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Signalling pathways regulating galactosaminoglycan synthesis and structure in vascular smooth muscle: implications for lipoprotein binding and atherosclerosis

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Short title: Cell signalling and proteoglycan synthesis

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Abstract

Atherosclerosis commences with the trapping of low density lipoproteins (LDLs) in blood vessels by modified proteoglycans (PGs) with hyperelongated glycosaminoglycan (GAG) chains. GAG chain synthesis and growth factor mediated hyperelongation regulates the composition and size of PGs in a manner that would cause low density lipoprotein (LDLs) retention in vessel wall. Galactosaminoglycans are a class of GAGs, commonly observed on PGs. Multiple enzymes are involved in galactosaminoglycan biosynthesis. Galactosaminoglycan synthesis is regulated by various signalling pathways which are amenable to pharmacological manipulation to treat atherosclerosis. Receptor mediated signalling pathways including protein tyrosine kinase receptors (PTKRs), serine/threonine kinase receptors (S/TKRs) and G-protein coupled receptors (GPCRs) pathways regulate galactosaminoglycan synthesizing enzyme expression. Increased expression of these enzymes modify galactosaminoglycan chain structure by making them hyperelongated. This review focuses on the signalling pathways regulating the expression of genes involved in galactosaminoglycan synthesis and modification. Furthemore, there are multiple other processes for inhibiting the interactions between LDL and galactosaminoglycans such as peptide mimetics of ApoB100 and anti-galactosaminoglycan antibodies and the therapeutic potential of these strategies is also addressed.

Keywords

Atherosclerosis, proteoglycans, galactosaminoglycans, signalling pathways

Abbreviations

Ang-II, Angiotensin-II

CDKs, Cyclin-dependent kinases

ChGn, Chondroitin N-acetylgalactosaminyltransferase

ChPF, Chondroitin polymerizing factor

ChSy, Chondroitin synthase

CS, Chondroitin sulfate

CVDs, Cardiovascular diseases

C4S, Chondroitin-4-sulfate

C6S, Chondroitin-6-sulfate

C4ST, Chondroitin 4-O-sulfotranferase

C6ST, Chondroitin 6-O-sulfotranferase

DS, Dermatan sulfate

DSE, Dermatan sulfate epimerase

D4ST, Dermatan 4-O-sulfotranferase

EGF, Epidermal growth factor

EGFR, Epidermal growth factor receptor

Erk1/2, Extracellular signal-regulated kinase 1/2

ET-1, Endothelin-1

GAGs, Glycosaminoglycans

Gal, Galactose

GalT, β-1,3-galactosyl transferase

GalNAc, N-acetyl-D-galactosamine

GalNAcT, N-acetylgalactosyl transferase

GalNAc4S-6ST, N-Acetylgalactosamine 4-sulfate 6-O-sulfotransferase

GlcA, Glucuronic acid

GlcNAc, N-acetyl-D-glucosamine

GlcAT, β-1,3-glucuronosyl transferase

GPCR, G-protein coupled receptors

HMGCoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A

IdoA, Iduronic acid

IP_3, Inositol 1,4,5 triphosphate

LDLs**,** Low density lipoproteins

mAb, Monoclonal antibody

MMPs, Matrix metalloproteinases

PCSK9, Proprotein convertase subtilisin/kexin type 9

PDGF, Platelet derived growth factor

PDGFR, Platelet derived growth factor receptor

PGs, Proteoglycans

PI3K, Phosphoinositide 3-kinase

PKC, Protein kinase C

PLC, Phospholipase C

PTKRs, Protein tyrosine kinase receptors

Ser, Serine

Sos, Son of sevenless

S/TKRs, Serine/threonine kinase receptors

TGF-β, Transforming growth factor-β

TGFBR, Transforming growth factor-β receptor

Thr, Threonine

Tyr, Tyrosine

UST, Uronosyl 2-O-sulfotranferase

VSMCs, Vascular smooth muscle cells

Xyl, Xylose

XT, Xylosyltransferase

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1. Introduction

Cardiovascular diseases (CVDs) are a heterogeneous group of conditions afflicting heart and blood vessels which share common underlying pathological mechanisms (Aghamohammadzadeh, Ormandy, & Heagerty, 2015). CVDs are the largest single cause of death globally and more people die annually from CVDs than from any other cause (Mendis, Puska, & Norrving, 2011; WHO, 2017). Atherosclerosis is a progressive disease characterized by lipid accumulation in the large arteries and it constitutes the single most important contributor to this enormous burden of disease and it is accelerated in the presence of diabetes (Libby, 2002; Nigro, Osman, Dart, & Little, 2006; Yang, et al., 2010). Atherosclerosis commences with the pre-inflammatory stage occurring as accumulation of lipids in the blood vessel wall followed by an inflammatory stage which is manifest ultimately as the formation of atherosclerotic plaques (Libby, 2002; Ross, 1999; Virmani, et al., 2005). Plaques may be stable or unstable with plaque rupture, causing the acute clinical syndrome of a life threatening myocardial infarction or stroke (Davies, 1996; Falk, 1989). The major pathological mechanisms currently under investigation as causes of atherosclerosis are the retention of low density lipoproteins (LDLs) (Skalen, et al., 2002), endothelial dysfunction (Davignon & Ganz, 2004), oxidative stress (Witztum, 1994), inflammation (Libby, 2002; Ross, 1999) and immune system defects (Fernández-Ruiz, 2016; Little, Chait, & Bobik, 2011). In human coronary arteries, atherosclerosis commences with the retention of LDLs by galactosaminoglycans on proteoglycans (PGs), specifically biglycan and this is followed by the immune response and the inflammatory cascade (Nakashima, Fujii, Sumiyoshi, Wight, & Sueishi, 2007). Utilization of genetically modified apolipoprotein knockout mice demonstrates the primary role of LDLs and PGs in atherogenesis (Skalen, et al., 2002). Boren *et al.* (Kijani, Vázquez, Levin, Borén, & Fogelstrand, 2017) confirmed that, vascular LDL retention is almost exclusively dependent on electrostatic interactions between

galactosaminoglycans and LDLs. In a murine model of atherosclerosis, the increased expression of galactosaminoglycan synthesizing genes is associated with lipid accumulation in the vessel wall (Anggraeni, et al., 2011).

In early atherogenesis, negatively charged sulfate and carboxyl groups of galactosaminoglycan chains on PGs in the extracellular matrix of the arterial intima bind and trap LDLs via electrostatic interactions with positively charged aminoacyl residues in the apoB moiety of an LDL particle (Borén & Williams, 2016; Ohira & Iso, 2013). LDL particles normally flux into and out of the arterial wall, most LDL particles that enter the arterial wall leave without contributing to the development of the atherosclerotic lesion (Borén & Williams, 2016; Schwenke & Carew, 1989; Tran-Lundmark, et al., 2008). LDL particles become trapped in the vessel wall because of the changes in the properties of both the LDLs and the extracellular matrix, particularly galactosaminoglycan chains on PGs (Haberland, Olch, & Folgelman, 1984; Little, Osman, & O'Brien, 2008; Nakashima, et al., 2007; Tabas, 1999).

Multiple signalling pathways are involved in regulating galactosaminoglycan synthesizing enzyme gene expression, which direct structural changes in galactosaminoglycan chains on PGs and increase their binding affinity to LDLs in the vessel wall. These signalling pathways represent potential targets for therapeutic intervention of atherosclerosis and thus are the subject of the current review.

2. Galactosaminoglycan structure contributes to LDL binding

2.1 Strucutural features of galactosaminoglycans

The major glycosaminoglycans (GAGs) in vertebrates include glucosaminoglycans (heparan sulfate (HS), keratan sulfate (KS), heparin (Hep)) and galactosaminoglycans (chondroitin sulfate (CS) and dermatan sulfate (DS)) (Kjellén & Lindahl, 1991). The GAG

chains are composed of alternating units of an amino sugar, either N-acetyl-D-glucosamine (GlcNAc) or N-acetyl-D-galactosamine (GalNAc) and a hexauronic acid either glucuronic acid (GlcA) or iduronic acid (IdoA). The presence of either type of amino sugar differentiates between glucosaminoglycans and galactosaminoglycans. GlcNAc or GalNAc is found to be present in disaccharide units of glucosaminoglycans and galactosaminoglycans, respectively. Although the polysaccharide backbones are simple, linear chains of repetiting disaccharide units, Hep/HS and CS/DS acquire a considerable degree of structural variability by extensive modifications involving sulfation and uronic acid epimerization (Kjellén & Lindahl, 1991; Yamada, Sugahara, & Özbek, 2011).

Galactosaminoglycan chains on PGs are complex, long, unbranched polysaccharides with important roles in cell growth, differentiation, morphogenesis, cell migration and microbial infection (Yamada, et al., 2011). Galactosaminoglycan chains are covalently attached to the core protein to form PGs, which are ubiquitously distributed in the extracellular matrix (Kjellén & Lindahl, 1991). Particularly, galactosaminoglycan chains are attached to a serine (Ser) residue on the core protein through the common linkage region tetrasaccharide, which is made up of xylose-galactose-galactose-glucuronic acid (Xyl-Gal-Gal-GlcA) (Fig 1). The galactosaminoglycan chains are generated principally by similar modifications to glucosaminoglycans with an initial polymerization product of the disaccharide unit GlcA/IdoA-GalNAc. The structural diversity of galactosaminoglycans is less pronounced than heparin related species as the GalNAc residues remain acetylated (Kjellén & Lindahl, 1991). The range of normal galactosaminoglycan chain lengths vary widely and extends from 20 kDa to approximately 60 kDa corresponding to approximately 40 to 120 disaccharides (Little, Ballinger, Burch, & Osman, 2008). Epimerization at the carbon-5 (C-5) position of GlcA in precursor chondroitin backbone results in the formation of repeating disaccharide unit GalNC-IdoA or dermatan backbone (A Malmström & Fransson,

1975). Both CS and DS galactosaminoglycans are fully developed through sulfation by the action of sulfotransferases. Galactosaminoglycans are mainly sulfated at C-4 and C-6 positions of their GalNAc moieties. C-2 and C-3 positions of GlcA and IdoA are other positions available for sulfation of galactosaminoglycan chains and therefore various types of di and trisulfated disachharide units may form (Karamanos, Syrokou, Vanky, Nurminen, & Hjerpe, 1994).

2.2 Galactosaminoglycan structure and LDL binding

There are three major structural properties of galactosaminoglycans that are regulated by signalling pathways and can influence their binding to apolipoproteins on LDLs. These include modification in the length of the chains (number of disaccharide units), the extent and pattern of sulfation and the isoform of the uronic acid moiety on the galactosaminoglycan chain (Ballinger, Nigro, Frontanilla, Dart, & Little, 2004). Treatment of VSMCs with multiple vascular agonists stimulates cells to produce elongated galactosaminoglycan chains which is associated with an increased binding to LDL (Figueroa & Vijayagopal, 2002; Getachew, Ballinger, Burch, Reid, et al., 2010). In aortic smooth muscle cells, oxidized LDL causes biglycan galactosaminoglycan chain elongation with increased LDL binding (Chang, Potter-Perigo, Tsoi, Chait, & Wight, 2000). Comparative studies with CS and DS PGs indicate that DS PGs are more efficient at LDL binding. Oversulfation is considered as the prime feature involved in increased LDL binding to DS molecules (Sambandam, Baker, Christner, & Ekborg, 1991). Both the degree of sulfation and the position of the sulfate groups on the galactosaminoglycans, determines their binding ability to LDLs (Ballinger, et al., 2004; Sambandam, et al., 1991). In early atherosclerosis, the ratio of deltadi-mono-6-sulfate/deltadi-mono-4-sulfate (Δdi-mono6S/Δdi-mono4S) in galactosaminoglycan chains is increased which reflects the similar increase in the ratio of CS/DS, since CS is mainly composed of C-6 sulfated disaccharides, whereas DS is mainly

composed of C-4 sulfated units (Theocharis, Theocharis, De Luca, Hjerpe, & Karamanos, 2002). These changes may facilitate the LDL accumulation in the aortic wall. Similar pattern of increases in C-6 sulfation (6S)/4S of galactosaminoglycan chains in the aorta of rats fed a high cholesterol diet have also been reported (Oberkersch, et al., 2014). Enzymatic epimerization of the carboxylic acid group on the C-5 position of the GlcA generates the isomer IdoA, by definition, the galactosaminoglycan chain changes from CS to DS. These two molecules show different levels of structural flexibility and rigidity that determine LDL binding. GlcA exists in a single well defined chair conformation, on the other hand IdoA residues exist in multiple isoenergetic conformers. IdoA containing galactosaminoglycan chains bind more avidly to LDL than GlcA containing galactosaminoglycans (Gigli, Ghiselli, Torri, Naggi, & Rizzo, 1993). Although the overall charge densities of the molecules are similar, IdoA containing galactosaminoglycans exhibit local concentrations of charge for ionic binding of galactosaminoglycan to the apolipoprotein on LDLs (Iverius, 1972).

Animal models of atherosclerosis have provided strong evidence to support the role of galactosaminoglycan in lipid binding and atherosclerotic lesion development (L Delgado-Roche, et al., 2013; Livan Delgado-Roche, et al., 2015; Sarduy, et al., 2017). Mice immunized with chimeric mouse/human antibodies against galactosaminoglycans and fed a high-fat, high cholesterol diet showed a 40% reduction in aortic lesion area due to inhibition of LDL-CS binding and reduction of oxidative stress (Víctor Brito, et al., 2012).

3. Enzymes involved in galactosaminoglycan biosynthesis and modification

Biosynthesis of galactosaminoglycan chains on PGs involves the concerted action of multiple enzymes (Fig 1). The initiation of the linkage region requires the xylosyltransferase-1 (XT-1) enzyme, to transfer a Xyl to Ser residue of PG core protein (Götting, Kuhn, & Kleesiek, 2007). Subsequently, the combined action of three enzymes β-1,3-galactosyl transferase-1 (GalT-1), GalT-2 and β-1,3-glucuronosyl transferase-1

(GlcAT-1) add two Gal and one GlcA residues to form the linkage region (Uyama, Kitagawa, & Sugahara, 2007). This linkage region connects the galactosaminoglycan chain to the core protein. Polymerization of repeating disaccharide units (GalNAc-GlcA) in CS chains occurs by the action of six chondroitin synthase enzymes including chondroitin synthase-1 (ChSy-1), ChSy-2, ChSy-3, chondroitin polymerizing factor (ChPF), chondroitin N-acetylgalactosaminyltransferase-1 (ChGn-1) and ChGn-2 (Izumikawa, et al., 2008; Izumikawa, Uyama, Okuura, Sugahara, & Kitagawa, 2007). The precursor CS backbone is matured and modified by sulfation with the action of a number of enzymes including uronosyl 2-O-sulfotranferase (UST), chondroitin 4-O-sulfotranferase (C4ST), chondroitin 6-O-sulfotranferase (C6ST) (Uyama, et al., 2007). CS backbone is modified to the DS with the epimerization action of the DS epimerase (DSE) enzyme at C-5 position of GlcA in CS backbone. DS backbone is fully matured by sulfation with the action of a different set of sulfotransferases, UST and dermatan 4-O-sulfotranferase (D4ST) (Evers, Xia, Kang, Schachner, & Baenziger, 2001). An enzyme N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST) transfers sulfate to the C-6 position of N-acetylgalactosamine 4-sulfate (GalNac($4SO_4$)) in both growing CS and DS chains (Ohtake, Ito, Fukuta, & Habuchi, 2001). All these sulfotranferases (regardless CS and/or DS) transfer sulfate groups to the different corresponding positions of GlcA and IdoA (Anders Malmström, Bartolini, Thelin, Pacheco, & Maccarana, 2012; Uyama, et al., 2007). ChSy-1 and ChSy-2 are bifunctional glycosyltransferases that exhibit GlcAT-2 and N-acetylgalactosyl transferase-2 (GalNAcT-2) activity (Izumikawa, et al., 2008; Izumikawa, et al., 2007). ChSy-3 exhibits only GlcAT activity (Izumikawa, et al., 2008). Whereas ChSy-1 and ChSy-2 are responsible for CS chain elongation, ChGn-1 and ChGn-2 catalyse both the chain initiation and elongation (Sato, et al., 2003). ChGn-1 and ChGn-2 enzymes exhibit GalNAcT-1 and GalNAcT-2 activity (Gotoh, et al., 2002; Sato, et al., 2003).

Chondroitin polymerization can be achieved by the combined action of enzyme complexes composed of any of two ChSys and ChPF (Izumikawa, et al., 2008; Izumikawa, et al., 2007). This enzyme complexes with chondroitin polymerase activity and interacts with C4ST-1 to regulate the length of CS chains (Izumikawa, Okuura, Koike, Sakoda, & Kitagawa, 2011). ChGn-1 regulates the chain numbers and level of CS and co-operates with C4ST-2 to increase the number of CS moieties (Izumikawa, Koike, & Kitagawa, 2012). Therefore, biosynthesis and chain length of galactosaminoglycans are not only regulated by chondroitin synthase family members but also CS sulfotranferases. Although an array of enzymes is involved in galactosaminoglycan chain elongation, ChGn-2 and C4ST-1 play a critical role in the regulation of galactosaminoglycan chain length *in vitro* (Izumikawa, et al., 2011). An increase in the CS/DS synthesis was found in a atherosclerotic mouse model study accompanied by an increased expression of these two enzymes (Anggraeni, et al., 2011). Thus the critical role of ChGn-2 and C4ST-1 in galactosaminoglycan chain elongation regulation was demonstrated both *in vivo* and *in vitro*.

4. Signalling pathways regulating galactosaminoglycan synthesis and structure

4.1 Protein tyrosine kinase receptor (PTKR) pathways

The PTKRs are transmembrane glycoproteins that are activated by the binding of their ligands (Hubbard & Till, 2000). PTKRs are involved in activation of numerous signalling pathways leading to cell proliferation, differentiation, migration or metabolic changes (Schlessinger, 2000). The PTKR family includes insulin receptors and numerous growth factor receptors such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor, vascular endothelial growth factor and nerve growth factor (Hubbard & Till, 2000). Growth factors such as PDGF and EGF are involved in the hyperelongation of galactosaminoglycan chains by altering gene expression utilizing classical PTKR signaling (Getachew, Ballinger, Burch, Reid, et al., 2010; Yang, et al., 2009).

PDGF via its cognate receptor stimulates the elongation of galactosaminoglycan chains on PGs (Getachew, Ballinger, Burch, Reid, et al., 2010; Schonherr, Jarvelainen, Kinsella, Sandell, & Wight, 1993). In human VSMCs PDGF stimulates autophosphorylation and activation of the PDGFRα and PDGFRβ in their kinase activation domain tyrosine^{849} (Tyr^{849}) and Tyr^{857}, respectively (Getachew, Ballinger, Burch, Reid, et al., 2010). Imatinib, a small molecule tyrosine kinase inhibitor blocks PDGFRβ by inhibiting autophosphorylation on Tyr^{857}, thus inhibiting galactosaminoglycan hyperelongation and LDL retention (Ballinger, et al., 2010). Tyrosine kinase inhibitor, Ki11502 blocks Tyr^{857} autophosphorylation on PDGFRβ, and is more potent than imatinib in inhibiting galactosaminoglycan elongation (Getachew, Ballinger, Burch, Little, & Osman, 2010). In contrast, genistein, a tyrosine kinase inhibitor does not block PDGFR autophosphorylation. Genistein inhibits PG core protein synthesis but has no effect on galactosaminoglycan chain elongation in VSMCs (Little, et al., 2012). These studies demonstrated that PDGFRβ autophosphorylation in Tyr^{857} is crucial to PDGF stimulated galactosaminoglycan chain elongation. PDGF also stimulates phosphorylation in Tyr^{751} and Tyr^{1021} on PDGFR, these are associated with downstream signalling via phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC). Although neither of these sites mediate galactosaminoglycan elongation however both sites are associated with increased PG core protein synthesis (Getachew, Ballinger, Burch, Reid, et al., 2010).

Phosphorylation of PDGFRβ Tyr^{857} initiates extensive autophosphorylation of 11 Tyr residues within the cytoplasmic region of the receptor. These sites serve as tyrosine kinase docking sites for a number of adaptor molecules containing SH2 domains such as Grb and Shc. For instance, Grb and Shc can bind to Tyr^{740} and Tyr^{771}, respectively on PDGFRβ. These molecules form complexes with son of sevenless (Sos), a guanine nucleotide exchange factor to activate Ras (Heldin & Westermark, 1999). Activated Ras sequentially activates

extracellular signal-regulated kinase 1/2 (Erk1/2) through the Ras/Raf/MEK/Erk pathway. Erk1/2 is associated with galactosaminoglycan elongation in VSMCs (Getachew, Ballinger, Burch, Reid, et al., 2010). Transcription factor Sp1 is phosphorylated by Erk1/2 on threonine^{453} (Thr^{453}) (Tan & Khachigian, 2009). PDGF treatment in VSMCs, produced peak of Thr^{453} Sp1 phosphorylation which is blocked in the presence of Erk inhibitors. This suggests the possible role of the Sp1 in galactosaminoglycan chain elongation (Getachew, Ballinger, Burch, Reid, et al., 2010). Barre *et al.* (Barré, et al., 2006) demonstrated that Sp1 phosphorylated and activated by Erk1/2 is a transactivator of GlcAT-1 gene promoter leading to increased expression of the GlcAT-1 enzyme in HeLa cells. In a recent study, ChSy-1 expression was found to be induced by Erk1/2 activated Sp1 in rat nucleus pulposus cells (Hu, et al., 2015). Together these results indicate an involvement of Sp1 in PDGF stimulated expression of CS enzymes and subsequent hyperelongation of CS chains (Fig 2).

PDGF leads to modifications in the sulfation pattern of CS chains. An increase in the the chondroitin-6-sulfate (C6S) to chondroitin-4-sulfate (C4S) ratio was found in PDGF treated monkey arterial smooth muscle cells. PDGF utilizes protein kinase C (PKC) signaling leading to the activation Ras/Raf/MEK/Erk pathway to mediate the changes in sulfation pattern of CS chains, but PKC activity alone is insufficient to generate this response (Cardoso, et al., 2010). The serine/threonine kinase, Akt is found to be phosphorylated in response to PDGF stimulation. Akt phosphorylation is associated with PG core protein synthesis but it has no effect on PTKR stimulated galactosaminoglycan elongation or the expression of C4ST-1 gene (Osman, et al., 2014). Another PTKR, EGF receptor (EGFR) stimulates the mRNA expression of galactosaminoglycan synthesizing gene ChSy-1 and C4ST-1 via downstream signaling intermediate Erk1/2, which is inhibited by EGFR antagonists AG1478 in VSMCs (Kamato, et al., 2016). PTKR mediated signaling pathways in response to their agonist stimulation are directly involved in inducing expression of

galactosaminoglycan synthesizing genes leading to the synthesis of hyperelongated galactosaminoglycan chains. These hyperelongated galactosaminoglycan chains render the vessel wall "more sticky" to trap more LDLs and initiates the pre-inflammatory stage of atherosclerosis.

4.2 Serine/threonine kinase receptors (S/TKRs) pathways

A family of transmembrane receptors that contain intracellular serine/threonine kinase domain is known as S/TKRs and ligands of these receptors include TGF-β superfamily members such as TGF-β, activin, nodal and others (ten Dijke, et al., 1994). TGF-β is a pleiotropic growth factor associated with the development of all stages of atherosclerosis. TGF-β family members consist of dimeric ligands and usually remain in a latent form bound to their polypeptide. When released from their polypeptide, these ligands become active and bind to dimers of membrane TGF-β receptors (TGFBRs). Following ligand binding, TGFBR1 dimers combine with TGFBR2 dimers to form heterotetrameric receptor complexes. Phosphorylation of TGFBR1 by TGFBR2 leads to the formation of active heterotetrameric receptor complexes (Massague, Seoane, & Wotton, 2005).

TGF-β1 has a very specific action on galactosaminoglycan elongation and has no effects on the C6S to C4S ratio (Schonherr, et al., 1993). Transcription factor Smad2 is an immediate downstream target of TGFBR1 in the TGF-β stimulated signalling pathway. Smad2 consists of an amino-terminal or "Mad-homology 1" (MH1) domain, a linker region and a carboxy-terminal or MH2 domain (Massague, et al., 2005). The canonical TGF-β signalling pathway via the TGFBR1 involves the phosphorylation of the Smad2 carboxy-terminal, which forms a complex with Smad4 to translocate to the nucleus (Burch, Zheng, & Little, 2011; Derynck, Zhang, & Feng, 1998; Massague, et al., 2005). In addition to carboxy terminal phosphorylation, the Smad2 linker region can undergo phosphorylation on Thr^{220}, Ser^{245}, Ser^{250}, Ser^{255} residues (Kamato, et al., 2014; Rostam, et al., 2016). Smad linker

region phosphorylation can occur by multiple serine/threonine kinases such as MAP kinases (Erk and p38), PI3K, cyclin-dependent kinases (CDKs), Rho associated protein kinase (ROCK) and glycogen synthase kinase 3 (GSK3) (Burch, Yang, et al., 2010; Kamato, Burch, Piva, et al., 2013; Matsuzaki, 2013; Rezaei, Kamato, Ansari, Osman, & Little, 2012). Highly specific signalling pathways from TGF-β to Smad2 linker region phosphorylation exist in human VSMCs (Rostam, et al., 2016). Serine/threonine kinases Erk, p38, PI3K and CDK mediate the phosphorylation of the Ser^{245}/Ser^{250}/Ser^{255} residues whereas only CDK is found to phosphorylate the Thr^{220} residue in Smad2 linker region. Phosphorylation of three Ser residues (Ser^{245}, Ser^{250} and Ser^{255}) in Smad2 linker region induces expression of two galactosaminolycan synthesizing enzymes ChSy-1 and C4ST-1, however phosphorylation of Thr^{220} is associated with induction of XT-1, the enzyme involved in the initiation of the galactosaminoglycan linkage region (Rostam, et al., 2016) (Fig 3). Phosphorylation of any of the three Ser residues on Smad2 linker region is involved in galactosaminoglycan chain elongation. In contrast, Thr^{220} phosphorylation on Smad2 linker region can induce galactosaminoglycan chain initiation. XT-1 expression is up-regulated by TGFβ1 in cardiac fibroblasts with a corresponding increased synthesis of galactosaminoglycans in cardiac tissues. A specific knock-down of the XT-1 mRNA with siRNA resulted in a decreased XT-1 activity and lower galactosaminoglycan synthesis *in vivo* (Prante, et al., 2007).

S/TKR signalling pathways play a crucial role in galactosaminoglycan synthesizing enzyme expression via Smad2 linker region phosphorylation. TGF-β stimulated overexpression of these genes involved in both galactosaminoglycan chain initiation and elongation render the vessel wall more susceptible to LDL binding.

4.3 G-protein coupled receptors (GPCRs) pathways

Seven transmembrane GPCRs are the most prolific and polyfunctional group of cell surface receptors in mammalian biology and are heavily implicated in physiology and

pathology. These receptors are coupled to G proteins with α, β and γ subunits which act as effectors of GPCR signalling (Pierce, Premont, & Lefkowitz, 2002). Downstream mediators of GPCR signaling include inositol 1,4,5 triphosphate (IP_3) leading to the increased cytosolic calcium and activation of protein kinase C (PKC) (Gilman, 1987).

Vasoactive GPCR agonists endothelin-1 (ET-1) (Little, Burch, Getachew, Al-aryahi, & Osman, 2010), angiotensin-II (Ang-II) (Figueroa & Vijayagopal, 2002) and thrombin (Burch, Ballinger, et al., 2010) stimulate PG synthesis in VSMCs. ET-1 stimulates PG synthesis with longer galactosaminoglycan chains and increased C6S to C4S ratio (Ballinger, Ivey, Osman, Thomas, & Little, 2009). Ang-II induces an increase in PG synthesis in human thoracic aorta smooth muscle cells, which is due to galactosaminoglycan chain hyperelongation and increased sulfation (Figueroa & Vijayagopal, 2002). Previous studies targeted the involvement of classical GPCR signaling pathways in PG synthesis and galactosaminoglycan chain elongation (Burch, Ballinger, et al., 2010; Survase, Ivey, Nigro, Osman, & Little, 2005). IP_3 and cytosolic calcium ions do not effect PG synthesis in VSMCs (Burch, Ballinger, et al., 2010; Survase, et al., 2005). Although, PKC can play an important role in PG synthesis and galactosaminoglycan chain elongation (Cardoso, et al., 2010). GPCR agonists do not stimulate PG synthesis and galactosaminoglycan chain elongation via classical GPCR mediated signalling pathways.

GPCRs can transactivate both PTKRs (Daub, Weiss, Wallasch, & Ullrich, 1996) and S/TKRs (Burch, Ballinger, et al., 2010; Kamato, et al., 2017; Kamato, Burch, Osman, Zheng, & Little, 2013). Indeed, the recognition of the ability of GPCRs to transactivate PTKRs and S/TKRs was discovered in the context of galactosaminoglycan chain elongation (Kamato, et al., 2016). Thrombin can stimulate PG synthesis in VSMCs which utilizes both PTKRs and S/TKRs transactivation pathways (Burch, Getachew, Osman, Febbraio, & Little, 2013). Thrombin elicits dual transactivation-dependent signaling pathways to stimulate ChSy-1 and

C4ST-1 mRNA expression (Kamato, et al., 2016) (Fig 4). Thrombin stimulation of galactosaminoglycan synthesizing enzyme expression occurs entirely via transactivation-dependent pathways and conversely does not involve transactivation-independent pathways (Kamato, et al., 2015). The transactivation of PTKR involves matrix metalloproteinases (MMPs) and the phosphorylation of Erk. In contrast, the S/TKR mediated response involves phosphorylation of Ser residues on Smad2 carboxy terminal and does not inolve MMPs. Chung *et al.* (Chung, Ramachandran, Hollenberg, & Muruve, 2013) showed similar results with TGFBR1 transactivation by proteinase-activated receptor-2 (PAR-2), except transactivation of TGFBR1 was reported to be dependent on MMPs. Smad2 linker region phosphorylation via thrombin stimulation is inhibited by the MMP inhibitor, GM6001 (Kamato, et al., 2016). MMPs are involved in GPCR transactivation of EGFR but not TGFBR1. Therefore, thrombin mediated Smad2 linker region phosphorylation occurs possibly via GPCR transactivation of PTKR not GPCR transactivation of S/TKRs. Smad2 linker region phosphorylation is of considerable interest because identifying a common target molecule downstream to distinct receptor mediated signaling pathways for the regulation of PG synthesis and galactosaminoglycan hyperelongation would be a potential therapeutic target for the prevention of atherosclerosis.

5. Potential of the response to retention hypothesis for providing a therapeutic agent for the prevention or treatment of atherosclerosis

The response to retention hypothesis proposes that atherosclerosis commences with the entry, binding and trapping of atherogenic LDLs by modified PGs in the vessel wall. So what are the potential therapeutic modalities to exploiting this hypothesis therapeutically?

5.1 Lowering plasma LDL cholesterol

LDL cholesterol lowering is the current gold standard and recommended treatment for preventing atherosclerosis. Simply lowering plasma cholesterol should reduce the cholesterol

flux into the vessel wall. Statins were consistently successful in multiple clinical trials and have been very successful in reducing CVD risk and events in real world therapeutic use for over two decades. There are multiple statins available and their actions and outcomes have been extensively reviewed (Collins, et al., 2016; Ehrenstein, Jury, & Mauri, 2005; Taylor, et al., 2013).

More recently proprotein convertase subtilisin kexin 9 (PCSK9) inhibitors have been developed – these agents bind to PCSK9 preventing its action in degrading LDL receptors leading to major upregulation of LDL receptors and an increased uptake of cholesterol and lowered plasma cholesterol (Yadav, Sharma, & Ferdinand, 2016). With statins expressing such high efficacy the rationale for PCSK9 inhibitors is less than clear. Both statins and PCSK9 inhibitors cause mild hyperglycaemia meaning an increase in diabetes depending upon the starting glycaemia status and susceptibility of the patient cohort (Schmidt, et al., 2017; Shah & Goldfine, 2012).

The efficacy of statins in preventing CVD is such that some clinical guidelines (Lloyd-Jones, Goff, & Stone, 2014) even indicate the use of a statin without ongoing reference to the plasma cholesterol level. This is related to the demonstrated clinical efficacy but also at least in part the wide range of favourable, pleiotropic, cardiovascular protective actions which have been demonstrated for statins (Collins, et al., 2016; Ford, Murray, McCowan, & Packard, 2016; Taylor, et al., 2013). Importantly, in the current context, statins have no effects on PG synthesis in VSMCs (Little and Osman, unpublished observations) so such an action is not in the group of anti-inflammatory, anti-oxidation and other actions which add to the cholesterol lowering actions of statins and thus targeting PG-LDL interactions is a bona fide approach in combination with a statin.

5.2 Targeting the interaction between LDLs and PGs

5.2.1 Small peptide mimetics of ApoB100

One potential mechanism to block the interaction between amino acids on ApoB100 and sulfate groups on galactosaminoglycan chains is to saturate the galactosaminoglycan chain binding sites with small pepetide mimetics and this has been successfully demonstrated in a mouse model (Skalen, et al., 2002). The rationale underlying this approach can be demonstrated by mutating the residues on ApoB100 Thus, as the LDL and PG interaction involves clusters of amino acids in the apoB100 that bind to the negatively charged sulfate groups on the galactosaminoglycan chain, site A at residues 3148-3158 and site B residues 3359-3369 (Boren, et al., 1998; Olsson, et al., 1997). Site A in apoB100 becomes functional and cooperates with site B to increase the PG binding activity in phospholipase A2 modified LDL (Flood, et al., 2004). Transgenic mice expressing mutated site B apoB100 at the PG binding site fed a hypercholesterolemic diet for 20 weeks displayed significant protection from atherosclerotic lesion development as compared to transgenic mice expressing wild-type apoB100 under the same conditions (Skalen, et al., 2002)..

5.2.2 Galactosaminoglycan directed antibodies

Administration of a chimeric mouse/human monoclonal antibody (mAb) to induce responses against galactosaminoglycans has been shown to be effective at preventing the progression of atherosclerotic lesions (Víctor Brito, et al., 2012; V. Brito, et al., 2017; L Delgado-Roche, et al., 2013; Sarduy, et al., 2017; Soto, et al., 2012). The anti-atherosclerotic antibody was based on the chimeric mouse/human immunoglobulin G1 (IgG1) variant of P3 mAb that recognises glycolylated gangliosides and sulfatides. The ChP3R99 antibody has an additional arginine at position 99 displaying a higher reactivity with antigens. The initial work to characterise ChP3R99 as an anti-atherogenic antibody shows that it did not have direct impact on the oxidized LDL or foam cell formation, however in an in vitro binding

assay, ChP3R99 inhibited 70% of LDL binding to CS (Soto, et al., 2012). Pre-treatment with the mAb in an atherosclerosis induced rabbit model completely inhibited lesion progression and resulted in lower macrophage infiltration (Soto, et al., 2012). ChP3R99 was compared with ChP3S98, a mutant variant with lower reactivity to the antigen in a murine model of atherosclerosis fed a hypercholesteraemic diet (Víctor Brito, et al., 2012). Multiple administration of ChP3R99 at biweekly or weekly intervals prevented lesion formation in apoE-/- mice as compared to treatment with ChP3S98 which had no apparent reduction in aortic lesion formation (Víctor Brito, et al., 2012). This can be explained with ChP3S98's lower capacity to generate antibody response against galactosaminoglycans. Pre and post immunization of ChP3R99 in apoE-/- induced galactosaminoglycan antibody production as compared to the low reactive mAb (Víctor Brito, et al., 2012).

In 2017, two major studies in this area were published (V. Brito, et al., 2017; Sarduy, et al., 2017). Earlier work with the anti-galactosaminoglycan antibodies was in young and adolescent apoE-/- male mice (Víctor Brito, et al., 2012). However as age and sex are major risk factors in the development of atherosclerosis, Vazquez *et al.* (Sarduy, et al., 2017) asses the influence of these variable on the capacity of the ChP3R99 mAb to generate anti-galactosaminoglycan response. Immunization with ChP3R99 mAb reduced the mean aortic lesion area by 31% and 38% in male and female mice, respectively compared with their respective control-treated groups and to 31% in middle aged female mice with spontaneous lesions (Sarduy, et al., 2017). Thus neither of the two major risk factors had an impact on the anti-galactosaminoglycan properties induced by immunization with this mAb. In the latter study the ChP3R99 mAb was used in established atherosclerosis in apoE-/- mice. Atherosclerosis was allowed to develop for 11 weeks with apoE-/- mice fed a high fat diet followed by subcutaneous injections of ChR3R99 mAb (Sarduy, et al., 2017), atherosclerotic lesion progression was reduced by 88% as compared to the control treated group.

Collectively these studies define the use of an anti-galactosaminoglycan antibody for the prevention of lesion development and as a treatment to halt lesion progression in advanced atherosclerosis (Víctor Brito, et al., 2012; V. Brito, et al., 2017; L Delgado-Roche, et al., 2013; Sarduy, et al., 2017; Soto, et al., 2012). Both of the above strategies are valid approaches but are not easily converted into a small chemical entity suitable for long term therapy in humans as is required for the treatment of a chronic disease such as atherosclerosis for the prevention of CVDs. Nevertheless, there are emerging pharmaceutical delivery techniques being developed, so a therapeutic agent is possible and as above such a drug would be used in combination with a statin for a two pronged attack on the development of this life threatening disease.

5.3 Targeting the changes in PGs in the vessel wall

With the position of statins as the drug of first choice for the prevention of atherosclerosis, new therapeutic modalities must include the ability to lower cardiovascular risk on top of a statin such that dual therapy would be indicated. It is necessary to have a mechanism which is different from cholesterol lowering and most likely a vessel wall directed therapy addressing the disease process directly. The working hypothesis in the area of targeting galactosaminoglycan chain synthesis in that a statin would lower blood cholesterol and a "PG (galactosaminoglycan) inhibitor" would reduce the "stickiness" of the vessel wall. Proof of concept for this approach has been provided in two studies with imatinib (Ballinger, et al., 2010; Getachew, Ballinger, Burch, Reid, et al., 2010). Imatinib was developed as a tyrosine kinase Abl inhibitor for the treatment of Chronic Myeloid Leukaemia. Imatinib inhibits a very small number of tyrosine kinases including in the current context PDGF receptor tyrosine kinase (Getachew, Ballinger, Burch, Reid, et al., 2010). Treatment with imatinib results in the inhibition of PDGF stimulated radiosulfate incorporation into PGs, elongation of galactosaminoglycan chain and reduced xyloside

binding to LDL (Ballinger, et al., 2010; Getachew, Ballinger, Burch, Reid, et al., 2010). Furthermore, in an in vivo model of atherogenesis, high-fat fed apoE-/- mice, imatinib reduced lipid deposition in the vessel wall (Ballinger, et al., 2010). The action of imatinib to reduce cholesterol deposition in the vessel wall, occurred without any effect on circulating lipid levels in the mice.

The optimum target in this area is the signalling pathways that controls the synthesis of galactosaminoglycan chians on biglycan (Ballinger, et al., 2009; Burch, Ballinger, et al., 2010; Burch, et al., 2013; Burch, Yang, et al., 2010; Little, et al., 2013; Rostam, et al., 2016; Yang, et al., 2010). The actual enzymes which mediate galactosaminoglycan elongation (Izumikawa, et al., 2012; Izumikawa, et al., 2011) are not targets themselves because those enzymes will be involved in multiple physiological processes and undoubtedly unwanted side effects would arise from targeting those enzymes. Furthermore, lipoprotein metabolism is very important in organs such as the liver and targeting those enzymes directly might perturb endogenous lipoprotein metabolism but targeting the signalling pathways is likely to be VSMC specific. In this review we have described in detail signalling pathways which mediate galactosaminoglycan hyperelongation in VSMCs and thus present as potential therapeutic targets (Burch, Ballinger, et al., 2010; Burch, et al., 2013; Burch, Yang, et al., 2010; Kamato, et al., 2016; Rostam, et al., 2016).

6. Conclusions

Therapies for atherosclerosis have thus far targeted blood borne risk factors and blood pressure. Although this has produced some of the most efficacious cardiovascular therapies, specifically the cholesterol-lowering statins, there remains a large burden of potentially preventable atherosclerosis-related ischemic CVDs. An additional target is the disease process in the vessel wall, an area which had been extensively researched over the past three decades. There are practical and regulatory limitations in clinical studies based on vascular

wall directed therapies but it is possible that great advances in imaging technologies may open up this area in a way that allows for the development of new classes of agents. Of the major areas of vascular wall directed therapies, this review proposes that a prime target is the potentially vascular-specific signalling pathways of vasoactive agonists that lead to an increase in the "stickiness" of vascular wall PGs for apolipoproteins on atherogenic LDL. The two critical processes of lipid retention and inflammation (represented by macrophage infiltration and foam cell formation) may pathologically be in series or parallel. Thus, if the processes are in series then slowing or preventing lipid retention will disrupt the atherosclerotic cascade. Hence, the combination of a statin to reduce blood cholesterol and an inhibitor of galactosaminoglycan hyperelongation aimed at stopping LDL accumulation in the vessel wall represents a potential strategy for attaining the requisite quantum increase in efficacy required to prevent atherosclerosis and thus the burden of CVDs.

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Conflict of Interests

Nothing to declare

Figures

Fig 1. Scematic representation of the role of enzymes in galactosaminoglycan biosynthesis and modification: Galactosaminoglycan linkage region is made up of xylose-galactose-galactose-glucuronic acid (Xyl-Gal-Gal-GlcA). The xylosyltransferase-1 (XT-1) enzyme transfers a Xyl to serine (Ser) residue of PG core protein. Two Gal residues are added by the action of two enzymes, β-1,3-galactosyl transferase-1 (GalT-1) and GalT-2. The β-1,3-glucuronosyl transferase-1 (GlcAT-1) adds a GlcA residues to complete the linkage region. Six chondroitin synthase enzymes including chondroitin synthase-1 (ChSy-1), ChSy-2, ChSy-3, chondroitin polymerizing factor (ChPF), chondroitin N-acetylgalactosaminyltransferase-1 (ChGn-1) and ChGn-2 catalyse subsequent addition of repeating disaccharide unit (GalNAc-GlcA) in CS chain. The modification of this precursor chondroitin backbone is occur by sulfation with the action of sulfotransferase enzymes including uronosyl 2-O-sulfotranferase (UST), chondroitin 4-O-sulfotranferase (C4ST), chondroitin 6-O-sulfotranferase (C6ST). Dermatan sulfate-glucoronyl C5-epimerase (DSE) enzyme catalyse epimerization at fifth carbon (C-5) position of GlcA in precursor chondroitin backbone to synthesize dermatan backbone. The dermatan backbone is then matured and modified by action of another set of sulfotransferases including UST and dermatan 4-O-sulfotranferase (D4ST). N-Acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST) transfers sulfate to C-6 position of N-acetylgalactosamino 4-sulfate (GalNac($4SO_4$)) in both growing CS and DS chain.

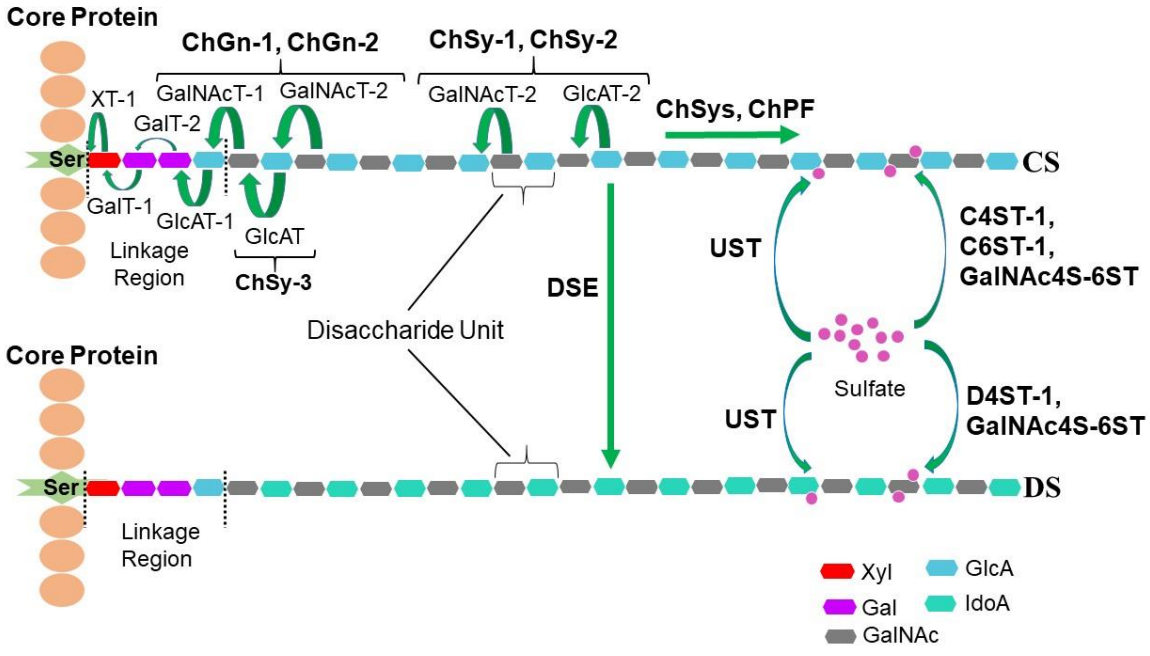


Fig 2. Platelet derived growth factor (PDGF) mediated signalling pathway leading to galactosaminoglycan genes expression: PDGF binding to the PDGF receptor β (PDGFRβ) induce receptor dimerization and autophosphorylation on Tyr^{857}. Tyr^{857} Phosphorylation initiates autophosphorylation of downstream tyrosine residues within the cytoplasmic region of the receptor. Adaptor molecules such as Grb and Shc contain SH2 domain that can bind these tyrosine kinase docking sites on PDGFR. These adaptor molecules form complex with son of sevenless (Sos), a guanine nucleotide exchange factor. Sos mediate the exchange for GDP bound to inactive Ras to GTP and activates Ras. Subsequent activation of Raf leading to phosphorylation and activation of mitogen activated protein kinase (MAPK) Erk kinase (MEK). Activated MEK sequentially phosphorylates and activates extracellular signal-regulated kinase 1/2 (Erk1/2). Sp1 transcription factor is phosphorylated on threonine^{453} (Thr^{453}) by Erk1/2 and induces expression of galactosaminoglycan synthesizing enzymes GlcAT-1 and ChSy-1.

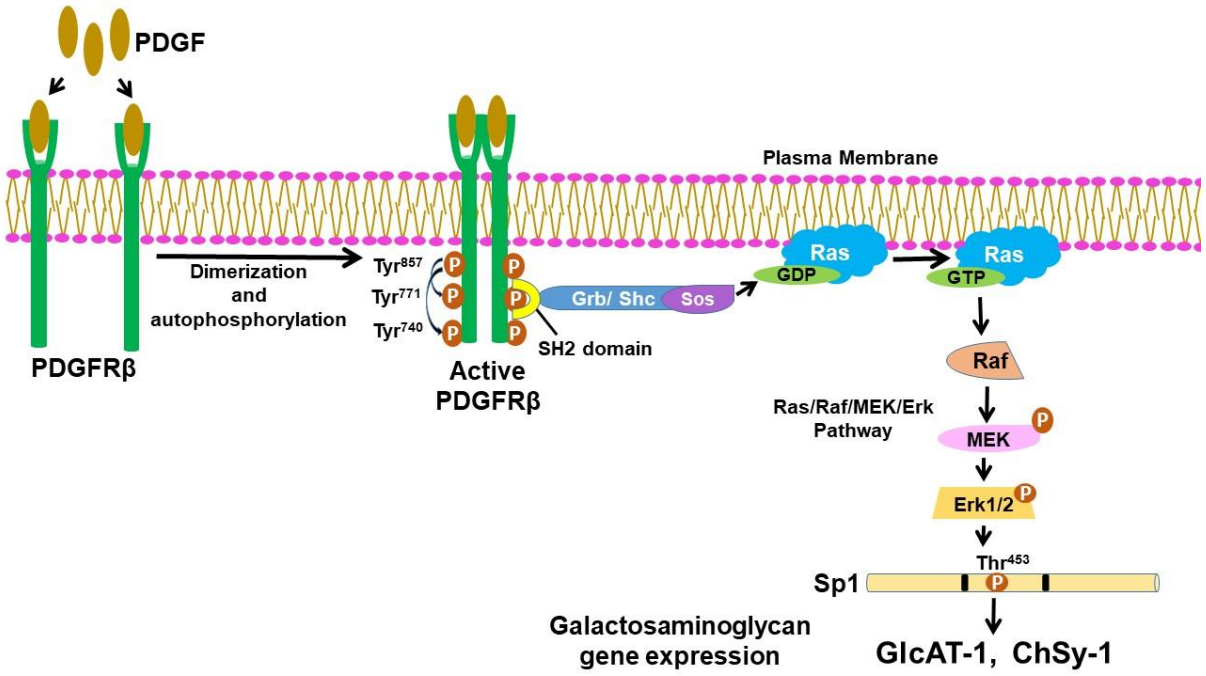


Fig 3. Transforming growth factor-β (TGF-β) mediated signalling pathway leading to galactosaminoglycan genes expression: Ligand binding induces TGF-β receptor 1 (TGFBR1) and TGFBR2 dimers to form heterotetrameric complex followed by the activation of TGFBR1. TGFBR1 can induce the activation of multiple Ser/Thr kinases. Individual Smad2 linker region sites can be specifically phosphorylated by these serine/threonine kinases. Phosphorylation of three Ser residues on Ser^{245}, Ser^{250} and Ser^{255} leading to induce the mRNA expression of two crucial galactosaminoglycan synthesizing enzyme ChSy-1 and C4ST-1. Thr^{220} phosphorylation is associated with expression of galactosaminoglycan chain initiating enzyme XT-1. These indicates that phosphorylation on one of the three Ser residues play key role in galactosaminoglycan chain elongation. In contrast, threonine^{220} (Thr^{220}) phosphorylation plays critical role in initiation of galactosaminoglycan chain in TGF-β stimulated galactosaminoglycan synthesis.

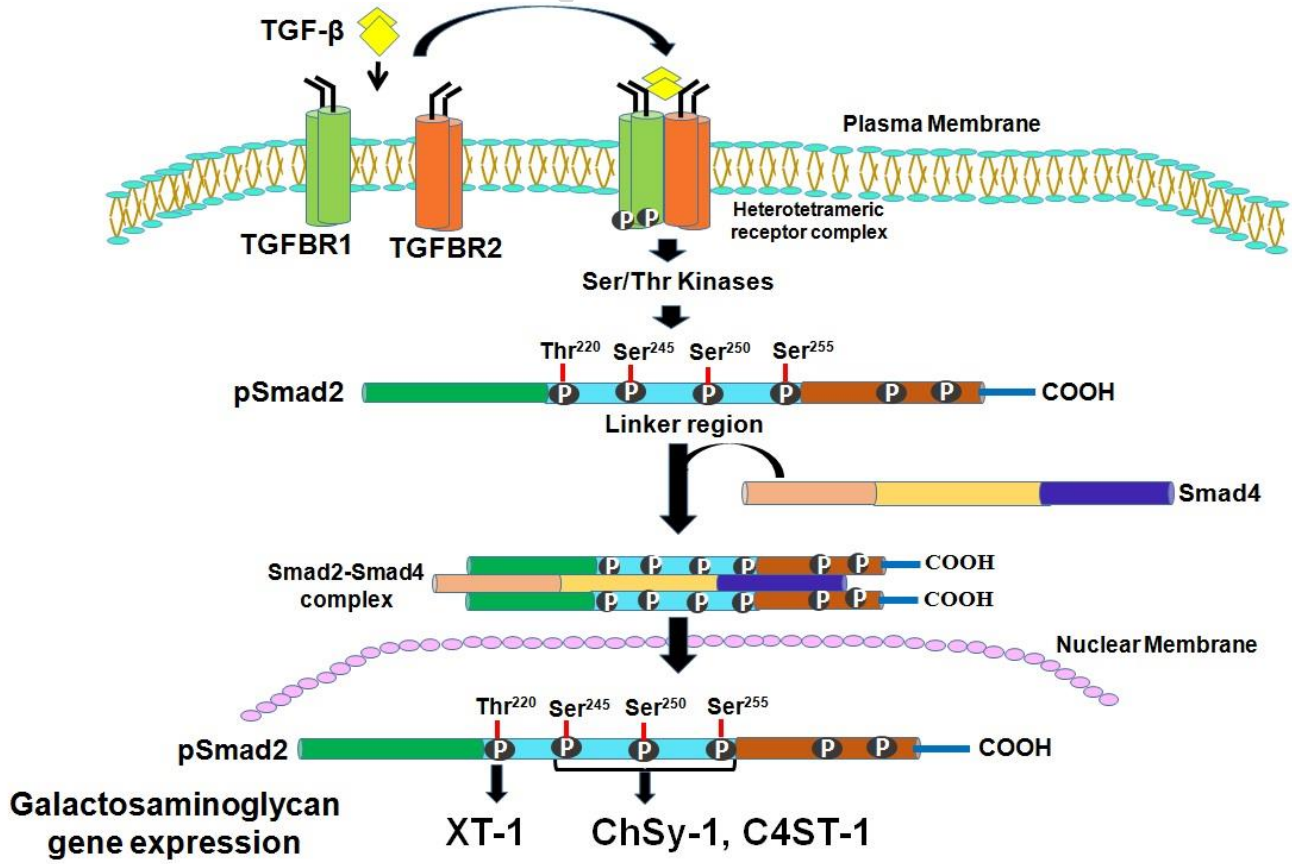
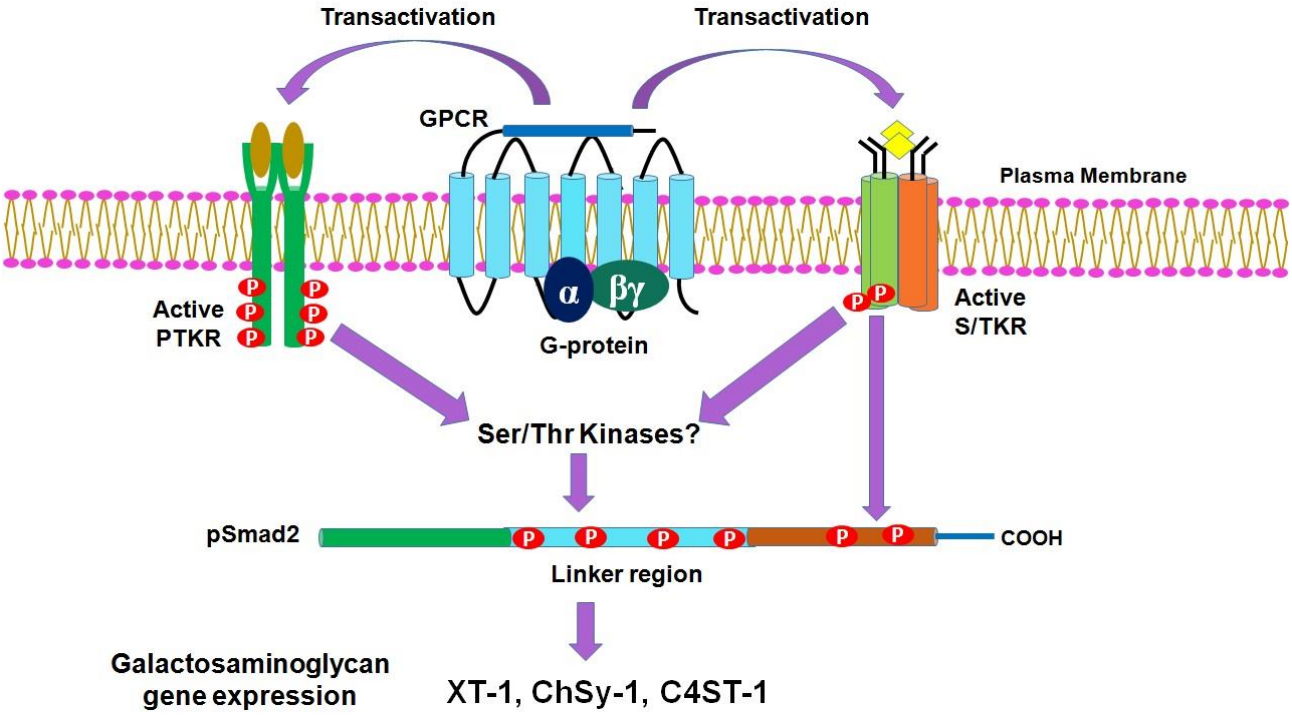


Fig 4. G-protein coupled receptor (GPCR) transactivation of PTKR and S/TKR leading to galactosaminoglycan gene expression: Thrombin mediated galactosaminoglycan chain elongation does not involve classical GPCR signalling rather it utilizes transactivation pathways for both PTKR and S/TKR to elicit response. GPCR transactivation of PTKR involve matrix metalloproteinases (MMPs), but S/TKR does not. Activated S/TKRs can directly phosphorylate transcriptiom factor Smad2 on its carboxy terminal. S/TKRs also phosphorylates the linker region of Smad2 via serine/threonine kinases (Ser/Thr kinases). Combined speculation of different studies also provide an indication to the Smad2 linker region phosphorylation by PTKRs. Smad2 linker region phosphorylation can induce the expression of galactosaminoglycan synthesizing enzymes XT-1, ChSy-1 and C4ST-1.



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