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Chapter I

Endogenous Angiogenesis Inhibitors: Is the List Ever Ending?

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Abstract

Angiogenesis is essential for embryonic development, growth and adult physiology. It is also critical in various pathological conditions such as cancer, rheumatoid arthritis and diabetic retinopathy. A delicate balance of angiogenesis throughout the body ensures physiological homeostasis and disease-free condition. This balance is regulated through the local availability of endogenous proangiogenic and antiangiogenic molecules in the tissue microenvironment. To date, a plethora of endogenous angiogenic inhibitors have been discovered and characterized. Nevertheless, the list of endogenous angiogenic inhibitors is continuously growing. In the past few years, many new proteins such as Parstatin, EPLIN-α, β2-glycoprotein-1, ICAP-1, Isthmin, AIP1, Semaphorin3A have been discovered to possess antiangiogenic properties. In particular, several members of the ADAMTS family of matrix metalloproteinase have been identified as endogenous inhibitors of angiogenesis. Furthermore, microRNA as negative regulator of angiogenesis is gaining increasing significance. An understanding of these new endogenous inhibitors is essential in unraveling the complexity of angiogenesis regulation and paves way towards developing mechanism-based medicine in treating angiogenesis-related diseases. Thus, this review will particularly focus on the recently characterized endogenous angiogenesis inhibitors and their significance in the biology of angiogenesis.

Keywords: Antiangiogenesis; endogenous angiogenesis inhibitors; Parstatin; Semaphorin3A; Isthmin; β_2GPI; EPLIN-α; ICAP-1; AIP1; ADAMTS; endothelial cells; miRNA

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1. Introduction

Angiogenesis, the formation of new vasculature from pre-existing ones, is an important and essential process in various pathological processes such as cancer, rheumatoid arthritis, as well as physiological processes such as tissue repair, wound healing, organ development and reproduction [1]. While the majority of the angiogenic processes occur during development, angiogenesis is a tightly regulated process occurring in selected niches under normal physiological conditions [2, 3]. A large number of signaling molecules including endothelial specific and non-specific growth factors, cytokines, chemokines play a role in angiogenesis regulation. Evidently, both angiogenic and antiangiogenic factors need to act together to maintain the physiological homeostasis.

In 1971, Dr. Judah Folkman proposed that angiogenesis is an important hallmark of tumor progression and a tumor could not grow beyond 2 mm without recruiting its own blood supply. This implied that tumor progression is dependent on angiogenesis, and the inhibition of angiogenesis can suppress tumor growth [4]. Since then, the search for angiogenesis inhibitors has been the focus of many research groups, including Dr. Folkman's laboratory. Many endogenous angiogenic inhibitor proteins have been identified such as Thrombospondin 1 (TSP1), platelet factor 4 (PF4), pigment epithelial derived factor (PEDF) etc [5-7]. Subsequently, a large number of angiogenic inhibitor proteins were identified as proteolytic fragments of parent proteins which themselves do not have any antiangiogenic activity (reviewed in [8]). Angiogenesis inhibitors in this group include angiostatin (fragment of plasminogen), endostatin (fragment of collagen XVIII), tumstatin (fragment of collagen IV) and many others [9-11]. Over the years, more and more endogenous angiogenic inhibitors have been identified and the list continues to grow. Recently, several members of the ADAMTS family of matrix metalloproteinase have emerged as novel angiostatic molecules, contrary to the previous belief that matrix metalloproteinase are predominantly proangiogenic and proinvasive [12]. Most notably, microRNAs have been identified as regulators in a number of processes related to tissue-repair in the cardiovascular system [13]. Several microRNAs have been identified to regulate angiogenesis directly or indirectly [14, 15].

It is increasingly clear that angiogenesis regulation is highly complex and diverse; hence a large number of angiostatic regulators may be required to balance the extent of angiogenesis in different tissues, various developmental stages, and different pathological conditions. The identification of endogenous players in angiogenesis will give us a better understanding of the delicate balance that exists between angiogenic and angiostatic molecules and improve in mechanism-based drug design for angiogenesis-related diseases such as cancer. In this chapter, we will focus on the recently identified endogenous angiogenesis inhibitors. The antiangiogenic molecules described here are representative but not exhaustive.

2. Secreted Angiogenesis Inhibitors

2.1. Parstatin

Parstatin is an N-terminal 41-amino acid peptide of the Protease-activated receptor-1(PAR-1) [16]. PARs are members of the G-Protein coupled receptor (GPCR) family [17]

that are activated by the proteolytic cleavage of their N-terminal domains. Parstatin is released from PAR-1 as a consequence of proteolytic cleavage at Arg41/Ser42 [18] by many serine proteinases such as thrombin [18], Factor Xa [19], activated protein C [20], matrix metalloproteinase 1 (MMP1) [21] etc. PAR-1 is expressed in various cell types such as platelets in blood [22], cardiomyocytes and vascular endothelium [23], astrocytes and neurons of nervous system [24] and osteoblasts of bone [25] . It is also expressed in various human cancers such as renal cell carcinoma [26], pancreatic adenocarcinoma [27], melanoma [28] and breast cancer [21].

Thrombin, the proteinase involved in the coagulation cascade, is a main mediator of PAR-1 activation by releasing parstatin [29]. Cleavage of parstatin peptide from the inactive PAR-1 results in a new N-terminus of PAR-1. This new N-terminus of PAR-1 serves as a tethered ligand and binds to the receptor site of PAR-1, leading to PAR-1 activation [29]. Thus PAR-1 is also referred to as thrombin receptor and helps thrombin to regulate a wide variety of physiological processes through PAR-1 activation and downstream cellular signaling. PAR-1 mediated functions include platelet aggregation, mitogenic activity, endothelial barrier dysfunction and inflammation [17].

The name “parstatin” derives from its parental molecule PAR-1 and its angiostatic function [16]. This peptide can be divided into two distinct regions, the N-terminal hydrophobic region (1-23 amino acids) and the C-terminal hydrophilic region (24-41 amino acids). The hydrophobic region is a part of the signal peptide that is 26 amino acids long [18]. Zania and colleagues coined the term “Parstatin” and were the first to discover the potential antiangiogenic function of this peptide through a series of *in vitro* and *ex vivo* experiments [16]. Endothelial cells (ECs) form a characteristic capillary network on surfaces coated with Matrigel. Matrigel is the gelatinous extracellular matrix (ECM) protein mixture derived from Engelbreth-Holm-Swarm mouse sarcoma cells [30]. Parstatin was shown to inhibit EC capillary tube-like structure formation *in vitro* on Matrigel and fibrin. It also reduced EC migration in a dose-dependent manner. Parstatin also suppressed both vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) stimulated EC proliferation and induced cell apoptosis [16].

Parstatin was shown to inhibit new blood vessel formation in the widely used *ex vivo* chick chorioallontoic membrane (CAM) assay and rat aortic ring assay. In CAM assay, parstatin was effective at nanomolar concentrations when stimulated with vascular growth factors such as VEGF and FGF thus re-emphasizing the fact that it is a potent endogenous inhibitor of angiogenesis. While the truncated parstatin (24-41 amino acids) did not show any antiangiogenic function, full-length parstatin with the hydrophilic stretch scrambled (24-41 amino acids) could retain the angio-inhibitory function. Hence the hydrophobic domain of parstatin is absolutely essential for its angiostatic function. Similar to CAM assay, parstatin also inhibited the formation of new microvessels in collagen-embedded rat aortic rings [16]. The angio-inhibitory function of parstatin involves the modulation in mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) cascade and caspase-dependent apoptotic pathway. MAPK/ERKs are key players in cell cycle regulation. Parstatin inhibited EC proliferation by inhibiting ERK1/2 signaling. Upon treatment with parstatin, there was a marked reduction in the phosphorylated ERK1/2 level. The inhibitory effect was dose-dependent and was observed upon stimulation with VEGF and basic fibroblast growth factor (bFGF). Inhibition of FGF mediated cell proliferation was more evident and reversible. Parstatin up-regulated caspase-3 in a dose-dependent manner, thus enhancing EC apoptosis.

The broad range caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) could reverse the effect of parstatin, confirming the caspase-dependent cell death induced by parstatin [16].

Fluorescein isothiocyanate (FITC) labeled parstatin was used to investigate if this peptide can enter ECs and where is it localized using flow cytometry and imaging. Parstatin was internalized in a cell-receptor independent mechanism as early as 1 min after incubating with cells, hence making it a member of cell-penetrating peptides (CPP) [16]. The peptide was localized in cell membrane in the initial time points and later diffused into the cytosol. This is in accordance with most of the CPPs which have a characteristic hydrophobic domain that helps the peptide to penetrate through the lipid barrier [31]. The truncated parstatin lacking the hydrophobic domain could not penetrate the cell thus confirming the importance of this domain for its angiostatic function [16].

Parstatin was evaluated as a therapeutic agent for the treatment of ocular neovascularization and inflammation [32]. Ocular neovascularization can be subdivided into three types- retinal, choroidal and corneal neovascularization based on the region of neovascularization in the eyes. This involves aberrant tissue invasion of ECs and inflammatory cells [33]. Using animal models for all the three kinds of neovascularization, parstatin was shown to be an effective agent against excessive formation of the blood vessels in the eye [32].

One of the strategies to analyze if a given molecule can inhibit ocular inflammation is to analyze if it can overcome the chemoattractant ability of VEGF to attract and adhere monocytes to retinal endothelium [34]. Thus VEGF-induced retinal leukostasis was compared between parstatin injected and control groups. There was a significant reduction in the VEGF-induced retinal leukostasis upon intravitreal injection of parstatin with VEGF compared to VEGF alone. In addition, the corneas of the parstatin-treated group had lower infiltrated neutrophils compared to control. Thus, parstatin is also anti-inflammatory. In choroid neovascularization, both angiogenic and inflammatory responses play a vital role in inflammatory invasion [33]. All the above experiments in adult animals did not illicit any detectable immune response nor cytotoxicity in the eye. This opens up a new therapeutic application against inflammation for this novel peptide.

In vivo, parstatin is cardioprotective after ischemia and reperfusion injury [35]. It also has vasodilatory properties which are mediated through a nitric oxide (NO) dependent pathway. The cardioprotective function of parstatin was attributed to the hydrophobic domain (1-26 amino acids) and this peptide was more potent than the full-length parstatin in this function [36].

However, there are still questions that remain to be answered. For example, it would be important to investigate the fate of parstatin in physiological conditions and specific tissue microenvironment. In addition, more studies to evaluate the stability of parstatin *in vivo* and whether it undergoes proteolytic cleavage need to be conducted. Using parstatin's hydrophobic domain alone to inhibit angiogenesis will enhance its potential as a therapeutic drug. As of now, the hydrophobic domain by itself is said to aggregate and hence there is a need for protein engineering to come up with specific modifications or addition of hydrophilic amino acids to make it soluble and yet effective. Also, several studies have shown that up-regulation of PAR1 leads to a proangiogenic effect in both physiological as well as pathological conditions such as carcinoma [37, 38]. Therefore it is important to understand how both pro- and antiangiogenic signals are translated in the cellular microenvironment.

2.2. Semaphorin3A

Semaphorin 3A/SEMA3A/Collapsin, a prototypic vertebrate semaphorin, belongs to the class 3 of the Semaphorin family, initially known for its axon guiding functions [39]. Subsequent studies indicated that semaphorins can modulate the behaviour of cancer cells and ECs. Various semaphorins either promote or inhibit tumor angiogenesis and tumor progression through multiple mechanisms. SEMA3As involvement as a modulator of tumor cell behaviour has been reported *in vitro*. However, only recently SEMA3A has been shown to be an endogenous angiogenesis inhibitor that blocks *in vivo* tumor growth [40].

Like the other vertebrate class 3 semaphorins, SEMA3A is a secreted glycoprotein. Western blot analysis has revealed that the full-length SEMA3A is 95 kDa and a furin processed form is 65 kDa [41]. They are synthesized as disulphide linked homodimers and dimerization is essential for their biological activity [42]. A typical Class 3 Semaphorin has a domain structure characterized by the presence of an amino-terminal 500 amino acid-long sema domain. A 70 amino acid stretch within this domain is responsible for its biological activity and binding specificity [43]. The carboxy-terminal of the sema domain bears a conserved stretch of 54 amino acid residues designated as the PSI (Plexin, Sema and Integrins) domain [44]. This is followed by an immunoglobulin loop and a conserved basic domain at the C-terminus. X-ray structure of the sema domain of Sema3A revealed a conserved seven-blade propeller structure [45] .

SEMA3A is predominantly expressed in neuronal cell types, while non-neuronal SEMA3A expression has also been widely reported. For example, high levels of SEMA3A expression have been observed in ECs, blood cells and platelets [46, 47]. In addition, increased expression of SEMA3A has also been reported in many types of cancer cells. It has been associated with anti-tumor functions such as in malignant mesothelial cells [48], myeloma [49] and leukemic T cells [50] via negative control of VEGF-mediated angiogenesis.

The primary signal transducers of Semaphorins are the Plexins. However SEMA3A does not directly bind to plexins. Instead it binds to another family of receptors called neuropilins. The neuropilin (NP) family comprises of 2 members NP1 and NP2. SEMA3A binds and signals exclusively through NP1 [51] . Although SEMA3A do not bind directly to plexins, Plexin A1/ Plexin D1 associates with NP1 and acts as the signal transduction unit for SEMA3A signaling.

Class 3 Semaphorins are expressed by both arterial as well as venous ECs, perineural blood vessels and more importantly in all neo-vessels responding to growth factor stimulation [52]. Many reports have shown these molecules to be clearly involved in both physiological as well as tumor angiogenesis.

Vascular remodelling is an essential requirement for normal embryonic development. The primary capillary plexus formed during vasculogenesis needs to undergo extensive morphogenetic changes to form the mature vascular tree. This process requires a dynamic regulation of the adhesive interactions between ECs and the ECM [53]. It has been shown that SEMA3A aids in this process by negatively regulating integrin function [52]. Studies have shown that SEMA3A co-localizes to integrin rich focal adhesions, which are known to be one of the most dynamic structures during cell migration. *In vitro* studies using dominant negative forms of SEMA3A receptors show that by inhibiting integrin function in these structures, SEMA3A may mediate transient adhesiveness without causing complete de-adhesion [52]. In

addition, it also regulates EC migration and cell reorientation. Together these effects result in regulated adhesive and locomotory events permitting proper angiogenesis. *In vivo* studies with developing chick embryo or mouse embryos has also confirmed the involvement of SEMA3A in angiogenic remodelling via the suppression of integrin function. In addition to its role in neuronal and vascular development, knockdown of *Sema3A or plexinD1* also lead to skeletal defects. This suggests that SEMA3A signaling may also be particularly important for the control of bone angiogenesis and endochondral ossification [54].

Keeping in mind that SEMA3A is expressed by ECs, several clues pointed to the possibility of SEMA3A to act as an endogenous angiogenesis inhibitor. SEMA3A inhibits bFGF and $VEGF_{165}$-induced EC proliferation and migration [55]. They also repel ECs by inducing EC contraction, accompanied by loss of focal contacts and actin stress fibers [55, 56]. This effect was found to be mediated through plexin A1 and RAC1 activation by SEMA3A, ultimately leading to either inactivation of β1 integrins or activation of actin degrading enzyme cofilin [57]. A report also records that SEMA3A regulates E-cadherin and integrin expression at the transcriptional level, suggesting its role in modulating cell adhesion, with possible implications during tumor progression [58]. An early report recorded that SEMA3A inhibits EC migration and capillary sprouting by causing retraction of EC lamellipodia [59]. In addition, it can also promote the apoptosis of ECs [55]. *In vitro* angiogenesis assays showed that SEMA3A prevents the spontaneous organization of ECs into tube-like structures on fibrin gels and inhibited sprouting in rat aortic ring assay [55, 59]. *In vivo*, SEMA3A inhibited VEGF-induced angiogenesis in the CAM assay [46]. Interestingly, SEMA3A is unique among class 3 semaphorins, to be a vascular permeability factor despite being a selective suppressor of VEGF-induced angiogenesis [46]. SEMA3A and VEGF share a common receptor NP1 [59, 60] and potentially SEMA3A may interfere with VEGF signaling. SEMA3A inhibits VEGF-mediated angiogenesis via the suppression of early integrin mediated signals associated with FAK and Src [46]. Since VEGF is also a vascular permeability factor [61], SEMA3A was also hypothesized to inhibit VEGF-induced vascular permeability. However, very interestingly, not only did SEMA3A enhances VEGF-mediated permeability, it also independently stimulated vascular permeability with more potency on its own. [46].

SEMA3A has been reported to be expressed in high amounts in various human tumor cell lines [62], and has an inhibitory effect on the behaviour of tumor cells *in vitro*. SEMA3A inhibits the migration and spreading of breast cancer cells, as well as the adhesion and invasiveness of prostate cancer cells in *in vitro* assays [58, 63]. These studies suggest that SEMA3A may be implicated in regulating tumor metastasis. In addition, it was also clearly demonstrated that inhibition of *in vivo* tumor growth by SEMA3A is associated with the appropriate expression of its specific receptors in these tumor cells [64]. In some tumor cells, tumor-derived SEMA3A functions as a paracrine negative regulator of T-cell activation [62]. In mesothelioma cells as well as in myeloma, SEMA3A has been shown to execute negative feedback of VEGF-mediated signals such as angiogenesis [48, 49]. One explanation for this effect could be that VEGF might induce the secretion of antiangiogenic semaphorins in a negative feedback loop to prevent unwarranted angiogenesis. Surprisingly, a clinical study has identified SEMA3A as a prognostic marker for poor patient survival in pancreatic cancer [65]. In another study, SEMA3A was shown to be a proinvasive factor of human colon cancer cells via collagen gel invasion assays [66].

A recent study investigated the role of SEMA3A in multiphase tumorigenesis and tumor angiogenesis using transgenic mouse models such as RipTag2 and K14-HPV/E_2 which recapitulate progressive development of human cancers. RipTag2 mice express SV40 T antigen under the control of the insulin promoter. These mice develop hyperplastic/dysplastic islets that eventually become angiogenic, form invasive carcinomas, and metastasize [67]. The K14-HPV/E_2 is a progressive cervical carcinogenesis model. K14-HPV16 mice express HPV16 early oncogene under the control of the keratin-14 promoter and develop squamous cell carcinomas eventually. When these mice are dosed with estrogen (E_2) capsules to sustain the estrus phase, a synchronous progression leads to uterine cervical cancer [68]. Both these models are characterized by an angiogenic switch that occurs in pre-tumoral stages and then persists throughout tumor growth. This study unveiled the existence of endothelial SEMA3A autocrine loops in premalignant lesions that are lost in overt cancer. This work thus provided conclusive proof of SEMA3A as an endogenous angiogenesis inhibitor. It demonstrated that SEMA3A reduces late-stage tumor volume without inducing enduring hypoxia or interfering with normal vessels. In addition, it was also observed that SEMA3A induces vessel normalization and stable disease making it a promising anti-cancer agent [40].

2.3. Isthmin

Isthmin (ISM) is a secreted protein which was first identified as part of the *Fgf-8* synexpression group in the *Xenopus* midbrain-hindbrain organizer (xIsm) [69]. Although Isthmin exhibited a distinct and complex expression pattern, it was postulated to be involved in the same biological process as Fgf-8 and other members of this group. This protein was secreted as a 60 kDa protein with a centrally located thrompospondin type 1 repeat (TSR) domain and a C-terminal domain called adhesion-associated domain in MUC4 and other proteins (AMOP). Lawler and Hynes originally described the TSR domain to be involved in cell-cell and cell-matrix interactions[70] and ever since, TSR domains have been observed in a wide number of secreted as well as transmembrane proteins including TSP1 and TSP2, F-spondin family, the brain-specific angiogenesis inhibitor (BAI), members of the semaphoring 5 family and many others [71]. The TSR of these proteins contain specific conserved motifs that attribute to its functions in cell-cell and cell-matrix interactions. The AMOP domain is a predicted protein domain which contains eight invariant cysteine residues and like TSR, is mainly found in secreted and transmembrane proteins. Although the precise function of the AMOP domain is unknown, it is speculated to be involved in cell adhesion due to its presence in cell-adhesion molecules [72]. Besides the common cysteine residues, the AMOP in ISM contains a 'KGD' motif which is commonly found in many antagonists of platelet aggregation and involved in integrin-dependent cell adhesion and tumor metastasis [73-75] .

Sequence analysis revealed the mammalian orthologs of *xIsm*. Human ISM was found to share 78% overall amino acid identity with xIsm, sharing strong sequence conservation in the TSR domain (88%)[69]. Pera and colleagues studied the temporal and spatial distribution of *xIsm* RNA by whole-mount in situ hybridization in *Xenopus* embryos. During the neuronal stage, *xIsm* was found to be highly expressed in the isthmus organizer, with additional expression being observed in the paraxial mesoderm and neural folds in the tail bud stage as well as in notochord in the neuronal stage. Work by our lab on zebrafish established that similar to Xenopus, *ism* is expressed at high levels in the developing midbrain-hindbrain

boundary and notochord [76]. The notochord is known to play an important role in zebrafish development including vascular development. Morpholino knockdown of the *ism* gene in zebrafish embryos resulted in obvious patterning defects of the trunk intersegmental vessels (ISV). In contrast, early blood vessel formation through vasculogenesis such as the dorsal aorta and axial vein were not affected, which implies that *ism* may specifically influence angiogenesis.

We identified that ISM is an endogenous angiogenesis inhibitor. Recombinant mouse ISM inhibits angiogenesis both *in vitro* and *in vivo* [76]. The full-length ISM suppressed EC capillary network formation on Matrigel in a dose-dependent manner. However, this inhibition was observed only when exogenous ISM was added at earlier time points (0-2 h) following cell seeding, which suggested that ISM most likely influenced early stages of the *in vitro* angiogenic process. Through a domain deletion approach, the C-terminal AMOP domain alone was sufficient to inhibit tube-formation on Matrigel, suggesting that the anti-tube formation activity of ISM is largely mediated by the AMOP domain [76].

ISM inhibited VEGF, bFGF as well as serum stimulated EC proliferation in a dose-dependent manner. In contrast, no significant effect on migration was observed. ISM also induced EC apoptosis in the presence of VEGF, bFGF or serum. At a concentration of 1 μM, ISM was able to induce apoptosis to the same extent as that induced by serum withdrawal. This apoptosis was found to be caspase-dependent as the Pan-caspase inhibitor Z-VAD-FMK abolished the effects of ISM on EC apoptosis. Caspase-3 was also found to be activated in a dose-dependent manner. ISM, however, had no effect on tumor cell apoptosis and induced only mild apoptosis of fibroblasts in culture. These results indicated an EC-specific effect of ISM. However, none of the ISM truncates could induce apoptosis which suggested that the full-length protein is required for this function [76].

ISM did not influence EC attachment and subsequent spreading on several matrix molecule-coated surfaces such as vitronectin, fibronectin, gelatin and diluted Matrigel. Interestingly, surfaces coated with recombinant ISM supported EC attachment and spreading in a manner similar to gelatin and fibronectin. With increasing evidence of the involvement of integrins in angiogenesis [77], interactions of ISM with EC surface integrins were studied. The most prominent integrins expressed by ECs are αvβ3, αvβ5 and β1 integrins. ISM was found to bind exclusively to αvβ5 integrin. Indeed, knocking down β5 in ECs abolished the death-promoting and antiangiogenic function of ISM [78].

Recombinant ISM inhibited VEGF/bFGF-induced *in vivo* angiogenesis in a modified Matrigel plug assay. However, under the same experimental condition, the C-terminal AMOP domain alone was unable to exert antiangiogenic effects. This result differs from the *in vitro* EC tube-formation result where the AMOP domain alone (ISM-C) is sufficient for suppression. The discrepancy may be due to the instability of this truncate *in vivo*. In a pathological setting, B16 melanomas stably overexpressing ISM showed significantly reduced xenograft tumor growth. Analysis of vascular density and tumor cell apoptosis in the tumors showed a marked reduction in vascularisation of the tumor and an increase in the rate of apoptosis. Increased apoptosis as well as the reduction is vascular density would contribute to the reduction in tumor size and volume in ISM-overexpressing B16 melanomas.

From previous studies, it is evident that ISM inhibits angiogenesis through its effects on EC survival. Current studies in our lab characterize ISM as a matricellular molecule which has differential effects on EC survival, based on its immobilization status. Similar to vitronectin (an abundant matricellular protein), ISM, when immobilized, promotes EC

attachment, integrin clustering, FAK activation and thus EC survival, functioning as an αvβ5 agonist. However, the soluble, non-immobilized form of ISM induces EC apoptosis and suppresses EC proliferation through its membrane receptor αvβ5, thus functioning as an antagonist [78]. This study opens up the possibility that endogenous angiogenesis inhibitors may have contrasting functions depending on their physical states *in vivo*.

2.4. β2-glycoprotein-I (β2GPI)

The discovery of β_2GPI as a novel angiogenesis inhibitor is an interesting story in itself. It reminds us of the discovery of the most well studied endogenous inhibitors angiostatin and endostatin by Judah Folkman's lab [9, 10]. These discoveries were based on the hypothesis that primary tumors secrete antiangiogenic peptides to inhibit the distant metastasis [79]. However, once the tumor is resected from the primary site, distant metastases grow up in due course due to the absence of such inhibitory factors [1]. β_2GPI's discovery as an antiangiogenic molecule is a result of the above observation in bladder cancer [80]. Transition cell carcinoma of the bladder turns invasive after complete transurethral excision from the primary site. The recurrence rate of the transition cell carcinoma is up to 70% of all the cases [81].

Beecken and colleagues were interested in isolating the possible endogenous antiangiogenic inhibitor that helps bladder cancer in maintaining this "tumor dormancy". Using selective *in vivo* passaging, they developed a variant of UMUC-3 human transitional cell carcinoma cell line. The tumor originating from this cell line (UMUC-3i) was able to suppress the growth of any secondary implant on the opposite flank in mice [82]. Hence, systematic analysis of the secreted proteome from UMUC-3i was undertaken to investigate the reason for this antitumorigenic property. Using reversed-phase high-performance liquid chromatography (HPLC), they checked the ability of each fraction to inhibit Human Umbilical Vein Endothelial Cell (HUVEC) proliferation. By performing mass-spectrometry (MS) and N-terminal amino acid sequencing of the fraction that potentially inhibited the EC proliferation, they identified that it was β_2GPI which conferred UMUC-3i its antitumorigenic property.

β_2GPI is a 50 kDa lipid binding monomeric glycoprotein [83] which is abundantly present in plasma (200 μg/ml) . About 40% of the total β_2GPI in the plasma are bound to lipoproteins and hence also known as apolipoprotein H [84]. Human β_2GPI gene was mapped to chromosome 17 [85]. This heavily glycosylated 326 amino acid long protein has 5 domains [83]. The first four domains are referred to as sushi domains. Each sushi domain comprise of 60 amino acids with highly conserved cysteine and proline residues. The fifth domain is known as kringle domain. It is 82 amino acid long with a positively charged phospholipid binding sequence (^{281}CKNKEKKC^{288}) [86]. It can bind to many negative charged entities including heparin [87, 88], anionic phospholipid vesicles [86, 87, 89, 90], platelets [91] etc. The C-terminal end of the 5th domain is surface exposed and is susceptible to cleavage by proteinases such as plasmin [92], Factor XI [93] etc. These proteinases cleave β_2GPI in the 5th domain at K^{317}-T^{318} [94] which shuts off β_2GPI's lipoprotein binding ability. β_2GPI is also classified under the complementary control protein superfamily due to the presence of the characteristic five short consensus repeats [95].

The physiological function of β_2GPI is still unclear. Many reports suggest that β_2GPI is a regulatory protein of the blood coagulation system. β_2GPI exhibits both pro- and anti-coagulant functions. There was a marked decrease in plasma thrombin generation from the β_2GPI null mice *in vitro* [96]. β_2GPI seems to interfere with the coagulation cascade at various steps. The anti-coagulant properties of β_2GPI are conferred by its inhibitory action on the prothrombinase activity of platelets [97], downregulating the release of serotonin during platelet aggregation [98], impedes the generation of factor Xa by activated platelets [99]. Its pro-coagulation action is brought about by inhibiting the anti-coagulant activity of activated protein C. Even though β_2GPI has both pro- and anti-coagulatory outcomes, all are brought about by the same mechanism of competing with phospholipid binding activity on the cell surface. However, β_2GPI is better known for its pathophysiological function than its physiological functions. β_2GPI is the primary target antigen in the auto-immune disorder called anti-phospholipid syndrome (APS)[100]. APS causes frequent recurrent thrombosis, miscarriage, thrombocytopenia, haemolytic anaemia etc [101].

Further exploration into its vessel inhibiting property lead to the discovery that it's not the native β_2GPI (nβ_2GPI) but only the clipped form (cβ_2GPI) which is able to mediate the angiostatic function [80]. Also, they found out that cβ_2GPI accomplishes its action on ECs through annexin II cell surface receptor. Subsequent *in vivo* studies by Sakai and colleagues assessed the ability of both nβ_2GPI and cβ_2GPI in inhibiting VEGF-dependent neovascularization in subcutaneously implanted Matrigel and Gelfoam plugs [102]. They found that both nβ_2GPI and cβ_2GPI can suppress new blood vessel formation. The ability of the β_2GPI to act as a tumor suppressor also was assessed using murine prostate cancer model. Endogenous angiogenic inhibitor was delivered intraperitoneally and subcutaneously through Alzet osmotic pump. Surprisingly, only cβ_2GPI and not nβ_2GPI could exert significant reduction in tumor size. Authors hypothesize that this discrepancy between Matrigel and osmotic pump based assay can be a result of difference in the proteolytic status of β_2GPI. In Matrigel, nβ_2GPI might get cleaved into cβ_2GPI due to localized production of plasmin. This in turn induces antiangiogenic effect. However, in the case of osmotic pump, the nβ_2GPI is delivered directly into the body at a distant place from the incision and hence less chances of plasmin led proteolytic cleavage [102].

Further investigations revealed that cβ_2GPI can bind to plasminogen [102]. This in turn can lead to the inhibition of plasmin production. Since plasmin is also known to initiate angiogenesis, inhibiting its formation can lead to an indirect inhibition of neovascularization [103]. In order to further understand the domains responsible for this antiangiogenic effect and the downstream regulators, Yu *et al.,* employed a domain deletion strategy and investigated the possible downstream regulators of β_2GPI [104]. They created two domain deletions in this five domain protein. One lacks the first domain (DII-V) and the second lacks the last domain (DI-IV). They showed that the first domain is absolutely essential for the inhibition of VEGF and FGF mediated EC proliferation, tube formation and migration. One contrasting observation in this independent study to the earlier study is that both the nβ_2GPI and cβ_2GPI could mediate antiangiogenic function. This discrepancy is hard to explain. One possible reason might be the difference in the native conformation after the protein purification. Another reason can be that the proteinase secreted from ECs might cleave nβ_2GPI on the cell surface thus leading to antiangiogenesis. Also, the possibility of nβ_2GPI being antiangiogenic at higher molar concentration cannot be excluded.

The molecular mechanism of action of this protein on VEGF/bFGF-stimulated ECs was explored. Upon β_2GPI treatment in the presence of VEGF/bFGF, it down-regulates VEGF receptor-2 (VEGFR2), but not VEGF receptor-1 (VEGFR1) at the transcriptional level. VEGFR1 is involved in recruitment of hematopoietic precursors and migration of lymphocytes such as monoctyes and macrophages whereas VEGFR2 play an important role in vascular and lymph ECs function such as proliferation, migration etc [105, 106]. This gives a hint on why there is a down-regulation of VEGFR2. In addition, Yu and colleagues explored the plausible regulators of the VEGFR2 mediated pathways. The inhibition of EC proliferation and migration can result from deregulation of downstream signaling in MAPK/ERK [107, 108]. Upon treatment with β_2GPI on VEGF/bFGF stimulated ECs, there was a decrease in phosphorylated ERK compared to control. Also, VEGF and bFGF are known to activate phosphotidylinositol 3-kinase (PI3K) through phosphorylation. This kinase can subsequently activate its downstream regulators such as Akt and Glycogen synthase kinase-3β (GSK-3β) [109]. These downstream regulators are essential for preventing cyclin D1 degradation [110, 111]. Hence, β_2GPI conveys cell survival signals and forms the main component of the PI3K/Akt/GSK-3β pathway. β_2GPI also leads to the decrease in phosphorylated PI3K, Akt and GSK-3β. As a result of down-regulation of these key players EC apoptosis takes place.

Nakagawa and colleagues discovered one of the binding partners of cβ2GPI – angiostatin4.5 (AS4.5) [112]. AS4.5, one of the angiostatin isoforms, possesses the first four kringle domains as that of parental anigostatin but differs in its 5th kringle domain. AS4.5 results from the proteolytic cleavage in the 5th kringle domain [113]. This cleavage is a result of autoproteolysis [114, 115] or by proteinases such as MMP-3,-7, -9 and -12 [116-118]. In spite of the cleavage at the 5th kringle domain, AS4.5 retains 85% of this domain [113]. AS4.5 is the only isoform of angiostatin found in plasma and it interacts with the cβ2GPI [119][105]. The interaction between AS4.5 and cβ2GPI was found to be comparable with that of the cβ2GPI and plasminogen. The dissociation constant between cβ2GPI and AS4.5 was found to be 3.27 X 10^5 M^{-1}. This interaction abolished the antiangiogenic function of the AS4.5. It neutralized the anti-proliferation, anti-migration and inhibition of ECs upon treatment with cβ2GPI and AS4.5 in vitro. It is interesting to note that nβ2GPI does not interact with the AS4.5. Dose dependent decrease in angiogenesis inhibition was observed when cβ2GPI was premixed with AS4.5 in the angioreactor tubes which were implanted in the 6-8 week old athymic nude female mice [112]. The results of this study are quite surprising due to the fact that it shows that one endogenous inhibitor (cβ2GPI) can attenuate the function of another (AS4.5) in physiological scenario, thus re-emphasizing the complex physiological functions and interactions of the various endogenous inhibitors.

Passam and colleagues further investigated the angiogenic potential in mice lacking β2GPI [120]. They performed angiogenic assays that included angioreactor implantation and tumor growth studies. In β2GPI knockout mice, there was a significant increase in the microvascular density in the Matrigel mixed with VEGF implanted in β2GPI knockout mice ($\beta2GPI^{-/-}$) compared to $\beta2GPI^{+/+}$ mice. In addition, mouse tumorigenesis assay showed that $\beta2GPI^{+/+}$ mice could suppress tumor growth compared to $\beta2GPI^{-/-}$ mice when subcutaneously injected with B16F10 mouse melanoma cells. Intriguingly, subcutaneous injection of nβ2GPI through an osmotic pump did not confer any survival benefit to $\beta2GPI^{-/-}$ mice injected with B16F10 mouse melanoma cells. This conflicting result might be due to the inability of these mice to cleave the nβ2GPI to generate sufficient amount of cβ2GPI in such a short time *in*

vivo [120]. All of the above data clearly suggest a possibility of β2GPI being exploited in treating excessive angiogenesis related complications such as cancer.

2.5. ADAMTS: A Family of Antiangiogenic Metalloproteinases

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) is a family of multidomain, extracellular metalloproteinases. The family comprises of 19 members in humans [121]. The metalloproteinase domain shares structural similarity with metalloproteinase domain of reprolysin, a snake venom proteinase. Thus they have been classified into a subgroup called M12B ADAM [122]. They all have the following domains in the order from N-terminus: i) A signal peptide that directs the protein into endoplasmic reticulum and secretary pathway; ii) a prodomain which helps in maintaining enzyme latency (with the exception of ADAMTS7 and ADAMTS13) [123, 124]. This prodomain is usually cleaved by furin and/or other proprotein convertases [125]. iii) a metalloproteinase domain with a zinc ion covalently bound to conserved histidines and an aspartate residue in the catalytic pocket. This is characteristic of reprolysin-type zinc binding motif [126]. In addition, this domain possesses a 'met-turn' downstream of these histidines, characterized by the presence of a methionine residue. This turn helps in maintaining the structural integrity of these metzincins [127]. The domains C-terminal of the metalloproteinase domain are referred to as ancillary domains. These domains regulate the interaction of ADAMTS with its substrate in the ECM. iv) a disintegrin domain shares sequence similarity with the disintegrin domain of the ADAM family. They are known to interact with integrin. However, in the case of ADAMTS this is a misnomer. There is no evidence till date of any ADAMTS interaction with integrin through its disintegrin domain. This is because ADAMTS lacks the signature RGD integrin recognition sequence [128]. v) a central thrombospondin type 1 repeat (TSR). This domain possesses sequence similarity of type 1 repeats of TSP1 and TSP2. vi) a cysteine-rich region which as the name suggests is rich in cysteine residues. vii) the spacer region. The cysteine rich and spacer regions are the main domains binding to ECM. viii) At the C-terminal end of the ADAMTS, there are variable numbers of TSRs. ADAMTS4 lacks this C-terminal TSR whereas ADAMTS9 and ADAMTS20 have 14 TSRs [121].

Metalloproteinases were thought to promote angiogenesis by mediating vascular basement degradation and remodeling of the ECM to pave the way for the new angiogenic sprouts [129]. However, now it is now clear that metalloproteinases can have diverse roles in angiogenesis. Several members of the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) families are known to contribute to angiostatic action through cleaving and release of endogenous angiogenic inhibitors from the ECM [130, 131]. For ADAMTS, the presence of a central TSR domain and variable number of C-terminal TSRs in all 19 members offered potential antiangiogenic tendencies. TSR domain is a well-established antiangiogenic domain mediating angiostatic functions in TSP1 and others [132, 133]. Based on the hypothesis that TSR containing proteins might possess antiangiogenic function, Iruela-Arispe's lab identified ADAMTS1 and ADAMTS8 as antiangiogenic proteins [134]. In recent years, more and more members of this family are being demonstrated to possess angiostatic function thus making it a family of novel endogenous antiangiogenic proteinases [135, 136]. However, it is important to note that the mechanism of action for its angio-inhibitory function is not the same for all ADAMTS members. A schematic diagram

showing the structural organization of the currently known angiostatic ADAMTS members are depicted in Figure 1.

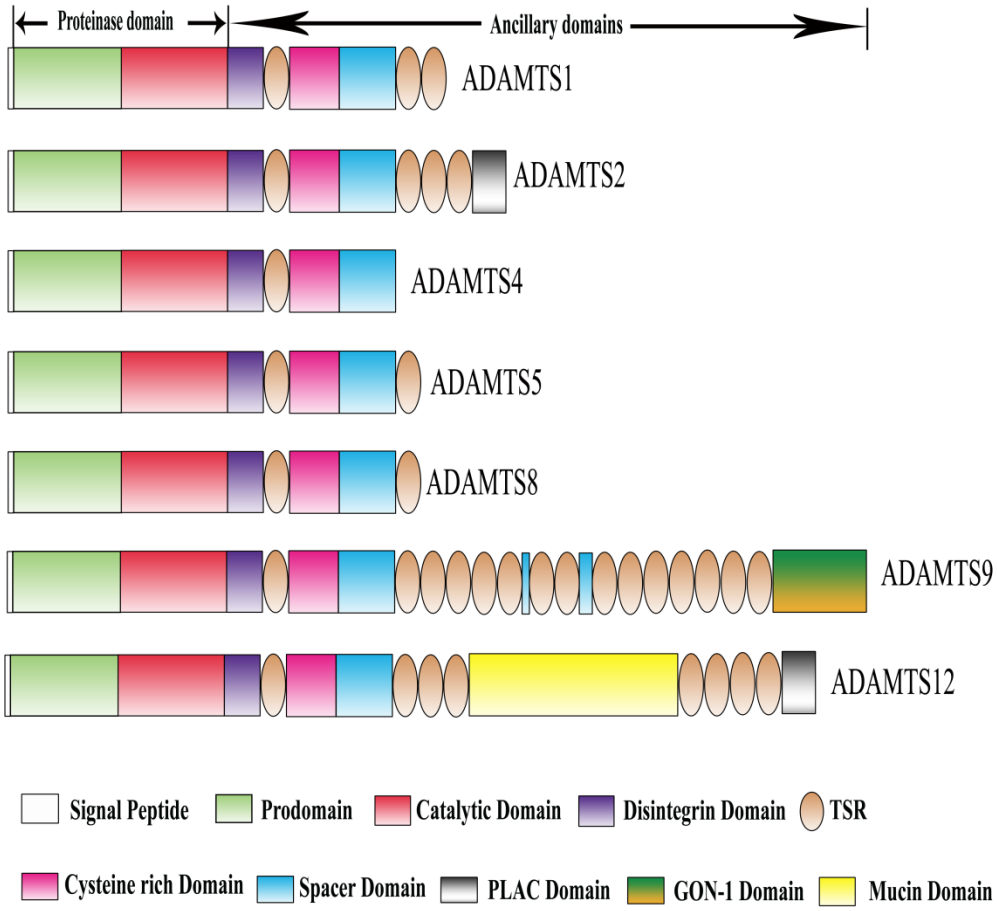

Figure 1. Domain structure of ADAMTS family members involved in regulating angiogenesis.

2.5.1. ADAMTS1

ADAMTS1 is the extensively studied molecule in the family for its antiangiogenic and antitumorigenic behavior. It is also known as METH1- a metalloproteinase with thrombospondin domains. This protein has a central TSR and two C-terminal TSRs. It undergoes prodomain cleavage mainly mediated by furin and also by other protein convertases [125, 137]. Additional processing at its C-terminal end in the spacer region also occurs [137]. ADAMTS1 is localized in the ECM with the help of its spacer and C-terminal TSR domains [138]. *Adamts1* knockout mice studies lead to the discovery of its importance in development and the urogenital system [139-141]. Northern analysis of human adult tissues shows high expression of *ADAMTS1* in adrenal, heart and placenta. This gene is also expressed at a high level in kidney in embryos [134]. However, expression data in various tumor tissues shows downregulation of this gene. For example, ADAMTS1 is down-regulated

in breast [142], prostate [143, 144] and pancreatic cancer [145]. Aberrant methylation of this gene is observed in lung cancer [146] and colorectal cancer [147].

ADAMTS1 is known to cleave proteoglycan substrates such as aggrecan [148, 149] and versican [150, 151]. Proteomic analysis using differential gel electrophoresis of conditioned medium isolated from mammalian cells transfected with ADAMTS1 revealed 5 possible additional substrates: basement membrane proteins nidogen-1, nidogen-2, desmosomal protein desmocollin-3 and the ECM glycoproteins such as dystroglycan-1 and Mac-2-binding protein [152].

Vasquez and colleagues showed that ADAMTS1 is antiangiogenic *in vitro* and *ex vivo*. Recombinant ADAMTS1 inhibited human dermal EC proliferation *in vitro*. It also suppressed bFGF induced neo-vascularization in the cornea pocket assay and CAM assay [134]. Most importantly, the potency of ADAMTS1s antiangiogenic effect was far greater than endostatin or TSP1 at the same molar concentration [134]. This discovery was quite surprising since most MMPs and ADAMs are proangiogenic due to their metalloproteinase activity. One possible explanation was that auto-catalytic cleavage of ADAMTS1 resulted in the segregation of its antiangiogenic C-terminal TSRs from the metalloproteinase domain. Iruela-Arispe's lab showed that the angiostatic function of ADAMTS1 is due to its C-terminal TSRs [153]. ADAMTS1 can bind to VEGF through its C-terminal region, thereby inhibiting angiogenesis by VEGF sequestration and suppression of VEGF downstream signaling. This was evident by the decreased phosphorylation of VEGF receptor2 (VEGFR2) and decreased EC proliferation. They also showed that ADAMTS1 can bind to $VEGF_{165}$ but not to $VEGF_{121}$ through cross-linking and co-immunoprecipitation studies. $VEGF_{121}$ lack the heparin interacting region that is present in its longer isoform $VEGF_{165}$. Collectively, these results confirm that ADAMTS1 suppress angiogenesis by regulating the bioavailability of VEGF through its C-terminal region [153].

In vivo tumorigenesis studies in mice by overexpressing the C-terminal region of ADAMTS1 containing the central and C-terminal TSRs in Chinese hamster ovary (CHO) led to the suppression of tumor growth [154]. In addition, overexpressing the C-terminal truncate reduced the metastatic potential of CHO cells. Perplexingly, this group also observed that overexpression of full-length ADAMTS1 did not suppress tumor growth *in vivo* [154]. To add to the complexity, the C-terminal ADAMTS1 enhanced metastatic potential in spite of its tumor growth suppression function. The intriguing behavior of ADAMTS1 in tumorigenesis and metastasis was clarified in a detailed study by Liu and colleagues [155]. They overexpressed full-length ADAMTS1, N-terminal and C-terminal truncates as well as the catalytically inactive mutant of ADAMTS1 (E386Q) in TA3 mammary carcinoma and Lewis lung carcinoma (LLC) respectively. Only the cell lines overexpressing catalytically active ADAMTS1 promoted lung metastasis. The cell lines overexpressing catalytically inactive mutant, N-terminal and C-terminal truncates suppressed metastasis. Immunohistochemical studies of the lung tumor showed that except for the catalytically active ADAMTS1, the rest of the truncates retained their antiangiogenic potential. This in turn led to decreased tumor cell proliferation and induced cell death [155]. They further demonstrated that ADAMTS1 acts as a sheddase and helps in cleaving two transmembrane precursors of the epidermal growth factor family that bind to heparin: amphiregulin (AR) and heparin-binding epidermal growth factor (HB-EGF) [155]. This leads to increases in the bioavailability of these growth factors, activation of EGFR and ErbB-2 receptor and hence the enhancement of tumor cell proliferation. In contrast, the catalytically-inactive ADAMTS1-E/Q mutant as well as N-

terminal and C-terminal truncates suppressed tumor metastasis and tumor angiogenesis. This study clearly demonstrated the contrasting functions of the catalytically-active ADAMTS1 and its catalytically-inactive mutant or truncates in tumor angiogenesis and metastasis. Therefore, whether ADAMTS1 promotes or suppresses tumorigenesis/metastasis would depend on the net balance of its full-length vs. proteolytically cleaved fragments in a particular tumor environment.

The complexity of ADAMTS1 in cancer is further exemplified by several cancer studies described below. ADAMTS1 was shown to promote tumor development in an epithelial tumor setting [156]. Overexpression of ADAMTS1 in bronchial epithelial tumor cell line (BRZ) did not inhibit angiogenesis in an *in vitro* rat aorta ring assay, however, there was an increase in tumor growth of the ADAMTS1 overexpressing BRZ cells in immunodeficient mice. This enhanced tumor growth was attributed to the increase in stromal reaction in ADAMTS1 overexpressing tumor milieu. This stromal reaction includes fibroblasts recruitment and collagen deposition. ADAMTS1 upregulates several factors that are involved in recruiting myofibroblasts including MMP-13, fibronectin, IL-1β etc. On the other hand, Gustavsson and colleagues presented that overexpression of ADAMTS1 is antiangiogenic and antitumorigenic in an experimental model of prostate cancer [157]. When ADAMTS1 was knocked-down in an ADAMTS1 overexpressing human prostate cancer cell line (LnCaP), blood vessels with smaller diameters were generated accompanied by a reduction of TSP1, a sign of more aggressive prostate cancer. In another study, ADAMTS1 was shown to play a critical role in conferring tumor plasticity [158]. This tumor plasticity helps the tumor cells to acquire endothelial-like phenotype and hence promoting tumor progression. This study cautions us against the classic view that tumor neovascularization can arise only from the pre-existing vessels through angiogenesis. Alternative mechanism of neovascularization through tumor cell adoption may influence tumor angiogenesis and progression. Indeed, a recent study of human glioma proves that cancer stem-like cells can differentiate into tumor ECs and contribute to tumor vessel formation [159].

The function of ADAMTS1 in angiogenesis is further complicated by the discovery that TSP1 and TSP2 are also substrates of ADAMTS1 [160]. Nevertheless, cleavages of TSP1/TSP2 did not result in loss of their antiangiogenic functions. Instead, it releases the antiangiogenic TSR peptides from the trimeric parent molecule and enhances its antiangiogenic function. Using *Adamts1* knockout mice, they further showed that the absence of ADAMTS1 lead to a delay in wound healing accompanied by the absence of ADAMTS1-mediated release of antiangiogenic fragments from TSP1 and TSP2 and an increase in blood vessel density [160]. This result again leads to the question of whether ADAMTS1 is proangiogenic or antiangiogenic. Furthermore, how an increase in angiogenesis could suppress wound healing is puzzling. Therefore, the function of ADAMTS1 in wound healing and its relationship with TSP1 require further investigations. Whether proteolytic fragments of ADAMTS1 were generated in wound healing have not been explored. In another study, Xu and colleagues showed that ECs upregulate ADAMTS1 expression upon treatment with VEGF both *in vitro* and *in vivo* [161]. They claim that overexpression of ADAMTS1 might be a possible feedback mechanism of regulating angiogenesis by ECs.

In conclusion, ADAMTS1 plays a key role in angiogenesis. However, whether it is proangiogenic or antiangiogenic depends on various factors including the type of tissue, the type of extracellular milieu, availability of the substrates, the extent to which it undergoes autocatalytic cleavage etc. The antiangiogenic function is a result of both metalloproteinase

dependent and independent mechanisms. The catalytic domain can cleave TSP1 and TSP2 resulting in the enhanced bioavailability of angiostatic peptides. The metalloproteinase independent mechanisms involve the C-terminal ancillary region and TSRs.

2.5.2. ADAMTS2

ADAMTS2 is involved in procollagen processing. This 135 kDa protein undergoes furin processing to generate the active 105 kDa proteinase. It possesses a central TSR and three C-terminal TSRs. A proteinase and lacunin domain (PLAC) is embedded at the C-terminal end of ADAMTS2. ADAMTS2 is an aminoprocollagen peptidase that can cleave procollagen type I, II, III and V [162-164]. It is expressed in skin, lung, aorta etc and gene knockout study indicated that it is the main procollagen 1 N-proteinase in the skin [165, 166]. Mutation in ADAMTS2 in humans is implicated in a connective tissue disorder called dermatosparaxis also known as Ehlers-Danlos syndrome [167]. This disease is a result of accumulation of unprocessed aminoprocollagen leading to skin fragility [165].

Recently, its role in antiangiogenesis was explored using mouse xenograft model [136]. This study was based on the hypothesis that TSRs of ADAMTS2 are antiangiogenic. Interestingly, unlike ADAMTS1, the antiangiogenic effect of ADAMTS2 was independent of its catalytic function. Mammalian-expressed ADAMTS2 inhibited angiogenesis *in vitro* in various aspects such as EC capillary tube formation, proliferation, altered cytoskeletal structures and induced apoptosis. In addition, it also inhibited the formation of vascular sprouts in embryoid bodies produced from undifferentiated embryonic stem cells upon induction by VEGF [136]. Using a model of choroidal neovascularization following LASER burn injury, ADAMTS2's antiangiogenic potency in forming new blood vessels was assessed in *Adamts2* knockout mice. There was a significant increase in corneal angiogenesis in *Adamts2* null mice compared to the wild type. Furthermore, overexpressing ADAMTS2 and its domain deletion constructs in HEK293-EBNA cells followed by xenograft assay in nude mice showed that the C-terminal TSRs are responsible for its antitumorigenic and antiangiogenic function. More importantly, the catalytically inactive ADAMTS2 also retains this antiangiogenic ability. This proves that the catalytic activity of this metalloproteinase is not required for its antiangiogenic function [136]. ADAMTS2 was shown to reduce ERK1/2 and FAK phosphorylation in HUVECs. Localization studies of recombinant ADAMTS2 interaction with HUVECs revealed that this proteinase binds to the cell surface and may interact with a cell surface receptor. Using ADAMTS2 as the bait and EC membrane fraction proteins as the source, nucleolin was identified as its receptor [16].

There are still many questions that need to be answered about ADAMTS2. For example, what is the expression spectrum of ADAMTS2 in human cancers and other angiogenesis-related diseases and what roles does it play in these diseases? Does it play a role in metastasis? In addition to corneal angiogenesis, what other physiological angiogenesis this proteinase is involved? What is the relationship between its procollagen proteinase activity and the proteinase-independent antiangiogenic function?

2.5.3. ADAMTS4

ADAMTS4, popularly known as aggrecanase-1 due to its up-regulation in cartilage degenerative diseases such as osteoarthritis and rheumatoid arthritis, is the only member in the family that possess a single TSR – the central TSR [168-170]. This secreted extracellular metalloproteinase also undergoes furin mediated zymogen activation and C-terminal autocatalytic cleavage [171, 172]. In addition to aggrecan, this proteinase is also involved in

brevican and versican cleavage [150, 173]. *Adamts4* knockout mice presented no phenotypic deformities and were not susceptible to experimentally induced arthritis [174]. It's possible that other members of ADAMTSs compensate for the loss of aggrecanase-1 in mouse. This gene is known to be up-regulated in human glioblastomas, helping the cancer cells to invade and metastasize [175]. Adamtsostatin-4, a peptide derived from central TSR of ADAMTS4 is shown to be antiangiogenic *in vitro* [176]. It inhibited *in vitro* HUVEC cell proliferation and migration. Ongoing work in our lab has shown that the single central TSR of ADAMTS4 is antiangiogenic *in vivo* in a mouse tumor xenograft model. However, the full-length metalloproteinase is proangiogenic and pro-tumorigenic, similar to ADAMTS1 (Ge lab, unpublished data). What is the role of ADAMTS4 in human cancer and other angiogenesis-related diseases remain to be determined.

2.5.4. ADAMTS5

Also known as aggrecanase-2, ADAMTS11 and implantin in mouse, ADAMTS5 was established to be the major aggrecanase from studies of *Adamts5* single and *Adamts4 /Adamts5* double knockout mice [177-180]. This metalloproteinase comprises of one central TSR and one C-terminal TSR. ADAMTS5 also undergoes zymogen activation mediated by furin and other protein convertases which cleaves the prodomain to release the active enzyme [181]. Expression pattern studies in mice showed that ADAMTS5 is expressed in nerve tissue and thus could regulate neuronal development. In adult mouse, it is expressed mostly in smooth muscle cells, suggesting a possible role in versican degradation [182]. In addition, it cleaves the brain proteoglycan, brevican [183]. Therefore, ADAMTS5 is not only a major aggrecanase, but also a proteoglycanase with a broader spectrum of substrates. This proteinase is also up-regulated in glioblastomas. Overexpression of this proteinase promoted glioma cell invasion in vitro [183]. On the other hand, we have previously demonstrated that the central TSR of ADAMTS5, but not the C-terminal TSR, is antiangiogenic *in vitro* [184]. Work in our lab also indicates that the full-length ADAMTS5 is also antiangiogenic and antitumorigenic *in vivo* (unpublished data). These data suggest that overexpression of ADAMTS5 might regulate angiogenesis and have a tumor-protective function. Thus, the role of ADAMTS5 in human cancer and other angiogenesis-related conditions warrant further investigation.

2.5.5. ADAMTS8

ADAMTS8, also known as METH2 in humans, is a zinc metalloproteinase having a central TSR and one C-terminal TSR [185]. It shares high homology and domain structure with ADAMTS5. This gene was mapped to chromosome 9 in mouse and chromosome 11 in humans [185]. Northern analysis of *Adamts8* showed a low expression during embryonic development and in adult mouse tissues. This protein is highly expressed in the human heart and lungs [185]. Multiple tissue expression array mRNA dot-blot confirmed the narrow tissue distribution of *ADAMTS8* with high expression in lung and aorta [186]. ADAMTS8 is an aggrecanase that can cleave aggrecan *in vitro* and its expression is up-regulated in the articular cartilage of osteoarthritic patients [186].

Parallel studies undertaken by Vasquez *et al.,* showed that METH2 is also antiangiogenic, similar to METH1 [134]. Recombinant ADAMTS8 inhibits FGF-2 induced CAM angiogenesis and cornea pocket assay. Although ADAMTS8 is not as potent as ADAMTS1, both of these proteinases are more potent than endostatin and TSP1 at same

molar concentration [134]. This gene was down-regulated significantly in breast carcinoma compared to the non-neoplastic breast tissue, suggesting a possible tumor suppressor role [187]. ADAMTS8 was down-regulated in most of the brain tumor tissues, affirming a possible tumor suppressor role of this gene. In addition, promoter hypermethylation was also observed in gliomas [188]. All these studies suggest that ADAMTS8 is a potent antiangiogenic metalloproteinase with tumor-protective function. Surprisingly, there are no publications reporting its role in *in vivo* angiogenesis up to now. *In vivo* studies are critical to understand its function in angiogenesis or antiangiogenesis.

2.5.6. ADAMTS9

ADAMTS9 is also an extracellular metalloproteinase comprising of an extra C-terminal GON-1 domain which shares homology with the *gon-1* gene of *C.elegans* [189]. *gon-1* is responsible for the gonadal development and hence the name. ADAMTS9 possesses fourteen C-terminal TSRs, the highest in the ADAMTS family, similar to ADAMTS20 [190]. ADAMTS9 also undergoes zymogen to proteinase conversion mediated by protein convertases such as furin at the cell surface [190, 191]. Surprisingly, this cleavage results in reduction of its catalytic activity which might be a regulatory mechanism for the enzyme activity [191]. Northern blot analysis in mouse showed that *Adamts9* is highly expressed in heart, placenta and skeletal muscle [190]. *In situ* hybridization studies in mouse showed that this gene is expressed during skeletogenesis in adult tissue and most importantly in vascular endothelium [192]. This metalloproteinase is also able to cleave versican and aggrecan [190]. *Adamts*9 is thought to be a tumor suppressor gene in esophageal carcinoma and nasopharyngeal carcinoma [193]. It is epigenetically silenced in these cancers through promoter hypermethylation [194].

ADAMTS9 is a cell-autonomous antiangiogenic metalloproteinase [135]. *Adamts*9 knockout mice resulted in embryonic lethality and die before gastrulation. Hence Apte's group studied it in *Adamts9*$^{+/-}$ mice congenic with the C57Bl/6 mice [135]. Spontaneous corneal opacity and neovascularization was observed in 80% of the *Adamts9*$^{+/-}$ mice after 25 weeks. In addition, irregular pupil and disorganized corneal stroma were observed. Immunohistochemistry showed a distinct capillary EC staining in various adult mouse tissues. In addition, there is an increased vasculature in xenograft tumor in *Adamts9*$^{+/-}$ mice compared with the wild type C57Bl/6 mice, indicating an antiangiogenic function of this gene. Furthermore, siRNA mediated knockdown of ADAMTS9 in ECs enhanced the angiogenic response of these cells *in vitro* with a significant increase in capillary tube-like structure formation, migration and microvascular spreading. Similar antiangiogenic function was observed from ECs of various human origin such as microvasculature of dermis, heart and umbilical vein. The expression of this antiangiogenic metalloproteinase in the endothelium suggests a possible negative regulatory mechanism to control angiogenesis.

As mentioned above, ADAMTS9 was down-regulated in esophageal and nasopharyngeal cancer and hence thought to be a tumor suppressor gene. Very recently, Lo and colleagues found that it is the antiangiogenic function of this metalloproteinase that is leading to its tumor suppressive function in a mouse xenograft model [195]. Hence ADAMTS9 is an antiangiogenic metalloproteinase with tumor-suppressive function.

However, the antiangiogenic mechanism of ADAMTS9 is different from that of ADAMTS1, ADAMTS2 or ADAMTS8. The metalloproteinase activity of ADAMTS9 is essential for its antiangiogenic function. Catalytically-inactive ADAMTS9 (E435Q) loses the

antiangiogenic function. Unlike ADAMTS1, ADAMTS9 does not cleave TSP1 or TSP2. In addition, the C-terminal ancillary domains of ADAMTS9 do not sequester VEGF thus excluding the possibility of these domains in antiangiogenesis through VEGF sequestration. Thus, ADAMTS9s antiangiogenic function is due to its metalloproteinase activity [135]. Further studies are required to unravel the molecular mechanisms of this angiostatic metalloproteinase.

2.5.7. ADAMTS12

ADAMTS12 is a 175 kDa secreted metalloproteinase with unknown biological function. This multi-domain protein comprises of eight TSRs arranged in three groups. One central TSR, three TSRs adjacent to the spacer region followed by a mucin domain that separates the four C-terminal TSRs. [196]. In addition, ADAMTS12 possess a proteinase and lacunin domain (PLAC) that is embedded in its C-terminal end and comprising of 6 conserved cysteine resudes. This domain is involved in epithelial cell morphogenesis in embryos and developing wings of the moth *Manduca sexta* [197]. However, the function of this domain in ADAMTS12 still remains unkown. ADAMTS12 gene maps to chromosome 5 in human [196]. It also undergoes furin based prodomain cleavage and additional processing at its C-terminal mucin domain resulting in two truncated fragments which separates the C-terminal TSRs from the rest of the ADAMTS12 proteinase. Northern blot analysis revealed that it is expressed largely in human fetal lungs but not other tissues. Studies have suggested its possible function in degrading cartilage oligomeric matrix protein (COMP) [198, 199]. COMP is a pentameric glycoprotein and forms a part of the non-collagenous component of cartilage. Degradation of COMP leads to degenerative diseases such as arthritis [200]. Interestingly, *Adamts*12 knockout mice presented no phenotypic abnormalities with a normal gestation, growth and fertility [201].

The antitumorigenic and antiangiogenic properties of ADAMTS12 were reported by Llamazares and colleagues [202]. Conditioned medium collected from ADAMTS12 overexpressing cells inhibited tubular structure formation of bovine aortic endothelial cells (BAEC) in 3D collagen gel. ADAMTS12 overexpressing A549 cells (human lung adenocarcinoma) showed a significantly reduced tumor growth [202]. In addition, overexpression of ADAMTS12 in Madin-Darby canine kidney (MDCK) cell line inhibited hepatocyte growth factor (HGF) mediated tumorigenic properties by interfering with the HGF signaling pathway. Overexpression of ADAMTS12 decreased the phosphorylation of ERK in MDCK cell line upon activation by HGF. Using domain deletion approach, the same group showed that the metalloproteinase and disintegrin domain devoid of ancillary domains cannot inhibit the HGF activation of MDCK cell line. This shows that the TSR containing ancillary domains play a key role in ADAMTS12s tumor-suppressor function.[202].

Even though *Adamts12*$^{-/-}$ mice had no apparent defects, they were prone to excessive angiogenesis related complications [201]. *Adamts12* deficiency led to an increased vasculature and increased tumor invasion into the host tissue when malignant keratinocytes were implanted. Angiostatic influence of ADAMTS12 in other pathological angiogenesis was confirmed using Matrigel and rat aortic ring assays. The catalytic activity of this metalloproteinase is not indispensable for its antiangiogenic function. Conditioned medium from MCF7 cell lines overexpressing either ADAMTS12 or the catalytically inactive mutant (H465Q/E466A) inhibited the capillary outgrowth from the Wistar rat aortic explants [196].

This study shows that ADAMTS12 is an endogenous angiogenesis inhibitor, which mediates its action independent of its catalytic activity similar to ADAMTS2.

ADAMTS12 was also shown to be a tumor suppressor gene in colon cancer [203]. Considering its antiangiogenic and antitumorigenic function, this is not surprising. Moncada-Pazos and colleagues showed that ADAMTS12 was epigenetically silenced in tumor cells by hypermethylation in CpG islands. Interestingly, they also observed a transcriptional activation of this tumor-protective gene in intratumor stroma. This might be a stromal attempt to contain primary tumor progression [203]. In summary, *Adamts*12 is a tumor-suppressor gene with angio-inhibitory function most likely mediated through its TSR containing ancillary domains.

Taken together, multiple members of the ADAMTS family of matrix metalloproteinase are endogenous antiangiogenic proteins. Although the mechanisms of action overlap in several members, it is not the same for all. While the antiangiogenic function is mediated through the metalloproteinase activity of ADAMTS9 [135], others carryout this function through their TSR domains such as ADAMTS2 [136], ADAMTS4 [204], ADAMTS5 [184], and ADAMTS12 [202]. In the case of ADAMTS1, both the metalloproteinase domain and the TSR ancillary domains contribute to its angiostatic function [153, 154, 160]. A few of the antiangiogenic ADAMTS members are also known to be tumor suppressors such as ADAMTS1, ADAMTS9 and ADAMTS12 [146, 194, 203], possibly through their antiangiogenic functions. Other ADAMTS such as ADAMTS15 and ADAMTS18 have also been characterized as tumor-suppressor genes [205, 206]. However, no studies have been undertaken to explore their antiangiogenic potential. It is likely that future studies will expand the list of ADAMTS members with antiangiogenic functions.

3. Cytoplasmic Angiogenesis Inhibitors

3.1. Integrin Cytoplasmic Domain Associated Protein-1 (ICAP-1)

ICAP-1, also known as ITGB1BP1 or Bodenin, is a phosphoprotein widely expressed during embryonic development [207]. Originally identified as an interaction partner of β1 integrin cytoplasmic domain, ICAP-1 protein in humans exists in two isoforms due to alternative splicing of the mRNA: a 200 amino acid ICAP-1α and a 150 amino acid ICAP-1β [208]. The carboxy-terminal 21 amino acid region has been shown to be crucial for the binding of ICAP-1α to β1 integrin. This region, which contains two conserved NPXY motifs, is absent in ICAP-1β. The cytoplasmic domains of integrins have important roles in ligand-binding affinity regulation and the localization of integrins to focal contacts. ICAP-1β is unable to bind to β1-integrins and its expression is low or absent in most cells when compared to the expression of ICAP-1α. The expression of ICAP-1α is ubiquitous, similar to the expression of its interacting partner β1 integrin. The presence of multiple Ser and Thr residues in ICAP-1α makes it a heavily phosphorylated protein. The phosphorylation status of ICAP-1α is regulated during cell-matrix interaction, i.e., phosphorylation is enhanced upon integrin-dependent cell adhesion on β1 substrates. However, the cellular mechanism that regulates the phosphorylation of ICAP-1α is still unclear.

Although detailed cellular functions have not been assigned to this protein, there are reports that it negatively regulates β1 integrin function. Overexpression of ICAP-1 led to a

reduction in cell adhesion and enhancement of cell migration on fibronectin surfaces, where β1 integrin activity is essential [209]. In a study by Millon and colleagues, ICAP-1 competed with TALIN1 for binding to the cytoplasmic domain of β1 integrin and thus inhibits focal adhesion assembly [209, 210]. TALIN is a large cytoplasmic protein which links the actin cytoskeleton to the ECM via interactions with integrin [210]. Another study suggests that ICAP-1 might act as a GDI (guanidine dissociation inhibitor), molecules which inhibit the small GTPase RAC1 and CDC42. This may also explain the spreading defects caused by ICAP-1 overexpression [209, 211]. In CHO cells, ICAP-1 functioned as an activator of cell migration which may be mediated by interaction with the small G-protein Ras-related protein-1 (RAP1) and the Rho kinase ROCK1 [212, 213].

In another study, ICAP-1 was found to strongly interact with the cerebral cavernous malformation (CCM) protein CCM1 (also called KRIT1) [214]. CCM is an autosomal disorder characterized by enlarged and disorganized microvessels in the brain parenchyma which lead to headaches, seizures and hemorrhagic stroke [215]. ICAP-1 had a stronger affinity for CCM1 than for β1 integrin. CCM1 could replace β1 integrin in ICAP-1 complexes and thus strengthen focal adhesion contacts. In humans, loss of function mutations in CCM1 very often causes CCM. CCM1 builds a protein complex comprising CCM2 and CCM3 at the cell membrane, and the lipids phosphatidylinositol (4, 5)-bisphosphate (PIP2) and phosphatidylinositol (1, 4, 5)-trisphosphate (PIP3) are part of this complex. A disruption of this complex by loss of any one of the CCM proteins leads to CCM, however, the CCM proteins are not related to each other [215]. Based on the relationship between this microvascular disease and ICAP-1, Brütsch and colleagues hypothesised that ICAP-1 may play a role in blood vessel development or maintenance [216].

RT-PCR and microarray analysis revealed a moderate level of ICAP-1 expression in ECs. Forced overexpression of ICAP-1α inhibited the formation of a capillary network on Matrigel. In addition, HUVEC spheroids overexpressing ICAP-1α inhibited VEGF/FGF2-induced sprouting angiogenesis in a 3D collagen bed. Interestingly, although the number of initial sprouts increased, these tubules stopped growing and remained significantly smaller than the controls. A cell-autonomous effect was observed, i.e., only the cells overexpressing ICAP-1α (in a mixed population of cells) were unable to form well-defined and elongated sprouts. Loss-of-function studies also strengthened the fact that ICAP-1α inhibited sprouting angiogenesis *in vitro*. *In vivo* angiogenesis was also studied using implantable Matrigel/Fibrin matrices containing human ECs in which ICAP-1α was either knocked-down or overexpressed, along with VEGF and FGF2. In line with the *in vitro* studies, knockdown of ICAP-1α in ECs led to an increase in blood vessel density while overexpression of ICAP-1 reduced blood vessel formation. Interestingly, ICAP-1α silencing led to the formation of blood vessels with larger lumen, similar to the vessels seen in CCM1-silenced ECs *in vitro* [217]. Also, ICAP-1 overexpression showed an increased accumulation of the CCM1 protein. Together, this implies that the ICAP1/CCM1 complex is necessary to stabilize both proteins.

Overexpression of ICAP-1 in ECs greatly reduced proliferation, accompanied by reduced ERK phosphorylation, as well as induction of p21 and p27 (cell-cycle inhibitors). ICAP-1 overexpression also increased Akt phosphorylation, thus protecting the cells from damage-induced apoptosis and supporting cell survival. In all, it appears that ICAP-1 expression is required to maintain the quiescent state of the endothelium.

The angiostatic function of ICAP-1 seemed to be similar to DLL4 and NOTCH1. Wüstehube and colleagues reported that that CCM1 protein activates DELTA-NOTCH

signaling [217]. The main support of the hypothesis that ICAP-1 may regulate DELTA-NOTCH signaling was the finding that ICAP-1 activates and stabilizes CCM1 [218]. The study conducted by Brütsch and colleagues clearly demonstrated that ICAP-1 acts genetically upstream of NOTCH, activating the DELTA-NOTCH pathway. Overexpression of ICAP-1α in HUVECs caused an increase in cleaved, active NOTCH1 protein. Consequently, expression of classical downstream NOTCH target genes *HEY1, HEY2* and *HES5* were increased. While expression levels of *JAG1* remained unchanged, ICAP-1 induced expression of the DELTA ligands, *DDL1* and *DDL4*. Blockade of NOTCH could alleviate the effects of ICAP-1 overexpression in impaired sprouting capacity. However, the complete mechanism by which ICAP-1 induces NOTCH is not clarified. Recent reports establish that Akt strongly induces NOTCH signaling and DELTA ligand gene expression [219]. Since ICAP-1 expression led to the increase in phospho-Akt levels, this effect could explain the activation of NOTCH signaling.

Identification of downstream targets of ICAP-1 by microarray analysis revealed that Endothelial cell-Specific Molecule-1 (ESM-1) was one of the strongest correlated genes that was down-regulated upon ICAP-1 overexpression [216]. ESM-1 (also known as Endocan) is an early target of VEGF stimulation [220, 221]. To establish a direct link between ESM-1 and ICAP-1, both siRNA silencing and adenovirus-mediated overexpression of ESM-1 in HUVECs were carried out. As expected, silencing of ESM-1 impaired sprouting angiogenesis, whereas overexpression of ESM-1 enhanced angiogenesis. Thus, ICAP-1 inhibits angiogenesis by down-regulating or repressing ESM-1.

Icap-1 deficient mice do not show classic angiogenesis defects [222]. Instead, ICAP-1 plays an essential role in bone development. Many endogenous angiogenesis inhibitors play important physiological functions that are distinct from its angiostatic function. It is important to note that in *Icap-1*deficient mice, close to 20% of newborn mice die from unknown causes. It is possible that ICAP-1 may have a vital function in the vasculature under stress conditions. Further studies are required to fully understand the function of this gene in angiogenesis.

3.2. Epithelial Protein Lost In Neoplasm (EPLIN)

Epithelial protein lost in neoplasm (EPLIN) is an epithelial protein which was initially identified as a gene differentially expressed in oral cancer cells [223]. The studies, which involved comparison of genes that were differentially expressed in normal oral epithelial cells (NHOK) in comparison to a human papilloma virus (HPV)-immortalized oral epithelial cell line (HOK18A), revealed a cDNA fragment corresponding to a gene preferentially expressed in normal epithelial cells. This gene fragment contained a partial ORF characterized by a central LIM domain. Proteins containing LIM-domain are known to participate in cell differentiation through protein-protein interactions [224] and are also frequently part of the cytoskeleton [225]. EPLIN cDNAs isolated from a HeLa cell line revealed two isoforms; 600 amino acid EPLIN-α and 759 amino acid EPLIN-β [226]. The two isoforms were generated by alternative promoter usage from a single gene on human chromosome 12 [227]. EPLIN-β extended an additional 160aa at the amino terminus and required translation of all 11 exons of the gene while EPLIN-α required exons 4-11 [227]. Although the precise functions and regulation of these two isoforms is still unknown, it was found that the expression of the two isoforms could be independently regulated.

In human, EPLIN was found to be expressed in various tissue types, predominantly expressed in epithelial cells [226]. In mouse, RT-PCR demonstrated high levels of EPLIN-α in the adult lung and the spleen. Lower levels were detected in other adult tissues. EPLIN-β was highly expressed in the kidney, lung, liver and testis [228]. This preferential expression of the α and β isoforms in a tissue specific manner suggests that each of these isoforms have specific, non-overlapping functions. In a pathological setting, the expression of EPLIN was found to be down-regulated in a majority of oral, prostate and breast cancer cells [226]. An extensive study of EPLIN-α expression in human breast cancer and its impact on clinical prognosis was conducted by Jiang and colleagues, where lower EPLIN-α levels were associated with poor prognosis and lower patient survival in higher grade tumor samples [229]. They also demonstrated a direct link between EPLIN-α levels and tumor progression and metastasis in xenograft mouse models. Overexpression of EPLIN-α in cancer cells rendered them less invasive, less motile and slower in migration both *in vitro* and *in vivo*.

EPLIN-α is the predominant form in cultured epithelial cells. In contrast, EPLIN-α is often down-regulated in cancer cells while EPLIN-β expression is maintained with no significant change, or in certain cases is even up-regulated [226]. Though the precise function of EPLIN is not known, subcellular localization studies showed that both EPLIN-α and –β were localized to the cytoplasm in a fibrillar pattern, towards the periphery of the cell. Overexpression studies of both the isoforms in cancer cell lines suggested that this protein is a cytoskeletal protein involved in cell-matrix interactions, strengthening cell attachment and inhibiting growth, thus may function as a tumor suppressor. Colocalization of EPLIN and actin stress fibres seemed to be important for the functions of EPLIN [230]. It was found that EPLIN stabilizes actin stress fibres and thus regulating their turnover. Further studies found that EPLIN mediated the linkage between the cadherin-catenin complex and F-actin through its interaction with α-catenin [231]. All these studies shed light on the potential mechanisms by which loss of EPLIN in cancer cells may drive tumor progression and metastasis. A recent study by Chircop and colleagues has demonstrated a role for EPLIN in cytokinesis, where a loss of EPLIN led to inefficient localization of cytokinesis proteins to the cleavage furrow. This in turn leads to multinucleation and aneuploidy, thus increasing genomic instability and cancer [232].

EPLIN is also expressed in certain ECs such as human HECV cell line and HUVEC although at extremely low levels [226, 229]. Overexpression of EPLIN-α caused a dramatic reduction in the migratory rate of HECVs, as well as in the attachment of these cells on Matrigel [233]. Further studies on the role of EPLIN-α in cell-matrix adhesion revealed an increased paxillin staining in the EPLIN-α overexpressed cells. This, although contradictory to the loss of adhesion observed, could possibly explain the reduction in migratory capacity of these cells, by strengthening focal adhesions. However, the role of other cellular signaling events in the above observed effects cannot be excluded.

Both *in vitro* and *in vivo* models tested support the role of EPLIN-α as an angio-inhibitory molecule. In comparison to control cells, EPLIN-α over-expressing cells failed to form tubules in an *in vitro* tube-formation assay. Previous studies have established a link between ERK and EPLIN, wherein, phosphorylation of EPLIN by ERK led to the disassembly of actin stress fibres and enhanced cellular motility [234]. In the study by Sanders and colleagues, a link was established between these two molecules in ECs, where treatment with an ERK inhibitor was performed simultaneously on control HECV cells and ELPIN-α overexpressed HECV cells. ERK inhibition was found to cause a reduction in cell

migration in control cells. However, in EPLIN-α overexpressed cells, a partial rescue of migratory inhibition was observed. In addition, a complete rescue of tube-formation was observed when EPLIN-α overexpressed cells were treated with ERK-inhibitor, similar to control cells. These data shows that ERK signaling is important in EPLIN-α inhibition of tube-formation in ECs. Consistent with *in vitro* data, co-injection of MDA-MB-231 breast cancer cells with EPLIN-α over-expressing HECVs into athymic nude mice led to a significant decrease in tumor size along with significant reductions in tumor development at later stages. Taken together, these *in vitro* and *in vivo* data suggest that EPLIN-α may negatively impact tumor development through disruption of angiogenesis and formation of tumor vasculature.

To summarize, as an endogenous angiogenesis inhibitor, higher EPLIN-α level may be sufficient to reduce EC-matrix interactions, migration and the formation of new vasculature, especially in a pathological setting. Further investigations are needed to fully understand its molecular mechanisms and the relationship between its function in epithelial cells and ECs *in vivo*.

3.3. ASK1-Interacting Protein1 (AIP1)

AIP1 (ASK1-interacting protein 1), also known as DAB2-interacting protein (DAB2IP), is a recently identified member of the Ras GTPase-activating protein (GAP) family. Studies have shown AIP1 to have a role in inhibiting cell survival and growth. Highly expressed in vascular ECs, AIP1 has been recently characterized as an endogenous inhibitor of VEGFR2-mediated signaling and inflammatory angiogenesis.

AIP1 produces two different mRNA transcripts with different 5' upstream sequences, one about 6.9 kb and another of 9.6 kb [235]. Corresponding to the 2 differential transcripts, protein bands of approximately 110 kDa and 135 kDa were detected in tissues. The protein possesses several conserved structural domains: a pleckstrin homology domain, a PKC-conserved region 2, a Ca^{2+} binding motif with 2 lysine clusters K1 and K2, and a RasGAP domain at the N-terminal half. At the C-terminal half, it contains a period-like domain, a stretch of 10 proline repeats, which binds to SH3 domain containing proteins and a leucine zipper motif, probably acting as a dimerization domain [236].

An expression profile analysis revealed that the 6.9 kb transcript of AIP1 was expressed in several tissues such as brain, lung, thymus, liver, bladder and skeletal muscle [235]. The expression of the 9.6 kb transcript was restricted to brain and kidney. Such diverse expression suggests that AIP1 has distinct physiological functions in each of these organs. There were no detectable levels of the protein in the urogenital organs, such as ventral prostate, dorsal lateral prostate, seminal vescicle and coagulating gland. However normal prostatatic basal epithelial cells but not the stromal cells exhibited an increased level of AIP1 expression. Notably, AIP1 is expressed in high amounts in primary vascular ECs, such as HUVECs and BAECs, in the vascular endothelium of mouse muscle as well as in vascular smooth muscle cells of aorta, epithelial cells of the lung and neurons of the brain [236, 237].

AIP1, as its name suggests, has been shown to interact with ASK1 (Apoptosis signal-regulating kinase 1), a member of the MAPK kinase kinase (MAP3K) family [237]. ASK1 activation triggers various biological responses such as apoptosis, inflammation, differentiation and even cell survival in various cell types. Interaction of AIP1 with ASK1

resulted in ASK1 activation and apoptosis of cultured BAECs. AIP1 also induced cell apoptosis in response to ER stress by activating IRE-1 [238]. AIP1 is also known as DAB2IP and has been shown to interact with the N-terminus of DOC2/DAB2 (Differentially expressed in ovarian carcinoma2/disabled-2), a potential signaling molecule and tumor suppressor [235]. AIP1 through its RasGAP domain, acts as a negative regulator of Ras mediated signaling pathway. The biological effect of its interaction with DOC2/DAB2 suggests a role for this protein as a potent growth inhibitor.

Multiple studies have provided data that suggests AIP1 may function as a tumor suppressor. AIP1 expression has been reported to be down-regulated in various human cancers through epigenetic mechanisms. Analysis of expression status of AIP1 in breast and lung cancers showed that there was a downregulation of AIP1 expression as the disease progresses towards metastasis [239, 240]. Such downregulation was shown to be mediated by aberrant methylation of AIP1 gene promoter as the major mechanism and a critical event in tumorigenesis of breast and lung cancer. In prostate cancer also AIP1 is down-regulated by both aberrant methylation as well as histone modification of its promoter region [241]. In addition to downregulation in cancerous tissues, AIP1 has also been identified as a novel fusion partner with a t(9;11)(q34;q23) chromosomal translocation. The t(9;11) translocation has been described in patients with acute myeloid leukemia. In this case, the intron 9 of the *MLL* gene is translocated into the exon 2 of *AIP1* gene, resulting in a disruption of the pleckstrin homology domain. This is a conserved domain in AIP1 involved in inducing EC apoptosis and cause cell growth inhibition via its ability to regulate RAS interaction with AIP1 [238, 242]. Thus, identification of AIP1 as an MLL fusion partner implies that it could be involved in leukemic transformation [242]. Genome association studies have also reported that AIP1 gene polymorphisms act as risk predictors for aggressive prostate cancer [243, 244].

The expression of AIP1 in vascular smooth muscle cells (VSMC) prompted an investigation into the possible physiological role of AIP1 in the vasculature. It was shown that AIP1 acts as a negative regulator in flow-mediated vascular remodelling in part, by downregulating IFN-γ-JAK-STAT-dependent proliferative signaling in VSMC [245]. AIP1 knockout mice showed normal vascular development and adult mice also showed proper growth and bred normally [236]. The ischemic hind limb model can be used to study angiogenesis in response to ischemia, multiple cytokines and altered systemic blood flow [246] . The simpler sponge granuloma model represents response to inflammation [247]. In both models, AIP1 knockout mice showed increased neovascularisation, indicating ASK1 as an angiogenesis inhibitor. Further investigations using other *in vivo* models such as ear, retina and cornea vascularisation assays, where a single proangiogenic factor can be tested, demonstrated that AIP1 is a specific inhibitor of VEGF-induced angiogenesis. Mechanistic studies revealed that AIP1 acts as an endogenous scaffold by associating with VEGFR2 via its first K1 cluster in the C2 domain and associating with the SH3 domain of PI3K via its PR domain. Thus, AIP1 inhibits VEGFR2-dependent signaling by associating with an active form of VEGFR2 at the late phase of VEGF response in conjunction with PI3K[236]. *In vitro* studies on cultured ECs also demonstrated that AIP1 inhibits VEGF-induced EC migration and tube formation. Taken together, these studies demonstrated that AIP1 function as a endogenous inhibitor of VEGFR2-mediated signaling and inflammatory angiogenesis in mice [236].

3.4. microRNA

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression at the post transcriptional level by either degradation or translational repression of target mRNA [248]. Increasing evidence shows that miRNAs play important roles in regulating angiogenic processes. miRNA biogenesis begins with miRNA gene transcribed by RNA polymerase II to generate long primary transcripts (pri-miRNAs). Subsequently the RNAseIII enzyme Drosha processes pri-miRNA to a 70 nt hairpin precursor pre-miRNA in the nucleus. The pre-miRNA is then transported to the cytoplasm and processed by the Dicer enzyme into unstable 19-25 nt mature miRNA duplex structures. The importance of miRNA as angiogenesis regulators was revealed with Dicer and Drosha knockdowns that resulted in profound dysregulation of angiogenesis related genes *in vitro* and *in vivo* [249-251].

An expression profiling of miRNA in ECs revealed that miR-221/222, miR-21, the let-7 family, the miR-17-92 cluster, the miRNA-23-24 cluster, and miR-126 are highly expressed in ECs [249, 250, 252]. Several other miRNAs are also highly expressed such as miR-16, miR-29, miR-99a, miR-100, miR-181a and miR-320 [249, 250, 252]. However, only a few have been characterized functionally.

The antiangiogenic miRNAs are summarized in Table 1. This long list includes miR-221/miR222, miR-15, miR-16, miR-21, miR-34a, miR-519c, miRNA-107 and the miR 17-92 cluster. Surprisingly, although many of these miRNAs showed an antiangiogenic function in ECs, they are also highly expressed in a large number of cancers and found to favour tumor growth and invasion [253]. For example, miR-221/222 promotes the proliferation of cancer cells by targeting the cell cycle regulator p27 [254]. Silencing miR-21 results in increased apoptosis in glioblastoma cells, reduced the proliferation, migration and invasion of hepatocellular carcinoma cells as well as reduction in metastasis in breast and colorectal cancer [253]. These opposing effects on tumor cells and the endothelium may be due to a difference in miRNA gene targets in different cell types. Whether these miRNAs indeed function as antiangiogenic molecules in the tumor endothelium in the cancers mentioned above, need to be clarified.

3.4.1. miRNA-221/222

These two miRNAs are expressed as a common pri-miRNA in HUVECs suggesting that they are regulated transcriptionally in a coordinated manner [252]. Overexpression of miR-221/222 in ECs resulted in blockage of *in vitro* tube formation [252], inhibition of EC migration and a reduction of wound healing. The antiangiogenic effects resulted from the targeted blockage of protein translation of a cell surface receptor c-Kit [255]. Activation of c-Kit by stem cell factor (SCF) has been previously shown to promote tube formation, migration and survival of HUVECs [256]. Another target of miR-221/222 is the proangiogenic EC function regulator eNOS, whose expression is indirectly reduced by miR-221/222. Hematopoietic progenitor cells respond to paracrine factors through the c-Kit receptor, resulting in vasculogenesis [249]. miR-221/222, by inhibiting cell proliferation by suppressing c-Kit at both transcriptional and translational levels, may interfere with this process.

3.4.2. miRNA-15 and miRNA-16

The miR-15 group of miRNA genes include among other members, miR-15a, miR-15b and miR-16 [257]. No studies have determined the direct effect of miR-15 and miR-16 on ECs. However, one study has shown that miR-15 and miR-16 may have the ability to block VEGF-induced angiogenesis [258] as miR-15b and miR-16 downregulate VEGF expression. It was observed that both miRNAs are down-regulated in response to hypoxia in tumor cell lines, suggesting their potential involvement for tumor sustenance by increased VEGF production and subsequent angiogenesis. Besides this, miR-15 and miR-16 are also frequently deleted or down-regulated in chronic lymphocytic leukemias. Incidentally, it has been shown that both miRNAs induce apoptosis of leukemic cells by targeting the anti-apoptotic protein Bcl-2 [259, 260].

3.4.3. miRNA-21

Identified as one of the key miRNAs highly dysregulated in many cancers, miR-21 is also one of the miRNAs highly expressed in ECs [261]. A recent study on the function of this molecule in ECs has revealed miR-21 as a new angiomiR which negatively regulates angiogenesis [253]. *In vitro*, miR-21 has been shown to reduce HUVEC vascular network formation. In addition, miR-21 also inhibits HUVEC migration towards serum or bFGF in boyden chamber assays as well as reduced the ability to close scratch wounds. *In vivo*, miR-21 has been shown to inhibit pathological angiogenesis in a mouse model of choroidal neovascularisation, which mimics age-related macular degeneration (AMD). A search for potential miR-21 target genes related to angiogenesis regulation revealed two candidates: Ras homolog gene family member B (RhoB) and Sprouty1 (SPRY1). RhoB modulates cell migration through the induction and assembly of actin stress fibers and through control of cell morphology [262]. miR-21 targeting of RhoB resulted in loss of actin stress fiber network and a rounded cell morphology. RhoB was shown to be a direct target of miR-21 which interacts with a conserved miR-21 binding site in the 3'UTR of RhoB mRNA. On the other hand, miR-21 also targets SPRY1, an antiangiogenic molecule [263]. These apparently conflicting functions of miR-21 in angiogenesis remain to be resolved.

3.4.4. miRNA-34a

Characterized as a tumor suppressor, miR-34a, has been found to be either down-regulated or lost in many cancers and tumor cell lines [264-268]. miR-34a has been shown to regulate apoptosis, induce cell cycle arrest and senescence in various cancer cells [269, 270]. However, no known role of miR-34a in angiogenesis has been reported to date. One of the principal targets of miR-34a is Silent Information Regulator 1 (Sirt1) [270]. Sirt1 is a histone deacetylase with multiple functions such as stimulation of autophagy and an important regulator of metabolic regulatory transcription networks [271-273]. Sirt1 has been recently implicated with a new role as a novel regulator of vascular EC homeostasis [274]. miR-34a regulation of apoptosis was found to be mediated by a direct binding to Sirt1. An *in vitro* study demonstrated that Sirt1 silencing in ECs resulted in impaired angiogenesis. Studies on vascular regenerative medicine have recently focussed on a promising therapeutic approach based on endothelial progenitor cell (EPC)-based neovascularisation. EPCs are a population of bone marrow derived cells which home to sites of new vessel formation. EPCs induce neovascularization through the secretion of cytokines and growth factors, which act in a

paracrine fashion and induce sprouting angiogenesis by the surrounding endothelium [275]. A recent study integrates miR-34a, Sirt1 and EPCs to demonstrate that miR-34a impairs EPC-mediated angiogenesis and induces senescence via inhibiting Sirt1, as demonstrated in a Matrigel tube formation assay and through senescence associated β-gal staining [276].

3.4.5. miRNA-519c and miRNA-107

Both these miRNAs have been recently discovered as regulators of tumor angiogenesis via the suppression of hypoxic signaling. Tumor angiogenesis is essential for tumors to grow beyond a certain size [4]. One of the chief inducer of this process is hypoxia, which triggers the production of angiogenic factors such as VEGF, bFGF etc by the tumors [277]. Hypoxia inducible factor-1 (HIF-1) is a transcription factor mediating the transcriptional response to hypoxia. It is a heterodimer composed of the subunits HIF-1α and HIF-1β. Recent studies demonstrate that HIF-1 subunit is regulated by miR-519c and miR-107.

miR-519c regulates HIF-1α in a post-transcriptional manner. A recent study demonstrated an angio-inhibitory role for miR-519c in HIF-1α induced angiogenesis as observed through decreased levels of angiogenic factors [278]. In addition, *in vitro* this miRNA also caused 75% reduction of HUVEC tube formation on Matrigel. *In vivo*, miR-519c caused a reduction in angiogenic activity in a Matrigel plug assay. Moreover,a reduction in tumor size and decreased blood vessels in tumor sections was also observed in xenograft mouse tumorigenesis assays upon overexpression of miR-519c. Additionally, miR-519c was demonstrated to be a regulator of tumor invasion and metastasis. Using CLI-5, a lung adenocarcinoma subline with higher migratory potential , it was shown that there was a significant inhibition of the migratory and invasive capabilities of these cells *in vitro*. *In vivo*, miR-519c suppresses CLI-5 metastasis as demonstrated via a tail vein metastasis assay [278].

miRNA-107 was discovered as a HIF-1β regulator whose effects on hypoxic signaling were shown via its decreased ability to stimulate tumor cell- induced EC proliferation and its ability to regulate VEGF release from tumor cells upon induction of hypoxia. miR-107 also has effects on cell cycle causing growth arrest at G1 phase and induces apoptosis. *In vivo*, miR-107 has the ability to influence tumor angiogenesis in mice. Reduced tumor size and vasculature were observed in miR-107 overexpressed tumors. In addition, it also causes a decreased expression of the hypoxia regulated VEGF in tumors. Thus, miR-107 seems to act by a bi-partite mechanism regulating both the cell cycle as well as hypoxic signaling [279] .

3.4.6. miRNA-17~92 Cluster

The miR-17-92 cluster was one of the miRs linked to tumor angiogenesis. It is a polycistronic miR cluster encoding miR-17, miR-18a, miR-19a/b, miR-20a and miR-92a. miR-17-92 cluster was earlier implicated with a proangiogenic function as overexpression of the cluster increased angiogenesis in myc-induced tumors. This effect was attributed to a downregulation of antiangiogenic molecules [14].

Recent evidence however implicates individual members of this cluster with a cell-intrinsic antiangiogenic function in ECs. miR-92a suppressed angiogenic sprout formation in ECs, inhibited tube formation in Matrigel assays, reduced EC migration and impaired EC adhesion to fibronectin in *in vitro* assays. *In vivo,* miR-92a blocks angiogenesis in Matrigel plug assay and interfered with intersegmental vessel (ISV) formation in zebrafish model [15]. Tg(fli1:EGFP) zebrafish line were injected with pre-miR-92a at one cell stage and analysed for ISV defects at 30 hours post fertilization (hpf) and 48 hpf. These effects of miR-92a were

likely to be mediated partially through its target gene integrin subunits α5 (ITGA5), known to play a pivotal role in vascular development and angiogenesis [15].

Another study revealed that overexpression of the individual members of the miR-17-92 cluster inhibited EC sprouting [280]. Similar to miR-92a, the other members in the cluster were also capable of inhibiting vascular network formation and EC migration. Amongst the members of this cluster, miR-17 and miR-20a was found to be the most efficient as an angiogenesis inhibitor both *in vitro* and *in vivo*. These two miRNAs have large sequence overlap differing only by two nucleotides and can be inhibited by a common antagomir designated antagomir-17/20 [281]. Although miR-17/20a suppressed neovascularisation in Matrigel plugs, it surprisingly has no effect on tumor angiogenesis as shown by their inability to affect tumor angiogenesis in LLC1 tumors. A search for miR-17 targets that might mediate its antiangiogenic function revealed p21, a cell cycle inhibitor and the tyrosine kinase Jak1. p21 reduction may disturb the balance of coordinated vessel growth by activating cell proliferation but reducing migration. The Jak/Stat pathway plays a crucial role in vascular homeostasis and disease [282]. Jak1 was efficiently down-regulated at both mRNA and protein levels by miR-17. Furthermore, Jak1 silencing reduced angiogenesis *in vitro* [280].

Table 1. Antiangiogenic microRNAs

miRNAs	Function in angiogenesis	Relevant targets	References
miR-221/222	Impairs SCF induced angiogenesis *in vitro*	c-Kit, eNOS	[48]
miR-15 miR-16	Blocks VEGF-induced angiogenesis	VEGF	[54]
miR-21	Negative regulator of angiogenesis *in vitro* and inhibits pathological angiogenesis *in vivo.*	RhoB, Spry1?	[49]
miR-34a	Impairs EPC mediated angiogenesis via induction of cell senescence.	Sirt1	[72]
miR-519c miR-107	Antiangiogenic via regulation of hypoxia signaling.	HIF-1α, HIF-1β	[75, 76]
miR-17~92 cluster	Cell-intrinsic antiangiogenic function in endothelial cells.	ITGA5,Jak1,p27	[78, 79]

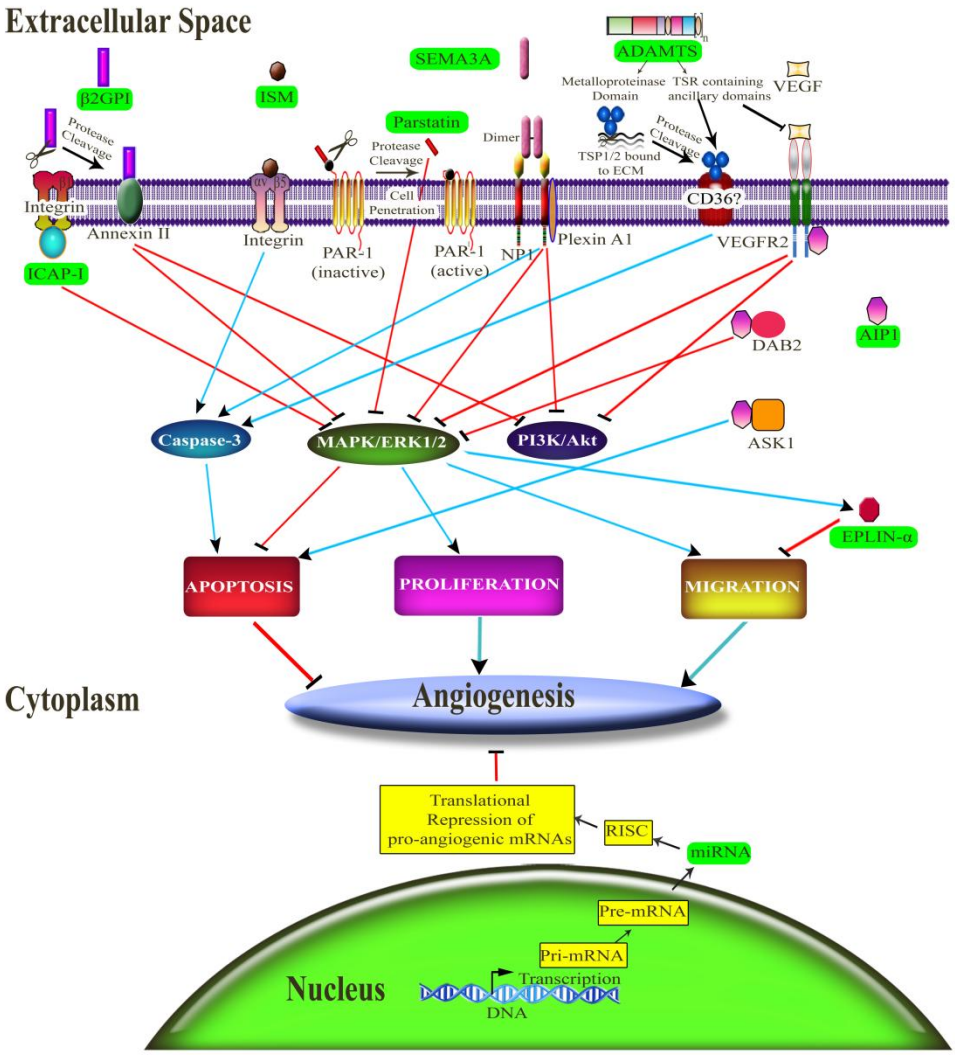

Figure 2. Mechanism of action of endogenous angiogenesis inhibitors on endothelial cells. Angiogenesis inhibitors target three processes crucial for angiogenesis-apoptosis, proliferation and migration of endothelial cells. Secreted angiogenesis inhibitors exert their angiostatic functions either by activating specific cell-surface receptors, as in the case of β2GPI, ISM, SEMA3A and some of the ADAMTSs; or, in the case of parstatin, by cell-surface penetration. Cytoplasmic angiogenesis inhibitors discussed in this chapter act through interaction with intracellular binding partners. Ultimately, both groups of molecules act either directly or indirectly to regulate common downstream effectors such as the caspases, MAPK/ERK, PI3K/Akt, to name a few. Another class of endogenous angiogenesis inhibitors is the miRNAs, which act through transcriptional control of angiogenic regulators. (The endogenous inhibitors reviewed in this chapter are highlighted in green).

Conclusion

Angiogenesis inhibitors constitute an important class of molecules in regulating both physiological as well as pathological angiogenesis. The mechanisms of action of the

endogenous inhibitors discussed in this chapter have been summarized in Figure 2. The maintenance of the delicate balance between angiogenic and angiostatic factors is essential for maintaining homeostasis. The reason for the growing interest in endogenous angiogenesis inhibitors lies in the fact that treatment of angiogenesis-related diseases with endogenous inhibitors may be less toxic than conventional therapies available today and also have a lower risk of drug resistance. Endogenous angiogenesis inhibitors have been reported to block pathological neovascularisation while keeping the established ones in healthy tissues unaffected like TSP1, TSP2, PEDF [283, 284].

Of the endogenous angiogenesis inhibitors known, thrombospondin, endostatin and angiostatin are the molecules that have reached clinical trials for anti-cancer therapy [285-287]. Although these compounds have shown greatly reduced toxicity compared to chemotherapeutic drugs and radiation therapy, clinical efficacy in human are still lacking. The disappointing outcome of clinical trials emphasis that the need to elucidate the mechanisms of action of these endogenous inhibitors including their targets.

It is known that tumors tend to develop resistant to antiangiogenic agents[288]. Thus the current trend is to combine antiangiogenic therapy with conventional chemotherapy. Patients with solid tumors (breast, colorectal, lung cancers) showed better prognosis using this approach[289]. With a better understanding of tumor angiogenesis and by targeting multiple signaling pathways in cancer, it is conceivable that better therapeutic options for the treatment of cancer will be available in future.

Non-Standard Abbreviations and Acronyms

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AIP1	ASK1-interacting protein 1
AMD	Age-related macular degeneration
AMOP	Adhesion-associated domain in MUC4 and other proteins
APS	Anti-phospholipid syndrome
ASK1	Apoptosis signal-regulated kinase 1
BAEC	Bovine aortic endothelial cell
BAI	Brain-specific angiogenesis inhibitor
bFGF	Basic fibroblast growth factor
BRZ	Bronchial epithelial tumor cell line
CAM	Chorioallantoic membrane
CCM	Cerebral cavernous malfromation
CDC42	cell division control protein 42 homolog
CHO	Chinese hamster ovary
COMP	Cartilage oligomeric matrix protein
CPP	Cell penetrating peptide
CRMP	Collapsing response mediator proteins
DAB2IP	DAB2-interacting protein
DLL4	Delta-like protein 4
DOC2/DAB2	Differentially expressed in ovarian carcinoma2/disabled-2
EC	Endothelial cell

ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
EPLIN-α	Epithelial protein lost in neoplasm–α
ERK	Extracellular signal-regulated kinase
ESM-1	Endothelial cell-specific molecule-1
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FITC	fluorescein isothiocyanate
GAP	GTPase activating protein
GDI	Guanine dissociation inhibitor
GPCR	G-protein coupled receptor
GSK	Glycogen synthase kinase
HB-EGF	Heparin binding-epidermal growth factor
HGF	Hepatocyte growth factor
HIF1	Hypoxia inducible factor 1
HOK18A	Human papilloma virus (HPV)-immortalized oral epithelial cell line
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
ICAP-1	Integrin cytoplasmic domain associated protein-1
ISM	Isthmin
ISV	Intersegmental vessels
ITGA5	Integrin subunits alpha 5
LLC	Lewis lung carcinoma
LnCaP	Human prostate cancer cell line
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney
MICALs	Molecules interacting with casL
miRNA	microRNA
MMP1	Matrix metalloproteinase 1
NP1, NP2	Neuropilin 1 & 2
PAR-1	Proteinase-activated receptor-1
PI3K	Phosphotidyl-inositol-3 kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (1,4,5)-trisphosphate
RAC1	GTPases ras-related C3 botulinum toxin substrate
RAP-1	Small G-protein ras-related protein-1
RhoB	Ras homolog gene family member B
ROCK1	Rho kinase1
RRAS	Ras related protein
RT-PCR	Reverse transcriptase – polymerase chain reaction
SEMA3A	Semaphorin3A
Sirt1	Silent information regulator 1
TSP1	Thrombospondin1
TSR	Thrombospondin type -1 repeat

VEGF	Vascular endothelial growth factor
VEGFR-1,VEGFR-2	Vascular endothelial growth factor receptor -1 & -2
VSMC	Vascular smooth muscle cells
Z-VAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone
β_2GP1	β_2 -glycoprotein-1

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