HHS Public Access

Author manuscript

Biochim Biophys Acta. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

Biochim Biophys Acta. 2015 December ; 1850(12): 2422–2438. doi:10.1016/j.bbagen.2015.09.007.

Endostatin's Emerging Roles in Angiogenesis, Lymphangiogenesis, Disease, and Clinical Applications

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Abstract

Background—Angiogenesis is the process of neovascularization from pre-existing vasculature and is involved in various physiological and pathological processes. Inhibitors of angiogenesis, administered either as individual drugs or in combination with other chemotherapy, have been shown to benefit patients with various cancers. Endostatin, a 20-kDa C-terminal fragment of type XVIII collagen, is one of the most potent inhibitors of angiogenesis.

Scope of review—We discuss the biology behind endostatin in the context of its endogenous production, the various receptors to which it binds, and the mechanisms by which it acts. We focus on its inhibitory role in angiogenesis, lymphangiogenesis, and cancer metastasis. We also present emerging clinical applications for endostatin and its potential as a therapeutic agent in the form a short peptide.

Major conclusions—The delicate balance between pro- and anti-angiogenic factors can be modulated to result in physiological wound healing or pathological tumor metastasis. Research in the last decade has emphasized an emerging clinical potential for endostatin as a biomarker and as a therapeutic short peptide. Moreover, elevated or depressed endostatin levels in diseased states may help explain the pathophysiological mechanisms of the particular disease.

General significance—Endostatin was once sought after as the 'be all and end all' for cancer treatment; however, research throughout the last decade has made it apparent that endostatin's effects are complex and involve multiple mechanisms. A better understanding of newly discovered mechanisms and clinical applications still has the potential to lead to future advances in the use of endostatin in the clinic.

Keywords

Matricryptin; Tumor angiogenesis; Type XVIII collagen; Anti-angiogenic factor; MMP; Short endostatin peptide

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1. INTRODUCTION

Research over the last three decades has greatly clarified the process of angiogenesis and its role in cancer, with more than 78,000 articles published on this concept. Angiogenesis is the process of neovascularization from pre-existing vasculature and is involved in various basic physiological and pathological processes [1–4]. The discovery of endostatin in a murine hemangioendothelioma cell line in 1997 was a major breakthrough in our understanding of angiogenesis [5]. Endostatin, a 20-kDa C-terminal fragment of type XVIII collagen, is one of the most potent endogenously produced inhibitors of angiogenesis [5]. After 18 years of research on endostatin, the paradigm of bench-to-bedside research has not culminated in an effective FDA-approved drug. However, the State Food and Drug Administration of China did approve Endostar, a modified recombinant human endostatin, in 2005 for the treatment of non-small-cell lung carcinoma [6]. Since then, phase II studies of recombinant endostatin in the United States have failed to show any activity related to the inhibition of angiogenesis [7]. The evidence is inconclusive and circumstantial regarding why Endostar failed in clinical trials in the United States, but was approved quickly in China. Despite extensive preclinical and clinical studies on endostatin therapy, the specific mechanisms responsible for its anti-angiogenic and anti-tumoral activities are far from completely understood. Originally, endostatin was thought to only block new blood vessel growth, but emerging data suggests that various mechanisms and roles account for the efficacy of endostatin in *in vitro* and *in vivo* models. Greater insight into the mechanisms and physiological roles associated with the anti-angiogenic activity of endostatin may help improve the current treatments, uncover other factors with similar activities, identify predictive markers for therapy, and potentially help with the discovery of novel therapeutics. In this review, we will present the various receptors to which endostatin binds and the related mechanisms of action. We will discuss the biological actions and production of endostatin and briefly mention other peptides with activity similar to that of endostatin. Other anti-angiogenic peptides derived from collagen include arresten, canstatin, tumstatin, and restin, which were recently reviewed [8] and will therefore not be the focus of this review. Endostatin and the fragments of collagen IV are referred to as matrikines [8] or as matricryptins [9, 10]. After establishing a framework for endostatin biology, we present current *in vitro* and *in vivo* models for studying angiogenesis and the potential that endostatin has for clinical application. Understandably, the hype surrounding endostatin in the early 2000s as the cure-all for various cancers is gone [11], but several research groups have persisted in their efforts to achieve a better understanding of endostatin. The scope of this review is to discuss endostatin's role in regulating angiogenesis, lymphangiogenesis, and cancer metastasis in the hopes of better explaining why continued research into the application of endostatin is worthwhile.

2. THE ROLES OF VARIOUS COLLAGEN TYPES IN ANGIOGENESIS

The basement membrane (BM), a specialized form of the extracellular matrix (ECM), has been recognized for its multi-faceted functions as a regulator of cell interactions, cell structure, and cell assembly. For example, specific components of the vascular BM have been found to regulate angiogenesis. The vascular BM contains collagens, a heterogeneous

family of proteins, which contain at least one triple-helical domain made of the repeating Gly-X-Y sequence with the presence of a glycine residue as every third residue [12]. To date, 28 different collagen types have been identified and described in mammalian species, and six of these, type I, type IV, type VIII, type XV, type XVIII, and type XIX, have been implicated in the regulation of angiogenesis.

2.1. COLLAGEN TYPES INVOLVED IN REGULATION OF ANGIOGENESIS

In 1994, O'Reilly et al. discovered the first anti-angiogenic peptide, angiostatin [13]. Angiostatin is a 38-kDa fragment from plasminogen that was first extracted from murine urine and shown to mediate the suppression of murine tumor metastasis by inhibiting endothelial cell proliferation [13]. Since the discovery of angiostatin, other anti-angiogenic peptides associated with collagen have been found and are the topic of this section. Collagens can be categorized as fibrillar or nonfibrillar. Fibrillar collagens form collagen fibrils and are composed of an uninterrupted collagenous domain. Collagen fibrils contribute to the structure, strength, and tensile properties of tissues. This is in contrast to nonfibrillar collagens, which have interruptions in their collagenous domain and structurally do not form fibril bundles. The three primary collagens that have been implicated in the regulation of angiogenesis (type IV, type XV, and type XVIII collagen) are non-fibrillar collagens. In addition, type XV and type XVIII collagens form the multiplexin subfamily of nonfibrillar collagens because both contain multiple alternating collagenous (COL) and noncollagenous (NC) domains [14]. Type I collagen, a fibrillar collagen, has been shown to stimulate angiogenesis *in vivo* and *in vitro* [15–17]. Type VIII collagen, a network-forming collagen that forms hexagonal networks, releases vastatin, an anti-angiogenic fragment located at its C-terminus NC1 domain [18]. Type XIX collagen, a member of the fibril-associated collagens with interrupted helices (FACIT) family, also possesses anti-angiogenic properties at its C-terminus NC1 domain [19].

2.1.1. TYPE IV COLLAGEN—Type IV collagen is the most abundant component of the BM and serves as the scaffold that binds to laminin, fibronectin, entactin, and proteoglycans to form the mesh-like structure of the BM [20, 21]. Type IV collagen is composed of six different α chains (α1–α6) that are encoded on six different genes (COL4A1-COL4A6) [22]. The three primary anti-angiogenic fragments released from the α1, α2, and α3 chains of type IV collagen are arresten, canstatin, and tumstatin, respectively. Further information on matrikines released from type IV collagen can be found in a review published by Monboisse et al. [8].

2.1.2. TYPE XV COLLAGEN—Type XV collagen is classified as a chondroitin sulfate proteoglycan and a member of the multiplexin and non-fibrillar collagen subgroups [23]. Type XV is highly homologous to type XVIII collagen; the two share homology in seven of their COL domains, in their NC11 domains, and in their NC1 domains [24–28]. Cleavage of the C-terminal NC1 domain of type XV collagen on its α_1 chain results in the production of restin, a 22-kDa anti-angiogenic factor similar to endostatin [28]. Similar to endostatin, restin inhibits bFGF-induced endothelial cell migration *in vitro* and exhibits anti-angiogenic properties *in vivo* in xenograft carcinoma mouse models [28, 29]. Endostatin and restin are

both capable of suppressing tumor growth, but endostatin has a stronger antitumorigenic effect [28].

2.1.3. TYPE XVIII COLLAGEN—Type XVIII collagen is the only heparan sulfate proteoglycan collagen and is found in various epithelial and vascular BMs. Type XVIII collagen is a non-fibrillar collagen and a member of the multiplexin subfamily [30]. Type XVIII collagen contains 10 collagenous domains interspersed in 11 non-collagenous domains as shown in Figure 1 [31]. Overall, this structure is flanked by a N-terminal NC11 domain and a C-terminal NC1 domain [31]. The NC1 domain is composed of an association domain involved in oligomerization of three α_1 chains to form a homotrimeric type XVIII collagen, a hinge domain that serves as a protease target, and a 20-kDa anti-angiogenic endostatin domain [32]. Three tissue-specific variants are found in mice and two variants in humans, located specifically within the NC1 domain [30, 33]. The two different human variants of type XVIII collagen are the short isoform and the long isoform. The short isoform is found in the human heart, kidney, placenta, ovary, skeletal muscle, and small intestine, whereas the long isoform is highly specific to the liver [33]. Type XVIII collagen has a significant presence in the BM of various components of the eye, which explains some of the pathologies and *in vitro* and *in vivo* models used to study endostatin. Overall, type XVIII collagen is primarily localized in epithelial and endothelial BMs as the short isoform [34–36].

2.2. DISCOVERIES MADE IN COLLAGEN KNOCKOUTS

In this section, we will discuss the different mouse models that have been created via knockout of the α_1 chain of type XVIII collagen, the α_1 chain of type XV collagen, and the α_1, α_2, and α_3 chains of type IV collagen. The physiological changes in these knockout mice provide insight on the specific functions of these collagens and their anti-angiogenic components.

2.2.1. TYPE IV COLLAGEN KNOCKOUT MODEL—Because type IV collagen contains three unique anti-angiogenic fragments on separate α chains, three separate knockout murine models have been created and observed. The six different α chains (α1–α6) of type IV collagen are encoded on six different genes (*COL4A1–COL4A6*).[22] Homozygous knockout of either *COL4A1* or *COL4A2* is lethal, with death occurring approximately 11 days into embryonic development [45]. This suggests that the α_1 and α_2 chains of type IV collagen, the main components of BMs, are not required for early BM development, but become essential later on [45, 46]. *Col4a3*−/− mice have thickened glomerulus BMs and are used as a model of Alport's syndrome [47]. Additionally, Hamano et al. reported that Col IVα3/tumstatin-deficient mice had accelerated tumor growth associated with enhanced pathological angiogenesis, while angiogenesis associated with development and tissue repair were unaffected [48].

2.2.2. TYPE XV COLLAGEN KNOCKOUT MODEL—*Col15a1*−/− mice are viable and fertile and present with normal development [49]. The most prominent abnormalities are seen in the heart and skeletal muscle and are first detected at three months of age [49]. Histological observations of the skeletal muscles of *Col15a1*−/− mice show muscle cell

degeneration and atrophy, macrophage infiltration, increased muscle cell regeneration, and varied muscle fiber lengths [49]. In addition to these findings, *Col15a1*−/− mice are more prone to exercise-induced muscle injury than their wild-type counterparts [49]. Despite the anti-angiogenic properties of restin, which is encoded in a region of the *COL15A1* gene, *Col15a1*−/− mice display overall normal vascular development; however, there is an increase in vascular permeability and extravascular extracellular space in their striated muscles [49, 50]. *Col15a1*−/− mice present with significant cardiac defects, because type XV collagen is normally highly expressed in the heart. Specifically, the capillaries in the heart are irregularly shaped and show evidence of endothelial cell degeneration and swelling [49]. This suggests that type XV collagen plays a larger role in impacting the microvasculature instead of overall vascular development [49]. Furthermore, cardiac hypotrophy is observed in older *Col15a1*−/− mice and increased myocardial stiffness is present independent of age [49]. *Col15a1*−/− mice also display impaired peripheral nerve maturation, which can be attributed to the presence of type XV collagen in the BM of peripheral nerves [51, 52].

2.2.3. TYPE XVIII COLLAGEN KNOCKOUT MODEL—The closest natural human representation of type XVIII collagen knockout is Knobloch syndrome [53]. Knobloch syndrome is caused by a DNA mutation in *COL18A1*, the gene that encodes the α_1 chain of type XVIII collagen, and is characterized phenotypically by severe myopia, vitreoretinal degeneration, retinal detachment, early-onset cataracts, and occipital encephalocele [53–61]. Due to the prevalence of type XVIII collagen in a variety of organs, *Col18a1*−/− mice present with a diverse set of abnormalities [62]. In the human eye, type XVIII collagen is located in the retina, lens capsule, corneal epithelium, epithelial BM, and Descemet's membrane [63, 64]. The hyaloid artery is a branch off the ophthalmic artery that provides nutrients to the lens in the developing human fetus and typically regresses naturally by the 10^{th} week of development [65]. Without the anti-angiogenic effects of endostatin, *Col18a1*−/− mice have delayed hyaloid vessel regression after birth and subsequent abnormal retinal vasculature growth [66–68]. Furthermore, ocular defects, anterior eye abnormalities, ciliary body atrophy, weakened irises, and an abnormal retinal pigment epithelium are detected [68–71]. All of these defects contribute to an abnormal loss in visual function with age [71]. *Col18a1*−/− mice also present with hyperlipidemia due to a decrease in plasma lipoprotein lipase levels, suggesting that type XVIII collagen plays a role in triglyceride metabolism *in vivo* [72]. Also, both short and long isoforms of type XVIII collagen are located in distinct regions of the kidney [73]. *Col18a1*−/− mice display decreased proximal tubule integrity, softened glomeruli, and effacement of their podocytes [73]. Overall, the vascular effects of the lack of type XVIII collagen are increases in blood flow and vessel permeability [50].

2.2.4. TYPE XVIII COLLAGEN AND TYPE XV COLLAGEN DOUBLE KNOCKOUT MODEL—To determine whether type XVIII collagen and type XV collagen have compensatory functions, Rasi et al. created *Col15a1*−/− × *Col18a1*−/− double null mice, which are viable and present with no gross abnormalities [74]. The double null mice present with muscle atrophy similar to the *Col15a1*−/− mice, suggesting that type XVIII collagen does not compensate for type XV collagen's role in muscles [74]. Both type XV and type

XVIII collagens are involved in the normal regression of the vasa hyloid propria (VHP), the embryonic vasculature serving the vitreous humor, in mice between postnatal days 6–10 [74, 75]. The amount of VHP regression was similar in wild-type and *Col15a1*−/− mice, but significantly increased in comparison to *Col18a1*−/− and double null mice, suggesting that type XVIII collagen can compensate for type XV collagen to some extent [74]. Overall, the separate biological roles of these two types of collagen indicate that compensation between them is only minor [74]. The biological roles of type XV collagen and type XVIII collagen are separate, with type XV collagen functioning primarily in muscles and type XVIII in the eye [74].

3. ENDOGENOUS FORMATION OF ENDOSTATIN

Endostatin is a 20-kDa fragment located at the C-terminal of the NC1 domain of the type XVIII collagen α_1 chain. Many different proteases are able to cleave the 34-kDa NC1 domain to produce endostatin or endostatin-containing fragments [76]. The efficiencies of these proteases are evaluated based on their ability to both generate and degrade endostatin. Various cathepsins [76] (a family of lysosomal endopeptidases) and elastase [77] (a family of proteases that break down connective tissue) cleave the Ala-His linkage in the NC1 hinge region of type XVIII collagen to release endostatin (Figure 2). The three specific types of cathepsins that are capable of generating endostatin are L, B, and K; and of these, cathepsin L is the most efficient and cathepsin K is the least efficient [76]. Tumor cells secrete cathepsin L, which degrades the ECM and BM of tissues to create the ideal environment for tumor invasion and metastasis [78]. With this known role in metastasis, cathepsin L levels have been shown to have potential as a prognostic marker for hepatocellular carcinoma [79], breast cancer [80], colorectal cancer [81], nasopharyngeal carcinoma [82], oral squamous cell carcinoma [83], gastrointestinal stromal tumors [84], bladder urothelial carcinoma [85], and pancreatic adenocarcinoma [86].

To date, 23 vertebrate members of the matrixin family, zinc-dependent proteases known to be involved in tissue remodeling and ECM degradation, have been identified [87]. There are two forms of MMPs: a secreted form and a membrane-type form (abbreviated as MT-MMP). Secreted MMPs, including collagenases, gelatinases, and stromelysins, are tightly regulated and secreted as zymogens [88]. MT-MMPs are transmembrane enzymes that specialize in cleaving ECM components and are important in cell migration due to their location close to the cell surface [89]. MMPs have been shown to also cleave the NC1 hinge region of type XVIII collagen to produce endostatin-containing fragments and other anti-angiogenic fragments [90]. Recent studies have shown that interruption in MMP activity does not affect the amount of endostatin produced by cathepsin L, indicating that cathepsin L's actions are likely independent of MMP [91]. Some members of the MMP family exhibit pro-angiogenic properties, whereas others exhibit anti-angiogenic properties [91]. MMP-3, -9, -12, -13, and -20 are capable but inefficient at cleaving the NC1 hinge region to produce endostatin-containing fragments [76]. MMP-2 and MMP-14 are even less effective at cleaving the NC1 hinge region than the aforementioned 7 MMP enzymes and produce a lower amount of endostatin-like fragments [76].

On the other hand, MMP-7 (also known as matrilysin and a secreted MMP) can efficiently cleave NC1 *in vitro* to form neostatin-7 (28-kDa) (Figure 2) [63, 92]. Aside from cleaving type XVIII collagen, MMP-7 also cleaves various ECM components during matrix remodeling in the wound healing process. Research has shown that MMP-7 is upregulated in the corneal epithelium and basement membrane in corneal wound-healing process after excimer laser keratectomy *in vivo* [93]. Another substrate of MMP-7 is plasminogen, which is cleaved into angiostatin, another anti-angiogenic factor. MMP-2 and MMP-9, integral proteases for ECM degradation, produce fragments with pro-angiogenic activity and are upregulated in angiogenesis [94, 95]. Endothelial cells bearing MMP-2 or MMP-9 also have the ability to degrade the type IV collagen and laminin components of the ECM to allow for blood vessel invasion [96]. Endostatin partly exerts its anti-angiogenic effects by inhibiting the enzymatic activity of MMP-2 [97], MMP-9 [98], and MT1-MMP [97] and by blocking the activation of MMP-2, -9, and -13[98], illustrating the complex interplay between endostatin and the proteases that release it.

4. ENDOSTATIN RECEPTORS

Endostatin has been shown to bind to a variety of receptors (Table 3). In this section, we will summarize the endostatin's physiological effects and mechanisms upon binding to VEGFR-2, VEGFR-2, members of the integrin family, glypican-1, and glypican-4.

4.1. VEGFR-1, VEGFR-2, AND VEGFR-3

VEGF exerts pro-angiogenic effects by binding to several endothelial cell surface receptors, most notably, VEGF receptor (VEGFR)-1 (also known as flt-1) and VEGFR-2 (also known as flk-1/KDR). Generally, VEGF ligand binding to the VEGF receptor tyrosine kinases activates a distinct network of downstream signaling pathways [100]. More specifically, once VEGF binds to VEGFR-2, it immediately activates an ERK/p38/MAPK signaling cascade [101]. VEGFR-2 expression is restricted to the vasculature and is a key mediator of angiogenesis. VEGFR-1 is also present on cells of the vasculature; however, its role is unclear, and VEGFR-1 may serve as a VEGF trap and negatively regulate angiogenesis. Endostatin inhibits angiogenesis by directly binding to both VEGFR-1 and VEGFR-2 and blocking VEGF interaction with Flt-1 and Flk-1 to prevent VEGF-induced tyrosine phosphorylation of VEGFR-1 and VEGFR-2 and all downstream signaling events [102]. VEGFR-3 (also known as flt-4), another receptor tyrosine kinase, is expressed primarily on lymphatic endothelial cell surfaces [103]. Endostatin competitively inhibits VEGF binding to VEGFR-3 *in vitro* [104]. Once bound, endostatin serves as an anti-lymphangiogenic factor by inhibiting VEGF-stimulated lymphatic endothelial cell proliferation and migration [104].

4.2. INTEGRIN $\alpha_5\beta_1$ AND $\alpha_V\beta_3$

Integrins are a family of transmembrane cell surface receptors that aid in cell–cell or cell–ECM interactions. Each integrin is composed of an α and a β subunit, and the combination of the two determines the specificity and signaling properties of each integrin. Humans have 18 different α subunits and 8 different β subunits [105]. Endostatin has been shown to associate with various different surface integrins: $\alpha_3\beta_1$ [106], $\alpha_5\beta_1$ [107–109], $\alpha_v\beta_3$ [108,

109], $\alpha_v\beta_5$ [108, 109]. $\alpha_5\beta_1$ primarily binds to endothelial cell fibronectin [108, 110], but also binds to other ECM components. Endostatin competes with fibronectin, pro-angiogenic ligand, to bind to integrin $\alpha_5\beta_1$ in order to disrupt cell migration [107]. Once bound, endostatin causes $\alpha_5\beta_1$ integrins to cluster and co-localize with endothelial caveolin-1 [111]. Caveolin-1 is a transmembrane anchor protein that couples integrins to signaling cascades and has been found to be an essential regulator of angiogenesis [112, 113]. Another consequence of endostatin binding to $\alpha_5\beta_1$ is the initiation of the tyrosine phosphorylation cascade, which activates cytoplasmic Src [111]. Phosphorylated Src then directly associates with caveolin-1 [111]. This phosphorylated Src plays a role in disassembling focal adhesion fibers and actin stress fibers to disrupt fibronectin matrix deposition, resulting in inhibition of cell migration [111]. Endostatin binds to both $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrins with similar affinities (K_D = 17.5 nM and 18.3 nM, respectively) [109]. When VEGF binds to endothelial cell surface VEGFR-2, it can induce VEGFR-2 association with $\alpha_V\beta_3$ and lead to downstream events that stimulate angiogenesis. Tumstatin, an anti-angiogenic factor from type IV collagen, also binds to integrin $\alpha_V\beta_3$ to inhibit cell proliferation, but does not displace fibronectin in the process [107]. This suggests that the anti-angiogenic effects of endostatin and tumstatin are achieved via different mechanisms [107]. Figure 3 summarizes the downstream signaling effects of these anti-angiogenic factors binding to their respective integrins.

4.3. GLYPICAN-1 AND -4

Glypicans are a family of glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans located on the endothelial cell surface. Karumanchi et al. discovered that endostatin binds weakly to the heparan sulfate region of both glypican-1 and glypican-4 (two endothelial cell surface receptors) and strongly to an ‘unknown receptor’ [114]. Their study showed that endostatin binding to glypicans is necessary for endostatin to bind to its ‘unknown receptor’ and proposed that binding of endostatin to glypican induces a conformational change in endostatin to bind strongly with its ‘unknown receptor’ [114].

The heparan sulfate domain is likely important for endostatin’s anti-angiogenic activity because recombinant endostatin that is unable to bind to heparan sulfate is also unable to inhibit VEGF- and bFGF-induced angiogenesis [115]. Interestingly, the heparan sulfate domain of glypican binds both pro-angiogenic factors (e.g., VEGF and bFGF) and anti-angiogenic factors (e.g. endostatin). Endostatin competes with bFGF to bind to heparan sulfate *in vivo*, though endostatin has a greater affinity for heparan sulfate than bFGF [116].

5. ENDOSTATIN CELL SURFACE-ASSOCIATED PROTEINS

5.1. THROMBOSPONDIN-1 AND SPARC

Thrombospondin-1 and SPARC (secreted protein acidic and rich in cysteine) are matricellular proteins, which serve as endogenous inhibitors of angiogenesis [117]. Matricellular proteins are ECM proteins that regulate cell function without contributing to the structural integrity of the ECM [118]. Endostatin binds to both thrombospondin-1 and SPARC and has also been shown to upregulate thrombospondin-1 gene expression [117, 119].

5.2. ENDOREPELLIN

Perlecan is a BM-specific heparan sulfate proteoglycan that contains an endorepellin fragment at its C-terminal end in domain V [120]. Endostatin binds to endorepellin and approximately half of endogenously produced endostatin has been found to be co-localized with perlecan *in vivo* [121]. Endorepellin's actions are complicated because it exhibits anti-angiogenic properties while also indirectly exhibiting pro-angiogenic properties [120]. Four direct mechanisms to inhibit angiogenesis via endorepellin have been described thus far: (1) inhibition of endothelial cell migration, (2) inhibition of collagen-induced endothelial tube morphogenesis, and (3) inhibition of blood vessel growth in both Matrigel plugs *in vitro* and (4) in chorioallantoic membranes [120]. When endostatin binds to endorepellin, endorepellin inhibits endostatin's anti-angiogenic properties by preventing endothelial cells from attaching to the ECM fibronectin and type I collagen [120]. While inhibiting endostatin's anti-angiogenic properties via an indirect mechanism, endorepellin is simultaneously carrying out its own four anti-angiogenic properties via direct mechanisms [120]. In comparison to the anti-angiogenic potency of endostatin and endorepellin individually, the overall anti-angiogenic capabilities of combined endostatin and endorepellin were found to be decreased when endostatin is bound to endorepellin [120].

5.3. TRANSGLUTAMINASE-2

Transglutaminase-2 (TG-2) is an enzyme located on the endothelial cell surface and is responsible for the multimerization of proteins and the stabilization of the BM [122]. TG-2 is co-localized with endostatin in the ECM secreted by endothelial cells *in vitro* [123]. Endothelial cells serve as a large source of tissue TG-2 [124]. Research suggests that TG-2 binds to VEGFR-2 at the endothelial cell surface and in the cytoplasm; and in the presence of VEGF, TG-2 helps translocate VEGFR-2 to the nucleus [125]. Although the downstream effects of the interaction between endostatin and TG-2 have not been delineated, endostatin binds strongly to TG-2 *in vitro* [117, 123].

5.4. BIGLYCAN AND LOW-DENSITY LIPOPROTEIN

Biglycan is a dermatan sulfate proteoglycan associated with the ECM and is implicated in the initiation of atheroma, which is an accumulation of fat in the intima layer of blood vessels that can result in atherosclerosis [126]. In atherosclerosis-prone mice models, biglycan levels are elevated, and the increased biglycan binds to and retains low-density lipoprotein (LDL) in the intima layer of the blood vessel [126]. Zeng et al. found that endostatin binds to biglycan *in vitro* and serves as an anti-atherosclerotic agent by preventing biglycan from retaining LDL [127]. In addition to binding biglycan, endostatin also directly binds to LDL, but the resulting effects have not been entirely identified [127]. Both *in vivo* and *in vitro* experiments with endostatin, LDL, and LDL-retaining matrix molecules (e.g. biglycan) show that endostatin is anti-atherosclerotic [127]. The mechanism by which endostatin can suppress atheroma initiation through binding to LDL is unclear, and the current hypothesis is that endostatin can change the conformation of LDL to decrease its affinity for biglycan [127].

5.5. AMYLOID PEPTIDE Aβ-(1–42)

Deininger et al. found that the amyloid plaques found in Alzheimer's disease patients are co-localized with insoluble endostatin that forms amyloid fibrils *in vitro* [117, 128]. When endostatin is partially denatured, cross-linking of its secondary structure of β-sheets tends to occur, forming an overall insoluble endostatin protein [129]. The insoluble form of endostatin, along with other proteins that form cross-β-sheets, has a tendency to aggregate with itself to form a fibrillar structure [129, 130]. This fibrillar endostatin can bind to neuronal cells and induce neuronal death, similar to the effects of β-amyloid on neuronal cells in Alzheimer's patients [129].

5.6. NUCLEOLIN

Nucleolin is a protein with angiogenic activity, that is found primarily in the nucleolus and possesses the ability to interact with many other proteins and RNA [131]. During angiogenesis, when endothelial cells adhere to ECM components, VEGF mobilizes nucleolin from the nucleus to the endothelial cell surface [132]. Nucleolin has been found in the nucleus, cytoplasm, and on the cell surface of angiogenic endothelial cells [133]. Nucleolin only appears on the cell surface of tumor-induced angiogenic endothelial cells and not on cells of mature endothelial vessels [133]. When nucleolin is inhibited by anti-angiogenic factors, endothelial cell migration and tube formation are suppressed [132]. Nucleolin internalizes and transports endostatin into the endothelial cell nuclei [134].

6. SHORT ENDOSTATIN PEPTIDES

Although endostatin has proven to be an important endogenous inhibitor of angiogenesis, its therapeutic use has been limited by several factors. The production of endostatin has been challenging due to the high costs associated with synthesis and difficulty in storage and handling [138]. Endostatin has a remarkable secondary structure that requires the correct pairing of two disulfide bonds in a nested pattern [139]. This secondary structure is very stable and requires a very low pH to induce unfolding. However, once endostatin is synthesized, it is rather difficult to promote the ideal conditions for proper folding [140]. The shortcomings of using native peptides as therapeutics have been long known and include limited oral bioavailability, short half-lives, and limited selectivity [141].

Recently, several research groups have attempted to determine the structural basis for the activity of endostatin in the hopes of discovering a short peptide that may be able to overcome the challenges of using a large protein. The general approach has involved synthesizing different internal fragments of endostatin and evaluating their specificity for anti-tumoral activity *in vitro* and *in vivo*. Table 4 shows some of the most significant studies that have helped determine the function of some sequences of endostatin. Because the endostatin peptide is large, it has various sequences with distinct activities [142]. The current hypothesis is that two important functional sequences of endostatin, an angio-stimulatory sequence and an angio-suppressive sequence, have opposing activities [143]. Depending on the disease process in question and the pathophysiology involved, inhibitors and stimulators of angiogenesis could be developed for therapeutic applications.

Zinc binds to endogenously produced endostatin at its N-terminus via three histidine residues [144]. The importance of zinc binding for endostatin's function as an anti-angiogenic factor is highly debated [144–147]. Cho et al. synthesized endostatin mutant H5, from which the N- and C-terminal pentapeptide sequences are removed, and showed that this mutant retains its anti-angiogenic activity *in vitro* and *in vivo* [146]. Figure 4 shows the entire sequence of recombinant human endostatin with some of the sequences that had improved activity highlighted. Because the zinc-binding domain is on the N-terminus of endostatin, zinc binding may not be required for endostatin's anti-angiogenic properties [146]. However, zinc binding may improve endostatin's structural stability by protecting it from rapid proteolysis, denaturants, and extreme temperature [148]. Using surface plasmon resonance (SPR) assays, molecular modeling, and an *in vitro* model of angiogenesis (i.e., embryonic stem cell-derived embryoid body secondary cultures in collagen I gel), Ricard-Blum et al. found that zinc is crucial for the multimerization of endostatin and significantly contributes to the *in vitro* anti-angiogenic activity of endostatin on endothelial cells when they are activated by fibroblast growth factor-2 [149].

Cattaneo et al. and Chillemi et al. divided the full-length endostatin protein into four separate peptides, A-I through A-IV, each containing 40–50 amino acid residues to better elucidate the roles of the individual functional sequences of endostatin [150]. Two of the peptides, A-I and A-IV, showed greater potency and efficacy in inhibiting angiogenesis than the full-length endostatin protein [150]. Surprisingly, A-III had angiogenic activity similar to endogenously produced VEGF, suggesting a possible homology between the sequence of endostatin and VEGF [150].

Becker et al. randomly separated the endostatin protein sequence into eight fragments and synthesized eight distinct 27-amino acid peptides with some overlapping segments (mP-1 through mP-8) [151]. They tested each peptide's ability to inhibit angiogenesis during endometriosis in *in vitro* and *in vivo* models [151]. The criteria used to determine the 27 amino acid as the ideal length for the potential therapeutic peptide included cost of production and storage, production time, ease of handling, and ease of potential delivery to patients by care providers [151]. The significant peptides included ones that had both strong activity and specificity in inhibiting VEGF-induced migration of endothelial cells and ones that had no activity [151]. mP-1 and mP-6 were the only peptides that had improved activity compared to endogenously produced endostatin. Becker et al. also synthesized a mutant of mP-1 by substituting the alanine residues in the 1 and 3 positions with histidine residues to determine specifically where the anti-angiogenic activity was located. They found that the alanine residues at the 1 and 3 position are critical for the anti-angiogenic activity [151]. Because mP-1 was one of the most active in Becker et al.'s analysis, Tanabe et al. measured its activity in a different *in vivo* model using peritoneal sclerosis secondary to injections, characterized primarily by angiogenesis and fibrosis, and found that these short peptides had significant anti-angiogenic activity, but did not compare the activity of the short peptide with the full-length endostatin protein [152].

Wickström et al. used surface-exposed sequences determined by the crystal structure of endostatin to design shorter peptides of 11–13 amino acid residues in length: ES-1, ES-2, ES-3, ES-4 and ES-5 [153]. ES-2 effectively inhibited angiogenesis and endothelial cell

migration at low concentrations, and when the arginine residues on ES-2 were substituted for alanine, the anti-angiogenic activity was eliminated, suggesting the importance of these basic arginine residues for endostatin's activity [153]. Through the use of molecular dynamics and computational analysis, Pieraccini et al. determined an active epitope of six amino acid residues (R-R(G)-A-D-R-A) that may be important for endostatin's anti-angiogenic role [154]. The six-amino acid epitope was also present in both A-I and A-IV and may be useful in designing small therapeutic peptides [154]. Although the epitope is present within the primary sequence, the activity may be due to the manner in which the protein is folded so that residues far apart in the primary structure may be close together and form an active epitope upon folding [154].

Our research group has employed SPR assays to evaluate the activity of various short endostatin peptides and further characterize how endogenous endostatin binds to its receptor [104]. We evaluated four short endostatin peptides (mEP, mEP-CA, mEP-AC, and mEP-AA), each 27 amino acids in length, and their specific affinity for the VEGFR-3 receptor *in vitro* [104]. The binding of short peptides to VEGFR-3 required two cysteine residues approximately seven amino acids apart, which are likely critical for both the structural and functional activity of the peptide [104]. Substitution of either of the two cysteine residues in the motif prevented peptide binding to VEGFR-3 [104]. Unsurprisingly, a similar cysteine motif is also present on VEGF-C, an endogenous agonist of VEGFR-3 [104]. Even though the roles of several domains within the endostatin protein have been determined, additional characterization of endostatin sequences that bind to its various receptors is required to synthesize novel therapeutics that effectively inhibit angiogenesis or lymphangiogenesis.

7. *IN VITRO* AND *IN VIVO* MODELS OF ENDOSTATIN'S ROLE IN ANGIOGENESIS AND LYMPHANGIOGENESIS

We have described the receptors to which endostatin binds, and in this section, we will outline the *in vitro* and *in vivo* effects of endostatin along with some of the assays used to determine the activity of endostatin.

7.1. IN VITRO ACTIVITIES OF ENDOSTATIN *In vitro*

pull down assays show that endostatin competes with VEGF-C to bind to VEGFR-2 on the endothelial cell surface. Once endostatin is bound to VEGFR-2, it prevents VEGF-C phosphorylation of VEGFR-2 and initiation of downstream signaling pathways that lead to angiogenesis [101, 102]. *In vitro* migration assays and cell proliferation evaluations have shown that full-length human endostatin inhibits bFGF- and VEGF-induced endothelial cell proliferation and migration [68]. When Endostar, a recombinant human endostatin with nine additional amino acids (MGGSHHHHH) added to its N-terminus, is added to cultured human umbilical vein endothelial cells (HUVEC) *in vitro*, VEGF-induced migration, proliferation, and tube formation is inhibited [156, 157]. These results were obtained through migration assays and tube formation assays [156]. The inhibition on aortic ring vessel branching was observed *in vitro* through an aortic ring assay [156].

In addition to its anti-angiogenic activity, endostatin also exhibits anti-lymphangiogenic activities that are primarily executed through binding to VEGFR-3 [104]. An SPR assay was used to characterize the binding between endostatin and VEGFR-3 [104]. Endostatin competes with VEGF-C for binding to VEGFR-3 and was found to have a lower affinity for this receptor than VEGF-C [104]. By blocking VEGF-C binding to VEGFR-3, endostatin inhibits lymphatic endothelial cell proliferation and migration as seen through a scratch migration assay and Bromodeoxyuridine (BrdU) assay [104]. In general, research has shown that endostatin can inhibit endothelial cell proliferation and migration and can induce cell apoptosis *in vitro* and *in vivo* [158–160].

7.2. IN VIVO EFFECTS

Endostatin's anti-angiogenic effects have been observed *in vivo* through a chick chorioallantoic membrane assay for neovascularization and through a Matrigel plug assay for tube formation [158, 161]. The Matrigel plug assay is a widely used technique for observing *in vivo* angiogenesis. In this assay, a pro-angiogenic factor, such as bFGF, is added to a Matrigel liquid, which solidifies after subcutaneous injection and allows for host cells to induce angiogenesis in the region of injection [162]. Both endogenously produced endostatin and Endostar, recombinant human endostatin, can inhibit angiogenesis *in vivo* [156]. In mouse models, endostatin has been shown to suppress and even completely inhibit the tumor mass growth rate though inhibition of angiogenesis [163, 164]. Although not extensively studied, the results of *in vivo* experiments support endostatin's anti-lymphangiogenic role based on its inhibition of bFGF-induced lymphangiogenesis [165]. Endostatin has recently been found to have intrinsic ATPase activity *in vivo*, which mediates its anti-angiogenic and anti-tumoral activities by further inhibiting endothelial cell proliferation, migration, tube formation, and adhesion [166].

8. ENDOSTATIN'S ROLE IN LYMPHANGIOGENESIS

The anti-lymphangiogenic action of endostatin has been widely accepted, yet largely understudied in terms of its clinical significance. Lymphangiogenesis involves the coordination of events that are similar to angiogenesis: cell proliferation, migration, sprouting, and tube formation [167]. However, the mechanism responsible for lymphangiogenesis is distinct from angiogenesis and depends upon the binding of VEGF-C [168] or VEGF-D [169] with VEGFR-2 or VEGFR-3 to activate extracellular-signal-regulated kinases that induce phosphorylation of Akt (also known as protein kinase B) and ultimately lead to lymphangiogenesis [170, 171]. Although lymphatic endothelial cells (LECs) share many characteristics with blood vascular endothelial cells, they differ in their gene expression and functional characteristics, which gives LECs distinct physiological and molecular behaviors [172–175]. With particular cancers, such as oral squamous cell carcinoma [176] and malignant pleural effusion [177], the generation of lymphatic vasculature and remodeling of the existing lymphatic vasculature may be critical to cancer metastasis. Fukumoto et al. first investigated the role of endostatin in lymphangiogenesis by examining the correlation between inhibition of lymph node metastasis and the administration of endostatin *in vivo* and *in vitro* [176]. They found that a crucial mechanism for endostatin's anti-lymphangiogenic activity is the downregulation of VEGF-C expression,

which subsequently leads to a reduction in lymph node metastasis [176]. Brideau et al. attempted to clarify the mechanism of endostatin inhibition and identified tumor-associated inflammatory mast cells as the primary source of VEGF-C expression [136]. Elevated endostatin levels are correlated with a reduction in the number of VEGF-C–producing inflammatory mast cells, which ultimately leads to the inhibition of lymphangiogenesis [136]. Human mast cells have been found both to express functional β_1 integrin and to adhere to vitronectin via its $\alpha_V\beta_3$ integrin receptor [178, 179]. As discussed in an earlier section of this review, endostatin binds to integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$ to inhibit angiogenesis [106, 108, 109, 142]. When endostatin is bound to integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$, it serves as an inhibitor of mast cell adhesion and migration to prevent release of VEGF-C, an endogenous agonist of lymphangiogenesis [136]. Elevated endostatin levels are also correlated with reduced VEGFR-3 levels, suggesting that endostatin inhibits the gene expression of VEGFR-3, the primary receptor located on LECs for regulating lymphangiogenesis [136]. Although there have been no clinical trials of endostatin treatment for any diseases associated with lymphangiogenesis, the efficacy of endostatin for the diseases for which its use is approved may be due to unknown anti-lymphangiogenic mechanisms. We are only beginning to understand how endostatin employs various coordinated mechanisms unique to its anti-lymphangiogenic role.

9. DISEASES ASSOCIATED WITH ALTERED ENDOSTATIN LEVELS

Elevated levels of endostatin have been associated with several diseases, some of which are discussed here. A comprehensive list of these diseases is provided in Table 5. Because endostatin is one of the most potent anti-angiogenic factors, a thorough understanding of its involvement in the pathology of the diseases discussed in this section can support the development of novel therapeutics. It is not possible to incorporate every study reporting a role for endostatin in a disease in the present review, and thus, we focused on diseases that help explain some of the mechanisms by which endostatin functions and the diseases that have not been emphasized in previous reviews.

9.1. CANCER METASTASIS

Because endostatin in an inhibitor of angiogenesis and lymphangiogenesis, it may initially seem counterintuitive that levels of endostatin are elevated in cancer, given that tumorigenesis requires pro-angiogenic factors [5]. O'Reilly et al. hypothesized in 1997 that endostatin is a "left-over" byproduct created during the transformation of a phenotypically normal cell into its angiogenic phenotype [5]. They also provided a second hypothesis that elevated endostatin levels may be due to random, nonspecific proteolytic activity of enzymes necessary to mobilize promoters of angiogenesis [5]. In contrast, the currently accepted hypothesis emphasizes the regulatory role of endostatin in tumorigenesis [180]. During tumor metastases, the balance between angiogenic factors and anti-angiogenic factors is disrupted and shifted towards the production of angiogenic factors to favor the growth of the primary tumor [158] Because inhibitors of angiogenesis have longer half-lives than angiogenic proteins, they tend to circulate in the bloodstream longer than pro-angiogenic factors [13, 181, 182]. Furthermore, surgeons have found that plasma levels of endostatin are reduced upon removal of the primary tumor, which facilitates angiogenesis in

secondary tumors [183]. Thus, the primary tumor may actually maintain the dormant state of secondary tumors by secreting angiogenic inhibitors such as endostatin [184]. Elevated levels of endostatin have been implicated in several cancer types as shown in Table 5.

9.2. DIABETES MELLITUS AND CORONARY ARTERY DISEASE

Sodha et al. reported that coronary artery disease patients with diabetes have greater levels of myocardial endostatin than coronary artery disease patients without diabetes [185]. Endostatin levels are positively correlated with hyperglycemia (measured by HgbA1c levels) and negatively correlated with coronary collateralization, the body's natural formation of blood vessels to bypass ischemic areas and provide an alternative blood supply [185]. During chronic myocardial ischemia, coronary collateralization is upregulated to limit the size of the infarct region [186– 189]. Cathepsin L, the protease that cleaves endostatin from type XVIII collagen, was also found to be elevated in the myocardium of diabetes patients [185, 190]. By inhibiting endostatin production, pharmaceuticals may be developed to improve outcomes in patients with diabetes and coronary artery disease [185].

9.3. ALZHEIMER'S DISEASE

Deininger et al. determined that endostatin accumulation occurs in the brains of patients with Alzheimer's disease [128]. One hypothesis for the etiology of Alzheimer's disease focuses on chronic cerebral hypoperfusion and endothelial cell abnormalities as important components that lead to the progressive nature of the disease [191, 192]. Endostatin accumulates in the cortical and perivascular plaques to disrupt endothelial vessel migration and growth [128]. Hypoxia likely induces the release of endostatin and its accumulation in amyloid plaques, and therefore, inhibition of endostatin release may be a potential avenue for novel treatment approaches for Alzheimer's disease [128].

9.4. CHRONIC KIDNEY DISEASE (CKD)

The balance between pro-angiogenic factors and anti-angiogenic factors determines the properties of the filtration barrier and glomerular capillary structure. If there is an insult to either the filtration barrier or the glomerulus, Lerman et al. found the balance between pro- and antiangiogenic factors to be disturbed [43, 193]. CKD results from the progressive deterioration of renal microvasculature that leads to tubulointerstitial fibrosis and glomerulosclerosis [43, 193]. Both *in vivo* and *in vitro* studies have shown that endostatin expression may result in the rarefaction of renal microvasculature [194, 195]. In addition, Chen et al. found increased plasma levels of endostatin in patients with CKD and demonstrated a concentration-dependent relationship between the severity of CKD and plasma endostatin levels [196]. Although the causal relationship between endostatin levels and the risk of CKD is unknown, novel therapeutics that target endostatin may reduce the risk of developing CKD [196].

10. CONCLUSIONS AND PERSPECTIVES

10.1. CLINICAL APPLICATIONS OF ENDOSTATIN

Despite all the funding that has been allocated to studying endostatin in recent decades, we are still at the initial stages of determining potential clinical uses for endostatin in a variety

of diseases and disorders. Significant progress has been made in recent years to determine the important binding sequences of endostatin, and several research groups appear to be close to identifying the ideal small peptide with the greatest activity towards inhibiting angiogenesis. Understandably, the next step in endostatin-related research will focus on drug delivery and determining whether combinatory therapies can be more efficacious than endostatin monotherapy [227, 228].

10.1.1. ENDOSTATIN THERAPY AS AN INTERVENTION AGAINST ANGIOGENESIS—

Almost two decades of research on endostatin has unraveled only some of its basic physiological functions in the regulation of different organ systems. Soon after the discovery of endostatin's critical role in inhibiting angiogenesis, the pharmaceutical development of endostatin became the focus of much research [229]. However, the reality is that drug design for clinical use is not as simple and predictable as *in vitro* and *in vivo* research in the laboratory. We have also come to learn that endostatin's role is more complex than we initially believed [230].

Endostatin, under the trade name Endostar, was approved for clinical use in China for the treatment of non-small-cell lung carcinoma in 2005, but unfortunately, human recombinant endostatin largely failed phase II clinical trials in the United States due to significantly low potency [7, 231]. One hypothesis to explain this failure in clinical trials in the United States is that the recombinant human endostatin (as shown in Figure 4) used in these trials did not contain the additional nine amino acids (MGGSHHHHH) present at the N-terminus of Endostar [157]. The His-tag added to the N-terminus of recombinant human endostatin increases its zinc-binding and may explain the improved stability achieved with Endostar [147].

A significant issue with Endostar in China has been compliance with the necessary dosage of intravenous therapy for 3–4 hours daily during a 14-day cycle [232]. More recently, combinatory therapy using standard chemotherapy and endostatin has shown better preliminary clinical outcomes with reduced side effects compared to endostatin or chemotherapy alone [233]. A short endostatin peptide that can overcome some of the issues of Endostar may have significant potential for treating angiogenesis- and lymphangiogenesis-related disorders when combined with current treatment plans. Although Endostar has not been approved in China for most of the cancers presented in Table 5, it has shown efficacy in clinical trials for breast cancer [234], prostate cancer [235], colorectal cancer [210], cervical cancer [205], and nasopharyngeal carcinoma [212].

Endostatin's antitumorigenic effects vary depending on the dosage and target endothelial cell type. It demonstrates a biphasic antitumor dose-response, where high and low dosages are less effective than the established ideal dosage [236]. This behavior is common among many antiangiogenic agents, and the reduced effectiveness of antiangiogenic agents at high doses may be due to the suppression of new vessel growth that would potentially carry the agent to the critical region surrounding the tumor [236]. Endostatin has also been found to have different and sometimes opposite effects depending upon which cell type it is acting upon [237]. For example, Schmidt et al. reported that endostatin decreased proliferation of HUVEC, but increased proliferation in differentiated embryonic stem cells (eESC) with the

same dosage [237]. Thus, determining both the ideal dosage of endostatin and cell type that it will act on is crucial in improving clinical applications of endostatin.

Recently, Wang et al. found that endostatin may be used to reduce obesity and metabolic syndrome. From both *in vitro* and *in vivo* experiments, they concluded that that endostatin may be able to effectively reduce the risk of developing insulin resistance, hepatic steatosis, and glucose intolerance by inhibiting the pathway of adipogenesis [238]. Adipogenesis plays a crucial role in determining the metabolic profile, the number of adipocytes, and body weight in the homeostatic state [238].

Shariati et al. explored the therapeutic role of endostatin in a schistosomiasis murine model created through infection with the helminth *Schistosoma mansoni* [239]. Schistosomiasis is a disease associated with the formation of schisotosome granulomas that cause inflammation and induce angiogenesis in a wound healing response [239]. Mice infected with *S. mansoni* and subsequently treated with endostatin were examined and found to have a decreased number of adult worms, worm eggs in their livers, and granulomas present compared to infected mice that were not treated with endostatin [239]. These results indicate that endostatin is able to reduce the injury characteristic of schistosomiasis [239].

Because corneal transplantation is the most widely performed solid organ transplantation, developing a treatment to prevent graft rejection will be valuable [240]. Tan et al. found that endostatin can potentially be used to prevent corneal allograft rejection *in vivo*. They found that endostatin levels must remain elevated for either an allograft or syngeneic corneal graft to survive [221]. Hence, they recommend the possibility of monitoring endostatin levels to predict potential failure of corneal grafts [221]. Because the cornea is normally avascular, it is important to maintain the avascularity after transplant [241, 242]. Unfortunately, after transplantation, allospecific T cells enter the graft and destroy the endostatin-producing cells, resulting in the loss of immune privilege within the cornea and ultimately leading to corneal neovascularization [221, 243]. The loss of immune privilege and initiation of corneal neovascularization results in the infiltration of effector T cells and thus graft rejection [221]. By inhibiting both angiogenesis and maturation of T cells into effector T cells, immunological failure of transplantation may be prevented [221].

Although endostatin has not been approved for clinical use in the United States, there are a variety of diseases for which it has therapeutic potential based on *in vivo* models. Kojima et al. explained the potential of endostatin's ability to treat disorders related to lymphangiogenesis, such as lymphedema [165]. Becker et al. and Zhang et al. used endostatin to treat endometriosis without affecting the estrous cycles in *in vivo* and *in vitro* models [151, 244]. Several disorders and diseases for which the therapeutic potential of endostatin has been demonstrated include melanoma [245], glioblastoma [246], fibroproliferative disorders [247], pancreatic cancer [248], non-Hodgkin's lymphoma [249], retinoblastoma [250], hypertension [251], and renal cell carcinoma [252]; even more diseases are included in Table 5.

10.1.2. ENDOSTATIN MODIFICATION AND DELIVERY AS A DRUG—Endostatin's anti-tumoral properties make it a very attractive therapeutic agent in the treatment of

cancers. The primary challenge in using endostatin as a drug is its short half-life *in vivo*, which makes it difficult and expensive to create large amounts of biologically active endostatin protein to maintain therapeutically active serum levels [253]. There are many different approaches used to improve the delivery of endostatin and its efficacy as a drug. N-terminus PEGylation of endostatin, the covalent attachment of a polyethylene glycol (PEG) polymer, improves the half-life by 86% compared to non-PEGylated endostatin by protecting it from proteolysis and decreasing its renal excretion rate, which overall enhances its antitumor activity [254, 255]. To target endostatin to the tumor vascular endothelium, an RGD (Arg-Gly-Asp) sequence that is present in integrin ligands can be attached to the carboxy- or amino-terminus of endostatin [256]. When the RGD-motif is added to endostatin, there is an increase in the drug's ability to attach to the endothelial cell surface and inhibit bFGF-induced endothelial cell proliferation and migration [256]. Another modification to endostatin is the addition of an iRGD (internalization RGD) sequence that allows endostatin to penetrate further into tumor tissue and increase its distribution volume in the tumor [257]. Fusing endostatin to the Fc region of IgG also increases its half-life from 2 hours to more than 2 weeks, and the addition of zinc can even further decrease the Fc-endostatin degradation [182, 258]. The use of nanoparticles for endostatin delivery allows for controlled drug release. Hu et al. attached Endostar to nanoparticles and observed an increase in Endostar's half-life by 26 hours, decreased tumor growth rate and dosing requirements, and improved antitumor effects [259]. A limitation of many of these therapeutic strategies is that chronic treatment would require the administration and pharmaceutical production of large amounts of protein. Gene therapy offers an approach to overcome this obstacle by targeting endogenous long-term production and secretion of endostatin protein [260]. The endostatin DNA can be delivered through both viral and nonviral vectors. Previously employed viral vectors for endostatin gene therapy include adenovirus [261, 262], adeno-associated virus [164], lentivirus [263], retroviruses [264], and Semliki Forest virus [265]. Sauter et al. found that endostatin delivered through an adenovirus vector decreased Lewis lung carcinoma tumor volume by 78% [262]. Lee et al. reported a significant tumoricidal and anti-angiogenic activity with a nonviral *Salmonella choleraesuis* vector [266]. Two other nonviral vectors include polymerized plasmids and DNA cationic liposomes, which both inhibit the growth of primary tumors and metastatic lesions in mice [267–269].

10.1.3. ENDOSTATIN AS A BIOMARKER—Recent studies have proposed that endostatin may serve as a marker of prognostic value in some cancers. Because endostatin and VEGF are the primary regulators of angiogenesis, measurements of both may explain the balance between inhibition and stimulation of angiogenesis. Zhou et al. tested the utility of examining levels of endostatin to differentiate malignant pleural effusions from tuberculous pleural effusions, because both present with similar clinical manifestations that are difficult to distinguish [226]. Both endostatin and VEGF individually have particularly low sensitivities and specificities for diagnosing malignant pleural effusion, but measurement of the combination of VEGF and endostatin levels has a significantly greater sensitivity of 81% and specificity of 97% [226]. Thus, the diagnostic efficiency of using the combination of endostatin and VEGF may prove to be very valuable in clinical practice [225]. These studies further illustrate the roles and balance of both positive and negative

regulatory factors in tumor angiogenesis [5]. Studies have also indicated the potential of endostatin as a marker of prognostic value in hepatocellular carcinoma [197], bladder cancer [200], cervical cancer [204], colorectal cancer [208], nasopharyngeal carcinoma [211], bronchopulmonary dysplasia [219], pulmonary arterial hypertension [214], traumatic brain injury [217], intermittent claudication in peripheral vascular disease [270], benign vs. malignant ascites [271], Alzheimer's disease [216], and chronic kidney disease [196].

10.2. FUTURE DIRECTIONS

A large number of preclinical studies using *in vitro* and *in vivo* models have produced several hypotheses to explain the relationship between the structure and activity of endostatin. Nonetheless, application of these mechanisms in the form of pharmaceuticals in clinical studies has proven difficult. We have outlined some of the shortcomings of using endostatin as a pharmaceutical in this review. Although endostatin's functional role is no longer a 'black box', we are still only beginning to understand the plethora of receptors and mechanisms by which endostatin exerts its various activities. As discussed in this review, studies on endostatin's role in lymphangiogenesis are limited, though we now understand that inhibiting lymphangiogenesis may be effective in the treatment of certain types of cancers. For the clinical use endostatin as a marker of prognostic value, we need to comprehensively explain and understand the mechanism of action of endostatin in the angiogenic or lymphangiogenic process of the particular disease. The only active study of clinical endostatin use in the United States is currently in phase I clinical trials for age-related macular degeneration. Currently, most clinical trials of endostatin are being conducted in China. However, the single clinical trial on endostatin in the United States is not a reflection of our interest in targeting angiogenesis. In fact, more than 500 current clinical trials in the United States focus on targeting angiogenesis. Several research groups, including ours, believe that there still may be hope for the clinical utility of endostatin in the form of a short peptide, as a potential prognostic marker, or as part of combination regimens for the treatment of disease. A methodical approach to understanding the mechanisms by which endostatin inhibits angiogenesis and lymphangiogenesis will help identify the domains required for its activity.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health [EY10101 (D.T.A.), R01EY018594 (M.I.R), EY023691, EY021886, I01 BX002386 (J.H.C), EY01792, and UL1TR000050] and an unrestricted grant from Research to Prevent Blindness, New York, NY.

Abbreviations

NC	noncollagenous
COL	collagenous
MMP	matrix metalloproteinase
bFGF	basic fibroblast growth factor
VEGF	vascular endothelial growth factor

VEGFR	vascular endothelial growth factor receptor
SPARC	secreted protein acidic and rich in cysteine
TG-2	transglutaminase-2
mP	mini peptide
ES	endostatin
mEP	mini endostatin peptide
HUVEC	human umbilical vein endothelial cell

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Highlights

- Endostatin is located at the C-terminal end of type XVIII collagen
- Endostatin has anti-angiogenic, anti-lymphangiogenic and anti-tumorigenic activity.
- Other matrikines also exhibit anti-angiogenic and anti-tumorigenic activity.
- Various recently discovered receptors bind endostatin to induce widespread effects.
- Endostatin's therapeutic potential as a short peptide or biomarker is promising.

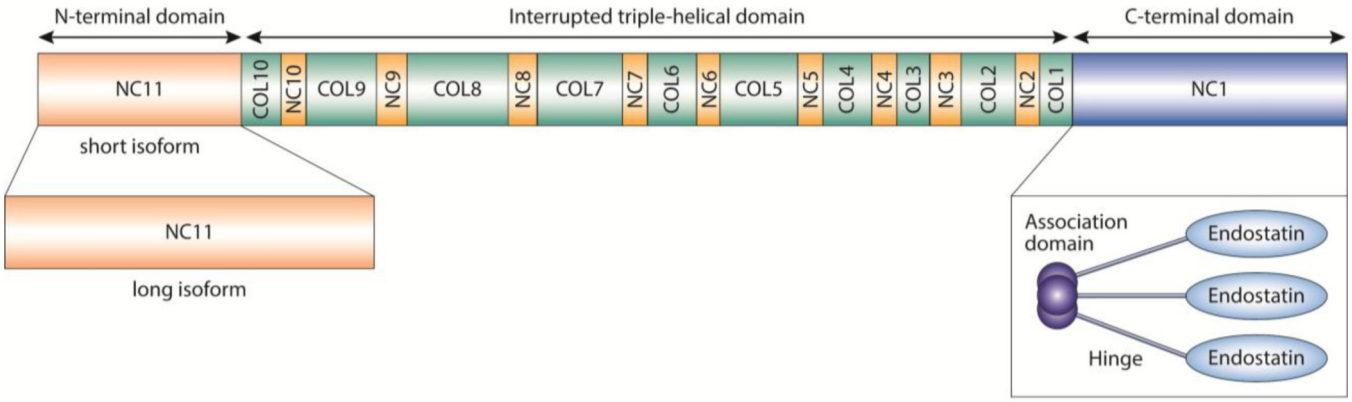

FIGURE 1.

Schematic model of the α1 chain of human collagen XVIII. The human collagen XV α1 chain is structurally homologous. They belong to a collagen subfamily, the multiplexins, on the basis of their central triple-helical domain (green boxes) interrupted by non-collagenous sequences. They contain an extended non-collagenous N-terminal domain, which, in collagen XVIII, can undergo alternative splicing (pale orange boxes), and a non-collagenous C-terminal domain (NC1; blue). The homotrimers (dark blue circles); a hinge domain (blue lines), which is highly susceptible to proteolytic processing; and a C-terminal endostatin domain (blue ovals), which has angiostatic properties. [Adapted from Iozzo [37] with permission from Nature Publishing Group.]

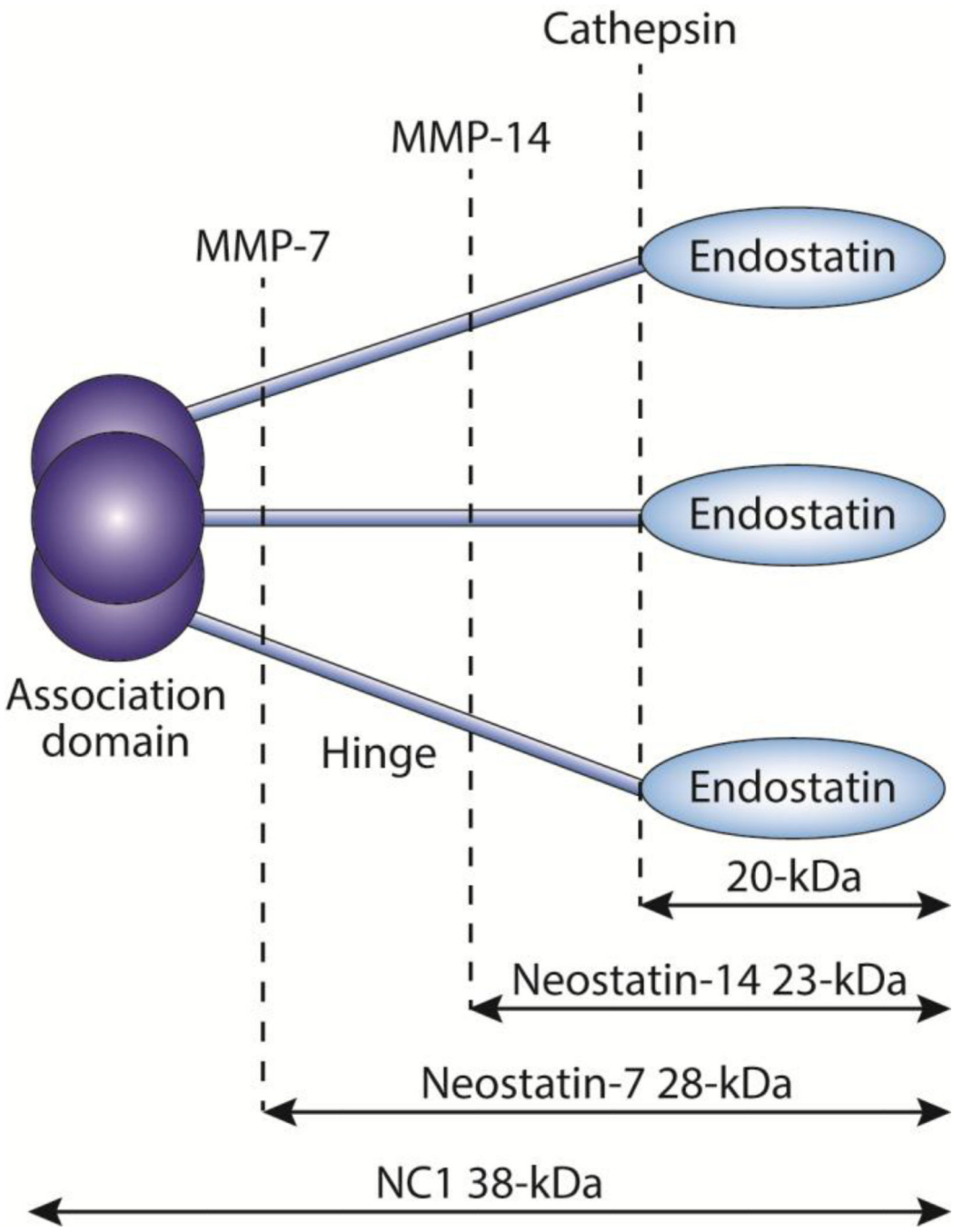

FIGURE 2.

The NC1 domain of type XVIII collagen can be cleaved into endostatin by cathepsin L, into neostatin-14 by MMP-14, and into neostatin-7 by MMP-7. [Adapted from Schenk et al. [99] with permission from Elsevier].

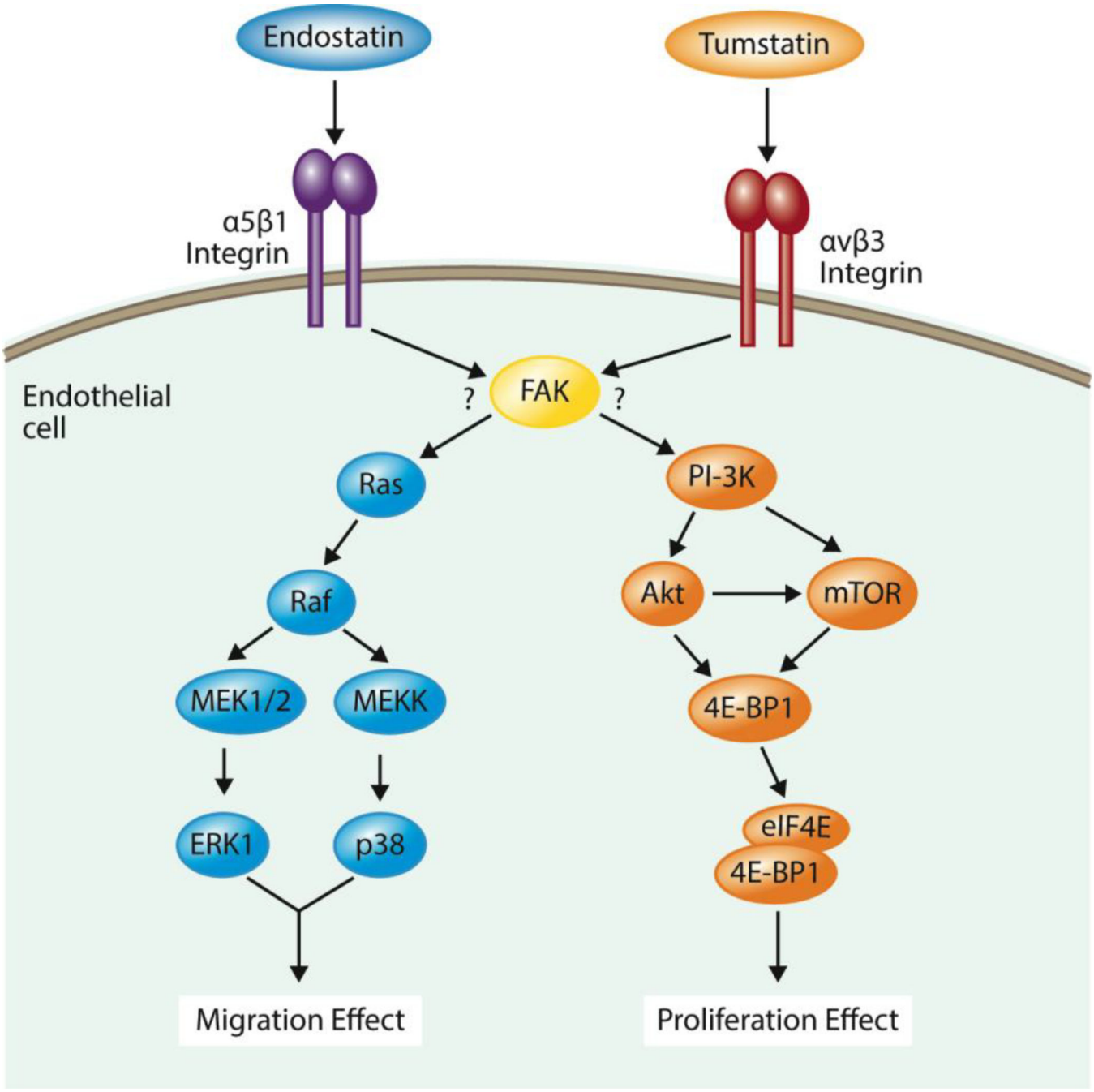

FIGURE 3.

Schematic illustration of distinct signaling pathways induced by rhTum and rhEndo. rhTum binds to $\alpha_v\beta_3$ integrin, whereas rhEndo binds to $\alpha_5\beta_1$. Both rhEndo and rhTum inhibit phosphorylation of FAK (yellow). Downstream of FAK, rhTum inhibits PI3-K/Akt/mTor/4EBP1 pathway, resulting in inhibition of endothelial protein synthesis and proliferation. MAP kinase pathways are not affected by rhTum. In contrast, inhibition of FAK activation by rhEndo binding to $\alpha_5\beta_1$ integrin leads to inhibition of ERK1/p38 MAP kinase pathways

with no effect on PI3-K/Akt/mTOR/4EBP1 pathways, resulting in inhibition of endothelial cell migration. [Adapted from Sudhaker et al. [107]]

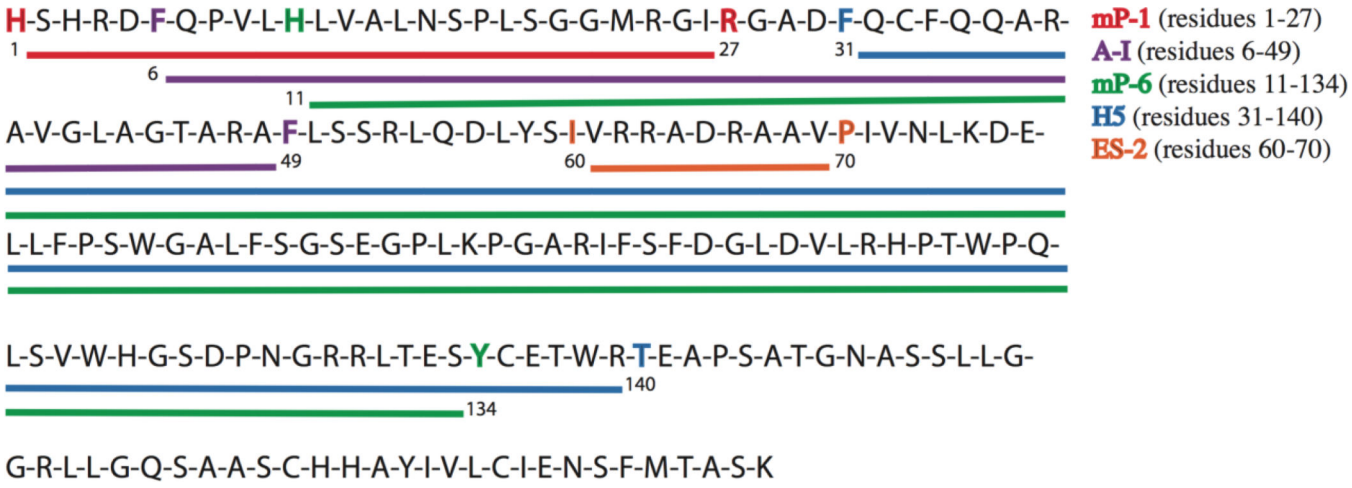

FIGURE 4.

Complete amino acid sequence of recombinant human endostatin. The sequences with improved activity compared to wild type, unmodified endostatin are highlighted and labeled according to the original study, as listed in Table 4.

TABLE 1

Anti-angiogenic peptides released from collagen in basement membranes

Collagen chain	C-terminal domain	MW	Function	Reference
α1(IV)	Arresten	26 kDa	Inhibits endothelial cell proliferation and migration. Suppresses tumor growth. Stimulates apoptosis.	Colorado et al.[38] Nyberg et al.[39]
α2(IV)	Canstatin	24 kDa	Inhibits endothelial cell proliferation and migration. Suppresses tumor growth. Stimulates apoptosis.	Kamphaus et al.[40] Panka and Mier[41]
α3(IV)	Tumstatin	28 kDa	Inhibits endothelial cell proliferation. Suppresses tumor growth. Stimulates apoptosis.	Maeshima et al [42, 43].
α1(VIII)	Vastatin	18 kDa	Inhibits endothelial cell proliferation. Stimulates apoptosis.	Xu et al [18].
α1(XV)	Restin	22 kDa	Inhibits endothelial cell migration. Suppresses tumor growth.	Ramchandran et al [28]. John et al [29].
α1(XVIII)	Endostatin	20 kDa	Inhibits endothelial cell proliferation and migration. Suppresses tumor growth.	Sasaki et al [32].
α1(XIX)	NC1 domain	2 kDa[a]	Inhibits endothelial cell migration. Suppresses tumor growth. Inhibits endothelial cell pseudotube formation.	Ramont et al [19].

[a] Predicted using 19 amino acid sequence of type XIX collagen's NC1 domain [19] with ProtParam [44]

TABLE 2

Phenotypes of collagen knockout mouse models.

Collagen Knockout Model	Phenotype	References
Col4a1−/−	Lethal by embryonic days 10.5–11.5.	Poschl et al. [45]
Col4a2−/−	Lethal by embryonic days 10.5–11.5.	Poschl et al. [45]
Col4a3−/−	A murine model for Alport's syndrome.	Cosgrove et al. [47]
Col15a1−/−	Skeletal muscle degeneration, atrophy, and macrophage infiltration. Increased vulnerability to exercise-induced injuries. Irregularly shaped cardiac endothelial cells, cardiac hypotrophy, and increased myocardial stiffness. Impaired peripheral nerve maturation.	Eklund et al. [49] Rasi et al. [52]
Col18a1−/−	Delayed hyaloid vessel regression with abnormal retinal vasculature. Ocular defects, anterior eye abnormalities, ciliary body atrophy. Abnormal loss in visual function with age. Weakened kidney proximal tubule and podocyte effacement. Hyperlipidemia.	Fukai et al. [66] Hurskainen et al. [67] Chang et al. [68] Marneros and Olsen [69] Ylikarppa et al. [70] Marneros et al. [71] Bishop et al. [72] Kinnunen et al. [73]
Col15a1−/− × Col18a1−/−	Phenotype similar to both *Col15a1−/−* and *Col18a1−/−* mice. More severe hyaloid vessel detachment from retina than in either *Col15a1−/−* or *Col18a1−/−* mice.	Ylikarppa et al. [74]

TABLE 3

A summary of the receptors that endostatin binds to and its subsequent effects.

	Location	Effects of Endostatin Binding	References
Receptor			
VEGFR-2 (flk-1/KDR)	Blood vessel and lymphatic vessel surface	Inhibits angiogenesis by competitively inhibiting VEGFR-2. Prevents VEGF-C induced ERK/p38/MAPK signaling cascade.	Pedram et al. [101] Kim et al. [102]
VEGFR-3 (flt-4)	Lymphatic vessel surface	Inhibits lymphangiogenesis via direct and indirect mechanisms.	Han et al. [104]
Integrin $\alpha_5\beta_1$	Cell surface	Disrupts cell migration. Causes integrin $\alpha_5\beta_1$ to cluster and associate with caveolin-1. Causes phosphorylated Src to associate with caveolin-1 and disrupt focal adhesion factor and actin stress fibers. Hypothesized to prevent mast cells from binding to fibronectin.	Wary et al. [113] Gille et al. [135] Rehn et al. [108] Wickstrom et al. [111] Sudhakar et al. [107] Morais et al. [112] Brideau et al. [136]
Integrin $\alpha_V\beta_3$	Cell surface	Inhibits angiogenesis. Hypothesized to prevent mast cell from binding to vitronectin.	Rehn et al. [108] Brideau et al. [136]
Glypican-1 and -4	Blood vessel surface	Binding is necessary for endostatin to bind to its high-affinity receptor to elicit anti-angiogenic effects via unclear mechanism.	Karumanchi et al. [114] Reis et al. [116]
Cell Surface-Associated Protein			
Thrombospondin-1	ECM	Upregulates thrombospondin-1 expression	Abdollahi et al. [119] Faye et al. [117]
SPARC	ECM	Unknown mechanism.	Abdollahi et al. [119] Faye et al. [117]
Endorepellin	Blood vessel surface	Endorepellin inhibits endostatin's anti-angiogenic activities, but carries out its own anti-angiogenic properties.	Miosge et al. [121] Mongiat et al. [120]
Transglutaminase-2	Blood vessel surface	Unknown mechanism.	Dardick [125] Faye et al. [117] Faye et al. [123]
Biglycan	ECM	Prevents biglycan LDL retention and atheroma formation.	Zeng et al. [127]
Amyloid Peptide	Brain	Implicated in Alzheimer's disease.	Deininger et al. [128] Kranenburg et al. [129] Faye et al. [117]
Nucleolin	Nucleolus	Inhibits cell migration and tube formation.	Srivastava et al. [137] Huang et al. [132] Shi et al. [134]

TABLE 4

Activity and origin of short endostatin peptide fragments.

Peptide Name	Endostatin Residues[a]	Activity compared to wild type, unmodified endostatin[b]	Origin	Reference
H5	31–140	+14%	C- and N-terminal ends removed from human endostatin.	Cho et al. [146]
A-I	6–49	+24%	Human endostatin protein divided into 4 synthetic peptides.	Cattaneo et al. [150] Chillemi et al. [155]
A-II	50–92	No activity		
A-III	93–133	No activity		
A-IV	134–178	+16%		
mP-1	1–27	+27%	Murine endostatin sequence was used to split the endostatin protein into 8 synthetic peptides with overlapping segments of the sequence.	Becker et al. [151] Tanabe et al. [152]
mP-2	23–47	−59%		
mP-3	45–69	−43%		
mP-4	67–91	−31%		
mP-5	89–113	−55%		
mP-6	11–134	+19%		
mP-7	135–159	−12%		
mP-8	157–184	−48%		
ES-1	23–34	−44%	Crystal structure of human endostatin protein was used to divide the sequence into 5 synthetic peptide fragments.	Wickström et al. [153]
ES-2	60–70	+8.7%		
ES-3	99–111	−56%		
ES-4	127–139	−27%		
ES-5	171–183	−56%		
mEP	N/A	+55%	27 amino acid endostatin peptide from C-terminal endostatin sequence.	Han et al. [104]
mEP-CA	N/A	No activity	Cysteine residue on mEP is substituted for alanine.	
mEP-AC	N/A	No activity	Different cysteine residue from mEP-CA is substituted for alanine in mEP.	
mEP-AA	N/A	No activity	Both cysteine residues are substituted with alanine on mEP.	

[a] The endostatin residues are numbered from N-terminus to C-terminus in human endostatin,

[b] Activity reported when available as percentage change from wild type, unmodified endostatin where a positive value is improvement in activity and a negative value is a reduction in activity

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TABLE 5

Diseases and disorders associated with variations in endostatin levels

Disease/Disorder	Endostatin Levels		Endostatin used for Treatment		Endostatin as a Potential Prognostic Marker	References
	Percentage Change from Normal Levels	Tissue or body fluid where endostatin has been measured	Clinical Trial	Animal Model		
Cancer						
Hepatocellular Carcinoma	≥ +169%	Liver Tissue	-	X	X	Hu et al. [197] Musso et al. [198, 199]
Bladder Cancer	≥ +42%	Serum	-	X	X	Cheng et al. [200] Pan et al. [201] Du et al. [202] Szarvas et al. [203]
Cervical Cancer	≥ +13%	Serum	X, Phase I	X	X	Landt et al. [204] Ke et al. [205] Jia et al. [206]
Colorectal Cancer	≥ + 10%	Serum	X, Phase II	-	X	Xu et al. [207] Kantola et al. [208] Lee et al. [209] Chen et al. [210] ClinicalTrials.gov NCT01529164
Nasopharyngeal Carcinoma	≥ +23%	Serum	X, Phase III	-	X	Mo et al. [211] Ye et al. [212] Xu et al. [213] ClinicalTrials.gov NCT01915134
Vascular Disease						
Pulmonary Arterial Hypertension	≥ +76%	Serum	-	-	X	Damico et al. [214]
Coronary Artery Disease with Diabetes Mellitus	≥ +68%	Myocardial Tissue	-	-	-	Sodha et al. [185] Felbor et al. [190] Goldberg et al. [186] Helfant et al. [187] Hansen [188] Habib et al. [189]
Intermittent Claudication in Peripheral Vascular Disease	≥ +22%	Serum	-	-	X	Golledge et al. [215]
Chronic Kidney Disease	≥ +76%	Serum	-	-	X	Maeshima et al. [43] Lerman et al. [193] Stoessel et al. [194]

Disease/Disorder	Endostatin Levels		Endostatin used for Treatment		Endostatin as a Potential Prognostic Marker	References
	Percentage Change from Normal Levels	Tissue or body fluid where endostatin has been measured	Clinical Trial	Animal Model		
						O'Riord an et al. [195] Chen et al. [196]
Neurological Disease						
Alzheimer's Disease	≥ +257%	Cerebrospinal Fluid and Brain Tissue[a]	-	-	X	Deininger et al. [128] Salza et al. [216]
Traumatic Brain Injury	≥ +36%	Cerebrospinal Fluid	-	-	X	Chen et al. [217] Mueller et al. [218]
Other						
Bronchopulmonary Dysplasia	≥ +13%	Cord Plasma	-	-	X	Mohamed et al. [219] Janer et al. [220]
Corneal Graft Rejection	≤−46%	Cornea	-	X	X	Tan et al. [221]
Rheumatoid Arthritis	No change	Joint Fluid	-	X	-	Huang et al. [222] Nagashima et al. [223]
Retinopathies	≤−32%	Vitreous Fluid	-	X	X	Bai et al. [145] Funatsu et al. [224]
Malignant Pleural Effusion	≥ +24%	Serum and Pleural Fluid	-	X	X	Ma et al. [177] Zhang et al. [225] Zhou et al. [226]

[a] Salza et al. [216] measured endostatin levels in the cerebrospinal fluid, and Deininger et al. [128] measured endostatin levels by immunohistochemistry in brains of patients with Alzheimer's disease.