The multifaceted roles of perlecan in fibrosis

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Abstract

Perlecan, or heparan sulfate proteoglycan 2 (HSPG2), is a ubiquitous heparan sulfate proteoglycan that has major roles in tissue and organ development and wound healing by orchestrating the binding and signaling of mitogens and morphogens to cells in a temporal and dynamic fashion. In this review, its roles in fibrosis are reviewed by drawing upon evidence from tissue and organ systems that undergo fibrosis as a result of an uncontrolled response to either inflammation or traumatic cellular injury leading to an over production of a collagen-rich extracellular matrix. This review focuses on examples of fibrosis that occurs in lung, liver, kidney, skin, kidney, neural tissues and blood vessels and its link to the expression of perlecan in that particular organ system.

Keywords

Abbreviations

apoE, apolipoprotein E; α-SMA, α -smooth muscle actin; BMP1, bone morphogenic protein-1;CCL, CC chemokine ligand; COPD, chronic obstructive pulmonary disease; CS, chondroitin sulfate; CSPG4, chondroitin sulfate proteoglycan 4; CXCL, CXC chemokine ligand; DS, dermatan sulfate; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; HS, heparan sulfate; HSC, hepatic stellate cell; HSPG2, heparan sulfate proteoglycan 2; IL, interleukin; IPF, idoiopathic pulmonary fibrosis; KS, keratan sulfate; LDL, low density lipoprotein; LG, laminin G-like domain; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NGF, neural growth factor; NG2, neural/glial antigen 2; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase, PRELP, proline-arginine-rich end leucine-rich repeat protein; PTEN, phosphatase and tensin homolog; SMC, smooth muscle cell; STAT, signal transducer and activator of transcription; SULF, sulfatase; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

1.   Introduction

Fibrosis is a complex and finely tuned process that occurs in response to either inflammation, traumatic cellular injury or as a result of unknown or congenital issues affecting the structure of the extracellular matrix (ECM). Fibrosis is generally associated with pathological changes where significant increases in the expression and assembly of the ECM, notably the collagen content of the tissues. This has significant longer term impacts on the structural and functional properties of the tissues that may lead to impairment and failure of that organ. While the primary attributes seen in these diseases lie in the over-production of collagen in the tissues, changes in perlecan, also known as heparan sulfate proteoglycan 2 (HSPG2), levels also occur [1-3].

It is now well accepted that fibrosis is not a static event, but a dynamic process involving several phases that are also common to the wound healing response. Initially, an influx of inflammatory cells are seen that become activated resulting in the downstream activation of tissue resident ECM secreting cells followed by a significant change in the structure of ECM from an increased synthesis and decreased proteolytic turnover, further immune cell infiltration and delayed apoptosis [4, 5]. Many of these phases involve heparin-binding growth factors, cytokines and chemokines suggesting that heparan sulfate (HS) proteoglycans play important roles in this process [6]. Despite the range of biological activities of HS, there are only a few HS proteoglycans present in the ECM including perlecan, collagen type XVIII and agrin [7]. The abundance of perlecan in basement membranes and connective tissues that exhibit fibrosis [7, 8] as well as the key roles that it plays in many tissues suggests that it may play important roles in fibrosis. Recent evidence that will be reviewed in the following sections supports the hypothesis that perlecan plays key roles in the regulation of fibrogenesis.

1.2 Overview of perlecan

1.2.1 Structure of perlecan

Perlecan is a ubiquitous HS proteoglycan synthesized by most cells and present in most pericellular and extracellular matrices and is recognized as an essential component of basement membranes [7, 9]. Perlecan has also been localized to connective tissues and is produced by leukocytes [10]. The protein core of human perlecan is 460 kDa and composed of five distinct domains with glycosaminoglycan attachment sites located in both the N- and C-terminal domains (Fig. 1). Perlecan exerts tissue-specific activities through its glycosaminoglycan chains as the structure of these depends on the context and cell source. Typically, the three glycosaminoglycan attachment sites in domain I are decorated with HS, which has been shown for endothelial cell derived perlecan [11]. Perlecan may also be decorated with chondroitin and/or keratan sulfate (CS and KS respectively) in addition to HS as has been demonstrated for smooth muscle and epithelial cell derived perlecan [12-14] and these may also utilize the glycosaminoglycan attachment site in domain V [15]. There are many biological functions ascribed to perlecan including playing central roles in regulating the assembly of the ECM and the binding and presentation of growth factors to cells [16].

1.2.2 Functions of the glycosaminoglycan chains

The HS chains that decorate perlecan electrostatically interact with growth factors enabling their storage in the ECM, protection from proteolytic degradation and potentiation of their activity when bound to their cognate receptors [17, 18]. The HS chains of perlecan, bind and modulate the signaling of fibroblast growth factors (FGF) 1, 2 and 18 [13, 14, 19, 20] and also bind to other heparin-binding growth factors that have been shown to be involved in the fibrotic process and include vascular endothelial growth factor (VEGF) isoforms 165, 189 and 206, epidermal growth factor (EGF), transforming growth factor (TGF) α, connective tissue growth factor and hepatocyte growth factor (HGF) [6, 21]. Fine structural changes in the HS chains on perlecan can control growth factor signaling. For example, FGF1 signals when complexed with endothelial cell derived perlecan HS but not smooth muscle cell derived perlecan HS [13]. Additionally, cells have the ability to dynamically change the structure of their glycosaminoglycan chains as a result of the microenvironment [22] enabling temporal control over heparin-binding growth factor activities. The FGF2/perlecan complex is important in specifying the spatial distribution of the growth factor while degradation of perlecan destabilizes the complex causing FGF2 to lose its defined localization [23] in the ECM enabling it to interact with the syndecan and glypican families of cell surface HS proteoglycans [24]. The HS chains that decorate perlecan modulate the activity of mitogenic growth factors that support the proliferation of a wide variety of cell types including those involved in fibrosis [25-28]. At high cell densities in culture, the proliferation of many mesenchymal cell types was reduced by perlecan [25, 29, 30], suggesting that the role of perlecan in these situations is complex and not well understood.

1.2.3 Enzymatic regulation by heparanase and sulfatases

The activity of heparin binding growth factors can be affected by the action of HS modifying enzymes including heparanase and the sulfatases. Heparanase cleaves HS both on the cell surface and in the basement membrane and has been shown to modulate FGF signaling [31, 32]. Heparanase expression is reduced during liver fibrosis and increased in atherosclerosis [14, 17, 33-35]. Sulfatases (SULF)-1 and -2 selectively remove 6-O-sulfate mainly from trisulfated disaccharide units (IdoA2S-GlcNS6S) within S-domains of heparin/HS, where many growth factors, including FGFs, bind. Pretreatment of heparin with SULF-2 has been shown to inhibit the binding of many heparin binding growth factors while treatment of complexes formed between heparin and growth factors can be released by treatment with SULF-2 [36]. In addition, SULF-1 inhibits growth factor binding to its receptor [37]. Interestingly, both SULF-1 and -2 are regulated by transforming growth factor (TGF)-β1 and are reported to be upregulated in pulmonary fibrosis [38]. Together these studies suggest a role for HS modifying enzymes in the regulation of growth factor signaling events in fibrosis.

1.2.4 Roles of the protein core

Perlecan possesses an α2β1 integrin binding site in domain V that mediates cell adhesion, while other putative cell adhesion sites reside in domains III and IV [13, 39, 40]. In addition, domain III of mouse perlecan contains an RGD sequence that may interact with integrins [41]. Perlecan can support the proliferation of a wide variety of cell types including those involved in fibrosis [25-28]. Perlecan can also be anti-proliferative for cell types such as smooth muscle cells (SMCs) via both HS and protein

core, particularly domain III [13, 42], that is mediated by the tumor suppressor PTEN [43]. Perlecan can also bind some growth factors via its protein core including platelet-derived growth factor (PDGF) to domain III [44] and keratinocyte growth factor (also known as FGF7) to both domains III and V [45]. Low density lipoprotein (LDL) binds to perlecan domain II and this binding is modulated by HS [35, 46].

Proteolytic processing of perlecan by chymase, MMPs, and cathepsins which can cleave in perlecan domain IV has been reported to generate perlecan fragments encompassing endorepellin [10], which is a recombinant form of perlecan domain V named due to its anti-angiogenic activities [47, 48]. Although endorepellin contains a glycosaminoglycan attachment site in its protein sequence it is devoid of a glycosaminoglycan chain [47], thus its activities are ascribed to the protein core. Endorepellin can be cleaved by BMP1/Tolloid-like proteases and cathepsin L to release the laminin G-like domain (LG)3 region that includes the α2β1 integrin binding site [49, 50]. Inflammatory cells including mast cells produce alternatively spliced forms of perlecan with a relative increase in the amount of transcripts corresponding to domain V [51]. Together the various roles of the perlecan protein core suggest that perlecan may exert its roles in fibrosis through both the parent molecule and biologically active fragments.

1.2.5 Roles in ECM organization

Perlecan is an essential molecule in tissue and organ development with roles in ECM stabilization and the maintenance of the functional status of mature connective tissues [52]. Perlecan interacts with a diverse range of ECM components including fibronectin, laminin and many of the collagens coordinating the organization of a diverse array of structural glycoproteins and ECM proteins [17, 53]. For example, perlecan assists in collagen fibrillogenesis, largely through its pendant glycosaminoglycan chains [54, 55].

2   Manifestations of fibrosis and the fundamental roles of perlecan

Fibrosis can affect a number of different tissues and organs in the body including the lung, liver, skin, kidney, neural and vascular tissues. The fibrogenic response involves several phases of cell-based processes such as fibroblast and immune cell activation, immune cell infiltration and delayed apoptosis resulting in the accumulation of ECM, which is the accepted hallmark of the condition. The many roles of perlecan described above suggest that it has important roles to play in both modulating and promoting fibrogenesis. The roles of perlecan in fibrogenesis are reviewed in the following sections drawing upon evidence in the literature of tissue-specific examples. A summary of the known roles of perlecan and their impact on fibrogenesis are summarized in Fig. 1.
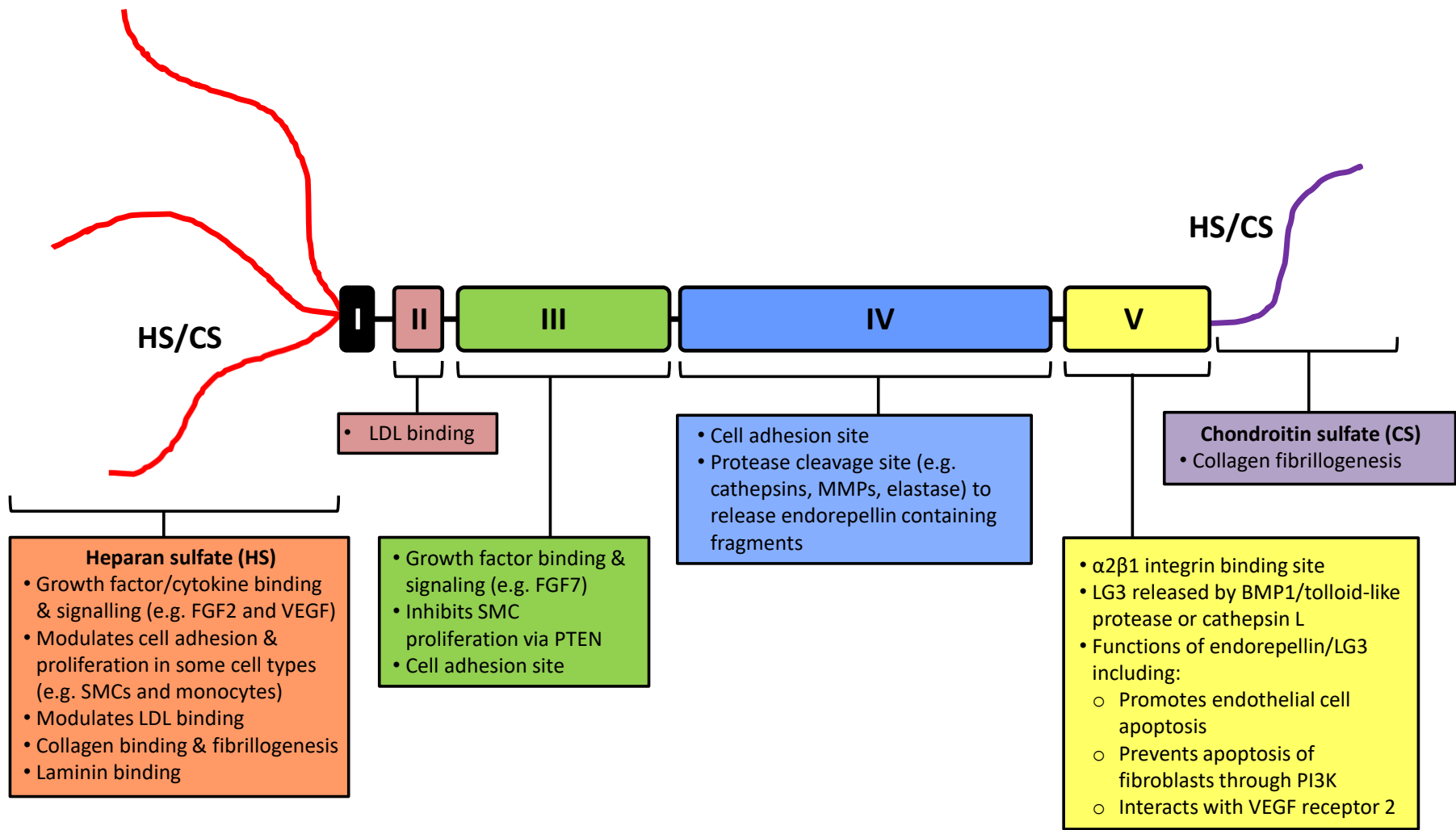
HS/CS

HS/CS

| I | II | III | IV | V |

Heparan sulfate (HS)
- Growth factor/cytokine binding & signalling (e.g. FGF2 and VEGF)
- Modulates cell adhesion & proliferation in some cell types (e.g. SMCs and monocytes)
- Modulates LDL binding
- Collagen binding & fibrillogenesis
- Laminin binding

- LDL binding

- Growth factor binding & signaling (e.g. FGF7)
- Inhibits SMC proliferation via PTEN
- Cell adhesion site

- Cell adhesion site
- Protease cleavage site (e.g. cathepsins, MMPs, elastase) to release endorepellin containing fragments

- $\alpha2\beta1$ integrin binding site
- LG3 released by BMP1/tolloid-like protease or cathepsin L
- Functions of endorepellin/LG3 including:
  - Promotes endothelial cell apoptosis
  - Prevents apoptosis of fibroblasts through PI3K
  - Interacts with VEGF receptor 2

Chondroitin sulfate (CS)
- Collagen fibrillogenesis

Fig. 1. Roles of perlecan in fibrogenesis mediated by both its protein core and glycosaminoglycan chains.

A key event in the initiation of fibrosis is the activation of fibrocyte and fibroblast cells and their trans-differentiation to myofibroblastic-like cells that express α-smooth muscle actin (α-SMA). Interestingly, the activation of fibroblasts is not unique to fibrosis as it occurs in cancer, decidualization and infection [56-58]. The net accumulation of ECM in injured tissues results from the increased synthesis of ECM components including collagen types I and III, perlecan and laminins by cells [59]. Cells that infiltrate the injured site, including circulating inflammatory cells and fibrocytes, may also contribute to the synthesis of ECM locally; for example, monocyte-derived macrophages express perlecan in response to hypoxia mediated via the HIF-1α pathway [60] while macrophages and granulocytes have also been shown to produce perlecan [10, 60]. Another example is the infiltration of mesenchymal stem cells and pericytes that potentially contribute to lung fibrosis that express the PDGF receptor α as well as fibrocytes of bone marrow origin [61] that home to sites of remodeling via CXC chemokine ligand (CXCL)12 and CXCR4 involving HS [62]. In ECM remodeling and repair, the myofibroblasts and fibroblasts are cleared through apoptosis [63]. However, during fibrosis the wound healing phase fails to terminate due to the lack of apoptosis of these cells and it has been hypothesized that the C-terminal fragment of perlecan directs cell apoptosis [64, 65].

Perlecan is involved in the generation of chemotactic growth factor and morphogen gradients which recruit cells to sites of ECM remodeling, primarily through interactions of the cytokines with its HS chains [66-70] but also including interactions of growth factors with its protein core [45]. Numerous cytokines including CC chemokine ligand (CCL) -2, -3 and -5 and CXCL-1, -8 (also known as interleukin (IL)-8), -9 and -10 and -12 (also known as stromal cell derived factor 1α) bind HS/heparin [71-78] and their gradients in tissues are likely to be affected by the structure of the perlecan HS chains. These factors promote the migration of fibrogenic cells to the site of injury thereby amplifying inflammation [79]. This selective action suggests that perlecan may be involved in recruiting cell types that contribute to the organization of provisional matrix during tissue repair, and not those with a profibrotic phenotype, which would limit fibrosis [80].

TGFβ1 is one of the major cytokines involved in upregulating perlecan expression and it is expressed by many of the cell types involved in fibrogenesis in tissues including hepatic stellate cells (HSCs), fibroblasts, astrocytes and microglia, specialized brain macrophages [81-84]. The latent form of TGFβ1 is activated by plasmin that in turn upregulates perlecan expression [85, 86]. There is also evidence of TGFβ1 regulating perlecan expression when endothelial cells are plated at high density, which may promote the repair of the injured endothelium *in vivo* [87].

Perlecan interacts with type IV collagen and a number of structural proteins to help form the basement membranes [88-90]. It assists in collagen fibrillogenesis, largely through its pendant CS chains [54], but there is evidence that this may also involve HS-dependent mechanisms [55]. Perlecan facilitates the interactions of laminin and collagen in conjunction with proline-arginine-rich end leucine-rich repeat protein (PRELP), a collagen anchorage small leucine-rich proteoglycan, to create the basement membrane present in many tissues including those discussed in more detail in the next sections [91, 92].

2.1 Lung

In lung, chronic obstructive pulmonary disease (COPD), asthma and idiopathic pulmonary fibrosis (IPF) affect the parenchymal tissue manifesting as a thickening of the subepithelial ECM [93, 94]. While both COPD and IPF exhibit a progressive loss of alveolar parenchyma, COPD is an obstructive disease with airway inflammation and IPF is a restrictive disease [95]. The major ECM producing cells are fibroblasts and myofibroblasts while airway epithelial and SMCs are also involved [94, 96, 97] (Fig. 2A). Airway SMCs obtained from donors with COPD showed enhanced perlecan production following stimulation with TGFβ1 that was driven by intracellular SMAD activation [86]. Furthermore, this study demonstrated that COPD airway SMCs adhered to perlecan via epitopes in domains IV and V [86]. Interestingly, the ECM is degraded in the alveoli and excessive deposition in the bronchi and bronchioles that is correlated with differences in the fibroblast morphology, proliferation and ECM production [98]. Phenotypically different fibroblasts have been reported in central airways and lung parenchyma in COPD [99]. Fibroblasts in the parenchymal tissue were more sensitive to TGFβ1 expression than in the central airways, which resulted in increased VEGF synthesis, proliferation and increased the synthesis of perlecan [99, 100]. Fibrotic cells from biopsies taken from patients with asthmatic symptoms showed an increased secretion of perlecan in culture media compared to cells from normal patients [101]. In addition, cigarette smoke extract increased perlecan expression by COPD fibroblasts *in vitro*, however the mechanisms involved have not been explored [102].

In a rat model of bleomycin-induced pulmonary fibrosis, total proteoglycan content was significantly increased, including the expression level of perlecan [103]. Furthermore, primary lung fibroblast cultures established from rats after bleomycin induction exhibited increased production of perlecan, compared with normal lung fibroblasts together with a significant increase in the amount of TGFβ1[104]. Incubation with an anti-TGFβ1 antibody inhibited proteoglycan expression in a dose-dependent manner, indicating that TGFβ1 was responsible in part for the increase in proteoglycan production in fibrotic cells [104]. Nitric oxide has been reported to downregulate perlecan expression in asthmatic lung fibroblasts [105]. Fibulin-1 peptides can stimulate the production of perlecan in lung derived airway SMCs and fibroblasts from patients with pulmonary fibrosis and COPD [106]. The augmented deposition of cell-associated perlecan together with fibronectin modulated by fibulin-1 may enhance fibroblast activity and extend fibroblast induced lung remodeling [106].

The structures of the HS chains decorating perlecan in lung tissue from patients with IPF showed increased levels of glycosaminoglycans as well as increased sulfation of the HS in the areas of increased matrix deposition linking these changes in glycosaminoglycan structure to the altered growth factor activity in fibrosis [107]. Immunohistochemical staining showed co-localization of perlecan and the highly sulfated HS, indicating perlecan may be the core protein that harbors these highly sulfated HS chains [107]. The altered HS composition in IPF may thus suggest that the ECM landscape in fibrosis favors growth factor activity and tissue remodeling [107].
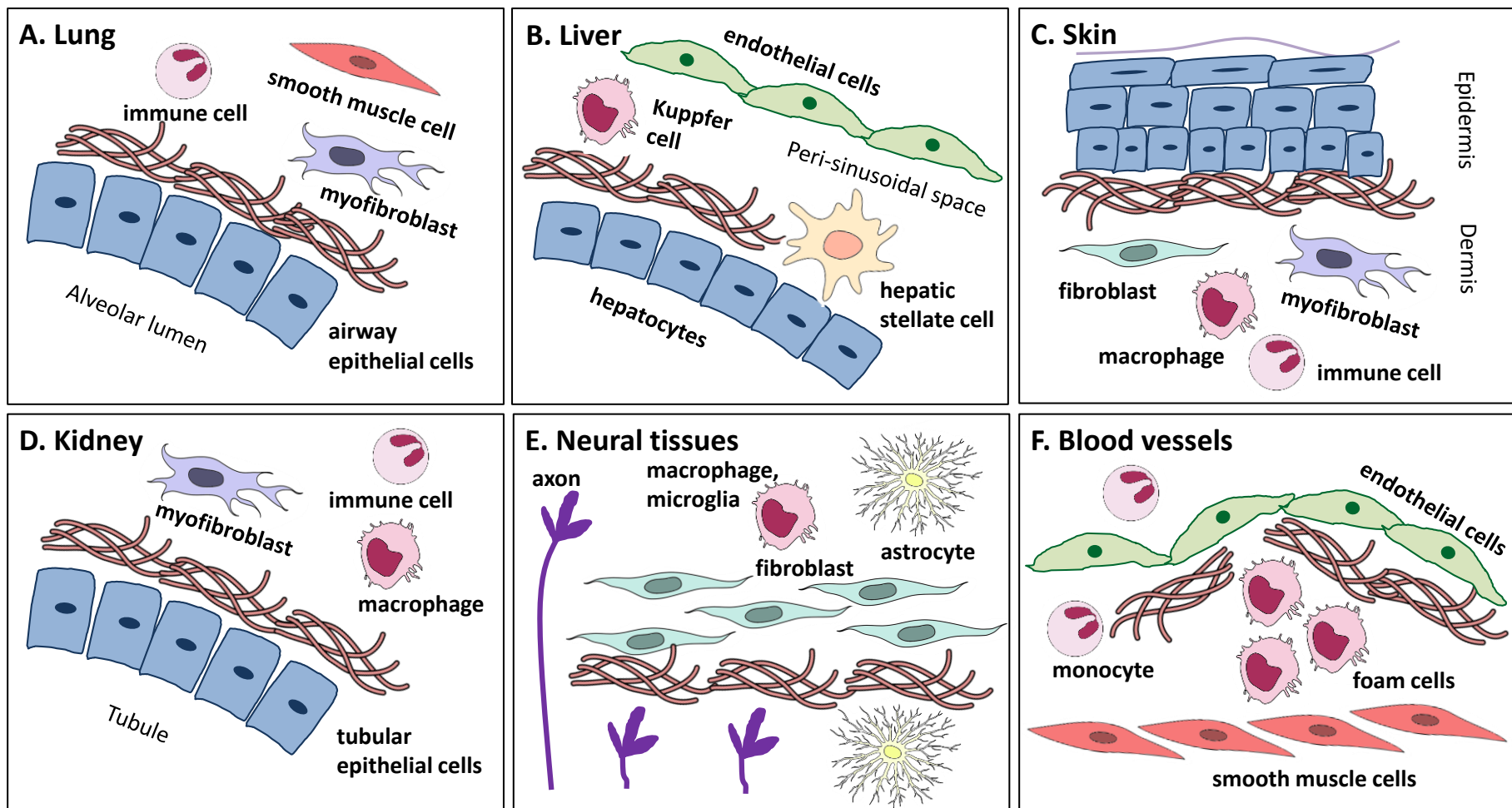
Fig. 2. Schematic of where fibrogenesis occurs and the cell types involved in (A) lung, (B) liver, (C) skin, (D) kidney, (E) neural tissues and (F) blood vessels.

2.2 Liver

Hepatic endothelial cells line the space of Disse, which contains a non-continuous basement membrane due to a lack of polymerized laminin, which presumably facilitates the movement of cells and blood throughout and across the liver sinusoids (Fig. 2B). Despite the absence of polymerized laminin, this discontinuous membrane contained significant amounts of other ECM components including collagen, perlecan and fibronectin [2, 24, 108, 109]. In contrast, laminin is present together with collagen type IV in classical continuous basement membranes around liver biliary ducts and blood vessels [110]. In fibrosis, peri-sinusoidal basement membranes are synthesized and it has been shown that these contain laminin and collagen fibrils that are deposited, along with increased perlecan expression [2, 85, 108, 110-113]. The production of these basement membrane structures can cause functional changes that impact upon the exchange of macromolecules between the endothelial sinusoids and hepatocytes in the liver [93, 114, 115]. While perlecan is a major component of the perisinusoidal basement membrane in the liver, it along with type XVIII collagen is upregulated in fibrosis [2, 116].

The primary cells that are responsible for the deposition of the ECM in liver fibrosis are HSCs [4] that are resident peri-sinusoidal cells in the subendothelial space residing between the hepatocytes and sinusoidal endothelial cells. These cells become activated at sites of injury by factors produced by hepatocytes and endothelial cells, as well as infiltrating inflammatory cells including macrophages [117-121]. Once activated HSCs exhibit increased mitogenic activity in the presence of PDGF-BB, a heparin binding growth factor that binds to perlecan HS and signals cells via the Ras-mitogen-activated protein kinase (MAPK) pathway via an upregulation of the PDGF-β receptor [17, 29]. The increased production and activity of cytokines are required for the continued activation of HSCs, which amplify inflammation through the release of neutrophil and monocyte chemo-attractants, such as monocyte chemotactic protein 1 (MCP-1) and colony-stimulating factor [122]. This positive feedback system results in an accumulation of HSCs and macrophages at the site of injury and it is interesting to note that activated HSCs respond to MCP-1 [123] that binds perlecan [124], demonstrating a level of cross talk in the system resulting in an accumulation of HSCs at the site.

Perlecan is upregulated in the periportal parenchyma during chronic cholestatic disease [24] and there is an increase in the HS and CS/dermatan sulfate (DS) content [125, 126] together with an upregulation of some of the enzymes involved in sulfate modification of HS including the 6-O-sulfotransferases and glucosamine 3-O-sulfotransferase 1 enzymes involved in producing heparin-like sequences [126]. This supports the hypothesis that an increased abundance of these highly sulfated glycosaminoglycan sequences promote both collagen fibrillogenesis and laminin polymerization [54, 55, 127].

Matrix metalloproteinase (MMP) activity is increased in fibrosis, particularly MMP-2,-3 and -9, that degrades basement membrane components, including perlecan [94, 128-131], and replace this ECM with type I collagen which further activates cell growth and MMP production [132]. MMPs are derived from tissue resident cell populations, activated HSCs and Kupffer cells which are specialized liver macrophages [133].

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In congenital hepatic fibrosis, there is a marked infiltration of mast cells scattered throughout the portal structures. These are more abundant than in other chronic liver diseases such as chronic viral hepatitis and alcoholic fibrosis [134] suggesting that mast cells might have a specific role in the congenital condition. It is tempting to speculate that it might involve the expression of the shorter forms of perlecan previously shown to be produced by mast cells [10, 51] which are produced by the actions of proteases secreted by mast cells including chymase or are a result of mRNA splicing and processing as shown for the mast cell line, HMC-1 [10].

2.3 Skin

The initiation of fibrosis in skin requires the epithelial cells in the epidermis to undergo an epithelial to mesenchymal transition, which converts the cell into an ECM expressing cell [94] (Fig. 2C). Perlecan is expressed throughout the epidermis and dermis of skin with a peri-cellular pattern with little demonstrable in the epithelial basement membrane. Aged keratinocytes have been reported to be perlecan deficient and were unable to form a multilayered epidermis with defects in cell proliferation and differentiation, suggesting that perlecan is a key modulator of cell proliferation [135]. Endorepellin has cell-signaling roles and was implicated as a key fibrogenic mediator [64]. Apoptosis of endothelial cells induces ECM proteolysis, and subsequently acts through phosphoinositide 3-kinase (PI3K) to prevent apoptosis in fibroblasts [65], implicating C-terminal fragments of perlecan in delaying fibroblast apoptosis. However, an increase in collagen secretion was not observed in response to endorepellin alone, indicating that additional apoptotic factors must act in a coordinated fashion to induce all features of myofibroblast differentiation [64]. Interestingly, human fibroblasts derived from sclerodermia skin lesions were more sensitive to the action of endorepellin than fibroblasts derived from healthy control skin [64], indicating a disease specific response to perlecan signaling.

2.4 Kidney

Renal fibrosis occurs in chronic nephropathies, leading to changes to the tubular and glomerular basement membranes and interstitial ECM resulting in decreased kidney function and eventually failure [136] (Fig. 2D). Kidney infections, toxins, mechanical obstruction, immune complexes resulting from autoimmune diseases or chronic infections and genetic disorders lead to cellular injury while type-2 diabetes mellitus and ischemic/hypertensive nephropathy also contribute to kidney fibrogenesis [137]. Fibrotic changes in the kidney can also be caused by genetic mutations such as Denys-Drash syndrome from mutations in the WT1 gene that causes an initial decrease in perlecan expression that is later reversed and increases along with collagen type IV, laminin, fibronectin and tenascin and results in mesangial sclerosis [96]. In the kidney, the major HS proteoglycan in the glomerular basement membrane is agrin [138] while perlecan plays a major role in filtration in the glomeruli [139] and has been correlated with a slower progression of IgA nephropathy [140]. Decreased amounts of perlecan have been shown in animal models of diabetic nephropathy as well as in diabetic patients [141]. Perlecan is also decreased in glomerular nephritis and IgA nephropathy whereas biglycan and decorin are both up regulated with a concomitant upregulation of TGFβ suggesting that a positive autocrine loop plays a major role in the expansion of the mesangial matrix [140]. Further, in an animal model of

diabetic nephropathy streptozotocin elevated levels of TGFβ and was a major factor in the up-regulation of tissue fibrosis [142]. An increase in collagen types I, III and IV and fibronectin accompanied the decreased expression of perlecan in this model [143].

In sclerotic lesions of focal glomerulosclerosis in a rat renal transplant model, an induction of perlecan expression in the glomerular basement membrane was detected compared with its expression in control kidneys [144]. The observed induction of perlecan expression was also observed in the interstitium in a capillary-like pattern. Kidney allografts also presented extensive lymphangiogenesis, associated with induced perlecan expression underneath the lymphatic endothelium. The magnitude of lymphangiogenesis and perlecan expression both correlated with severity of interstitial fibrosis and impaired graft function [144].

2.5 Glial scarring in neural tissues

Glial scarring can arise in neural tissues that have undergone physical injury due to trauma including injury to the spinal cord or an hypoxic insult as a result of ischemia or cerebral stroke. In neural tissues, astrocytes, oligodendrocytes, Schwann cells and microglia, specialized brain macrophages, are the major cell types involved in the production of ECM components and assembly of myelin [145, 146] (Fig. 2E). The microglia and astrocytes have major roles in fibrosis after becoming activated in response to the release of inflammatory cytokines such as IL-1β and TGFβ [145, 146]. These cytokines cause further release of proinflammatory cytokines and an upregulation of ECM molecules, most notably CS lectican proteoglycans including aggrecan, versican, neurocan, brevican [147] and phosphacan [145, 146]. Perlecan has a central role in the developing brain by regulating neurogenesis through the control of neural cell position and phenotype in regions of the developing brain [148, 149].

The CS proteoglycans present in the glial scar surrounding the lesion site prevent neurite outgrowth *in vitro* and nerve regeneration *in vivo* [150]. A recent transcriptomic analysis of astrocytes stimulated with IL-1β showed no evidence of upregulation of any of the CS proteoglycan genes suggesting that they were not the only proteoglycans in the glial scar [151]. Rat cortical astrocytes were shown to produce more HS than CS in culture with a relatively higher content of unsubstituted glucosamine disaccharides. The glycosaminoglycan chains of higher negative charge were more effective at stimulating nerve growth factor (NGF) signaling in PC12 cells but the protein core to which the HS was attached was not investigated [152]. The heparin binding domain of laminin is responsible for neurite outgrowth together with NGF [127] implicating HS proteoglycans in this process. The C-terminal domain V region of perlecan delayed the onset of glial scarring in rat models by decreasing the expression of neurocan and phosphacan and stimulating the activity of NGF [153]. These data suggest that the balance between CS and HS proteoglycans in the ECM and the alteration in this balance changes the nature of the surrounding environment, which can either inhibit or stimulate neurite outgrowth and nerve regeneration [154].

While the laminin-like G domain (LG)3 fragment, as yet, has not been associated with the formation of glial scarring, mice deficient of neural/glial antigen 2 (NG2)/chondroitin sulfate proteoglycan 4 (CSPG4) had a reduced amount of glial scarring and were more permissive to axonal regrowth [155]. Interestingly these animals had a similar phenotype to the progranulin deficient animals [155].

Progranulin is a neuroprotective molecule [156] that binds to the C-terminal region of perlecan and specifically to the first two laminin like repeats LG1 and LG2 [157]. A recent study indicated that the C-terminal region of perlecan binds to CSPG4 [158] and it is tempting to speculate that this binding involves an intermediate interaction with progranulin. Interestingly, this same C-terminal region of perlecan is neuroprotective and pro-angiogenic in a rat ischemic model [159].

2.6 Blood vessels and vascular tissues

Atherosclerosis is characterized by intimal thickening due to an over production of ECM and lipid accumulation in the intima as well as macrophage activation and foam cell formation [160, 161] (Fig. 2F). Monocyte recruitment, retention and differentiation play a central role in the development of atherosclerosis [161] and removal of HS increased the association of monocytes with the subendothelial matrix [35] suggesting that HS may inhibit monocyte interactions with the ECM by inhibiting cell-surface integrin interactions [162, 163]. The initial activation of SMCs after arterial injury is coupled with a decrease in the expression of laminin and basement membrane-like structures, whereas fibronectin accumulates around proliferating cells in the arterial media and intima [164]. Perlecan can act directly on fibrocyte-like cell activation through promotion of cell adhesion or indirectly via binding and potentiating the signaling of growth factors and cytokines involved in cell activation and proliferation [165]. Non-human primates fed a diet to induce hypercholesterolemia demonstrated atherosclerotic lesions with perlecan associated with the media in normal vessels and early stages of lesion formation and was prominent in the SMC matrix near the plaque core of more advanced lesions [166]. These observations indicate that basement membrane components, including perlecan, may take part in the regulation of the differentiated state of ECM producing cells [167].

The progression and organization of the developing plaque in blood vessels, including migration and proliferation of SMCs into the intima, results in the secretion of collagen and the formation of a fibrous cap that surrounds the lipid rich core of the plaque. Perlecan is thought to have pro-atherogenic effects by retaining lipoproteins in the matrix in the early phase of atherosclerosis [46, 168]. Perlecan supports low density lipoprotein (LDL) sequestration via its protein core, notably domain II, while HS modulates this binding [35, 46], a key effect in the progression of atherogenesis.

Vascular SMC proliferation occurs via FGF2 and suggests a role for perlecan in the downregulation of the FGF2 mediated proliferative response as demonstrated by the HS chains that decorate SMC derived perlecan did not potentiate the growth signal of FGFs [13]. Removal of HS, through the action of the HS degrading enzyme heparanase [169], from late vascular lesion explants supported the proliferation of neointimal SMCs and rendered them responsive to PDGF [17, 170]. FGF2 is also induced by PDGF treatment of SMCs, further supporting the role of FGF2 in the proliferation of SMCs [171]. While perlecan is often thought of as a pro-atherogenic molecule [172], vascular SMC activation and proliferation is inhibited by perlecan [170, 173, 174] and it is the lack of perlecan expression in early initiation of atherosclerosis that enables SMC activation, proliferation and ECM synthesis [170]. When SMCs are present in high density, thrombin can promote perlecan synthesis [175, 176] and may act in concert with mechanical strain and angiotensin II [177-179]. This suggests that perlecan expression is tightly linked to limiting SMC activation and proliferation. In the vasculature this suppression of SMC

activation is mediated by thrombin and factor Xa [177]. The mechanisms by which perlecan is anti-proliferative for vascular SMCs is via both HS and protein core, particularly domain III [13, 42], that is mediated by the tumor suppressor phosphatase and tensin homolog (PTEN), which inhibits growth factor receptor signaling [43]. Furthermore, perlecan has been implicated in inhibiting growth of vascular SMCs via Oct-1, a member of a family of transcription factors involved in cell growth processes and suggest that breakdown of the basement membrane and loss of interaction with perlecan activates cells from their quiescent state [173]. Plaque development is also attributed to the HS chains on perlecan as demonstrated by mice lacking HS in the N-terminal domain of perlecan on an ApoE-deficient background exhibit reduced plaque development [172]. Thus the ability of perlecan to suppress SMC proliferation may contribute to plaque instability as it is the SMCs that produce the fibrous cap that stabilizes the lipid plaques.

Cadmium is an environmental risk factor for atherosclerosis and has been found to induce changes in the expression of endothelial perlecan with markedly shorter HS chains that were able to stimulate the proliferation of vascular SMCs in the presence of FGF2 [180]. Heparin has also been reported to upregulate perlecan expression in vascular SMCs [42] and hypothesized to promote FGF2 signaling. Perlecan expression is increased in late stages of atherosclerosis and modulated by apolipoprotein E (apoE) [59, 174] as demonstrated by reduced perlecan expression in apoE deficient mice [160]. A murine model of atherosclerosis exhibited enhanced perlecan expression in apoE deficient mice fed a chow diet and was expressed in both early and advanced plaques [181]. Nitric oxide may be an important intermediate signaling molecule in the induction of perlecan by apoE in SMCs as both are required for the modulation of SMC proliferation [174].

Myeloperoxidase and peroxynitrous acid released from activated leukocytes selectively degrade the core protein of perlecan [182, 183]. The precise degradation site(s) on the core protein await identification however, there is evidence for the presence of peroxynitrite-modified perlecan fragments in human atherosclerotic lesions [184]. Proteolytic processing of perlecan by chymase, MMPs, and cathepsins which can cleave in perlecan domain IV has been reported to generate perlecan fragments encompassing endorepellin [47]. Endothelial cell apoptosis triggers a caspase-dependent release of the LG3 containing fragment of perlecan suggesting that proteases are responsible for the cleavage of perlecan to release this bioactive fragment. This fragment supports the proliferation of vascular SMCs and fibroblasts and inhibits their apoptosis [185] through PI3K [65], whilst increasing the expression of αSMA through OI3K and collagen types I and III expression in these cells [49, 63, 64]. Interestingly, the EGF peptide motif in perlecan domain V together with 4-sulfated CS could replicate this inhibition of apoptosis of fibroblasts and vascular SMC [64]. However, an increase in collagen secretion was not observed in response to endorepellin alone, indicating that additional apoptotic factors act coordinately to induce all features of myofibroblast differentiation [185].

Endorepellin interacts with VEGF receptor 2 providing evidence for its ability to control cell adhesion and VEGF-mediated mitogenic activities [186]. The C-terminal domain V of perlecan has either anti-angiogenic [187, 188] or angiogenic [10, 15] activities in line with the action of the parent molecule. Endorepellin has been shown to be anti-angiogenic when added to cultures of endothelial cells by either interfering with the interactions between VEGF receptor 2 and the LG1/2 regions in domain V [189] or

sterically competing with the α2β1 integrin mechanism of attachment in domain V [39]. Endorepellin binds to the N-terminal fragment of type XVIII collagen, known as endostatin, where it counteracts its anti-angiogenic activities [47].

Interestingly, TGFβ is upregulated during the initiation of early atherosclerosis when perlecan expression is downregulated, potentially by PDGF that decreases perlecan expression in the vasculature [190], suggesting that in the vasculature, perlecan expression is regulated independently of TGFβ expression [191]. Concomitant with this decrease in perlecan expression is an increase in the levels of CS/DS proteoglycans [160]. Oxidized LDL and lysolecithin, a product of LDL oxidation, both decrease endothelial HS proteoglycan expression [35]. While the mechanism is unclear, activation of signal transducer and activator of transcription 1 (STAT1) are key signaling molecules involved in inhibition of perlecan transcription by oxidants and inflammatory cytokines [35].

Restenosis is a scarring that occurs in the vasculature following a vascular intervention and involves a change in phenotype of the quiescent contractile SMCs to proliferate and produce ECM [192]. This is accompanied by a downregulation of their contractile proteins, which is in contrast to fibroblasts in fibrosis which upregulate αSMA to become myofibroblasts [193]. Perlecan expression has been reported to be low in uninjured blood vessels and immediately following injury which corresponded to maximal SMC proliferation, while perlecan has been reported to increase later in advanced lesions that coincided with an arrest of SMC proliferation [170]. Interestingly, heparinase treatment promoted SMC growth [170] suggesting that the temporal expression of perlecan is important for fibrogenesis.

The length and sulfation pattern of perlecan HS chains are cell type specific [194], and it is therefore likely that these features play a role in the tissue-specific functions of perlecan. Deletion of the glycosaminoglycan attachment sites in domain I of mouse perlecan supported the proliferation of SMCs following flow cessation of the carotid artery [195] further supporting the role of both the HS and perlecan protein core in the modulation of SMC activation and proliferation. In a separate study, HS proteoglycans were more effective in inhibiting neointima formation in a rabbit model of restenosis than heparin [196] supporting the synergistic role of HS together with the perlecan protein core. These studies indicate that during tissue remodeling perlecan promotes desirable cell proliferation whilst simultaneously inhibiting undesirable proliferation activities of SMCs.

3. Therapies for fibrosis that alter perlecan expression

While therapies exist which delay fibrogenesis, the contribution of these treatments to the structure or abundance of perlecan in fibrosis is less clear. Of the fibrosis treatments explored, sulodexide, a mixture of non-anticoagulant low molecular weight heparin/HS and DS, has been shown to increase perlecan expression in a rat model of diabetic nephropathy, whilst the expression of collagens I and IV decreased suggesting that TGFβ may be controlled by interacting with the glycosaminoglycans [143]. Additionally, i*n vitro* culture of tubular cells treated with sulodexide reduced expression of αSMA, heparanase-1 and MMP9 suggesting that this treatment inhibited the epithelial-mesenchymal transition characteristic of fibrosis [197]. Additionally, perlecan expression is upregulated via treatment of murine atherosclerosis with telmisartan, an angiotensin II receptor antagonist [198].

With the concept of the multifaceted roles of perlecan in fibrosis, the effect of the following fibrosis treatments warrant further investigation for their effect on perlecan expression. Nintedanib, a tyrosine kinase inhibitor, is approved for the treatment of IPF [199]. It inhibits a variety of receptors including the PDGF receptor, VEGF receptors 1 and 2, and FGF receptors 1–3 [200] and affects the binding partners for perlecan that are involved in cell proliferation in fibrosis, although analysis of the effect on perlecan expression has not been examined. Nintedanib inhibits the progression of processes in fibrosis including the migration of fibroblasts, their transformation into myofibroblasts and the deposition of extracellular matrix [201, 202] thus it is possible to speculate that this treatment affects perlecan expression. Through a similar approach, sorafenib targets the PDGF receptor and is effective in reducing fibrosis in animal models of liver fibrosis [203] and many also affect perlecan expression.

Adenoviral vectors expressing an antisense mRNA of the PDGF β-chain delivered to an animal model of induced liver fibrogenesis down-regulated endogenous PDGF β -chain expression [204, 205], which is likely to lead to increased perlecan expression [190] with a positive outcome for modulating the progression of fibrosis. Pirfenidone inhibits TGFβ1 stimulated collagen expression [206] and *in vitro* experiments have shown that it can inhibit the proliferation of HSCs and lung fibroblasts [207-209] and is thus worthy of further exploration for its effect on perlecan expression.

Statins are effective in lowering LDL cholesterol [210] and also reduced plaque volume when used at high dose [210]. ApoE deficient mice treated with statins also display reduced plaque volume with increased decorin and biglycan expression and a more condensed collagen rich ECM, however perlecan expression was not altered in these mice [211] even though it has a major role in LDL retention and has been shown to modulate HS proteoglycan expression [35]. The renin-angiotensin system (RAS) is a central regulatory system for cardiovascular function and critically connected to atherosclerosis and mediated by angiotensin II. RAS blockade has been shown to attenuate the progression of atherosclerotic lesions in animals and is thus a promising therapy for human cardiovascular diseases [191]. A novel perlecan-inducing compound, RUS310, is a potent inhibitor of SMC activity and has been trialed for the treatment of in-stent restenosis [212]. So far this compound has not been trialed for the treatment of fibrosis, but may be a rewarding approach to modulate perlecan expression.

4.   Final Considerations

The literature provides ample evidence for the roles of perlecan in controlling / modulating fibrogenesis (Fig. 1). Perlecan has key roles in promoting tissue repair whilst limiting damaging fibrosis even though many of the tissue repair activities can also contribute to fibrogenesis if not resolved in a timely fashion (Fig. 3). The anti-fibrotic activities of perlecan are mediated through modulating cell adhesion, proliferation and migration of key inflammatory and ECM producing cells. In contrast, perlecan contributes to fibrogenesis through growth factor binding and signaling, LDL binding, ECM interactions and activities of the C-terminal region of perlecan that inhibit the apoptosis of fibroblasts. These multifaceted roles of perlecan suggest that it plays a role in each phase of fibrogenesis, with particularly important roles in modulating the activity of growth factors, chemokines and cytokines for

cell activation, infiltration and proliferation. These activities can be attributed to either the glycosaminoglycan or protein core components of perlecan. The glycosaminoglycan chains change dynamically as a result of changes in the microenvironment enabling temporal and spatial control over growth factor, chemokine and cytokine activity. While the precise mechanisms involved in the roles of perlecan involved in fibrogenesis remain unclear, the current evidence suggests that it is an important candidate worthy of further development of more effective treatments for fibrosis.
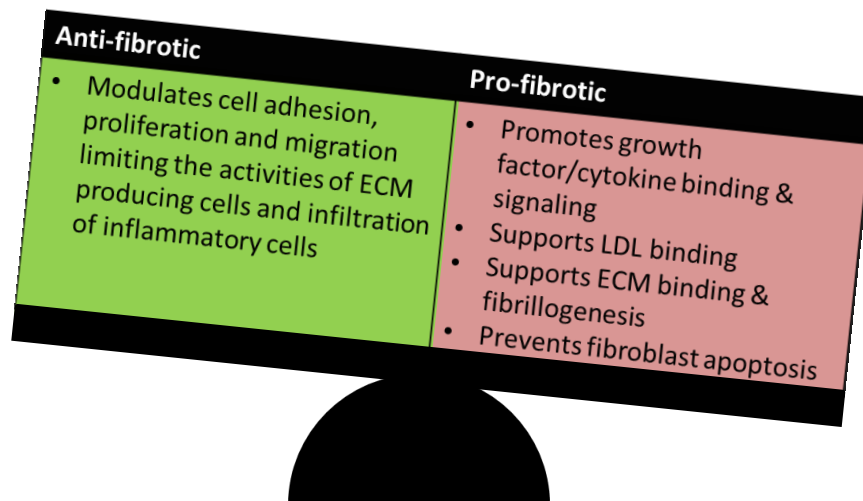
Fig. 3. The anti- and pro-fibrotic activities of perlecan that are open to therapeutic manipulation.

Acknowledgements

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