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Protein Phosphatase 1 Beta is Modulated by Chronic Hypoxia and Involved in the Angiogenic Endothelial Cell Migration

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Key Words
Protein phosphatase 1 • Endothelial cell migration • Angiogenesis • Hypoxia

Abstract
\textbf{Background/Aim:} Endothelial cell migration is required for physiological angiogenesis, but also contributes to various pathological conditions, including tumour vascularization. The mRNA expression of PP1c\(\beta\), the beta isoform of the catalytic PP1 subunit, was shown to be upregulated in chronic hypoxia. Since hypoxia is a major regulator of angiogenesis, the potential role of PP1c\(\beta\) in angiogenesis was investigated. \textbf{Methods:} We examined PP1c\(\beta\) protein level in pediatric heart following chronic hypoxia and found PP1c\(\beta\) upregulation in cyanotic compared with acyanotic myocardium. By treating HUVEC cells with hypoxia mimicking agent, PP1c\(\beta\) protein level increased with maximum at 8 hours. The effect of PP1c\(\beta\) pharmacological inhibition, knockdown and overexpression, on endothelial cell migration and morphogenesis, was examined using \textit{in vitro} wound healing scratch assay and endothelial tube formation assay. The PP1c\(\beta\) knockdown effects on F-actin reorganization (phalloidin staining), focal adhesion formation (vinculin) and focal adhesion kinases (FAK) activation, were evaluated by immunocytochemical staining and immunoblotting with specific antibodies. \textbf{Results:} PP1c\(\beta\) knockdown significantly reduces endothelial cell migration, but does not have any significant effect on endothelial tube formation. Endothelial cell migration in the knockdown group is restored to the control level upon consecutive transfection with PP1c\(\beta\) cDNA. PP1c\(\beta\) overexpression does not significantly affect endothelial cell migration. Furthermore, PP1c\(\beta\) knockdown induces profound cytoskeletal reorganization, loss of focal adhesion sites and impairment of focal adhesion kinases (FAK) activation. \textbf{Conclusions:} PP1c\(\beta\) is regulator of endothelial cell migration, which is critical in the angiogenic process. PP1c\(\beta\) inhibition reduces endothelial cell migration through focal adhesion turnover and actin polymerization pathways.
Introduction

Angiogenesis is a complex multistep process that involves endothelial cell (EC) proliferation, degradation of the basement membrane and extracellular matrix (ECM), EC migration and tube formation [1, 2]. These steps are required in the physiological angiogenic process, but also contribute to various pathological conditions, including tumor vascularization.

Protein phosphatase 1 (PP1) belongs to a major family of serine/threonine specific phosphatases and is universally expressed in all eukaryotic cells. The roles of PP1 have been well established in diverse cellular processes, including cell division, glycogen metabolism, mRNA splicing and apoptosis [3]. Furthermore, PP1 plays a role in multiple signaling pathways that are involved in angiogenic processes and its activity is likely mediated by VEGF-A-induced gene expression of phosphatase actin regulating protein-1 (Phactr-1) [4].

Each functional PP1 enzyme consists of a catalytic (PP1c) and regulatory/targeting (R) subunit. Three mammalian PP1c genes have been identified, encoding PP1α, PP1β/δ and PP1γ1 and PP1γ2, the latter two being splice variants of the PP1γ gene [5]. The roles of each isoform are yet to be determined. The low number of PP1c isoforms, and their high amino acid sequence identity, supports the notion that it is the R subunits that modulate substrate specificity of PP1c and target the catalytic subunits to particular subcellular localizations [5, 6, 7]. Therefore, the interactions between catalytic and regulatory subunits produce a variety of differentially localized holoenzymes and are fundamental for PP1 to achieve its required functional specificity.

PP1cβ is a specific isoform of the catalytic subunit of PP1 enzyme and its gene is localized to chromosome 2p23 in humans [8].

If the physiological PP1cβ activity contributes to the normal maintenance of some cellular functions such as endothelial cell barrier protection, cytoskeleton structure support [9, 10, 11] and DNA repair [12], its overexpression has been observed in certain cancers, like metastatic melanoma [13], malignant pancreatic tumors [14, 15], and breast cancers [16, 17]. Additionally, we have recently shown that hypoxia, a major regulator of angiogenesis [18], upregulates the mRNA of protein phosphatase type 1 beta (PP1cβ) in cyanotic children with tetralogy of Fallot (TOF) [19]. Furthermore, mRNA expression of HIF-1α, and the expression of its target genes VEGF and EPO (erythropoietin) is markedly increased in newborns with persistent pulmonary hypertension of the newborn (PPHN), and cyanotic congenital heart disease (CCHD) [20]. Since children with CCHD commonly present with widespread formation of collateral blood vessels [21], and VEGF is a potent stimulator of angiogenesis that is upregulated in hypoxia, PP1cβ mRNA upregulation in CCHD implies its potential role in vascular growth.

The finding that PP1cβ is also overexpressed in several cancers, and that angiogenesis is a hallmark of cancer development, with certain tumours developing a hypoxic microenvironment to further potentiate its vascularization and growth [22], suggest that PP1cβ might play an essential role in angiogenesis.

Hence, we investigated the role of PP1cβ in angiogenesis. We first showed that PP1cβ protein is upregulated in cyanotic compared with acyanotic myocardium. Then we showed that treating HUVEC cells with CoCl₂, a hypoxia mimicking agent, increases PP1cβ protein level in a time-dependent manner. By using different PP1 pharmacological inhibitors and through the knockdown of PP1cβ by siRNA transfection we found that PP1cβ downregulation significantly reduced endothelial cell migration in vitro, which was restored by the consecutive transfection with PP1cβ cDNA. No significant difference in endothelial tube formation between PP1cβ siRNA transfected HUVEC and the control group was observed, implying that PP1cβ is involved in the migration process of angiogenesis rather than in the subsequent step of tube formation. We also demonstrated that PP1cβ role in endothelial cell migration involves cytoskeleton reorganization through focal adhesions and focal adhesion kinase (FAK) signaling.
Materials and Methods

The collection of human right ventricle specimens used in this study, was approved by the North Somerset and South Bristol Research Ethics Committee (REC reference 07/H0106/172), The National Research Ethics Service, England. Parental informed written consent was gained for all patients. Twelve patients with a diagnosis of cyanotic (n=6) or acyanotic (n=6) Tetralogy of Fallot undergoing surgical repair at the Bristol Royal Hospital for Children were studied.

Immunohistochemistry

Right ventricular specimens were fixed in 4% paraformaldehyde, washed in PBS, and embedded in paraffin, and 4-μm sections were obtained. Immunohistochemistry was performed with the ABC-Kit from DakoCytomation (Glostrup, Denmark). Photos were taken at 20× magnification.

Cell culture

HUVECs were purchased from Lonza and were cultured at 37°C in humified 5% CO₂ in endothelial growth medium containing 2% FBS (Lonza, CC-4176). HUVECs between passage eight and twelve were used in the experiments. To mimic hypoxic conditions, CoCl₂ [23] was added to the culture medium (100 µM) for the indicated times.

In vitro DNA and siRNA transfection and co-transfection

After seeding in a 24-well plate, HUVECs (~ 35000/well) were allowed to settle for 24h (37°C, 5% CO₂). For PP1cβ overexpression, HUVECs were transfected with PP1cβ full-length cDNA encoding plasmid (1µg/well), pCMV-SPORT6.cdb vector (Thermo Scientific). The pmaxGFP plasmid (Green Fluorescent Protein, Amaxa) was used as a control. Transfection was performed using JetPEI™-HUVEC (PolyPlus Transfection) according to the manufacturer’s instructions.

For PP1cβ knockdown, HUVECs were transfected with PP1cβ siRNA (10 nM, Qiagen) and with scrambled siRNA (10 nM, Qiagen) using commercially available transfection agent INTERFERin (PolyPlus Transfection).

DNA/siRNA co-transfection was performed by siRNA transfection followed by DNA transfection 4 hours later. Twenty-four hours later, cells were subjected to migration and network formation assays, as below.

Viability assay

Viability and apoptosis of cultured cells in presence or absence of the PP1 inhibitor Calyculin (1nM, Sigma) was detected using the Calcein AM Cell Viability Assay Kit (Biotium) following manufacturer’s instructions. Briefly, after 6h treatment with Calyculin or vehicle, cells were incubated with a solution containing 2.0 µM Calcein AM and 4.0 µM EthD-III in PBS for 30 min at 37°C, 5% CO₂ and then visualized under inverted fluorescent microscope (Leica DM IRB). Live cells were distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the nonfluorescent cell-permeant calcein AM to the green fluorescent Calcein. Cell death was detected by the EthD-III dye, which enters dead cells, thereby producing a bright red fluorescence. Percentage of live and dead cells was achieved by counting the ratio of green/red positive cells to the total number of cells (given by the Hoechst staining of nuclei).

Proliferation assay

To assess the effect of Calyculin on proliferation, HUVECs were seeded onto 96 wells plate at increasing density and allowed to settle overnight (37°C, 5% CO₂). Then, cells were incubated with Calyculin 1 nM or vehicle for 18h, after which the old media was replaced with fresh warm media (100 uL/well) and the number of viable cells in proliferation assay was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation (MTS) Kit (Promega). 20µL of the MTS substrate was added to each well and the plate was incubated at 37°C for 3 hours, then the absorbance at 490 nm was recorded using an ELISA plate reader. The correlation coefficient of the curve was used to assess whether the absorbance proportionally correlated with the relative number of cells, as suggested by the Promega manufacturer’s instruction.
**Migration assay**

After reaching the confluence, the cell monolayer was scratched using a P1000 tip at the centre of the well. Then, the medium was exchanged to complete EGM with 2 mM of Hydroxyurea (Sigma) to induce growth arrest. In order to investigate the involvement of PP1 on cell migration, the assay was performed in the presence of the PP1 inhibitor Calyculin (1 nM, SIGMA), Okadaic Acid (1 nM, Enzo Life Sciences), Tautomycin (10 nM, Enzo Life Sciences) or vehicle, which were added 6h before the scratch.

The same protocol was performed with transfected HUVEC. 24h after siRNA and/or DNA transfection, confluent HUVEC monolayer was scratched using a P1000 tip at the centre of the well. Images were taken using a bright-field inverted microscope (at 10X). The percentage of gap closure was calculated using the Zeiss Axiovert software (Carl Zeiss Microscopy, Jena, Germany), measuring 5 distances per field of view of the leading edges at the time of the scratch and 24h later. Each experiment was performed in triplicate and repeated 3 times.

**In vitro matrigel assay**

Six hours Calyculin pre-treated HUVEC or 24 h siRNA transfected-HUVECs were seeded in 96 well plates, previously filled with 40 µL of complete or reduced-growth factors Matrigel (BD Bioscience) and placed at 37°C for 20 min to allow matrix solidification. HUVECs vehicle-treated or pre-treated with scrambled siRNA served as normalization control. For each condition, triplicate wells were set up and experiments were repeated in three independent experiments. The cells were carefully dispensed onto the solidified Matrigel and allowed to settle and form networks for 9h, after which pictures were taken using a bright-field inverted microscope. The length of network was quantified using the Image-Pro Plus software (Media Cybernetics, UK). All images were taken at 5X magnification.

**Staining for Cytoskeletal Proteins**

Control and PP1cβ siRNA transfected HUVECs were grown until approximately 60% confluence on glass coverslips. Then, cells were fixed at room temperature for 20 minutes in 4% paraformaldehyde and permeabilized for 10 minutes with 0.1% Triton X-100 in PBS, prior to incubation with primary antibodies provided by the Actin Cytoskeleton and Focal Adhesion Staining kit (Millipore). For F-actin staining, cells were incubated with tetrarhodamine isothiocyanate (TRITC)-Phalloidin, while for detection of focal adhesions, cells were immunostained, with anti-vinculin antibody, followed by incubation with FITC-conjugated anti mouse secondary antibody. Coverslips were mounted on glass slides with Vectashield Hardset Mounting Medium with DAPI (Vector Laboratories), and images were obtained using a confocal fluorescence microscope (Leica SP5) with a 63x oil immersion objective.

**Proteins extraction and Western Blotting**

Right ventricular specimens were homogenised in the presence of RIPA buffer, containing protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 (Sigma). HUVECs were washed with ice-cold PBS after wound healing scratch assay and removed by scraping in the presence of RIPA buffer containing the same protease phophatase inhibitors. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, UK). Detection of proteins by western blot analysis was done following separation of equal amount of cell extracts on 12% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane and probed with the anti PP1cβ (1:20000, Abcam), anti ph-FAK (1:1000, Cell Signaling) and anti FAK (1:1000, Cell Signaling) antibodies. GADPH (1:1000, Cell Signaling) or Laminin (1:1000 Cell Signaling) were used as loading control. For detection, secondary antibody goat anti rabbit (1:5000, Santa Cruz) was used and proteins were detected using an ECL™ detection system (Amersham, GE Healthcare).

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical analysis was performed using SigmaPlot 12.2. All data was tested for normality using Shapiro-Wilk test. Samples were analyzed by Student’s t test. Results were considered significant if p ≤ 0.05.
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Results

PP1cβ protein is upregulated in cyanotic compared with acyanotic heart

In a previous study [19], hundreds of genes were identified as differentially expressed in cyanotic versus acyanotic heart. In particular, a number of upregulated genes were involved in apoptosis, cell movement, morphogenesis, and development, indicating genetic remodeling of the cyanotic myocardium in response to chronic hypoxia. Among these, PP1cβ isoform was significantly upregulated in the cyanotic heart (3.8x fold change). Hence, we evaluated the protein levels of this gene in the myocardium of cyanotic and acyanotic children by Western blotting. As shown in Fig. 1 a, a significant increase of the protein level in the cyanotic, and therefore hypoxic tissue, was observed. By immunohistochemistry we confirmed the overexpression of PP1cβ protein in the cyanotic specimens (Fig. 1 b). Interestingly, the positive staining was located mainly in the endothelial wall of small vessels that perfuse the myocardium. This might explain why PP1cβ protein expression difference between cyanotic and acyanotic hearts wasn’t so great in the Western blotting experiment, as it reflected only the expression changes in endothelial cells.

In order to reproduce the hypoxic environment in vitro, HUVECs were incubated for different times with the hypoxia-mimicking agent CoCl$_2$. Cobalt has been widely used as a hypoxia mimic in cell culture and it is known to activate hypoxic signalling by stabilizing the hypoxia inducible transcription factor 1α (HIF1α) [24, 25]. As shown in Fig. 1 c, CoCl$_2$ time-dependently induced the expression of PP1cβ, with the highest expression observed at 8h.

Inhibition of PP1cβ activity by pharmacological and siRNA approach reduces HUVEC migratory capacity

To assess the role of PP1cβ in cell migration, we performed a scratch assay in presence of different protein phosphatase 1 and 2 (PP1 and PP2) pharmacological inhibitors. The pretreatment of HUVEC with Okadaic Acid reduced the percentage of gap closure after the wounding, although not significantly, likely because of its higher selectivity for the isoform 2 of the protein phosphatase [26]. However, with the more specific PP1 inhibitors Calyculin and Tautomycin, cell migration was significantly delayed, in comparison with untreated cells (Fig. 2 a-b), with Calyculin being more effective than Tautomycin. In order to ensure that the treatment did not impair the vital functions of the cells, viability and proliferation assays were performed in presence and absence of Calyculin. Exposure to calyculin did not...
affect cell viability, as the capacity of cells to enzymatically convert the nonfluorescent cell-permeable calcein AM to the green fluorescent calcein was unaltered in either the control or calyculin treated cells. The same ratio of dead cells was equally detected in both groups (Fig. 3 a-b). Likewise proliferation capacity was not affected by the pharmacological treatment, compared to the untreated cells (Fig. 3 c).

Since all of the available inhibitors non-specifically inhibit PP1 and PP2 activity [26], we used the siRNA approach to selectively knock down the expression of PP1cβ. Successful downregulation of PP1cβ protein level was determined following the scratch assay by using

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**Fig. 2.** PP1cβ pharmacological inhibition effect on HUVEC migration. A, Confluent HUVEC monolayers were incubated for 6h with Calyculin (1 nM), Tautomycin (10 nM) or Okadaic Acid (1 nM) and then scratched in the presence of Hydroxyurea (2 mM) to induce growth arrest. Phase contrast images were collected after 24h. All the inhibitors delayed HUVEC migration, with Calyculin and Tautomycin exerting a significant effect. B, Representative histograms indicating the percentage of gap closure: Values are means ± SEM of 3 independent experiments, *P<0.05.

**Fig. 3.** Calyculin treatment does not affect cell viability and proliferation. A, Simultaneous fluorescent imaging on live cells showed that the same ratio of viable (green) and dead (red) cells were detected in the untreated and calyculin-treated groups. B, Proliferation curve of control and calyculin-treated cells. The correlation coefficient of the line was > 0.98 in both groups, indicating a linear response between cell number and absorbance.
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Western blotting analysis (Fig. 4 a).

Wound healing scratch assay was repeated 24 h post transfection with PP1cβ siRNA or scrambled siRNA (control). The average wound closure at 24h following the scratch was still reduced in PP1cβ siRNA transfected group compared to the control (Fig. 4 b-c), even though the reduction was not as effective as the calyculin treatment, maybe due to the low transfection efficiency (30%). However, when PP1cβ expression was re-established by the subsequent transfection with PP1cβ cDNA encoding plasmid, the cell migration of the co-transfected HUVEC was restored nearly to the migration level observed in the control group (Fig. 4 d-f).

**PP1cβ is not involved in the endothelial tube formation process of angiogenesis**

Since angiogenesis is a complex process with multiple steps and PP1cβ is involved in the early phase of cell migration, we set out to investigate whether this protein phosphatase has also a role in the later process of tube formation. To this end, calyculin and vehicle pre-treated HUVECs were seeded onto complete (data not shown) or reduced-growth factors Matrigel matrix to allowed forming capillary-like structures. No difference between the experimental and control groups were found by quantifying the total tube length (Fig. 5 a-b). Similarly, transfection with PP1cβ siRNA showed no effect on HUVEC tube formation capability (Fig. 5 c-d).
Fig. 5. PP1cβ is not involved in the endothelial tube formation process of angiogenesis. A, HU-VECs pre-incubated with Calyculin (1nM, 6h) or vehicle (control) were plated onto Matrigel matrices, and endothelial tube formation assessed after 24h of incubation. Representative images of capillary-like structures from each group are shown. B, Graph shows average total tube length per well, data presented as mean±SEM (Student’s t test, p>0.05 vs. control, n=5). C, HU-VECs, pre-transfected with PP1cβ siRNA and scrambled siRNA (control), were plated onto Matrigel matrices, and endothelial tube formation assessed after 24h of incubation. Representative images of capillary-like structures from each group are shown. D, Graph shows average total tube length per well, data presented as mean±SEM (Student’s t test, p>0.05 vs. control, n=5).

Fig. 6. PP1cβ knockdown induces cytoskeletal reorganization in endothelial cells. A, Immunofluorescent staining of scrambled siRNA and PP1cβ siRNA HUVEC was performed, 24h after transfection, with TRITC-Phalloidin (red) and anti-vinculin antibody (green). Yellow arrows in scrambled siRNA cells point to focal adhesions anchoring to stress fibers. Compared with the control cells, PP1cβ-knockdown cells showed a dramatic loss of actin stress fibers and focal adhesion sites (vinculin staining). B, Immunoblot analysis of FAK and phosphorylated FAK in control and knockdown groups. In PP1cβ knockdown cells lysate, a reduction in Tyr-397 (FAK) was found, compared to the control cells lysate. Intensity levels of ph-FAK (Tyr-397) and total FAK were normalized to GADPH and ratio of normalized ph-FAK and FAK were plotted in arbitrary units.

PP1cβ knockdown induces morphological and cytoskeletal changes in endothelial cells
Since cell migration involves cytoskeleton reorganization through focal adhesions and focal adhesion kinase (FAK) signaling pathway, we examined the effect of PP1cβ knockdown on cell morphology and actin cytoskeleton arrangement, whose organization is essential to
give structural support to the cells and mediate intra- and extra-cellular movement. Confocal imaging revealed that, compared with controls, PP1cβ depletion resulted in a complete loss of the regular stress fibers organization, as detected by Phalloidin staining for the F-actin filaments. In addition, focal adhesions, that serve as nucleation sites for actin filaments and therefore support the cell structure, were examined in control and knockdown cells. The large pointed focal adhesions anchoring stress fibers, observed in the control group (detected by staining for vinculin), were completely lost in the PP1cβ silenced cells (Fig. 6 a). Furthermore, PP1cβ knockdown triggered a significant reduction in Tyr-397 phosphorylation of focal adhesion kinase (FAK), which is essential for FAK activation. These findings suggest a role for PP1cβ in the signalling pathway that drives cell adhesion and migration (Fig. 6 b-c).

Discussion

Angiogenesis refers to the formation of new capillaries from already existing vessels through a complex process that involves multiple steps, including degradation of ECM surrounding the parent vessel, migration and proliferation of endothelial cells, assembly of the new blood vessel, lumen formation and construction of the mural layer of the vessel wall [27]. This mechanism is essential during embryonic development and wound healing. However, if dysfunctional, it may have important pathogenic consequences, as reported in vascular diseases and cancer growth.

The current study aimed to determine the contribution of the PP1cβ isoform in the regulation of angiogenesis, through investigation of endothelial cell migration and tube formation in vitro.

Previous studies have reported PP1cβ localization to focal adhesions and association of PP1cβ with focal adhesion [28]. However no direct role for PP1cβ in cell migration has been shown. Focal adhesions are membrane-associated macromolecules involved in the linkage of integrin adhesion molecules to the actin cytoskeleton and are associated with protein complexes including vinculin, paxillin and focal adhesion kinases (FAK) [29, 30], all of which play a crucial role in cell adhesion and motility. In particular, FAK signalling pathway is essential for cell migration and its activation is orchestrated by a complex mechanism resulting from the interaction of several kinases and phosphatases [28, 31]. A previous study showed that PP1 is involved in FAK activation through its dephosphorylation of Ser-722 residue. This event led to the activation of FAK catalytic activity by increasing the phosphorylation of the Tyr-397 autokinase, ultimately resulting in FAK activation [28]. Our finding that PP1 knockdown reduced the phosphorylation status of FAK supports the above studies. FAK-mediated turnover of focal adhesions, together with p38-mediated actin polymerization, allow cell contraction through formation of stress fibres, consequently enabling endothelial cell migration [32]. Thus, FAK is a positive regulator of cell motility [33]. Whether PP1cβ is also involved in the endothelial tube formation phase of the angiogenic process was also assessed. Our results showed no effects of knocking-down PP1cβ on HUVEC tube formation. The presence of growth factors in either complete or reduced-growth factors Matrigel may explain the absence of effect in network formation. Different growth factors are involved in the tube formation of vascular endothelial cells, such as transforming growth factor-α (TGF-α) and -β (TGF-β), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) [34]. Besides such a variety of growth factors, laminin, which is one of the major vascular basement membrane components, is also shown to be a crucial factor in endothelial tube formation in cultured HUVECs [35]. Laminin is also the main component of Matrigel followed by collagen IV, heparin sulfate proteoglycans, entactin and nidogen [36]. These ECM proteins control vascular morphogenesis through specific integrin-dependent signaling pathways [37] that may counterbalance the effect of PP1cβ silencing, where PP1cβ may be part of an unrelated signaling pathway.
Conclusions

Our findings show for the first time that PP1cβ, the catalytic isoform of PP1 plays a role in endothelial cell migration, whereby PP1cβ knockdown and pharmacological inhibition reduce the migration of HUVEC cells in culture. The mechanism, by which PP1cβ regulates endothelial cell migration, involves the interplay of actin cytoskeleton proteins and focal adhesion molecules signaling. Our study helps elucidating endothelial cell migration mechanism, specifically in the context of cancer-induced angiogenesis, and highlights PP1cβ as a possible promising target for antiangiogenic therapy and inhibition of tumor growth.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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