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Modulation of VEGF Receptor 2 signaling by Protein Phosphatases

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Abstract

Phosphorylation of serines, threonines, and tyrosines is a central event in signal transduction cascades in eukaryotic cells. The phosphorylation state of any particular protein reflects a balance of activity between kinases and phosphatases. Kinase biology has been exhaustively studied and is reasonably well understood, however, much less is known about phosphatases. A large body of evidence now shows that protein phosphatases do not behave as indiscriminate signal terminators, but can function both as negative or positive regulators of specific signaling pathways. Genetic models have also shown that different protein phosphatases play precise biological roles in health and disease. Finally, genome sequencing has unveiled the existence of many protein phosphatases and associated regulatory subunits comparable in number to kinases. A wide variety of roles for protein phosphatase roles have been recently described in the context of cancer, diabetes, hereditary disorders and other diseases.

In particular, there have been several recent advances in our understanding of phosphatases involved in regulation of vascular endothelial growth factor receptor 2 (VEGFR2) signaling. The receptor is the principal signaling molecule mediating a wide spectrum of VEGF signal and, thus, is of paramount significance in a wide variety of diseases ranging from cancer to cardiovascular to ophthalmic. This review focuses on the current knowledge about protein phosphatases' regulation of VEGFR2 signaling and how these enzymes can modulate affect its biological effects.

Graphical abstract

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Keywords

Protein phosphatases; VEGFR2 signaling; endothelial cells; blood vessels; angiogenesis

1. Introduction

The human genome encodes 518 protein kinases and approximately 150 protein phosphatases [1, 2]. Members of both groups are historically classified according to their amino acid preference, serine (Ser)/threonine (Thr) or tyrosine (Tyr). A comparison between these categories shows that Protein Ser/Thr Kinases (PSKs) are roughly 10 times more numerous than their counterparts Protein Ser/Thr Phosphatases (PSPs) (~ 428 vs 40) [1, 3]. However, there is a comparable number of Protein Tyr Kinases (PTKs) and Protein Tyr Phosphatases (PTPs) (~ 90 vs 107) [1, 4]. Recent classifications have indicated a slightly different number of PTPs [5, 6]. A classification that considers only enzymatically active phosphatases (and includes nonprotein phosphatases) limits PTPs number to 96 [5]. However, a broader PTP definition, which includes criteria of function, structure and sequence similarities, has increased the total number of PTPs to 125 (namely the extended human PTPome)[6].

Classical PTPs only recognize p-Tyr residues and are divided into receptor PTPs (PTPR), which are localized at the cell membrane, and non-receptor intracellular PTPs (PTPN). A distinct PTP subgroup (~ 65 members) recognizes both p-Tyr and p-Ser/p-Thr residues and are referred to as Dual Specificity Phosphatases (DUSPs) [7]. The PTP catalytic domain contains the conserved sequence *HC-X₅-R* whose Cys-residue is necessary for the phosphatase activity and is susceptible to inactivation by reactive oxygen species (ROS)-mediated oxidation. PTPRs contain an extracellular domain that may bind soluble ligands or interact with extracellular matrix components, while the catalytic domain is located in the intracellular part of the protein. Conversely, PTPNs are found in the cytosol or bound to intracellular membrane compartments and presents a variety of regulatory domains that are critical for correct spatial and temporal localization.

PSPs mainly exist as holoenzymes consisting of a catalytic subunit and one or more regulatory subunits. PSP catalytic mechanisms are less conserved compared to PTPs and are usually classified into three families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases. Although there is a limited

number of PSP catalytic subunits (~30) [3], a large number of regulatory subunits that can associate with the same catalytic subunit may provide substrate and localization specificity. The reader is referred to recent reviews on this subject for a more exhaustive description of phosphatases classification, mechanism of action and molecular structure, [2, 3, 7].

VEGFR2 is a Receptor Tyrosine Kinase (RTK) predominantly expressed in endothelial cells (ECs) and their embryonic precursors [8-10], although its expression can be detected in neuronal cells and hematopoietic stem cells [11]. Deletion of VEGFR2 [12] or its main ligand VEGFA [13, 14] leads to early embryonic lethality in mice (E8.5/9.5 and E9.5/10.5, respectively) due to severe impairment of vascular development and hematopoietic cell maturation. Furthermore, deletion of a single VEGFA allele is sufficient to induce embryonic death at E11-12 [13, 14]. VEGFA was initially named as the Vascular Permeability Factor (VPF) after its ability to induce Evans Blue extravascular leakage in guinea pig back skin [15], however, it is now evident that VEGFA plays a larger role in both embryonic development and adult life [16, 17]. The activation of VEGFR2 by VEGFA can initiate multiple signaling pathways that orchestrate a variety of complex biological effects, such as endothelial cells maturation, vessel lumen formation, vascular permeability, vasodilation, angiogenesis and arteriogenesis [18-21]. Furthermore, knock-in mice which express only selected VEGFA splicing isoforms show marked phenotype differences; this suggests some degree of function segregation between the isoforms [16, 19]

Mounting evidence during the last decade demonstrates that phosphatases do not merely “switch-off”, but can finely modulate molecular and cellular responses by regulating phosphorylation events in negative or positive nodes of a signaling pathway [22]. A number of recent reviews have addressed the role of protein phosphatases in animal disease models and human diseases such as cancer [23, 24], diabetes [25], antiplatelet therapy [26], and hereditary disorders [27]. This review will focus on phosphatases known to affect VEGFR2 signaling (and its biological effects) by means of targeting phosphorylation of the receptor itself (Fig.1) or that of its downstream signal transducers (Fig. 2). VEGFR2 signaling plays an important role in diseases such as cancer [16], cardiovascular disease [28], retinopathies (age-related macular degeneration, AMD; diabetic macular edema, DME; retinopathy of prematurity, ROP; and others) [29], cerebrovascular disorders [30] and stroke [31], therefore phosphatases that modulate its signaling (Tables 1-2) may represent novel pharmacological targets to improve therapy of these diseases.

2. Cell membrane phosphatases

Binding of VEGFA to VEGFR2 induces receptor dimerization and autophosphorylation at multiple tyrosine sites including Y1054/1059, Y1175, Y951, and Y1214 and others less characterized [32]. Each site is thought to promote unique downstream signaling pathways, which are linked to different cellular responses such as proliferation, migration, survival and permeability [20, 21]. Both receptor and non-receptor protein phosphatases can regulate VEGFR2 signaling and will be discussed in turn.

2.1 VE-PTP (Vascular Endothelial PTP)

VE-PTP (also called hPTP- β , R-PTP β) is an endothelial-specific receptor PTP [33, 34]. It is found in the endothelium of all blood vessels and in the endocardium (with a high level of expression in arteries and cardiac valves) [33, 35]. VE-PTP substrates include angiopoietin-1 receptor TIE2 [34], VE-Cadherin [36], γ -catenin (Plakoglobin) [37], and VEGFR2 [38].

An association between VE-PTP and VEGFR2 has been shown by a proximity ligation assay [38]. However, binding of a VE-PTP substrate-trapping mutant (a phosphatase mutant that allows stable binding with its substrate without dephosphorylation [39]) to VEGFR2 requires formation of a trimeric complex with Tie2 [40]. The VE-PTP catalytic domain dephosphorylates immunoprecipitated VEGFR2 [40] while silencing of VE-PTP in ECs increases VEGFA-induced VEGFR2 phosphorylation at multiple sites (Y951, Y1175) in immortalized microvascular EC [38]. Baseline VEGFR2 phosphorylation is not affected by VE-PTP silencing [38, 40]. VE-PTP silencing also enhances VEGFA-induced EC proliferation *in vitro* [38]. In addition, embryonic bodies that lack VE-PTP form excessive vessel sprouts and filopodia in response to VEGFA due to persistent VEGFR2 Y1175 phosphorylation in stalk cells and increased ECs proliferation [40].

VE-PTP activity is important in regulation of VEGFA-driven lumen formation and endothelial cell polarity. Vessel sprouts from VE-PTP null embryonic bodies show disorganized podocalyxin (a marker for vessel luminal side) distribution and impaired lumen formation [40]. This defect is rescued by lowering VEGFA concentration, suggesting that this phenotype is due to VEGFR2 hyper-activation in the absence of VE-PTP. Similarly, VE-PTP knockdown in zebrafish markedly increases the frequency of lumen-less intersomitic vessels that lack blood flow [40].

Generation of VE-PTP null mice has been achieved with two different strategies. In one case truncation of a VE-PTP sequence led to production of a secreted protein, thereby eliminating its plasma membrane expression [33]. A second strategy involved replacing the VE-PTP gene with a LacZ reporter gene [35]. In both cases VE-PTP null mice died by E9.5-E10 due to severe cardiovascular defects. These include failure of the primary vascular plexus to remodel into hierarchical branched structures, lack of functional blood vessels in the yolk sac, and multiple heart defects (e.g. lack of trabeculation and pericardial edema) [33, 35]. In contrast, VE-PTP heterozygous mice appear normal and phenotypically identical to wild type mice.

In the endothelium of adult vasculature, VE-PTP localizes in a stable junctional complex with VE-Cadherin, which contributes to maintain VE-Cadherin in an unphosphorylated state, thus enhancing its barrier function [36, 37]. This complex is disrupted following VEGFR2 activation [37]. The importance of this event is demonstrated by the loss of VEGFA-induced permeability in a knock-in mouse in which VE-PTP and VE-Cadherin are unable to dissociate [41]. Nevertheless, postnatal deletion of VE-PTP results in viable mice with no evident defects in basal permeability and with a generalized reduction in permeability response to multiple factors, including VEGFA and histamine [42]. This effect is linked to a marked increase in Tie2 phosphorylation (a VE-PTP substrate) and formation of highly stabilized endothelial junctions. Notably, Tie2-mediated enhancement of barrier

integrity overrides the simultaneous loss of VE-CAD stabilization due to VE-PTP deletion. Inhibition of VE-PTP by a small molecule (AKB-9778) or a VE-PTP blocking antibody inhibits retinal and choroidal neovascularization. This may represent a novel therapeutic tool for multiple ocular diseases [43]. The mechanism of this effect has also been linked to enhanced activation of Angiopoietin-1/Tie2 signaling and improved vascular integrity. A similar mechanism has been proposed for a beneficial effect of VE-PTP inhibition on tumor vascular normalization [44].

2.2 DEP-1 (Density Enhanced Phosphatase 1)

In the vascular system, DEP-1 (also called CD148, PTP η , SCC1) is expressed in endothelial cells, smooth muscle cells, leukocytes, and platelets [26, 45-48]. DEP-1 substrates include PDGFR β [49, 50], VEGFR2 [51], insulin receptor [52-55], p120 [56], β -catenin [57] and Src [58]. As suggested by its name, DEP-1 expression is markedly upregulated when cells reach confluency [59].

DEP-1 substrate-trapping mutant binds to phosphorylated VEGFR2 [51]. Silencing DEP-1 in HUVEC or mouse ECs enhances VEGFA-induced VEGFR2 phosphorylation at all major phosphorylation sites [51, 60]. DEP-1 is involved in contact inhibition of cell growth as shown by high VEGFA-induced BrdU incorporation in confluent endothelial cells that lack DEP-1 [60]. Finally, DEP-1 peptide activators inhibit VEGFA-induced VEGFR2 phosphorylation and proliferation in HUVEC [61]. Despite its dephosphorylation of VEGFR2, DEP-1 favors VEGFA-induced Src activation (Y416 phosphorylation) [51, 62] due to its ability to dephosphorylate Src inhibitory site (Y527). Consistent with this role, silencing of DEP-1 in HUVEC inhibits VEGFA-induced *in vitro* permeability [62], a process that is dependent on Src activation [63, 64]. This effect is also seen *in vivo* as evidenced global DEP-1 null mice display impaired VEGFA-induced skin permeability. The defect is specific to VEGFA because both histamine-induced and basal tissue permeability are normal in DEP-1 null mice [65].

Multiple genetic strategies have been used to assess DEP-1 functions *in vivo*. DEP-1 global null mice are viable, fertile and do not display any gross phenotype [66]. Similarly, mice expressing a modified DEP-1 that lacks a transmembrane domain (which results in a secreted extracellular DEP-1) are viable and fertile [67, 68]. Finally, an in-frame replacement of DEP-1 intracellular domain with eGFP leads to early embryonic lethality (E10.5/E11.5) due to a number of severe angiogenesis defects. Among these are a collapse of the dorsal aorta, absence of mature intersomitic vessels, disorganized and enlarged peripheral vessels, disrupted extraembryonic circulation, and endothelial cells hyperproliferation in the yolk sac [69]. The reason for these phenotype differences has not been defined, but may include GFP-driven disruption of endothelial junctional complexes and hyperactivation of VEGFR2.

Characterization of the DEP-1 role in adult vasculature has generated discordant data. A monoclonal antibody, which targets DEP-1 extracellular domain and promotes its phosphatase activity, inhibits angiogenesis in a cornea pocket assay; thus suggesting that DEP1 may act as negative regulator of pathological angiogenesis [70]. Conversely, global

DEP-1-null mice display reduced VEGFA-induced angiogenesis in a Matrigel plug assay; thus indicating that DEP-1 expression can enhance angiogenesis *in vivo* [65].

DEP-1 global null mice are unable to increase cerebrovascular blood flow (CBF) following permanent occlusion of the common carotid artery [71]. The same study did not detect differences in blood flow recovery and collateral vessel formation after seven days in a hind limb ischemia model. However, a recent report shows that DEP-1 global null mice have reduced blood flow recovery and arteriogenesis fourteen days following the induction of hind limb ischemia [65].

In general, the role of DEP-1 in the vasculature appears complex and context-dependent. This is probably the consequence of its opposite actions upon two key molecules such as Src and VEGFR2. Furthermore, DEP-1 is expressed in multiple cell types in the vasculature, including smooth muscle cells in which it regulates PDGFR β signaling. Future studies using conditional knockout models will help to dissect the role of DEP-1 in angiogenesis and arteriogenesis.

3. Intracellular Phosphatases

Intracellular phosphatases lack a transmembrane domain and can be found in the cytosol or bound to specific subcellular structures [7, 72]. Shortly after dimerization, VEGFR2 is internalized via a classical clathrin-dependent mechanism and moves across the cytoplasm in a series of endosomal vesicles [73, 74]. During endocytosis and the subsequent intracellular trafficking, the VEGFR2 C-terminal domain retains full or partial phosphorylation and continues to signal [75, 76]. Consequently, in addition to plasma membrane, VEGFR2 can also be exposed to intracellular phosphatases that sit in specific subcellular compartments providing a more complex spatiotemporal regulation of its signal [77].

3.1 PTP1B

PTP1B, a ubiquitously expressed PTP, anchored to the endoplasmic reticulum (ER) membrane with the catalytic domain facing the cytosol [78]. PTP1B substrates include the insulin receptor [79, 80], VEGFR2 [81], and β -catenin [82].

PTP1B dephosphorylates immunoprecipitated VEGFR2 and silencing of PTP1B in HUVEC leads to increased VEGFA-induced VEGFR2 phosphorylation (tested Y1175 only) and proliferation [81]. Similar findings show that primary mouse EC isolated from PTP1B null mice display increased VEGFA-induced VEGFR2 phosphorylation and augmented proliferation and migration in response to VEGFA [83].

Mice carrying a global homozygous deletion of PTP1B are viable, fertile, and show enhanced insulin sensitivity and resistance to obesity [84, 85]. These mice also exhibit accelerated skin wound-associated angiogenesis and a faster rate of wound closure compared to wild-type mice [86]. Endothelial-specific PTP1B deletion leads to increased angiogenesis, as demonstrated in multiple *in vivo* models including developmental retinal angiogenesis, *in vivo* Matrigel assay and wound healing [83].

Importantly, PTP1B is a negative regulator of arteriogenesis [83]. This role of the phosphatase came to light during studies of dysfunctional arteriogenesis in myosin-VI [87] and synectin null mice [88], as well as mice carrying a knock-in of a mutant Nrp1 gene with a deleted cytoplasmic domain [89]. All three mutants are characterized by abnormally slow cytoplasmic trafficking of internalized VEGFR2, which leads to a prolonged contact between the internalized receptor in early endosomes and the endoplasmic reticulum-bound PTP1B. This leads to a partial down regulation of the VEGFR2-PLC γ -MAPK cascade that plays a crucial role in arteriogenesis [90]. Defects in arteriogenesis and angiogenesis reported in endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN) null mice have also been linked to PTP1B action [91].

3.2 Shp1 (Src Homology-2 domain-containing Phosphatase 1)

Shp1 (also called HCP, PTP-1C, SH-PTP1, SHP) is highly expressed in hematopoietic cells, but is also found in other tissues [92-94]. Shp1 has been implicated in negative regulation of various signaling pathways initiated by RTKs, integrins, and cytokine receptors [94-96]. This phosphatase contains two N-terminal Src homology-2 (SH2) domains and a C-terminal PTP domain. SH2 domains allow Shp1 to bind phosphotyrosine residues in receptors and adapters. This event leads to activation of its PTP domain which can then dephosphorylates downstream targets [94, 97].

A role for Shp1 in VEGFR2 signaling modulation was initially proposed after the observation that it associates with VEGFR2 after VEGFA stimulation [98]. Subsequent studies have confirmed and extended this observation. Silencing of Shp1 in HUVEC increases VEGFA-induced phosphorylation at Y1175 and Y054/59, but not Y951 [99]. Functionally, VEGFA-induced proliferation is increased after Shp1 knockdown, but migration is not affected [99]. Several ECM-associated proteins with antiangiogenic properties (e.g. TSP-1, Thrombospondin-1; TIMP-2, Tissue Inhibitor of Metalloproteinase-2; CCN1, CCN family member 1) exert their effect by regulating Shp1 activity and/or expression. In microvascular EC (MVEC), binding of TSP-1 to its receptor CD36 promotes an association between Shp1 and VEGFR2, which leads to inhibition of VEGFA-induced signaling and migration [100]. Indeed, silencing of Shp1 renders the TSP-1/CD36 axis unable to exert its inhibitory action toward VEGFR2 signaling [100]. TIMP-2 antiangiogenic activity *in vitro* and *in vivo* is also Shp1-dependent [101, 102]. Mice that lack Shp1 expression ("motheaten mice or me/me mice") suffer from a chronic systemic inflammation and die of interstitial pneumonia within three weeks of birth [103, 104]. EC-specific Shp1 deletion has not been reported, however indirect evidences points to an important role of this phosphatase in VEGFR2 signaling and angiogenesis *in vivo*. Silencing of Shp1 via siRNA injection *in vivo* results in increased capillary density in a hind limb ischemia model [105]. Furthermore, CCN1 (a matricellular protein that can bind integrins and proteoglycans) promotes Shp1 expression, which results in negative regulation of VEGFR2 signaling [106]. Retinas from mice with a postnatal endothelial CCN1 deletion have ~60% reduction in Shp1 expression in EC and display a general VEGFR2 signaling augmentation : This results in retinal EC hyperproliferation and formation of a highly dense and immature vasculature network with delayed vessel outgrowth [106].

3.3 Shp2 (Src Homology-2 domain-containing Phosphatase 2)

Shp2 (also called PTP-1D, SH-PTP2, SH-PTP3, Syp, PTP-2C) is a ubiquitously expressed PTP with a structure similar to that of Shp1, but with distinct molecular and functional roles [94, 97, 107]. Shp2 substrates include GAB1 [108], RasGAP [109], β -Catenin [110, 111], and RTKs such as PDGFR β [112] and VEGFR2 [113]. Shp2 is a positive regulator of the Ras-ERK pathway and multiple lines of evidence indicate that Shp2 is upstream of RAS [114]. Indeed, removal of Shp2 impairs ERK activation downstream of various RTKs, integrins, and cytokines [97, 115, 116]. Finally, expression of Shp2 in the endothelium reduces baseline phosphorylation of junctional proteins (e.g. VE-cadherin and β -catenin), thus contributing to maintenance of endothelial barrier integrity [117, 118].

Similar to Shp1, Shp2 was initially shown to associate with VEGFR2 upon VEGFA stimulation [98]. Shp2 dephosphorylates immunoprecipitated VEGFR2 [113] while silencing of Shp2 in HUVEC enhances VEGFA-induced VEGFR2 phosphorylation [119]. Expression of a Shp2 dominant-negative mutant (C459S) leads to an increase in both VEGFA-induced VEGFR2 phosphorylation and VEGFA-induced cell migration [113]. Interestingly, VEGFA-induced ERK1/2 activation is enhanced following silencing of Shp2 in HUVEC [120], which is in contrast with observations of Shp2 as a positive regulator of RAS-ERK pathway. One potential explanation for this difference is experimental evidence, which indicate that VEGFR2-induced ERK1/2 activation is RAS independent [121].

Finally, silencing of Shp2 in HUVEC leads to decreased activation of Src (Y416) and downstream AKT in response to VEGFA [120]. The positive effect on Src activation is indirect and is the consequence of Shp2-mediated dephosphorylation of PAG/Cbp, a regulator of the C-terminal SRC kinase (Csk). In the absence of Shp2, Csk becomes more active and promotes hyperphosphorylation of the C-terminal Src inhibitory site (Y527), thus resulting in a general Src inhibition [122].

Currently, there are no *in vivo* data regarding the role of Shp2 in VEGFR2 signaling. A global deletion of the phosphatase leads to early embryonic lethality [123], while overexpression of a constitutive active Shp2 in the endothelial cells leads to severe cardiac defects (myocardial thinning, valves and AV septum defects) and early embryonic lethality [124]. The phenotype recapitulates the major defects present in the Noonan syndrome, which is associated with a Shp2 gain-of-function mutation in 50% of the cases [125]. It remains to be defined whether this phenotype is partly due to aberrant VEGFR2 signaling remains to be defined.

2.3 PTEN (Phosphate and tensing homolog deleted on chromosome 10)

PTEN (also called MMAC, TEP1) was initially identified as a dual specificity protein phosphatase [126, 127]. However, further characterization showed that PTEN also has a lipid phosphatase activity, which allows efficient dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) generated at the plasma cell membrane [128]. Due to its ability to inhibit PI3K/AKT signaling, PTEN is a potent tumor suppressor and it is frequently mutated in tumors [129]. PTEN is largely expressed in the cytoplasm and nuclei, but it is quickly recruited to the plasma membrane where it converts PIP3 back to

PIP2 [130]. VEGFR2-mediated PI3K activation promotes PIP3 formation and AKT recruitment/activation at the plasma membrane, an event that is thought to play an important role in tumor angiogenesis [131], adult arteriogenesis [132], and developmental angiogenesis [133]. In recent years, PTEN protein phosphatase activity has also emerged as a potential regulator of biological effects such as cell migration [127]. A number of novel PTEN substrates for this function have been proposed; they include FAK, Src, and their upstream regulators [127]. The protein phosphatase activity of PTEN in EC biology needs to be defined.

Although PTEN does not directly dephosphorylate VEGFR2, it counteracts PIP3 formation at the plasma membrane level and indirectly inhibits VEGFR2-induced AKT activation [134]. PTEN overexpression in EC inhibits VEGFA-induced migration, proliferation, and pro-survival effect *in vitro* [134]. Furthermore, VEGFA-induced angiogenesis is decreased in the aortic ring assay after adenovirus-mediated PTEN overexpression [134].

A global deletion of PTEN leads to embryonic lethality at E9.5 [135, 136]. Embryonic bodies that lack PTEN develop larger and longer vessel sprouts upon VEGFA stimulation [137]. A constitutive deletion of endothelial PTEN leads to embryonic mortality at E11.5 due to endothelial cells hyperproliferation, enlarged capillary network, failure of vascular plexus remodeling, severe hemorrhage, and cardiac failure [138]. The loss of a single allele of PTEN in the endothelium results in viable mice that manifest increased VEGFA-induced angiogenesis in Matrigel and tumor angiogenesis assays [138]. Mice with a postnatal endothelial PTEN deletion display a phenotype characterized by excessive branching and larger vessel diameter, but have normal radial extension and number of vessel sprouts in the developing retinal vasculature [137]. The phenotype is the consequence of increased EC proliferation at the sprouting front due to loss of the Notch antiproliferative effect in stalk cells [137].

Recent findings show that PTEN is a crosstalk node between Activin-receptor Like Kinase 1 (ALK1) and VEGFR2 signaling pathways. Indeed, silencing of ALK1 or blocking its ligands, BMP9 and 10 in ECs, leads to decreased PTEN activity at the plasma membrane and hyperactivation of the VEGFR2/PI3K pathway. This mechanism is responsible for the excessive angiogenesis and arteriovenous malformations observed in deficient mice in BMP signaling. Finally, inhibition of PI3K in this model rescues vascular malformations and suggests a novel therapeutic target for Hereditary Hemorrhagic Telangiectasia 2 (HHT2) [139].

4. Additional phosphatases involved in regulation of VEGFR2 biological effects

In addition to the extensively studied protein phosphatases discussed above, a certain number of other phosphatases have also been implicated in VEGFR2 signaling. These are discussed below.

4.1 LMW-PTP (Low Molecular Weight-PTP)

LMW-PTPs (also called HCPTP, Red Cell Acid Phosphatase) are a group of five small cytosolic tyrosine phosphatases (~18 KDa) derived from splice variants of same gene [140, 141]. LMW-PTPs are ubiquitously expressed and conserved phosphatases [4] that have attracted particular interest for the development of inhibitors to use in cancer therapy [141, 142]. The isoform HCPTP-A has been shown to interact with VEGFR2 in a yeast two-hybrid system and to dephosphorylate the recombinant VEGFR2 [143]. Overexpression of HCPTP-A in endothelial cells inhibits VEGFA-induced phosphorylation (tested anti-pTyr only), migration, and proliferation [143]. Similarly, overexpression of HCPTP-A in rat aortic rings leads to reduced number and length of VEGFA-induced sprouts [143]. LMW-PTP null mice are viable, fertile, and display protection from heart failure [144].

4.2 PTP-MEG2

PTP-MEG2 (also called MEG2) is found in the cytosol, membrane fractions, and secretory vesicles [145, 146]. It is widely expressed in most organs and is detected in lymphocytes, platelets, and endothelial cells [93, 147, 148]. PTP-MEG2 substrate-trapping mutant is able to bind phosphorylated VEGFR2 [149]. Overexpression of PTP-MEG2 reduces VEGFA-induced VEGFR2 phosphorylation in HUVEC (tested Y1175 only), while silencing PTP-MEG2 leads to an increase in VEGFR2-downstream signaling [149]. The majority of PTP-MEG2 null mice (~90%) do not survive *in utero* development and die during late embryonic stages due to incomplete neuronal tube development and extensive hemorrhages [147].

4.3 TC-PTP (T cell PTP)

TC-PTP is a ubiquitously expressed PTP localized in the endoplasmic reticulum, nuclei, and the cytoplasm [150]. It is highly expressed in various hematopoietic cell lineages, but is also found in blood vessels [150, 151]. Although TC-PTP shares 70% sequence identity with PTP1B and shows certain redundancy of functions, the two phosphatases have remarkably different roles and a distinct set of substrates [152]. PTP1B has a well known role in metabolism and regulation of insulin signaling [153], while TC-PTP is a critical regulator in inflammation and plays an important role in immune system development and response [150].

TC-PTP dephosphorylates immunoprecipitated VEGFR2 (affected sites Y1054/59 and Y1214; no changes in Y1175) and a TC-PTP substrate-trapping mutant binds phosphorylated VEGFR2 [151]. Silencing of TC-PTP in HUVEC increases VEGFA-induced proliferation, while TC-PTP overexpression abrogates VEGFA-induced migration [151]. A global deletion of TC-PTP in mice leads to death within five weeks of birth due to anemia, severe immunosuppression, and systemic inflammation [154, 155].

4.4 Calcineurin (CN)

CN (also called PP2B, i.e. Protein Phosphatase 2B) is a Calcium/Calmodulin (Ca^{2+} /CaM)-dependent Ser/Thr phosphatase that consists of a catalytic subunit (CnA) and a regulatory subunit (CnB) [3, 156]. CN dephosphorylates the members of the NFATc transcription factor family (c1-c4). Unphosphorylated NFATc translocate to the nucleus where it drives expression of target genes. Classical immunosuppressants such as cyclosporine and FK-506

are potent CN inhibitors and lead to a block in NFATc dephosphorylation/nuclear translocation. The major consequence of the block is a decreased expression of T-cell immune response genes that otherwise would drive rejection of transplanted organs [157]. CN also plays an important role in the development of the cardiovascular system [158].

The CN/NFATc axis is activated by VEGFA-induced increase in intracellular Ca^{2+} levels (via the VEGFR2/PLC γ /IP3 cascade) [159-161]. This launches context-dependent gene programs that contribute to modulation of EC behavior during heart development [162, 163] and angiogenesis in inflammation and cancer [164-166].

CN inhibition reduces VEGFA-induced migration/proliferation *in vitro* [167, 168] and VEGFA-induced angiogenesis in a cornea pocket assay [168]. These effects correlate with loss of cyclooxygenase-2 (COX-2) upregulation which is driven by VEGFA-induced NFATc nuclear translocation [168]. COX-2 is a known positive regulator of angiogenesis in chronic and acute inflammation due to its ability to increase synthesis of proangiogenic molecules such as PGE2 [169, 170] and other prostanoids [171]. Intravitreal injection of CN inhibitors has been shown to decrease pathological neovascularization in a ROP model in rats [172].

CN inhibition reduces VEGFA-induced NFATc1 nuclear translocation and proliferation in human pulmonary valve endothelial cells *in vitro* [173]. In line with these data, global homozygous NFATc1 deletion in mice results in impaired development of pulmonary and aortic valves [174, 175] partly due to decreased proliferation of the valve endocardial lineage [162]. This mice also display early embryonic lethality ($\sim$ E14.5) [174, 175].

Knock-in mice carrying a global inactive CN (CnB $^{*/}$) die at E10.5/E11.5 due to disorganized remodeling of the vascular plexus although the initial endothelial cell differentiation and vasculogenesis appear normal [176]. Double NFATc3/c4 null mice recapitulate most features of CnB $^{*/}$ mice and die at $\sim$ E11.5 [176]. Both phenotypes appear to be due, at least in part, to a dysregulated local VEGFA expression [176]. CN inhibition (between E9.5 and E12.5) also impairs correct patterning of coronary endothelial cells [163], a coordinated process that relies on myocardial VEGFA to drive ventricular endocardial cells to form the coronary arterial network [177]. Tie2-cre driven CnB-deletion (begin $\sim$ E8.0) recapitulates the coronary EC patterning defects, which are observed with CN inhibitors [163].

4.5 PP2A (Protein Phosphatase 2A)

PP2A is a ubiquitously expressed, highly conserved Ser/Thr phosphatase [3]. The active enzyme is a heterotrimeric complex that contains a scaffold subunit (A) bound to catalytic (C) and regulatory (B) subunits. The existence of numerous B isoforms permits the formation of multiple holoenzyme combinations ($\sim$ 80), which can have different substrate specificities or subcellular localizations [3, 178]. PP2A substrates include central transducers of VEGFR2 signaling such as AKT [179], RAF [180], and eNOS [181].

Prolonged exposure of endothelial cells to fatty acids or lipid metabolites (e.g. ceramide) increases baseline PP2A activity, which in turn suppresses major VEGFA-induced responses such as AKT, eNOS and ERK activation [182]. This effect may contribute to the onset of

endothelial dysfunction and arteriogenesis defects observed in high-fat diet fed mice [182, 183]. Vasoinhibins, a family of prolactin-derived antiangiogenic peptides, promote PP2A activation in EC and inhibit VEGFA-induced eNOS activation and permeability *in vitro* [184]. Intravitreal injections of vasoinhibins also blocks VEGFA-induced permeability in rat retinas [184]. Finally, inhibition of VEGFA-induced eNOS activation by antiangiogenic peptide endostatin requires PP2A activity [185].

4.6 PRL-3 (Protein of Regenerating Liver-3)

PRL-3 is a dual specificity phosphatase that localizes at the plasma membrane and endosomes following prenylation at the C-terminal end [186]. PRL-3 is highly expressed in heart and blood vessels during development [187] and is upregulated in tumor endothelium [188-191]. It may act as a positive regulator of VEGFR2 signaling. Primary mouse EC isolated from PRL-3 null mice display reduced VEGFA-induced VEGFR2 phosphorylation and Src activation, although the mechanism behind this defect has not been clarified [192]. A PRL-3 inhibitor reduces VEGFA-induced migration in HUVEC [192]. Finally, VEGFA-induced permeability is impaired in PRL-3 null mice [192].

4.7 MKP-1 (Mitogen activated Kinase Phosphatases-1)

MKP-1 (also called CL100) is a dual specificity nuclear phosphatase that acts on multiple MAPKs (i.e. ERK, p38, c-Jun N-Terminal Kinase) [193]. Silencing of MKP-1 reduces VEGFA-induced EC migration and proliferation *in vitro* [194]. VEGFA-induced sprout formation is reduced in aortic rings isolated from MKP-1 null mice [195]. MKP-1 null mice also show delayed distal blood flow recovery in a hind-limb ischemia model due to reduced angiogenesis and arteriogenesis [194]. In the absence of MKP-1, its substrate histone H3 Serine 10 (H3S10) [196] remains phosphorylated and inhibits VEGFA-induced CX3CL1 transcription [194], a proangiogenic gene [194, 197, 198].

4.8 RPTP ζ (Receptor Protein Tyrosine Phosphatase zeta)

RPTP ζ (also called phosphacan, RPTP β/ζ , PTPRZ) is a receptor tyrosine phosphatase that may play a positive role in VEGFR2-Src activation. RPTP ζ dephosphorylates the Src inhibitory site (Y527) [199] and silencing of RPTP ζ inhibits VEGFA-induced migration in HUVEC [200]. Pleiotrophin (PTN, HARP), a natural PTPRZ ligand that inhibits its tyrosine phosphatase activity [201], reduces VEGFA-induced proliferation in HUVEC and VEGFA-induced angiogenesis in a Matrigel plug assay *in vivo* [202]. However, this effect is linked to the PTN ability to bind and neutralize VEGFA rather than RPTP ζ inhibition [202].

5. Therapeutic opportunities

During the last 15 years, a plethora of studies in animal models has highlighted the diversity of VEGFR2 biological effects in health and disease [16]. Furthermore, the role of VEGFR2 in human biology has been further clarified with approval of anti-VEGFA monoclonal antibodies in clinical settings [203]. While beneficial in a number of settings, the experience with these antibodies has shown that a blockade of VEGFR2 signaling can lead to a number of adverse effects such as hypertension, renal toxicity, bowel perforation, hemorrhages, wound healing delay, and others [204, 205]. Thus, it has become evident that activation of

VEGFR2 signaling can have both positive and negative consequences [30, 206, 207] because various VEGFR2 downstream pathways promote different biological effects [21]. Conceptually, future therapeutic approaches should aim to modulate some, but not other, VEGFR2 signaling pathways in order to increase therapeutic efficacy and decrease side effect potential.

In this scenario, targeting phosphatases may offer a new way to fine-tune VEGFR2 signaling. Mechanisms through which different phosphatases can modulate the net effect of a signal are multiples and includes phosphosite specificity, differential subcellular localization, different level of expression and activity, modulation by ligands and regulatory subunits [3, 7, 72]. Below are some examples of disease where targeting phosphatases to modulate VEGFR2 downstream effects may prove to be therapeutically useful.

5.1 Ischemic stroke

Occlusion of a cerebral artery leads to focal ischemia and VEGFA upregulation as early as six hours after vessel occlusion [31, 208]. This results in VEGFR2 signaling activation throughout stroke progression with both deleterious and beneficial consequences [31, 209, 210]. The harmful effects include BBB disruption, vascular leakage, and brain edema [211-213]. On the positive side, VEGFR2 activation enhances tissue oxygenation by promoting vasodilation, increases arteriogenesis in the penumbra area (the less viable area around the necrotic core), and exerts certain neuroprotective and neurogenic effects [31, 211]. Preclinical studies have consistently shown that VEGFA administration immediately after a vessel occlusion can result in severe adverse effects such increased brain edema, injury, and hemorrhagic stroke transformation [31]. Conversely, VEGFA administration following the acute window (two to three days after occlusion) can lead to important beneficial effects including improved vascular function, decreased ischemic injury and enhanced neurological recovery [31]. Although promising, administration of VEGFA (especially systemic) still poses elevated risk of acute adverse effects that can overcome long-term benefits [30, 31]. In order to achieve safety and maximize benefits, novel strategies aim to dissociate VEGFA permeability effects from its neurogenic and proangiogenic ones [30].

Inhibition of VEGFR2 signaling in ischemic stroke has been also linked to therapeutic benefits such as reduced edema and infarct volume [31, 212, 214]. These beneficial effects are mainly attributed to inhibition of VEGFA-induced vascular permeability. In agreement, inhibition of Src (which drives VEGFA-induced permeability) has been shown to reduce edema and infarct volume together with an improved neurological score [215]. This led to the idea of Src inhibition as a potential treatment in cerebral ischemia and stroke [64, 216]. Small molecule inhibitors with broad target for multiple kinases, including Src family kinases, have been approved for treatment of various oncological diseases [217]. However, therapeutic applications of these new molecules in the settings of stroke still need to be investigated.

Interestingly, deletion of certain protein phosphatases leads to specific inhibition of VEGFR2- induced Src activation, resulting in impairment of VEGFA-induced permeability (i.e. DEP-1, PRL-3) [65, 192]. Thus, it is conceivable that safety of VEGFA administration

following ischemic stroke could be improved by contemporaneous inhibition of these phosphatases. Alternatively, inhibition alone during the acute stroke phase may be considered in order to block permeability effects of endogenously produced VEGFA. Finally, inhibition of other VEGFR2-related phosphatases (e.g. Shp1, LMW-PTPs) has been shown to improve the VEGFA-induced proangiogenic response [105, 143] with no reported impairment in either basal or VEGFA-induced permeability [218, 219]. Post-acute inhibition of these phosphatases may be feasible to boost long-term VEGFA proangiogenic and neuroprotective activities with minimal risk of adverse effects.

Animal studies demonstrated that several other neurological disorders (e.g. Amyotrophic Lateral Sclerosis, Parkinson Disease, and others) could benefit from VEGFA therapy with a proper control of its on- vs. off-target different biological effects [30]. Thus, a fine tailoring of VEGFR2 activation by phosphatases could represent a valuable therapeutic option in multiple neurological disorders in addition to stroke.

5.2 Peripheral artery disease

Peripheral artery disease (PAD) is caused by reduction of arterial blood flow in major arteries in the limbs and in the brain. The disease is associated with an increased risk of stroke, myocardial infarction, cardiovascular death [220]. It often progresses to critical limb ischemia (CLI), a condition characterized by severe impairment of blood flow to peripheral tissues in the legs leading to gangrene and/or necrosis. It can further lead to complications such as skin ulceration, severe pain, claudication, and limb amputation. In these settings, proangiogenic therapy has been tested as a means to promote blood flow restoration in the affected limbs. Various trials of VEGFA therapy failed to show meaningful functional benefits, such as decreased limb amputation frequency or cardiovascular mortality [220, 221]. The reasons for such a sub-optimal response to VEGFA therapy are poorly understood, but include decreased expression and impaired activation of VEGFR2 [222, 223].

Recent advances in our understanding of VEGFR2 signaling [21] include realization of the central role played by endothelial activation of ERK signaling in arteriogenesis [90, 224], an event negatively regulated by PTP1B [83], that is upregulated in the ischemic tissue [81]. Indeed, specific endothelial deletion of PTP1B increases VEGFA-induced VEGFR2 phosphorylation at Y1175 and correlates with improved arteriogenesis and blood flow recovery following hind limb ischemia (a mouse model for PAD), even in mice with impaired arteriogenic response [83]. Alternatively, inhibition of phosphatases inactivation of ERK may also achieve the same result. Thus, inhibition of phosphatases that selectively regulate VEGFR2/ERK signaling may offer a potential new therapeutic target to improve proarteriogenic therapy in PAD.

5.3 Cancer

The idea of “starving cancer to death” idea has driven the development of antiangiogenic therapies. The advent of therapies that block VEGFA/VEGFR2 signaling, such as TKIs and monoclonal antibodies, and inhibit tumor angiogenesis represents a significant improvement in treatment of certain cancers [203]. Yet, this approach has not met the highest expectation,

the complete arrest of tumor growth [206, 225, 226]. A multitude of clinical trials has shown that the benefits of antiangiogenic therapy are limited to an increase in progression-free survival and sometimes overall survival on the order of months [206, 225, 226]. Moreover, certain cancer types completely fail to respond to antiangiogenic therapy [203, 226]. The current view is that aggressive antiangiogenic therapy confers a transitory slow-down because tumors put in place alternative mechanisms to escape hypoxia. Multiple reasons have been proposed to explain acquired resistance to antiangiogenic therapy in cancer. These have been widely described in recent reviews [206, 226-228]. A plethora of phosphatases have now been associated with either oncogenic (e.g. Shp2, PTP1B) or tumor suppressor (e.g. PTEN, PP2A) functions [23, 229]; they have attracted interest as novel pharmacological targets in cancer therapy [23, 24, 230]. Similarly, phosphatases that control VEGFR2 signaling or other proangiogenic pathways may be targeted to improve the efficacy of antiangiogenic therapies.

Furthermore, experimental evidences suggests that normalization of the tumor vasculature rather than complete VEGF antagonism and vessel disruption could achieve better therapeutic effects [231]. The general idea is to develop new therapeutic strategies to balance the pruning of leaky immature vessels while stabilizing a sufficient healthy vessel network to insure uniform and enhanced chemotherapy delivery with minimal hypoxia (thus limiting the onset of resistance) [206, 232, 233]. VEGFA is naturally present in tumor microenvironment, therefore, phosphatases that control VEGFR2 signaling could be targeted to promote or modulate vascular normalization. Indeed, certain phosphatases have already been implicated in tumor angiogenesis and vascular normalization (e.g. VEPTP and Shp1) [44, 234]. Further data from mouse tumor models are required in order to understand the role of the different phosphatases in shaping the vascular tumor network.

5.4 Other diseases

In addition to the diseases described above, regulation of VEGFR2 signaling has been the focus of multiple clinical trials for cardiovascular disease [221, 235] and retinopathies [236]. Furthermore, animal studies have shown that VEGFR2 signaling is involved in heart failure [237] and atherosclerosis [238]. Mouse mutants of phosphatases that target VEGFR2 signaling are now available, thus preclinical studies using animal models of these diseases could help to unveil novel therapeutic targets and shed light into the relative contribution of VEGFR2 and related phosphatases.

6. Perspectives and challenges

The common idea that phosphatases are housekeeping genes which are unable to influence the net effect of signaling and not able to achieve complex signaling modulation has turned out to be incorrect (see Ref. [22, 239] for a complete historical perspective). Multiple experimental evidence over the years has demonstrated that: first, dephosphorylation does not necessarily lead to deactivation of the targeted molecule (e.g. Src activation requires Y527 dephosphorylation); second, inactivation of a pathway node by dephosphorylation can still result in a global positive effect (there are double inhibition circuits); third, dephosphorylation cannot be associated *a priori* to termination of a pathway with functional

outcomes (e.g. Shp2 activating mutations promote RAS/ERK pathway and are associated to Noonan syndrome and multiple cancer types); fourth, diseases and pathological events can arise from both increased or decreased activity of specific phosphatases, thus phosphatase inhibitors as well as activators are desirable. Furthermore, the relatively high number of phosphatase genes [1, 2], the wide spectrum of phenotypes in mouse knockout models, and a degree of substrate specificity [240] suggest that phosphatases have well defined biological roles, which make them potential candidates for pharmacological targeting.

Unfortunately, the R&D process for phosphatase-targeting drugs has proved to be difficult due to the necessity of designing highly negatively charged molecules that target the catalytic domain [241]. These compounds do not move well across cell membranes, therefore, they result in low bioavailability and poor therapeutic effect. However, progress has been made and more efficient ways to target phosphatases are emerging. These include molecules that can bind to allosteric sites or regulatory subunits. Identification of both selective inhibitors and activators using these strategies has been recently reported [230, 242-244]. Novel inhibitors that can target the catalytic domain with different mechanisms are also being developed [245, 246]. An update on molecules that modulate the activity of phosphatases involved in VEGFR2 signaling regulation is given in Table 3. The list includes only compounds that have shown biological effects in vascular-related functions or that have been tested in advanced preclinical models or clinical trials.

VEGFR2, the main VEGFA receptor, can initiate multiple signaling pathways that orchestrate a variety of biological effects including endothelial cell maturation, sprouting, migration, vascular permeability, vasodilation, and angiogenesis [18-20, 73]. A significant amount of experimental evidence have demonstrated that phosphatases can act either as a positive or negative regulator of signaling. This notion also appears valid for VEGFR2 signaling, as described in this review. A plethora of phosphatases have now been associated with either oncogenic or tumor suppressor functions [23, 229] and have attracted interest as novel pharmacological targets in cancer therapy [23, 24, 230]. Similarly, phosphatases that control VEGFR2 signaling or other proangiogenic pathways may be targeted to improve the efficacy of antiangiogenic therapies. Conversely, certain diseases may benefit from better proangiogenic effects (e.g. peripheral artery disease [220]), thus modulation of phosphatase activity may be a useful tool to sustain local VEGFR2 activation.

Further studies will improve our knowledge of phosphatase function in physiological and pathological processes; these enzymes may soon provide a novel set of pharmacological targets in the context of multiple diseases.

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Abbreviations

AMD	age-related macular degeneration
BrdU	bromodeoxyuridine
Cys	cysteine
DR	diabetic retinopathy
DUSP	dual specificity phosphatase
E	embryonic day
EC	endothelial cells
ECM	extracellular matrix
ER	endoplasmic reticulum
HUVEC	human umbilical vein endothelial cells
p-Ser/p-Thr	phospho-serine/phospho-threonine
p-Tyr	phospho-tyrosine
PTP	protein tyrosine phosphatase
R&D	research and development
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SMC	smooth muscle cells
Src	tyrosine kinase c-SRC
TKI	tyrosine kinase inhibitor(s)
VEGFR2	vascular endothelial growth factor receptor type 2
Y	tyrosine

Direct VEGFR2 dephosphorylation by PTPs

Fig. 1. Summary of PTPs that dephosphorylate VEGFR2

Multiples PTPs can dephosphorylate VEGFR2 and modulate its biological effects. Protein phosphatases often have domains that dictate their interaction partners or specific localization to subcellular compartments. DEP-1 and VE-PTP contain fibronectin type-III repeats implicated in cell adhesion. PTP1B and TC-PTP contain a C-terminal ER-anchoring motif. Shp1 and Shp2 have two SH2 domains that can bind phosphotyrosine residues and control activation of its PTP domain. PTP-MEG2 contains an N-terminal Sec14p-homology domain that targets this PTP to secretory vesicles.

Fig. 2. Summary of phosphatases that modulate VEGFR2 downstream effectors

Multiple protein phosphatases can regulate VEGFR2 signaling and its biological effects by modulating phosphorylation of its downstream effectors. The figure shows VEGFR2 signaling nodes where dephosphorylation (by different phosphatases) results in a negative (left panel) or a positive (right panel) effect. Negative phosphatases oppose a VEGFR2-induced downstream effect, while positive phosphatase enhance/promote it. Detailed description of each phosphatase role can be found in the text.

Table 1

Role of protein phosphatases in VEGFR2 signaling

Protein Name	Gene name	Type	Expression (vascular system)	Effect on VEGFR2 signaling	Ref.
Cell Membrane Phosphatases					
VE-PTP	PTPRB	PTPR	Endothelium only	<u>Negative</u> : dephosphorylates VEGFR2	[38, 40]
DEP-1	PTPRJ	PTPR	Endothelium, SMC, platelets, white blood cell lineages	<u>Negative</u> : dephosphorylates VEGFR2 <u>Positive</u> : dephosphorylates Src inhibitory site (Y527)	[51, 60] [51, 62]
RPTPζ	PTPRZ1	PTPR	Endothelial cells (and likely other cell types)	<u>Positive</u> : dephosphorylates Src inhibitory site (Y527)	[199]
Intracellular Phosphatases					
PTP1B	PTPN1	PTPN	All tissues	<u>Negative</u> : dephosphorylates internalized VEGFR2 and inhibits PLC- γ /ERK pathway	[81, 83, 89]
Shp1	PTPN6	PTPN	All tissues (high in hematopoietic lineages)	<u>Negative</u> : dephosphorylates VEGFR2	[99]
Shp2	PTPN11	PTPN	All tissues	<u>Negative</u> : dephosphorylates VEGFR2 <u>Positive</u> : inhibits Csk activation which in turn leads to reduced phosphorylation of Src inhibitory site (Y527)	[113] [120]
PTEN	PTEN	DUSP/lipid phosphatase	All tissues	<u>Negative</u> : dephosphorylates PIP3 thus antagonizing VEGFA-induced PI3K/AKT pathway	[134]
LMW-PTP	ACP1	PTPN	All tissues	<u>Negative</u> : dephosphorylates VEGFR2	[143]
PTP-MEG2	PTPN9	PTPN	White blood cell lineages, platelets, endothelium	<u>Negative</u> : dephosphorylates VEGFR2	[149]
TC-PTP	PTPN2	PTPN	All tissues (high in hematopoietic lineages)	<u>Negative</u> : dephosphorylates VEGFR2	[151]
Calcineurin	PPP3	Ser/Thr	All tissues	<u>Positive</u> : dephosphorylates NFATc which can translocate to nucleus and promotes VEGFR2-downstream gene expression	[161, 173]
PP2A	PPP2	Ser/Thr	All tissues	<u>Negative</u> : dephosphorylates RAF, eNOS and AKT	[182, 184]
PRL-3	PTP4A3	DUSP	Endothelial cells (high in development and tumor EC)	<u>Positive</u> : Increases VEGFR2 phosphorylation and Src activation (Y416)	[192]

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Protein Name	Gene name	Type	Expression (vascular system)	Effect on VEGFR2 signaling	Ref.
MKP-1	DUSP1	Ser/Thr	All tissues	Positive: enhances VEGFA-induced CX3CL1 upregulation (a proangiogenic gene)	[194]

Table 2
Summary of phenotypes in phosphatase mutant mice

Name	Mouse phenotype of phosphatase mutants	Ref.
VE-PTP	- Global deletion: death at E9.5-10 due to failure of vascular plexus remodeling	[33, 35]
	- Postnatal EC deletion: increased Tie2 phosphorylation and higher stability of EC junctions, decreased vascular permeability to various stimuli (e.g. thrombin, VEGFA, histamine)	[42]
	- Mutant mice in which VE-PTP forms a stable complex with VE-Cadherin: impaired VEGFA-induced vascular permeability	[41]
DEP-1	- Global deletion: viable and fertile	[67, 68]
	- Global deletion: decreased cerebrovascular flow recovery after carotid occlusion; normal arteriogenesis at day 7 of hind limb ischemia (HLI)	[71]
	- Global deletion: decreased VEGFA-induced permeability, normal basal permeability and response to histamine; decreased arteriogenesis at day 14 of HLI	[65]
	- Knockin mice expressing DEP-1 with eGFP in place of the intracellular domain: death at E10.5/11.5 with severe angiogenesis defects	[69]
PTP1B	- Global deletion: viable and fertile with enhanced insulin sensitivity and no obesity	[84, 85]
	- EC-specific deletion: increased arteriogenesis	[83]
Shp1	- Global deletion: death within 3 weeks of birth due to chronic systemic inflammation	[103, 104]
	- 60% reduced expression (mice with CCN1 postnatal EC-deletion): increased VEGFR2 activation, EC hyperproliferation and delayed vessel outgrowth in retina	[106]
Shp2	- Global deletion: early embryonic lethality	[123]
	- Expression a constitutive active mutant in EC leads to cardiac defects similar to Noonan Syndrome (myocardial thinning, septal and valves defects)	[124]
PTEN	- Global deletion: death at E9.5	[135, 136]
	- Single allele deletion in EC: viable mice with increased pathological angiogenesis	[138]
	- Homozygous deletion in EC: severe angiogenesis defects and death at E11.5	[138]
	- Postnatal deletion in EC: excessive vasculature branching and larger diameter vessels in the retina	[137]
LMW-PTP	- Global deletion: mice are viable and protected from heart failure	[144]
PTP-MEG2	- Global deletion: death during late embryonic stages due to incomplete neuronal tube development and extensive hemorrhages	[147]
TC-PTP	- Global deletion: death within 5 weeks of birth due to anemia, severe immunosuppression and systemic inflammation	[154, 155]
Calcineurin	- Knockin mice expressing an inactive CN die at E10.5/11.5 due to disorganized remodeling of the vascular plexus	[176]
	- EC specific deletion (Tie2 promoter) leads to defective coronary EC patterning and death at ~E13.5	[163]
PRL-3	- Global deletion: viable but show impaired VEGFA-induced skin permeability	[192]
MKP-1	- Global deletion: viable but display reduced adult arteriogenesis	[194]
RPTPζ	- Global deletion: viable and fertile with increased number of hematopoietic stem cells	[247, 248]

Table 3
Status of compounds that target phosphatases described in this review

Target	Compound	Status and therapeutic applications	Ref / Clinical trial ID
VE-PTP	AKB-9778 (small molecule inhibitor)	<u>Clinical trials</u> : Diabetic Macular Edema, Diabetic Retinopathy (Phase 2a completed)	[249]
		<u>Preclinical studies</u> : improved vascular normalization; decreased tumor growth and metastasis	[42, 44]
DEP-1	Nonapeptides (activators); monoclonal antibody (activator)	<u>Preclinical studies</u> : inhibition of cancer cells and EC proliferation; inhibition of angiogenesis in a mouse cornea pocket assay	[61, 70]
PTP1B	IONIS-PTP1B _{Rx} (antisense oligomer that reduces PTP1B expression)	<u>Clinical trials</u> : Type-2 diabetes (Phase 2 completed)	[250, 251] NCT01918865
	Trodusquemine/MS-1436 (small molecule inhibitor)	<u>Clinical trials</u> : metastatic breast cancer (Phase 1 on-going)	NCT02524951
	(3-bromo-7-cyano-2-naphthyl)(difluoro)methyl phosphonic acid	<u>Preclinical studies</u> : decreased tumor growth and metastasis; antidiabetic effect; increased arteriogenesis in hind-limb ischemia	[87, 252, 253]
Shp1	Sodium Stibogluconate (small molecule inhibitor used for treatment of Leishmaniasis; preferential Shp1 but also inhibits Shp2 and PTP1B)	<u>Clinical Trials</u> : advanced melanoma or other cancer in combination with classical chemotherapeutic agents (Phase 1 completed)	[254] NCT00498979
	TP1 (small molecule inhibitor)	<u>Preclinical studies</u> : decreased tumor growth	[255]
	siRNA (decreased Shp1 expression)	<u>Preclinical studies</u> : improved angiogenesis in hind-limb ischemia	[105]
Shp2	II-B08 (small molecule inhibitor)	<u>Preclinical studies</u> : increased mouse survival in a model of mast-cell leukemia	[256]
	SHP099 (small molecule inhibitor, selective and orally bioavailable)	<u>Preclinical studies</u> : tumor growth inhibition in an orthotopic model of human-derived acute myeloid leukemia	[244]
PTEN	Bisphosphonate-based compounds (inhibitors)	<u>Preclinical studies</u> : decreased myocardial infarct size, reduced reperfusion damage in liver surgery and transplantation	[257, 258]

Target	Compound	Status and therapeutic applications	Ref / Clinical trial ID
LMW-PTP	Various lead compounds (natural and synthetic)	<u>Proposed therapeutic applications:</u> cancer, diabetes, infectious disease, heart failure	[141, 142, 144, 259]
PTP-MEG2	PTP-MEG2-Inhibitor 7	<u>Preclinical studies:</u> increased insulin signaling in primary hepatocytes, improved insulin sensitivity in a diet-induced obese mouse model	[260]
TC-PTP	Spermidine and related compounds (activators)	<u>Preclinical studies:</u> negative regulation of EGFR and VEGFR2 signaling; reduced VEGFA-induced sprout formation <i>in vitro</i>	[261]
PP2A	FTY720 and related compounds (activators)	<u>Proposed therapeutic applications:</u> tumor suppression, inhibition of tumor growth and tumor angiogenesis	[262, 263]
PRL-3	Various lead compounds (inhibitors)	<u>Proposed therapeutic applications:</u> cancer	[24, 241, 264]
MKP-1	Various lead compounds (inhibitors and activators)	<u>Proposed therapeutic applications:</u> inflammatory and autoimmune diseases, neurological disorders, cancer, cardiovascular disease	[265-268]
RPTP ζ	SCB4380 (small molecule inhibitor)	<u>Preclinical studies:</u> decreased tumor growth in a glioblastoma model	[269]