TIMAP promotes angiogenesis by suppressing PTEN-mediated Akt inhibition in human glomerular endothelial cells

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Obeidat M, Li L, Ballermann BJ. TIMAP promotes angiogenesis by suppressing PTEN-mediated Akt inhibition in human glomerular endothelial cells. Am J Physiol Renal Physiol 307: F623–F633, 2014. First published July 9, 2014; doi:10.1152/ajprenal.00070.2014.—The function of TIMAP, an endothelial cell (EC)-predominant protein phosphatase 1-regulatory subunit, is poorly understood. We explored the potential role of TIMAP in the Akt-dependent regulation of glomerular EC proliferation, survival, and in vitro angiogenesis. To deplete TIMAP, the EC were transfected with TIMAP-specific or nonspecific small interfering (si) RNA. The rate of electrical impedance development across subconfluent EC monolayers, a measure of the time-dependent increase in EC number, was 93 ± 2% lower in TIMAP-depleted than in control EC. This effect on cell proliferation was associated with reduced DNA synthesis and increased apoptosis. TIMAP silencing reduced 5-ethyl-2'-deoxyuridine incorporation by 38 ± 2% during the exponential phase of EC proliferation, and cleaved caspase 3 as well as caspase 3 activity increased in TIMAP-depleted relative to control cells. Furthermore, TIMAP depletion inhibited the formation of angiogenic sprouts by glomerular EC in three-dimensional culture. TIMAP depletion strongly diminished growth factor-stimulated Akt phosphorylation without altering ERK1/2 phosphorylation, suggesting a specific effect on the PI3K/Akt/PTEN pathway. Endogenous TIMAP and PTEN colocalized in EC and coimmunoprecipitated from EC lysates. The inhibitory PTEN phosphorylation on S370 was significantly reduced in TIMAP-depleted relative to control cells. Furthermore, TIMAP depletion inhibited the formation of angiogenic sprouts by glomerular EC in three-dimensional culture. TIMAP depletion strongly diminished growth factor-stimulated Akt phosphorylation without altering ERK1/2 phosphorylation, suggesting a specific effect on the PI3K/Akt/PTEN pathway. Endogenous TIMAP and PTEN colocalized in EC and coimmunoprecipitated from EC lysates. The inhibitory PTEN phosphorylation on S370 was significantly reduced in TIMAP-depleted compared with control EC, while phosphorylation of PTEN on the S380/T382/T383 cluster remained unchanged. Finally, the PTEN inhibitor bpV(phen) fully reversed the suppressive effect of TIMAP depletion on Akt phosphorylation. The data indicate that in growing EC, TIMAP is necessary for Akt-dependent EC proliferation, survival, and angiogenic sprout formation and that this effect of TIMAP is mediated by inhibition of the tumor suppressor PTEN.

endothelial cells; PTEN; protein phosphatase 1-regulatory subunit; PPP1R16B; apoptosis; electric cell-substrate impedance sensing

MECHANISMS THAT REGULATE ANGIOGENESIS have received massive attention since Folkman (15) first advanced the theory that tumor growth depends on new blood vessel formation. Angiogenesis involves the sprouting and elongation of new blood vessels from preexisting vessels followed by stabilization and vessel specification (28). This process is crucial for normal blood vessel formation and patterning during embryonic development, for postembryonic blood vessel repair (5), and remodeling in response to hypoxia, in the female reproductive cycle, and for the establishment of the placental vasculature. Pathological retinal and tumor vascularization also depend on angiogenesis. While endothelial cells (EC) at the tip of the angiogenic sprout determine the direction and pattern of new blood vessel growth, elongation of the new vessel stalk requires EC proliferation (13). A complex array of growth factors and their intracellular signaling systems regulate angiogenesis, including vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), the angiopoietins, and the transforming growth factor-β (TGF-β) family (28).

Precise regulation of EC proliferation depends, in part, on control over the phosphatidylinositol 3-kinase (PI3K)/Akt/phosphatase and tensin homolog deleted on chromosome 10 (PTEN) signaling cascade and is critical for the establishment of a normal vasculature and inhibition of pathological angiogenesis (29). Akt is a Ser/Thr kinase that regulates cell survival, proliferation, migration, and angiogenesis via phosphorylation of diverse downstream proteins (12, 19, 37). Constitutively active Akt induces abnormal blood vessel formation (41), and Akt-deficient mice exhibit impaired fetal development and vascularization (1, 59). Akt is activated by phosphorylation on T308 in its kinase domain by phosphoinositide-dependent kinase (PDK1) and on S473 in the regulatory domain by the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) (7, 25, 45, 55). These phosphorylation events occur at the plasma membrane after phosphatidylinositol (3,4,5) triphosphate (PIP3)-mediated recruitment of Akt and PDK1 (4). In turn, PIP3 is generated by PI3K-mediated phosphorylation of PIP2 in response to activation of receptor tyrosine kinases (2, 16). Activation of Akt is opposed by PTEN, which dephosphorylates PIP3 and consequently blocks Akt activation (8, 21). PTEN deletion in mice results in EC hyperpolarization and disordered angiogenesis (23). In patients with chronic kidney disease (CKD), hypoxic stimuli do not result in a sufficiently robust angiogenic response to produce collateral vessels that might mitigate ischemia. Instead, microvascular rarefaction in the peritubular compartment contributes significantly to renal fibrosis in many forms of progressive CKD, in transplant nephropathy, and in ischemic injury in the aging kidney. It has been postulated that failed angiogenesis may be central to progressive renal fibrosis (51). Furthermore, while EC sprouts invade the capillary cleft of developing nephrons during renal development (34), glomerular EC injury in glomerulonephritis does not lead to a sprouting angiogenesis response. Instead, the glomerular capillary loop number increases through intussusceptive growth (40, 56), proliferating glomerular EC remain confined within the existing glomerular capillary basement membrane, and angiogenic sprouts do not penetrate the mesangium. Finally, even though podocytes continually produce vastly more VEGF than any other differentiated cells in vivo (6, 49), podocyte-derived VEGF does not serve as a stimulus for sprouting angiogenesis in mature glomeruli, but instead is required for the formation and maintenance of a properly differentiated, fenestrated endothelium (14). Hence, controls over the angiogenic response in the kidney are more complex than those in

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F623
the hypoxic tumor environment and may not allow an angiogenic response in the face of hypoxia or injury.

TIMAP was first identified in glomerular EC and is highly expressed in peritubular vessels and glomeruli during kidney development. TIMAP is also highly enriched in proliferating EC in culture, where it localizes to the plasma membrane in EC projections (9). Based on its amino acid sequence and on structural modeling, TIMAP is a protein phosphatase 1 (PP1c)-regulatory subunit in the myosin phosphate-targeting subunit (MYPT) family (9, 22, 48). The C-terminal CAAX motif of TIMAP is modified by prenylation, leading to plasma membrane association (9, 30). In EC, TIMAP interacts directly with the non-integrin laminin receptor (LAMR1) and regulates its phosphorylation (30, 48). The LAMR1 is upregulated during EC proliferation and angiogenesis (35, 50) and is required for capillary formation in vitro and angiogenesis in vivo (18, 31).

TIMAP also regulates moesin phosphorylation in EC (11) and can dephosphorylate MLC2 in vitro (48), but its actual mechanism of action in EC is poorly understood.

In this study, we describe a previously unknown function of TIMAP in EC, namely that TIMAP expression is required for EC proliferation and angiogenesis (35, 50) and is required for in vitro angiogenic sprout formation. Our data suggest that TIMAP supports PI3K-dependent Akt phosphorylation by suppressing PTEN activity in glomerular EC.

MATERIALS AND METHODS

Reagents. All chemicals were of reagent grade and purchased from Sigma-Aldrich Canada unless otherwise specified. Polyclonal rabbit anti-TIMAP antibodies were prepared as previously described (9). Monoclonal mouse anti-β-tubulin AA2 (catalog no. 05–661) were from EMD Millipore (Billerica, MA). Monoclonal mouse anti-β-actin AC-15 (catalog no. A5441) were from Sigma (Canada). Rabbit anti-cleaved caspase 3 D175 (catalog no. 9661S), rabbit anti-phospho-Akt S473, 193H12 (catalog no. 40585), rabbit anti-phospho-Akt T308 (catalog no. 92755), monoclonal rabbit anti-phospho-P44/P42 MAPK T202/Y204 E10 (catalog no. 91065), and rabbit anti-P44/p42 MAPK (catalog no. 9102) antibodies were from Cell Signaling Technology (New England Biolabs). Monoclonal mouse anti-Akt, B-1 (catalog no. Sc-25928), mouse anti-phospho-Akt, A2B1 (catalog no. Sc-7974), rabbit anti-phospho-PENT S370 (catalog no. Sc-101787), and rabbit anti-phospho-PENT S380/T382/383 (catalog no. Sc-101789) antibodies were from Santa Cruz Biotechnology (Dallas, TX). Alexa Fluor 680 goat anti-mouse antibody for LI-COR imaging was from Molecular Probes (Eugene, OR). The IR Dye 800-conjugated A goat anti-rabbit (product no. 611–132–122) was from Rockland Immunocchemicals (Gilbertsville, PA). The PTEN inhibitor bpV(phen), catalog no. sc-221378, was from Santa Cruz Biotechnology. Small interfering (si) RNA transfection reagent HiPerFect (catalog no. 301705) was from Qiagen. Human recombinant proteins VEGF165A (GF094) and basic FGF (GF003) were from Milipore.

Cell culture. Primary human glomerular EC, free of mycoplasma contamination, were purchased from Angio-Proteomie (Boston, MA) and maintained in EGM-2 MV Bulletkit growth media (Lonza, Walkersville, MD). Cell passages 2–7 were used for the experiments, and cells were cultured in culture dishes coated with Quick Coating Solution cAP-01 from Angio-Proteomie. Human foreskin fibroblast (HFF1) cells were a gift from Dr. Allan Murray (Univ. of Alberta) and maintained in EGM-2 MV Bulletkit growth media (Lonza). Cells were cultured in culture dishes coated with 0.1% gelatin.

RNA interference. Nonspecific control siRNA (AllStars Neg, catalog no. 1027281, Qiagen) was used as a nonspecific siRNA. To silence endogenous glomerular EC TIMAP, TIMAP-specific siRNAs (Hs_PPP1R16B_5) targeting the PPP1R16B gene (TIMAP) were purchased from Qiagen: siRNA no. 1 (catalog no. SI0011468): target sequence: 5′-CAGGCAATTTCGTTCCTGGAA-3′; sense strand: 5′-GGCAAUUUGUCCUGAGAAATT-3′; antisense strand: 5′-UCCCAGGAAGAAAUGUGCTT-3′; and siRNA no. 2, (catalog no. SI03115469): target sequence: 5′-TCCGCTCAGCTTACCCAGTA-3′, sense strand: 5′-CGGUCAUGGUCCACAGGUATT-3′, antisense strand: 5′-UCAGGUGAGCUGACCGAGA-3′.

Glomerular EC were cultured 1 day before transfection at 50–60% cell density and transfected twice on consecutive days with 100 nM nonspecific control or TIMAP siRNA using HiberFect according to the manufacturer’s instructions. Fresh EGM-2 media was replaced 4–6 h after transfection. TIMAP protein expression was optimally inhibited by 72 h after the first transfection, and monitored by Western blotting. All experiments were started at 72 h after the first transfection.

DNA synthesis and cell proliferation. DNA synthesis was examined using a Click-iT 5-ethyl-1H-deoxyuridine (EdU) flow cytometry assay kit (Life Technologies, Burlington, ON), according to the manufacturer’s instructions. Briefly, subconfluent siRNA-transfected glomerular EC were incubated with 10 μM EdU in fresh EGM-2 media overnight. One plate of glomerular EC was cultured in EGM-2 media without EdU to provide a negative control. The EC were harvested using 0.05% trypsin EDTA, sedimented for 5 min at 2,000 rpm, and prepared for flow cytometry according to the kit’s instructions. Five thousand cells were examined for each condition using FACScalibur. To obtain a direct measure of cell number, human glomerular EC transfected with control or TIMAP-specific siRNA were plated on 24-well tissue culture plates at a density of 20,000 cells/well in complete media, and the cell number was determined daily in triplicate using a hemocytometer counting chamber.

Electrical cell-substrate impedance sensing. Glomerular EC were plated onto 8W10E electrical cell-substrate impedance sensing (ECIS) arrays (Applied Biophysics) in 400 μl EGM-2 medium at a cell density of 25,000 cells/well and incubated in humidified 5% CO2-air at 37°C. Electrical impedance was determined every 5 min using the ECIS model 1600 (Applied Biophysics).

Apoptosis. Staurosporine-stimulated apoptosis of EC was performed as described by Thuret et al. (52). Briefly, glomerular EC transfected with anti-TIMAP siRNA or nonspecific siRNA were plated into EGM-2 medium for 24 h. Staurosporine (250 nM) was added in DMSO (0.05%) or DMSO vehicle was added for 4–6 h in endothelial basal medium (EBM). Attached and floating cells were harvested into ice-cold medium followed by sedimentation for 2–5 min at 14,000 g in a refrigerated centrifuge (4°C). The pellet was then denatured in 2× Laemmli buffer at 99°C for 10 min followed by Western blot analysis for cleaved caspase 3.

The activity of caspase 3/7 was studied using an Apo-ONE homogeneous caspase-3/7 assay kit from Promega (Madison, WI), according to the manufacturer’s instructions. siRNA-transfected glomerular EC were treated with 200 nM staurosporine for 4 h in EBM. Attached and floating cells were harvested by scraping them into ice-cold medium, and sedimented for 2–5 min at 14,000 g in a refrigerated centrifuge (4°C). The cell pellets were then resuspended in EBM, and 40-μl aliquots of the cell suspension were distributed into individual wells of a 96-well plate. Forty microliters of caspase 3/7 reagent mix, containing the fluorogenic substrate, was added and mixed by gentlyagitating the plates. The assay plate was then covered and allowed to freeze at −80°C for 1 h. Kinetic analysis of caspase 3/7-mediated substrate cleavage was then detected using a Fluoroskan Ascent microplate fluorometer (Thermo Scientific) at wavelengths of 485/527-nm excitation/emission, with 200-ms integration at 30-min intervals for 20 h. The assay was done for triplicate wells for each condition. Background activity was determined using the same media: reagent ratios and volumes, but without cells.

3-Dimensional angiogenesis. Angiogenesis in vitro was performed as previously described by Nakatsu and Hughes (39). siRNA-transfected glomerular EC were harvested with trypsin/EDTA to make a
single-cell suspension and incubated with Cytodex 1 beads (GE Healthcare, Canada) at a density of 400 cells/bead for 4 h in humidified air-5% CO2. The beads were then resuspended in basolateral EGM containing 2 mg/ml fibrinogen and 0.15 U/ml aprotinin. Five hundred microliters of the fibrinogen/bead mixture was then added to individual wells of 24-well plates with thrombin (final concentration 0.625 U/ml) to initiate fibrin polymerization. Fibrin polymerization was allowed to proceed for 30 min. Five hundred microliters EGM-2 complete medium was then added to each well, followed by incubation in humidified air-5% CO2. Angiogenic sprouts were visualized 18 h later using a Leica DMRB microscope (Micsysystem, Ontario, Canada) at ×10 magnification. Images for 40 consecutive beads were obtained with a QImaging Retiga EX camera and evaluated using OpenLab Improvision software (Lexington, MA). Each data point represents the mean number of sprouts per bead, determined from 40 consecutive, unselected beads. Data from three independent experiments were used for statistical analysis. To determine the effect of TIMAP silencing on capillary tube formation, HFF1 cell feeder layers were plated on the surface of the fibrin gel, and angiogenesis was allowed to proceed for 5 days.

**Western blot analysis.** Glomerular EC monolayers were washed overnight at 4°C in 1:1 PBS to Odyssey blocking buffer, probed with specific primary antibodies at 4°C followed by incubation with the species-specific secondary antibody (Aves) overnight at 4°C. The beads were extensively washed by incubation with the specific primary antibodies at 4°C for 2 h followed by incubation with goat anti-chicken IgG-coated beads (Aves) overnight at 4°C. The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33). The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33). The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33). The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33). The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33). The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33). The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33). The soluble cell fraction was incubated with control chicken anti-IgY antibody or chicken anti-TIMAP antibody at 4°C for 2 h (33).

**Immunoprecipitation.** Endogenous TIMAP was immunoprecipitated from glomerular EC as previously described (33). Briefly, EC monolayers were was washed once with ice-cold PBS and harvested immediately in 200 μl hot × 2 Laemmli buffer and heat-denatured for 10 min at 99°C. Proteins were separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). Levels of total and phosphorylated proteins were detected by probing the membranes with the specific antibodies overnight at 4°C in 1:1 PBS to Odyssey blocking buffer, visualized using LI-COR Odyssey imaging (LI-COR Biotechnology, Lincoln, NE).

**TIMAP encoding adenovirus.** The DNA fragments of EGFP and EF1-GFP-TIMAP were amplified from the corresponding constructs in pEGFP-C1 vector by PCR using the following primers: forward (both constructs): 5'-CCAGGTACCTACCATGCGGACCAGTTGTGAG-CA-3', reverse (EF1): 5'-CCAGGTCACTAGTTAATGTTA-ACCTCGTGTACAGCCGAG-3', reverse (EGFP-TIMAP): 5'-CCAGGTACCTACCATGCGGACCAGTTGTGAG-CA-3'. The PCR products were subcloned into the KpnI/SalI site of pShuttle-CMV. Sequence fidelity was confirmed by sequencing for both constructs. The pShuttle-CMV-EGFP or pEGFP-TIMAP constructs were linearized by digestion with restriction endonuclease PmeI and then transformed into Escherichia coli (B5813) containing the adenoviral backbone plasmid pAdEasy-1 for homologous recombination. The recombinant plasmids were confirmed by PciI restriction endonuclease analyses. The linearized recombinant plasmid was transfected into the adenovirus packaging cell line HEK293A. The adenoviruses were propagated, harvested, purified, and the viral titer was established (24). For infection, glomerular EC were replated at 70% cell density 1 day before infection. They were infected with 20 MOI of adenovirus in complete EGM-2 media containing μg/ml polybrene. Experiments were performed 72 h after infection.

**Statistics.** Data are shown as means ± SE, and n represents the number of independent experiments. Statistical analysis was performed by Student’s t-test for comparison between two values and by one-way ANOVA for time course experiments. P values <0.05 were considered significant.

**RESULTS**

**Inhibition of TIMAP expression attenuates EC proliferation, survival, and angiogenesis.** To study the function of TIMAP, its expression in human glomerular EC was inhibited with two distinct TIMAP-specific siRNAs or nonspecific control siRNA. Western blot analysis (Fig. 1A) showed that TIMAP-specific siRNA reduced TIMAP protein abundance on average by 76 ± 7.7% (means ± SE, n = 3) relative to nonspecific siRNA. While EC transfected with control siRNA formed confluent monolayers in complete media 72 h after transfection, the cell density of EC transfected with TIMAP-specific siRNA remained sparse (Fig. 1B). Numerous rounded, nonadherent cells were observed in cultures transfected with TIMAP-specific siRNA, but not in cultures transfected with nonspecific control siRNA. We also used ECIS, which provides a measure of cell density when monolayers are subconfluent (57) to obtain an independent measure of cell number. The human glomerular EC transfected with control or TIMAP-specific siRNA were plated on 8W10E ECIS arrays at a density of 25,000 cells/well in complete media, and electrical impedance was monitored for 40 h. Inhibition of TIMAP expression reduced the rate of electrical impedance development, on average by 93 ± 2% (P < 0.05, n = 4) (Fig. 1C and D). Glomerular EC proliferation, determined by direct cell counting, was also significantly suppressed in cells transfected with TIMAP siRNA compared with control (nonspecific siRNA, Fig. 1E), indicating that inhibition of TIMAP expression suppresses EC proliferation and/or survival.

To determine whether TIMAP alters sprouting angiogenesis, the human glomerular EC were transfected with TIMAP-specific or nonspecific control siRNA and seeded on collagen-dextran (Cytodex 1) beads to produce confluent monolayers. The beads were suspended in fibrin gels and complete medium, and the number of angiogenic sprouts was quantified 24 h later. Angiogenic sprout formation was significantly lower in EC transfected with TIMAP-specific siRNA than in EC transfected with nonspecific siRNA (5.8 ± 1.3 vs. 10.2 ± 0.8 sprouts/bead, P < 0.05, n = 3) (Fig. 2, A and B). Angiogenic sprout length was also significantly reduced in glomerular EC transfected with TIMAP-specific siRNA (129.76 ± 6.69) compared with EC transfected with control siRNA (161.56 ± 1.27, P < 0.05) (Fig. 2C). Essentially, identical results were observed with the second TIMAP-specific siRNA2 (data not shown). As shown in Fig. 2D, glomerular EC formed capillaries with lumens (arrow) when angiogenesis was allowed to progress for 5 days. In glomerular EC transfected with TIMAP-specific siRNA, fewer capillaries were observed. These results are consistent with the reduced number and length of angiogenic sprouts observed at earlier stages of sprouting angiogenesis. These data are consistent with the observation in cultured EC monolayers that TIMAP depletion inhibits EC...
growth and/or survival and suggest that TIMAP is a positive regulator of EC angiogenesis.

To determine whether reduced TIMAP expression alters cell proliferation, we next quantified DNA synthesis by determining the incorporation of EdU using flow cytometry. In EC transfected with TIMAP-specific siRNA, EdU incorporation was 38% lower than in cells transfected with nonspecific control siRNA (Fig. 3A and B), indicating that DNA synthesis was attenuated when TIMAP expression was suppressed. Essentially identical results were obtained with the second, distinct TIMAP-specific siRNA2 (data not shown).

Given that the effect of TIMAP depletion on DNA synthesis was much less than the effect on electrical impedance development (Fig. 1, C and D), and since rounded cells were observed in the monolayer of EC transfected with TIMAP-specific siRNA (Fig. 1B), we determined whether TIMAP silencing may also accentuate EC apoptosis by evaluating the abundance of cleaved caspase 3 and caspase 3/7 activity. To sensitize cells to apoptosis, the EC were treated with staurosporine (200 nM, 4 h) or vehicle. Compared with EC transfected with nonspecific (scramble) or TIMAP siRNA (TIMAP), Values are means ± SE; n = 4 independent experiments. *P < 0.05.

Fig. 1. TIMAP deficiency impairs endothelial cell (EC) growth. A: TIMAP protein expression after transfection with TIMAP targeting small interfering (si) RNA1 (lane 2) and siRNA2 (lane 3) and nonspecific control siRNA control (scramble, lane 1). B: brightfield images of glomerular EC transfected with control (scramble) or TIMAP siRNA, taken at 0 and 72 h after transfection. C: electrical cell-substrate impedance sensing (ECIS) analysis of glomerular EC growth. The lines show time-dependent changes in electrical impedance across EC monolayer transfected with nonspecific control siRNA (scramble) or TIMAP siRNA (TIMAP). Values are means ± SE; n = 4 independent experiments. *P < 0.05. E: mean cell count of glomerular EC transfected with nonspecific control siRNA (scramble) or TIMAP siRNA (TIMAP) at 1, 2, 3, and 4 days after plating. The experiment was repeated two more times with similar results. Values are means ± SD of triplicate data points. *P < 0.05.
therefore evaluated whether TIMAP depletion alters the phosphorylation of Akt in EC. In human glomerular EC transfected with TIMAP-specific siRNA and cultured in serum-replete medium, there was a striking reduction of S473 Akt phosphorylation relative to that observed in cells transfected with nonspecific control siRNA (Fig. 4A). Total Akt abundance did not differ between EC transfected with TIMAP-specific or nonspecific siRNA, and the phosphorylation of ERK1/2 was similar in EC transfected with TIMAP-specific or nonspecific control siRNA (Fig. 4A). Conversely, Akt phosphorylation increased when wild-type TIMAP was overexpressed in glomerular EC using adenoviral gene transfer (Fig. 4B). The seemingly different basal pS473 Akt between Fig. 4A and B reflects the fact that exposure time of the blot in 4B was reduced to compensate for the massive increase pS473 Akt in adenoviral GFP-TIMAP-transfected cells. Phosphorylation of Akt on T308 was also significantly reduced in EC transfected with TIMAP-specific siRNA, compared with cells transfected with control siRNA (n = 4, P < 0.05) (Fig. 4C). In glomerular EC transduced with increasing MOI of GFP-TIMAP-adenovirus, a concentration-dependent increase in S473 and T308 Akt phosphorylation proportional to the level of overexpressed TIMAP was observed. No effect on S473 or T308 Akt phosphorylation was observed when EC were transduced with GFP-adenovirus (Fig. 4D). These results indicate that TIMAP depletion suppresses, and TIMAP overexpression stimulates, the PI3K/Akt pathway in glomerular EC.

Both FGF-2 and VEGF-A act on EC tyrosine kinase receptors, activating the PI3K/Akt and the ERK1/2 MAPK signaling pathways, and both control EC proliferation and survival. To determine whether TIMAP depletion alters FGF-2 and/or VEGF-A signal transduction in human glomerular EC, cells were made quiescent by withdrawing serum and were then stimulated with FGF-2 or VEGF-A followed by determination...
Fig. 3. TIMAP silencing impairs EC proliferation and increases caspase 3 activity. A: flow cytometry of 5-ethyl-2'-deoxyuridine (EdU) incorporation in glomerular EC transfected with nonspecific siRNA (scramble) or TIMAP siRNA (TIMAP). B: mean EdU incorporation. Values are means ± SE; n = 3 independent experiments. *p < 0.05. C, left: Western blot of cleaved caspase 3 in glomerular EC transfected with nonspecific siRNA (lanes 1 and 3) or TIMAP siRNA (lanes 2 and 4) without (lanes 1 and 2) or with (lanes 3 and 4) staurosporine (200 nM). Right: quantification of the fold-change in cleaved caspase 3 induced by staurosporine. Values are means ± SE; n = 3 independent experiments. *p < 0.05. D: quantification of the fold-change in caspase 3/7 activity in glomerular EC transfected with nonspecific siRNA (scramble) or TIMAP siRNA (TIMAP) in the presence of 200 nM staurosporine. Values are means ± SE; n = 3 independent experiments. *p < 0.05.

of Akt and ERK1/2 phosphorylation. Not shown, pilot studies revealed that FGF-2 (100 ng/ml)-mediated Akt phosphorylation was maximum after 5 min, while VEGF-A (100 ng/ml)-stimulated Akt phosphorylation peaked after 10 min. In human glomerular EC transfected with TIMAP-specific siRNA, both FGF-2 and VEGF-A stimulated S473 Akt phosphorylation, but Akt phosphorylation was consistently less than that in EC transfected with nonspecific siRNA (Fig. 4, E and F). Similar to the findings in EC cultured in replete media (Fig. 4A), total Akt abundance as well as ERK1/2 phosphorylation were unaffected by TIMAP silencing. These results indicate that activation of the PI3K pathway can occur in EC depleted of TIMAP but that T308 and S473 Akt phosphorylation is blunted when TIMAP is silenced.

Akt phosphorylation is restored by PTEN inhibition in TIMAP-deficient EC. Since Akt phosphorylation depends on PI3K-mediated production of PIP3, which is reversed by PTEN activation (8), we next determined whether TIMAP might suppress PTEN activity in EC. We have previously shown that TIMAP localizes to EC projections (9). In untransfected human glomerular EC, endogenous TIMAP and PTEN strongly colocalized at the tip of EC projections (Fig. 5A). Furthermore, endogenous TIMAP and PTEN could be coimmunoprecipitated from human glomerular EC lysates (Fig. 5B). PTEN activity and localization are regulated by phosphorylation on several Ser/Thr residues in the C-terminal tail (54). Since TIMAP is a PP1c-regulatory subunit, we determined whether TIMAP silencing alters PTEN phosphorylation. In human glomerular EC, TIMAP depletion resulted in significantly less PTEN phosphorylation on S370 (ratio phosphorylated PTEN:total PTEN; 0.56 ± 0.13 in control siRNA vs. 0.12 ± 0.01 in TIMAP siRNA-transfected cells) (P < 0.05, n = 5), while phosphorylation on S380/S382/S383 remained unchanged (Fig. 5C). The S370 residue of PTEN is phosphorylated by casein kinase 2 (CK2), and this phosphorylation reduces PTEN activity (36, 53). We therefore determined whether bpV(phen), a potent inhibitor of PTEN (32), relieves the effect of TIMAP silencing on Akt phosphorylation. Pilot experiments showed that bpV(phen) increased S473 Akt phosphorylation in a concentration-dependent fashion in TIMAP-depleted and in cells treated with control siRNA, with a maximal effect at a concentration of 20 μM (not shown). Treatment of glomerular EC with 20 μM bpV(phen) eliminated the inhibition of Akt phosphorylation by TIMAP depletion (Fig. 5, D and E). This...
Fig. 4. TIMAP silencing impairs Akt phosphorylation in EC. A, left: S473-Akt and ERK1/2 MAPK phosphorylation in glomerular EC transfected with nonspecific control siRNA (scramble) or TIMAP siRNA (TIMAP). Middle: mean ratio of pS473 Akt to total Akt. Values are means ± SE; n = 4 experiments. **P < 0.01. B, left: S473 Akt phosphorylation in glomerular EC expressing AdGFP-TIMAP [multiplicity of infection (MOI) = 20]. Values are means ± SE; n = 4 experiments. *P < 0.05. C, left: T308 Akt phosphorylation in glomerular EC transfected with nonspecific control siRNA (scramble) or TIMAP siRNA (TIMAP). Right: mean ratio of pT308 Akt to total Akt. Values are means ± SE; n = 4 experiments. *P < 0.05. D: S473 and T308 Akt phosphorylation in glomerular EC expressing increasing AdGFP control, or AdGFP-TIMAP at increasing MOI (0–20). A dose-dependent increase in S473 and T308 Akt phosphorylation is observed in EC transduced with AdGFP-TIMAP, but not AdGFP (representative of 2 experiments). E, top: S473 Akt and ERK1/2 phosphorylation as a function of time in glomerular EC transfected with nonspecific control siRNA (scramble) or TIMAP siRNA (TIMAP) and treated with 100 ng/ml VEGF-A. Bottom left: quantification of the ratio of pS473-Akt to Akt for 3 independent experiments. Values are means ± SE; n = 3. *P < 0.05.
finding suggests that impaired Akt phosphorylation in response to TIMAP silencing is due to PTEN activity.

DISCUSSION

This study describes a previously unknown function of TIMAP in EC. TIMAP silencing in glomerular EC impairs angiogenic sprout formation in vitro, an effect associated with reduced EC proliferation and increased caspase 3 activation. The mechanism by which TIMAP silencing alters EC growth is attributed to impaired Akt activation while the MEK/ERK cascade remains intact. We observed that TIMAP colocalizes with PTEN in EC protrusions and that TIMAP silencing suppresses the inhibitory S370 PTEN phosphorylation. Given that PTEN inhibition during TIMAP depletion rescues Akt phosphorylation, we postulate that TIMAP is an inhibitor of PTEN, in turn blocking Akt-driven EC proliferation and survival pathways.

The possibility that TIMAP supports EC proliferation in vitro was initially entertained because glomerular EC consistently failed to produce confluent monolayers in vitro after TIMAP silencing (Fig. 1B). That this effect is biologically significant is suggested by the observation that angiogenic sprout formation in vitro was also strongly inhibited by TIMAP silencing in glomerular EC (Fig. 2) and is consistent with our previous observations that TIMAP is expressed in developing blood vessels (9).

Angiogenic sprouting requires formation of guiding EC tip cells and elongation due to EC proliferation in the new vessel stalk (20). Stabilization of the newly formed vessel and inhibition of regression depends critically on EC survival (3). TIMAP silencing inhibits both EC proliferation and survival, shown by the findings that TIMAP depletion reduced DNA synthesis and increased caspase 3 cleavage as well as caspase 3 activity (Fig. 3).

Binding of VEGF, FGF-2, and other angiogenic agonists to their specific receptor tyrosine kinases results in activation of the Raf/MEK/ERK and the PI3K-Akt signaling cascades (38, 58). Both cascades are required for angiogenic sprout formation and for survival of EC in the newly formed vessel (38, 58). However, we observed that ERK1/2 phosphorylation was...
essentially unaffected by TIMAP depletion, whereas phosphorylation of Akt on S473 and T308 was profoundly reduced by TIMAP silencing in EC grown in serum-replete medium. This observation suggests that only the PI3K/Akt pathway is suppressed by TIMAP silencing while signaling via the Raf/MEK/ERK pathway remains intact. The findings that VEGF-A or FGF-2 fully activates ERK1/2 phosphorylation when TIMAP is silenced (Fig. 4) also imply that tyrosine kinase receptor activation by FGF-2 and VEGF-A remains intact when TIMAP expression is inhibited.

Akt activation requires its recruitment to the plasma membrane by PIP₃, which is generated by receptor tyrosine kinase-stimulated PI3K (45). PIP₃ is dephosphorylated by the powerful tumor suppressor PTEN, in turn inhibiting Akt recruitment and activation (8). Overexpression of PTEN in EC was previously shown to inhibit angiogenic sprout formation, and disordered angiogenesis due to hyperproliferation is observed when PTEN is deleted or mutated to reduce activity in EC (23). We found that TIMAP colocalizes with PTEN in EC projections and that some PTEN is coimmunoprecipitated with TIMAP from EC lysates. These observations suggest that TIMAP and PTEN can closely associate, although further studies will have to be done to determine whether there is any direct interaction. Nonetheless, a functional interaction between PTEN and TIMAP is evident as TIMAP silencing strongly reduced S370 phosphorylation of PTEN without altering phosphorylation of the S380/T382/T383 cluster or PTEN protein abundance.

PTEN activity is regulated at several levels, including protein expression, subcellular distribution, and phosphorylation on several Ser/Thr residues, especially in its C terminus (47). CK2 constitutively phosphorylates the C terminus of PTEN on residues S370, S380, T382, and T383 (53). Phosphorylation of these sites regulates PTEN activity by modulating intramolecular interactions (43), membrane localization, and proteasome-mediated degradation (44). Our results indicate that TIMAP does not alter PTEN protein abundance but that it specifically regulates PTEN phosphorylation on S370. The fact that the remaining CK2-dependent sites on PTEN are phosphorylated in TIMAP-deficient EC also implies that TIMAP is unlikely to interfere with CK2 kinase activity directly. A recent nuclear magnetic resonance-based study suggests that two temporally independent phosphorylation events, both mediated by CK2, serve to phosphorylate PTEN on the S380-S385 and S361-S370 clusters (10). Our findings suggest that TIMAP plays a role in the phosphorylation of the S361-S370 cluster, but not the S380-S385 cluster. The fact that Akt phosphorylation is strongly induced by the PTEN inhibitor bpV(phen) (Fig. 5D) is in keeping with constitutive PTEN activity in the cultured glomerular EC. Furthermore, since PTEN inhibition fully restores Akt phosphorylation in EC in which TIMAP was silenced, it seems plausible that TIMAP represses PTEN activity in EC and that depletion of TIMAP disinhibits PTEN.

TIMAP is a member of the MYPT family of PP1c-targeting subunits (33, 48). The question therefore arises whether the effects of TIMAP silencing observed here can be attributed to reduced TIMAP/PP1c phosphatase activity. If TIMAP/PP1c acted as a phosphatase toward Akt and/or PTEN, TIMAP silencing would have been expected to increase phosphorylation of these targets. However, T308 and S473 Akt as well as S370 PTEN phosphorylation were inhibited when TIMAP was silenced, indicating that these residues are not TIMAP/PP1c phosphatase targets. A limitation of this study is that we have not established whether TIMAP/PP1c is acting as a phosphatase on an unknown target that can suppress PTEN action. More work will be required to define the direct molecular target(s) of TIMAP in the PI3K/Akt/PTEN cascade.

Finally, in Dictyostelium cells undergoing chemotaxis, PI3K and Akt are found at the leading edge, segregated from PTEN, which is localized to the trailing edge (17). Also, during cytokinesis of dividing D. discoideum cells, PTEN and PI3K/AKT segregate (27), ensuring continuous, localized Akt activation. Furthermore, PTEN and myosin II colocalize under conditions of chemotaxis and cytokinesis in these cells (42). Since TIMAP is a membrane-associated MYPT family member that suppresses the action of PTEN on Akt in EC, it is conceivable that TIMAP, by controlling localized myosin II function, could spatially segregate PTEN from the PI3K/Akt in EC. Clearly, much more work will be needed to investigate this possibility.

TIMAP is an EC-predominant protein, expressed in cultured glomerular and aortic EC (9), in proliferating human umbilical vein EC (Obiedat M, unpublished observations), and in developing glomerular and in renal peritubular vascular EC in vivo (9). During renal development, angiogenic sprouts invade the capillary cleft of developing nephrons in response to cues from podocyte-derived VEGF. Taken together with the findings in this study, it is therefore plausible that TIMAP, by inhibiting PTEN, promotes EC proliferation and survival during glomerular capillary development. Since TIMAP is EC predominant, but not glomerular EC specific, and since PTEN is known to regulate angiogenesis (23), TIMAP may also inhibit PTEN during tumor angiogenesis. It is furthermore tempting to speculate that the limited angiogenic response to VEGF in glomeruli, and during progressive renal fibrosis in the renal interstitium, may be due, in part, to unopposed PTEN action in glomerular and peritubular EC.

In summary, the EC-predominant PP1c-regulatory subunit TIMAP is required for angiogenesis, at least in vitro. TIMAP is permissive for S473/T308 Akt and S370 PTEN phosphorylation in EC. Since TIMAP is expressed predominantly in EC, these findings raise the intriguing possibility that TIMAP might serve as a new target for antiangiogenic therapy in tumors. Conversely, if a mechanism could be found to activate TIMAP, it could enhance EC-specific proliferation in chronically hypoxic tissues, for instance during progressive renal fibrosis.

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Author contributions: M.O. and B.J.B. provided conception and design of research; M.O. and L.L. performed experiments; M.O., L.L., and B.J.B. analyzed data; M.O., L.L., and B.J.B. interpreted results of experiments; M.O. prepared figures; M.O. drafted manuscript; M.O., L.L., and B.J.B, approved final version of manuscript; B.J.B. edited and revised manuscript.

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