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REVIEW PAPER

Soluble FLT-1 in angiogenesis: pathophysiological roles and therapeutic implications

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Abstract

Fine-tuning angiogenesis, the development of new blood vessels, is essential for maintaining a healthy circulatory and lymphatic system. The small glycoprotein vascular endothelial growth factors (VEGF) are the key mediators in this process, binding to their corresponding membrane-bound VEGF receptors (VEGFRs) to activate angiogenesis signaling pathways. These pathways are crucial throughout human life as they are involved in lymphatic and vascular endothelial cell permeability, migration, proliferation, and survival. Neovascularization, the formation of abnormal blood vessels, occurs when there is a dysregulation of angiogenesis and can result in debilitating disease. Hence, VEGFRs have been widely studied to understand their role in disease-causing angiogenesis. VEGFR1, also known as Fms-like tyrosine kinase-1 (FLT-1), is also found in a soluble form, soluble FLT-1 or sFLT-1, which is known to act as a VEGF neutralizer. It is incorporated into anti-VEGF therapy, designed to treat diseases caused by neovascularization. Here we review the journey of sFLT-1 discovery and delve into the alternative splicing mechanism that creates the soluble receptor, its prevalence in disease states, and its use in current and future potential therapies.

Keywords Soluble FLT-1 · FLT-1 · VEGF · VEGFR · RNA splicing · Angiogenesis · Gene therapy

Abbreviations

AAV	Adeno-associated virus
AD	Alzheimer's disease
ATP	Adenosine triphosphate
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
C-lobe	Carboxyl terminus distal kinase domain
CNV	Choroidal neovascularization
CRISPR-Cas	Clustered regularly interspaced short palindromic repeats—CRISPR-associated proteins
CSF-1R	Colony-stimulating-1 receptor
C-terminus/terminal	Carboxyl terminus/terminal
ECD	Extracellular domain
FCR	Fragment crystallizable region
FDA	U.S. Food and Drug Administration
FMS	FELINE McDonough Sarcoma
HSPGs	Heparan sulfate proteoglycans
ICD	Intracellular C-terminus domain
IgD	IG-like domain
Ixo-vec	Ixoberogene soroparvovec
JMD	Juxtamembrane domain
KD	Kinase domain
kDa	Kilodalton
KDEL	Lys-Asp-Glu-Leu
KDI	Kinase domain inserts
mFLT-1	Membrane-bound vascular endothelial growth factor receptor 1
MMP	Matrix metalloproteinase
MMP14	Matrix metalloproteinase 14
N-lobe	Amino terminus proximal kinase domain
N-terminus/terminal	Amino terminus/terminal
PDGF-R	Platelet-derived growth factor receptor

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PI3K	Phosphoinositide 3-kinase
PIGF	Placenta growth factor
PolyA	Polyadenylation
sVEGFR	Soluble vascular endothelial growth factor receptor
sVEGFR1/sFLT-1	Soluble vascular endothelial growth factor receptor 1
TMD	Transmembrane domain
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VEGFR	Vascular endothelial growth factor receptor
VEGFR1/FLT-1	Fms related receptor tyrosine kinase 1
VEGFR2/KDR	Kinase insert domain receptor
VEGFR3/FLT-4	Fms related receptor tyrosine kinase 4
vg	Viral genome

Introduction

Blood vessel formation is a fundamental aspect of development, resulting in a circulatory system that delivers blood and nutrients, removes waste, and works together with the human defense system to ensure the body is pathogen-free. Angiogenesis, the development of new blood vessels from pre-existing vessels, is a key process in the formation and maintenance of the circulatory and lymphatic systems. To date, the most important angiogenesis-tuning protein discovered is called vascular endothelial growth factor (VEGF). VEGFs are protein ligands that bind to their corresponding VEGF receptors (VEGFRs), triggering their activation and subsequent angiogenesis signal transduction [1]. VEGF receptor binding is a process modulated by pro- and anti-angiogenic factors that work in opposition to ensure a balance of vessel formation while preventing an abnormal overgrowth of vessels. Due to the delicate nature and importance of the angiogenesis process, deviation from normal signal transduction can result in devastating consequences including but not limited to heart failure, cancer, and blindness [2].

VEGFRs are usually membrane-bound, receiving angiogenesis/lymphangiogenesis signals from various ligands in their extracellular domains. To date, three VEGFRs have been identified; VEGFR1/Fms-like tyrosine kinase-1 (FLT-1), VEGFR2/Kinase insert domain receptor (KDR), and VEGFR3/FLT-4 (Fig. 1). Human FLT-1 was the first of the three to be identified in a placental cDNA (complementary DNA) library. It was named FLT-1 as it has similarities with the FMS family of receptors, including a conserved cysteine motif and a tyrosine kinase domain [3]. Soon after, two more VEGF receptors were discovered; KDR was isolated from a human endothelial cell cDNA library, and FLT-4 was first cloned from leukemia cell cDNA [4, 5]. The three receptors can dimerize to form homodimers or heterodimers in various combinations upon ligand binding. For instance, FLT-1 dimerizes in response to placenta growth factor (PIGF), VEGF-A, VEGF-B, and snake venom VEGF (also known as VEGF-F). KDR will be bound by VEGF-A, VEGF-C, VEGF-D, VEGF-E, and VEGF-F, while FLT-4 will be bound by VEGF-C and VEGF-D [6]. Although similar in structure, the three receptors have very different functions regarding lymph and blood vessel angiogenesis. For instance, FLT-4 is involved in lymphangiogenesis, mainly lymphatic endothelial cell proliferation, migration, and survival, while, FLT-1 and KDR both modulate vascular angiogenesis [7]. KDR has a very active tyrosine kinase domain

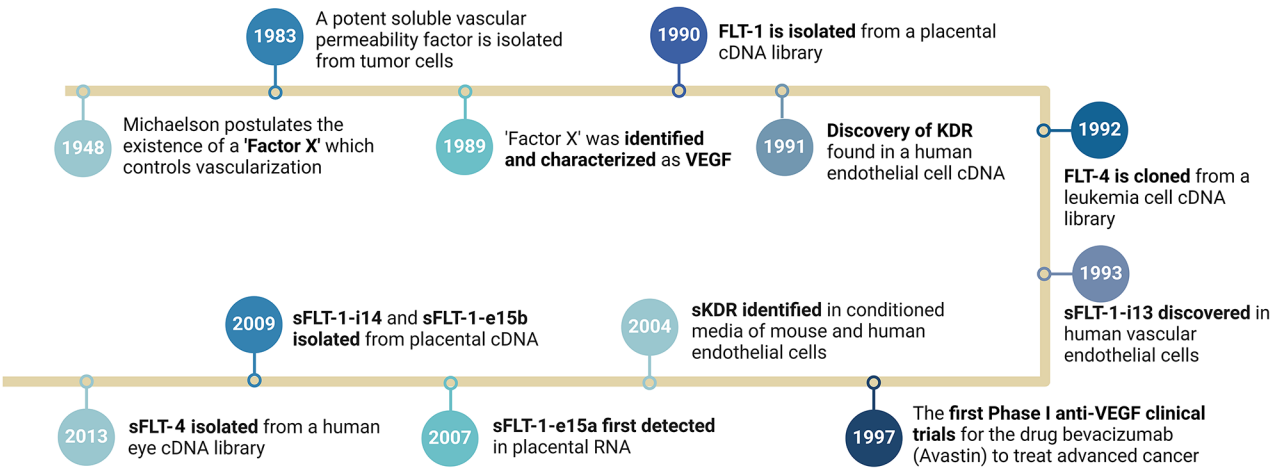

Fig. 1 Timeline of significant events concerning the discovery of vascular endothelial growth factor and its full-length and soluble receptors [1, 3–5, 12, 13, 15–19]. Figure created with Biorender.com

which elicits many different downstream cascades for regulating vascular endothelial cell permeability, proliferation, migration, and survival [8]. KDR knockdown mice live no more than 9.5 days postcoital before dying from failed vasculogenesis [9]. FLT-1 is also essential for development, as FLT-1-deficient homozygotic mice do not develop further than embryonic day 9. Unlike KDR, FLT-1 has weak tyrosine kinase domain activity and limited angiogenesis signaling potential. It functions as an anti-angiogenesis ligand binding receptor recognized for its importance in vascular organization in early development and the maintenance of angiogenesis in adulthood [10]. Furthermore, FLT-1 was also found to be essential for blocking E.coli invasions in the blood-brain barrier [11].

A pronounced anti-angiogenic factor that holds relevance in many diseases where angiogenesis is implicated is the soluble vascular endothelial growth factor receptor (sVEGFR). sVEGFRs are not membrane-bound or signal-transducing but still preserve their ligand-binding and receptor-interacting capabilities. Out of the three VEGFRs, FLT-1 has the most soluble isoforms, and these isoforms are observed in many more pathogenic states than the other two sVEGFRs. There have also been soluble forms of KDR and FLT-4 discovered, although they are not as highly associated with pathogenesis compared to sFLT-1 [12, 13]. The first soluble FLT-1 (sFLT-1) isoform to be discovered was sFLT-1-i13, which was found in vascular endothelial cell cDNA [14]. Three other isoforms of FLT-1 were discovered shortly after, sFLT-1-i14, sFLT-1-e15a, and sFLT-1-e15b [15]. They act as angiogenesis-inhibiting factors by trapping proteins and forming non-signaling heterodimers with KDR [16]. This review will focus on the physiological and pathological aspects of sFLT-1. It will review the history of the discovery of sFLT-1, the ligands they attract, and the mechanism of alternative splicing that forms the four isoforms. It will also explore the disease states associated with sFLT-1 and the current and potential angiogenesis-targeting therapies that involve sFLT-1.

A closer look at the structure and signal transduction of FLT-1

To understand the physiological significance of soluble FLT-1, it is important to first understand the function of its membrane-bound counterpart, FLT-1 [3]. Membrane-bound FLT-1 is primarily expressed on the surface of endothelial cells through all stages of life, but it can also be found in other cell types such as monocytes, macrophages, dendritic cells, and some cancer cells [20]. Cell-specific expression is regulated in the transcriptional promoter region by an E26 transformation-specific-binding motif as well as a cyclic adenosine monophosphate (cAMP) response element [21]. Its role in angiogenesis is indirect but crucial. Unlike KDR, which is directly involved in angiogenesis through tyrosine kinase activation of the intracellular domains, FLT-1 has a very weak tyrosine kinase and functions as normal even with a loss of the intracellular domains. In contrast, FLT-1 has a role in guiding the tip cells of the vessel into appropriate migration patterns but also limits growth by neutralizing VEGF binding to KDR [22]. Despite FLT-1 having weak tyrosine kinase domain activity, FLT-1 and KDR share the most homology in this kinase region, with sequences matching a 70.1% homology, while the extracellular domain and C-terminus demonstrate a weaker homology at 33.3% and 28.1%, respectively [23].

The *FLT-1* gene is located on chromosome 13q12 and consists of 30 exons that encode for the ~180 kilodalton (kDa) membrane-spanning protein (Fig. 2a). FLT-1 is a type IV receptor tyrosine kinase that is composed of three segments: the extracellular domain (ECD) at the amino terminus (N-terminus), the transmembrane domain (TMD), and the intracellular domain (ICD) at the carboxyl terminus (C-terminus) (Fig. 2b). It has features that closely resemble the FMS family of receptors, including a conserved cysteine motif for proper folding and function and a tyrosine kinase domain for phosphorylation of tyrosine residues on the receptor and downstream signaling events [3].

The extracellular immunoglobulin-like domains

The extracellular domain of FLT-1 contains seven Ig-like domains (IgD), which all have different roles in the ligand binding and receptor activation process (Fig. 2c). IgD1 may play a role in regulating the binding of the VEGF dimers [24]. IgD2 and IgD3 are the binding sites of the dimeric ligands (Fig. 2d). FLT-1 interacts with the ligands VEGF-A, VEGF-B, and PlGF and upon ligand interaction at the binding site, receptor dimerization and activation of signal transduction pathways occur. Interestingly, of the three VEGFRs, the ligand binding domains of FLT-1 have the highest affinity for VEGF. Despite a higher affinity to the signaling molecule, FLT-1 displays 10-fold lower kinase activity than KDR [25]. IgD5 and IgD6 have been linked to the unbinding of the ligand from the receptor after activation. Lastly, IgD4 and IgD7 have been associated with allosteric regulation of the receptors to induce downstream receptor signaling [26, 27].

The transmembrane and intracellular domain

Juxtamembrane domains (JMDs) flank both sides of the TMD and are instrumental in aligning the TMD and receptor activation. JMDs are composed of basic amino acid residues commonly seen between the ECD and ICD on both

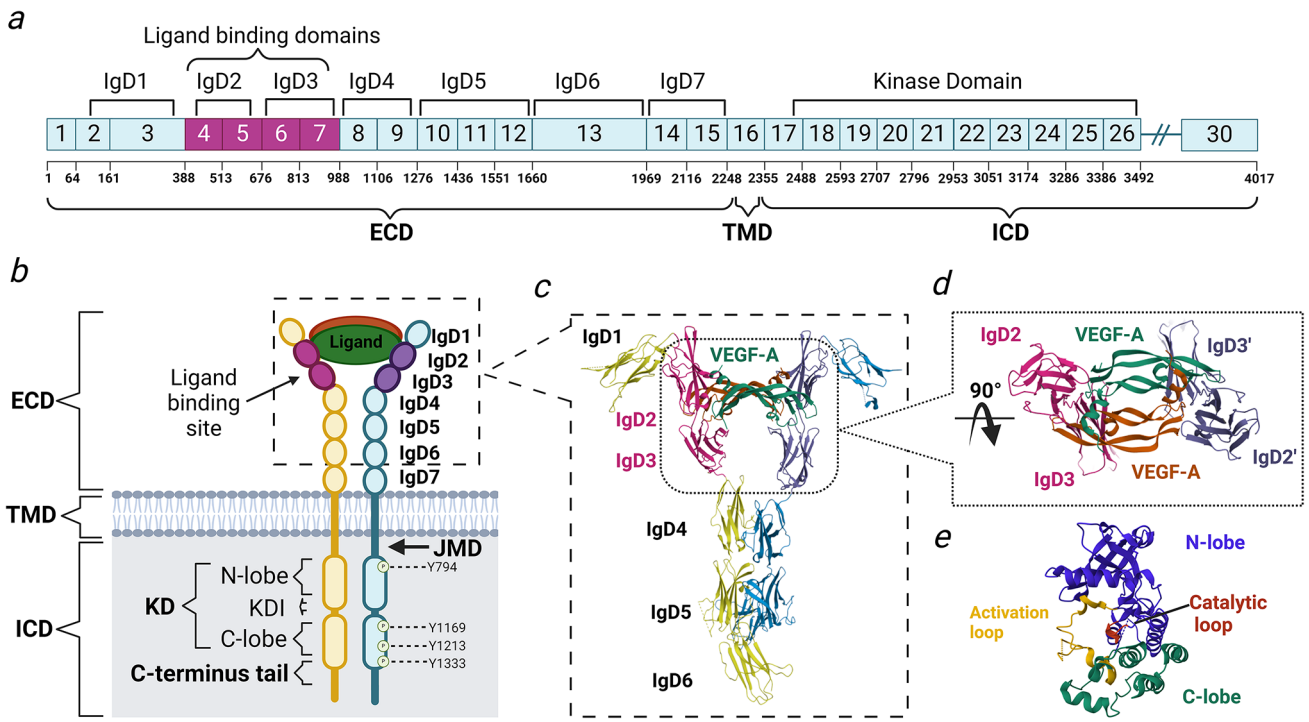

Fig. 2 Schematic of the structure of FLT-1 showing (**a**) the mature mRNA gene sequence and the corresponding domain coding regions, (**b**) the FLT-1 homodimer protein structure bound to VEGF homodimer, (**c**) The 3D protein map (PDB ID:5T89) of IgD 1–6 of an activated FLT-1 homodimer in a complex with a VEGF-A homodimer [40], (**d**) 90º view of the FLT-1 homodimer bound to a VEGF-A ligand homodimer and (**e**) the 3D protein map (PDB ID: 3HNG) of the kinase domain of FLT-1, highlighting the catalytic and activation loop [38]. ECD: extracellular domain, TMD: transmembrane domain, ICD: intracellular domain, JMD: juxtamembrane domain, KD: kinase domain, KDI: kinase domain inserts and IgD: Ig-like domain, C-lobe: C-terminus distal kinase domain, N-lobe: N-terminus proximal kinase domain. Figure created with Biorender.com

sides of the TMD of transmembrane receptors. The FLT-1 JMD has been demonstrated to repress signaling abilities, which could contribute to the FLT-1's lower kinase activation competency compared to KDR [28].

The intracellular domain of all receptor tyrosine kinases is composed of the JDM, two tyrosine kinase domains (proximal kinase domain/N-lobe, and distal kinase domain/C-lobe) interrupted by an activation loop and a C-terminus tail (Fig. 2e). The 70 amino acid insert in the activation loop between the N- and C-lobes kinase domain is characteristic of other type III receptor tyrosine kinases such as platelet-derived growth factor receptor (PDGF-R) and colony-stimulating-1 receptor (CSF-1R) [4]. The kinase insert functions as a flexible region between the N- and C-lobes in the kinase domain, allowing it to bend in the proper conformation to create an adenosine triphosphate (ATP) binding pocket. Flanked by subdomains VII and VIII of the kinase domains, the kinase insert is not highly conserved between VEGFRs. FLT-1 kinase domain deficient mice, which lose receptor activation abilities but maintain ligand binding function, develop their vasculature without complications other than suppressed macrophage migration across the endothelium [29]. This suggests that the kinase domain of FLT-1 is not as essential for development. In fact, under physiological conditions, FLT-1 exhibits weak tyrosine phosphorylation and has minimal capacity to phosphorylate substrates to cause a downstream cascade. Despite having fewer tyrosine residues (sites at which phosphorylation events occur, creating docking sites for downstream signaling proteins) than KDR and weak tyrosine phosphorylation, FLT-1 has still demonstrated an ability to activate multiple pathways using four main residues: Y794, Y1169, Y1213, and Y1333. The pathways are associated with changes in blood vessel hemodynamics, cell proliferation, and FLT-1 endocytosis, resulting in receptor degradation [30–34]. It was more recently discovered that FLT-1 has no ligand-independent autophosphorylation; if there are no ligands around, FLT-1 will not phosphorylate independently and remain inactive [35]. Instead, FLT-1 is deemed to be essential for its VEGF neutralization capabilities. It regulates angiogenesis by either interacting with KDR in a heterodimer to limit its activation potential or by competitively binding to VEGF [36].

Furthermore, the ICD also contains several elements such as a Glycine-rich loop/GXGXXG sequence (provides the flexibility to change conformation during activation), an ATP binding site (which initiates conformational change),

and a catalysis motif (stabilization, ATP alignment, and ATP hydrolysis) [37]. All VEGFR ICDs feature a flexible carboxyl tail, which consists of approximately 200 residues. This region is the least conserved region of the intracellular domains and has the potential to regulate tyrosine kinase activity both positively and negatively. At least 57 amino acids at the C-terminus tail of FLT-1 are required for activation to occur, as without it, the receptor cannot form stable active or inactive structures. Additionally, replacing the KDR C-terminus with that of FLT-1 restored the ability of KDR to activate signaling proteins. Hence, the C-terminus does not account for the differences in kinase activity between FLT-1 and KDR [38, 39].

FLT-1 ligands

For signal transduction to occur, VEGF must first bind and interact with FLT-1. VEGF is a small glycoprotein that plays a critical role in blood and lymphatic vessel growth. The VEGF family of cytokines is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, snake venom VEGF (VEGF-F), and PIGF [41]. VEGF-A to -D and PlGF are formed naturally in the human body, while VEGF-E and VEGF-F are exogenously found in the Orf-virus and snake venom, respectively. All seven VEGF ligands form homodimers of two identical subunits. They can be alternatively spliced to produce isoforms with different properties [42]. The proteins consist of an amino-terminal (N-terminal) domain and a carboxyl-terminal (C-terminal) domain. At the N-terminus exists a signal peptide, which allows the protein to be secreted from cells [43]. Once ligands are secreted, they bind to the extracellular domain of their specific receptor/s. The ligand-receptor interactions are dependent on the saturation of the ligand and co-receptor interactions such as heparan sulfate proteoglycans (HSPGs) and Neuropilin1/2 interactions [44]. The ligands that bind FLT-1 are VEGF-A, VEGF-B, PIGF, and VEGF-F.

VEGF-A

Vascular endothelial growth factor-A (VEGF-A) is a member of the VEGF family of proteins and plays a key role in promoting angiogenesis. Discovered nearly three decades ago from a cDNA library of folliculostellate cells, it has been extensively studied for its role in cancer and other pathologies [1]. The *VEGF-A* gene is located on chromosome 6p23.1 and the 3D protein structure resolved by X-ray crystallography showed it to be a member of the cysteine-knot growth factor superfamily, which contains disulfide bridge links. The two disulfide bridges on VEGF-A covalently link the two identical polypeptide chains that make up the homodimer glycoprotein [45].

VEGF-A has many isoforms that range in size depending on the alternative splicing pattern. There are 14 different isoforms of VEGF-A discovered to date. Four of the most studied are VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}. These four isoforms have the same receptor binding domains; however, they differ in their N-terminal domains (the heparin-binding and neutrophil domains), resulting in different biological activity and receptor affinities [46, 47]. For example, VEGF-A_{189} and VEGF-A_{206} contain a non-interrupted heparin binding site on exons 6 and 7. Therefore, they are bound to cell surface HSPG and are not easily diffusible in the extracellular matrix (ECM). On the other hand, exons 6 and 7 are absent in VEGF-A_{121}, so this isoform does not bind to the HSPGs, which could account for its dramatically lower endothelial cell mitotic activity [48]. Furthermore, the most abundant and biologically active isoform of VEGF-A, VEGF-A_{165}, is composed of 165 amino acid residues and has a molecular weight of approximately 38 kDa. It contains only exon 7 but not 6, and acts as an intermediary to the previously mentioned isoforms, as it is both freely diffusible and still able to bind to HSPGs [49].

VEGF-A binds to two receptors, FLT-1 and KDR. It has the highest affinity for FLT-1, a weak affinity for KDR, and no binding to FLT-4. The FLT-1 binding site of VEGF-A_{165} differs slightly from that of KDR. Specifically, the amino acid residues Asp^{63}, Glu^{64}, and Glu^{67} were found to be crucial for FLT-1 binding, while Arg^{82}, Lys^{84}, and His^{86} were discovered to be important for binding to KDR [50]. The interaction between VEGF-A and its receptors triggers downstream signaling pathways that promote angiogenesis and vascular permeability. It is known as the most potent pro-angiogenic protein and is involved in vascularization, wound healing, cutaneous angiogenesis, tumor growth, and cancer cell survival [41]. Furthermore, VEGF-A released by astrocytes also contributes to complications of brain irradiation, by eliciting barrier leakage [51].

VEGF-B

VEGF-B was first cloned from a human fetal brain cDNA library and is located on chromosome 11q13 [52, 53]. It contains eight exons, of which the first 5 are in common with VEGF-A. Alternative splicing leads to two isoforms, VEGF-B_{186} and VEGF-B_{167}, which have the same N-termini but a differing C-termini. VEGF-B_{167} contains a heparin-binding domain, while VEGF-B_{186} does not. The polypeptide chains of VEGF-B_{186} and VEGF-B_{167} are 186 or 167 amino acids long, respectively. The homodimer molar masses of VEGF-B_{186} and VEGF-B_{167} are 60 kDa and 42 kDa, respectively [54, 55].

VEGF-B binds only to FLT-1 and has been demonstrated to be dispensable at the embryogenesis stage [56]. However, in adults, VEGF-B has been linked to adaptive responses to tissue injury, endothelial cell function, and cell survival. VEGF-B has a role in the development of collateral vessels in the heart and is upregulated in response to myocardial infarction and ischemic injury [57, 58]. Furthermore, it is also crucial for lipid metabolism in adipose and liver tissue, as well as regulating glucose uptake in skeletal muscles. A study in type 2 diabetes using a rodent model showed a restoration of insulin sensitivity and improvement in the rodent's tolerance to glucose with decreased VEGF-B signaling [59]. It has also been shown to play a role in tumor growth, metastasis, and neuroprotection [60–62]. For instance, when FGFR1 is expressed in abundance, VEGF-B can bind to it, preventing the formation of the FGF2/FGFR1 complex and downstream activation of the Erk pathway. In turn, FGF2-induced Erk activation is suppressed, reducing angiogenesis and inhibiting tumor growth [63].

PIGF

Discovered from a cDNA library of full-term placental cells, the placenta growth factor (*PIGF*) gene is found on chromosome 14q24 and spans a 13.7 kb region to encode a 149 amino acid protein. Like the other VEGF ligands, PIGF acts as a glycosylated homodimer [64]. There are 4 known isoforms of PIGF which are $PIGF_{131}$ or PIGF1, $PIGF_{152}$ or PIGF2, $PIGF_{203}$ or PIGF3, and $PIGF_{224}$ or PIGF4. PIGF1 and PIGF3 lack a heparin-binding domain, while PIGF2 and PIGF4 have the additional 21 amino acids that enable them to bind to heparin [65]. The expression of PIGF is prominent in the placenta and is a biomarker for predicting preeclampsia diagnosis [66]. It is also expressed in smaller amounts in other tissues, including lungs, heart, liver, bone, and thyroid [67, 68]. Like VEGF-B, PIGF is dispensable in the early stages of development. Its role becomes prevalent after the early embryogenesis stage as loss of function adult mice exhibit a compromised proliferation of capillaries in ischemic areas, blood vessel leakage, increased inflammation, delayed wound healing, and cancer growth. Furthermore, PIGF was found to have a synergetic relationship with VEGF-B. In PIGF-deficient mice, VEGF-B was unable to rescue adult development, however, once PIGF was introduced exogenously, it amplified the amount of VEGF-B binding to FLT-1. Therefore, PIGF was found to amplify, but not determine the response of VEGF-B [56].

Snake venom VEGF or VEGF-F

Snake venom VEGF or VEGF-F is a unique form of the VEGF family found in the snake venom of different snake species. Depending on the snake species, they vary in binding affinities, receptor preferability, and pathway activation ability. For example, Vammin (the venom of *Vipera a. ammodytes*) and VR-1 (the venom of *Daboia r. russelli*) are heparin-binding homodimeric cytokines that only bind to KDR. They induce the proliferation of endothelial cells with stronger mitotic activity than $VEGF\text{-}A_{165}$ [69]. On the other hand, *Tf-sv*VEGF (the venom of *Trimeresurus flavoviridis*) and Pm-VEGF (the venom of *Protobothrops mucrosquamatus*) have a stronger affinity to bind to FLT-1, with weaker binding to KDR, and no binding to FLT-4 [70].

Soluble FLT-1

Soluble VEGF receptors are shorter non-membrane binding receptors. They are often referred to as natural VEGF inhibitors, as they maintain their ability to bind and neutralize ligands. The soluble form of FLT-1 or sFLT-1 was first discovered in a human vascular endothelial cell library and it plays an important role as a VEGF trapping anti-angiogenic factor, as well as a mediator of endothelial cell migration and adhesion by interacting with α5β1 integrin in the extracellular matrix [71]. In humans, sFLT-1 can naturally arise by proteolytic cleavage or alternative splicing, creating a shorter protein missing the transmembrane and intracellular domains (Fig. 3) [72].

Proteolytic cleavage of VEGF receptors is crucial for the maintenance and regulation of angiogenesis. Proteolytic cleaving can occur by ECM modifiers which cleave the receptor after the full-length protein has been integrated into the membrane. An example of ECM modifiers is matrix metalloproteinases (MMPs). These are zinc-dependent endopeptidases found on the ECM, which can remodel the ECM by modifying other components around it [73]. Some MMPs are involved in the proteolysis of the ECM components, which is crucial for angiogenesis [74]. For instance, membrane-bound matrix metalloproteinase 14 (MMP14), has demonstrated the capacity to bind and cleave the extracellular domain of FLT-1 at two points leaving behind three small fragments which are 59.8, 35, and 21 kDa in size. The 59.8 kDa fragment contains IgD 1–5 and is released as a soluble form with ligand binding domains. The remaining attached intracellular domain becomes redundant [74]. FLT-1 can also be cleaved by β-Secretase which releases a 90 kDa ectodomain. This cleavage is a prerequisite for γ-secretase which cleaves and releases a 100 kDa intracellular domain into the cytosol of the cell, leaving behind a 10 kDa transmembrane segment [75].

The other mechanism used to create sFLT-1 is alternative splicing, which occurs before the mRNA is translated to a protein. After DNA transcription into pre-mRNA, post-transcriptional modifications occur to mature the mRNA.

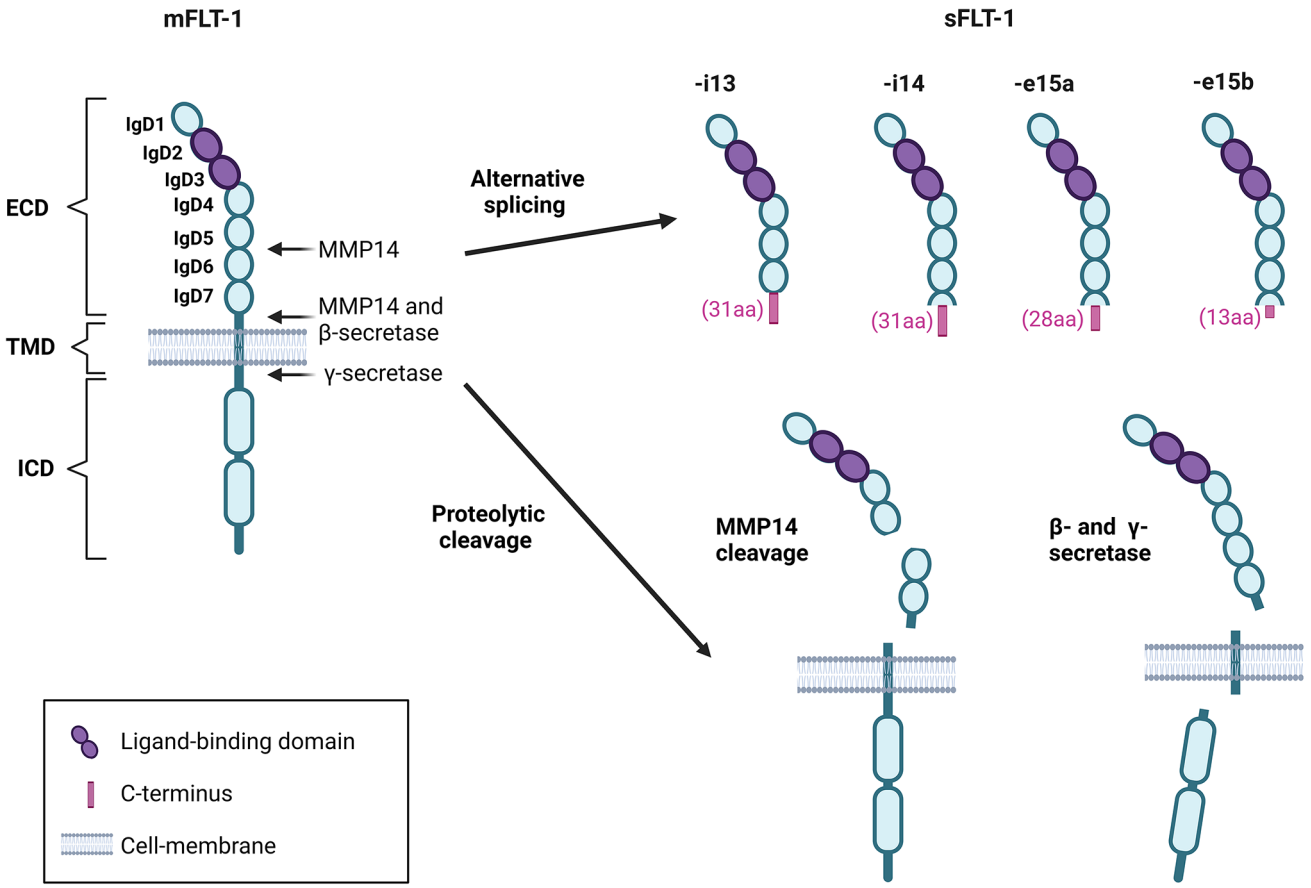

Fig. 3 Schematic of protein structures of membrane-bound FLT-1 (mFLT-1), and all forms of soluble FLT-1 produced either by alternative splicing or proteolytic cleavage. Figure created with Biorender.com

These modifications include capping, splicing, and polyadenylation (polyA). The splicing of introns (or, in some cases, exons) is regulated by splicing signals, specifically the 5′ splice site, the branch site, and the 3′ splice site [76]. Alternative splicing occurs when mRNA is spliced in different combinations from the common, most abundant form, resulting in variably spliced mRNA. When the splicing changes, this can change the 3' UTR (untranslated region) at the tail end of the protein-coding mRNA [19]. Besides the cleaved form of sFLT-1, there are four known forms of alternatively spliced sFLT-1: sFLT-1-i13, sFLT-1-i14, sFLT-1-e15a, and sFLT-1-e15b (Fig. 4). All isoforms retain most of the extracellular domain, but not the transmembrane and intracellular domain which are transcribed by exon 16 onwards. Therefore, they can capture their corresponding ligands but not activate any signal transduction pathways [15].

sFLT-1-i13

sFLT-1-i13 was first isolated from spongiotrophoblast cells in the placenta and is an important factor in regulating angiogenesis [16]. The first 6 IgDs of FLT-1 are entirely conserved in isoform sFLT-1-i13, however, the regular intronic 5' splicing site of intron 13 is skipped leading to the inclusion of intron 13. Intron 13 contains an in-frame stop codon 31 bp (base pairs) into the intron and subsequent polyA sites required for termination of translation. This isoform will recognize new intronic PolyA signals in a process referred to as alternative polyadenylation, which dictates where the mRNA is cleaved at the 3' end [77]. Intron 13 contains six polyA sites in total, but only three major polyadenylation sequences (one proximal and two distal) produce a short and long sFLT-1-i13 pre-mRNA transcript as shown in Fig. 5b and c, respectively. The proximal polyA site is 90 bp from the beginning of intron 13. The distal polyA sites are 4104 bp and 4126 bp from the 3' end of exon 13. The short sFLT-1-i13 transcript is around 2.6 kb, while the long sFLT-1-i13 that is produced by the recognition of the distal PolyA is around 7 kb in length. This is because the distal PolyA is near the 3' end of the 4 kb long intron 13, adding 4 kb to the 3' UTR [19]. Once modified and matured, the final sFLT-1-i13 mRNA has a unique 31 bp C-terminus. The distal polyA sites are necessary for sFLT-1 modulation as deletion

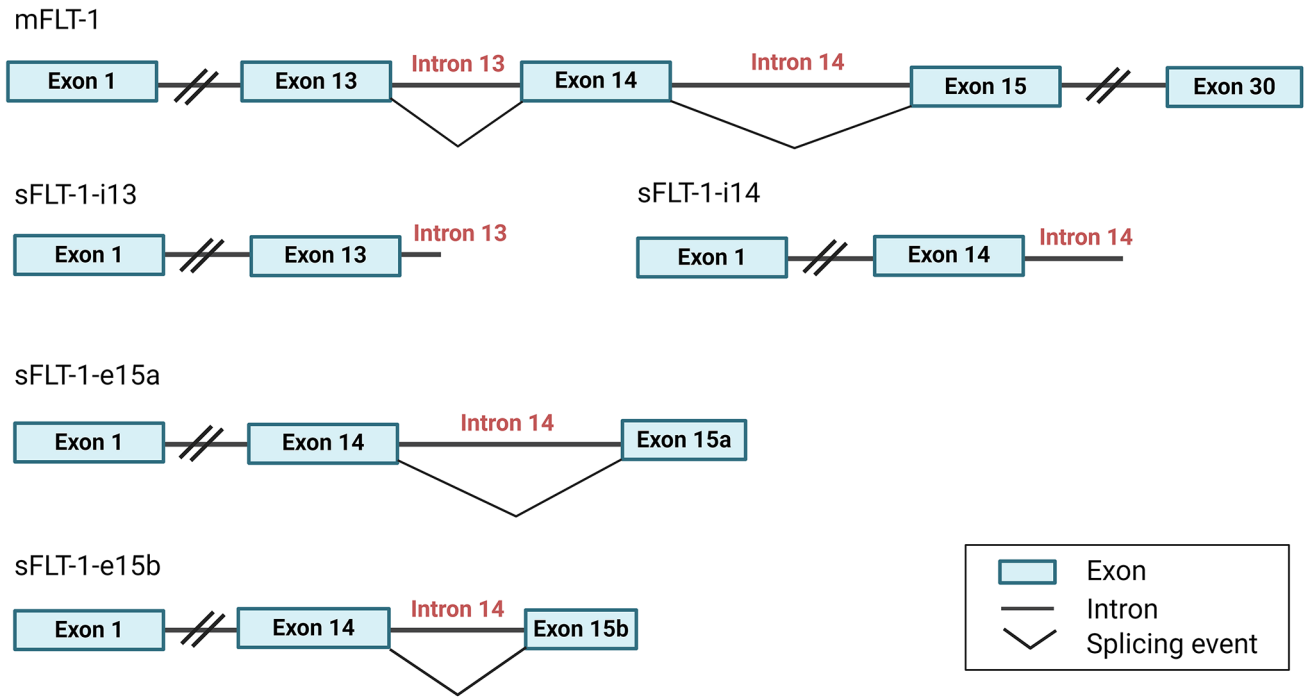

Fig. 4 Schematic of the mature mRNA transcripts of membrane-bound FLT-1 (mFLT-1), soluble FLT-1 (sFLT-1)-i13, sFLT-1-i14, sFLT1-e15a and sFLT1-e15b. Figure created with Biorender.com

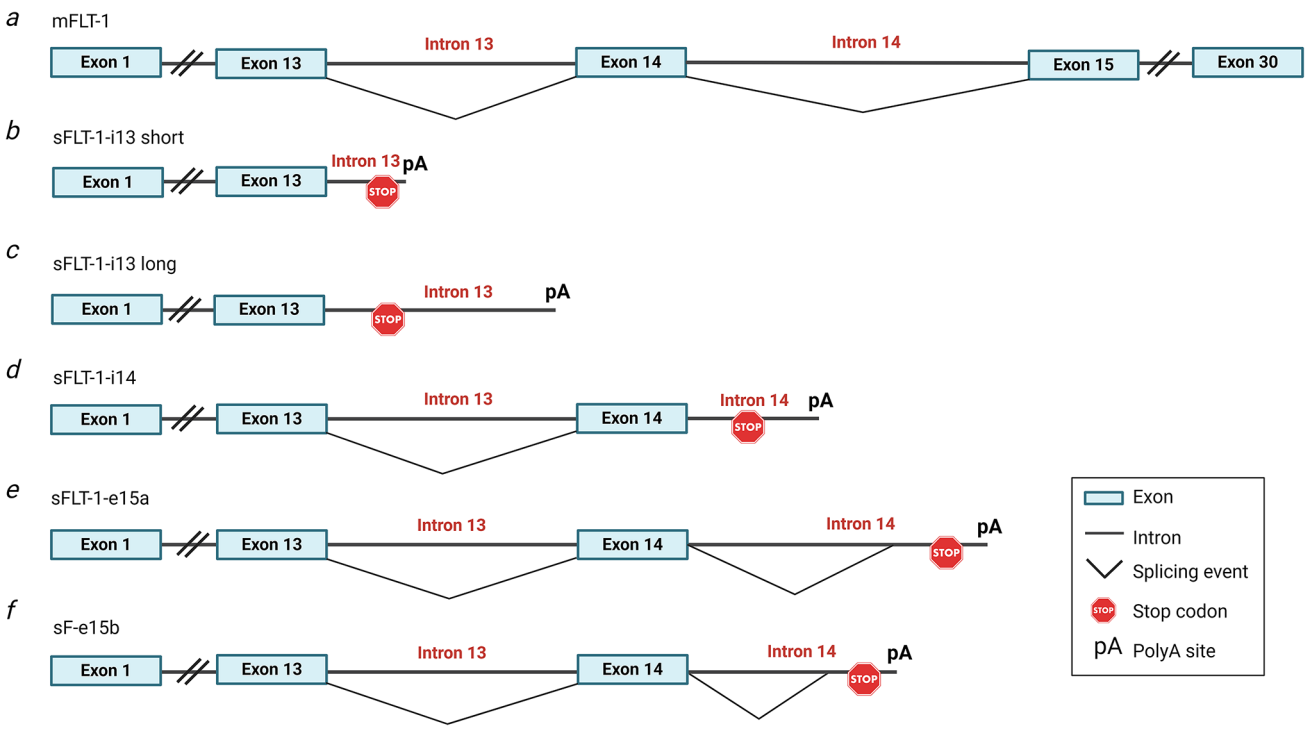

Fig. 5 The pre-mRNA transcripts of (**a**) full length of FLT-1, (**b**) soluble FLT-1(sFLT-1)-i13 short, (**c**) sFLT-1-i13 long, (**d**) sFLT-1-i14, (**e**) sFLT-1-e15a and (**f**) sFLT-1-e15b isoforms. PolyA = polyadenylation. Figures created with Biorender.com

of the two distal polyA sites resulted in membrane-bound FLT-1 receptors becoming soluble in an FLT-1 minigene system [78]. Besides cis-element regulation of splicing, it was discovered that $VEGF_{165}$ binds to trans-elements, such as transcription factor SOX2 and the splicing factor SRSF2 to form a network that induces the formation of sFLT-1-i13 in squamous lung carcinoma cells [79].

sFLT-1-i14

sFLT-1-i14 was first predicted by bioinformatic analysis and then confirmed in human placental cDNA. The splicing mechanism which forms sFLT-1-i14 is very similar to that which forms sFLT-1-i13, except in this case the intron 14 5' splice site is skipped, instead of intron 13, leading to the extension of the transcript for 270 bp into intron 14. The open reading frame extends for 93 bp where an in-frame stop codon is located (Fig. 5d). Therefore, the final protein product is the first 6 IgDs of FLT-1, a partial 7th IgD, and a unique 31 amino acid C-terminus. Because this isoform does not appear to be very prevalent in pathogenesis (except for a non-significant increase in preeclampsia patients), it has not been extensively explored like the other three soluble FLT-1 isoforms [15].

sFLT-1-e15

The last of the sFLT-1 splice variants, sFLT-1-e15, appears in two isoforms: sFLT-1-e15a (Fig. 5e), and sFLT-1-e15b (Fig. 5f). First discovered in placental RNA, these isoforms express the first 14 exons of FLT-1, however during post-transcriptional modification, spliceosomes identify two cryptic 3' splice sites, resulting in alternative splicing [15, 80]. Within the cryptic splice-acceptor regions are in-frame stop codons and PolyA sites, resulting in the translation of a 'false' or cryptic exon 15. sFLT-1-e15a and -e15b proteins contain a 28 and 13 amino acid unique c-terminus, respectively. sFLT-1-e15a translates to a 733 amino acid protein with a serine-rich tail, while sFLT-1-e15b translates to a shorter 718 amino acid protein. The mechanism behind this isoform's alternative splice site recognition is still unknown [15, 81].

Soluble FLT-1 as a biomarker for pathological conditions

Soluble variants of FLT-1, or sFLT-1, are prominent in normal physiology and can aggravate or alleviate certain pathologies. sFLT-1 has been reported to be expressed in vascular endothelial cells, human peripheral blood monocytes, vascular smooth muscle cells, human umbilical tissue-derived cells, human proximal tubule cells, human corneal epithelium, and human cancer cell lines [82–87]. Many studies use sFLT-1 levels independently or in combination with VEGF or PIGF, as prognostic or diagnostic markers of disease [88]. The various pathological conditions under which varying levels of sFLT-1 are observed are listed in Table 1. In some instances, soluble forms of VEGFRs have evolved to act as natural competitors of their corresponding membrane-bound receptors, neutralizing VEGF to reduce angiogenesis in areas where it is deleterious. This is seen with MMP14 proteolytic cleavage of FLT-1 into sFLT-1, which was found to be an important process in the cornea for corneal transparency maintenance by preventing aberrant vascularization [89]. If levels of sFLT-1 decline, some diseases can be aggravated due to a lack of anti-angiogenic factors preventing irregular blood vessel formation. This is observed in conditions such as diabetic retinopathy, and age-related macular degeneration (AMD) where sFLT-1 levels are lower than normal controls and levels of VEGF are higher (Fig. 6) [90]. A study comparing both sFLT-1 in the aqueous humor of 27 diabetic patients and 33 control patients found that sFLT-1 was significantly lower ($p < 0.05$) in non-proliferative diabetic retinopathy patients [91]. A similar narrative is observed in patients with neovascular AMD (nAMD; also called wet AMD or exudative AMD). A study observing sFLT-1 levels in 97 late nAMD, 53 early nAMD, and 57 control participants found that serum concentrations of sFLT-1 in late nAMD patients were significantly lower ($p < 0.01$) compared to the other two groups. The study also found that age along with sFLT-1 levels can be used as a biomarker for disease, as patients above the age of 73 years old with sFLT-1 levels less than 80 pg/mL were 6 times more likely to develop nAMD [92].

In some pathologies, sFLT-1 can alleviate pathological outcomes in pre-existing disease states. For instance, in patients with cancers including acute myeloid leukemia, myelodysplastic syndromes, and breast cancer, a lower level of sFLT-1 is associated with poor prognosis and lower overall survival [93, 94]. This is because sFLT-1 is an anti-angiogenic factor, and higher levels indicate the body's ability to reduce vascular supply to tumors and therefore interfere with their growth. For instance, a study measuring the intratumoral levels of sFLT-1 in 202 primary breast cancer tissue found that the ratio of sFLT-1:VEGF was a significant indicator of disease-free progression ($p = 0.008$) and overall survival rate ($p = 0.0002$) [94]. Other conditions such as sepsis, trauma, rheumatoid arthritis, and psoriasis also inhibit increased levels of the anti-angiogenic factor sFLT-1, as less blood supply to affected regions helps reduce inflammation and unnecessary blood loss from injury [95–97].

In some cases, sFLT-1 can aggravate disease states. This is observed in preeclampsia, where higher levels of soluble FLT-1 variants, sFLT-1-e15a/b, and sFLT-1-i13, have been

Table 1 Soluble receptors and their association with human pathologies

Pathology	Soluble receptor associated	Soluble receptor levels	Proposed mechanism of action	Reference
Cancer progression	sFLT-1	An inverse correlation between sFLT-1 levels and complete remission. Lower sFLT-1 and higher VEGF are associated with poor prognosis	sFLT-1 may be acting as a tumor angiogenesis inhibitor	[93, 94]
Myocardial infarction and severe acute heart failure	sFLT-1	Higher sFLT-1 patients with acute myocardial infarction. Significantly higher sFLT-1 in patients who later developed severe acute heart failure	Hypoxic conditions could generate sFLT-1 by inducing alternative splicing	[102, 107, 108]
Alzheimer's disease	sFLT-1	Lower FLT-1 but higher sFLT-1 levels	sFLT-1 induced by hypoxia could be reducing microvessel density in Alzheimer's disease	[106]
Diabetic retinopathy	sFLT-1	Lower levels of sFLT-1	sFLT-1 is an inhibitor of angiogenesis. Less sFLT-1 results in more cellular permeability and vascularization	[91]
Neovascular age-related macular degeneration (nAMD)	sFLT-1	Lower levels of sFLT-1	sFLT-1 is an inhibitor of angiogenesis. Fewer inhibitors result in more vascularization observed in nAMD	[92, 109]
Preeclampsia	sFLT-1-i13, -e15a and -e15b	Higher levels of sFLT-1 receptors, with an exponential increase by weeks 29–32 of the preeclamptic pregnancy.	sFLT-1 competes with mFLT-1 for VEGF, resulting in endothelial dysfunction and therefore blood pressure dysregulation	[15, 66]
Pulmonary arterial hypertension (PAH)	sFLT-1	Higher levels of sFLT-1	Higher sFLT-1 levels may contribute to the abnormal angiogenesis observed in PAH due to its anti-angiogenic properties	[103]
Sepsis and Trauma	sFLT-1	sFLT-1 level increased with variable factors including the severity of the injury, shock, tissue damage, and inflammation in trauma patients	Lack of angiogenic stimulation due to sFLT-1 anti-VEGF inhibition leads to reduced blood flow to major organs to reduce blood loss and inflammation	[95]
Rheumatoid arthritis	sFLT-1	sFLT-1 level increases in line with inflammation	More inflammation induces more anti-angiogenic agents as an evolutionary mechanism to downregulate angiogenesis	[110, 96]
Psoriasis	sFLT-1	Higher sFLT-1 levels	sFLT-1 may help to control the increase in VEGF levels	[97]

linked to decreased blood vessel formation in the placenta which may cause hypertension in preeclamptic pregnancies (Fig. 6) [15]. sFLT-1 is naturally expressed by various cells in the placenta, and in normal pregnancies, sFLT-1 levels will initially decrease, then gradually increase at 26 weeks, followed by a rapid increase at 35 weeks. A concentration of 1000 pg/mL is abnormal in the early stages, however, by late pregnancy levels of 2000 pg/mL are in the normal range [13]. The major source of FLT-1 and sFLT-1 in the placenta are the vasculogenic and angiogenic precursor cells, while cytotrophoblasts Hofbauer cells strongly express VEGF [98]. It is proposed that higher sFLT-1 expression in pre-eclampsia has been linked to HIF1-α in hypoxic conditions [80]. A nested case-control study paired 120 women with pre-eclampsia with women with normal pregnancies and measured serum concentrations of sFLT-1, VEGF, and PIGF throughout the pregnancy. They found that women with pre-eclampsia have approximately 2.7 times more sFLT-1 ($p < 0.001$) in their serum compared to normotensive controls with fetuses of the same gestational age. Pre-eclamptic women also demonstrated lower levels of PIGF as early as 13 weeks into pregnancy, and this level would continue to decrease alongside the increase of sFLT-1 within 5 weeks before the onset of pre-eclampsia. Thus, the sFLT1/PIGF ratio can be helpful in the early diagnosis of pre-eclampsia [66]. However, a recent study found that pregnant women with obesity have significantly lower sFLT1 levels compared to normal and overweight women, reducing the performance of the sFLT1/PIGF ratio as a predictor of pre-eclampsia diagnosis [99]. Furthermore, there is a higher level of membrane-bound FLT-1 protein in the preeclamptic placenta. Despite this, the ratio of sFLT-1:FLT-1 is still significantly higher in the tissue of a pre-eclamptic placenta compared to a normotensive control placenta [100]. Hence, sFLT-1 can be used as an early biomarker to predict the development of pre-eclampsia during pregnancy.

Additionally, it is important to note that the role of sFLT-1 in some diseases is still under investigation. It is unknown whether higher levels of sFLT-1 in pulmonary arterial hypertension, myocardial infarction, and severe acute heart failure are a response to hypoxia and elevated blood pressure, or if they are contributors to the abnormalities in angiogenesis observed in these conditions [101–103]. The role of sFLT-1 in Alzheimer's disease (AD) is also non-conclusive [104].

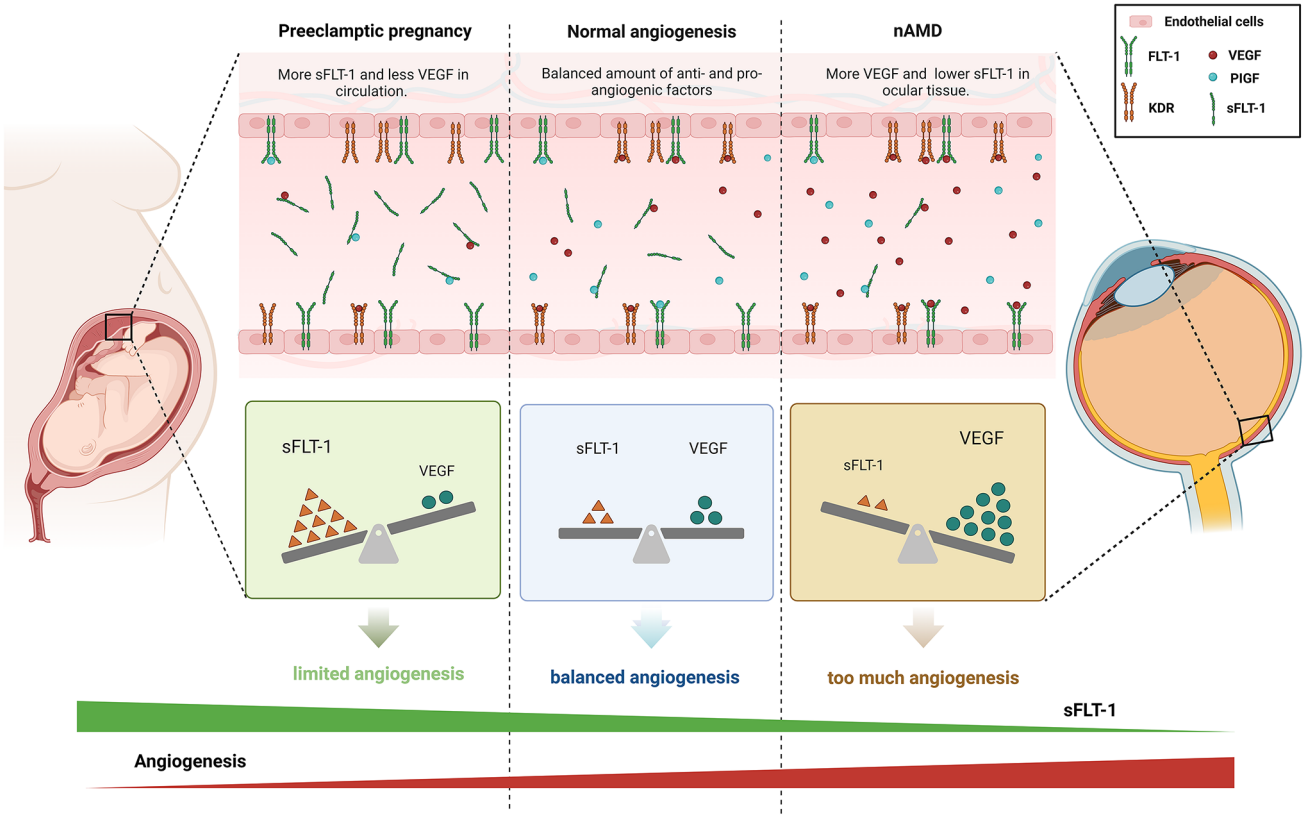

Fig. 6 A schematic of the balance of pro-angiogenesis and anti-angiogenesis factors and how they cause pathology in preeclampsia and neovascular age-related macular degeneration (nAMD). sFLT-1: soluble vascular endothelial growth factor receptor 1, VEGF: vascular endothelial growth factor, and PIGF: placental growth factor. Figure created with Biorender.com

The "angiogenesis hypothesis" proposed by Vagnucci and Li, 2003, states that the AD brain is in a hypoxic state and so it activates angiogenesis signaling pathways [105]. Hence, findings of higher sFLT-1 levels may indicate the body's natural mechanism to reduce angiogenesis that is stimulated by hypoxic conditions [106]. All in all, sFLT-1 is a useful biomarker for pathological prediction and progression, but there is still much to be learned about its prevalence and role in pathogenesis.

Therapeutic implications of soluble FLT-1 in pathological angiogenesis

The concept of using soluble FLT-1 as a therapy has been of interest for the past 3 decades. Clinical studies associated with the use of exogenous sFLT-1 have reported therapeutic potential, observing that the soluble receptor inhibits, slows, and reverses the progression of a variety of diseases, including cancers, choroidal neovascularization (CNV), and rheumatoid arthritis [96, 111–113]. Furthermore, many anti-VEGF therapies incorporating decoy VEGF receptors have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of neovascular diseases.

Anti-VEGF agents are used therapeutically to neutralize VEGF-A so that it can no longer bind to KDR and activate the angiogenesis pathway [13]. Hence, several exogenous anti-VEGF approaches have incorporated the direct use of all or part of sFLT-1 as the anti-angiogenic agent (Table 2). Exogenous anti-VEGF factors can be introduced in the form of protein, DNA, or RNA. A protein therapy that features sFLT-1 usually consists of soluble receptor fusion proteins that incorporate the receptors' ligand-binding domains into antibody fragments. For instance, Aflibercept (Eylea®) by Regeneron Pharmaceuticals, Inc./Bayer is formed by combining the ligand binding domain IgD2 of FLT-1 and IgD3 of KDR to the fragment crystallizable region (FCR), of human IgG1 [114]. This drug has been FDA-approved for the treatment of cancer and CNV [115]. When using Aflibercept (Zaltrap®) to treat various cancers, including advanced non-small cell lung cancer, metastatic pancreatic cancer, and metastatic colorectal cancer, patients show an improvement in survival rate and progression-free survival when compared to control groups. In a Phase I study focused on advanced solid tumors, 38 patients were administered aflibercept subcutaneously at different doses. The VEGF bound to aflibercept (Zaltrap®) in plasma increased

Table 2 Comprehensive list of therapy approaches that incorporate sFLT-1 as an anti-VEGF therapy. All strategies target ligands that bind to FLT-1: VEGF-A, VEGF-B, and PIGF. CNV: choroidal neovascularization. FDA: U.S. Food and Drug Administration. AAV: adeno-associated virus. nAMD: wet age-related macular degeneration

Strategy	Drug/company	Disease	Delivery vector and/or administration route	Mechanism of action	Clinical trial phase	Outcome	Frequency	Country	Trial Identifier/s	References
Transgene delivery	Ixo-vec (formerly known as ADVM-022) by Adverum Biotechnologies	choroidal neovascularization (CNV)	AAV.7m8 capsid carrying Aflibercept administered via intravitreal injection	AAV carrying cDNA of Aflibercept. Once AAV is delivered, Aflibercept will be transcribed and translated into the cells	II	Improved best-corrected visual acuity and central retinal thickness	One-off (for up to 1.7 years)	United States of America	NCT05536973 and NCT05607810	[125]
	rAAV2.sFLT-1 by Lions Eye Institute and Adverum Biotechnologies, Inc.		rAAV2 carrying sFLT-1 administered via subretinal injection	AAV carrying DNA transgene of a section of sFLT-1. Once AAV is delivered, sFLT-1 will be transcribed and translated into the cells	II	Sustained vision and less blood leakage	One-off	Australia	NCT01494805	[141, 128]
	AAV2-sFLT01 by Sanofi		AAV2 vector carrying domain 2 of FLT-1 linked by a polyglycine 9-mer to human IgG1-Fc administered via intraretinal injection		I	Improvement of vision	One-off	United States of America	NCT01024998	[142, 143, 129]
	AAV2.FLT23K		AAV2 carrying domains 2 and 3 of FLT-1 via subretinal injection	AAV2 carries the plasmid of ligand binding domains of FLT-1 with a KDEL sequence	In vivo (Murine laser-induced CNV model)	Prevented laser-induced CNV in a murine model	N/A	United States of America	N/A	[131]
Decoy soluble receptor fusion protein	Aflibercept (Zaltrap®) by Regeneron Pharmaceuticals, Inc./Bayer	Various cancers including metastatic colorectal, metastatic pancreatic, and advanced non-small cell lung cancer	Intravenous (IV) injection	Protein is designed by fusing antibody fragments with VEGFR ligand binding domains. Binds to and neutralizes all natural targets of VEGF binding domains	All phases and approved by the FDA	Improvement of patient survival rate and progression-free survival	Every 4–8 weeks	United States of America	NCT00036946	[116]
	Aflibercept (Eylea®) by Regeneron Pharmaceuticals, Inc./Bayer	CNV	Intravitreal injection			Increase in visual acuity and maintained vision gains			NCT00320775	[115]
	Conbercept (Lumitin®) by Chengdu Kanghong Biotech Company	nAMD, diabetes-related macular edema, and pathologic myopic CNV	Intravitreal injection		All phases and approved by the FDA.	Improves best-corrected visual acuity and reduces central retinal thickness	Every 4 weeks	People's Republic of China	NCT03426540	[144]

proportionally to the dose and 47% of patients demonstrated at least 10 weeks of disease stability (ClinicalTrials.gov Identifier: NCT00036946) [116]. In a crucial Phase III study, 1126 patients with metastatic colorectal cancer receiving chemotherapy were randomly administered Aflibercept or placebo. Overall survival was significantly improved ($p = 0$ 0.0032) in patients who received the Aflibercept, although a higher incidence of adverse effects typically associated with anti-VEGF therapy was reported, such as hypertension, venous thromboembolic events, hemorrhage, and arterial thromboembolic events stability (ClinicalTrials.gov Identifier: NCT00561470) [117]. Aflibercept was also developed to treat CNV. A Phase II study in which 159 patients with CNV received an intravitreal injection of either 0.5, 2, or 4 mg of Aflibercept (Eylea®) found that there was a decrease in central retinal thickness which correlated with an improvement in vision acuity, and their overall vision is either maintained or improved over time (ClinicalTrials.gov Identifier: NCT00320788) [118]. A recent Phase II/III study of Aflibercept (Eylea®) compared a higher dose of 8 mg with 2 mg on 660 patients with diabetic macular edema and found that a higher dose could extend dose intervals and was deemed well-tolerated [119]. Similarly, Conbercept (Lumitin®) by Chengdu Kanghong Biotech Company is formed by fusing IgD2 of FLT-1 and IgD3 and IgD4 of KDR to the FCR of human IgG1 [120]. In a Phase I study on proliferative diabetic retinopathy, 50 patients with non-clearing vitreous hemorrhage were divided equally into treatment and control groups. The treatment group received an intravitreal injection of Conbercept (10 mg/mL, 0.5 mg) immediately after a vitrectomy while the control group didn't receive any treatment. The treatment group demonstrated an improvement in best-corrected visual acuity ($p < 0.001$) as well as a reduction in central retinal thickness ($p = 0.012$) as early as 1 week after treatment compared to the control group (ClinicalTrials.gov Identifier: NCT03426540) [121]. The most recent Phase III trial of Concerbept aimed to evaluate the safety and efficacy of 0.5 mg of treatment injected intravitreally in 114 patients with CNV at a regiment of 3 monthly injections followed by one injection every 4 months. The results demonstrated a significant improvement ($p < 0.001$) in best-corrected visual acuity at month 3 and the regiment was considered to be an effective treatment for nAMD [122]. Although these protein therapies have been extensively successful, the transient nature of proteins means there is a requirement for continuous dosage, otherwise the pathologies will reoccur. Hence, Aflibercept has been adapted into ixoberogene soroparvovec (ixo-vec), formerly known as ADVM-022, by Adverum Biotechnologies which is an AAV.7m8 capsid carrying cDNA encoding for the drug. This form of treatment is classified as gene therapy. Gene therapy involves introducing, modifying, or manipulating genes to treat disease [123]. Conventional gene therapy involves introducing healthy gene copies to replace disease-causing gene mutations. An adeno-associated virus (AAV) vector is the leading delivery system for gene therapy as it can target specific subtypes of cells and allows for long-term gene delivery. The AAV delivers a whole gene, also known as a transgene, into a cell to be translated intracellularly by utilizing endogenous ribosomes [124]. Ixo-vec is now in Phase II clinical trials and shows promising results of improved or maintained best-corrected visual acuity and central retinal thickness in nAMD patients, for up to 1.7 years. Patients also reported a significantly reduced anti-VEGF injection burden as a result of the continuous expression design of ixo-vec (ClinicalTrials.gov Identifier: NCT05536973) [125].

Other therapies in the early phase of clinical trials involve the delivery of sFLT-1 transgenes with AAVs. Earlier studies established that when treated with an AAV delivering sFLT-1, the trVEGF029 mouse line which typically shows morphological changes similar to diabetic retinopathy demonstrated long-term regression of choroidal neovascularisation and the maintenance of structural morphology in the retina [126]. Currently, there are two human therapies incorporating the use of AAV to deliver sFLT-1 in early-phase trials; rAAV2.sFLT-1 and AAV2-sFLT01. Developed by Lions Eye Institute and Adverum Biotechnologies, Inc., rAAV2.sFLT-1 (Phase II) is an AAV carrying the sFLT-1 gene, which when delivered subretinally will express sFLT-1 and neutralize VEGF (Fig. 7). A three-year follow-up of the phase I dose escalation trial revealed that patients who were injected with either a high dose (1×10^{11} viral genome [vg]) or low dose (1×10^{10} vg) of rAAV2. sFLT-1 received less overall anti-VEGF intervention in a 36-month period. The treatment also demonstrated the stability of nAMD symptoms and was well tolerated (ClinicalTrials.gov Identifier: NCT01494805) [127]. The most recent Phase IIa trial showed very similar results, when injected with rAAV2-sFLT-1 (1×10^{11} vg), patients received less anti-VEGF retreatment. There was no sFLT-1 in bodily fluids and no adverse side-effect, suggesting the potential of co-treatment using an AAV-delivered sFLT-1 and anti-VEGF to lessen the burden of injections [128]. On the other hand, AAV2-sFLT01 (Phase I) by Genzyme, a Sanofi company utilizes an AAV2 vector to express domain 2 of FLT-1 linked by a polyglycine 9-mer to human IgG1-Fc, which can be administered via intraretinal injection. A phase I clinical trial observed 19 patients with advanced nAMD for a 52-week period after receiving an intravitreal injection of AAV2-sFLT01 to assess safety of treatment at multiple doses. The treatment was well-tolerated and 6 of the 11 patients with intraretinal or subretinal fluid at baseline, showed a reduction in fluid and improved vision (ClinicalTrials.gov Identifier:

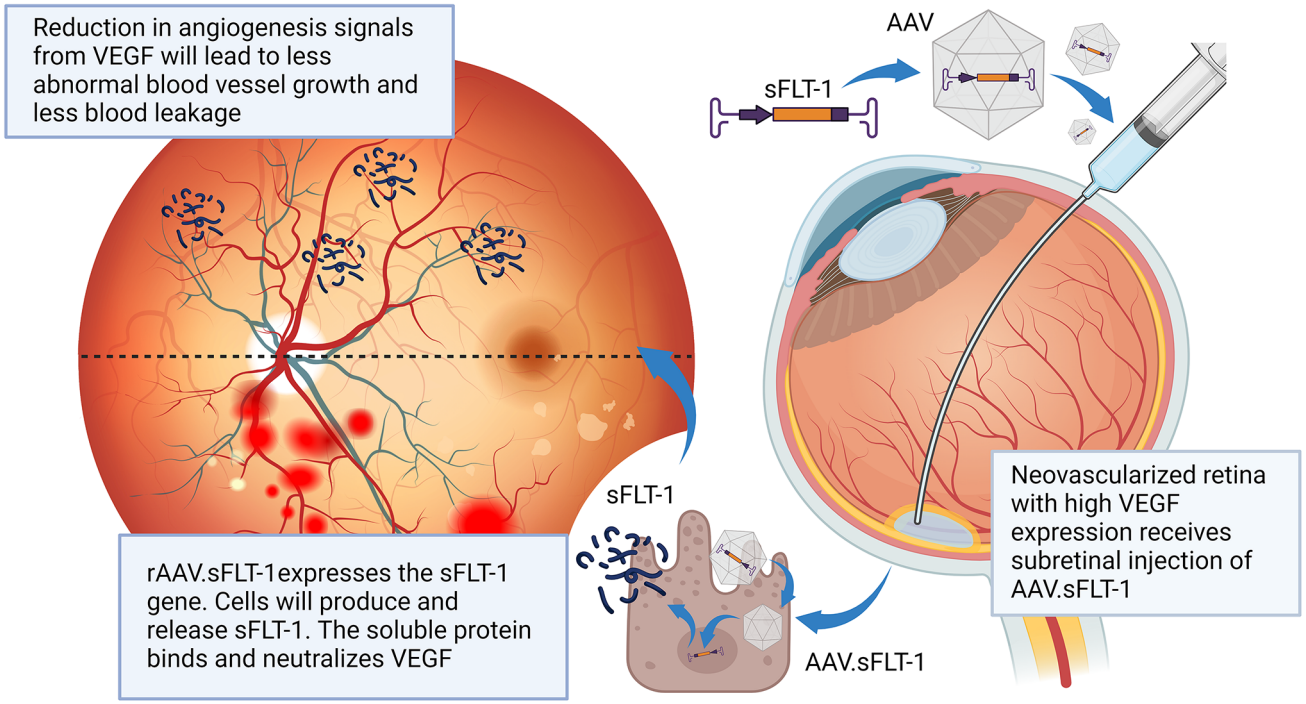

Fig. 7 The assembly and mechanism of action of rAAV.sFLT-1 by Lions Eye Institute and Adverum Biotechnologies, Inc. This AAV is designed to produce long-term expression of the soluble FLT-1 gene to manage angiogenesis signaling by VEGF. sFLT-1: soluble vascular endothelial growth factor receptor 1. VEGF: vascular endothelial growth factor. Figure created with Biorender.com

NCT01024998) [129]. Another transgene therapy adapted to AAV delivery is FLT23k. This plasmid was designed by combining the ligand binding domain IgD2 and IgD3 of FLT-1 to an endoplasmic reticulum retention peptide KDEL (Lys-Asp-Glu-Leu) [130]. After intraretinal injection into a murine laser photocoagulation-induced CNV model using male wildtype C57BL/6 mice, the AAV2.FLT23k was present for up to 6 months postinjection and prevented CNV after laser treatment [131]. It is important to note that there are a few challenges associated with using AAV as delivery vectors. Some AAV serotypes demonstrate a strong immune response which compromises treatment and patient safety [132, 133]. AAV therapies developed to treat retinal neovascularisation benefit from immune privilege, however the administration route of the AAV in the retina can affect the development of anti-AAV antibodies. It has been observed that intravitreal injections are more likely to interact with the immune system and cause an increase in anti-AAV antibodies compared to subretinal injections [134]. Furthermore, finding the optimal dosage of anti-VEGF treatment is critical, as a high dose comes with the risk of neutralizing VEGF which can have consequential side effects, while too low a dose can result in undertreatment. For instance, endogenous expression of VEGF is essential in the retina as it is neuroprotective and maintains the retinal structure and function, so anti-VEGF treatment in the retina should not completely neutralize VEGF [135]. In most cases, the optimal anti-VEGF dosage varies. Some trials have demonstrated positive clinical outcomes with higher doses, while others have identified no significant differences between higher and lower doses [136].

The anti-VEGF strategies mentioned above all involve the exogenous introduction of anti-VEGF factors. However, there have been a few attempts in endogenous sFLT-1 splicing modulation which have shown promising therapeutic potential. Endogenous manipulation of membrane-bound FLT-1 to sFLT-1 is in an earlier stage of validation. There are some benefits to the endogenous manipulation approach over the current anti-VEGF therapy. Firstly, this method will affect the baseline expression of both soluble and membrane-bound FLT-1; increasing sFLT-1 and decreasing mFLT-1 simultaneously may have a greater effect on reducing neovascularization, compared to increasing anti-VEGF factors alone [137]. Secondly, splicing modulation can be used to induce more or less sFLT-1, unlike exogenous manipulation which is constricted to the upregulation of anti-VEGF factors. This property means the treatment can be adapted to multiple disease models caused by dysregulation of sFLT-1 expression, such as preeclampsia [80]. Lastly, endogenous modulation of sFLT-1 using an introduced splice switching system can be adapted to a specific long-term delivery system such as AAVs. This means patients may only require a single injection without the need for ongoing anti-VEGF injections.

As sVEGFRs are formed due to alternative splicing, RNA-targeting gene therapy approaches have been a popular choice for inducing endogenous sFLT-1 isoforms. One strategy is to use RNA-targeting splice-switching oligonucleotides called morpholinos, which are short uncharged oligonucleotides that bind to complementary RNA sequences, blocking processing and translating machinery from binding or reading through introns [138]. One study designed a morpholino to target the exon 13-intron 13 boundary to block the 5' splice site and encourage the translation of sFLT-1-i13. Exposure of the morpholino in vivo on a laser-induced CNV mouse model, demonstrated a significant increase in sFLT-1:mFLT-1 ratio ($p < 0.001$) and reduced neovascularization by up to 50% ($p = 0.0179$). In female nude mice inoculated with MBA-MD-231 human breast adenocarcinoma xenograft tumors, this treatment successfully reverted tumor volume and significantly reduced the vascular density of the tumors ($p < 0.05$) [137]. Another more recent technology with notable splice-switching potential is the RNA-targeting CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats- CRISPR-associated proteins) system. In this system, a single guide RNA leads a Cas enzyme targeting the premature mRNA before it is processed into mature mRNA. RNA-targeting strategies are becoming increasingly popular due to their transient and reversible nature, as the original DNA sequence remains unaltered [139]. CRISPR Artificial Splicing Factors, also known as CASFx, were designed by merging an RNA-targeting Cas protein with a spliceosome. This system successfully induced exon inclusion and exclusion in spinal muscular atrophy (SMA) patient fibroblasts [140]. If adapted to encourage the inclusion of intron 13 in FLT-1, the system could be used to read through the intron and therefore increase endogenous levels of sFLT-1-i13. These endogenous splice modulation strategies show early potential; however, they are limited by the knowledge of the splicing mechanism which converts the full-length receptor to its soluble form.

Concluding remarks

Angiogenesis is the growth of new blood vessels from old ones; a vital process for developing the vascular system. Full-length VEGF receptors on the cell membrane interact with their corresponding VEGF ligands to activate the angiogenesis pathway. When angiogenesis is dysregulated, neovascularization can occur, causing irregular blood vessel formation which is accompanied by pathological side effects [145]. Hence, the body has also developed a system to naturally counteract pathogenic angiogenesis by producing a soluble anti-angiogenesis factor called sVEGFR. Soluble receptors are a shorter free-floating form of the receptor with VEGF binding and neutralizing properties [146]. sFLT-1 is present in normal conditions and has been observed to vary in expression in some diseases. However, in some instances, it is observed as a mechanism to prevent vascularization and therefore prevent disease establishment/progression [89]. Some therapies have been developed which incorporate all or part of sFLT-1. A well-established therapy that incorporates sFLT-1 is anti-VEGF therapy. This form of therapy utilizes exogenous anti-angiogenic factors to reduce the aberrant blood vessel formation caused by neovascularization through VEGF neutralization. Anti-VEGF therapy has achieved tremendous success in treating neovascular-induced or aggravated diseases and many of these therapies are also widely used off-label [147]. Alternatively, treatments designed to regulate endogenous sFLT-1, show promising early results as a potential therapy. Although there is a list of benefits for modulating endogenous factors, there needs to be more investigation on the splicing mechanism responsible for inducing the sFLT-1. Furthermore, most of the studies designed to understand sFLT-1 splicing are not directly translational to the human system i.e. they use minigene systems or rodent models [148]. Further investigation on sFLT-1 splicing modulation will be beneficial for the field of angiogenesis modulation, especially with the goal that this knowledge can be adapted and applied to similar receptor-ligand diseases with receptor imbalance.

Author contributions LEW and GSL developed the concepts described in this review. LEW performed literature research. LEW wrote the paper. LEW, GSL, and AW edited, reviewed, and gave their final approval.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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