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Signaling networks in focus

Perlecan signaling: Helping hedgehog stimulate prostate cancer growth

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Abstract

Perlecan, an extracellular matrix proteoglycan, regulates signaling by a variety of growth factors through protein–protein and protein–carbohydrate interactions. Recent evidence demonstrates that Perlecan modulates sonic hedgehog signaling during both development and neoplasia, in particular in prostate cancer. Perlecan directly binds to sonic hedgehog and is required for its signaling. Increased sonic hedgehog signaling due to Perlecan in aggressive and metastatic prostate cancer cells can be attributed to increased perlecan expression or changes in Perlecan glycan structure. Additional co-localization studies suggest that other tumor types may also have a Perlecan-modulated hedgehog signaling pathway. Inhibitors of Perlecan function at either the protein or glycan level would be ideal drug candidates for anti-cancer therapies.

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1. Introduction

Perlecan, a large (>400 kDa) protein, is part of the extracellular matrix in organisms from worms to humans (Lozzo, 2005). Perlecan's size is further increased by the

presence of heparan sulfate sugar chains at its amino terminal end. Together, the protein core and carbohydrate domains create a proteoglycan of 700–800 kDa. Besides being a structural component of the extracellular matrix, Perlecan also plays a role in modulating the signaling of various peptide growth factors through interactions with either its protein core or its sugar chains. Knock-down of Perlecan in multiple tissue culture studies results in decreased binding of growth factors and decreased signaling activity. Complementary genetic studies in mice and flies where Perlecan is lost reveal mutant phenotypes consistent with loss of signaling by multiple growth factors. While initial studies focused on Perlecan's role in development, these studies have been translated into cancer growth and metastasis. For example, in development

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Signaling network facts

- Perlecan modulates several growth factor pathways via its glycan and protein domains.
- Heparan sulfate chains are required for hedgehog signaling.
- Hedgehog signaling is critical for growth and metastasis of advanced prostate cancer.
- Perlecan regulates the level of hedgehog signaling activity.
- Perlecan modulated hedgehog signaling may be found in other tumor types.
- Further insight into Perlecan activity can be found at: <http://www.uku.fi/laitokset/anat/PG/perlecan.htm>.

Perlecan has been implicated in hedgehog signaling and neural stem cell division in the embryonic brain (Park et al., 2003). We noted an association between Perlecan and genetic mapping studies for families with high rates of brain and prostate cancers (Datta et al., 2006). Our subsequent studies demonstrated Perlecan's role in the growth and metastasis of human prostate cancer cells. These studies linked Perlecan's roles in development to subsequent functions in neoplasia. Similar links are being encountered with Perlecan, other growth factors, and different cancers.

2. Functions

Perlecan has a long history of affecting signaling by molecules ranging from integrins to the fibroblast growth factors (FGFs). Some molecules, such as FGF7, bind to the protein core of Perlecan (Mongiat et al., 2000), while others such as FGF2 bind to the heparin sulfate chains (Aviezer et al., 1994) (Fig. 1). The FGF2–Perlecan complex binds with high affinity to the FGF receptor, resulting in more sensitive growth factor

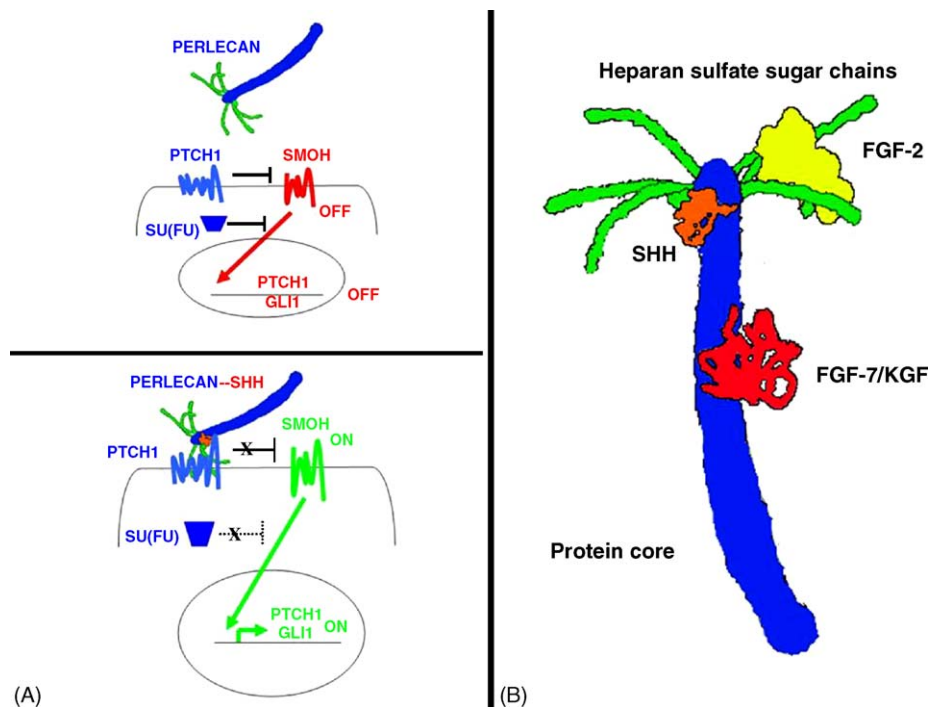


Fig. 1. Hedgehog signaling, Perlecan structure and growth factor binding. (A) A simplified representation of the hedgehog pathway indicating a proposed role for Perlecan as an extracellular co-receptor for hedgehog. PTCH1 = Patched1, the sonic hedgehog receptor. SMOH1 = Smoothened1, a cell surface protein. SU(FU) = Suppressor of fused, an intracellular inhibitor of sonic hedgehog signaling. PTCH1 and GLI1 are sonic hedgehog response genes, their transcription is activated by hedgehog signaling. (B) Perlecan is composed of a protein core and heparan sulfate sugar structures attached at the amino terminus. Growth factors bind to Perlecan through its protein core (fibroblast growth factor-7/keratinocyte growth factor, FGF-7/KGF), heparan sulfate sugars (basic fibroblast growth factor, FGF-2), or both (sonic hedgehog, SHH).

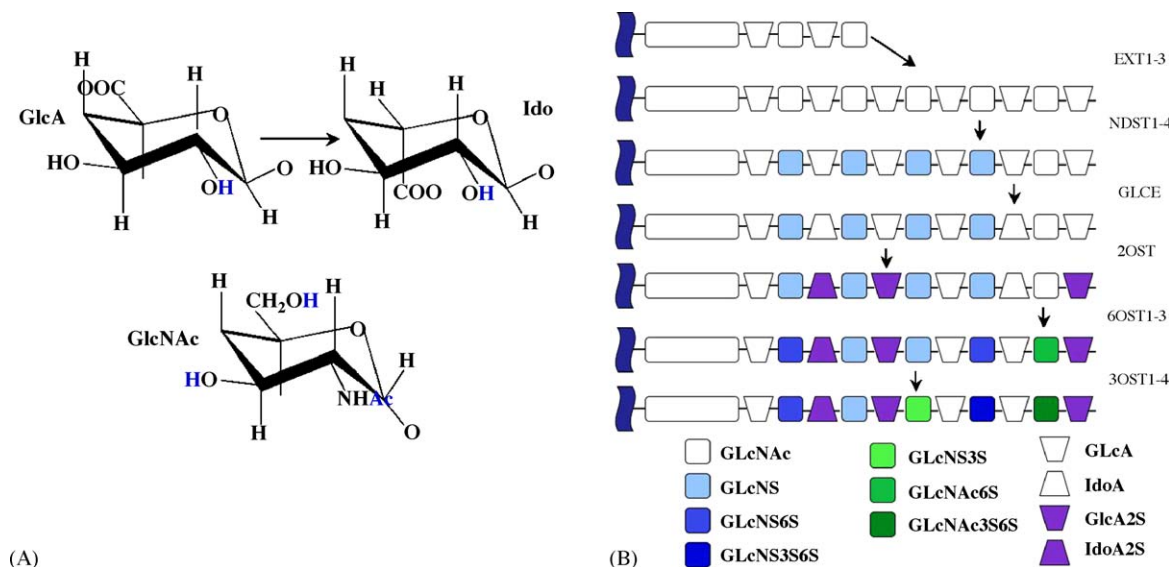


Fig. 2. Heparan sulfate structure and modification. Synthesis and modification of heparan sulfate chains are diagrammed. (A) Structure of the different saccharides that make up heparan sulfate. Note that glucuronic acid (GlcA) can be converted to iduronic acid (Ido) by an epimerase during heparan sulfate synthesis. GlcNAc = *N*-acetyl glucosamine. In blue are marked the positions that may be modified by sulfation. (B) General synthesis and modification pathway of mammalian heparan sulfate. The enzymes that catalyze specific steps are indicated as well as the number of isozyms known.

signaling. The heparan sulfate chains are key to this complex formation, determining both the affinity of binding to the FGF-receptor and also the specificity for FGF receptor subtypes (Knox, Merry, Stringer, Melrose, & Whitelock, 2002).

Heparan sulfate chains (Fig. 2) are unbranched polymers of alternating glucuronic acid and *N*-acetylglucosamine that undergo enzymatic modification by removal of the *N*-acetyl group and addition of sulfate groups at the 2-, 3- and 6-O positions (Fig. 2A). The glucuronic acid residue can also undergo epimerization to form iduronic acid. This results in a polymer with the potential for four different monosaccharides in the glucuronic/iduronic acid position and six monosaccharides in the *N*-acetyl glucosamine position. Thus Perlecan's heparan sulfate chains are subject to modification by acetylation, sulfation and epimerization by multiple enzymes (Fig. 2B) (Lin, 2004), each with different tissue distributions. Therefore it is not surprising that Perlecan isolated from different tissues or even from the same cell types grown under different conditions has different glycan structures, and thus different growth factor binding characteristics and functions (Knox et al., 2002). Since there are multiple proteoglycans that express heparan sulfate chains, sorting out which proteoglycan regulates a specific growth factor-receptor interaction in a given tissue must be undertaken on a case-by-case basis. Developmental studies on stem

cell proliferation in the *Drosophila* brain demonstrated that Perlecan modulates hedgehog signaling (Park et al., 2003). However, investigation of other fly systems implicated a different proteoglycan—the glypican Dally-like (Desbordes & Sanson, 2003). In rodent studies chondrogenesis is regulated by Syndecan-3, which modulates the growth factor Indian hedgehog (Shimo et al., 2004). Finally, we have recently shown that in human prostate cancer Perlecan modulates tumor cell response to sonic hedgehog (Datta et al., 2006). We propose that the identity of the proteoglycans, their differing sugar structures, and their tissue or cell-type localization will produce layers of nuanced regulation that affect growth factor signaling and their disparate effects on tumor progression.

3. Cascades

The structure of Perlecan with its multiple protein and carbohydrate domains, lends itself to a variety of functional mechanisms (Iozzo, 2005) (Fig. 3). The type of mechanism used determines whether a cellular process is stimulated or inhibited. One example involves the cleavage of Perlecan Domain V into an 85 kDa fragment termed Endorepellin. Endorepellin binding to $\alpha 2\beta 1$ integrin results in activation of protein kinase A, eventually inhibiting angiogenesis. In contrast, the heparan sulfate chains on full length Perlecan bind and

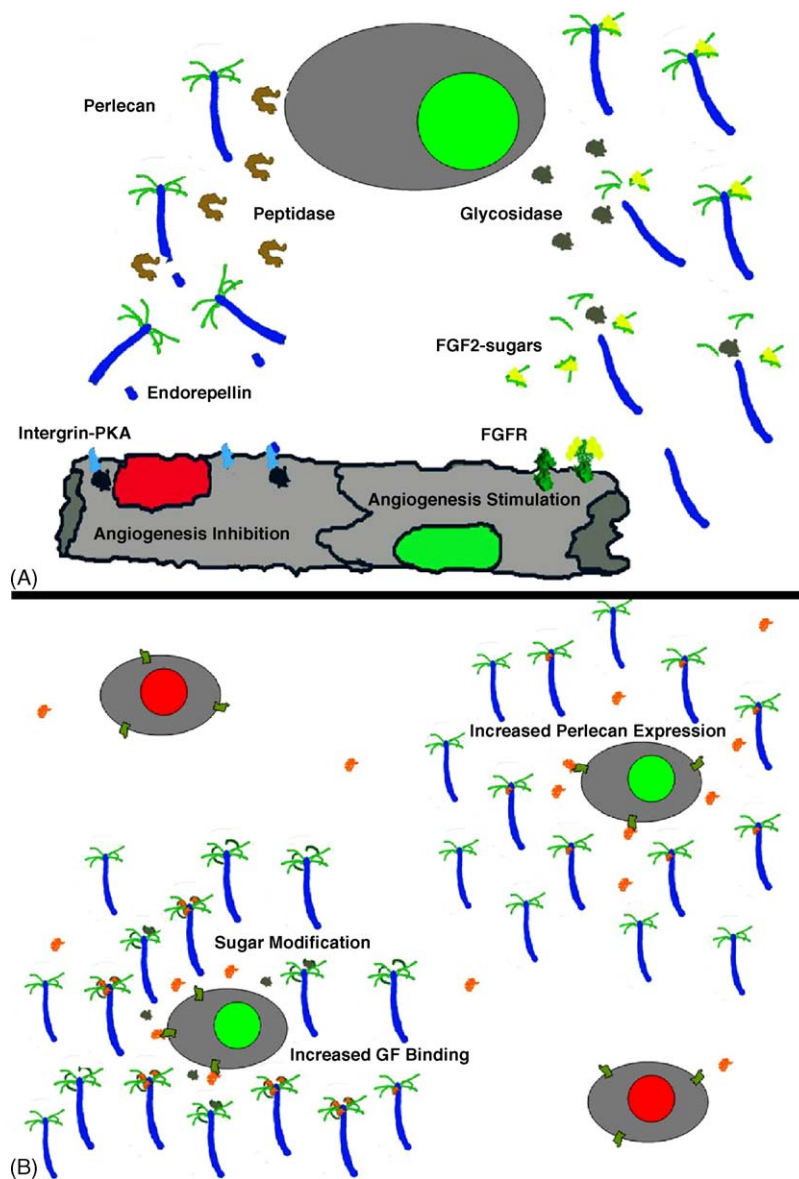


Fig. 3. Mechanisms of Perlecan based growth factor regulation. (A) Perlecan can undergo proteolysis to release peptide factors such as Endorepellin, which inhibit angiogenesis through integrin/protein kinase A (PKA) activation. The heparan sulfate sugar chains on Perlecan can also be cleaved by glycosidases that result in the release of basic fibroblast growth factor (FGF-2), resulting in angiogenesis. The balance of each enzymatic process achieves the overall control of tumor angiogenesis. (B) Localized expression (or increased expression) of *Perlecan* can result in increased local growth factor binding. Likewise, the secretion of sugar modification enzymes can alter Perlecan's heparan sulfate sugar chains, resulting in increased growth factor binding. Both mechanisms result in high microconcentrations of growth factors, supplying a selective growth advantage.

stimulate FGF2 signaling, thus promoting angiogenesis. A second means for altering Perlecan function involves changes in Perlecan's heparan sulfate chains. Alterations in sulfation and epimerization of sugars in the heparan sulfate chains clearly affect growth factor binding affinities (Lin, 2004). Releasing heparan sulfate chains and their associated growth factors through heparanase or sulfatase action would alter local growth factor concen-

trations near the cell surface. Finally, alterations in the level of Perlecan expression itself may have profound effects on signaling molecule activity. Our studies show that in human prostate tumors both increased expression of *Perlecan* and alteration of Perlecan functionality are used to regulate sonic hedgehog (SHH) signaling (Datta et al., 2006). It has already been shown that SHH activity drives the proliferation and metastasis of

advanced prostate cancer cells (Datta & Datta, 2006). Perlecan protein and SHH expression significantly colocalize in aggressive, rapidly growing tumors (Datta et al., 2006). Knock-down of Perlecan expression in metastatic prostate cancer cell lines results in decreased proliferation and decreased SHH signaling. Prostate cancer cell lines that are more metastatic produce a form of Perlecan that appears to have a higher binding affinity for SHH. This greater complex forming ability by Perlecan from metastatic cells correlates with increased SHH signaling in these cells, thereby stimulating proliferation.

Key molecules

The array of Perlecan-based mechanisms that regulate cell–cell signaling use a variety of key molecules to implement critical steps (Iozzo, 2005). The BMP–Toll-like family of proteases cleave Perlecan into functional protein subdomains such as Endorepellin. Heparan sulfate synthase enzymes of the exostose/tout-velu family and sugar modification enzymes including deacetylases, sulfotransferases and epimerases alter the sugars on Perlecan's heparan sulfate chains (Lin, 2004). The activity of heparanases release heparan sulfate chains from the Perlecan core. Varying the pattern of enzyme expression or activity within a tissue or over the time course of a disease would result in different patterns of heparan sulfate structures spatially across tissues or temporally during development or disease progression. Up-regulation of glycosyltransferases (enzymes that synthesize glycans) during oncogenesis and cancer progression with concomitant changes in glycan structures at the cell surface has been demonstrated in several types of cancers, notably, transcriptional up-regulation of *N*-acetylglucosaminyltransferase V in breast carcinomas (Guo, Lee, Kamar, Akiyama, & Pierce, 2002). Increases in the cell surface levels of the glycans synthesized by this enzyme result in decreased cell–cell and cell–matrix adhesion, increased invasiveness and migration, as well as altered levels of intracellular signaling activity involving both cadherin–catenin and integrin–ERK (Handerson, Camp, Harigopal, Rimm, & Pawelek, 2005). Similarly, changes in the patterns of glycan modifications on Perlecan during oncogenesis and tumor progression could regulate the interactions between Perlecan and a variety of growth factors, including SHH. This hypothesis is consistent with our observation that highly metastatic prostate cancer cell lines produce a form of Perlecan that more efficiently complexes with SHH, and demonstrates higher levels of SHH signaling (Datta et al., 2006).

5. Associated pathologies

Perlecan knock-out mice are embryonic lethal with severe abnormalities in cartilage and the long bones of the limbs as well as a lack of the neurotransmitter acetylcholinesterase at the neuromuscular junction (degrading enzyme Iozzo, 2005). The first observation led to the linkage of mutations in human *Perlecan* with Silverman-Handmaker type dyssegmental dysplasia, a lethal disease characterized by short limbs and aberrant vertebral structure. The second observation may explain the association of human *Perlecan* mutations with Schwartz-Jampel Syndrome, a myotonia characterized by lack of muscle tone. Analysis of Perlecan knock-out mice have led to insights on cardiac and cartilage development and the rate of tumor growth. Tissue culture studies have demonstrated that Perlecan is essential for the growth and/or viability of human colon cancer and metastatic melanoma cell lines (Cohen et al., 1994; Sharma et al., 1998). Perlecan's role in these cancer cells is due at least in part to its ability to modulate the signaling by growth factors such as FGF7 and FGF2. Further analysis of the role of Perlecan in cancer progression led to the observation that the human *Perlecan* gene maps to 1p36, the same region implicated in familial risk of brain and prostate cancers (Datta et al., 2006; Gibbs et al., 1999). Other genes encoding components of the SHH pathway also mapped to areas associated with increased prostate cancer risk (Datta & Datta, 2006; Sanchez et al., 2004). SHH signaling is necessary and sufficient for the increased growth, survival and spread of advanced prostate cancer cells (Karhadkar et al., 2004; Sanchez et al., 2004; Sheng et al., 2004). Our analysis of human prostate cancer showed that Perlecan levels correlate with increased aggressiveness of prostate cancer based on the tumor Gleason score (Datta et al., 2006). We demonstrated that Perlecan increases the activity of the SHH pathway in advanced prostate cancer cells, even when SHH levels are very low. This suggests that Perlecan gives metastatic cancer cells a survival advantage under growth factor limiting conditions such as might be found within a growing tumor (Fig. 4A). These data are also consistent with a model where up-regulation of Perlecan expression or function is a key step in cancer progression from a localized tumor to metastatic spread (Fig. 4B). In this model, increased Perlecan activity results in high SHH signaling which in turn causes the increased proliferation and invasiveness of advanced prostate cancer. Yet activation of the SHH pathway combined with Perlecan knock-down only partially restored cellular proliferation in the metastatic LNCaP subline, in contrast to the complete rescue observed in the more

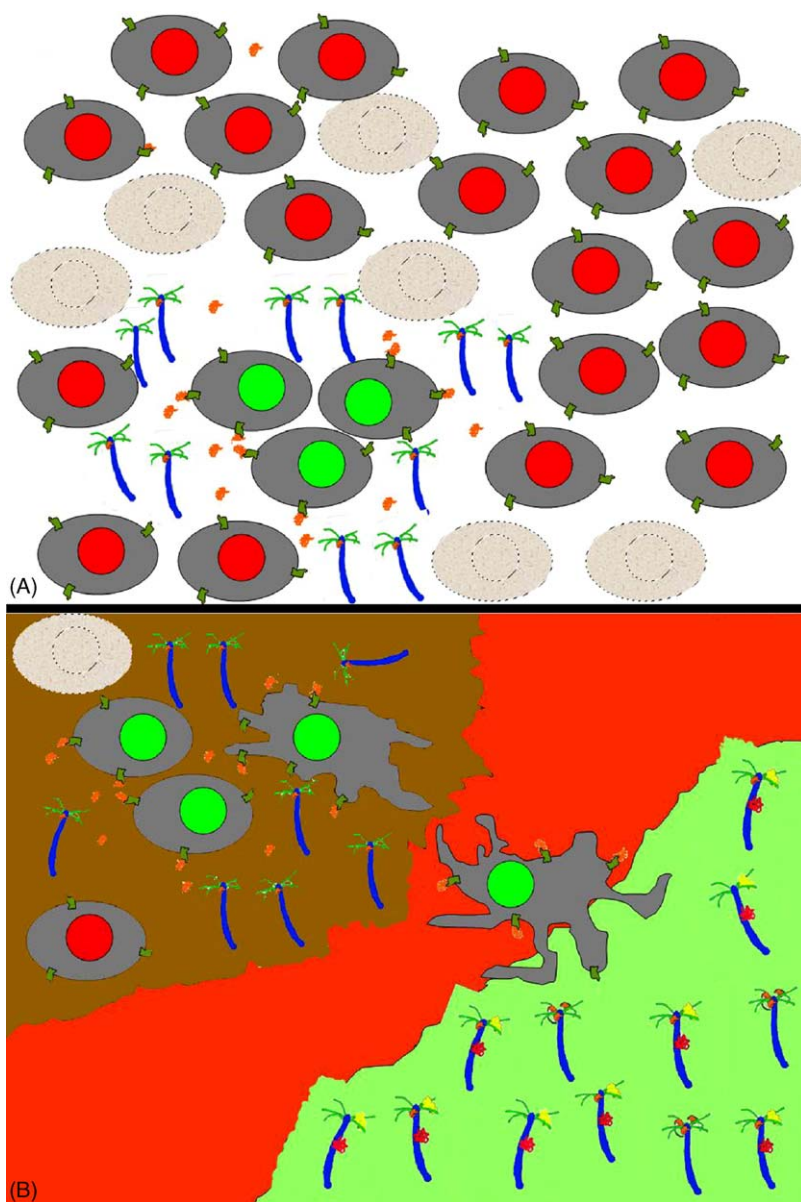


Fig. 4. Perlecan's effect on tumorigenesis. (A) Localized *Perlecan* expression confers a selective growth advantage to tumor cells in environments where growth factor concentrations are limiting, such as in a rapidly growing primary tumor. (B) The expression of *Perlecan* is also associated with increased metastatic potential in prostate cancer cells. Prostate cancer cells that utilize *Perlecan* to optimize growth under limiting growth factor conditions (such as a primary tumor site, or in the bloodstream), will thrive when they find an environment rich in *Perlecan* and growth factors such as the bone marrow stroma.

benign parental LNCaP cell line ((Datta et al., 2006), Datta, Schlicht, & Datta, unpublished). Interestingly, *Perlecan* knock-down in the highly metastatic LNCaP prostate cancer subline greatly diminished the ability of the cells to respond to the heparin-binding growth factors FGF2 and VEGF (Savore et al., 2005). These results indicate that as cancer cells become more metastatic, *Perlecan*'s role expands from regulation of SHH activity

alone to the control of multiple growth factor signaling pathways.

The results from several studies suggest that *Perlecan*, a secreted glycoprotein, has potential as a biomarker for the detection of advanced prostate cancer, and may also serve as a therapeutic target. In fact, given the number of cancers where SHH has been implicated, it is possible that *Perlecan* could provide a marker and drug

target for cancers ranging from medulloblastoma, glioma and melanoma to pancreatic, stomach and esophageal cancers (Datta & Datta, 2006). While Perlecan levels can be monitored in the blood stream, its expression in other tissues such as the kidney has limited its appeal as a biomarker. Perlecan's different glycoforms combined with the additional functions of its protein core may explain Perlecan-specific growth factor control. Thus assays will require the design of Perlecan-specific targeting strategies that do not cross-react with other proteoglycans. Similarly, an understanding of which modification enzymes catalyze the production of key sugar sequences responsible for up-regulating SHH signaling could also provide therapeutic insights. By using cancer targeted nanoparticles that contain inhibitors of key enzymes or blockers of Perlecan–SHH binding, therapies could be delivered to diseased cells with fewer side effects. Perlecan-based therapies might be most effective when combined with other treatments. Clinical studies repeatedly demonstrate the necessity for combination chemotherapy as drug monotherapy selects for treatment resistant tumor cells. Our addition of SHH to the signaling pathways modulated by Perlecan places Perlecan in a central position for the control of cell–cell communication in development and neoplasia. The ability of Perlecan to modulate numerous growth factor pathways, including SHH, demonstrates the significance of Perlecan inhibition, either as a target for multi-pathway blockage, or by selective inhibition in combination with other pathway specific inhibitors. Selective inhibition of specific signaling pathways could be accomplished by using individual domains of Perlecan to engineer molecules or glycopeptides as dominant-negative inhibitors for the targeting of specific pathways. The ability to control Perlecan function for our purposes could lead to therapies that inhibit tumor growth and prevent metastasis.

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