrying a SM22-cre transgene (SM22-cre^{tg}, The Jackson Laboratory, stock

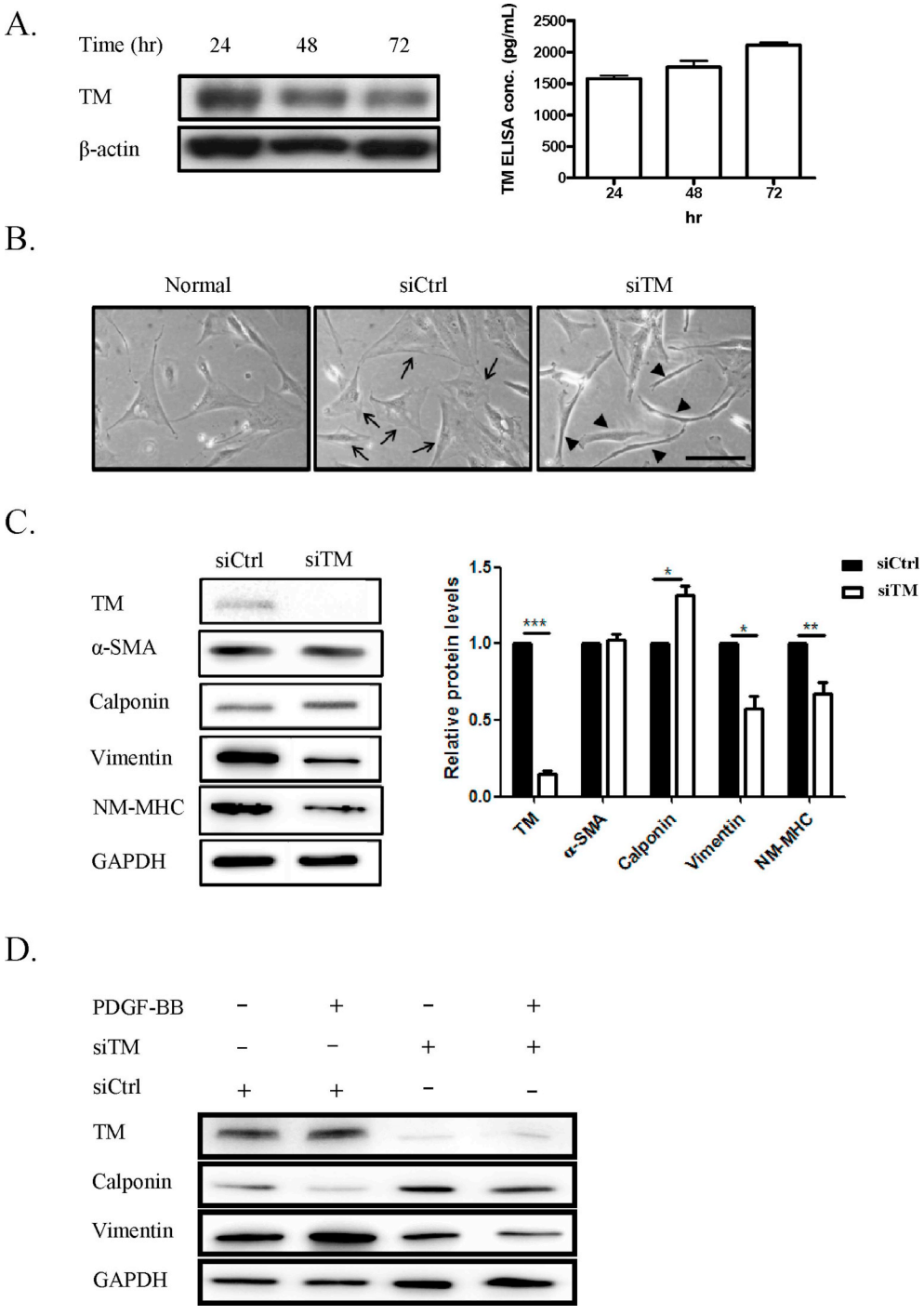

Fig. 1. Phenotype changes of aortic SMCs after TM knockdown.
(A) Cultured aortic SMCs expressed TM as shown by Western blot (left panel) of the cell lysates and ELISA of the cultured media (right panel). (B) Cultured aortic SMCs treated with TM-targeting siRNA (siTM) underwent morphological changes from a rhomboid (arrows) to spindle shape (arrowheads) compared with non-targeting siRNA controls (siCtrl). Scale bars, 20 μm. (C) Representative Western blot illustrating expression levels of TM, contractile and synthetic markers in aortic SMCs with (siTM) and without (siCtrl) TM knockdown. GAPDH was used as a loading control. Quantification of protein expression (n = 6) was normalized to GAPDH and expressed as mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$. α-SMA, α-smooth muscle actin; NM-MHC, non-muscle myosin heavy chain. (D) Representative Western blot illustrating the expressions of calponin and vimentin in aortic SMCs with the indicated treatment. GAPDH was used as a loading control.

number: 004746) to generate SM22-cre^{tg}/TM$^{flox/+}$ mice, which were subsequently interbred to yield SM22-cre^{tg}/TM$^{flox/flox}$ mice and their wild-type controls, SM22-cre^{tg}/TM$^{+/+}$ mice. All animal experiments were approved by the Institutional Animal Care and Use Committee, National Cheng Kung University (IACUC approval number:104215) and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication #85-23, revised 1996).

2.9. Mouse carotid ligation model

Carotid artery ligation was performed according to our previous study [9]. In brief, the mice were anesthetized by intraperitoneal injection of pentobarbital. Neck incision was performed and the left common carotid artery was ligated completely with a 6-0 silk suture near the carotid bifurcation. At 2 and 4 weeks after ligation, the mice were sacrificed and the segment of left common carotid artery distal to the ligation site was excised for further analysis.

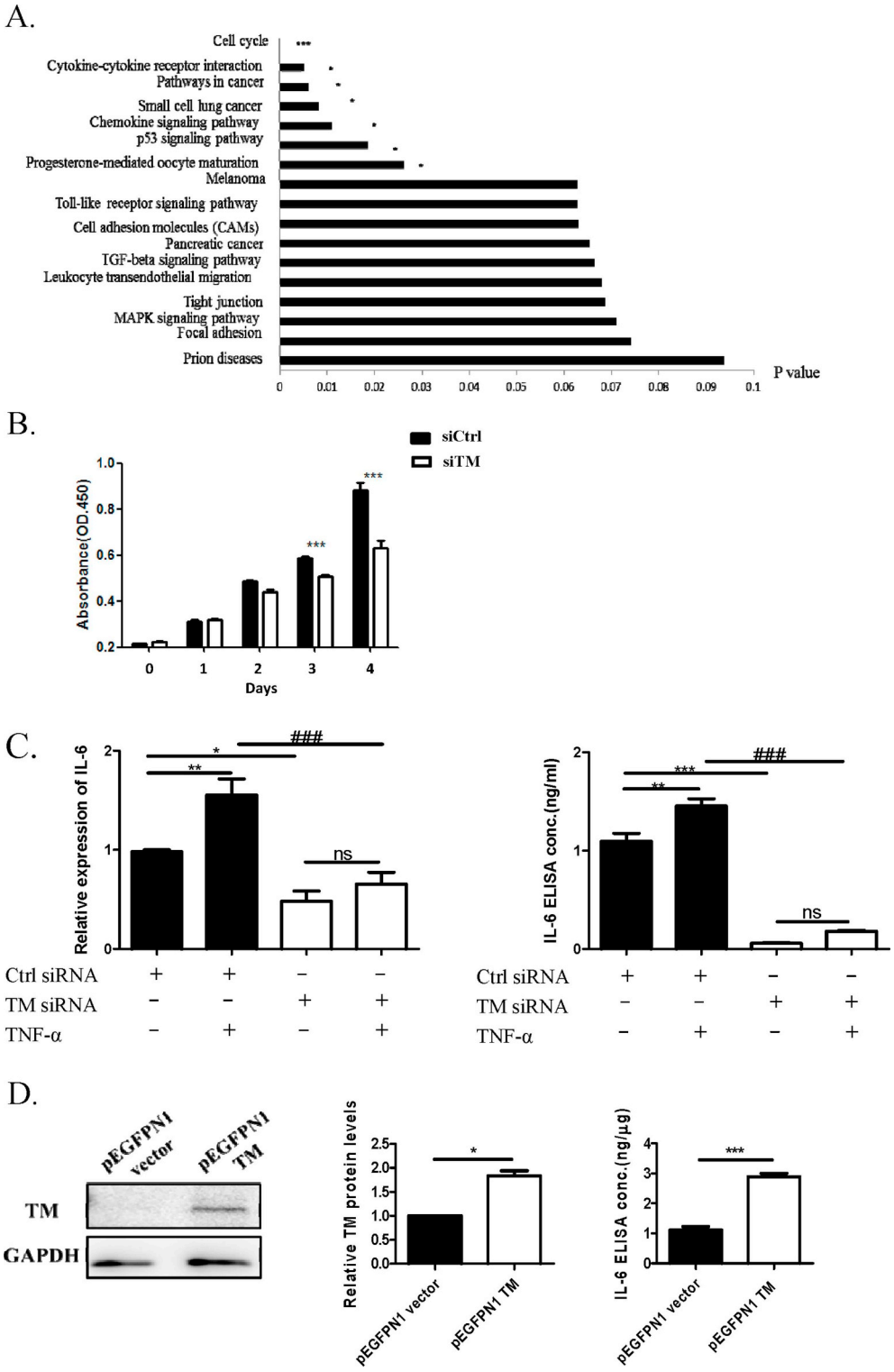

Fig. 2. Functional changes of aortic SMCs after TM knockdown.
(A) Microarray results showed significant changes in cell cycle and cytokine-cytokine receptor interaction pathway in aortic SMCs with TM knockdown (siTM). *p < 0.05, ***p < 0.001 compared with controls (siCtrl). (B) Cell proliferation was measured by WST-1 cell proliferation assays. The number of cells was expressed in units of optical density (OD450). All experiments were performed at least 3 times. ***p < 0.001. (C) *IL*-6 mRNA expression levels in aortic SMCs and IL-6 concentrations in conditioned media were measured. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ###p < 0.001, n.s. indicates not significant (n = 3). (D) Expression and quantification of TM protein in pEGFPN1 vector and pEGFPN1-TM transfected aortic SMCs with TM knockdown (n = 3). IL-6 concentrations in the conditioned media from TNF-α-treated pEGFPN1 vector and TNF-α-treated pEGFPN1-TM transfected cells were determined by ELISA. Data are expressed as mean ± SEM. *p < 0.05, ***p < 0.001. GAPDH was used as a loading control. Results were normalized to GAPDH and expressed as mean ± SEM.

2.10. Neointima severity evaluation

Carotid artery was excised and 5 transverse sections (5 μm thick) of each carotid artery were cut at 100 μm intervals. After staining with hematoxylin-eosin, the borders of the internal lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were traced on a digitizing board with Meta Imaging Series 5.0 (Adobe Inc). The luminal, IEL, and EEL areas were measured, and the neointima area was calculated by subtracting the luminal area from the IEL area. The media area was calculated by subtracting the IEL area from the EEL area, and the ratio of neointima to media area (N/M ratio) was calculated. The areas of the 5 sections were analyzed and averaged.

2.11. Staining of carotid artery

For immunofluorescence staining, carotid arteries isolated from the mice were embedded in optimum cutting temperature compound. Frozen sections (5 μm thick) were fixed and blocked with 5% goat serum. The sections were then stained with antibodies for TM (clone 1009, 1:150, Dako, Glostrup, Denmark), α-SMA (1:200), or Ki-67 (1:200, all from Abcam), followed by incubation with FITC-conjugated rabbit anti-mouse IgG (1:50, Millipore, Darmstadt, Germany) as the secondary antibody. DAPI (Sigma) was used to identify the nuclei.

2.12. Statistical analysis

Data were presented as mean ± standard error. Comparisons between 2 groups were made using the Mann-Whitney test. For multiple comparisons of groups, one-way ANOVA was used, followed by Bonferroni post hoc analysis. All statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA). A p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Changes in aortic SMCs after TM knockdown

As expected, cultured human aortic SMCs expressed TM as shown by Western blot of the cell lysates and ELISA of the cultured media (Fig. 1A). Most of the cultured aortic SMCs displayed a rhomboid-shaped morphology (Fig. 1B). When the cells were transfected with TM siRNA, they redifferentiated back into a spindle-shaped morphology (Fig. 1B). The expression of contractile phenotype marker, calponin, increased, while those of the synthetic phenotype markers, vimentin and NM-MHC, decreased after TM knockdown in the aortic SMCs (Fig. 1C). In cells without TM knockdown, PDGF-BB treatment decreased the expression of calponin and increased vimentin. In addition, an increased expression of calponin and decreased expression of

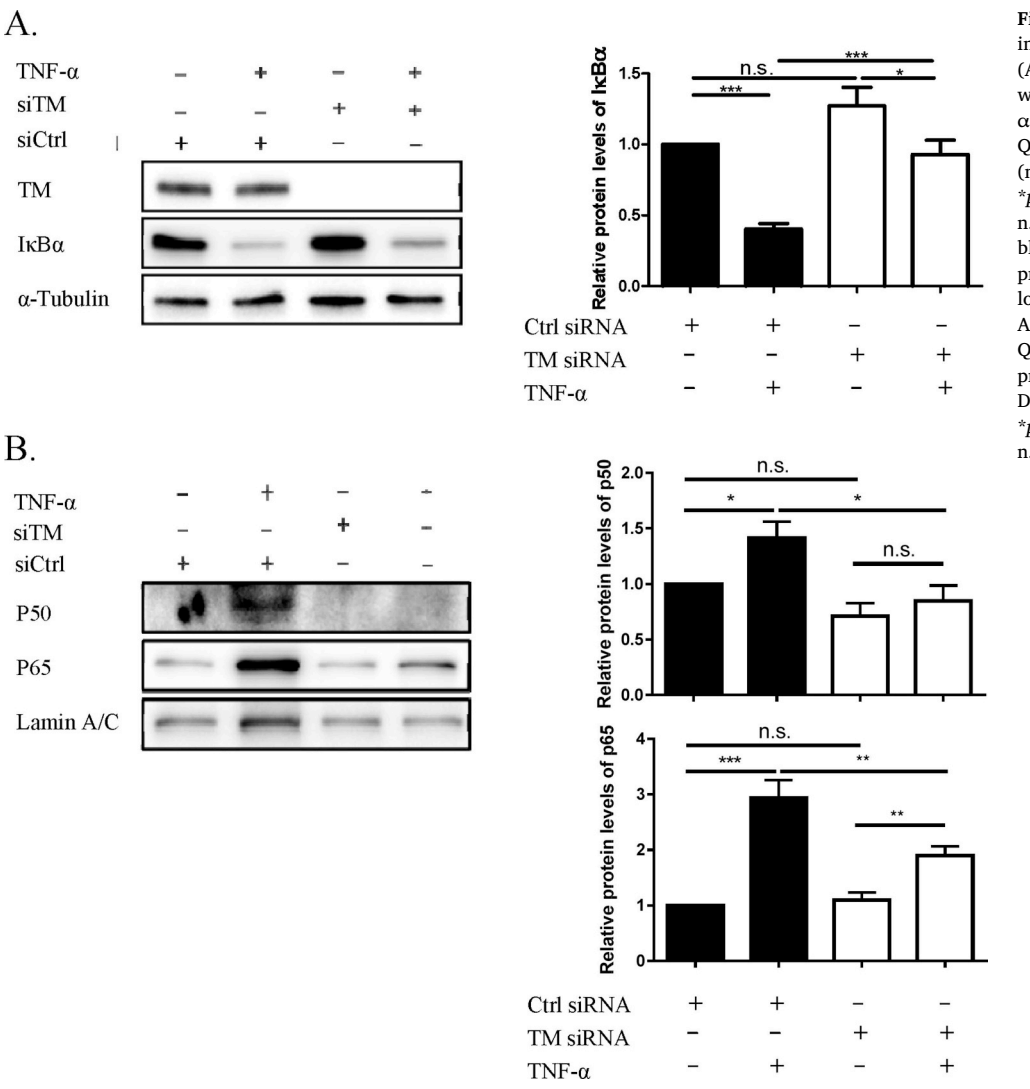

Fig. 3. TM knockdown attenuated TNF-α-induced NFκB activation in aortic SMCs. (A) Cell lysates with the indicated treatment were analyzed for TM and IκBα expressions. α-tubulin was used as a loading control. Quantification of IκBα protein expression (n = 3) was shown as the bar graph. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n.s. indicates not significant. (B) Western blot analysis of p50 and p65 protein expressions. Equality of nuclear sample loading was confirmed with control lamin A/C, a nuclear protein marker. Quantification of p50 and p65 protein expressions (n = 3) is shown as the bar graph. Data are expressed as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n.s., not significant.

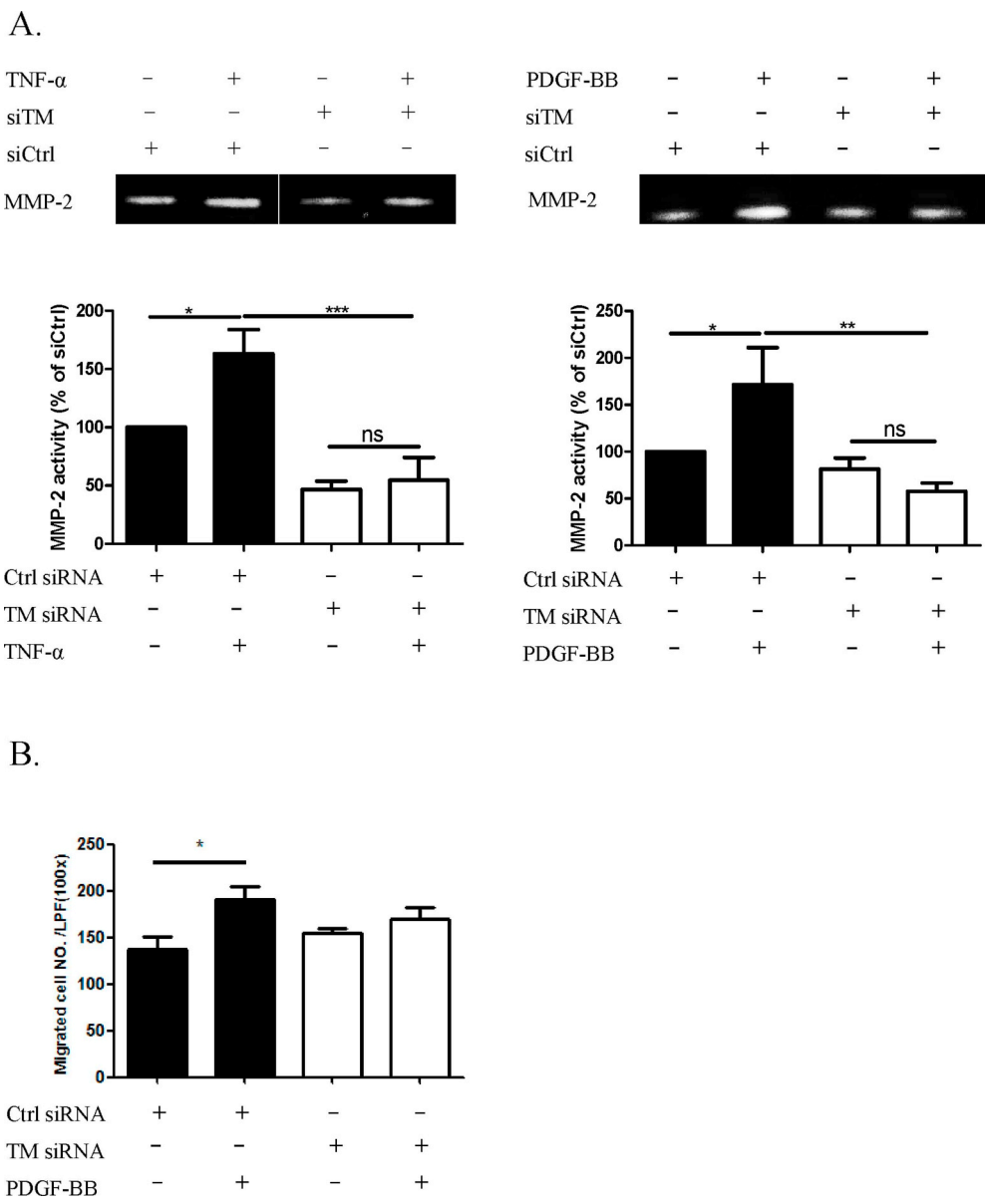

Fig. 4. Aortic SMC migration after TM knockdown.
(A) Aortic SMCs received the indicated treatment and MMP-2 activity was analyzed by zymography. Data are expressed as mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s., not significant (n = 3). (B) Migration of aortic SMCs with the indicated treatment was evaluated by Boyden chamber assay. The cells were allowed to migrate for 6 h in response to medium with or without PDGF (20 ng/mL) in the lower chamber. The number of migrated cells was counted in 5 random fields for each well. Data are shown as mean ± SEM; n = 3. $*p < 0.05$.

vimentin were noted in the TM knockdown cells with or without PDGF-BB treatment compared to the cells without TM knockdown (Fig. 1D).

3.2. Aortic SMC behaviors

Microarray analysis demonstrated that the most prominent effects of TM knockdown on the aortic SMCs were changes in gene expressions related to proliferation (cell cycle) and inflammation (cytokine-cytokine receptor interaction) compared to the non-targeting siRNA controls (Fig. 2A). Proliferation assays demonstrated that the aortic SMCs with TM knockdown had lower proliferation ability than the cells without TM knockdown (Fig. 2B). We then evaluated the cytokine-producing ability of the aortic SMCs. Treatment with tumor necrosis factor (TNF)-α induced greater IL-6 mRNA expression and IL-6 protein production in the aortic SMCs, whereas the IL-6 producing ability was significantly reduced in the aortic SMCs with TM knockdown (Fig. 2C). In contrast, the forced expression of TM in the aortic SMCs with TM knockdown increased IL-6 production after TNF-α stimulation (Fig. 2D). These results suggested that membrane-bound TM might be responsible, at least in part, for TNF-α-mediated inflammation in aortic SMCs.

Since NF-κB transcription factor is the most important signal integration step in vascular inflammation, we further investigated the influence of TM knockdown on NF-κB activation in the aortic SMCs. Exposure of the human aortic SMCs to TNF-α significantly decreased the expression of NF-kB inhibitor, IκBα, and TM knockdown augmented the expression of IkBα (Fig. 3A). Treating the aortic SMCs with TNF-α caused significant increases in p50 and p65 protein levels in the nuclear

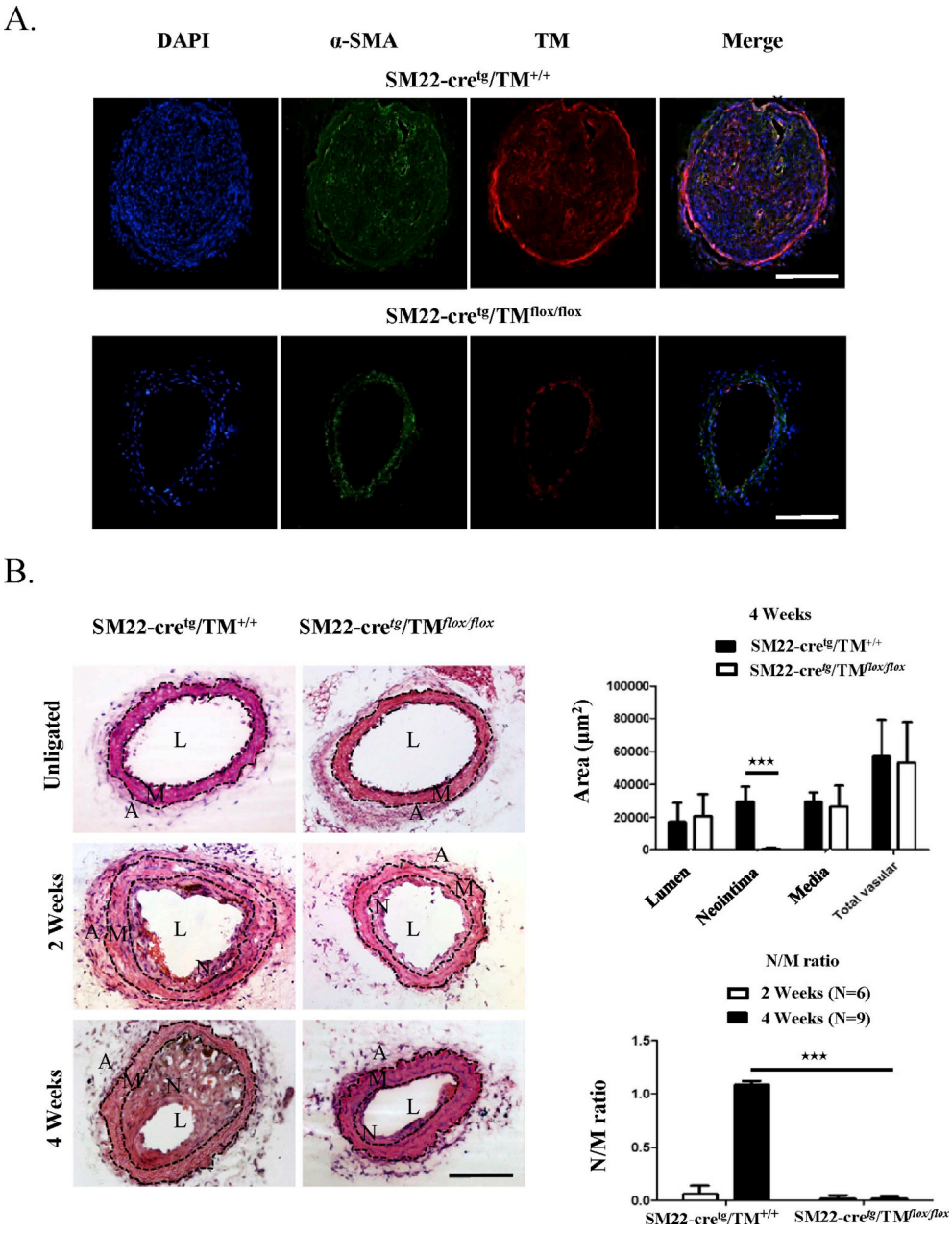

Fig. 5. Neointima formation in mice after carotid artery ligation.
(A) Double immunofluorescent staining for α-SMA and TM in the ligated carotid arteries. Nuclei were counterstained with DAPI. TM expression was detected in the media and neointima in SM22-cre^{tg}/TM$^{+/+}$ mice, but only minimal TM staining was observed in the endothelium of the ligated carotid arteries of SM22-cre^{tg}/TM$^{flox/flox}$ mice. Scale bar, 100 μm. (B) Hematoxylin–eosin staining of carotid arteries after carotid ligation. The dashed lines indicate borders of neointima/media and media/adventitia. A, adventitia; L, lumen; M, media. Scale bar, 100 μm. Quantification of the neointima was calculated and shown as the bar graph. M, media; N, neointima. ***$p < 0.001$. (C) Double immunofluorescent staining for α-SMA and Ki67 (arrows) in the carotid arteries after ligation. The dashed lines indicate borders of the neointima and media. Scale bar, 50 μm. M, media; N, neointima. The ratio of Ki positive cells to total cells and the absolute number of Ki67 positive cells was calculated and shown as the bar graphs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (D) Immunohistochemical staining of α-SMA in the carotid arteries at 2 weeks after carotid ligation. Scale bar, 100 μm. The α-SMA positive staining intensity was quantitated and shown as the bar graph. **$p < 0.01$. ***$p < 0.001$.

fraction of the aortic SMCs without TM knockdown, whereas TM knockdown decreased the levels of p50 and p65 (Fig. 3B) in the aortic SMCs.

Although microarray analysis did not show significant changes in migration-related genes in the aortic SMCs with TM knockdown, we still examined the influence of TM expression on aortic SMCs migration. TNF-α or PDGF-BB stimulation increased MMP-2 activity in the aortic SMCs, and TM knockdown significantly decreased MMP-2 producing activity of the aortic SMCs (Fig. 4A). The Boyden chamber assay showed that aortic SMC migration in response to PDGF-BB stimulation was slightly attenuated in cells with TM knockdown (Fig. 4B).

3.3. Vascular SMC-specific TM-deletion mice

Transgenic mice with TM specifically deleted in vascular SMCs (SM22-cre^{tg}/TM$^{flox/flox}$ mice) and their wild-type controls (SM22-cre^{tg}/TM$^{+/+}$ mice) were generated. Immunoblotting confirmed that TM was eliminated from vascular SMCs isolated from the SM22-cre^{tg}/TM$^{flox/flox}$ mice (Supplemental Fig. 1B). The blood pressure and body weight were similar between the SM22-cre^{tg}/TM$^{flox/flox}$ mice and their wild-type

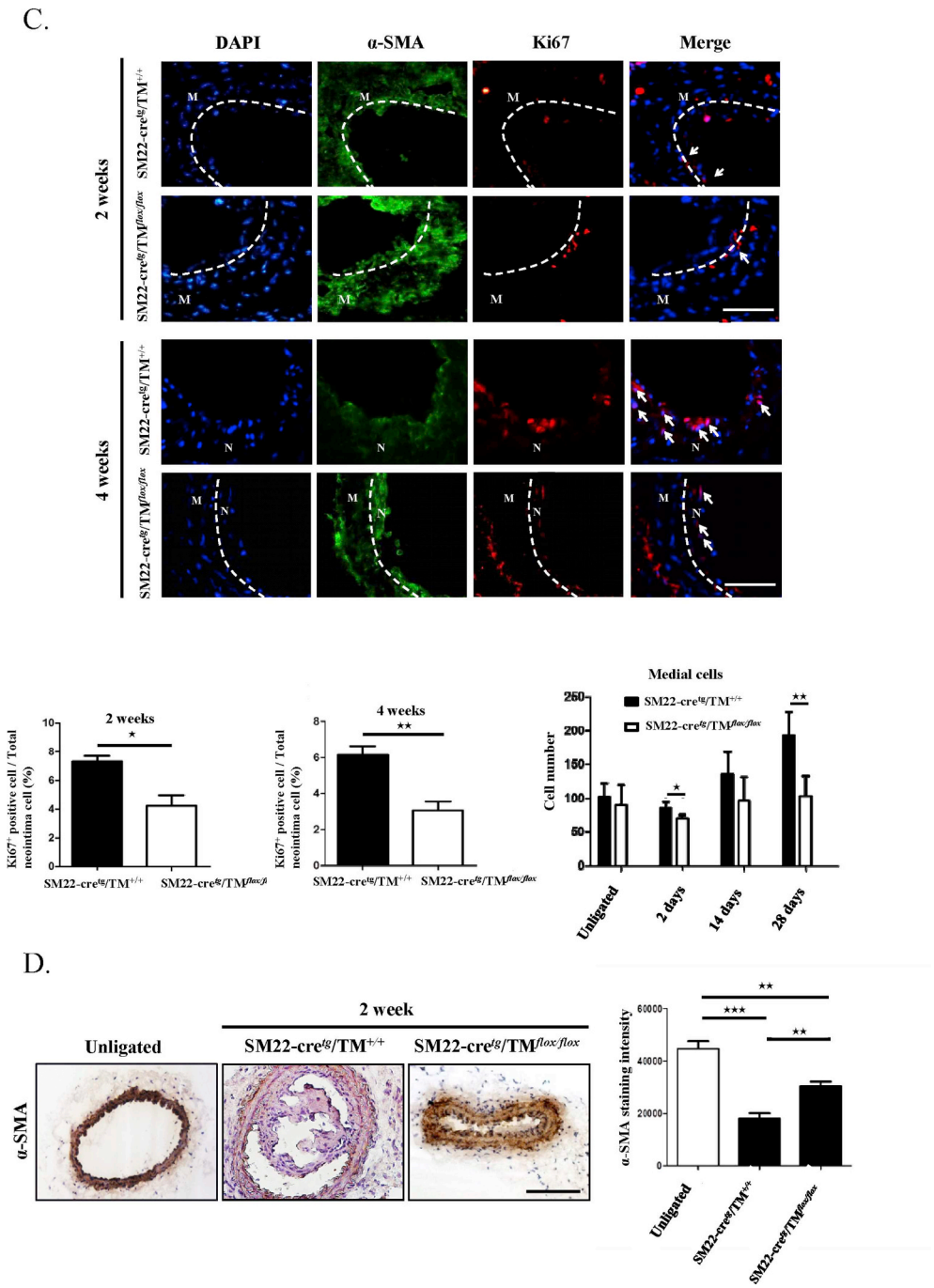

Fig. 5. (*continued*)

controls (Supplemental Figs. 1C and D). Carotid ligation caused neointima formation in the mice. Immunofluorescence staining showed high TM expression in the medial and neointimal cells at 4 weeks in the SM22-cre^{tg}/TM$^{+/+}$ mice after carotid ligation, whereas no TM staining was found in the SM22-cre^{tg}/TM$^{flox/flox}$ mice (Fig. 5A). There was a progressive increase in the neointima area from 2 to 4 weeks after carotid ligation in both the SM22-cre^{tg}/TM$^{flox/flox}$ and SM22-cre^{tg}/TM$^{+/+}$ mice (Fig. 5B). The neointima area and neointima/media area ratio were significantly smaller in the SM22-cre^{tg}/TM$^{flox/flox}$ mice than in the SM22-cre^{tg}/TM$^{+/+}$ mice (Fig. 5B). Immunofluorescence staining showed fewer Ki67-positive cells in the media and neointima in the SM22-cre^{tg}/TM$^{flox/flox}$ mice compared to the SM22-cre^{tg}/TM$^{+/+}$ mice (Fig. 5C). The α-SMA positive staining intensity was also stronger in the SM22-cre^{tg}/TM$^{flox/flox}$ mice (Fig. 5D), suggesting that TM deficiency in the vascular SMCs in medial lesions resulted in a more contractile status after carotid ligation.

4. Discussion

In this study, we found that aortic SMC membrane-bound TM played a role in SMC phenotype and changes in behavior. TM affects cell behavior differently in different cell types. In epidermal epithelial A431 cells, cells expressing TM were shown to maintain compact cell colonies with an epithelial morphology, whereas TM knockdown induced the formation of cell protrusions at the edges of the colonies and increased cell migration [23]. TM expressed on monocyte works as an adhesion molecule and mediates monocytes adhesion to vascular endothelium [24]. With regards to SMCs, Tohda et al. demonstrated that

exogenous recombinant TM protein treatment significantly increased the proliferation of rat vascular SMCs [10]. However, the effects of endogenous expression of TM on vascular SMCs have rarely been studied. Several studies have demonstrated that cultured vascular SMCs express TM, and this was associated with the down-regulation of contractile phenotype markers such as α-actin and tropomyosin [25,26]. In addition, Ramachandran et al. found that knockdown of the endogenous expression of TM in cultured human urinary bladder SMCs, a visceral SMC, did not affect bladder SMC growth but significantly attenuated PDGF-induced bladder SMC migration [27]. In the current study, we demonstrated that the TM expression on aortic SMCs was mainly associated with proliferative and proinflammatory phenotype transition, and also mildly increased aortic SMC migration. SMCs have extensive functional diversity depending on the specific demand within a given organ. Phasic and rhythmic contraction is the characteristic of visceral SMCs, while vascular SMCs in arteries usually maintain continuous contraction to preserve vascular tone [28]. The mechanisms underlying the different effects of TM expression on different cell types, including vascular and visceral SMCs, are unclear and need further exploration.

Our *in vivo* experiments showed that neointima formation was more severe in the wild-type mice with TM expression compared to vascular SMC-specific TM-deficient mice. Previous studies have shown that systemic injections of exogenous recombinant TM protein or local incubation of recombinant adenoviral constructs overexpressing TM within injured arterial lumen decreased vascular injury-induced neointima formation [9,29,30]. The anti-thrombotic and anti-inflammatory effects of recombinant TM protein on the endothelium are the major mechanisms underlying the anti-atherosclerosis effect of TM. Recombinant TM has been shown to decrease thrombin-induced PAR-1 activation and inflammation on the endothelium after vascular injury [31]. Recent studies have further demonstrated that recombinant TM protein has a thrombin-independent effect on the endothelium and can directly decrease the stress-induced apoptosis of endothelial cells [32,33] and reduce leukocyte transmigration to the endothelium [34]. The accumulation of vascular SMCs in the subintima is a major feature of atherosclerosis and neointima. Co-culturing SMCs with endothelial cells has been shown to cause a shift in SMCs to the synthetic phenotype and induced the expressions of inflammatory genes in endothelial cells [35]. Although recombinant TM has beneficial effects on the endothelium, the potential impact of vascular SMC-bound TM on endothelial cells is unknown. Monocyte-expressed TM enhances adhesion of monocytes to endothelium and myeloid-specific TM-deficient mice had less neointima formation after carotid ligation [24]. The interplay between TM expression on monocytes, vascular SMCs and endothelial cells is unclear. The effects of TM on individual vascular cell types are different and further investigations are needed to elucidate its role in cross talk between different cell types during atherosclerotic process. Another limitation of our study is the specificity of SM22 as a SMC-specific deletion Cre. Although commonly used to study smooth muscle specific expression, it is not the most specific marker and could be expressed in non-muscular cells [36]. This could influence the experimental results of our study.

In conclusion, although previous studies have documented the expression of TM on neointimal SMCs and hypothesized its effect in protecting injured arteries from thrombosis, the present study showed that vascular SMC-bound TM had an important physiological effect on vascular SMC biology beyond its traditional anti-thrombotic effect.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Wang KC, Chung HC, Tseng SY, Huang TY, and Lin YL performed the experiments and collected the data. Wang KC, Chen PS, Chao TH, and Li YH designed the study and analyzed the data. Li YH and Wang KC drafted the manuscript. Luo CY, Shi GY, and Wu HL revised the manuscript for important intellectual content.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.05.019.

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