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The role of perlecan and endorepellin in the control of tumor angiogenesis and endothelial cell autophagy

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Abstract

During tumor growth and angiogenesis there is a dynamic remodelling of tissue architecture often accompanied by the release of extracellular matrix constituents full of biological activity. One of the key constituents of the tumor microenvironment is the large heparan sulfate proteoglycan perlecan. This proteoglycan, strategically located at cell surfaces and within basement membranes, is a well-defined pro-angiogenic molecule when intact. However, when partially processed by proteases released during cancer remodelling and invasion, the C-terminal fragment of perlecan, known as endorepellin, has opposite effects than its parent molecule. Endorepellin is a potent inhibitor of angiogenesis by exerting a dual receptor antagonism by simultaneously engaging VEGFR2 and $\alpha 2\beta 1$ integrin. Signaling through the $\alpha 2\beta 1$ integrin leads to actin disassembly and block of endothelial cell migration, necessary for capillary morphogenesis. Signaling through the VEGFR2 induces dephosphorylation of the receptor via activation of SHP-1 and suppression of downstream proangiogenic effectors, especially attenuating VEGFA expression. A novel and emerging role of endorepellin is its ability to evoke autophagy by activating Peg3 and various canonical autophagic markers. This effect is specific for endothelial cells as these are the primary cells expressing both VEGFR2 and $\alpha 2\beta 1$ integrin. Thus, an endogenous fragment of a ubiquitous proteoglycan can regulate both angiogenesis and autophagy through a dual receptor antagonism. The biological properties of this natural endogenous protein place endorepellin as a potential therapeutic agent against cancer or diseases where angiogenesis is prominent.

Keywords

Perlecan; LG domains; angiogenesis; autophagy; Peg3

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Declaration of interest

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Introduction

The extracellular matrix (ECM) is a biological structure that is found in all multi-cellular organisms. It provides physical scaffolding through adhesive interactions between adjacent cells, and also biochemical support by mediating a number of signalling processes through the direct interaction with various growth factor receptors located on ECM-associated cells (1-3). The ECM is the primary feature that dictates organismal architecture and many of its biological roles are maintained within various animal lineages. These roles are predominantly cell adhesion and cell-to-cell communication. However, due to an independent evolution rate between multi-cellular lineages, ECM constituents often differ among organisms and animals of differing species (4) and have the ability to utilize a wide variety of biological roles, which they use to fit the biological task they require (5). A major class of molecules found within the ECM is proteoglycans, an expanding family of gene products that now encompass 43 distinct genes belonging to four families (6). Deregulated expression of many proteoglycans has been closely linked to the onset of a number of pathologies including wound healing, tumor growth, fibrosis and abnormal angiogenesis (7). This emphasizes the importance of a balanced and controlled expression of these biologically active proteoglycans. This minireview will focus on the roles of the proteoglycan perlecan and its C-terminal fragment, endorepellin, in cancer, angiogenesis and autophagy.

Perlecan genetics

Heparan sulfate proteoglycans (HSPGs) are a special class of proteoglycans, which govern crucial events in embryonic development, inflammation, wound repair and cancer. Perlecan, also known as HSPG2, is one of the largest proteoglycans found in the body (8,9). It was originally isolated from mouse basement membrane (10) and given its eponym from its appearance of a “beads on a string” on rotary shadow electron microscopy. It was later discovered that the cell-surface HSPG of human colon carcinoma cells was essentially the same as perlecan (11-13). It has a protein core of ~470 kDa and contains three HS chains located at the N-terminus (Figure 1A), each weighing approximately 30 kDa. Perlecan is encoded by the *HSPG2* gene located on the short arm (p) of chromosome 1, specifically 1p36 (13). This large gene covers >120 kb of continuous DNA and contains 97 protein-encoding exons (14). The *HSPG2* gene has a complex organization at the promoter level (15,16), and is an early response gene that is transcriptionally inhibited by interferon γ (17) and induced by TGF β (18) and phorbol ester (19). Its promoter complexity is further enhanced by the potential generation of alternatively-spliced mRNA variants reported to occur in mast cells (20,21).

The expression profile of perlecan during development follows a non-random and defined pattern. Within early phases, perlecan can be found within the endothelial cells of the cardiac tissue such as the heart and blood vessels, followed later by being expressed in the liver, kidney and spleen (22). Lack of perlecan in developing *Hspg2*^{-/-} mouse embryo leads to severe brain, heart, and skeletal dysplasia responsible for death between embryonic stage E10.5 and birth, primarily because of blood leakage into the pericardial cavity (23,24). Importantly, this is around the stage of development when perlecan expression is induced.

Moreover, *Hspg2*^{-/-} mice develop severe skeletal dysplasia characterized by shortened bones and craniofacial abnormalities, and die shortly after birth of respiratory failure due to the cartilage defects of the rib cage (23,24).

Mutations in the human perlecan gene can cause at least two autosomal recessive skeletal disorders, the dyssegmental dysplasia Silverman-Handmaker type (DDSH) (25,26), and the Schwartz-Jampel syndrome, with myotonic myopathy and chondrodysplasia (SJS) (27-29). DDSH new-borns display massive skeletal abnormalities and die shortly after birth. SJS is represented by a range of mutations with most patients reaching adulthood exhibiting a spectrum of disorders related to the degree of haplo-insufficiency, notably severe muscle stiffness (29). In addition to the disorganization of the growth plate and chondro-osseous junction of bones caused by the absence of perlecan, there are other distortions in the growth of joints including the temporo-mandibular joint, myotonia and electrical disturbances of muscle and modifications to the structures of facial bones (30). Moreover, perlecan deficiency in hypomorphic mutant mice influences bone formation by enhancing osteogenesis and concurrently affects mineralization, leading to increased brittleness at older ages (31).

The absence of perlecan affects FGF signalling in the growth plate (23). *Hspg2* mutant mice with a knock-in mutation Cys1532Tyr, found in human SJS, have a phenotype similar to SJS (32). These findings provide robust genetic evidence demonstrating the critical need for perlecan expression and also the importance of the protein core in cardiovascular development. Recent studies designed to unravel the cause of the leakage into the pericardial cavity have shown that basement membranes lacking perlecan deteriorate in the heart with accompanying loss of cell-cell attachment in the ventricle and outflow tract (33).

Perlecan protein core and its functions

The protein core of perlecan is composed of five distinct modules which are schematically illustrated in Figure 1A. Perlecan regulates several biological processes by interacting with growth factor receptors and soluble growth factors through its HS chains or protein core (34-36). Perlecan is widely conserved across animals and is one of the few gene products that are found in both vascular and avascular tissues (22,37-40). Due to its large size and complex structure, perlecan has many roles. Among these are: cell adhesion and invasion (41-45), inhibition of smooth muscle cell proliferation (46-48), cardiovascular development (24), lipid metabolism (49-51), corneal epithelial structure (52), epidermal and osteophyte formation (53,54), cartilage homeostasis (55), endochondral ossification (56), apoptosis (57), lens capsule homeostasis (58), and synaptogenesis (59,60). However the most notable role is its ability to promote vessel formation (40,61-65) and angiogenesis (9,66-71). This occurs through the binding of several pro-angiogenic factors to the HS chains or the protein core (61,64,72-75). Moreover, perlecan binds to several growth factors (76-78), including progranulin (79), a protein inducing angiogenesis and cancer growth (80,81). Perlecan can be processed by MMP7 at the invasive tumor microenvironment, thereby acting as a molecular switch to alter prostate carcinoma and favoring cell invasion (45).

One of the most intriguing functions of perlecan is its involvement in blood vessel formation (1,82). The mRNA levels of perlecan are high in endothelial cells of the developing mouse embryo (22), and further increase after recruitment of pericytes to the endothelial tubes. Perlecan also supports the maintenance of brain and skin subendothelial basement membrane and promotes vascular formation and angiogenesis by modulating FGF2 activity (83). *Hspg2*^{3/3} mice harboring a 45-bp in-frame deletion of *Hspg2* exon 3, which removes the attachment sites for HS chains in Domain I, are viable but have small eyes and their lenses degenerate within 3 weeks of birth (58). *Hspg2*^{3/3} mice also possess impaired angiogenesis, delayed wound healing and retarded tumor growth (84). Thus, perlecan HS chains play an important role in the regulation of wound healing and tumor growth. Perlecan knockdown in zebrafish reveals abnormal phenotypes with a pronounced curvature of the tail and trunk caused by severe defects in the musculoskeletal system and inhibition of angiogenesis (40). Recently, it has been shown that perlecan deficiency promotes endothelial cell dysfunction by reducing the expression of endothelial-specific nitric oxide synthase (85), thereby controlling vascular relaxation.

The pro-angiogenic activity of perlecan is thought to be achieved primarily through interacting with FGF receptors using the three HS chains present in the N-terminal Domain I (61). This interaction actively increases cell proliferation, motility, and adhesion (44,61). Often, the HS chains of perlecan can be liberated by either partial proteolysis of the protein core (86) or by the activity of heparanase (87). The latter enzyme is known to cleave HS generating relatively large fragments (88) that are full of HS-binding mitogens and pro-angiogenic factors (89). Perlecan HS chains are key factors for angiogenesis in an experimental animal model of hind-limb ischemia (71). In this animal model, the HS side chains in perlecan are important mediators of the angiogenic response to ischemia through a mechanism that involves induction of VEGF expression (71). On the other hand, the HS chains of perlecan, which are processed by heparanase released by tumor and inflammatory cells (90-94), have a profound inhibitory role on smooth muscle cell proliferation *in vivo* (46,47). Thus, there is a fine balance of activator and inhibitor effects at the N-terminus of perlecan, further stressing the biological complexity of this macromolecule.

Deregulated expression of perlecan is reported to be instrumental in cancer progression (84,95,96). For instance, in some cancers of the ovaries perlecan expression is lost from the basement membrane (97). In others, such as melanomas, oral squamous carcinomas and hepatocellular carcinomas, perlecan is markedly upregulated (95,98,99). In the latter case, knockdown of perlecan in carcinoma cells isolated from various tumor sites reduces cell migration and adhesion (98). Moreover, knockdown of perlecan in metastatic prostate cancer cells reduces *in vivo* tumor growth (100). Perlecan is overexpressed in the desmoplastic prostate cancer stroma and this is mediated by TNF α -mediated transcriptional induction of perlecan (101). Thus, perlecan transcription could be a part of an innate response of the tumor stroma to cancer invasion.

Collectively, these findings posit perlecan and its HS chains as key regulators of angiogenesis during tumor progression. Because perlecan acts as a pro- and anti-angiogenic HSPG, aberrant perlecan levels and/or abnormal processing during cancer invasion could have profound effects on tumor progression and metastasis.

Endorepellin, the C-terminal angiostatic fragment of perlecan

The most notable region of this HSPG is its C-terminal Domain V (Figure 1A), also known as endorepellin to signify its anti-endothelial properties (102). Endorepellin is an 85-kDa glycoprotein consisting of three laminin-like globular (LG) repeats separated by epidermal growth factor (EGF)-like module doublets. The crystal structure of the LG3 domain has been recently solved (103). This region is made up of about 200 amino acids and is constructed as a 15-stranded anti-parallel β sandwich forming a jellyroll fold, characteristic of LG domains (Figure 1B). Notably, Asp⁴¹⁹⁷ located near the N-terminus of LG3, might be a potential binding site for the $\alpha 2\beta 1$ integrin, as mutation of this residue LG3 activity (103).

Endorepellin is not produced alone but must be proteolytically cleaved from secreted perlecan, and as such endorepellin has its own repertoire of actions. Generation of bioactive endorepellin is mediated by the activity of Cathepsin L (104), the same enzyme that is also involved in the generation of endostatin. One can envisage a scenario where, during remodelling of basement membranes, Cathepsin L can dually affect angiogenesis. In contrast, LG3 is highly sensitive to BMP1-Tolloid-like family of MMPs (105). Once cleaved, endorepellin is found in similar regions as perlecan, primarily within the basement membrane of epithelial-lining organs and along the cardiovascular system (106). Notably increased levels of endorepellin have been found in sarcopenia, the age-related loss of skeletal muscle mass and function (107). Endorepellin works by binding with high affinity to receptors located on endothelial cells and, therefore, influences the cells lining of the vessels. The difference in the receptors targeted by LG modules and the actions they provoke are critically assessed in the next sections.

Endorepellin signaling

One of the key features of liberated soluble endorepellin is its ability to signal through various receptors including integrins and VEGFRs (108). This signaling ability of perlecan/endorepellin is also extended to its perlecan ortholog in *Drosophila* named *Trol*, for terribly reduced optical lobes. *Trol* regulates multiple developmental stages (109) as well as FGF-signaling output during mesoderm development (110). Signaling through perlecan regulates prostate cancer progression through Sonic Hedgehog (111,112). In mammals, both perlecan and endorepellin can bind VEGFR1 and VEGFR2, at sites independent of the VEGFA binding site (113). VEGFA is able to bind to either receptor at picomolar concentrations; however VEGFA has approximately 5-fold higher binding capacity for VEGFR1 ($K_d \sim 100$ pM) than VEGFR2 ($K_d \sim 480$ pM) (114). Notably, endorepellin binds with a similar affinity to both receptors as VEGFA (114) and this binding capacity is greater than the whole glycanated parental proteoglycan. In competition binding assays, highly-purified, human recombinant endorepellin cannot be displaced from either VEGFR1 or VEGFR2 by molar excesses of recombinant VEGFA (114). This demonstrates that endorepellin binds to a discrete region of VEGFR2 ectodomain that doesn't overlap with the VEGF ligand binding site.

Initially, it was demonstrated that endorepellin binds to the $\alpha 2\beta 1$ integrin on endothelial cells (115-119) and platelets (117) where it enhances collagen-platelet response. In addition,

endorepellin affects the global proteomic profile of endothelial cells (120). It is well established that the $\alpha 2\beta 1$ integrin is an important receptor regulating various aspects of angiogenesis (121-123), and is required for endorepellin's biological activity, both *in vitro* and in tumor xenografts (124). However, it is now known that endorepellin binds to both VEGFR2 and $\alpha 2\beta 1$ on endothelial cells (114,125). This causes internalization and downregulation of both receptors within 10 minutes of binding; a concept referred to as "dual receptor antagonism" (114). Importantly, only the LG1/2 domain of endorepellin can interact with VEGFR2, whereas, only the LG3 domain is capable to interact with the $\alpha 2\beta 1$ integrin (125,126). Indeed, cells expressing $\alpha 2\beta 1$ integrin, but lacking VEGFR2, are incapable of responding to endorepellin (114). LG1/2 and the LG3 domains are able to elicit different responses independently of each other (126). This suggests that cells that lack $\alpha 2\beta 1$ but do express VEGFR2, may actually be able to respond to endorepellin but specifically only to the LG1/2 domain.

The downstream cascades from the dual signalling by endorepellin have been investigated using an antibody array capable of probing the phosphorylation status of numerous receptor tyrosine kinases (RTKs) (119). True to its angiostatic activity, endorepellin reduces the phosphorylation of key receptors involved with angiogenesis including VEGFR1/2, EGFR and ROR2 (119). These findings were further validated when the effects of endorepellin were hampered following functional blockage of the key endorepellin receptor, $\alpha 2\beta 1$. Following up to 30 minutes of endorepellin treatment, the $\alpha 2$ subunit was immunoprecipitated and revealed that Src homology protein phosphatase-1 (SHP-1) was functionally linked to the $\alpha 2$ subunit (119). Moreover, following endorepellin treatment, SHP-1 phosphorylation also increases, suggesting that SHP-1 is a key downstream target of $\alpha 2\beta 1$ following VEGFR2 engagement by soluble endorepellin. Endorepellin also causes SHP-1 to actively translocate from the plasma membrane to the nucleus where it is known to influence the transcription of early response genes (119). These findings were validated *in vivo*, as endothelial cells isolated from $\alpha 2\beta 1^{-/-}$ mice following endorepellin treatment have significantly reduced VEGFR2 phosphorylation (119).

Endorepellin signalling is able to influence the activity of two important signalling branches, the PI3K/Akt/mTOR and PKC/JNK/AP-1 pathways (Figure 2A), both of which are thought to be primarily governed by SHP-1 bioactivity (113). Under normal physiological conditions, PLC γ and the adaptor protein Shb are bound to VEGFR2 at Tyr¹¹⁷⁵. When these proteins become phosphorylated they activate the PI3K/Akt/mTOR and PKC/JNK/AP-1 pathways, respectively (Figure 2A). As well as being known to translocate to the nucleus to regulate genes, SHP-1 is able to dephosphorylate VEGFR2 at Tyr¹¹⁷⁵, which in turn causes the physical dissociation of Shb and PLC γ from VEGFR2, resulting in decreased signalling of PI3K/Akt/mTOR and PKC/JNK/AP-1 pathways (113). Normal signalling of VEGFA is able to promote the recruitment of PLC γ to VEGFR2. However, treatment with endorepellin can inhibit the effects of VEGFA on PLC γ (113). As endorepellin and VEGFA do not compete for the same binding region on VEGFR2, this would suggest that endorepellin carries a greater signalling potential than VEGFA, likely by allosterically inhibiting the receptor or preventing its dimerization. More studies need to be performed to decipher the complexity of downstream signalling and its components.

Anti-angiogenic properties of endorepellin

Due to the high level expression in cardiovascular tissues and vessels, and the ability of endorepellin to signal through VEGFR2, angiogenesis is the most widely reported process endorepellin is able to influence (113,114,119). Despite being a key region within the pro-angiogenic parent molecule perlecan, endorepellin is a potent mediator in repressing angiogenesis both *in vitro* (113,114) and *in vivo* (119).

HIF-1 α is a key transcription factor that is commonly upregulated when cells detect reduced oxygen content. This, in turn, promotes angiogenesis to replenish hypoxic regions. Endorepellin suppresses HIF-1 α levels in porcine aortic endothelial cells (PAE) stably overexpressing VEGFR2 (113). Importantly, this downregulation is transcriptionally regulated as cells transfected with luciferase driven by the human *HIF1A* promoter have a dose- and time-dependent suppression of the promoter activity following endorepellin treatment (113). In support of these findings, blocking VEGFR2, via either small molecule inhibitor or siRNA, abrogates the effects of endorepellin, demonstrating that this bioactivity is mediated through this receptor. Importantly, soluble endorepellin suppresses *HIF1A* and *VEGFA* mRNA and inhibits the secretion of VEGFA under hypoxic conditions (113).

There is robust *in vivo* evidence that supports a role for endorepellin as anti-cancer and anti-tumor angiogenic factor. When mice bearing either squamous cell or Lewis lung carcinomas, are systemically treated with human recombinant endorepellin at clinical dosages (5 mg/kg), the exogenously administered endorepellin specifically localizes to the tumour vascular system and peri-vascular regions where it remains for several days (116). This leads to regression of tumour angiogenesis and also induces a more hypoxic tumour microenvironment, whilst decreasing the tumour metabolism and the mitotic index (116). These findings put forward bold evidence that endorepellin is able to inhibit tumour growth through a number of angiogenic-mediated processes, but also that systemic administration of endorepellin is able to specifically target the tumor vascular system, thereby providing a proof for targeted tumour specificity. In support of these studies in mammals, exogenous administration of endorepellin to zebrafish with morpholino-mediated suppression of perlecan expression, can rescue the severe vascular phenotype (40). Although this again provides evidence to suggest the effects of endorepellin on angiogenesis, it also suggests that endorepellin may have potential therapeutic effects on cardiovascular diseases as well as cancer.

Pro-autophagic role of endorepellin

We recently discovered that endorepellin acts as a potent inducer of an intracellular catabolic process known as autophagy (126). These effects are also regulated through interaction with VEGFR2 and $\alpha 2\beta 1$ (Figure 2B) suggesting that these receptors also hold the downstream signaling machinery to regulate several pathways outside of angiogenesis (126).

Autophagy is a catabolic lysosomal process that is conserved across all eukaryotes (127). It is a highly-controlled process, which can often be described as pro-survival, where a cell uses self-eating as an alternate energy source by degrading and recycling cytosolic components (128). Autophagy is stimulated during various pathological and physiological

states, such as starvation. In cells starved for nutrients or growth factors, autophagy provides crucial nutrients and energy that enhance cell survival through the breakdown of cytosolic components. Other cellular stresses, including hypoxia, growth factor withdrawal, and ER stress, can also induce autophagy, often to enhance cell survival (129-131). The autophagic pathway, essential for embryonic development and organogenesis (132,133), can be deregulated in several disorders, including metabolic and infectious diseases, neurodegenerative disorders, angiogenesis and cancer (134). According to an emerging hypothesis, autophagy provides an anti-carcinogenic function in primary cells by safeguarding against metabolic stress through the homeostatic turnover of mitochondria and the clearance of protein aggregates. In established tumors, autophagy may confer a survival advantage on tumor cells that are under metabolic stress, as a result of a high proliferation rate and exposure to hypoxia from insufficient vascularization (135). However, there is also strong evidence that sustained angiogenesis evoked by angiostatic proteins negatively affect tumor angiogenesis and cancer growth (136).

Notably, other secreted ECM products, including decorin, endostatin and kringle 5, have been shown to trigger autophagy (137-139). Endorepellin causes the formation of well-developed, large autophagosomes in HUVEC that are visible using differential interference contrast (DIC) microscopy (Figure 3B), that are similar to those generated by canonical autophagy-inducing factors such as rapamycin (Figure 3C), and nutrient deprivation (126). Importantly, the number of these structures increases in a dose-dependent manner following endorepellin treatment (126). Two key markers of autophagy are Beclin1 and LC3 (140). Beclin1 is an important binding partner of class III PI3K-Vps34. Under normal conditions Vps34 is found located at the plasma membrane; however, when autophagy is initiated, Vps34 is mobilized to the phagophores, the initial formation stage of autophagosomes (141). LC3 exists in two forms, LC3-I and LC3-II. The latter is the cytosolic form which is conjugated to phosphatidylethanolamine and is recruited to the autophagosomal membranes (140). The stimulatory effects of endorepellin on these canonical catabolic markers provide the strongest evidence of endorepellin-mediated autophagy.

Following treatment with endorepellin, there is a change in the distribution of Vps34 from the plasma membrane to the cytoplasm (126). Interestingly, once redistributed, endorepellin evokes the movement of Vps34 into large intracytoplasmic vacuoles, which are Beclin 1 and LC3-II positive (126). Although the levels of Vps34 do not significantly change, the levels of Beclin 1, LC3 and Peg3 are all increased (126). We previously reported (142) that Peg3 is required for decorin-mediated induction of autophagy and proposed that Peg3 should be regarded as a marker of autophagy. Interestingly, when Peg3 is depleted, the ability of endorepellin to increase Beclin 1 and LC3 is totally abrogated, consistent with a primary role for Peg3 in the autophagic process (126). Notably, endorepellin causes co-localization of Peg3 with both autophagic markers LC3-II and Beclin 1 (126). These data suggest that not only may Peg3 be a novel autophagic protein, but it may also be one of the most reliable markers as it is responsible for the induction of Beclin1 and LC3.

As described above, endorepellin has three main LG domains exhibiting profound differences in the receptors they target and effects they provoke. LG1/2 binds with high affinity to VEGFR2, whereas, the LG3 domain binds $\alpha 2\beta 1$ (114). The importance of these

three domains is profound in directing whether endorepellin induces autophagy or inhibits angiogenesis. LG1/2, independently of LG3, induces the expression of *PEG3*, *BECN1* and *MAPLC3A* genes. In contrast, signalling by LG3 alone actually reduces the levels of all the aforementioned genes (126). This demonstrates that only endorepellin domains LG1/2 are able to promote autophagy, which is solely dependent on VEGFR2 (Figure 2B). In contrast, the LG3 domain signals through $\alpha 2\beta 1$ and works to repress autophagy. Therefore, the ability of endorepellin to induce autophagy is based on VEGFR2, while its anti-angiogenic property is based upon dual receptor antagonism, involving VEGFR2 and $\alpha 2\beta 1$ integrin.

Since full length endorepellin is able to induce autophagy (126), it would suggest that signalling of LG1/2 through VEGFR2 carries a greater signalling potential and can override the effects of LG3 and the $\alpha 2\beta 1$ to reduce autophagy. Further evidence of this signalling being solely dependent on VEGFR2 is provided when $\alpha 2\beta 1$ is blocked using anti- $\alpha 2\beta 1$ blocking antibodies. In this setting, there are no changes in the endorepellin-induced increases in *PEG3*, *BECN1* or *MAPLC3A* (126). However, when VEGFR2 is silenced, the ability of endorepellin to increase *PEG3*, *BECN1* and *MAPLC3A* is markedly suppressed (126). Thus, endorepellin evokes a catabolic program in endothelial cells and this aberrant autophagy could be detrimental to the formation of cancer-associated blood vessels.

Conclusions

Angiogenesis is an imperative aspect of normal human development. However, this process is often aberrant in many forms of cancer where the tumors induce the development of a florid blood supply in order to support their progressive increase in growth. The proteoglycan perlecan and its C-terminal fragment endorepellin best describe the fundamental balance required to maintain the angiogenic system. Perlecan proteoglycan is, overall, a pro-angiogenic molecule that is expressed in the cardiovascular system during development and in the adult life. In contrast, endorepellin which is found in similar regions as perlecan, is strongly anti-angiogenic. Perlecan is a large multidomain proteoglycan that has major effects in the development of the cardiovascular system from an embryonic stage of development, and into adulthood where perlecan is necessary for proper blood vessel maintenance. Perlecan-derived endorepellin signals through a dual receptor antagonism by modulating the activity of both VEGFR2 and $\alpha 2\beta 1$ integrin, a process that ultimately leads to a suppression of powerful pro-angiogenic cues, including HIF-1 α and VEGFA. The effects of endorepellin have been demonstrated in a number of *in vitro* and *in vivo* models placing it in the category of a molecule with true therapeutic potential. More importantly, exogenous endorepellin specifically targets the tumour vasculature thereby retarding cancer growth, metabolism and angiogenesis. Moreover, as endorepellin targets the $\alpha 2\beta 1$ integrin, a powerful pro-angiogenic receptor that is overexpressed in actively-proliferating endothelial cells, it exerts a profound effect on tumor angiogenesis vis-à-vis normal blood vessels which usually express only low levels of this integrin. As more protein-based therapies to treat various pathologies start to be utilized in the clinics, a natural endogenous inhibitor of angiogenesis and pro-autophagic factor such as endorepellin could be of potential advantage. The use of endorepellin would carry very low risk to patients, as it is non-toxic and does not target normal vasculature (116).

Already the potential diagnostic and prognostic values of LG3 domain of endorepellin have been utilized, as LG3 has been reported to be a potential clinical biomarker in patients with premature rupture of fetal membranes (143), renal failure (144,145), and in vascular allograft rejection (146). Endorepellin and LG3 have been detected in the urine of children with sleep apnea (147), in the media conditioned by apoptotic endothelial cells (57,148,149), and in the secretome of pancreatic and colon carcinoma cells (105,150-152). Notably, circulating LG3 levels are reduced in breast cancer patients, suggesting that LG3 might be a useful biomarker for cancer progression and invasion (153). Although the discovery of endorepellin is still in its early stages, increasing evidence on the potential anti-cancer implications this protein can have is progressing. Granted that before endorepellin can be considered in the clinics, much more field work is required. The novel approach of targeted therapy using an endogenous protein that bares little toxicity, and aims to use the body's own defence to directly curtail tumors nutrients, certainly represents an exciting avenue to explore in cancer treatment.

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Nomenclature

ECM	extracellular matrix
GAG	glycosaminoglycan
HS	heparan sulfate
HSPG	HS proteoglycan
GAG	glycosaminoglycan
VEGF	vascular endothelial cell growth factor
VEGFR	VEGF receptor
MMP	matrix metalloproteinase
EGF	epidermal growth factor
LG	laminin-like globular domain
SHP1	Src homology protein phosphatase-1
PLCγ	phospholipase C γ
Shb	SH2 domain-containing adaptor protein B
PI3K	phosphatidyl inositide 3-kinases
mTOR	mammalian target of rapamycin
JNK	c-Jun N-terminal kinase
PKC	protein kinase C

AP1	activating protein 1
HIF-1α	hypoxia-inducible factor 1 α
DIC	differential interference contrast microscopy
HUVEC	human umbilical vein endothelial cell
LC3	microtubule-associated protein 1A/1B-light chain 3
VPS34	class III phosphoinositide kinase
Peg3	paternally expressed gene 3
PAE	porcine aortic endothelial cells
DDSH	dyssegmental dysplasia Silverman-Handmaker type
SJS	Schwartz-Jampel syndrome
RTK	receptor tyrosine kinase

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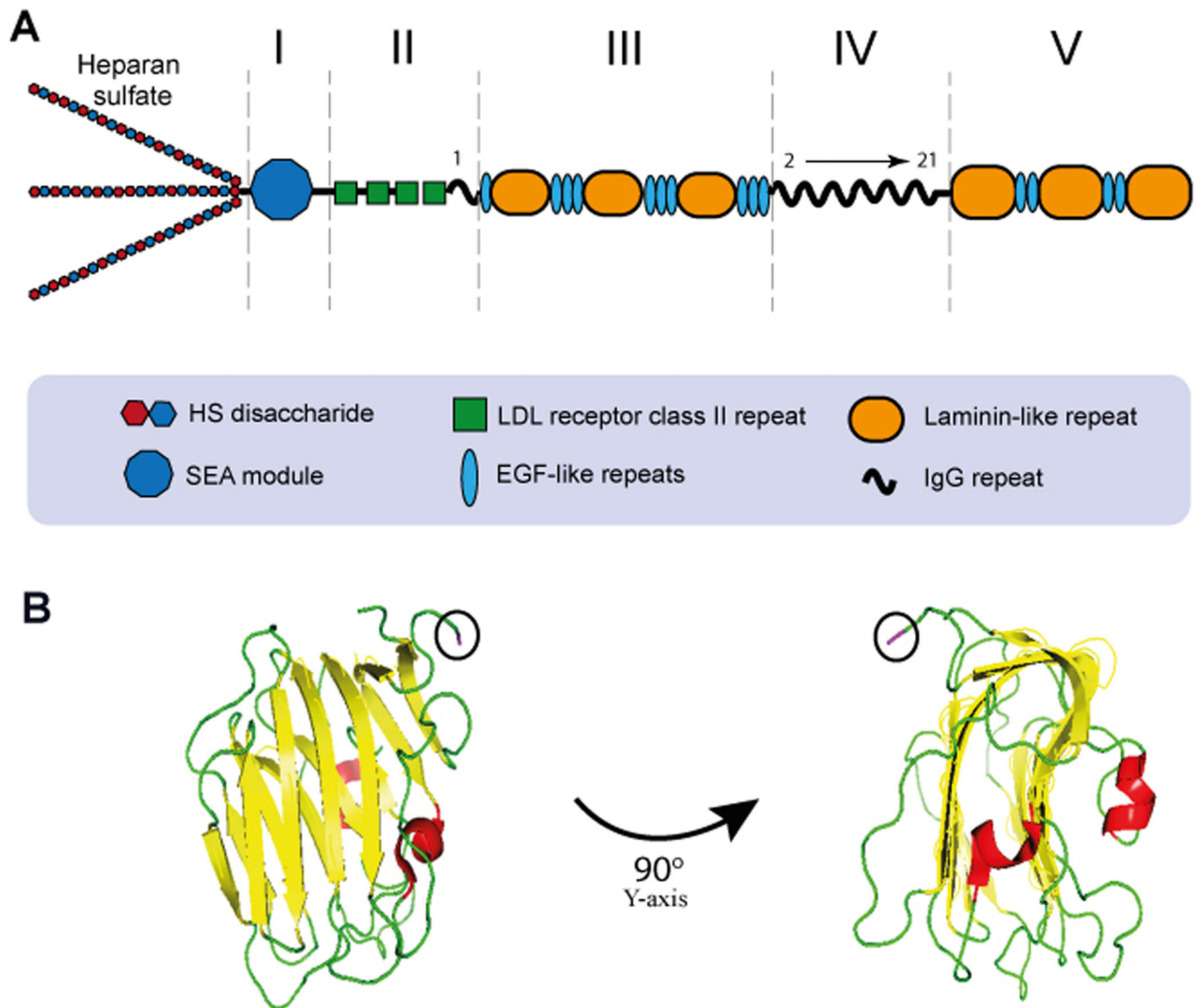


Figure 1. Schematic representation of the multimodular perlecan and its C-terminal endorepellin. (A) Perlecan is large multidomain proteoglycan consisting of 5 domains. It contains 3 HS chains towards the N-terminus. Domain I contains a SEA module. Domain II is made up of four LDL repeats and the first IgG. Following this domain, is Domain III which is made up of 3 LG repeats, each being separated by 3 laminin-like EGF repeats. Domain IV solely contains a further 20 IgG repeats. The C-terminal Domain V, termed endorepellin, has a similar structure to Domain III as it contains 3 laminin repeats; however, each of these is separated by 2 laminin-like EGF repeats. (B) Refined crystal structure of LG3 domain of endorepellin. Image was generated using PDB:3SH4 and presented using PyMol software. LG3 is constructed as a 15-stranded anti-parallel β sandwich forming a jellyroll fold characteristic of LG domains. The right image represents a 90° rotation along the y axis. This region is known to interact with $\alpha 2\beta 1$ integrin located on endothelial cells. The two α helices are illustrated in red, the 12 β sheets are yellow and the loops are green. Of particular note,

Asp⁴¹⁹⁷ is highlighted in magenta (circled) and is found at the N-terminus of the protein. This amino acid is suggested to be important in the processing of endorepellin. A mutation in this amino acid inhibits endorepellin activity. Thus, this site is hypothesised to be a potential binding site for the $\alpha 2\beta 1$ integrin.

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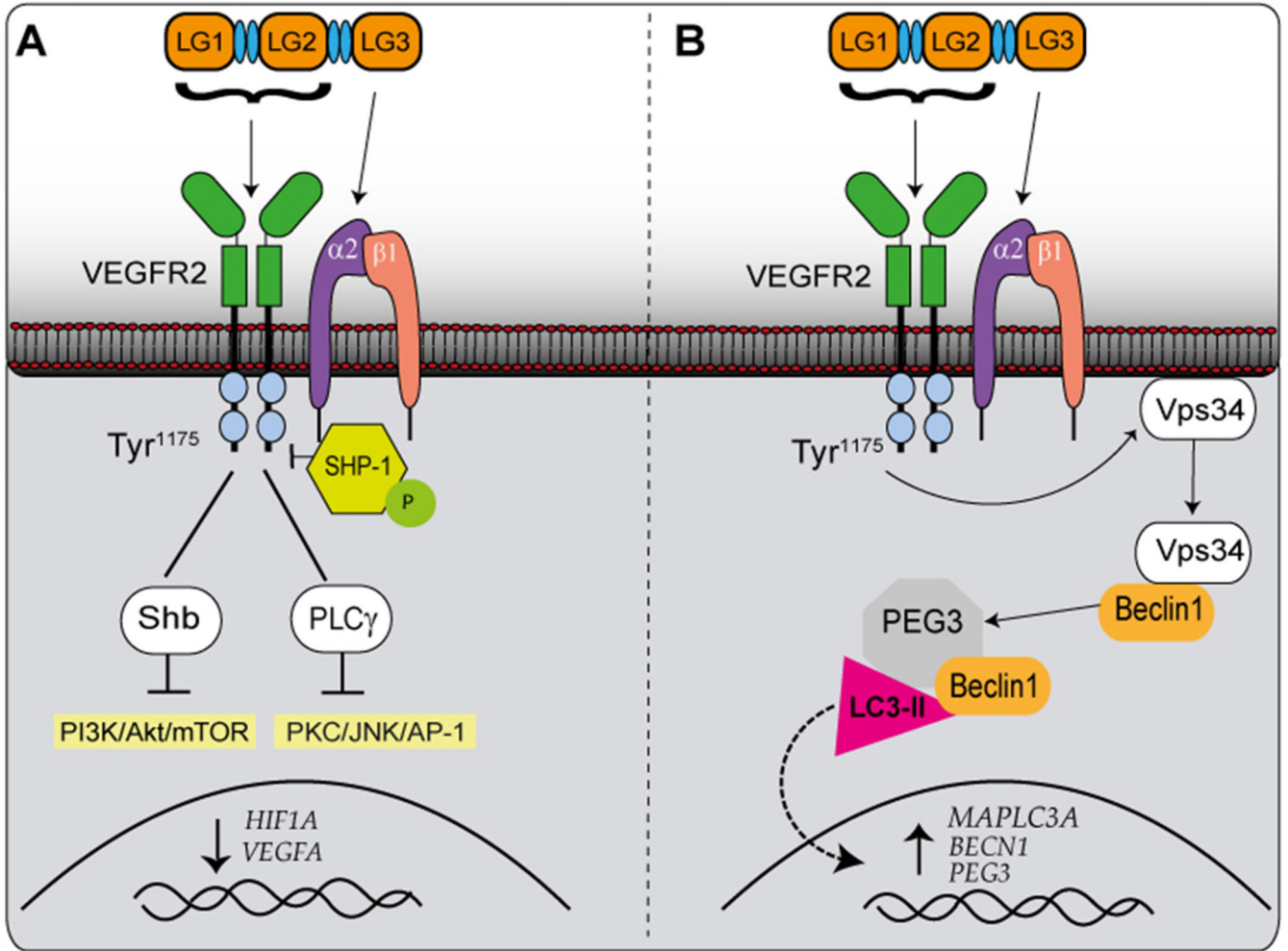


Figure 2. Schematic representation of the signalling pathways involved in endorepellin-evoked intracellular signalling network. (A) The anti-angiogenic effects of endorepellin. Endorepellin signals through VEGFR2 via its LG1/2 domain and $\alpha 2\beta 1$ via LG3. This causes phosphorylation of SHP-1, which relocates from the plasma membrane to the $\alpha 2$ subunit of the integrin. This subsequently causes dephosphorylation of VEGFR2 at Tyr¹¹⁷⁵, which prevents the Shb and PLC γ from binding to VEGFR2 and therefore prevents the activation of PI3K/Akt/mTOR and PKC/JNK/AP-1 pathways. These pathways prevent the transcription of *HIF1A* and *VEGFA*. (B) The pro-autophagic effects of endorepellin. Endorepellin signals through VEGFR2 and $\alpha 2\beta 1$ in the same manner as previously described. Although the involvement of SHP-1 is not yet elucidated, Tyr¹¹⁷⁵ is again dephosphorylated causing Vps34 to re-locate from the plasma membrane and interact with Beclin1 in autophagosomes. Beclin1 then co-localizes with Peg3 and LC3-II. The signalling of endorepellin also results in increased mRNA expression of *MAPLC3A*, *BECN1* and *Peg3*.

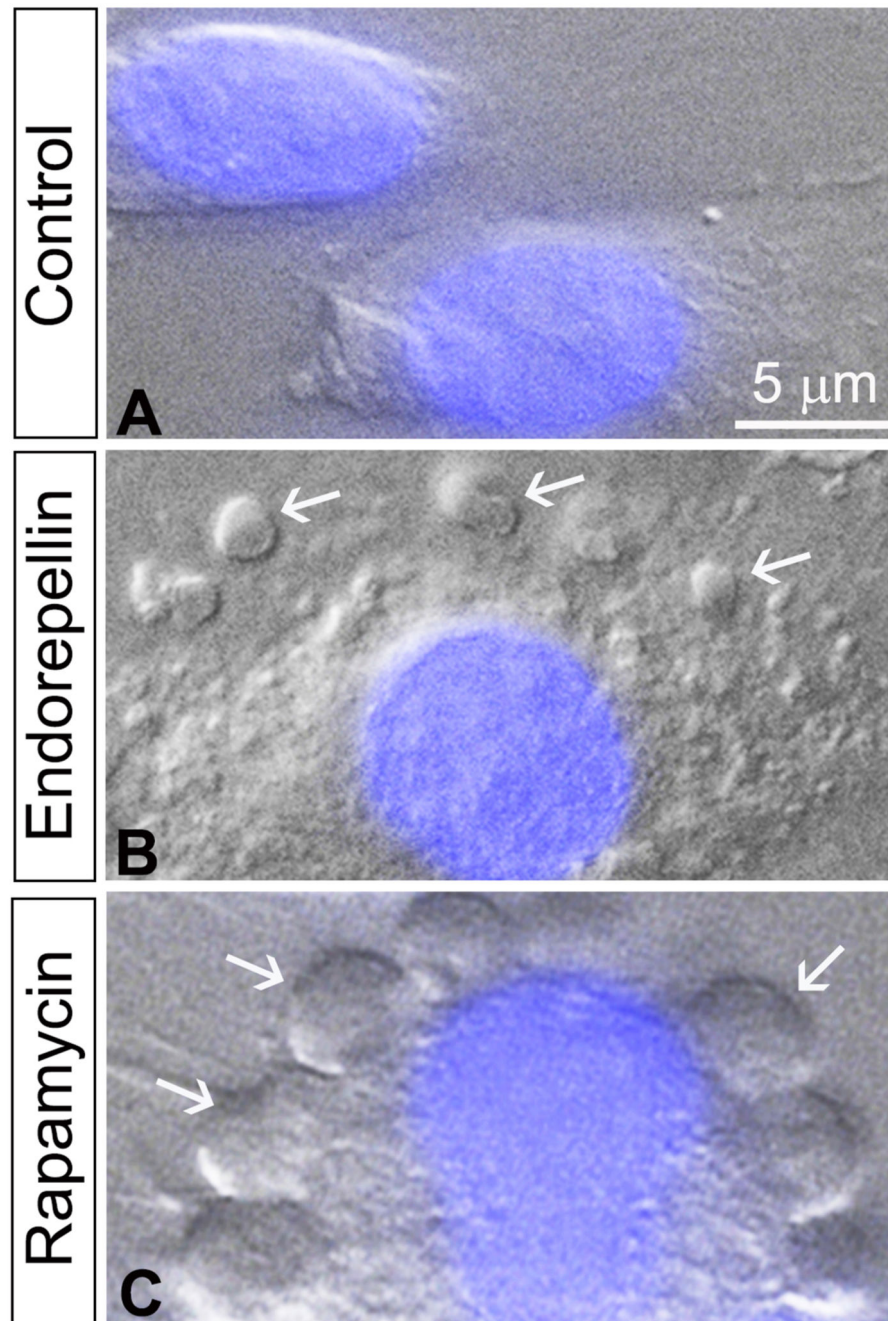


Figure 3. Endorepellin induces autophagy in endothelial cells through an mTOR-dependent pathway. Representative images of HUVECs following 6 h treatment with vehicle (A) 200 nM endorepellin (B) or 100 nM rapamycin (C) in nutrient-enriched conditions. The images were captured with differential interference contrast microscopy and fluorescence to detect the nucleus stained in blue by DAPI. Notice the formation of multiple autophagosomes (white

arrows) in the cytoplasm of the cells in both endorepellin- and rapamycin-treated endothelial cells vis-à-vis vehicle-treated controls.

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