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Endostatin and endorepellin: a common route of action for similar angiostatic cancer avengers

Chiara Poluzzi^a, Renato V. Iozzo^b, and Liliana Schaefer^{a,*}

^aPharmazentrum Frankfurt/ZAFES, Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der Goethe-Universität Frankfurt am Main, Frankfurt am Main, Germany

^bDepartment of Pathology, Anatomy and Cell Biology, and the Cancer Cell Biology and Signaling Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107 USA

Abstract

Traditional cancer therapy typically targets the tumor proper. However, newly-formed vasculature exerts a major role in cancer development and progression. Autophagy, as a biological mechanism for clearing damaged proteins and oxidative stress products released in the tumor milieu, could help in tumor resolution by rescuing cells undergoing modifications or inducing autophagic-cell death of tumor blood vessels. Cleaved fragments of extracellular matrix proteoglycans are emerging as key players in the modulation of angiogenesis and endothelial cell autophagy. An essential characteristic of cancer progression is the remodeling of the basement membrane and the release of processed forms of its constituents. Endostatin, generated from collagen XVIII, and endorepellin, the C-terminal segment of the large proteoglycan perlecan, possess a dual activity as modifiers of both angiogenesis and endothelial cell autophagy. Manipulation of these endogenously-processed forms, located in the basement membrane within tumors, could represent new therapeutic approaches for cancer eradication.

Graphical abstract

*Corresponding author: Liliana Schaefer, M.D., Pharmazentrum Frankfurt, Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der JW Goethe-Universität Frankfurt am Main, Haus 74, Z. 3.108a, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany; Tel: +49 69 6301-7899; Fax: +49 69 6301-83027; Schaefer@med.uni-frankfurt.de.

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Keywords

proteoglycan; angiogenesis; autophagy; collagen type XVIII; perlecan

1. Introduction

The development of new blood vessels from pre-existing vasculature, known as angiogenesis, is a complex mechanism involving the concerted actions of endothelial cells, smooth muscle cells and pericytes. Due to the intrinsically high proliferative rate of cancer cells, the supply of nutrients and oxygen via angiogenesis is a *sine qua non* for the overall expansion of cancers [1]. Conventional therapy, exerting a cytotoxic action, has been commonly focused on targeting the mass of growing cancer cells; however, drug resistance to single agent therapies is often an adverse outcome [2].

Proteoglycans are large molecules with complex modular structures that reside in strategic positions, within the extracellular matrix and basement membranes, and are in close contact with vascular endothelia. By virtue of their particular architecture, they directly interact with ligands and receptors involved in the regulation of tumor growth and new vasculature formation [3]. The modular nature of proteoglycans results in their susceptibility to proteolytic attack by diverse enzymes in the extracellular environment thereby releasing individual modules with biological activity, often with opposite effects than the parental protein core [4, 5].

Autophagy is an emerging field in the context of cancer progression. It is a mechanism exerted through the action of lysosomes that allows cells to maintain a homeostatic balance between *de novo* generated and degraded molecules, under normal conditions. Often, it is physiologically induced to counteract the lack of available nutrients in high metabolic situations, where an energetic supply is needed [6–10]. Autophagy can evoke apoptotic cell death [11–13] but, in response to cytotoxic stimuli, can promote autophagic programmed cell death (PCD) in cells that are instead protected against apoptosis [14]. Hence, autophagy exhibits duality, in that it may be cytoprotective or cytotoxic.

Many factors combine to orchestrate and regulate angiogenesis and autophagy, and since aberrations of these programs are often seen in tumors, its modulation holds clinical value in cancer therapy [15]. Recent evidence suggests that several constituents of the extracellular

matrix can regulate autophagy via interaction with cell surface receptors [16]. Thus, together with the ability to regulate angiogenesis [17], proteoglycans and other matrix constituents can harbor pro-autophagic activity that can be beneficial in suppressing cancer growth [18–22]. Recent discoveries have pointed out a new activity for endogenously-released fragments of the extracellular matrix, not only as anti-angiogenic factors but also as autophagy inducers [23–25].

In this review, we will critically assess the role of two well-known fragments derived from heparan sulfate proteoglycan (HSPG) protein core, namely endostatin derived from collagen XVII and endorepellin, derived from perlecan. After several years of investigating the biological effects of these two anti-angiogenic factors there is new evidence indicating that both bioactive molecules converge on a common theme of action: dual receptor antagonism leading to angiostatic and pro-autophagic activity.

2. Collagen XVIII

Collagen XVIII belongs to a group of collagen-like proteins of the extracellular matrix also known as multiplexins, which include collagen XV as its closest relative [26]. It was subsequently discovered that collagen XVIII is substituted with HS chains and thus it is a true HSPG [27]. Collagen XVIII possesses a trimeric structure with a central area of three homologous $\alpha 1$ chains, and it harbors ten collagen regions interrupted by eleven non-collagenous (NC) domains [27, 28] (Fig. 1A). Collagen XVIII and XV share an N-terminal thrombospondin-like module. In addition, the N-terminus of collagen XVIII can contain a cysteine-rich domain related to the frizzled module of *Drosophila* and/or an acidic segment A, based on alternative splicing. These multiplexins components can be modified by chondroitin sulfate chains, on collagen XV, or HS side chains, on collagen XVIII [27, 29, 30]. They not only share structural homology but also a C-terminal NC1 module containing the endostatin protein with intense angiostatic activity (Fig. 1A). Localized to chromosome 21 [31], the gene of human collagen XVIII possesses 43 exons and two promoters. Variants of its transcription generate a total of three different isoforms. One short form of this collagen is NC11-303, whereas another promoter activity is responsible of the other two longer isoforms [32–36].

Collagen XVIII is widely distributed and it is one of the main constituents of epithelial and vascular basement membranes [26]. Mice deficient in *Col18* show abnormal eye development [37] and abnormal ocular vessel formation and maturation [38–40]. Additionally, during atherosclerosis collagen XVIII plays a role in neovascularization and in preserving the permeability of blood vessels [41, 42]. Collagen XVIII has been suggested not only as an anti-atherosclerotic factor but also as a negative regulator of angiogenesis. Indeed, aortic explants isolated from *Col18a1*^{−/−} mice show increased angiogenesis compared to wild-type mice [43]. Recently, collagen XVIII has been implicated in the pathogenesis of renal ischemia/reperfusion as a mediator of leukocytic influx [44], and in hyperlipidemia associated with fatty liver and visceral obesity, suggesting that it might play a role in the adipose tissue formation [45].

2.1 Prognostic relevance of collagen XVIII in cancer

In humans, a mutation in *COL18A1* gene results in an autosomal recessive disease, the Knobloch syndrome, which in turn leads to blindness at birth because of abnormal retinal development [36, 46]. Similarly, a pathology in which the retina is not well vascularized has been also reported in *Col18a1*^{-/-} mice [47]. Notably, in *C. elegans*, deletion of the NC1 domain of *cle-1*, the orthologue of human collagen XVIII, induces defects in cell migration and axonal guidance, and this phenotype can be rescued by ectopic expression of this domain [48].

In spite of an accumulating wealth of information on the role of collagen XVIII in various pathological states, the biological role of collagen XVIII in human cancer is not well defined. There are several studies reporting abnormal levels of endostatin (see also below) in gastrointestinal cancer [49, 50]. High expression of collagen XVIII, as the precursor of this angiogenesis inhibitor, could adversely impacts prognosis [51].

In pancreatic cancer, characterized by a florid desmoplastic response, over-expression of both type collagens IV and XVIII in the tumor vasculature has been reported. Interestingly, the circulating levels of both collagens are elevated at the time of the diagnosis in pancreatic cancer patients compared to controls, and these levels are normalized following treatment. These results suggest a role for type XVIII collagens as a potential tumor marker for pancreatic cancer [52–54]. Similarly, poor outcome associated with over-expression of collagen XVIII, and high endostatin levels in the serum, are found in non-small cell lung cancer [55, 56]. Collagen XVIII over-expression closely correlates with poor clinical response and is an independent prognostic factor [56].

Collectively, these studies support a role for this HSPG as a potential prognostic biomarker for gastrointestinal and pulmonary carcinomas.

2.2 Endostatin, the processed NC1 domain of collagen XVIII

The history of the angiogenic field is marked by two seminal developments. The first is Folkman's concept that tumors need angiogenesis to survive [1] and the second is the discovery of the vascular endothelial growth factor [57]. Additional milestones in this field include the discovery of novel anti-angiogenic fragments derived from extracellular matrix proteins, such as the identification of an anti-angiogenic thrombospondin fragment [58] and angiostatin, the first endogenous angiogenesis inhibitor that specifically inhibits endothelial cell proliferation [59]. Few years later, a more powerful endothelial cell inhibitor and tumor-induced angiogenesis suppressor, endostatin, was characterized [60]. The N-terminal hinge domain of collagen XVIII is processed with high efficiency by the proteases cathepsin L and elastase, releasing the 22-kDa fragment endostatin as a monomer [61–64] (Fig. 1A). Endostatin-like fragments of ~25 kDa have been detected in human tissue and sera that are similarly cleaved from the NC1 domain, although the cleavage sites are not the same between human and mouse [65, 66]. Due to the high identity of the terminal NC1 domains of collagens XVIII and XV, endostatin-like fragments are also generated from the latter, showing an analogous globular architecture [67]. The crystal structure of endostatin is composed of ~180 amino acid residues, folding into 2 α -helices, 16 β -sheets and 2 disulfide

bridges. In addition, monomers released from collagen XVIII harbor a sequence of 11 arginine residues that serves as HS-binding site, which has significantly lower complexity in collagen XV-endostatin forms, thereby explain the differential affinity for heparin/HS between the two endostatins [62, 68]. Notably, endostatin harbors 3 N-terminal histidine residues that allow its binding to zinc ions. This region could also be involved in HS-binding and modulating endostatin bioactivity [69, 70].

Endostatin has multiple interacting receptors (Fig. 1B). Initially, it was discovered that recombinant endostatin binds to $\alpha 5\beta 1$ - and $\alpha V\beta 3/\alpha V\beta 5$ -integrins antagonizing their biological activities on endothelial cells [71, 72]. Endostatin can also inhibit the binding of the αV_3 -integrin-ligand, gelatin, to endothelial cell [73]. Further studies have shown that endostatin can enhance its angiostatic activity by binding to HS chains of proteoglycans via its heparin-binding region [68, 74]. Utilizing tagged endostatin, the cell surface HSPG glypican was identified as a low-affinity binding partner, potentially playing a role as a co-receptor [75]. Furthermore, endostatin has been found to bind tropomyosin [72, 76], caveolin-1 [77, 78] and directly to the fibronectin receptor $\alpha 5\beta 1$ - integrin [72]. Recently, binding to the vascular endothelial growth factor receptor (VEGFR)1 and VEGFR2 has also been detected [79, 80] (Fig. 1B). In addition, endostatin affects the Wnt signaling pathway and can also interact with nucleolin, see below [81, 82].

The presence of five different receptors for endostatin underlines the emerging concepts that extracellular matrix constituents have multiple partners in their outside-in signaling. This is in contrast with natural secreted ligands for various receptors, which are highly specific for one receptor or class of receptors. As shown below, endorepellin has also multiple receptors with relatively high affinity (Kd in the nanomolar range) similar to their natural ligand counterparts.

2.3 Anti-angiogenic properties of endostatin

The first evidence for the angiostatic nature of endostatin was identified with its isolation in 1997 [60]. A protein with the same sequence of the C-terminal domain of collagen XVIII contained in conditioned media of murine hemangio-endothelioma cells inhibited bovine capillary endothelial cell proliferation. The “shrinking” of blood vessels formation by endostatin was also detected *in vivo* through the chick chorioallantoic membrane (CAM) assay and proved by systemically administration of endostatin in tumor-bearing mice [60]. Later on, recombinant mouse endostatin was found to have similar function on the endothelium of blood vessels as the native form [83–85]. In contrast, a variety of non-endothelial cells are not inhibited in the proliferation by native or recombinant purified endostatin [60].

Endostatin has a broad spectrum of angiostatic activities on endothelial cells [86]. One of the proposed mechanisms is via inhibition of matrix metalloproteinases (MMPs). During angiogenesis, endothelial cell migration and invasion are facilitated by MMPs-mediated proteolytic degradation of the extracellular matrix [87, 88]. In particular, MMP-2, MMP-9 and MMP-13 have been identified as targets for endostatin inhibitory action [61, 73, 89, 90]. Furthermore, initial reports described inhibition of human vascular endothelial cell migration by endostatin through its binding to $\alpha 5$ - and αV -integrins [71, 72, 91]. Other

angiostatic functions of endostatin can be summarized in three major downstream effects: actin disassembly *via* Src-dependent-p190RhoGAP activation [77, 78], inhibition of the FAK/Ras/p38-MAPK/ERK signaling cascade through $\alpha_5\beta_1$ -integrin binding [91, 92], with suppression of HIF-1 α /VEGFA, and Wnt signaling-dependent down-regulation of β -catenin [93] (Fig. 2). Moreover, endostatin binds directly to VEGFR2, without binding to its ligand, and inhibits VEGF-induced phosphorylation with consequent down-regulation of this receptor [79, 80, 94]. The VEGF-mediated downstream signaling pathway involving p125FAK, ERK and p38MAP kinase is suppressed by endostatin in human endothelial cells [79].

It is possible that the direct binding to VEGFR2 by endostatin is responsible for the inhibition of VEGF activity, through competitive blockade of VEGF, thereby promoting endostatin's angiostatic effects. In line with this hypothesis is the observation that endostatin blocks vascular endothelial tube formation by suppressing nitric oxide synthase (eNOS) [95]. Specifically, endostatin blocks VEGF-induced eNOS phosphorylation at Ser1177 but not VEGF-mediated Akt phosphorylation and Akt-stimulated endothelial cell migration. When a dominant negative inactive construct of the catalytic domain of the phosphatase PP2A, known to inactivate eNOS, is used, endostatin suppresses VEGF-induced endothelial cell migration by activating PP2A, therefore inhibiting eNOS phosphorylation and consequent angiogenesis. Moreover, endostatin down-regulation of the Wnt pathway has been demonstrated in HUVEC [93, 96]. Endostatin inhibits the Wnt signaling *via* down-regulation of β -catenin in a GSK-3 β -independent manner [93]. Degradation of β -catenin leads to the suppression of the transcription of important genes involved in cell cycle, like Myc and cyclin D1. Furthermore, G1 arrest of endothelial cell by endostatin, down-regulation of Myc mRNA expression and decreased cyclin D1 have been also detected [97]. Importantly, several studies report endothelial cell apoptosis as a common mechanism of endostatin anti-angiogenic activity [86, 98, 99].

Another important player in endostatin's actions is nucleolin [82], a ubiquitous protein involved in several activities as chromatin organization, cell proliferation and ribosome assembly [100–102] (Fig. 1B and 2). Nucleolin is present at the cell surface of endothelial cells and acts as a receptor for endostatin [81, 103]. Upon the binding of endostatin *via* its heparin-binding site, nucleolin induces its internalization and translocation to the nuclei [81]. This receptor is present only in the endothelium of new blood vessels and its phosphorylation, important for cell proliferation and mediated by VEGFA [104], can be suppressed by nuclear translocated-endostatin. Co-localization of nucleolin with endostatin at the cell surface of endothelial cells within tumor tissues provides further evidence for a potential role of nucleolin in cancer [81, 103]. Endostatin activity has been further described to be dependent on E-selectin expression, which may regulate its efficacy *in vivo* [105].

The degree of complexity of endostatin molecular network has been revealed by a study using a custom microarray platform covering almost the entire human genome. It was found that ~12% of all human genes are modulated by endostatin in human endothelial cells [106]. These changes involve down-regulation of TNF- α , NF- κ B, STAT, ephrin, HIF-1 α , AP-1, and several others, but also up-regulation of endogenous angiogenesis inhibitors like

thrombospondin-1 [106]. Clearly, endostatin plays a key role in endothelial cell formation, survival and migration, as well as cell adhesion, proliferation and apoptosis.

2.4 Modulation of autophagy/apoptosis balance by endostatin in endothelial cells

An emerging new function for endogenous angiogenesis inhibitors released from matrix molecules is the ability to evoke autophagy in endothelial cells. Commonly increased under stress or nutrient deprivation, autophagy is the physiological catabolic mechanism by which excess components, undegraded proteins or damaged molecules, are processed to generate new macromolecules [7]. Soluble endostatin induces autophagy in endothelial cells by directly interacting with the $\alpha 5\beta 1$ integrin and modulating Beclin 1 and β -catenin levels, thereby potentiating its angiostatic activity [24]. It has also been reported that endostatin induces autophagic cell death in the Eahy926 human endothelial cell line [107], a lung cancer cell line fused with HUVEC. Both native and a mutant form of endostatin, P125A-endostatin with increased anti-angiogenic properties, induce autophagy in endothelial cells [24]. Thus, it is possible that endostatin-evoked autophagy could occur not only *via* the binding to the $\alpha 5\beta 1$ integrin but also by down-regulating the Wnt/ β -catenin canonical pathway (Fig. 2). Indeed, when β -catenin levels are silenced by the use of small interfering RNAs, endostatin induces an increased autophagic rate, demonstrated by higher Beclin 1 levels and autophagic vacuoles in endothelial cells vis-a-vis cells treated with endostatin. In contrast, over-expression of β -catenin down-regulates Beclin 1 levels in presence of endostatin [24]. Endostatin can also evoke autophagy in a PI3K- and Wnt-dependent mechanism [24].

Another endogenous inhibitor of angiogenesis, kringle 5, which is a processed form of plasminogen, can induce autophagy under normoxia and nutrient-rich conditions [25]. As with endostatin, a common feature of the two treatments is the up-regulation of Beclin 1 levels leading to alterations in the Beclin 1/Bcl-2 complex in favor of their disassociation and consequent pro-autophagic stimulus. Interestingly, angiogenesis inhibitors induce autophagy in endothelial cells independent of nutritional or hypoxic stress, and it takes place even in the presence of endothelial-specific survival factors such as VEGF.

Endostatin-induced autophagy could represent an attempt by the cell to rescue itself from endostatin-mediated anti-angiogenic action [24]. Indeed, it has been previously proposed that endostatin's angiostatic effect on tumor endothelium could be the result of its ability to induce apoptosis of endothelial cells [98]. Down-regulation of the endogenous levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL has been reported in cow pulmonary artery endothelial cells with consequent endostatin-evoked apoptosis [98]. Likewise, endostatin evokes apoptosis in murine brain endothelial cells via a tyrosine kinase-induced mechanism mediated by the Shb adaptor protein [99], and can induce endothelial cell apoptosis by activating caspase-3 thereby reducing the anti-apoptotic protein Bcl-2 and MAP kinase activities [98]. Indeed, Bcl-2 dephosphorylation and intracellular levels are also modulated by P125A-endostatin in HUVEC [24]. Finally, interfering with the autophagic response by knocking down Beclin 1 dramatically increases apoptosis of endothelial cells. This balance between endostatin-mediated autophagy and apoptosis could be modulated in favor of one or the other depending on the particular cellular context.

Endothelial cells with a senescence phenotype have impaired homeostatic functions as loss of NOS generation and increased peroxy-nitrite formation. Enhanced endostatin, physiologically released from collagen XVIII, in mice repeatedly exposed to NOS inhibitors, induces transformation of these defective endothelial cells maintained under stress-induced premature senescence conditions (SIPS). This process leads to a reduction of renal microvasculature as a default mechanism of suppression of damaged cells in which autophagy is impaired [108]. Thus, manipulations focused on increasing the autophagic rate of endostatin in these cells *versus* endostatin-induced cell death, could prevent or reverse the SIPS phenotype of endothelial cells. In contrast, endostatin-evoked apoptosis has been shown in Eahy929 cells and described as an autophagy-induced, caspases-independent cell death [107]. Therefore, we speculate that autophagy in vascular endothelium may be a novel mechanism that specifically targets endothelial cells and could improve the angiostatic therapeutic potential of endostatin. Modulation of autophagy exerted by this angiostatic factor could represent a novel therapeutic agent used to potentiate its intrinsic anti-angiogenic activity in the context of a tumor microenvironment.

3. Perlecan, a large multimodular heparan sulfate proteoglycan

Perlecan is a large pericellular proteoglycan with a ~470-kDa protein core and three HS chains at its N-terminus [109–112] (Fig. 3A). Perlecan is a ubiquitous HSPG that is expressed in most basement membranes, but it has a unique feature, that is, perlecan is one of the few molecules that is expressed in both vascular and avascular tissues [4, 113–116]. Originally perlecan was isolated from the basement membrane of Engelbreth-Holm-Swarm (EHS) mouse sarcoma revealing a molecule that was first hypothesized to function as an anionic filter, but later described as the multimeric proteoglycan perlecan [117]. The HSPG2 gene is located on chromosome 1p36, and is composed of 97 exons, covering over 120 kb of DNA [118, 119]. It possesses a complex structure organized in an array of five bioactive modules with high homology to a multitude of adhesion and signaling proteins, and growth factors, accounting for its broad biological activity [110]. The ~470 kDa protein core harbors a SEA module at its N-terminal domain I, four LDL receptor repeats in domain II, three laminin-like and nine EGF-like repeats in domain III, and a large array of IgG-like repeats in domain IV [110, 112] (Fig. 3A). Domain V contains endorepellin (see below) and is composed of three Laminin-like globular (LG) modules interspersed by four EGF repeats.

Perlecan is highly conserved across animal lineages and interacts with a variety of partners via either its HS chains or its protein core domains [113, 114]. Laminin, collagen IV, nidogen-1, fibronectin, angiopoietin-3, fibrillin-1 and thrombospondin-1 are just a few of the partners interacting with its domain I [110, 120, 121]. Perlecan-binding molecules include several growth factors of the fibroblast growth factor family (FGF-1, FGF-2, FGF-7, FGF-9 and FGF-18), FGF-binding protein, PDGF, VEGF, HGF and several others [122–130]. In addition, perlecan promoter can be positively regulated by TGF- β [131, 132] and negatively regulated by interferon- γ [133], making perlecan an early response gene. A novel interaction of this proteoglycan has been found at nodes of Ranvier, where perlecan directly interacts with the neurofascin-glia ligand, gliomedin [134].

As in the case of collagen XVIII, perlecan has multiple receptors, surprisingly all interacting with domain V/endorepellin (Fig. 3B). Domain V has a high-affinity binding site for the laminin receptor α -dystroglycan, higher than laminin α 1 and α 2 chains [5, 135, 136]. Interestingly, perlecan and laminin α 1 chain are major components of the Reichert's membrane [137], and a complex between perlecan and dystroglycan has been described to occur at the neuromuscular junction [138–140]. As mentioned above, gliomedin has been recently discovered to participate in this interaction. One of the strongest evidence for a perlecan receptor is the interaction of domain V/endorepellin with α 2 β 1-integrin. This is based on genetic and biochemical evidence pointing to a binding to the α 2 I subunit of the integrin for downstream signaling activity [141]. More recently, the C-terminal fragment of perlecan has been found to interact with both VEGFR1 and VEGFR2 at the endothelial cell surface, directly modulating the intracellular signaling of the latter receptor [142] (Fig. 3B). Perlecan as a whole molecule can interact with the endothelial cell receptor VEGFR2 through its ligand VEGF, which is bound to its HS chains, therefore influencing the VEGFA-VEGFR2 signaling axis [15, 143].

During murine development, *Hspg2* mRNA levels appear early, at embryonic day 4.5, and increase with time being detectable at day 10 in early endothelial tissues (e.g. the heart/ major blood vessels), followed shortly by the mesenchyme. Later in development, its expression in organs such as the liver, kidney and spleen is required for correct tissue differentiation [144, 145]. Perlecan has crucial relevance also in the mature animal as it is retained in adult life in all basement membranes and in some mesenchymal tissues and it is continually expressed. Indeed, mutations in *HSPG2* gene are associated with several hereditary disorders. For example, Schwartz-Jampel Syndrome type 1 (chondrodystrophic myotonia), an autosomal recessive disorder, is caused by missense and splicing mutations in *HSPG2* [146, 147].

The roles that perlecan may play are vast. For example, perlecan is involved in regulating skin and endochondral bone formation [127, 148], modulation of lipid metabolism [149], cell adhesion and cell death [150–152], and osteophyte formation [153]. Furthermore, it plays a role in maintaining the biomechanics of cartilage and vasculature [154–156] and it regulates the Sonic Hedgehog pathway during development [157]. Perlecan possesses adhesive [150] or anti-adhesive [158] properties depending on the interactions of its different domains with the α 2 β 1 integrin receptor. The recent report that mast cells can also release perlecan in a shorter form, as well as its domain V, open new horizons to an involvement of perlecan or its terminal domain in tissue repair and inflammation [159, 160].

Perlecan can regulate murine neurogenesis during telencephalon development [161] and can intensify clustering of components of nodes of Ranvier, by double binding to both dystroglycan and gliomedin [134]. Thus, perlecan might be involved in normal or pathological processes relating to peripheral nodes, since Na⁺-channel accumulation and fast conduction are mediated by nodes of Ranvier. HSPG2 has been further suggested to modulate lipid retention and atherogenesis. In fact, perlecan domain II can bind low-density lipoproteins via its O-linked oligosaccharides, and could therefore induce endocytosis and catabolism of this protein by acting as a specific lipoprotein receptor [149, 162].

3.1 The role of perlecan in angiogenesis and tumor growth

While perlecan has a variety of roles outside the circulatory system, the first evidence for its involvement in developmental angiogenesis and vasculogenesis was based on studies performed in murine and zebrafish models [144, 163–165]. Although vasculogenesis appears normal, absence of perlecan in zebrafish, evoked by specific morpholinos targeting *Hspg2* mRNA, reveals not only circulatory flow deprivation in morphant vessels but also marked inhibition of the sub-intestinal and intersegmental vessels [165]. The latter vessels are generated by angiogenic sprouting emanating from the dorsal aorta, thus genetically confirming a primary role for perlecan in angiogenesis and correlating well with the murine mutants. Most of the *Hspg2*^{-/-} mice are embryonic lethal due to intracardiac hemorrhage at ~day 10.5, around the time of maximal perlecan expression; the few that survive birth, die perinatally because of breathing impairment secondary to profound chondrodysplasia and serious defects in the cardiovascular system, due to a profound remodeling of great vessels and coronary arteries [166, 167]. Interestingly, apart from cardiovascular defects, strong abnormal developmental angiogenesis was not evident in these null-mice, suggesting a compensatory role exerted by collagen XVIII. This hypothesis was corroborated by studies carried out in double deficient mice for perlecan HS chains and collagen XVIII [168]. In parallel to these findings, experiments performed in *Drosophila* with loss-of-function mutations in *Trol* gene (“terribly reduced optical lobes”), the ortholog of perlecan, reveal embryonic lethality and larval defects in the central nervous system [157, 169, 170]. Further manipulations of this gene reveal a role for *Trol* not only in modulating FGF and Hedgehog signaling, as its human ortholog, but also in key steps of the larva development [157, 169, 170].

Perlecan’s pro-angiogenic activity is exerted principally through the modulation of the FGF-2 pathway [171]. A major role in this process is played by its HS side chains, which present this ligand to its receptor, thereby inducing a complex downstream signaling cascade [172]. By modulating FGF2 signaling, HSPG2 promotes cell proliferation, motility and adhesion, and helps in maintaining endothelial integrity and barrier function [122, 151]. Likewise, perlecan positively affects angiogenesis through the modulation of the VEGFR2-Neuropilin-1 signaling axis, which is achieved by directly presenting its HS-bound VEGFA to the VEGFR2 or indirectly bringing VEGFA to its receptor upon heparanases-mediated cleavage [15, 143, 173]. Moreover, perlecan is involved in inhibiting *in vivo* thrombosis following vascular injury and contributes to the control of intimal hyperplasia mediated by endothelial cell [174].

During cancer growth, the basement membranes of the vasculature are repeatedly subjected to remodeling, a process in which growth factors are liberated from perlecan *via* MMPs and heparanase [175–177]. The multimodular perlecan located along the pathways of migrating endothelial cells, which interact with the proliferating cancer cells, could represent one of the best candidates to modulate angiogenesis in favor or against cancer progression [116, 178]. Indeed, a large number of growth factors and pro-angiogenic proteins are trapped by perlecan in this zone, and because of its large structure it might link cells with other extracellular matrix constituents providing a network for vessels to grow and expand [179]. Aberrant perlecan expression or fluctuations of its levels can predictably happen during the

progression of some cancers, generally leading to enhanced invasiveness and metastatic potential [180, 181]. Consistent with these findings, abundant perlecan deposition has been observed in the stroma of human breast cancer tissue [182, 183], whereas a 15-fold increase in the mRNA levels of perlecan have been found in metastatic melanoma tumor samples [184]. Just after a 10-minute stimulation with neurotrophin, there is a robust induction of perlecan levels suggesting that, in the context of tumor invasion, perlecan is also an early response gene. Similarly, utilizing antisense perlecan cDNA in human melanoma cells, autocrine and paracrine FGF-2 activities are inhibited. Presumably, with lower perlecan HS chains there is a low bioactivity for FGFs resulting in attenuation of cell proliferation and invasion, further emphasizing a strong mechanistic link between cancer prognosis and the tumor micro-environment [185]. In support of this cross-talk between perlecan and the surrounding tumor, colon carcinoma mouse models in which perlecan is suppressed result in inhibition of new vessels formation and cancer growth [186]. This role of perlecan in enhancing the progression of the tumor is further exhibited in prostate cancers, where higher level of perlecan has been found, associated with rapid cell proliferation independently of androgen signaling [187]. In addition, correlation between perlecan expression and aggressiveness of the tumor has been measured by Gleason scores, revealing a strong association between the two. In contrast, perlecan can also function as a negative modulator of cancer progression and invasion in fibrosarcoma cells and xenografts [188], and in human Kaposi's sarcoma cells.

In summary, for the most part, over-expression of the perlecan parent molecule is pro-angiogenic and pro-tumorigenic, with just a few examples leading to the opposite conclusion. Thus, the cellular context must play a role in the biology of this fascinating macromolecule.

3.2 Endorepellin, the angiostatic terminal “pearl” of perlecan: dual receptor antagonism

Following on the heels of endogenous anti-angiogenic cleaved fragments, the C-terminal domain of the pericellular proteoglycan perlecan was shown to be a potent endothelial cell repellent, therefore called endorepellin. The origin of endorepellin traces back to the seeking for new binding partners of perlecan by a yeast two-hybrid screening. Soon it was discovered that a segment of the NC1 domain of collagen XVIII, containing endostatin, can act as an interacting partner and immediately recognized that domain V of perlecan had similar activity as endostatin [189] (Fig. 3A). Endorepellin is produced by proteolytic cleavage from its parental molecule and it resembles the short end of a linear bracelet consisting of three laminin-like globular (LG) domains interspersed by two epidermal growth factor (EGF)-like repeats [159, 160, 190].

As previously described, promoting angiogenesis is an inherent characteristic of the parent perlecan molecule. However, its cleaved terminal fragment possesses a complete opposite activity on blood vessel formation and spreading. Moreover, endorepellin binds to two additional functional receptors (Fig. 3B). It was first discovered that endorepellin binds to the $\alpha 2 \beta 1$ -integrin with downstream cytoskeleton disassembly and inhibition of endothelial cell migration [189, 191]; then endorepellin-mediated down-regulation of VEGFR2 was found to give its contribution to this signaling cascade [142], therefore enhancing

endorepellin anti-angiogenic activity on endothelial cells by dual receptor antagonism [192] (Fig. 4).

3.3 The C-terminal fragment of perlecan: an endogenous ligand of the $\alpha 2\beta 1$ -integrin receptor

Perlecan and its parts have dual activity, consisting in either acting as pro-angiogenic or angiostatic molecules [15]. Precisely, perlecan possess the ability to modulate not only the VEGFA/VEGFR2 pathway but also the $\alpha 2\beta 1$ integrin cascade. In parallel to its pro-angiogenic downstream signaling *via* interaction with the VEGFR2, perlecan can act as an angiogenesis inhibitor by the binding through its domain V, endorepellin, or its C-terminus processed form, LG3, to the $\alpha 2\beta 1$ integrin (Fig. 4). Indeed, the 85-kDa endorepellin results from the cleavage activity of proteases, whereas BMP-1/Tolloid-like family of metalloproteases can further liberate the C-terminal 25-kDa LG3 [160, 193]. In serum starved apoptotic endothelial cells, caspase-3 activation induces lysosomal destabilization and consequent extra-lysosomal release of cathepsin-L, which has also been shown to produce the LG3 fragment of endorepellin. The serine protease tPA translocated at the periphery of the cell, but not released in soluble form by endothelial cells undergoing apoptosis, can induce the cleavage of LG3 as well, although to a lower extent [194]. Fragments of domain IV extending into domain V and thus, containing endorepellin, have been observed previously in the blood [195]. Endorepellin has been also identified in the fetal bovine rib growth plate [196], and in proteomic studies of chronic allograft nephropathy [197]. More recently, full-length endorepellin has been identified in pancreatic cysts [198].

The first evidence of endorepellin's anti-angiogenic bioactivity showed a dramatic suppression of endothelial cell migration as well as collagen-mediated capillary morphogenesis; in addition, a sustained repression of blood vessel maturation was also observed through CAM and Matrigel plug assays [189]. It is not known whether endorepellin is presented to cells in solution or bound to the matrix. This is physiologically important as the activities of the domain V are also reported to be pro-angiogenic through promoting both endothelial cell adhesion and proliferation when presented to the cells bound to a surface [160]. During these studies, endorepellin/LG2 module was found to specifically bind endostatin, whereas the interaction with the α -dystroglycan was already shown to be exerted by both LG1/2 domains, with lower affinity detected for LG3 [135]. In addition, in surface plasmon resonance assays, endorepellin was detected to bind to fibulin-2, nidogen in two different nidogen epitopes, and the laminin-nidogen complex [5, 120, 135]. However, the extracellular matrix protein (ECM)1 and progranulin represent the unique binding partners of domain V of perlecan [199, 200].

It is well established that perlecan co-localizes with endostatin in several basement membranes [4, 27, 201]; however, several assays have subsequently demonstrated that endorepellin not only binds to endostatin, but also their activities are counteracted when concurrently present on endothelial cell surface. Notably, the effects of the two anti-angiogenic factors are not additive. Endostatin's actions on blood vessel cells are blocked by endorepellin, and similarly endorepellin functions are affected by endostatin with the

exception of endorepellin-mediated inhibition of tube formation [189]. Thus, these *in vitro* interactions suggest that *in vivo* and, especially during tissue remodeling such as tumor angiogenesis, the endorepellin/endostatin interactions might be very complex.

Endorepellin's function on endothelial cells triggers a signaling cascade that ultimately leads to dissolution of the actin cytoskeleton and disruption of focal adhesions [191]. Soluble endorepellin increases cyclic AMP levels which activates protein kinase A (PKA), followed by sustained activation of focal adhesion kinase (FAK), but not ERK1/ERK2, and transient phosphorylation of heat shock protein 27 (HSP27) and p38 mitogen-activating protein kinase (MAPK) (Fig. 4). This cascade is mediated by a specific interaction between the LG3 domain and $\alpha 2\beta 1$ integrin [191]. Subsequently it was shown that the LG3 module alone can induce disruption of the cytoskeleton and focal adhesions, a process that requires Ca^{2+} but not HS chains [191]. Moreover, following endorepellin or LG3 treatment, there is redistribution of the $\alpha 2\beta 1$ integrin with disrupted actin fibers, and this process is mediated at the molecular levels by a specific interaction of LG3 with the I domain of the $\alpha 2$ integrin subunit [189, 191]. Further analysis has shown that endorepellin evokes activation of the tyrosine phosphatase Src homology-2 protein phosphatase-1 (SHP-1) in an $\alpha 2\beta 1$ integrin-dependent manner [202]. This leads to dephosphorylation of various receptor tyrosine kinases (RTKs), including VEGFR2 [202] (Fig. 4). Notably, endorepellin causes not only activation but also translocation of SHP-1 to endothelial cell nuclei [202], probably interfering with transcriptional activity of several genes.

Several functional assays have demonstrated the importance of the $\alpha 2\beta 1$ integrin binding for endorepellin's biological activity on endothelial cells [141, 191, 202, 203]. Notably, endorepellin needs to be in solution for proper binding to the I domain of the $\alpha 2\beta 1$ integrin subunit [191], while no response is detected upon endorepellin stimulation of microvascular endothelial cells from $\alpha 2\beta 1^{-/-}$ mice [141]. In zebrafish, $\alpha 2\beta 1$ -integrin knockdown induces a vascular phenotype similar to the perlecan morphants with no functional intersegmental vessels [204].

These findings further underline the double function of perlecan as a strong angiogenic molecule within the basement membranes of endothelial cells, through interaction with a plethora of growth factors and signaling receptors, and as a potent angiostatic effector *via* the specific interplay of its domain V, or better its LG3 fragment, with the $\alpha 2\beta 1$ -integrin.

3.4 Endorepellin-mediated down-regulation of VEGFR2 signaling axis

Concurrent with binding to the $\alpha 2\beta 1$ integrin, endorepellin interacts with the VEGFR2 at the surface of endothelial cells. Endorepellin binds via the two proximal LG modules (LG1 and LG2) to the ectodomain of VEGFR2 between Ig-repeats 3 and 5, a site that only partially overlaps with the major binding site of its natural ligand VEGFA [205] (Fig. 4). Endorepellin binding to the Ig3-5 of VEGFR2 attenuates both the two main VEGFR2 signaling axis. In normal conditions, the phosphatidylinositol-3 kinase (PI3K) class I axis of VEGFR2 downstream signaling leads to the activation of the transcription factor hypoxia inducible factor 1 α (HIF1 α) through phosphoinositide-dependent kinase 1 (PDK1), Akt1 and mammalian target of rapamycin (mTOR). On the other hand, PLC γ recruitment to Tyr1175 allows the hydrolysis of PIP2 into inositol trisphosphate (IP3); this in turn causes

an increase in calcium allowing calcineurin to dephosphorylate NFAT1. Translocation of NFAT1 to the nucleus then activates HIF1 α . Additionally, this axis also induces activation of the JNK/AP1 pathway by activated protein kinase C (PKC), which eventually provokes VEGFA transcription [206]. Importantly, endorepellin possesses the ability to attenuate both signaling routes [142] (Fig. 4). Thus, this proteolytic fragment of the perlecan protein core is capable of blocking the feed forward loop created by the interaction between VEGFA and its receptor.

The binding of endorepellin to VEGFR2 is concurrent with its binding to $\alpha 2\beta 1$ integrin, two key receptors involved in the angiogenesis process. Thus, the concept of dual receptor antagonism: down-regulation of VEGFR2 axis occurs in concert with repression of the $\alpha 2\beta 1$ integrin downstream signaling, leading to inhibition of VEGFA transcription and concurrent disassembly of actin stress fibers and focal adhesions. This double negative suppression exerted by endorepellin on both receptors enhances its activity on endothelial cells and is responsible for the intense inhibition of blood vessels maturation and endothelial cell migration. This is in stark contrast to the parent molecule (Fig. 4).

Recently, the crystal structure of LG3 as well as the binding site for the $\alpha 2\beta 1$ integrin has been unveiled [207]. Based on a jellyroll fold, LG3 is constituted of 200 amino acids with an architecture formed by a β -sandwich composed of 15 anti-parallel β -strands. Other element belonging to the EF loop could be also involved in the angiostatic activity of LG3. Moreover, Ca²⁺ is needed for this function but it is not required for the binding to the $\alpha 2\beta 1$ integrin [207]. We previously proposed that the DAPGQYG sequence, located at the N-terminus of LG3, could be involved in the processing of endorepellin by Asp-N endoproteinases [189]. Subsequently, it was confirmed as the main cleavage site mediated by BMP-1/Tolloid-like metalloproteases [193]. New surface plasmon resonance analyses have identified this site, in particular the Asp4197, as a key residue involved in the anti-migratory effects exerted by endorepellin [207].

Collectively, these findings put forward the concept of released endogenous fragments of major matrix components within the basement membranes as important angiostatic effectors on endothelial cells.

3.5 Endorepellin and VEGFR2-dependent autophagy in endothelial cells

A new role for endorepellin has been recently revealed as a potent autophagic inducer [23]. Similar to another well-known anti-angiogenic proteoglycan, decorin [208], this basement membrane proteoglycan fragment evokes autophagy specifically in endothelial cells under nutrient enriched conditions. Only one of the two endorepellin-binding receptors has been shown to be involved in the regulation of the autophagy cascade: endorepellin binds VEGFR2 on endothelial cells through its LG1/2 domains to remove the repression on autophagy induced by mTOR (Fig. 4). The novelty of this discovery is that endorepellin-evoked autophagy is independent of its engagement with the $\alpha 2\beta 1$ integrin receptor [209]. Endorepellin induces not only several key markers of autophagy, such as Vps34, Beclin 1, LC3 and p62, in endothelial cells, but also the newly discovered master regulator of autophagy Peg3 (Fig. 4). Endorepellin causes a redistribution of the class III PI3K, Vps34, from the plasma membrane onto large intracytoplasmic Beclin 1-positive autophagosomes, a

process that is blocked by a small molecule inhibitor of VEGFR2 tyrosine kinase, but not by $\alpha 2\beta 1$ integrin-blocking antibodies.

As previously described, LG3 is the domain of endorepellin, which carries the majority of the angiostatic effect; however only LG1/2 domains are able to evoke autophagy through the VEGFR2. It has been suggested that LG3 alone would instead repress autophagy due to its ability to down-regulate mRNA levels of *PEG3*, *BECN1* and *MAPLC3A* genes. The therapeutic implications of this mechanism are evident: the entire endorepellin is needed for the fulfillment of its angiostatic and pro-autophagic activity. It is predictable that the potent signaling carried by its LG1/2 domains would counteract the suppression of autophagy exerted by its LG3 domain. As mentioned before, endostatin, comparable to endorepellin, has also been recognized to promote autophagy in endothelial cells in addition to its angiostatic activity [24]. However no direct linkage between endostatin roles in autophagy and angiogenesis has been reported yet. Importantly, in Matrigel-based angiogenesis assays, endorepellin inhibits capillary tube morphogenesis and concurrently enhances autophagy. Thus, there is a link between the potent anti-angiogenic action of endorepellin and its strong autophagic induction on human endothelial cells [23, 209].

4. Therapeutic agents or tumor targets?

A common pathway emerging from studies of these two pericellular HSPGs is their ability to signal as whole proteoglycan or by the release of processed C-terminal fragments. This double action is emphasized for perlecan which behaves as a pro-angiogenic molecule maintaining blood vessels integrity and promoting endothelial cell proliferation, adhesion and motility, both in normal and neoplastic conditions. In contrast, endorepellin and LG3 are effective angiostatic factors, involved in powerful anti-tumor vasculature responses. Collagen XVIII, however, functions as a negative regulator of blood vessel remodeling, and its released C-terminal domain endostatin is also a negative modulator of pathological angiogenesis. In the settings of the tumor microenvironment, differential processing of the two parental molecules by MMPs could result in diverse outcomes, either positive or negative, for tumor progression and metastasis. Therapeutically, targeting a large pro-angiogenic proteoglycan like perlecan could represent a big challenge. On the contrary, both endorepellin and endostatin are able to evoke an autophagic signaling cascade specifically in endothelial cells that increases their angiostatic role. In light of these findings, a reasonable question that arises, besides the implications of these cleaved angiostatic autophagy-inducers in cancer, is how to manipulate endorepellin and endostatin as therapeutic agents in cancer. Where traditional chemotherapy fails or where tumors relapse due to cancer resistance to single-agent treatment, these angiostatic and pro-autophagic agents could represent, alone or in combination with conventional therapy, a novel horizon to combat cancer. Below, we will focus on the potential utilization of endostatin and endorepellin as cancer therapeutics.

4.1 Endostatin in cancer biology

Since its original discovery [60], inhibition of tumor angiogenesis, growth and metastasis by endostatin has been proven in several publications. Recombinant endostatin, subcutaneously injected into tumor-bearing mice, dramatically suppresses the growth of Lewis Lung Carcinoma primary tumors showing a 7-fold increase in apoptotic rate [60]. Several types of

malignant tumors, such as hemangioendotheliomas, fibrosarcomas and melanomas, can also be inhibited by systemic administration of recombinant endostatin [60]. Notably, continuous administration of endostatin results in a state of tumor dormancy that persists as long as the therapy is continued. Endostatin not only potently suppresses tumor angiogenesis and tumor growth, but also maintains metastases at an undetectable size [60]. Endostatin has been utilized in more than 100 reports for the treatment of many types of cancer, with a broad heterogeneity of doses, schedules and ways of administration. Notably, diverse recombinant forms of endostatin have been generated and some of these have enhanced angiostatic activity and improved stability. Endostatin chemically modified with low molecular weight heparin (LMWH) exhibits higher activity and better heat tolerance than native endostatin [210]. In addition, local administration of endostatin modified with polyethylene glycol (PEG) results in a less toxic protein with increased anti-angiogenic activity and no adverse reactions [211]. Several studies have been performed utilizing Endostar, a modified endostatin with harboring an additional nine amino acids [212]. Another genetic modification of endostatin is the P125A endostatin which apparently enhances its binding to endothelial cells and its anti-angiogenic properties [213]. Notably, P125A endostatin evokes also autophagy in endothelial cells [24]. Furthermore, adding an Asn-Gly-Arg (NGR) sequence to the N-terminus of human endostatin can enhance tumor growth suppression, endothelial cell homing, and its angiostatic activity [214]. Finally, an RGD (Arg-Gly-Asp) sequence usually present in several integrin-binding ligands has confirmed to possess an increased angiostatic activity [215], especially if bound to the N- or C-terminus of P125A-endostatin [216].

Although several reports have suggested that the N-terminal region, the zinc-binding site of endostatin, serves only as a structural role, it has been demonstrated that endostatin requires Zn^{2+} for the binding to heparin and heparan sulfates and that this capacity is critical for its biological functions [217]. Indeed, a 27-amino-acid region at the N-terminal domain of endostatin, containing the zinc-binding site, is necessary for the anti-angiogenic and anti-permeability activities of endostatin [70]. Mutations in the zinc-binding region block anti-endothelial migration activities but not its anti-permeability effect. However, modified recombinant Endostar, which harbors an additional zinc-binding peptide at its N-terminus, binds its receptor nucleolin with higher affinity and faster rate than normal recombinant endostatin purified from *Pichia pastoris* [217].

These modified proteins combined with gene therapy could represent powerful therapeutic agents in the treatment of cancer. They have been utilized as new angiogenesis inhibitors in several therapeutic applications in which the route of administration play also a role. For example, results obtained from the treatment of ectopic tumors differ from the therapy performed on orthotopic ones. An emerging concept is that endostatin requires prolonged delivery due to its short half-life [218]. An increase of 1.6-fold of endostatin in the blood has been demonstrated to protect against the development of tumors in transgenic mice over-expressing endostatin [219].

Viral-vector systems utilizing retrovirus, adenovirus or adeno-associated virus have shown reduction of tumor growth and metastases by endostatin [220–222]. However, overall these reports have shown lower anti-tumor effects than the ones originally obtained by

systemically administered endostatin. Notably, endostatin secretion into the circulation has been ensured for many days using polyvinyl pyrrolidone and liposome complexes, thereby inhibiting not only the growth of the primary tumor but also its metastatic spread [223–226] (Table 1). Cell encapsulated endostatin, continuously released by these cells and implanted into tumor-bearing mice, has shown reduction of human and murine gliomas [227–229] (Table 1).

Overall, *in vivo* tumor responses to endostatin varies from 47% to 91% inhibition with a range of doses between 10 and 100 mg/kg/day. NGR-endostatin delivered into nude mice bearing ovarian cancer markedly inhibits tumor burden [214], comparable to the inhibition seen in murine acute myelogenous leukemia by microencapsulated murine endostatin [230]. By direct microinfusion of recombinant endostatin, a 74% suppression of human orthotopic brain tumors (U87) has been observed [231]. Similar therapeutic results have been obtained in mouse models of colorectal cancer [232], orthotopic breast cancers [233], murine melanomas [234], pancreatic cancer [235], and rat brain tumors [236]. Moreover, in murine C51 colon cancers and human HT29 xenografts recombinant endostatin not only inhibits tumor angiogenesis but also suppresses tumor cell proliferation [237, 238].

Besides endostatin as a single agent, administered either as recombinant protein or via viral vectors, several combinatorial strategies have also pursued. For example, human testicular xenografts treated with a combination of endostatin, and carboplatin or thrombospondin-1, show decreased primary tumors and metastases when compared to each individual treatment [239]. Moreover, in Lewis Lung Carcinomas endostatin gene therapy potentiates the outcome of ionizing radiation [240] and double treatment with endostatin gene therapy and gemcitabine causes higher effects in mice carrying human lung carcinoma xenografts [241]. All these preclinical animal studies underline the importance of combinatorial therapies, especially in tumors resistant to individual therapies and suggest that angiostatic therapeutic agents that target the genomically-stable endothelial cells could enhance the outcome of conventional therapies.

Only a few reports have described a lack of activity by either endostatin gene therapy or recombinant endostatin [242–247]. Potential explanations for these negative results include a variable self-aggregation of *E.coli*-isolated endostatin [246], a too short duration of the treatment or abnormal secretion of VEGF from tumor cells [244]. Indeed, the therapeutic efficacy of endostatin exhibits a biphasic dose-response curve [248], that is, the endostatin anti-tumor and anti-angiogenic effects possess a U-shaped curve whereby circulating levels of endostatin that are too high or too low are actually inactive. The range of endostatin in the normal murine blood is between 5 and 15 ng/ml. Therapeutic levels that are up to 80–450 ng/ml are effective, but higher levels may not be so efficacious [17].

Importantly, elevated levels of endostatin have been observed in several human cancer types. Circulating serum endostatin varies from 10 to 50 ng/mL in healthy individuals, corresponding to 0.5–2.5 nM [249–251]. In patients with head and neck squamous cell [252], hepatocellular [253, 254] and vulvar carcinomas [255], endostatin levels are increased, as well as for soft tissue sarcomas [256], clear cell renal [257] and breast [258] carcinomas. Higher levels of endostatin were also found in osteosarcomas [259], NSCLCs

[55], ovarian and endometrial cancers [260], similarly to acute myeloid leukemias [261], bladder [262] and colorectal cancers [263]. These increased circulating levels of endostatin have been implicated as prognostic factors and utilized as a diagnostic marker for clinical studies. Recently, correlation of circulating levels of endostatin with the stage of gastric cancers has been proposed as an useful prognostic biomarker [50]. Endostatin levels were also identified in the brain tissue and serum of glioblastoma patients implying an immune response to endogenous produced endostatin [264] and in pathological diseases as rheumatoid arthritis [265] and diabetic retinopathy [266].

Notably, high levels of circulating endostatin have been detected in patients with Down Syndrome carrying trisomy of chromosome 21 [251]. As *COL18A1* gene maps to chromosome 21, it is likely that the increased circulating levels might be due to increased gene dosage. Indeed, patients with Down syndrome possess a lower risk of tumor development likely as a consequence of this high level of serum endostatin. These findings are in line with the observation that genetically over-expressing endostatin by 2-fold in the endothelium, thus enhancing the circulating levels of endostatin, efficiently inhibits tumor growth [267]. Therefore, manipulations aimed to increase the levels of endogenous angiogenic inhibitors in the blood, without reaching too high serum levels comparable to the ones detected in cancer patients, could potentiate the anti-tumor effects exerted by these molecules [17]. A good example of this mechanism is shown by celecoxib, a nonsteroidal anti-inflammatory drug, that can increase endogenous levels of endostatin in the serum [268].

A number of phase I and II clinical trials have been performed before endostatin was approved as an anti-cancer drug. The first phase I trial was performed in 1999 when a biopharmaceutical company (EntreMed Inc.) used recombinant endostatin at the Dana-Farber Cancer Center [269]. This trial was then followed by other three [270–273] and, after detection of no toxicity and evidence of some therapeutic benefit, in 2002, a multicenter phase II study was brought into clinic for the treatment of carcinoid or advanced pancreatic neuroendocrine tumors [274]. However, while minimal toxicity was observed, no significant anti-tumor activity was detected in this study. Therefore, because of the high costs and poor clinical outcome, this trial did not reach the approval for phase III. In contrast to these initial clinical trials, Chinese studies showed good tolerance and an increased response rate when Endostar was administered in combination with chemotherapy for the treatment of advanced NSCLC patients [275]. These encouraging results were the main reason for the approval of Endostar in China for ordinary use in cancer therapy. A possible explanation for these contrasting responses could be ascribed to the different recombinant drugs utilized by the studies. The American trials utilized a recombinant endostatin purified from yeast, whereas the Chinese clinical trials administered recombinant Endostar, which has been shown to possess higher activity, as described above, due to the modification of its structure [217]. Combination of anti-angiogenic inhibitors and chemotherapeutics is another reason to consider looking at this successful study.

Several studies have analyzed the efficacy of Endostar in combination with conventional chemotherapy versus chemotherapy alone in the treatment of this type of lung cancers. A meta analysis and comprehensive review of these clinical trials reported 88 potentially-

relevant trials of which, based on several reasons such as limited cases or lack of controls, only 15 have been selected [276]. Accordingly, Endostar, when administered in combination with platinum-based doublet chemotherapy (PBDC), has a better outcome than PBDC alone, with similar adverse reactions [276].

In conclusion, by slowly shrinking tumor blood vessels instead of targeting cancer cells, angiostatic proteins exert a cytostatic action that increases the access of ordinary chemotherapy into growing tumors, favoring their cytotoxic activity.

4.2 Endorepellin and its role in cancer

The importance of endorepellin as a key modulator in health and disease is becoming apparent. Endogenous endorepellin has been identified in zebrafish embryos, and exogenous endorepellin was found to rescue most of the vascular/somatic muscle defects seen in morphants where perlecan expression was suppressed, thereby providing evidence for a role for endorepellin in vascular angiogenic development [165]. In a large study investigating the human blood serum proteome, perlecan-derived endorepellin has been observed as a major circulating protein of human blood [195]. Additionally, whole endorepellin has been recovered in the growth plate of fetal cartilage specifically in the upper zone where the proliferation rate is higher [196] (Table 2). The importance of this finding leads to speculate that endorepellin could be involved in the inhibition of angiogenesis within the cartilage or impede blood vessels invasion into this mesenchymal tissue, therefore serving as a biomarker for fetal ischemia and vascular injury.

As mentioned above, during remodeling and tumor cell invasion, the basement membrane-associated endorepellin is processed by the ubiquitous BMP1/Tolloid-like protease to release its bioactive terminal domain, LG3. In line with these observations, LG3 has been found in the amniotic fluid of pregnant women, especially enhanced in women with symptoms of premature rupture of fetal membranes [277, 278] or carrying Down Syndrome fetuses [279] (Table 2). In addition, LG3 forms are detectable in the urine of children with sleep apnea [280], suggesting LG3 as a potential biomarker not only for abnormal fetal development, or fetal ischemia, but also for transient brain ischemia. Moreover, LG3 can be released in condition of hypoxia and exert a neuroprotective role in brain infarcts [281, 282].

As a trigger of atherosclerosis, endothelial cell apoptosis is followed by over-expression of anti-apoptotic factors by smooth muscle cells and fibroblasts, which accumulate within the intima. Released LG3 by pre-apoptotic endothelial cells increases the levels of Bcl-2 and Bcl-xL in these cells, therefore preventing apoptosis of the surrounding cells and leading to the progression of the sclerotic response [152, 194, 283]. In this context, elevated LG3 levels have been detected in the urine of renal transplant recipients with chronic nephropathy and in the urine of end-stage renal patients [197, 284] (Table 2). Collectively, these findings suggest that urinary LG3 levels might represent a useful biomarker for nephropathy and allograft rejection [197].

Recently, LG1/2 fragments of ~63 kDa have been detected increased in murine aged *gastrocnemius* muscle extracts [285]. Apoptosis of endothelial cells within the aged skeletal muscle correlates with the release of endorepellin fragments. Once again, released

perlecan's fragments have been found associated with a disease, which in this case belongs to sarcopenia. Likewise, elevated LG3 levels were recently described also in the circulation of refractory cytopenia patients with multilineage dysplasia [286]. A recent observation of endorepellin and shorter fragments of perlecan, generated by mast cells, implicates endorepellin also in inflammation and tissue repair [160]. In addition, endorepellin fragments have been found also in the secretome of pancreatic and colon carcinoma cells [193, 287]. Notably, lower circulating levels of LG3 have been detected in patients with breast cancer [183], suggesting a potential role of endorepellin's LG3 in suppressing tumor progression and metastasis (Table 2). In this case, LG3 could also serve as a biomarker for cancer development and invasion.

The inhibition of blood vessel formation, endothelial cell migration and survival exerted by endorepellin has been investigated *in vivo* [141, 203]. In murine orthotopic xenografts, after acute or chronic systemic administration, endorepellin localized to the vasculature of A431-induced squamous carcinoma xenografts or syngeneic Lewis Lung Carcinoma tumors, where it remains for several days [203]. Several *in vivo* assays, including positron emission tomography and computed tomography, have demonstrated that endorepellin inhibit tumor angiogenesis, tumor metabolism and growth in these mouse models, further suggesting that in mammals, endorepellin is a potent anti-tumor agent [203]. The specificity of this domain to specifically target tumor blood vessels and to concentrate in the tumor central areas has also been found by intraperitoneal injection of endorepellin labeled with the near-infrared dye IR800CW [203]. Interestingly, tumors treated with endorepellin not only exhibit hypoxia and decreased metabolism, but also show decreased cell proliferation, although not inducing apoptosis. In addition, endorepellin is internalized by tumor endothelial cells with consequent redistribution and association of the tumor $\alpha 2\beta 1$ integrin within the same perivascular area where endorepellin was found to accumulate [203]. More importantly, systemic administration of endorepellin into tumor-bearing $\alpha 2\beta 1^{-/-}$ mice show no effect on tumor growth, tumor vessels or metabolism [141], therefore providing robust genetic evidence that the $\alpha 2\beta 1$ integrin is a required cell surface receptor for endorepellin's biological activity. As most tumor blood vessels express high levels of $\alpha 2\beta 1$ integrin, it is likely that the therapeutic activity of endorepellin and LG3 are mediated by this integrin, favoring the specific targeting of tumor blood vessels. Interestingly, intraperitoneal injections of recombinant endorepellin in C57Bl/6 mice bearing syngenic Lewis Lung Carcinomas evoke not only inhibition of tumor growth and microvasculature density but also induce SHIP-1 tyrosine phosphatase levels in wild-type mice but have no effect in $\alpha 2\beta 1^{-/-}$ mice [202].

The newly identified role of this potent angiostatic fragment in inducing autophagy in endothelial cells provides a new layer of complexity and opens new perspectives for the treatment of tumor angiogenesis. In contrast to other angiostatic proteins, such as endostatin, endorepellin causes no increase in the apoptotic tumor cell rate, suggesting that this C-terminal fragment of perlecan inhibits cancer growth in a novel fashion. In the case of endostatin and kringle 5, their ability to induce endothelial cell autophagy and consequent endothelial cell death in part facilitates their angiostatic action [288]. Accordingly, autophagy induction by endorepellin would likely enhance its angiostatic properties.

Several *in vivo* studies are still needed before considering endorepellin as a therapeutic agent entering the clinics. Dose-response assays are necessary for understanding the correct concentration of endorepellin to be delivered *in vivo*. The route of administration could be also important. Up to now, endorepellin homing to tumor blood vessels has been proven only by intraperitoneal administration. In addition, the over-expression of $\alpha 2\beta 1$ integrin by the proliferative tumor endothelial cells could represent one of the mechanisms through which endorepellin is specifically trapped in the perivascular region for several days. This might not occur for all forms of solid tumors. Monitoring the long term effects of exogenously-delivered endorepellin in tumor-bearing mice could give insights into the possibility of tumor escaping from endorepellin suppression and relapsing, or provide further evidence in support of its tumor angiostatic activity. In light of the new pro-autophagic role of endorepellin on endothelial cells, transgenic mice expressing GFP-LC3 could offer exciting and valuable information as to the progression of autophagy in the animal. Using this model endorepellin's efficacy could be detected in a wider variety of tissues, which may provide insights into its function in normal physiology.

With its unique capacity of binding to the $\alpha 2\beta 1$ integrin *in tandem* with the VEGFR2 specifically at the surface of endothelial cells, the only cells concurrently expressing these two receptors, endorepellin can target *in vivo* areas of enhanced angiogenesis, where over-expression of $\alpha 2\beta 1$ integrin by proliferating endothelial cells acts as a trap for this fragment. As both autophagy and angiogenesis heavily impinge on cancer progression, endorepellin may present a novel agent, which by the combinatorial effect of angiostasis and autophagy, could modulate the tumor stroma and enhance chemotherapy efficacy, ultimately resulting in tumor growth retardation.

5. Conclusions and perspectives

An increased gamut of processed extracellular matrix components has emerged in recent years as potent anti-angiogenic factors [17, 289, 290]. Attenuation of tumor angiogenesis was a property initially discovered for angiostatin and endostatin by Folkman and co-workers, who first proposed the concept that cleaved fragments originating from larger proteins and possessing potent anti-angiogenic activity, but with no effect on tumor cell proliferation, could cross-talk with tumor cells in reducing growth and metastasis [1, 60]. The new emerging feature is that these proteolytically-released proteins from the extracellular milieu could act specifically on blood vessel formation in an anti-angiogenic manner and inducing autophagy in endothelial cells. The protracted autophagy induced by endostatin and endorepellin could lead to autophagic-induced apoptosis, with consequent suppression of tumor growth and metastasis. Thus, autophagy could act in concert with angiostasis in the confinement and later eradication of tumors. Indeed, increased apoptotic rate in tumor cells was found in the original work describing endostatin [60].

These two anti-angiogenic cleaved fragments of major HSPGs, endostatin and endorepellin, share common biological properties. Both proteins bind to main endothelial cell receptors such as integrins, inducing cytoskeleton disassembly and angiostasis. Both proteins bind to VEGFR2, through which autophagy is evoked. In addition, both endostatin and endorepellin, are internalized by endothelial cells. Endostatin utilizes nucleolin, as the main

receptor for its internalization. In contrast, endorepellin is internalized by tumor-derived endothelial cells via the $\alpha 2\beta 1$ integrin. Both endostatin and endorepellin are found in the circulation in human tissues and fluids, and specifically localize at the site of the tumor, where they interact with their respective over-expressed receptors and mediate potent anti-tumor action. Finally, both fragments evoke autophagy in both macrovascular and microvascular endothelial cells. This new activity could potentiate their biological function as angiostatic molecules. Other endogenous fragments released from extracellular matrix proteoglycans could exert a similar activity on tumors. Agrin, for example, could be another candidate that shares an LG3 domain at its C-terminus resembling the one of endorepellin, and shows similar activities as the latter endogenous processed fragment.

Future gene therapies aimed to target the release of endorepellin or endostatin from their parental molecules, specifically at the site of tumor development, could represent an effective treatment for cancer patients. The expected toxicity would be minimized since these endogenous molecules are able to specifically target tumor endothelial cells, and the immune response would be minimal with the use of native recombinant proteins, as seen for endostatin *in vivo* therapy. Nucleolin, specifically located at the surface of endothelial cells on tumor tissues, is a valid receptor for endostatin internalization. Thus, a new therapeutic approach utilizing a combination of modified recombinant endostatin and nucleolin-guided delivery could represent a new direction in cancer therapy. Moreover, the combination of these diverse endostatin treatments with the use of normal chemotherapeutics could enhance the efficacy of the therapy and improve clinical outcomes.

We feel that, in the light of the cumulative data presented in this review, the interest should now shift toward developing new modified therapeutic forms of these processed fragments to be utilized in new clinical trials. Moreover, future efforts should be put in testing the efficacy of these factors in human pathological conditions and cancers, and toward the identification of other natural matrix-derived proteins potentially involved in similar mechanisms of action.

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Abbreviations

PCD	programmed cell death
NC1	non-collagenous sequence 1
VEGFR1/2	vascular endothelial growth factor receptor 1/2
CAM	chick chorioallantoic membrane
MMPs	matrix metalloproteinases
GAP	GTPase-activating protein

FAK	focal adhesion kinase
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
HIF-1α	hypoxia-inducible factor 1-alpha
VEGFA	vascular endothelial growth factor A
VEGF	vascular endothelial growth factor
Wnt	wingless-type MMTV integration site family
eNOS	endothelial nitric oxide synthase
Akt	protein kinase B
PP2A	serine/threonine-protein phosphatase 2A
GSK-3β	glycogen synthase kinase 3 beta
TNF-α	tumor necrosis factor alpha
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
STAT	signal transducer and activator of transcription
AP-1	activator protein-1
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
SIPS	stress-induced premature senescence conditions
EGF	epidermal growth factor
LG	laminin-like globular domain
GAG	glycosaminoglycan
EHS	engelbreth-holm-swarm
HSPG2	heparan sulfate proteoglycan 2
SEA	sperm protein, enterokinase and agrin
LDL	low-density lipoprotein
FGF	fibroblast growth factor
PDGF	platelet-derived growth factor
HGF	hepatocyte growth factor
TGF-β	transforming growth factor beta
Trol	terribly reduced optic lobes
HS	heparan sulfate
cDNA	complementary deoxyribonucleic acid

LG3	laminin-like globular domain 3
BMP-1	bone morphogenetic protein 1
tPA	tissue plasminogen activator
ECM1	extracellular matrix protein 1
AMP	adenosine monophosphate
PKA	protein kinase A
FAK	focal adhesion kinase
HSP27	heat shock protein 27
SPR	surface plasmon resonance spectroscopy
TIMP-2	tissue inhibitor of metalloproteinase 2
SHP-1	Src homology-2 protein phosphatase-1
RTK	receptor tyrosine kinases
EGFR	epidermal growth factor receptor
PLCγ	phospholipase C gamma
PI3K	phosphatidylinositol-3 kinase
PDK1	phosphoinositide-dependent kinase 1
mTOR	mammalian target of rapamycin
PIP2	phosphatidylinositol 4,5-bisphosphate
IP3	inositol trisphosphate
NFAT1	nuclear factor of activated T-cells 1
JNK	c-Jun N-terminal kinase
PKC	protein kinase C
Vps34	vacuolar protein sorting-associated protein 34
LC3	microtubule associated light chain 3
Peg3	paternally expressed gene 3
LMWH	lower molecular weight
PEG	polyethylene glycol
NGR	asparagine-glycine-arginine
RGD	arginyl-glycyl-aspartic acid
SCID	severe combined immunodeficiency
NSCLC	non-small-cell lung carcinoma
FDA	food and drug administration

PBDC	platinum-based doublet chemotherapy
PET	positron emission tomography
CT	computed tomography
EGFP	enhanced green fluorescent protein

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Fig. 1. Collagen type XVIII and endostatin: essential modules and receptors

(A) Schematic illustration of the structure of collagen XVIII and its domains. Endostatin is shown as the terminal domain of this pericellular proteoglycan. (B) Schematic diagram depicting the cell surface receptors interacting with collagen XVIII and endostatin.

Fig. 2. Schematic model of endostatin activity on endothelial cells

A comprehensive model of angiostatic and pro-autophagic activities exerted by endostatin in endothelial cells.

Fig. 3. Perlecan and endorepellin: five modular domains and respective receptors
 (A) Representation of the large architecture of perlecan and its five modules. Endorepellin is displayed as the C-terminal processed form of this multimodular proteoglycan. (B) Schematic diagram depicting the cell surface receptors interacting with perlecan and endorepellin.

Fig. 4. A view on endorepellin's angiostatic and pro-autophagic signals on endothelial cells
In addition to the autophagic signaling induced by endorepellin, a summary of its main anti-angiogenic pathways is provided. Notice the similarity of action of endorepellin and endostatin, both processed forms originated by two fundamental pericellular proteoglycans.

Table 1Tumor inhibitory activity exerted by endostatin *in vivo*.

Tumor	Origin	Therapy	References
Lewis Lung carcinoma	Murine	Recombinant endostatin	[60, 291]
Ovarian	Murine	Recombinant endostatin	[214]
Acute myelogenous leukemia	Murine	Recombinant endostatin	[230]
Colorectal carcinoma	Murine	Recombinant endostatin	[232]
B-16 melanoma	Murine	Recombinant endostatin	[234]
Glioblastoma (U87)	Human	Recombinant endostatin	[231]
Breast carcinoma	Human	Recombinant endostatin	[233]
Pancreatic carcinoma	Human	Recombinant endostatin	[235]
Testicular carcinoma	Human	Combination therapy with recombinant endostatin	[239]
Lewis Lung carcinoma	Murine	Gene therapy	[221]
B16 brain tumors	Murine	Gene therapy	[292]
Renal carcinoma (Renca)	Murine	Gene therapy	[293]
Gliosarcoma (9L)	Rat	Gene therapy	[236]
Mammary carcinoma MCa-4	Murine	Gene therapy	[226]
Hepatocellular carcinoma	Human	Gene therapy	[241, 294–296]
Lung cancer	Human	Endostatin gene therapy and gemcitabine	[241]
Bladder carcinoma (KU-7) orthotopic	Human	Gene therapy	[297]
Non-small-cell lung cancer (KNS 62)	Human	Gene therapy	[298]

For simplicity, only a selection of murine and human tumors significantly inhibited (by more than 70%) by recombinant endostatin is presented. Some examples are described in details in the text.

Table 2

Perlecan's processed forms in biological tissues and fluids.

Tissue/Fluid	Condition	Endorepellin and its fragments	Biomarker for	References
Murine aged <i>gastrocnemius</i> muscle extracts	Sarcopenia	LG1/2	Age-related loss of skeletal muscle mass and function	[285]
Secretome of mast cells	Inflammation	Endorepellin and shorter forms	Inflammation	[160]
Secretome of endothelial cells	Apoptotic endothelial cells	LG3 and other endorepellin's fragments	Fibrosis	[194]; [152, 283]; [299]
Secretome of pancreatic and colon carcinoma cells	Colon carcinomas and pancreatic cancer	LG3 and other endorepellin's fragments	High turnover rate of cancer cells	[193]; [287]
Secretome of neurons and neurovasculature cells	Brain infarcts	LG3	Oxygen and glucose deprivation in neuronal cells	[281, 282]
Growth plate of fetal cartilage (upper zone)	Blood vessel invasion within cartilage	Endorepellin	Fetal ischemia and vascular injury	[196]
Amniotic fluid	Premature rupture of fetal membranes	LG3	Fetal ischemia and vascular injury	[277]; [278]
Amniotic fluid	Mothers carrying Down Syndrome fetuses	LG3	Abnormal fetal development and vascular injury	[279]
Urine	End-stage renal disease	LG3	Vascular injury	[284]
Urine	Chronic allograft nephropathy	LG3	Immune-mediated vascular injury	[197]
Urine	Children with sleep apnea	LG3	Brain ischemia	[280]
Blood	Patients with refractory cytopenia with multilineage dysplasia	LG3	Refractory cytopenia with multilineage dysplasia	[286]
Blood	Normal subjects and breast cancer patients	LG3	Breast cancer	[183]