New insights into the role of ubiquitination in angiogenesis (Review)

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Abstract. Angiogenesis is a dynamic and complex mechanism for generating new blood vessels from existing ones. Angiogenesis occurs through all life stages and involves several physiological processes. It has an important physiological and pathological role including in cancer, wound healing and inflammation. The emerging role of ubiquitination in regulating angiogenesis highlights the importance of studying this pathway in an angiogenic setting. In angiogenic events, imbalances between pro- and anti-angiogenic factors, induction of hypoxic signaling and stimulation of angiogenic signaling pathways play a central role. This review provides a comprehensive overview of the role of ubiquitination in angiogenesis. This includes angiogenic factors [VEGF, platelet-derived growth factor, (basic) fibroblast growth factor and angiopoietin], vascular cells (pericytes, endothelial cells, vascular smooth muscle cells) and extracellular matrix and cell adhesion molecules, all of which have important roles in angiogenesis, hypoxic signaling (hypoxia-inducible factor), which induces angiogenesis, and important vascular signaling pathways (Wnt and Notch). In addition, the molecular biological basis of angiogenesis is discussed and the potential therapeutic value of ubiquitination in angiogenesis-related diseases is highlighted.

Contents

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1. Introduction

Ubiquitination is the sequential binding of ubiquitin (Ub) molecules by three enzymes (E1 activator, E2 conjugating enzyme and E3 ligase) and the transfer of the small modified protein Ub to lysine residues within the substrate protein. Proteins can be modified with a single Ub at one or more lysine residues or with a Ub chain formed through one of their lysine residues or an N-terminal methionine residue (M1). In humans, there are two Ub-specific E1 proteins, ~35 Ub-specific E2 binding enzymes and nearly 1,000 identified E3 ligases (1,2). Ubiquitination is a general term for several different types of Ub modifications, each having a different effect on the target protein. Polyubiquitin chains allow proteins to be degraded by the proteasome. Another form of ubiquitination is monoubiquitination, which can alter subcellular localization. These different types of Ub modifications represent the different effects of ubiquitination on protein function (3). As a post-translational modification (PTM), ubiquitination can affect the stability, interaction, localization and/or activity of thousands of proteins by forming conjugates of different topologies, thereby controlling a large number of signaling events in the cell, including proteasomal degradation, DNA damage repair and cell cycle progression, amongst others (4,5). Ubiquitination plays an important role in all aspects of cellular physiology, including angiogenesis, which is the focus of this review. The process of ubiquitination, on the other hand, is the process by which ubiquitin molecules, in the presence of a series of special enzymes, classify proteins in the cell and select from them target protein molecules for specific modification (Fig. 1).

2. Molecular basis of angiogenesis

Angiogenesis is the process by which new capillaries are formed from existing capillaries and occurs via two primary

mechanisms: Sprouting and non-sprouting. Angiogenesis aims to respond to changes in the cellular machinery or metabolic environment, and appears in the form of endothelial cell (EC) sprouting and longitudinal division. The sprouting form is considered the primary mechanism of angiogenesis during physiological development and in cancer, where new capillaries branch and protrude from existing ones, and this requires the excessive proliferation of ECs (6). The non-budding process appears to be more efficient compared to the budding approach. In the non-budding approach to angiogenesis, the developed vasculature is halved by longitudinal splitting within the capillaries, turning one capillary into two. This reduces the need for EC proliferation (7). Angiogenesis is a complex, multi-step process involving the close interplay of cell proliferation, differentiation, migration and signaling. Blood vessels are composed of two main cell types, ECs and pericytes. ECs constitute the main body of the blood vessel, forming a hollow tube known as the lumen, which is lined with a specialized extracellular matrix (ECM) known as the basement membrane. Pericytes are attached to the lumen's surface and regulate the vessel's permeability and stability as required by the environment, whilst also serving as important support cells for the vessel (8). Angiogenesis begins when ECs receive pro-angiogenic signals in the form of growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Fibroblast growth factors (FGFs) promote migration and proliferation in angiogenesis by stimulating VEGF-mediated integrin levels and activating proteolytic activity through tight binding to their respective receptors (FGFRs) (9). Activated ECs then invade the basement membrane and ECM, disrupting integrin-mediated interactions. When ECs are activated, MMPs are secreted from the tip cells to degrade the ECM and break down the basement membrane in the region where they are located. By digesting the basement membrane, ECs migrate and proliferate. Angiopoietin (Ang) is then activated to initiate lumen formation (10). In addition, cell adhesion molecules (CAMs) play a central role in angiogenesis. ECs use CAMs to achieve homogeneous and heterogeneous adhesion and to adhere to and migrate through the ECM, a critical step in angiogenesis. Increased expression of activated CAMs can also lead to their detachment or release from ECs. Several soluble forms of CAMs have been identified, such as intercellular adhesion molecule-1 (ICAM-1), vascular CAM 1 (VCAM-1) and E-selectin (11). Then, when the ECs converge, they begin to interconnect and form tubular structures, termed luminalization. Intercellular interactions, adhesion and alignment of cells are particularly critical at this stage (11). The EphB/ephrinB system is involved in regulating the process of lumen formation, while the involvement of pericytes and smooth muscle cells is required to stabilize the newly formed vessels (12). Finally, the process of vascular maturation requires the involvement of multiple cells and signals. The interaction between ECs, SMCs and pericytes is particularly important in this process. The encapsulation of SMCs and pericytes not only enhances the structural strength of the vessel but also regulates blood flow and vascular reactivity. Reconstruction of the ECM and modulation of biosignals ensures that the vessel is sufficiently resilient and functional to cope with the various changes in pressure in the body. In addition, the neovasculature needs to interface with the existing vascular network to form a stable circulatory network (11,12). In this process, cell-to-cell communication and microenvironmental regulation determine whether the blood vessel can be truly 'generated' and function effectively (11,12). There is also a process in angiogenesis called arteriogenesis. This is the enlargement of an artery's diameter and wall thickness. It occurs primarily in large arteries and small arterioles. This process requires the proliferation of SMCs and ECs. The most important stimulus involved in the process of arteriogenesis is the hemodynamic stimulus of shear stress (13). Acute changes in fluid shear stress induce ECs to express chemokines and CAMs, and chemokines trigger the recruitment of circulating monocytes that adhere to activated endothelium-expressing CAMs. Monocytes then migrate through the endothelium into the subendothelial space, where they transform into macrophages and produce inflammatory cytokines and growth factors that diffuse into the intima-vascular layer and ultimately mediate the transformation of the SMC phenotype by modulating SMC signaling pathways (Fig. 2A and B) (14).

Ubiquitination and tumor angiogenesis. There is increasing evidence that several diseases are dependent on angiogenesis, including atherosclerosis, pulmonary hypertension, inflammatory diseases, neurodegenerative diseases, aneurysms and cancer (15). Although these diseases have different etiologies and pathogenesis in several respects, they all have dysregulated angiogenesis in common. The most extensive research in the field of angiogenesis is currently focused on tumors. Angiogenesis is one of the hallmarks of cancer and angiogenesis begins during the early stages of tumor development. For tumors to grow, they must acquire angiogenic capabilities, which are usually achieved through hypoxia-induced expression of angiogenesis-inducing molecules. To support expanding tumor growth, the angiogenic switch is flipped, allowing the proliferation and germination of ECs that are normally quiescent (16). Angiogenesis is therefore an important component of tumor growth and metastasis. Once the angiogenic switch is activated, a series of responses are triggered, including activation of various proteases by activated ECs, leading to degradation of the basement membrane surrounding existing vessels, migration and proliferation of ECs, formation of vascular sprouting lumens, generation of new basement membranes by recruiting pericytes and finally generation of new fusion vessels. Under certain circumstances, when cells within the tissue respond to hypoxia, certain oncogenes together with other hypoxia-inducible genes induce the expression of VEGF, thereby inducing tumor angiogenesis (17,18). However, ubiquitination occurs throughout almost all steps of tumor angiogenesis. For instance, in the hypoxic microenvironment of tumor cells, hypoxia-inducible factor (HIF) induces angiogenesis to increase oxygen delivery by controlling the transcription of multiple genes (e.g. VEGF). Tumor cells produce angiogenic factors to stimulate the development of new blood vessels. Several transcription factors (such as HIF) and signaling pathways (such as Notch and Wnt) are involved in angiogenesis (19). Among them, VEGF and its associated receptors (VEGFR) are important players. Deubiquitinating enzymes regulate the stability of VEGFR, affecting EC responses and vascular physiology (20).

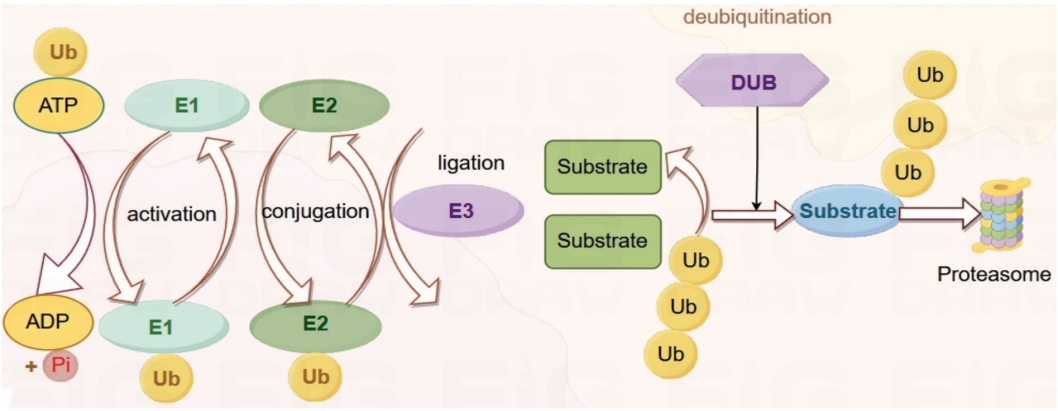

Figure 1. Ubiquitination modification process. The process of ubiquitination typically requires the synergistic action of three enzymes: E1 Ub-activating enzyme, E2 Ub-conjugating enzyme and E3 Ub ligase. First, the E1 Ub-activating enzyme activates the Ub molecule in the presence of ATP-supplied energy. Next, the activated Ub molecule is delivered to the E2 Ub-conjugating enzyme. Finally, the E3 Ub ligase attaches the Ub molecule to the target protein. Proteins to be degraded are first modified by ubiquitination and then degraded by the proteasome. Ubiquitination is a reversible process and specific DUBs remove ubiquitin from the target protein. DUB, deubiquitinating enzymes; Ub, ubiquitin.

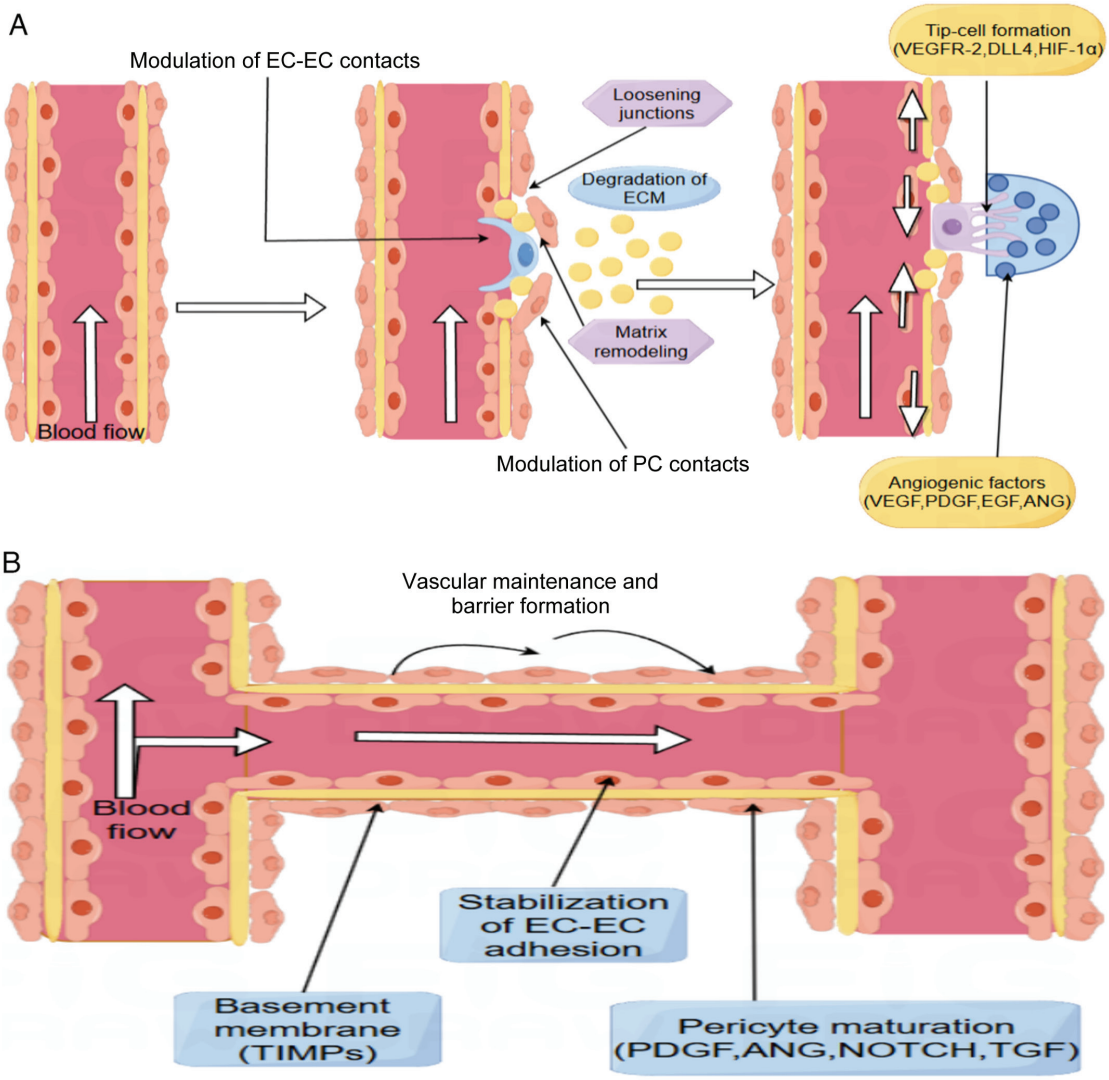

Figure 2. The process of sprouting angiogenesis. (A) Sprouting angiogenesis is the process by which new shoots form from existing vessels. The key biological process of neovascularization involves a balance between the formation of 'tip' and 'stalk' ECs. (B) Tip cells primarily play the role of directional navigation, guiding the direction of microvascular outgrowth. The tip cells maintain their phenotypic stability through VEGF and Notch signaling pathways in response to various factors in the extracellular environment. In addition, tip cells can specifically enhance glycolytic pathways to adapt to the low-oxygen environment during neovascularization, while non-tip cells can specifically use fatty acid metabolites to maintain their proliferative capacity, which drives the tip cells to extend forward. VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; EC, endothelial cell; ECM, extracellular matrix; PDGF, platelet-derived growth factor; ANG, angiopoietin; HIF, hypoxia-inducible factor; DLL4, delta-like 4; TGF, transforming growth factor; PC, pericyte; TIMP, tissue inhibitor of metalloproteinases.

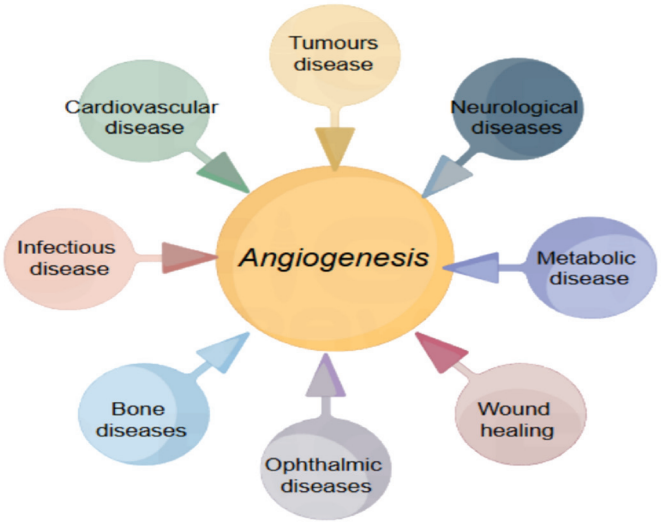

Figure 3. Angiogenesis is involved in human diseases. Angiogenesis is involved in the majority of diseases, including tumors, cardiovascular diseases, neurological diseases, metabolic diseases, infectious diseases, diseases of the bones, and physiological processes such as wound healing. Theoretically, targeted stimulation or inhibition of angiogenesis may provide novel therapeutic options for these diseases.

In addition, ubiquitination is involved in basement membrane degradation, EC migration and proliferation, vascular sprouting lumen formation, pericyte recruitment and other angiogenic processes in tumors and other diseases (discussed further below). Thus, angiogenesis is important in tumorigenesis and other human diseases, and tubulogenesis is considered a promising target for the treatment of related diseases. Thus, angiogenesis is considered a promising target for the management of related diseases (Fig. 3).

3. Ubiquitination and angiogenic factors

In angiogenesis, growth factors (GFs) enable blood vessel formation by promoting EC proliferation and differentiation, and recruitment of SMCs and fibroblasts (21). To date, several angiogenic GFs have been identified, the most important of which are VEGF, PDGF, basic FGF (bFGF/FGF2) and Ang. Ubiquitination regulates a wide range of cellular functions, including protein processing and transcriptional regulation, and these core pro-angiogenic proteins, including angiogenic signaling proteins, as well as other non-VEGF proteins, are an important basis for angiogenesis, and ubiquitination plays a central role in regulating the function of these signaling proteins and pathways.

Ubiquitination and VEGF. Circulating angiogenesis-stimulating factors are mediators of the angiogenic switch and VEGF is one of the most important. At least five ligands associated with VEGF have been identified: VEGF-A, -B, -C, -D and -E. VEGF-A is considered the most important in stimulating angiogenesis (22). The effects of VEGF are mediated by binding to three closely related receptor tyrosine kinases (RTKs): VEGFR-1, -2 and -3 (23). VEGFR-2 belongs to the family of RTKs, which play key roles in physiological and pathological angiogenesis (24). Structurally, the extracellular region of VEGFR-2 consists of seven N-glycosylation-modified immunoglobulin-like regions, and the intracellular region is affected by a variety of PTMs such as Tyr and Ser/Thr phosphorylation, Arg and Lys methylation, acetylation and ubiquitination (25). The Ub-proteasome system (UPS) is the major pathway for protein degradation in eukaryotic cells. This system controls a wide range of cellular regulatory proteins, including transcription factors, as well as cell cycle regulators, and has a major impact on the onset of angiogenic responses through signaling via the VEGFR-2 pathway (26). The UPS consists of two major components: Substrate-recruiting enzymes (E1, E2 and E3) and substrate-degrading enzymes. E1 activates the peptide Ub in an ATP-dependent manner, allowing it to be translocated to the Ub-carrying enzyme E2 (27). Inhibition of the proteasome significantly suppressed VEGFR-2 mRNA accumulation. In addition, treatment with a proteasome inhibitor significantly reduced the transcriptional activity of the VEGFR-2 promoter gene with a deletion at the 5' end. Proteasome inhibition-mediated repression was controlled by a GC-rich region containing a consensus Sp1 binding site. Proteasome inhibition reduces structural Sp1-dependent DNA binding (28). In addition, the presence of the PEST structural domain in the carboxylated structural domain of VEGFR-2 and the EST sequence, which is hypothesized to be an unstructured region in certain protein sequences, may act as a phospho-degrading agent that recruits the F-box-containing Ub E3 ligase, leading to ubiquitination and degradation, which may be the key role of this carboxylated structural domain in the degradation of VEGFR-2 (29). Of note, ubiquitination can also affect cell signaling by targeting activated proteins in a protein degradation-independent manner (30). VEGF-A, which can be secreted by several cell types, exerts its pro-angiogenic effects primarily through interaction with VEGFR-2 (31). The levels of VEGFR-2 in the plasma membrane significantly modulate VEGF-A-mediated signaling. Activation of the MAPK (32), phospholipase C (PLC)γ1 (33) and AKT (34) signaling pathways was also significantly increased by VEGF-A stimulation. E2 Ub-conjugating enzymes (UBE2D1 and UBE2D2) form a complex with VEGFR-2, downregulating VEGFR-2 expression in ECs (35). A net pool of cellular regulatory VEGFR-2 in the plasma membrane controls RTK-mediated signaling and cellular responses to extracellular VEGFR-2. This ligand-independent regulatory pathway mediates the availability of VEGFR-2 at the plasma membrane for VEGF-A-stimulated signaling functions. At the same time, the E1 enzyme UBA1 can regulate basement membrane VEGFR-2 levels and influence VEGF-A-stimulated activation of the PLC-ERK1 and γ/2 signaling pathways (36). Phosphorylation of RTKs such as VEGFR or VEGFR-2 is followed by ubiquitination and regulated intracellular trafficking. This endocytotic process depends on the interaction between the ubiquitinated receptor and carrier proteins with Ub-interacting motifs such as epsin, EGFR pathway substrate 15 and hepatocyte growth factor-regulated tyrosine kinase substrate (37). In addition, the Ub E3 ligase stem cell factor (SCF)-β-transducin repeat containing E3 ubiquitin protein ligase pseudogene 1 (β-TRCP) can promote ubiquitination and degradation of VEGFR-2 in a casein kinase I (CKI)-dependent manner. Knockdown of the β-TRCP gene or inhibition of CKI resulted in the accumulation of VEGFR-2, which led to an increase in VEGFR-2 downstream signaling. By contrast, β-TRCP-deficient ECs exhibited

enhanced angiogenesis *in vitro* (38). Hect E3 Ub ligases are a class of E3 Ub ligases characterized by the HECT structural domain (homologous to the C-terminus of E6AP), a conserved C-terminal catalytic structural domain through which ubiquitination of substrate proteins is achieved. NEDD4 E3 ubiquitin protein ligase (Nedd4) belongs to the E3 proteins containing the Hect domain and the NEDD4 family all contain three structural domains: A single C2 domain at the N-terminal end for membrane binding, 1-4 WW domains in the center for specific recognition of PY motif substrate proteins and the C-terminal HECT domain for Ub-protein attachment. This feature allows the transfer of Nedd4 Ub from E2 to the HECT domain of E3 and then to the substrate (39,40). Nedd4 expression induces intracellular degradation of VEGFR-2. While GF receptor-bound protein 10 (Grb10) is a positive regulator of the VEGF signaling pathway, Grb10 can stimulate vascular VEGFR-2 expression by inhibiting Nedd4-mediated degradation of VEGFR-2 (40). Thus, ubiquitination can target VEGFR-2 in a proteasome catabolism-dependent manner (the UPS body pathway) and indirectly regulate VEGF by modulating gene expression and/or spatial localization of VEGFR-2.

Ubiquitination and PDGF. PDGFs belong to the family of GFs and five isoforms have been identified to date; PDGF-AA, -AB, -BB, -CC and -DD. These isoforms play a critical role in stimulating cell growth and directing changes in cell shape and motility (21). Regarding angiogenesis, they are critical for pericyte recruitment and differentiation (21). In the tumor microenvironment, PDGF-BB is widely expressed in ECs and perivascular-like cells, and there is a strong association between the expression of angiogenesis-related genes, the infiltration of all vascular-associated cells including pericytes and the enrichment of angiogenesis-associated gene sets that play an important role in tumor angiogenesis (41). PDGF-BB binds to all PDGF receptors (PDGFR), leading to PDGFR autophosphorylation, pathway activation and internalization (42). PDGF-BB not only improves angiogenesis in bone marrow endothelial progenitor cells (EPCs), but also effectively promotes human umbilical vein EC (HUVEC) angiogenesis (43). The Cbl family is a group of E3 ligases that share the same structural domain. These molecules consist of an N-terminal tyrosine kinase binding domain, a linker region, a RING finger domain for recruitment of the E2 enzyme, a proline-rich unfolded region for binding to proteins containing the SH3 domain and a C-terminal Ub-associated domain (44). The RING finger domain has been implicated in the ubiquitination of RTKs, including PDGFRs, and stimulation of PDGFR promotes phosphorylation of Cbl proto-oncogene C (c-Cbl) and their interactions. In turn, overexpression of c-Cbl accelerates ligand-induced ubiquitination and subsequent degradation of PDGFR-A and PDGFR-B, and inhibits PDGF pro-proliferative and survival-dependent, negative regulation of PDGF-BB-induced chemotaxis (45,46). The PDGF isoforms also bind to α- and β-RTKs (PDGFRα and PDGFRβ, respectively) and promote receptor dimerization, which leads to receptor autophosphorylation and subsequent ubiquitination and internalization from the cell membrane (47). PDGFRα and PDGFRβ signaling play equally important roles in angiogenesis (48). Inhibition of PDGFRα phosphorylation inhibits angiogenesis in HUVECs (49), whereas overexpression of PDGFRβ not only promotes angiogenesis in HUVECs *in vitro*, but also regulates tumor angiogenesis (50). The Cbl family plays a key role in the Ub-mediated internalization of PDGFRβ and PDGFRα. Depletion of Cbl significantly reduces PDGFRβ ubiquitination (49) and overexpression of Cbl increases PDGFRα ubiquitination (51). Most tripartite motif (TRIM) family proteins are a type of E3 Ub ligase and TRIM proteins are characterized by their N-terminal TRIM-containing RING finger structural domain, one or two zinc-finger structural domains named box B (box B1 and box B2), and the associated coiled-coil regions. The multiple heterogeneous heterodimeric forms of TRIM proteins may contribute to the recognition of different substrates and play a role in enzyme regulation through molecular interactions or dominant negative effects in enzyme regulation (52). In addition, TRIM21 (an E3 ligase) also promotes PDGFRβ ubiquitination (53). Thus, the PDGF family members are primarily associated with pericyte recruitment during angiogenesis, and the degradation of PDGF requires ubiquitination to promote, so targeting the ubiquitination degradation process of PDGF may affect its role in angiogenesis.

Ubiquitination and FGFs. Among members of the FGF family, bFGF is the most well-studied and common pro-angiogenic factor. The expression of bFGF is increased at sites of chronic inflammation, after tissue injury and in several types of cancer (54). bFGF exerts its pro-angiogenic effects through the activation of FGFRs, including FGFR1, -2, -3 and -4 (55). It binds tightly to the ECM of the vascular basement membrane under physiological conditions and promotes bFGF production under certain conditions of the angiogenic process. Certain conditions stimulate the production of bFGF. For instance, during wound healing, bFGF is released from the ECM via heparan sulfate-mediated enzymatic degradation. Under hypoxic conditions, bFGF can exert a remodeling effect on the perivascular ECM via MMPs (16). bFGF induces angiogenesis through its effect on SMCs and ECs, and also induces chemotaxis and proliferation of fibroblasts and ECs (56). Conversely, bFGF can indirectly promote angiogenesis at wounds by increasing the expression levels of VEGF, MMP2 and MMP9 (57). Angiogenesis is associated with inflammation in a variety of physiological and pathological conditions, including cancer. bFGF is expressed by inflammatory cells, and inflammatory mediators activate the synthesis and release of bFGF from the vascular endothelium, stimulating angiogenesis by autocrine secretion (54). For instance, the long non-coding RNA taurine upregulated 1 is ubiquitylated following bFGF ubiquitination, downregulating bFGF expression, and this ultimately promotes osteogenic differentiation of tendon stem/progenitor cells (58). The UPS degradation pathway is important in controlling bFGF expression in angiogenesis in the tumor microenvironment. By promoting ubiquitination of bFGF, bFGF expression is downregulated, ultimately inhibiting angiogenesis (55,59). However, there are fewer studies on the role of ubiquitination in bFGF-induced angiogenesis and the exact mechanism remains to be fully elucidated (60). Studies have confirmed that bFGF plays an important role in angiogenesis under pathological and physiological conditions, and the study of the ubiquitination process of bFGF served as

a major breakthrough point for targeting bFGF to counteract angiogenesis (60,61).

Ubiquitination and Ang. Ang1-4 are important GFs whose activity is mediated by the RTKs tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1) and Tie2. The best-characterized members are Ang1 and Ang2 (62). Ang1 is a potent angiogenic factor that is essential for angiogenesis *in vivo* and functions differently from VEGF (63). Ang1, secreted by perivascular cells, has been identified as a major ligand for Tie2, and binding of Ang1 to Tie2 induces several downstream signaling pathways, primarily regulated by Akt, which induces a pro-angiogenic response (64). Activation of Ang1-Tie2 signaling promotes interactions between ECs and peri-EC supporting cells, which stabilizes the vasculature. In addition, Ang1 stimulates EC migration and promotes EC sprouting and tube formation, which are key events in angiogenesis (65). Ang2 is another key regulator of angiogenesis and its effect on vascular EC production is relevant to the local environment. Ang2 binds to Tie2 and acts as a negative regulator of Ang1-Tie2 signaling during angiogenesis, which in turn controls the response of ECs to exogenous cytokines. The binding of Ang2 to VEGF induces vascular sprouting, whereas in the absence of VEGF, Ang-2 induces EC apoptosis (66). It has been suggested that Ang2 binds to integrins in Tie2 EC and subsequently induces phosphorylation of the integrin adaptor protein focal adhesion kinase in a Tie2-independent manner, leading to angiogenesis (67). Furthermore, in cancer, the angiogenic process is significantly accelerated when the Ang2/Ang1 ratio is downregulated (68). During angiogenesis, Ang4 expression in ECs is induced by hypoxia, and Ang4 is required to maintain the metabolic balance necessary for EC proliferation and migration. Conditional Ang4 deficiency in vascular ECs *in vivo* reduces vascular EC proliferation, thereby reducing pathological angiogenesis (69). Therefore, studying the role of Ang regulation during angiogenesis is of particular importance. Prior to receptor-mediated endocytosis, RTKs are often modified by the addition of Ub. The addition of a single Ub molecule to RTKs is sufficient for receptor-targeted internalization and degradation (70). The E3 Ub ligases known to be involved in the ubiquitination of most RTKs are members of the Cbl protein family (71). Stimulation of Tie2-expressing cells with Ang1 resulted in their ubiquitination, suggesting that this may be a necessary signal for receptor conversion. c-Cbl interacts with the Tie2 signaling complex in a stimulus-dependent manner and this interaction is required for Tie2 ubiquitination, internalization and degradation (72). Attenuation of Tie2 trafficking by eliminating Tie2 ubiquitination may result in the maintenance of the signaling cascade for this receptor on the cell surface, increasing vascular stability and integrity, and potentially inhibiting angiogenesis (73). In addition, HECW2 is an EC Ub E3 ligase that plays a key role in stabilizing inter-EC junctions by regulating the stability of angiomotor protein, and depletion of HECW2 stimulates Yes-associated protein (YAP) translocation to the nucleus, thereby promoting EC outgrowth during angiogenesis by increasing ANG-2 expression (74). In summary, several GFs, including VEGF, PDGF, FGF and ANG, play key roles in angiogenesis *in vivo*. It is clear that ubiquitination plays an important role in regulating the signaling and trafficking of several GFs in angiogenesis, and that ubiquitination is an important pathway for their internalized degradation. The study of the ubiquitination process is important in the identification of novel targets of GFs in angiogenesis.

4. Ubiquitination and vascular cells

Vascular physiology relies on the synergistic movements of multiple cell types, including pericytes, ECs and vascular smooth muscle cells (VSMCs). This interaction between cell types is inherently dynamic (75). Pericytes are wall cells that support vascular development, remodeling and homeostasis, and are implicated in a variety of pathological conditions, including cancer (76). ECs are the primary cells responsible for the expansion of the new vasculature. During angiogenesis, ECs proliferate, migrate against the blood flow and differentiate into neovascular tips, capillaries and arterial ECs (77). In addition, during arteriogenesis, EC channels are covered by pericytes or VSMCs, providing stability and controlling perfusion (78).

Ubiquitination and pericytes. When resting vessels sense angiogenic signals (e.g. VEGF, VEGF-C, ANG-2, FGF or chemokines) released following hypoxia, inflammation or by tumor cells, pericytes first detach from the vessel wall and are released from the basement membrane by proteolytic degradation (78,79). The currently known effects of ubiquitination in pericytes are primarily focused on the nervous system. Brain pericytes surround the ECs of cerebral capillaries and form the neurovascular unit responsible for maintaining the integrity of the blood-brain barrier and controlling blood flow (80). When pericytes are exposed to Ub-proteasome inhibitors, severe cytotoxicity occurs due to the production of reactive oxygen species leading to apoptosis (81). Dynamic interactions between pericytes and ECs are the basis of angiogenesis. Pericytes are recruited to developing capillary buds by proliferation, migrate independently of ECs and can proliferate on growing capillaries (75). It has been shown that Japanese encephalitis virus-infected pericytes release biologically active molecules (such as IL-6) that activate the Ub-proteasome. While expression of the E3 Ub ligase Ubr1 is dependent on STAT activity induced by the IL-6/gp130 pathway, proteasomal degradation of Ubr1 zona occludens 1-expressing proteasomes is stimulated by IL-6 and leads to disruption of endothelial barrier integrity (82). NEDD8 is a Ub-like protein and MLN4924, a small-molecule inhibitor of NEDD8-activating enzymes that can be activated by inhibiting cullin RING E3 ligase, preserves chronic hypoperfusion-mediated loss of pericyte coverage in callosal capillaries (83). In addition, seven in absentia homolog 1 (Siah1) (an E3 ubiquitination ligase) may be involved in protein ubiquitination and protease-mediated degradation (84). As pericytes are perivascular cells closely associated with retinal capillaries, Siah1 association with GAPDH nuclear translocation may partially mediate pericyte death secondary to high glucose exposure (85). In addition, the effect of ubiquitination on pericytes has been demonstrated in the tumor microenvironment. TGF-β induces the transformation of quiescent pericytes into myofibroblasts, which promote tumor growth and metastasis, whereas the E3 Ub ligase-SMAD ubiquitination regulatory factor/recombinant mothers

against decapentaplegic homolog 7 (SMURF/SMAD7) complex inhibits tumor growth by antagonizing TGF-β signaling by promoting ubiquitination of the TGF-β receptor complex and inhibiting pericyte transformation (86). Furthermore, in non-small cell lung cancer, glioma-associated oncogene 1 (Gli1) can promote pericyte and EC motility required for angiogenesis by promoting bFGF expression, whereas inhibition of the Gli1-bFGF axis markedly reduces pericyte motility for tumor angiogenesis (55). This shows that the ubiquitination process has a wide range of effects on pericytes, but the effects on pericytes during angiogenesis need to be further investigated.

Ubiquitination and the endothelium. ECs play an important role in angiogenesis. First, tightly coordinated activation/inactivation of Rho GTPase signaling, a key molecule controlling actin dynamics, is essential for the stabilization, disruption and reconstitution of endothelial junctions (87). While localization of Rho GTPases is critical for their activation and downstream signaling, they are regulated by a variety of PTMs, including ubiquitination (88). Signaling by Rac family small GTPase 1 (rac1), a Rho GTPase, is critical for the regulation of EC diffusion, and there is a strong positive correlation between rac1 activity and its ubiquitination level (89). In studies of the mechanisms of EPC-mediated repair of damaged endothelium, Cbl was found to increase the ubiquitination of Janus tyrosine kinase 2 (JAK2) and decrease the expression of JAK2 and STAT4, which increases the expression of Runt-associated transcription factor 3 (Runx3) by regulating the level of histone H3 lysine 4 trimethylation. JAK2 and overexpression of STAT4 or Runx3 increased apoptosis of HUVECs and abrogated the effect of Cbl on endothelial function (90). In addition, ubiquitination modifications play an important role in response to oxidative stress in ECs, which are likely to be dominated by certain E3 ligase family members. The E3 Ub ligase Smurf2 regulates the stability of poly(ADP-ribose) polymerase 1, a highly conserved protein associated with vascular EC injury, to attenuate oxidative stress-induced HUVEC injury (91). In addition, E3 Ub protein ligase 2 (WWP2), which contains the WW structural domain, can regulate the ubiquitination and stability of specific substrate proteins in the following ways (92). WWP2 has been shown to regulate angiotensin II-induced oxidative stress in ECs by inducing ubiquitination and proteasome-mediated degradation of Septin 4 (93). In addition, WWP2 attenuates endothelial injury by targeting K63-linked polyubiquitination and proteasomal degradation (94). It was found that TRIM47E3 (a Ub ligase), a novel EC activator, can also induce inflammatory responses in ECs by enhancing K63-linked ubiquitination of tumor necrosis factor receptor-associated factor (TRAF)2, which in turn activates NF-κB and MAPK signaling pathways (95). In addition to studies showing the involvement of ubiquitination in oxidative stress (96) and inflammation (97), it also plays an important role in cellular senescence. It has been indicated that the E3 Ub ligase STIP1 homology and U-box containing protein 1 promotes ubiquitination of basic helix-loop-helix ARNT-like 1, thereby delaying cellular senescence (98). Inhibition of USP7 (a deubiquitinating enzyme) via inhibits glycosylation end product-induced cell cycle arrest and cellular senescence in HUVECs by promoting p53 ubiquitination (99). In addition, YAP/tafazzin, phospholipid-lysophospholipid transacylase (TAZ) regulates sprouting angiogenesis during development and tumor growth (100). Deletion of endothelial FAT atypical cadherin 1 (FAT1) interferes with the degradation of the Hippo signaling pathway effector protein YAP/TAZ, leading to increased YAP/TAZ protein expression levels and expression of canonical YAP/TAZ target genes, which, in turn, leads to increased EC proliferation. By contrast, the E3 Ub ligase mind bomb-(Mib)2, a FAT1-interacting protein, mediates FAT1-induced ubiquitination and degradation of YAP/TAZ, leading to this effect (101,102). A study of gallocatechin gallate (EGCG)-induced changes in the human EC proteome found a significant increase in Ub-proteasome activity in EGCG-treated cells. Interestingly, studies on angiogenic properties showed that EGCG primarily reduced EC proliferation, migration/invasion, endothelial tube formation, barrier function, transendothelial resistance and the expression of VEGF-2 (103).

Ubiquitination and VSMCs. In angiogenesis, the effects of ubiquitination modifications on VSMCs are primarily observed during arteriogenesis. During arteriogenesis, collateral vascular function requires the maintenance of a contractile state of VSMCs to regulate vascular tone and blood flow, while synthesized VSMCs grow and remodel in the middle layer of collateral branches to form more stable ductal arteries (14). Timely VSMC phenotypic switching requires the coordinated action of several molecules and cellular mediators, resulting in dilatory remodeling of the side branches to efficiently restore blood flow to downstream ischemic tissues (14). Not only does arteriogenesis require timely VSMC phenotypic switching, but maintenance of normal VSMC numbers and function is also critical. TRIM is the most abundant subfamily of the E3 Ub ligase family, and TRIM proteins are involved in EC injury, inflammation, angiogenesis, oxidative stress, and SMC proliferation and migration under conditions associated with vascular disease (104). TRIM65 and TRIM32 are more recently discovered E3 ligases that interact with and ubiquitinate a variety of substrates. Overexpression of TRIM65 and TRIM32 was found to activate PI3K/Akt signaling, leading to loss of the contractile phenotype of VSMCs, as well as cell proliferation and migration (105,106). In addition, high expression of TRIM59 (other TRAM family members) promotes the proliferation of pulmonary artery SMCs (PASMCs) in pulmonary hypertension (PH), and this effect can be enhanced by YAP1/TEAD4 (107). In addition, YAP expression was upregulated and activated in a rat model of MCT-induced PH, accompanied by PASMC proliferation. Siah2 Ub ligase inhibited excessive PASMC proliferation by promoting proteasomal degradation, which, in turn, upregulated YAP expression, reduced its phosphorylation and promoted its nuclear translocation (108). USPs cleave Ub chains from specific protein substrates, thereby inhibiting the degradation of the targeted substrate or modulating its subcellular localization (109). USP7 was found to upregulate MDM2, an E3 Ub ligase, to promote ubiquitination of forkhead box O4 and subsequently increase cyclin D1 expression to mediate PDGF-induced proliferation of PASMCs (110). In addition to the findings on PASMCs, the ubiquitination process affects atherosclerotic VSMCs. For instance, carboxyl terminus of the

Hsp70 interacting protein (CHIP) is a co-chaperone protein that interacts with the C-terminus of chaperone proteins such as heat shock proteins 70/90, and exerts E3 ligase activity on its substrate proteins. CHIP interacts with troponin, reduces troponin stability, inhibits troponin-dependent SMC differentiation and inhibits troponin-independent SMC differentiation by promoting Ub-mediated degradation of troponin in SMC differentiation (111,112). In addition, nuclear receptor 6 subfamily A group member 1 (NR6A1) is involved in promoting the apoptotic process in VSMCs, and the linear Ub chain assembly complex inhibits VSMC apoptosis by inhibiting its expression by promoting linear ubiquitination of NR6A1, leading to dephosphorylation of amino/threonine protein kinase 3 (113). In addition, by stimulating arterial SMCs, overexpression of TRIM37 reduced the expression of B-connexin, c-Myc and cell cycle protein cyclin D1. In addition, TRM37 inhibits arterial SMC proliferation and invasion by inhibiting the Wnt/β-catenin signaling pathway (114). TRAF6 maintains VSMC viability by stimulating cytoskeletal-associated smooth muscle protein Sm22α ubiquitination, possibly by increasing glucose-6-phosphate dehydrogenase activity and production (115). However, the above findings in different VSMCs are not sufficient to address the true regulatory role of ubiquitination on SMCs during angiogenesis. Therefore, the regulatory role of ubiquitination on VSMC function in angiogenesis remains to be validated by further studies.

5. Ubiquitination and the ECM

The vascular ECM consists of the vascular basement membrane and the interstitium, and is considered an essential component of the vasculature (116). Interactions between the cellular matrix and cells to control and influence angiogenesis and the balanced activity between specific angiogenic molecules that initiate the process and specific inhibitory molecules that block the process are hypothesized to be critical for an optimal angiogenic response. The ECM orchestrates complex signaling cascades within the cell and influences several fundamental aspects of its biology, including proliferation, migration, cytoskeletal organization, cell shape, survival and ultimately vascular stabilization (116,117). The ECM plays a critical role in the regulation of vascular processes, which are driven by a variety of positive and negative regulators. Degradation of the ECM results in the degradation or partial modification of matrix molecules, the release of soluble factors and the exposure of cryptic sites with pro- and/or anti-angiogenic activity. ECM molecules and fragments produced by proteolytic hydrolysis can also act directly as inflammatory stimuli, thus exacerbating angiogenesis (117,118). The MMPs are a group of metal (calcium and zinc) -dependent protein hydrolases that are capable of degrading the ECM. MMPs are secreted intracellularly and extracellularly as zymogen pro-MMP, which is activated by a series of protease cascades in order to become hydrolytically competent. MMP-2 has been implicated in angiogenesis; it proteolytically hydrolyses ECM components, causing EC budding, fragmentation and release of matrix-bound angiogenic factors, and interacts with integrin αvβ3 (119). JWA is a multifunctional microtubule-binding protein that is important in regulating tumor metastasis by inhibiting MMP-2. JWA was found to inhibit Sp1 activity through a Ub proteasome-dependent mechanism and downregulate the expression of pro-angiogenic MMP-2, providing new evidence for the inhibition of tumor angiogenesis in gastric cancer (120). In addition, inhibitor of growth family member 4 (ING4), a potential tumor suppressor, has also been implicated in angiogenesis. ING4 was found to inhibit the expression and transcriptional activity of Sp1 through Ub degradation and to downregulate the expression of the pro-angiogenic gene MMP-2 downstream of Sp1, potently inhibiting angiogenesis in colorectal cancer (121). TRIM25 can also inhibit angiogenesis in gastric cancer by promoting the ubiquitination of SP1 at K610, which further inhibits the expression of MMP-2 (122). In addition, anti-angiotensin small inhibitory RNA reduced endogenous angiotensin expression in HUVECs, which reduced MMP-2 activity and expression. Downregulation of angiotensin and its analogs may be achieved by inhibiting the degradation of the Ub-proteasome pathway (123). Although, to the best of our knowledge, to date, no study has directly demonstrated the involvement of ubiquitination processes in the effects on angiogenesis, it is clear from the available evidence that ubiquitination plays a key role in the regulation of the ECM in angiogenesis under both physiological and pathophysiological conditions, and this relationship needs to be confirmed by further studies.

6. Ubiquitination and CAMs

Angiogenesis is a multifactorial process initially influenced by the disassembly of endothelial junctions, then by the detachment, proliferation and migration of ECs, and finally by the re-establishment of cell-cell and cell-matrix contacts (124). Endothelial junctional CAMs, such as platelet EC adhesion molecule (PECAM) (125) and junctional adhesion molecule A (126), promote EC-cell contact through homophilic binding and are involved in the regulation of EC homeostasis and angiogenesis through various mechanisms. VCAM-1 plays a role in promoting angiogenesis during oxidative stress-induced neovascularization. In retinal neovascular disease, there are interactions between adhesion molecules (VCAM-1 and ICAM-1) and pro-angiogenic factors (e.g. VEGF and PDGF) that regulate angiogenesis (127). Interestingly, ubiquitination of several CAMs associated with angiogenesis has been identified. Membrane-associated ring-CH-type finger IX, an E3 ligase, controls the expression of ICAM-1, a key CAM (128). In addition, the E3 ligase TRIM65 selectively targets VCAM-1 and promotes its ubiquitination and degradation (129). PECAM-1 may mediate the response to vascular remodeling and collateral vessel formation during angiogenesis by sensing shear stress (130). The cell cyclosome (APC) can catalyze the ubiquitination of key molecules to regulate cell cycle progression. APC/CDh1 (chromodomain helicase DNA binding protein, one of the co-activators of APC) has been shown to promote K48-linked polyubiquitination of PECAM-1, which maintains EC function by degrading PECAM-1 under pulsatile shear stress (131). In addition, calcineurin 4, a major adhesion molecule in adherens junctions, can induce angiogenesis in papillary thyroid carcinoma by inhibiting the E3 Ub ligase β-TrCP-dependent ubiquitination of β-catenin, an important structural component of the calcineurin adherens junction (132). Thus, it is evident that different

types of CAMs affect the angiogenic process in a similar manner, primarily through their effects on the endothelium, and the role and mechanism of ubiquitination in this process need to be verified by further studies.

7. Ubiquitination and hypoxia signaling

Angiogenesis is a multi-step process that requires the involvement of several biological signals and stimuli under both physiological and pathological conditions. For example, vascular injury, occlusion and reduced blood flow can lead to a reduction in oxygen supply, a state known as hypoxia. Hypoxia is a potent angiogenic trigger that stimulates the activity of pro-angiogenic factors (133). HIF is an important transcription factor of the cellular response to changes in oxygen concentration. Under hypoxic conditions, HIF is activated, inducing the expression of numerous genes necessary for adaptation to hypoxia (134). HIF signaling mediates the response to hypoxia by cell-autonomous mechanisms, with non-autonomous effects on angiogenesis. The expression of HIF-stimulated pro-angiogenic factors (e.g. VEGF, VEGFR, MMPs and IL-8) is critical for angiogenesis in physiological and pathophysiological settings (135,136). In eukaryotic cells, HIF-1 is a major transcriptional mediator of the hypoxic response and a key regulator of oxygen homeostasis. Under normoxic conditions, hydroxylation of two proline residues and acetylation of one lysine residue on the oxygen-dependent degradation structural domain of HIF-1 triggers its binding to the von Hippel-Lindau (VHL) E3 ligase complex, leading to degradation of HIF-1 via the Ub-proteasome pathway. Under hypoxic conditions, the HIF-1 subunit is stabilized and interacts with coactivators to regulate target gene expression (134,137). Due to the rapid degradation of the HIF-1α subunit via the proteasome pathway, its stability is markedly reduced under normoxic conditions. In addition to hypoxia, ubiquitination can also regulate the stability and transcriptional activity of HIF-1α. In addition to the oxygen-dependent Ub E3 ligase VHL, which is involved in the oxygen-dependent regulation of HIF degradation, there are also E3 Ub ligases involved in the oxygen-dependent regulation of HIF-1α degradation, such as receptor for activated C kinase 1, hypoxia-associated factor, TRAF6 and CHIP (138). Specifically, ubiquitination modifications are involved in multiple processes that are affected by hypoxia during angiogenesis. For instance, the transcriptional co-activator YAP1 is an important oncogenic component of the Hippo signaling pathway that contributes to the development and progression of several tumors (139). Induction of tumor angiogenesis is primarily mediated by HIF, particularly HIF-1α (140). There may be a novel means by which YAP1 is regulated under normal hypoxic conditions; for example, it may interact with prolyl hydroxylase 2 (PHD2). This leads to the hydroxylation of specific proline residues, which in turn leads to its interaction with VHL and degradation by the proteasome. Under hypoxic conditions, the interaction of YAP1 with PHD2 and VHL is lost, and YAP1 interacts with HIF-1α and/or E2F1, inducing a variety of genes involved in the angiogenic process, including VEGF, as well as MMPs (141). In addition, leucine-rich repeat protein 16 in breast cancer (142), ETS transcription factor ELK3 in glioma (143), homeodomain interacting protein kinase 2 in hepatocellular carcinoma (144) and histone deacetylase 1 in colorectal cancer (145) can directly bind to HIF-1α and induce its ubiquitination and degradation, thereby controlling tumor angiogenesis. The inhibitory member of ASPP of p53 family can bind directly to the β-structural domain of VHL, which is also involved in the binding of HIF-1α, and thus block the binding of VHL and therefore the degradation of the HIF-1α protein in the normoxic state (146). In summary, the effect of HIF-1α on angiogenesis in tumor cells may be influenced by the presence of VEGF and regulated by the ubiquitination process. In addition to the above findings in tumor cells, in a model of retinopathy of prematurity, the circular RNA of phosphodiesterase 4B can inhibit VEGF-A expression and ultimately pathological angiogenesis by promoting VHL-mediated Ub degradation of HIF-1α (147). Taken together, these findings lead to the conclusion that HIF orchestrates the angiogenic process by participating in each step of angiogenesis and that ubiquitination modifications play an important role in this process. In addition, the interaction between HIF-1 and proangiogenic factors is fundamental in the angiogenic process under hypoxic conditions.

8. Ubiquitination and signaling molecules

The signaling molecules Wnt and Notch are important in angiogenesis. Wnt signaling can regulate angiogenesis through cell proliferation and migration, and Notch signaling can regulate the transcription of a series of genes involved in angiogenesis. Notch signaling also plays a crucial role in the recruitment and tight interaction of vascular ECs with pericytes and VSMCs. In addition, Wnt and Notch are the most important signaling pathways and downstream transcription factors determining EC arteriovenous differentiation, driving endothelial progenitor cell differentiation to arterial or venous EC through the coordinated action of different family members (148,149). More importantly, tip cell migration and stem cell proliferation require the involvement of Notch and Wnt signaling. Cells neighboring the tip cells occupy auxiliary positions as stem cells, which divide to lengthen the stem (stimulated by Notch, Wnt and other signals) and establish the lumen. Tip cells are equipped with filamentous pseudopods to sense environmental guidance cues, while stem cells convey spatial information about the position of their neighbors, thereby allowing stem elongation (150). Therefore, PTMs of receptors and ligands ensure precise signaling regulation. From receptor synthesis to maturation, ubiquitination plays a role in a series of processes to ensure the correct outcome of signal transduction (151).

Ubiquitination and Wnt signaling. Wnt signaling controls a wide range of cellular functions, including cell proliferation and migration. Wnt signaling can be divided into classical/β-catenin-dependent pathways and non-classical pathways (including Wnt signaling through Ca^{2+}, planar cell polarity and other signaling mechanisms that do not involve β-catenin), which both regulate and control angiogenesis (152,153). Wnt ligands trigger these pathways by binding to appropriate receptors. These signaling pathways are triggered by Wnt ligands by binding to appropriate receptors, which belong to the family of seven-pass transmembrane proteins called Frizzled. Activation of certain Wnt signaling pathways requires a co-receptor, LDL receptor-related protein (LRP)5/6, which is

blocked when LRP5/6 binds to the secreted inhibitory protein Dickkopf-1 (154). The binding of Wnt to these receptors has now been shown to play a role in angiogenesis in human ocular vascular disease (149). During tumor angiogenesis, overactivation of the Wnt/β-catenin signaling pathway consistently induces upregulation of pro-angiogenic factors (VEGF-A and VEGF-C) (155). In addition to the classical signaling pathways, non-classical signaling pathways also play an important role in angiogenesis. Exogenous Wnt family member 5A (Wnt5a) expression in vascular EC can promote angiogenesis by inducing the proliferation and survival of vascular ECs *in vitro*. The underlying mechanism is related to regulation of MMP-1 and Tie2 by the non-classical Wnt5a signaling pathway. In pathological angiogenesis, macrophage-derived Wnt5a can stimulate angiogenesis through inflammatory responses. Wnt5a released by infiltrating monocytes is particularly important in driving angiogenesis in inflammatory vessels (156). In addition to Wnt5a, Wnt1 and Wnt3a have been shown to control EC migration and proliferation (157). Secreted frizzled-related protein can activate the Wnt/Ca^{2+} pathway to stimulate angiogenesis in tumors (158). Various signaling factors have been shown to mediate the up- or downregulation of Wnt signaling through PTMs. Among the numerous PTMs involved, most Wnt signaling factors are regulated by ubiquitination and deubiquitination. Ubiquitination by E3 ligases attaches Ub to target proteins and usually induces proteasomal degradation of Wnt signaling factors such as β-catenin, Axin and glycogen synthase kinase (GSK)3 (159-162). In the Wnt/β-catenin signaling pathway, the Wnt ligand is the initiator, while β-catenin is the key mediator. In the absence of the Wnt ligand, cytoplasmic β-catenin is continuously phosphorylated by the destruction complex. Phosphorylated β-catenin is recognized by the E3 Ub ligase, β-TrCP, and then degraded by the proteasomal complex to reduce nuclear translocation of β-catenin (163). Axin is recognized by four proteasomal ligands, Siah-1, ring finger protein (RNF)146, Smurf1 and Smurf2, and four other E3 ligases. Ubiquitination of Siah-1 is achieved by binding to the VxP motif of Axin, which is a positive regulator of the Wnt signaling pathway. RNF146 induces degradation and facilitates K48-linked ubiquitination of Axin. Smurf1 ubiquitination occurs at residue K505 of Axin, leading to its degradation, while Smurf1 ubiquitination does not lead to Axin degradation, but interferes with the interaction between Axin and LRP6, acting as a negative regulator of the Wnt pathway (159,160). GSK3κ is a negative regulator of the Wnt/κ-c-catenin pathway and activates the Wnt/κ-c-catenin pathway by promoting the ubiquitinated degradation of GSK3κ, thereby stimulating angiogenesis in tumors (164). In the absence of Wnt ligands (Wnt-off phase), regulation of transcriptionally active nuclear catenin is essential to ensure controlled induction of Wnt target genes (165). In ECs, active catenin is ubiquitinated by c-Cbl, and Wnt activation promotes c-Cbl phosphorylation at tyrosine 731 (Y731), which increases c-Cbl dimerization and binding of Y731 to β-catenin. Y731 phosphorylation and dimerization mediates the nuclear translocation of c-Cbl and leads to the degradation of nuclear-active β-catenin in the Wnt-on phase. In the Wnt-off phase, termination of dimerization then disrupts the binding of 731 to β-catenin (166). The activity of c-Cbl also inhibits the pro-angiogenic Wnt targets IL-8 and VEGF levels and inhibits angiogenesis independently of β-catenin (167). In colorectal cancer, regulation of nuclear β-catenin by c-Cbl requires phosphorylation of c-Cbl at Tyr371 and the Y371H mutant interacts with nuclear β-catenin but does not ubiquitinate it. The nuclear localization of c-Cbl Y371H mutants contributes to their dominant negative effect on nuclear β-catenin (168). LRP5 is an essential coreceptor in the Wnt/β-catenin signaling pathway. sp90ab1 exerts these functions by interacting with LRP5 and inhibiting Ub-mediated degradation of LRP5 (169). In addition, YAP is essential for ubiquitination and proteasomal degradation of β-catenin. In the cytoplasm, YAP directly interacts with β-catenin and restricts its nuclear translocation. YAP is also critical for the recruitment of β-TrCP to the destruction complex, promoting the degradation of β-catenin (170). Given the potent angiogenic activity of Wnt signaling, components of the Wnt pathway have long been recognized as important targets in the control of angiogenesis. Further studies on the regulation of Wnt by ubiquitination may help to identify drug targets that are not limited to angiogenesis in tumor pathology.

Ubiquitination and Notch signaling. The Notch signaling pathway is a highly evolutionarily conserved signaling mechanism that controls cell fate specification, tissue morphology, cell proliferation, survival and differentiation in a wide range of cell types in all multicellular organisms (171). Notch ligands can be structurally classified into two groups: δ-like ligands (δ-like 1, 3 and 4) and serrate-like ligands (Jagged-1 and -2). Both classes of ligands are transmembrane proteins with numerous tandem EGF-like repeats in their extracellular structural domains and unique δ-, Serrate- and Jagged-2-binding structural domains at the amino terminus, which are required for receptor interaction (172). Classical Notch signaling requires the interaction of Notch ligands on the membrane of the cell sending the signal with Notch receptors on the cell receiving the signal, which induces Notch receptor protein hydrolysis (173). Notch signaling also plays an important and complex role in angiogenesis. It has been suggested that Notch signaling may regulate angiogenesis directly or indirectly through VEGFR (174). There appears to be a feedback loop between Notch signaling and VEGF, in which Notch signaling is downstream of VEGF, and when Notch signaling is activated, it downregulates the expression of VEGFR2. Increased δ-like 4/Notch signaling results in transcriptional repression of VEGFR2 and its co-receptor neuropilin 1 (175). δ-like 4 is expressed in ECs and is associated with tip and stem-cell differentiation during angiogenesis. δ-like 4 acts as a negative regulator to inhibit EC growth, proliferation and metastasis, and δ-like 4 upregulation inhibits excessive vessel sprouting and branching (176). In addition, the ligand Jagged 1, a potent pro-angiogenic regulator, antagonizes δ-like 4-mediated Notch signaling in angiogenesis (177). The effect of Notch on angiogenesis depends on the interplay between multiple angiogenic pathways. The Ang-1/Tie-2 pathway is an important angiogenic signaling pathway that enhances Notch signaling through Akt-mediated β-catenin activation. β-catenin activation enhances Notch signaling to regulate vasoreactivity (178). The magnitude and duration of the Notch response depend on the PTMs of the activated Notch receptor-Notch intracellular structural domain (NICD). Factor inhibiting HIF hydroxylates NICD1 under normoxic conditions, leading to USP10

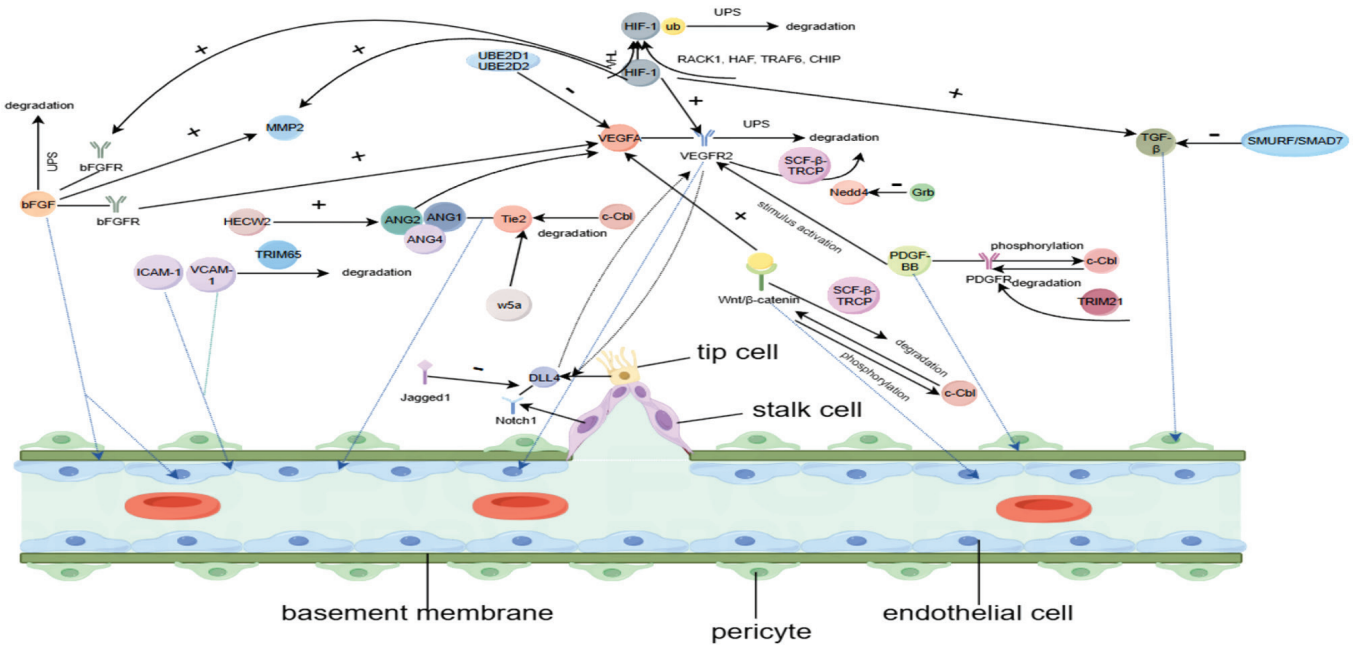

Figure 4. Ubiquitination during angiogenesis. During angiogenesis, imbalances between pro- and anti-angiogenic factors, increased hypoxic signals and stimulation of angiogenic signaling pathways have a central role, and the three enzymes of ubiquitination (E1-activating enzyme, E2-binding enzyme and E3 ligase) regulate the angiogenic process through various mechanisms. Angiogenesis begins with pro-angiogenic signaling in vascular ECs by growth factors such as VEGF, PDGF and EGF. Degradation of pro-angiogenic signals is inducible by ubiquitinating enzymes in the process. VEGF increases EC permeability and forms a temporary ECM skeleton. ECs migrate to the surface of the ECM. Proteases result in the release of angiogenic molecules stored in the ECM and remodel the ECM into a vasoactive environment. Cells neighboring the tip cells occupy auxiliary positions as stem cells, dividing to extend the stem and establish a lumen. A hypoxia-induced program driven by HIF-1α renders the ECs responsive to angiogenic signals, and the stability and transcriptional activity of HIF-1α is regulated by ubiquitination. By contrast, signals such as PDGF-B, Ang-1 and NOTCH induce the coverage of ECs by pericytes. As a consequence of the response to VEGF, activation of VEGFR-2 upregulates DLL4 expression in tip cells. VEGFR-2 is primarily degraded via the ubiquitin-proteasome pathway. Jagged1, another NOTCH ligand expressed by stem cells, promotes tip cell selection by interfering with reciprocal DLL4 and NOTCH signaling from stem cells to tip cells, and NOTCH and ligand formation can be ubiquitination-regulated. Ligand-activated Wnt signaling pathways are also activated in response to VEGF. Overactivation of Wnt-activated signaling pathways consistently induces upregulation of pro-angiogenic factors (VEGF-A and VEGF-C), which can be degraded by the proteasome complex. EC, endothelial cell; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PDGF, platelet-derived growth factor; EGF, epithelial growth factor; HIF-1α, hypoxia-inducible factor-1α; Ang, angiopoietin; DLL4, delta-like 4; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MMPs, matrix metalloproteinases; UPS, ubiquitin-proteasome system; bFGF, basic fibroblast growth factor; ANG, angiopoietin; TRIM, tripartite motif protein; UBE2D, ubiquitin-conjugating enzyme E2D; Tie2, tyrosine kinase receptor 2; Grb, GF receptor-bound protein; SMURF, SMAD ubiquitination regulatory factor; SMAD7, recombinant mothers against decapentaplegic homolog 7.

recruitment and subsequent deubiquitination and stabilization of NICD1. Under hypoxic conditions, this regulatory loop is disrupted, resulting in an attenuated Notch response (179). In addition, the cell fate determinant NUMB endocytic adaptor protein reduces Notch signaling by inhibiting the ubiquitination of the intracellular structural domain of Notch1. The E3 ligase Mdm2 can also bind to Notch1 through the RAM and ANK repeat regions of Notch1 and ubiquitinate Notch1 to promote its stability and signaling activity. It can also ubiquitinate NUMB, a negative regulator of Notch, for proteasomal degradation (151). The mature Notch receptor on the cell membrane is partly bound to ligands via the classical pathway and partly endocytosed, degraded in lysosomes or activated in nuclear endosomes (ligand-independent) to participate in non-classical pathways (180). By contrast, the E3 Ub ligases Deltex (Itch-associated protein) and suppressor of dx (mammalian Itch) ubiquitinate the intracellular structural domains of Notch, facilitating the process of endocytosis and thereby activating the non-classical pathway of Notch signaling (181). Typical δ/Serrate/LAG-2 (DSL) ligands are the most frequent activators accounting for the major inducer of Notch signaling. Two distinct families of RING-containing E3 ligases, Neuralised (Neur1 and -2) and Mib1 and -2 in mammals, directly promote mono-ubiquitination of DSL ligands and are also required for DSL ligand endocytosis (182,183). In addition, F-box and WD repeat domain containing 7 acts as a substrate recognition element for S-phase kinase-associated protein 1, cullin (CUL)1, protein F (SCF)-type E3 Ub ligases and is a potent positive regulator of angiogenesis, limiting Notch activity in the endothelium of growing vessels (184). BCL-6-associated zinc finger protein has been found to bind to the VEGF-A-induced Notch signaling factor CBF1 and promote degradation of CBF1 through polyubiquitination in the CBF1-cullin3 (CUL3) E3 ligase complex to downregulate Notch signaling (185). The mechanisms of Notch regulation in angiogenesis are complex and ubiquitination as a substrate recognition element is a potent positive regulator of Notch activity in the growing vascular endothelium. The regulatory mechanisms of Notch in angiogenesis are also complex, and ubiquitination acts at multiple levels of the Notch pathway and plays a critical role in maintaining Notch, its ligands, and the signaling activity. It is therefore particularly important to understand how ubiquitination leads to angiogenesis by affecting the Notch signaling pathway (Fig. 4, Table I).

Table I. Angiogenic factors or signals associated with ubiquitination during angiogenesis.

Angiogenic factors or signalling molecules	Main participating members	Main biological effects	Involved in ubiquitination enzymes or systems	(Refs.)
VEGF/R	VEGF-A, VEGFR-2	Promotes endothelial cell migration and proliferation, increases vascular permeability and alters the ECM	UPS, SCF-β-TrCP (E3 ligase), UBE2D1 and UBE2D2 (E2-binding enzymes), UBA1 (E1-activating enzyme), Nedd4 (E3 ligase)	(28-30,35-40)
PDGF/R	PDGF-B, PDGFRα, PDGFRβ	Promotion of angiogenesis-related gene expression, pericyte recruitment and differentiation	c-Cbl (E3 ligase), TRIM21 (E3 ligase)	(45-53)
FGF/receptor	bFGF, bFGFR	Promotion of endothelial cell migration, proliferation and differentiation, ECM degradation	UPS	(55,58,59)
Ang/receptor	Ang1, Ang2, Ang4, Tie1, Tie2	Promotes endothelial cell migration, differentiation, adhesion	c-Cbl (E3 ligase), HECW2 (E3 ligase)	(72-74)
MMPs	MMP-2	Promotes hydrolysis of ECM and endothelial cell sprouting	UPS	(122,123)
Cell adhesion molecule	PECAM, JAM-A, VCAM-1, ICAM-1, CDH4	Promotes endothelial cell adhesion and migration	K48-linked polyubiquitination, TRIM65 (E3 ligase), MARCH-IX (E3 ligase), SCF-β-TrCP (E3 ligase)	(128,129,131,132)
HIF	HIF-1	Stimulates expression of pro-angiogenic factors (e.g. VEGF, VEGFR, MMPs and IL-8)	VHL (E3 ligase), E3 Ub ligase (e.g. RACK1, HAF, TRAF6, CHIP), UPS	(134,137-139,141,146,147)
Wnt signalling pathway/ ligand	β-catenin, Axin, GSK3, Wnt5a, Wnt1, Wnt3a	Induces upregulation of pro-angiogenic factors (VEGF-A and VEGF-C), endothelial proliferation and migration	E3 ligases (Siah-1, RNF146, Smurf1 and Smurf2), SCF-β-TrCP (E3 ligase), c-Cbl (E3 ligase)	(159-164,166-168,170)
Notch signalling pathway/ ligand	Notch1, Delta-like1, Delta-like 4, Jagged1	Inhibition of endothelial cell migration, proliferation and differentiation, inhibition of tip cell and stem cell differentiation and vascular sprouting and downregulation of VEGFR2 expression	Mdm2 (E3 ligase), Deltex and Su(dx) (E3 ligases), Neuralized and Mind bomb (RING-containing E3 ligases), Fbxw7 (SCF-type E3 ubiquitin ligase), CBF1-cullin3 (E3 ligase complex)	(151,179,181-185)

VEGF, vascular endothelial growth factor; UPS, ubiquitin-proteasome system; PDGFR, platelet-derived growth factor receptor; bFGF, basic fibroblast growth factor; Ang, angiopoietin; MMP, matrix metalloproteinase; HIF, hypoxia-inducible factor; PECAM, platelet endothelial cell adhesion molecule-1; JAM-A, junctional adhesion molecule A; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular cell adhesion molecule-1; CDH4, cadherin 4; ECM, extracellular matrix; UBE2D, ubiquitin-conjugating enzyme E2D; TRIM, tripartite motif protein; SCF, stem cell factor; Tie2, tyrosine kinase receptor 2; MARCH-IX, membrane-associated ring-CH-type finger IX; CHIP, carboxyl terminus of the Hsp70 interacting protein; HAF, hypoxia-associated factor; VHL, von Hippel-Lindau.

9. Conclusions and future perspectives

Angiogenesis is an important physiological process involved in development and wound healing, as well as in pathological states, such as tumor growth, diabetes and ischemic heart disease. Studying the mechanisms and factors involved in this process is a major challenge, as angiogenesis is not limited to the imbalance between pro- and anti-angiogenic factors, but also involves the complex interplay of several signaling pathways that regulate these factors in different environments. As our understanding of cellular differentiation and tissue structure continues to expand, the study of *in vitro* models of angiogenesis has become more widespread. Through the use of *in vivo* and *in vitro* models, it has been established that several interrelated molecular pathways regulate angiogenesis, including angiogenic signaling, vascular cell stabilization and homeostasis, the cellular matrix and intercellular interactions. In addition, PTMs, including ubiquitination, tightly regulate these mechanisms and play a key role in controlling this process. Therefore, ubiquitination plays an important role in the regulation of angiogenesis, and understanding its mechanism is important in developing novel treatments for angiogenesis-related diseases. However, ubiquitination remains to be fully elucidated and its involvement in the regulation of angiogenesis remains largely uncharacterized. Unraveling the role of this important PTM and its contribution to angiogenesis has also become a major challenge. Further efforts are required to overcome this challenge to gain a more complete understanding.

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Competing interests

The authors declare that they have no competing interests.

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