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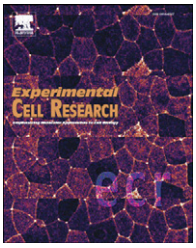

Research Article

Tumor endothelial marker 5 expression in endothelial cells during capillary morphogenesis is induced by the small GTPase Rac and mediates contact inhibition of cell proliferation

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ABSTRACT

Tumor endothelial marker (TEM) 5 is an adhesion G-protein-coupled receptor upregulated in endothelial cells during tumor and physiologic angiogenesis. So far, the mechanisms leading to upregulation of TEM5 and its function during angiogenesis have not been identified. Here, we report that TEM5 expression in endothelial cells is induced during capillary-like network formation on Matrigel, during capillary morphogenesis in a three-dimensional collagen I matrix, and upon confluence on a two-dimensional matrix. TEM5 expression was not induced by a variety of soluble angiogenic factors, including VEGF and bFGF, in subconfluent endothelial cells. TEM5 upregulation was blocked by toxin B from *Clostridium difficile*, an inhibitor of the small GTPases Rho, Rac, and Cdc42. The Rho inhibitor C3 transferase from *Clostridium botulinum* did not affect TEM5 expression, whereas the Rac inhibitor NSC23766 suppressed TEM5 upregulation. An excess of the soluble TEM5 extracellular domain or an inhibitory monoclonal TEM5 antibody blocked contact inhibition of endothelial cell proliferation resulting in multilayered islands within the endothelial monolayer and increased vessel density during capillary formation. Based on our results we conclude that TEM5 expression during capillary morphogenesis is induced by the small GTPase Rac and mediates contact inhibition of proliferation in endothelial cells.

Introduction

Angiogenesis, the sprouting of new blood vessels from pre-existing vessels, plays an important role in physiologic processes such as fetogenesis, wound healing, and the female reproductive cycle. It also contributes to several pathologic disorders such as diabetic retinopathy, tumor growth, metastasis, and rheumatoid arthritis. Endothelial cells that line the inner surface of blood vessels play a central role in angiogenesis which requires coordinated changes in endothelial cell morphology, function, and gene expression [1]. Angiogenesis is triggered by angiogenic factors such as VEGF and bFGF which activate quiescent endothelial cells. Upon activation endothelial cells loose cell–cell contacts, degrade the basement membrane, migrate into the tissue and expand by proliferation. In the tissue endothelial cells differentiate into new capillaries by forming specific cell–cell contacts and lumens. Eventually capillary morphogenesis results in an inhibition of cell migration and proliferation referred to as contact inhibition [2]. So far, vascular endothelial (VE)-cadherin which is localized in endothelial adherens junctions has been implicated in this process [3,4]. It has been shown that VE-cadherin engagement mediates activation of Rac, a member of the Rho family of small GTPases [5].

The Ras-related small GTPases of the Rho family play an important role in the regulation of the actin cytoskeleton, cell

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migration, cell proliferation, and gene expression in endothelial cells during angiogenesis [6]. Like all Ras-related proteins, Rho GTPases cycle between a GTP-bound active state at the plasma membrane and a GDP-bound inactive state in the cytosol. This cycling is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins. Rho GTPases can be divided into eight subfamilies: Rho, Rac, Cdc42, Rnd, RhoBTB, RhoD/RhoF, RhoH, and Wrch. The Rac subfamily consists of Rac1, Rac2, Rac3, and RhoG [7].

Rho, Rac, and Cdc42 are efficiently inhibited by toxin B from *Clostridium difficile* strain 10463 (TcdB) which acts as glucosyltransferase. UDP glucosylation in the effector region of these GTPases by TcdB blocks interactions with downstream targets [8]. Rho is specifically inactivated by the exoenzyme C3 transferase from *Clostridium botulinum* which acts as ADP ribosyltransferase. ADP ribosylation takes place in the effector region of Rho and inhibits binding to downstream targets [9]. The chemical compound NSC23766, an inhibitor of Rac, was identified by a structure-based virtual screening of compounds that fit into a surface groove of Rac1 known to be crucial for interactions with GEFs. NSC23766 has been shown not to cross-react with the closely related GTPases Rho and Cdc42 [10]. TcdB, C3 transferase, and NSC23766 are widely used as molecular tools to inhibit the function of small GTPases with high specificity in a variety of cellular systems including endothelial cells.

Tumor endothelial markers (TEMs) 1–8 have been identified as previously unknown and structurally unrelated genes that display upregulated expression in endothelial cells during tumor angiogenesis [11] as well as physiologic angiogenesis [12]. TEMs 1, 5, 7, and 8 encode transmembrane proteins and are therefore potential targets for the selective delivery of radioimmuno- or radiopeptide-conjugates into tumors.

TEM5 belongs to the adhesion family of G-protein-coupled receptors (GPCRs) and has therefore also been termed GPR124 [13]. Members of the adhesion family of GPCRs are characterized by: A long extracellular N-terminus bearing conserved domains

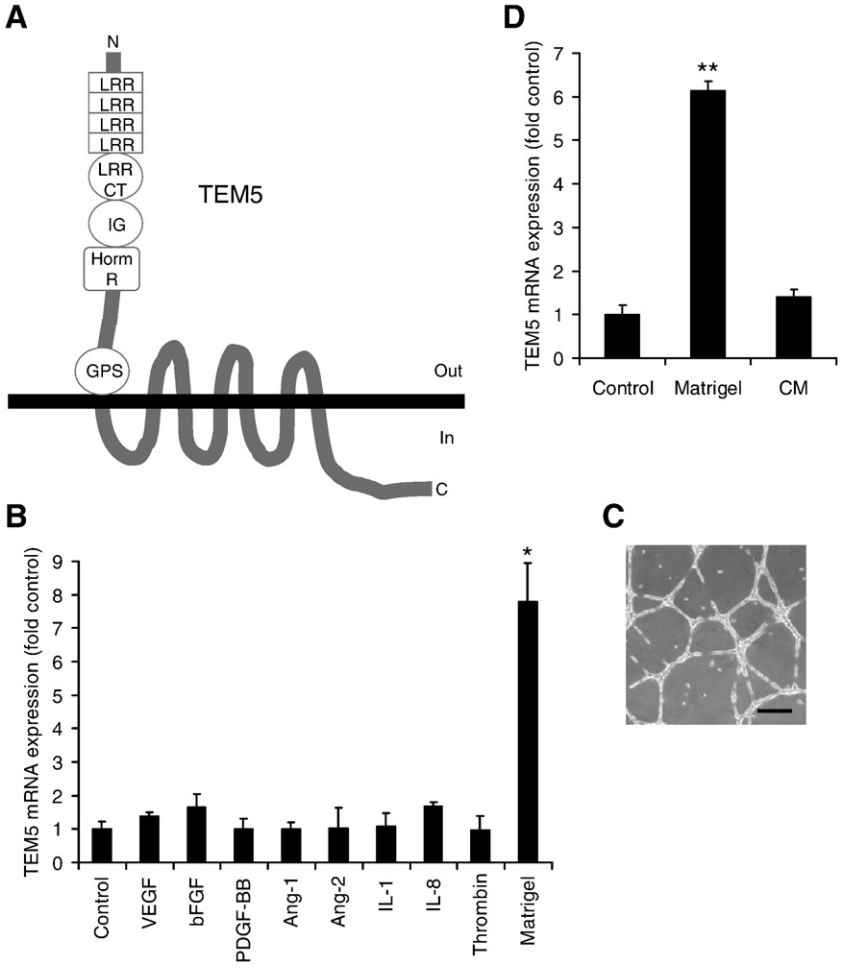

Fig. 1 – TEM5 upregulation in endothelial cells is not mediated by soluble factors. (A) Domain structure of TEM5. LRR, leucine-rich repeat domain; CT, C-terminal; IG, immunoglobulin domain; HormR, hormone receptor domain; GPS, GPCR proteolysis site domain. (B, D) Subconfluent HUVEC were stimulated for 24 h with VEGF, bFGF, PDGF- BB, Ang-1, Ang-2, IL-1, and IL-8 at 50 ng/ml, with thrombin at 1 U/ml, by incubating on Matrigel, with conditioned medium (CM) derived from HUVEC incubated on Matrigel for 24 h, or with medium only (control). Real-time PCR was performed to quantify TEM5 mRNA expression which was normalized against HPRT expression ($n = 2 \pm$ SD, $*p < 0.02$ vs. control, $**p < 0.001$ vs. control). (C) Phase contrast microscopic image of HUVEC incubated on Matrigel for 24 h. Scale bar: 220 μm.

involved in cell–cell and cell–matrix interactions, a GPCR proteolysis site (GPS) domain, a seven-pass transmembrane domain, and an intracellular domain. The TEM5 extracellular domain contains several conserved subdomains: Four leucine-rich repeat domains, a leucine-rich repeat C-terminal domain, an immunoglobulin domain, a hormone receptor domain, and a membrane proximal GPS domain (Fig. 1A). It has been reported that the TEM5 intracellular domain interacts with the tumor suppressor protein human disc large (hDlg) [14].

We previously reported that a soluble TEM5 fragment (sTEM5) encompassing the TEM5 extracellular domain is partially shed by endothelial cells during capillary-like network formation and upon growth factor stimulation. sTEM5 binds to glycosaminoglycans present in the extracellular matrix and contains a cryptic RGD motif which is exposed by proteolytic processing. Proteolytically processed sTEM5 immobilized on plastic or glycosaminoglycans mediates adhesion and survival of endothelial cells by activation of integrin $\alpha_v\beta_3$ [15].

So far, the mechanisms leading to upregulation of TEM5 and the function of full-length TEM5 during angiogenesis have not been identified. In the present study, we report that TEM5 is upregulated in endothelial cells during capillary morphogenesis in a three- dimensional collagen I matrix and upon confluence on a two-dimensional matrix. We found that TEM5 expression during angiogenesis is induced by the small GTPase Rac. Using a function blocking monoclonal TEM5 antibody or an excess of recombinant sTEM5 contact inhibition of cell proliferation in endothelial monolayers and during capillary morphogenesis was blocked.

Materials and methods

Reagents and antibodies

Recombinant human (rh) VEGF, bFGF, PDGF-BB, IL-1, and IL-8 were purchased from ImmunoTools (Friesoythe, Germany). rhAng-1 and rhAng-2 were from R&D Systems (Minneapolis, MN, USA). Human thrombin, PMA, and Protease Inhibitor Cocktail were from Sigma-Aldrich Chemie (Steinheim, Germany). Monoclonal HuCAL GFP antibody (clone MOR6391) was from AbD Serotec-MorphoSys (Düsseldorf, Germany). Function blocking monoclonal VE-cadherin antibody (clone BV9) was from Hycult biotechnology (PB Uden, Netherlands). Polyclonal TEM5 antibody (TEM5-CT) was from ProSci (Poway, CA, USA). Peroxidase-conjugated anti-rabbit IgG antibody was from GE Healthcare (Little Chalfont Buckinghamshire, England). NSC23766 was from Calbiochem (Darmstadt, Germany). BCA protein assay kit was from Pierce (Rockford, IL, USA). TcdB from *C. difficile* strain 10463 was provided by Prof. Martin Aepfelbacher. Recombinant C3 transferase from *C. botulinum* was provided by Prof. Stefan Linder.

Generation of recombinant TEM5 proteins

rhsTEM5 (aa 34–755) and rhTEM5 (aa34-1338, NCBI ID: NP_116166) were cloned and expressed as previously described [15]. Recombinant murine (rm) sTEM5 (aa 34-753, NCBI ID: Q91ZV8) was amplified by PCR from murine kidney cDNA and cloned and expressed as previously described for the human protein [15]. rhsTEM5 and rmsTEM5 were purified from cell supernatants and rhTEM5 was purified from cell lysates as previously described [15]. Where not indicated explicitly rhsTEM5 was used in this study.

Generation of monoclonal TEM5 antibody

Monoclonal TEM5 antibody was generated in cooperation with AbD Serotec-MorphoSys. Briefly, the HuCAL library was alternatingly panned against rhsTEM5 and rmsTEM5. The resulting antibody (clone AbD06669) bound to human and murine TEM5 with similar affinity and its epitope was mapped within the immunoglobulin domain of TEM5 (data not shown).

Cell culture

Pooled human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany) and maintained in Endothelial Cell Growth Medium (ECGM, PromoCell) at 37 °C and 5% CO_2. For experiments HUVEC from passages 1 to 4 were used.

Capillary-like network formation assay

6-well plates were layered with 0.5 ml ice- cold growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA) and Matrigel was allowed to polymerize for 30 min at 37 °C. 2.5×10^5 HUVEC resuspended in ECGM were seeded on the Matrigel and incubated. Cells were recovered from Matrigel by washing three times with PBS and incubating on ice for 1 h with PBS containing 5 mM EDTA.

Capillary morphogenesis assay

Capillary morphogenesis assay was adapted from Davis and Camarillo [16]. Briefly, ice-cold rat collagen type I (~10 mg/ml, BD Biosciences) was neutralized with NaOH and supplemented with 0.1 volumes 10× PBS. Neutralized collagen I was diluted in ice-cold assay medium (Opti-MEM I [Invitrogen, Carlsbad, CA, USA] containing 50 ng/ml of each VEGF, bFGF, and PMA) to a final concentration of 3.5 mg/ml and placed on ice. HUVEC were trypsinized and washed extensively with PBS. Pelleted cells were resuspended in collagen I solution at a concentration of 1×10^6 cells per ml. The cell-collagen mixture was seeded in 24- or 96-well plates and collagen was allowed to polymerize for 15 min at 37 °C. The cells were overlaid with assay medium and incubated for the indicated amount of time. For bright field microscopy cells were fixed with 3.7% formaldehyde in PBS and stained with 0.1% Toluidine Blue O.

Real-time PCR

Total RNA was isolated from cells using the High Pure RNA Isolation Kit from Roche Applied Science (Mannheim, Germany). mRNA was reverse transcribed into cDNA using the Protoscript First Strand cDNA Synthesis Kit from New England Biolabs (Ipswich, MA, USA). Real-time PCR was performed using the Applied Biosystems (Foster City, CA, USA) 7300 real-time PCR system and the qPCR MasterMix Plus for SYBR Green I from Eurogentec (Seraing, Belgium). The PCR program was as follows: 95 °C for 10 min; 95 °C for 15 sec and 64 °C for 1 min, 40 cycles. TEM5 primers had the sequence 5′-CCCACACACACTCCTTG-GAATGG-3′ (forward) and 5′-GCTCTCCA GTGCTAAGGCATTGG-3′

(reverse). HPRT primers had the sequence 5′-GCTTTCCTTGG TCAGGCAGTATAAT-3′ (forward) and 5′-AAGGGCATATCCTACAA-CAAATCCG-3′ (reverse). Primers were synthesized by MWG-Biotech (Ebersberg, Germany). TEM5 Ct values were normalized against HPRT Ct values and relative quantification was performed. Specificity of the primer pairs was verified by determining dissociation curves of the PCR products which only showed single peaks at the expected temperatures (data not shown).

Western blotting

Cells were scraped into lysis buffer (TBS containing 2% Triton X-100, 1 mM EDTA, and 1% Protease Inhibitor Cocktail) and incubated on ice for 30 min. Lysates were cleared by centrifugation and protein concentrations were determined using the BCA protein assay kit. Lysates were supplemented with reducing Laemmli sample buffer and equal amounts of protein were separated by 8% SDS-PAGE. Proteins were blotted on PVDF membranes (Millipore, Billericia, MA, USA) and blots were blocked with 5% low-fat skimmed milk in TBS containing 0.1% Tween 20 (TBST) for 1 h. Membranes were incubated over night with polyclonal TEM5 antibody diluted 1:200 in blocking buffer. After washing with TBST blots were incubated for 1 h with peroxidase-conjugated anti-rabbit IgG antibody diluted 1:50000 in blocking buffer. Membranes were washed with TBST, incubated with Immobilon Western substrate (Millipore) and chemoluminescence was detected using Amersham Hyperfilm ECL films (GE Healthcare). Equal loading was verified by reprobing membranes with peroxidase-conjugated monoclonal β-actin antibody (Gen-Script, Piscataway, NJ, USA) diluted 1:10000 in blocking buffer.

Sandwich ELISA

Sandwich ELISA was performed using the standard protocol. Briefly, a 96-well ELISA plate was coated with monoclonal TEM5 antibody and blocked with 5% BSA. HUVEC were lysed in TBS containing 1% Triton X-100, 1 mM EDTA, and 1% Protease Inhibitor Cocktail and protein concentrations were determined using the BCA protein assay kit. Anti-TEM5-coated wells were incubated with the lysates and rhTEM5 serially diluted in lysis buffer as standard for 2 h at room temperature. Bound TEM5 was detected using polyclonal anti-TEM5 (1:500) as primary and peroxidase-conjugated anti- rabbit IgG (1:5000) as secondary antibody and TMB (AbD Serotec-MorphoSys) as substrate. Chromogenic reaction was stopped with H_2SO_4 and absorbance at 450 nm was

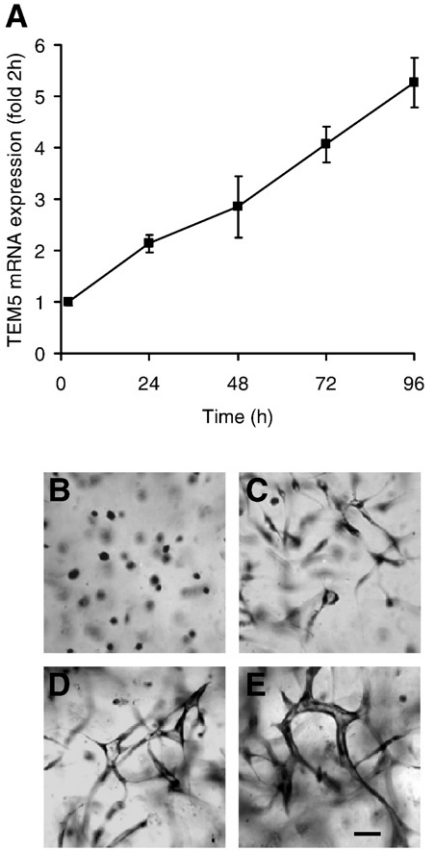

Fig. 2 – TEM5 is upregulated during capillary morphogenesis. HUVEC were induced to undergo capillary morphogenesis in a three-dimensional collagen I matrix. (A) Real-time PCR was performed to quantify TEM5 mRNA expression at indicated time points. TEM5 was normalized against HPRT ($n = 3 \pm$ SD). (B–E) Bright field microscopic images of the cells stained with Toluidine Blue O after 1 h (B), 24 h (C), 48 h (D), and 96 h (E). Scale bar: 55 μm.

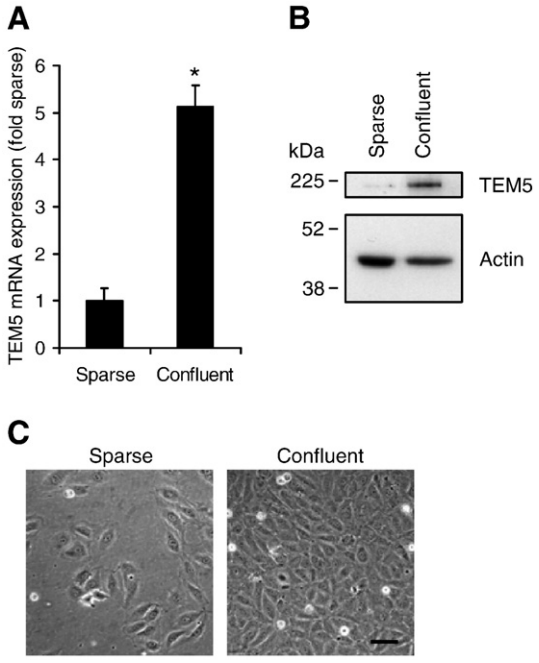

Fig. 3 – TEM5 mRNA and protein expression is induced in confluent endothelial cells. HUVEC were seeded sparsely (2.6×10^4 cells/cm^2) and confluently (1×10^5 cells/cm^2) on TC plastic and incubated for 24 h and 48 h, respectively. (A) Real-time PCR was performed to quantify TEM5 mRNA expression which was normalized against HPRT expression ($n = 2 \pm$ SD, $^*p < 0.01$ vs. sparse). (B) Western blotting was performed to visualize TEM5 protein expression (upper panel). Blot was reprobed with an actin antibody to verify equal loading (lower panel). (C) Phase contrast microscopic images of the cells. Scale bar: 55 μm.

measured. TEM5 protein concentrations were determined and normalized against the total protein concentrations.

Cell proliferation assay

7.5×10^3 HUVEC resuspended in ECGM and 2.5×10^4 HUVEC resuspended in ice-cold collagen I (3.5 mg/ml) were seeded in 96-well plates and incubated for 24 h and 2 h, respectively. Medium was changed and cells were treated with indicated antibody or recombinant protein. At indicated time points cell supernatants were supplemented with 0.1 volumes AlamarBlue (AbD Serotec-MorphoSys) and cells were incubated for 1 h at 37 °C. Supernatants were transferred to new plates and fresh medium containing antibody or recombinant protein was added to the cells. Absorbances of the supernatants at 570 nm and 600 nm were measured and reduction of the AlamarBlue dye was calculated according to the manufacturer's instructions.

Immunofluorescence staining

Cells cultured in 96-well plates were fixed with 3.7% formaldehyde in PBS for 10 min and washed with PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min. After washing with PBS cells were blocked with 10% fetal calf serum (FCS) in PBS for 1 h. Cells were incubated over night at 4 °C with polyclonal VE-cadherin antibody (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:50 in blocking buffer. After washing with PBS cells were incubated for 1 h with FITC-conjugated anti-rabbit IgG antibody (Sigma-Aldrich Chemie) diluted 1:100 in PBS containing 2% FCS. Cells were washed with PBS, layered with Vectashield Mounting Medium for Fluorescence (Vector Laboratories, Burlingame, CA, USA), and VE-cadherin staining was visualized using a fluorescence microscope.

Image analysis

Cells cultured in 96-well plates were fixed with 3.7% formaldehyde in PBS and stained with 0.1% Toluidine Blue O. Cell number per field was determined by counting cell nuclei in three independent bright fields (320×) using the ImageJ software. To quantify multilayered cells, overlapping cell nuclei were counted in three wells using bright field microscopy. Capillary density was determined by measuring the area fraction of the capillaries in three independent bright fields (50×) using the ImageJ software.

Statistical analysis

Statistical significance was determined using the Student *t* test.

Results

TEM5 upregulation is not mediated by soluble factors

Soluble angiogenic factors play an important role in the regulation of gene expression in endothelial cells during angiogenesis. Therefore, we tested several angiogenic factors as potential stimuli for the upregulation of TEM5 in subconfluent HUVEC. Interestingly, none of the soluble factors (VEGF, bFGF, PDGF-BB, Ang-1, Ang-2, IL-1, IL-8, thrombin) tested by us resulted in increased TEM5 mRNA expression. Only differentiation of the cells into capillary-like networks on Matrigel mediated eight fold upregulation of TEM5 mRNA after 24 h (Fig. 1B, C). Even though growth factor-reduced Matrigel was used in our experiment, we could not

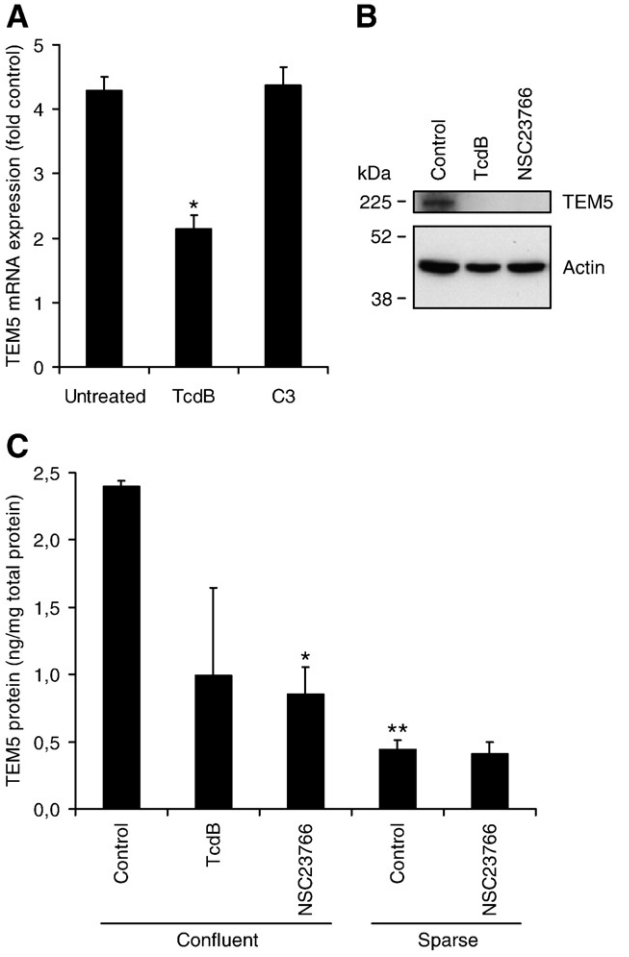

Fig. 4 – TEM5 upregulation is mediated by Rac. (A) HUVEC were induced to undergo capillary-like network formation on Matrigel and treated with 50 ng/ml TcdB, 3 μg/ml C3 transferase, or left untreated for 48 h. Cells that received C3 transferase were pre-treated with 3 μg/ml C3 transferase for 18 h. Control cells were incubated on TC plastic for 48 h. Real-time PCR was performed to quantify TEM5 mRNA expression which was normalized against HPRT expression ($n=2\pm$SD, $*p<0.01$ vs. untreated). (B) HUVEC were seeded confluently (1×10^5 cells/cm^2) and incubated. After 24 h cells were treated with 10 ng/ml TcdB, 100 μ_M NSC23766, or left untreated (control) for 48 h. Western blotting was performed to visualize TEM5 protein expression (upper panel). Blot was reprobed with an actin antibody to verify equal loading (lower panel). Data shown represent one out of three independent experiments with similar results. (C) HUVEC were seeded confluently (1×10^5 cells/cm^2) or sparsely (1.3×10^4 cells/cm^2) and incubated. After 24 h cells were treated with 10 ng/ml TcdB, 100 μM NSC23766, or left untreated (control) for 48 h. Sandwich ELISA was performed to determine TEM5 protein expression ($n=3\pm$SD, $*p<0.01$, $**p<0.001$, p values vs. control [confluent]).

completely exclude the involvement of residual angiogenic factors in the upregulation of TEM5. Furthermore, soluble factors released from the cells during capillary-like structure formation might mediate upregulation of TEM5 in a paracrinic manner. To determine whether Matrigel- or cell-derived soluble factors are responsible for upregulation of TEM5, conditioned medium (CM) from cells differentiating into capillary-like networks on Matrigel was used to stimulate subconfluent cells. No upregulation of TEM5 mRNA was observed using this CM indicating that upregulation is due to signaling pathways activated during endothelial network formation but not due to soluble factors (Fig. 1D).

TEM5 expression is induced during capillary morphogenesis

Differentiation of endothelial cells on Matrigel results in rapid formation of capillary-like networks without lumens within 16 h [15]. Therefore, we investigated whether TEM5 is also upregulated in an *in vitro* model of angiogenesis mimicking angiogenesis *in vivo* where capillary networks containing lumens are formed within days. Indeed, TEM5 mRNA expression linearly increased in HUVEC during capillary morphogenesis in a three- dimensional collagen I matrix and was five fold elevated after 96 h when differentiation was morphologically completed (Fig. 2A). Upregulation of TEM5 correlated with the formation of cell–cell contacts and lumens during capillary morphogenesis (Fig. 2B–E).

TEM5 expression is induced in confluent endothelial cells

Since TEM5 expression is not induced by soluble factors in subconfluent endothelial cells and correlates with the formation of cell–cell contacts during capillary morphogenesis we investigated whether expression of TEM5 correlates with cell density, i.e. the formation of cell–cell contacts in endothelial monolayers. Indeed, TEM5 mRNA and protein expression was upregulated in confluent HUVEC compared to sparse cells (Fig. 3).

TEM5 upregulation is mediated by the small GTPase Rac

Rho GTPases play an important role in the regulation of gene expression in endothelial cells during angiogenesis [5]. In order to determine whether Rho family proteins modulate TEM5 expression in endothelial cells we used different highly specific inhibitors of Rho GTPases. We found that TcdB from *C. difficile* strain 10463 which inhibits Rho, Rac, and Cdc42 [8] blocked upregulation of TEM5 mRNA during capillary-like network formation on Matrigel, whereas C3 transferase from *C. botulinum* which inhibits Rho but not Rac and Cdc42 [9] did not affect TEM5 upregulation (Fig. 4A). TcdB also inhibited upregulation of the TEM5 protein in confluent endothelial cells (Fig. 4B). These results indicate an involvement of Rac and/or Cdc42 in the upregulation of TEM5. To further investigate which Rho GTPase mediates upregulation of TEM5

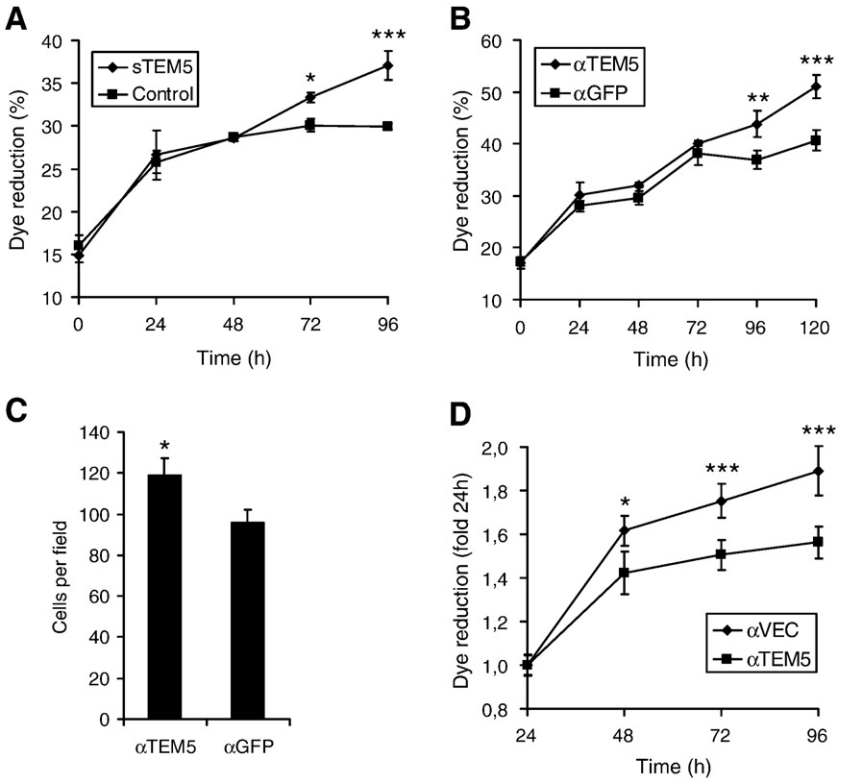

Fig. 5 – TEM5 mediates contact inhibition of cell proliferation in endothelial monolayers. (A) HUVEC were seeded subconfluently and treated with 12 μg/ml sTEM5 or left untreated (control). (B–D) HUVEC were seeded subconfluently and treated with 25 μg/ml function blocking monoclonal VE-cadherin antibody (αVEC), function blocking monoclonal TEM5 antibody (αTEM5), or monoclonal GFP antibody (αGFP) as isotype control. (A, B, D) Cell proliferation was determined at indicated time points using the AlamarBlue dye which is reduced by viable cells ($n = 3 \pm$ SD, *$p < 0.05$ vs. control/αTEM5, **$p < 0.02$ vs. αGFP, ***$p < 0.01$ vs. control/αGFP/αTEM5). (C) After 120 h cells were stained with Toluidine Blue O and cell number per bright field (320×) was determined by counting cell nuclei in three independent fields using the ImageJ software (mean values ± SD, *$p < 0.02$ vs. αGFP).

we made use of the inhibitor NSC23766 which specifically blocks Rac but not Rho and Cdc42 [10]. Inhibition of Rac by NSC23766 blocked upregulation of the TEM5 protein in confluent endothelial cells (Fig. 4B, C) but did not reduce basal TEM5 protein expression in sparse cells (Fig. 4C). Endothelial monolayer morphology and integrity was not affected by NSC23766 (data not shown).

TEM5 mediates contact inhibition of cell proliferation in endothelial monolayers

It has been reported that the TEM5 intracellular domain interacts with the tumor suppressor protein hDlg [14] and hDlg has been shown to negatively regulate cell proliferation [17]. Based on these reports and our finding that TEM5 is upregulated in confluent endothelial cells we hypothesized an involvement of TEM5 in contact inhibition of endothelial cell proliferation. To test our hypothesis HUVEC were seeded subconfluently and treated with an excess of recombinant sTEM5 to competitively inhibit TEM5 binding to its extracellular ligand. Cell proliferation was monitored every 24 h. Upon confluence untreated cells (control) ceased to proliferate as expected, in contrast to sTEM5-treated cells which continued to grow (Fig. 5A). To further confirm our results HUVEC were seeded subconfluently and treated with a function blocking monoclonal TEM5 antibody and a monoclonal GFP antibody of the same isotype as control. Upon confluence anti-GFP control-treated cells became contact inhibited, whereas anti-TEM5-treated cells continued to proliferate (Fig. 5B, C). HUVEC were seeded subconfluently and treated with a function blocking monoclonal VE-cadherin antibody and anti-TEM5 to compare VE-cadherin- with TEM5-mediated contact inhibition. Cells treated with anti-VE-cadherin proliferated faster than anti-TEM5-treated cells even before confluence was reached (Fig. 5D). Immunofluorescence staining for VE-cadherin as well as Toluidine Blue O staining after 120 h revealed that the cells treated with TEM5 antibody formed a monolayer containing islands of multilayered cells (Fig. 6A, left panels). Such islands were not found in control antibody-treated cells (Fig. 6A, right panels). Overlapping cell nuclei were counted to quantify multilayered cells which were highly abundant in anti-TEM5-treated and almost absent in anti-GFP-treated cells (Fig. 6B).

TEM5 mediates contact inhibition of cell proliferation during capillary morphogenesis

To determine whether TEM5 also mediates contact inhibition of endothelial cell proliferation during angiogenesis HUVEC were induced to undergo capillary morphogenesis in a three- dimensional collagen I matrix and treated with an excess of recombinant sTEM5 or a function blocking monoclonal TEM5 antibody. Indeed, inhibition of TEM5 resulted in significantly higher levels of cell proliferation during capillary morphogenesis for 72 h (Fig. 7A, B). After 72 h proliferation ceased and cell death became predominant. Toludine Blue O staining of the cells after 120 h revealed an increase in capillary density by treatment with anti-TEM5 compared to control treatment with anti-GFP (Fig. 7C, D).

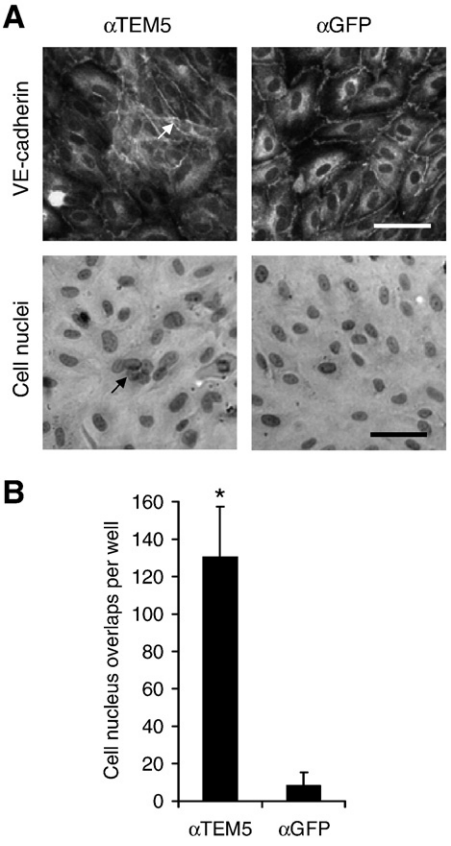

Fig. 6 – Inhibition of TEM5 results in multilayered growth of endothelial cells. HUVEC were treated with 25 μg/ml function blocking monoclonal TEM5 antibody (αTEM5) or monoclonal GFP antibody (αGFP) as isotype control for 120 h. (A) Immunofluorescence staining for VE-cadherin (upper panels) and Toluidine Blue O staining of the cell nuclei (lower panels) was performed. Arrows indicate multilayered cells. Scale bar: 55 μm. (B) Toluidine Blue O staining of the cell nuclei was performed and multilayered cells were quantified by counting overlapping cell nuclei per 28 mm ^{2}-well ($n = 3 \pm$ SD, $*p < 0.01$ vs. αGFP).

Discussion

In this study we investigated the expression and function of TEM5 in endothelial cells during angiogenesis. We found that TEM5 expression in endothelial cells is not induced by soluble angiogenic factors but during capillary-like network formation on Matrigel, during capillary morphogenesis in a three-dimensional collagen I matrix and upon confluence on a two- dimensional matrix. While the cells may be in different functional conditions in these models they have at least one essential process in common: The formation of cell–cell contacts. Cell–cell contacts may activate the signaling pathways that lead to enhanced transcription of the TEM5 gene.

Rho family GTPases have been shown to play an important role in the formation of cell–cell contacts and in the signaling pathways activated by these contacts [18]. To determine whether Rho GTPases are involved in the regulation of TEM5 expression we used different highly specific inhibitors of Rho family proteins. We found that TcdB from *C. difficile* strain 10463, an inhibitor of Rho, Rac, and Cdc42 [8], inhibited upregulation of TEM5 during capillary-like network formation and in confluent endothelial cells. In contrast, C3 transferase from *C. botulinum*, an inhibitor of Rho [9], had no effect on TEM5 expression. NSC23766 which inhibits Rac [10] blocked upregulation of TEM5 in confluent cells.

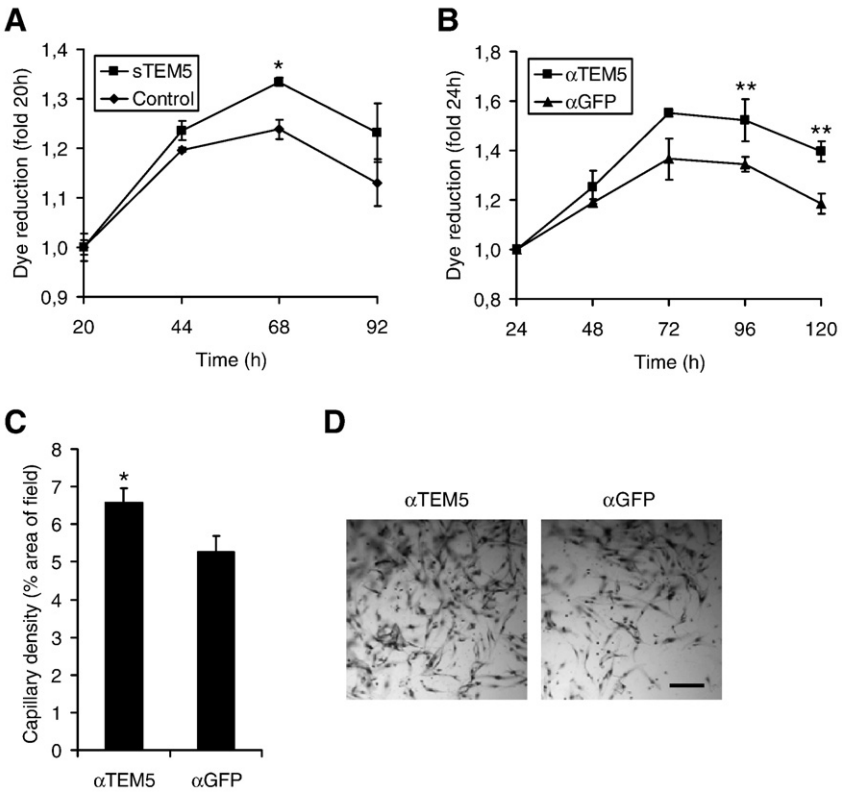

Fig. 7 – TEM5 mediates contact inhibition of proliferation during capillary morphogenesis. HUVEC were induced to undergo capillary morphogenesis in a three-dimensional collagen I matrix. (A) Cells were treated with 25 μg/ml sTEM5 or left untreated (control). (B) Cells were treated with 25 μg/ml function blocking monoclonal TEM5 antibody (αTEM5) or monoclonal GFP antibody (αGFP) as isotype control. (A, B) Cell proliferation/death was determined at indicated time points using the AlamarBlue dye which is reduced by viable cells ($n = 3 \pm$ SD, *$p < 0.001$ vs. control, **$p < 0.05$ vs. αGFP). (C, D) Cells were treated with indicated monoclonal antibodies at 25 μg/ml. After 120 h cells were stained with Toluidine Blue O. (C) Capillary density was determined as % area fraction of bright field (50×) using the ImageJ software ($n = 3 \pm$ SD, *$p < 0.01$ vs. αGFP). (D) Bright field microscopic images of the cells. Scale bar: 220 μm.

Inhibition of Rac in subconfluent endothelial cells had no effect on TEM5 expression indicating that basal TEM5 expression is regulated by another mechanism. These results indicate that Rac mediates upregulation of TEM5 in endothelial cells during angiogenesis.

It has been shown that Rac1 is activated during capillary-like network formation on Matrigel and its activation correlates with the formation of cell–cell contacts [19]. Furthermore, it has been reported that Cdc42 and Rac1 are activated during capillary morphogenesis in three-dimensional collagen I matrices [20]. These results support our finding that TEM5 expression during capillary-like network formation is induced by Rac. Cadherin engagement mediates activation of Rac in confluent endothelial and epithelial cells [5,21–23] which supports our finding that Rac mediates upregulation of TEM5 in confluent endothelial cells. These results also suggest that cadherins, notably VE-cadherin, may regulate TEM5 expression upstream of Rac. It has been shown that Rac1 activates the transcription factors c-jun and serum response factor which may mediate enhanced transcription of the TEM5 gene [24–26].

Growth factors such as VEGF mediate activation of Rac [27]. However, none of the growth factors we tested induced upregulation of TEM5 in subconfluent endothelial cells. Growth factor-induced activation of Rac is short and transient reaching basal levels again after 60 min [27]. In contrast, activation of Rac during capillary formation and in confluent endothelial cells is sustained [5,19,20] which may explain why TEM5 is upregulated under these conditions but not by stimulation with growth factors.

TEM5 is an orphan adhesion G-protein-coupled receptor. However, using a function blocking monoclonal TEM5 antibody or an excess of recombinant sTEM5 we found that suppression of TEM5 binding to its extracellular ligand blocks contact inhibition of proliferation in confluent endothelial cells and during capillary morphogenesis. The lack of contact inhibition resulted in multi-layered cell islands within the endothelial monolayer and in increased vessel density during capillary morphogenesis. So far, VE-cadherin has been described as the only receptor that mediates contact inhibition in endothelial cells [3,4]. Interestingly, we found that VE-cadherin negatively regulates cell proliferation even before confluence is reached. In contrast, TEM5 only inhibits proliferation in confluent cells. This difference may be explained by the constitutive expression of VE-cadherin in endothelial cells and the induced expression of TEM5 upon confluence. TEM5 may represent a novel independent mechanism that mediates contact inhibition in endothelial cells or function downstream of VE-cadherin.

Since TEM5 is expressed on the surface of tumor endothelial cells it may be used as a molecular target for the selective delivery of radioimmuno- or radiopeptide-conjugates into tumors. However, blocking the function of TEM5 may not be beneficial for tumor treatment as we show here that inhibition of TEM5 results in increased capillary formation.

It has been reported that the TEM5 intracellular domain interacts with the tumor suppressor protein hDlg [14]. hDlg has been implicated in negative regulation of cell proliferation [17] and is associated with E-cadherin in epithelial cells [28]. Extracellular ligation of TEM5 may lead to activation of hDlg and thereby negatively regulate cell proliferation. Further studies on the potential role of hDlg in TEM5-mediated contact inhibition appear justified. Contact inhibition in endothelial cells is not only required for capillary morphogenesis during angiogenesis but also in mature blood vessels. However, TEM5 is not upregulated in endothelial cells of mature blood vessels in normal tissues [11]. This indicates that TEM5 is only required to mediate contact inhibition during angiogenesis but not to maintain it in mature vessels.

Based on our results we conclude that TEM5 is upregulated in endothelial cells at a late stage of angiogenesis when endothelial cells differentiate into new capillaries and Rac is activated by the formation of cell–cell contacts. Activation of TEM5 by its extracellular ligand which has yet to be identified mediates contact inhibition of endothelial cell proliferation in these early capillaries.

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