The Ubiquitin-Proteasome System Meets Angiogenesis

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Abstract

A strict physiological balance between endogenous proangiogenic and antiangiogenic factors controls endothelial cell functions, such that endothelial cell growth is normally restrained. However, in pathologic angiogenesis, a shift occurs in the balance of regulators, favoring endothelial growth. Much of the control of angiogenic events is instigated through hypoxia-induced VEGF expression. The ubiquitin-proteasome system (UPS) plays a central role in fine-tuning the functions of core proangiogenic proteins, including VEGF, VEGFR-2, angiogenic signaling proteins (e.g., the PLCγ1 and PI3 kinase/AKT pathways), and other non-VEGF angiogenic pathways. The emerging mechanisms by which ubiquitin modification of angiogenic proteins control angiogenesis involve both proteolytic and nonproteolytic functions. Here, I review recent advances that link the UPS to regulation of angiogenesis and highlight the potential therapeutic value of the UPS in angiogenesis-associated diseases.

Introduction

Angiogenesis, the growth of new blood vessels from preexisting vessels, is an important physiological process in the body that is required for normal wound-healing and female reproduction. Pathologic angiogenesis (excessive or insufficient) is now recognized as a common denominator underlying a number of deadly and debilitating human diseases, including cancer, age-related macular degeneration (AMD), diabetic retinopathy, and cardiovascular diseases (1, 2). Although in many ways these diseases have distinct etiologies and mechanisms of development, they all share abnormal angiogenesis. For example, although cancer has nothing to do with AMD, it shares one of its characteristics, angiogenesis, with AMD (Fig. 1). Acquisition of angiogenesis by tumor cells is considered the most critical step in tumor growth and metastasis. To grow beyond 2 mm in diameter, a tumor must acquire angiogenesis, which is often established by hypoxia-induced expression of VEGF-A and other angiogenesis-inducing molecules (3). To support the growth of the expanding tumor, an angiogenic switch is turned on, causing normally quiescent endothelial cells to proliferate and sprout (4). It is now clear that induction of VEGF-A and other related angiogenesis inducers, and a reduction in the expression of angiogenesis inhibitors, such as thrombospondin (TSP-1), govern the tumor-induced angiogenic switch (5, 6). Although it was initially thought that angiogenesis plays a substantial role only when the tumor mass reaches a macroscopic size, it is becoming increasingly apparent that angiogenesis is instigated in the early stage of tumor development (5, 7), further supporting angiogenesis as a vital component of tumor growth and metastasis.

VEGF-A also plays a central role in the development of choroidal neovascularization, and indeed is responsible for both neovascularization and vascular leakage in wet AMD (8). The hallmarks of wet AMD are drusen formation [i.e., the focal deposition of debris between the retinal pigment epithelium (RPE) and Bruch’s membrane], choroidal neovascularization, RPE cell detachment, fibrovascular scarring, and vitreous hemorrhage. Aberrant blood vessel growth and blood vessel leakage subsequently result in the loss of central vision (9). Many cell types in the eye, including RPE cells, pericytes, endothelial cells, glial cells, Müller cells, and ganglion cells, synthesize and secrete VEGF. In addition to the vital importance of VEGF in the pathology of AMD, elevated VEGF levels also strongly correlate with retinal ischemia-associated neovascularization in diabetic retinopathy and retinopathy of prematurity (8, 10).

Once the angiogenic switch is activated, different sequential steps take place, including the activation of various proteases from activated endothelial cells resulting in the degradation of the basement membrane surrounding the existing vessel, migration of the endothelial cells into the interstitial space, endothelial cell proliferation, sprouting, lumen formation, generation of new basement membrane with the recruitment of pericytes, and fusion of the newly formed vessels (11). In general, and in most pathologic conditions, angiogenesis starts when cells within a tissue respond to hypoxia (i.e., low oxygen) or in certain circumstances when oncogenic gene products such as Ras and Myc induce expression of VEGF along with other hypoxia-inducible genes (12). The VEGF family of growth factors includes placental growth factor.
VEGF-A isoforms result from alternative splicing of messenger RNA (mRNA), and VEGF-165 is considered to be the most common VEGF-A isoform (14). Three different VEGF gene products (PIGF, VEGF-A, and VEGF-B) have been identified as ligands for VEGFR-1. VEGF-A, VEGF-D, and VEGF-C bind to and activate VEGFR-2 (13). VEGF-C and VEGF-D are also known to recognize VEGFR-3 (also called FLT4), a receptor tyrosine kinase (RTK) that is expressed predominantly by lymphatic endothelial and hematopoietic progenitor cells (15). VEGF-E is a virally encoded VEGF-like protein that selectively binds to VEGF-2 (16). VEGF family proteins also interact with non-RTK cell surface receptors, including neuropilin-1 and neuropilin-2, which are characterized as coreceptors for VEGF family ligands (13, 17). Among the VEGF receptors and coreceptors, activation of VEGFR-2 by VEGF family ligands is considered the most critical event in angiogenesis (18, 19). Beyond VEGF-mediated VEGFR-2 activation, recent studies have shown that under certain circumstances, VEGFR-2 can also be activated by non-VEGF family ligands, including heparan sulfate proteoglycans (20) and galectin-3, a glycan-binding protein (21). Heparan sulfate proteoglycans have been proposed to potentiate VEGFR-2 activation through cis- and trans-binding with VEGFR-2 and VEGF complex (20), where galectin-3 is thought to potentiate VEGFR-2 activation by prolonging its presence in the plasma membrane (21). Although VEGF family proteins are prominent regulators of angiogenesis, several other growth factors and cytokines, including angiopoietin-1, Del-1, fibroblast growth factor, hepatocyte growth factor, interleukin-8 (IL-8), and leptin, are known to stimulate angiogenesis (22), adding further complexity to the regulation of angiogenesis.

The role of VEGFR-2 in angiogenesis is well established, and more-comprehensive reviews on the role of VEGFR-2 in angiogenesis were recently published.
Figure 2 summarizes VEGF superfamily ligands and their interactions with VEGF receptors (Fig. 2). It is increasingly evident that angiogenic signaling is established through an elegant and complex system of VEGF and non-VEGF ligands, and VEGF receptors and coreceptors, resulting in homo- and heterodimeric activation of VEGF receptors and in turn the complex processes of angiogenesis.

Ubiquitin-Proteasome System

Ubiquitin is an evolutionarily conserved polypeptide that consists of 76 amino acids and was named for its ubiquitous expression in eukaryotes. Ubiquitin is activated by a ubiquitin-activating enzyme, E1, in an ATP-dependent manner and is transferred to a ubiquitin-conjugating enzyme, E2. Eventually, a ubiquitin-protein ligase, E3, specifically attaches the ubiquitin molecule to a target protein through the ε-amino group of a lysine residue (Fig. 3). E3 ubiquitin ligases are a large family of proteins (with almost 700 in the human genome) that are known to be involved in regulating the turnover and activity of many target proteins (23). E3 ligases are divided into 2 large groups: the homology to the E6-associated protein carboxyl terminus (HECT) domain-containing E3 ligases, and the Really Interesting New Gene (RING) domain-containing E3 ligases. The RING-type E3 ligases include single subunit E3 ligases (such as Cbl family E3 ligases) and multisubunit E3 ligases (such as Cullin-RING ubiquitin ligases). In recent years, additional E3 ligases have been identified that use different domains to recognize E2-conjugating enzymes, such as plant homeodomain domain-containing E3 ligases and the U-box E3 ligases (23, 24).

Although conjugation of ubiquitin to target proteins was initially recognized as a signal for protein degradation by the 26S proteasome, it is now recognized that ubiquitination regulates a broad range of cellular functions, including protein processing, membrane trafficking, and transcriptional regulation (23, 25). Recent
studies showed that ubiquitination can also influence cell signaling by targeting activation of proteins in a proteolysis-independent manner (26–28).

Multiple ubiquitin molecules can be attached to a target protein by means of monoubiquitination (i.e., attachment of a single ubiquitin to 1 or multiple lysine residues). Mono-ubiquitination is regarded as a signal for nonproteolytic events such as endocytosis, histone regulation, DNA repair, virus budding, and nuclear export (25). Alternatively, ubiquitin can be attached to a target protein in the form of polyubiquitination, where multiple ubiquitin molecules are attached to a single lysine residue. Ubiquitin contains 7 different lysine residues that potentially can be used for ubiquitin-chain assembly. Lys48- and Lys29-linked polyubiquitination generally is associated with degradation of target proteins by the 26S proteasome, whereas Lys63-linked polyubiquitination is involved in DNA repair, signal transduction, and endocytosis, but not degradation (23, 25). Clearly, the ubiquitin machinery has evolved to play a versatile role in protein functions ranging from protein turnover to subcellular localization and kinase activation, and consequently it is highly relevant to the pathobiology of many human diseases.

The ubiquitin-proteasome system (UPS) consists of 2 major components: substrate-recruiting enzymes (E1, E2, and E3) and substrate-degrading enzymes. E1 activates the polypeptide ubiquitin in an ATP-dependent manner, enabling its transfer onto the ubiquitin carrier enzyme, E2. Activated ubiquitin is then transferred by the ubiquitin protein ligase, E3, to a substrate protein (29). The substrate-recruiting components of UPS then catalyze the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of a substrate protein lysine residue. Continual addition of ubiquitin moieties onto substrate (i.e., polyubiquitination) facilitates recognition of the substrate by the proteolytic machinery of the UPS, the 26S proteasome (29, 30). The 26S proteasome complex is essentially composed of 1 20S and 2 19S units (Fig. 3). The 19S complex has 2 multisubunit components, often described as the base and the lid. The base contains 6 ATPases, which belong to the TRIPLE-A family of ATPases, and 2 non-ATPase subunits, which bind to the 20S catalytic core. The lid contains up to 10 non-ATPase subunits (31, 32). Together, the base and lid function in the recognition of ubiquitinated substrates and their subsequent binding. The 20S complex is composed of 28 related subunits (14 different subunits) that are arranged as 4 heptameric staggered rings. The 2 outer rings contain the α subunits (α1–α7). The 2 inner rings contain 2 copies of the β subunits (β1–β7). Within the 20S proteasome, subunits β1, β2, and β5 exhibit postglutamyl peptide hydrolyzing, trypsin-like, and chymotrypsin-like
Regulation of VEGF expression by von Hippel-Lindau E3 ubiquitin ligase

The molecular mechanism by which hypoxia sets off expression of VEGF has been extensively studied (12, 34). In normoxic conditions (i.e., normal oxygen levels), VEGF expression is generally inhibited by the interaction of von Hippel-Lindau (pVHL) E3 ubiquitin ligase with hypoxia-induced transcription factor 1α (HIF-1α), leading to its ubiquitination and targeting HIF-α for degradation by 26S-proteasome. Two of the 3 HIF-α isoforms, HIF-1α and HIF-2α, are closely related and can interact with hypoxia response elements to induce VEGF expression (35, 36), whereas HIF-3α appears to be involved in the negative regulation of hypoxia-induced gene expression (37).

Oxygen-mediated posttranslational modification of HIF-α through non-heme and iron-dependent oxygenases that uniquely hydroxylate specific HIF-α at proline residues regulates its transcriptional activity. Hydroxylation of human HIF-1α at 2 proline residues (Pro402 and Pro564) by prolyl hydroxylase domain (PHD) proteins creates binding sites for the pVHL E3 ubiquitin ligase complex that targets HIF-1α for proteasomal degradation (38, 39). These proline hydroxylation sites contain a conserved LXXLAP (where X indicates any amino acid) motif that is recognized by PHD proteins, leading to HIF-1α hydroxylation by PHD (38, 40). Of interest, hydroxylation of an asparagine residue (Asn803) in the C-terminal activation domain of HIF-1α by HIF asparagine hydroxylase, termed factor inhibiting HIF (FIH), inhibits HIF-1α activity by blocking interaction of the HIF-1α C-terminal activation domain with the transcriptional coactivator, p300 (41). Under hypoxic conditions, however, VEGF is generally overproduced, which leads to pathologic angiogenesis. In response to low oxygen, pVHL E3 ubiquitin ligase is S-nitrosylated (the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine), which blocks HIF-α interaction with pVHL E3 ubiquitin ligase. HIF-1α escapes from ubiquitin-mediated degradation as a consequence of S-nitrosylation of pVHL (Fig. 4). Nitric oxide–mediated S-nitrosylation of HIF-1α at the cysteine residue (C800) permits interaction of HIF-α with p300, prompting its transcription activity and VEGF expression (42).

Another important aspect and additional complexity of HIF-α regulation is the function of heat shock protein 90 (Hsp90). Hsp90 is often upregulated under cellular stress conditions, such as hypoxia (43), which appears to prevent HIF-1α degradation in a pVHL-independent manner (43–45). Consistent with the protective role of Hsp90 in HIF-1α, agents that inhibit Hsp90 activity have also been shown to promote ubiquitin-mediated degradation of HIF-1α (44, 46). A recent study indicated that inhibition of Hsp90 by hemin, a derivative of the protoporphyrin compound, increases HIF-1α ubiquitination and hence angiogenesis (47).

Figure 4. Role of pVHL in expression of VEGF and angiogenesis. A, in the presence of oxygen, proline residues in the oxygen-dependent degradation domain of HIF are hydroxylated. This allows HIF-α to interact with pVHL. The interaction between HIF and pVHL causes degradation of HIF through ubiquitination. B, in response to low oxygen (i.e., hypoxia), pVHL is S-nitrosylated, preventing HIF-α from interacting with pVHL, and the degradation of HIF-α is disallowed. HIF-α stimulates expression of VEGF, which in turn stimulates angiogenesis, as manifested in cancer progression. S-nitrosylation is the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine.
β-Transducin repeat-containing protein ubiquitin E3 ligase controls ubiquitination and degradation of VEGFR-2

Activation of VEGFR-2 by VEGF family ligands mediates most of the known VEGF cellular responses (13, 19, 48). Central to proper regulation of the angiogenic activity of VEGFR-2 is the process by which VEGFR-2 triggers its own internalization and degradation, consequently terminating its angiogenic signaling. Upon stimulation with VEGF family proteins, VEGFR-2 is removed from the cell membrane and undergoes clathrin-dependent endocytosis (49), which initiates its degradation (50) and recycling (51). Of interest, VEGFR-2 internalization is stabilized by cadherin-5 (52). Cadherin-5-dependent stabilization of VEGFR-2 is established by reducing the tyrosine phosphorylation of VEGFR-2, perhaps by recruiting tyrosine phosphatases to VEGFR-2 (49, 53). Ligand-mediated degradation of VEGFR-2 requires tyrosine kinase activity, and activation of the protein kinase C (PKC) pathway accelerates its degradation (50). On the other hand, activation of p38 mitogen-activated protein kinase (p38MAPK) has been shown to stabilize VEGFR-2 (52), suggesting that the stability and degradation of VEGFR-2 in endothelial cells are highly fine-tuned by the activities of the PKC and p38 MAPK pathways. Initial studies showed that the carboxyl terminal of VEGFR-2 plays a pivotal role in VEGFR-2 stability and degradation. Progressive deletion of the carboxyl terminal of VEGFR-2 was shown to inhibit ligand-dependent degradation of VEGFR-2 (48, 50). A recent study identified the presence of a PEST domain in the carboxyl domain of VEGFR-2, which may account for the critical role of the carboxy terminal in VEGFR-2 degradation (52). The PEST motif [rich in proline (P), glutamic acid (E), serine (S), and threonine (T)] is considered to be a signature of short-lived proteins that are degraded by the ubiquitin pathway (54). It is thought that PEST sequences are unstructured regions in certain protein sequences, which may serve as a phoshodegron for the recruitment of F-box–containing ubiquitin E3 ligases leading to ubiquitination and degradation (55, 56). Phosphorylation of Ser1188 and Ser1191 of VEGFR-2, leading to SCFβ-TrCP1–dependent ubiquitination and degradation of VEGFR-2. Degradation of VEGFR-2 is mainly attained through Lys48-linked polyubiquitination (52).

β-TrCPs (also called FWD1) belong to a larger family of Fbw (F-box/WD40 repeat containing) proteins that are generally characterized by the presence of a 42–48 amino-acid F-box motif at the N-terminus and 7 WD40 repeats at the C-terminus (57). β-TrCPs are highly conserved across species, in particular within the F-box motif and WD40 repeats motif. Human β-TrCP1 and β-TrCP2 exist in multiple isoforms due to alternatively spliced mRNA, but all are conserved in F-box and all 7 WD40 repeats (58, 59). The most notable differences in sequences between β-TrCP1 and β-TrCP2 are found in their N-terminal regions, which are proximal to the F-box motif. It is thought that the N-terminal sequences of β-TrCP1 and β-TrCP2 allow these proteins to undergo homo- and heterodimerization (60). It appears that both β-TrCP1 and β-TrCP2 can promote ubiquitination of VEGFR-2, which suggests that to some extent they may act in a redundant fashion in VEGFR-2 ubiquitination (52). A redundant role of mammalian β-TrCP1 and β-TrCP2 in ubiquitination and degradation of other proteins, including IκB and β-catenin, has also been suggested (61, 62).

Role of ubiquitination in phospholipase Cγ1 activation and angiogenesis

Activation of phospholipase Cγ1 (PLCγ1) in endothelial cells is considered to be one of the chief mediators of the angiogenic signaling of VEGFR-2. It catalyzes the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol from phosphatidylinositol 4,5-bisphosphate (PIP2). Phosphorylation of PLCγ1 on mouse VEGFR-2 (corresponding to Tyr1175 on human VEGFR-2) has been identified as the primary site responsible for the recruitment of PLCγ1 to VEGFR-2 (26, 63, 64). Substantial information obtained from animal models links PLCγ1 to angiogenesis. The initial evidence linking PLCγ1 to endothelial cell function and angiogenesis was provided by targeted deletion of PLCγ1, which resulted in early embryonic lethality between embryonic days 9.5 and 10.5 due to significantly impaired vasculogenesis and erythropoiesis (65). Inactivation of PLCγ1 in zebrafish was also shown to be required for VEGF function and arterial development (66). Additional evidence of the importance of PLCγ1 in angiogenic signaling of VEGFR-2 was obtained by pharmacological inhibition of PLCγ1. U73122, a potent PLCγ1 inhibitor, was shown to inhibit endothelial cell tube formation in vitro (64) and angiogenesis in vivo in a chorioallantoic membrane assay (67). Silencing the expression of PLCγ1 in primary endothelial cells by an siRNA strategy also inhibits VEGF-mediated endothelial cell tube formation and proliferation (68), further underscoring the importance of the PLCγ1 pathway for angiogenic signaling of VEGF.

PLCγ1 is a multidomain protein that consists of 2 SH2 domains and 1 SH3 domain between the catalytic domains. The SH2 domains recognize phosphorylated Tyr1173 on VEGFR-2 (64), whereas the SH3 domain recognizes proline-rich sequences (PXXP motifs). In addition to its SH2 domains, PLCγ1 also contains a C2 domain, EF hand, and 2 putative PH domains. The presence of both N- and C-terminal SH2 domains is required for optimal binding of PLCγ1 to VEGFR-2 (64). PLCγ1 also interacts with c-Cbl through its proline-rich motif in a noninducible manner (50). As a result of activation by VEGFR-2, c-Cbl is recruited to VEGFR-2 and distinctly inhibits phosphorylation of Tyr783 on PLCγ1 in an ubiquitination-dependent manner (26, 68). c-Cbl negatively regulates PLCγ1 activation in a proteolysis-independent manner. Instead of targeting it for degradation, c-Cbl distinctively mediates ubiquitination of PLCγ1 and suppresses its
phosphorylation on Y783 (26). How PLCγ1 can escape from ubiquitin-mediated degradation but then enter into a less enzymatic active state is a conundrum that warrants further investigation.

Endothelial cells derived from c-Cbl knockout mice also showed that loss of c-Cbl results in an increased phosphorylation of PLCγ1 with no apparent effect on its half-life (68). Overexpression of c-Cbl in endothelial cells has also been shown to inhibit tube formation and sprouting of endothelial cells. Conversely, overexpression of c-Cbl (70Z/3-Cbl), an E3 ligase-deficient variant form of c-Cbl, or silencing its expression by siRNA elevated sprouting of endothelial cells (26). Recent studies showed that VEGF- and tumor-induced angiogenesis is highly elevated in c-Cbl nullizygous mouse (67, 68). It appears that the role of c-Cbl in angiogenesis is widespread, because laser-induced angiogenesis in c-Cbl knockout mice also results in enhanced retinal neovascularization (67).

Role of ubiquitination in the PI3 kinase/AKT pathway
The phosphoinositide 3-kinase (PI3K) signal transduction pathway is one of the main signaling routes that VEGFR-2 uses to stimulate endothelial cell survival and proliferation (69–71). VEGFR-2 activates PI3K through recruitment of p85 of PI3K involving Tyr799 and Tyr1173 (67, 68). PI3K consists of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. It is a lipid kinase that converts the plasma membrane lipid PIP2 to phosphatidylinositol-3,4,5-triphosphate (PIP3). Proteins with pleckstrin-homology (PH) domains, such as protein kinase B (PKB/AKT), phosphoinositide-dependent kinase-1 (PDK-1), and PDK-2, bind to PIP3. AKT is activated by PIP3, PDK1, and PDK2, leading to phosphorylation of a host of other proteins that affect cell proliferation, cell cycle progression, and cell survival (73). The Cbl-b ubiquitin E3 ligase is known to interact with the p85-SH2 domain and catalyze p85 polyubiquitination (27,74). Of interest, the Cbl-mediated ubiquitination does not lead to degradation of p85 (27).

AKT, a serine/threonine protein kinase, is one of the key PI3K substrates that play a central role in mediating VEGFR-2-dependent cellular events in endothelial (75, 76). It was recently shown that the carboxyl terminus of Hsc-70-interacting protein (CHIP) interacts with AKT and induces its ubiquitination (77). In addition, tetratricopeptide repeat domain 3 (TTC3) containing E3 ligase was recently linked to AKT ubiquitination and degradation (78, 79). Of interest, TTC3 interacts only with active AKT, and not inactive AKT, in the nucleus (78, 79), indicating that perhaps TTC3-mediated AKT ubiquitination is important for controlling AKT signaling in the nucleus. TTC3 itself is the target of AKT and is phosphorylated at S378 by AKT, and this phosphorylation appears to be necessary for TTC3 E3 ligase activity (78, 79). BRCA1 is another E3 ligase that also interacts with activated AKT and targets it for ubiquitination and degradation (80). Recent studies also showed that AKT is ubiquitinated by TRAF6 E3 ligase. TRAF6 directly interacts with and induces AKT ubiquitination (28). TRAF6-mediated AKT ubiquitination takes place through the K63-linked modification and does not trigger AKT degradation. K63 chain polyubiquitination of AKT contributes to its membrane localization, where it is phosphorylated (28). Figure 5 summarizes various ubiquitin E3 ligases that are involved in fine-tuning the abundance and activation of key angiogenic proteins. Some ubiquitin E3 ligases, such as Cbl family proteins, target multiple angiogenic proteins,

![Figure 5](https://mct.aacrjournals.org/content/11/3/544/F5.large.jpg)

Figure 5. Regulation of core angiogenic proteins by ubiquitin E3 ligases. Expression of VEGF is regulated by activity of pVHL. βTrcp ubiquitates VEGFR-2 and subjects it to proteasomal degradation. c-Cbl catalyzes ubiquitination of numerous angiogenic signaling proteins, including VEGFR-1, Eph, Tie2, and PLCγ1. AKT abundance and activity are regulated by TRAF6, CHIP, and TTC3. Itch regulates degradation of the Notch1 receptor.
whereas in other cases more than 1 ubiquitin E3 ligase is involved in the ubiquitination of an angiogenic protein, as illustrated for AKT (Fig. 5).

Role of Ubiquitination in Wnt Signaling

The Wnt pathway is another key player in angiogenesis. Binding of Wnt to its 7-span transmembrane receptor, Frizzled (Fz), and its coreceptor, Lrp5/6, at the cell surface initiates a signaling cascade that mediates angiogenesis and other key developmental processes, including stem cell maintenance, growth, and cell-fate specification, and cell migration (81). Deregulation of the activation of Wnt/β-catenin signaling has been linked to a range of human diseases, including cancer (81, 82). During the resting state of canonical Wnt signaling, several key Wnt-associated signaling proteins, including β-catenin, are targeted via ubiquitination for degradation. Initially, the adenomatous polyposis coli protein forms a complex with glycogen synthase kinase 3β (GSK-3β) and axin. This complex then binds to β-catenin in the cytoplasm, which leads to phosphorylation of β-catenin by casein kinase 1 (CK1) and GSK-3β. Phosphorylation leads to the creation of a phosphodegron motif on β-catenin that allows the ubiquitin E3 ligases (e.g., β-Trcp) and Jade-1 to recognize β-catenin. As a result, β-catenin is targeted for ubiquitination, leading to its 26S-proteasome–mediated degradation (83, 84).

In addition, other ubiquitin E3 ligases (e.g., Siah1 and Ozr) also target β-catenin for degradation in a cell-type– or context-specific manner (85, 86).

Removal of cytosolic β-catenin through the UPS prevents β-catenin from translocating into the nucleus, where it acts as a transcription factor for genes associated with various angiogenic events, such as proliferation of endothelial cells. In contrast, activation of the canonical Wnt signaling pathway results in inhibition of β-catenin degradation, leading to increased cytosolic β-catenin, which then translocates to the nucleus. In the nucleus, β-catenin associates with at least one of a family of Tcf/Lef transcription factors and induces the expression of numerous genes, such as cyclinD1 and c-myc (87), which are implicated in cellular proliferation.

In addition to Wnt–mediated ubiquitination of β-catenin, the stability of the β-catenin protein is regulated by ubiquitination of cadherins. For example, the c-Cbl–related ubiquitin E3 ligase Hakai associates with E-cadherin, promoting its degradation and resulting in destruction of the cadherin–β-catenin complex and its degradation (88).

Of interest, in addition to regulation of β-catenin protein levels by ubiquitination, the levels and subcellular functions of Dvl are tightly regulated via multiple ubiquitin-dependent pathways.

Binding of the Kelch-like 12 (KLHL12) E3 ligase to Dvl is regulated by Wnt stimulation. Subsequent ubiquitination of Dvl leads to its proteasomal degradation (89), suggesting that KLHL12 acts as a Wnt-mediated negative regulator of the Wnt pathway by inducing the degradation of Dvl. Surprisingly, in neuronal cells, Dvl uniquely is ubiquitinated by the HECT-type E3 ligase NEDL1, and not by KLHL12 (90), suggesting a cell-type–specific regulation of Dvl by the UPS.

The ubiquitination system as a potential target for antiangiogenesis and anticancer therapy

Given that the expression and degradation of core proangiogenic proteins are regulated by the UPS, selectively targeting the different components of this pathway may prove to be an effective strategy for antiangiogenesis treatments. Our increasing understanding of the ubiquitination system and its role in angiogenesis is generating great interest in the development of novel strategies to block pathologic angiogenesis. The role of the ubiquitination pathway in human diseases in general, and in angiogenesis in particular, is still unclear because the molecular mechanisms and gene products involved in the UPS are not fully understood. Moreover, there has been no comprehensive analysis of the functional importance of the UPS in angiogenesis-associated human diseases. The UPS has many different components that potentially could be targeted for inhibition or stimulation in the milieu of angiogenesis. For example, the therapeutic value of the proteasome inhibitor bortezomib (Velcade; Millennium Inc.), the first UPS-targeting drug to be approved by the U.S. Food and Drug Administration for treatment of relapsed or refractory multiple myeloma (91), could be explored in angiogenesis-associated diseases.

Recent studies have linked the potential therapeutic value of inhibition of the proteasome pathway to angiogenesis. Indeed, treatment of endothelial cells in a cell culture system with proteasome inhibitors was shown to inhibit capillary tube formation of endothelial cells and blood vessel formation in an embryonic chick choroidallantoic membrane assay (92, 93). Moreover, bortezomib was shown to inhibit tumor angiogenesis in a murine xenograft model (94). More recently, it was shown that small molecules such as nutlins (Roche, Inc.) and RITA can block p53 ubiquitination by inhibiting the activity of MDM2 ubiquitin ligase, which is known to mediate p53 ubiquitination (95–97). Loss of p53 activity is linked to both tumor growth and angiogenesis, suggesting that, in principle, the application of nutlins in cancer treatment could target both tumor cells and angiogenesis. PR-171 (Proteolix, Inc.), a synthetic analog of epoxomicin, is another proteasome inhibitor that was reported to irreversibly inhibit the chymotryptic site of the 26S proteasome, and initial studies suggested that it has more potent anticancer activity than bortezomib (98). Moreover, recent patent applications indicate that the ubiquitin activating enzyme, E1, could also be targeted for possible therapeutic use (99, 100). More-comprehensive reviews on inhibition of the 26S proteasome and drug discoveries were recently published (101, 102). Numerous ubiquitin E3 ligases are involved in the regulation of angiogenesis; however, it remains to be determined whether a particular ubiquitin E3 ligase can be exploited as a target for...
molecular therapeutic approaches in angiogenesis-associated diseases. Regardless of whether a particular ubiquitin E3 ligase can be targeted for therapeutic approaches in angiogenesis-associated diseases, broad studies are clearly required to increase our understanding of their role in angiogenesis. In light of the current drug-discovery activities involving the UPS, it is evident that by harnessing the UPS we may be able to design strategies for different components of the UPS to selectively target proteins for ubiquitination/degradation or inhibit protein degradation. Hence, it is not unreasonable to expect more drug-discovery efforts based on ubiquitin with the aim of targeting proteins with pro- and antiangiogenesis activities in the near future.

Conclusions and Perspectives

The emerging role of the UPS in regulating angiogenesis highlights the importance of investigating this pathway in the milieu of angiogenesis. The reports outlined in this review provide examples of regulation of angiogenesis by various components of the UPS; however, these studies represent only the beginning of our attempt to understand how this important pathway functions in the regulation of angiogenesis. Characterizing the nature of this system in angiogenesis and the complexity of the UPS will undoubtedly have many therapeutic applications. Understanding the molecular basis of the UPS and the target protein substrates in endothelial cells may also provide a foundation for learning to stimulate or inhibit angiogenesis. Finally, it is reasonable to envision the UPS as a key component of the angiogenic switch in cancer and other types of pathologic angiogenesis.

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## The Ubiquitin-Proteasome System Meets Angiogenesis

Nader Rahimi


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