Individual Smad2 linker region phosphorylation sites determine the expression of proteoglycan and glycosaminoglycan synthesizing genes

Author

Kamato, Danielle, Burch, Micah, Zhou, Ying, Mohamed, Raafat, Stow, Jennifer L, Osman, Narin, Zheng, Wenhua, Little, Peter J

Published

2019

Journal Title

Cellular Signalling

Version

Accepted Manuscript (AM)

DOI

10.1016/j.cellsig.2018.11.005

Individual Smad2 linker region phosphorylation sites determine the expression of proteoglycan and glycosaminoglycan synthesizing genes

Danielle Kamato[a,b,*] d.kamato@uq.edu.au, Micah Burch[c], Zhou Yang[a], Raafat Mohamed[a], Jennifer L Stow[d], Narin Osman[a,e,f] and Peter J. Little[a,b]

[a]School of Pharmacy, University of Queensland, Pharmacy Australia Centre of Excellence, Woolloongabba, QLD, 4102, Australia

[b]Department of Pharmacy, Xinhua College of Sun Yat-sen University, Tianhe District, Guangzhou, 510520, China

[c]Dept. Of Cardiovascular Medicine, Brigham and Harvard Medical School, Boston, MA 02115, USA

[d]Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD, 4067, Australia

[e]School of Health and Biomedical Sciences, RMIT University, Bundoora, VIC, 3083 Australia

[f]Department of Immunology and Pathology, Monash University, Melbourne, VIC, 3004 Australia

Correspondence author at: School of Pharmacy, The University of Queensland, 20 Cornwall Street, Woolloongabba, QLD 4102, Australia

Abstract

Growth factors such as thrombin and transforming Growth Factor (TGF)-β facilitate glycosaminoglycan (GAG) chain hyperelongation on proteoglycans , a phenomenon that increases lipoprotein binding in the vessel wall and the development of atherosclerosis. TGF -β signals via canonical carboxy terminal phosphorylation of R-Smads and also non-canonical linker region phosphorylation of R-Smads. The G protein coupled receptor agonist, thrombin, can transactivate the TGF-β receptor leading to both canonical and non-canonical Smad signalling. Linker region phosphorylation drives the expression of genes for the synthesis of the proteoglycan, biglycan. Proteoglycan synthesis involves core protein synthesis, the initiation of GAG chains and the subsequent elongation of GAG chains. We have explored the relationship between the thrombin stimulated phosphorylation of individual serine and threonine sites in the linker region of Smad2 and the expression of GAG initiation xylosyltransferase-1 (XT-1) and GAG elongation chondroitin 4-sulfotransferase-1(C4ST-1) and chondroitin synthase-1 (CHSY-1) genes. Thrombin stimulated the phosphorylation of all four target residues (Thr220, Ser245, Ser250 and Ser255 residues) with a similar temporal pattern – phosphorylation was maximal at 15 min (the earliest time point studied) and the level of the phospho-proteins declined thereafter over the following 4 hours. Jnk, p38 and PI3K, selectively mediated the phosphorylation of the Thr220 residue whereas the serine residues were variously phosphorylated by multiple kinases. Thrombin stimulated the expression of all three genes – XT-1, C4ST-1 and CHSY-1. The three pathways mediating Thr220 phosphorylation were also involved in the expression of XT-1. The target pathways (excluding Jnk) were involved in the expression of the GAG elongation genes (C4ST-1 and CHSY-1). These findings support the contention that individual Smad linker region phosphorylation sites are linked to the expression of genes for the initiation and elongation of GAG chains on proteoglycans. The context of this work is that a specific inhibitor of GAG elongation

represents a potential therapeutic agent for preventing GAG elongation and lipid binding and the results indicate that the specificity of the pathways is such that it might be therapeutically feasible to specifically target GAG elongation without interfering with other physiological processes with which proteoglycans are involved.

Keywords: Transactivation signalling, G-protein coupled receptors, Smad, Smad linker region, G proteins, Serine/threonine kinase receptors

Abbreviations

CDK cyclin dependent kinase

CHSY-1 chondroitin synthase

C4ST-1 chondroitin 4-O-sulfotranferase

EGFR epidermal growth factor receptor

GAG glycosaminoglycan

GalT-1 β-1,3-galactosyl transferase-1

GlcAT-1 β-1,3-glucuronosyl transferase-1

GPCRs G protein coupled receptors

MAPK mitogen activated protein kinase

PI3K phosphatidylinositide 3-kinase

PAR protease activated receptor

PTKR protein tyrosine kinase receptors

S/TKR serine/threonine kinase receptors

TGF-β transforming growth factor beta

TGFBR1 transforming growth factor beta receptor

VSMCs vascular smooth muscle cells

XT-1 xylosyltransferase 1

1. Introduction

Transforming Growth Factor (TGF)-β is a pleiotropic growth factor with broad involvement in physiological and pathophysiological processes. TGF-β has a very strong effect to stimulate the hyperelongation of glycosaminoglycan (GAG) chains on the major lipid binding proteoglycan biglycan and as it is associated with lipid deposition in atherosclerosis then this process represents a potential therapeutic target [1, 2]. TGF-β signals via canonical carboxy terminal phosphorylation of R-Smads (Smad2 in vascular smooth muscle cells (VSMC)) and non-canonical linker region phosphorylation of R-Smads [3, 4]. The role of TGF-β signalling in cell biology was greatly expanded when we showed that the paradigm of G Protein Coupled Receptor (GPCR) transactivation of protein tyrosine kinase receptors extended to the transactivation of serine/threonine kinase receptors and specifically the Type I TGF-β Receptor (TGFBR1) [5-8].

GPCRs such as Protease Activated Receptor (PAR) -1 and endothelin receptors can engage TGFBR1 and activate downstream signalling commencing with Smad phosphorylation [9-12]. The PAR-1 agonist thrombin can transactivate the TGFBR1 in VSMCS leading to both canonical and non-canonical Smad signalling and downstream effects on the synthesis, specifically elongation of the GAG chains on the lipid-binding chondroitin sulfate/dermatan sulfate (CS/DS) proteoglycan, biglycan. Proteoglycan synthesis is a complex process which involves core protein synthesis, the initiation of GAG chain synthesis and the subsequent elongation and maturity of GAG chains [13-17]. Whereas Smad linker region phosphorylation was initially described to inhibit Smad nuclear translocation [18] and hence TGF-β signalling, in an *in vitro* model of atherosclerosis we showed that linker region phosphorylation drives the expression of genes for the enzymes and transferases responsible for the synthesis of the biglycan [19-21].

Chondroitin sulfate GAG chain synthesis commences with the synthesis of the tetra-saccharide linkage region by the initial action of xylosyltransferase 1 (XT-1) [22] to transfer a xylose to the serine residue on the core protein, followed by 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) to add two galactose and one glucuronic acid residues. GAG chain synthesis involves the combination of more than one GAG synthesizing enzyme for the elongation of the GAG chain. An increase in GAG chain length and an increase in atherosclerotic lesion progression in vivo is associated with an increase in GAG enzyme expression [23].

The cell signalling mechanisms involved in GAG synthesis and elongation are distinct [6, 24]. The canonical TGF-β signalling pathway comprises direct carboxy-terminal phosphorylation of Smad2 by TGFBR1. In addition, Smads can also be phosphorylated in the linker region [3, 4, 25, 26]. Smad linker region is phosphorylated via multiple serine/threonine kinases including mitogen activated protein kinase (MAPK) (Erk, Jnk and p38), phosphatidylinositide 3-kinase (PI3K) and cyclin dependent kinase (CDK) [19, 20, 27]. There are multiple phosphorylation sites in the human Smad2 linker region: threonine at position 220 (Thr220) and three serine residues at positions 245, 250, 255 (Ser245, Ser250, Ser255) [3, 26]. TGF-β mediated GAG chain hyperelongation [20] and GAG synthesizing gene expression [19] are regulated by the Smad2 linker region. The role of Smad2 linker region phosphorylation in thrombin signalling has not been elucidated but it represents an interesting model to study the implications of TGFBR1 transactivation and the broader relationship between GPCRs and cell surface kinase receptor transactivation.

The Smad2 linker region plays a central integrating point for GPCR mediated signalling for the expression of the genes for the enzymes, which mediate the initiation, and elongation of GAGs on proteoglycans. With the complexity of the synthesis of proteoglycans involving more than ten transferases and elongation factors, this is a suitable model to study the

relationship between the individual sites of phosphorylation of serine/threonine residues in the linker region of Smad2 and the resultant expression of specific genes for GAG initiation and elongation transferases.

2. Materials and Methods

2.1 Materials

Foetal bovine serum (FBS) was purchased from Invitro Technologies Pty. Ltd. (VIC, Australia). Dulbecco's Modified Eagle Medium (DMEM) (0mM and 25mM glucose), Trypsin-Versene, penicillin and streptomycin were from GIBCO BRL (Ne, USA). Human TGF-β, anti-rabbit IgG horseradish peroxidase (HRP), anti-GAPDH antibodies were purchased from Cell Signalling Technology (MA, USA). Flavopiridol, SP600125, SB202190 and LY294002 were purchased from Sigma-Aldrich (St Louis, USA). UO126 was from Promega (WI, USA). CHSY-1 rabbit polyclonal antibody was purchased from Abcam (Vic, Australia). C4ST-1 rabbit polyclonal antibody was purchased from ThermoFishes Scienctific (Vic, Australia). Amersham ECL Prime chemiluminescence detection reagent was purchased from GE Healthcare (Paramatta, Australia). The primers (forward and reverse) for C4ST-1, CHSY-1, XT-1, EXT-1, EXT-2, EXT-3, NDST-1 and 18S, RNeasy Mini Kit, the QuantiTect reverse transcription kit, QuantiFast SYBR green PCR kit and the Rotor Gene Q series software were from Qiagen (Vic, Australia). Anti-phospho-Smad2L(Thr220) rabbit IgG polyclonal, anti-phospho-Smad2L(Ser245) rabbit IgG polyclonal, anti-phospho-Smad2L(Ser250) rabbit IgG polyclonal and anit-phospho-Smad2L(Ser255) rabbit IgG polyclonal were a gift from Professor Koichi Matsuzaki (Kansai Medical University, Osaka, Japan)

2.2 Cell culture

Primary cultures of human vascular smooth muscle cells (VSMCs) were obtained from discarded sections of saphenous veins from patients undergoing surgery at the Alfred Hospital

(Melbourne, Australia) the acquisitions of the vessels was approved from the Alfred Hospital Ethics Committee. VSMCs were grown in DMEM (5mM glucose, 10% FBS and 1% antibiotics at 37°C in 5% CO_2). VSMCs were seeded in 60 mm dishes and 6 well-plates. Cells were grown to confluence then rendered quiescent by serum deprivation for 48 h. Inhibitors were pre-incubated for 30 min prior to treatment with agonists. Incubation times and concentrations are given in detail in the figure legends.

2.3 Western blotting

Whole cell lysates (30µg of protein) were resolved on 10% SDS-PAGE and semi-dry transferred onto PVDF. Membranes were blocked with 5% bovine serum albumin and incubated with primary antibody targeting protein of interest followed by HRP-anti-rabbit IgG and ECL detection. The protein of interest was normalised with GAPDH to determine equal loading. Blots were imaged using the Bio-Rad gel documentation system and densitometry analysis was performed with Quantity One imaging software.

2.4 Assessing mRNA gene expression

The mRNA level of the GAG enzymes was determined by quantitative real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from VSMCs treated as described. RNA was extracted from 5×10^5 cells using RNeasy Mini kit (Qiagen) according to the manufacturers' instructions. RNA purity was checked by spectrophotometry (260/280 nm) using Nanodrop2000 spectrophotometer (Thermo Fisher Scientific). First strand cDNA was synthesized from 1µg RNA using Quantitect reverse transcriptase kit (Qiagen) according to the manufacturers' instructions. Quantitative RT-PCR was performed using Qiagen Rotor Gene Q and QuantiFast SYBR green PCR master mix kit (Qiagen). Data was normalised to the ribosomal 18S housekeeping gene to adjust for control variations between individual

experiments. Relative expression of mRNA levels was quantified using comparative ΔΔ Ct method. Experiments were performed in duplicate at least three times.

2.5 Statistical analysis

Normalised data is expressed as the mean ± standard error of the mean of three independent experiments, unless stated otherwise. A one-way ANOVA was used to calculate statistical significance of normalised data as stated followed by least significant difference *post-hoc* analysis. Results were considered significant when the probability was less than 0.05 (*$p<0.05$) and 0.01 (**$p<0.01$).

3. Results

3.1 Thrombin stimulates the phosphorylation of individual Smad2 linker region residues in VSMCs

There are at least four important phosphorylation sites on the human Smad2 linker region being Thr220, Ser245, Ser250 and Ser255 [3]. To determine whether site-specific linker region phosphorylation is mediated by thrombin signalling in VSMCs, a time course study was preformed with antibodies specific for the individual phosphorylated linker region residues [28]. Thrombin treated VSMCs showed a rapid increase in the phosphorylation of the Thr220 residue. Thrombin mediated phosphorylation peaked at 15 min with a 2.5-fold ($p<0.01$) change when compared to basal (Figure 1A). Phosphorylation was sustained although there was a gradual decline over the 4h treatment. There was also a rapid increase in the phosphorylation of Ser245 reaching a peak of 1.9-fold ($p<0.01$) at 15 min when compared to basal (Figure 1B). This was sustained for 4h. Similarly the Ser250 residue was rapidly phosphorylated in the presence of thrombin with a peak phosphorylation of 2.5-fold ($p<0.01$) at 15 min (Figure 1C). The phosphorylation of this residue was sustained for 60 min. The phosphorylation of the Ser255 residue followed the same trend as the other linker region residues in which thrombin

caused a rapid increase with a peak at 15 min of 2.5-fold ($p<0.01$) when compared to basal and this gradually declined over 4h. These results demonstrate that in VSMCs thrombin treatment rapidly phosphorylates Thr220, Ser245, Ser250 and Ser255 in the Smad2 linker region. The challenge then is to determine the kinases mediating these specific phosphorylations.

3.2 The involvement of serine/threonine kinases in thrombin stimulated phosphorylation of Smad2 linker region residues

Smad2 linker region phosphorylation involves activation of serine/threonine kinases. Here we investigated kinases Erk, p38, Jnk, PI3K and CDK on thrombin phosphorylation of individual Smad2 linker region residues. VSMCs treated with thrombin for 30 min increased the phosphorylation of the Thr220 to 2.7-fold ($p<0.01$) (Figure 2A) which was unaffected in the presence of Erk and CDK inhibitors, UO126 and flavopiridol. Inhibitors to p38 and Jnk SB202190 and SP600124 partially inhibited thrombin mediated phosphorylation of Thr220 and in the presence of PI3K inhibitor LY294002 this response was completely inhibited (Figure 2A).These results demonstrate that phosphorylation of the Thr220 residue via thrombin dependent mechanisms involves p38, Jnk and PI3K pathways but not Erk and CDK.

Thrombin treatment of VSMCs caused an increase in the phosphorylation of Ser245 to 2-fold ($p<0.01$) at 30 min (Figure 2B). The presence of inhibitors to Erk, p38 and Jnk had no effect on the thrombin mediated phosphorylation of Ser245. PI3K inhibitor, partially inhibited thrombin mediated phosphorylation of Ser245, and in the presence of CDK inhibitor there was complete inhibition ($p<0.01$) (Figure 2B). Thus, thrombin stimulated Ser245 phosphorylation requires the activation of PI3K and CDK.

In cells treated with thrombin, the level of the phosphorylation of Smad2 linker region residue Ser250 was increased to almost 3-fold ($p<0.01$) (Figures 2C). Erk inhibitor, UO126 partialy inhibited Ser250 phosphorylation to 2-fold ($p<0.05$). In the presence of inhibitors to

p38, Jnk, PI3K and CDK the thrombin mediated phosphorylation of Ser250 is unnaffected (Figure 2C). Thus showing that Erk is the only kinase involved in thrombin mediated phosphorylation of Smad2 linker region residue Ser250.

Thrombin mediated phosphoryaltion of the Ser255 residue was completely inhibited in the presence of UO126 ($p<0.01$) (Figure 2D). SB202190, LY294002 and flavopiridol partially inhbited this response however SP600125 had no effect (Figure 2D). Thus showing that thrombin mediated phosphorylation of Ser255 occurs via the activation of the Erk, p38, PI3K and CDK pathways. Taken together the results demonstrate that individual Smad2 linker region residues are phosphorylated by different serine/threonine kinases, with the PI3K being a common kinase for Thr220, Ser245 and Ser255 and Jnk is involved in regulating the Thr220 residue.

3.3 Thrombin stimulates the expression of genes associated with GAG initiation (XT-1) and elongation of CS GAG chains (C4ST-1 and CHSY-1)

To investigate if thrombin can stimulate the mRNA expression of GAG biosynthesizing genes, specifically CHSY-1, C4ST-1 and XT-1, VSMCs were treated with thrombin (10 units/ml) and the mRNA expression of these genes was measured over a 24 hour time period (Figure 3). During the biosynthesis of GAG chains, GalNAc residues of the repeating disaccharide units are sulfated at C4 by C4ST-1. Treatment of VSMCs with thrombin generated a parabolic time dependent increase in the mRNA expression of C4ST-1 (Figure 3A). At 2 hours post thrombin treatment, a 1.4-fold increase ($p<0.05$) in the mRNA expression of C4ST-1 was detected and reached a peak of 3-fold at 6-8 hours post stimulation ($p<0.01$) (Figure 3A). The addition of a monosaccharide to an acceptor oligosaccharide is referred to as chondroitin polymerization which occurs with the action of glycosyltransferase CHSY-1 [14]. Thrombin mediated mRNA expression of CHSY-1 followed a similar pattern to the expression of C4ST-1 where it caused a parabolic time dependent increase in the mRNA expression (Figure 3B).

Thrombin treated VMSCs caused a 2-fold increase ($p<0.05$) in the mRNA expression of CHSY-1 at 4 hours and this was sustained reaching a peak of 3-fold ($p<0.01$) at 8 hours (Figure 3B).

XT-1 is an enzyme that catalyses the attachment of xylose, the first residue of the linkage region that links the CS GAG chain backbone to proteoglycan core protein. When examining the mRNA expression of XT-1 we observed a slight increase of 1.8-fold ($p<0.01$) at 4 hours post treatment (Figure 3C). However from 4 hours onwards there was no effect on the mRNA expression of XT-1. Together these results demonstrate that thrombin treatment of VSMCs leads to an increase in the mRNA expression of GAG chain initiation gene XT-1 and GAG elongation genes C4ST-1 and CHSY-1.

3.4 Thrombin stimulates the synthesis of C4ST-1 and CHSY1 proteins.

The protein expression of C4ST-1 and CHSY-1 was measured using western blotting to investigate whether the increase in the mRNA expression resulted in the increase in the protein expression of the respective GAG synthesizing enzyme. Treatment with thrombin stimulated the synthesis of C4ST-1 from 16-24 h (Figure 4A). The peak protein expression of C4ST-1 of 2-fold ($p<0.01$) was observed at 20 h. Thrombin treatment stimulated the expression of CHSY-1. An increase protein expression of CHSY-1 was observed from 16-24 h ($p<0.01$) (Figure 4B) with a peak of 2-fold reached at 20 h. Taken together these results demonstrate that the change in mRNA expression of C4ST-1 and CHSY-1 correlated with the protein expression of the respective enzymes.

3.5 Thrombin regulates the mRNA expression of genes associated with the elongation of heparin sulphate GAG chains

GAG chain synthesis requires the combined action of enzymes associated with polymerization and sulphation [29]. To investigate whether thrombin is involved in regulating the genes

associated with the elongation of heparin sulphate GAG chains the mRNA expression of extostatin-like glycosoltransferase (EXT)-1,2,3 and N-sulfotransferase-1 (NDST-1) was measured over an 8 hour time period (Figure 5). Treatment of VSMCs with thrombin generated a modest increase in the mRNA expression of EXT-1 to 1.5 fold ($p<0.05$)(Figure 5A) at 6 hours. VSMCs treated with thrombin had no effect on the mRNA expression of EXT-2 (Figure 5B). However thrombin treatment generated an increase in the mRNA expression of the EXT-3 enzyme (Figure 5C). A gradual stimulation was observed at 2-hours, which reached a peak stimulation of 1.4-fold ($p<0.01$) (Figure 5C) at 6-8 hours with thrombin treatment. NDST-1 involved in the sulphation of the heparin GAG chain was investigated. Treatment with thrombin over an 8 hour period had no effect on the mRNA levels of NDST-1. Taken toghether these results show that thrombin plays a modest role in regulating heparin sulphate synthezing enzymes EXT-1 and EXT-2 and is not involved in regulating EXT-2 and NDST-1 expression in VSMCs.

3.6 Serine/threonine kinases differentially regulate thrombin mediated mRNA expression of genes involved in the initiation and elongation of GAG chains

Our final aim was to evaluate the downstream consequences of Smad2 linker phosphorylation by measuring the expression of genes involved in GAG synthesis. Smad2 linker region phosphorylation occurs via cytosolic serine/threonine kinases [25]. Hence, we hypothesized that the serine/threonine kinases involved in phosphorylation of the Smad2 linker region will regulate thrombin mediated mRNA expression of C4ST-1 and CHSY-1.

VSMCs treated with thrombin for 6 hours showed a 2.9-fold increase in the mRNA expression of C4ST-1 compared to non-treated cells (Figure 6A). In the presence of UO126, SB202190, LY294002 and flavopiridol thrombin stimulated mRNA expression of C4ST-1 was

almost completely inhibited (Figure 6A). In the presence of the Jnk inhibitor SP600125 there was no inhibition of C4ST-1 mRNA expression. This data shows that Erk, p38, PI3K and CDK pathways are involved in thrombin stimulation of C4ST-1 mRNA expression.

Thrombin treatment of VSMCs showed a 2-fold increase of CHSY-1 mRNA expression (Figures 6B). Thrombin mediated mRNA expression of CHSY-1 was inhibited in the presence of Erk, p38, PI3K and CDK inhibitors (Figure 6B). Thrombin stimulated CHSY-1 mRNA expression was not blocked by Jnk inhibitor, SP600125. These data demonstrate that thrombin stimulation of CHSY-1 mRNA expression involved Erk, p38, PI3K and CDK but not Jnk. The serine/threonine kinases involved in thrombin mediated mRNA expression of GAG enzymes C4ST-1 and CHSY-1 are the same for both genes.

Thrombin mediated mRNA expression of C4ST-1 and CHSY-1 was not regulated by Jnk. We have previously demonstrated that Jnk, was not involved in TGF-β mediated proteoglycan synthesis and GAG elongation however its role in GAG chain initiation has not yet been investigated [20]. Our earlier results (Figure 2) showed that the Jnk inhibitor SP600125 has no effect on thrombin mediated phosphorylation of the serine residues (Ser245/250/255). However Jnk played a role in thrombin mediated phosphorylation of the Thr220 residue. Here we explored the role of the kinases which regulate the Thr220 residue and their role in the initiation of the GAG chain by studying mRNA expression of XT-1. VSMCs treated with thrombin showed a 3 fold increase in the mRNA expression of XT-1 (Figure 6C). In the presence of SP600125, LY294002 and SB202190 thrombin mediated mRNA expression of XT-1 was inhibited by approximately 50 percent to 2 fold (Figure 6C). This data shows that the Jnk, PI3K and p38 are involved in thrombin mediated mRNA expression of XT-1 and GAG chain initiation demonstrating the exquisite specificity of the signalling pathways regulating the synthesis of a single molecule – biglycan.

4. Discussion

Proteoglycan core protein synthesis as well as the hyperelongation of GAG chain results in increased binding to LDL causing increased lipid retention and accelerated atherosclerosis [2, 30]. Signalling pathways regulating proteoglycan synthesis and GAG chain elongation have therapeutic potential as targets for early onset of atherosclerosis [31]. Thrombin signalling pathways leads to proteoglycan synthesis, GAG elongation [10] and an increase in the mRNA expression of GAG synthesizing genes [11]. TGF-β mediated proteoglycan synthesis [20, 32] and GAG gene expression [19, 21] are regulated by the phosphorylation of transcription factor Smad2 in the linker region. Multiple signalling pathways are involved in Smad2 linker region phosphorylation [3, 8]. In this study we investigated the role of the phosphorylation of individual Smad2 linker region residues in thrombin mediated expression of GAG synthesising genes involved in the initiation and elongation of GAG chains.

The key findings of this study are that in VSMCs, treatment with thrombin can rapidly increase the phosphorylation of the 4 residues of the Smad2 transcription factors in its linker region. Thrombin treatment activated Erk, p38, PI3K and CDKs but not Jnk to mediate GAG chain hyperelongation measured as CHSY-1 and C4ST-1 mRNA expression. The same serine/threonine kinases activate the serine residues of the Smad2 linker region. Interestingly phosphorylation of the Thr220 residue and the mRNA expression of XT-1 were regulated by Jnk, p38 and PI3K, thus showing a correlation between the phosphorylation of the Thr220 and the initiation of GAG chain synthesis.

In VSMCs, TGF-β mediated Smad2 linker region phosphorylation of the serine residues occurs via the activation of Erk and p38 but not Jnk [19, 20]. In our study thrombin phosphorylation of the serine residues required Erk and P38 but not Jnk. However a Jnk inhibitor abolished thrombin phosphorylation of theThr220 residue. Thrombin signals via transactivation of a protein tyrosine kinase and a serine/threonine kinase receptor [33]. In our

earlier work we have shown that thrombin mediated Smad2 linker region phosphorylation partially occurs via transactivation of the EGFR [11]. There have been a number of studies showing that tyrosine kinase receptor agonists activate the Jnk pathway, leading to the phosphorylation of the Smad2/3 linker region. Hepatocyte growth factor treated RGM-1 cells activated the Jnk pathway, inducing phosphorylation of Smad2 linker region [34]. PDGF treated rat hepatic stellate cells rapidly increase the phosphorylation of Smad2/3 threonine residue which was inhibited in the presence of a Jnk antagonist. In human keratinocytes Jnk had no effect on thrombin mediated phosphorylation of the serine residues of the Smad2 linker region [35]. However in a rat model of liver injury, activation of Jnk was accompanied by phosphorylation of Smad2 Ser249 and 254 [36]. This shows that Jnk activation of the threonine residue of the Smad linker region may be specific for PTKR signalling in VSMCs.

The MAPK Erk regulated thrombin mediated phosphorylation of the Ser250 and Ser255 residues. In AKR-2B [37] and NIH/373 fibroblasts [38] TGF-β mediated phosphorylation of Ser245/250/255 is dependent on Erk. In CD4+ T helper cells Erk drives the phosphorylation of only the Ser255 residue [39]. A study from our lab showed that in BAECs, TGF-β activation of Erk leads to the phosphorylation of Ser245, Ser250 and Ser255 [27]. In TGF-β mediated phosphorylation of the Smad2 linker region in human VSMCs, Erk was involved in the phosphorylation of Ser245, Ser250 and Ser255 residues but not the phosphorylation of the Thr220 [19]. Interestingly, the Thr220 residue was not regulated by TGF-β mediated Erk phosphorylation in both BAECs and VSMCs and in our current thrombin model. Thrombin mediated mRNA expression of the two GAG genes (C4ST-1 and CHSY-1) involved in the elongation of GAG chains was regulated by thrombin. Thus these results are in agreement with our previous work that Erk driven pathways are associated with proteoglycan synthesis and in driving the expression of GAG synthesising genes in VSMCs [19, 20].

The present work identifies that thrombin phosphorylation of Ser245 and Ser255 is regulated by CDK. Similarly, in BAECs, CDK phosphorylates the Ser245 and Ser255 residues [27]. This shows that in these two cell models, CDK does not activate the Ser250 leading to PAI-1 expression or C4ST-1 and CHYS1 expression in VSMCs. Consistent with our results, CDK is not involved in the phosphorylation of the Thr220 residue in a melanoma model [40]. Direct activation of the TGFBR1 in VMSCs reveals that CDK is only involved in the phosphorylation of the Thr220 residue of the Smad2 linker region [19, 21]. Interestingly, unlike the model proposed by our own data, TGF-β stimulation of CDK family members has a greater incidence for the phosphorylation of the Thr220 than the serine residues [41-43]. PI3K could act as an upstream mediator for CDK activation [44]. Thrombin mediated phosphorylation of the Smad2 linker region residues Ser245 and Ser255 occurs via PI3K and CDK however Thr220 is only dependent on PI3K activation. PI3K was also shown to participate in thrombin mediated mRNA expression of C4ST-1, CHSY-1 and XT-1. PDGF and thrombin are known to activate PI3K via Akt [45]. Data from our lab (unpublished) shows that PI3K is partially involved in thrombin mediated proteoglycan synthesis. VSMCs treated with thrombin increased the phosphorylation of the serine residues of Smad2 linker region, this was unaffected by Akt inhibitor [45]. These results demonstrate that, in VSMCs, thrombin regulation of the Smad2 linker serine residues does not involve the PI3K/Akt signalling but instead requires the PI3K/CDK pathways, correlating with the results showing that thrombin mediated linker region phosphorylation does not involve Akt [45].

Thrombin mediated mRNA expression of C4ST-1 and CHSY-1 occurs via transactivation of TGFBR1 and the EGFR [11]. The mRNA expression of the GAG synthesizing enzymes correlates with proteoglycan synthesis and GAG elongation [46]. TGF-β mediated GAG chain elongation is associated with Smad2 linker region phosphorylation. The CHSY-1 enzyme is directly involved in addition of repeated monosaccharide units on

GAG chains, the sulfation enzymes C4ST-1 is involved in the sulfation of the GAG chain to provide docking sites for the addition of disaccharide units [47]. Thrombin can activate Erk, p38, PI3K and CDK to stimulate an increase in the mRNA expression of C4ST-1 and CHSY-1. Among the MAPKs involved in TGF-β signalling in VSMCs, Erk and p38 but not Jnk have been identified to modify GAG chains. In nucleus pulposus cells of invertebral discs, Erk participates in TGF-β regulation of CHSY-1 expression [48]. The data presented in this study is consistent with these two models.

Jnk did not regulate TGF-β mediated GAG gene [49] and GAG chain hyperelongation [20] in VSMCs. Thrombin phosphorylation of the Thr220 residue is regulated by Jnk, PI3K and p38. Interestingly thrombin mediated expression of the rate limiting genes involved in the elongation of GAG chains is not regulated by Jnk, however, the mRNA expression of the enzyme involved in the initiation of the GAG chains is regulated by Jnk, PI3K and p38. TGF-β mediated phosphorylation of the Smad2 Thr220 residue regulates XT-1 mRNA expression [19]. Knock down of XT-1 mRNA reduced GAG chain synthesis in cardiac fibroblasts [50]. Akt phosphorylation had no effect on TGF-β mediated GAG chain elongation however it played a role in proteoglycan synthesis [45]. In the current model of thrombin mediated GAG chain elongation as measured by the increase in the mRNA expression of C4ST-1 and CHSY-1, the PI3K/CDK not the PI3K/Akt pathway regulates GAG chain elongation, however the phosphorylation of the Thr220 residue was unaffected in presence of CDK inhibitor thus the Thr220 requires the activation of PI3K/Akt. Taken together the data indicates that phosphorylation of Thr220 residue regulates the expression of XT-1 gene.

5. Conclusions:

We have shown that PAR-1 mediated mRNA expression of two GAG synthesizing genes associated with the elongation of GAG chains on biglycan correlated with the phosphorylation

of the Smad2 linker region. However, PAR-1 mediated initiation GAG chain synthesis, as measured by the mRNA expression of XT-1, correlates to the phosphorylation of Thr220. Thrombin mediated GAG chain elongation requires pathways which activate Erk, p38 and PI3K/CDK but not Jnk, however the initiation of GAG chains require Jnk activation. These findings represent an example and a model of a concurrent signalling cascade which may provide a therapeutic target for human diseases, such as atherosclerosis, glomerulosclerosis and age related macular degeneration which have the underlying mechanism of lipid binding and entrapment by modified chondroitin sulfate/ dermatan sulfate proteoglycans.

Funding:

This work was supported by the University of Queensland Early Career Grant (Grant No. 1832825).

Competing interests

There are no competing interests

Figure 1: Time course of thrombin mediated phosphorylation of four specific Smad2 linker region residues. Western blots probed with antibodies specific to **A** phospho-Smad2/3(Thr220/179) **B** phospho-Smad2(Ser245) **C** anti-phospho-Smad2(Ser250) and **D** anti-phospho-Smad2(Ser255). Blots shown are representative of three independent experiments. Histograms represent band density expressed as fold per basal from three independent experiments (*$p<0.05$ vs basal) (**$p<0.01$ vs basal) using one-way ANOVA followed by least significant difference *post-hoc* analysis.

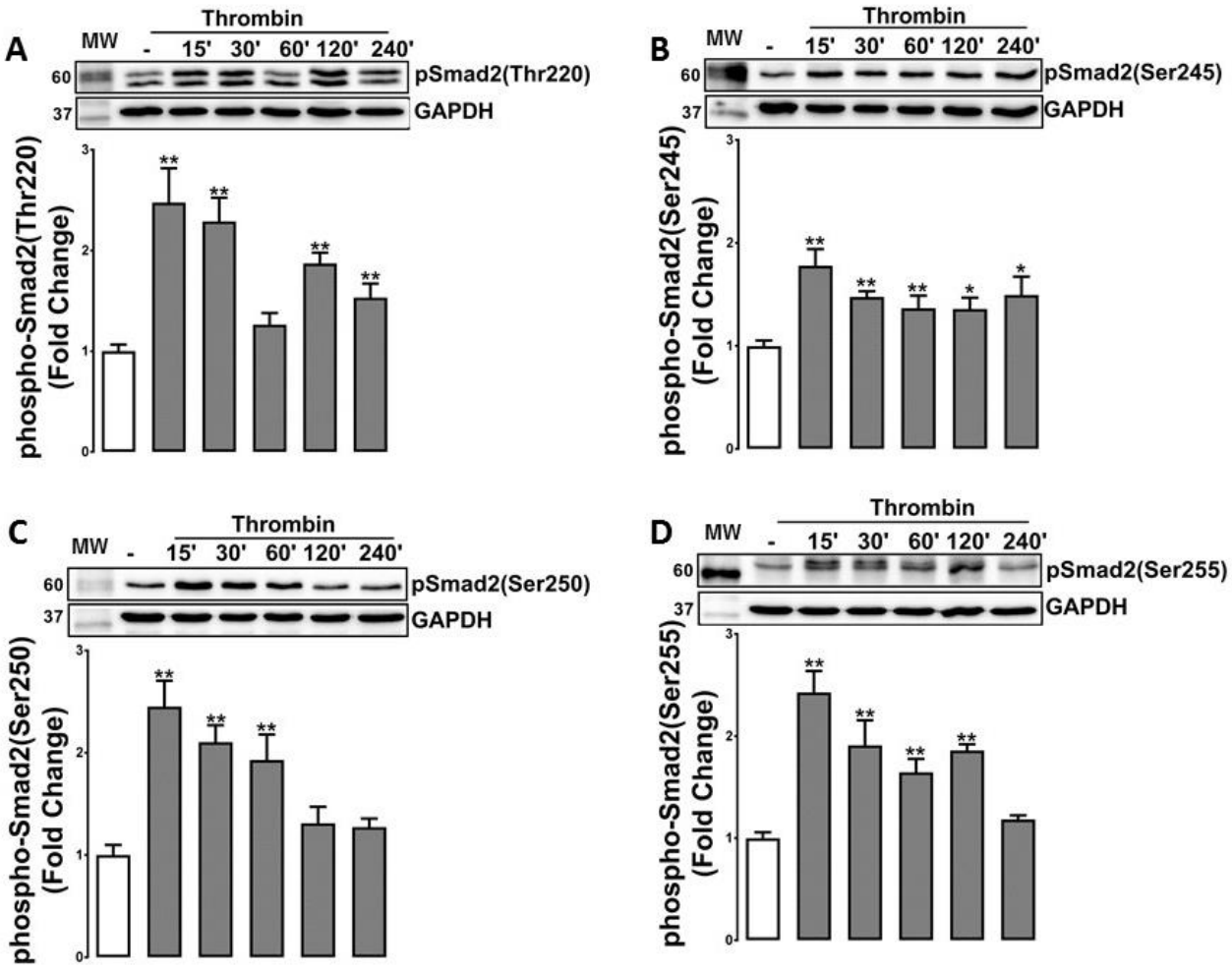


Figure 2: Effects of inhibitors to Erk, p38, Jnk, CDK and PI3K on thrombin mediated Smad2 linker phosphorylation via the transactivation of the EGFR. VSMCs were treated with thrombin for 15 min in the presence and absence of each of the serine/threonine kinase inhibitors. Each blot was probed with antibodies specific to **A.** phospho-Smad2/3(Thr220/179) **B.** phospho-Smad2(Ser245) **C.** phospho-Smad2(Ser250) and **D.** phospho-Smad2(Ser255). Blots are representative of three-independent experiments. Histogram represents band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis ##,$p<0.01$ versus basal and **, $p<0.01$ agonist versus antagonist.

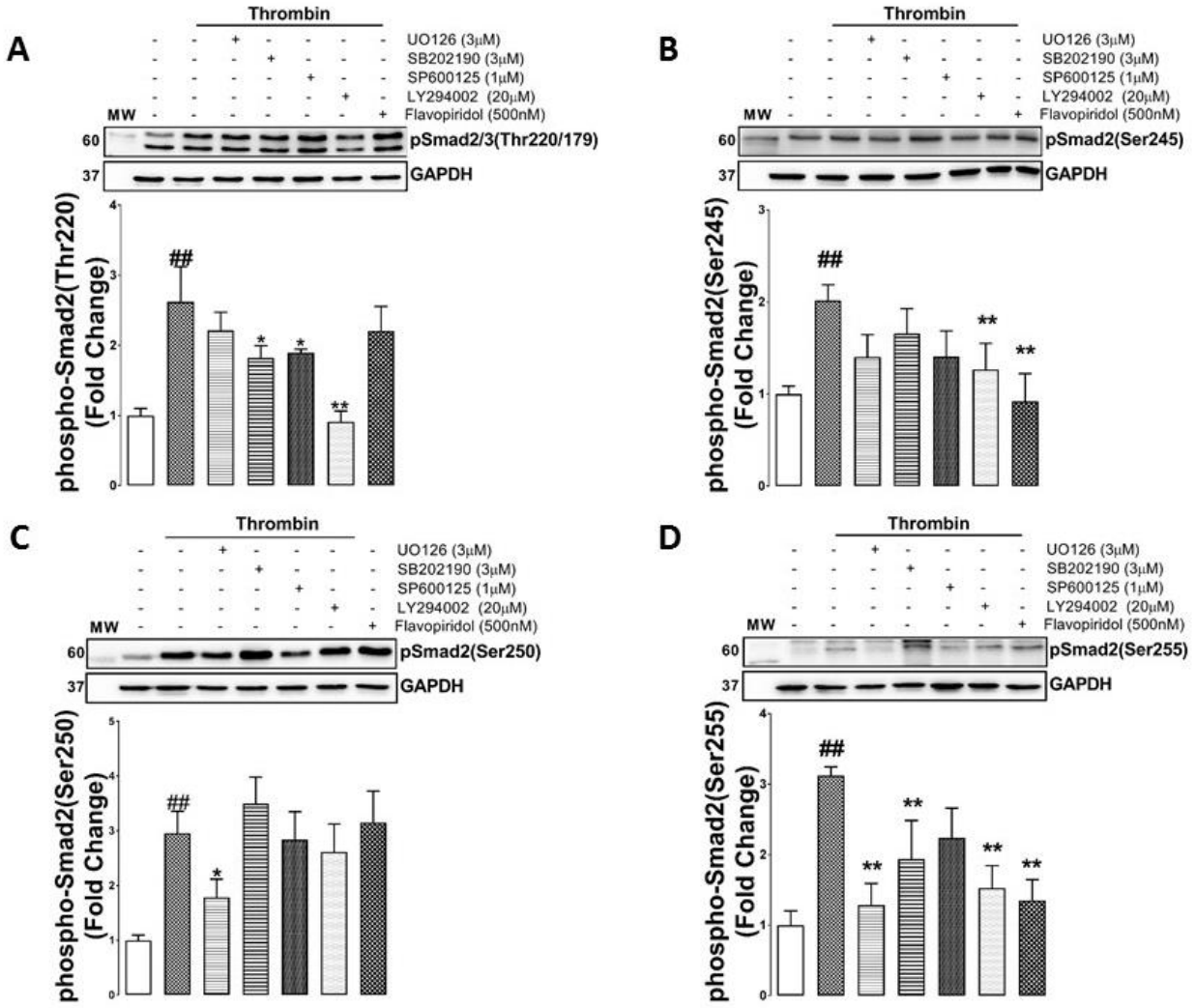
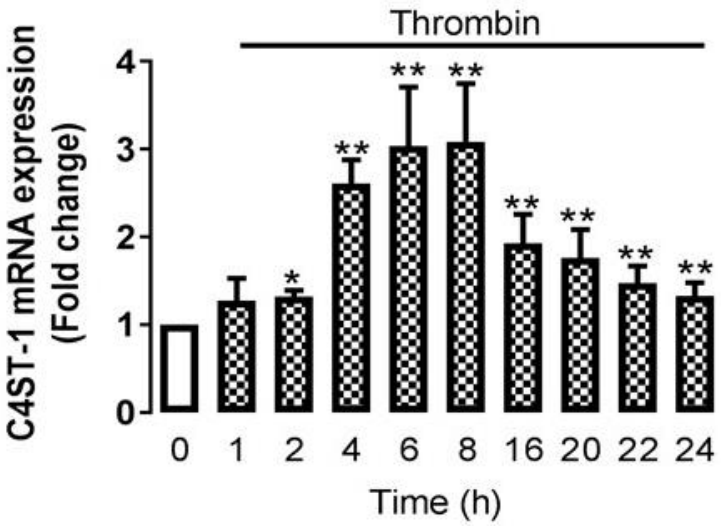
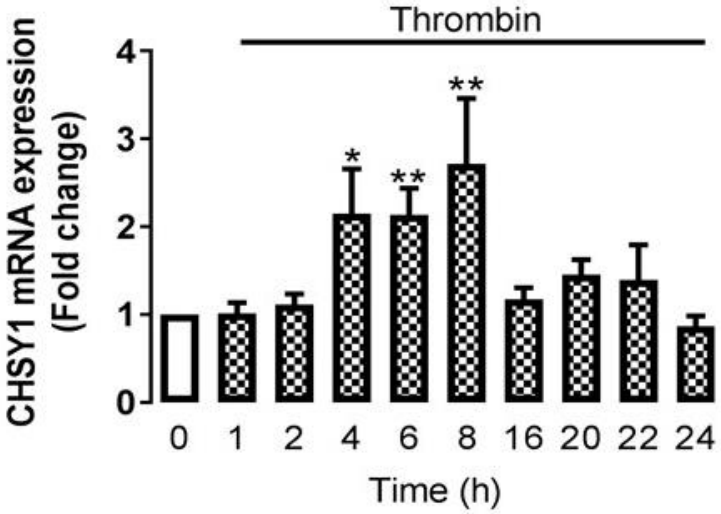


Figure 3: Time course of thrombin mediated mRNA expression of GAG synthesizing genes C4ST-1, CHSY-1 and XT-1. VSMCs were treated with thrombin (10units/ml) to investigate the effect of the mRNA expression of **A** C4ST-1, **B** CHSY-1 and **C** XT-1. Total RNA was harvested, cDNA was synthesized and the mRNA expression of these genes was analysed using quantitative RT-PCR. 18S was used as a house keeping gene. Results are expressed as the mean ± standard error from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by the least significance *post* hoc analysis, **$p<0.01$ and * $p<0.05$ basal versus agonist.

A



B



C

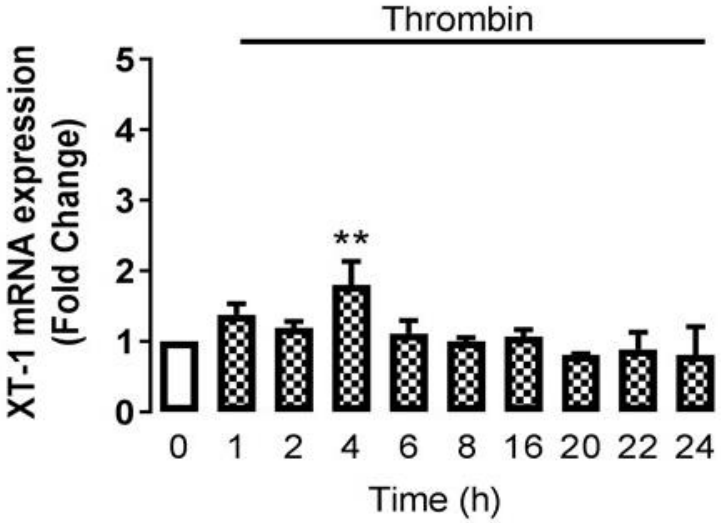


Figure 4: Thrombin time course of GAG synthesizing enzyme C4ST-1 and CHSY-1 protein expression. VSMCs were treated with thrombin (10 units/ml) for 16, 18, 20 and 24 hr. Western blots probed with antibodies specific to **A** C4ST-1 and **B** CHSY-1**.** Blots shown are representative of three independent experiments. Histograms represent band density expressed as fold per basal. Statistical significance was determined by one-way ANOVA followed by least significant difference *post-hoc* analysis ** $p<0.01$ versus non treated basal.

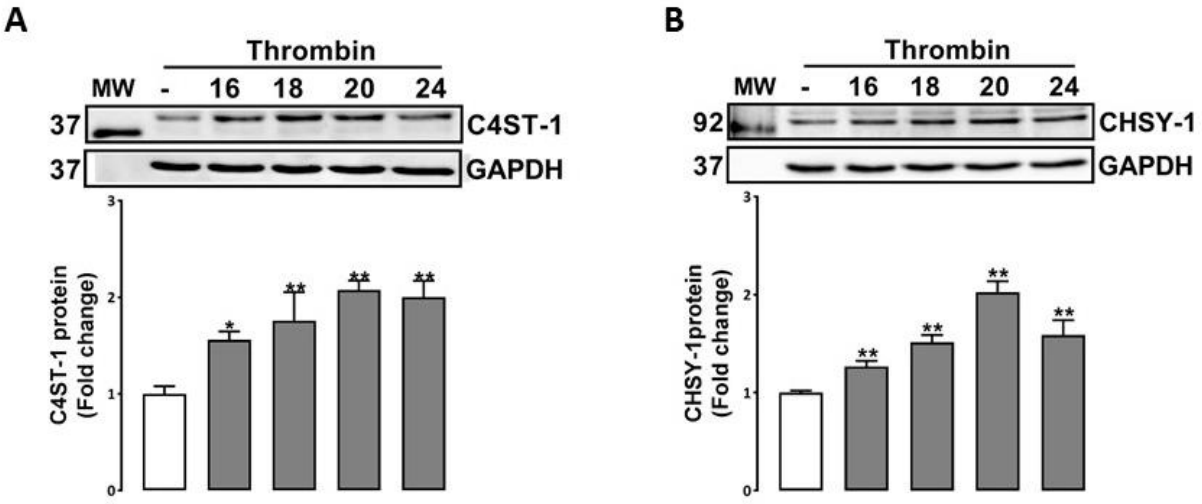


Figure 5: Time course of thrombin mediated mRNA expression of heparin sulfate synthesizing genes EXT1, EXT2, EXT3 and NDST1. VSMCs were treated with thrombin (10units/ml) to investigate the effect of the mRNA expression of **A** EXT1, **B** EXT2, **C** EXT3 and **D** NDST1. Total RNA was harvested, cDNA was synthesized and the mRNA expression of these genes was analysed using quantitative RT-PCR. 18S was used as a house keeping gene. Results are expressed as the mean ± standard error from three independent experiments. Statistical significance was determined by one-way ANOVA, followed by the least significance *post* hoc analysis, **$p<0.01$ and * $p<0.05$ basal versus agonist.

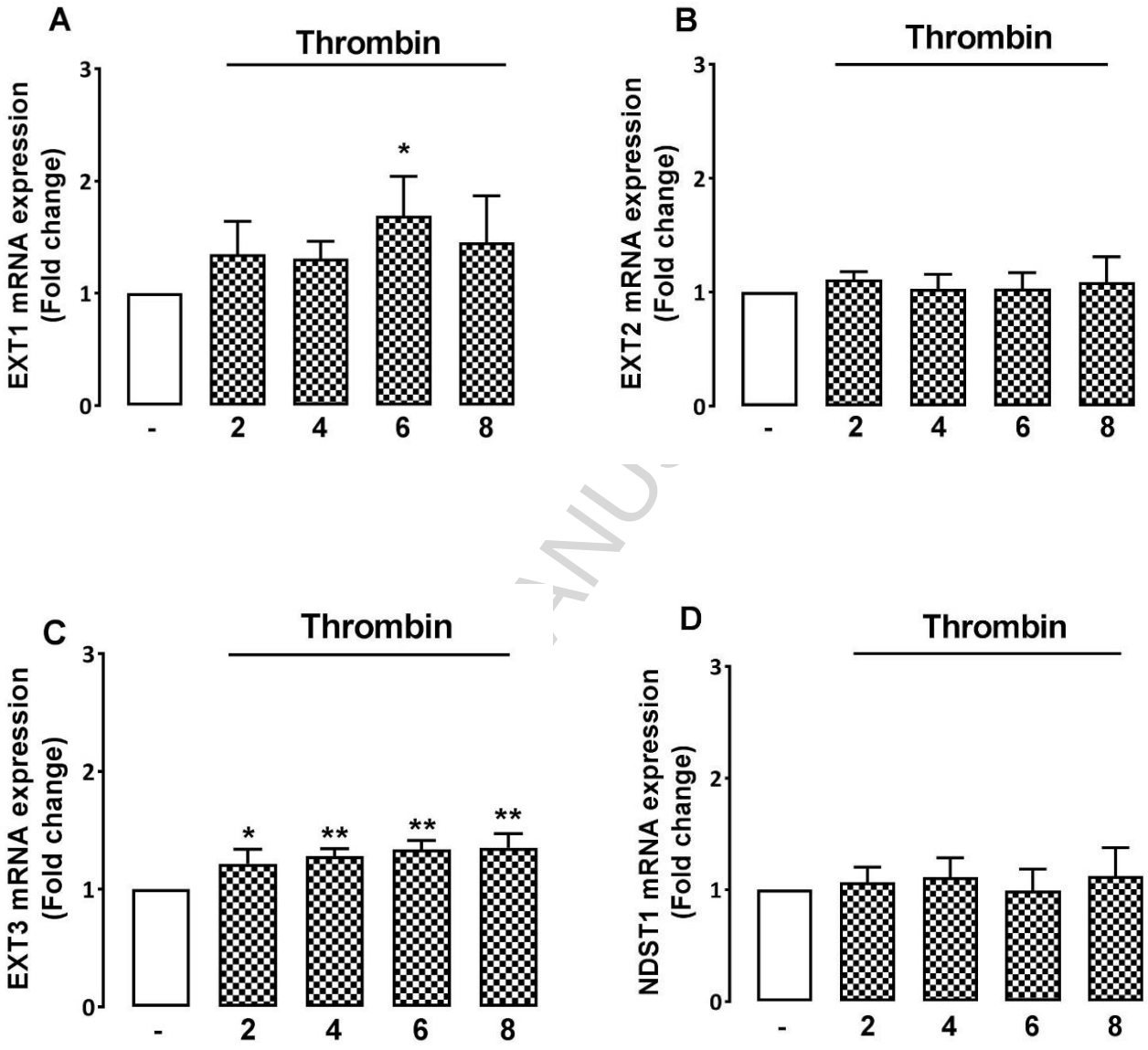
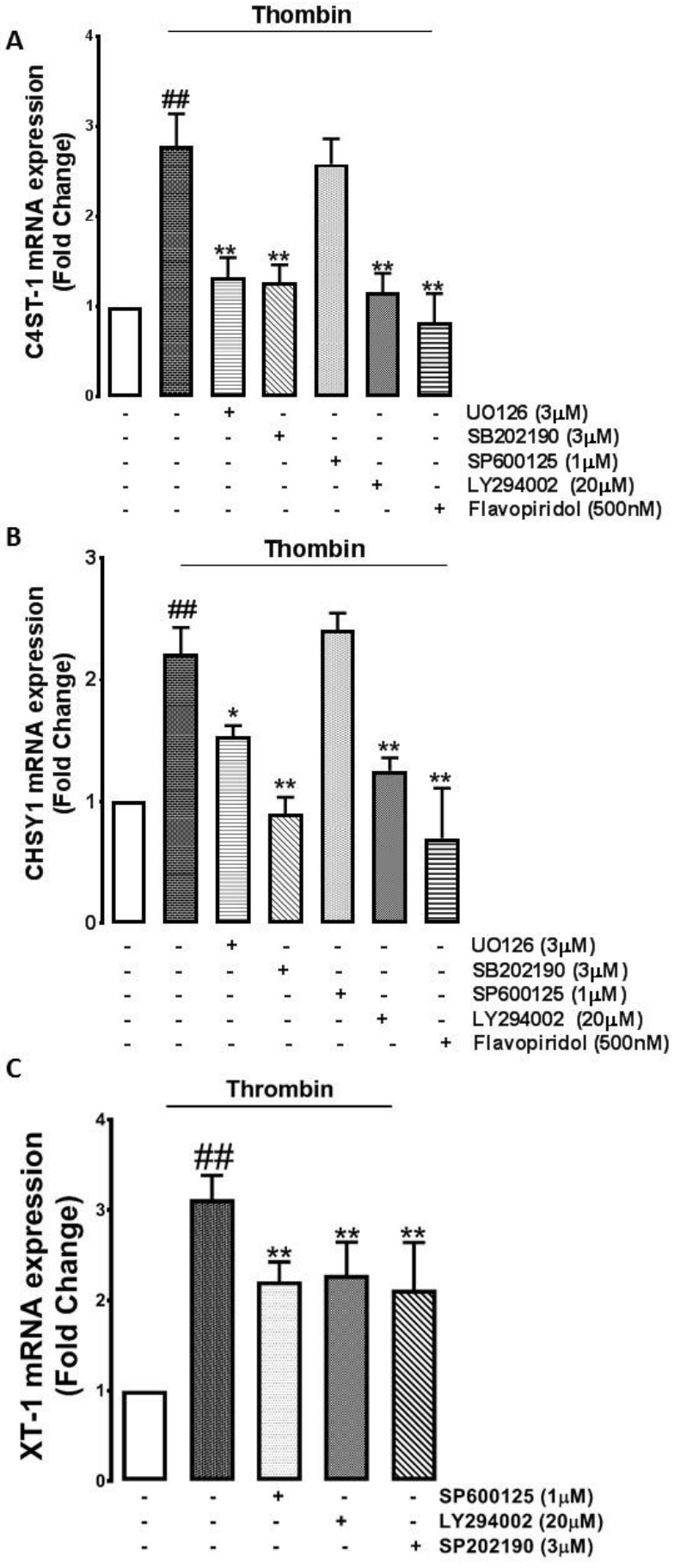


Figure 6: Serine/threonine kinases differentially regulate thrombin mediated mRNA expression of GAG synthesizing enzymes. VSMCs were pre-treated with serine/threonine kinase inhibitors in the presence of thrombin to determine the effects on the mRNA expression of **A.** C4ST-1, **B.** CHSY-1 and **C.** XT-1. Data are expressed as the mean ± standard error from three experiments. Statistical significance was determined by one-way ANOVA, followed by least significant difference *post-hoc* analysis. ##,$p<0.01$ basal versus thrombin and *,$p<0.01$ and **,$p<0.01$ thrombin versus antagonist was used for statistical analysis.



References

[1] P.J. Little, L. Tannock, K.L. Olin, A. Chait, T.N. Wight, Proteoglycans synthesized by arterial smooth muscle cells in the presence of transforming growth factor-beta1 exhibit increased binding to LDLs, Arterioscler Thromb Vasc Biol 22(1) (2002) 55-60.
[2] M.L. Ballinger, N. Osman, K. Hashimura, J. de Hann, K. Jandeleit-Dahm, T.J. Allen, L.R. Tannock, J.C. Rutledge, P.J. Little, Imatinib inhibits vascular smooth muscle proteoglycan synthesis and reduces LDL binding *in vitro* and aortic lipid deposition *in vivo*, J of Cell and Mol Med 14 (2010) 1408-1418.
[3] D. Kamato, M.L. Burch, T.J. Piva, H.B. Rezaei, M.A. Rostam, S. Xu, W. Zheng, P.J. Little, N. Osman, Transforming growth factor-beta signalling: Role and consequences of Smad linker region phosphorylation, Cell Signal 25(10) (2013) 2017-2024.
[4] H.B. Rezaei, D. Kamato, G. Ansari, N. Osman, P.J. Little, Cell biology of Smad2/3 linker region phosphorylation in vascular smooth muscle, Clin Exp Pharmacol Physiol 39(8) (2012) 661-7.
[5] M.L. Burch, N. Osman, R. Getachew, S. Al-Aryahi, P. Poronnik, W. Zheng, M.A. Hill, P.J. Little, G protein coupled receptor transactivation: extending the paradigm to include serine/threonine kinase receptors, Int J Biochem Cell Biol 44(5) (2012) 722-7.
[6] R. Chaplin, L. Thach, M.D. Hollenberg, Y. Cao, P.J. Little, D. Kamato, Insights into cellular signalling by G protein coupled receptor transactivation of cell surface protein kinase receptors, Journal of cell communication and signaling 11(2) (2017) 117-125.
[7] D. Kamato, M.A. Rostam, R. Bernard, T.J. Piva, N. Mantri, D. Guidone, W. Zheng, N. Osman, P.J. Little, The expansion of GPCR transactivation-dependent signalling to include serine/threonine kinase receptors represents a new cell signalling frontier, Cell Mol Life Sci 72(4) (2015) 799-808.
[8] P. Dayati, H.B. Rezaei, N. Sharifat, D. Kamato, P.J. Little, G protein coupled receptors can transduce signals through carboxy terminal and linker region phosphorylation of Smad transcription factors, Life Sci 199 (2018) 10-15.
[9] M.L. Burch, M.L. Ballinger, S.N. Yang, R. Getachew, C. Itman, K. Loveland, N. Osman, P.J. Little, Thrombin stimulation of proteoglycan synthesis in vascular smooth muscle is mediated by protease-activated receptor-1 transactivation of the transforming growth factor beta type I receptor, J Biol Chem 285(35) (2010) 26798-805.
[10] M.L. Burch, R. Getachew, N. Osman, M.A. Febbraio, P.J. Little, Thrombin-mediated proteoglycan synthesis utilizes both protein-tyrosine kinase and serine/threonine kinase receptor transactivation in vascular smooth muscle cells, J Biol Chem 288(10) (2013) 7410-9.
[11] D. Kamato, L. Thach, R. Getachew, M. Burch, M.D. Hollenberg, W. Zheng, P.J. Little, N. Osman, Protease activated receptor-1 mediated dual kinase receptor transactivation stimulates the expression of glycosaminoglycan synthesizing genes, Cell Signal 28(1) (2016) 110-9.
[12] P.J. Little, M.L. Burch, R. Getachew, S. Al-aryahi, N. Osman, Endothelin-1 stimulation of proteoglycan synthesis in vascular smooth muscle is mediated by endothelin receptor transactivation of the transforming growth factor-[beta] type I receptor, J Cardiovasc Pharmacol 56(4) (2010) 360-8.
[13] S.N.Y. Yang, N. Osman, M.L. Burch, P.J. Little, Factors affecting proteoglycan synthesis and structure that modify the interaction with lipoproteins, Clin Lipidol 4(4) (2009) 479-492.
[14] T. Mikami, H. Kitagawa, Biosynthesis and function of chondroitin sulfate, Biochim Biophys Acta 1830(10) (2013) 4719-33.
[15] K. Sugahara, H. Kitagawa, Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans, Curr Opin Struct Biol 10(5) (2000) 518-27.
[16] H. Kitagawa, K. Tsutsumi, M. Ujikawa, F. Goto, J. Tamura, K.W. Neumann, T. Ogawa, K. Sugahara, Regulation of chondroitin sulfate biosynthesis by specific sulfation: acceptor specificity of serum beta-GalNAc transferase revealed by structurally defined oligosaccharides, Glycobiology 7(4) (1997) 531-7.
[17] K.J. Grande-Allen, N. Osman, M.L. Ballinger, H. Dadlani, S. Marasco, P.J. Little, Glycosaminoglycan synthesis and structure as targets for the prevention of calcific aortic valve disease, Cardiovasc Res 76(1) (2007) 19-28.

[18] M. Kretzschmar, J. Doody, I. Timokhina, J. Massague, A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras, Genes Dev 13(7) (1999) 804-16.
[19] M.A. Rostam, D. Kamato, T.J. Piva, W. Zheng, P.J. Little, N. Osman, The role of specific Smad linker region phosphorylation in TGF-beta mediated expression of glycosaminoglycan synthesizing enzymes in vascular smooth muscle, Cell Signal 28(8) (2016) 956-66.
[20] M.L. Burch, S.N. Yang, M.L. Ballinger, R. Getachew, N. Osman, P.J. Little, TGF-beta stimulates biglycan synthesis via p38 and ERK phosphorylation of the linker region of Smad2, Cell Mol Life Sci 67(12) (2010) 2077-90.
[21] M.A. Rostam, A. Shajimoon, D. Kamato, P. Mitra, T. Piva, R. Getachew, Y. Cao, W. Zheng, N. Osman, P.J. Little, Flavopiridol inhibits TGF-beta-stimulated biglycan synthesis by blocking linker region phosphorylation and nuclear translocation of Smad2, J Pharmacol Exp Ther (2018).
[22] C. Ponighaus, M. Ambrosius, J.C. Casanova, C. Prante, J. Kuhn, J.D. Esko, K. Kleesiek, C. Gotting, Human xylosyltransferase II is involved in the biosynthesis of the uniform tetrasaccharide linkage region in chondroitin sulfate and heparan sulfate proteoglycans, J Biol Chem 282(8) (2007) 5201-6.
[23] V.Y. Anggraeni, N. Emoto, K. Yagi, D.S. Mayasari, K. Nakayama, T. Izumikawa, H. Kitagawa, K. Hirata, Correlation of C4ST-1 and ChGn-2 expression with chondroitin sulfate chain elongation in atherosclerosis, Biochem Biophys Res Commun 406(1) (2011) 36-41.
[24] D. Kamato, M.A. Rostum, R. Bernard, T.J. Piva, N. Mantri, D. Guidone, W. Zheng, O. N., P.J. Little, The expansion of GPCR transactivation-dependent signalling to include serine/threonine kinase receptors represents a new cell signalling frontier, Cellular and Molecular Life Sciences In press (2014).
[25] K. Matsuzaki, Smad phosphoisoform signaling specificity: the right place at the right time, Carcinogenesis 32(11) (2011) 1578-88.
[26] K. Matsuzaki, C. Kitano, M. Murata, G. Sekimoto, K. Yoshida, Y. Uemura, T. Seki, S. Taketani, J. Fujisawa, K. Okazaki, Smad2 and Smad3 phosphorylated at both linker and COOH-terminal regions transmit malignant TGF-beta signal in later stages of human colorectal cancer, Cancer Res 69(13) (2009) 5321-30.
[27] D. Kamato, M.A. Rostam, T.J. Piva, H. Babaahmadi Rezaei, R. Getachew, L. Thach, R. Bernard, W. Zheng, P.J. Little, N. Osman, Transforming growth factor beta-mediated site-specific Smad linker region phosphorylation in vascular endothelial cells, J Pharm Pharmacol 66(12) (2014) 1722-33.
[28] G. Sekimoto, K. Matsuzaki, K. Yoshida, S. Mori, M. Murata, T. Seki, H. Matsui, J. Fujisawa, K. Okazaki, Reversible Smad-dependent signaling between tumor suppression and oncogenesis, Cancer Res 67(11) (2007) 5090-6.
[29] J. Kreuger, L. Kjellen, Heparan sulfate biosynthesis: regulation and variability, J Histochem Cytochem 60(12) (2012) 898-907.
[30] P.J. Little, N. Osman, K.D. O'Brien, Hyperelongated biglycan: the surreptitious initiator of atherosclerosis, Current Opinion in Lipidology 19 (2008) 448-454.
[31] R. Afroz, Y. Cao, M.A. Rostam, H. Ta, S. Xu, W. Zheng, N. Osman, D. Kamato, P.J. Little, Signalling pathways regulating galactosaminoglycan synthesis and structure in vascular smooth muscle: Implications for lipoprotein binding and atherosclerosis, Pharmacol Ther (2018).
[32] D. Kamato, H. Babaahmadi Rezaei, R. Getachew, L. Thach, D. Guidone, N. Osman, B. Roufogalis, C.C. Duke, V.H. Tran, W. Zheng, P.J. Little, (S)-[6]-Gingerol inhibits TGF-beta-stimulated biglycan synthesis but not glycosaminoglycan hyperelongation in human vascular smooth muscle cells, J Pharm Pharmacol 65(7) (2013) 1026-36.
[33] D. Kamato, M.L. Burch, N. Osman, W. Zheng, P.J. Little, Therapeutic implications of endothelin and thrombin G-protein-coupled receptor transactivation of tyrosine and serine/threonine kinase cell surface receptors, J Pharm Pharmacol 65(4) (2013) 465-73.
[34] S. Mori, K. Matsuzaki, K. Yoshida, F. Furukawa, Y. Tahashi, H. Yamagata, G. Sekimoto, T. Seki, H. Matsui, M. Nishizawa, J. Fujisawa, K. Okazaki, TGF-beta and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions, Oncogene 23(44) (2004) 7416-29.

[35] N. Talati, D. Kamato, T.J. Piva, P.J. Little, N. Osman, Thrombin promotes PAI-1 expression and migration in keratinocytes via ERK dependent Smad linker region phosphorylation, Cell Signal 47 (2018) 37-43.
[36] K. Yoshida, K. Matsuzaki, S. Mori, Y. Tahashi, H. Yamagata, F. Furukawa, T. Seki, M. Nishizawa, J. Fujisawa, K. Okazaki, Transforming growth factor-beta and platelet-derived growth factor signal via c-Jun N-terminal kinase-dependent Smad2/3 phosphorylation in rat hepatic stellate cells after acute liver injury, Am J Pathol 166(4) (2005) 1029-39.
[37] C. Hough, M. Radu, J.J. Dore, Tgf-beta induced Erk phosphorylation of smad linker region regulates smad signaling, PLoS One 7(8) (2012) e42513.
[38] F. Li, B. Zeng, Y. Chai, P. Cai, C. Fan, T. Cheng, The linker region of Smad2 mediates TGF-beta-dependent ERK2-induced collagen synthesis, Biochem Biophys Res Commun 386(2) (2009) 289-93.
[39] J.H. Yoon, K. Sudo, M. Kuroda, M. Kato, I.K. Lee, J.S. Han, S. Nakae, T. Imamura, J. Kim, J.H. Ju, D.K. Kim, K. Matsuzaki, M. Weinstein, I. Matsumoto, T. Sumida, M. Mamura, Phosphorylation status determines the opposing functions of Smad2/Smad3 as STAT3 cofactors in TH17 differentiation, Nat Commun 6 (2015) 7600.
[40] K.A. Cohen-Solal, K.T. Merrigan, J.L. Chan, J.S. Goydos, W. Chen, D.J. Foran, F. Liu, A. Lasfar, M. Reiss, Constitutive Smad linker phosphorylation in melanoma: a mechanism of resistance to transforming growth factor-beta-mediated growth inhibition, Pigment cell & melanoma research 24(3) (2011) 512-24.
[41] K. Yumoto, P.S. Thomas, J. Lane, K. Matsuzaki, M. Inagaki, J. Ninomiya-Tsuji, G.J. Scott, M.K. Ray, M. Ishii, R. Maxson, Y. Mishina, V. Kaartinen, TGF-beta-activated kinase 1 (Tak1) mediates agonist-induced Smad activation and linker region phosphorylation in embryonic craniofacial neural crest-derived cells, J Biol Chem 288(19) (2013) 13467-80.
[42] I. Matsuura, N.G. Denissova, G. Wang, D. He, J. Long, F. Liu, Cyclin-dependent kinases regulate the antiproliferative function of Smads, Nature 430(6996) (2004) 226-31.
[43] C. Alarcon, A.I. Zaromytidou, Q. Xi, S. Gao, J. Yu, S. Fujisawa, A. Barlas, A.N. Miller, K. Manova-Todorova, M.J. Macias, G. Sapkota, D. Pan, J. Massague, Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways, Cell 139(4) (2009) 757-69.
[44] M.L. Major, R. Lepe, R.H. Costa, Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators, Mol Cell Biol 24(7) (2004) 2649-61.
[45] N. Osman, R. Getachew, M. Burch, G. Lancaster, R. Wang, H. Wang, W. Zheng, P.J. Little, TGF-beta stimulates biglycan core protein synthesis but not glycosaminoglycan chain elongation via Akt phosphorylation in vascular smooth muscle, Growth Factors 29(5) (2011) 203-10.
[46] M.L. Burch, W. Zheng, P.J. Little, Smad linker region phosphorylation in the regulation of extracellular matrix synthesis, Cell Mol Life Sci 68(1) (2011) 97-107.
[47] T. Izumikawa, Y. Okuura, T. Koike, N. Sakoda, H. Kitagawa, Chondroitin 4-O-sulfotransferase-1 regulates the chain length of chondroitin sulfate in co-operation with chondroitin N-acetylgalactosaminyltransferase-2, Biochemical Journal 434(2) (2011) 321-331.
[48] B. Hu, C. Shi, Y. Tian, Y. Zhang, C. Xu, H. Chen, P. Cao, W. Yuan, TGF-β Induces Up-Regulation of Chondroitin Sulfate Synthase 1 (CHSY1) in Nucleus Pulposus Cells Through MAPK Signaling, Cellular Physiology and Biochemistry 37(2) (2015) 793-804.
[49] M.A. Rostam, D. Kamato, T.J. Piva, W. Zheng, P.J. Little, N. Osman, The role of specific Smad linker region phosphorylation in TGF-β mediated expression of glycosaminoglycan synthesizing enzymes in vascular smooth muscle, Cellular signalling 28(8) (2016) 956-966.
[50] C. Prante, H. Milting, A. Kassner, M. Farr, M. Ambrosius, S. Schon, D.G. Seidler, A.E. Banayosy, R. Korfer, J. Kuhn, K. Kleesiek, C. Gotting, Transforming Growth Factor beta1-regulated Xylosyltransferase I Activity in Human Cardiac Fibroblasts and Its Impact for Myocardial Remodeling, J Biol Chem 282(36) (2007) 26441-9.

Highlights

- Thrombin stimulates Smad linker region phosphorylation
- Thrombin stimulates the expression of glycosaminoglycan synthesising genes
- Serine residues of Smad linker region correlated with GAG chain elongation
- Threonine residue of Smad linker region correlated with GAG chain initiation