Effect of Extracellular Matrix and Mechanical Strain on Airway Smooth Muscle

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~ Abstract ~

Airway remodeling in asthma includes alterations in extracellular matrix and airway smooth muscle (ASM) mass. For this study, ASM cells were obtained from rats that were challenged with ovalbumin (OVA) or saline (SAL) as control. OVA and SAL cells were seeded on plastic control (PC) or on plates coated with decorin or biglycan. OVA cell number was significantly increased versus SAL cells, for cells seeded on PC (48 h). A significant decrease in cell number was observed for both OVA and SAL cells seeded on decorin compared to PC cells (48 h). OVA cells, however, showed a more modest reduction in cell number. Furthermore, biglycan decreased SAL cell number only. Compared to no strain (NS), mechanical strain (S) reduced cell number for OVA and SAL cells on all matrices. In addition, S up-regulated expression of β_1-integrin relative to NS controls. Results suggest an ability of ASM cells to be modulated by matrix and mechanical stimulation.

~ Abrégé ~

Le remodelage de l'asthme inclut des changements dans la matrice extracellulaire et dans la masse du muscle lisse des voies respiratoires (MLVR). Pour cette étude, des cellules du MLVR sont provenues du rats administrés avec de l'ovalbumine (OVA), ou de saline (SAL). Le nombre de cellules OVA et SAL ont été semées sur un contrôle plastique (CP) ou sur des plats enduits de decorin ou biglycan. Le nombre de cellules OVA, semées sur le CP (48 h), avaient augmenté considérablement comparé aux cellules SAL. Une réduction importante a été observée pour le nombre de cellules OVA et SAL semées sur le decorin comparé à celles du CP. De plus, le biglycan a réduit le nombre cellulaire du SAL. Comparé à aucune contrainte (AC), une tension mécanique (TM) a réduit le nombre de cellules pour OVA et SAL sur toutes les matrices. En plus, TM hausse l'expression de l'intégrine β_1 par rapport à AC. Les résultats suggèrent donc une capacité des cellules MLVR d'être modulée par la matrice et une stimulation mécanique.

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"Wise is the one who collects the wisdom of others".

- Juan Guerra Caceras

~ Abbreviations ~

airway hyperresponsiveness	AHR
airway smooth muscle	ASM
Arg–Gly–Asp	RGD
basic fibroblast growth factor	bFGF
bovine serum albumin	BSA
Brown Norway	BN
c-Jun N-terminal kinases	JNK
Dulbecco's Modifed Eagle Medium	DMEM
endothelin-1	ET-1
epidermal growth factor	EGF
extracellular matrix	ECM
extracellular-regulated kinase	ERK
fetal bovine serum	FBS
glycosaminoglycan	GAG
Hanks' Balanced Salt Solution	HBSS
hertz	Hz
hours	h
interleukin	IL
intraperitoneally	ip
kiloDalton	kDa
mitogen-activated protein kinase	MAPK
ovalbumin	OVA

phosphate buffered saline	PBS
phosphoinositide 3'-kinase	PI3K
platelet-derived growth factor	PDGF
prostaglandin E_2	PGE_2
saline	SAL
small leucine rich proteoglycans	SLRP
smooth muscle myosin light chain kinase	sm-MLCK
transforming growth factor-beta	TGF-β
tumor necrosis factor-alpha	TNF-α

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~ *INTRODUCTION* ~

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~ Introduction ~

1.1 Asthma and airway remodeling

1.1.1 Asthma defined

Asthma, which affects approximately 20 million Americans, ranks among the most common chronic conditions in the United States, making it a major public health concern (1). In fact, according to the American Lung Association, it is the cause of 5000 deaths annually (1). Asthma is a syndrome that may affect people of all ages and race and its prevalence is rising, predominantly among children (2). Causative factors include inhaled allergens, viral infections, exercise, smoking, air pollution and a small number of drugs (3).

Asthma is a respiratory condition characterized by "chronic airway inflammation, episodic airway obstruction and airway hyperresponsiveness (AHR)" (4). The inflammation is thought to play a significant role in structural alterations of the asthmatic airway wall (5). Many cytokines and growth factors have been shown to be pro-inflammatory factors which work to augment the pathological features typical of asthma through repeated episodes of injury and repair (6). While described as a chronic disease with reversible airway obstruction, a small percentage of severe asthmatics experience fixed airflow obstruction, low-grade lung function and may be unresponsive to inhaled bronchodilators (for review see (3)). This is a crucial finding for reasons that permanent structural alterations resulting from airway remodeling may be a causal factor in the persistent symptoms and resistivity observed in these patients.

Finally, the precise mechanisms as to the underlying causes of asthma remain unclear, however it appears to involve many of the structural cell types present in the lung, namely epithelial cells, fibroblasts and airway smooth muscle (ASM) cells.

1.1.2 Airway remodeling

The structural alterations, collectively termed airway remodeling, include epithelial denudation, subepithelial fibrosis, mucous gland hyperplasia, bronchial angiogenesis, alterations and increased deposition of extracellular matrix (ECM) proteins within and around the ASM and, finally, an increase in the ASM mass (7). Although this will be discussed in further detail, it is important to note that airway remodeling has been evidenced through human studies in addition to animal and *in vitro* models of asthma. Furthermore, airway remodeling may be detrimental for numerous reasons. For example, airway remodeling is thought to amplify luminal narrowing in response to ASM shortening and therefore is an important factor in AHR (8). In addition, deposition of ECM in the outer layers of the airways, such as the adventitia, may have an uncoupling effect (9). That is, the ASM may become detached from the surrounding parenchymal attachments that work to prevent airway closure. Although less researched, structural alterations may, as well, have beneficial effects. For example, the increased ECM deposition and subsequent thickening may allow the airways to better resist dynamic compression (9). In addition, deposition of ECM within and around the ASM can act to constrain movement of ASM cells (9). Data has shown that treatment with the enzyme, collagenase, of ECM surrounding an ASM preparation *in vitro*

resulted in an increased shortening velocity (10). Taken together, these data suggest a very important role for ASM and ECM in the pathogenesis of asthma. In fact, computational modelings suggest that this enhanced ASM may be the most significant contributor to AHR typical of asthma (11).

1.2 Airway smooth muscle in asthma

The etiology behind the increase in ASM mass in asthmatics has become the focus of research as these cellular changes are believed to be the most significant in the remodeled airway. With increasingly more evidence, it has become apparent that ASM cells serve a far greater role than simply contraction and relaxation, especially within the asthmatic airways (Figure 1).

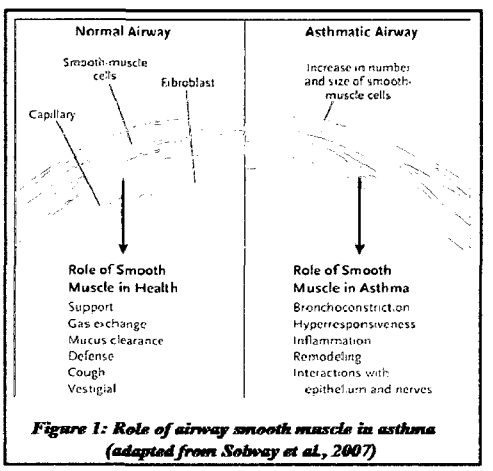

Figure 1: Role of airway smooth muscle in asthma (adapted from Solway et al., 2007)

1.2.1. Proliferation

There is considerable evidence showing an increase in ASM mass in asthmatic patients. For example, research has found a larger quantity of ASM in cases of fatal asthma when compared to controls or asthmatics dying of non-respiratory causes (12, 13). Furthermore, evidence has shown the increase to be due to hypertrophy or, more convincingly, hyperplasia of ASM (13). Finally, the

increase in numbers of ASM cells may occur through increased rates of cellular division or decreased rates of cellular apoptosis (14).

Studies have revealed the capacity of ASM to proliferate in response to mitogenic stimuli such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) (for review see (14)) and transforming growth factor-beta (TGF-β) (15). In addition, through *in vitro* studies, Johnson et al. (16) have demonstrated that ASM cells isolated from asthmatics proliferate faster than cells from non-asthmatic patients. Furthermore, ASM cells obtained from asthmatic patients proliferate faster in response to secreted matrix factors than cells from non-asthmatic patients (17) suggesting an autocrine modulation of proliferation.

Increased proliferation has been reported *in vivo,* where detection of proliferating cell nuclear antigen was observed in rats (18) and mice (19) following ozone and repeated allergen exposure, respectively. Numerous studies have also shown an increased incorporation of the proliferative marker, 5-bromodeoxyuridine, within the ASM layer of Brown Norway (BN) rats following an ovalbumin (OVA) sensitization and challenge regimen (20, 21).

Research has demonstrated that the dominant pathways involved in proliferation of ASM cells may implicate phosphoinositide 3'-kinase (PI3K) and extracellular-regulated kinase (ERK) activation in response to the major mitogenic groups such as polypeptide growth factors, contractile agonists and pro-inflammatory cytokine stimulation (22). Considering the evidence, it appears likely that the enhanced ASM volume in asthma may be attributable to proliferation.

1.2.2 Migration

Evidence has shown that ASM cells are capable of migrating in response to various chemoattractents *in vitro* (23); many of these stimuli, such as PDGF, are present in increased amounts in asthmatic airways. The place of origin of migrating ASM cells *in vivo* remains unclear, however, recent studies have reported potential origins such as the original ASM layer, circulating myofibroblasts and lung- or bone marrow-derived mesenchymal cells (for review see (23)). Thus, migration could, in part, explain the ASM hyperplasia observed in asthma.

1.2.3 Airway smooth muscle phenotypic plasticity

The notion that ASM plays a passive role in the lung no longer remains. In addition to functioning as a structural and contractile cell producing airway narrowing, results from recent *in vitro* studies have shown a phenotypic instability of ASM. That is, when proliferating, ASM cells undergo a phenotypic change from a contractile, quiescent phenotype to one that is synthetic and proliferative. The former remains relatively inactive in terms of protein synthesis and is rich in contractile proteins such as alpha-smooth muscle actin, smooth muscle-myosin heavy chain, and calponin (24). In fact, the existence of a hypercontractile phenotype characterized by increased contractile protein expression and velocity of shortening has been recognized (25). The latter phenotype shows a reduced contractile protein expression, an ability to synthesize and secrete various growth factors as well as matrix proteins and, finally, the capacity to proliferate in response to stimuli (26). Any one of these phenotypes, together with an increase

in ASM mass, may complicate ASM contractility in asthma. ASM cells have been shown to actively synthesize and release pro-inflammatory mediators and ECM proteins capable of further influencing ASM and thus promoting airway remodeling. For example, the ASM cell has the ability to synthesize and secrete TGF-β, which in turn is capable of inducing ASM proliferation and promoting the production of collagen and fibronectin in an autocrine manner (for review see (22)) and may, therefore, contribute to subepithelial fibrosis. Collagen and fibronectin, as will be discussed in further detail (see section 1.3), are pro-proliferative matrix components. *In vitro* studies have demonstrated that serum from asthmatic patients, when compared to serum from non-asthmatics, increases ASM secretion of matrix proteins (27). In addition, ASM is capable of actively synthesizing interleukin (IL)-4, -5, and -13, which have been shown to increase the contractile response through an autocrine mechanism (for review see (28)). Furthermore, exposure of human ASM cells to tumor necrosis factor-alpha (TNF-α) induced release of IL-8, which plays a role in recruitment of neutrophils (29). TNF-α and IL-1β have also been shown to decrease ASM responsiveness to β-agonists (30), thus cytokines are capable of altering both contraction and relaxation. Taken together, the data suggests that the inflammatory conditions observed in the asthmatic airways may favor the proliferative, synthetic phenotype thus further promoting airway remodeling (Figure 2).

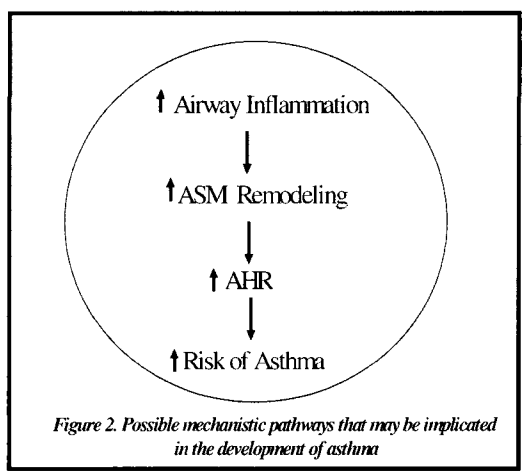

Figure 2. Possible mechanistic pathways that may be implicated in the development of asthma

There is also evidence that shows ASM is capable of producing bronchoprotective factors. For instance, in response to bradykinin, various pro-inflammatory cytokines and acetylcholine, the ASM releases prostaglandin-E_2 (PGE_2) (for review see (26)). PGE_2, in turn, has an inhibitory effect on airway bronchoconstriction as well as other inflammatory responses. In fact, it has been suggested that the major source of PGE_2 in the airways is the ASM (26). Therefore, the role of this prostaglandin may be biologically relevant.

1.2.4 Role of β_2-Agonists and glucocorticoids

Short-term, fast-acting β_2-agonists, long-acting β_2-agonists and glucocorticoids have all been used in the treatment of asthma. The first two have been used to resolve acute bronchospasmic episodes and to control symptomatic asthma and the latter as the major anti-inflammatory relief (31). ASM cells are responsive to both β_2-agonists and glucocorticoids and this response appears to be mediated through β_2-adrenergic and glucocorticoid receptors located on the cell surface and intracellularly of ASM cells, respectively (28).

β_2-agonists inhibit DNA synthesis of ASM cells in culture (14) by decreasing activity of cyclin D1, a critical regulator of cell cycle progression (22). In addition, β_2-agonists may also affect gene transcription of ERK and p38 mitogen-activated protein kinase (MAPK) pathways (32). Although they have been shown to inhibit proliferation, β_2-agonists may not be as effective in treating inflammation (33).

Glucocorticoids exert their anti-inflammatory effects in the nucleus upon binding of the intracellular glucocorticoid receptor. The activated receptor then

translocates to the nucleus where it suppresses gene transcription of pro-inflammatory cytokines and induces production of anti-inflammatory mediators (31). Glucocorticoids have also been shown to exert anti-proliferative effects in that they are able to reduce production of cyclin D1 proteins and inhibit ASM cell cycle in the G1 phase (34). However, several reports have shown that the beneficial effects of glucocorticoids are limited. Roth et al. (35), for example, have reported the failure of glucocorticoids to inhibit proliferation of cells from asthmatic patients *in vitro*. This effect appears to be mediated through the absence of the anti-proliferative transcription factor, CCAAT/enhancer binding protein-alpha, in asthmatic ASM cells.

Recent studies have shown that a combination of both β_2-agonists and glucocorticoids is most effective against proliferative actions and that a certain synergy may exist between the two. That is, glucocorticoids have been shown to up-regulate expression of β_2-agonist receptors and, conversely, β_2-agonists allow for enhanced glucocorticoid receptor translocation (36). In addition, a combination of the two has shown a beneficial effect against the synthetic capabilities of the ASM cell. For example, Pang et al. (29) reported that a combination of salmeterol and fluticasone propionate inhibited TNF-α-induced release of IL-8 in a synergistic manner compared with either treatment alone.

1.3 Extracellular Matrix

The ECM is a well organized complex network of macromolecules that surrounds tissue cells and whose chief function is to provide structural support to the tissues of the body. The various elements that comprise the ECM include collagen types I, II, III, elastin, non collagenous-glycoproteins such as laminin and fibronectin as well as proteoglycans (37). These ECM proteins are capable of influencing cell functions as varied as proliferation and apoptosis, differentiation, migration, localization, and release of mediators from cells originating from a variety of tissues including the airway (38). Together, cells and matrix are able to influence each other in a bidirectional manner. That is, ECM is capable of modulating the behavior of the cells, and the cell in turn can modulate the surrounding ECM.

1.3.1 Proteoglycans: general introduction

The role of proteoglycans, being major constituents of the ECM, has become a recent focus of attention in areas of lung physiology and pathophysiology. Proteoglycans are macromolecules consisting of a protein core and glycosaminoglycan (GAG) side chains which include chondroitin sulfate, keratin sulfate, heparan sulfate, dermatin sulfate and, finally, hyaluronic acid, a GAG not bound to the protein core (39). Proteoglycans have been divided into several families and different subclasses have been described. For instance, the lecticans, such as aggrecan and versican, are large molecules weighing approximately 1000 kiloDaltons (kDa) with numerous GAG sidechains that form

aggregates with hyaluronic acid (39). Furthermore, there are the small leucine rich proteoglycans (SLRP). Well known family members include decorin, biglycan, fibromodulin and lumican (40). Decorin and biglycan show very high homology (40) and are 40-60 kDa molecules that can adopt a horse-shoe shape which allows for close protein-protein interactions (37). Finally, a class of heparan sulfate-bound proteoglycans has been described which includes perlecan, a crucial component of basement membranes, and agrin (37).

Proteoglycans have been identified in cartilage, bone, vasculature and, most recently, the lung and airways. Versican and aggrecan are present in the lung interstitium and tracheobronchial cartilage, respectively (41, 42), whereas lumican has been identified within the normal airway wall (4). Decorin is evident in both human and rat lungs, mainly in the subepithelial connective tissue and, more so, in the adventitial layers of the airways (43, 44). Biglycan, on the other hand, is found mainly in and around the ASM layer and the subepithelial regions (4, 43).

These molecules have been shown to subserve a number of biologically important roles. For example, both decorin and biglycan, members of the SLRP subclass, play a role in basic lung biology. Both are able to bind to collagen, one of the major load bearing components of the lung scaffold, and are capable of affecting collagen fibrillogenesis and matrix assembly (40). Specifically, due to its horseshoe-like structure, a close interaction may be predicted between the concave surface of decorin and collagen fibrils, thus allowing the proteoglycan to stabilize the fibrils and orient fibrillogenesis during axial growth (40). In fact, animal studies using decorin-knockout mice have shown disruption in the organization of collagen fibers when compared to wild-type mice (45).

In addition, these molecules bind different growth factors; decorin has an especially high affinity for TGF-β (46) allowing it to act as a TGF-β storage site. In this way, proteoglycans can influence TGF-β bioavailability and sequester its activities in the ECM. Furthermore, proteoglycans are capable of modulating the ability of growth factors to influence cell proliferation and protein deposition, as TGF-β is often implicated in fibrotic diseases (40).

Finally, the GAG side chains of proteoglycans are exceptionally hydrophilic and are capable of attracting ions and thus fluid into the ECM (40). Versican has largely been identified in the lung parenchyma and has many GAG side chains (41, 42); therefore, it is reasonable to assume that this proteoglycan plays a major role in the viscoelastic properties of the lung. In fact, Al Jamal et al. (47) demonstrated this by obtaining parenchymal strips from rat lungs and exposing them to the digestive enzymes, chondroitinase and heparitinase, which degrade GAG side chains. These strips showed alterations in their mechanical behavior such that a decrease in dynamic tissue elastance was observed compared to control strips. In addition, decorin-deficient mice have demonstrated altered lung mechanics when compared to wild-type mice (48).

1.3.2 Proteoglycans in asthma

Research has shown alterations in the ECM of asthmatic airways and these changes may very well involve proteoglycans. Deposition of collagen and fibronectin in the subepithelial basement membrane of asthmatic airways (49) has been well established. However, recent data supports the notion that proteoglycans also contribute to airway remodeling.

Human studies have greatly contributed to the body of literature on proteoglycans and asthma. For example, Huang et al. (4) have shown enhanced proteoglycan deposition in the airway walls of atopic asthmatics. When compared to non-asthmatic individuals, airways of asthmatics showed an increase in lumican, biglycan and versican in the subepithelial region. In fact, the degree of deposition for each protein correlated with AHR. Proteoglycan alterations have also been described in fatal asthmatics. A hallmark study by Roberts et al. (50) showed prominent staining of proteoglycans decorin, biglycan and versican in airways of fatal asthmatics. A thickened airway wall has functional implications as it may contribute to the increased resistance, luminal narrowing and hypersensitivity present in the asthmatic airways (9). Conversely, de Medeiros et al. (51) compared the content and location of proteoglycans in airways of fatal asthmatics to individuals that had died from non-respiratory causes. The main differences were observed in the smaller airways wherein the outer areas showed a reduction of decorin and lumican content. These observations are significant as they may help to explain the disruption of alveolar-parenchymal attachments and thus relate to the associated changes in lung function characteristic of asthma. Furthermore, a study comparing mild with severe asthmatics showed prominent staining for proteoglycans in biopsies from both groups of asthmatic airways (52). However, an increased deposition of biglycan and lumican was observed in the smooth muscle layer of moderate versus severe asthmatics or controls. This is suggestive of a protective role for airway remodeling in asthma in that this particular distribution of ECM may act to impede ASM shortening.

In vivo studies and animal models of asthma have further strengthened these findings. Studies using the BN rat model of asthma have shown that deposition of decorin, biglycan and collagen I were all increased in allergen-challenged rats (43). Interestingly, biglycan was detected in the smooth muscle layer, while decorin and collagen deposition spared the smooth muscle and were localized in the adventitia. In addition, a mouse model of asthma found increased deposition of decorin in airways following challenge with the allergen OVA (53).

Finally, data from *in vitro* studies has offered further insight into the role of proteoglycans in asthma. For example, Johnson et al. (17) have shown that ASM cells obtained from asthmatic patients increased production of perlecan and collagen I compared with cells from non-asthmatic patients and are capable of enhancing proliferation in an autocrine manner.

Collectively, these data suggest that although the exact role for proteoglycans in asthma remains unclear, it certainly is deserving of further investigation.

1.3.3 Extracellular matrix and airway smooth muscle interactions

The ECM is capable of influencing many aspects of cellular behavior and research has shown a role for the various ECM components in differentially affecting ASM cell proliferation. For example, Hirst et al. (54) have shown that collagen and fibronectin increase ASM proliferation and promote a synthetic phenotype while laminin decreases proliferation and promotes a more contractile phenotype. That is, when human ASM cells were seeded onto a collagen and fibronectin matrix, the authors observed increased proliferation in response to

PDGF stimulation when compared to cells seeded on a laminin matrix. Concurrently, they found a decreased expression of contractile marker proteins such as alpha-smooth muscle actin, calponin and smooth muscle myosin heavy chain in those cells showing an increased proliferative index.

In addition, compared with non-asthmatic extracellular proteins, proteins from asthmatic cells enhanced ASM cell proliferation in an autocrine fashion (17). Studies have also shown that the ECM may exert a major role in human ASM cell survival (55). In this particular study, cells received strong survival signals when seeded on various extracellular matrices or provided with a substratum for integrin binding. Moreover, the ECM environment was able to modulate cell morphology.

Of all the proteoglycans, the role for decorin in the control of cellular growth has been most studied. While evidence has shown that biglycan is able to enhance proliferation and migration of vascular smooth muscle cells (56), it has been reported that decorin may have anti-proliferative and/or pro-apoptotic effects depending on the cell type studied. For instance, in both carcinoma and vascular cells, decorin has been shown to decrease proliferation through its interaction with the EGF receptor (57, 58). In fact, Zhu et al. (59) have demonstrated that decorin is able to bind directly to the EGF receptor and cause its dimerization. In this way, decorin evokes receptor internalization and subsequent degradation through caveolar-dependent endocytosis. In addition, evidence has shown that decorin-induced cell cycle arrest in the G1 phase is related to p21-induced inhibition of cyclin dependent kinase activity (60). Moreover, evidence has presented a pro-apoptotic role for decorin in squamos cell carcinoma lines (61), although an anti-

apoptotic role has been reported in various cell types including macrophages (62), endothelial cells (63) and renal epithelial cells (64).

Recently, the anti-proliferative and pro-apoptotic effects of decorin were further elucidated through experiments from our laboratory. D'Antoni et al. (65) showed the differential regulation of human ASM cells from non-asthmatic subjects by ECM components. On plastic wells coated with a decorin matrix, a significant decrease in cell number was observed at 0, 48 & 96 hours (h) following stimulation with PDGF. Collagen, on the other hand, increased cell number, whereas biglycan had a more modest affect, decreasing cell number only at the 0 h time point. Subsequent experiments showed that the decorin-induced reduction in cell number was primarily due to an increase in apoptotic rates. The decrease in proliferation was more modest.

The anti-mitogenic role of glucocorticoids has been well studied, yet their affects on ECM deposition remain controversial. Although some studies have shown that glucocorticoid treatment was able to reduce thickness of bronchial collagen deposition in the subepithelial layer (66, 67), others have not found any effects of anti-asthma medication in regulating deposition of ECM in the airways (68). In fact, di Kluivjer et al. (69) found an increase in the mean density of decorin and biglycan following steroid treatment. This lack of a consistent effect underscores the need for the development of novel treatments for asthma.

1.4 Mechanical Strain

It is well established that inflammation plays a major role in the structural alterations of the asthmatic airway wall. Another causative factor, however, may be that of mechanical stimulation. The major structural cells of the lung, which include epithelial cells, fibroblasts and ASM cells, are all capable of sensing and responding to their mechanical environment (70). In disease states such as asthma, it seems possible that airway remodeling may modify the mechanical behavior of the airways.

1.4.1 Mechanical forces in the normal lung

The lung is a unique and complex organ in that it is continuously subjected to an array of physical forces, beginning from fetal development through to adulthood. These forces are crucial to the normal development of the lung (72). The mechanical environment is able to modulate cellular proliferation, differentiation and migration during fetal growth (for review see (71)). For instance, studies have shown fluid secretion by epithelial cells is capable of transmitting significant transpulmonary pressure throughout the airway lumen (71). In response, epithelial and mesodermal cells rapidly proliferate and continue to shape the lung; proliferation and migration themselves also exert certain forces (71). Furthermore, research has shown reduced number of airway generations and lung hypoplasia in fetal human lungs developing in the presence of decreased lung volumes or distention (72).

At birth, the airway environment changes in that an air-liquid interface is formed. The mechanical environment becomes more complex and dynamic and is influenced by a multitude of factors that include volume changes, surface tension, and ASM tone (71). In turn, mechanical forces continue to influence growth, proliferation, vasculogenesis in addition to pulmonary surfactant synthesis and secretion throughout adulthood (for review see (70)). Collectively, this body of knowledge demonstrates the fundamental linkage between mechanical forces and cellular function.

1.4.2 Mechanical stimulation in the asthmatic airway

It is apparent that mechanical forces play a vital role in normal lung physiology; however, excessive mechanical stimuli may lead to pathophysiological conditions in the lung. The asthmatic airway walls are likely subject to increased mechanical stimulation because of enhanced baseline tone as a result of an increased state of ASM activation. In addition, due to the heterogeneous nature of bronchoconstriction in asthma, there is uneven airflow distribution throughout the lungs (73). Hence, some airways may be subjected to abnormal volume alterations which transmit greater mechanical strain to airway structural cells.

Cell growth has been widely studied in respect to mechanical loading and studies have confirmed that mechanical forces influence cell growth and remodeling in various tissues. There have been a number of devices developed in order to subject cells to various types of mechanical stimulation. For instance, Tschumperlin et al. (74) exposed epithelial cells to mechanical stress by seeding

them on transwells and applying an apical to basal transcellular pressure in order to compress cells. In addition, a unique model was devised that allows for the selective, single cell application of stress through a method called optical magnetic twisting (75). Specifically, cells are bound to Arg-Gly-Asp (RGD)-coated ferromagnetic beads which are then magnetized in certain directions to allow for distortion of the cell membrane. Xu et al. (76) employed a method to mechanically stretch cells wherein mixed fetal lung cells were embedded in a three dimensional gel. In order to strain cells in this experimental system, one end of the gelfoam sponge remains fixed to an immovable surface while the other is attached to a movable metal bar controlled by a magnetic force and driven by solenoid valves. Finally, numerous studies have used the Flexercell strain unit to subject cells to cyclical mechanical strain (77). This is ideal for investigations of airway function as the cyclical nature of the device simulates the pulsatile airflow experienced while breathing.

1.4.3 Mechanotransduction

Mechanotransduction is the biological process wherein cells respond to mechanical stimuli by altering protein metabolism (78). How mechanical forces applied to whole tissues can be sensed by cells and transduced into biochemical signals at the molecular level remains unclear. Various factors that may be involved include stretch-activated ion channels, mechanoreceptors and, finally, matrix-integrin-cell interactions (for review see (70)). The latter is most interesting because it provides a direct route for mechanical signals to be transmitted into the cell.

Many studies have demonstrated the effects of mechanical forces on the various resident cells of the lung. Results are in line with data that have been observed in human asthma or in cells obtained from asthmatic patients. For example, Tschumperlin et al. (74) exposed bronchial epithelial cells to compressive stresses similar to that occurring *in vivo* as a result of bronchoconstriction. In response, cells showed an enhanced release of TGF-β and endothelin. Both of these peptides play a role in fibrosis and elevated levels have been found in asthmatic airways (79, 80). Ma et al. (81) found increased levels of smooth muscle-myosin light chain kinase (sm-MLCK), a marker of contractility, in endobronchial biopsies obtained from asthmatic patients versus control. Concurrently, Smith et al. (82) observed an increase in sm-MLCK following cyclical mechanical strain of ASM cells. In addition, these same two groups observed enhanced shortening capacity of ASM cells from asthmatic patients (81) and following stretch *in vitro* (83). Mechanical strain has also been shown to result in increased proliferation of ASM cells *in vitro* (77), thus mimicking results from human studies showing hyperplasia *in vivo* (13) and enhanced proliferation of asthmatic cells in culture (16).

The effects of mechanical stimulation on remodeling of the ECM have been investigated. Mechanical strain has been shown to differentially regulate gene expression of the major ECM components in lung cells, including increased secretion of collagen I, IV and biglycan compared with static controls (84). Another study found increased mRNA levels of procollagen in pulmonary fibroblasts seeded on elastin and laminin matrices and exposed to mechanical strain when compared to controls (85). This, however, was not observed when

fibroblasts were seeded on fibronectin. Furthermore, Ludwig et al. (86) reported that fibroblasts obtained from asthmatic patients and subjected to cyclic mechanical strain on collagen-coated wells increased gene expression of decorin and versican compared with control cells. These authors hypothesized that enhanced message for versican may serve as a protective mechanism for the cell. Secretion of versican, being a highly hydrophilic proteoglycan and attracting fluid into its surrounding, may act as a kind of "shock absorber" to absorb and disperse excessive mechanical forces. Although there have been reports of increased ASM cell proliferation in response to cyclic mechanical stretch (77), Bonacci et al. (87) have shown a decrease in cell number in response to strain. Specifically, bovine ASM cells were seeded on a collagen matrix and subjected to a strain regimen in the presence of bFGF. Strain decreased cell number and these observations were not found in static counterparts nor cells seeded on laminin and mechanically strained. This is an interesting observation given that the culture conditions which resulted in a decrement in cell number were a collagen matrix, presence of growth factor, and abnormal mechanical stimulation; all features characteristic of an asthmatic airway. This may again be a protective mechanism working to prevent enhanced ASM volume. Mechanical strain has also been shown to inhibit proliferation and induce apoptosis in rat lung fibroblasts (88).

Studies investigating ventilation-induced lung injury have also reported pathological changes resulting from excessive mechanical forces. This is exemplified in a study by Vaneker et al. (89) who demonstrated an increase in pro-inflammatory cytokines IL-1α, IL-6 and TNF-α in murine model of mechanical ventilation. Interestingly, one group reported that following

mechanical ventilation of healthy rats, proteoglycan fragmentation was observed (90). Moreover, this group showed that the presence of GAG fragments preceded any changes in respiratory mechanics suggesting this is an early response to mechanical ventilation. Data from our laboratory have also demonstrated an up-regulation of the proteoglycans versican, perlecan and biglycan in response to excessive mechanical ventilation (91). Essentially, abnormal mechanical stimulation and subsequent mechanotransduction may affect a variety of factors, all of which may contribute to airway remodeling in asthma.

1.4.4 Integrins and mechanical stimulation

How cells are able to act as mechanosensors remains unclear but may involve a variety of factors including the ECM, specifically through integrins. Integrins are transmembrane receptors consisting of non-covalently linked αβ-subunits which act as the site for ECM attachments (75). Specifically, the α-subunit allows integrins to bind to the ECM by recognizing RGD sequences, whereas the β-subunit functions as a signal transducer (92). Once activated, the β-subunit interacts with numerous proteins to form a focal adhesion complex (93). In this way, various signal transduction pathways are activated including c-Jun N-terminal kinases (JNK), ERK and p38 MAPK which coordinate many cellular functions, including survival and apoptosis (for review see (93)). Freyer et al. (55) have reported that functionally blocking the $\alpha_5\beta_1$ integrin resulted in the loss of ECM-derived cell survival signals in human ASM cells. Moreover, integrins are able to transduce mechanical changes from the ECM directly to the actin cytoskeleton through interactions with adaptor proteins such as talin and α-actinin

which, in turn, bind other structural proteins including vinculin, paxillin and tensin (94). Integrins are thus able to alter cytoskeletal organization and therefore influence the behavior of cytoskeleton-associated signaling molecules (95) (Figure 3).

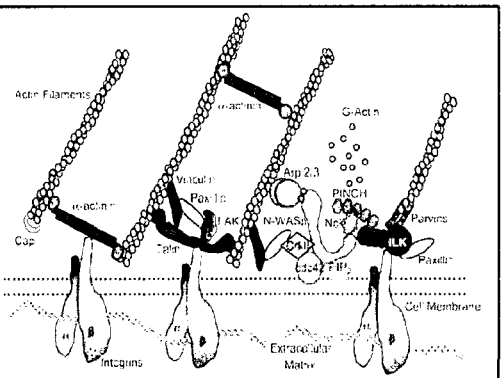

Figure 3. Model of molecular organization of cytoskeletal-integrin-ECM interactions (adapted from Zhang et al., 2008)

Within the family of integrins, 24 α- and 9 β-subunits have been described to date (38). Little is known about the expression of integrins on ASM cells, yet recent evidence suggests that differences do exist between expression in smooth muscle cells *in vivo* and in culture. For example, healthy human ASM cells *in situ* express α_1, α_3, β_1 and β_3, while in culture these cells have been shown to express subunits $\alpha_{1\text{-}5}$, α_V, β_1, β_3, and β_5 (for review see (38)). Furthermore, reports have demonstrated a differential expression of integrins between asthmatic and non-asthmatic cells in response to cytokine stimulation (96).

Various studies have confirmed the role of integrins in mediating cellular responses to mechanical stimulation. For example, one study in which vascular smooth muscle cells were subjected to mechanical stretch showed increases in both expression of β_1-integrin and rates of apoptosis (97). Subsequent experiments revealed that functional blocking of the β_1-integrin inhibited this response, thus suggesting a role for the integrin in mediating stretch-induced apoptosis. Furthermore, ligament fibroblasts exposed to cyclic strain showed greater strain-dependent growth inhibition on fibronectin versus collagen substrates (98), indicating that cellular responses to mechanical strain are

conferred through ECM-specific interactions. Expression for β_1 and α_5 integrin subunits was also increased following strain. In addition, strain was shown to induce a mitogenic response in rat vascular smooth muscles cells when seeded on collagen, fibronection or vitronectin substrates but not elastin or laminin (99). This response, however, was abrogated by blocking integrin subunit β_3 and integrin $\alpha_V\beta_5$.

Le Bellego et al. (100) reported that asthmatic fibroblasts responded differently to mechanical strain than non-asthmatic fibroblasts. During strain on collagen matrices, phosphorylation of p38 was increased in non-asthmatic fibroblasts, while strain increased JNK phosphorylation and decreased ERK phosphorylation in asthmatic fibroblasts. Activation of these signaling pathways may be mediated through various integrins. Strain has also been shown to regulate localization of focal adhesion proteins in cultured smooth muscle cells (101, 102), reorganize cytoskeletal elements (82) and induce clustering of β_1-integrins (103).

Taken together, these data suggest that mechanical forces could induce unique cellular responses that may be dependent on the cell phenotype and microenvironment. Depending on the type of stimulus, cell and integrins present, different pathways could be activated that may either induce survival or promote apoptosis.

1.5 Animal Models of Asthma

Observational studies from human asthmatics have provided detailed reports supporting a critical role for ASM and ECM remodeling in asthma. This

approach, however, limits investigations into the mechanisms involved in airway remodeling. Research has, therefore, employed the use of *in vivo* models of asthma, as invasive methods for measuring changes in the airway wall can be performed and may thus allow for a more comprehensive assessment of asthma pathophysiology.

Several animal models of asthma have been used which have broadened our understanding concerning the effects of chronic allergic inflammation on airway remodeling. Considerable attention has been given to the BN rat model of allergic asthma. Studies have shown that this rat strain is capable of replicating several of the pathological features observed in human asthma, such as early and late AHR, infiltration of eosinophils and increased ASM mass (for review see (104)). In addition, mouse models have been widely employed in order to examine airway responses resulting from allergen challenge (for review see (105)). Horses (106), cats (107), canines (108, 109) and rhesus monkeys (110) have all been shown to reproduce many characteristics typical of asthma (for review see (18)).

1.5.1 Brown Norway rat model of allergic asthma

The BN rat is a well established model used to investigate ASM hyperplasia and hypertrophy. For example, Panettieri et al. (21) administered repeated OVA challenges to sensitized BN rats. In order to determine whether hyperplasia accounted for the observed increase in ASM mass in large and medium airways, the authors used bromodeoxyuridine, an indicator of DNA synthesis. Results showed that OVA inhalation induced DNA synthesis in ASM

cells when compared to saline (SAL)-challenged controls. Similarly, Salmon et al. (18) noted a greater than 3-fold increase in ASM cell DNA synthesis in the large airways in addition to eosinophilic and lymphocyte recruitment in rats following repeated OVA challenge. To further investigate this concept, Xu et al. (111) employed unbiased stereological methods to determine whether ASM hyperplasia occurred in these allergen-challenged rats. This direct and effective method for counting cells demonstrated an increase in ASM cell number 7 days following the fourth OVA challenge and this was associated with airway inflammation and transient increases in AHR. Interestingly, administration of an endothelin receptor antagonist has been shown to attenuate the allergen-induced proliferative and inflammatory response in BN rats (112). Reports have shown that endothelin-1 (ET-1) is able to cause potent contraction and proliferation of ASM cells *in vitro* in addition to possessing various pro-inflammatory properties (for review see (112)). Therefore, Salmon et al. (112) were able to show a role for ET-1 in the proliferative and inflammatory responses to allergen challenge *in vivo*; although its role in AHR remains unclear. Collectively, these data suggest that allergen-induced increases in ASM mass in BN rats may be attributed to hyperplasia.

There is considerable debate concerning phenotypic modulation of ASM cells in asthma, i.e., alterations among contractile, synthetic or hypercontractile cells. Moir et al. (113) repeatedly challenged BN rats with OVA in order to investigate phenotypic modulation of ASM. Twenty-four hours after the first challenge, results from isolated bronchioles showed that ASM content was significantly increased in OVA-challenged rats when compared to SAL-challenged controls. However, an increase in the number of cell nuclei was not

detected. In addition, bronchiolar smooth muscle explants demonstrated an increase in maximal tension development *ex vivo*. Moreover, at this same time point, semi-quantitative western blotting demonstrated a decrease in smooth muscle contractile, cytoskeletal and regulatory proteins such as alpha-smooth muscle actin, smooth muscle-myosin heavy chain , calponin and smoothelin-A. Interestingly, these differences were not observed 7 days following the last challenge. However, at day 35, increases in force generation and reductions in the majority of the contractile proteins were still noted in OVA-challenged rats. Results from this study are difficult to interpret and many conclusions may be drawn. Although hyperplasia was not shown to account for the increase in ASM content, results suggest that OVA inhalation induces a phenotypic modulation of ASM cells *in vivo* and enhanced responsiveness *ex vivo* that resemble changes in asthma.

Notably, BN rats also develop alterations in ECM. Vanaker et al. (114) reported increased fibronectin deposition following repeated allergen exposure. Interestingly, reports have also shown that OVA challenge results in concomitant alterations of collagen, biglycan and decorin in BN rats (52).

Taken together, these data indicate that the BN rat model of allergic asthma is useful in examining structural changes typical of asthmatic airways in addition to studying underlying mechanisms that may play a role in airway remodeling.

1.5.2 Limitations

Although the aforementioned animal models have all proven useful in studying the pathophysiology of asthma *in vivo*, the relevance of these experiments is still limited as there may be discrepancies at the cellular and molecular levels. In addition, differences exist in the anatomy of the respiratory tree between humans and animals and results extrapolated may not be indicative of human conditions. Finally, considerable attention must be given to that fact that allergic disease in these models has been relatively acutely induced and this may create confounding factors when representing the chronic, naturally occurring disease that is asthma (106).

1.6 Objectives and Hypotheses of this study

The purpose of the present study will be to further characterize cell-matrix interactions and their effects on ASM growth and survival. Specific aims include investigation of the effects of proteoglycans (decorin & biglycan) and mechanical strain on ASM proliferation. In addition, any synergistic effects between these two factors will be examined (Figure 4). Finally, analyses will be performed in order to determine differential responses to matrix and mechanical stimulation of ASM cells isolated from OVA-challenged animals compared to SAL-challenged control animals. We hypothesized

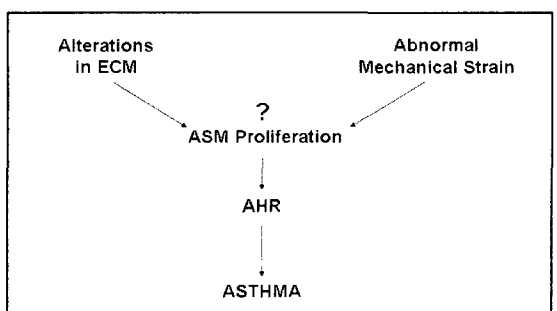

Figure 4. Proposed mechanistic pathways that may lead to asthma

that the anti-proliferative/pro-apoptotic effects of decorin and biglycan on ASM cells will be modulated by mechanical strain. Furthermore, OVA ASM cells will be less sensitive to the effects of these proteoglycans.

Chapter two
~ *MATERIALS & METHODS* ~

Chapter two
~ Materials & Methods ~

2.1 Materials

The following reagents were purchased from Gibco-Brl-Invitrogen (Burlington, Ont. Canada): Dulbecco's Modifed Eagle Medium (DMEM), F-12 Nutrient Mix (HAM), Antibiotic-Antimycotic, Fetal Bovine Serum (FBS), Trypsin-EDTA solution, Hanks' Balanced salt solution (HBSS). Decorin, biglycan, collagen Type 1, transferrin, insulin, absorbic acid, bovine serum albumin (fraction V) (BSA), monoclonal anti-alpha smooth muscle actin, anti-calponin, anti-pan cytokeratin and tetramethylethylenediamine (TEMED) were purchased from Sigma (Oakville, Ontario, Canada). To detect antibodies, Alexa Fluor 568 signal amplification kit was purchased from Molecular Probes (Burlington, Ontario, Canada). PDGF was purchased from R&D Systems (Minneapolis, MN, USA). β_1-Integrin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, U.S.A). Molecular markers, HRP Chemiluminescence Kit and precision strep tactin HRP were purchased from Bio-Rad laboratories (Hercules, California, U.S.A). BCA Protein Assay Kit was purchased from Pierce (IL, USA)

2.2 Airway Smooth Muscle Cell Isolation & Cell Culturing Experiments

2.2.1 Brown Norway rat model of allergic asthma

Cells used in *in vitro* studies were obtained from the BN rat model of allergic asthma as previously described (43). Male BN rats were obtained from Harlan, United Kingdom. To induce allergen-driven experimental asthma (Figure 5), allergic sensitization was performed on 7-8 week-old rats by a single subcutaneous injection of 1 mg of OVA with 100 ug aluminum hydroxide adjuvant in phosphate buffered saline (PBS) solution. Heat-killed bacteria bordetella pertussis (2×10^9) was given intraperitoneally (ip). Two weeks later, subsequent airway OVA challenge (or SAL control) was delivered as an aerosol through orotracheal intubation repeatedly on days 14, 19 and 24. On the day of challenge a 5 % wt/vl solution of OVA was prepared in sterile saline. Xylazine (7 mg/kg ip) was used to sedate the rats, after which the animals were anesthetized with sodium pentobarbital (30 mg/kg ip). The polyethylene tubing used to intubate the rats was inserted into a Plexiglas box into which a micromist nebulizer (Hudson RCI; Teleflex Medical, Temecula, CA) delivered aerosols of OVA or SAL for five minutes at a rate of 8 liters/min. BN rats were either sensitized and challenged with OVA or challenged with SAL as control. Forty-eight hours following the last antigen challenge, animals were sacrificed with 1 ml injection (ip) of sodium pentobarbital and smooth muscle cells isolated from the trachea. All experimental studies conducted on animals were performed using

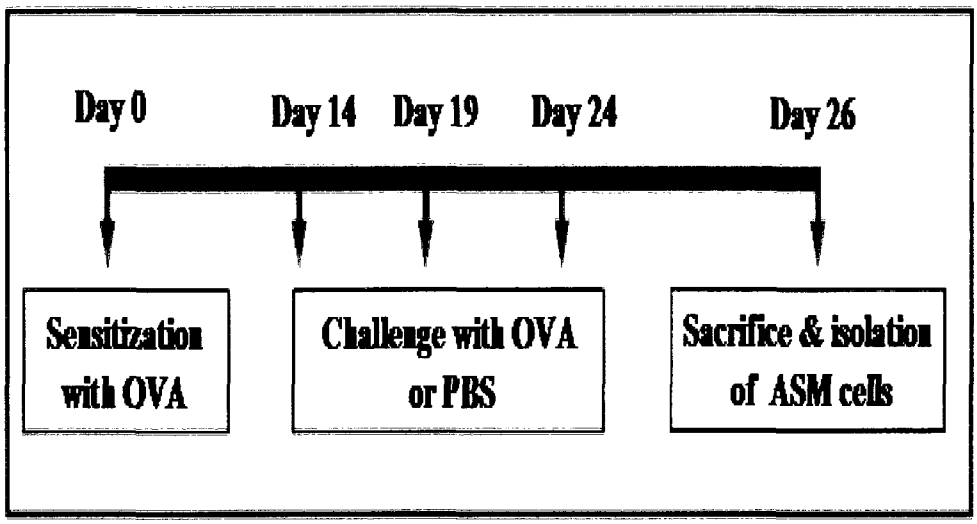

Figure 5: Timeline for induction of ovalbumin-driven experimental asthma. BN rats were sensitized with ovalbumin (OVA) on day 0. Two weeks later, antigen challenges were delivered at 5 day intervals. Forty-eight hours following the last challenge, animals were sacrificed and ASM cells were isolated.

protocols approved by McGill University Animal Ethics Committee in compliance with the Guide to the Care and Use of Experimental Animals devised by the Canadian Council of Animal Care (see Appendix).

2.2.2 Cell isolation and cell culture

Following sacrifice, tracheas were removed from both groups under aseptic conditions, incubated in an enzyme solution containing HBSS with collagenase type IV (6 mg) and elastase type IV (1.5 mg) and then placed in a water bath. These enzymes were specifically chosen due to the high content of collagen and elastin in the matrix surrounding ASM (115). Following digestion, the connective tissue was filtered out and the remaining cell suspension was centrifuged in order to separate out ASM cells. The cell pellet was re-suspended in 10% FBS in DMEM (supplemented with L-glutamine, 110 mg/ml sodium pyruvate, and pyroxidine hydrochloride)-F-12 medium (supplemented with l-glutamine) containing an antibiotic-antimyotic cocktail and plated in a 25 cm^2 culture flask. The medium was changed every 48 h. For all experiments, passages 1-5 were used.

2.2.3 Indirect immunofluorescence to stain for smooth muscle-specific proteins

The presence of rat ASM cells was confirmed by positive immunocytochemical staining for smooth muscle specific alpha-actin and calponin. These proteins were chosen as they have been shown to be specific to ASM and are not present in fibroblasts and epithelials cells (two cell types which may contaminate cell cultures upon isolation of smooth muscle cells) (24).

Contamination of cultures by epithelial cells was also ruled out by negative staining for keratin. Cells were grown to post-confluence on glass chamber wells in the presence of DMEM-F12 medium containing 0.5% FBS, 0.4 ug/ml insulin, 5ug/ml transferrin, 100uM absorbic acid, 0.1% BSA and antibiotic-antimiotic cocktail (0.5% FBS medium). Cells were serum starved in order to maximize expression of contractile proteins (24). Following a growth arrest period, medium was removed and glass chambers containing cells were gently washed with PBS solution. In order to permeabalize cells, 300 ul of ice-cold acetone was added to each chamber well and kept for 20 minutes at -20°C. After which, acetone was removed and replaced with 70% ethanol for 5 minutes also at -20°C. The following steps were performed at room temperature. The chamber wells were removed leaving only a glass slide to which the cells had been fixed. The slides were rinsed 3 times with PBS for 5 minutes/rinse in order to rehydrate cells. The slides were then immersed in 1% BSA solution for 30 minutes in order to block any unspecific binding sites. The previous washing step was then repeated. Slides were dried using blotting paper and primary antibodies for alpha-smooth muscle actin, calponin and keratin were added (diluted 1:400, 1:10000, 1:400 in PBS solution, respectively). Slides were incubated in a humidified plastic container for 1 h. During this time secondary antibody was prepared. Following the incubation period, slides were washed, blot-dried and the secondary antibody (10 ug/ml recommended concentration for rabbit anti-mouse IgG) was applied for 30 minutes followed by a 30 minute incubation period with 10 ug/ml goat anti-rabbit IgG in order to enhance the fluorescence signal. Positive cells were visualized using Image Pro-Plus technology (Figure 6).

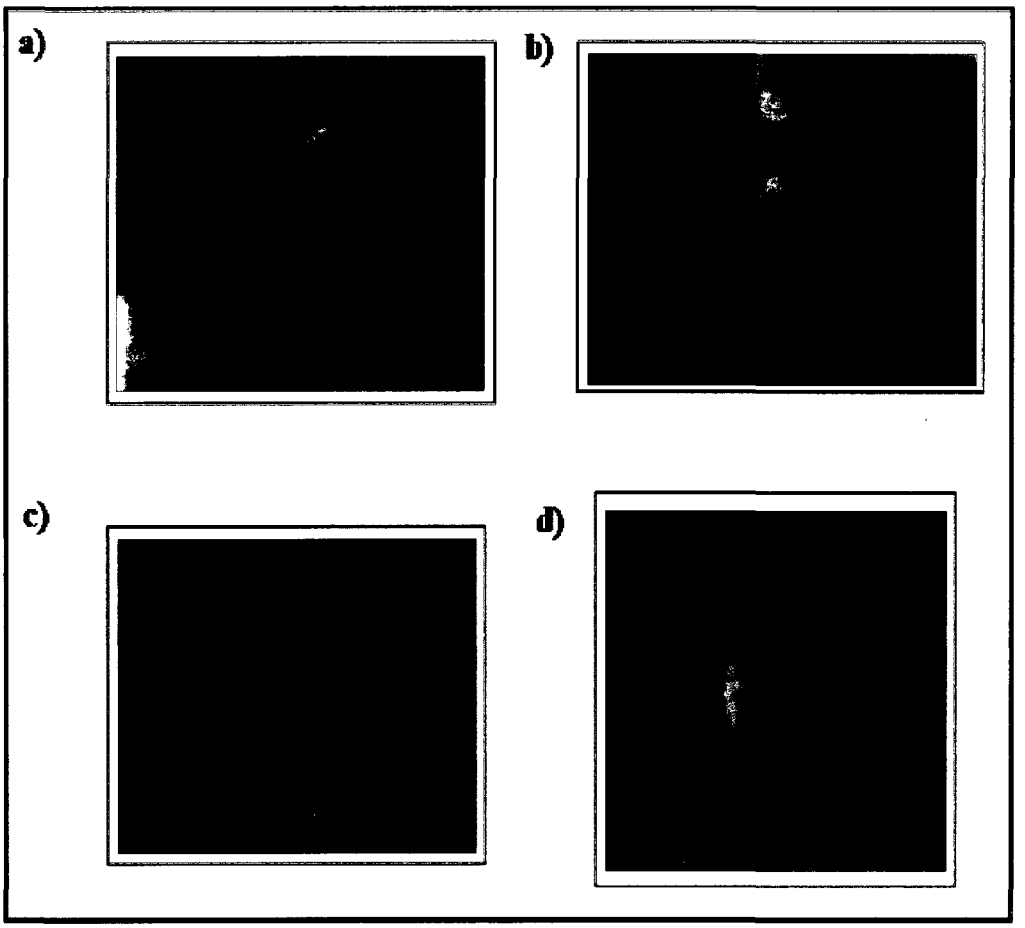

Figure 6: Indirect immunofluorescence to stain for smooth muscle-specific proteins. Visualization of positive staining for both alpha-smooth muscle actin in SAL (**a**) and OVA (**b**) cells and calponin in SAL (**c**) and OVA (**d**) cells.

2.2.4 Surface coating of culture well with extracellular matrix

Decorin and biglycan, purified from articular bovine cartilage, were diluted in PBS to concentrations of 0.1, 0.3, 1.0 ug/ml. Members of our laboratory have successfully used matrix concentrations of 1, 3 and 10 ug/ml upon which to seed human ASM cells (65), however these dilutions resulted in no measurable growth for rat ASM cells. Therefore we tested the above concentrations of matrix proteins and achieved results comparable to those of the human ASM cells. The diluted proteins were adsorbed to six well plastic culture plates and incubated over night at 37°C. Plates were then washed with PBS to remove unbound proteins followed by a 30 minutes incubation period at room temperature with 0.1% BSA in PBS to block any unoccupied binding sites. This protocol was adopted from other research groups as described (54, 65). Experiments in our laboratory have confirmed, through western blot analysis, that this method is able to successfully adsorb matrix protein onto a glass slide in a graded fashion.

2.2.5 Cell culturing protocol on plastic culture plates

Cells were seeded onto 6-well culture plates (growth area per well is 9.6 cm^2) wells at a density of 4167 cells/cm^2 in the presence of 0.5% FBS medium and allowed a growth arrest period of 48 h (Figure 7). Earlier experiments in our laboratory have shown that rat ASM cells will achieve confluence more rapidly than human ASM cells. For this reason, we chose to seed cells at a lower density than the conventional 6250 cells/cm^2. Following serum starvation, cells were stimulated with 10 ng/ml of the mitogen PDGF in 0.5% FBS medium

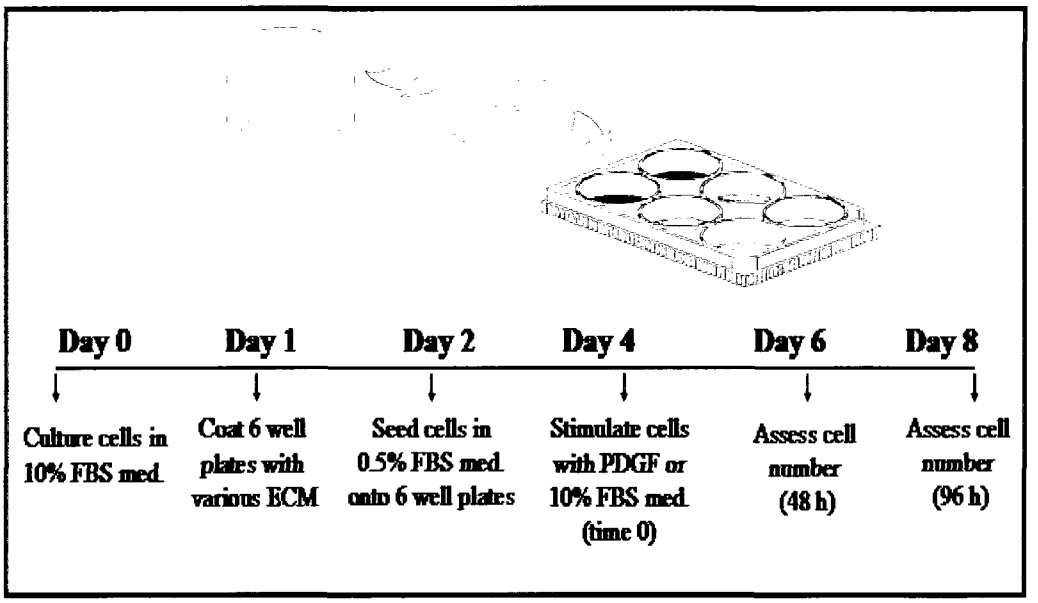

Figure 7: Experimental timeline for cell culturing and counting of ASM cells. Cells were seeded onto matrix-coated plastic wells (day 2) and allowed a 48 h growth arrest period. On day 4, cells were stimulated with PDGF and cell number was assessed on day 6 and day 8.

(0 h time point) and medium was replenished every 48 h thereafter. PDGF is widely established as a potent stimulator of ASM cell proliferation (116). Our culture conditions consisted of cells grown on varying concentrations of matrix-coated wells. The baseline control consisted of cells seeded on plastic in the presence of 0.5% FBS medium. Cells were seeded on plastic in 10% FBS medium as a positive control. Cells in all culture conditions were stimulated with PDGF at 0 and 48 h, except for our positive control. At 0, 48 and 96 h ASM cells were harvested by washing twice with PBS to remove medium and adding trypsin-EDTA solution to detach cells. Neutralization of trypsin was achieved via the addition of 1:5 (vol/vol) of 10% FBS medium. Following centrifugation (5 minutes, 1500 rpm), cells were re-suspended in 0.5% FBS medium and counted.

2.2.6 Cell enumeration

Cell number was assessed at 0, 48 and 96 h using the trypan blue viability test and a hemocytometer chamber. This test uses a blue stain that will selectively color dead cells. Viable cells with intact membranes are not permeable to the dye and will not stain blue. Cells were then counted using a hemocytometer, which consists of a thick glass slide divided into nine sections each consisting of sixteen smaller squares and a coverslip. Cells were introduced into the hemocytometer via a small chamber and each square represents a volume of 0.1 mm^3. Through a series of calculations one is then able to determine the number of cells per sample. Cell number was assessed at the 0 h time point in order to verify whether the initial number of cells seeded (40 000 cells per well) persisted following serum starvation and mitogenic stimulation. Forty-eight and 96 h time points were

chosen for the reason that at these points cell growth remains in the log phase (linear portion of growth curve) rather than the plateau phase of cell growth (24).

2.3 Mechanical stimulation of airway smooth muscle cells

2.3.1 Cell culturing protocol on silastic culture plates

Six-well culture plates composed of a flexible silastic bottom membrane were coated with biglycan, decorin (both 0.1 ug/ml) and collagen (rat tail, 1.0 ug/ml) as described above. We were not able to apply the same culturing protocol that was described for experiments on plastic (see section 2.2.5) on the silastic membranes. These membranes are made of silicone and plastic and it was extremely difficult to initiate cell attachment and growth when cells were seeded initially in the presence of 0.5% FBS medium. In addition, experiments showed that a membrane without matrix was not sufficient for cell attachment. Specifically, the cells required a matrix coating onto the silastic membrane prior to seeding for attachment and spreading. For this reason, a baseline and positive control similar to the one in the plastic experiments could not be used. Collagen coated wells were therefore chosen as a positive control. We found that concentrations of 0.1 and 1.0 ug/ml collagen yielded comparable results for ASM cell number. Furthermore, it was shown that cells seeded on silastic wells did not respond to stimulation with PDGF in the same manner as compared to when seeded onto plastic wells. Taking all of these factors into consideration and through a series of preliminary experiments, a modified protocol was created

(Figure 8). Cells were seeded onto culture plates in the presence of 10% FBS medium for 48 h in order to allow for appropriate attachment and spreading onto the silastic membrane. Cells remained in 0.5% FBS medium for 24 h prior to strain initiation, after which cells were mechanically strained for 48 h in the presence of 10% FBS medium.

2.3.2 Regimen for mechanical strain of airway smooth muscle cells

The Flexercell Strain Unit (FX-3000, Flexercell McKeesport, PA, USA) was used in order to generate strain on cells (Figure 9). Following a 24 h period of serum starvation (0 h time point) culture plates were placed onto a vacuum manifold controlled by computer software and a solenoid valve. The system uses a vacuum source to apply a negative pressure causing a downward deformation of the membrane to which the cells have attached. Studies have shown that the strain applied over the loading-post regions is approximately equal in the radial and circumferential directions (117). At strain initiation, cells were replenished with 10% FBS medium and subjected to an equibiaxial strain of 17% amplitude for 48 h at a frequency of 0.20 Hz. This strain regimen was chosen in order to apply an excessive strain on the cells. Previous research has shown that if muscle stretches isotropically as the cube root of lung volume, then a 17% strain would appear to be within the physiological range for airway distension during deep inspiration (118). A frequency of 0.2 Hz was specifically chosen to allow for the maximum amount of strain possible; that is, an inverse relationship exists between frequency

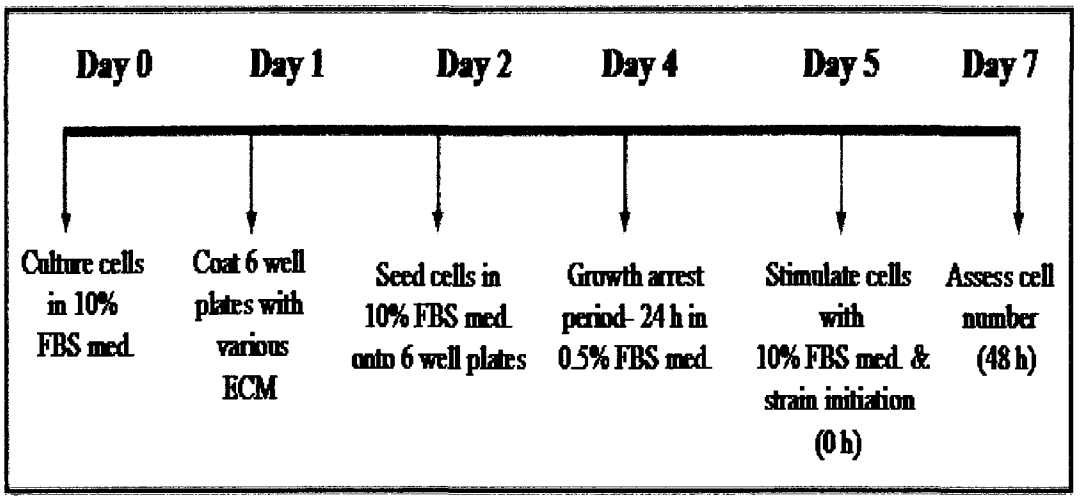

Figure 8: Experimental timeline for mechanical stimulation of ASM cells. Cells were seeded onto matrix-coated silastic wells on day 2 in the presence of 10 % FBS medium. Following a growth arrest period of 24 h, mechanical stimulation was initiated on day 5 and cell number was assessed on day 7.

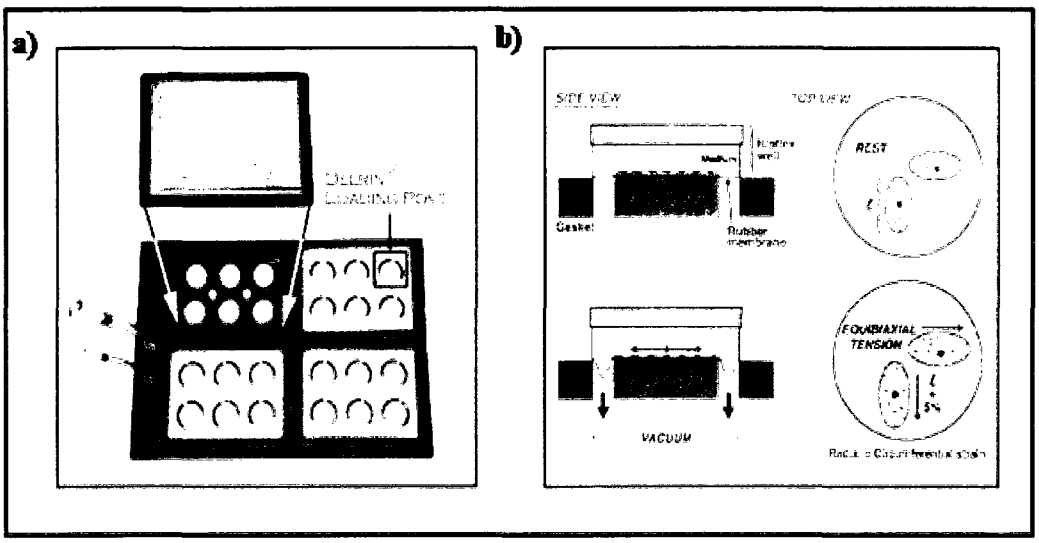

Figure 9: Flexercell Strain Unit

Flexible silastic-bottom six-well culture plates containing cells were placed on a vacuum manifold controlled by computer software and a solenoid valve (**a**). The use of loading-posts allows for an equibiaxial strain in the circumferential and radial directions (**b**). FX-3000, Flexercell McKeesport, PA, USA

and percent strain.

2.3.3 Cell enumeration

Cell number was assessed 48 h following mechanical strain using the trypan blue exclusion test and hemocytometer as described above (see section 2.2.6). In addition, cell layer and culture medium were collected and stored at -80°C.

2.4 Western Blot Analysis for expression of β_1-integrin in airway smooth muscle cells

2.4.1 Protein assay

BCA protein kinase assay was used in order to measure protein content in cell samples. Cells harvested from mechanical strain experiments were centrifuged and the cell pellet washed twice with PBS solution to ensure medium free conditions. Cells were re-suspended in lysis buffer (contaning 20 mM tris-HCL, 150 mM NaCl, 1 mM Na_2–EDTA, 1 mM EGTA and 1% Triton in addition to protein inhibitor cocktail). Sonic Dismembrator (Fischer Scientific) was used to help dissolve the cell membrane. The cell lysate was then centrifuged at 1300 rpm for 15 minutes after which supernatant containing protein content was collected and measured using a microplate reader.

2.4.2 Western blot analysis

Equal amounts of protein in each lane were run on a 10% SDS-PAGE gel, after which separated proteins were transferred to a nitrocellulose membrane. In order to prevent non-specific binding, the membrane was incubated with 5% skim

milk in Tris Buffered Saline Solution (TBS; 0.02 M Tris; 0.5 M NaCl; ph 7.6) with 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were then probed with primary antibodies against β_1-integrin (diluted 1:200) overnight at -4°C. Membranes were then washed with TBS-T and probed with horseradish peroxidase-labelled secondary antibodies (diluted 1:5000) for 1 h at room temperature followed by further washing with TBS-T. Enhanced chemiluminescence was performed to visualize immunoreactive bands. Preliminary experiments were performed on a test group of rat ASM cells grown on plastic in 10% FBS medium in order to determine appropriate antibody dilutions. Figure 10a shows the relationship between band density and the number of cells at a 1:200 dilution. Densitometric analysis of β_1-integrin was performed using image analyzer software (Fluorchem; Alpha Innotech, San Leandro, CA) (Figure 10b). Software measures the sum of all pixel values following background correction.

2.5 Statystical analysis

Graphpad Software was used to analyze these data and results were expressed as +/- SE. Anova with Dunnett's Multiple Comparison Test was used to analyze difference between matrix concentrations compared to baseline control. A two-way Anova was used to identify differences between cell groups. An independent T-test was performed to verify for differences between strain and no strain groups.

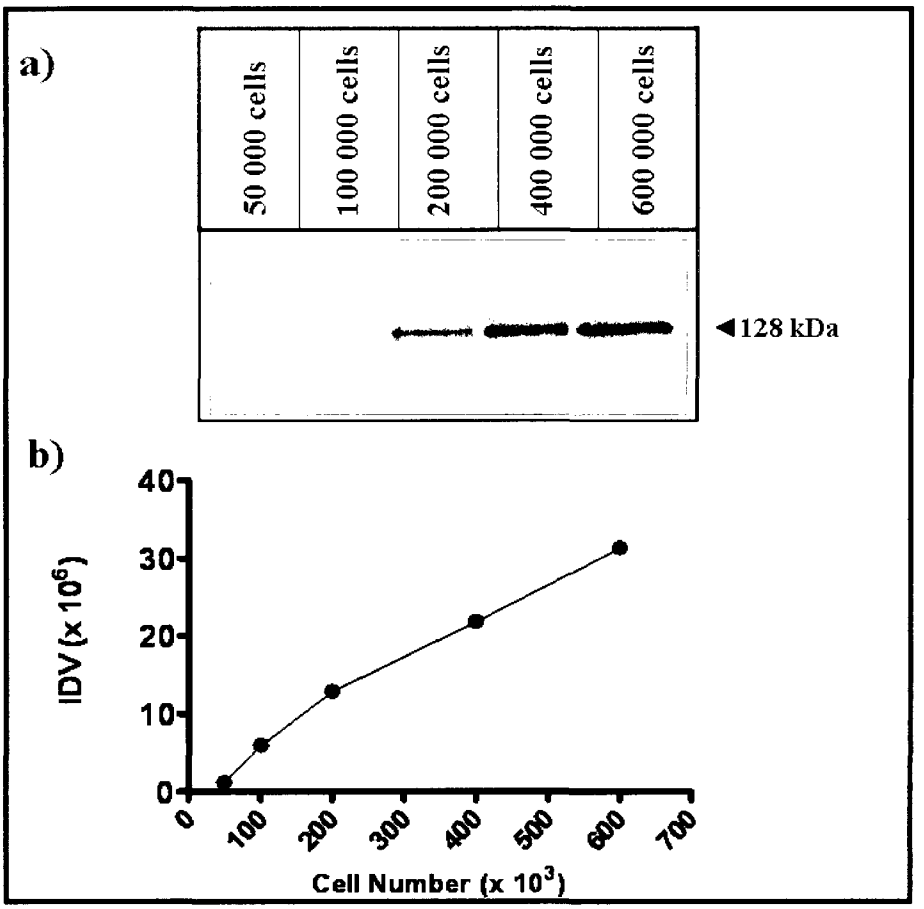

Figure 10: Western Blot analysis for expression of β_1-integrin in ASM cells. Visualization of expression for β_1-integrin in various cellular concentrations of OVA cells (**a**). Densitometric analysis shows the expression of β_1-integrin increases in a graded fashion according to cell number (**b**). Measurements presented as integrated density value (IDV), arbitrary units.

Chapter three
~ *RESULTS* ~

Chapter three
~ Results ~

3.1 Cell number is increased in OVA cells when compared to SAL cells

Analysis of the data showed that, when cultured under baseline conditions, cell number for OVA cells was significantly higher when compared to SAL cells at 48 h (Figure 11). These differences were also apparent when cells were seeded on 0.1 and 1.0 ug/ml decorin matrices.

3.2 Effect of extracellular matrix proteins on airway smooth muscle cell number

3.2.1 Effect of decorin on airway smooth muscle cells

Rat ASM cells were seeded onto 6-well culture plates coated with decorin (0.1, 0.3, 1.0 ug/ml). Cell number was assessed initially at the point of stimulation with PDGF (0 h) and 48 and 96 h post-stimulation. At the 0 h time point, cell numbers did not differ compared to 0.5% FBS medium (baseline control), regardless of cell group or matrix coating. For SAL cells, a significant reduction in cell number was observed compared to 0.5% FBS medium for cells seeded onto a 1.0 ug/ml matrix at 48 h (Figure 12a). In addition, seeding OVA cells onto the same concentration of decorin significantly reduced cell number compared to baseline control also at 48 h (Figure 12b).

3.2.2 Effect of biglycan on airway smooth muscle cells

A 1.0 ug/ml biglycan matrix had a similar effect on SAL cells, in that it significantly reduced cell number compared to baseline control at 48 h (Figure 13a). Seeding OVA cells on a biglycan matrix, however, did not affect cell number relative to baseline, regardless of matrix concentration and time point (Figure 13b).

3.2.3 OVA cells may be more resistant to the decorin-induced reduction in cell number

Interestingly, not only was cell number significantly increased for OVA cells, they also may be more resistant to the decorin-induced reduction in cell number. That is, SAL cells grown on a 1.0 ug/ml decorin matrix were less resilient and showed a 70% decrement in cell number when compared to baseline control (Figure 14a), while OVA cells showed only a 30% reduction in cell number on the same concentration of decorin (Figure 14b). Moreover, biglycan caused a 52% reduction in cell number for SAL cells (Figure 14c), whereas OVA cells were not affected by this proteoglycan (Figure 14d).

3.3 Effect of mechanical strain and extracellular matrix on airway smooth muscle cells

3.3.1 SAL cells respond to mechanical strain

In our experiments, both SAL and OVA cells were subjected to an equibiaxial strain of 17% amplitude applied for 48 h at a frequency of 0.20 Hz. Cells were either strained using this apparatus or not strained as a control.

Figure 15a shows that SAL cells responded to mechanical strain. When compared to NS, 48 h of mechanical strain resulted in a significant decrease in cell number for cells seeded on decorin, biglycan and positive control collagen.

3.3.2 OVA cells respond to mechanical strain

In addition, Figure 15b shows OVA cells respond in a very similar manner; that is, 48 h of strain resulted in a significant reduction in cell number for cells seeded on all matrices.

3.3.3 Decorin may enhance the strain-induced reduction in cell number for OVA cells

We compared the ratios of decrement in cell number as a result of mechanical strain and ECM. It was found that decorin matrix did not affect response of SAL cells to mechanical strain (Figure 16a), although decorin was able to significantly enhance the strain-induced reduction in cell number for OVA cells when compared to positive control collagen (Figure 16b). There was no differential response to mechanical strain for either SAL or OVA cells when seeded on biglycan relative to collagen control (Figure 16c & d, respectively).

3.4 Effect of mechanical strain on expression of β_1-integrin in airway smooth muscle cells

Relative to no strain (NS) control, western blot analysis (Figure 17a) and densitometry (Figure 17b) revealed that OVA cells responded to mechanical strain (S) with an up-regulation of β_1-integrin expression when seeded on decorin, biglycan and collagen matrices.

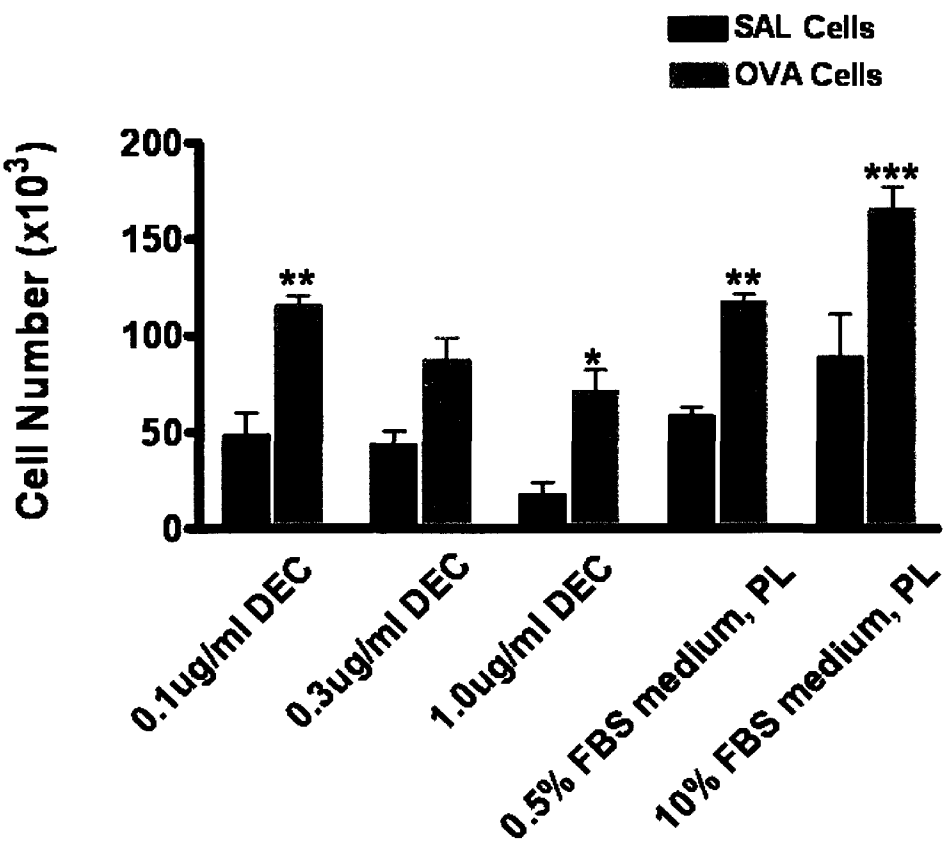

Figure 11: Comparison of cell number between OVA and SAL cells

Following 48 h of PDGF stimulation, cell number was assessed for OVA and SAL cells under plastic (PL) control conditions and decorin (DEC) matrix. For all experiments n=3 and data is represented as ± SE. *P<0.05 vs SAL cells and **P<0.01 vs SAL cells and ***P<0.001 vs SAL cells.

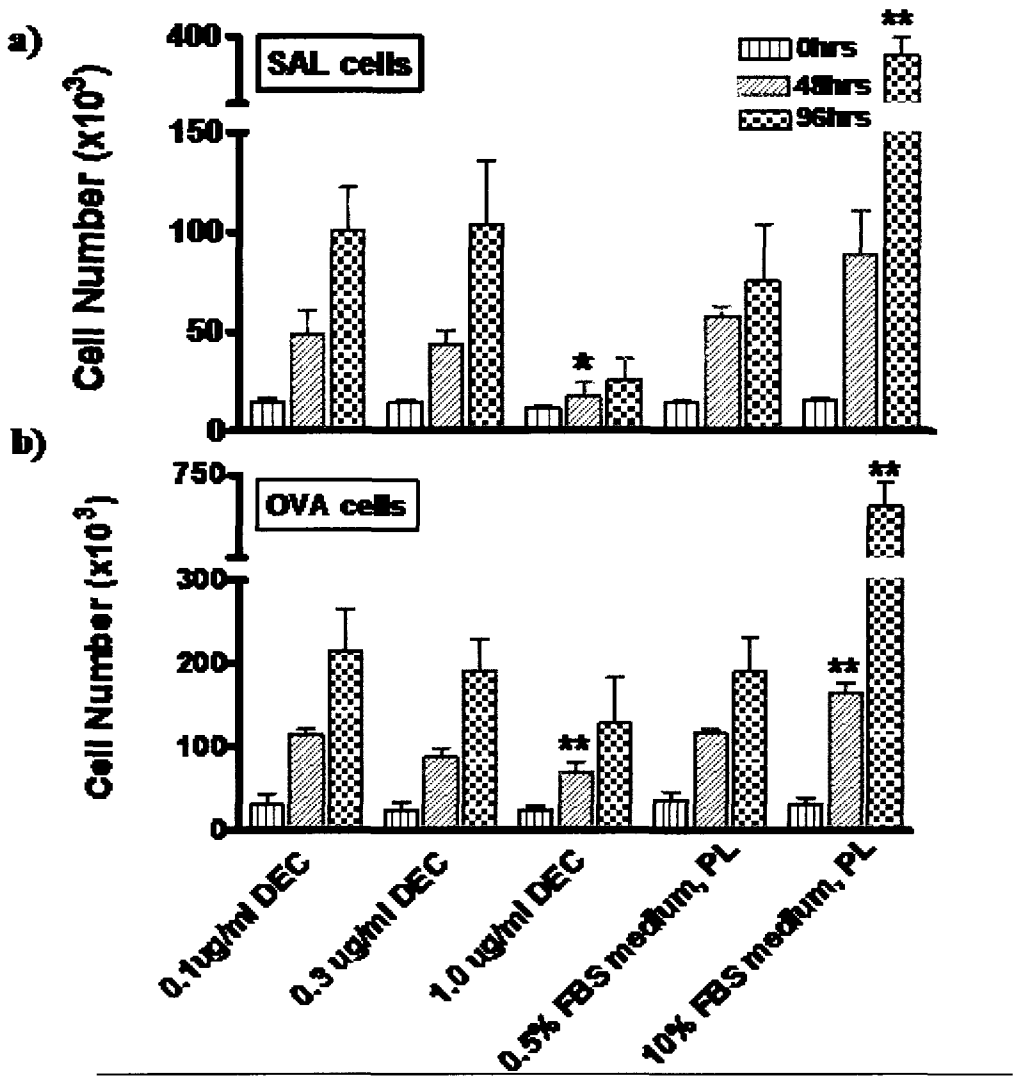

Figure 12: Effect of decorin on ASM cell number

SAL cells (**a**) and OVA cells (**b**) were seeded on decorin (DEC) matrix and cell number was assessed at 0, 48 & 96 h following PDGF stimulation. For all experiments n=3 and data is represented as ± SE. *P<0.05 vs 0.5% FBS medium, PL and **P<0.01 vs 0.5% FBS medium, PL at equivalent time point.

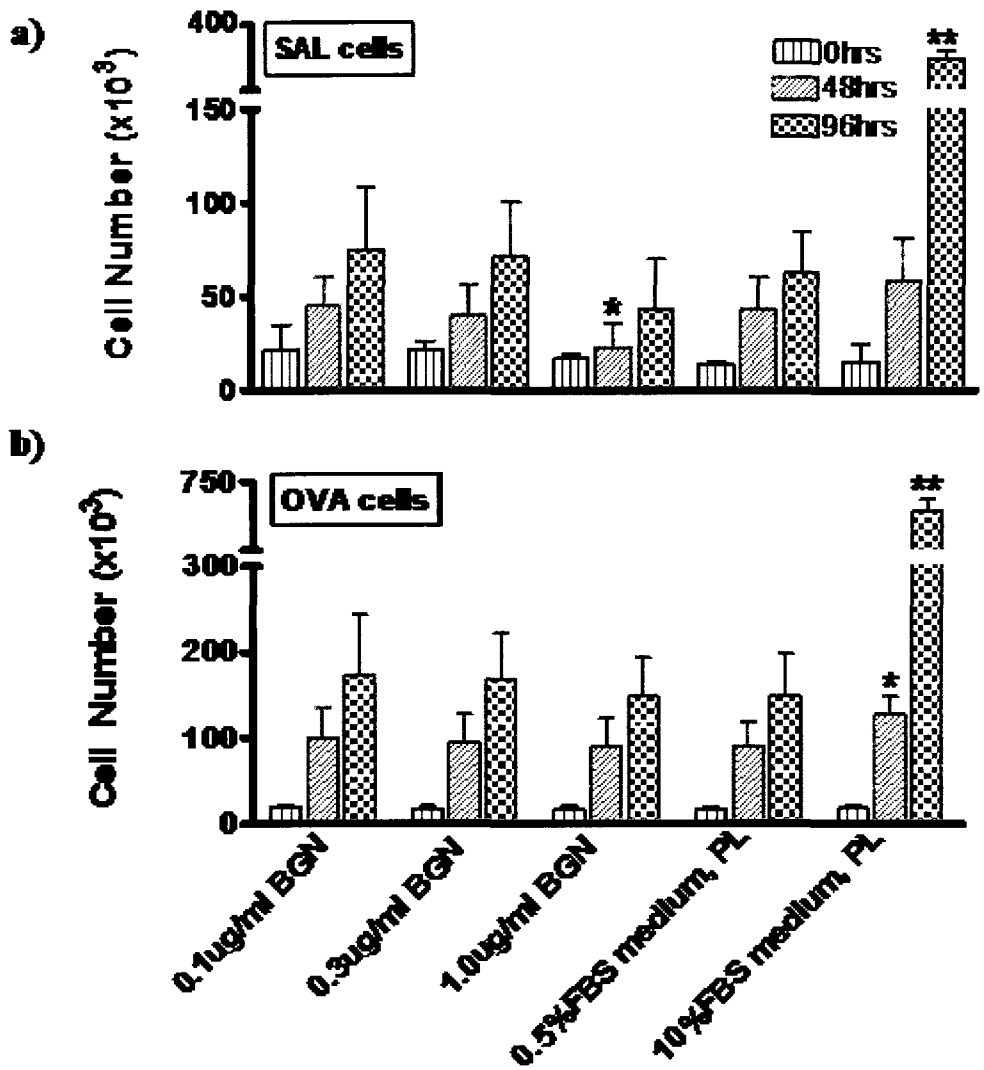

Figure 13: Effect of biglycan on ASM cell number

SAL cells (**a**) and OVA cells (**b**) were seeded on a biglycan (BGN) matrix and cell number was assessed at 0, 48 & 96 h following PDGF stimulation. For all experiments n=3 and data is represented as ± SE. *P<0.05 vs 0.5% FBS medium, PL and **P<0.01 vs 0.5% FBS medium, PL at equivalent time point.

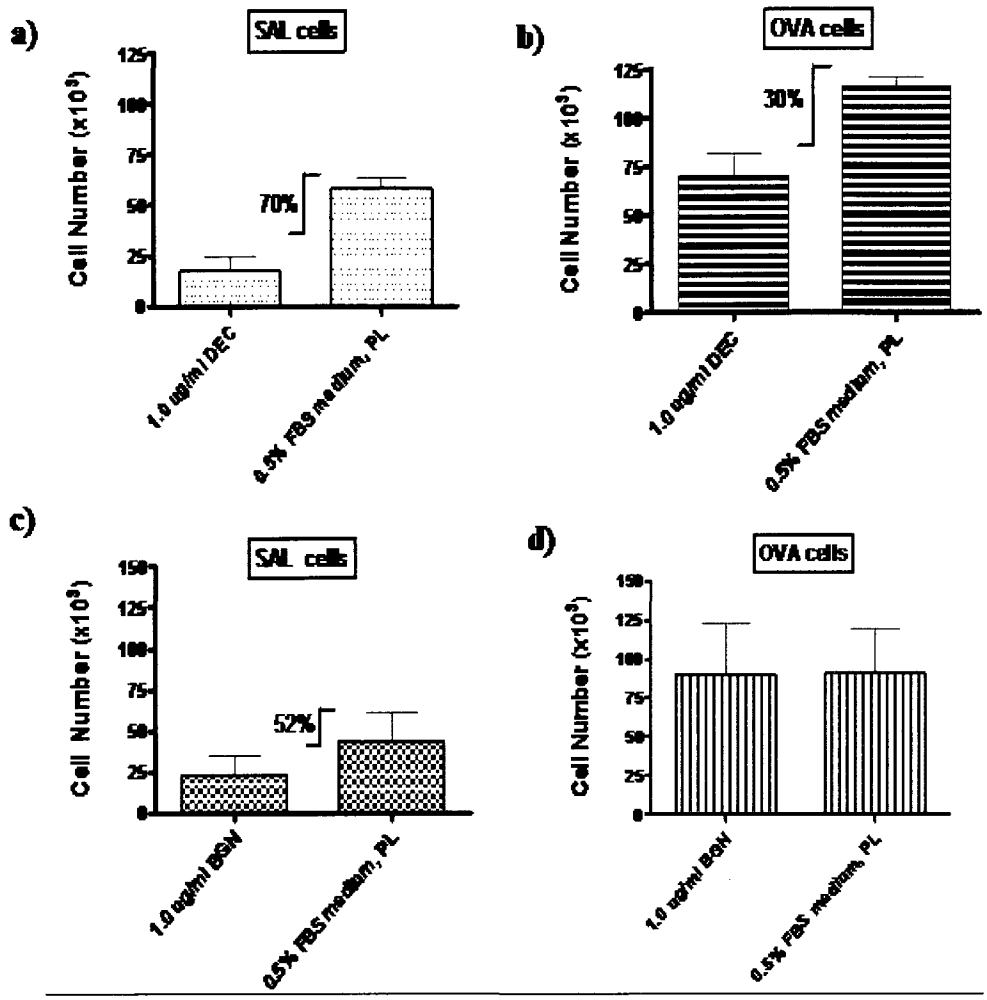

Figure 14: OVA cells may be more resistant to decorin-induced reduction in cell number. Ratios of decrement in cell number were compared for rat ASM cells seeded on matrix protein relative to baseline control. Decorin (DEC) decreased cell number for SAL cells **(a)** and OVA cells **(b)**. Biglycan (BGN) decreased cell number for SAL cells only **(c)** and did not affect OVA cells **(d).** For all experiments *n=3*.

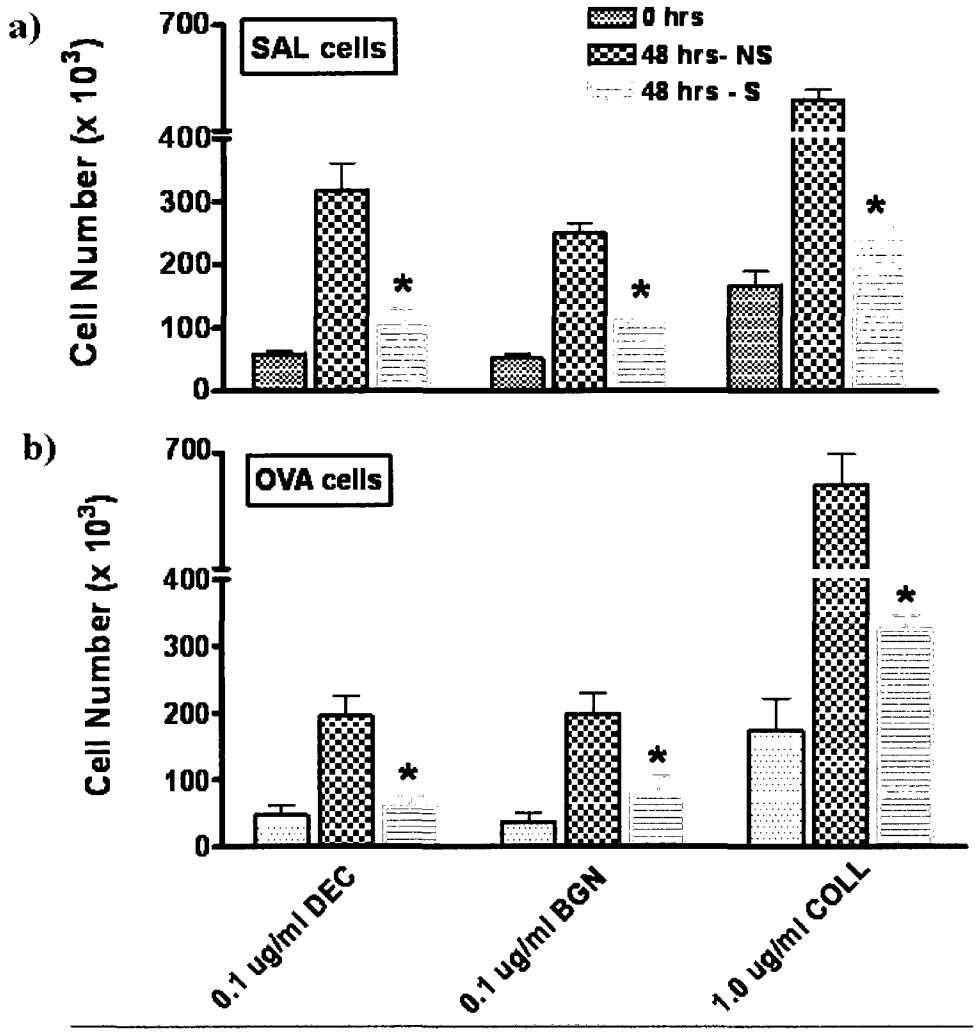

Figure 15: Effect of mechanical strain and extracellular matrix on ASM cells. Cell number was assessed for SAL cells **(a)** and OVA cells **(b)** following 48 h of mechanical strain **(S)** on decorin (DEC), biglycan (BGN) and positive control collagen (COLL). For all experiments n=4 and data is represented as ± SE. *P<0.05 vs 48 h no strain **(NS)** for equivalent matrix.

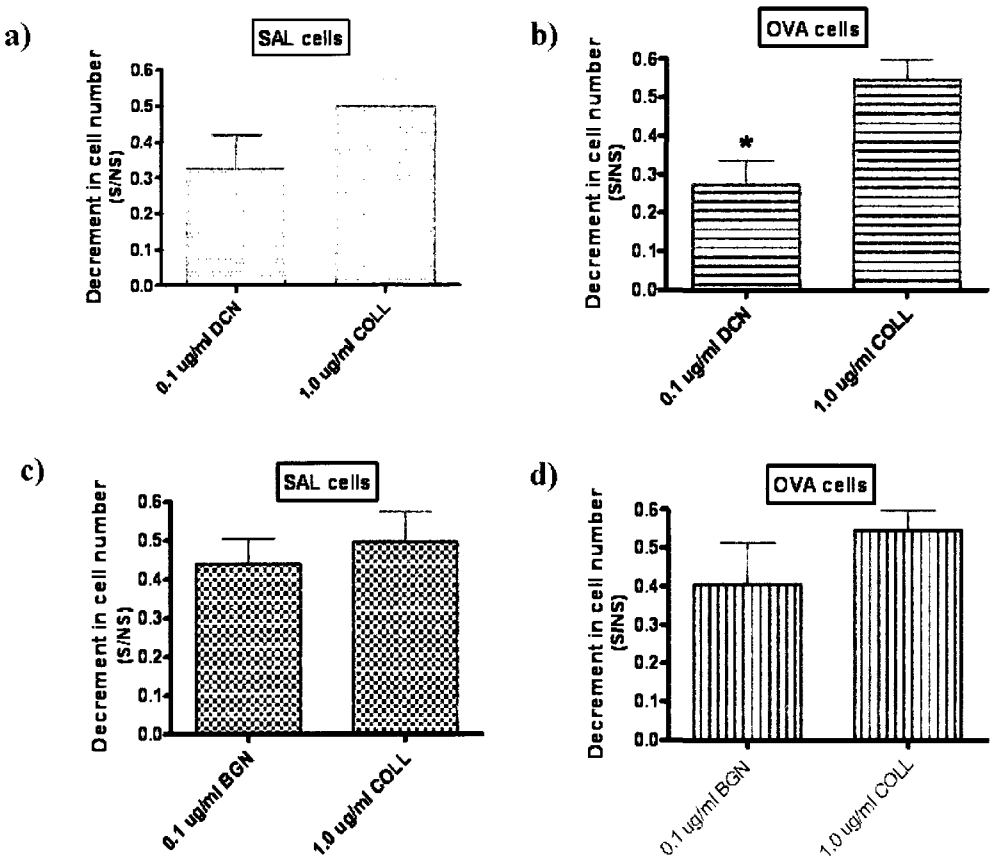

Figure 16: Mechanical strain may be conferred through specific interactions with the extracellular matrix. Decrements in cell number were compared for the effects of mechanical strain on SAL cells **(a & c)** and OVA cells **(b & d)** seeded on decorin (DEC) and biglycan (BGN). *P<0.05 vs positive control collagen (COLL).

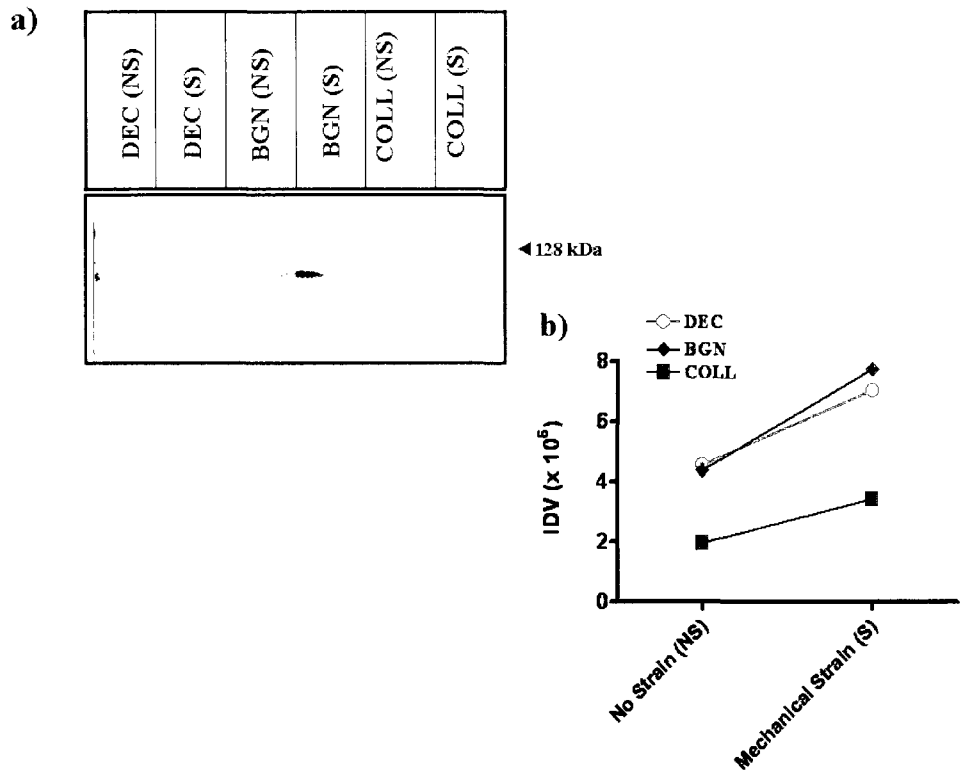

Figure 17: Western Blot analysis for β_1-integrin expression in ASM cells subjected to mechanical strain. Mechanical strain (S) resulted in an up-regulation of β_1-integrin in OVA cells cultured on decorin (DEC), biglycan (BGN) and collagen (COLL) relative to no strain (NS) controls **(a).** Densitometric analysis presented as measurements in integrated density value (IDV), arbitrary units **(b).**

Chapter four
~ *DISCUSSION* ~

Chapter four
~ Discussion ~

Alterations in proteoglycans in asthmatic disease have been noted in the scientific literature, but how these matrix components affect ASM is poorly understood. The present study shows the differential regulation of rat ASM cell proliferation by decorin and biglycan. Culture of rat ASM cells on a decorin matrix resulted in a reduction in cell number for both SAL and OVA cells at the 48 h time point. Furthermore, SAL cells showed a 70% decrease in cell number compared to baseline control, whereas cell number was only reduced 30% for OVA cells. These results were limited to decorin, given that culture on biglycan reduced cell number for SAL cells only.

These data are consistent with what has been reported in the literature. That is, various studies have shown an anti-proliferative and/or pro-apoptotic role for decorin in cells of different origin including carcinoma cell lines (61), vascular cells (57, 58) and, most recently, human ASM cells (65). In addition, evidence showing deposition of decorin in the subepithelial region (44, 52) and the adventitial layer (52) of asthmatic airways suggests a potential protective role for this proteoglycan. Essentially, decorin may serve to limit the enhanced ASM volume by restricting it to its usual compartment, as studies have reported the encroachment of ASM into the subepithelial region in asthmatic airways (119). In our study, results showed that, despite a decrease in cell number for both SAL and OVA cells, OVA cells seemed to be more resistant to the anti-proliferative/pro-apoptotic effects of decorin.

Evidence suggests that asthmatic ASM cells may be more proliferative than non-asthmatic ASM cells and this may contribute to the characteristic increase in ASM in asthmatic airways. Our studies revealed that cells obtained from OVA-challenged rats showed an increase in cell number when compared to control. The literature also reports a phenotypic instability of ASM cells in that they may modulate from a relatively, inactive contractile phenotype to a synthetic, proliferative phenotype. It is possible in our experiments that the increase in OVA cell number is due to an enhanced proliferative capacity of the cells. In turn, these cells may become more synthetic, actively synthesizing and secreting growth factors and pro-proliferative matrix components. In fact, Johnson et al. (17) reported that human asthmatic cells increased production of perlecan and collagen I and proliferated faster in response to these matrix proteins relative to non-asthmatic cells. Hirst et al. (54) have shown that human ASM cells increased proliferation when grown on collagen and fibronectin matrices. In addition, ASM cells can synthesize and secrete TGF-β, a mitogen capable of inducing ASM proliferation (120). Perhaps, OVA cells are more resilient to the anti-mitogenic/pro-apoptotic effects of decorin because they are more proliferative and thus better able to produce growth factors and matrix proteins that promote cellular growth. In this way, the OVA cells used in our experiments would be capable of offsetting, to a certain extent, the decorin-induced reduction in cell number (relative to control). This may also help to explain the results observed at 96 h. It is possible that at this time point the cells have had enough time to deposit their own matrix, thereby altering the response to decorin. Taken together, these observations suggest that, although decorin may be up-regulated in asthmatic

airways, asthmatic ASM cells may be better suited to resist the effect of this matrix protein. A scenario such as this may result in an enhanced ASM volume and subsequent AHR.

This notion is also supported by results showing that SAL cells seeded on a biglycan matrix decreased in cell number while OVA cells were not affected. Little information is available in the literature on the role for biglycan in cell cycle control. One study showed that this proteoglycan enhanced proliferation and migration of vascular smooth muscle cells (56). Conversely, our laboratory has previously shown that biglycan has a modest effect of reducing human airway smooth muscle cell number (65). Those experiments showed that, although decorin reduced cell number at all time points, biglycan decreased cell numbers only at the early time point (0 h) of experiments; by 48 and 96 h cell number had recovered. It is possible that in the present study, OVA cells are more synthetic, thus more robust, and capable of resisting any anti-proliferative or pro-apoptotic signals biglycan may convey.

We have also investigated the impact of mechanical strain and ECM on growth of ASM cells. Airway remodeling plays a key role in the pathogenesis of asthma. It is believed to amplify luminal narrowing in response to ASM shortening and is therefore an important factor in AHR (8). Moreover, although mechanical forces are important in normal lung physiology, altered deposition of ECM and enhanced bronchomotor tone may lead to excessive mechanical stimulation of airways and airway resident cells. We performed *in vitro* studies in an attempt to simulate these aspects of the asthmatic airway. Rat ASM cells were cultured on matrix-coated flexible membranes and subjected to a mechanical

strain regimen. Specifically, cells were exposed to an equibiaxial strain of 17% amplitude applied for 48 h at a frequency of 0.2 Hz. Previous studies have reported that a 17% strain correlates with physiological levels of airway distention during deep inhalation (118). In order to mimic the pulsatile airflow experienced while breathing, various studies have employed cyclical mechanical stimulation and have shown differential affects of strain in regards to ASM cell growth, function and phenotype. Our studies revealed that, relative to no strain conditions, both OVA and SAL cells responded to cyclical mechanical strain with a reduction in cell number and this was regardless of culture on decorin, biglycan or positive control, collagen. These data suggest an important role for strain in the abrogation of cell growth and survival signals. In addition, it is interesting to note that the mechanical stimulus was able to override the pro-proliferative signals normally provided by collagen (54, 87).

Analysis of the ratios of decrement in cell number, revealed that for OVA cells only, mechanical strain reduced cell number to a greater extent for cells seeded on decorin relative to those seeded on positive control collagen. This is noteworthy for the reason that, on plastic wells, OVA cells were less affected by decorin than SAL cells. Although a clear explanation for this discrepancy cannot be deduced, some suggestions may be offered. Differences may result simply for the reason that in one set of experiments cells were cultured on plastic wells, whereas for the other set of experiments, cells were cultured on silastic wells. In addition, for both sets of experiments, different culturing protocols were employed (see Chapter two-Materials & Methods). Another plausible explanation may be that mechanical strain alters the way OVA cells would normally respond

to decorin. To better illustrate this notion, consider the study of Wernig et al. (97), where a regimen of strain similar to the present study was shown to induce apoptosis in vascular smooth muscle cells and this response was mediated via an up-regulation of β_1-integrin. In *in vitro* culture systems, transmission of forces occurs through the substratum, thus implicating integrins in mechanotransduction (121). Our studies have shown that OVA cell number is more affected by mechanical strain on a decorin matrix relative to positive control. Furthermore, these cells respond to strain with an up-regulation of the β_1-integrin on all matrices. Interestingly, results from densitometric analysis show a trend suggesting that expression of this integrin is enhanced for cells seeded on decorin and biglycan matrices relative to collagen matrix. It possible that cells could not adhere to decorin as firmly as to collagen and, in order to compensate, the cells increased β_1-integrin expression. However, counting experiments of unattached cells in the supernatant showed no observable differences in cell numbers following mechanical strain for cells seeded on decorin compared to collagen. While no data is available for ASM cells, platelets have been shown to interact with decorin through the $\alpha_2\beta_1$ integrin (122). Although integrins are normally associated with cell survival (55), it is possible that overstretching induces signaling that results in growth suppression and apoptosis (Figure 18). For example, one study showed that strain-induced β_1-integrin activation and subsequent p38 MAPK signaling resulted in apoptosis in vascular smooth muscle cells (97). Other studies have shown that, depending on the cell type and stimulus, this pathway may also be capable of inducing cell survival (for review see (93)). Depending on the type of signal present, activation of various integrins and

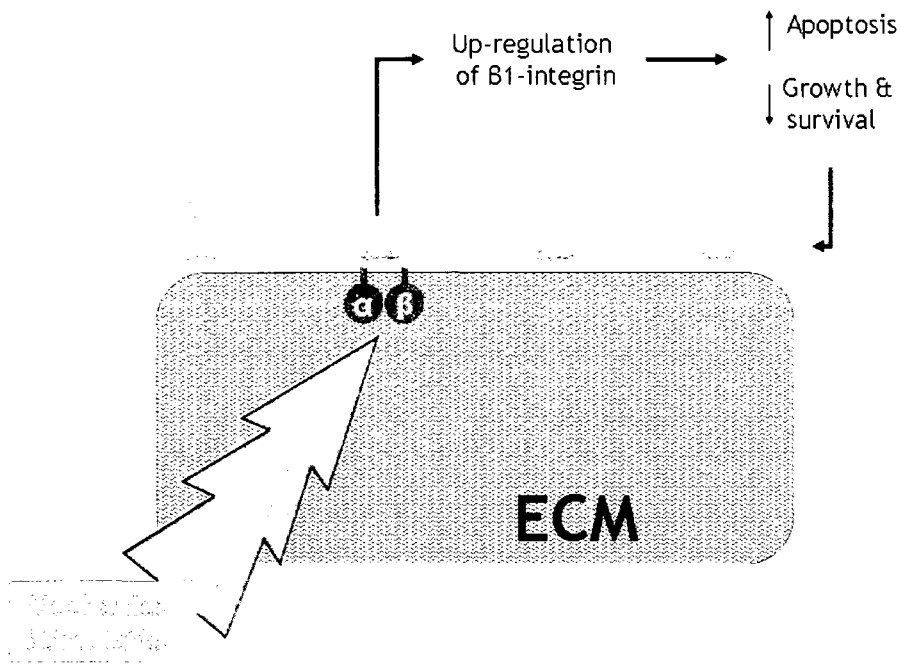

Figure 18: Hypothetical schema of mechanical strain-induced reduction in cell number. Mechanical stimuli are conveyed from the ECM through integrins into the cell. This may induce signaling cascades which could result in growth suppression and apoptosis leading to decrements in cell number.

signaling pathways may occur. These findings may also help to explain results showing an increase in proliferation of canine ASM cells as a response to mechanical strain (77). In these experiments, culture conditions and strain regimens differed from our experimental setup. Presumably, this may have led to the activation of a combination of integrins and signaling pathways which promote cell growth and survival.

A number of studies have reported the regulation of protein synthesis and release by cells in response to mechanical forces. Asthmatic fibroblasts respond to mechanical strain with an up-regulation of message for decorin and versican (86). In addition, ASM cells are capable of synthesizing and secreting various growth factors and pro-proliferative matrix components such as collagen (for review see (22)). Much in the same way that strain has been shown to directly promote cell survival or apoptosis, in the present experiments, excessive mechanical stretch may have led to the production of decorin. Deposition of decorin may, in turn, be a causative factor in the reduction of cell number. Conversely, this proteoglycan may interact with strain in a synergistic manner to further inhibit cell growth. Finally, the notion that OVA cells may be more synthetic supports our data showing that strain-induced decrements in cell number did not differ for SAL cells regardless of matrix. SAL cells, for instance, may not respond to mechanical strain with decorin production and therefore would not contribute to the strain-induced reduction in cell number.

Conditions characteristic of asthmatic airways such as enhanced collagen deposition, presence of growth factors and pro-inflammatory mediators may all promote enhanced ASM volume. Cells may be responding to excessive stretch

with programmed cell death or an inhibition of growth. Accordingly, this may serve as a protective mechanism working to prevent an increase in ASM growth, in a milieu that would otherwise encourage growth. Therefore, a reduction in cell number in response to abnormal mechanical stimulation may have important functional implications.

In conclusion, it is hoped that the results which comprise this thesis are able to encourage more research into the realm of airway remodeling and asthma. The present experiments could be helpful in designing future studies investigating multiple factors involved in the pathogenesis of asthma. It should be understood that the etiology of airway remodeling is complex, involving a multitude of potential factors acting at numerous sites that extend beyond what is possible to achieve through *in vitro* experiments. Nevertheless, our data highlights the importance of alterations in the airways, be it at the mechanical, tissue, or cellular level. Once it is possible to identify the major factors in airway remodeling, steps may be taken towards the development of novel treatments for asthma. This knowledge would have significant implications for improving functional outcomes of asthmatic patients.

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~ APPENDIX ~

ACTION	✓	DATE
COs		
DB	✓	July 30/08
APPROVED		

MCL

www.mcgill.ca/researchoffice/compliance/animal/forms/

McGill University Animal Care Committee

RENEWAL of Animal Use Protocol

For Office Use Only:
Protocol #: 3325
Approval end date: June 30, 2009
Facility Committee: RVH
Renewal#: (1st) 2nd

For: Research ☒ Teaching ☐ project

Principal Investigator: Mara Ludwig
Protocol # 3325
Protocol Title: Proteoglycans and asthmatic airway function
Category: C
Unit, Dept. & Address: Meakins Christie Laboratories, Dept of Medicine 3626 St. Urbain Street Montreal QC H2X 2P2
Email: mara.ludwig@mcgill.ca **Phone:** 541 398-3164 **Fax:** 514 398-7483
Funding source: CIHR MOP 57876 MOP 57466 6933 10441 83949
Start of Funding: Oct 2005 **End of Funding:** Sept. 2010
Emergency contact #1 + work AND home phone #s: Venkatesan Narayanan 514 398-3864 (00128); (514) 933-4962
Emergency contact #2 + work AND home phone #s: Mara Ludwig 514 398-3864 (00142); 514 482-9141

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). Indicate if the Principal Investigator is not handling animals. If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature *"Has read the original full protocol"*
		UACC on-line Theory course	Workshops + others		
Mara Ludwig	PI	yes	no (will not be handling animals directly)		
Cinzia Marchica	graduate student	yes	yes		
Venkatesan Narayanan	research associate	yes	yes		
Jaryd Zummer	undergraduate student	yes	yes		
Stephanie Pasternyk	graduate student	yes	yes		

Role of the undergraduate student: Mr. Zummer is involved in experiments in which changes in airway responsiveness in allergen induced asthma in decorin deficient mice is examined. He is working as a summer student and his experiments will be completed by mid-August. He is working under the direct supervision of one of the graduate students (Cinzia Marchica) and myself.

* Indicate for each person, if participating in the local Occupational Health Program see http://www.mcgill.ca/researchoffice/compliance/animal/occupational/ for details.

Approved by:

2. Approval Signatures

I will ensure that all collaborators and staff are aware of all changes to this protocol. Date: July 15/08

30 JUIL. 2008

3375

Principal Investigator/ Course Director		
Chair, Facility Animal Care Committee	Marc Laviolette	Date: 22 JULY 2008
Animal Compliance Office		Date: July 30 '08
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*		Date:
Approved Animal Use Period	Start: July 1, 2008	End: June 30, 2009

☐ **Renewal requires submission of full Animal Use Protocol form**

Form version January 2008

3. Summary (in language that will be understood by members of the general public)
AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(use section 5a in main protocol).*

The overall aim of the study is to better understand the mechanisms behind remodeling of the airway wall in asthma. We will focus on how lack of a specific extracellular matrix molecule, ie, the proteoglycan, decorin, will impact on development of the asthmatic phenotype in a murine model of allergic asthma. This study has important implications for understanding basic disease mechanisms and, ultimately, may offer new targets for paharmacologic intervention. A second study looks at allergen induced asthma in a rat model. We will characterize changes in the protein in the airway wall and also how airway smooth cells from "asthmatic" rats change their behavior in response to various stimuli.

4. Has there been any animal care issues?	YES ☐	NO ☒	if yes, supply details:

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form.*
If mice expressing new phenotype have been produced, submit a *Phenotype Disclosure form.*
Blank forms at *http://www.mcgill.ca/researchoffice/compliance/animal/forms/*

6. Procedures

a) For B and C level of invasiveness.

The procedures are the same as the original protocol: YES ☒ NO ☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol, including amendments *(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):*

b) For D level of invasiveness.