FGFR1 mediates recombinant thrombomodulin domain-induced angiogenesis

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Aims

The recombinant epidermal growth factor-like domain plus the serine/threonine-rich domain of thrombomodulin (rTMD23) promotes angiogenesis and accelerates the generation of activated protein C (APC), which facilitates angiogenesis. The aim of this study was to elucidate the molecular mechanisms underlying the angiogenic activity of rTMD23.

Methods and results

We prepared rTMD23 and its mutants that did not possess the ability to promote APC generation and investigated their angiogenic activities in vitro and in vivo. rTMD23 mutants promoted proliferation, migration, and tube formation of human umbilical vein endothelial cells in vitro and induced neovascularization in vivo; these effects were similar to those exerted by wild-type rTMD23. To investigate its interaction with rTMD23, Type I fibroblast growth factor receptor (FGFR1) was precipitated along with syndecan-4 by rTMD23-conjugated Sepharose in human umbilical vein endothelial cells and FGFR1-expressing human embryonic kidney 293 cells. Additionally, the kinetics of the interaction between rTMD23 and FGFR1 were analysed using surface plasmon resonance. rTMD23-induced FGFR1 activation and tube formation were inhibited by an FGFR1-specific tyrosine kinase inhibitor, PD173074, or by knockdown of FGFR1 using siRNA technology. We observed an improvement in rat hindlimb recovery in an ischaemic model following rTMD23 treatment, and this was associated with increased neovascularization and FGFR1 phosphorylation.

Conclusion

rTMD23 induced angiogenesis via FGFR1, a process that is independent of the APC pathway.

Keywords

Thrombomodulin • Type I fibroblast growth factor receptor • Activated protein C • Angiogenesis • Syndecan-4

1. Introduction

The generation of vascular systems mediated by vasculogenesis, angiogenesis, and arteriogenesis is important for normal development and tissue repair. Angiogenesis is the formation of new capillaries from pre-existing capillaries. This process involves several cellular reactions, including the angiogenic switch; endothelial cell activation, proliferation, and migration; and vascular tube formation. Angiogenesis is controlled by angiogenic promoters and inhibitors directly produced by different types of cells or generated from proteolytic modification of precursors, such as heparin-binding epidermal growth factor (EGF) and angiostatin. Several mechanisms underlying the regulation of angiogenesis have been proposed and demonstrated; however, the need to more accurately determine these mechanisms remains.

The Type I fibroblast growth factor receptor (FGFR1) belongs to the FGFR family, which participates in a plethora of biological functions during embryo development and adult tissue homeostasis. FGFR1 interacts with several molecules, including fibroblast growth factors (FGFs), heparan sulfate proteoglycans (HSPGs), heparin, and neural cell adhesion molecules. Heparin can reconstitute a low affinity receptor for basic FGF (bFGF) and it is required for the binding of bFGF to its high-affinity receptor, FGFR, especially syndecan-4, function as a reservoir of FGFs to facilitate the interaction between bFGF and FGFR1, thereby regulating the activation and signalling of FGFR1. At least 22 structurally related FGF members and 4 conserved high-affinity tyrosine kinase receptors, identified in humans and mice, are implicated in governing a wide range of biological functions. Because of its pleiotropic actions on a variety of cell types involved in neovascularization,
a complicated FGF/FGF system that regulates angiogenesis has been proposed and positioned upstream of other growth factor systems.  

Thus, several pharmaceutical inhibitors of FGF have been developed for treating cancer. Among those, the compound PD173074, when administered in a nanomolar range, inhibits FGFR1 signalling, and thereby suppresses the proliferation, migration, and differentiation of oligodendrocyte lineage cells, as well as angiogenesis.

Thrombomodulin (TM) is a membrane glycoprotein that forms a complex with thrombin. The TM–thrombin complex activates protein C and triggers an anticoagulation cascade. Activated protein C (APC) possesses several cellular functions besides anticoagulation, including anti-inflammation and angiogenesis. TM is believed to possess functions besides anticoagulation due to its extracellular expression and the association between soluble TM and vascular diseases. Indeed, novel functions of TM have been revealed in recent studies. For example, TM participates in cell-to-cell adhesion, and regulates inflammation as well as angiogenesis. The lectin-like domain of TM inhibits inflammation through interactions with the Lewis Y antigen. The recombinant EGF-like domain of TM promotes angiogenesis and the association between soluble TM and vascular diseases. For example, TM participates in cell-to-cell adhesion, and regulates inflammation as well as angiogenesis. Further, in TM, the recombinant EGF-like domain plus the serine/threonine-rich domain (together known as rTMD23) augments therapeutic angiogenesis by enhancing the angiogenic potential of human early endothelial progenitor cells (EPCs) through the activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Additionally, soluble TM that contains the EGF-like domain acts as a paracrine anti-apoptotic factor that exerts a cytoprotective effect on the vascular endothelium during stress-induced endothelial damage. However, the receptor for rTMD23 in angiogenesis has yet to be identified. In this study, we generated several rTMD23 proteins lacking the ability to facilitate APC formation and analysed their angiogenic activity. Our results demonstrated the FGFR1-dependent mechanism underlying rTMD23-induced angiogenesis.

2. Methods

2.1 Expression and purification of various rTMD23 proteins

To investigate whether APC is required for the angiogenic activity of rTMD23, we prepared rTMD23 mutants that lack protein C activation activity, including rTMD23E357A, rTMD23R385S, and rTMD23D400A, using previously reported methods and the QuickChange Site-Directed Mutagenesis kit (Stratagene) with paired primers (see Supplementary material online, Table S1). The recombinant proteins, which have a C-terminal 6-His tag and c-Myc epitope, were expressed in a suppression system (Invitrogen) and purified with a nickel-chelating Sepharose Lewis Y antigen. The recombinant EGF-like domain of TM promotes angiogenesis and the association between soluble TM and vascular diseases.

2.2 Protein C activity assay

The thrombin-dependent activation of protein C activity in various rTMD23 proteins was measured as previously described.

2.3 Cell culture and FGFR1 inhibition

Human umbilical vein endothelial cells (HUVECs; Invitrogen) from the second to fourth passage were used in all experiments. HUVECs were grown in M199 medium (M199) supplemented with 16% foetal bovine serum (FBS) and endothelial cell growth supplement (MilliPore). Human embryonic kidney 293 (HEK293) cells were grown in DMEM (D5648, Sigma-Aldrich) supplemented with 10% FBS. The starvation of HUVECs was performed by incubating cells in M199 containing 1% FBS for 12 h or serum-free M199 for 2–4 h. To investigate the effect of FGFR1 activation in rTMD23-induced angiogenic activity, we used a specific inhibitor to FGFR1: the compound PD173074 (Calbiochem). FGFR1-knockdown HUVECs were used to study the specificity of FGFR1 in rTMD23-induced angiogenic activity in vitro. The FGFR1-specific siRNA (FGFR1 siGenome SMARTpool) and its negative control (siGenome Non-Targeting siRNA pool 2) were purchased from Dharmacon. The HUVECs were transfected with siRNA using DharmaFECT1 Transfection Reagent (Fisher Scientific) 1 day before performance of in vitro angiogenic assays.

2.4 Cell proliferation assay

To evaluate the effect of rTMD23 proteins on DNA synthesis in HUVECs, 5-bromo-2′-deoxyuridine (BrdU) incorporation was measured using a labelling kit (Roche) according to the manufacturer’s protocol.

2.5 Cell migration assay

The cell migration assay was performed using a 48-multiiwell Boyden chamber (Neuro Probe) with 8 µm pore-size polycarbonate filters (Neuro Probe) coated with 0.1% gelatin (Sigma-Aldrich). We placed 50 µL of cell solution (4 × 10^5 cells/mL) in the upper well, and used rTMD23 proteins placed in the bottom well as chemoattractants. Either recombinant EGF or bFGF (R&D Systems) was used as a positive control. The chamber was incubated for 4 h at 37 °C and 5% CO2. The migrated cells were enumerated following staining with Liu’s stain.

2.6 Endothelial cell tube formation assay

An in vitro tube formation assay was performed using growth factor-reduced Matrigel (BD Biosciences). We used 200 µL (2.5 × 10^4 cells) of HUVECs in M199 containing 1% FBS as the negative control, and EGF or bFGF as the positive control. Various rTMD23 proteins were examined in the presence or absence of PD173074. We used the MetaMorph software (Leica) to measure tube length 6 h after incubation.

2.7 rTMD23-Sepharose pull-down assay

rTMD23 or BSA was immobilized in cyanogen bromide (CNBr)-activated Sepharose 4B (Amersham Pharmacia Biotech AB) according to the manufacturer’s protocol. HEK293 cells were transfected with FGFR1 expression plasmid (MHS1010-7429517, Thermo Scientific Open Biosystems) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The HUVECs or FGFR1-expressing HEK293 cells that had been starved with serum-free medium for 24 h were lysed with a lysis buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM CaCl2, ethylenediaminetetraacetic acid-free protease inhibitor cocktail (Roche Diagnostic GmbH), and 1% NP-40. We incubated 200 µg of cell lysate with immobilized rTMD23- or BSA-Sepharose at 4 °C for 16 h with rotation. Subsequently, Sepharose beads were washed three times with lysis buffer without a protease inhibitor cocktail, and then analysed by western blot with antibodies against FGFR1 (sc-121, Santa Cruz Biotechnology) and syndecan-4 (sc-15350, Santa Cruz Biotechnology).

2.8 SPR assay

The binding parameters for the interaction between rTMD23 and FGFR1 were measured using a BIACore 3000 SPR-based biosensor system. The chip was activated with 1-ethyl-3-(3-dimethyl aminopropyl) carbodimide/N-hydroxysuccinimide and then blocked with ethanolamine. Using the
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amine coupling protocol, the recombinant human FGFR1 (FGFR1-316H, Creative BioMart) was immobilized on a CMS chip (Biacore, GE Healthcare). Various concentrations of rTMD23 in PBS containing 0.05% Tween-20 were passed through the flow cells at a flow rate of 20 μL/min. Kinetic analysis was performed, and the association and dissociation periods were each 180 s. All data were corrected for the response obtained by using a blank reference flow cell. Binding data were evaluated using the BIACore assessment program version 4.1 (Biacore), and the binding activity of rTMD23 and tag-deleted rTMD23 was compared.

2.9 FGFR1 phosphorylation assay
To analyse the activation of FGFR1 by rTMD23, serum-deprived HUVECs or FGFR1-transfected HEK293 cells were stimulated with various rTMD23 and bFGF for 30 or 60 s. Cells were lysed with a lysis buffer containing 1% NP-40, protease inhibitor cocktail (Roche Diagnostic GmbH), 2 mM Na3VO4, 10 mM NaF, 137 mM NaCl, and 20 mM Tris–HCl (pH 7.4). The cell lysates were analysed by western blot with antibodies against phosphorylated FGFR1 tyrosine residue 653/654 (sc-30262, Santa Cruz Biotechnology), FGFR1 (sc-121, Santa Cruz Biotechnology), and α-tubulin (Abcam).

2.10 Corneal angiogenesis assay
Uniformly sized pellets were made from the slow release polymer Hydron (polyhydroxyethylmethacrylate; Sigma), with sucralfate (Sigma) as a stabilizer, as previously described. Using an aseptic technique, we performed an intrastromal keratotomy on the corneas of 8- to 10-week-old BALB/c mice. The mice were anaesthetized by an intraperitoneal injection of tiletamine/zolazepam 50 mg/kg (Zoletil, Virbac), and the eye was topically anaesthetized with 0.5% proparacaine ophthalmic solution (Alcon). With the aid of a dissecting microscope, we used a Slit Knife (BD Beaver) to make a micropocket in the mouse cornea into which a pellet could be inserted. Smooth fine forceps were used to guide the pellet, which was placed 1 mm from the limbal vascular plexus, into the pocket. To prevent infection after surgical operation, we immediately applied gentamicin ophthalmic ointment (ALCON CU5). Pellets contained either 100 ng bFGF or 200 ng various rTMD23 proteins. We measured the maximal vessel length extending from the limbal vessel to the pellet, and estimated a circumferential zone of neovascularization in clock hours. Neovascularization was observed 7 days after implantation under a stereomicroscope. The angiogenic index was calculated using the following formula (as previously described):

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\text{Vessel area} = 0.02 \times \pi \times x \times \text{vessel length}
\]

The mice were sacrificed by inhalation of CO2. The eye globes were enucleated, fixed in acetone for 30 min, and then washed in PBS. The cornea was removed and stained with FITC-conjugated antibody against CD31 (Abcam).

2.11 Rat hindlimb ischaemic model
The Sprague–Dawley rats (225–275 g) were anaesthetized by an intraperitoneal injection of tiletamine/zolazepam 50 mg/kg (Zoletil, Virbac) and xylazine 0.2 mL/kg (Rompun, Bayer) before surgery. During and after the surgery, and until awaking, an assistant monitored respiration rate, muscle relaxation, and different reflexes to ensure that anaesthesia and analgesia were adequate. The ischaemic surgical procedures were performed as previously described with a few modifications that are detailed in Supplementary material online. A total of 40 rats were divided into four groups as follows: (i) Sham group, which included skin incision only without artery ligation and treatment; (ii) Ischaemia group, in which subjects underwent operations that constituted all surgical procedures without treatment injection; (iii) PBS group, in which subjects underwent ischaemic operations, before 200 μL of PBS was injected intramuscularly; and (iv) rTMD23 group, in which subjects underwent ischaemic operations and received an intramuscular injection of 125 μg/kg rTMD23. All treatments immediately followed ischaemia, and multiple injections containing treatment agents or a placebo were injected into the ischaemic main adductor muscle using a 30-gauge needle. To isolate muscle samples, rats were euthanized using CO2 inhalation.

2.12 Clinical score and micro-lightguide spectrophotometer (O2C) perfusion analysis
Treatment effectiveness was evaluated by gross examination and measuring blood flow to distal thigh muscles on Day 28 after treatment. Limb condition was categorized into several levels on Day 28. The micro-lightguide spectrophotometer O2C (Oxygen to See; LEA Medizintechnik) was used to analyse regional microvascular blood flow, which was recorded from both limbs. The overlying skin was excised to expose the muscle surface for perfusion measurements. The data for limb condition were presented as the ratio of the ischaemic limb (right) to the normal limb (left).
2.13 Histological analysis
The adductor magnus muscles were obtained from the ischaemic leg and processed for histological evaluation (described in detail in Supplementary material online). The capillary was stained with the antibody against von Willebrand Factor (Santa Cruz Biotechnology), and we measured capillary density from at least 15 files (original magnification of × 20) containing randomly selected ischaemic areas in each sample. The assessor was blinded to the specimen identity.

2.14 Animal care
The animal studies were complied with the guidelines in Guide for the Care and Use of Laboratory Animals (8th edition, National Institutes of Health Publication, USA). The Institutional Animal Care and Use Committee of the National Cheng Kung University (Tainan, Taiwan) approved animal care conditions and design of experiments.

2.15 Statistical analyses
Data are mean ± S.E.M. Comparisons between > 2 groups were made by one-way analysis of variance followed by a Bonferroni’s multiple comparison test, and comparisons between groups with two variables were made using two-way analysis of variance. P-values of < 0.05 were considered statistically significant.

3. Results
3.1 Expression, purification, and characterization of rTMD23 proteins
The purified rTMD23 (Ala224 through Ser497) and the three rTMD23 mutants lacking the ability to activate protein C (rTMD23E357A, rTMD23R385S, and rTMD23D400A) showed a single band of 63 kDa in SDS–PAGE and in western blot analysis with a mAb against the c-Myc epitope (Figure 1A). Consistent with previous studies showing a decreased cofactor activity of TM caused by missense mutations in the TM gene, whereas wild-type rTMD23 did as expected, none of the rTMD23 mutants possessed the ability to enhance protein C activation in the presence of thrombin (Figure 1B). The analyses from mass spectrometry and NH2-terminal sequencing showed that all rTMD23

Figure 2 Various rTMD23 proteins promote HUVEC proliferation, migration, and tube formation in vitro. HUVECs were treated with equal amounts of rTMD23 variants. (A) After 24 h of incubation, cell proliferation was measured by a BrdU incorporation assay. (B) Cell chemotaxis was analysed by a Boyden chamber migration assay. M199 with 1% FBS was used as a control. After 4 h of migration towards rTMD23 variants, migrated cells were enumerated. (C) Representative images of tube structures in Matrigel after 6 h of incubation with rTMD23 variants are shown. (D) Statistical analysis of the tube length in each group. The data represent mean ± S.E.M. (n = 6). ***P < 0.001 compared with the control.
proteins had the starting sequence at the fusion peptide, Glu-Phe, followed by the expected sequence of rTMD23.

3.2 rTMD23 proteins promote HUVEC proliferation, migration, and tube formation in vitro, and angiogenesis in vivo

The angiogenic activity of rTMD23 mutants was assayed and compared with that of wild-type rTMD23 using in vitro and in vivo systems. rTMD23 mutants exhibited comparable angiogenic activity with wild-type rTMD23 and a positive control, EGF, in promoting DNA synthesis in HUVEC culture (Figure 2A), directional migration (Figure 2B), and HUVEC tube formation in Matrigel (Figure 2C and D). In addition, a corneal micropocket assay in mice was performed to evaluate the angiogenic activity of several rTMD23 proteins. Various rTMD23 proteins exhibited similar angiogenic activity in terms of the time for vessel induction, the maximal length of the induced neovessels, and the area of neovascularization (Figure 3). These results suggested that rTMD23 induced angiogenesis through a pathway independent of APC. Therefore, we investigated its action.

3.3 rTMD23 directly interacts with FGFR1

We proposed that rTMD23 might interact with HSPG system because the angiogenic activity of rTMD23 in vivo is dependent on heparin. Syndecan-4 has been shown to potenti ate the biological activity of bFGF–FGFR1 in endothelial cells. The interaction between HSPGs or FGFR1 and rTMD23 was analysed using an rTMD23-immobilized Sepharose gel and cell lysates from HUVECs or FGFR1-transfected

**Figure 3** Various rTMD23 proteins induce neovascularization in the mouse cornea. A micropocket in the cornea was surgically generated and implanted with a pellet containing 100 ng bFGF or 200 ng various rTMD23 proteins. (A) Corneal photographs were taken 7 days after implantation. The corneal whole mount staining of CD31 was performed with FITC-conjugated antibody against CD31. Representative images are shown. (B) Statistical analysis of corneal neovascularization. The neovascularization area was calculated using the following formula: vessel area = 0.02π × h × vessel length. The data represent mean ± S.E.M. The number in the open bar indicates the number of corneas of each group. ***p < 0.001 compared with the vehicle control, PBS.
HEK293 cells that express FGFR1 and syndecan-4. Syndecan-4 and FGFR1 were co-precipitated from the lysate of HUVECs (Figure 4A) or FGFR1-transfected HEK293 cells by immobilized rTMD23 (Figure 4B). In addition, the interaction kinetics between rTMD23 and FGFR1 was investigated using an SPR assay. The tag-deleted rTMD23 was used to exclude any possible interaction from the C-terminal tag of rTMD23 in the SPR assay. Since the cell is a dynamic system, we monitored binding kinetics over a range of concentrations (Figure 4C). Based on SPR sensorgram data, we applied rate equations for 1 : 1 kinetics and calculated the association rate ($k_a$) and the dissociation rate ($k_d$). The on and off rates of rTMD23 were $1.86 \times 10^4$ (M$^{-1}$ s$^{-1}$) and $2.8 \times 10^{-2}$ (s$^{-1}$), respectively. The affinity ($K_D$) between rTMD23 and immobilized FGFR1 calculated from the SPR result was 1.51 M. These results suggested that rTMD23 directly interacts with FGFR1 and forms an rTMD23/FGFR1/syndecan-4 complex; thus, it may trigger the activation of FGFR1 in an angiogenic reaction.

### 3.4 Activation of FGFR1 by rTMD23

The autophosphorylation of the tyrosine residues at the 653rd and 654th positions of FGFR1 is the critical step in activating tyrosine kinase activity of FGFR1, and is essential for FGFR1-dependent biological responses. We proposed that rTMD23 could activate FGFR1 because rTMD23 can interact with the complex of syndecan-4 and FGFR1. Indeed, rTMD23 increased the phosphorylation of FGFR1 on the 653rd and 654th tyrosine residues in HUVECs in a dose-dependent manner (Figure 5A), indicating that FGFR1 was activated by adding rTMD23 proteins in a serum-deprived condition. Additionally, we used HEK293 cells that were transiently transfected with FGFR1 to test this hypothesis. After starvation by serum depletion, the FGFR1-transfected HEK293 cells were stimulated with rTMD23 proteins or bFGF. As a result, bFGF, wild-type rTMD23, and rTMD23 mutants induced FGFR1 activation within 1 min of stimulation. Furthermore, the rTMD23-mediated activation of FGFR1 was suppressed by pre-treatment with an FGFR1 kinase inhibitor, PD173074 (Figure 5B and C).

### 3.5 FGFR1 mediates rTMD23-induced angiogenesis in vitro

To test whether the activation of FGFR1 was necessary for rTMD23-induced angiogenesis, we investigated the effect of PD173074 on rTMD23-induced endothelial cell tube formation, using bFGF as the positive control. Results showed that rTMD23 stimulated endothelial cell tube formation in Matrigel in the same way as bFGF (Figure 6A and B). In addition, pre-treatment of HUVECs with PD173074 inhibited both rTMD23- and bFGF-stimulated endothelial cell tube formation (Figure 6A and B). The specificity of FGFR1 in rTMD23-induced angiogenic activity in vitro was further investigated using FGFR1-knockdown HUVECs. FGFR1 was decreased by 50% using siRNA, whereas knockdown of FGFR1 suppressed HUVEC migration (Figure 6C) and tube formation.

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**Figure 4** rTMD23 proteins interact with syndecan-4 and FGFR1. rTMD23 pull-down assay. The pull-down complex was analysed by western blot with antibodies against FGFR1, syndecan-4, and α-tubulin. (A) Pull-down assay in HUVECs. (B) Pull-down assay in FGFR1-expressing HEK293 cells. (C) SPR assay. The recombinant human FGFR1 was immobilized on the CM5 chip. Various concentrations of tag-deleted rTMD23 were passed through the flow cells at a flow rate of 20 µL/min.
formation (Figure 6D) induced by rTMD23 or mutant rTMD23. These results indicated that FGFR1 mediates rTMD23-induced angiogenesis.

### 3.6 rTMD23 improves limb recovery from ischaemia

To further assess the therapeutic potential of rTMD23 and FGFR1 activation by rTMD23 in vivo, we performed hindlimb ischaemia assay in rats. Local administration of rTMD23 immediately after surgery promoted limb salvage after ischaemia (Figure 7A and B). Enhanced blood perfusion of ischaemic limb was observed in the rTMD23-treated group (Figure 7C). We also analysed capillary density and FGFR1 activation in tissue sections and tissue homogenates, respectively. Increased vascular density (Figure 7D) and elevated FGFR1 activation (Figure 7E and F) were consistently observed in the rTMD23-treated group.

### 4. Discussion

TM is found in endothelial cells and acts as an anticoagulant via APC. APC directly activates gelatinase A expression in human endothelial cells, and promotes endothelial cell proliferation in vitro and angiogenesis in vivo. In addition, APC induces the production of angiogenic factors in human skin cells and promotes cutaneous wound healing. Previously, we demonstrated that rTMD23 stimulated similar signalling pathways to APC in the promotion of angiogenesis. In this study, we demonstrated that rTMD23 and its mutants could stimulate HUVEC proliferation, migration, and tube formation, as well as induce angiogenesis in vivo. Although the cornea of a mouse comprises avascular tissue, it is thought that thrombin and protein C should still be present in the system during neovascularization. Therefore, if APC is required for rTMD23-induced neovascularization in vivo, wild-type and mutant
rTMD23 proteins should cause different angiogenic responses in a mouse cornea assay; however, this result was not demonstrated in our study. In addition, with an investigation into the interaction and kinetics between rTMD23 and FGFR1, we demonstrated that rTMD23 induces FGFR1 activation, whereas PD173074 reverses rTMD23-induced FGFR1 activation and tube formation in HUVECs. Furthermore, knockdown of FGFR1 suppressed the HUVEC migration and tube formation induced by rTMD23 and its mutants. These results indicate that rTMD23 can induce angiogenesis independent of the APC pathway, and that FGFR1 mediates this angiogenic activity.

EGF binds to its specific membrane receptor, EGFR, to initiate cellular activities that include proliferation and migration. In previous studies, several molecules containing an EGF-like domain were compared with EGF for regulation of cell proliferation and migration, and different conclusions were presented. For example, laminin, isolated from an Engelbreth–Holm–Swarm tumour, and its domains, which contain EGF-like repeats, stimulate thymidine incorporation in Swiss 3T3 cells, whereas these mitogens do not compete with EGF in binding to the cells. Coagulation factor XII, which contains two EGF-like structures, binds to uPA receptor, and induces angiogenic signalling via EGFR. Conversely, the EGF-like domain of laminin-5 is capable of binding to EGFR, and thereby stimulating MMP-2 expression and cell migration. The recombinant EGF-like domain of TM is a mitogen to several types of cells, including human dermal fibroblasts, A549, U937, HepG2, and Swiss 3T3 cells, but competition with [125I]EGF for cell binding does not occur. Consistent with these reports, our results showed that rTMD23 stimulated DNA synthesis, directional migration, and endothelial tube formation of HUVECs, but it did not induce EGFR activation (data not shown). Instead, rTMD23 proteins activated FGFR1. Taken together, these studies indicate that the EGF-like domain-containing peptides have mitogenic activity similar to EGF; however, they may act via different receptors and thus have distinct molecular mechanisms.

Heparin is composed of highly sulfated GAGs and is used as an anticoagulant. Heparin-Sepharose has successfully been used to purify coagulation factors, DNA-binding proteins, and growth factors. Syndecan-4 is a cell surface HSPG that contributes to the biological

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**Figure 6** FGFR1 mediates rTMD23-induced angiogenesis in vitro. An FGFR1 tyrosine kinase inhibitor, 100 nM PD173074 compound, was used to inhibit the activation of FGFR1. HUVECs were stimulated with 100 ng/mL of rTMD23 or bFGF in Matrigel. An in vitro Matrigel tube formation assay was performed. (A) Representative pictures of a tube structure after 6 h of incubation. (B) Statistical analysis of tube length of each group. The data represent mean ± S.E.M. (n = 6). ***P < 0.001 compared with the control. ###P < 0.001 compared with the group without PD173074 compound. w/o, without. (C) Knockdown of FGFR1 aboliishes rTMD23-induced HUVEC migration. Western blot analysis of FGFR1 expression and statistical analysis of Boyden chamber migration assay. (D) Knockdown of FGFR1 suppresses rTMD23-induced HUVEC tube formation. The data represent mean ± S.E.M. (n = 5 – 6). P < 0.001 compared with the control. ###P < 0.001 and ##P < 0.01 when compared with siRNA-treated one.
Figure 7 rTMD23 promotes limb salvage after ischaemia. (A) Gross appearance of rat hindlimb at 4 weeks after surgical excision of the right femoral artery. (B) Ischaemic functional scores of rats at 4 weeks after hindlimb ischaemia. 0 = normal, 1 = mild claudication without muscular dystrophy, 2 = moderate claudication with muscular atrophy but without limb cyanosis, 3 = severe claudication with muscular atrophy with limb cyanosis, 4 = toe gangrene, 5 = foot gangrene, 6 = limb amputation. The data represent mean ± S.E.M. (n = 10). **P, 0.01 compared with ischaemia or PBS group. (C) Regional blood flow in the ischaemic hindlimb. Blood flow was measured by laser Doppler spectrophotometer. Superficial regional blood flow was measured by a 2 mm depth probe. The data represent mean ± S.E.M. (n = 10). ***P < 0.01 compared with ischaemia or PBS group. (D) rTMD23 increases capillary density in the rat ischaemic hindlimb. Representative images of capillary stained with anti-vWF at the mid-thigh level. Magnifications at ×200. The data represent mean ± S.E.M. (n = 10). **P < 0.01 compared with ischaemia or PBS group. (E) Western blot analysis of FGFR1 phosphorylation in ischaemic hindlimb. The data represent mean ± S.E.M. (n = 5). *P < 0.05 compared with the PBS group. (F) Immunohistochemistry analysis of FGFR1 activation in ischaemic hindlimb. The data represent mean ± S.E.M. (n = 4). **P < 0.01 compared with the PBS group.
effect of bFGF. In our previous study, we observed that heparin is required for rTMD23-induced angiogenesis in Matrigel plugs injected in mice. In the current study, we demonstrated that rTMD23 formed a complex with syndecan-4 and FGFR1 in HUVECs. Additionally, the role of syndecan-4 in our hypothesis was evaluated in syndecan-4-knockdown HUVECs. The interaction between syndecan-4 and rTMD23 in syndecan-4-knockdown HUVECs was reduced by 75% in the rTMD23-Sepharose pull-down assay (see Supplementary material online, Figure S1B); however, rTMD23 still stimulated HUVEC tube formation in Matrigel (see Supplementary material online, Figure S1C) when syndecan-4 was reduced by 80% (see Supplementary material online, Figure S1A). These results indicated that syndecan-4 did not play a major role in rTMD23-induced angiogenesis in vitro. The multiple bands of syndecan-4 that were detected in our pull-down assays may represent the SDS-resistant dimeric form or various levels of glycanation on the core protein. We note that the interaction kinetics estimated from results of SPR were the same for rTMD23 and tag-deleted rTMD23 when binding to FGFR1 (data not shown), which indicates that the C-terminal tags of rTMD23 did not interfere with the interaction. Furthermore, results of binding kinetics for rTMD23 and FGFR1 showed a fast association and dissociation rate in comparison to the values obtained in a previous study of bFGF and FGFR1. Taken together, these results imply that the interaction between rTMD23 and FGFR1 could occur in a fast and tuneable manner in which signalling could be reversibly fine-tuned. Additionally, it is possible that HSPGs regulate and enhance the interaction between rTMD23 and FGFR1 in vivo.

The rhomboids are a highly conserved family of intramembrane serine proteases that mediate the release of membrane-tethered EGF-like ligands, and thereby regulate several biological functions. Recently, rhomboid-like-2 (RHBDL2), a human rhomboid analogue, was shown to mediate the intramembrane cleavage of TM that releases its ectodomain of TM. Additionally, the MMPs are thought to mediate TM lectin-like domain shedding. Therefore, in certain biological conditions, soluble TM proteins can be generated and could have an activity similar to that of rTMD23. The concentration of soluble TM fragments in plasma and urine is elevated in several pathological conditions. However, the biological function of soluble TM fragments in the human body is currently obscure, and the regulation of RHBDL2 is unclear. Recently, we demonstrated that an autocrine/paracrine circuit of soluble TM mediated by RHBDL2 could promote cutaneous wound healing. Additionally, soluble TM was released by human early EPCs, where it promotes EPC proliferation and augments therapeutic angiogenesis. Ileezoe et al. showed that recombinant TM protein protects endothelial cells against apoptosis through induction of anti-apoptotic protein. Consistent with this finding, in our recent study, we suggested that soluble TM generated from endothelial cells may provide a protective action against stress-induced apoptosis. These cytoprotective effects of soluble TM/recombinant TM proteins are independent of the APC pathway; therefore, we suggest that FGFR1 may contribute to this activity.

The pleiotropic effects of the FGFs/FGFRs system on different cell types are dependent on cellular context and the combination of ligands/receptors. FGFR1-deficient mice are developmentally retarded and they die during gastrulation. However, mice with Fgf1 and Fgf2 deficiency are viable and fertile, and they do not display gross phenotypic defects, indicating the extensive redundancy of the ligand system. Recently, the expression of FGFR1 and FGFR2, as well as TM and RHBDL2, in keratinocytes was functionally associated with the epidermal barrier and cutaneous homeostasis. Furthermore, the change of FGFRs expression in skin before and after cutaneous wound is correlated with aging in mice, suggesting that low expression of FGFR1 and FGFRs may contribute to age-related slow wound healing in mice. TM is essential for normal embryonic development, and its expression in keratinocytes is associated with epidermal differentiation and regulated during cutaneous wound healing. In summary, this study showed that rTMD23 interacted with FGFR1 and induced the activation of FGFR1. The angiogenic activities of rTMD23 were inhibited by an FGFR1 inhibitor or by FGFR1 knockdown, indicating that FGFR1 could be one of the receptors that mediate rTMD23-induced angiogenesis. rTMD23 induces angiogenesis in cornea assay as well as promotes limb salvage, which is associated with elevated phosphorylation of FGFR1 and capillary density in situ after ischaemic damage, suggesting that rTMD23 may have therapeutic potential in peripheral artery disease. Genetically modified animal, either FGFR1 knockout or dysfunction, can be used to further evaluate if FGFR1 is the only receptor for rTMD23 in vivo. Soluble TM fragments that contain the EGF-like domain plus the serine/threonine-rich region may act as a ligand to FGFR1, and perhaps coordinate biological activity during development and regeneration with FGFs. Therefore, we suggest that this hypothesis, which has potential relevance in embryogenesis and regenerative processes, warrants further investigation.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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