Role of non-Smad signaling pathways in transforming growth factor beta (TGFβ)-induced expression of chondroitin sulfate proteoglycans (CSPGs) by reactive astrocytes

By

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Abstract

Chondroitin sulphate proteoglycans (CSPGs) from the glial scar inhibit axonal regeneration following spinal cord injury. CSPG expression can be induced by transforming growth factor β (TGFβ), which suggests that inhibition of TGFβ may reduce CSPG levels. Astrocytes were treated with cyclic AMP (cAMP), which reduced TGFβ signaling protein Smad2 in astrocytes. However, cAMP-treated astrocytes showed strong neurocan expression following TGFβ treatment, which suggests that TGFβ may mediate CSPG expression through non-Smad pathways. Smad2 or Smad4 were knocked down in astrocytes using siRNA and TGFβ-induced neurocan, brevican and aggrecan expression were still observed, indicating that Smad signaling is not required for CSPG expression. Administration of a PI3K/Akt inhibitor produced significant reductions in neurocan, brevican and aggrecan expression in astrocytes, which suggests that PI3K/Akt pathway mediates CSPG expression. Erk1/2 inhibitor treatment did not reduce CSPG expression significantly. Targeting non-Smad signaling pathways may therefore be effective strategies to reduce CSPG expression following injury.

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Dedication

I would like to dedicate this thesis to my parents MD. Harunur Rashid and Jesmin Jahan who made a lot of sacrifices for my future and always inspired me.

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List of Acronyms and Abbreviations

AA	Arachidonic Acid
BDNF	Brain Derived Neurotrophic Factor
BNP	Brain Natriuretic Peptide
CCI	Cortical Compression Injury
CGN	Cerebellar Granular Neuron
chABC	Chondroitinase ABC
CNS	Central Nervous System
CS	Chondroitin Sulfate
DMEM	Dulbecco's Modified Eagle’s Medium
DMSO	Dimethyl Sulfoxide
DRG	Dorsal Root Ganglion
DSPG	Dermatan Sulfate Proteoglycan
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial-mesenchymal Transition
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GDNF	Glial cell line Derived Neurotrophic Factor
GF	Growth Factor
GFAP	Glial Fibrillary Acidic Protein

GPI	Glycophosphatidylinositol
HSPG	Heparan Sulfate Proteoglycan
IFN	Interferon
JNK	c-Jun N-terminal kinases
KSPG	Keratan Sulfate Proteoglycan
MAG	Myelin Associated Glycoprotein
MAPK	Mitogen Activated Protein Kinase
mTOR	Mammalian Target of Rapamycin
OPCs	Oligodendrocyte Precursor Cells
PDE4	Phosphodiesterase-4
PDGF	Platelet Derived Growth Factor
PDK1	Phosphoinositide-dependant kinase-1
PGE2	Prostaglandin E2
PIP2	Phosphatidylinositol 4,5 bisphosphate
PI3K	Phosphoinositide 3-Kinase
PTEN	Phosphatase and tensin homolog
SARA	Smad anchor for receptor activation
SEMA 3	Semaphorin 3
siRNA	Small interference RNA
TGF β	Transforming Growth Factor beta
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Introduction

1.1 Astrocytes

Astrocytes are a type of glial cell and are the most abundant type of cell found in the human central nervous system (CNS). They are derived from a heterogeneous population of glial stem cells of the embryonic neuroepithelium (Gariano et al., 1996). There are two major subtypes of astrocytes, namely fibrous and protoplasmic astrocytes (Sofroniew and Vinters, 2010). Fibrous astrocytes are predominantly found in the white matter and protoplasmic astrocytes are distributed in the grey matter (Bushong et al., 2002). Morphological variations are observed between these two subtypes. Fibrous astrocytes have long thread like processes with which they make contact with surrounding astrocytes and these processes also establish connection between the blood vessels and nodes of Ranvier (Sofroniew and Vinters, 2010). Protoplasmic astrocytes are found as a globe shaped structure because of abundant branching of their processes (Sofroniew and Vinters, 2010). There are some specialized astrocytes such as Muller glia in the retina and Bergmann glia in the cerebellum that have morphologies and antigenic properties similar to protoplasmic astrocytes (Barres BA., 2008).

Astrocytes express glial fibrillary acid protein (GFAP) which acts as a marker for their immunohistochemical identification (Eng et al., 2000). GFAP expression is not exclusive to astrocytes and it can be expressed by some other cells of the CNS such as radial neural stem cells (Sofroniew and Vinters, 2010). GFAP is also expressed by other non neural tissues such as enteric glial cells, mesenchymal stellate cells, hepatic stellate cells and stellate cells present in kidney and lung (Sofroniew and Vinters, 2010). Some other astrocyte markers that have been identified include glutamate synthase and S100β (Goncalves et al., 2008), but they are non specific and not exclusive to astrocytes. Therefore GFAP is being used as a common astrocyte marker (Sofroniew and Vinters, 2010).

1.1.1 Roles in synapse formation and synaptic transmission

Astrocytes play an important role in formation of synapses by releasing thrombospondin. Thrombospondin induces formation of synapses that contain active presynaptic protein, synapsin and silent postsynaptic protein, PSD-95 (Barres BA, 2008). Astrocytes also secrete another protein, not yet identified, which induces glutamate sensitivity in the post synaptically silent synapses and makes them active (Barres BA, 2008). They play a direct role in synaptic transmission by releasing neurotransmitters such as glutamate, purines, GABA and D-serine and thereby astrocytes are considered essential elements of synapse in the ‘tripartite synapse’ hypothesis (Sofroniew and Vinters, 2010). According to tripartite synapse theory, a synapse must contain 3 elements which are presynaptic components, post synaptic components and the astrocytes (Halassa et al., 2006).

1.1.2 Roles in fluid, ion and pH homeostasis

Astrocytic processes are rich in Aquaporin4 (AQP4) water channel that increases permeability to water (Kimelberg and Nedergaard, 2010). Having AQP4, astrocytes form the major route for water transport into and out of the brain and thus astrocytes maintain fluid homeostasis in neurons (Kimelberg and Nedergaard, 2010). Astrocytes from the blood brain barrier region contain carbonic anhydrase enzymes and they have sodium proton co transporters (3 HCO_3^- plus 2 Na^+) and Cl^-/ HCO_3^- exchangers through which they maintain ionic balance of the CNS (Kimelberg and Nedergaard, 2010).

1.1.3 Regulation of blood flow to CNS and roles in CNS metabolism

Astrocytic processes come in direct contact with the CNS vasculature and release molecular mediators like prostaglandins (PGE), nitric oxide (NO) and arachidonic acid (AA) which can increase or decrease CNS blood vessel diameter and thus they can control CNS blood flow in response to changes in neuronal activity (Zonta et al., 2003). Astrocytes are the primary site for glycogen storage in the brain and they play significant role in CNS metabolism by transporting glucose metabolites and glutamate through astrocytic gap junctions during periods of increased neuronal activity (Sofroniew and Vinters, 2010). In hypoglycaemic conditions, stored glycogen from astrocytes breaks down to lactate and serves as the preferred energy substrate for neurons (Sofroniew and Vinters, 2010).

1.1.4 Other functions of astrocytes

During development, astrocytes form molecular boundaries that repel the developing axons and neuroblasts and keep them in the right tract to their targets and thus they guide the migration of these axons (Powell and Geller, 1999). Within synapses, astrocytes induce expression of complement C1q and mark them for removal by microglia which helps in developmental pruning of synapses (Barres BA., 2008). Astrocytes contain several anti oxidants such as superoxide dismutases and catalases which can neutralize reactive oxygen species and protect neurons from free radical injury (Kimelberg and Nedergaard, 2010). Astrocytic processes ensheathe groups of neurons and retraction of this ensheathement causes increased interaction between the neurons (Kimelberg and Nedergaard, 2010). As a result of this interaction, amount of hormone secretion from the neurons are also increased (Kimelberg and Nedergaard, 2010).

1.2 Astrocytes in relation to CNS pathologies

Astrocytic structural or functional changes are related to different CNS pathologies. Functional alteration of astrocytes is linked with different neurodegenerative diseases like amyotrophic lateral sclerosis, in which defective glutamate transportation by astrocytes occurs in the spinal cord and in the cerebral cortex (Rothstein et al., 1992). Multiple sclerosis is another degenerative disease where interaction between astrocytes and microglia are disrupted which leads to demyelination of axons (Markiewicz and Lukomska, 2006). In Alzheimer's disease, astrocytic release of cytokines leads to the

formation of amyoloid β particle and this amyloid deposition initiates neuronal damage (Markiewicz and Lukomska, 2006). Astrocytes also play a role in Huntington's disease where astrocytes fail to uptake excess glutamate and accumulated glutamate leads to neurodegeneration in the cerebellar cortex (Markiewicz and Lukomska, 2006). Another important example of a degenerative disease is glaucoma, where lack of astrocytic antioxidant functions results in increased production of reactive oxygen species. This leads to axonal damage in the optic nerve head and can give rise to glaucomatous condition (Sofroniew and vinters, 2010). Certain metabolic diseases are also associated with structural abnormalities of astrocytes such as aceruloplasminemia, Niemann-pick type C, congenital glutamate synthatase deficiency and pyruvate carboxylase deficiency (Oide et al., 2006; Patel et al., 1999). In stroke, loss of astrocytic water channel AQP4 function may generate vasogenic oedema in the brain (Kimelberg and Nedergaard, 2010). Astrocytes can modulate calcium signaling pathways and alteration in this signaling process may generate seizure and epileptic conditions (Tian et al., 2005). Some genetic disorders are also associated with developmental alteration of astrocytes such as Alexander disease or fibrinoid leukodystrophies where the main pathology is mutation of the gene encoding GFAP (Aoki et al., 2001).

1.3 Astrogliosis

Astrogliosis is defined as an increase in the number and size of astrocytes. Reactive astrogliosis refers to a wide range of structural, functional and molecular changes that occur in astrocytes as a consequence of CNS injuries and it is regulated by different signaling molecules (Sofroniew MV, 2009). Molecules that can trigger astrogliosis include cytokines and growth factors such as interleukins, neurotransmitters like glutamate, noradrenaline, molecules of oxidative stress such as NO, reactive oxygen species and amyloid β (Sofroniew MV, 2009). Many different intracellular signaling pathways are associated with astrogliosis, such as STAT3, NFκB, SOCS3, Nrf2 and cyclic AMP pathways (Sofroniew MV, 2009).

Astrogliosis is not a single isolated process, but a highly coordinated process in terms of expression of genes and cellular changes (Sofroniew MV, 2009). Based on its microscopic appearances, reactive astrogliosis has been classified into three broad categories which are mild to moderate reactive astrogliosis, severe diffuse reactive astrogliosis, and severe reactive astrogliosis with compact scar formation (Sofroniew and Vinters, 2010). In mild to moderate astrogliosis, GFAP expression can vary from low to high and cells are hypertrophied with preservation of normal cellular orientation (Sofroniew and Vinters, 2010). In severe diffuse astrogliosis, there is modest up regulation of GFAP and other genes, and cellular hypertrophy becomes prominent with disruption of normal cellular architecture (Sofroniew and Vinters, 2010). The third variety of astrogliosis includes changes associated with mild and severe diffuse forms of

astrogliosis along with compact scar formation (Sofroniew and Vinters, 2010). In its mild to moderate grade, astrocytes retain the potential to resolve the triggering stimulus and can return to normal healthy state (Bushong et al., 2002). At its extreme level of activation, reactive astrogliosis leads to formation of a glial scar with newly proliferated cells and astrocytes permanently lose the features of their normal uninjured state (Sofroniew MV, 2009).

A large number of studies have reported neuroprotective roles of reactive astrocytes. Reactive astrocytes have been reported to protect CNS tissues from oxidative stress and prevent neuronal death via glutathione production (Chen et al., 2001). After stroke or in obstructive hydrocephalus, ablation of reactive astrocytes results in aggravation of vasogenic edema and faulty BBB repair which indicates its essential defensive role (Bush et al., 1999). Selective ablation of reactive astrocytes following moderate contusion injury induced by controlled cortical impact (CCI) in mice was found to increase lesion size, enhance neuronal damage and cause massive demyelination (Myer et al., 2006). Clinical studies have also reported that certain disease conditions are precipitated by loss of reactive astrocytes, such as multiple sclerosis where autoimmune destruction of astrocytes disrupts connection with blood vessel and induces CNS inflammation (Markiewicz and Lukomska, 2006).

Besides its beneficial roles, reactive astrogliosis also has a number of detrimental effects. Production of the transcription factor nuclear factor (NF)-kappa B by astrocytes induces inflammation and secondary damage following spinal cord injury and hampers functional

recovery after contusive injury to the spinal cord (Brambilla et al., 2005). Reactive astrocytes can initiate formation of excessive free radicals and toxic nitric oxide (NO) by cytokine stimulation which propagates tissue damage (Hamby et al., 2006). It should be noted that other studies have reported that cytokine stimulated NO production was primarily caused by microglial cells and that astrocytes just accelerate NO production by microglia (Possel et al., 2000; Sola et al., 2002). Reactive astrocytes contribute seizure generation by releasing excitotoxic glutamate that can initiate synchronized firing of neurons (Tian et al., 2005). Evidence has shown that in autoimmune encephalitis, reactive astrocyte-derived vascular endothelial growth factor (VEGF) leads to disruption of the BBB (Argaw et al., 2009).

1.4 Glial scar

The most well known feature of reactive astrogliosis is the formation of the glial scar. The term glial scar describes the structure that is formed in regions of CNS injury by the glial cells of the CNS in response to a chronic injury (Sofroniew MV, 2009). The glial scar is a dynamic structure where different cells participate in its formation at different times following injury (Sofroniew MV, 2009). The main cell types involved in scar formation are astrocytes, microglia with a few oligodendrocytes and meningeal cells (Fawcett and Asher, 1999). The first cells to form the scar are microglia and ultimately the scar is predominantly astrocytic (Fawcett and Asher, 1999).

The most important function of the glial scar is sealing off injured areas of the CNS to limit spreading of lesions and to prevent leaking of reactive oxygen species and free radicals into the uninjured environment (Rolls et al., 2009). Another function of the glial scar is to control the activity of immune cells. Astrocytes within the glial scar secrete immune modulating molecules like transforming growth factor beta (TGFβ), tumor necrosis factor alpha (TNFα) and proteoglycans at sites of neuronal injury to reduce immune mediated tissue damage (Rolls et al., 2009). Reactive astrocytes and secreted chondroitin sulfate proteoglycans (CSPGs) also aid in neurogenesis by regulating proliferation and differentiation of neural progenitor cells (Rolls et al., 2009). All these examples represent the beneficial effects of glial scar in CNS repair following injury; however, the glial scar is better known for its inhibitory effects on axonal regeneration after injury.

1.5 Glial scar limits CNS regeneration

The glial scar contains reactive astrocytes and extracellular matrix molecules which act as a physical barrier for regenerating axons at sites of injury (Mckeon et al., 1991). Initially, it was considered only a mechanical barrier but in recent times multiple studies have reported that the molecular composition of the glial scar is responsible for regeneration failure in the CNS (Fawcett and Asher, 1999; Silver and Miller, 2004). Reactive astrocytes within the glial scar up regulate growth inhibitory molecules like tenascin, semaphorin 3, ephrin B2, Slit proteins and chondroitin sulfate proteoglycans (CSPGs) (McKeon et al., 1995). Tenascin is an extracellular matrix molecule produced by

astrocytes and oligodendrocytes, which is up regulated after CNS injury and can inhibit axon growth by interaction with the specific cell surface molecule F3/11 (Fawcett and Asher, 1999). Semaphorin 3 (SEMA 3) is expressed by astrocytes and fibroblasts after injury and acts as a chemorepellent. Regenerating axons are repelled from the area containing SEMA3 (Silver and Miller, 2004). Ephrin B2 is a transmembrane glycoprotein which binds to Eph receptors and regulates tissue patterning, cell migration and axonal guidance during development (Bundesen et al., 2003). Ephrin B2 is also expressed after spinal cord injury by reactive astrocytes which interact with ephrin expressing meningeal fibroblasts and form a basal lamina called the glia limitans, which acts as a barrier for axonal regeneration (Bundesen et al., 2003). Among all these factors, proteoglycans of the glial scar play the most important role to hamper regeneration (McKeon et al., 1995). Astrocytes, the main cell type of glial scar, are capable of producing four classes of proteoglycans, including heparan sulfate proteoglycans (HSPGs), dermatan sulfate proteoglycans (DSPGs), keratin sulfate proteoglycans (KSPGs) and chondroitin sulfate proteoglycans (CSPGs) (Johnson-Green et al., 1991). CSPGs are the major inhibitory molecules from this family which has been evidenced by reduction of neural outgrowth both *in vivo* and *in vitro* (McKeon et al., 1991).

1.6 Chondroitin sulfate proteoglycans (CSPGs)

CSPGs are proteoglycans consisting of a protein core with a varying number of sulfated glycosaminoglycan (GAG) side chains. The core protein is usually a glycoprotein and the GAG side chains are attached to the core by covalent bonds (Bartus et al., 2012). One single GAG chain can consist of up to 50 disaccharide subunits and the length of the side chain varies depending upon the type of CSPG (Kwok et al., 2012). The core protein is synthesized in the Golgi apparatus and CS-GAG chains are synthesized in endoplasmic reticulum (Kwok et al., 2012). Then, enzymatic modification occurs in the form of phosphorylation or sulfation in the linkage or in the CS chain region (Kwok et al., 2012).

CSPGs perform a wide variety of functions during development by aiding in cellular migration, cell adhesion, receptor binding, synaptogenesis and axonal guidance (Bartus et al., 2012). With maturation of the CNS, levels of CSPG expression are reduced and their activity is decreased (Bartus et al., 2012). The growth inhibitory activity of CSPGs was demonstrated *in vitro* by reduced neurite outgrowth in an explant culture. The culture substrate was collected from a filter paper that was inserted into the rat cortex to produce injury and growth of neurites was inhibited in specific areas of culture where inhibitory molecules like CSPGs were upregulated (McKeon et al., 1991). CSPGs are inhibitory *in vivo* as well which was shown by reduced extension of transplanted adult sensory axons in the adult rat spinal cord after a dorsal column lesion. Dorsal root ganglion neurons were injected into the injured white matter of the spinal cord, a few millimeters rostral to the lesion site and outgrowth of sensory axons was found at the transplantation site. No growth was observed at the lesion epicenter where the immunoreactivity for CSPGs was

high (Davies et al., 1999). This suggests that CSPGs are a major impediment for regeneration *in vivo*. *In vivo* expression of CSPGs following injury was also measured in a model of glial scarring after cortical injury. A piece of Millipore filter paper was surgically implanted into the rat cerebral cortex and kept for 14 days to induce *in vivo* gliosis. High immunoreactivity for CSPGs was found in that filter paper compared with the uninjured cortex (McKeon et al., 1999). CSPGS are not only inhibitory for regeneration; they also hamper sensory and motor functions of animal following spinal cord injury. Enzymatic degradation of CSPGs by chondroitinase ABC treatment has been shown to promote recovery of locomotor functions and proprioceptive behaviours (Bradbury et al., 2002). Another study showed that Sox 9 knock-out mice expressed reduced levels of CSPGs following injury, which was accompanied by improved hind limb function and locomotor recovery (Mckillop et al., 2013).

It has been shown that CSPG mediates its inhibitory function by binding to a receptor called protein tyrosine phosphatase sigma (PTPσ). PTPσ has a specific immunoglobulin like domain which binds with the chondroiton sulfate (CS) chain and PTPσ fusion proteins colocalize with areas of CSPG expression in the injured adult CNS (Shen et al., 2009). PTPσ knock-out neurons showed less inhibition of neurite outgrowth by CSPGs in culture, which indicates that PTPσ is a functional receptor for CSPGs (Shen et al., 2009). The leukocyte common antigen related phosphatase (LAR) is another functional receptor, identified for CSPGs and neurons from LAR knock-out mice were shown to overcome inhibition by CSPGs (Fisher et al., 2011).

More than 15 CSPGs have been identified, including members of the the lectican family such as neurocan, aggrecan, brevican and versican (Morgenstern et al., 2002). NG2 and Phosphacan are structurally unrelated CSPGs which are synthesized as transmembrane proteins but can undergo proteolytic cleavage like other CSPGs (Morgenstern et al., 2002). NG2 is primarily expressed by oligodendrocyte precursor cells (OPCs) in the normal CNS and very few astrocytes express NG2 following injury (Morgenstern et al., 2002). Expression of NG2 is upregulated 4 to 7 days following a lesion and returns to normal levels by 14 days post injury. Therefore this molecule does not exert much inhibitory effect following spinal cord injury (Morgenstern et al., 2002).

Phosphacan is only expressed by neural tissues and reactive astrocytes have been shown to express phosphacan in an *ex vivo* model of the glial scar (Mckeon et al., 1991). Phosphacan may exert inhibitory or promoting effects, which is determined by various factors like neuronal age and type, molecular nature of its side chains and also upon the pattern of its glycosylation (Morgenstern et al., 2002). Phosphacan has been shown to inhibit outgrowth of dorsal root ganglion (DRG) neurons on a laminin substrate and this inhibitory effect was not decreased by chondroitinase ABC treatment (Morgenstern et al., 2002). Phosphacan was shown to promote neurite outgrowth of rat hippocampal neurons as well and this growth promoting effect was abolished by removal of GAG side chains with chondroitinase ABC treatment (Morgenstern et al., 2002). Expression of phosphacan decreases 3 days after spinal cord injury, starts to increase 4 weeks after injury and remained elevated up to 8 weeks post injury (Jones et al., 2003).

Versican is expressed by both neural and non-neural tissues (Asher et al., 2000). Within the CNS, it has been shown to be expressed by OPCs, not by astrocytes or microglia (Asher et al., 2002). Immunoreactivity of versican is upregulated from its basal level by 24 hours after injury, peaked at 2 weeks and remained elevated up to 4 weeks after injury (Jones et al., 2003). No evidence has been found showing axonal growth inhibition by versican (Fawcett and Asher, 1999).

1.7 Neurocan

Neurocan is a member of the lectican family and a cellular constituent of normal extracellular matrix (ECM) (Rauch et al., 2001). It is composed of two homologous domains of N-terminal and C-terminal regions with a central mucin like region (Rauch et al., 2001). The N-terminal domain consists of one immunoglobulin (Ig) moiety and two linker fragments (Rauch et al., 2001). The C-terminal domain is composed of two epidermal growth factor (EGF) like domains, a C-type lectin, and a Sushi fragment with a C-terminal extension of 45 amino acids (Rauch et al., 2001). The central region contains the core protein, which is 600 amino acids long (Rauch et al., 2001). Four forms of neurocan have been detected. They are intact neurocan, neurocan C at C-terminal fraction, neurocan 130 and neurocan 90 at N-terminal end; the latter 3 arise from proteolytic cleavage of intact neurocan (Asher et al., 2000). The molecular weight of full length neurocan is 220 KDa and the molecular weight of neurocan C is 150 KDa (Asher et al., 2000). Regarding its cellular origin, neurocan is primarily expressed by astrocytes and also by OPCs (Morgenstern et al., 2002). Expression of neurocan is significantly

increased from its basal level by 1 day after injury at the lesion site, peaks at 2 weeks after injury throughout the lesioned tissue, and remains elevated up to 4 weeks post injury (Jones et al., 2003). Levels of neurocan start to decrease at 8 weeks after injury (Jones et al., 2003). The inhibitory action of neurocan was shown in an *in vitro* assay where laminin-coated coverslips were coated with stripes of neurocan and neurite outgrowth was strongly inhibited on those stripes (Asher et al., 2000). Neurocan shows molecular interaction with ECM molecules like heparin, hyaluronan, tenascin C and tenascin R (Rauch et al., 2001). It can also interact with cell surface molecules such as neural cell adhesion molecule (N-CAM), neuron-glia cell adhesion molecule (Ng-CAM), Tag1/axonin1, tenascin and PTPσ through which it can inhibit axon growth (Fawcett and Asher, 1999; Shen et al., 2009). Neurocan expression by astrocytes is regulated by different cytokines like transforming growth factor β (TGFβ) and epidermal growth factor (EGF), where TGFβ and EGF have been shown to upregulate its expression and interferon gamma (IFN γ) and platelet derived growth factor (PDGF) were found to decrease the expression of neurocan (Asher et al., 2000).

1.8 Aggrecan

Aggrecan is a major constituent of the articular cartilage and belongs to the lectican family of proteoglycans (Kiani et al., 2002). It is a heavy protein having a molecular mass of more than 2500 kDa where the molecular weight of its core protein is 200-250 KDa (kiani et al., 2002). It contains more than 100 chondroitin sulphate chains of 20 KDa each (Kiani et al., 2002). The core protein of aggrecan is composed of three globular domains:

G1, G2 and G3, and three extended domains: inter globular domain (IGD), keratan sulfate (KS) and chondroitin sulfate (CS) (Kiani et al., 2002). Aggrecan is found in the CNS and in the spinal cord during embryonic development and also in adult life (Lemons et al., 2001). Aggrecan is predominantly expressed by neurons and it has been shown to be expressed by astrocytes in an *in vitro* motility assay where aggrecan inhibited motility of Schwann cells on astrocytic mono layers (Afshari et al., 2010). Aggrecan can inhibit axonal growth which was demonstrated in a rat spinal cord hemisection injury model where aggrecan inhibited the growth of implanted DRG neurons (Lemons et al., 2003). Following spinal cord injury, expression of aggrecan was markedly decreased in the lesion site and no elevation was observed by 28 days post injury (Andrews et al., 2012). Expression of aggrecan can be modulated by cytokine stimulation and TGFβ was shown to induce aggrecan expression in a chondrogenic cell line (Lemons et al., 2001; Watanabe et al., 2001).

1.9 Brevican

Brevican is a brain specific CSPG and is the smallest member of the lectican family of proteoglycans (Morgenstern et al., 2002). The core protein of brevican has a molecular weight of about 145 KDa and contains a G1 domain at the N terminal end, a G3 domain in the C terminal end and no G2 domain (Morgenstern et al., 2002; Frischknecht and Seidenbecher, 2012). Brevican is the only lectican which can exist as a splice variant: a glycophosphotidylinositol (GPI) anchored form which lacks the G3 domain (Frischknecht and Seidenbecher, 2012). Brevican is expressed by astrocytes, neurons and

OPCs but the GPI anchored isoform is expressed by mature oligodendrocytes only (Morgenstern et al., 2002). Brevican usually localizes to neuronal surfaces and being a substrate for a number of matrix metalloproteases, it plays an important role in formation of perineuronal net (Frischknecht and Seidenbecher, 2012). Brevican is a potent growth inhibitor which was shown to inihibit neurite extension in an *in vitro* stripe assay where stripes were coated with brevican on a laminin substrate (Yamada et al., 1997).

In the intact spinal cord, expression of brevican is low but following injury, brevican expression is markedly increased from its basal level by 24 hours after injury. Expression peaks at 2 weeks and remains elevated up to 2 months after injury (Jones et al., 2003). Brevican expression can be induced in astrocytes *in vitro* and TGFβ has been shown to upregulate the expression of brevican in astrocytes (Hamel et al., 2005).

1.10 Transforming growth factor β (TGFβ)

Transforming growth factor beta (TGFβ) is a multifunctional protein which belongs to the TGFβ superfamily, consisting of the activin/inhibin family, bone morphogenetic proteins (BMPs), TGFβ subfamily, growth differentiation factors (GDFs) and glial cell line derived neurotrophic factors (GDNF) family (Kubiczkova et al., 2012). Three different isoforms of TGFβ have been identified. TGFβ1 is an isoform localized in cartilage, bone skin and low levels in the CNS (Kubiczkova et al., 2012; Wilcox and Derynck, 1988). TGFβ2 is another isoform which is expressed by embryonic neurons and astroglial cells (Kubiczkova et al., 2012). TGFβ3 is the third isoform, which was detected

in adenocarcinamatous lung, kidney carcinoma cell line and in umbilical cord (Kubiczkova et al., 2012). TGFβ plays a crucial role in normal cell physiology by regulating cell growth, tissue homeostasis, cell proliferation, differentiation and apoptosis (Kubiczkova et al., 2012).

1.10.1 Role of TGFβ in spinal cord injury

TGFβ has been found to play an important role in spinal cord injury. After injury, levels of both TGFβ1 and TGFβ2 mRNA are increased in the lesioned area and high levels of TGFβ2 were found in the reactive astrocytes of the glial scar (Lagord et al., 2002). This increased TGF β has been associated with excessive glial scar formation and impaired locomotor function after SCI, as administration of TGFβ neutralizing antibody at the injury site in rat spinal cord was shown to promote locomotor recovery with suppressed gliosis (Kohta et al., 2009). TGFβ has been shown to induce expression of inhibitory CSPG molecules like neurocan, phosphacan,brevican and aggrecan in astrocytes (Asher et al., 2000; Smith and Strunz, 2005; Hamel et al., 2005; Watanabe et al., 2001).

1.10.2 TGFβ receptors and TGFβ-Smad signaling

Three types of receptors have been shown to mediate TGFβ signaling. They are TGFβ receptor type I (TβR I), type II (TβR II) and type III (TβR III) (Cheifetz et al., 1987). Astrocytes have been shown to express TβR I & TβR II (Vivien et al., 1998). Active TGFβ at first binds to TβR II and forms a complex with TβR I, which leads to

phosphorylation of TβR I and activates it (Massague et al., 1998). Activated TβR I induces phosphorylation of Smad2/3 and phosphorylated Smad2/3 form a heterotrimeric complexes with the Co Smad, Smad4 and then this complexes translocates into the nucleus (Massague et al., 1998). This mechanism is common to all of the receptors for the TGFβ family.

1.11 Smad signaling pathway

Smads are intracellular signaling proteins which regulate gene expression via transcription. Smad proteins consist of 500 amino acids with 2 domain structures connected by a linker region (Massague et al., 2005). The N terminal region, also called mad homology 1 (MH1) domain, serves as the DNA binding module, whereas the C-terminal domain or MH2 domain, acts as the binding site for different transcriptional activators or repressors (Massague et al., 2005). The linker region acts as a phosphorylation site for the serine/threonine kinases (Massague et al., 2005). There are 3 classes of Smads, which are receptor regulated Smads (R Smads), inhibitory Smads (I Smads) and common mediator Smads (Co Smads) (Massague et al., 1998). R Smads include Smad1, Smad2, Smad3, Smad5 and Smad8 and they act as substrates for the TGFβ family of receptors (Massague et al., 2005). More specifically, Smad2 and Smad3 act as substrates for the TGFβ and activin receptors, while Smad1, Smad5, and Smad8 act as substrates for the BMPs receptors (Massague et al., 2005). Smad6 and Smad7 are I Smads which inhibit receptor binding to R Smads and also prevent Smad-Smad interactions (Massague et al., 2005). Smad4 is the only Co-Smad and it serves as a

common and essential binding partner for all Smads in order to facilitate internalization into the nucleus where they can regulate transcription (Massague et al., 2005).

Smad signaling is initiated by phosphorylation of R Smads, which facilitates a continuous shuttling of RSmads into the nucleus (Massague et al., 2005). This shuttling is mediated by decreasing affinity of RSmads for cytoplasmic anchors and increasing their affinity for nuclear factors (Massague et al., 2005). The most important cytoplasmic anchor for Smad2 and Smad3 is the protein Smad anchor for receptor activation (SARA) (Massague et al., 2005). SARA is a multi domain protein containing a Smad binding domain (SBD) and phospholipid binding domains. Interaction of SARA with Smad2/3 inhibits their translocation into the nucleus; but receptor mediated phosphorylation of RSmads decreases the affinity of Smad2/3 for SARA, allows them to form complex with Smad4, and allows them to translocate into the nucleus (Massague et al., 2005). Inside the nucleus, they form transcriptional complexes which can either induce gene expression by recruiting transcriptional coactivators or inhibit gene expression by recruiting corepressors to the target promoters (Massague et al., 2005). As a result of concomitant dephosphorylation of RSmads, they are transported back to the cytoplasm and start another cycle of receptor mediated phosphorylation (Massague et al., 2005).

1.12 Involvement of cyclic AMP (cAMP) in Smad signaling pathway

In addition to inhibitory molecules of the glial scar, there are some extrinsic and intrinsic factors in the CNS environment which can contribute to regeneration failure (Afshari et

al., 2009). CNS myelin is an important extrinsic fator that inhibits regeneration after spinal cord injury (Caroni and Schwab, 1988). Three major inhibitors of growth have been identified within myelin, which are myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp) and Nogo A (Domeniconi and Filbin, 2005). MAG is a transmembrane protein present in the CNS and peripheral nervous system (PNS) that inhibits axonal growth through interaction with Nogo receptors and gangliosides (Afshari et al., 2009). OMgp is also found in the CNS, where it causes growth cone collapse and prevents regeneration (Afshari et al., 2009). Nogo A was the first inhibitor to be isolated from myelin and it induces growth cone collapse through a calcium dependent mechanism (Fawcett et al., 1999). Application of the Nogo-A-blocking monoclonal antibody IN-1 promotes axonal sprouting in the injured adult CNS both *in vitro* and *in vivo* (Schnell and Schwab, 1990).

Different strategies have been applied to overcome myelin mediated inhibition of axonal regeneration. One of the most successful approaches has been the elevation of intracellular cyclic AMP, which was shown to promote cerebellar neurite outgrowth on a substrate of myelin in a dose dependant manner (Cai et al., 1999). Cyclic AMP can be elevated in different ways, such as using cyclic AMP analogues like dibutyryl cyclic AMP, performing peripheral conditioning lesion of the sciatic nerve, priming with neurotrophins like brain derived neurotrophic factor (BDNF) or by treating with phosphodiesterase inhibitors, such as rolipram (Hannila and Filbin, 2008). Further studies established that elevation of intracellular cyclic AMP is sufficient not only to overcome myelin inhibition, but it is also capable of inducing spinal axon regeneration

(Qiu et al., 2002). Therefore cyclic AMP is considered a key player for promoting regeneration through the hostile CNS environment following injury.

In 2008, Dr. Azad Bonni's lab identified an important association between the TGFβ signaling protein Smad2 and CNS myelin inhibition (Stegmuller et al., 2008). They showed that siRNA knockdown of Smad2 results in enhanced neurite outgrowth on myelin, which indicates that Smad2 plays an important role in myelin mediated inhibition (Stegmuller et al., 2008). This finding was further confirmed in Dr. Filbin's lab where they showed increased outgrowth of cerebellar granular neurons (CGN) on a myelin substrate after treating with Smad2 siRNA (Hannila et al., 2013). They have also demonstrated that CGN treated with MAG or Nogo have increased phosphorylation of Smad2, which suggests that activation of Smad2 is an important step for myelin mediated inhibition (Hannila et al., 2013). Taken together, this evidence suggests that suppression of Smad2 expression might be a useful approach to stimulate axonal growth on myelin.

Furthermore, the Filbin lab discovered an effective way to suppress Smad2 expression in neuron by elevating levels of cyclic AMP. They showed that treatment with dbcAMP could significantly reduce expression of Smad2 in CGN, cortical and DRG neurons (Hannila et al., 2013).

1.13 Non-Smad pathways for TGF β

Smad pathways are the canonical mediators for the signaling response from TGFβ (Zhang YE, 2009), but TGFβ can exert cellular responses through non Smad pathways as well. For apoptosis in epithelial cells and in hepatocytes, type II TGFβ receptor was found to interact with an adaptor protein called Daxx that initiates activation of JNK pathway and induces apoptosis (Moustakas and Heldin, 2005). Epithelial mesenchymal transition (EMT) is a crucial event during embryogenesis which is usually regulated by TGFβ through Smad pathways. Activated TGFβ was found to phosphorylate a non-Smad protein: par 6, which recruits ubiquitin ligase Smurf 1 for dissolution of the actin cytoskeleton and thus induces EMT in epithelial cells (Moustakas and Heldin, 2005). In migratory breast cancer cells, large amounts of TGFβ are produced locally which activates phosphatidylinositol-3-kinase (PI3K)/Akt and Erk pathways for cell motility with no actions via Smads (Moustakas and Heldin, 2005). This suggests that non-Smad pathways are important for mediating cellular responses from TGFβ and the most studied non-Smad pathways include the PI3K/Akt pathway and branches of mitogen activated protein kinase (MAPK) pathways (Zhang Y E, 2009).

1.14 Phosphatidylinositol-3kinase (PI3K)/Akt pathway

Phosphatidylinositol is a phospholipid of the cell membrane and the enzymes that phosphorylate the phosphatidylinositol molecules are called phosphoinositide kinases (Fruman et al., 1998). Phosphatidylinositol-3 kinase (PI3K) is one of these enzymes and

is further divided into three classes designated as class I PI3K, class II PI3K and class III PI3K (Fruman et al., 1998). Class I PI3K is again subdivided into Class IA and class IB which consists of a catalytic subunit p110 and the regulatory subunits p85α, p85β, p55α, p55γ and p50α (Engelman et al., 2006). Class IA PI3K is activated by receptor protein tyrosine kinases (RPTK) and class IB is activated by G protein coupled receptors (Vara et al., 2004). Activation of Class I PI3K results in generation of phosphatidylinositol-3, 4, 5-triphosphate (PIP3) from phosphatidyl inositol bisphosphate (PIP2). PIP3 is a second messenger which leads to recruitment of an important mediator, Akt, in the plasma membrane (Vara et al., 2004). Within the plasma membrane, Akt is phosphorylated and activated by phosphoinositide dependant kinase (PDK-1) (Nicholson and Anderson, 2002). Phosphatase and tensin homolog (PTEN) is a phosphatase which can reverse this process by converting PIP3 into PIP2 (Engelman et al., 2006). Mutation or deletion of PTEN causes hyperactivation of PI3K/Akt signaling, leading to increased cellular proliferation (Engelman et al., 2006). Once the PI3K/Akt signaling pathway is activated, Akt mediates activation of different target genes resulting in cellular growth, cell proliferation, cell survival, cytoskeletal organization, vesicle trafficking, glucose transport and regulation of platelet function (Nicholson and Anderson, 2002; Fruman et al., 1998). Akt can also regulate protein synthesis via activation of mammalian target of rapamycin (mTOR), a serine/threonine kinase which is a downstream target of PI3K/Akt signaling (Bhatt and Damania, 2013). Activation of the PI3K/Akt/mTOR signaling pathway has been shown to promote neuroprotection by decreasing death of motor neurons, increasing sparing of tissue, and improving functional recovery in rats following spinal cord injury (Liu and Xu, 2012).

1.15 Extracellular signal regulated kinases (Erk 1/2 pathway)

Extracellular signal regulated kinases (Erk) 1and 2 are members of the mitogen activated protein kinase (MAPK) superfamily which can induce cellular transcriptional and post translational responses upon activation by extracellular stimuli (Cheng et al., 2003). Erk 1/2 is expressed in almost all tissues and is activated by external stimuli such as serum, growth factors, lipopolysacharides, ultraviolet light and heat shock proteins, although the pattern of activation varies among different cells (Zhimin and Suchian, 2006). Erk1/2 activation occurs in a stepwise process which is usually initiated by small G protein Ras-Raf family members and then by MAPK kinase (MEK1/2) which in turn activates Erk1/2 (Zhimin and Suchian, 2006). Activated Erk1/2 phosphorylates various substrates which can be categorized into protein kinases, transcription factors, protein phosphatases, cytoskeletal proteins, cell surface receptors and signaling molecules, as well as apoptosis related proteins (Zhimin and Suchian, 2006). Activated Erk1/2 is important for maintaining normal cellular survival, cellular growth, cell proliferation, long term potentiation and synaptic plasticity (Cheng et al., 2003). Activated Erk1/2 generally acts as an anti-apoptic factor but sometimes it can also act as a pro-apoptic molecule as determined by the activation or inhibition of its downstream effectors (Zhimin and Suchian, 2006). Activation of Erk1/2 was found following SCI (Cheng et al., 2003) and increased levels of phosphorylated Erk (pErk) were detected in microglia and oligodendrocytes following dorsal rhizotomy in the spinal cord (Cheng et al., 2003). Following rat spinal cord injury, Erk 1/2 activation was shown to occur initially in neurons, then in microglia and in astrocytes (Wang et al., 2011).

Chapter 2

Hypothesis & Aims

Previous studies have demonstrated that TGFβ is a major factor that induces CSPG expression and it has been suggested that TGFβ mediates this effect via the canonical Smad signaling pathway (Asher et al., 2000; Susarla et al., 2011). PI3K and Erk1/2, two non Smad pathways, are also activated by TGFβ, but the role of these pathways in CSPG expression remains to be elucidated. This study was conducted to explore the effects of non Smad pathways in mediating TGFβ induced expression of CSPGs. The recent finding of downregulation of Smad2 in neurons by dbc AMP initially led us to examine the effects of dbc AMP on astrocytes. Our data showed significant downregulation of Smad2 in astrocytes by dbc AMP and so we expected that dbcAMP would reduce TGFβ mediated expression of CSPGs through downregulation of Smad2. However, we found that downregulation of Smad2 does not reduce TGFβ-mediated neurocan expression in astrocytes. Therefore we hypothesize that TGFβ may mediate CSPG expression through non-Smad pathways.

Aim # 1: To determine if Smad signaling is required for TGF-β to mediate CSPG expression in astrocytes.

Aim # 2: To identify the non-Smad pathways that are involved in CSPG expression.

Chapter 3

Materials & Methods

All animal procedures were approved by the Protocol Management and Review Committee of the University of Manitoba (protocol #10-057/1).

3.1 Astrocyte cultures

Astrocytes were obtained from both male and female Long Evans rat pups at postnatal day 6 to 7 (P6-P7). Rat pups were anaesthetized using 30% Isoflurane in propylene glycol and decapitated. The brains were isolated and kept in Neurobasal (NB) (Life Technologies) media. The meninges and blood vessels were removed from the brains using fine forceps under a dissecting microscope and the cerebral cortices were collected in a conical tube containing chilled NB media. The tissues were trypsinized with 0.1% trypsin and 50 µg/ml DNAse I for 15 minutes at 37ºC. After 15 minutes, tissues were triturated using 10 ml, 5 ml and 2 ml pipettes and the trypsin was inactivated by adding Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies) with 10% dialyzed fetal bovine serum (dFBS; Life Technologies) with 1% antibiotic- antimycotic (AA; Life Technologies). The solution was strained using a 70 µm strainer and the filtered cell suspension was plated on 75 cm^2 tissue culture flasks coated with 100 µg/ml poly-L-lysine (PLL; Sigma). Flasks were kept in the incubator at 37^oC for 1 hour and then the

media was aspirated and replaced with 15 ml of fresh DMEM with 10% dFBS and AA. The next day, the flasks were given a half change of media and media changing continued every 2 days until the cells became confluent.

When the cells became confluent, the media was aspirated from the flasks and replaced with 10 ml of NB with 1% dFBS. The cells were equilibrated in the incubator for 30 minutes. The flasks were put on a horizontal shaker at 350 rpm, 37ºC for 6 hours to detach oligodendrocytes and microglia. After completion of shaking, the media was replaced with DMEM containing 10% dFBS and AA and the flasks were put back to the incubator. The next day, the media was aspirated and the flasks were washed with warm 1X phosphate buffered saline (PBS). The cells were trypsinized with 0.1% trypsin and 50 µg/ml DNAse I in Neurobasal-A media for 5 minutes at 37ºC, and then triturated. The trypsin was inactivated using DMEM with 10% dFBS and then the collected cells were centrifuged at 1000 rpm for 5minutes. The cell pellet was resuspended in DMEM with 10% dFBS and AA and cells were counted using a haemocytometer. The cells were diluted to a concentration of 30,000 to 35,000 cells/ ml in DMEM and plated in PLL-coated 60 mm dishes (3 ml per plate).

3.2 Immunostaining

Purified astrocytes were plated in 8 chamber culture slides (Thermo) coated with PLL and incubated at 37^{o}C for 3days. Then, the slides were fixed with 4% paraformaldehyde in 1X PBS for 20 minutes. After fixation, slides were blocked using 10% donkey serum diluted in 0.25% Triton X-100 in PBS for 1 hour at room temperature. Following blocking, slides were rinsed with 1x PBS and incubated with primary antibodies diluted in 3% donkey serum in 0.25% Triton X-100. Slides were incubated overnight at 4^{o}C, rinsed, and then incubated with secondary antibodies for 2 hours at room temperature. Primary antibodies were: mouse anti-GFAP (1:500; Cell Signaling) and rabbit anti-Iba-1 (1:500; Wako chemicals USA, Inc). Secondary antibodies were FITC anti-mouse IgG (1:200; Jackson Immuno Laboratories Inc; USA) and Alexa Fluor 647 anti-rabbit IgG (1:400; Jackson Immuno Laboratories Inc; USA). Secondary antibodies were diluted in 3% donkey serum. Nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole; 1:10,000) for 5 minutes and the slides were cover slipped. Following immunostaining, cells were observed under the microscope and counted. The total number of cells was counted by detecting DAPI positive nuclei. The number of GFAP stained astrocytes and Iba-1 stained microglia were counted and their percentages were calculated relative to the total cell population. A single field from each well was counted. Total cell count was 4626 in which 4407 cells were astrocytes and 219 cells were microglia. In our culture 95.26% cells were astrocytes and 4.5% microglia which indicate the purity of our cell culture. Images (multidimensional acquisition) from the slides were obtained using Zeiss Axiovision software.

3.3 Treatments

3.3.1 Dibutyryl cyclic AMP treatment

Astrocytes were serum starved for 24 hours with plain DMEM (2 ml in each plate) before starting the treatment. Cells were treated with dibutyryl cyclic AMP (dbcAMP) (Calbiochem), which is a cell-permeable, non-hydrolysable analogue of cAMP. The concentration of dbcAMP was 1 mM and the cells were treated for 1 hour or 24 hours at 37^{o}C. To test the effects of dbcAMP treatment on neurocan expression, astrocytes were treated with 1mM dbcAMP for 1 hour, followed by 10 ng/ml recombinant human TGFβ1 (R&D Systems) to induce CSPG expression. Treatment continued for 5 days with no media change and therefore both dbcAMP and TGFβ treatment persisted for the entire 5 days. Cells were lysed after 5 days and conditioned media was collected.

3.3.2 TGFβ receptor inhibitors treatment

Astrocytes were serum starved for 24 ho urs with plain DMEM and then treated with two selective TGFβ receptor type I inhibitors: SB431542 and SB505124 (Sigma). Dimethyl sulfoxide (DMSO) was used as a vehicle and stock concentrations of SB431542 (5 mM) and SB505124 (5 mM) were achieved by diluting them in DMSO. Following 24 hours serum starvation cells were treated with 20 µM SB431542 or 5 µM SB505124. Cells were treated with the inhibitors for 1 hour at 37^{o}C, and then 10 ng/ml TGFβ was added. Remaining plates were treated with 10 µl/ml DMSO alone or with 10 µl/ml DMSO for 1

hour followed by 10 ng/ml TGFβ. This treatment continued for 5 days at 37^{o}C with no media changes and then cells were lysed after collecting conditioned media.

3.3.3 PI3 kinase inhibitor treatment

To investigate the involvement of the PI3 Kinase/Akt pathway in TGFβ mediated CSPG expression, astrocytes were treated with 5 or 10 μM LY294002 (Sigma). A stock concentration of LY294002 (1 mM) was achieved by diluting in DMSO. Cells were incubated for 1 hour with 5 or 10 μM LY294002 at 37^{o}C, and then treated with 10 ng/ml TGFβ. Remaining cell plates were treated with 10 μl/ml DMSO for 1 hour followed by 10 ng/ml TGFβ, or 10 μl/ml DMSO alone. Cells were incubated for 5 days with no media change and then lysed after collecting the conditioned media.

3.3.4 MEK inhibitor treatment

To test the involvement of the MAPK/Erk pathway in TGFβ mediated CSPG expression, astrocytes were treated with 5 or 10 μM of UO126 (MEK1 inhibitor) (Sigma). A stock concentration of UO126 (1 mM) was achieved by diluting in DMSO. Cells were incubated with the inhibitor for 1 hour at 37^{o}C, and then treated with 10 ng/ml TGFβ. Remaining cell plates were treated with 10 μl/ml DMSO for 1 hour followed by 10 ng/ml TGFβ, or 10 μl/ml DMSO alone. This treatment continued for 5 days with no media change and then cells were lysed after collecting the conditioned media.

3.3.5 Small interference RNA (siRNA) assay

To achieve knock down of Smad2 and Smad4 expression in astrocytes, Accell SMARTpool rat Smad2 siRNA, Accell SMARTpool rat Smad4 siRNA and Accell Non Targeting siRNA (Dharmacon) were used. Stock concentrations of siRNA agents were made at 100 µM using 1x siRNA buffer (Dharmacon). Astrocytes were treated with 1 µM Smad2 siRNA, Smad4 siRNA, or non-targeting siRNA in Accell delivery media (Dharmacon) for 3 days at 37^{o}C. After 3 days, the media was aspirated from the cell plates and 2 ml of fresh plain DMEM was added. 10 ng/ml TGFβ was added to half of the plates and all the plates were then incubated for 5 days at 37^{o}C with no media changes. Cells were lysed after 5 days and the conditioned media was collected before lysing.

3.4 Cell lysis and Western blotting

Cells were lysed using 50 µl of 1X RIPA buffer (Millipore). Before Western blotting, protein concentrations were determined using Bio-Rad *DC* protein assay (Bio-Rad Laboratories Inc, USA). Samples containing 30 µg of protein were mixed with 5X sample buffer containing dithiothreitol (DTT; Thermo) and boiled for 5 minutes. The samples were run on 8% polyacrylamide gels and transferred into nitrocellulose membrane at 75V for 1 hour at room temperature. The membranes were blocked for 1 hour in 10% milk made in PBS containing 0.05% Tween-20, and then incubated overnight with primary antibodies at 4^{o}C. Primary antibodies were rabbit anti-phospho Smad2 (Ser465/467; 1:1000; Cell Signaling), rabbit anti-Smad2/3 (1:1000; Cell Signaling), rabbit anti-Smad4 (1:1000; Cell Signaling), rabbit anti-phospho Akt (Ser473; 1:1000; Cell Signaling), rabbit

anti-Akt (1:1000; Cell Signaling), rabbit anti-phospho Erk1/2 (Thr202/Tyr204; 1:1000; Cell Signaling), rabbit anti-Erk1/2 (1:1000; Cell Signaling), mouse monoclonal anti GFAP (1:1000; Cell Signaling), rabbit anti- glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; Cell Signaling), and rabbit anti-actin (1:1000; Sigma). Incubation with secondary antibodies was for 1 hour at room temperature and secondary antibodies were: horseradish peroxidase (HRP) conjugated anti-rabbit IgG and HRP conjugated anti-mouse IgG (both 1:2000; Cell Signaling). To visualize the protein bands, membranes were reacted with Pierce ECL Western Blotting Substrate (Thermo) or Super Signal West Femto Maximum Sensitivity Substrate (Thermo) and nitrocellulose membranes were exposed to autoradiographic films (HyBlot CL, Denville Scientific Inc. New Jersey). Membranes were stripped using 20 ml of Restore Western Blot Stripping buffer (Thermo) and then reprobed with other antibodies in succession.

For the conditioned media, 1 ml samples of conditioned media were treated with chondroitinase ABC (0.01U/ml) (Sigma) for 3 hours at 37^{o}C to remove GAG chains. The protein concentration of media samples was measured using the Bio-Rad *DC* protein assay. Media samples containing 30 μg of protein were mixed with 5x sample buffer and boiled for 5 minutes. Samples were run on 6% polyacrylamide gels and transferred to nitrocellulose membranes at 4^{o}C for 16 hrs at 150 mA. Membranes were blocked with 10% milk for 1 hour at room temperature and probed with mouse monoclonal anti-neurocan (1:4000; Sigma), mouse monoclonal anti-brevican (1:4000; BD Biosciences) and rabbit anti-aggrecan (1:500; Abcam) overnight at 4^{o}C. HRP-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG (both 1:2000; Cell Signaling) were used as

secondary antibodies and incubation was done for 1 hour at room temperature. Membranes were reacted with Pierce ECL Western Blotting Substrate (Thermo) or Super Signal West Femto Maximum Sensitivity Substrate (Thermo) and exposed to autoradiographic films.

3.5 Image acquisition

Radiographic films were scanned and densitometric analyses of proteins were done using Image J software (NIH). Figures were prepared using Adobe Photoshop CS5.

3.6 Statistical analyses

All analyses were done using Graph Pad Prism software, version 5. Comparison between two data sets was done by paired, one tailed Student's *t* test. Comparisons of three or more data sets were made using one way ANOVA with Bonferroni's multiple comparison test. Significance was determined at the $p < 0.05$ level. All data are represented as mean ± standard error of mean (SEM).

Chapter 4

Results

4.1 Effects of elevation of cyclic AMP on TGFβ signaling protein Smad2/3 in astrocytes

A previous study of intracellular cyclic AMP elevation demonstrated that dbcAMP treatment significantly reduces levels of Smad2/3 in neurons (Hannila et al., 2013). Based on this finding, we wanted to determine if elevated cyclic AMP could reduce Smad2/3 in astrocytes. Astrocytes were treated with 1 mM dbcAMP for 24 hours and we found an 85% reduction in levels of Smad2/3 in these cells compared to the control (paired t tests, ****p <0.0001, n=4) (Fig.1 A). Then, to test how quickly this reduction can be achieved, astrocytes were treated with 1 mM dbcAMP for 1 hour and a 77% reduction in levels of Smad2/3 compared to the control was found (paired t tests, ***p <0.001, n=3) (Fig.1 B). This suggests that dbcAMP mediates this reduction rapidly. The two time points of treatment were chosen from the previous study that showed downregulation of Smad2/3 in cerebellar granular neuron (CGN), cortical and dorsal root ganglion (DRG) neuron by treating with dbcAMP (Hannila et al., 2013). These data indicate that elevation of intracellular cyclic AMP in astrocytes can suppress Smad2/3 protein expression.

4.2 Effects of elevated cyclic AMP on neurocan expression in astrocytes

Having shown that dbcAMP treatment can reduce Smad2/3 in astrocytes and since TGFβ has been shown to induce neurocan expression in astrocytes (Asher et al., 2000), we hypothesized that by downregulating Smad2, dbcAMP would inhibit TGFβ-Smad signaling and decrease neurocan expression. Astrocytes were treated with 1 mM dbc AMP for 1 hour followed by 10 ng/ml TGFβ for 5 days with no media change. We observed a significant increase in neurocan expression in astrocytes following TGF-β treatment (one way ANOVA, $*p < 0.05$, n=3) (Fig. 4.1 C). Expression of Smad2/3 was strongly suppressed in astrocytes treated with dbcAMP and TGFβ but no reduction in neurocan was observed in these astrocytes compared to the astrocytes treated with TGFβ alone (Fig.1 C). This finding indicates that the reduction of Smad2/3 does not affect expression of neurocan by TGFβ, which directed us to investigate if signaling molecules other than Smads are involved in TGFβ mediated expression of neurocan and other CSPGs in astrocytes.

4.3 Activation of TGFβ receptor is required for expression of CSPG in astrocytes

Having observed that reduction of neurocan was not achieved following downregulation of Smad2 by dbcAMP, we tested whether targeting the TGFβ receptor can affect levels of CSPG in astrocytes. We aimed to exclude the possibility that TGFβ is working

through other receptors to mediate CSPG expression in astrocytes. Astrocytes were treated with two selective TGFβ receptor type I inhibitors: 20 μM SB431542 or 5 μM SB505124 for 1 hour followed by 10 ng/ml TGFβ for 5 days with no change of media. The doses for SB431542 and SB505124 were based on those used in previous studies (Stegmuller et al., 2008; DaCosta et al., 2004). Western blotting revealed decreased TGFβ-induced Smad2 phosphorylation following treatment with 20 μM SB431542 or 5 μM SB505124, which confirmed receptor blockade activity. A significant increase (3 fold) in neurocan expression following TGFβ treatment was found and this was significantly reduced in response to treatment with 20 μM SB431542 (83% reduction) or 5 μM SB505124 (84% reduction) (one way ANOVA, ****$p < 0.0001$, n=4) (Fig. 2). This data indicates that activation of the TGF-β receptor is required for mediating neurocan expression in astrocytes. In addition to neurocan, we found a significant increase in the expression of brevican (2 fold) and Aggrecan (5 fold) by TGFβ, followed by significant reductions of brevican (78% reduction) and aggrecan (60% reduction) expression after treatment with 20 μM SB431542 or 5 μM SB505124 (one way ANOVA, **$p < 0.01$, ***$p < 0.001$) (Fig. 2). These data strongly suggest that TGFβ receptor activity is essential to mediate expression of different CSPGs.

4.4 Contributions of Smad signaling to TGFβ-induced expression of CSPG in astrocytes

Numerous studies have shown that TGFβ regulates cell fate by signaling through the Smad pathway (Massague et al., 2005; Kretzschmar et al., 1997). For neurocan expression, a previous study has reported that TGFβ mediates neurocan expression through the Smad signaling pathway (Susarla et al., 2011). In contrast, our experiments with dbcAMP suggest that TGFβ mediates neurocan expression in the absence of Smad. To resolve this, we investigated the specific role of Smad signaling in TGFβ-mediated expression of CSPGs in astrocytes using siRNA gene silencing. For the Smad siRNA experiments, astrocytes were incubated with 1 μM non-targeting, Smad2 or Smad4 siRNA for 3 days. Once Smad2 and Smad4 were suppressed in astrocytes, cells were exposed to 10 ng/ml TGFβ for 5 days with no media change. Western blots showed clear evidence of Smad2 and Smad4 knockdown (Fig. 3). A significant increase in neurocan (4 fold), brevican (5.4 fold) and aggrecan (2 fold) expression was found in the non targeting sample following TGFβ treatment (paired t test, $**p < 0.01$, n=3) (Fig. 3). Neurocan expression was significantly increased (4.5 fold) following treatment with TGFβ in the samples where expression of Smad2 was suppressed (paired t test, $*p < 0.05$, n=3) (Fig. 3). In addition to neurocan, we observed similar significant increases in brevican (5 fold) and Aggrecan (3 fold) expression following TGFβ treatment in samples where Smad2 was knocked down (paired t tests, $**p < 0.01$, n=3) (Fig. 3). This demonstrates that TGFβ mediates CSPG expression in the absence of Smad2, which is consistent with our previous result from the dbcAMP experiments. This suggests that Smad2 is not required

for CSPG expression. For further investigation, the expression of neurocan, brevican and aggrecan was measured in the samples that received Smad4 siRNA. Significant increases in neurocan (4.9 fold), brevican (4.5 fold) and Aggrecan (2 fold) were found in these samples following treatment with TGFβ (paired t tests, $*p < 0.05$, n=3) (Fig. 3). This indicates that TGFβ is inducing high levels of CSPG expression in the absence of Smad4, which is the only common mediator for Smad signaling (Lagna et al., 1996). The absence of Smad4 excludes the possibility of any other R Smads acting in this pathway. These data strongly suggest that TGFβ does not require Smad signaling to mediate expression of CSPGs in astrocytes. We also observed a significant increase in GFAP expression indicating increased reactive astrogliosis in the absence of Smad signaling (paired t tests, $*p < 0.05$, $**p < 0.01$, n=3) (Fig. 3), which suggests that the Smad signaling is not required for astrogliosis as well.

4.5 Involvement of non-Smad pathways in TGFβ-mediated CSPG expression

Having shown that Smad signaling is not required for CSPG expression, we investigated if there are other pathways acting in response to TGFβ. TGFβ is known to activate non Smad signaling pathways such as the Phosphoinositide 3 kinase (PI3K/Akt), the Rho-like GTPase, and the Mitogen activated protein kinase (MAPK/Erk1/2) pathways (Wrzesinski et al., 2007; Zhang YE, 2009). Based on the results from our previous experiments, we hypothesized that TGFβ may mediate expression of CSPGs via non-Smad pathways. We

decided to test the involvement of two major non-Smad pathways for TGFβ – PI3K/Akt and MAPK/Erk1/2 – in mediating expression of CSPGs in astrocytes.

4.6 Role of PI3K/Akt signaling in the expression of CSPGs: non-Smad pathways for TGFβ

To determine the contribution of the PI3K/Akt pathway to TGFβ-mediated CSPG expression, astrocytes were treated with a PI3 kinase inhibitor, 5 or 10 μM LY294002, for 1 hour followed by 10 ng/ml TGF-β for 5 days with no media change. Western blotting showed reduced levels of Akt phosphorylation which confirmed the efficacy of the inhibitor (Fig. 4). Neurocan expression was significantly increased after treatment with TGFβ compared to vehicle treated astrocytes (one way ANOVA, ****$p < 0.0001$, n=4) (Fig. 4). Expression of brevican and aggrecan were also increased significantly after TGFβ treatment (Fig. 4). Compared to the astrocytes treated with TGFβ and DMSO, there was a significant decrease in the expression of neurocan, brevican and aggrecan in astrocytes treated with TGFβ and LY294002 particularly at the 10 μM concentration (one way ANOVA, **$p < 0.01$, n=4) (Fig. 4). Expression of neurocan, brevican and aggrecan were decreased by 5 μM LY294002 treatment but that reduction was not statistically significant. This data strongly suggests that TGF-β mediates expression of CSPG via the PI3K/Akt signaling pathway and that PI3K/Akt may serve as a common pathway for CSPG expression.

4.7 Role of Erk1/2 signaling in expression of CSPGs: non Smad pathways for TGFβ

To see whether the Erk 1/2 pathway is involved in TGFβ mediated CSPG expression, astrocytes were treated with a MEK 1/2 inhibitor, 5 or 10 μM UO 126, for 1 hour followed by 10 ng/ml TGFβ treatment for 5 days with no media change. Western blots revealed reduced Erk1/2 phosphorylation in response to treatment with 5 or 10 μM UO126, which again shows the efficacy of the inhibitor (Fig. 5). Following quantification, a significant increase in the expression of neurocan and brevican, and non significant increase in aggrecan expression was found in astrocytes treated with TGFβ compared to the vehicle treated astrocytes (one way ANOVA, $*p < 0.05$, ns=non significant, n=4) (Fig. 5). No significant reduction in levels of neurocan, brevican or aggrecan was found in astrocytes treated with TGFβ and 5 or 10 μM UO126 compared to the astrocytes treated with TGFβ and DMSO. While the presented Western blots show a trend of decrease in CSPG expression, particularly for the neurocan, these reductions were not significant, which suggests that the Erk1/2 pathway does not play a significant role in TGFβ- mediated expression of CSPGs in astrocytes.

Figure 1. dbcAMP significantly reduces Smad2/3 protein in astrocytes but does not reduce TGFβ-induced neurocan expression. (A) Western of cell lysates from P6-7 astrocytes treated with 1 mM dbcAMP for 24 hours. There is a highly significant reduction in Smad2/3 levels by dbcAMP treatment (B) Western blots of cell lysates from P6-7 astrocytes treated with 1 mM dbcAMP for 1 hour. These astrocytes show a significant reduction in Smad2/3 levels. (C) Western blots of conditioned media and cell lysates from P6-7 astrocytes treated with 1 mM dbcAMP for 1 hour followed by 10 ng/ml TGFβ treatment for 5 days. There is a significant increase in neurocan expression following TGFβ exposure. No reduction in neurocan expression was found in astrocytes treated with dbcAMP and TGFβ, even though Smad2 is strongly downregulated. Images are from a single, matched experiment. Graphs represent average densitometric measurements ± SEM (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, ns = non significant). The data in each graph was obtained from 3 independent experiments.

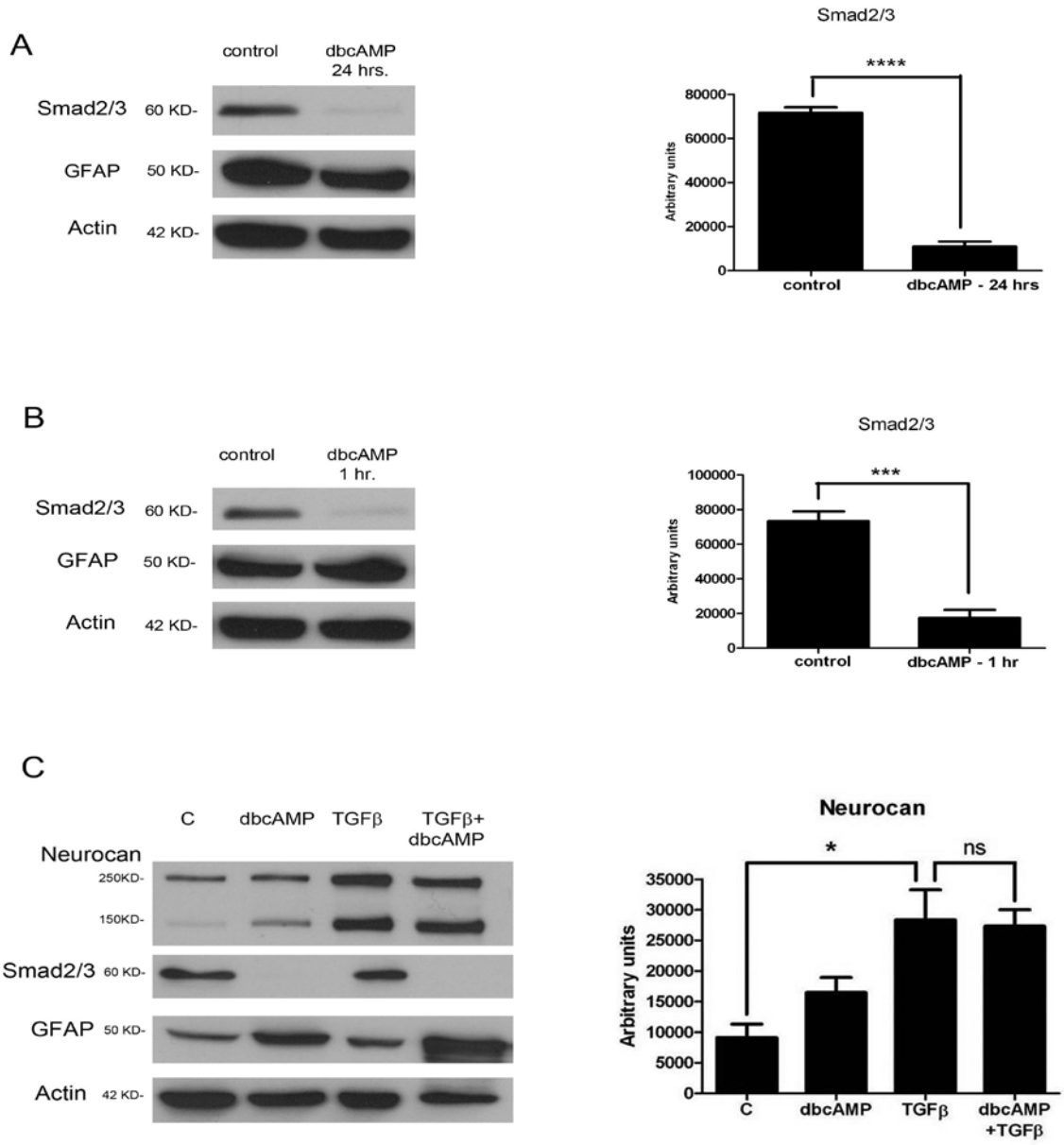

Figure 1: dbcAMP significantly reduces Smad2 protein in astrocytes but does not reduce TGFβ-induced neurocan expression.

Figure 2. Activation of TGFβ receptor is required to mediate CSPG expression. Western blots of conditioned media and cell lysates from P6-7 astrocytes treated with 20 μM SB431542 or 5 μM SB505124, for 1 hour followed by 10 ng/ml TGFβ treatment for 5 days. There is a significant increase in the expression of neurocan, brevican and aggrecan in astrocytes treated with TGFβ. Astrocytes treated with SB431542 and SB505124 followed by TGFβ treatment show significant reductions in neurocan, brevican and aggrecan expression. Images are from a single, matched experiment. Graphs represent average densitometric measurements ± SEM (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). The data in each graph was obtained from 4 independent experiments.

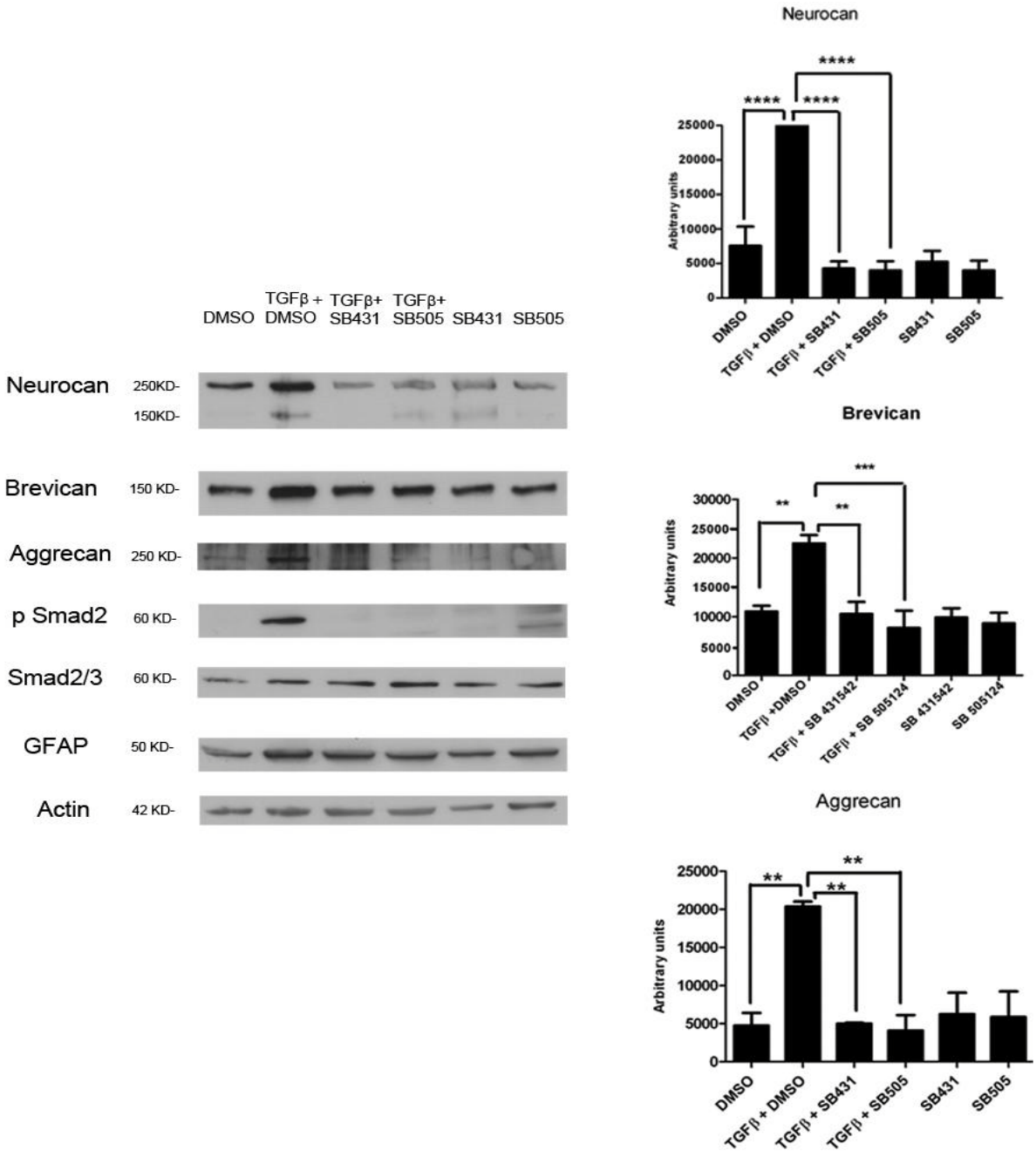

Figure 2: Activation of TGFβ receptor is required to mediate CSPG expression in response to TGFβ.

Figure 3. Smad signaling is not required for TGFβ-mediated expression of CSPG. Western blots of conditioned media and cell lysates from P6-7 astrocytes treated with non-targeting (NT), Smad2 or Smad4 siRNA for 3 days, followed by 10 ng/ml TGFβ for 5 days. TGFβ treatment in the astrocytes that were transfected with NT, Smad2 siRNA and Smad4 siRNA show significant increases in the expression of neurocan, brevican, aggrecan and GFAP. Images are from a single, matched experiment. Graphs represent average densitometric measurements ± SEM (*$p < 0.05$, **$p < 0.01$). The data in each graph was taken from 3 independent experiments.

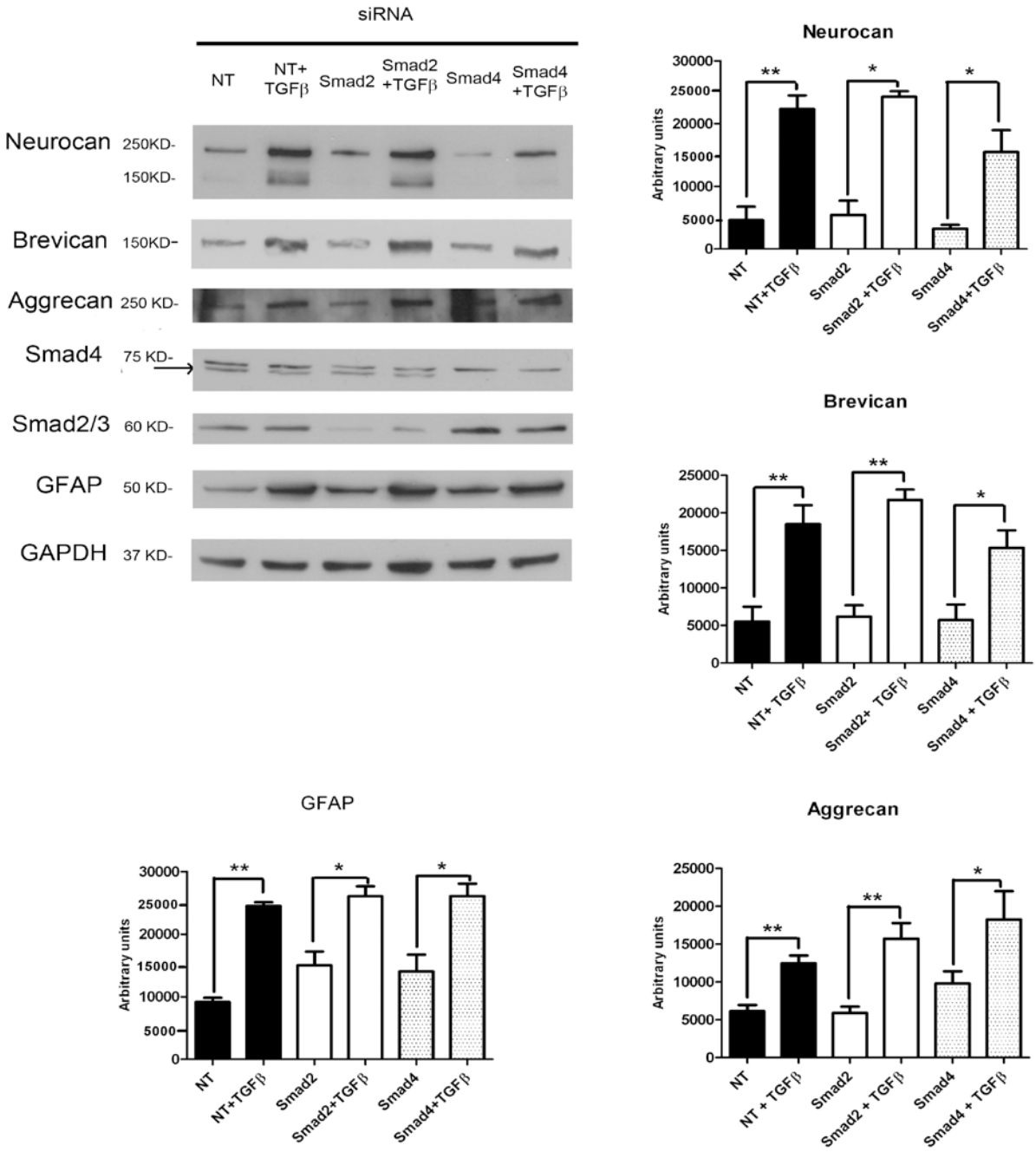

Figure 3: Smad signaling is not required for TGFβ-mediated expression of CSPG.

Figure 4. TGFβ mediates CSPG expression via the PI3K/Akt pathway. Western blots of conditioned media and cell lysates from P6-7 astrocytes treated with 5 or 10 μM LY294002 for 1 hour, followed by 10 ng/ml TGFβ treatment for 5 days. Significant increases in neurocan, brevican and aggrecan expression were found following treatment of astrocytes with TGFβ. Astrocytes treated with 10 μM LY294002 and TGFβ show significant decreases in neurocan, brevican and aggrecan expression. Images are from a single, matched experiment. Graphs represent average densitometric measurements ± SEM (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). The data in each graph was taken from 4 independent experiments.

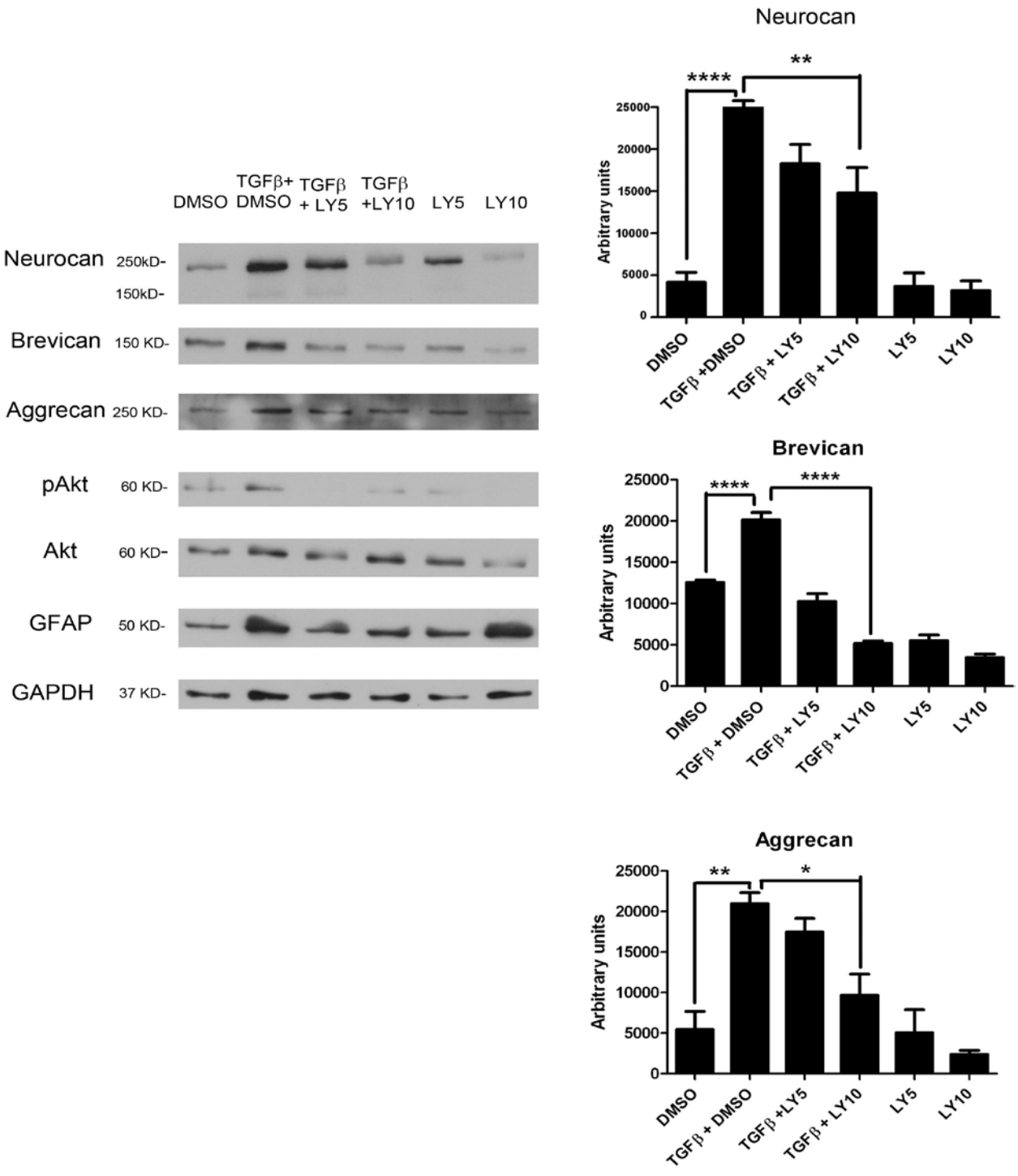

Figure 4: TGFβ mediates CSPG expression via the PI3K/Akt pathway

Figure 5. Inhibition of Erk1/2 signaling does not have significant role in reducing TGFβ-mediated expression of CSPG. Western blots of conditioned media and cell lysates from P6-7 astrocytes treated with 5 or 10 μM UO126 for 1 hour, followed by 10 ng/ml TGFβ treatment for 5 days. There was no significant decrease in neurocan, brevican and aggrecan expression in astrocytes compared to the astrocytes treated with TGFβ only. Images are from a single, matched experiment. Graphs represent average densitometric measurements ± SEM (*$p < 0.05$, ns = non significant). The data in each graph was taken from 4 independent experiments.

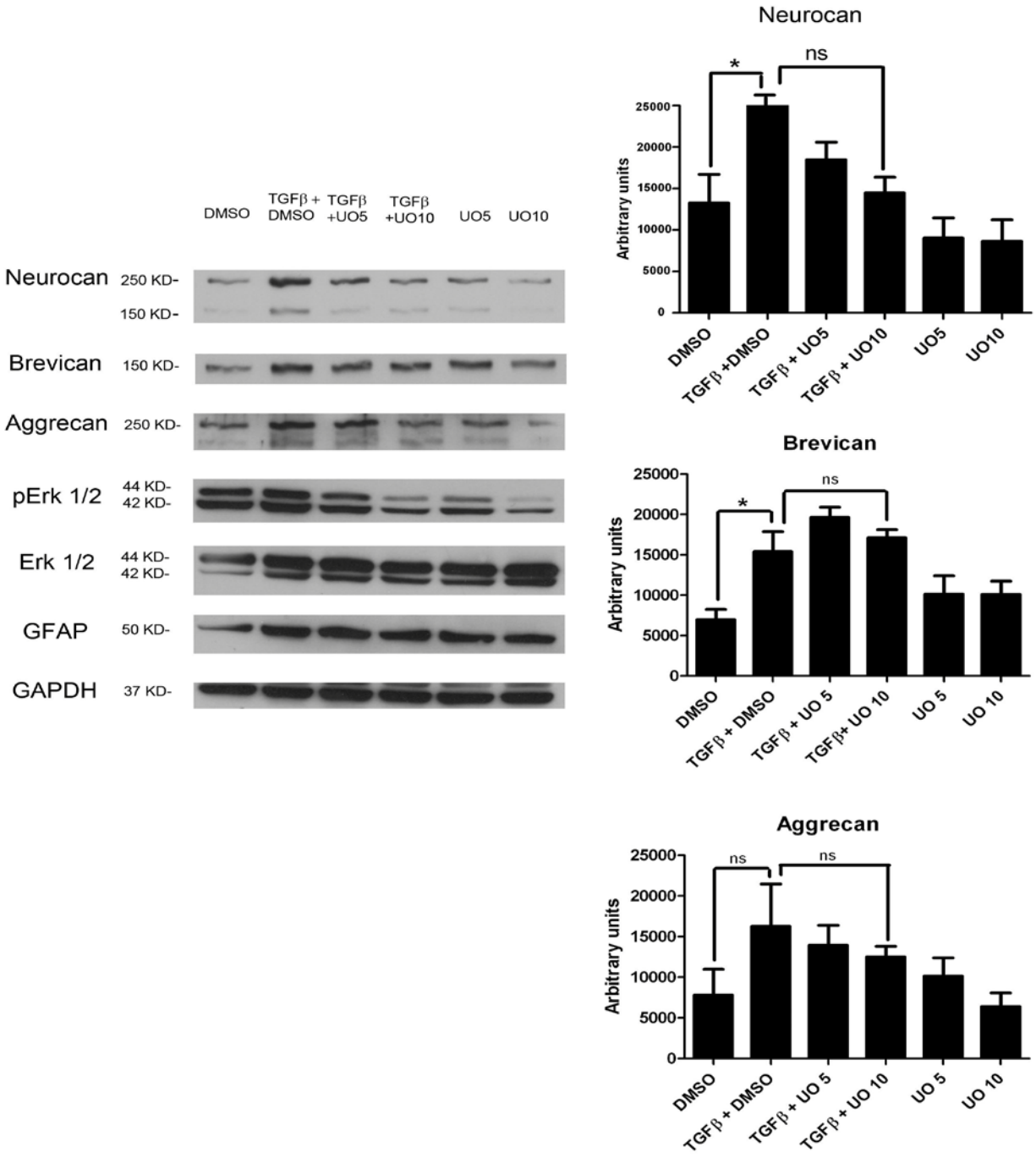

Figure 5: Inhibition of Erk1/2 signaling does not have a significant effect on TGFβ-mediated expression of CSPG

Chapter 5

Discussion

Spinal cord injury is an important clinical problem that results in different forms of disability and has long term impact with no specific therapy available for the injured person. This injury leads to astrogliosis and formation of a glial scar which plays a central role in the inhibition of regeneration (Silver and Miller, 2004). CSPGs are one of the major constituents of the glial scar and their expression is up regulated by reactive astrocytes following spinal cord injury (Fawcett and Asher, 1999; Mckeon et al., 1999). Following injury, CSPGs inhibit axonal regeneration which was evidenced by reduced neurite outgrowth in an *in vitro* model of glial scarring (Mckeon et al., 1991). This reduction in growth was accompanied by massive upregulation of CSPGs in the scar (Mckeon et al., 1991). Expression of CSPGs also affects sensory and motor functions as degradation of CSPG was found to improve locomotion and proprioception following injury to the rat spinal cord (Bradbury et al., 2002). Here, the GAG chain moiety of the CSPG molecule was degraded by administration of chondroitinase ABC (Bradbury et al., 2002), which suggests that molecular alteration of CSPG can overcome its inhibitory effects. Therefore it is conceivable that reducing the amount of CSPGs at the injury site might be a way to promote axonal growth following injury.

Different strategies have been applied to attenuate CSPG actions. These include preventing CSPG's binding to its receptor PTPσ by genetic knockdown of PTPσ in mice (Shen et al., 2009) and inhibiting deposition of CSPGs using decorin (Davies et al., 2004). The timing of CSPG inhibition must also be considered when attempting to promote regeneration following spinal cord injury. Inhibition of CSPG synthesis immediately following injury was found to exacerbate tissue loss and impaired functional recovery (Rolls et al., 2008). Immediate inhibition of CSPG synthesis results in alteration of spatial organization of infiltrating myeloid cells, elevation of TNF-α levels and reduction of insulin like growth factor (IGF) production by microglia, which results in tissue damage (Rolls et al., 2008). Delayed inhibition of CSPG expression, such as inhibiting 2 days following injury, was found to improve locomotor function (Rolls et al., 2008). In this context, our strategy for reducing CSPG expression is to suppress the downstream signaling pathways responsible for CSPG expression in astrocytes during the sub acute phase of injury.

TGFβ is an important cytokine for cellular growth and survival, and upregulation of TGFβ mRNA levels was found in the spinal cord following injury (Lagord et al., 2002). TGFβ has been shown to enhance expression of neurocan and brevican in astrocytes (Asher et al., 2000; Smith and Strunz, 2005; Hamel et al., 2005) and previous studies have found that TGFβ also induces expression of aggrecan in a chondrocyte cell line (Watanabe et al., 2001). Improved locomotion and reduced astrogliosis are seen after treating with TGFβ neutralizing antibody in a model of rat spinal cord injury, suggesting that increased levels of TGFβ contribute to glial scarring (Kohta et al., 2009). This

suggests that interference in TGFβ signaling might simultaneously reduce glial scar formation and CSPG expression.

TGFβ signaling is initiated after binding to the serine/threonine kinase receptor TβR II which activates TβR I. Both TβR II and TβR I are expressed by astrocytes (Kubiczkova et al., 2012; Vivien et al., 1998). In this study, we have shown that TGFβ receptor activation is essential for mediating CSPG expression. First, we have confirmed TGFβ-induced expression of neurocan, brevican and aggrecan in our cultured astrocytes. Next, our experiments demonstrated that administration of TGFβ receptor inhibitors significantly reduces TGFβ-mediated expression of neurocan, brevican and aggrecan by astrocytes.

Through receptor mediated activation, TGFβ induces phosphorylation of R Smads and activates the Smad signaling pathway. TGFβ-Smad signaling has been suggested as the primary pathway for mediating CSPG expression (Susarla et al., 2011). Suppressing expression of Smad2 and Smad3 by transfecting with lentiviruses expressing short hairpin RNA (LV-shRNA) for Smad2 and 3 was shown to block TGFβ-mediated expression of neurocan in astrocytes (Susarla et al., 2011). Their data showed significant reductions in neurocan and phosphacan expression following Smad2 suppression, while suppression of Smad3 reduced neurocan only. No effect on brevican expression was found following suppression of either Smad2 or Smad3 (Susarla et al., 2011). When Smad2 is knocked down, two Smad3 proteins can form a complex with Smad4 and translocate to the nucleus (Massague et al., 2005). Conversely, when Smad3 is knocked

down, Smad2 can form a complex with Smad4 in the same manner (Massague et al., 2005) and therefore, their data would be more convincing if they showed the same effect after knocking down Smad4 (Susarla et al., 2011). In our study, expression of Smad2/3 in astrocytes has been significantly downregulated by treatment with dbcAMP. However, TGFβ-induced neurocan expression in our astrocytes remained unaffected by the downregulation of Smad2/3. This suggests that Smads are not an essential factor in the process of neurocan expression. To further investigate the role of Smad signaling in mediating CSPG expression, our astrocytes were transfected with non targeting, Smad2 or Smad4 siRNA to suppress the expression of Smad2 or Smad4. Our data revealed a significant increase in the expression of neurocan, brevican and aggrecan in the absence of Smad2 or Smad4 following TGFβ exposure. Smad4 is the only and essential co Smad for the canonical Smad signaling mechanism (Lagna et al., 1996) and hence, expression of CSPGs in the absence of Smad4 eliminates the possibility of action through R Smads. Our study is the first to show that expression of CSPGs increases after Smad4 knockdown, which excludes TGFβ-Smad signaling as the source of CSPG expression in astrocytes. Therefore, our data present distinct evidence that Smad signaling is not required by TGFβ to mediate CSPG expression.

It is well known that TGFβ activates non-Smad pathways like PI3 kinase, Erk 1/2, Rho like GTPase, P38 and JNK (Moustakas and Heldin, 2005; Zhang YE, 2009). The PI3K/Akt pathway has been shown to be activated following spinal cord injury and it suppresses inflammation in a cord compression injury model in mice (Paterniti et al.,

2011). To explore the role of the PI3K/Akt pathway in mediating CSPG expression, an inhibitor of PI3K, LY294002, was used in this study to inactivate the PI3K/Akt pathway in our astrocytes. Our data show that treatment with LY294002 significantly reduces expression of neurocan, brevican and aggrecan by astrocytes following TGFβ stimulation. This finding suggests that TGFβ mediates expression of CSPGs via the PI3K/Akt pathway and inhibition of this pathway might be a beneficial approach to reduce CSPG expression in astrocytes. It is important to note that inhibition of PI3K/Akt might aggravate tissue damage, as PI3K/Akt activation was shown to promote neuroprotection following spinal cord injury in rats (Liu and Xu, 2012). Another study reported that inhibition of PI3K/Akt pathway by pre treatment with LY294002, promoted inflammation and apoptosis following spinal cord injury (Paterniti et al., 2011). To avoid these effects after PI3K/Akt inhibition, an alternative approach could be targeting the downstream signaling molecules of PI3K/Akt pathway such as mammalian target of rapamycin (mTOR). mTOR acts as a downstream effector for the PI3K/Akt pathway (Laplante and Sabatini, 2009) and inhibition of mTOR by treatment with rapamycin was found to reduce astrogliosis in cultured astrocytes (Codeluppi et al., 2009). Besides reducing astrogliosis, inhibiting mTOR with rapamycin might affect expression of CSPGs in the glial scar, and we have preliminary data suggesting that this is the case.

Our study also focused on elucidating the role of Erk1/2 signaling in mediating CSPG expression in astrocytes. The Erk1/2 pathway is activated following spinal cord injury and it has been suggested that phospho Erk (pErk) is responsible for generation of pain sensation following injury (Wang et al., 2011). In this study we have used UO126, an

inhibitor of MEK, to investigate the role of Erk1/2 in mediating CSPG expression. Expression of neurocan, brevican and aggrecan was reduced following inhibition of Erk1/2 signaling, but this reduction was not statistically significant. This data suggests that the TGFβ-induced CSPG expression in astrocytes may be partly mediated via the Ras-MEK-Erk1/2 signaling but this pathway is not essential like the PI3K/Akt pathway.

Apart from TGFβ, there are other cytokines that are upregulated following spinal cord injury and are involved in deposition of CSPGs in the glial scar. Epidermal growth factor (EGF) is a cytokine related to CNS injury. Upregulation of EGF mRNA was found as early as one day after injury at the lesion sites in rat spinal cord (Ahn et al., 2006) and activation of EGF receptor (EGFR) was found following CNS injuries that cause astrocytes to become reactive (Liu et al., 2006). EGF was shown to enhance expression of neurocan and phosphacan in cultured astrocytes (Asher et al., 2000; Smith and Strunz, 2005), but no reports were found showing the effects of EGF on brevican or aggrecan expression. Interferon gamma (IFNγ) is another cytokine which is related to modification of CSPG expression. Administration of IFNγ was shown to reduce TGFβ induced upregulation of neurocan expression by astrocytes and promoted functional recovery following spinal cord injury in mouse (Fujiyoshi et al., 2010). Interestingly, IFNγ treatment could not reduce neurocan expression when it was induced by EGF and underlying reason for this difference is not known (Fujiyoshi et al., 2010).

As an extension to our work, it would be interesting to study if inactivation of the PI3K/Akt pathway could create a more permissive substrate for neurite outgrowth on

reactive astrocytes. To investigate this, expression of Akt could be knocked down in cultured astrocytes by treating with Akt siRNA and astrocytes could be treated with TGFβ for 5 days. Then a layer of CGN could be plated on the astrocyte monolayers and axonal outgrowth of CGN could be observed. We would expect there to be less CSPG expression as TGFβ would not be able to induce CSPG in the absence of Akt and so more axonal outgrowth would be expected following Akt knockdown. Another direction could be conducting experiment on Akt knockout mice. Akt knockout mice showed mild growth retardation during embryonic and early post natal period but their growth and survival was normal in adult life (Cho et al., 2001). These knockout mice may have impaired glucose tolerance and they may exhibit depressive behaviour, but these factors were not shown to affect functional assessment of animals (Leibrock et al., 2013). After performing contusion injury to the spinal cord of the Akt knockout mice, their motor functions could be assessed over a period of one month using the Basso Mouse scale (Basso et al., 2006). Then those mice could be sacrificed and the spinal cord sections could be immunostained for CSPGs and GFAP to see if there was less CSPG expression or less astrogliosis. In an experimental context, targeting Akt is a viable approach but in a clinically relevant model, it might not be the best possible target, as inhibition of Akt could disrupt multiple signaling pathways necessary for cellular survival and growth that are mediated through the downstream effectors of Akt.

In summary, our study presents evidence that Smad signaling is not required for TGFβ to mediate expression of CSPGs in astrocytes. In addition, our data demonstrate that levels of neurocan, brevican and aggrecan are significantly reduced by inhibition of PI3K. Our

data suggest that TGFβ-mediated expression of CSPGs occurs through non-Smad pathways, particularly via the PI3K/Akt pathway, and that it might act as a common pathway for mediating CSPG expression. Overall, this study provides new insight into the mechanisms underlying CSPG expression, and targeting these signaling pathways might be an effective approach to develop new therapies for patients suffering from disabilities after spinal cord injury.

Chapter 6

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