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A motif within the N-terminal domain of TSP-1 specifically promotes the proangiogenic activity of endothelial colony-forming cells

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ABSTRACT

Thrombospondin-1 (TSP-1) gives rise to fragments that have both pro- and anti-angiogenic effects *in vitro* and *in vivo*. The TSP-HepI peptide (2.3 kDa), located in the N-terminal domain of TSP-1, has proangiogenic effects on endothelial cells. We have previously shown that TSP-1 itself exhibits a dual effect on endothelial colony-forming cells (ECFC) by enhancing their adhesion through its TSP-HepI fragment while reducing their proliferation and differentiation into vascular tubes (tubulogenesis) *in vitro*. This effect is likely mediated through CD47 binding to the TSP-1 C-terminal domain. Here we investigated the effect of TSP-HepI peptide on the angiogenic properties of ECFC *in vitro* and *in vivo*. TSP-HepI peptide potentiated FGF-2-induced neovascularisation by enhancing ECFC chemotaxis and tubulogenesis in a Matrigel plug assay. ECFC exposure to 20 μg/mL of TSP-HepI peptide for 18 h enhanced cell migration ($p < 0.001$ *versus* VEGF exposure), upregulated alpha 6-integrin expression, and enhanced their cell adhesion to activated endothelium under physiological shear stress conditions at levels comparable to those of SDF-1α. The adhesion enhancement appeared to be mediated by the heparan sulfate proteoglycan (HSPG) syndecan-4, as ECFC adhesion was significantly reduced by a syndecan-4-neutralising antibody. ECFC migration and tubulogenesis were stimulated neither by a TSP-HepI peptide with a modified heparin-binding site (S/TSP-HepI) nor when the glycosaminoglycans (GAGs) moieties were removed from the ECFC surface by enzymatic treatment. *Ex vivo* TSP-HepI priming could potentially serve to enhance the effectiveness of therapeutic neovascularisation with ECFC.

1. Introduction

Angiogenesis triggered by vascular injury or tissue ischemia is a multistep process involving the mobilisation, migration, proliferation, adhesion and differentiation of endothelial cells and the release of cytokines, soluble growth factors, proteases, and extracellular matrix proteins [1,2]. Endothelial progenitor cells (EPC) from bone marrow migrate to ischemic tissues and participate in neovascularisation [3], representing a potential tool for autologous cell therapy of vascular diseases such as heart and leg ischemia [4]. EPC targeting to sites of neovascularisation involves their migration, adhesion, and differentiation into mature endothelial cells [5,6]. Various cell populations play a role in

Abbreviations: EPC, endothelial progenitor cells; ECFC, endothelial colony-forming cells; FGF-2, basic fibroblast growth factor; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell; PBS, phosphate buffered saline; VEGF, vascular endothelial growth factor.

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angiogenesis [7,8], but only one subset, endothelial colony-forming cells (ECFC), has been shown to possess all of the characteristics of a true endothelial progenitor capable of forming neovessels *in vivo* [9].

Thrombospondin-1 (TSP-1) is a homotrimeric 180-kDa cell matrix protein first identified as a natural inhibitor of angiogenesis. The anti-angiogenic activity of TSP-1 is mainly exerted through the binding of type I repeats (TSR) to the CD36 receptor on microvascular cells and also by its pro-collagen homology domain [10,11]. The C-terminal domain of TSP-1, which contains the integrin-associated protein/CD47-binding sites, may also play a role [12]. However, there is growing evidence that TSP-1 also possesses pro-angiogenic properties both *in vitro* and *in vivo*. In particular, TSP-1 expression is enhanced at the sites of vascular injury [13]. In addition, we have previously reported that TSP-1 is expressed in newly formed vessels in patients with peripheral arterial disease who received local injections of bone marrow mononuclear cells, suggesting a possible modulation of tissue ischemia by TSP-1 [14]. We also found that TSP-1 had a dual effect on ECFC: its N-terminal domain enhanced ECFC adhesion, while the whole protein reduced ECFC proliferation and differentiation into vascular tubes *in vitro*. These last effects were most likely mediated by CD47 binding to the TSP-1 C-terminal domain [14].

We and others have independently attributed the pro-angiogenic activities of TSP-1 to its N-terminal heparin-binding domain or HBD [15–20] through its binding to several receptors including calreticulin/LDL-related receptor [15], integrins $\alpha_3\beta_1$ [16], $\alpha_4\beta_1$ [17], and $\alpha_6\beta_1$ [18] and, as reported by our group, the cell-surface heparan sulfate proteoglycan (HSPG) syndecan-4 [19]. We characterised two specific motifs within the HBD of TSP-1, namely TSP-HepI/A1 (spanning residues 17–35) and TSP-HepII/A2 (spanning residues 78–94), that can interact with mature human umbilical cord endothelial cells (HUVEC) and induce their differentiation into vascular tube-like structures when immobilised in fibrin matrices [20] or Matrigel [19]. These pro-angiogenic peptides both exhibit high affinity for glycosaminoglycans (GAG) and interact with syndecan-4 [19].

In vitro studies have shown that the HBD is readily released from TSP-1 and can be detected in platelet aggregation supernatants [21,22] as well as in endothelial cell-conditioned medium [22,23]. Thrombin, plasmin, cathepsins and matrix metalloproteinases, specifically ADAMTS1, cleave TSP-1 and release its HBD *in vitro* [21,23,24]. A 25-kDa fragment of the HBD has also been shown to induce angiogenesis *in vivo* (rabbit cornea model) by enhancing the effect of FGF-2 [25].

In keeping with our finding that recombinant human TSP-1 acts as an adhesion molecule for ECFC and reduces ECFC proliferation and differentiation into vascular tubes, we subsequently showed that under the same experimental conditions, TSP-HepI peptide, mimicking part of the TSP-1 N-terminal domain, strongly increased ECFC adhesion similarly to TSP-1, but without affecting cell proliferation [14]. The aim of the present study was to investigate the effects of TSP-HepI peptide on the angiogenic properties of ECFC *in vitro* and *in vivo*.

2. Material and methods

2.1. Animals

Animal care conformed to French guidelines (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture, Paris, France), and all experiments were performed in accordance with the guidelines set by the Université Paris Descartes and the Institutional Committee on Animal Care and Use (C75.06.02).

2.2. Reagents

Recombinant human TSP-1 was purchased from R&D Systems Europe (Lille, France, 3074-TH-050). The peptides TSP-HepI and S/TSP Hep I, derived from the N-terminus of TSP-1 and modified at amino acid positions essential for GAG binding, were synthesised at the Department of Biophysics at UNIFESP (Federal University of São Paulo, Escola Paulista de Medicina, Brazil) using an automated bench-top simultaneous multiple solid-phase peptide synthesiser (PSSM 8 System; Shimadzu, Tokyo, Japan), followed by HPLC purification. The molecular weight was determined by MALDI-TOF mass spectroscopy, and the sequences were verified with a PPSQ-23 (Shimadzu) sequencer. Matrigel was purchased from Becton-Dickinson Biosciences (BD; Le Pont de Claix, France). Basic fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), and stromal cell-derived factor-1 alpha (SDF-1α) were purchased from R&D Systems Europe (Lille, France). Heparinase II (Heparin lyase; EC4.2.2.7), heparinase III (HS lyase; EC4.2.2.8), and chondroitinases ABC (chondroitinases ABC lyase; EC4.2.2.4) were purchased from Sigma–Aldrich (Lyon-Saint Exupéry, France). R-PE-conjugated monoclonal antibody directed against α6-integrin (CD49f, clone G0H3), R-PE-conjugated mouse anti-human IgG_2a, secondary R-PE-conjugated anti-mouse and R-PE conjugated mouse anti-human IgM were purchased from BD Biosciences (Le Pont de Claix, France). Monoclonal antibody directed against human heparan sulfate (clone F-58-10E4) was purchased from AMS Biotechnology (Abingdon, UK). R-PE-conjugated monoclonal antibody directed against the ectodomain of human syndecan-4 (SDN4, clone 5G9) and mouse anti-human IgG_2a were purchased from Santa Cruz Biotechnology (Tebu-Bio, Le Perray en Yvelines, France).

2.3. Cell isolation, culture and pretreatment

Umbilical cord blood was collected with the mothers' consent ($n = 20$). The study was approved by the ethics committee of Hôpital des Instructions et des Armées de Begin (France) (201008043234797), and the protocol conformed to the ethical guidelines of the Declaration of Helsinki. ECFC were isolated from human umbilical cord blood, expanded and characterised as previously described [26]. HUVEC were isolated by enzymatic digestion as previously described [27]. Endothelial cells were defined as being positive for both DiI-AcLDL uptake (Invitrogen, Molecular Probes, Saint Aubin, France) and BS-1 lectin binding (Sigma–Aldrich, Saint-Quentin Fallavier, France). Expression of the following cell-surface antigens of the endothelial lineage was assessed by FACS analysis (FACSCalibur, Becton Dickinson, BD Biosciences, Le Pont de Claix, France): CD31 and CD34 (Immunotech, Marseille, France), Tie-2 and Flt-1 (BD Biosciences, Le Pont de Claix, France), CD144 (Tebu-Bio, Le Perray en Yvelines, France) and KDR (Sigma–Aldrich, Saint-Quentin Fallavier, France). One day before experiments, cells were growth-arrested for 18 h in EBM-2 with 2% FCS and released from growth arrest by adding EBM-2 with 5% FCS, with or without 20 μg/mL of TSP-HepI, for 18–20 h at 37 °C. They were then washed, detached with accutase (Sigma–Aldrich, Saint-Quentin Fallavier, France) and washed twice with Hank's buffered salt solution with 0.5% BSA before use in angiogenesis assays *in vitro*. All assays were performed in triplicate with cells cultured for less than 30 days.

2.4. Migration assay

A directional migration (chemotaxis) assay was carried out in modified Boyden chambers as previously described [26]. The choice of an optimal dose, 20 μg/mL (10 μM), was based on preliminary assays performed with HUVEC and ECFC. All

conditions used in a given experiment were tested in triplicate. A migration index was calculated as the ratio of the number of cells that migrated in the test conditions, as compared to the control (M199 supplemented with 2% BSA).

2.5. Shear-flow adhesion assays

Flow adhesion experiments were conducted with a parallel-plate flow chamber in physiological shear stress conditions as previously described [28]. HUVEC monolayers (3×10^5) were seeded on cover slips, maintained at 37 °C for 4 days, placed in the flow chamber, and then stimulated by exposure to a shear rate of 50 s^{-1} for 30 min. To distinguish between adherent pre-treated ECFC and detached endothelial cells (HUVEC), the ECFC were stained with calcein (Interchim, FluoroProbes, Montluçon, France). The calcein-labelled ECFC (3×10^6) in adhesion buffer (cation-free HBSS, 10 mmol/L HEPES, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 2 mg/mL BSA, pH 7.4) were perfused over HUVEC monolayers for 15 min at 37 °C at a shear rate of 50 s^{-1}, and the coverslips were washed with adhesion buffer for 10 min. Adherent cells were examined by phase-contrast microscopy. All experiments were observed in real-time and videotaped for offline analysis. Fluorescence micrographs of 40 random microscopic fields (10 × objective) were collected with Replay software (Microvision Instruments, France). Data were expressed as the number of adherent cells per field. The results from three different experiments were pooled for each study. Adherent ECFC were tested for resistance to detachment from the model endothelium by increasing the flow rate from 50 to 5000 s^{-1} for over one minute and counting the number of remaining adherent cells at one-minute intervals.

2.6. FACS analysis

Cell-surface antigen expression on TSP-HepI-treated and untreated ECFC was analysed after immunofluorescent labelling in an FACS-SORT flow cytometer (BD Biosciences). Labelling with mouse anti-human heparan sulphate antibody (1 μg/mL) was visualised with a PE-conjugated anti-mouse antibody. A PE-conjugated anti-α6 antibody was used to visualise human α6-integrin. In each immunofluorescence experiment, an isotype-matched IgG antibody was used as a control, and the fluorescence intensity of stained cells was gated according to established methods. Data were analysed with CellQuestTM software (BD Biosciences).

2.7. In vitro *tube formation assay*

Basement-membrane gels used for three-dimensional assays were prepared by Matrigel polymerisation (8 mg/mL) for 30 min at 37 °C. When required, 10 μg/mL of TSP-HepI were included in the gels before polymerisation. ECFC (10^5 cells/cm^2) diluted in EBM-2 containing 2% FCS were seeded and allowed to form pseudotubes for 6 h at 37 °C with 5% CO_2. The cells were then fixed with 1.1% glutaraldehyde for 15 min and stained with Giemsa. The total length of the tube structures was quantified with Videomet software (Microvision Instruments, France). The results are reported as the average of three different experiments in each condition. When appropriate, the ECFC were pretreated for 2 h at 37 °C with a mixture of 0.5 U/mL heparinase I, 0.1 U/mL heparinase III and 0.2 U/mL chondroitinases ABC before the inclusion of TSP-HepI in the Matrigel.

2.8. Murine angiogenesis assay

Ice-cold Matrigel (8 mg/mL) mixed with PBS and FGF-2 (350 ng/mL), alone or supplemented with TSP-HepI peptide (200 μg/mL), was prepared and maintained in liquid form at 4 °C. The solution was injected subcutaneously in C57Bl/6 mice (8 weeks old, from Janvier, France), and the Matrigel plug was recovered 14 days later. Haemoglobin content was measured as previously described [29]. Functional vessels were identified as vessels containing red blood cells by light microscopy.

2.9. Statistical analysis

Significant differences between mean values were identified by ANOVA with the Tukey post-test for multiple comparisons. The results are expressed as the mean ± SEM of at least three experiments. *P*-values less than 0.05 were considered significant.

3. Results

The ECFC were isolated from human umbilical cord blood on the basis of CD34 expression. The presence of Weibel–Palade bodies and the combined expression of endothelial markers (CD31, Tie-2, KDR, Flt-1, CD144) unequivocally confirmed the endothelial phenotype of the isolated ECFC (data not shown). Furthermore, the ECFC did not express leuko-monocytic markers such as CD14 and CD45 [14].

3.1. TSP-HepI peptide enhances FGF-2-induced neoangiogenesis in vivo

To evaluate the proangiogenic potential of TSP-HepI *in vivo*, we used a mouse model of implanted Matrigel plugs. In this model, host endothelial cells and ECFC, attracted by growth factors such as FGF-2 and/or peptides included in the gel, migrate into the implanted plugs and form a capillary network within two weeks [30]. Matrigel (0.5 mL) containing PBS (control), TSP-HepI alone (200 μg/mL), FGF-2 (350 ng/mL) alone, or a mixture of TSP-HepI and FGF-2 was injected into the flank of C57BL/6 mice. As shown in Fig. 1A and B, plugs from the PBS control group and plugs containing TSP-HepI alone were mostly translucent and pale in colour, indicating little or no vessel formation after two weeks. In contrast, plugs containing FGF-2 alone were redder (Fig. 1C), indicating new vessel formation. Surprisingly, plugs treated concurrently with FGF-2 and TSP-HepI had an intense red colour, indicating the presence of abundant new capillary vasculature formation (Fig. 1D). An analysis of haemoglobin content confirmed that TSP-HepI and FGF-2 together enhanced neoangiogenesis compared to TSP-HepI alone ($p < 0.001$, Fig. 1E). Thus, TSP-HepI peptide appeared to enhance the FGF-2-induced recruitment of circulating cells, suggesting a synergistic effect on angiogenesis *in vivo*.

3.2. TSP-HepI and HSPG promote ECFC chemotaxis and tubulogenesis

Having previously shown that TSP-1 is present at sites of neovascularisation [14] and that TSP-HepI potentiates FGF-2-induced neoangiogenesis, we hypothesized that the local release of TSP-1 N-terminal domain fragments by protease cleavage might participate in the recruitment of circulating progenitor cells and in their differentiation into mature endothelial cells at neovascularisation sites. Therefore, we first examined whether the TSP-HepI peptide promoted ECFC chemotaxis by a standard migration assay. We found that the TSP-HepI peptide (20 μg/mL) significantly enhanced ECFC migration (Fig. 2) to a similar extent as the proangiogenic chemotactic factor FGF-2. Under the same experimental conditions, we showed that the S/TSP-HepI peptide, which was modified in the GAG-binding consensus motif, does not have any effect (Fig. 2). We then evaluated the effect of TSP-HepI on ECFC tubulogenesis *in vitro*. While capillary-like tube formation

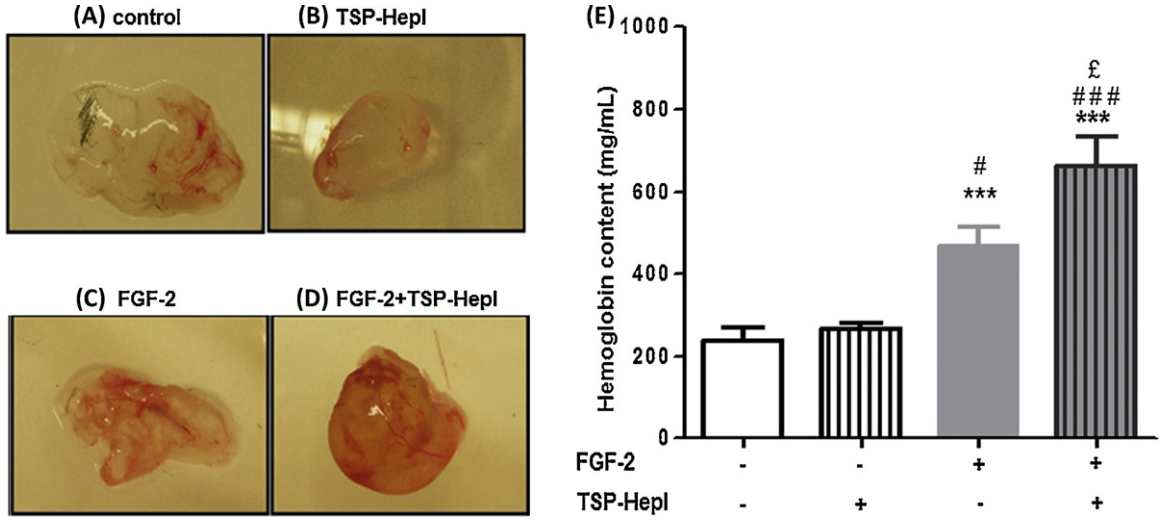

Fig. 1. TSP-HepI enhances FGF-2-induced angiogenesis *in vivo*, in a mouse model of Matrigel plug assay. (A–D): Representative photographs of plugs excised on day 14 containing (A) PBS, (B) TSP-HepI alone (200 μg/mL), (C) FGF-2 (350 ng/mL) alone or (D) FGF-2 (350 ng/mL) + TSP-HepI (200 μg/mL). (E) Haemoglobin quantification: values represent mean ± SEM of the haemoglobin content in Matrigel plugs excised from C57Bl/6 mice ($n = 10$ per experimental group); ** $p < 0.01$ and *** $p < 0.001$ *versus* PBS; # $p < 0.05$, ### $p < 0.001$ *versus* TSP-HepI and £ $p < 0.05$ *versus* FGF-2.

was enhanced when 10 μg/mL of TSP-HepI was added to the Matrigel prior to polymerisation, TSP-1 significantly inhibited capillary-like tube formation under the same conditions (Fig. 3A). Because we had previously shown that the effect of TSP-HepI on HUVEC tubulogenesis involved HSPG syndecan-4 binding [19], we then examined whether the proangiogenic effects of TSP-HepI on ECFC involved GAG binding. The ECFC were incubated for 2 h at 37 °C with enzymes that selectively removed heparan and chondroitin sulfates (Fig. 3B), and then they were seeded on the Matrigel containing TSP-HepI peptide (Fig. 3C). As shown in Fig. 3A and 3C, ECFC treatment with enzymes that specifically removed heparan sulfate reduced tube formation in the Matrigel by 45% ($p < 0.001$). Similarly, ECFC treatment with the same enzymes reduced tubulogenesis in TSP-HepI-containing Matrigel by 72% ($p < 0.001$). Our S/TSP-HepI peptide reduced pseudotube formation by 30% ($p < 0.01$) under the same experimental conditions, as compared to TSP-HepI (Fig. 3A). Thus, the interaction of TSP-HepI peptide with HSPG seems essential for the promotion of ECFC migration and tubulogenesis, likely through the activation of specific HSPG-dependent signalling pathways.

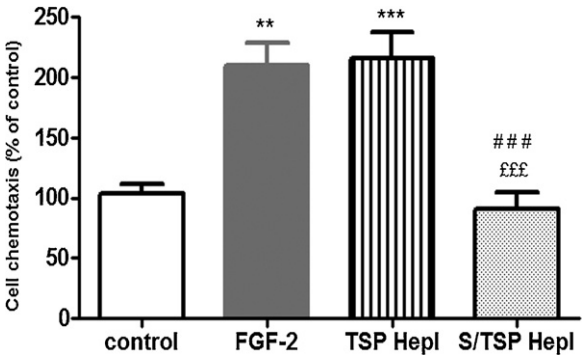

Fig. 2. TSP-HepI stimulates ECFC chemotaxis. Migration assays were performed using chemotaxis chambers, towards M199/2% BSA (control medium), FGF-2 (10 ng/mL, positive control), TSP-HepI (20 μg/mL) or S/TSP-HepI peptide (20 μg/mL) in the lower chamber. ECFC (7×10^4) were seeded in the upper chamber and incubated for 6 h at 37 °C. Data are expressed as a percentage of the control group value. Values are a mean ± SEM of six determinations. ** $p < 0.01$ and *** $p < 0.001$ *versus* control, £££ $p < 0.001$ *versus* FGF-2 and ### $p < 0.001$ *versus* TSP-HepI.

3.3. TSP-HepI peptide preconditioning promotes ECFC adhesion to activated HUVEC monolayers under physiological shear stress conditions

We then investigated whether TSP-HepI had a direct effect on ECFC adhesion to endothelium, which is one of the key steps of endothelial progenitor recruitment to ischemic sites. Prior to adhesion, the ECFC were incubated overnight in EBM-2 with 2% FCS and then stimulated for 18 h with various concentrations of TSP-HepI in medium supplemented with 5% FCS. We used a flow-based adhesion assay using HUVEC monolayers to investigate the binding of TSP-HepI-stimulated ECFC to activated endothelium. The experimental conditions mimicked the shear forces encountered by ECFC adhesion to vascular endothelial cells *in vivo*. In pilot experiments, we found that TSP-HepI at 20 μg/mL resulted in the greatest adhesion to activated endothelium, under shear stress conditions (Fig. 4A). Compared to control cells, ECFC pretreated with TSP-HepI adhered more rapidly to HUVEC (Fig. 4B), while TSP-1 pretreatment did not affect ECFC adhesion. As shown in Fig. 4C, TSP-HepI treatment increased the percentage of adhered ECFC (265% treated *versus* 100% control after 10 min; $p < 0.001$), a result similar to what was observed with SDF-1 pretreatment. Only 110% of TSP-1-treated ECFC adhered under the same conditions ($p < 0.01$; 100% of control) (Fig. 4C). Additionally, TSP-HepI-stimulated ECFC adhered more strongly and were more resistant to washing than control untreated cells at shear rates of up to 2000 s^{-1} (Fig. 4D).

3.4. Syndecan-4 is involved in TSP-HepI-stimulated ECFC adhesion to HUVEC monolayers

To assess the possible role of syndecan-4 (SDN4) in the effects of TSP-HepI under dynamic flow conditions, we first used FACS analysis to examine SDN4 surface expression on ECFC. As shown in Fig. 5A (right panel), ECFC expressed similar SDN4 levels as HUVEC (Fig. 5A, left panel). We then pre-incubated ECFC with anti-SDN4 or control antibody prior to TSP-HepI stimulation and adhesion assay. The ECFC stimulated with TSP-HepI adhered similarly to the previous experiment (Fig. 5B and C), and anti-SDN4 preincubation reduced their adhesion by 84% ($p < 0.001$, Fig. 5C). The control antibody had no significant effect on ECFC adhesion after TSP-HepI stimulation (Fig. 5B and C).

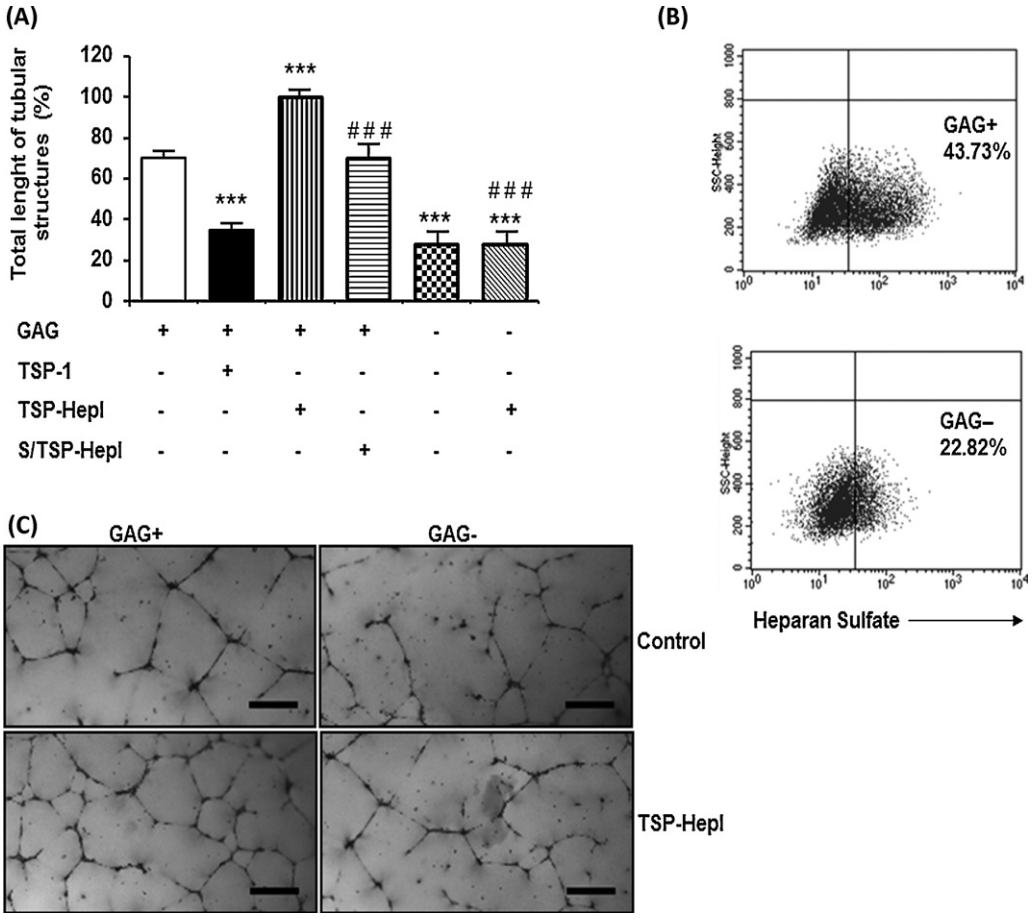

Fig. 3. TSP-HepI-HSPG interaction mediates vascular tube formation by ECFC. ECFC treated with a cocktail of heparinases and chondroitinases (GAG−) or untreated (GAG+) were seeded (1×10^5 cells/cm^2) in Matrigel containing 10 μg/mL of TSP-HepI. In addition, ECFC (1×10^5 cells/cm^2) untreated (GAG+) were seeded in Matrigel mixed with TSP-1 (10 μg/mL) or S/TSP-HepI peptide (10 μg/mL). (A) Quantification of tubular structures in Matrigel. Data are expressed as a percentage of the TSP-HepI group value. Values are a mean ± SEM of three determinations. *** $p < 0.001$ *versus* control, ### $p < 0.001$ *versus* TSP-HepI. (B) Flow cytometry analysis of heparan sulfate expression on (GAG+) and (GAG−) ECFC surface. (C) Morphological aspect of Matrigel assays after 6 h, for untreated (GAG+) and treated (GAG−) ECFC, in the presence or absence of TSP-HepI.

3.5. TSP-HepI preconditioning enhances ECFC expression of the α6-integrin subunit.

We previously reported that increased vascular tube formation by HUVEC is associated with α6-integrin subunit overexpression [26]. Furthermore, we demonstrated that α6-integrin plays a major role in the proangiogenic properties of ECFC [31]. Therefore, we suspected that α6 subunit modulation might also occur after ECFC stimulation with TSP-HepI. We used flow cytometry to measure α6-subunit surface expression on ECFC before and after 18 h of TSP-HepI stimulation (Fig. 6). Expression of the α6-subunit measured by fluorescence intensity was 1.5-fold higher on the TSP-HepI-stimulated ECFC than on the control ECFC ($p < 0.001$), suggesting that TSP-HepI might also enhance the proangiogenic properties of ECFC by modulating α6-subunit expression. FGF-2 treatment, also used in this analysis as a positive control for α6-integrin upregulation [26], led to a near three-fold increase of α6 integrin expression on ECFC surface.

3.6. TSP-HepI preconditioning enhances ECFC motility

Finally, we examined the effect of TSP-HepI pretreatment for 18 h on ECFC motility. As shown in Fig. 7, TSP-HepI-treated ECFC migrated more rapidly than untreated ECFC and approximately twice as fast as VEGF-pretreated ECFC ($p < 0.001$). To determine the role of GAG in this effect, we used the S/TSP-HepI peptide devoid of the GAG-binding consensus motif. Indeed, the S/TSP-HepI-stimulated ECFC had a reduced migratory capacity compared to the TSP-HepI-stimulated ECFC ($p < 0.001$). Thus, TSP-HepI binding to GAGs may contribute to the observed enhancement of ECFC angiogenic potential.

4. Discussion

The N-terminal domain of TSP-1 has a proangiogenic effect on mature endothelial cells, whereas the C-terminal region and the intact protein have antiangiogenic effects [10,12,13]. We have previously reported that TSP-HepI, a 2.3-kDa synthetic peptide derived from the N-terminal domain (HBD) of TSP-1, markedly enhances ECFC adhesion to a similar extent as intact TSP-1, but the peptide has no effect on ECFC proliferation [14]. Here, we demonstrate that TSP-HepI also modulates the angiogenic properties of ECFC. Additionally, the data presented here demonstrate that TSP-HepI potentiates FGF-2-induced neovascularisation in an *in vivo* Matrigel plug model, suggesting that the synergy between the pro-angiogenic factor FGF-2 and TSP-HepI would lead to better vascularisation.

Several groups have reported that Matrigel plug model support a vascular response when supplemented with significant doses of growth factors [32–34] and/or heparin [35,36]. The *in vivo* pro-angiogenic activity of the N-terminal domain of TSP-1 was evaluated by only four groups so far [16,17,25,37] Among these, Staniszewska et al. demonstrated that a fragment of N-terminal domain, NOC1 (1-356), could stimulate *in vivo* formation of new

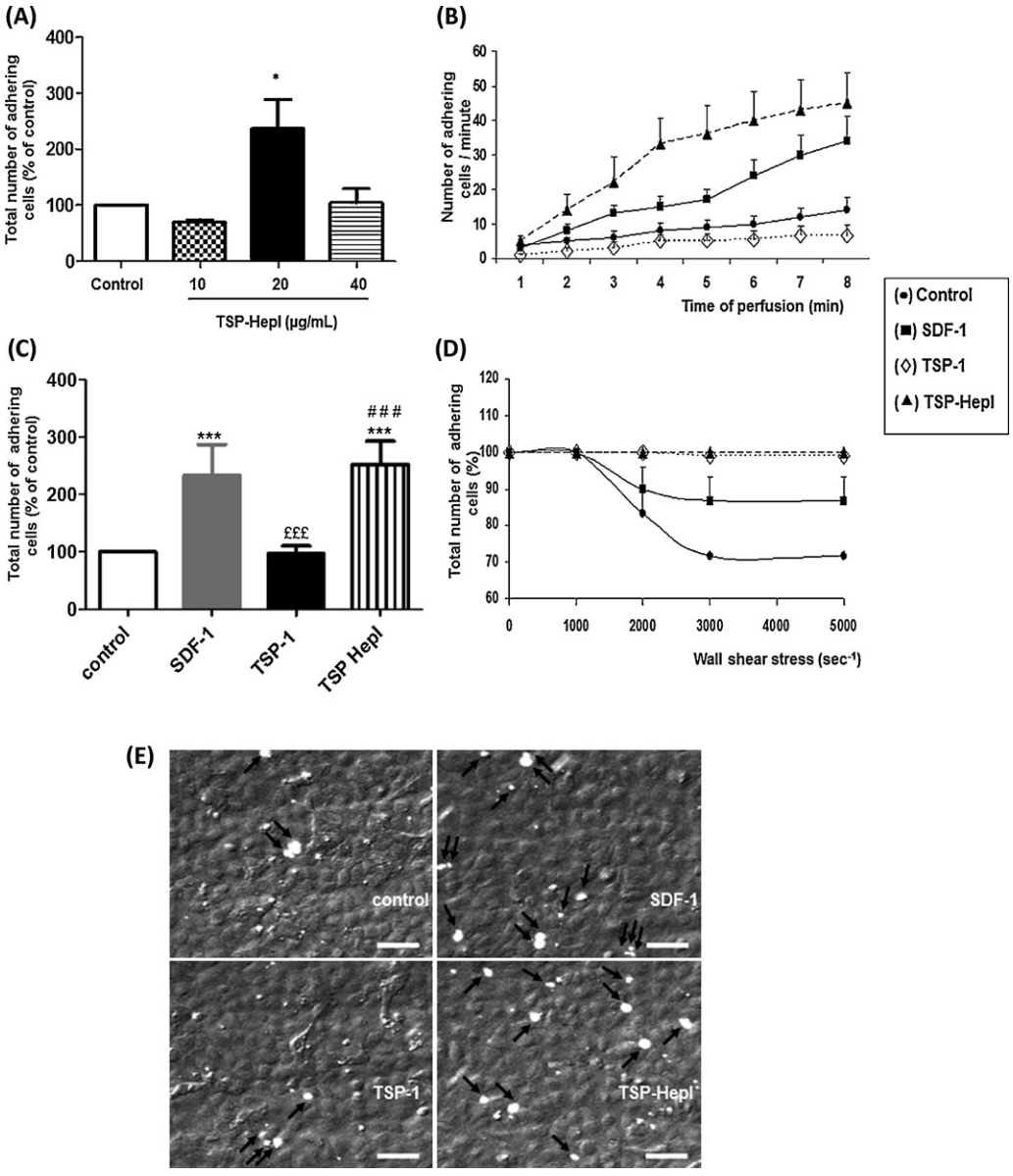

Fig. 4. TSP-HepI-pretreated ECFC showed enhanced adhesion to activated endothelium, at a shear rate of 50 s^{-1}. Before flow perfusion, ECFC were incubated for 18–20 h (overnight) with EBM-2/5% FCS (control medium), TSP-HepI peptide (20 μg/mL) or TSP-1 (10 μg/mL). SDF-1 stimulation (100 ng/mL) for 30 min was used as a positive control. (A) Dose-response analysis of ECFC adhesion to activated HUVEC: calcein-labelled ECFC suspensions (3×10^6 cells) were pretreated overnight with varying concentrations (10, 20 and 40 μg/mL) of TSP-HepI peptide and then perfused over confluent HUVEC monolayers for 15 min. Adhering ECFC were quantified as described in Section 2. (B) Time-course of ECFC adhesion to activated HUVEC, following overnight pretreatment of ECFC with TSP-HepI peptide (20 μg/mL). (C) ECFC adhesion to HUVEC monolayers, after ECFC overnight pretreatement with TSP-HepI peptide (20 μg/mL), as compared with SDF-1 and TSP-1. (D) Analysis of the ECFC resistance to detachment after overnight pretreatment with TSP-HepI peptide (20 μg/mL), as compared with SDF-1 and TSP-1, under increasing shear stress rates (from 50 to 5000 s^{-1}). (E): Fluorescent micrographs calcein-labelled ECFC (black arrows) over a HUVEC monolayer (gray). The scale bar represents 30 μm in the photomicrographs. Control (●), SDF-1 (■), TSP-1 (◇) and TSP-HepI (▲). Data are expressed as a percentage of the control group value. Values are a mean ± SEM of four determinations. * $p < 0.05$ *versus* control; *** $p < 0.001$ *versus* control; £££ $p < 0.001$ *versus* SDF-1, ### $p < 0.001$ *versus* TSP-1.

vessels in the absence of growth factors, but in the presence of heparin [37]. In our experimental conditions, we choose FGF-2 as a positive control for angiogenesis promotion and tested it *in vivo* at the concentration of 350 ng/mL, based on dose-response curves obtained in pilot experiments (unpublished results). TSP-HepI peptide was used at 200 μg/mL (corresponding to 100 μM), based on several *in vivo* studies using synthetic peptides at concentrations 10-times higher than those used *in vitro* assays [38,39]. Despite keeping these guidelines, we could not see any positive effect of TSP-HepI peptide when tested alone.

A number of factors can be considered as possible contributors to the observed *in vivo* outcome. Firstly, based on the ratio between the *in vitro* FGF-2 concentration (10 ng/mL) *versus* the *in vivo* one (350 ng/mL), we should have tested a dose 35-fold higher for the TSP-HepI peptide – about 700 μg/mL – which was inconceivable. Thus, the concentration of TSP-HepI peptide used *in vivo* was most probably too low to allow the detection of the potent direct stimulatory effects observed with endothelial cells *in vitro*. Nevertheless, we cannot rule out other aspects expected to affect TSP-HepI availability to cells, such as the rate of peptide turnover/clearance from Matrigel plugs after 14 days of assay.

However, the concentration of TSP-HepI peptide used in our work clearly boosted FGF-2 angiogenic response *in vivo*, and this observation is largely in accordance with the available literature. Taraboletti et al.

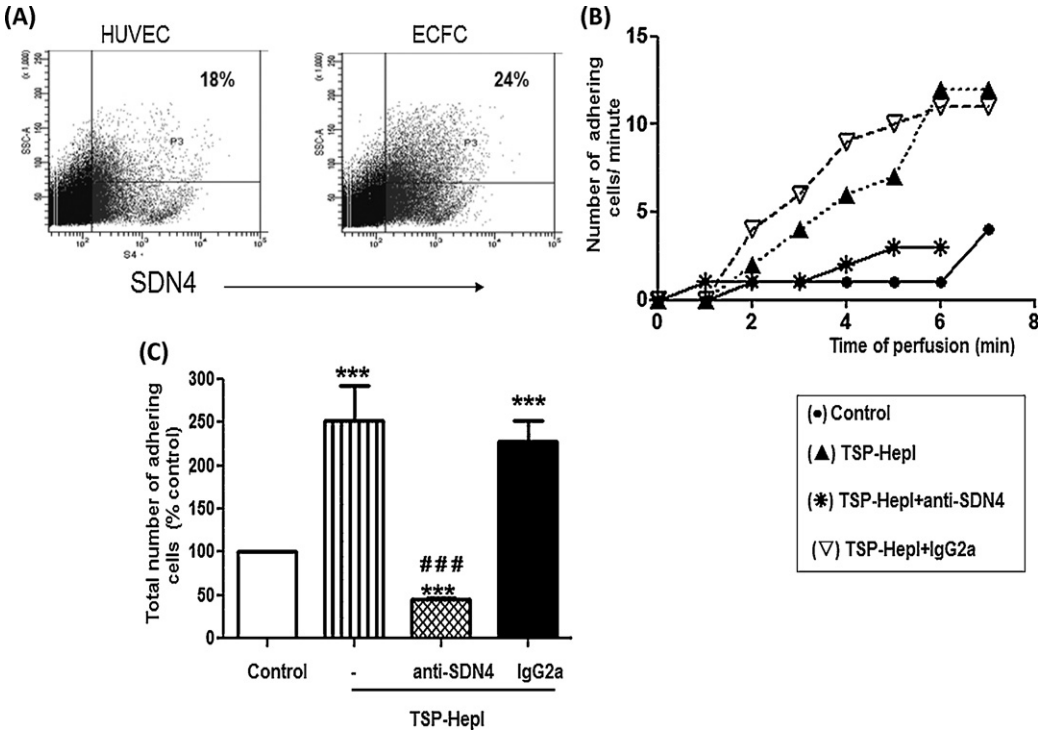

Fig. 5. A monoclonal antibody directed against the ectodomain of syndecan-4 (SDN4) inhibits TSP-HepI stimulation of ECFC adhesion to activated endothelium. ECFC were incubated with either an anti-SDN4 monoclonal antibody or a non-immune IgG2a isotype control (both at 1 μg/mL) and then overnight exposed to TSP-HepI (20 μg/mL), before being perfused over HUVEC monolayers at 50 s^{-1}, as described in Section 2. (A): Flow cytometric analysis of SDN4 expression by ECFC and HUVEC. (B) Time-course of ECFC adhesion: control (●), TSP-HepI (▲), TSP-HepI + anti-SDN4 (*) and TSP-HepI + IgG2a (▽). (C) Total number of ECFC adhering to activated HUVEC. Data are expressed as a percentage of the control group value, which was considered as 100%. Values are a mean ± SEM of four determinations. *** $p < 0.001$ *versus* control; ### $p < 0.001$ *versus* TSP-HepI.

reported that the 140-kDa carboxy-terminal fragment of TSP-1 – but not 25-kDa N-terminal heparin-binding fragment – fully retained a FGF-2 binding capacity, and that this interaction led to inhibition of proliferation and chemotaxis induced by FGF-2 in mature endothelial cells [40]. Accordingly, the 140 kDa C-terminal portion bear the anti-angiogenic motifs of TSP-1 [41–43]. Another study by Taraboletti and colleagues [25] showed that the 25 kDa N-terminal domain indeed potentiated *in vivo* FGF-2-induced angiogenesis. In this study, the 25 kDa fragment was effective alone, but it should be noted that it is 10 times larger than our peptide (2.3 kDa).

In addition to being able to bind FGF-2 through HS chains and to present it to FGF tyrosine kinase receptors, syndecan-4 directly initiates a number of intracellular signaling events. From our

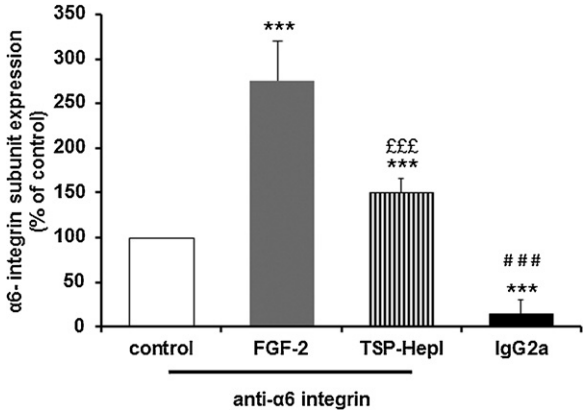

Fig. 6. TSP-HepI pretreatment of ECFC induces α6-integrin subunit expression. ECFC were incubated overnight with EBM-2/5% FCS (control medium), FGF-2 (5 ng/mL, positive control) or TSP-HepI peptide (20 μg/mL) and then quantified by flow cytometry for analysis of surface expression of the α6-integrin chain. Data are expressed as a percentage of the mean fluorescence intensity in each condition, as compared with the control condition, considered as 100% (mean ± SEM of four experiments). *** $p < 0.001$ *versus* control; £££ $p < 0.001$ *versus* FGF-2; ### $p < 0.001$ *versus* TSP-HepI.

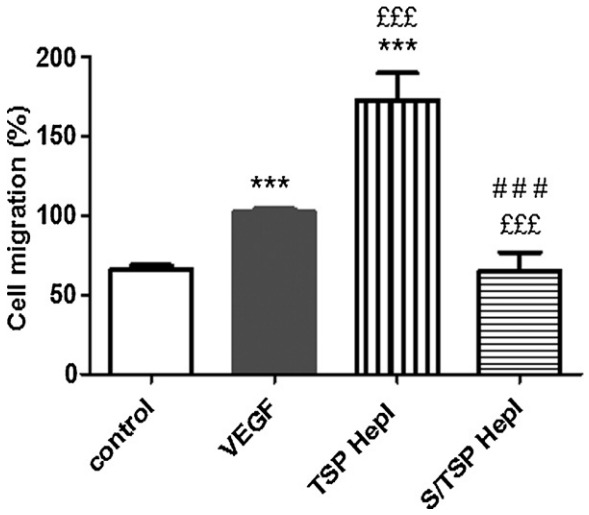

Fig. 7. TSP-HepI pretreatment of ECFC enhances cell migration. Before the migration assay, ECFC were incubated overnight with EBM-2/5% FCS (control medium), TSP-HepI peptide (20 μg/mL), S/TSP-HepI peptide (20 μg/mL) or VEGF (10 ng/mL – positive control). Migration was assayed in a chemotaxis chamber, as described in Section 2. ECFC (7×10^4) were seeded in the upper chamber and incubated for 6 h at 37 °C. Data are expressed as a percentage of the VEGF condition value. Values are a mean ± SEM of three determinations. *** $p < 0.001$ significantly different from control; £££ $p < 0.001$ *versus* VEGF, ### $p < 0.001$ *versus* TSP-HepI.

experience, it has been clearly established that syndecan-4 is a major receptor for the pro-angiogenic motifs within the N-terminal domain of TSP-1, in both mature and progenitor endothelial cells [19,20 and the present work]. The engagement of syndecan-4 by TSP-HepI peptide directly activates PKC and Akt signalling pathways [19], both of them essential for angiogenic morphogenesis [44–46].

It has been proposed that syndecan-4 also increases FGF-2 internalization and targeting to the nucleus, where additional and powerful stimulatory gene regulation will further activate cell proliferation and migration [47–49]. A strong piece of evidence in support of such a mechanism was provided by a recent paper by Jang et al., who showed that syndecan-4-enriched lypossomes improved the revascularization of ischemic hind limbs, by enhancing FGF-2 signaling and targeting to the nucleus, when this angiogenic factor was administered to lesions as a therapeutic agent [50]. Thus, we hypothesize that a synergistic action could be also triggered by the simultaneous engagement of tyrosine-receptors and syndecan-4 by FGF-2 and TSP-HepI, respectively, leading to a more vigorous downstream pro-migratory and proliferative response of endothelial cells. This suggests that TSP-HepI peptide, even in sub-optimal concentrations – which would preclude potent direct effects on endothelial cells *in vivo* – could be an useful tool to intensify FGF-2 pro-angiogenic activity.

TSP-HepI modulated the main angiogenic properties of ECFC *in vitro*. The peptide stimulated ECFC chemotaxis with a similar potency to FGF2, an effective stimulator of cell motility. In addition, it enhanced ECFC differentiation into vascular tubes when incorporated into Matrigel, whereas TSP-1 had no such effect. Furthermore, TSP-HepI-pretreated ECFC adhered tightly to activated endothelium (HUVEC monolayers) under dynamic conditions and were resistant to high shear rates, while their adhesion was reduced by pretreatment with the whole TSP-1 molecule. Importantly, the effect of TSP-HepI pretreatment in this assay was comparable to that obtained with SDF-1, a chemokine which potently recruits progenitor cells to ischaemic lesions [51]. Finally, ECFC migrated faster when treated with TSP-HepI than with VEGF, a well-known growth factor essential for endothelial cell recruitment.

We have reported that the HSPG syndecan-4 contributes to the proangiogenic activity of HUVEC by interacting with motifs within the HBD of TSP-1 [19]. Additionally, Roberts and colleagues identified several endothelial integrins such as $\alpha3\beta1$, $\alpha4\beta1$ and $\alpha6\beta1$ involved in the pro-angiogenic effects of the TSP-1 N-terminal domain on microvascular endothelial cells [16–18]. There is a possibility that the cooperation between syndecan-4 and such integrins are able to modulate the response of HBD to angiogenesis. The importance of TSP-HepI-HSPG interaction is highlighted in our present findings as follows: (i) S/TSP-HepI peptide (possessing a modified GAG-binding site) reduced chemotaxis and vascular tube formation; (ii) pre-incubation with a monoclonal antibody directed against syndecan-4 reduced TSP-HepI-pretreated ECFC adhesion to HUVEC monolayers; and (iii) pretreatment with S/TSP-HepI had no effect on ECFC motility. A study of the crystal structure of the TSP-1 N-terminal domain showed that the R29 residue at the TSP-HepI region is exposed and available for interaction [52]. Thus, TSP-HepI peptide could effectively mimic HBD in its interaction with HSPG such as syndecan-4.

The $\alpha6$-integrin subunit has been implicated in ECFC recruitment to sites of ischemia [31], and $\alpha6$-integrin over-expression by ECFC enhances *in vitro* vascular tube formation [26]. We observed a moderate increase in ECFC $\alpha6$-integrin expression after TSP-HepI stimulation although the functional relevance of this effect remains to be established. The interaction of $\alpha6$-integrin with HSPG is known to induce proangiogenic activity in HUVEC [53]. Interestingly, it has been recently shown that TSP-1 induces $\alpha6$-integrin chain expression on breast carcinoma cells, which in turn become more adherent to laminin-rich matrices [54]. Thus, one could expect that $\alpha6$-integrin also plays a role in ECFC adhesion to target sub-endothelial matrices exposed by activated endothelium in sites of neovascularisation. These authors were not able to block this induction effect with an antibody against anti-TSP-1 type I repeat, suggesting that other TSP-1 domains are responsible for the increase in $\alpha6$-integrin chain expression [54].

Taken together, our results suggest that the interaction of TSP-HepI with syndecan-4, with a possible involvement of $\alpha6$-integrin, enhance the proangiogenic activity of ECFC. Syndecan-4 is strongly expressed in ischemic tissues and at sites of vascular injury. It acts as a co-receptor in focal adhesion *via* heparin-binding growth factors (*e.g.*, VEGF and FGFs) and extracellular matrix proteins, and it binds to integrins such as $\alpha4\beta1$ and $\alpha5\beta1$ [55]. Two studies have suggested that syndecan-4 enhances HUVEC motility by activating Rac1 in a protein kinase-C-α (PKC-α) activation-dependent manner [56,57]. On the other hand, we have previously observed that the adhesion of HUVEC activate PKC-dependent Akt phosphorylation [19]. Nevertheless, we did not find any evidence that TSP-HepI affects the pERK-1/2 or pAkt signalling pathway in ECFC (data not shown), although both are known to be activated by syndecan-4 and are involved in endothelial cell adhesion, migration and survival [19,58,59]. We showed that PKC-α inhibition led to a 4-fold decrease in the spreading ratio of HUVEC adhesion to TSP-HepI. These findings support the participation of syndecan-4 in intracellular signalling in response to the N-terminal domain of TSP-1 [19]. Further work is needed to determine whether TSP-HepI also activates other signalling pathways in ECFC.

The dual effect of TSP-1 on angiogenesis seems to depend on the availability of its soluble proteolytic products, whether free in plasma or bound to extracellular matrix proteins and/or HSPG. We have previously observed elevated plasma TSP-1 levels in patients with peripheral artery disease [14]. Furthermore, despite its anti-angiogenic properties, TSP-1 is expressed on newly formed vessels following local injections of bone marrow mononuclear cells. We demonstrated here that the soluble proteolytic fragments of HBD of TSP-1 may modulate local angiogenesis. The HBD is rapidly cleaved by proteases relevant to the vascular process into 20–40 kDa fragments recognised by specific antibodies [22]. However, the presence of smaller peptides could not be addressed because monoclonal antibodies directed against the TSP-1 N-terminal domain were unable to recognize the smaller fragments and peptides derived from HBD. No TSP-1 isoforms resulting from alternative splicing have so far been identified [60]. Therefore, the known active TSP-1 fragments appear to arise from *in vivo* proteolysis of the mature protein. Indeed, TSP-1 cleavage that produces the N-terminal fragment (40 kDa) was shown to occur *in vivo* in a wound healing situation [24]. The physiological relevance of these fragments remains to be formally demonstrated.

In summary, local release of TSP-HepI during neovascularisation could be an important factor in ECFC recruitment to sites of ischemia by enhancing their capacity to adhere to the endothelium, migrate and form an extensive tubular network. TSP-HepI priming might be an interesting strategy to improve the efficiency of therapeutic neovascularisation using bone-marrow-derived endothelial precursors. Additionally, the combined use of TSP-HepI and FGF-2 could be designed as a new approach for increasing the efficacy of angiogenic growth factor therapies. Our results also present new perspectives for understanding the clinical significance of TSP-1 at sites of angiogenesis.

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Author contributions

JVD, ZBA, ME, CMT, VM and CBV designed and performed the research, analysed the data and wrote the manuscript. ZBA, JVD and CBV performed the Matrigel plug assays, data collection and image analysis. FG performed the zymography, data collection and image analysis. AL provided technical assistance with the cell cultures. IGF was the cell culture supervisor. LJ synthesised and characterised the peptide. VM and CBV obtained funding. BLB and OBB provided a critical revision of the manuscript for important intellectual content.

Conflicts of interest

None to declare.

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