Introduction

Related Transcriptional Enhancer Factor-1 (RTEF-1), also known as TEAD4 (TEA domain family member 4), is a member of the Transcription Enhancer Factor family, and plays important roles in a variety of physiological and pathological conditions. RTEF-1 targets the promoters of many genes and shares a highly conserved domain capable of binding to the MCAT element CATN(T/C)(T/C) [1,2] in the promoter region of genes expressed in endothelial [3], cardiac [4], skeletal and smooth muscle cells [5], as well as myofibroblasts [6]. In endothelial cells, RTEF-1 is involved in the stimulation of angiogenesis under hypoxia via transcriptional regulation of its target genes [7]. RTEF-1 is shown to transcriptionally regulate Hypoxia inducible factor (HIF)-1 and accelerate recovery rates from hind limb ischemic injury [3]. In addition, RTEF-1 impacts the Fibroblast growth factor (FGF)/FGFR system through the eNOS pathway in the regulation of angiogenesis and vasodilation [8]. We have recently found that endothelial specific RTEF-1-deficient mice lack the ability to form normal capillary networks suggesting that a loss of RTEF-1 signaling leads to the disintegration of the mature vasculature and that RTEF-1 is required for endothelial connections and capillary network formation [9]. However, the direct effect of RTEF-1 on angiogenesis in endothelial cells and new target genes that might be involved in RTEF-1 driven angiogenesis has not been fully understood.

Endothelial differentiation gene 1 (Edg-1), also known as sphingosine-1-phosphate receptor 1 (S1PR1 or S1P1) is a G-protein-coupled receptor. It binds the ligand sphingosine-1-phosphate (S1P) with high affinity and high specificity and is suggested to be involved in the processes that regulate the differentiation of endothelial cells. Activation of this receptor by S1P induces cell–cell adhesion [10], migration and proliferation in endothelial cells [11]. Edg-1−/− mice exhibit embryonic hemorrhage leading to intrauterine death between E12.5 and E14.5 due to a deficiency in vascular maturation [12]. These findings suggest that Edg-1 plays an important role in angiogenesis. However, transcription factors that regulate Edg-1 in angiogenesis have not been elucidated.

Here, we report that the transcription factor RTEF-1 is involved in enhancing angiogenesis in endothelial cells. Furthermore, we show that RTEF-1 regulates Edg-1 gene expression as a transcriptional activator upon binding to Edg-1 promoter. In addition, the angiogenesis enhanced by RTEF-1 is associated with the Edg-1 and Pertussis Toxin (PTX)-sensitive Gi/Go protein pathway.

Materials and Methods

Ethics Statement

The animal study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of
Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center (049-2011).

Cell Culture
Human dermal microvascular endothelial cells-1 (HMEC-1; Center of Disease Control) were cultured in MCDB-131 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone and 2 mM L-glutamine. HEK293 (human embryonic kidney 293; ATCC) and HEK293T (a derivative of HEK293 which constitutively expresses the simian virus 40 large T antigen; ATCC) cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum.

Generation of RTEF-1 Transgenic Mice
RTEF-1 transgenic mice were generated at the BIDMC Transgenic Core Facility using the vascular endothelial (VE)-cadherin promoter to drive endothelial-specific expression of human RTEF-1 [13]. RTEF-1 transgenic mice were genotyped by PCR as described previously [14]. A conditional knockout (KO) line of RTEF-1 was generated by crossing homozygous TEA-Dfflox/lox mice (a gift from Dr. Andres Buonanno, NICHHD, NIH) with transgenic mice expressing Cre recombinase under control of the endothelial cell-specific Tie2 promoter/enhancer (a gift from Dr. Anthony Rosenzweig, BIDMC) [15]. Mice were screened by PCR to verify germline transmission, using primers P1, P2, P3 and/or P4 as described previously [16].

Retroviral Transduction
The stable over-expression of RTEF-1 in HMEC-1 cells was performed as previously described [3]. In brief, the coding sequence of RTEF-1 (NM_003213) was subcloned into the pBMN-GFP vector (Origene, San Diego, CA). In 10-cm dishes, 6x10^6 HEK293T cells were transfected with pBMN-GFP-RTEF-1, pMD-VSVG, pJK3, or pCMV-tat using Polyethyleneimine (Carlsbad, CA) and confirmed by real-time PCR and western blotting. Two duplexes of RTEF-1 siRNA (5'-GGG CAG ACC UCA ACA CCA ATT-3', 5'-UUG GUG UUG AGG UCU GCC CAG-3' and 5'-ACC CAA GAU GGU GUG UAU UTT-3', 5'-AAU ACA CAG CAU CAU GGG UTT-3') and two duplexes of Edg-1 siRNA (5'-GCC AUU AGU UAG GUG CTT-3' and 5'-GCG CAC UUU AUC UAA AUG ATT-3', 5'-UCA AGA UAA AGU GGG CTT-3') were used in the experiment. A duplex of RNA (5'-UAG UGC GUA GCG UUA GCC ATT-3', 5'-UCG UGA CAC GGU CCG AGA ATT-3') that is not targeted to any human gene was used as a negative control.

Small Interfering RNA Transfection
Small interfering RNA (siRNA) encoding human RTEF-1 or Edg-1 (Genepharm Shanghai, China) at a final concentration of 50 nM was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and confirmed by real-time PCR and western blotting. Two duplexes of RTEF-1 siRNA (5'-GGG CAG ACC UCA ACA CCA ATT-3', 5'-UUG GUG UUG AGG UCU GCC CAG-3' and 5'-ACC CAA GAU GGU GUG UAU UTT-3', 5'-AAU ACA CAG CAU CAU GGG UTT-3') and two duplexes of Edg-1 siRNA (5'-GCC AUU AGU UAG GUG CTT-3' and 5'-GCG CAC UUU AUC UAA AUG ATT-3', 5'-UCA AGA UAA AGU GGG CTT-3') were used in the experiment. A duplex of RNA (5'-UAG UGC GUA GCG UUA GCC ATT-3', 5'-UCG UGA CAC GGU CCG AGA ATT-3') that is not targeted to any human gene was used as a negative control.

RNA and Protein Analyses
Total RNA was extracted from cells or lungs of mice that were euthanized by carbon dioxide. A total of 2.0 µg of RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) with random primers according to the manufacturer’s protocol. Quantitative real-time PCR amplification was done using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instruction. Gene expression was analyzed with the primers for Edg-1 (5'-CTCCACAACGGGAGCAATAC-3' and 5'-GAGCGGCGTAGTGCAGTC-3'), RTEF-1 (5'-CCACGAAGGTCGTCTTTC-3' and 5'-CTCACCTGGCTGACACCTCAA-3') and Actin (5'-TCTTTATGTGACG-CAGGATT-3' and 5'-TGACCCACACTGTGGCGAT-3').

Proteins were isolated from cells or tissue samples lysed in cold RIPA buffer (Boston BioProducts, Worcester, MA) supplemented with a cocktail of protease inhibitors (Roche Applied Science, Branford, CT). Protein concentration was determined with the DC Protein Standard Assay (Bio-Rad, Hercules, CA). Samples were subjected to 10–12% SDS-PAGE, transferred to polyvinylicidene fluoride membranes (Millipore, Billerica, MA) and subsequently blocked in Tris-buffered saline-Tween 20 containing 8% nonfat milk. The membranes were then incubated with the indicated primary antibodies: anti-Edg-1 (NOVUS Biologicals, Littleton, CO; 1:1000), anti-RTEF-1 (Genemed Synthesis Inc, San Antonio, TX; 1:10,000) and anti-Vinculin (Sigma-Aldrich, St. Louis, MO; 1:100,000), respectively, followed by incubation with horseradish peroxidase-conjugated secondary antibodies anti-rabbit IgG with 1:3000 dilution (Calbiochem, La Jolla, CA) or anti-mouse IgG with 1:20,000 dilution (Vector Labs, Burlingame, CA). Blots were developed using the chemiluminescence detection system according to the instructions of the manufacturer (Thermo Fisher, Pittsburgh, PA).

Matrigel Analysis
Growth Factor-Reduced BD Matrigel Matrix (BD Biosciences, San Jose, CA) was coated on a pre-chilled 24-well culture plate on ice. After Matrigel solidification for 30 minutes, RTEF-1 over-expression (RTEF-1 o/e) HMEC-1 cells and Edg-1 (50 nmol/ml each) treated control HMEC-1 cells were plated at 10^3 cells per well. The extent of network formation was observed and photographed at 6 h and 24 h of culture in Matrigel. In a second set of experiments, RTEF-1 siRNA or corresponding negative control siRNA (50 nmol/ml each) transfected control HMEC-1 cells and Edg-1 siRNA or corresponding negative control siRNA (50 nmol/ml each) transfected RTEF-1 o/e HMEC-1 cells and control HMEC-1 cells were plated in Matrigel and the capillary network was observed at 24 h. In a tertiary experiment, the capillary network formation was tracked at 24 h Matrigel in RTEF-1 o/e HMEC-1 or control HMEC-1 with or without PTX (200 ng/mL, Tocris Biosciences, Minneapolis, MN) treatment. Tube length ratio, branch number per field and aggregation area were analyzed using Image J software.

Migration Analysis
Cell migration in RTEF-1 siRNA (50 nmol/ml) treated control HMEC-1 cells and Edg-1 siRNA (50 nmol/ml) treated RTEF-1 o/e HMEC-1 cells and control HMEC-1 cells were compared to their corresponding negative siRNA transfected controls. The comparison was also performed in RTEF-1 o/e HMEC-1 cells...
Figure 1. RTEF-1 induces angiogenesis in endothelial cells. A. RTEF-1 was over-expressed in HMEC-1. RTEF-1 mRNA (left) and protein (right) were detected in control HMEC-1 and RTEF-1 o/e HMEC-1. (** = p < 0.01, vs control HMEC-1) B. RTEF-1 expression was knocked down by RTEF-1 siRNA in control HMEC-1. (** = p < 0.01, vs control HMEC-1; left: mRNA; right: protein) C. RTEF-1 enhanced endothelial cell connections. The tubule length (left) and branch number (middle) were increased by RTEF-1 at 6 h in Matrigel compared to control. Notably, tube formation was more apparent at 24 h than at 6 h in Matrigel. The aggregation area (right) were increased by RTEF-1 both at 6 h and 24 h in Matrigel and pronounced at 24 h in
and control HMEC-1 cells treated with or without PTX (200 ng/ml). The cell migration rate was measured using the wound healing assay. Briefly, equal numbers (1×10⁴) of RTEF-1 o/e HMEC-1 and control HMEC-1 cells were plated in 12-well plates and serum-starved in 0.5% serum medium for 24 hours. The monolayer of cells was wounded by manual scratching with a 200 μl pipette tip and the cells were then cultured in complete growth medium. Matched wound regions were photographed with a Nikon microscope using phase contrast at 0 h and 12 h migration. The migration rate was assessed by the quotient of the cell migration distance divided by the original scratched width.

Cell Growth Assay

Crystal violet assay was used to detect cell growth. A total of 10⁴ cells of control HMEC-1 and RTEF-1 o/e HMEC-1 treated with or without RTEF-1 siRNA (50 nmol/ml) and Edg-1 siRNA (50 nmol/ml) were plated in each well of a 24-well plate. At 5 h and on 2, 3, and 4 days after plating, cells were fixed in 100% ethanol and subsequently stained with 0.1% crystal violet dissolved in 10% ethanol. After staining and thorough washing, the dye was extracted with 10% acetic acid, and absorbance was measured at 590 nm.

Immunofluorescence

Lungs were removed from transgenic and littermate control mice euthanized by carbon dioxide, embedded in O.C.T. compound (Sakura Finetek USA Inc., Torrance, CA), and frozen at −80°C. Tissues were sectioned with a Cryostat CM Model 3050S-3-1-1 (Leica, Wetzlar, Germany), fixed in 4% paraformaldehyde, and stained with antibodies against Edg-1 (Novus Biologicals, Littleton, CO) with a 1:500 dilution and Lectin Alexa Fluor 595 (Leica, Wetzlar, Germany), fixed in 4% paraformaldehyde, and stained with antibodies against Edg-1 (Novus Biologicals, Littleton, CO) with a 1:500 dilution and Lectin Alexa Fluor 595 (Leica, Wetzlar, Germany) at a dilution of 1:500. Immunofluorescence-stained sections were visualized with a DM5000B upright microscope (Leica, Wetzlar, Germany).

Promoter Activity and Chromatin Immunoprecipitation (ChIP)

A construct containing nucleotide fragment (~831 bp to 174 bp of human sequence at GeneBank: NM_001400.4) encompassing basal elements of the human Edg-1 promoter was obtained from Switchgear Genomics (Menlo Park, CA). HEK293 cells were transfected with the construct and different doses of pXHJ0/RTEF-1 and pXJH0 (gifted from Dr. Alexandre Stewart, University of Ottawa) using Polyethyleneimine. The addition of pXHJ0 was used to maintain the same cDNA background. Luciferase activity was determined using the dual luciferase assay system (Switchgear Genomics, Menlo Park, CA) 24 hours after transfection. Chromatin immunoprecipitation was performed with the ChIP-IT Express Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. The Edg-1 and Actin promoters were amplified with the primer pairs 5'−AAA CCC CTC GCC CGG CAA CAG CAA−3' , 5'−GCC AGA GGT AGC GAG GAG ACA AGC−3' , and 5'−TCT TTA ATG TCA CGC CGCATT−3' , 5'−TCA CCC AGA CTG TGC CCA T−3' , respectively. Edg-1 primers were designed to the putative MCAT element binding site for RTEF-1 and the amplification sequence was −595 bp to −418 bp from the transcription start site.

Statistical Analysis

Results are expressed as the mean value±SD. Student’s t test was used for two samples test and multiple comparisons among three or more groups were carried out by one-way ANOVA. A value of P<0.05 was considered significant.

Results

RTEF-1 Promotes Endothelial Cell Aggregation, Migration and Cell Growth

To determine the direct effect of RTEF-1 on angiogenesis in endothelial cells, we constructed the stable over-expressing RTEF-1 (RTEF-1 o/e) HMEC-1 (Figure 1A) and the transient RTEF-1 siRNA-transfected HMEC-1 (Figure 1B). In the Matrigel assay, RTEF-1 o/e HMEC-1 cells showed a significant cell aggregation both at 6 h and 24 h and tube formation mostly at 6 h (Figure 1C) while RTEF-1 siRNA-transfected HMEC-1s in which endogenous RTEF-1 was knocked down attenuated the formation of normal capillary network at 24 h (Figure 1D). In addition, RTEF-1 o/e HMEC-1 demonstrated a rapid migration in the scratch assay (Figure 1E) and a rising cell growth in the crystal violet assay (Figure 1G) whereas RTEF-1 siRNA retarded endothelial cell migration (Figure 1F) and growth (Figure 1H). The observation indicated that RTEF-1 promotes angiogenesis in endothelial cells.

RTEF-1 Regulates Edg-1 Gene Expression in vitro and in vivo

In a DNA microarray data from human umbilical vein endothelial cells (HUVEC), we found that Edg-1 was significantly down-regulated following RTEF-1 knockdown by RTEF-1 siRNA (p = 0.0002, vs negative control siRNA treated HUVEC) (Figure S1). To test our hypothesis that Edg-1 might be a potential target gene of RTEF-1, RTEF-1 o/e HMEC-1 as well as RTEF-1 knockdown HMEC-1 by RTEF-1 siRNA was examined for Edg-1 expression. Edg-1 mRNA increased to 1.80 fold in RTEF-1 o/e HMEC-1 cells and decreased to 0.13 fold and 0.30 fold in RTEF-1 knockdown HMEC-1 cells compared to their corresponding controls (Figure 2A). Edg-1 protein expression in HMEC-1 cells with or without RTEF-1 showed a similar trend to that of Edg-1 mRNA (Figure 2B). Moreover, expression of Edg-1 in lung tissue dramatically increased in VE-Cad; RTEF-1−/− mice compared to their littermate controls (mRNA expression shown in Figures 2C and protein expression shown in 2D). We further confirmed that Edg-1 was localized in the endothelium by co-staining with antibodies against CD31 and Edg-1 in the lungs of control and transgenic mice (Figures 2E and 2F). The expression of Edg-1 was apparently associated with the RTEF-1 gene level in these mice.

To determine whether Edg-1 is regulated by RTEF-1 on a transcriptional level, the Edg-1 promoter was co-transfected with...
RTEF-1 Up-Regulates Edg-1 in Endothelial Cells

A

![Bar graph showing Edg-1 mRNA levels in Control, RTEF-1 o/e, and RTEF-1 siRNA.](image)

B

![Western blots showing Edg-1 and Vinculin levels in Control, RTEF-1 o/e, NC siRNA, RTEF-1 siRNA1, and RTEF-1 siRNA3.](image)

C

![Bar graph showing Edg-1 mRNA levels in Control, VE-Cad:RTEF-1, and RTEF-1 -/- .](image)

D

![Western blots showing Edg-1 and Vinculin levels in Control and RTEF-1 -/- .](image)

E

![Images of Edg-1, CD31, Merge, and DAPI staining for Control, VE-Cad:RTEF-1, and RTEF-1 -/- .](image)

F

![Graph showing luciferase activity with hEdg-1 promoter, RTEF-1 cDNA, and Control vector concentrations.](image)

G

![Gel showing Edg-1 and Actin bands for Input, RTEF-1 Ab, IgG, Mock, and Marker.](image)
RTF-1 cDNA and subjected to luciferase assay. The Edg-1 promoter exhibited an increased activity in an RTF-1 dose-dependent manner, with a maximum 4.0 fold enhancement (Figure 2F). The physical interaction of RTF-1 with the Edg-1 promoter was examined by ChIP assay in RTF-1 o/e HMEC-1. As shown in Figure 2G, a specific DNA fragment was detected in RTF-1 immunoprecipitated chromatin. Together, these data indicated that RTF-1 up-regulated Edg-1 expression in endothelial cells by binding with Edg-1 promoter and increasing Edg-1 promoter activity.

Edg-1 Takes Part in RTF-1-driven Endothelial Cell Aggregation, Migration and Growth

To determine whether Edg-1 is involved in RTF-1 induced angiogenesis in endothelial cells, we transiently transfected Edg-1 siRNA into RTF-1 o/e HMEC-1 and control HMEC-1 and observed endothelial cell aggregation, migration and growth. As shown in Figure 3B, Edg-1 siRNA significantly decreased the aggregation area both in RTF-1 o/e HMEC-1 and control HMEC-1, while the inhibitory effect on tube formation was obvious in control HMEC-1 but not in RTF-1 o/e HMEC-1. Knockdown of Edg-1 also retarded cell migration in both RTF-1 o/e HMEC-1 and control HMEC-1 compared with negative control siRNA (Figure 3C). Crystal violet assay demonstrated that RTF-1 o/e HMEC-1 cell growth had been inhibited by Edg-1 siRNA after culture for 2 days (Figure 3D). These results showed that Edg-1 participates in RTF-1 stimulated angiogenesis in endothelial cells.

PTX Inhibited RTF-1 Driven Endothelial Cell Tube Formation and Migration

To further confirm that the Edg-1 post-receptor signaling pathway plays a role in RTF-1 stimulated endothelial angiogenesis, RTF-1 o/e HMEC-1 and control HMEC-1 cells were treated with or without PTX, a Gi/Go protein inhibitor. The result showed that PTX had an obvious inhibitory effect on tube formation and aggregation in both RTF-1 o/e HMEC-1 and control HMEC-1 at 6 h and 24 h Matrigel (Figure 4A). Furthermore, treatment with PTX showed a significant retardation of migration in RTF-1 o/e HMEC-1 and control HMEC-1 (Figure 4B).

Discussion

Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels. Sprout angiogenesis generates from the activation of endothelial cells by angiogenic growth factors. The activated endothelial cells proliferate into the surrounding matrix and form solid sprouts connecting neighboring vessels. After that, endothelial cells migrate in tandem and form loops to become a full-fledged vessel lumen. An increase in capillary network formation, endothelial cell proliferation and migration are the most important features of angiogenesis. We have observed an increase capillary formation at quadriceps [3] and heart [14] in VE-Cad/RTEF-1 mice compared with wild-type mice in our previous work. It demonstrates that RTF-1 has a proangiogenic effect in vivo. In this study we found that stable over-expression of RTF-1 cDNA in endothelial cells enhanced cell growth, migration and capillary network formation, while knockdown of RTF-1 by its siRNA slowed down these processes of angiogenesis. These data indicate that RTF-1 plays an important role in angiogenesis by activating endothelial cells in vivo.
1 receptor coupled pathways are involved in RTEF-1 driven angiogenesis is needed to be clarified.

Previous investigation revealed that Edg-1 coupled with members of the Gi family (sensitive to PTX) but not Gs, Gq, or G12/13 (insensitive to PTX) [18]. Observations also found that PTX suppressed endothelial cell DNA synthesis and migration in response to S1P, a specific ligand of Edg-1. It suggested that the activated Edg-1 function is mediated through PTX-sensitive Gi/Go –proteins [11]. Our results showed that PTX interrupted RTEF-1 o/e HMEC-1 capillary network formation and migration, indicating that PTX-sensitive Gi/Go -proteins coupled with the Edg-1 pathway are involved in RTEF-1 driven angiogenesis in endothelial cells.

In summary, we have shown for the first time that RTEF-1 binds to the Edg-1 promoter in mature endothelial cells, induces Edg-1 transcription, and is required for endothelial angiogenesis. Edg-1 is a new target gene of RTEF-1 involved in angiogenesis. Furthermore, PTX-sensitive Gi/Go -protein pathway coupled with Edg-1 was discovered to play an important role in RTEF-1 driven angiogenesis in endothelial cells. These findings would be helpful to elucidate the role of RTEF-1 as a transcription factor in angiogenesis and have important potential in development of...
agents capable of intervening endothelial angiogenesis through RTEF-1 during relevant pathophysiological events.

Supporting Information

Figure S1 DNA microarray of RTEF-1 gene knockdown by RTEF-1 siRNA in HUVEC. Compared to negative control siRNA, knockdown of endogenous RTEF-1 by three different duplexes of RTEF-1 siRNA in HUVEC decreased Edg-1 gene expression. Data was analyzed by Ingenuity Pathway Analysis software (Ingenuity System, Redwood City, CA).

(TIFF)

Figure S2 Cx43 (A) and Edg-1 (B) mRNA in RTEF-1 o/e and control HMEC-1s in 6 h and 24 h Matrigel. Total RNA was extracted from cell lysates obtained from dissolved cell networks in 6 h and 24 h Matrigel. Quantitative real-time PCR amplification was performed. The results were quantified based on networks in 6 h and 24 h Matrigel. Quantitative real-time PCR RNA was extracted from cell lysates obtained from dissolved cell and control HMEC-1s in 6 h and 24 h Matrigel.

References


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Author Contributions

Conceived and designed the experiments: PH JL. Performed the experiments: PH MJP. Analyzed the data: PH. Contributed reagents/materials/analysis tools: PH MJP XA JW AFM. Wrote the paper: PH MJP JL.