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REVIEW

Matricellular protein thrombospondin-1 in pulmonary hypertension: multiple pathways to disease

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Abstract

Matricellular proteins are secreted molecules that have affinities for both extracellular matrix and cell surface receptors. Through interaction with structural proteins and the cells that maintain the matrix these proteins can alter matrix strength. Matricellular proteins exert control on cell activity primarily through engagement of membrane receptors that mediate outside-in signaling. An example of this group is thrombospondin-1 (TSP1), first identified as a component of the secreted product of activated platelets. As a result, TSP1 was initially studied in relation to coagulation, growth factor signaling and angiogenesis. More recently, TSP1 has been found to alter the effects of the gaseous transmitter nitric oxide (NO). This latter capacity has provided motivation to study TSP1 in diseases associated with loss of NO signaling as observed in cardiovascular disease and pulmonary hypertension (PH). PH is characterized by progressive changes in the pulmonary vasculature leading to increased resistance to blood flow and subsequent right heart failure. Studies have linked TSP1 to pre-clinical animal models of PH and more recently to clinical PH. This review will provide analysis of the vascular and non-vascular effects of TSP1 that contribute to PH, the experimental and translational studies that support a role for TSP1 in disease promotion and frame the relevance of these findings to therapeutic strategies.

Keywords Thrombospondin-1 • CD47 • Pulmonary hypertension • Nitric oxide • eNOS • Endothelin-1 • ROS • Nox1 • cMyc • Vasorelaxation

1. Introduction

Matricellular proteins are a diverse group of molecular entities that are secreted into the extracellular milieu from where they modify cell responses. The term matricellular protein has been employed for over 20 years and was coined to embrace the properties of secreted proteins that were noted to alter cell attachment to matrix and cell-cell attachments.[1] Contained within the term is the idea that these proteins interact with matrix, other secreted proteins including growth factors, enzymes such as proteases, calcium, and with the cell membrane and membrane-located receptors, but do not serve as structural elements of the extracellular matrix.[2] A founding member of this group is thrombospondin-1 (TSP1), a trimeric glycoprotein composed of 150-KDa monomers linked through disulfide bonds.[3] These later play a role in TSP1-mediated adhesion via integrins,[4] in growth factor signaling[5] and in Ca^{2+} binding.[6] Each TSP1 monomer consists of an N-terminal globular domain that binds heparin, type I, II, and III repeats, and a C-terminal globular domain.[3] TSP1 was first described as a protein released from thrombin-activated platelets a characteristic incorporated in its name.[7] TSP1 regulates multiple cellular activities, in part, due to its multi-domain structure permitting interaction with several cell receptors and matrix proteins[8] (*Table 1*). Initially assigned a role in modifying the tumor micro-environment, further work suggests a role for TSP1 in vascular[9] and metabolic diseases,[10] although it also has a role in health and homeostasis. This review will consider the systemic and the pulmonary vascular effects of TSP1 signaling and the implications of these for pulmonary hypertension (PH).

2. Thrombospondin-1 (TSP1)–in health and homeostasis

TSP1 has under-appreciated functions in health including the maintenance of vascular structure and homeostasis through the regulation of cell proliferation, apoptosis, and adhesion.[3,11] In development TSP1 supports synapse formation in the central nervous system,[12] while in adult mice absence of TSP1 is associated with increased seizure susceptibility.[13] TSP1

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Table 1 TSP1 domains with receptor and interacting partners

	N-terminal domain	TSRs domains	C-terminal domain
Molecular interaction	• Integrins • ($\alpha3\beta1$,$\alpha4\beta1$, $\alpha6\beta1$, $\alpha9\beta1$)[121–124] • Fibrinogen[125] • HSPG/Heparin[126] • LRP[127] • Decorin[128] • Calreticulin[129]	• $\beta1$ integrins[121] • CD36[130] • Fibronectin[131] • MMP-2[132] • TGF-β[133] • HSPG	• CD47[48] • Integrins ($\alpha v\beta3$, $\alpha IIb\beta3$)[134] • Cathepsin G and Elastase[135]
Functions	• Platelet aggregation • Focal adhesion disassembly • Cell attachment, spreading, proliferation and migration	• Inhibits endothelial cell (EC) proliferation and angiogenesis • Induces EC apoptosis • Cell attachment • Matrix interactions • Platelet aggregation • Immune responses • Neurite outgrowth	• Cell attachment and spreading • Cell migration • Platelet aggregation • Smooth muscle cell proliferation • T cell activation • Inhibition of NO signaling • Protease inhibition

is necessary for maintaining normal lacrimal gland homeostasis[14] and the immune privileged status of the ocular region.[15] TSP1 influences von Willebrand Factor (VWF) multimer size and may control vWF release.[16] TSP1 has a beneficial role in modulating inflammation through tempering macrophage[17] and T cell[18,19] responses. In left ventricle pressure overload induced by aortic constriction, mice lacking TSP1 showed more injury compared to mice expressing TSP1,[20] whereas after cutaneous thermal injury the rate of wound closure was decreased in the absence of TSP1.[21] Together these findings speak to a homeostatic role for TSP1 in wound healing.

3. TSP1–a natural anti-angiogenic

TSP1 limited endothelial cell chemotaxis to fibroblast growth factor β (FGF-β) and FGF-β-stimulated angiogenic outgrowth in rat corneas.[22] Concurrently, it was reported that TSP1 limited endothelial cells proliferation[23] identifying it as a natural inhibitor of angiogenesis. In endothelial cells TSP1 inhibited the effects of vascular endothelial growth factor (VEGF) by suppressing VEGF's[24] ability to increase cyclic guanosine monophosphate (cGMP), a downstream effector of the biogas nitric oxide (NO).[25] This latter activity was thought to be through engagement of cell receptor CD36 on endothelial cells.[25] However the specificity of this interaction was in doubt as other CD36-interacting partners including collagen and oxidized LDL mimicked the inhibitory actions of TSP1 on endothelial cells.[26] TSP1 can also bind soluble VEGF and displace cell membrane-bound VEGF[27] to limit endothelial cell angiogenic activity. Insight was provided through the observation that minimal concentrations of TSP1 (0.2–2 nM TSP1) interacting with the cell receptor CD47 inhibited VEGF-mediated activation of receptor VEGF-R2 on endothelial cells,[28] and this was absent in endothelial cells lacking CD47. Overexpression of TSP1 in mice confirmed the anti-angiogenic activity of TSP1.[29,30] Not surprisingly, enhancing TSP1 signaling with domain-derived peptides and molecules that enhance specific activities of the protein are in development as therapies for excessive ocular angiogenesis,[31] and to inhibit tumor-driven angiogenesis.[32]

4. TSP1 and nitric oxide (NO)

Nitric oxide (NO) is a gaseous transmitter produced under physiologic and pathologic conditions by several synthetic enzymes (reviewed in Ref. 33). In response to blood flow, increased NO production at low concentrations by endothelial nitric oxide synthase (eNOS)[34] is rapid, intermittent and limits endothelial inflammation,[35] thrombosis formation[36,37] and vasoconstriction[38] to increase blood flow. Low concentrations of NO regulate cell signaling by binding to soluble guanylate cyclase to increase soluble guanylate cyclase (sGC).[39] In inflammation, NO produced by inducible nitric oxide synthase[40] is sustained, substantial and invokes transcriptional changes in cells that are distinct from those mediated by eNOS-derived NO.[41]

The effect of TSP1 to inhibit NO was first appreciated in experiments that assessed the anti-angiogenic activity of TSP1. Skeletal muscle from young male *Thbs1*$^{-/-}$ mice explanted in collagen matrix had increased vascular cell outgrowth compared to wild type (TSP1$^{+/+}$) samples following treatment with an NO donor.[25] Further, TSP1 inhibited NO-induced cell outgrowth in explants from wild type mice.[25] *In vitro*, NO-mediated effects on endothelial cell proliferation, cGMP levels and cGMP-sensitive kinase activation were inhibited by 1 μg/mL (2.2 nM) TSP1.[25] Thus, in endothelial cells TSP1 exerts upstream and downstream control of NO (*Figure 1*). Subsequent studies found that TSP1 inhibited NO signaling in other vascular cells including arterial vascular smooth muscle cells (VSMC)[42] and platelets.[43] As noted, TSP1 binds to several cell receptors including calreticulin/low-density lipoprotein (LDL) receptor-related protein, CD148, syndecan-3, neuroligin-1, very-low-density-lipoprotein receptor, six different integrins, CD36 and CD47 (reviewed in Ref. 44). It was not clear which of these receptors was responsible for transducing TSP1 inhibition of NO. Initial attention again focused on CD36. NO-stimulated increases in endothelial cell cGMP were inhibited by treating with TSP1, a CD36 agonist antibody and a domain of TSP1 that binds to CD36.[25] Proximate to NO activation of sGC, TSP1 limits eNOS activation and production of NO through alteration in calcium signaling[45] and in some circumstances via CD36 restricting uptake of the fatty acid myristate.[46] However, TSP1 also inhibits NO signaling in *CD36*$^{-/-}$ VSMC[47] that express the TSP1 receptor CD47.[48]

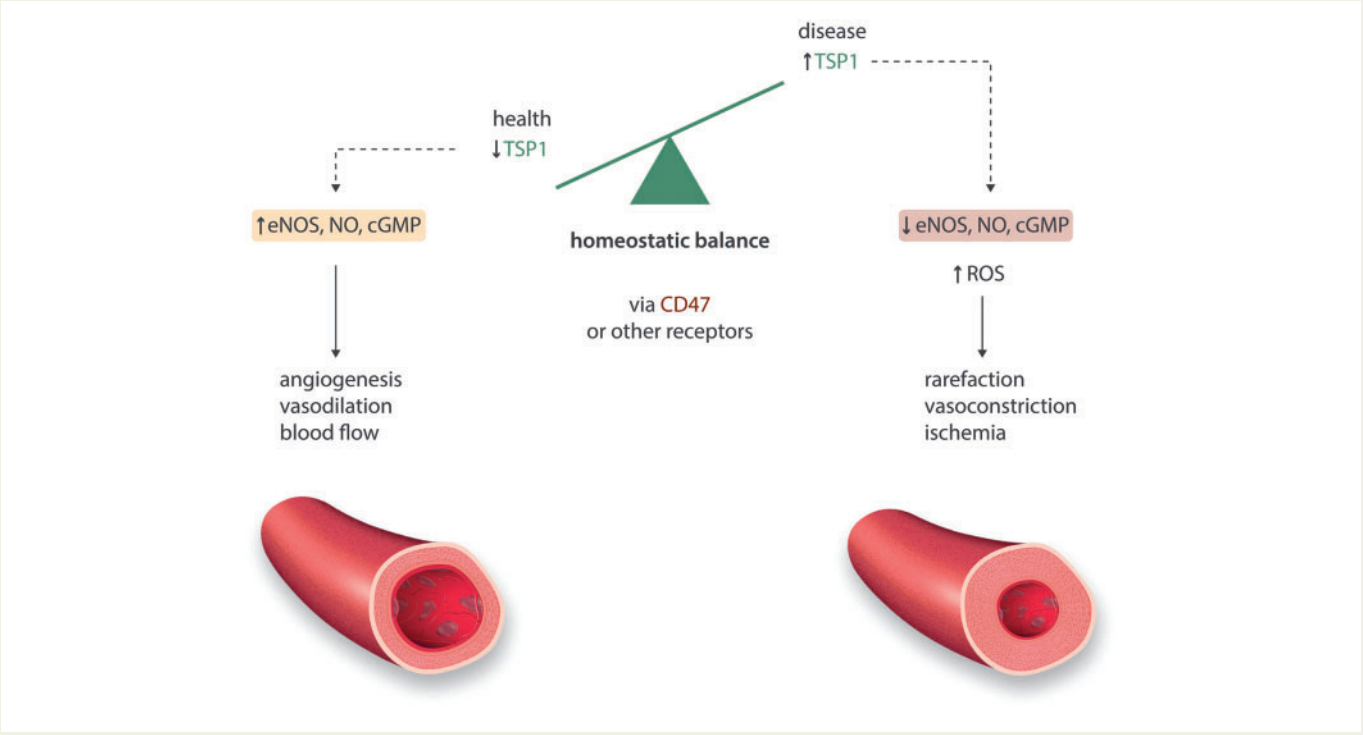

Figure 1 Thrombospondin-1 negatively intersects vascular NO-ROS. Under healthy conditions TSP1 is almost undetectable. In acute and chronic injury and disease, TSP1 is induced in the systemic vasculature to engage CD47 and limit NO signaling through decreasing eNOS activity, and separately by rendering sGC and PKG resistant to activation. Separately, TSP1 limits cAMP signaling. TSP1 also activates NADPH oxidase 1 (Nox1) increasing superoxide production. Acting via these several pathways TSP1 promotes vascular rarefaction, vasoconstriction and ischaemia.

This finding was important, as vascular cells from *Cd47*$^{-/-}$ mice, that express CD36, were insensitive to TSP1-mediated inhibition of NO signaling.[47] Studies employing the recombinant CD47 binding domain of TSP1, agonist and antagonist CD47 antibodies, and vascular cells and tissues from *CD36*$^{-/-}$ and *Cd47*$^{-/-}$ mice confirmed that while CD36 is sufficient for TSP1 inhibition of NO only CD47 is necessary[47] (*Figure 1*). It was noted that TSP1 did not bind to a bacterial-derived recombinant CD47 extracellular domain raising concerns that TSP1 was not a ligand of CD47.[49,50] Subsequent studies demonstrated that TSP1 binds with high affinity to CD47 to limit NO signaling[48] and that in some cells this binding requires a glycosylation that is absent in bacterial-expressed CD47.[51] At baseline *Thsp1*$^{-/-}$ and *Cd47*$^{-/-}$ mice have elevated levels of phosphorylated (active) eNOS,[45] cGMP, and cAMP in tissues compared to controls.[52] Conversely, cultured human umbilical vein endothelial cells (HUVEC) treated with the NO donor (DETA/NO, 0.1 μM, 24 h) showed a 50% decrease in TSP1 protein vs. untreated.[53] The finding of NO-mediated suppression of TSP1 has yet to be confirmed *in vivo*. Still these findings are important in light of the broad anti-angiogenic activity of TSP1 and have implications in regard to pulmonary hypertension (PH) as this is a disease of low NO signaling and elevated TSP1.

5. TSP1 and reactive oxygen species (ROS)

NO has a half-life of seconds[39] and is more transient in conditions of inflammation where the reactive oxygen species (ROS) superoxide is abundant. Treating U937 cells, a human monocyte cell line, with TSP1 (20 μg/mL) enhanced phorbol myristate-mediated superoxide production, and this was abrogated by superoxide dismutase or a α6β1 integrin blocker.[54] Aortic VSMC from young *Cd47*$^{-/-}$ mice had lower ROS production compared to wild type cells, when quantified by dihydroethidium (DHE), dichlorofluorescein and Mitosox fluorescence.[55] Human pulmonary arterial endothelial cells challenged with hypoxia (1% O_2, 12 h) displayed increased TSP1 protein expression and increased superoxide production quantified by DHE fluorescence.[56] Treatment with a human CD47 antibody (clone B6H12, 1 μg/mL) that blocks TSP1 binding,[48] suppressed the hypoxia-mediated increase in superoxide.[56] In hearts from 30 month old mice TSP1 was increased compared to hearts from 2 month old animals,[57] while in the skin of aged wild type mice TSP1 and CD47 protein expression was increased and associated with decreased blood flow vs. young animals.[58] Together, these findings suggest that TSP1 promotes ROS production and that aging may upregulate TSP1-CD47 signaling. Expanding upon this, studies in arterial VSMC[59] and renal tubule epithelial cells (rTEC)[60] demonstrated that treatment with TSP1 at relevant concentrations (2.2 nM) increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1-derived superoxide production (Nox1). Further, TSP1 via both CD47[59] as well as cell membrane receptor signal regulatory protein-α (SIRP-α)[60] phosphorylated the key Nox1 organizer subunit $p47^{phox}$ and increased superoxide production. It remains to be determined if these ligand-receptor interactions stimulate Nox1 in a mutually dependent or independent manner. Nonetheless, involvement of SIRP-α[61] in vascular and epithelial cell signaling anticipates a role beyond phagocytosis.

6. TSP1 inhibits transplant healing and blood flow

Skin grafting is a standard model of angiogenic tissue healing.[62] In patients with skin grafts to burn wounds, soluble TSP1 was found in the wound fluid,[63] however it was not clear if this was associated in any way with graft healing. Skin grafts transplanted from wild type C57Bl/6 mice to wounds in *Thsp1*$^{-/-}$ and *Cd47*$^{-/-}$ mice (both strains on a C57BL/6 background) resulted in improved healing vs. transplants of wild type skin grafts to wild type recipients.[64] Treatment with a TSP1-CD47 antagonist antibody or a CD47 targeting morpholino oligonucleotide that decreased CD47 protein levels increased healing rates of wild type grafts.[64] Curiously, skin grafts from *Cd47*$^{-/-}$ mice displayed better healing when transplanted onto wild type recipients vs. wild type grafts. In light of the ability of wild type (SIRP-α +) macrophages to phagocytize circulating cells lacking CD47,[65] these data suggest that SIRP-α-CD47-mediated phagocytosis is less relevant for parenchymal cells.

A role for TSP1 in regulating blood flow was hinted at in relation to Sickle Cell Disease (SCD). Flow chambers coated with TSP1 had increased adhesion of SCD red blood cells (RBCs).[66] In a translational study, plasma TSP1 levels positively correlate with some vascular complications in SCD patients.[67] Under flow conditions TSP1 via CD47 promoted platelet adhesion to endothelial cells[68] and contributed to endothelial cell apoptosis.[69] Also, TSP1 promoted platelet adhesion under pathologic shear stress.[70] In other experiments, TSP1 expression increased in endothelial cells maintained in static (no-flow) conditions but was suppressed under laminar flow.[71] Finally, HUVEC exposed to shear stress (13 dynes/cm^2, 2 h) demonstrated increased TSP1 expression in the extracellular matrix compared to matrix from cells under static conditions.[72] These results suggest the intriguing idea that lowering of blood flow (and hence eNOS-derived NO production), through upregulation of TSP1 promotes vascular occlusion.

Evidence that TSP1 has a role in acutely controlling blood flow was first shown in young male wild type and *Thsp1*$^{-/-}$ mice. Challenging anesthetized animals with the NO donor (DEA/NO) resulted in increased hind limb skeletal muscle blood flow (quantified by BOLD-MRI) that was faster and greater in *Thsp1*$^{-/-}$ as compared to wild type mice.[73] In 14–18 month old male wild type, *Thsp1*$^{-/-}$ and *Cd47*$^{-/-}$ mice subjected to surgically-induced soft tissue ischaemia, laser Doppler demonstrated enhanced tissue blood flow immediately after injury in mice lacking TSP1 and CD47.[74] Interestingly, BOLD-MRI found that aged *Thsp1*$^{-/-}$ mice had improved NO-mediated blood flow changes 72 h after femoral artery ligation compared to aged wild type.[74] Potential differences in capillary density among mouse strains might have accounted for some of these effects. Nonetheless, young[75] as well as aged male wild type mice[74] and young pigs[76] treated with a CD47 suppressing morpholino oligonucleotide had less tissue injury and improved blood flow after ischaemia compared to controls. Acute ischaemia followed by reperfusion (IR) increases TSP1 expression in organs,[77] while animals lacking TSP1[77] and CD47[78,79] and wild types treated with CD47 blocking agents[78,80,81] are resistant to IR injury. Similarly, intravenous TSP1 impedes reperfusion in rats and this is corrected by pre-treatment with a CD47 blocking antibody (clone OX101).[59] In keeping with a role for TSP1 to acutely inhibit blood flow *in vivo*, studies in isolated systemic arteries found that TSP1 (0.22 nM), as well as a recombinant portion of the protein that contains the CD47 binding domain, inhibited vasorelaxation by the eNOS activator acetylcholine (Ach)[45] in arteries from wild type mice, whereas TSP1 inhibited Ach-stimulated vasorelaxation in arteries from *Thsp1*$^{-/-}$ but not *Cd47*$^{-/-}$ mice.[45] In other studies, TSP1 inhibition of Ach-mediated vasorelaxation in wild type vessels was partially ameliorated by treatment with the ROS scavenger Tempol.[60] Conversely, TSP1$^{-/-}$ and CD47$^{-/-}$ arteries were less sensitive to phenylephrine-mediated vasoconstriction compared to wild type arteries.[45] TSP1 also inhibited NO-mediated vasorelaxation of coronary arterioles from 24 month old, but not 4 month old, female rats.[82] Treatment with a CD47 blocking antibody (clone OX101) and ROS scavengers limited inhibition by TSP1, although the CD47 blocking antibody was more effective at increasing NO sensitivity.[82] Finally, TSP1 mRNA levels were increased in amputated limbs from individuals with peripheral vascular disease (PVD),[83] while plasma TSP1 levels positively correlated with PVD.[84]

7. Pulmonary hypertension–a disease of lung ischaemia

Pulmonary hypertension (PH) is a progressive fatal process characterized by among other things deterioration in NO signaling, hyperactive ROS production, progressive loss of micro-vascularity, ischaemia, absence of angiogenesis, proliferative remodeling of pulmonary macrovessels and increased resistance to pulmonary blood flow (reviewed in Refs. 85 and 86). As the circulatory system is an integrated unit, PH is predicted to have systemic manifestations. Yet it remains to be understood why in PH ischaemia primarily presents in the lung and why angiogenesis is suppressed.

8. TSP1 and PH–cell and animal studies

Over the last decade an interest in TSP1 in relation to PH has been pursued. In a pig model of pulmonary arterial (PA) IR lung injury, PA sensitivity to Ach-mediated vasorelaxation was decreased post-IR and this was associated with increased TSP1 mRNA levels vs. sham PAs.[87] This study is important in linking TSP1 and vascular dysfunction in PAs. Proliferation of human PA VSMC was inhibited by treatment with TSP1 (25 ng/mL).[88] Chronic hypoxia increases pulmonary vascular resistance in mammals. Young male mice exposed to hypoxia (10% FiO_2) for 1 and 7 days showed increased TSP1 and decreased CD36 transcript in microdissected pulmonary vessels compared to normoxic mice.[89] Unfortunately, cardiopulmonary pressure and heart morphometric data was not provided. *Thsp1*$^{-/-}$ mice exposed to 10% FiO_2 for 6 weeks displayed less increase in right ventricular systolic pressure (RVSP) and Fulton index (weight of right ventricle/left ventricle and septum) vs. wild type.[90] Pulmonary vascular remodeling was also less pronounced in lungs from *Thsp1*$^{-/-}$ mice.[90] Normoxic *Thsp1*$^{-/-}$ mice had less elevation in RVSP after treatment with the thromboxane A2 receptor agonist U-46619 and no elevation in RVSP following 15 min of hypoxia (10% FiO_2) compared to wild type. In these studies, *Thsp1*$^{-/-}$ mice received sulfamethoxazole and trimethoprim putatively to preempt possible lung infection.[11] It remains unknown what effect treatment with these drugs had on the outcomes. Others confirmed that pulmonary TSP1 is increased in hypoxia-[56] as well as bleomycin-mediated[91] PH, and that *Thsp1*$^{-/-}$ mice are resistant to cardiopulmonary changes following chronic hypoxia.[56] However, in bleomycin-mediated PH the data require careful interpretation as rat neonatal pups were employed. Interestingly, in normoxic pulmonary arterial endothelial cells TSP1 limited VEGF-stimulated

angiogenic activity,[91] similar to results in systemic arterial vascular endothelial cells,[25,28] while treatment with a Rho-kinase inhibitor partially limited thrombin (10 U/mL)-stimulated TSP1 production in pulmonary cells.[91] Together, these data suggest that absence of TSP1 provides protection from acute and chronic pulmonary vascular changes and that TSP1 has a presser effect in the pulmonary circulation. *Ex vivo* myography studies of PAs confirmed the presser activity of TSP1. Normoxic PAs from mice and rats treated with TSP1 (2.2 nM) were less sensitive to endothelial (Ach)- and VSMC (sodium nitroprusside, SNP)-mediated vasorelaxation, and in the case of rat PAs more sensitive to a vasoconstrictor.[92] When PAs were studied under hypoxic conditions, endothelial-mediated vasorelaxation was reduced in vessels from wild type but not *Thps1*$^{-/-}$ mice[93] (*Figure 2*).

Under low oxygen conditions, cells respond through hypoxia inducible transcription factors (HIFs).[94] TSP1 mRNA and protein are increased in lungs from mice within 7 h of hypoxia[93] concurrent with increased pulmonary expression of hypoxia inducible factor 2 alpha (HIF-2α). *In vitro*, TSP1 expression was also increased in murine fibroblasts and murine and human PA endothelial and VSMC exposed to 1% FiO_2 for 24 h.[93] Mutant mice that lack the Von Hippel-Lindau (VHL) protein, which mediates proteasomal degradation of HIF-α protein under normoxia[95] and mimic wild type mice under hypoxia, showed upregulation of pulmonary TSP1.[93] In contrast, *vhl*$^{-/-}$/*hif-2α*$^{-/-}$ double mutant mice had decreased pulmonary TSP1 protein and mRNA levels that were restored in *vhl*$^{-/-}$/*hif-1α*$^{-/-}$ mice, while suppression of HIF-2α in pulmonary vascular cells abrogated hypoxia-stimulated upregulation of TSP1.[93] Chinese hamster ovarian cells transfected with a luciferase-TSP1 hypoxia response element and a constitutively active HIF-2α construct had significant luciferase induction.[93] These data give genetic evidence for HIF-2α in the pulmonary induction of TSP1 (*Figure 2*).

Loss of NO signaling in PH includes decreased eNOS expression in human PAs, and this correlates inversely with the extent of vasculopathy and the degree of elevation in pulmonary vascular resistance,[96] although eNOS expression may be increased in pulmonary plexi-form lesions.[97] Further, NO and NO byproducts are decreased in lungs of PH subjects.[98] In keeping with data from systemic vascular cells, eNOS activation, as characterized by phosphorylation at serine 1176 (the murine equivalent of the human residue serine 1179[99]), was constitutively increased in lungs from normoxic young male *Thps1*$^{-/-}$ vs. wild type mice.[56] However, challenging *Thsp1*$^{-/-}$ mice with hypoxia (10% FiO_2, 3 weeks) resulted in decreased levels of phosphorylated eNOS, suggesting TSP1 does not directly control eNOS activity in hypoxic PH lungs.[56] In pulmonary endothelial cells Cav-1 interacts with CD47 and this interaction is suppressed by TSP1 and hypoxia,[56] while in lungs from hypoxic *Thsp1*$^{-/-}$ and *Cd47*$^{-/-}$ mice Cav-1 protein levels are increased and ROS levels decreased compared to lungs from hypoxic wild type mice. This is important as loss of Cav-1 results in PH.[100,101] Conversely, blocking TSP1-CD47 signaling resulted in increased Cav-1 expression and decreased eNOS-derived superoxide production by hypoxic pulmonary arterial endothelial cells.[45] These findings indicate that TSP1 inhibits the ability of Cav-1 to mitigate eNOS dysfunction under chronic hypoxia. However, it remains to be determined if TSP1 is an activator of Nox1 in the pulmonary vasculature. This is a valid question as Nox-derived superoxide contributes to pre-clinical PH[102] while Nox1, a target of TSP1 in systemic vascular cells,[59,60] is increased in PAs from hypoxic piglets.[103] Interestingly, hypoxia-stimulated proliferation of human PA VSMC was inhibited by treatment with a TSP1 antibody and by rosiglitazone (a peroxisome proliferator-activated receptor gamma blocker), while treatment with exogenous TSP1 (2.2 nM) for 72 h increased Nox4 protein expression.[104] Conversely, overexpression of Nox1 in A549 cells resulted in accumulation of HIF-1α,[105] suggesting the hypothesis that Nox1 via HIFs may act in a feed-forward fashion to increase TSP1.

While experiments in isolated pulmonary vascular cells and rodents point to a role for TSP1 to promote PH, the possible receptors transducing these effects have only started to be confirmed. Initial information was derived from studies in rats given the pyrrolizidine alkaloid monocrotaline (mct, 50 mg/kg). This induces a delayed but significant injury to the pulmonary circulation with increased pulmonary vascular resistance and RV hypertrophy.[106] Four weeks after treatment male rats treated with mct displayed increased expression of pulmonary TSP1 and CD47 protein and elevated RVSP and Fulton index compared to controls.[56] Treating mct injured animals with a CD47 blocking antibody (clone OX101, 1 μg/gram body weight on day 1 and day 14 via i.p. injection) partially normalized RVSP and Fulton index and was associated with increased pulmonary phosphorylated eNOS and Cav-1 protein expression.[56] Also, *Cd47*$^{-/-}$ mice are resistant to hypoxia-mediated cardiopulmonary aberrations compared to wild type.[92] These results indicate that absence of ligand TSP1 and receptor CD47 or blockade of TSP1-CD47 signaling provides protection from hypoxic- and mct-derived PH. Studies in pulmonary arterial endothelial cells and whole lungs indicate that the protection gained in the absence of CD47 is, in part, secondary to suppression of ET-1 signaling. It was noted that TSP1, via CD47, acted to constitutively suppress pulmonary cMyc which, being a key transcription factor regulator, significantly inhibited ET-1 and its pulmonary VSMC receptor endothelin-A (ETA).[92] In the setting of high TSP1-CD47 signaling cMyc is suppressed allowing for upregulation of pulmonary ET-1/ETA and VSMC hypertrophy. In the setting of low or absent TSP1-CD47 signaling cMyc is upregulated leading to the suppression of ET-1/ETA (*Figure 2*). A repercussion of this is that treating pulmonary VSMC with a CD47 blocking antibody limits the hypertrophic effects of exogenous ET-1.[92] ET-1 modifies NO signaling in vascular cells to both increase and decrease NO effects.[107] It remains to be seen if TSP1-CD47, via cMyc and ET-1, alters pulmonary NO signaling. It also remains to be determined why in the face of chronically elevated pulmonary cMyc, *Cd47*$^{-/-}$ mice have minimal vascular remodeling and overgrowth.

9. TSP1 and PH–human data

Studies in isolated mammalian pulmonary vascular cells, and rodent and pig models, indicate a role for TSP1 in PH. Beyond this, several recent studies have linked TSP1 to clinical PH. Analysis of lungs and distal PA (5th order) segments from patients undergoing transplant for end-stage PH confirmed upregulation of TSP1 and CD47 protein and TSP1 mRNA compared to non-PH lungs and PAs.[56,92] Immunofluorescent TSP1 and CD47 were increased in the medial and adventitial layers of distal PAs from end-stage PH lungs along with increased matrix protein mRNA.[92] Of functional relevance, TSP1 (2.2 nM) inhibited SNP-mediated vasorelaxation and potentiated ET-1-stimulated vasoconstriction of healthy 5th order PAs. Diseased distal PAs from PH lungs were diminished in vasorelaxation to Ach and SNP, whereas treating diseased PAs with a CD47 antibody (clone B6H12, 1 μg/mL) improved endothelial- and VSMC-mediated vasorelaxation.[92] In 93 individuals with isolated PH and 19 healthy controls, plasma TSP1 levels were assessed.[108] The disease cohort included idiopathic pulmonary arterial hypertension, lung disease-associated and chronic thromboembolic PH. Plasma TSP1 levels were elevated in all PH groups compared to controls and increased with worsening World Health Organization functional classification.[108]

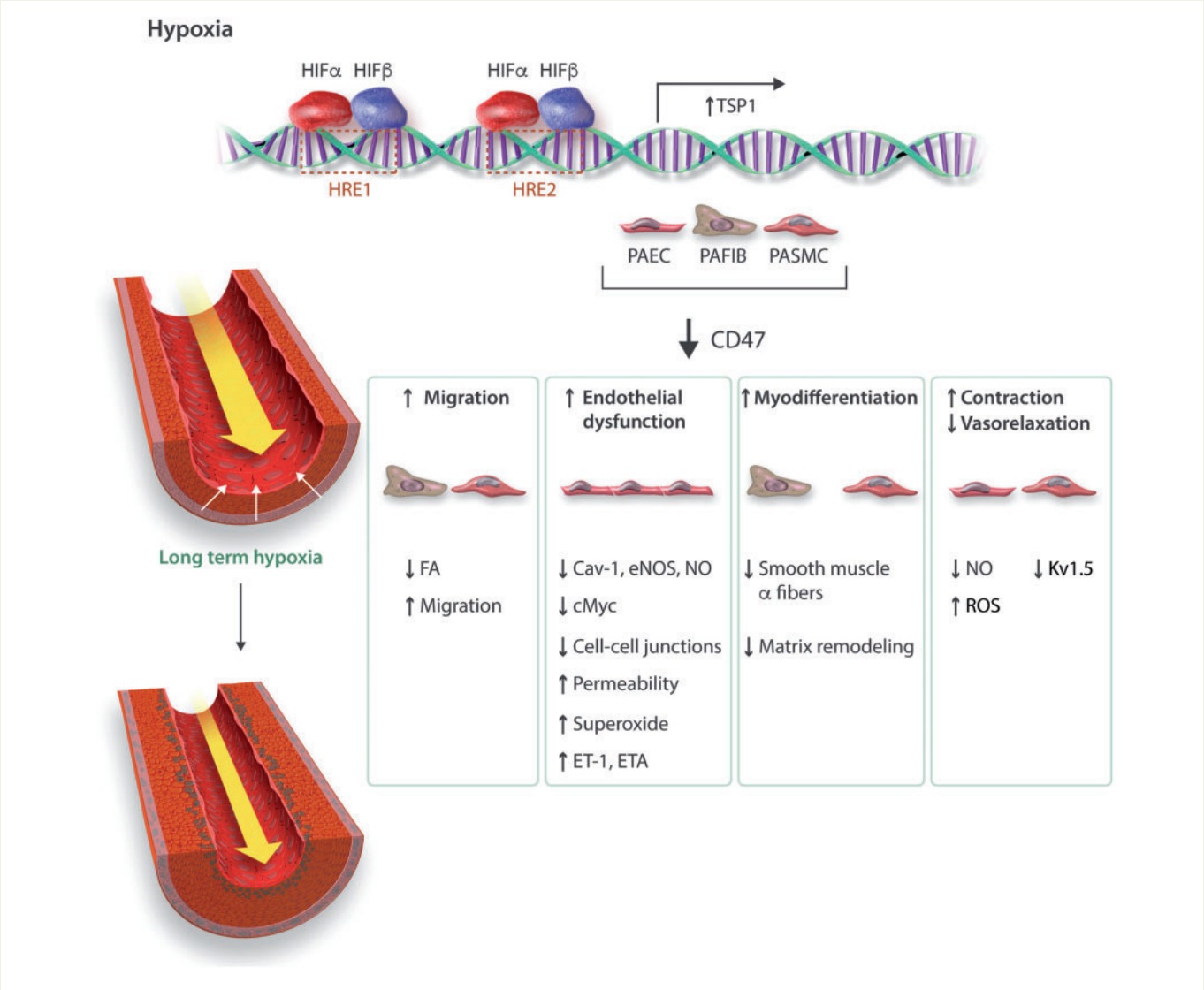

Figure 2 Thrombospondin-1 in PH. Hypoxia inducible factor-2α (HIF-2α) targets hypoxia response elements (HRE) to increase *THSP1* transcription in pulmonary vascular cells. In pulmonary arterial endothelial cells (PAEC) TSP1 via CD47 promotes PH by (1) targeting Cav-1 to dysregulate eNOS while increasing ROS, (2) suppressing cMyc to de-repress ET-1/ETA, and (3) decreasing cell-cell adhesion. TSP1 also increases pulmonary smooth muscle cell (PASMC) migration and fibroblast (PAFIB) activity. In pulmonary arteries, TSP1 inhibits vasorelaxation, increases vasoconstriction and in hypoxia decreases Kv1.5 ion channel effects.

Although 61% of PH subjects were women, no difference in TSP1 in relation to gender was found. Plasma TSP1 levels positively correlated with mean PA pressure and negatively correlated with cardiac output. Non-linear regression analysis found a bi-phasic pattern to plasma TSP1 levels with an initial increase as a function of increasing pulmonary vascular resistance that plateaued and then declined as resistance peaked, although the reason for this unclear. Noteworthy, chronically hypoxic wild type mice displayed a significant increase in plasma TSP1, whereas plasma TSP1 was not elevated in hypoxic *Cd47*$^{-/-}$ mice.[92] Finally, 5 year mortality in the PH cohort positively correlated with the degree in elevation in plasma TSP1.[108] Interestingly, TSP1 is increased in other pulmonary diseases including asthma,[109] idiopathic interstitial pneumonia,[110] and chronic obstructive pulmonary disease.[111] These and other studies (*Table 2*) suggest that TSP1 is a possible biomarker of pulmonary and cardiovascular disease.

Genetic links between *THBS1* and PH have also been reported. In familial pulmonary arterial hypertension several mutations of the type 1 repeat domain of TSP1 (which engages CD36 and mediates activation of latent transforming growth factor beta, TGF-β) were found in about 5% of individuals.[112] Recombinant mutant TSP1 protein or peptide mimics of the mutated region were less effective in activating latent TGF-β and at inhibiting cell proliferation.[112] Another report analyzed patients with homozygous haemoglobin SS (HbSS, SCD) from the multicentre walk-PHaSST (Treatment of Pulmonary Hypertension and Sickle Cell Disease with Sildenafil Therapy) trial and identified two single nucleotide polymorphisms (SNPs) of TSP1 that negatively associated with tricuspid value regurgitant velocity (TRV) ≥ 2.5 m/s, an echocardiographic marker of PH associated with increased mortality.[113] Both SNPs localized to the 5′ untranslated region. Limited but applicable data from explanted lungs from a SCD patient with end-stage PH found upregulation of TSP1 and

Table 2 Select studies implicating TSP1 as a possible biomarker

Target	Cohort	Readout	Correlation	Control for platelet activation	Reference
[CD142+/TSP1+] platelet microparticles from blood	clinical and genetic diagnosis of heterozygous Familial Hypercholesterolemia (FH) (n = 37) and non-FH secondary hypercholesterolemic patients (n = 37)	burden of atherosclerosis by MRI	TSP1+/CD142+ platelet-derived microparticles characterize young patients with high cardiovascular risk	unknown	[136]
TSP1 mRNA analysis - cardiac needle biopsies	11 cardiac allografts, 15 normal hearts	vasculopathy on angiography	+ correlation increased TSP1 mRNA and transplant vasculopathy	N/A	[137]
TSP1 in plasma	non-diabetics with fasting glucose, 2 h glucose and fasting insulin data, or who had type 2 diabetes	nano-liquid chromatography MS in SRM mode	+ correlation with TSP1 and pre-diabetes	unknown	[138]
TSP1 in cerebrospinal fluid	31 acute subarachnoid haemorrhage patients	transcranial Doppler of cerebral vasospasm	elevated TSP1 post subarachnoid haemorrhage correlated with cerebral vasospasm and poor outcome	N/A	[139]
TSP1 in plasma	61 chronic haemodialysis patients with diagnosis of cardiovascular disease including cerebrovascular, coronary and peripheral vascular disease	outcomes analysis of all-cause mortality and cardiovascular mortality	elevated TSP1 associated with CVD highest TSP1 level patients had a 5.32- and 6.75-fold more risk for all-cause and cardiovascular mortality	unknown	[140]

CD47 in parenchymal tissues, 5th order PAs and cultured VSMC from PAs.[114] Also, in 5th order PAs from the SCD lungs (that over-express TSP1-CD47) vasorelaxation to Ach was decreased compared to PAs from non-PH lungs.[114]

10. Therapeutic opportunities for targeting TSP1 signaling in PH

PH treatments focus on elevating NO signaling, limiting ET-1 signaling, increasing cyclic adenosine monophosphate (cAMP), and increasing cardiac function. TSP1 intersects NO-cGMP and ET-1 to promote adverse cellular responses in the pulmonary vasculature. TSP1 also dysregulates cAMP in arterial VSMC.[115] Thus, TSP1 exerts negative regulatory activity on current PH therapies, including heme-independent sGC activators.[116] However, TSP1 exists in large quantities pre-formed in platelets supporting targeting TSP1 receptors, and in particular CD47, in PH. CD47 antibodies are in clinical trials albeit for cancer (see: https://clinicaltrials.gov/ct2/results?term=CD47&Search=Search). These CD47 antibodies will likely have effects upon the vascular compartment in PH. Indeed, published *ex vivo* studies in diseased PAs from human PH lungs found that treatment with a commercially available human CD47 antibody enhanced vasorelaxation to endothelial and VSMC activators.[92] However, it is not known what effects targeting CD47 will have upon vascular remodeling and ischaemia in PH. Also, antibodies have low tissue penetration, although this may be circumvented by small molecule inhibitors or morpholino oligonucleotides. This strategy would have the additional benefit of avoiding the 'antibody sink' of CD47 expressed on circulating RBCs.[117] While small molecules targeting CD47 await development, morpholino oligonucleotides that effectively suppress CD47 have been demonstrated to provide injury protection in pre-clinical studies. As a class, these agents are in the clinic with the third-generation morpholino oligonucleotide Eteplirsen approved by the FDA for treating patients with Duchene muscular dystrophy.

11. Future directions and concluding remarks

TSP1 is emerging as a possible promoter of PH. Yet many important questions deserve attention. First, is TSP1 a primary or secondary contributor to PH? In animals, pulmonary TSP1 is upregulated rapidly following hypoxic challenge, well before gross cardiovascular and pulmonary injury, but what does this mean in relation to clinical disease. Second, the cellular source(s) of pulmonary TSP1 remain to be characterized. While *in vitro* results confirm that multiple pulmonary vascular cell types including endothelial, VSMC and fibroblasts can produce TSP1, it is also

possible that inflammatory cells and platelets contribute to pulmonary TSP1 expression. Cell-specific TSP1 and CD47 null mutant animals will be instrumental in addressing such questions. The role of other TSP1 receptors and signaling pathways warrants additional consideration. For example, Systemic Sclerosis is associated with PH and increased expression of CD36.[118] The finding of a link between TSP1 and pulmonary cMyc is possibly far reaching, but pre-clinical data should be complemented with clinical results. TSP1 is also an activator of latent TGF-β, and in rats mct-driven PH is partially ameliorated by treatment with the TGF-β blocker SD-208.[119] Interestingly, treatment of mice with a CD47 morpholino oligonucleotide decreased TGF-β mRNA levels associated with thermal injury,[21] suggesting a further benefit in PH from targeting TSP1-CD47. As a regulator of both NO and superoxide, TSP1 would be likely to have significant effects upon cardiac function. This notion is supported by the finding that *Cd47*$^{-/-}$ mice are protected from LV pressure overload secondary to transverse aortic constriction via regulation of Ca^{2+}-calmodulin-dependent protein kinase II.[120] The role of TSP1 to promote RV disease independent of pulmonary effects needs to be determined in a model of RV injury such as pulmonary arterial banding. Also, TSP1 has been involved in dysregulating Ca^{2+} signaling and this may have implication to cardiomyocyte function. The role of TSP1 to increase tissue remodeling and to alter matrix quality and quantity should be considered in relation to PH. Additional clinical studies of plasma TSP1 and mutations of TSP1 in PH are justified. In these future studies factors such as circadian rhythms, medications especially those that elevate NO and suppress ROS, sex, age and others would be useful to investigate. Lastly, it remains to be tested if TSP1 or one of the TSP1 receptors can be successfully suppressed, for how long and in which patients to obtain optimum therapeutic advantage in PH.

Published studies reviewed herein indicating a role for TSP1 in PH are relatively recent and are far fewer compared to the scientific literature implicating other factors promoting PH. Nonetheless, building upon a foundation of bench-top, animal and human studies from multiple groups it seems that TSP1 exerts negative effects in PH that are at the cross roads of angiogenesis, oxidative stress and hypertrophic growth signaling, supporting the need for continued basic, translation and clinical study.

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