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Review

Novel endogenous angiogenesis inhibitors and their therapeutic potential

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Angiogenesis, the formation of new blood vessels from the pre-existing vasculature is essential for embryonic development and tissue homeostasis. It also plays critical roles in diseases such as cancer and retinopathy. A delicate balance between pro- and anti-angiogenic factors ensures normal physiological homeostasis. Endogenous angiogenesis inhibitors are proteins or protein fragments that are formed in the body and have the ability to limit angiogenesis. Many endogenous angiogenesis inhibitors have been discovered, and the list continues to grow. Endogenous protein/peptide inhibitors are relatively less toxic, better tolerated and have a lower risk of drug resistance, which makes them attractive as drug candidates. In this review, we highlight ten novel endogenous protein angiogenesis inhibitors discovered within the last five years, including ISM1, FKBPL, CHIP, ARHGAP18, MMRN2, SOCS3, TAp73, ZNF24, GPR56 and JWA. Although some of these proteins have been well characterized for other biological functions, we focus on their new and specific roles in angiogenesis inhibition and discuss their potential for therapeutic application.

Keywords: anti-angiogenesis; endogenous angiogenesis inhibitors; ISM1; FKBPL; CHIP; ARHGAP18; MMRN2; SOCS3; TAp73; ZNF24; GPR56; JWA; cancer

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Introduction

Angiogenesis is the formation of new blood vessels from the existing vasculature. It is a tightly regulated process that is essential for embryonic development and several adult physiological processes such as wound healing and reproduction[1, 2]. Angiogenic regulation is achieved by a fine balance between stimulators and inhibitors that act together to maintain physiological homeostasis[3]. Pathological angiogenesis is a consequence of a disruption in this fine balance, resulting in diseases such as cancer, rheumatoid arthritis, and heart disease.

Angiogenesis is an important hallmark of cancer[4, 5]. The angiogenic switch, a process that signifies tumor development from an avascular stage to a vascularized stage through the initiation of angiogenesis, is essential for tumors to grow beyond 1-2 mm^3. Hence, angiogenesis has been actively explored as a drug target for cancer therapy. Over the years, a large number of angiogenesis inhibitors have been discovered and developed, ranging from endogenous angiogenesis inhibitors, such as proteins, protein fragments and microRNAs, to monoclonal antibodies and small molecule drugs.

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Many proteins have been identified as endogenous angiogenesis inhibitors including thrombospondins 1 and 2[6, 7], vasohibin[8], chondromodulin[9], pigment epithelial derived factor[10], platelet factor 4[11], and several members of the interleukin and interferon families. In addition, several proteins harbor or generate protein fragments that are anti-angiogenic including endostatin (fragment of collagen XVIII)[12], angiostatin (fragment of plasminogen)[13], tumstatin (fragment of collagen IV)[14], *etc*. Furthermore, a growing number of microRNAs have been identified as a new class of endogenous angiogenesis inhibitors[15].

Endogenous angiogenesis inhibitors have continuously been discovered in recent years. The identification of novel endogenous angiogenesis inhibitors and the elucidation of their biological functions are essential for our understanding of angiogenesis homeostasis in physiology and their dysregulation in pathology. This new knowledge will help us design new and better drugs for angiogenesis-related diseases such as cancer.

In this review, we discuss ten endogenous anti-angiogenic proteins discovered within the last five years. These anti-angiogenic proteins are representative but not exhaustive. The list includes isthmin1 (ISM1), FK506-binding protein-like (FKBPL), carboxy-terminus of Hsc70 interacting protein (CHIP), Rho GTPase activating protein 18 (ARHGAP18), multimerin-2 (MMRN2), suppressor of cytokine signaling-3

(SOCS-3), tumor protein 73-alpha (TAp73), zinc finger protein 24 (ZNF24), G-protein coupled receptor 56 (GPR56) and JWA. All of these endogenous proteins have proven anti-angiogenic functions, particularly in inhibiting pathological angiogenesis such as in cancer and retinopathy. Hence, these proteins have the potential to be developed into anti-angiogenic drugs for cancer or other diseases that involve excessive angiogenesis.

Isthmin 1 (ISM1)

Ism1 was first identified as a gene that is highly expressed in the isthmus organizer in the *Xenopus* midbrain-hindbrain boundary during embryonic development[16]. This secreted protein has an N-terminus signal peptide, a centrally located thrombospondin type-1 repeat domain (TSR) and a C-terminal adhesion-associated domain in Mucin 4 (MUC4) and other proteins (AMOP) domain. Although this gene is present in all major vertebrates, its biological function was completely unknown until recently.

We identified ISM1 to be a novel endogenous angiogenesis inhibitor[17]. Recombinant ISM1 (rISM) inhibited endothelial cell (EC) capillary network formation on Matrigel largely through its C-terminal AMOP domain. *In vivo*, ISM1 potently inhibited vascular endothelial growth factor (VEGF)/basic fibroblast growth factor (bFGF)-induced angiogenesis in an implanted Matrigel plug. ISM1 inhibited VEGF, bFGF and serum-induced EC proliferation without affecting EC migration. Significantly, ISM1 also induced EC apoptosis in the presence of EC survival factors such as VEGF through a caspase-dependent pathway. Both stable overexpression of ISM1 in cancer cells and systemic intravenous infusion of rISM potently suppressed xenograft tumor growth and angiogenesis in mice[17, 18].

Two EC cell-surface ISM1 receptors have been identified by our lab[18, 19]. While αvβ5 integrin serves as a low-affinity receptor that binds ISM1 with μM affinity, cell-surface glucose regulated protein 78 (GRP78) is a high-affinity receptor that binds ISM1 in the nM range. We demonstrated that ISM1 exerts its anti-angiogenic effect and induces EC apoptosis through two independent signaling pathways mediated by its two cell-surface receptors (Figure 1). ISM1 induces EC apoptosis through αvβ5 integrin-mediated death by direct recruitment and activation of caspase-8 without causing anoikis[19]. Further, ISM1-GRP78 interaction triggers endocytosis of this ligand-receptor complex. The internalized ISM-GRP78 complexes are targeted to the mitochondria, leading to mitochondrial dysfunction and cell death[18].

GRP78 is a member of the HSP70 heat shock chaperon family. It is a major endoplasmic reticulum (ER) stress response protein that is overexpressed in cells under stress and protects stressed cells from cell death[20]. In normal cells, GRP78 is dominantly localized to the ER lumen. However, in stressed cells, GRP78 is overexpressed, and a portion of GRP78 is translocated to the cell surface and serves as a signaling receptor. Knockdown of GRP78 expression by siRNA or blocking of cell-surface GRP78 by anti-GRP78 antibody both disrupt the pro-apoptotic function of ISM[18].

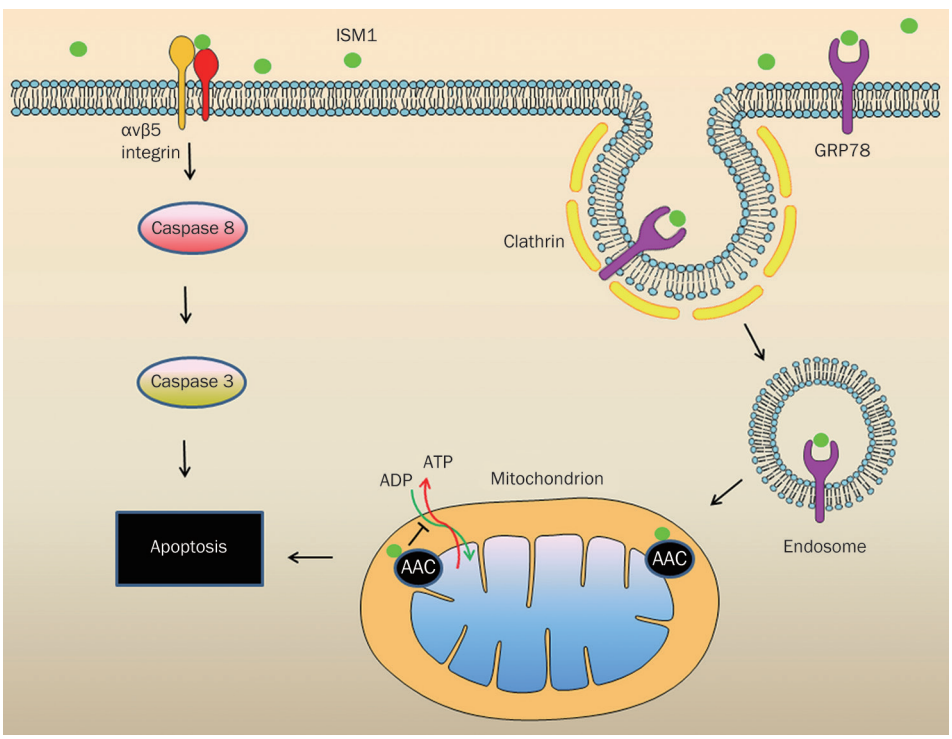

Figure 1. Mechanism of action of isthmin on endothelial cells. On the left, ISM1 binds to integrin αvβ5 and triggers apoptosis via the recruitment and activation of caspase-8. On the right, ISM1 binds to cell surface GRP78 and is internalized via clathrin-mediated endocytosis. ISM1-GRP78 is then trafficked to mitochondria where it binds to AAC and induces apoptosis by interfering with ADP/ATP exchange.

Upon interaction with cell-surface GRP78, ISM1 is internalized together with GRP78 through clathrin-dependent endocytosis. Interestingly, the internalized ISM-GRP78 complex is targeted to the mitochondria. Both cell fractionation and fluorescent imaging experiments have shown the mitochondrial targeting of ISM1 via GRP78. An analysis of ISM1's interaction partners within the mitochondria revealed members of the mitochondrial ATP/ADP carriers (ATP/ADP transporter), AAC2 and AAC3. AAC, also known as adenine nucleotide translocase or ADP/ATP translocase, is the major ADP/ATP transporter located in the mitochondrial inner membrane[21]. By interacting with AACs, ISM1 interfered with ADP/ATP exchange, blocking ATP transport from mitochondria to cytosol and ADP entry into the mitochondria from the cytosol. Nevertheless, ISM does not disrupt the mitochondrial membrane potential and integrity. Consistently, no release of the mitochondrial apoptotic pathway components such as cytochrome *c* into the cytosol was observed. These data indicate that ISM1 induces apoptosis mainly by blocking ATP/ADP exchange on the mitochondrial inner membrane[21]. The two ISM receptors, GRP78 and αvβ5, thus mediate independent signaling pathways without convergence inside the cell (Figure 1).

Cell-surface GRP78 is preferentially present in cancer cells and cancer ECs; thus it is an attractive target for cancer therapy[22-24]. High levels of cell-surface GRP78 result from its overexpression, which is associated with tumor progression and metastasis. Accordingly, ISM1 selectively induces apoptosis in cancer cells and active ECs, which harbor high levels of cell-surface GRP78. In contrast, ISM1 has no effect on normal cells or benign tumor cells, which harbor little or no cell-surface GRP78 protein. Indeed, systemic delivery of rISM potently inhibited xenograft melanoma and breast cancer growth in mice[18]. Recombinant adenovirus expressing ISM1 significantly suppressed orthotropic glioma growth through intracerebral delivery[25].

Thus, ISM, or its peptide derivatives, has the potential to be developed into anticancer drugs that target cell surface GRP78. To date, no drug that specifically targets cell surface GRP78 has been developed. Development in this direction is highly anticipated in the next few years.

FK506 binding protein like (FKBPL)

FKBPL (FK506 binding protein like) was initially discovered as DIR1 with a potential role in induced radioresistance[26]. It was later renamed FKBP-like due to its similarity to the FKBP family of heat-shock related proteins known as immunophilins. FKBPL has been implicated in cellular stress responses and control of the cell cycle[27]. It has a C-terminally located tetratricopeptide repeat domain (TPR), which is important for its interaction with Hsp90[28]. It has also been shown to interact with the steroid receptor family members, glucocorticoid receptors and androgen and estrogen receptors, and it plays a role in cellular signaling[29-31].

A recent study highlighted the importance of FKBPL as a prognostic and predictive marker of breast cancer[31]. FKBPL expression correlated with overall survival and distant metastasis-free survival in breast cancer patients. It increases the sensitivity to anti-estrogens such as tamoxifen in breast cancer cells[31]. Overexpression of Hsp90 is known to induce neovascularization *in vivo*[32], and Hsp90 inhibitors have been shown to possess anti-angiogenic properties[33]. As an Hsp90 intracellular co-chaperone, FKBPL may be an anti-angiogenic protein.

Indeed, a recent study demonstrated that overexpression of FKBPL or treatment with recombinant FKBPL (rFKBPL) inhibited angiogenesis both *in vitro* and *in vivo*. FKBPL suppressed migration and tube formation in human microvascular endothelial cell line HMEC-1, without affecting proliferation[34]. rFKBPL also dose dependently inhibited blood vessel formation in an *ex vivo* rat aortic ring angiogenesis assay and *in vivo* subcutaneously implanted sponge angiogenesis assay[34]. The antiangiogenic domain of FKBPL was mapped to amino acids 34 to 57 at the N-terminus of the protein. This region is outside of the Hsp90 binding region suggesting an alternative target for FKBPL. CD44, the cell surface receptor for hyaluronan, was identified as the target of the anti-migration function of extracellular FKBPL. CD44 is known to play important roles in cell adhesion, migration, *in vivo* angiogenesis and tumor progression[35, 36]. FKBPL inhibited HMEC-1 migration via the inhibition of CD44 and its downstream target, the small GTPase Rac. Furthermore, a 24 amino acid synthetic peptide spanning the anti-angiogenic domain of FKBPL from aa34-aa57, termed AD-01, functions similarly to full-length protein in inhibiting angiogenesis[34]. Direct intratumoral injection of *FKBPL* cDNA expression vector into DU145 prostate cancer xenografts exhibited dramatic reduction in tumor growth and angiogenesis compared to vector injected control tumors. In addition, systemic delivery of AD-01 peptide suppressed tumor growth in two different tumor models – DU145 prostate cancer and MDA-MB-231 breast cancer[34].

Several characteristics suggest the great potential for FKBPL and its derivative, AD-01 synthetic peptide, to be developed into anticancer drugs. First, treatment with AD-01 led to potent inhibition of tumor progression in both prostate and breast cancer models with a dose as low as 0.003 $mg \cdot kg^{-1} \cdot d^{-1}$. Extensive central necrosis of the tumor core resulting in an empty core with a viable tumor rim, which is a classical presentation similar to other angiogenesis inhibitors in clinical trials, was also observed upon AD-01 treatment[37]. Second, intravital microscopy of the tumor blood vessels revealed not only decreased blood vessel numbers but also increased vessel diameter, which is suggestive of vessel normalization and is favorable for drug delivery. Third, AD-01 treatment did not affect the normal vasculature as evidenced by the lack of any inhibitory effect on the retinal vasculature. Fourth, failure of angiogenesis inhibitors is often attributed to the drastic side effects associated with their anti-proliferative effects[38]. Because FKBPL and AD-01 have no effect on EC proliferation and showed no observable cytotoxicity in mice, they appear to be attractive anti-angiogenic compounds. Fifth, FKBPL's effects are mediated by CD44 in the tumor vasculature; hence, it offers broad applicability over a wide range of solid tumors.

Lastly, in addition to showing promise as a single agent, combination therapy with docetaxel showed significantly increased anticancer activity[34].

Carboxy-terminus of Hsc70 interacting protein (CHIP)

CHIP (carboxy-terminus of Hsc70 interacting protein) was first discovered as a chaperone-associated ubiquitin ligase[39]. The CHIP protein has a TPR domain at its N-terminus, through which it interacts with the molecular chaperones Hsc70, Hsp70 and Hsp90 and modulates their chaperone activity. At its carboxyl-terminus, CHIP has a U-box domain through which it acts as an E3 ubiquitin ligase, triggering proteosomal degradation of known chaperone substrates[40]. CHIP has been shown to ubiquitinate and degrade several oncogenic proteins, such as mutant p53[41], estrogen receptor α[42], c-ErbB2/neu[43], Dbl[44], Smad3[45], hypoxia inducible factor 1α[46], Runx1[47], Met receptor and steroid receptor coactivator-3 (SRC-3)[48, 49], which is supportive of a tumor suppressor role for the protein.

CHIP is a tumor suppressor for breast cancer and suppresses several oncogenic pathways. CHIP levels were negatively correlated with malignant breast tumors and survival of breast cancer patients. Interestingly, knockdown of CHIP in breast tumors resulted in increased vascularization, indicating an anti-angiogenic function for CHIP[49]. Surprisingly, Xu *et al* in 2011 reported that CHIP contributes to enhanced tumorigenesis of human glioma both *in vitro* and *in vivo*[50]. Malignant gliomas are characterized by a marked increase in blood vessel density[51]. However, the authors did not explore CHIP's role in tumor angiogenesis in glioma.

Recently, CHIP's role in inhibiting tumor angiogenesis in human gastric cancer (GC) was demonstrated[52]. Using human gastric cancer cell line BGC823, Wang *et al* showed that the stable overexpression of CHIP resulted in a significant reduction of microvessel density in these gastric tumors *in vivo* compared to vector controls. To study the specific effect on GC angiogenesis, conditioned media from stable CHIP overexpressing and knockdown BGC823 derivative cell lines were tested on human umbilical vein endothelial cells (HUVEC). Overexpression of CHIP resulted in 48% inhibition of HUVEC growth whereas CHIP knockdown caused a 1.82 fold increase in growth compared to corresponding vector controls. A marked reduction in EC tube formation upon CHIP overexpression and an increase in tube formation were observed when CHIP levels were knocked down[52]. CHIP interacts directly with p65 NF-κB via its U-box domain, causing ubiquitin-mediated proteosomal degradation of NF-κB. Consistently, NF-κB responsive genes such as interleukin-8 (IL-8), matrix metalloproteinase-2 (MMP-2) and VEGF[52] were inhibited in GC.

IL-8 production is closely linked to increased vascularization, malignant phenotype and poor prognosis in several cancer types[53, 54]. While CHIP reduced both IL-8 mRNA and protein secretion, IL-8 rescued the inhibitory effects of CHIP on GC angiogenesis[52]. By inhibiting NF-κB activation, CHIP triggered a wide array of downstream genes and had a profound impact on many cellular processes, such as adhesion and invasion, in addition to angiogenesis. The fact that reduced CHIP levels are correlated with malignant phenotype in both GC and breast cancer supports the notion that restoration of CHIP may be a novel strategy for anti-angiogenic therapy for human cancer.

Rho GTPase activating protein 18 (ARHGAP18)

ARHGAP18 was initially identified as Mac guanosine triphosphatase activating protein (MacGAP) expressed in the human epididymis[55]. ARHGAP18 contains the traditional RhoGAP domain in its C-terminus and is one of the crucial factors that regulate RhoA to control cell shape, spreading and migration. It is required for remodeling the actin cytoskeleton in response to integrin engagement[56].

The association of ARHGAP18 with angiogenesis was first noticed in an expression profiling screen for functionally important genes during *in vitro* angiogenesis[57]. Later, the same group identified ARHGAP18 as an endothelial senescence-associated gene termed SENEX[58]. Relative to other RhoGAPs, ARHGAP18 expression is the highest in ECs, and its localization is predominantly cytosolic[59]. The overexpression of ARHGAP18 in EC results in an induction of premature senescence in ECs. During *in vitro* HUVEC tube formation, ARHGAP18 mRNA was down-regulated during the early migration phase but was later upregulated during the stabilization phase[58].

Knockdown of ARHGAP18 promoted EC migration in a scratch wound assay. The migratory front was protrusive and irregular, with the proximal edge of the leading cell having disrupted cell junctions[60]. The increased migration was also observed in an *in vitro* 3D spheroid sprouting assay, with a significant increase in both sprout length and the number of sprouts. Interestingly, knockdown of ARHGAP18 in epithelial cells (MDA-MB-231 cells) resulted in delayed migration, indicating a possible cell-specific function for this protein[56].

In vivo studies with zebrafish embryos confirmed the above *in vitro* findings. Knockdown of ARHGAP18 lead to enhanced angiogenic sprouting of intersegmental vessels (ISV) in zebrafish embryos. The hyper-sprouting phenotype was further characterized by increased filopodia extensions and reduced lumen diameter at least in the dorsal aorta at 24 hpf. This phenotype was vascular specific as there were no effects on lymphatic vessels[60]. Using an *ex-vivo* aortic ring assay, it was revealed that loss of ARHGAP18 in ARHGAP18$^{-/-}$ knockout mice resulted in a hyper-sprouting phenotype with a propensity to form branches instead of linear sprouts, which is reminiscent of the tip-cell phenotype[61]. Knockdown of ARHGAP18 in cultured ECs caused a higher and prolonged activation of Akt and increased mRNA expression of the tip cell marker genes such as Dll4, Flk1 and Flt4, which is consistent with additional tip cell formation[60].

ARHGAP18 expression was localized to VE-cadherin expressing EC junctions in spheroid sprouts[62]. ARHGAP18 is dynamically regulated during angiogenesis. Upon thrombin stimulation, ARHGAP18 quickly re-localized from the cytosol to EC junctions within 2 min and was found at the junctional

edges in an active junction at 10 min. Upon achieving junctional maturity, ARHGAP18 was lost and returned to the cytosol within 60 min. ARHGAP18 knockdown was linked to RhoC activation but not the activation of RhoA, Rac1, Cdc42 and RhoJ. Thus, ARHGAP18 seems to act as RhoCGAP and control EC junctional integrity[60].

ARHGAP18$^{-/-}$ animals developed subcutaneous B16F10 melanoma faster than wildtype mice, and the tumors were also hypervascularized, which is consistent with ARHGAP18 acting as an angiogenesis inhibitor[60]. Furthermore, ARHGAP18 is a cancer risk locus and showed loss of copy number in 30%-56% of breast, lung and ovarian cancers[63-65]. ARHGAP18 was also down-regulated under conditions of chronic activation of rat sarcoma (RAS) and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE2), often found associated with hemangiomas and venous malformations, respectively[66]. Because ARHGAP18$^{-/-}$ mice are phenotypically normal, targeting this protein in pathological angiogenesis could be a therapeutic possibility.

Multimerin2 (MMRN2)

Multimerin2 (MMRN2), also known as endoglyx-1, is an ECM glycoprotein that is closely associated with the cell surface. It comprises four different disulfide bonded subunits of p125, p140, p110 and p200, with an approximate molecular mass of 500 kDa. It is an EMILIN-like protein with a signal peptide, an N-terminal EMI domain and a C-terminal C1q domain separated by a central coiled-coil rich region, and it is a member of the EDEN (EMI Domain ENdowed) protein family[67].

MMRN2 exhibits a pan-endothelial expression in both normal and tumor vasculature, including hot spots of neovascularization in some tumors[68]. It is specifically deposited in EC in tight juxtaposition to blood vessels and is also present in the luminal side of the vessels. Nevertheless, its function in angiogenesis and EC function has remained elusive until recently.

Lorenzon *et al* recently discovered an anti-angiogenic role for MMRN2: it inhibits EC migration and blood vessel organization without affecting proliferation[69]. MMRN2 dose-dependently impaired the formation of microvessels in a fibroblast/EC co-culture system and dramatically reduced vessel sprouting from rat aortic rings. *In vivo*, MMRN2 inhibits angiogenic sprouting towards a VEGF containing sponge in a CAM assay but not towards a b-FGF containing sponge[70]. Indeed, MMRN2 was found to directly bind VEGF-A with a Kd of 50 nM and to interfere with VEGF/VEGFR2 signaling in ECs.

MMRN2 displaced radiolabelled VEGF bound to HUVEC cells, indicating that the protein interfered with VEGF-VEGFR binding. Consistent with the fact that MMRN2 closely associates with the EC surface, it is likely that the pericellular concentration of MMRN2 is enriched and serves as an important competitor for VEGF binding to VEGFR2, sequestering and regulating VEGF activity. Stable overexpression of MMRN2 in HT1080 human fibrosarcoma cells dramatically inhibited xenograft tumor growth in nude mice through inhibiting tumor angiogenesis[69]. Direct intratumoral injection of recombinant MMRN2 adenovirus also led to tumor suppression and anti-angiogenic effects, although to a lesser extent[69].

Several key features implicate MMRN2 as an attractive angiogenesis inhibitor. First, although MMRN2 exhibits pan-endothelial expression, it does not seem to affect normal EC growth, proliferation or apoptosis. Second, with a unique mechanism of VEGF sequestration, this ECM molecule could serve to limit local angiogenesis to either maintain a quiescent state or in pathological conditions as a feedback regulator of VEGF signaling. Indeed, most ECM members that sequester VEGF also affect EC proliferation[71,72], but MMRN2 exhibits a unique inhibitory effect restricted to EC motility alone. Finally, the dramatic effects of MMRN2 on tumor growth and angiogenesis opens the possibility to the develop MMRN2 into a new anti-angiogenic drug for cancer therapy.

Suppressor of cytokine signaling-3 (SOCS3)

Suppressor of cytokine signaling-3 (SOCS3) belongs to the family of suppressors of cytokine signal transduction that regulate important cellular processes such as proliferation and differentiation[73]. Members of this family are transiently induced by inflammatory mediators such as lipopolysaccharide[74], interleukin-6 (IL-6)[74] and tumor necrosis factor-α (TNF-α)[75]. SOCS3 has been shown to disrupt JAK/STAT kinases and deactivates receptor tyrosine kinase signaling[73, 76].

Pathological vascular growth is often triggered by massive inflammatory and growth factor stimuli, which could be countered by endogenous angiostatic regulators. As a suppressor of inflammatory and cytokine signaling, SOCS3 is a newly identified endogenous negative regulator of angiogenesis that acts on both inflammation and growth factor–mediated vessel formation specifically in pathologic contexts[77].

SOCS3 expression is temporally associated with pathological retinal angiogenesis. In an oxygen-induced retinopathy (OIR) model[78, 79], hypoxia-induced pathological angiogenesis peaks on postnatal day 17 (P17). This pathological angiogenesis coincided with increased TNF-α and SOCS3 expression[80]. In particular, SOCS3 expression was highly and specifically localized to pathologic retinal vessels. Given that *Socs3* knockout mice are embryonic lethal[81], a Tie2-driven EC-specific *Socs3* knockout mouse (*Tie2-Socs3*^{ko}) line was generated. After being subjected to OIR, these conditional *Tie2-Socs3*^{ko} mice exhibited increased pathological neovascularization compared to littermate controls (*Socs3*$^{flox/flox}$) at P17. Notably, *Tie2-Socs3*^{ko} mice also exhibited normal retinal vascular development, similar rates of vascular loss during the first phase of OIR and normal vessel repair and regrowth, suggesting that SOCS3 specifically suppresses pathological angiogenesis[77]. Furthermore, when LLC (lung carcinoma) and B16F10 melanoma cells were injected into *Tie2-Socs3*^{ko} mice, enhanced tumor growth was observed in both xenograft models with a concomitant increase in tumor vascular density[77].

Both growth factor and inflammatory signals trigger pathological angiogenesis in OIR and cancer[82-85]. As a negative regulator of both signaling pathways, SOCS3 was shown to suppress the downstream signaling of both insulin growth

factor-1 (IGF-1) and TNF-α. Silencing of SOCS3 expression by siRNA in ECs increased EC proliferation when stimulated with both agents. Both mTOR and STAT-3 pathways exhibited transient activation upon *Socs3* silencing. This activation became sustained when ECs were pre-stimulated with TNF-α prior to IGF-1 treatment. Thus SOCS3 acted as a negative feedback regulator of growth factor signaling by modulating mTOR and STAT-3 activation[86].

SOCS3 represents a new class of angiogenic modulators that target an integrated endothelial response rather than suppressing individual growth factors or receptors. A recent report also closely links the loss of SOCS3 to an aggressive phenotype in breast carcinoma[87]. As a novel angiogenesis inhibitor, SOCS3 has particular application potential in both proliferative retinopathy and cancer due to its specific activity toward pathological angiogenesis.

Tumor protein 73-alpha (TAp73)

TAp73 or p73-alpha is a 636-amino-acid isoform of tumor protein 73 (TP73)[88]. TP73 generates several isoforms through both alternative splicing and alternative promoter usage[89, 90]. TAp73 along with the other TP73 variants are known as transactivation (TA) variants, as they all share an N-terminal transactivation domain.

The primary role of TAp73 is the transcriptional control of proapoptotic genes in the response to genotoxic stress by inhibiting p53[91, 92]. Unlike p53, a tumor suppressor gene whose mutations are widely found in human cancers, TAp73 is rarely mutated in cancer and instead is highly upregulated in several cancers[93, 94]. Thus, the role of TAp73 in tumorigenesis has been elusive. On one hand, isoform specific deletion of TAp73 resulted in spontaneous and carcinogen-induced tumors, with a high incidence of lung adenocarcinomas, indicating that TAp73 is a tumor suppressor[95]. Nevertheless, the high incidence of TAp73 protein expression in cancers suggests that the protein may afford proliferative advantages to tumor cells. Indeed, a recent report supports a role for TAp73 in regulating metabolism and promoting oncogenic cell growth[96].

TAp73 has been linked to VEGF expression with contradictory reports; however, the specific roles of TAp73 in angiogenesis are still unknown. One report attributes an inhibitory role for TAp73 in controlling VEGF expression by transcriptional repression of the VEGF promoter[97]. However, another report states that overexpression of TAp73 resulted in increased expression of VEGF at both the mRNA and protein levels[98]. A recent study by Amelio et al clarified the role of TAp73 in angiogenesis and demonstrated that TAp73 suppresses angiogenesis by promoting HIF-1α ubiquitination and degradation.

Using a two-stage chemical carcinogenesis model in mouse skin, Amelio *et al* showed that TAp73$^{-/-}$ mice displayed accelerated initial lesion development, large tumor sizes and increased progression to squamous cell carcinoma compared to TAp73$^{+/+}$ mice. Both peritumor vascularization and aortic ring angiogenesis increased in TAp73$^{-/-}$ mice compared to wildtype mice[99].

HIF-1 is a master regulator of tumor growth and angiogenesis, and its expression is under the tight control of oxygen levels within the microenvironment[100]. In the absence of p53, TAp73 knockdown resulted in enhanced HIF-1α expression. Nonetheless, TAp73 induced downregulation of HIF-1α in an oxygen-independent manner. TAp73 directly interacts with HIF-1α and promotes its ubiquitination and degradation, possibly functioning as a scaffold to bring HIF-1α in close proximity for subsequent ubiquitination and degradation[99].

Accordingly, the overexpression of TAp73 led to downregulation of both VEGF-A and VEGFR2 mRNA, while stable knockdown of TAp73 in tumor cells resulted in dramatic increases in subcutaneous tumor growth and vessel density[99]. In human lung adenocarcinoma, individuals with high TAp73 expression showed significantly better prognosis, whereas HIF-1α activity and angiogenic signatures were inversely correlated with TAp73 expression[99].

In conclusion, the tumor suppressor TAp73 has emerged as a novel inhibitor of tumor angiogenesis and plays a critical role in cancer pathogenesis. How it can be used effectively in therapeutics still requires investigation.

Zinc finger protein 24 (ZNF24)

ZNF24, also known as ZFP191 or KOX17, was identified as a member of the SCAN domain family of Krüppel-like C2H2 zinc finger transcription factors[101]. It possesses four C2H2 zinc finger domains at the C-terminus that function as DNA binding domains and one scan domain in its N-terminus[102, 103]. ZNF24 is ubiquitously expressed during embryonic development[104], and ZNF24 knockout mice die prematurely at 7.5 d post fertilization, suggesting its key functions in normal development[105]. At the cellular level, ZNF24 is involved in the regulation of the proliferation, migration, differentiation and invasion of cells of different lineages[104-108]. In addition to regulating normal cells, ZNF24 has been shown to play perplexing roles in cancer initiation and progression.

The ZNF24 gene is located on chromosome 18q12.1[109] and is frequently deleted in several human cancers[110-113]. In addition, down regulation of ZNF24 mRNA expression was also observed in malignant colon and breast carcinoma[114, 115]. However, ZNF24 has also been shown to promote cell proliferation of hepatocarcinoma cells[116]. ZNF24 represses VEGF transcription in MDA-MB-231 breast cancer cells *in vitro* and exhibits an inverse correlation with VEGF in angiogenic tumor nodules as well as malignant human colon and breast biopsies[114]. It also represses PDGFR-β transcription[117], a gene known to be essential for vascular stability[118, 119].

How ZNF24 represses VEGF transcription and the *in vivo* consequences of this repression were recently revealed by Jia *et al*[120]. ZNF24 binds to an 11 bp segment within the proximal promoter region of the VEGF gene and targets VEGF directly. This 11-bp VEGF proximal promoter region can serve as a decoy to abolish VEGF repression by ZNF24. ZNF overexpression in zebrafish resulted in decreased VEGF expression and severe vascular defects including pericardial edema, abnormal formation of caudal vascular plexus, defects in ISV formation

and impaired circulation[120]. These defects are consistent with the VEGF knockdown phenotype.

MDA-MB-231 breast cancer cells overexpressing ZNF24 exhibited significantly reduced VEGF levels both extracellularly and intracellularly. Using an *in vivo* tumor cell induced dermal angiogenesis assay, MDA-MB-231 cells overexpressing ZNF24 induced significantly lower numbers of blood vessels in the injected dermal tissue compared to control cells. In addition, a high percentage of human breast cancer tissues showed significantly lower levels of ZNF24 staining, correlated with a higher VEGF level compared to adjacent normal breast tissues. These resulted suggested that ZNF24 may play a suppressive role in the initiation and/or progression of human breast cancer[120].

Paradoxically, a recent paper from the same lab reported that ZNF24 positively regulates the angiogenic potential of human microvascular endothelial cells (HMVEC)[121]. They showed that knockdown of ZNF24 in HMVECs resulted in decreased cell migration, invasion, proliferation and decreased formation of vascular networks along with significantly impaired VEGFR2 signaling[121]. These results seem to suggest that ZNF24 serves as a positive stimulator of the angiogenic potential of microvascular ECs. How ZNF could exhibit divergent functions in different cell types (endothelial *vs* cancer cells) is very intriguing. Its role in tumor endothelium warrants further investigation.

Although ZNF24 seems to function as an inhibitor of angiogenesis in both zebrafish embryonic development and in human breast cancer by suppressing VEGF transcription[120], its therapeutic potential is still unclear. In addition, how ZNF24 regulates the transcription of other angiogenesis-related genes warrant future investigation. For example, ZNF24 also represses the transcription of PDGFR-β, a gene that is important in regulating angiogenesis[117]. Elucidating the mechanisms and consequences of PDGFR-β repression by ZNF24 would be important for fully understanding the function of this gene in angiogenesis.

G-protein-coupled receptor 56 (GPR56)

G-protein-coupled receptor (GPR56), or seven transmembrane molecule containing a long N-terminus (TM7XN1), is an atypical G-protein-coupled receptor (GPCR) that belongs to the subfamily of adhesion G-protein-coupled receptors (GPCRs). GPR56 was previously found to be anti-tumorigenic and anti-metastatic in melanoma, where overexpression of GPR56 suppressed tumor growth and metastasis[122]. The expression levels of GPR56 were inversely correlated with the progression of human melanomas[123]. GPR56 was reported to exert anti-angiogenic function by inhibiting VEGF secretion/release from melanoma cells[123]. This function requires the proteolytic cleavage fragments, the extracellular N-terminal fragment (GPRN) and the transmembrane C-terminal fragment (GPRC), to associate with each other. A 70-amino acid serine threonine proline-rich (STP) segment in the GPRN is required for this function. GPR56 inhibits VEGF secretion by inhibiting protein kinase Cα (PKCα)[123, 124]. PKCs are proteins that regulate VEGF release from specific granules in multiple cell types[125-127]. Hence, inhibiting PKCα prevents VEGF release from melanoma cells and inhibits angiogenesis.

GPR56 is the first adhesion GPCR attributed with anti-angiogenic function. Moreover, its anti-angiogenic mechanism of preventing VEGF release at the source is unique. Thus, GPR56 complements the current anti-angiogenic drugs and raises the efficacy of these drugs, which do not inhibit the production of VEGF. GPR56 can also be used as a prognostic marker as GPR56 down-regulation is correlated with melanoma progression.

JWA

JWA is also known as Prenylated Rab acceptor 1 domain family member 3 (PRAF3) and ADP-ribosylation factor-like 6 interacting protein 5 (ARL6IP5)[128]. It is part of the prenylated rab acceptor 1 domain family (PRAF), whose members are involved in intracellular protein transport. JWA functions as a microtubule-binding protein and is involved in the mitogen activated protein kinase (MAPK) signaling pathway, regulating cancer cell migration[129]. Tumor suppressor functions such as inducing apoptosis[129], inhibiting metastasis in melanoma[130], osteosarcoma, breast and cervical cancers have also been reported[128]. Recently, JWA was found to function as a tumor angiogenesis inhibitor in melanoma and gastric cancer[131, 132]. Overexpression of JWA in melanoma and gastric cancer cell lines inhibited angiogenesis *in vitro* and *in vivo*.

Different anti-angiogenic mechanisms for JWA exist in melanoma and gastric cancer. In melanoma, JWA suppresses angiogenesis by down-regulating integrin-linked kinase (ILK) expression through integrin αvβ3 and transcription factor Sp1[130]. ILK signaling activates NF-κB/IL-6/STAT3/VEGF angiogenic signaling pathways. JWA overexpression significantly inhibited IL6 and VEGF expression, but this inhibition is lost when ILK is also overexpressed. By suppressing ILK expression, JWA inhibits melanoma angiogenesis. Regarding gastric cancer, JWA down-regulates the expression of MMP-2, a pro-angiogenic molecule. Down-regulation of MMP-2 expression is also through inhibiting transcription factor Sp1 via an ubiquitin-proteasome dependent mechanism[132].

To the best of our knowledge, JWA is the first microtubule-binding protein that has been shown to have anti-angiogenic function. JWA thus has the potential to be developed into an attractive therapeutic drug for cancer and other angiogenesis-related illnesses. JWA expression correlates with melanoma survival. Patients with melanoma expressing high levels of JWA and low levels of ILK had significantly increased 5-year survival rates[133]. Similarly for gastric cancer, patients with high JWA and low MMP2 had better survival rates[132]. Hence, JWA can be both a prognostic marker and a potential therapeutic for melanoma and gastric cancer.

Conclusion

Angiogenesis inhibitors play important roles in regulating both physiology and pathology. Endogenous protein inhibitors have the advantage of low toxicity, high tolerance, low

Table 1. A brief summary of the endogenous angiogenesis inhibitors discussed in this review.

Protein	Function	Role in cancer	Role in angiogenesis	Mechanism of action
ISM1	Anti-angiogenic, Proapoptotic	- Suppresses B16 melanoma tumor xenograft growth. - Suppresses tumor growth of human glioma cells injected intracerebrally or subcutaneously. - Induces apoptosis in cancer bearing high cell-surface GRP78.	- Suppresses VEGF and b-FGF stimulated EC proliferation. - Induces EC apoptosis. - Inhibits vascular invasion in Matrigel plug assay. - Inhibits tumor angiogenesis in B16 melanoma and 4T1 breast cancer xenografts.	- Mitochondrial dysfunction via inhibiting ADP/ATP exchange
FKBPL	Cellular stress Response	- Prognostic and predictive marker in breast cancer. - Suppresses xenograft tumor growth of DU145 prostate cancer and MDA-MB-231 breast cancer.	- Suppresses HMEC-1 migration, tube formation and wound healing *in vitro*. - Disrupts growth factor induced angiogenesis in an *ex vivo* rat aortic ring angiogenesis and *in vivo* murine sponge implantation assay. - Suppresses tumor angiogenesis in DU145 prostate cancer xenografts.	- Inhibits CD44 and downstream Rac signaling.
CHIP	E3 ubiquitin ligase	- Suppresses tumor progression in breast cancer. - Associated with malignant breast tissue and poor survival in patients. - Enhances tumorigenesis of human glioma *in vitro* and *in vivo*. - CHIP expression decreased in gastric cancers. - Suppresses BGC823 xenograft gastric tumor growth *in vivo*.	- Enhances vascularization in CHIP knocked-down breast tumors. - Inhibits HUVEC proliferation and tube formation *in vitro*. - Suppresses tumor angiogenesis in BGC823 gastric tumor xenografts.	- Inhibits NFκB signaling by promoting ubiquitin-mediated proteosomal degradation of NF-κB.
ARHGAP18	Cell adhesion and migration	- Suppresses B16F10 melanoma tumor xenografts. - Loss of copy numbers in breast, lung and ovarian cancers. - Downregulated in RAS2, TIE2 activated hemangiomas and venous malformations.	- Inhibits EC migration *in vitro* and decreased angiogenesis in *ex vivo* spheroid sprouting assay. - ARHGAP-18 knockdown increased ISV lengths and promoted hypersprouting phenotype in zebrafish embryos. - Suppresses tumor angiogenesis in B16F10 melanoma tumor xenografts.	- Inhibits RhoC activation and controls VE-cadherin junctional stability.
MMRN2	Angiogenesis regulator	Suppresses HT1080 xenograft tumor growth	- Pan endothelial expression in normal and tumor vasculature. - Inhibited EC migration and *in vitro* tube formation. - Inhibited VEGF-induced angiogenesis in CAM assay and aortic ring assay. - Inhibited *in vivo* tumor angiogenesis of HT1080 tumor xenografts.	- VEGF sequestration. - Reduction of FAK phosphorylation and EC migration.

(To be continued)

Protein	Function	Role in cancer	Role in angiogenesis	Mechanism of action
SOCS3	Negative regulator of cytokine signaling	- Suppresses *in vitro* breast cancer cell proliferation and anchorage dependent growth. - Inhibits inflammatory cytokines in a triple negative breast cancer model. - SOCS-3 associated with inflammation and cancer.	- SOCS-3 inhibited angiogenesis in an oxygen-induced retinopathy model. - Suppressed tumor angiogenesis in LLC and B16F10 tumor xenografts. - Suppressed growth factor or cytokine stimulated vessel outgrowth in an aortic ring assay.	- Inhibits TNF-α and IGF-1 inflammatory and growth factor signaling.
TAp73	Tumor suppressor	- TAp73-specific knockout mice exhibited partial embryonic lethality, infertility and a marked increase in spontaneous and carcinogen-induced tumors. - TAp73 is rarely mutated but frequently overexpressed in multiple cancers.	- TAp-73 deficient mice exhibited enhanced vascularization in spontaneous, carcinogen-induced and xenograft tumors.	- Inhibits HIF-1α signaling by promoting HIF-1α ubiquitination and degradation.
ZNF24	Transcriptional regulation	Implicated with a tumor suppressor role via its suppression of PDGFR, MYO6 and BMPR2 expression.	Anti-angiogenic - Overexpression caused severe cardiovascular defects and impaired circulation in zebrafish embryos. - Suppresses *in vivo* tumor angiogenesis of MDA-MB-231 tumor xenografts. Proangiogenic - Promoted microvascular EC proliferation, migration and invasion *in vitro* and blood vessel formation *in vivo*.	- Represses VEGF transcription. - Enhances VEGFR-2 signaling.
GPR56	Tumor inhibition, Neuron development	- GPR56 mRNA expression down-regulated in highly metastatic melanoma cell lines. - Inhibits MC-1 melanoma tumor growth and metastasis.	- Inhibits melanoma tumor angiogenesis by preventing VEGF release.	- Inhibits VEGF release from intracellular granules.
JWA	Cell differentiation	- Inhibits melanoma metastasis by suppressing integrin αvβ3 signalling. - Regulates cancer cell proliferation, migration and apoptosis via MAPK cascades.	- Reduced JWA expression correlated with high CD31 in human gastric tumors. - Conditioned media from JWA overexpressing gastric cancer cell lines inhibited angiogenesis *in vitro* and *in vivo*.	- Inhibits Sp1 signaling. - Inhibits MMP. - Down-regulating integrin-linked kinase (ILK).

risk of drug resistance and a higher chance of specifically blocking pathological neovascularization, without affecting the normal vasculature[134, 135].

Over the years, many endogenous angiogenesis inhibitors have been discovered, and some of them have reached clinical trial stages or are already in the market; these include endostatin (Endostar) and angiostatin[136-139]. Other inhibitors have served as parent molecules from which derivative analogues have been developed and reached clinical trial stage, such as thrombospondin-1 and its analogue ABT-510[140]. Nevertheless, to date, clinical efficacy in humans is still questionable. Only endostar, a modified recombinant endostatin, has been approved as an anticancer drug in China. In addition, the anti-angiogenic drugs currently in clinical use are predominantly based on inhibiting VEGF signaling pathways. Lack of long-term therapeutic efficacy and the development of drug resistance are prevalent with the current drugs. This underscores the importance of discovering novel angiogenesis inhibitors to not only fully understand the biology of angiogenesis regulation but also identify unique mechanisms of action and additional cellular targets to design more effective drugs.

In this review, we selectively presented ten novel endogenous angiogenesis inhibitors discovered in the past five years. All of these inhibitors have demonstrated inhibitory roles in pathological angiogenesis such as in cancer, retinopathy or fracture healing (summarized in Table 1) but do not affect normal physiological angiogenesis. These proteins have various subcellular localizations including secreted/extracellular (isthmin, FKBPL and multimerin-2), transmembrane (GPR56), cytoplasmic (CHIP, ARHGAP18, SOCS-3, TAp73 and JWA) and nuclear (ZNF24). Each of the proteins has distinct and diverse mechanisms of action (summarized in Figures 1 and 2) including the prevention of VEGF release, VEGF sequestration, promoting proteosomal degradation of angiogenesis

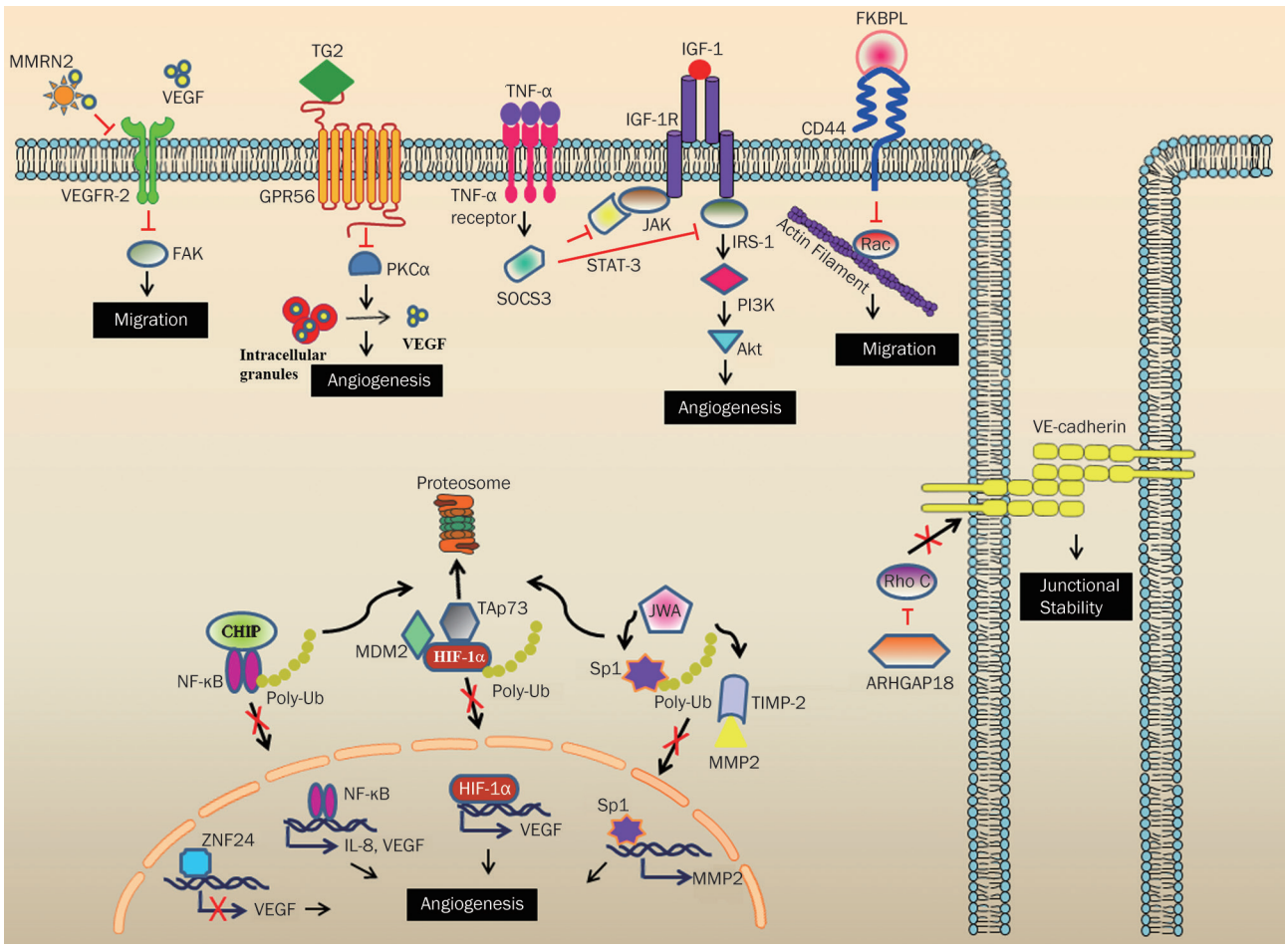

Figure 2. Mechanism of action of endogenous angiogenesis inhibitors. Angiogenesis inhibitors affect the fundamental processes leading to angiogenesis such as proliferation, survival, adhesion, migration and junctional stability. The secreted angiogenesis inhibitors such as MMRN2 and FKBPL act on specific cell surface receptors modulating their pro-angiogenic function. In the case of membrane proteins such as GPR56, binding to its specific ligand results in the activation of a signaling cascade leading to angiogenic inhibition. The cytoplasmic angiogenesis inhibitors such as CHIP, TAp73 and JWA function via a common mechanism: by promoting proteosomal degradation of their cellular targets, thereby preventing their nuclear translocation and subsequent action. ARHGAP18 achieves angiogenic inhibition via influencing EC junctional stability. Finally, ZNF24 acts via transcriptional repression of the angiogenic stimulator VEGF.

stimulators, or interfering with multiple angiogenic stimulator signaling pathways. Thus, these proteins have great potential to function as anti-angiogenic drugs. Further studies on these proteins will expand our understanding of their biology and help to design efficacious drugs for angiogenesis-related diseases. In addition, molecules such as CHIP, TAp73, GPR56 and JWA have been shown to have an inverse correlation with several types of human cancers and could potentially also serve as prognostic markers.

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