ESC European Society of Cardiology
Cardiovascular Research
doi:10.1093/cvr/cvy179

Human peroxidasin 1 promotes angiogenesis through ERK1/2, Akt, and FAK pathways

Hayfa Medfai[1,2], Alia Khalil[1], Alexandre Rousseau[1], Vincent Nuyens[1], Martina Paumann-Page[3], Benjamin Sevcnikar[3], Paul G. Furtmüller[3], Christian Obinger[3], Nicole Moguilevsky[1], Olivier Peulen[4], Michael Herfs[5], Vincent Castronovo[4],Mohamed Amri[2], Pierre Van Antwerpen[6], Luc Vanhamme[7†], and Karim Zouaoui Boudjeltia[1*†]

[1]Laboratory of Experimental Medicine (ULB 222 Unit), Faculté de Médecine, CHU de Charleroi, A. Vésale Hospital, Université Libre de Bruxelles, Hôpital André Vésale, 706, Rue de Gozée, 6110 Montigny-le-Tilleul, Charleroi, Belgium; [2]Department of Biological Sciences, Laboratory of Functional Neurophysiology and Pathology, UR/11ES09, Université de Tunis El Manar, Faculté des Sciences de Tunis, 20 Rue de Tolède, 2092 Manar II, Tunis,Tunisia; [3]Division of Biochemistry, Department of Chemistry, BOKU, University of Natural Resources and Life Sciences, Muthgasse 18, A-1190 Vienna, Austria; [4]Metastasis Research Laboratory, Giga-Cancer, University of Liege, Quartier Hopital, Avenue de l'Hopital, 11, 4000 Liège, Belgium; [5]Department of Pathology, Laboratory of Experimental Pathology, Giga-Cancer, University of Liege, Quartier Hopital, Avenue de l'Hopital, 11, 4000 Liège, Belgium; [6]Pharmacognosy, Bioanalysis and Drug Discovery and Analytical Platform of the Faculty of Pharmacy, Faculty of Pharmacy, Université Libre de Bruxelles, Campus de la plaine CP205/09, Boulevard du Triomphe, 1050 Bruxelles, Belgium; and [7]Laboratory of Molecular Parasitology, IBMM, Faculty of Sciences, Université Libre de Bruxelles, Rue Adrienne Bolland 8, 6041 Gosselies, Belgium

Received 12 April 2018; revised 4 June 2018; editorial decision 29 June 2018; accepted 3 July 2018

Time for primary review: 23 days

Aims The term angiogenesis refers to sprouting of new blood vessels from pre-existing ones. The angiogenic process involves cell migration and tubulogenesis requiring interaction between endothelial cells and the extracellular matrix. Human peroxidasin 1 (hsPxd01) is a multidomain heme peroxidase found embedded in the basement membranes. As it promotes the stabilization of extracellular matrix, we investigated its possible role in angiogenesis both *in vitro* and *in vivo*.

Methods and results We analysed the effects of peroxidasin 1 gene silencing and supplementation by recombinant hsPxd01 in TeloHAEC endothelial cells on cell migration, tubulogenesis in matrigel, and intracellular signal transduction as assessed by kinase phosphorylation and expression of pro-angiogenic genes as measured by qRT–PCR. We further evaluated the angiogenic potential of recombinant peroxidasin in a chicken chorioallantoic membrane model. RNA silencing of endogenous hsPxd01 significantly reduced tube formation and cell migration, whereas supplementation by the recombinant peroxidase promoted tube formation *in vitro* and stimulated vascularization *in vivo* through its catalytic activity. Moreover, recombinant hsPxd01 promoted phosphorylation of Extracellular signal-Regulated Kinases (ERK1/2), Protein kinase B (Akt), and Focal Adhesion Kinase (FAK), and induced the expression of pro-angiogenic downstream genes: Platelet Derived Growth Factor Subunit B (PDGFB), endothelial-derived Heparin Binding EGF-like growth factor (HB-EGF), CXCL-1, Hairy-Related Transcription Factor 1 (HEY-1), DNA-binding protein inhibitor (ID-2), Snail Family Zinc Finger 1 (SNAI-1), as well as endogenous hsPxd01. However, peroxidasin silencing significantly reduced Akt and FAK phosphorylation but induced ERK1/2 activation after supplementation by recombinant hsPxd01. While hsPxd01 silencing significantly reduced expression of HEY-1, ID-2, and PDGFB, it did not affect expression of SNAI-1, HB-EGF, and CXCL-1 after supplementation by recombinant hsPxd01.

Conclusion Our findings suggest a role of enzymatically active peroxidasin 1 as a pro-angiogenic peroxidase and a modulator of ERK1/2, Akt and FAK signalling.

Keywords Human peroxidasin 1 • Angiogenesis • Extracellular-signal-regulated kinase 1/2 • Akt • Focal adhesion kinase

* Corresponding author. Tel: +32 71 924705; fax: +32 71 92 47 10, E-mail: kzouaoui@ulb.ac.be

† The last two authors are co-directors of this work.

1. Introduction

During angiogenesis, new blood vessels sprout from pre-existing ones. This mechanism requires the co-ordination of multiple steps that involve dynamic interactions between endothelial cells, vascular smooth muscle cells, pericytes, extracellular matrix, and vascular growth factors.[1] Once proangiogenic factors activate endothelial surface receptors, proteases are released into the extracellular space to degrade the basement membrane underlying the endothelium. Subsequently, endothelial cells proliferate, migrate and differentiate into a capillary-like tube network.[2,3] As for many epithelias, the endothelium is supported by the basement membrane, a specialized form of extracellular matrix. Blood vessels sprouting is partially regulated by this basement membrane.[4] Indeed, the dynamic remodelling of the extracellular matrix, and its interactions with endothelial cells regulate sprouts behaviour during the different stages of angiogenesis including endothelial capillary morphogenesis and cell migration.[5–8] Under physiological conditions, angiogenesis is driven by a balance between pro- and anti-angiogenic factors, that regulate embryonic development, organ growth, wound healing, and reproduction.[3,9] However, the disruption of this angiogenic balance, leads to an abnormal vascularization that can contribute to pathological conditions including inflammation, autoimmune diseases, and cancer.[10]

Human peroxidasin 1 (hsPxd01) has recently been shown to contribute to stabilization and stiffness of this basement membrane. The multidomain heme peroxidase was originally found to be expressed in the cardiovascular system, but nowadays is known to be widely expressed.[11–14] The metalloenzyme is secreted into the extracellular space and the circulating plasma and embedded in the basement membranes.[11,14] In addition, a study has demonstrated the localization of peroxidasin 1 within the endoplasmic reticulum and in the cell surface of Cos 7 cells expressing human recombinant peroxidasin.[15] Peroxidasin 1 belongs to Family 2 of the peroxidase-cyclooxygenase superfamily.[16] It is a homotrimeric highly glycosylated heme enzyme which—in addition to the catalytic peroxidase domain—comprises a leucine-rich repeat domain (LRR), four C-like immunoglobulin domains (Ig) at the N-terminus, and a C-terminal von Willebrand factor type C module (VWC).[17] Those non-catalytic domains are known to mediate protein-protein interactions.[18]

Peroxidasin 1 was originally described as an enzyme essential for extracellular matrix consolidation and structural tissue integrity during the early steps of embryogenesis and larval development in *Drosophila melanogaster*.[19] This was related to its capacity to catalyze the formation of sulfilimine bonds (S = N) in the NC1 hexamers of collagen IV, the predominant constituent of basement membrane.[15,20,21] The biosynthesis of these covalent bonds was shown to depend on the presence of hydrogen peroxide and bromide.[20,21] Furthermore, recent studies have demonstrated the correlation between clinical conditions such as congenital cataract, corneal opacity, and developmental glaucoma; and hsPxd01 mutations that affect the immunoglobulin and the peroxidase domains,[22] suggesting a crucial role for hsPxd01 in the stabilization of the ocular basement membrane. Finally, hsPxd01 has been identified as a glioma, melanoma, and renal carcinoma marker or determinant,[23,24] suggesting its possible involvement in the regulation of tumour angiogenesis.

During angiogenesis, endothelial cells interact with extracellular matrix components, and form filopodia or lamellipodia. This process initially involves proteolytic degradation of the extracellular matrix, which allows endothelial cells to proliferate and invade the extracellular matrix. The latters establish capillary tubules covered with a newly assembled basement membrane matrix, providing stabilization for the neoformed vessels. As a consequence, this delicate balance between degradation and assembly of the extracellular matrix is critical for an optimal angiogenic response.[25,26] The angiogenic mechanism is mimicked in culture by the formation of tubule-like structures, a process called tubulogenesis.[27,28]

Although the enzymatic role of hsPxd01 in extracellular matrix remodelling is already characterized to some extent, its direct involvement in angiogenesis is not documented. This role has been proposed for homologous Family 1 peroxidases such as myeloperoxidase (MPO) and eosinophil peroxidase (EPO),[29] which are composed of peroxidase domains only and are typically stored within granules in leukocytes. This prompted us to investigate whether hsPxd01 is implicated in the regulation of the angiogenic process. In this article, we demonstrate that hsPxd01 exhibits a pro-angiogenic function both *in vitro* and *in vivo* through the modulation of ERK1/2, Akt, and Focal Adhesion Kinase (FAK) signalling pathways.

2. Methods

2.1 Cell culture

hTERT immortalized human aortic endothelial cells (TeloHAEC) were purchased from ATCC, and cultivated at 37°C, 5% CO_2 in vascular cell basal medium (ATCC), supplemented with vascular endothelial cell growth kit-VEGF (ATCC).

2.2 Cell transfection

TeloHAEC were grown to 70% confluency, and transiently transfected with universal negative control siRNA or siRNA targeting human hsPxd01 (Sigma), using GeneXPlus transfection reagent (ATCC). Cells were incubated at 37°C, 5% CO_2 for 48 h in culture medium devoid of heparin sulfate and antibiotics.

2.3 Cloning, expression, and purification of full length hsPxd01 and truncated constructs

Full length hsPxd01 was expressed and purified from a stable transfected HEK293 cells as described previously by Soudi *et al.*[17] DNA of hsPxd01-con4 (uniprotkb: Q92626, residues Pro246-Asp1314) was cloned into a modified gWiz vector (Genlantis) carrying an N-terminal His6 tag for protein purification.

Forward and reverse polymerase chain reaction (PCR) primers for preparation of hsPxd01-con4 DNA were as follows: 5′-GAGGCT CACCACCACCATCACCATCCCCGAATCACCTCCGAGCCC-3′ and 5′-TAGCCAGAAGTGATCTGGATCTCAGTCCTGCCACACCC GGAGGTC-3′.

Transformed *Escherichia coli* XL-10 cells were screened with colony-PCR. Positive clones were selected and validated by DNA sequencing. HsPxd01-con4 in gWiz vector served as template for site directed mutagenesis. In order to obtain inactive hsPxd01-con4 (hsPxd01-con4 Q823A) the highly conserved glutamine residue 823 in the active site (which is crucial for halide binding[30,31]) was exchanged by an alanine. Site directed mutagenesis primer were as follows: 5′-GCTGATGCA GTGGGGCGCCTTCCTGGACCACGACCTCG-3′ and 5′-CGAGGT CGTGGTCCAGGAAGGCGCCCCACTGCATCAGC-3′.

QuickChange Lighning Site Directed Mutagenesis Kit was used according to manufacturing guidelines (Agilent Technologies). Active and inactive hsPxd01-con4 were expressed in the HEK 293 F

(Invitrogen) suspension cell system. Cells were cultivated according to the 'FreeStyle 293F-Cells User Guide' and transfected as previously described.[32] Heme was added to the culture medium to a final concentration of 5 μg/mL, 4 h after transfection to improve heme incorporation. Supernatants were harvested 5 days after transfection. Harvested supernatant was filtrated with a 0.45 μm PVDF membrane (Durapore) and concentrated to 100 mL using a Millipore Labscale TFF diafiltration system. Subsequently, the sample was adjusted to 1 M NaCl and 20 mM imidazole. For purification of the His6-tagged protein, 5 mL HisTrap FF columns (GE Healthcare) loaded with nickel chloride were used. After equilibration of the column with 100 mM phosphate buffer, pH 7.4, 1 M NaCl, and 20 mM imidazole, sample was loaded and washed with equilibration buffer. Protein was eluted using two consecutive step gradients of 8% and 70% of 20 mM phosphate buffer, pH 7.4, 500 mM NaCl, and 500 mM imidazole. Eluted fractions were analysed by UV-visible spectroscopy and SDS-PAGE following standard procedures. Fractions were pooled and washed for five times with 100 mM phosphate buffer, pH 7.4, using Amicon Ultra-15, 30 kDa cut-off centrifugal filters (Merck Milipore). Bromination activities were tested spectrophotometrically by measuring the halogenation of NADH as described by Soudi *et al.*[17]

2.4 Tubulogenesis assay

Angiogenesis wells (Ibidi) were coated with 10 μL of growth factor reduced matrigel (ThermoFisher), then allowed to polymerize for 30 min at 37°C. Next, 10^4 cells were seeded per well, incubated at 37°C, 5% CO_2 and observed for tubule formation after 5 h. Pictures were taken from different fields with the ×10 objective using phase contrast microscopy (Nikon, Eclipse Ti). Vessel morphometric parameters including length of vessels and density of junctions were measured using AngioTool software.

2.5 Scratch assay

A total of 3×10^4 cells were seeded into two well silicone insert defining a cell-free gap (Ibidi) and incubated overnight in growth medium at 37°C, 5% CO_2 to allow cell attachment. Inserts were then removed allowing cells to migrate and fill the gap, and fresh growth medium was added. Photographs were taken from different fields with the ×10 objective using light microscopy (PixeLINK PL-A642 Megapixel FireWire Camera). The cell-free wound surface was measured using the ImageJ software.

2.6 Cell proliferation assay

Cell proliferation was evaluated using Cell Proliferation Reagent WST-1 (Sigma) based on the measurement of the glycolytic production of NADH directly correlated to the number of active cells. A total of 25×10^3 cells were cultured in growth medium (5% CO_2) for 24 h at 37°C, and 10 μL of WST-1 reagent were added per well.

2.7 Chicken chorioallantoic membrane angiogenesis assay

Fertilized eggs were opened at embryonic day E3. On embryonic day E7, 3 mm sterile PVA microsphere (Netcell surgical sponge Ref 30–380) loaded with 20 μL of full length hsPxd01, both active and inactive hsPxd01-con4 (C4 active and C4 inactive) at a concentration of 1 μM, were laid onto the egg chorioallantoic membrane. Vehicle or VEGF (5 μg/mL) (Sigma) were used as negative and positive controls, respectively. At embryonic day E10, chicken chorioallantoic membranes (CAMs) were collected. After image binarization, blood vessel density (relative % of blood vessel area) and length density (relative percentage of blood vessel length) were evaluated in a region located from 2 to 6 mm away from the microsphere boundary using Fiji. CAMs were then fixed (4% formalin) for 24 h and paraffin embedded. In order to identify blood vessels, immunohistochemical analyses were performed using the following primary antibody: anti-alpha smooth muscle actin (SMA) 1:600 (Abcam). The rabbit Envision kit (Dako, Glostrup, Denmark) was used for the secondary reaction. Five CAMs were analysed in each group. Whole tissue sections were finally digitalized (Ventana iScan HT, Ventana Medical Systems, Tucson, AZ, USA) and the relative alpha SMA-positive vessel areas were quantified by computerized counts QuPath 0.1.2 software.

2.8 RNA extraction and quantitative real-time–PCR

Total RNA was extracted from cells using spin columns (Qiagen) according to the manufacturer's instructions. Purity was assessed through absorption ratio (260/280 nm) measurements. One microgram of total RNA was treated with DNAase and reverse-transcribed using PrimeScript RT reagent Kit (Takara-Clontech). Quantitative real-time PCR (qRT–PCR) was performed using LightCycler® 480 SYBR Green I Master (Roche). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for gene expression quantification. The relative expression was calculated according to the comparative ΔΔCt method. Primer sequences are listed in Supplementary material online, *Table S1*.

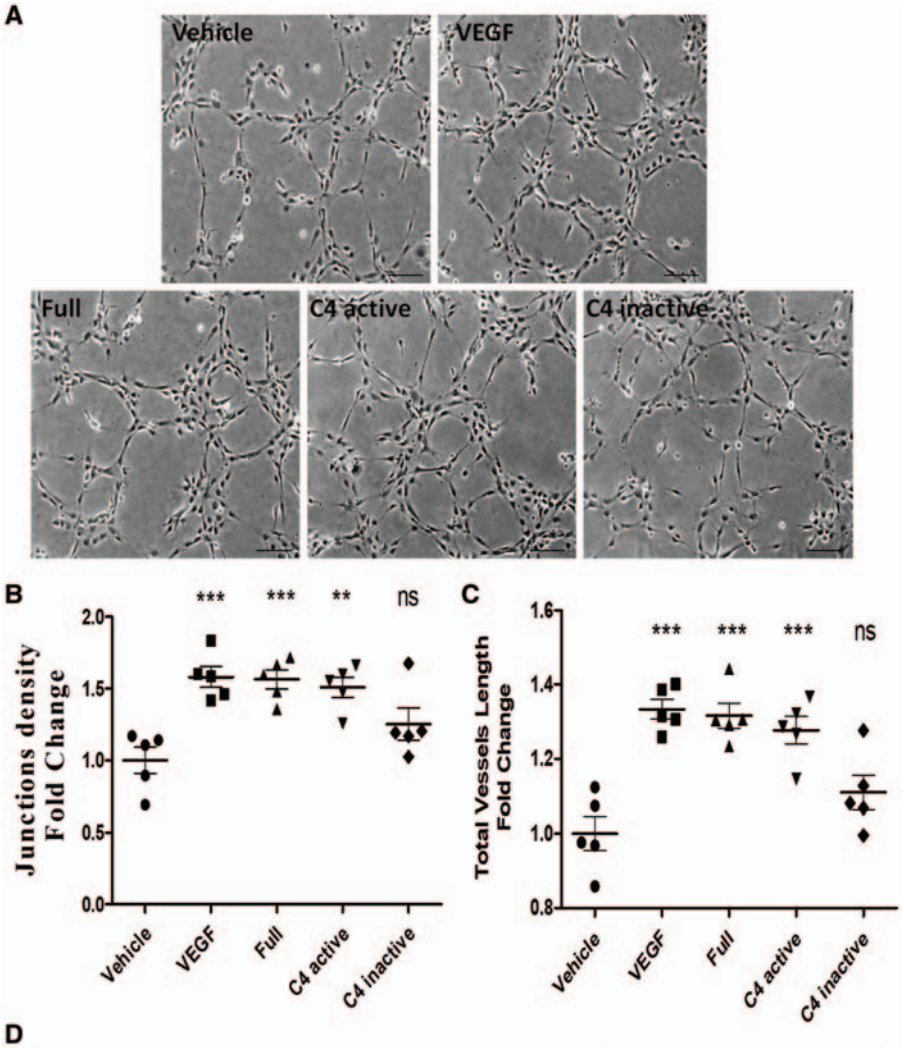
2.9 Western blot

Whole cell protein lysates were extracted in RIPA lysis buffer (Sigma) supplemented with protease inhibitor (Roche). They were centrifuged at 12 000 *g*, 4°C for 15 min, and protein concentration was measured using the bicinchoninic acid assay kit (ThermoFisher). Proteins were separated on SDS–PAGE under reducing conditions and transferred onto nitrocellulose membrane (GE Healthcare Life Sciences). After saturation with 5% milk, the membrane was incubated overnight at 4°C with primary antibodies: custom anti-hsPxd01 1:1000, or commercial anti-Phospho ERK1/2 1:5000, anti-total ERK 1/2 1:10 000, anti-Phospho Akt 1:5000, anti-total Akt 1:10 000, anti-Phospho FAK 1:1000, and anti-total FAK 1:1000 (Abcam), washed, incubated with (HRP-conjugated) secondary antibody for 1 h at room temperature and revealed using the ECL substrate (PerkinElmer). Monoclonal anti-β-actin-peroxidase antibody 1:80 000 (Sigma) was used for protein loading control.

2.10 Software tools and statistics

The AngioTool software[33] is an open source validated tool that allows quantifications of different morphological parameters by assessing the variation in foreground and background pixel density across a microscopic image. QuPath 0.1.2 software is an open source software for digital pathology image analysis. Image J software is a tool allowing image analysis and processing.

Data were expressed as mean ± standard error of mean (SEM). For comparaison of two groups, unpaired *t*-test was used. Differences between multiple groups were evaluated using one-way or two-way ANOVA as appropriate, followed by Bonferroni *post hoc* test for multiple comparaison. The significance level was chosen as *P*-value: <0.05 (*), <0.01 (**), and <0.001(***). Experiments were repeated five times independently. All calculations were performed with GraphPad Prism 5.01.



D

hsPxd01 (full length)	hsPxd01-con4 (active)	hsPxd01-con4 Q823A (inactive)
N-LRR-POX-VWC-C	N-POX-C	N-POX-C, Gln^{823} Ala
3 × 165 kDa	120 kDa	120 kDa
H_2O_2	H_2O_2	-
K_M = 18.6 ± 4.6 µM V_{max} = 0.12 ± 0.01 µM min^{-1} k_{cat} = 1.2 min^{-1} k_{cat}/K_M = 6.5 × 10^4 $M^{-1}min^{-1}$	K_M = 37.7± 6.0 µM V_{max} = 2.2 ± 0.5 µM min^{-1} k_{cat} = 22 min^{-1} k_{cat}/K_M = 5.8 × 10^5 $M^{-1}min^{-1}$	
Br^-	Br^-	-
K_M = 4.1 ± 0.5 µM V_{max} = 0.21 ± 0.01 µM min^{-1} k_{cat} = 2.1 min^{-1} k_{cat}/K_M = 5.1 × 10^2 $M^{-1}min^{-1}$	K_M = 4.4 ± 0.8 µM V_{max} = 3.4 ± 0.2 µM min^{-1} k_{cat} = 34 min^{-1} k_{cat}/K_M = 7.7 × 10^3 $M^{-1}min^{-1}$	

Figure 1 Peroxidasin 1 promotes tubulogenesis through its catalytic activity. (*A*) TeloHAEC cells (10^4) were treated with the indicated hsPxd01 protein constructs (*D*) at a concentration of 1 µM, or with VEGF-A (100 ng/mL) and seeded onto matrigel for 5 h. Micrographs were taken at ×10 magnification. Scale bars represent 200 µm. (*D*) Domain architecture and kinetic parameters of bromination activity of full length hsPxd01 and the truncated variants. Junction density (*B*) and total vessels length (*C*) were quantified using the AngioTool software. Results are expressed as fold change over the vehicle control. Results are expressed as mean ± SEM of five independent experiments. Statistics were analysed using one-way ANOVA, followed by Bonferroni's multiple comparison test (**$P < 0.01$, ***$P < 0.001$; $n = 5$). ns, non-significant.

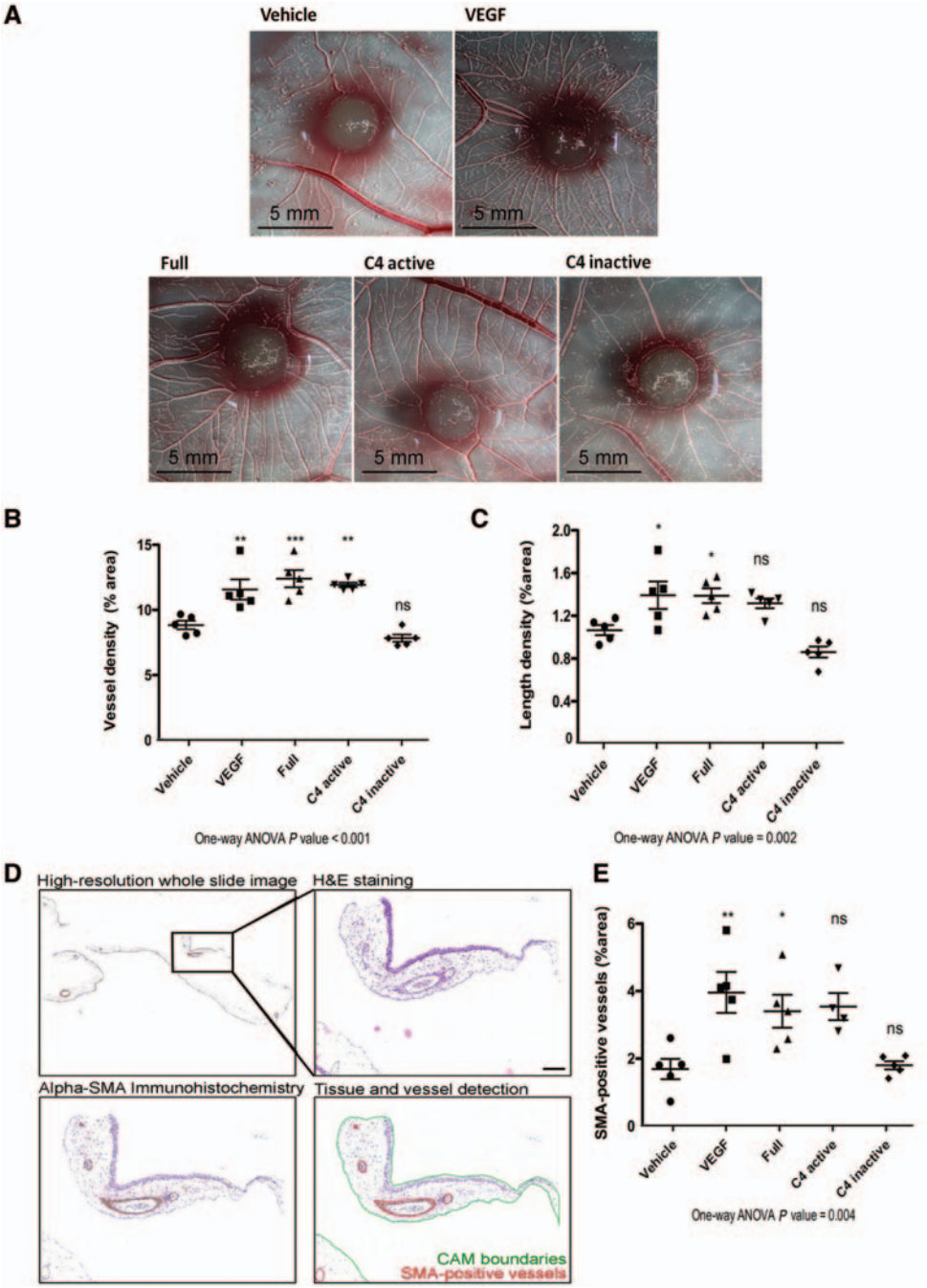


Figure 2 Peroxidasin 1 promotes angiogenesis in the CAM assay through its catalytic activity. (*A*) Merge of native and binarized macroscopic images of CAM at E10. Scale bars represent 5 mm (*B* and *C*) Fiji quantification of blood vessel density (relative % of blood vessel area) and blood vessel length density (relative % of blood vessel length). (*D*) Quantification procedure of SMA-positive vessel in CAM. Scale bars represent 200 µm. (*E*) SMA-positive vessels quantification. Results are expressed as mean ± SEM of five independent experiments. Statistics were analysed using one-way ANOVA, followed by Bonferroni's multiple comparison test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; $n = 5$). ns, non-significant.

3. Results

3.1 Peroxidasin 1 promotes tubulogenesis *in vitro* and *in vivo* via its catalytic activity

TeloHAEC cells were seeded on matrigel supplemented with the recombinant full length homotrimeric protein (3 × 163 kDa), and both active and inactive monomeric (120 kDa) truncated constructs (hsPxd01-con4 and hsPxd01-con4 Q823A: C4-active and C4-inactive) at a physiological dose (1 µM)[11] for 5 h (*Figure 1A*). The truncated variants lack the LRR and VWC domains as schematically depicted in *Figure 1D*.

It has to be noted that hsPxd01 efficiently oxidizes bromide to hypobromous acid but is unable to use chloride as electron donor.[17,34]

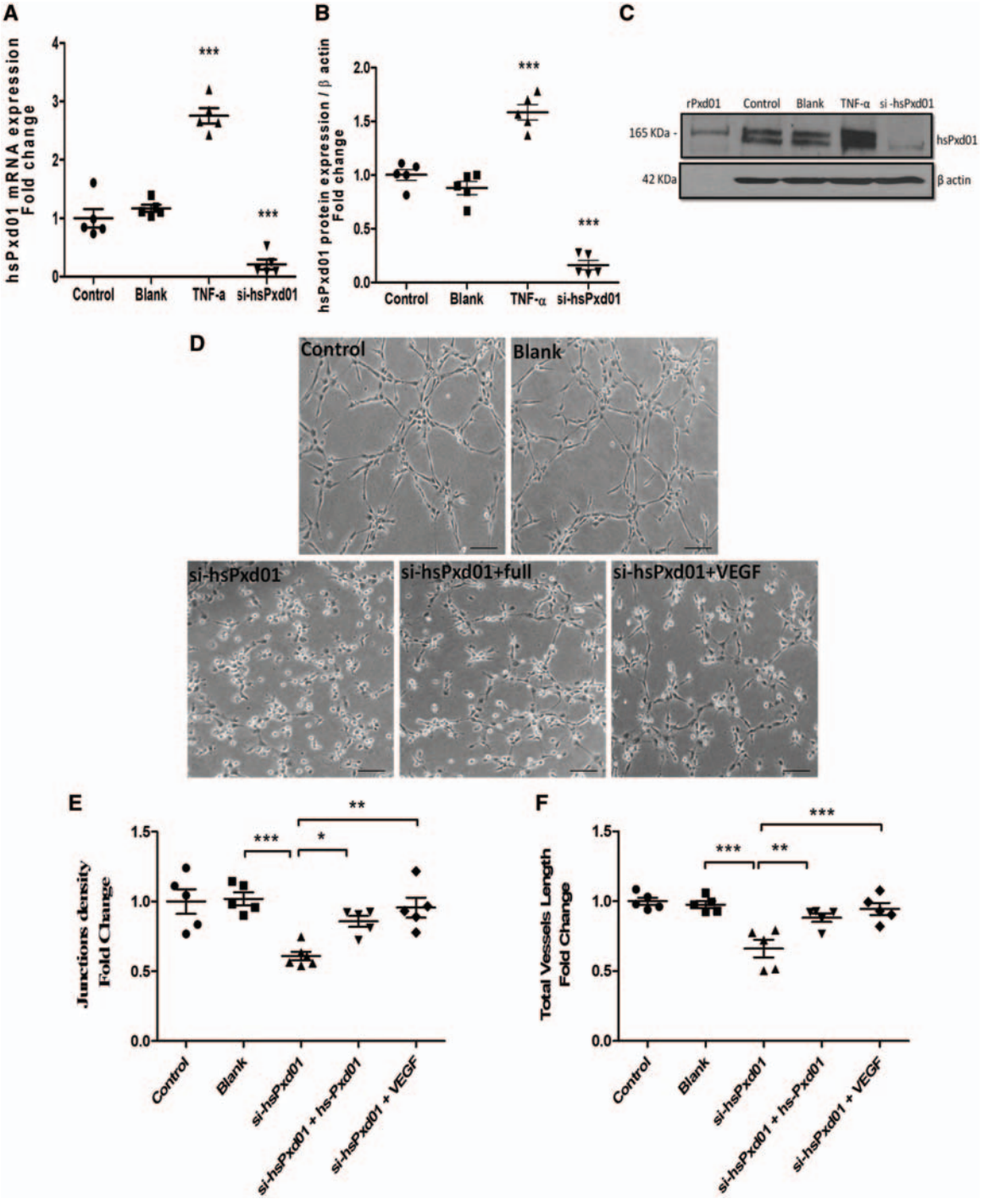


Figure 3 Effects of knockdown of peroxidasin 1 and supplementation by recombinant hsPxd01 on tubulogenesis. (*A–C*) Validation of knockdown efficiency using siRNA hsPxd01 in TeloHAEC cells. TeloHAEC cells (8×10^4) were transfected with siRNA targeting human hsPxd01 (si-hsPxd01) or scrambled siRNA (Blank). (*A*) mRNA were extracted at 48 h post-transfection and analysed by qRT–PCR. Relative hsPxd01 mRNA expression levels were normalized to GAPDH. Results are expressed as fold change to the control. (*C*) Representative western blot analysis using anti hsPxd01 antibody, and quantification (graph) (*B*) show the drop of hsPxd01 protein level normalized to β-actin. Recombinant hsPxd01 (rPxd01) and TNF-α stimulation (10 ng/mL, 24 h) were used as positive controls. (*D*) Effects of hsPxd01 silencing and supplementation on tube formation. TeloHAEC cells (10^4) were transfected with siRNA targeting hsPxd01 (si-hsPxd01) or scrambled siRNA (Blank), then treated with the full length hsPxd01 at a concentration of 1 µM, and seeded onto matrigel for 5 h. Micrographs were taken at ×10 magnification. Scale bars represent 200 µm. Junctions density (*E*) and total vessels length (*F*) were quantified using the AngioTool software, and expressed as fold change over the Blank. Results are expressed as mean ± SEM of five independent experiments. Statistics were analysed using one-way ANOVA, followed by Bonferroni's multiple comparison test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; $n = 5$).

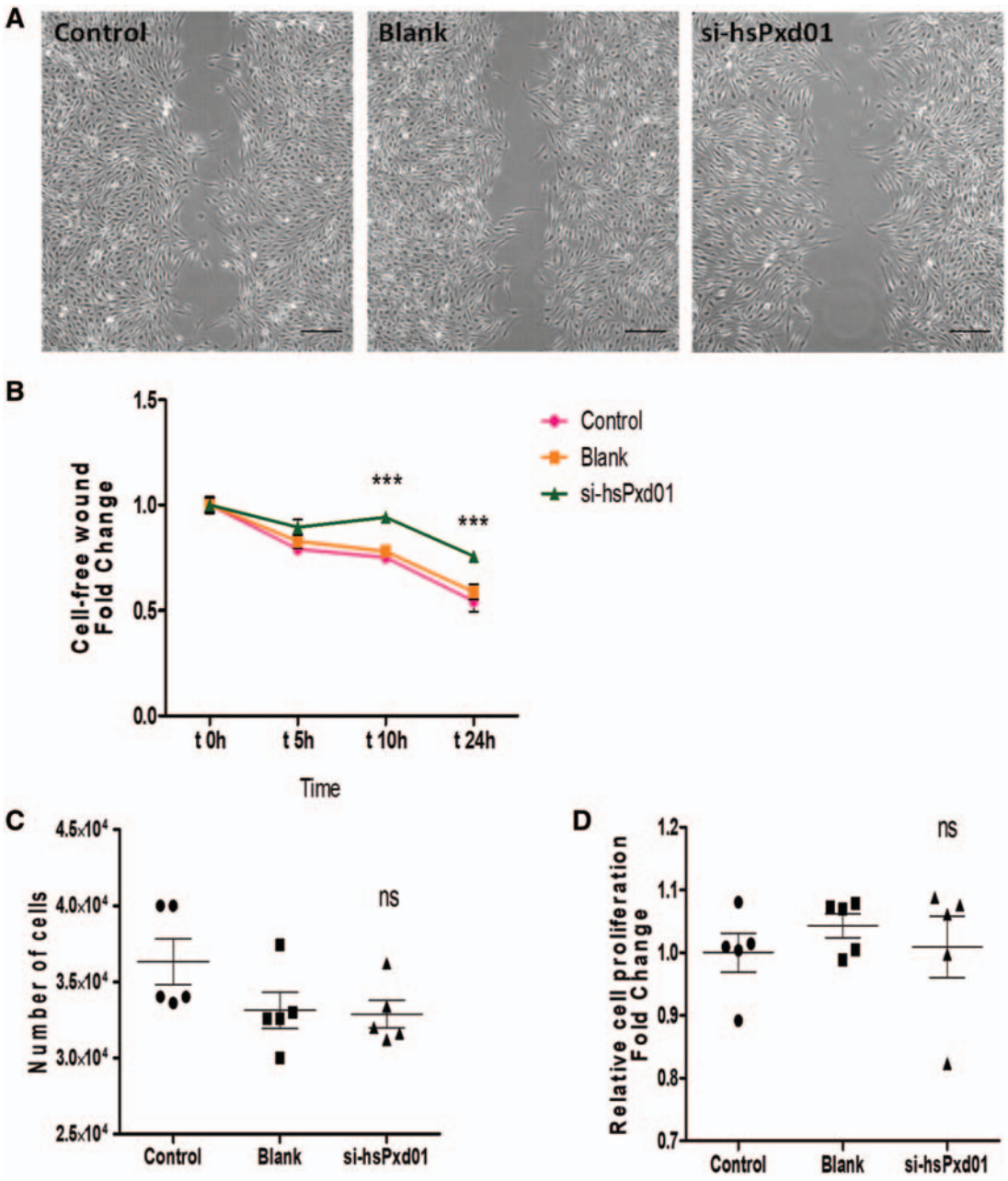


Figure 4 Peroxidasin 1 is required for endothelial cell migration. (*A*) TeloHAEC cells (3×10^4) were transfected with hsPxd01 siRNA (si-hsPxd01) or scrambled siRNA (Blank), then seeded into two well culture insert and allowed to migrate for 24 h. Micrographs were taken at ×10 magnification. Scale bars represent 200 µm. (*B*) The relative change in the cell-free gap surfaces was measured at different time points and expressed as fold change over the control. Cells were counted (*C*) and the proliferation rate was evaluated at t24 h (*D*). Results are expressed as fold change and are shown as mean ± SEM of five independent experiments. Statistics were analysed using two-way ANOVA, followed by Bonferroni's multiple comparison test (***$P < 0.001$; $n = 5$). ns, non-significant.

The K_M values for bromide were similar for both full length homotrimeric hsPxd01 (4.1 mM) and the monomeric construct hsPxd01-con4 (4.4 mM), which reflects similar heme cavity structure, whereas the catalytic efficiency for bromide oxidation (k_{cat}/K_M) increased upon truncation (*Figure 1D*).[17] Importantly, upon exchange of the highly conserved halide-binding glutamine residue at the position 823 by alanine, the construct hsPxd01-con4 completely lost its capacity to oxidize bromide (C4-inactive).[16] Treatment of TeloHAEC cells with recombinant full length hsPxd01 promoted tube formation with a maximal 1.5- and 1.3-fold increase in junctions density and total vessels length respectively when compared with vehicle control. Similarly, active hsPxd01-con4 (C4 active) induced tube formation with a maximal 1.5- and 1.2-fold increase in junctions density and total vessels length respectively. However, no significant effect was observed with C4-inactive when compared with

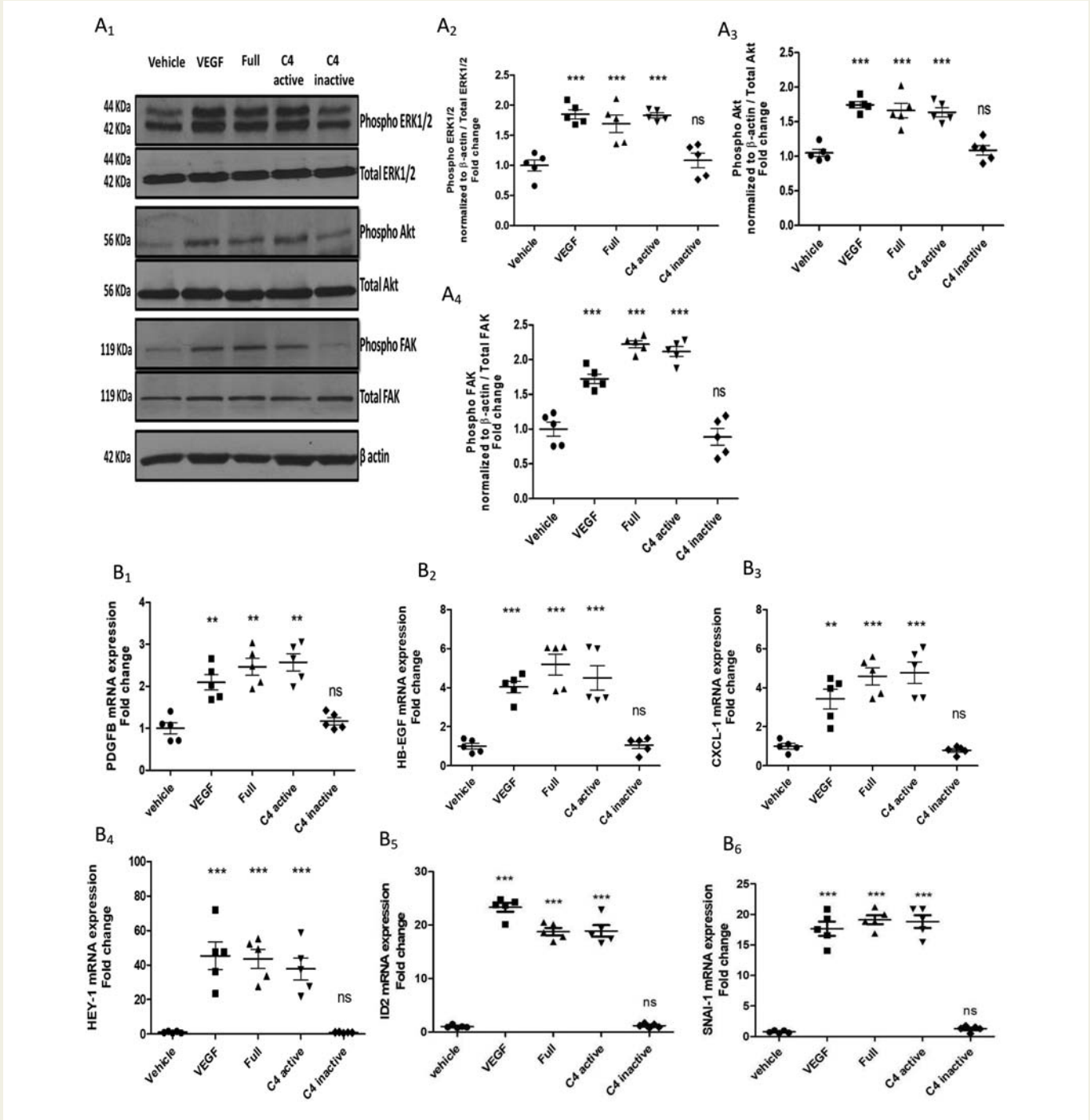


Figure 5 Peroxidasin 1 activates angiogenic signalling pathways and expression of pro-angiogenic downstream genes via its catalytic activity. (*A1*) TeloHAEC cells (4×10^5) were treated with the full length hsPxd01 and the truncated variants C4-active and C4-inactive (1 μM), or with VEGF (100 ng/mL) for 1 h. Representative western blots analysis of protein extracts were performed using the indicated antibodies. Graphs (*A2*, *A3*, *A4*) show the quantification of phosphorylation levels of the indicated kinases normalized to β-actin and total kinase expression. Results are expressed as fold change over vehicle control and shown as mean ± SEM of five independent experiments. (*B1*, *B2*, *B3*, *B4*, *B5*, *B6*) TeloHAEC cells (9×10^4) were treated with the full length hsPxd01 or the truncated variants C4-active and C4-inactive (1 μM), or with VEGF (100 ng/mL) for 1 h. mRNA were extracted and analysed by qRT–PCR using primers targeting the indicated genes. Relative mRNA expression levels were normalized to GAPDH. Results are expressed as fold change over vehicle control, and are shown as mean ± SEM of five independent experiments. Statistics were analysed using one-way ANOVA, followed by Bonferroni's multiple comparison test (**$P < 0.01$, ***$P < 0.001$; $n = 5$). ns, non-significant.

the vehicle control (Figure 1B and C). For comparaison, VEGF (100 ng/mL)—known to promote tube formation—increased junctions density and total vessels length by 1.5- and 1.3-fold, respectively (Figure 1B and C). These findings suggest that hsPxd01 promotes tubulogenesis via its catalytic activity. Neither loss of its oligomeric state nor of the LRR and VWC domains did affect hsPxd01-mediated tubulogenesis.

Next, we assessed the capacity of hsPxd01 to stimulate angiogenesis *in vivo* by using the chicken chorioallantoic membrane (CAM) assay. Macroscopic vessel and length densities were evaluated between 2 and 6 mm remote from the microsphere boundary. Upon treatment with full length hsPxd01 or C4-active vessel density increased when compared with vehicle control (Figure 2B). Interestingly, this increase in vessel density was similar to that observed upon treatment with VEGF (5 µg/mL) (Figure 2B). However, addition of C4-inactive (1 µM) did not induce angiogenesis (Figure 2B).

Increase in length density was only observed when CAMs were treated with full length hsPxd01 or VEGF (Figure 2C). Whereas treatment of CAMs with full length hsPxd01 significantly increased both vessel and length densities when compared with vehicle control (Figure 2B and C), addition of C4-active showed a significant increase in blood vessel density only (Figure 2B). Considering microscopic SMA-positive vessels, results are in agreement with those obtained at macroscopic level (Figure 2E). Taken together, our data show that full length recombinant hsPxd01 and the construct C4-active are able to induce angiogenesis *in vivo*.

3.2 Effects of knockdown of endogenous peroxidasin 1 and supplementation by recombinant hsPxd01 on tubulogenesis

In order to determine the role of endogenous hsPxd01 in endothelial tube formation, we transfected TeloHAEC cells with hsPxd01 siRNA. Knockdown was achieved to around 80% of the control level for both mRNA (Figure 3A) and protein levels (Figure 3B and C). Scrambled siRNA was used as a control and did not show any significant effect (Blank). In contrast, knockdown of hsPxd01 affected the formation of tubular structures on matrigel (Figure 3D). Both junctions density and total vessels length were decreased by ∼40% (Figure 3E and F) when compared with wild-type cells.

Next, we probed whether addition of recombinant hsPxd01 is able to counteract the effects of hsPxd01 knockdown. Consequently, si-hsPxd01 cells were seeded on matrigel and supplemented with recombinant full length hsPxd01 (1 µM) for 5 h (Figure 3D). As Figure 3D–F depict, addition of recombinant full length hsPxd01 partially restored tube formation in si-hsPxd01 cells with a maximal 1.4- and 1.3-fold increase in junctions density and total vessels length respectively when compared with untreated si-hsPxd01 cells. For comparaison, we found that VEGF (100 ng/mL) rescued tube formation in si-hsPxd01 cells with a maximal 1.6- and 1.4-fold increase in junctions density and total vessels length respectively when compared with untreated si-hsPxd01 cells (Figure 3E and F).

3.3 Peroxidasin 1 is required for endothelial cell migration

As angiogenesis *in vivo* also requires cell motility, we assessed the effect of knockdown of hsPxd01 on endothelial cell migration. TeloHAEC cells were seeded into two well culture insert and allowed to migrate (Figure 4A). Cell-free gap surfaces were measured at different time points. Figure 4B shows that knockdown of hsPxd01 as opposed to scrambled siRNA (Blank) and control cells, significantly slows down the closure of the cell free gap by 25% after 24 h. Knockdown of hsPxd01 did not affect cell proliferation (Figure 4C and D) excluding an impact of hsPxd01 knockdown on the cell cycle.

3.4 Peroxidasin 1 activates angiogenic signalling pathways and expression of pro-angiogenic downstream genes through its catalytic activity

As hsPxd01 affects endothelial cell migration and tube formation, which are characteristics for angiogenesis *in vivo*, we aimed to understand the underlying molecular mechanism. First, we investigated the ability of full length hsPxd01, C4-active and C4-inactive as well as VEGF to activate Extracellular signal-Regulated Kinases (ERK1/2), Protein kinase B (Akt), and FAK, actors of the major angiogenic signalling pathways. Treatment of TeloHAEC cells with both full length hsPxd01 and C4 active increased the phosphorylation of ERK1/2, Akt and FAK when compared with vehicle control. This effect is comparable to that seen with VEGF. However, no significant effect on kinases activation has been observed with C4-inactive (Figure 5A1, A2, A3, A4).

Next, we analysed the effect of recombinant full length hsPxd01, C4-active and C4-inactive on expression of downstream angiogenic genes encoding growth factors such as Platelet Derived Growth Factor Subunit B (PDGFB), endothelial-derived Heparin Binding EGF-like growth factor (HB-EGF), CXCL-1, a pro-angiogenic chemokine, and some of the "key" transcriptional regulators such as Hairy-Related Transcription Factor 1 (HEY-1), DNA-binding protein inhibitor (ID-2), and Snail Family Zinc Finger 1 (SNAI-1).

Treatment of TeloHAEC cells with the full length hsPxd01 and C4-active significantly enhanced the expression of PDGFB (2.4, 2.5)-fold, HB-EGF (5, 4.5)-fold, CXCL-1 (4.5, 4.7)-fold, HEY-1 (43, 37)-fold, ID-2 (17.8, 16.6)-fold, and SNAI-1 (19, 18)-fold, respectively. These genes were also induced with VEGF (100 ng/mL) used as a positive control. However, addition of C4-inactive had no impact on expression of these genes when compared with vehicle control cells (Figure 5B1, B2, B3, B4, B5, B6). Our findings clearly suggest that addition of active recombinant hsPxd01 (either full-length homotrimeric or truncated monomeric) stimulates ERK1/2, Akt and FAK phosphorylation, and induces the expression of downstream angiogenic genes via its catalytic activity.

In addition, we examined the phosphorylation level of ERK1/2, Akt, and FAK in si-hsPxd01 treated cells. Phosphorylation of Akt and FAK was reduced in si-hsPxd01 cells compared with scrambled siRNA treated cells upon hsPxd01 stimulation (Figure 6A1, A4, A5). Interestingly, there was a significant increase of ERK1/2 phosphorylation in si-hsPxd01 cells after addition hsPxd01 when compared with hsPxd01-treated scrambled siRNA cells (Figure 6A1, A3). However, silencing of hsPxd01 did not affect ERK1/2, Akt and FAK activation upon stimulation by VEGF (Supplementary material online, Figure S1 A1, A3, A4, A5). Furthermore, we probed whether endogenous hsPxd01 regulates downstream angiogenic gene expression. Knockdown of hsPxd01 did not alter the basal level of PDGFB, HB-EGF, CXCL-1, HEY-1, ID-2 and SNAI-1 expression (Figure 6B2, B3, B4, B5, B6, B7), whereas expression of HEY-1, ID-2, and PDGFB was reduced by 29-fold, 7-fold, and 1.3-fold respectively in hsPxd01-treated si-hsPxd01 cells when compared with hsPxd01-treated scrambled siRNA cells (Figure 6B5, B6, B2). Moreover, addition of recombinant hsPxd01 induced the expression of endogenous hsPxd01 both at mRNA (Figure 7A) and protein levels (Figure 7B and C) in wild-type cells. Taken together, these results suggest

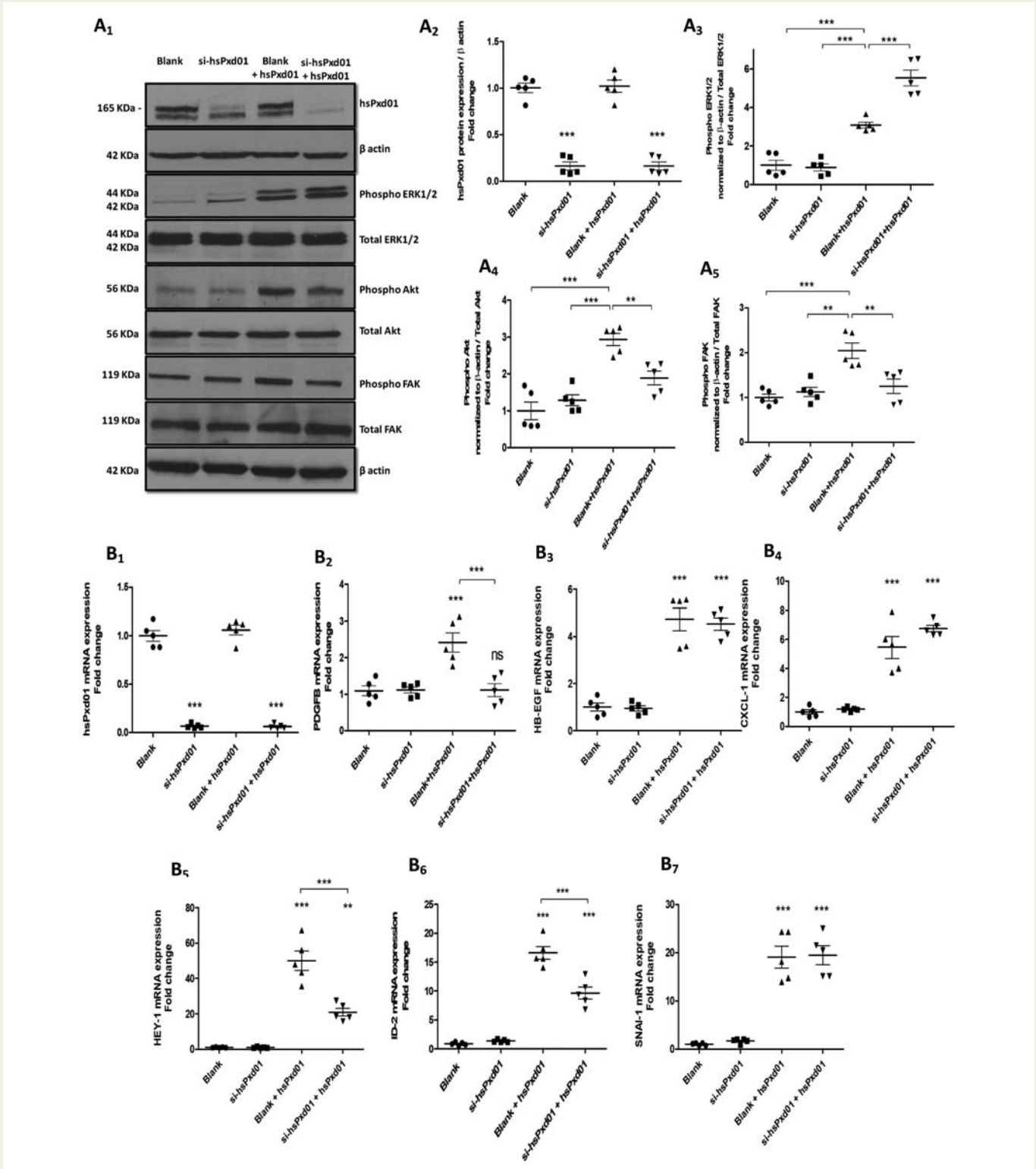


Figure 6 Peroxidasin 1 activates angiogenic signalling pathways and expression of pro-angiogenic downstream genes via endogenous peroxidasin 1. (*A1*) Scrambled siRNA (blank) and si-hsPxd01-transfected cells (4×10^5) were treated with the full length recombinant hsPxd01 (1 μM) for 1 h. Representative western blots analysis of protein extracts were performed using the indicated antibodies. Graphs (*A2*, *A3*, *A4*, *A5*) show the quantification of hsPxd01 protein level and phosphorylation levels of the indicated kinases normalized to β-actin and total kinase expression, and expressed as fold change over Blank. (*B1*, *B2*, *B3*, *B4*, *B5*, *B6*, *B7*) Scrambled siRNA (Blank) and si-hsPxd01-transfected cells (9×10^4) were treated with the full length recombinant hsPxd01 (1 μM) for 1 h. mRNA were extracted and analysed by qRT–PCR using primers targeting the indicated genes. Relative mRNA expression levels were normalized to GAPDH. Results are expressed as fold change over blank and are shown as mean ± SEM of five independent experiments. Statistics were analysed using one-way ANOVA, followed by Bonferroni's multiple comparison test. (**$P < 0.01$, ***$P < 0.001$; $n = 5$). ns: non-significant.

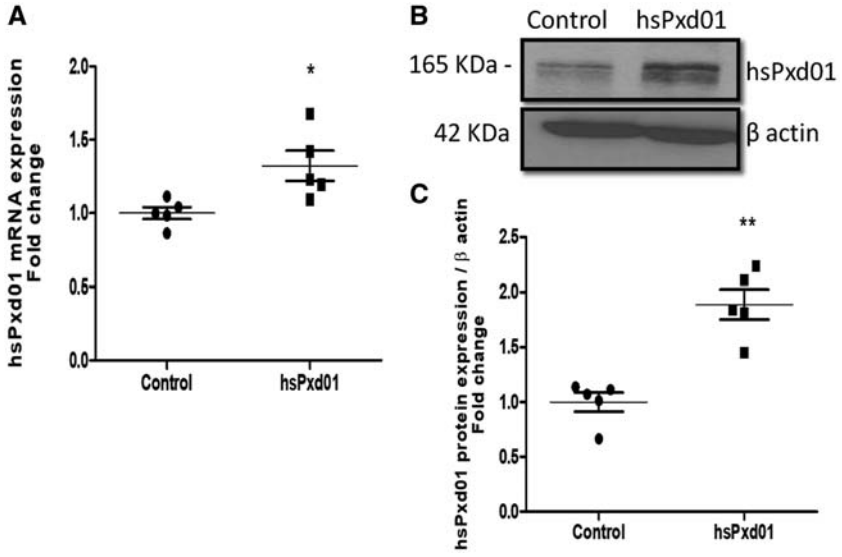


Figure 7 Extracellular peroxidasin 1 induces its own endogenous expression. TeloHAEC cells (3.5×10^5) were treated full length hsPxd01 (1 μM) for 48 h. (*A*) mRNA were extracted and analysed by qRT–PCR. Relative hsPxd01 mRNA expression levels were normalized to GAPDH. (*B*) Representative western blot analysis using anti-hsPxd01 antibody and quantification (graph) (*C*) show the induction of expression of endogenous hsPxd01 after 48 h of addition of full length recombinant hsPxd01. Results are expressed as fold change over control, and are shown as mean ± SEM of five independent experiments. Statistics were analysed using unpaired *t*-test (*$P < 0.05$, **$P < 0.01$; $n = 5$).

that extracellular hsPxd01 regulates the expression of some angiogenic genes (e.g. HEY-1, ID-2, and PDGFB) via expression of endogenous hsPxd01, while it controls another gene pack through a pathway independent of endogenous hsPxd01. In the same context, VEGF (100 ng/mL, 48 h) had no effect on the expression of the endogenous hsPxd01 (Supplementary material online, *Figure S2 A, B, C*). Moreover, hsPxd01 silencing did not affect downstream genes expression upon VEGF stimulation (Supplementary material online, *Figure S1 B2, B3, B4, B5, B6, B7*), suggesting that endogenous hsPxd01 does not interfere in pro-angiogenic response induced by VEGF. This may explain the rescue effect of exogenously added VEGF in hsPxd01 knockdown cells on both tube formation and cell migration (*Figure 3D–F* and Supplementary material online, *Figure S3A* and *B*, respectively). Taken together, our study suggests that hsPxd01 and VEGF promote angiogenesis independently.

4. Discussion

During angiogenesis, endothelial cells interact with extracellular matrix components. The present work proposes a role of human peroxidasin in this highly coordinated process. This multidomain peroxidase was originally described as an enzyme essential for extracellular matrix consolidation and remodelling by catalyzing the formation of sulfilimine bonds in collagen IV (the predominant constituent of basement membrane) via the H_2O_2-mediated oxidation of bromide to hypobromous acid.[15,19–21]

Among the many processes involved in angiogenesis, tube formation and cell migration are amenable to experimentation in culture through tubulogenesis and scratch assays. Our data clearly demonstrated that addition of recombinant hsPxd01 stimulated tube formation in endothelial cells as well as in the chicken embryo chorioallontoic membrane (CAM) assay. The recombinant enzyme significantly induced vascularization, associated with an increase of alpha-smooth muscle actin-positive vessels. Importantly, both full length homotrimeric hsPxd01 and the monomeric construct C4-active (which lacks the LRR and VWC domains) promoted tubulogenesis both *in vitro* and *in vivo*, whereas the enzymatically inactive construct (C4-inactive) had no impact. This corroborates recent studies demonstrating that LRR and VWC domains are not required for efficient sulfilimine cross-links formation in collagen IV mediated by various hsPxd01 constructs.[21] Moreover, it has been demonstrated that the proteolytic elimination of the VWC domain by proprotein convertase *in vivo* enhances the catalytic activity of hsPxd01 and represents a key regulatory process in the physiological function of human peroxidasin 1.[35] Along the same line, a recent report has shown that the pro-angiogenic effect of two other heme peroxidase family members MPO and EPO was reduced using a specific peroxidase inhibitor, highlighting the importance of the catalytic activity for peroxidase-mediated angiogenesis.[29] Furthermore, we confirmed the importance of endogenous hsPxd01 in angiogenesis, since its silencing reduced both tube formation and cell migration. These findings are supported by recent studies showing that melanoma and choriocarcinoma cells migratory behaviour and invasion were reduced as a consequence of hsPxd01 silencing.[36,37]

Active recombinant peroxidasin 1 (both full length hsPxd01 and C4-active) were demonstrated to promote the pro-angiogenic activity by activation of the extracellular signal-regulated kinases (ERK1/2), Protein kinase B (Akt), and FAK, which are known to regulate cellular events required for endothelial proliferation, migration and tube formation[38–40] similar to VEGF used as a positive control. In hsPxd01 knockdown cells, phosphorylation of Akt and FAK (but not ERK1/2) was reduced, suggesting that endogenous hsPxd01 is implicated in Akt and FAK, but not in ERK1/2 activation. A recent study showed that endogenous hsPxd01 acts upstream of the Akt pathway.[41] However, another study reported that

endogenous hsPxd01 could also regulate ERK1/2 activation in smooth muscle cells.[13] So far, no study has demonstrated a link between peroxidasin 1 and FAK phosphorylation. The sustained activation of ERK1/2 may explain the rescue effect of exogenously added recombinant hsPxd01 in knockdown cells on tube formation, complementary to other signalling cascades that could be implicated. It has to be noted that endogenous hsPxd01 has been shown to activate Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) in endothelial cells.[42,43]

Knockdown of hsPxd01 only affected the expression of PDGFB, HEY-1, and ID-2, which could be correlated to Akt or FAK but not ERK dysregulation in those cells. Indeed, the inhibition of Akt pathway in tumorigenic hepatic and melanoma cells, led to a decrease in both PDGFB and HEY-1 expression.[44,45] Furthermore, in a model of human embryonic stem cells, the inhibition of Src/FAK signalling suppresses ID-2 expression.[46] With respect to unaffected genes, the inhibition of the ERK1/2 significantly decreases CXCL-1 and HB-EGF expression in endometrial and airway smooth muscle cells respectively.[47,48] Moreover, SNAI-1 has been identified as a downstream effector of ERK signalling during tumour progression.[49]

In summary, our work supports a role of hsPxd01 as a pro-angiogenic secreted peroxidase capable of regulating vessel formation through its catalytic activity. Moreover, we suggest possible signal transduction cascades by which hsPxd01 may mediate its pro-angiogenic response. Thus, the extracellular hsPxd01 promotes Akt, FAK, and ERK1/2 activation. The activation of the two first kinases involves endogenous hsPxd01 as a link. We propose that the pro-angiogenic role of endogenous hsPxd01 may be mediated through its capacity to form hypohalous acids, known as intracellular angiogenic modulators during vascular formation and development.[50] Further investigations are required to precisely establish the mechanisms by which hsPxd01 activates pro-angiogenic signalling, using proteomic-based approaches to screen for intracellular post-translational modifications. Therefore, human peroxidasin 1 could be an interesting angiogenic therapeutic molecule that would enhance wound repair and vascular regeneration after ischaemic events. However, additional works are required to investigate a possible implication of endogenous peroxidasin 1 in angiogenesis through alternative signalling pathways.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

Funding

This work was supported by grants from the CHU Charleroi; the Fonds de la Chirurgie Cardiaque; Fonds de la Recherche Médicale en Hainaut (FRMH); a fellowship grant from the Tunisian Ministry of Higher Education and Scientific Research to H.M; L.V. is Director of Research at the Belgian National Fund for Scientific Research; the Austrian Science Fund (project number P25538) and the doctoral program BioToP—Biomolecular Technology of Proteins (FWF W1224). The Analytical Platform is supported by the FRS-FNRS.

References

1. Ucuzian AA, Gassman AA, East AT, Greisler HP. Molecular mediators of angiogenesis. *J Burn Care Res* 2010;**31**:158–175.
2. Kumar R, Yoneda J, Bucana CD, Fidler IJ. Regulation of distinct steps of angiogenesis by different angiogenic molecules. *Int J Oncol* 1998;**12**:749–757.
3. De Cicco-Skinner KL, Henry GH, Cataisson C, Tabib T, Gwilliam JC, Watson NJ, Bullwinkle EM, Falkenburg L, O'Neill RC, Morin A, Wiest JS. Endothelial cell tube formation assay for the *in vitro* study of angiogenesis. *J Vis Exp* 2014;**91**:e51312.
4. Grant DS, Kibbey MC, Kinsella JL, Cid MC, Kleinman HK. The role of basement membrane in angiogenesis and tumor growth. *Pathol Res Pract* 1994;**190**:854–863.
5. Kim WY, Lee HY. Brain angiogenesis: mechanism and therapeutic intervention in brain tumors. *FEBS J* 2009;**276**:4653–4664.
6. Liu Y, Senger DR. Matrix-specific activation of Src and Rho initiates capillary morphogenesis of endothelial cells. *FASEB J* 2004;**18**:457–468.
7. Senger DR, Claffey KP, Benes JE, Perruzzi CA, Sergiou AP, Detmar M. Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. *Proc Natl Acad Sci USA* 1997;**94**:13612–13617.
8. Lo C-M, Wang H-B, Dembo M, Wang Y-L. Cell movement is guided by the rigidity of the substrate. *Biophys J* 2000;**79**:144–152.
9. Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev* 2004;**56**:549–580.
10. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005;**438**:932–936.
11. Cheng G, Li H, Cao Z, Qiu X, McCormick S, Thannickal VJ, Nauseef WM. Vascular Peroxidase-1 is rapidly secreted circulates in plasma, and supports dityrosine cross-linking reactions. *Free Radic Biol Med* 2011;**51**:1445–1453.
12. Zhang YS, He L, Liu B, Li NS, Luo XJ, Hu CP, Ma QL, Zhang GG, Li YJ, Peng J. A novel pathway of NADPH oxidase/vascular peroxidase 1 in mediating oxidative injury following ischemia–reperfusion. *Basic Res Cardiol* 2012;**107**:266.
13. Shi R, Hu C, Yuan Q, Yang T, Peng J, Li Y, Bai Y, Cao Z, Cheng G, Zhang G. Involvement of vascular peroxidase 1 in angiotensin II-induced vascular smooth muscle cell proliferation. *Cardiovasc Res* 2011;**91**:27–36.
14. Péterfi Z, Donkó A, Orient A, Sum A, Prókai A, Molnár B, Veréb Z, Rajnavölgyi E, Kovács KJ, Müller V, Szabó AJ, Geiszt M. Peroxidasin is secreted and incorporated into the extracellular matrix of myofibroblasts and fibrotic kidney. *Am J Pathol* 2009;**175**:725–735.
15. Lázár E, Péterfi Z, Sirokmány G, Kovács HA, Klement E, Medzihradszky KF, Geiszt M. Structure-function analysis of peroxidasin provides insight into the mechanism of collagen IV crosslinking. *Free Radic Biol Med* 2015;**83**:273–282.
16. Soudi M, Zámocký M, Jakopitsch C, Furtmüller PG, Obinger C. Molecular evolution, structure, and function of peroxidasins. *Chem Biodivers* 2012;**9**:1776–1793.
17. Soudi M, Paumann-Page M, Delporte C, Pirker KF, Bellei M, Edenhofer E, Stadlmayr G, Battistuzzi G, Boudjeltia KZ, Furtmüller PG, Van Antwerpen P, Obinger C. Multidomain human peroxidasin 1 is a highly glycosylated and stable homotrimeric high spin ferric peroxidase. *J Biol Chem* 2015;**290**:10876–10890.
18. Cheng G, Salerno JC, Cao Z, Pagano PJ, Lambeth JD. Identification and characterization of VPO1, a new animal heme containing Peroxidase. *Free Radic Biol Med* 2008;**45**:1682–1694.
19. Nelson RE, Fessler LI, Takagi Y, Blumberg B, Keene DR, Olson PF, Parker CG, Fessler JH. Peroxidasin: a novel enzyme-matrix protein of Drosophila development. *EMBO J* 1994;**13**:3438–3447.
20. Bhave G, Cummings CF, Vanacore RM, Kumagai-Cresse C, Ero-Tolliver IA, Rafi M, Kang JS, Pedchenko V, Fessler LI, Fessler JH, Hudson BG. Peroxidasin forms sulfilimine chemical bonds using hypohalous acids in tissue genesis. *Nat Chem Biol* 2012;**8**:784–790.
21. Ero-Tolliver IA, Hudson BG, Bhave G. The ancient immunoglobulin domains of peroxidasin are required to form sulfilimine cross-links in collagen IV. *J Biol Chem* 2015;**290**:21741–21748.
22. Khan K, Rudkin A, Parry DA, Burdon KP, McKibbin M, Logan CV, Abdelhamed ZI, Muecke JS, Fernandez-Fuentes N, Laurie KJ, Shires M, Fogarty R, Carr IM, Poulter JA, Morgan JE, Mohamed MD, Jafri H, Raashid Y, Meng N, Piseth H, Toomes C, Casson RJ, Taylor GR, Hammerton M, Sheridan E, Johnson CA, Inglehearn CF, Craig JE, Ali M. Homozygous mutations in *PXDN* cause congenital cataract, corneal opacity, and developmental glaucoma. *Am J Hum Genet* 2011;**89**:464–473.
23. Liu Y, Carson-Walter EB, Cooper A, Winans BN, Johnson MD, Walter KA. Vascular gene expression patterns are conserved in primary and metastatic brain tumors. *J Neurooncol* 2010;**99**:13–24.
24. Castronovo V, Waltregny D, Kischel P, Roesli C, Elia G, Rybak JN, Neri D. A chemical proteomics approach for the identification of accessible antigens expressed in human kidney cancer. *Mol Cell Proteomics* 2006;**5**:2083–2091.
25. Van Hinsbergh VW, Koolwijk P. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. *Cardiovasc Res* 2008;**78**:203–212.
26. Neve A, Cantatore FP, Maruotti N, Corrado A, Ribatti D. Extracellular matrix modulates angiogenesis in physiological and pathological conditions. *BioMed Res Int* 2014;**2014**:1.
27. Herbert SP, Stainier DY. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol* 2011;**12**:551–564.
28. Davis GE, Koh W, Stratman AN. Mechanisms controlling human endothelial lumen formation and tube assembly in three-dimensional extracellular matrices. *Birth Defects Res C Embryo Today* 2007;**81**:270–285.
29. Panagopoulos V, Zinonos I, Leach DA, Hay SJ, Liapis V, Zysk A, Ingman WV, DeNichilo MO, Evdokiou A. Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. *Int J Biochem Cell Biol* 2015;**68**:128–138.
30. Zámocký M, Jakopitsch C, Furtmüller PG, Dunand C, Obinger C. The peroxidase-cyclooxygenase superfamily: reconstructed evolution of critical enzymes of the innate immune system. *Proteins* 2008;**72**:589–605.

31. Zámocký M, Hofbauer S, Schaffner I, Gasselhuber B, Nicolussi A, Soudi M, Pirker KF, Furtmüller PG, Obinger C. Independent evolution of four heme peroxidase superfamilies. *Arch Biochem Biophys* 2015;**574**:108–119.
32. Longo PA, Kavran JM, Kim M-S, Leahy DJ. Transient mammalian cell transfection with polyethylenimine (PEI). *Methods Enzymol* 2013;**529**:227–240.
33. Zudaire E, Gambardella L, Kurcz C, Vermeren S. A computational tool for quantitative analysis of vascular networks. *PLoS One* 2011;**6**:e27385.
34. Paumann-Page M, Katz RS, Bellei M, Schwartz I, Edenhofer E, Sevcnikar B, Soudi M, Hofbauer S, Battistuzzi G, Furtmüller PG, Obinger C. Pre-steady-state kinetics reveal the substrate specificity and mechanism of halide oxidation of truncated human peroxidasin 1. *J Biol Chem* 2017;**292**:4583–4592.
35. Colon S, Bhave G. Proprotein convertase processing enhances peroxidasin activity to reinforce collagen IV. *J Biol Chem* 2016;**291**:24009–24016.
36. Jayachandran A, Prithviraj P, Lo P-H, Walkiewicz M, Anaka M, Woods BL, Tan B, Behren A, Cebon J, McKeown SJ. Identifying and targeting determinants of melanoma cellular invasion. *Oncotarget* 2016;**7**:41186–41202.
37. Tauber S, Jais A, Jeitler M, Haider S, Husa J, Lindroos J, Knöfler M, Mayerhofer M, Pehamberger H, Wagner O, Bilban M. Transcriptome analysis of human cancer reveals a functional role of heme oxygenase-1 in tumor cell adhesion. *Mol Cancer* 2010;**9**:200.
38. Srinivasan R, Zabuawala T, Huang H, Zhang J, Gulati P, Fernandez S, Karlo JC, Landreth GE, Leone G, Ostrowski MC. *Erk1* and *Erk2* regulate endothelial cell proliferation and migration during mouse embryonic angiogenesis. *PLoS One* 2009;**4**:e8283.
39. Tavora B, Batista S, Reynolds LE, Jadeja S, Robinson S, Kostourou V, Hart I, Fruttiger M, Parsons M, Hodivala-Dilke KM. Endothelial FAK is required for tumour angiogenesis. *EMBO Mol Med* 2010;**2**:516–528.
40. Jiang BH, Liu LZ. AKT signaling in regulating angiogenesis. *Curr Cancer Drug Targets* 2008;**8**:19–26.
41. Tang Y, Xu Q, Peng H, Liu Z, Yang T, Yu Z, Cheng G, Li X, Zhang G, Shi R. The role of vascular peroxidase 1 in ox-LDL-induced vascular smooth muscle cell calcification. *Atherosclerosis* 2015;**243**:357–363.
42. Li DJ, Zhao T, Xin RJ, Wang YY, Fei YB, Shen FM. Activation of α7 nicotinic acetylcholine receptor protects against oxidant stress damage through reducing vascular peroxidase-1 in a JNK signaling-dependent manner in endothelial cells. *Cell Physiol Biochem* 2014;**33**:468–478.
43. Bai YP, Hu CP, Yuan Q, Peng J, Shi RZ, Yang TL, Cao ZH, Li YJ, Cheng G, Zhang GG. Role of VPO1, a newly identified heme-containing peroxidase, in ox-LDL induced endothelial cell apoptosis. *Free Radic Biol Med* 2011;**51**:1492–1500.
44. Lau CK, Yang ZF, Ho DW, Ng MN, Yeoh GC, Poon RT, Fan ST. An Akt/hypoxia-inducible factor-1 alpha/platelet-derived growth factor-BB autocrine loop mediates hypoxia-induced chemoresistance in liver cancer cells and tumorigenic hepatic progenitor cells. *Clin Cancer Res* 2009;**15**:3462–3471.
45. Bedogni B, Warneke JA, Nickoloff BJ, Giaccia AJ, Powell MB. Notch1 is an effector of Akt and hypoxia in melanoma development. *J Clin Invest* 2008;**118**:3660–3670.
46. Afrikanova I, Yebra M, Simpkinson M, Xu Y, Hayek A, Montgomery A. Inhibitors of Src and focal adhesion kinase promote endocrine specification: impact on the derivation of β-cells from human pluripotent stem cells. *J Biol Chem* 2011;**286**:36042–36052.
47. Baston-Büst DM, Schanz A, Böddeker SJ, Altergot-Ahmad O, Krüssel JS, Rein D, Hess AP. CXCL1 expression in human decidua in vitro is mediated via the MAPK signalling cascade. *Cytokine* 2013;**64**:79–85.
48. Kumasawa F, Hashimoto S, Mizumura K, Takeshita I, Onose A, Jibiki I, Maruoka S, Gon Y, Kobayashi T, Takahashi N. Mitogen-activated protein kinase (MAPK) regulates leukotriene D4-induced HB-EGF and ADAM12 expression in human airway smooth muscle cells. *Asian Pac J Allergy Immunol* 2013;**31**:58–66.
49. Wang S, Cheng Y, Zheng Y, He Z, Chen W, Zhou W, Duan C, Zhang C. PRKAR1A is a functional tumor suppressor inhibiting ERK/Snail/E-cadherin pathway in lung adenocarcinoma. *Sci Rep* 2016;**6**:39630.
50. Zhou Y, Yan H, Guo M, Zhu J, Xiao Q, Zhang L. Reactive oxygen species in vascular formation and development. *Oxid Med Cell Longev* 2013;**2013**:374963.