Role of Caveolae and the Dystrophin Glycoprotein Complex in Airway Smooth Muscle Phenotype and Lung Function

by

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DEDICATION

Dedicated to my loving and beautiful daughter "PARI" who brings smile and happiness in every moment of my life…..love you dear!!

&

To my "PARENTS" for their untiring love and endless blessings …thank you for making me what I'm today …..love you always!!

ACKNOWLEDGEMENTS

This body of work could not exist without the pivotal support and able guidance of some very special people. First, I would like to thank my PhD mentor, Dr. Andrew HALAYKO, for his dedicated mentorship. Under his guidance, I have not only learned nuts and bolts of academic research but he has immensely helped me in making the right career choices at a given point in time during my graduate training. I am grateful to have him as my mentor as his passion for research has hugely motivated me and left an indelible mark on me to continue research and contribute to this field.

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LIST OF ABBREVIATIONS

ASM - airway smooth muscle

β-DG - β-dystroglycan

Cav-1 - caveolin-1

CSD - caveolin scaffolding domain

COX-2 - cyclooxygenase-2

Cy3 - indocarbocyanine

DGC - dystrophin glycoprotein complex

DMD - duchene muscular dystrophy

EDTA - ethylenediarninetetraacetic acid

ERK - extracellular signal-regulated kinase

ETYA - eicosatetraynoic acid

FITC - fluorescein isothiocyanate

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GPCR - G-protein coupled receptor

GSK3β - glycogen synthase kinase 3β

GR - golden retriever

GRMD - golden retriever muscular dystrophy

hTERT - human telomerase reverse transcriptase

INDO - indomethacin

ILK - integrin linked kinase

IP_3R - inositol 1,4,5-trisphosphate

kDa -	kilodalton
LGMD -	limb girdle muscular dystrophy
MAP kinase -	mitogen-activated protein kinase
MCh -	methacholine
mTOR -	mammalian target of rapamycin
5-LO -	5-lipooxygenase
NFAT -	nuclear factor of activated T-cells
PAGE -	polyacrylamide gel electrophoresis
PDK2 -	3-phosphoinositide dependent protein kinase 2
PI3K -	phosphatidylinositol 3-kinases
SR -	sarcoplasmic reticulum
SDS -	sodium dodecyl sulphate
SG -	sarcoglycan
smMHC -	smooth muscle myosin heavy chain
TEM -	transmission electron microscopy
XeC -	xestospongonC
TpG -	thapsigargin

ABSTRACT

Smooth muscle is a primary determinant of physiology as its ability to contract affords dynamic control of diameter of the hollow organs it encircles including the airways. Mature airway smooth muscle (ASM) cells are phenotypically plastic, enabling them to subserve contractile, proliferative, migratory and secretory roles that relates to its function in health and disease. ASM cells can control airway diameter both acutely, *via* reversible contraction, and chronically, by driving fixed changes in structure and function properties of the airway wall. However, the scope of research on ASM biology and function has broadened greatly in the past two decades, embracing the now recognized dynamic and multifunctional behavior, but there is always a need to investigate the role of new proteins regulating ASM phenotype *in vitro* and lung function *in vivo*. The multimeric dystrophin-glycoprotein complex (DGC) links the extracellular matrix (ECM) and actin cytoskeleton while caveolae form membrane arrays on ASM cells. Using ASM cells and tissues from human and canine and intact mouse for lung physiology, we investigated the role of DGC in phenotype maturation. We also investigated the mechanism for the organization of DGC with caveolae and further tested whether this is functionally important in mobilizing intracellular Ca^{2+} in ASM cells, contraction of ASM tissue and finally its role in airway physiology. Our data demonstrate that the expression of DGC is an integral feature and a key determinant for phenotype maturation of human ASM cells. Our new data reveals an interaction between caveolin-1 and DGC and indicate that this association, in concert with anchoring to the actin cytoskeleton, underpins the spatial organization of caveolae on the membrane and has a functional role in receptor-mediated Ca^{2+} release in ASM *in vitro*, ASM contraction *ex vivo*

and lung function *in vivo*. Collectively our study indicates that the organization of caveolae and DGC, and its link from ECM to the actin cytoskeleton with in caveolae are a determinant of phenotype and functional properties of ASM, which underpins its role in physiology and pathophysiology of chronic airway diseases such as asthma.

Chapter 1

INTRODUCTION

The principal function of smooth muscle cells encircling various hollow organs is to contract [1]. Smooth muscle cells carry out this specialized function by evolving a repertoire of necessary contractile proteins and signaling molecules. Similarly airway smooth muscle (ASM), which encircles the airways, has long been known to be a primary determinant of airway physiology in health and disease by virtue of its ability to contract, and thereby control the diameter of the airway lumen [2]. Apart from its principal contractile function ASM is also capable of a multitude of other functions at various stages of tissue development, repair and in disease. The remarkable plasticity of these cells allows them to migrate, proliferate, and secrete extracellular matrix (ECM), growth factors, cytokines, and chemokine's, thereby contributing to fibroproliferative remodelling of the airways during the pathogenesis of chronic airway diseases such as asthma and COPD [2, 3].

Airway Smooth Muscle Phenotype in Cell Culture

The primary role of the mature ASM cells is contraction; hence, the vast majority of ASM cells in an adult are thought to exist, more or less, in a contractile state [4]. Cell culture studies have demonstrated the presence of stable, phenotypically heterogeneous ASM subpopulations [5-7]. These subpopulations may differ in proliferative capacity to serum stimulation [8] and it has been shown that prolonged serum deprivation induces a discrete subset of ASM cells to a functional contractile phenotype with abundant contractile proteins such as smooth muscle myosin heavy

chain (smMHC) and sm-α-actin, and functionally coupled muscarinic M3 receptors [5, 9, 10].

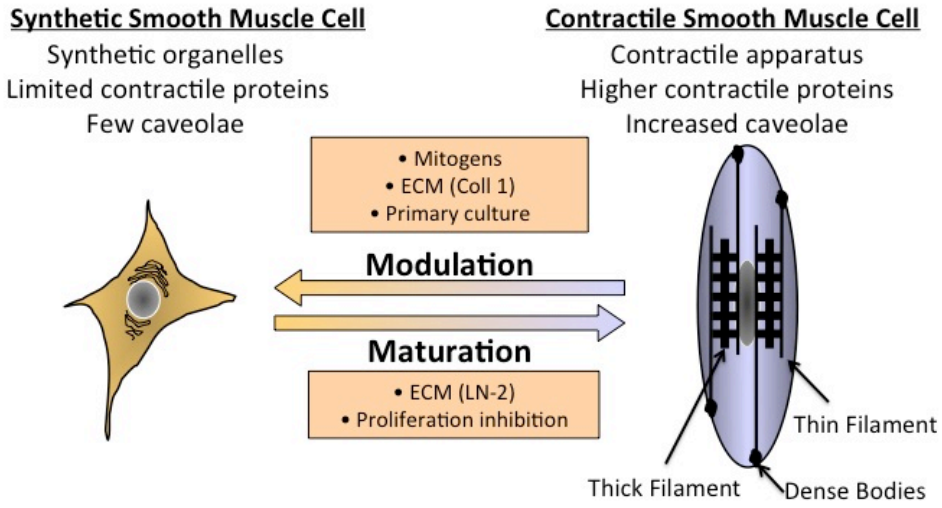

Figure 1. Schematic representation showing the phenotype plasticity of smooth muscle. Phenotypic plasticity results from reversible modulation and maturation of smooth muscle cells (SMC) between a synthetic and contractile state as defined by cell response to defined stimuli and by the presence of molecular and ultrastructural markers. Reproduced with permission from "Phenotype and functional plasticity of airway smooth muscle: role of caveolae and caveolins"; Volume 5, pp. 80-88, © 2008 Proceedings of the American Thoracic Society, HighWire Press, USA.

The contractile and synthetic phenotypic states have been described to represent idealized extremes in which most ASM cells likely exist in specific organs and species [11] (depicted in Fig. 1). Some of ECM surrounding smooth muscle cells is likely synthesized by them indicating that they are capable of sustaining both a contractile and synthetic phenotype [12]. It is also well established in primary culture, that native, contractile smooth muscle cells from a variety of organs rapidly modulate to a mitotically active, synthetic phenotype in the presence of serum [4, 13, 14]. ASM exhibits phenotype plasticity through the reversible *modulation* and *maturation* of individual airway myocytes both *in vitro* and *in vivo* [5, 6, 14] (schematic described in

Fig. 1). Primary smooth muscle cell culture models have revealed that contractile myocytes undergo spontaneous switching towards a more proliferative/synthetic phenotype when seeded at subconfluence in the presence of mitogens [4, 14, 15] and/or a specific subset of ECM proteins (e.g. laminin, fibronectin and collagen type I) [16, 17]. This phenotype switching from a contractile to a proliferative/synthetic phenotype is defined as modulation [4] and has been reported to be a characteristic response of mature smooth muscle cells derived from a variety of origins, including the airways [6, 18, 19]. A number of recognized molecular markers are abundant in contractile phenotype smooth muscle cells, in particular intracellular contractile apparatus- and cytoskeleton-associated proteins such as smooth muscle myosin heavy chain (smMHC), SM22, calponin, smooth muscle-α-actin, and desmin [2, 14, 15]. Expression of these proteins can be used as a marker for smooth muscle function in tissue biopsies taken from the airways, vasculature or other hollow organs [20].

Mature smooth muscle cells generally approximate a contractile state characterized by the presence of a high volume fraction of myofilaments and the expression of numerous smooth muscle-specific genes encoding contractile proteins which regulate contraction [4, 13, 14]. The contractile apparatus of mature ASM cells is the dominant ultrastructural component [4], consisting of actin and other actin-binding proteins and thick myofilaments, occupying 80-90% of total cytoplasmic volume [21]. Myofilament abundance appears to be correlated with the content of myosin, actin and myofilament-associated proteins [14, 22, 23]. Linear arrays of flask-shaped invaginations

of the sarcolemma, called caveolae, are seen between membrane dense bodies - the caveolae increase in number during ASM maturation and are a distinctive feature of the mature smooth muscle cells [24, 25].

Synthetic smooth muscle cells have an abundance of organelles for protein processing and synthesis, including rough endoplasmic reticulum and Golgi apparatus, numerous mitochondria and a limited number of myofilaments [11]. These cells have an increased proliferative capacity and exhibit a diminished abundance of contractile apparatus associated proteins, which is in line with a reduced responsiveness towards contractile agonists in vitro [4, 10, 16, 26]. ASM cells maintaining this phenotype replicate frequently in comparison to contractile ASM cells. They synthesize and secrete ECM proteins and proteases abundantly, and express genes for growth factors, cytokines and their requisite receptors [13, 27]. Mature ASM cells spontaneously modulate their phenotype in primary culture and their responsiveness to various spasmogenic and mitogenic stimuli differ significantly from those of freshly isolated, uncultured cells [7, 28, 29].

KEY REGULATORS OF CONTRACTILE PHENOTYPE

Acquisition of a contractile phenotype by the ASM cells requires the accumulation of contractile and regulatory proteins, such as SM22, sm-α-actin, smMHC, calponin, and desmin through regulated control of transcriptional and translational processes [30, 31]. A number of intracellular signaling cascades, including the Rho/Rho

kinase pathway and PI3K (phosphatidyl inositide 3-kinase) dependent pathways have been associated with transcription and translation of smooth muscle specific proteins, respectively [9, 32-35]. These pathways are illustrated in Figure 2, and some of these are discussed below. Mature ASM cells express elevated levels of active, phosphorylated kinases with known effects on protein translation, including PI3K, Akt1, mTOR (mammalian target of rapamycin), and p70 ribosomal S6 kinase [9].

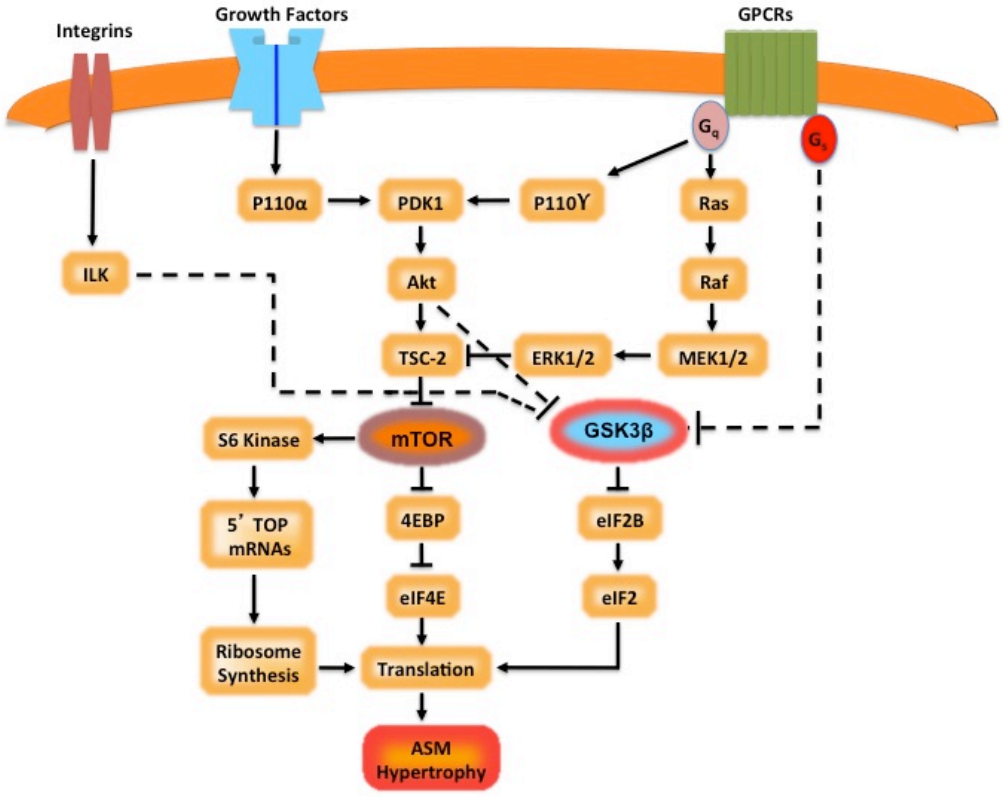

Figure 2. Schematic representation showing the key signaling pathways required for contractile protein accumulation in airway smooth muscle cells.

Signaling cascades downstream from PI3K play a principal role in mediating protein synthesis and hypertrophy of airway smooth muscle (8–10). Downstream targets of PI3K and Akt that are associated with promoting protein synthesis and

accumulation include glycogen synthase kinase-3β (GSK3β), p70S6 kinase (p70S6K), and PHAS-1/4E-BP [36, 37] (outlined in Figure 2). Akt1 can also phosphorylate, and in part activate, the rapamycin-sensitive threonine/serine kinase, mammalian target of rapamycin (mTOR). mTOR has downstream targets that include the mitogen and amino acid–sensitive serine/threonine kinase, p70S6K, and the translation repressor PHAS-1/4E-BP1 [38-40]. Activation of p70S6K regulates efficiency of protein translation by phosphorylating of the 40S ribosomal protein S6 [38, 41], and is required for the autocrine loop mediated ASM cell maturation and hypertrophy [5, 9]. Thus, PI3K may positively regulate cell size via activation of Akt, inactivation of TSC2, activation of Rheb, and activation of mTOR [42-45]. It is now known that mTOR exists in two distinct multi-protein complexes, one rapamycin-sensitive (mTOR complex 1) and one rapamycin-insensitive (mTOR complex 2) [46]. mTOR complex 1 includes mTOR and Raptor; mTOR complex 2 is comprised of mTOR-Rictor and mammalian stress-activated protein kinase interacting protein. Furthermore, as noted above, mTOR complex 2 appears to be identical to the proposed Akt kinase, PDK2, which phosphorylates serine 473 on Akt [47, 48]. Thus, Akt acts as both an upstream activator of mTOR complex 1, and is a target for activation by mTOR *via* mTOR complex 2 to permit high-level PI3K/Akt signaling [49].

GSK3β is a constitutively active serine/threonine kinase that phosphorylates multiple substrates including eIF2Bε, cyclin D1, and p21 [50-53]. Phosphorylation by Akt inactivates GSK3β, leading to dephosphorylation and the activation of eIF2B, as well as a general enhancement of ribosomal 43S pre-initiation complex formation [50]. GSK3β

also negatively regulates transcription factors involved in muscle-specific gene expression, including nuclear factors of activated T cells (NFAT), GATA4, and β-catenin [54-58] suggesting a critical role in ASM growth. Recent studies also implicate regulation of GSK3β as a key downstream mechanism for the effects of integrin-mediated effects of extracellular matrix proteins on cell growth; this involving the signaling intermediate, integrin linked kinase (ILK) [59-61].

AIRWAY SMOOTH MUSCLE: ROLE IN ASTHMA

The broad diversity in the sensitivity and reactivity of mature ASM tissues to chemical and physical stimuli that trigger contraction and relaxation still exists [62, 63]. A number of mechanisms for such diversity of function of ASM are being proposed such as mechanical [64-67] and functional plasticity [68-70]. One of the key contributing factors to functional diversity among mature smooth muscle types is a significant variation in the expression profile of proteins that mediate cell responses to environmental signals. This fine-tuning in function is achieved by the repertoire of proteins expressed as receptors, receptor regulators, signaling effectors, and ion channels [71-77]. This multifunctional capacity of ASM cells enables them to contribute directly both to reversible, intermittent bronchial spasm, and to structural changes in the airway wall that are associated with irreversible loss of lung function in patients with obstructive airways diseases [31].

ASM cells have the capacity to synthesize and release pro-inflammatory biomolecules in response to a variety of stimuli and maintain ASM cells in a synthetic/proliferative phenotype, thereby contributing to asthma pathogenesis [78-82]. Cultured asthmatic ASM cells also produce an altered composition of ECM proteins leading to fibrotic changes associated with asthma that affect airway and lung function [83]. The thickening of ASM layer in asthma is a result of both cellular hyperplasia [78, 84, 85] and hypertrophy [20, 86]. Increased ASM mass in asthma, is accompanied by increased abundance of contractile protein markers, which underpins increased contractility of ASM tissue in asthma and in asthma models [20, 72, 87, 88]. Collectively, these reports indicate that expression of contractile phenotype marker proteins are consistent with the maturation of contractile myocytes that occurs during prolonged serum-free cell culture [5, 9].

ROLE OF CAEVOLAE IN AIRWAY SMOOTH MUSCLE PHENOTYPE

There has been a considerable progress since caveolae were first identified by electron microscopy as 50-100 nm membrane invaginations [89]. These specialized plasma membrane regions regulate cellular activity by sequestering numerous receptors, signaling proteins, and ion channels [89, 90]. Caveolae are formed from cholesterol and sphingolipid-rich, ‘‘liquid-ordered’’ lipid rafts that bind to and are stabilized by oligomeric complexes of distinct caveolae proteins, the caveolins (Figure 3 describes structural and functional domains in caveolin-1 (Cav-1)). Proteins other than

caveolins are also needed for caveolae formation, such as the recently discovered cavin (PTRF) [91].

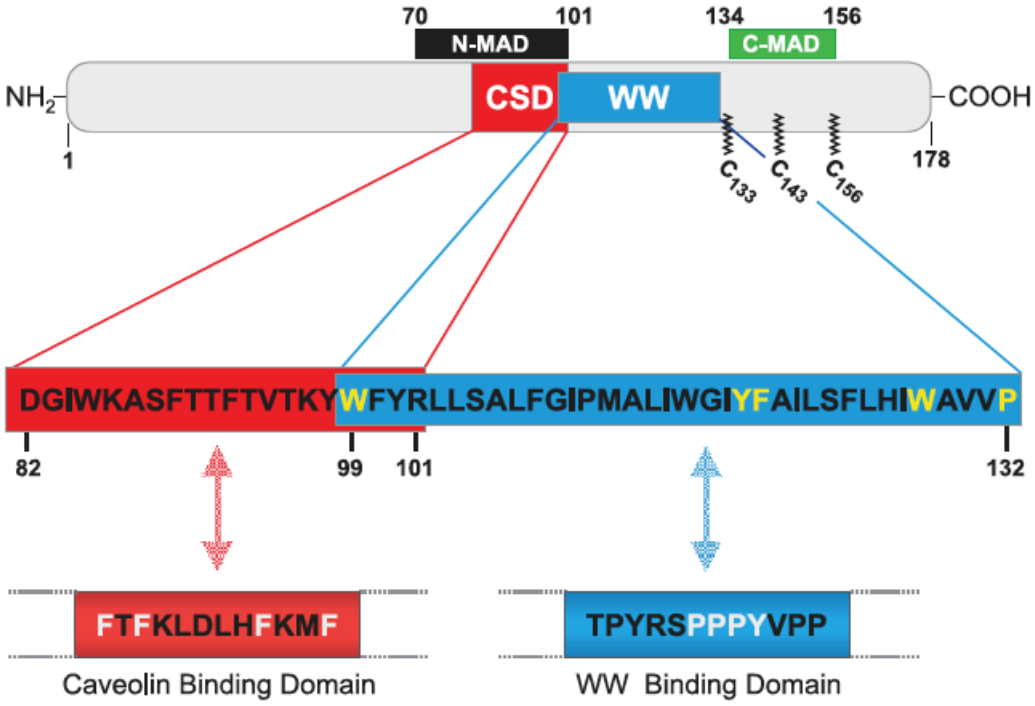

Figure 3. Schematic representation of structural and functional domains in caveolin-1. The 21-24 kDa caveolin-1 protein is composed of 178 amino acids (aa). It is anchored to the cytosolic leaflet of the plasmalemma bilayer by 2 membrane association domains; N-MAD (black, aa 70–101) and C-MAD (green, aa 134–156), N- and C-termini are cytoplasmic. The highly conserved caveolin-scaffolding domain (CSD) (shown in red) includes aa 82–101 of the N-MAD and is essential for interactions with signaling molecules such as Gα-subunits, PKC isozymes, and RhoA that carry a caveolin binding domain (CBD) sequence; the CBD sequence shown corresponds to $G\alpha i_{a1}$. Caveolin-1 also contains a WW domain (WW; shown in blue) from aa 99–132 that is marked by conserved aromatic and proline residues. The WW domain has selective binding affinity for proteins with a WW-binding domain that includes a conserved PPXY motif; the WW-binding domain shown corresponds to the C-terminal tail of β-dystroglycan. Reproduced with permission from "The association of caveolae, actin, and the dystrophin-glycoprotein complex: a role in smooth muscle phenotype and function? Can J Physiol Pharmacol. Oct; 83(10): 877-91''. © 2005 Canadian Science Publishing, operating as NRC Research Press.

It has been shown that acute disruption of plasma membrane caveolae suppresses G-protein-coupled receptor (GPCR) mediated contraction and Ca^{2+}

mobilization in ASM [92-94]. Cav-1, the primary structural protein of caveolae plays a key role in orchestrating activation of pathways that underpin cell proliferation, migration, and contraction [1, 95]. Recent research also suggests a role for Cav-1 in chronic lung diseases such as asthma, COPD and pulmonary fibrosis [25, 96, 97]. A number of signaling pathways that control transcription and translation of phenotype marker proteins are known to be associated with caveolae; this implies that these membrane structures, which themselves are significantly increased in number in contractile phenotype myocytes, may be involved with the integration of mechanisms that control myocyte differentiation. Cav-1 protein abundance increases greatly as vascular and ASM cells acquire a contractile phenotype, and this leads to their orientation into organized longitudinal rib-like plasma membrane arrays [1, 94, 98, 99]. Thus studying caveolin interaction with signaling proteins and their regulation may be important determinant of smooth muscle phenotype and function.

ROLE OF CAVEOLAE IN AIRWAY SMOOTH MUSCLE CONTRACTION

In a contractile smooth muscle cells, caveolae are highly organized into discrete longitudinal arrays in parallel with domains of adherens junctions and dense plaques [1, 100, 101]. Because of their proximity to organelles associated with intracellular Ca^{2+} flux, such as the peripheral sarcoplasmic reticulum and mitochondria, caveolae have been postulated to be involved with Ca^{2+} release that is required for receptor-mediated activation of the contractile apparatus [102, 103]. Notably, the signaling machinery required for mobilizing Ca^{2+} localize to myocyte caveolae [104-107]. Therefore caveolae

are functionally important in ASM, as they are needed to facilitate contraction and Ca^{2+} mobilization mediated by some GPCRs [92, 93]. The schematic in Figure 4 describes this paradigm for a complex and dynamic association of receptors and signaling proteins with caveolae and Cav-1.

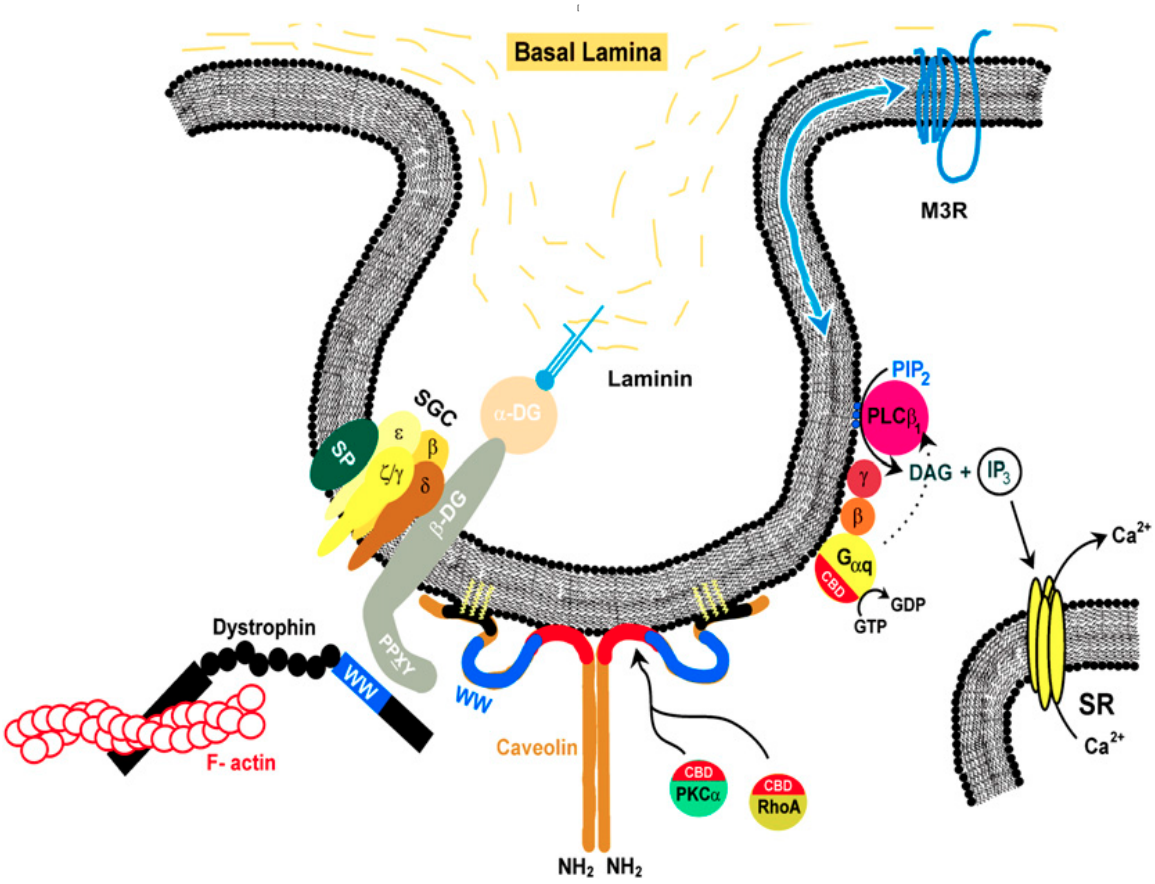

Figure 4. Relationship of caveolin-1 with the dystrophin glycoprotein complex (DGC) and association with G-proteincoupled receptor–mediated intracellular signaling. For simplicity a single caveolin homodimer is shown. Caveolin-1 is anchored to the cytosolic leaflet of the plasmalemma by N-MAD (red) and C-MAD (green) regions. The WW domain (blue) of both caveolin-1 and dystrophin has affinity for the WW binding domain with consensus PPXY motif of b-dystroglycan (β-DG). Dystrophin acts as a linker between the DGC and the filamentous (F)-actin cytoskeleton. The DGC is stabilized by other transmembrane subunits, including a tetrameric sarcoglycan complex (SGC) that includes, in airway and vascular smooth muscle, β-, δ-, ε-, and γ- or ζ-sarcoglycans, and sarcospan (SP). The DGC is also linked to the surrounding basal lamina *via* binding of its a-dystroglycan (α-DG) subunit to laminin-1 and -2. The caveolin scaffolding domain (CSD, shown in red) of caveolin-1 overlaps the N-MAD region, and serves as a anchor point for signaling effector proteins such as Gα-subunits, PKC isozymes, and RhoA that

carry a caveolin binding domain (CBD). Upon exchanging GDP for GTP, Gaq-subunits associate with and activate phospholipase C-β1 (PLCβ1), which catalyzes hydrolysis of phosphatidylinositol 4,5- bisphosphate (PIP2) to produce the intracellular messengers diacylglyceraol (DAG) and inositol 1,4,5 trisphosphate (IP_3). IP_3 binds to its receptor on proximal sarcolasmic reticulum (SR) membranes, and mediates a rapid release of SR Ca^{2+} into the cytosol. Mobilization of cytosolic Ca^{2+} is required to initiate contraction of smooth muscle cells. Reproduced with permission from "Phenotype and functional plasticity of airway smooth muscle: role of caveolae and caveolins"; Volume 5, pp. 80-88. Proceedings of the American Thoracic Society © 2008 HighWire Press, USA.

Caveolae and Dystrophin Glycoprotein Complex

It has been shown that in a contractile smooth muscle cells, caveolae and caveolin-1 are uniquely organized into rib-like longitudinal arrays [100, 101], which run parallel to regions that contain dense plaques and adheren junctions [101]. The dense plaque regions are enriched in proteins such as integrins, vinculin, and filamin that tether filamentous actin to the extracellular matrix and adjacent cells [66, 101, 108] and transmit mechanical forces across the plasma membrane during stretch and contraction [109-111]. In contractile smooth muscle cells, caveolae membrane domains are linked to actin cytoskeleton and ECM though 2 independent systems. First, caveolin-1 can bind to the cortical actin cross-linking proteins, filamin A and B, which localize to integrin-enriched dense plaques in smooth muscle cells [112]. Second, caveolin-1 binds to the intracellular tail of β-dystroglycan, the core transmembrane subunit of the dystrophin glycoprotein complex (DGC), which is linked both to intracellular actin by dystrophin and to extracellular laminin by α-dystroglycan [101, 113, 114]. The distribution of Cav-1 and dystrophin overlaps at the membrane of contractile smooth muscle cells [101]. Dystrophin is a large, flexible protein with an actin-binding domain at the amino-terminus that anchors the protein to cortical actin composed of β- and γ-actin. The

carboxyl region of dystrophin harbors a WW-like domain that binds the β-dystroglycan (Figure 3) [113-116]. In smooth muscle, the DGC includes dystrophin, β-dystroglycan (the core transmembrane subunit), α-dystroglycan (an extracellular subunit that serves as a selective binding site for laminin), a transmembrane sarcoglycan complex consisting of β-, δ-, ε-, and ζ- or γ-sarcoglycan, and sarcospan (Figure 4) [114, 117-119]. The sarcoglycan complex is stabilized by sarcospan, and in turn sarcoglycans (particularly the δ-subunit) interact with β-dystroglycan and provide structural stability to the DGC [120, 121]. One of the key roles for the DGC is thought to be in stabilizing the plasma membrane to protect it against damage that might occur from mechanical forces during contraction and stretch.

Dystrophin

Dystrophin is a rod-shaped cytoplasmic protein, and a vital part of a protein complex that connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix through the cell membrane. Full-length dystrophin (427 kDa) is expressed in skeletal muscle, cardiac muscle, and brain, each from a different promoter [122] (described in Fig. 4). The dystrophin protein has four major domains: an N-terminal region with homologies to α-actinin, a central rod domain composed of spectrin-like repeats, a cysteine-rich domain, and a C-terminal domain [123]. The cysteine-rich region and the C-terminal region contain several binding motifs, including a WW domain, two putative calcium-binding EF-hand motifs, a putative zinc finger ZZ domain, and a coiled-coil motif [124, 125]. There are multiple smaller dystrophin

isoforms, including Dp260, Dp140, Dp116, and Dp71 (designated according to molecular weights), that are transcribed from promoters within introns of the dystrophin gene and therefore share the same C-terminal domains. Dp260 is expressed primarily in the retina; Dp140 expression is restricted to the central nervous system and kidney; Dp116 is expressed in peripheral nerve; and Dp71 is most highly expressed in brain, lung, liver, and kidney [126, 127]. The N-terminal regions of dystrophin and utrophin associate with cytoskeletal actin [123, 124]. The central rod region of dystrophin also has actin-binding properties, [124]. The cysteine-rich regions of the dystrophin proteins bind to β-dystroglycan [128, 129], and this interaction is *via* the dystrophin WW domain [130]. Finally, there is a C-terminal domain that interacts with the dystrobrevins *via* coiled-coil motifs [130], and with the syntrophins *via* unique binding sequences [131-133]. In non-muscle tissues, the shorter dystrophin transcripts mentioned earlier appear to replace dystrophin and co-purify with components of the DGC that bind to the C-terminal region of dystrophin [134].

Dystroglycan Complex

Dystroglycan forms an essential core of the DGC as it connects the cytoskeletal components of the DGC to the ECM and is a product of a single gene post-translationally cleaved to produce α and β subunits [135] (described in Fig. 4). β-dystroglycan is a single-pass transmembrane protein, and its carboxyl-terminus interacts with the cysteine-rich conserved WW domains in dystrophin and caveolin-3 [115, 116, 136]. β-dystroglycan plays a key role in cell signaling through the WW-binding domain in its

intracellular tail while various signaling effectors such as Nck, Shc, Grb2, Src, FAK, Fyn, MEK, and ERK can also bind to its SH2 and SH3 domains [137-142]. Caveolin-3 also interacts with β-dystroglycan, and it may compete for the same binding site as dystrophin [116]. The amino-terminus of β-dystroglycan interacts with its extracellular binding partner α-dystroglycan, which forms an important connection to the extracellular matrix through its interactions with the α2chain of laminin 2. Thus, dystroglycan forges a link between the sarcolemma and the extracellular milieu. Studies suggests that β-dystroglycan appears to have the capacity to participate in and modulate signal transduction cascades involving effectors that are localized to caveolae membrane domains, which could have an impact on development and maintenance of a contractile phenotype through its direct binding with α-dystroglycan which interacts with laminin [17, 99, 143].

SARCOGLYCAN COMPLEX

The sarcoglycans are a family of homologous transmembrane proteins closely associated with dystroglycan and is thought to stabilize its interactions with the extracellular matrix and with dystrophin and its associated proteins [144, 145] (described in Fig. 4). Six sarcoglycan genes and gene products have been identified to date. Sarcoglycans can be divided into three subfamilies based on nucleic acid and protein similarity and evolutionary conservation. α-sarcoglycan and ε-sarcoglycan are highly similar, type-I transmembrane proteins with their amino termini on the intracellular face of muscle cells. β-sarcoglycan is a type- II transmembrane protein. γ-,

δ-, and ζ- sarcoglycan also are type-II glycosylated transmembrane proteins that are highly similar among themselves and weakly similar to β-sarcoglycan [146] . The gene structures for γ-, δ-, and ζ-sarcoglycan are identical, consistent with multiple gene duplication events. The sarcoglycans typically are found in a heteromeric complex including at least four different sarcoglycans. Growing evidence suggests that there are two types of sarcoglycan complexes; one containing α-, β-, δ-, and γ-sarcoglycans, and the other consisting of β-, γ-, ε-, and ζ-sarcoglycans [118, 147]. In addition to the four sarcoglycans (α, β, γ, and δ) that are highly, although not exclusively, expressed in skeletal muscle, [117, 144] a fifth sarcoglycan, ε, is expressed in a wide variety of tissues [148]. In smooth muscle, ε-sarcoglycan replaces α-sarcoglycan in a tetrameric complex with β-, γ-, and δ-sarcoglycan [117-119, 149]. There has been some debate about whether γ- or ζ-SG is the final member of the tetrameric sarcoglycan complex, however a recent report indicates that they are functionally indistinguishable [150]. Based on biochemical studies, Chan et al. proposed that δ-sarcoglycan might bind to the dystroglycan complex, linking the other components to the DGC [146]. Direct binding of components of the sarcoglycan complex to dystrophin has also been reported [151, 152]. The current belief is that the sarcoglycan complex is necessary for “stabilization” of the DGC in general and the dystrophin-dystroglycan interaction in particular [153]. This is based on the observation that disruptions in the sarcoglycan complex lead to secondary reductions in the levels of other DGC components at the sarcolemma.

SARCOSPAN

Sarcospan is a 25-kDa protein that belongs to the tetraspan family of proteins, so named because they have four membrane-spanning domains that are conserved among the family members [154]. To date, only a single sarcospan gene has been identified. A major transcript is expressed primarily in skeletal muscle, but a minor transcript is expressed in skeletal muscle and other tissues such as ovary, prostate, and intestine [154]. Studies of sarcospan protein expression have demonstrated its presence in skeletal, cardiac, and smooth muscle cells [154-156].

FUNCTIONAL SIGNIFICANCE OF DGC

Dystrophin deficiency in muscle results in a secondary deficiency of all of the components of the DGC and a severe phenotype of DMD [157]. It is also clear that deletions within the C-terminal region of dystrophin result in severe dystrophies, suggesting that it is the disruption of the DGC interactions in this region that predispose to muscle cell death [158]. Whether the severe phenotype in this case is due to a disruption of DGC signaling or to a disruption of some other functional property of the N-terminal region of dystrophin is not known. It is noteworthy that *mdx* transgenic mice expressing a dystrophin construct deleted for the actin-binding N-terminal domain have a very mild phenotype [159].

Mutations in the dystroglycan gene does not cause any identified muscular dystrophies in humans. Mice that are deficient in dystroglycan do not survive to birth, [160] but chimeric dystroglycan mice do survive postnatally and go on to develop a

muscular dystrophy [161]. Thus, expression of dystroglycan is crucial for normal development, consistent with the patterns of its expression in many tissues, and its role in muscle cell survival can be demonstrated only when its essential functions in other tissues during embryonic development are not disrupted.

Mutations in each of the four skeletal muscle sarcoglycans have been found to cause limb-girdle muscular dystrophies (LGMD) in humans, and deletions of each of these genes cause muscular dystrophies in mice [162]. In general, a primary deficiency in any one of the sarcoglycans causes a secondary deficiency of all the other sarcoglycans and sarcospan, suggesting that the biosynthesis and membrane targeting of these proteins are tightly linked [163, 164]. However, exceptions have been found in muscle of both humans and animals [165, 166]. These examples demonstrate that mutations of single sarcoglycans do not necessarily lead to a secondary deficiency in all the others. In humans, the mutations are homozygous, and loss of function in the genes encoding β-, γ-, or δ-sarcoglycan results in limb-girdle muscular dystrophy and cardiomyopathy [145, 167].

A primary deficiency of sarcospan does not produce any muscle phenotype and no human diseases have been identified due to sarcospan gene mutations [168], while its expression is secondarily reduced by deficiencies of other DGC proteins [154, 169].

ANIMAL MODELS AND DGC

Cat, dog, and mouse models have all been used to study the function of DGC

proteins with respect to muscular dystrophy.

Large Animal Models

The hypertrophic feline muscular dystrophy (HFMD) cat and the canine X-linked muscular dystrophy (CXMD) dog/ golden retriever muscular dystrophy (GRMD) dog, (widely used dog model) are being used to study the function of the DGC and the descriptions of phenotype and histopathology, are available [170-173]. Unlike the HFMD cat, the CXMD/GRMD dog is the closest model to DMD in phenotype and histopathology. The CXMD/GRMD dog muscles have a complete absence of dystrophin, and show early muscle degeneration. Dogs lose mobility and die by one year of age from respiratory failure. The CXMD/GRMD dog is the ideal model to study DMD, however, because of the cost and temporal issues associated with using the dog model, most researchers opt to use the mouse model.

Mouse Models

The *mdx* mouse model was discovered in 1984 in a colony of C57/BL10 mice. They were originally isolated because they had abnormally high levels of pyruvate kinase and abnormal muscle lesions. These mice have an X-linked dystrophy [174] that was later found to be due to a mutation at the base pair in position 3185 in the dystrophin gene [175, 176]. Unlike DMD patients, the *mdx* mouse appears to have little "clinical" manifestation of dystrophy. The mouse lives a relatively long life, although the lifespan is markedly 14 weeks shorter than that of normal C57/BL10 mice; the *mdx*

mouse rarely lives past two years of age [177, 178], while wild-type mice live two and a half to three years [179]. The muscle phenotype of *mdx* mice is similar to DMD, except for the severity; the difference in severity is especially notable early in life. However, histopathologically there are many similarities between dystrophin-deficient myopathies in *mdx* mice and DMD patients. The damage is more extensive in DMD and has greater functional consequences. Fibrosis and adipose tissue deposits are less extensive in *mdx* muscle than in DMD muscle. Therefore, both mdx muscle and DMD muscle show disease progression, although at different rates. *Mdx* muscles have been shown to generate less twitch and tetanic force per cross-sectional area than muscles in wild-type mice [180, 181]. The *mdx* model is said to have two limitations when compared to DMD; the first is its delayed progression compared to DMD, and the second is its milder tissue pathology and functional phenotype. There is no other natural mouse model available for studying the function of the DGC, though various knock out mice have been developed and used extensively to study the other subunits of DGC.

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Chapter 2

THESIS OVERVIEW

STUDY RATIONALE

Increased airway smooth muscle (ASM) mass and airway hyperresponsiveness (AHR) are features of airway remodelling, and asthma. ASM cells contribute greatly to asthma pathogenesis due to their phenotype plasticity, which gives them capacity for contraction, proliferation, cellular hypertrophy, migration, and the synthesis of extracellular matrix (ECM) and pro-inflammatory biomolecules. ASM cells express numerous G-protein coupled receptors (GPCRs) that control these cellular functions alone and in synergy with other receptor subtypes. Indeed, GPCRs are the primary target of asthma relief therapies, and recent work shows their inhibition reduces allergen-induced AHR and airway remodelling *in vivo*. ***The current research project focussed on molecular mechanisms unique to ASM that control GPCR function and intracellular signaling, the contraction of ASM, and ultimately the physiology of the airways and lung in health and disease.***

GPCRs also associate with and may be regulated by specialized cholesterol-rich lipid rafts called caveolae, which appear as 50-100 nm plasma membrane invaginations and are abundant in smooth muscle (described in Chapter 1). In smooth muscle cells caveolae are uniquely aligned into discrete arrays on the plasma membrane. The principal membrane protein of caveolae is caveolin-1 (Cav-1), which has two key functional regions; a caveolin scaffolding domain (CSD), which anchors signaling effectors such as Gα subunits associated with G-proteins, and a WW domain, which binds β-dystroglycan, the core subunit of the dystrophin-glycoprotein complex (DGC)

(described in Chapter 1). In vascular and skeletal myocytes the DGC is stabilized by sarcospan and a complex of four sarcoglycans, and it is linked both to cytoskeletal actin by dystrophin and to ECM laminin *via* α-dystroglycan (described in Chapter 1).

As discussed in detail in Chapter 1, *the role of DGC in terms of expression and function and for the transmission of mechanical forces across the plasma membrane between the actin cytoskeleton and the ECM has been extensively studied in striated, cardiac and vascular smooth muscles; this has not been investigated in ASM cells. Also to date the expression, subunit composition, and functional role of the DGC in particular to its relationship with caveolae-mediated regulation of ASM phenotype and function has not been investigated.*

The information summarized above has led to the derivation of a general hypothesis under which studies reported in this thesis were carried out.

GENERAL HYPOTHESIS

This project tested the hypothesis that the unique plasma membrane organization of caveolae in ASM results from the interaction of the caveolin-1 WW domain with the DGC, and that this organization is needed for caveolin-1 and DGC to act as an effector in pathways determining ASM cell phenotype and function.

RESEARCH PLAN:

General Objective: To characterize and study the organization and interaction of DGC subunits with Cav-1 in ASM cells and tissue, and then to determine the functional

significance of the DGC and caveolae in modulating ASM cell phenotype *in vitro* and lung function *in vivo*.

To test the above hypothesis and achieve the general objective studies were divided into following groups:

1. ***In vitro* studies**: Chapters 4, 5 and 6 outlines most of the *in vitro* work we carried out using airway smooth muscle cells from humans and canine. Detailed description of the rationale is described in the introduction section of these chapters. Chapter 4 describes the systematic characterization of DGC proteins with a context to their expression regulated in a contractile ASM cell by PI3K signaling and laminin-integrin interaction. Chapter 5 describes the interaction of DGC with Cav-1 in a contractile ASM cell and the functional significance of this interaction in mobilizing intracellular Ca^{2+} in ASM cell. Chapter 6 further investigates the role of DGC in modulating intracellular Ca^{2+} pools in human and canine ASM cells.

2. ***Ex vivo* and *in vivo* studies**: Chapter 7, 8 and 9 outlines the *ex vivo* and *in vivo* studies using canine and mouse models lacking various DGC subunits (namely dystrophin and ð-sarcoglycan) and Cav-1. Detailed description of the rationale is described in the introduction section of these chapters. Chapter 7 describes the role of dystrophin using a canine model: golden retriever muscular dystrophy (GRMD) lacking dystrophin and *mdx* (dystrophin knock out) mice to study its role on ASM phenotype, contraction and lung function. Chapter 8 investigates the tracheal

responsiveness to methacholine (MCh) in Cav-1 KO mice and the regulation of epithelium-derived lipid mediators by Cav-1 in this process. Chapter 9 investigates the *in vivo* role of DGC protein ð-sarcoglycan (ð-SG) using ð-SG KO mice in ASM contraction and lung function.

Chapter 3

GENERAL METHODS

Table I. List of antibodies used in the study.

S. No.	Antibody	Company
1	Calponin	Sigma, USA
2	smMHC	Sigma, USA
3	sm-α-actin	Sigma, USA
4	Caveolin-1	Santa Cruz & BD Transduction Labs, USA
5	PTRF	BD Transduction Lab, USA
6	α-dystroglycan	Gift from Dr. Kevin Campbell, USA
7	β-dystroglycan	Novocastra, UK
8	α-sarcoglycan	Novocastra, UK
9	β-sarcoglycan	Novocastra, UK
10	δ-sarcoglycan	Novocastra, UK
11	γ-sarcoglycan	Santa Cruz, USA
12	ε-sarcoglycan	Santa Cruz, USA
13	Dystrophin	Chemicon, USA
14	Clathrin HC	Santa Cruz, USA
15	Akt-1	Cell Signaling Technology, USA
16	p-Akt1 (Thr 308)	Cell Signaling Technology, USA
17	p42/44 MAPK	Cell Signaling Technology, USA
18	phospho-p42/44 MAPK	Cell Signaling Technology, USA
19	p-Akt1 (Ser 473)	Cell Signaling Technology, USA
20	PLCβ1	Santa Cruz, USA
21	Gαq	Santa Cruz, USA
22	pan-cadherin	Cell Signaling Technology, USA
23	Cyclooxygenase-2	Calbiochem, USA
24	5-Lipooxygenase	Santa Cruz, USA
25	Cyclooxygenase-1	Cell Signaling Technology, USA
26	β-actin	Sigma, USA
27	GAPDH	Sigma, USA
28	4EBP-1	Cell Signaling Technology, USA
29	GSK3β	Cell Signaling Technology, USA
30	FAK	Santa Cruz, USA
31	p-FAK	Santa Cruz, USA
32	Vinculin	Sigma, USA
33	Talin	Sigma, USA
34	mTOR	Cell Signaling Technology, USA
35	p-mTOR	Cell Signaling Technology, USA

Chemicals and reagents

Hoechst 33342 (H-3570), Latrunculin-A (L-12370), Texas Red-X Phalloidin (T7471) were from Molecular Probes. LY-294002 and Wortmannin were obtained from Calbiochem. Tissue-Tek O.C.T. embedding medium was from Sakura Finetek. Cell culture media (DMEM and Ham's F12) and supplements (fetal bovine serum, ITS-A, penicillin and streptomycin) were obtained from Invitrogen. All other chemicals were of analytical grade.

Animals used

Whole body knock-out (KO) mice were used for the entire study. Caveolin-1 (KO) $Cav1^{tm1Mls/J}$ (Cav-1 KO) and genetic control B6129SF2/J (wild-type) mice were used in this study. All mice were female, aged between 8 to 12 weeks and were purchased from Jackson Laboratory. *Mdx* mice (dystrophin deficient) were obtained from Dr. July Anderson, Faculty of Biological Sciences, University of Manitoba. For δ-SG KO mice breeding pairs were obtained from Jackson Labs for targeted mutation against δ-SG gene in B6.129-$Sgcd^{tm1Mcn}$/J mice. Breeding was performed at University of Manitoba, animal care facility and genotyping was performed to establish KO, heterozygous and wild-type animals. The University of Manitoba Animal Care Committee approved all protocols regarding the handling of animals. All animals were maintained on a 12 h dark and light cycle and were fed with regular laboratory chow while in house at the university facility. The canine ASM were cultures from control golden retriever and

dystrophic dogs (GRMD) obtained from Dr. MK Childers, Wake Forest University Health Sciences, Winston-Salem, NC, USA.

ð-Sarcoglycan Knockout Genotyping:

Tail DNA Extraction was done using Using EZ Tissue/Tail PCR Genotyping Kit, EZ BioResearch. Primers set used was as following:

1. 898F (common) TGCTCAGGCTAATGCCACATTG
2. W1295R (wild type) TGCTCCTGTGGTGGGAATACTG
3. M1896 (mutant) CCAGCTCATTCCTCCCACTCA

Product sizes: C898F/W1295R is 398 bp

C898F/M1896 is 587 bp

For n tail clips, (n+1) 20 µl Solution A was mixed with (n+1) 180 µl Solution B in a 15 ml tube. It was vortexed to mix. 200 µl extraction buffer was added (A+B) to each tube. Samples were put in the thermocycler for the following tissues: 55^{o}C for 10 min, 95^{o}C for 10 min, hold at 4^{o}C. It was followed by PCR with initial denaturation at 94^{o}C, 1 min (35 cycles), 94^{o}C for 30 sec, 60^{o}C for 30 sec, 72^{o}C for 2 min, Final Extension, 72^{o}C, 10 min. Samples (6–10 µl) of each reaction were run on a 2% agarose gel at 75 volts. Gel documentation system (AlphaEaseFC, Alpha Innotech Corporation, San Leandro, CA) was used to analyze the gel.

Immortalized human airway smooth muscle cell culture

For all studies at least four senescence-resistant human airway smooth muscle (ASM) cell lines were used; these cell lines were prepared using MMLV retroviral vectors

to facilitate stable integration of the human telomerase reverse transcriptase gene (hTERT) as we have previously described [1]. hTERT-expressing human ASM cell lines retain expression of contractile phenotype markers, including smMHC, calponin, sm-α-actin, and desmin to passage 10 and higher [1]. For all experiments, passages 10–17 hTERT ASM cultures were used. The primary cultured human ASM cells used to generate each cell line were prepared as we have described from macroscopically healthy segments of 2nd-to-4th generation main bronchi obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma [2, 3]. For some experiments we used primary cultured human tracheal smooth muscle cells that were prepared from healthy transplant donors; after micro dissection to isolate the trachealis muscle, myocytes were isolated using procedures mimicking that used to prepare bronchial ASM primary cultures [2, 3]. All procedures were approved by the Human Research Ethics Board (University of Manitoba). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS); medium was changed every 48 hours unless otherwise specified. To induce acquisition of a contractile phenotype, confluent cultures were maintained in DMEM supplemented with ITS (insulin 5 μg/ml; transferrin 5 μg/ml, selenium 5 ng/ml) for up to 4 days as we have described [4].

Primary human and canine airway smooth muscle cell culture

Primary airway myocytes for cell culture were obtained from dissociated canine or human trachealis as previously described [2]. Cells were plated onto 100 mm culture dishes or pre-cleaned sterile cover slips placed in 6 well culture clusters and grown to

confluence using Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum. At confluence, myocytes were serum deprived a further 7-10 days using Ham's F12 medium supplemented with insulin, transferrin and selenium (ITS-A, 1%) to induce a contractile phenotype. Cultures were maintained in a humidified chamber at 37°C/5% CO_2 and all media contained both 100 units/mL penicillin G and 100 μg/mL streptomycin sulfate. For experiments involving actin disruption, cells were incubated for 60-90 minutes in HBSS (1.26 mM $CaCl_2$, 0.493 mM $MgCl_2 \cdot 6H_2O$, 0.407 mM $MgSO_4 \cdot 7H_2O$, 5.33 mM KCl, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137.93 mM NaCl, 0.338 mM Na_2HPO_4 (anhydrous) and 5.56 mM) Dextrose) with or without 1μM latrunculin-A. Myocytes were used at passage 0 or 2 in these studies.

Preparation of protein lysates from human ASM tissue and cells

Intact ASM tissue was isolated from human bronchial specimens by microdissection at 4°C. Thereafter tissues were homogenized in ice cold in RIPA buffer (composition: 40 mM Tris, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1 mM NaF, 5mM β-glycerophosphate, 1 mM Na_3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 1 mM PMSF, pH 8.0) using a Polytron homogenize. The lysate was transferred to 1.5 ml plastic tube, centrifuged (760 × g, 5 min) and the supernatant stored at -20°C for subsequent protein assay and immunoblot analyses. Protein lysates were collected from human ASM cultures at three stages: serum-fed, 50-70% confluence (proliferative stage); serum-fed, 90-100% confluence (Day 0); and, four days (Day 4) after switching the media of confluent (Day 0) cultures to serum-free DMEM

supplemented with ITS. To prevent phenotype maturation, in some experiments, during serum-free culture cells were treated with PI3K inhibitor (20μM LY294002 or 100nM wortmannin) or with laminin competing peptide YIGSR (1μM) or an inactive scrambled peptide, GRADSP (1μM). For protein lysate preparation plates were washed twice with ice-cold PBS, cells homogenized by scrapping in ice-cold RIPA buffer. Lysates were transferred to 1.5 ml plastic tubes, centrifuged (760 × g, 5 min), and the supernatants stored at -20°C for subsequent protein assay and immunoblotting.

Preparation of protein lysates from human and canine ASM tissue and cells

Intact airway smooth muscle tissue was isolated from human bronchial or canine tracheal specimens by microdissection at 4°C. Smooth muscle tissues and primary cultured cells were homogenized in ice cold in RIPA buffer (composition: 40 mM Tris, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1 mM NaF, 5mM β-glycerophosphate, 1 mM Na_3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 1 mM PMSF, pH 8.0) using a polytron. The lysate was transferred to 1.5 ml plastic tube, centrifuged (760 × g, 5 min) and the supernatant stored at -20°C for subsequent protein assay and immunoblot analyses.

Preparation of lung & tracheal tissue lysates from Cav-1 KO and wild-type mice

Entire lungs were cut into small fragments and approximately half of the lungs were preserved in 200 μl of lysis buffer (composition: 40 mM Tris, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1 mM NaF, 5mM β-glycerophosphate, 1 mM

Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 1 mM PMSF, pH 8.0) and stored at – 20˚C for protein analysis. The other half of the lungs were put into RNAlater* buffer (10 ml/mg tissue) and kept at 4˚C over night and moved to -20˚C after 24 hrs. Frozen lung and tracheal tissues in the lysis buffer were slowly thawed in ice and were transferred into 5 mL tubes for homogenization using a polytron. The lysate was transferred to 1.5 ml plastic tube, centrifuged (760 × g, 5 min) and the supernatant stored at -20°C for subsequent protein assay and immunoblot analyses.

Immunoblotting

Protein content in supernatant samples was determined using the BioRad protein assay with bovine serum albumin as a reference (BioRad, Hercules, CA). Immunoblotting was performed using standard techniques [4]. Briefly, after reconstituting samples in denaturing buffer, 18–25 μg protein was loaded per lane and size-separated electrophoretically under reducing conditions using SDS-polyacrylamide gels. Thereafter proteins were electro-blotted onto nitrocellulose membranes, which were subsequently blocked with 5% w/v skim milk in Tris Buffered Saline (TBS) (composition: 10 mM Tris HCl, pH8.0, 150 mM NaCl) with (0.2%) or without Tween-20. Blocked membranes were incubated with primary antibodies diluted in TBS containing 1% w/v skim milk with (0.2%) or without Tween-20. The membranes were developed by subsequent incubation with HRP-conjugated secondary antibody, and then visualized on photographic film using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK). β-actin was used to correct for equal loading of all samples.

Densitometry and quantification of the relative protein abundance was performed using the Epson Perfection 4180 Station using either TotalLab TL100 software (Nonlinear Dynamics, Durham, NC) or a gel documentation system (AlphaEaseFC, Alpha Innotech Corporation, San Leandro, CA).

Isolation of caveolae-enriched membranes

Membrane caveolae from cells or tissue were isolated by sucrose density gradient ultracentrifugation using a detergent free protocol [1]. Briefly, cells were lysed by sonication in 500 mM carbonate buffer (pH 11) containing 2mM PMSF (after mincing, tissue was first homogenized and then sonicated in carbonate buffer). Sonicates were then combined with an equal volume of 90% sucrose in MES buffer (25 mM MES, 150 mM NaCl, pH 6.5) and the resulting 45% sucrose layer overlayed with a stepwise gradient of 30%, 20% and 5% sucrose buffers. The gradient was ultracentrifuged at 200,000 x g, 16h at 4°C. Sequential 1 mL fractions were taken from the top of the gradient for later analysis by SDS-PAGE and western blotting. Caveolae membranes were isolated as opaque light scattering bands at the 5%/20% and 20%/30% sucrose interfaces.

Immunoprecipitation

Protein-G conjugated sepharose beads (Protein G Sepharose™ - GE Healthcare, Sweden) were mixed with 500 µl of tissue lysate. After overnight incubation with anti-Cav-1 antibody at 4°C, beads were washed four times with TBS (pH 7.4) containing 0.1%

Tween 20 and once with PBS. Beads with immunoprecipitated proteins were stored at -80°C until used for protein blot analysis for either β-dystroglycan or COX-2 protein.

β-*dystroglycan shRNAi*

The β-dystroglycan lentiviral shRNA construct was purchased from Open-Biosystems (Huntsville, AL) distributed by the Biomedical Functionality Resource, University of Manitoba, as a bacterial culture (Clone Id: V2LHS_24095). Individual colonies were grown up in 2ml LB broth with 100μg/ml ampicillin (bacterial selection) for 8 hours at 37°C, with shaking at 280 RPM. The cultures were then added to 200 ml LB broth with ampicillin (100 μg/ml) and incubated as above overnight. The culture was centrifuged and plasmid purified with a Hi Speed Plasmid Maxi Kit (Qiagen cat# 12663). The plasmid was transfected into 293T cells using a Ca^{2+}-phosphate 3 plasmid transfection VSVG (envelope vector), 8.2Δvpr (packaging vector) and expression vector for β-dystroglycan to generate lentivirus by counting the puromycin resistant colonies, as previously described [5, 6]. A non-coding β-dystroglycan refractory shRNA (shRNAi non-code) was used as a transduction control. The cells were incubated for 3 days at 37ºC and supernatant containing virus was concentrated by ultracentrifugation. The virus was resuspended in DMEM 0.5% FBS for 24 hr at 4°C and aliquoted and stored at -80°C. For the transduction, human primary ASM cells were grown up to 70-80% confluence in twelve-well plates and virus was added to the cells at a varied multiplicity of infection (MOI) from 3-9. The transduction was repeated twice and then the cells were allowed to grow in complete media for 48 hours. The cells were then selected with

puromycin 4 μg/ml. Viability of the transduced cells undergoing experiment was >98% as assessed by trypan blue dye exclusion after completion of the experiment.

RNA isolation and RT-PCR

Total RNA was extracted using the Qiagen RNeasy Mini Kit in accordance with the manufacturer's recommendations (Qiagen, Mississauga, ON) from human bronchial tissue enriched in ASM that was microdissected from two different human donors. Total RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) for 2 h at 37°C followed by 5 min incubation at 95°C, and then diluted 1:10 with RNase-free water. The RT-PCR reactions for cDNAs of interest were carried out in a thermal cycler (Mastercycler, Eppendorf, Germany) using primer pairs listed in Table 1. The coding regions corresponding to the primers were taken from National Center for Biotechnology Information (NCBI) and than primers were designed using PRIMER-3 and IDT programs available online. Cycle parameters were: denaturation (94°C for 45 s), annealing (60°C for 45 s), and extension (72°C for 45 s). The initial denaturation period was 4 min and the final extension was 5 min. Glyceraldhyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Amplified products were analyzed by DNA gel electrophoresis in 2% agarose, and visualized by Gelstar staining under ultraviolet illumination using a gel documentation system (AlphaEaseFC, Alpha Innotech Corporation, San Leandro, CA).

Real-Time RT-PCR analysis

Total RNA was extracted from human ASM cells using the RNeasy Plus Mini Kit in accordance with the manufacturer's recommendations (Qiagen, Mississauga, ON). The RNA concentration and purity were assessed with optical density measurements [7]. Total RNA (1 µg) was reverse transcribed using the Quantitect Reverse Transcription Kit as recommended by the supplier [Qiagen, Mississauga, ON]. Real-Time PCR for cDNAs of interest were carried out with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.), using primer pairs for β-dystroglycan (as previously described), COX-2, 5-LO, Cav-1 and 18S ribosomal RNA (calibrator gene: NCBI X03205.1. Forward 5'-CGCCGCTAGAGGTGAAATTC -3', Reverse 5'- TTGGCAAATGCTTTCGCTC -3'). Each reaction contained the following: 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.), 0.4 µM of each primer and cDNA template in a final volume of 20 µl. After initial denaturation for 10min at 95°C, the reactions were cycled 40 times for 15 at 95°C, 1min at the annealing temperature of 60°C and 30 s at 72°C for extension of both β-dystroglycan and 18S ribosomal RNA. Product specificity was determined by melting curve analysis. Relative quantitation of gene expression was performed using the 7500 Sequence Detection software v.1.4 (Applied Biosystems, Foster City, CA, U.S.A.).

Immunofluorescence microscopy

Tissue

Segments of 2nd-to-4th generation mainstem bronchi from healthy portions of human lung removed during lung resection surgery were frozen fresh in OCT embedding

medium. Tissue was cryosectioned serially into thin slices (5 microns), transferred on to precleaned microscope slides, then air dried briefly and equilibrated in cold cytoskeletal buffer (CB): (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$ and 5 mM glucose at pH 6.1). Sections were fixed for 15 minutes in cold 3% paraformaldehyde and then permeabilized using 0.3 % Triton-X in CB buffer for 40 minutes. Sections were blocked for 2 hours using 3% donkey serum in cyto-TBS (20 mM Tris base, 154 mM NaCl, 20 mM EGTA and 20 mM $MgCl_2$ at pH 7.2) containing 0.1% Tween 20 (cyto-TBST), then incubated at 4 °C overnight in primary antibody diluted in cyto-TBST (0.1% Tween 20): anti-smMHC (1:200), anti-β-dystroglycan (1:50), anti-β-sarcoglycan (1:50), or anti dystrophin antibody (1:50). For negative controls, samples were incubated with isotype-matched mouse IgG. CY-3 conjugated secondary antibodies were used to detect primary antibody bound to tissues. Nuclei were stained with Hoechst 33342 (10 μg/ml). After staining coverslips were mounted using ProLong antifade medium (Molecular Probes, Inc. USA), and fluorescent imaging was performed using an Olympus BX-51 Fluorescencent microscope (Olympus America Inc, Melville, N.Y.).

Cells

Human ASM cells were plated onto pre-cleared glass coverslips in 6-well culture dishes. Cells were fixed for 15 minutes at 4^oC in CB buffer containing 3% paraformaldehyde (PFA). Cells were then permeabilized by incubation for 5 minutes at 4^oC in CB buffer containing 3% PFA and 0.3% Triton X-100. For immunofluorescence microscopy, fixed cells were first blocked for 2 hours at room temperature in cyto-TBS

buffer containing 1% BSA and 2% normal donkey serum. Incubation with primary antibodies occurred overnight at 4^{o}C in cyto-TBST using anti-smMHC (1:200), anti-β-dystroglycan (1:50), anti-β-sarcoglycan (1:50) or anti dystrophin antibody (1:50). For negative controls, samples were incubated with either isotype-matched mouse IgG or rabbit antiserum. Incubation with FITC- or Texas Red (TxR)-conjugated secondary antibodies was for 2 hours at room temperature in cyto-TBST. Coverslips were mounted using ProLong antifade medium (Molecular Probes, Inc. USA). Fluorescent imaging was performed by capturing a mid-cell section of 0.3µm focal depth using an Olympus LX-70 FluoView Confocal Laser Scanning Microscope (Olympus America Inc, Melville, N.Y.) equipped with a 40x objective.

Histology and immunohistochemistry

Paraffin embedded mouse tissue was used for IHC. Paraffin sections of 4µm thickness were prepared using a Shandon Finesse E Microtome Sectioner (Fisher Scientific, Ottawa, ON). Sections were mounted on Superfrost Plus slides (Fisher Scientific, Ottawa, ON) and, dried overnight at 50°C in a slide dryer. For immunostaining, slides were first de-paraffinized in xylene and hydrated in graded ethanol to distilled water, followed by washings with a solution of 1X PBS (pH 7.4). Heat-induced Epitope Retrieval (HIER) was performed by placing the slides in a pre-warmed steamer (60-90°C) with citrate buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 6.0) in a Coplin Jar for 30 min. The slides were then removed, and allowed to cool to room temperature for 20 min while they were remained in citrate buffer. They were then washed three times for

5 min in 1X PBS. Blocking solution (3% BSA, “Bovine Serum Albumin” in PBS) was added to each slide and the slides were incubated in a humidified chamber for 30 min at room temperature to decrease background staining. The slides were then washed three times for 5 minutes in 1X PBS and incubated with 3% H_2O_2 (in 1X PBS) for 10 min at room temperature. The slides were then incubated with the Avidin and Biotin blocking Kit (Vector Inc, Burlington, ON) for 15 min followed by three washes with 1X PBS for 5 min. Primary antibodies were then diluted in blocking solution and incubated with tissue sections at 4°C overnight in a humidified chamber. The next day, the slides were washed three times for 5 min with 1X PBS. When using mouse primary antibodies on mouse tissues, sections were first incubated with unconjugated AffiniPure Fab Fragment goat anti-mouse IgG (Jackson ImmunoResearch Labs) for 1 h to block endogenous mouse IgG. Next, biotinylated secondary antibody was diluted in blocking solution and incubated on the sections for 1 h at room temperature. The slides were then washed again three times for 5 min in 1X PBS and ABC solution (Vector Inc, Burlington, ON) was added to the slides and incubated for 30 min at room temperature. After washing, DAB substrate (3,3-diaminodbenzidine) was added to the sections and incubated for 2-5 min. The reaction was stopped by the addition of distilled water for 5 min. Nuclei were counter-stained with Mayer’s hematoxylin for 3 min followed by 30 sec in basic water (0.2% lithium carbonate in distilled water). The slides were then washed and dehydrated as described above and mounted with Permount (Thermo Fisher Scientific, Ottawa, ON). As a negative control, sections were processed as above but addition of primary antibody was omitted.

Transmission electron microscopy

The ultrastructure of intact canine trachealis was assessed as described previously with slight modification [8]. Specimens consisting of two cartilage rings with intact trachealis were prepared from the cervical segments using a sharp scalpel. Specimens were incubated at 37°C in oxygenated Krebs-Henseleit solution (KH; 117.5 mM NaCl, 5.6 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.28 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 5.55 mM D-glucose, gassed with 5% CO_2 and 95% O_2, 37°C, pH 7.4) for 1 h in the presence or absence of Latrunculin A (1 μM). Specimens were washed once with fresh KH buffer and fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 hr at 4°C, washed and fixed in 1% osmium tetroxide, before embedding in Epon. Thereafter, the smooth muscle layer was removed from each ring and subjected to postfixation with 1% osmium tetroxide and embedded in LX-112 acrylic medium. Ultra-thin cross-sections of the muscle tissue were then prepared, mounted onto coated grids, and stained with 1% uranyl acetate and lead citrate. Transmission electron microscopy was performed with a Philips CM10, at 80kV, on ultra thin sections (100 nm on 200 mesh grids) stained with uranyl acetate and counterstained with lead citrate.

Subcellular fractionation

Cytosolic and membrane fractions were generated using a subcellular fractionation technique at 4 °C as previously described [9]. Cells were scraped in ice cold buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and

protease inhibitor cocktail), sonicated on ice 3 times for 5 s, and then the homogenate was separated into cytoplasmic and membrane fractions by ultra-centrifugation ($100{,}000 \times g$ for 35 min). The membrane fraction was obtained by solubilizing in dissociation buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 1 mM dithiothreitol, 1% SDS, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail). For the detection of specific protein by immunoblotting, an equal amount of membrane and cytosolic protein fraction were subjected to standard SDS-PAGE and transferred to nitrocellulose membranes.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement

Real-time quantification of cytosolic Ca^{2+} in cultured ASM cells was performed using the Ca^{2+}-sensitive ratiometric fluorescent dye Fura-2 AM as we have described previously [8, 10]. All measurements were carried out using myocytes grown on glass coverslips or chamber slides. Myocytes were washed briefly with HBSS/HEPES buffer containing 0.1% BSA and then incubated with 5 μg/ml fura-2 AM (37°C, 1 h) in buffer supplemented with 0.01% pluronic acid. Cells were then washed three times and incubated in buffer for a further hour at room temperature to allow for fura-2 AM de-esterification. Real-time changes in $[Ca^{2+}]_i$ were recorded using an Olympus LX-70 inverted epifluorescent microscope (20x objective) coupled to a Nikon CCD camera controlled by NIS imaging software. The system was further coupled to a Sutter Instruments Lambda 10-2 filter wheel and controller with repeated 100 ms excitation at 340 and 380 nm; emission at 510 nm was recorded continually for up to 5 min after the

addition of contractile agonists. Maximum change in $[Ca^{2+}]_i$ was calculated as the average baseline value subtracted from the peak $[Ca^{2+}]_i$ response to agonist. The ratio of emission at 510 nm excited by 340- and 380-nm light was converted to $[Ca^{2+}]_i$ values from a calibration curve generated using Ca^{2+} standards and calculated by the method of Grynkiewicz [11]. For studies examining the effects of actin disruption on $[Ca^{2+}]_i$ mobilization induced by contractile agonist, Fura-2-loaded cells were incubated at room temperature for 1h in buffer containing 1μM latrunculin-A. Controls cultures were incubated for the same time period in buffer only.

Isolated tracheal ring preparation

Mice were anesthetized and euthanized using pentobarbital (90mg/Kg bodyweight) prior to dissection. For tracheal isolation, the chest cavity contents were removed en masse and placed in Krebs-Henseleit bicarbonate solution (K-H) of the following composition (in mM): 118 NaCl, 23.5 $NaCO_3$, 4.69 KCl, 1.18 KH_2PO_4, 1.00 $MgCl_2$, 2.50 $CaCl_2$, and 5.55 dextrose. The K-H was gassed with 95% O_2-5% CO_2 to maintain a pH between 7.3 and 7.5. Tracheal isolations were carried out in cold K-H (4˚C) by pinning the apex of the heart and the voice box of trachea to a dissecting dish and removing extraneous tissue. Lungs were removed and frozen for protein and RNA analysis (see below).

Each isolated trachea was cut into 4 segments; each segment containing 3 or 4 cartilage rings. Tracheal ring preparations were mounted between 2 pins- one pin firmly

fixed and the other attached to an isometric force transducer in one chamber of a Danish Myo Technology (Aarhus, Denmark) organ bath system. The paries membranaceous of the tracheal ring preparation (containing the smooth muscle layer) was placed between the 2 support pins. Tissue preparations were maintained in gassed K-H at 37°C and pH 7.3-7.5 for all subsequent studies. For epithelium-denuded studies, tracheal segments were threaded onto silk surgical threads (Ethicon P4888C size 5); then the rings were rolled 3 revolutions on a paper towel soaked with the K-H [12]. Randomly selected tracheal preparations were saved for histological evaluation of epithelial removal while others were used for immunohistochemical staining of Cav-1, 5-LO and COX-2.

Smooth muscle equilibration

To establish optimal resting tension, reference length, and stable baseline tracheal rings were equilibrated for 90-120 min with intermittent (~20 min) instillation of 63 mM KCl-substitiuted K-H (usually 3 exposures) in order to isometrically contract the tissues in a non-G-protein coupled manner. Reference resting tension for all preparations was established at ~ 0.6 mN. The isometric force developed for each smooth muscle preparation in response to the 3rd KCl-substituted K-H exposure was used as the reference force for subsequent contractions elicited through G-protein coupled receptor activation.

Methacholine (MCh) concentration-response studies

After equilibration, tracheal rings were incubated for 30 min with either: dimethyl sulphoxide (DMSO vehicle control; 1 μL/mL), Indomethacin (COX-2 inhibitor), INDO; 3 μM), eicosatetraynoic acid (phospholipase-A_2 (PLA_2) inhibitor, ETYA; 10 μM) alone or INDO in combination with either leukotriene (LTC_4 and LTD_4) receptor antagonist, Monteleukast (10 μM), or 5-lipoxygenase inhibitor, Zileuton (10 μM). Tracheal rings from Cav-1 KO and wild-type mice were randomly assigned to these various inhibitors of the arachidonic acid cascade. After incubation with these inhibitors, MCh concentration-response studies (1.0 nM to 1.0 mM) were performed. After the final administration of MCh, rings were washed with K-H. Preparations were preserved in lysis buffer and stored at -80°C until homogenization for western blot analysis. Tracheal rings were pooled but separated into wild-type and Cav-1 KO preparations with and without epithelium; i.e., 4 groups of pooled tracheal rings.

Measurement of Lung Mechanics:

Mice were anesthetized with intra-peritoneal sodium pentobarbital (95 mg/kg). The trachea was dissected using fine dissection scissors and a 20-gauge polyethylene catheter was inserted which was further connected to a flexiVent small animal ventilator (Scireq Inc. Montreal, PQ). Mice were ventilated with a tidal volume of 10 ml/kg body weight, 150 times per minute. A positive end expiratory pressure (PEEP) of 3 cmH_2O was used for all studies. Mice were subjected to an increased dose of nebulized methacholine (MCh) challenge protocol to assess concentration response characteristics of respiratory mechanics. For MCh challenge, ~ 30μL of saline containing

from 0 to 50 mg/ml MCh was delivered over 10 seconds using an in-line ultrasonic nebulizer.

To assess the effects of MCh challenge on respiratory mechanics we used a low frequency forced oscillation technique. Respiratory mechanical input impedance (*Zrs*) was derived from the displacement of the ventilator's piston and the pressure in its cylinder. Correction for gas compressibility, and resistive and accelerative losses in ventilator, tubing and catheter were performed according manufacturer instructions, using dynamic calibration data obtained from volume perturbations applied to the system in an open and closed configuration. By fitting *Zrs* to the constant phase model, flexiVent software calculated conducting airway resistance (R_{aw}), peripheral tissue and airway resistance (G), tissue elastance or stiffness (H); each parameter was normalized according to body weight. Values for each parameter were calculated as the mean of all 20 perturbation cycles performed after each MCh challenge.

Data analysis

Values reported for all data represent means ± standard error of means (SEM). For all studies, replicate data from atleast 3-4 different plated cell cultures or animals were obtained. The statistical significance of differences between two means was determined by an unpaired two-tailed Student's *t*-test, or when appropriate using one way ANOVA with Bonferroni's Multiple Comparison Test for comparison between

treatments or Tukey's multiple range test. Differences were considered to be statistically significant when $p < 0.05$.

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Chapter 4

Expression of the dystrophin glycoprotein complex is a marker for human airway smooth muscle phenotype maturation

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Abstract

Airway smooth muscle (ASM) cells may contribute to asthma pathogenesis through their capacity to switch between a synthetic/proliferative and a contractile phenotype. The multimeric dystrophin-glycoprotein complex (DGC) spans the sarcolemma, linking the actin cytoskeleton and extracellular matrix. The DGC is expressed in smooth muscle tissue, but its functional role is not fully established. We tested whether contractile phenotype maturation of human ASM is associated with accumulation of DGC proteins. We compared subconfluent, serum-fed cultures and confluent cultures subjected to serum deprivation, which express a contractile phenotype. Western blotting confirmed that β-dystroglycan, β-, δ - and ε-sarcoglycan, and dystrophin abundance increased 6-8 fold in association with smooth muscle myosin (smMHC) and calponin accumulation during 4-days serum deprivation. Immunocytochemistry showed that the accumulation of DGC subunits was specifically localized to a subset of cells that exhibit robust staining for smMHC. Laminin competing peptide (YIGSR, 1μM) and phosphatidylinositide-3-kinase (PI3K) inhibitors (20μM LY294002 or 100nM wortmannin) abrogated the accumulation of smMHC, calponin and DGC proteins. These studies demonstrate that the accumulation of DGC is an integral feature for phenotype maturation of human ASM cells. This provides strong rationale for future studies investigating the role of the DGC in ASM smooth muscle physiology in health and disease.

Introduction

The dystrophin glycoprotein complex (DGC) provides a strong mechanical link between the intracellular actin cytoskeleton and the extracellular matrix. The complex is composed of multiple transmembrane, cytoplasmic, and extracellular protein subunits, with dystrophin, a large intracellular rod-like protein, serving as a tether between cytoskeletal actin and β-dystroglycan, the core transmembrane subunit of the DGC (summarized in chapter 1) [1, 2].

Despite its recognized role in striated muscle, there is only marginal appreciation of the expression profile and role of DGC subunits in smooth muscle. Vascular smooth muscles reportedly express α- and β-dystroglycan (DG), dystrophin, and a complement of sarcoglycan (SG) s (β-, δ-, ε- and γ- or ζ- subtypes) that is unique from that of striated muscle [3-6]. Recent studies suggest this profile may be consistent in most smooth muscle tissues [3]. Mardini et al [7] reported that age-related loss of ASM mass occurs to a greater extent in dystrophic hamsters, and this correlates with reduced contractile agonist-induced force generation. In contractile smooth muscles dystrophin compartmentalizes to caveolae-rich linear arrays that associate with Ca^{2+} handling microdomains in the plasmalemma [8-11]. These data suggest an important role for the DGC in the function of contractile smooth muscle cells; however to date there has been little focus on the expression or role of this protein complex in ASM.

In the current study we characterized the profile of DGC subunits expressed in intact human ASM tissue and cultured myocytes. We also tested whether, in a manner akin to striated muscle differentiation, the expression of DGC proteins is associated with, and dependent upon, phenotype switching between contractile and proliferative states in human ASM cells. We

investigated bronchial smooth muscle tissue obtained from human subjects, human bronchial smooth muscle cell lines, and primary cultured human tracheal smooth muscle cells obtained from healthy transplant donors. To characterize DGC subunit expression we used reverse transcriptase (RT)-PCR, Western blotting, and fluorescent microscopy to assess cell and tissue distribution. To assess the relationship between DGC expression and myocyte phenotype we used established, prolonged serum-free culture protocols to induce ASM phenotype maturation [12, 13]. To directly assess whether DGC expression occurs as a consequence of myocyte maturation we compared DGC expression in response to phosphatidylinositide-3-kinase (PI3K) inhibition or blockade of the binding between laminin and integrins, two mechanisms that are required for myocyte maturation [12, 13]. Our studies provide the first assessment of the DGC subunit profile in human ASM and mechanism regulating their expression in association with phenotype plasticity.

Results

Characterization of dystrophin-glycoprotein complex subunits in human ASM tissue

We first addressed the question of whether DGC subunits are present in the human ASM tissue by assessing protein abundance using Western blotting. Protein lysates were collected from human bronchial smooth muscle tissue dissected from intact bronchial specimens. All lysates were characterized by abundant smMHC, a stringent marker for contractile smooth muscle tissue (Figure 1A). In addition, samples were probed for several DGC subunits, including β-DG, β-SG, δ-SG, ε-SG, and dystrophin; each of the DGC protein subunits were easily detected in all human ASM tissues analyzed (Figure 1A).

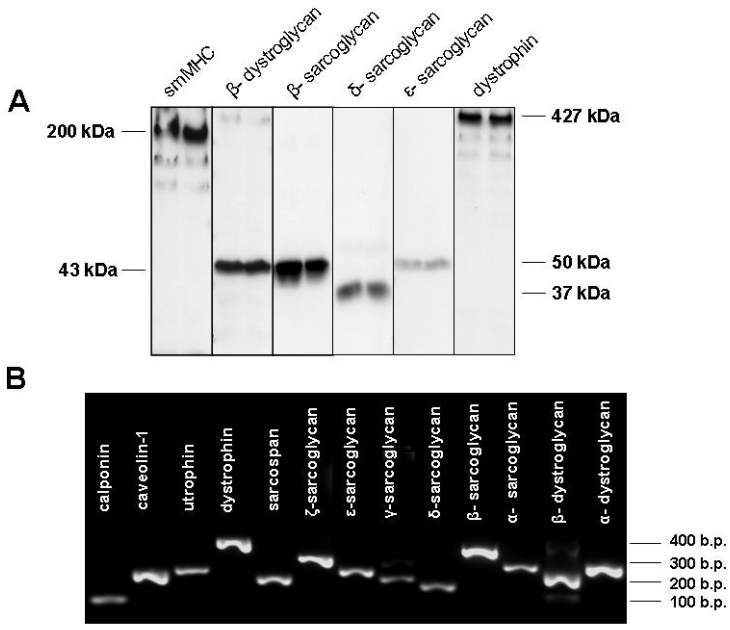


Figure 1. ***Characterization of dystrophin-glycoprotein complex subunits in human airway smooth muscle tissue****.* Western blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) were performed using protein and mRNA lysates obtained from isolated human bronchial smooth muscle tissue. **(A)** Western blot analysis showing abundance of smooth muscle myosin heavy chain (smMHC), β-dystroglycan (DG), β-sarcoglycan (SG), δ-SG, ε-SG and dystrophin. Blots shown include samples from 2 human donors, which appear in separate lanes for each protein assayed. Relative molecular mass of individual bands is shown. **(B)** RT-PCR showing expression of DGC subunits (α-DG, β-DG, α-SG, β-SG, δ-SG, ε-SG, γ-SG, ζ-SG, sarcospan, dystrophin, and utrophin), calponin, and caveolin-1. Gel shown is representative of an independent experiment using samples from 2 different human donors. Numbers on *right* indicate location and size of molecular mass markers.

Because reliable antibodies were not available for all DGC subunits, we complemented our immunoblot studies with RT-PCR to profile mRNA for DGC subunits in human ASM tissue. Consistent with our immunoblot analyses, RT-PCR confirmed the presence of transcripts for β-DG, β-SG, δ-SG, ε-SG and dystrophin, but also demonstrated the presence of mRNA for α-DG, α-SG, γ-SG, ζ-SG, sarcospan, and utrophin (Figure 1B). RT-PCR also confirmed the presence of mRNA for calponin, a contractile apparatus-associated smooth muscle marker, and caveolin-1

which we showed previously is abundant in contractile ASM cells [14], where it is thought to be associated with the DGC complex and involved in excitation-contraction coupling [8, 15].

Table 1. *List of primers for DGC subunits used in RT-PCR*

Gene Product	Accession No. (NCBI)	Primer Sequences	No. of Cycles	Product Size, bp
α-Dystroglycan*	NM004393	F: 5′-GTT CAA CAG CAA GAG CCA GCT CAT-3′ R: 5′-TGG TGC TAC AGT TTC GGT CTC CAA-3′	35	275
β-Dystroglycan*	NM004393	F: 5′-GCC TGA CTT TAA GGC CAC AAG CAT-3′ R: 5′-CAA TGA TGC CAG CAA TGA GCA GGA-3′	35	221
α-Sarcoglycan	NM000023	F: 5′-TCT ACT TGC CTG AAG ATG GTG GCA-3′ R: 5′-TGA CCA GAG CAT CCA CCA AGA AGT-3′	35	259
β-Sarcoglycan	NM000023	F: 5′-TGG TGC TGA CCA TCA CAG ATC CAT-3′ R: 5′-AAG CAC AGT AGG AGG AAG GCG AAA-3′	35	425
δ-Sarcoglycan	NM172244	F: 5′-ACA TTA CTG CCG GGA GTG TTG AGT-3′ R: 5′-GCA TCG TTT CCG CCA GCC ATA AAT-3′	35	143
γ-Sarcoglycan	NM000231	F: 5′-AAA ATT GAG GCG CTT TCT CA-3′ R: 5′-GGT ACA GCT TCC CAT CTG GA-3′	40	172
ε-Sarcoglycan	NM003919	F: 5′-ATG CAA ACA CCA GAC ATC CA-3′ R: 5′-TCT GAT GTG GCA AGT TCT GC-3′	35	220
ζ-Sarcoglycan	NM139167	F: 5′-ATC TCA GGC TTG AAT CAC CCA CCA-3′ R: 5′-ACA GGC AGA TGT TGC TAC TGG ACT-3′	40	318
Sarcospan	NM005086	F: 5′-CGC AGC TCA CAC AGT TTA CC-3′ R: 5′-GCC GCA GAC CAA ATT AAG AA-3′	35	176
Dystrophin	NM004010	F: 5′-TGT CAC TCG GCT TCT ACG AAA GCA-3′ R: 5′-ACC CTG GAC AGA CGT GGA AAG AAA-3′	35	481
Utrophin	NM007124	F: 5′-CAA ACA CCC TCG ACT TGG TT-3′ R: 5′-TGG TGG AGC TGC TAT CAG TG-3′	35	224
Caveolin-1	BC082246	F: 5′-GAG CTG AGC GAG AAG CAA GT-3′ R: 5′-ACA GCA AGC GGT AAA ACC AG-3′	35	208
Calponin	NM001299	F: 5′-TGT TTG AGA ACA CCA ACC ATA CAC A-3′ R: 5′-GTT TCC TTT CGT CTT CGC CAT-3′	35	77
GAPDH	NM014364	F: 5′-AGC AAT GCC TCC TGC ACC ACC AAC-3′ R: 5′-CCG GAG GGG CCA TCC ACA GTC T-3′	35	136

F, forward primer; R, reverse primer. *α-Dystroglycan (DG) and β-DG are derived by posttranslational cleavage of a peptide synthesized from the same mRNA transcript. Although the PCR primers we used target short, nonoverlapping sequences that correspond to the specific mRNA coding regions for each protein product, they do recognize the same full-length cDNA product (which carries coding sequence for both α-DG and β-DG).

To further characterize DGC expression in human ASM tissue, we performed immunohistochemistry of bronchi obtained from human donors (Figure 2). Consistent with our immunoblot and RT-PCR analyses, marked labeling of β-DG, β-SG and dystrophin was seen in the ASM tissue layer, which was clearly identifiable from smMHC staining. Collectively, our experiments demonstrate that DGC subunits are abundant in human ASM tissue.

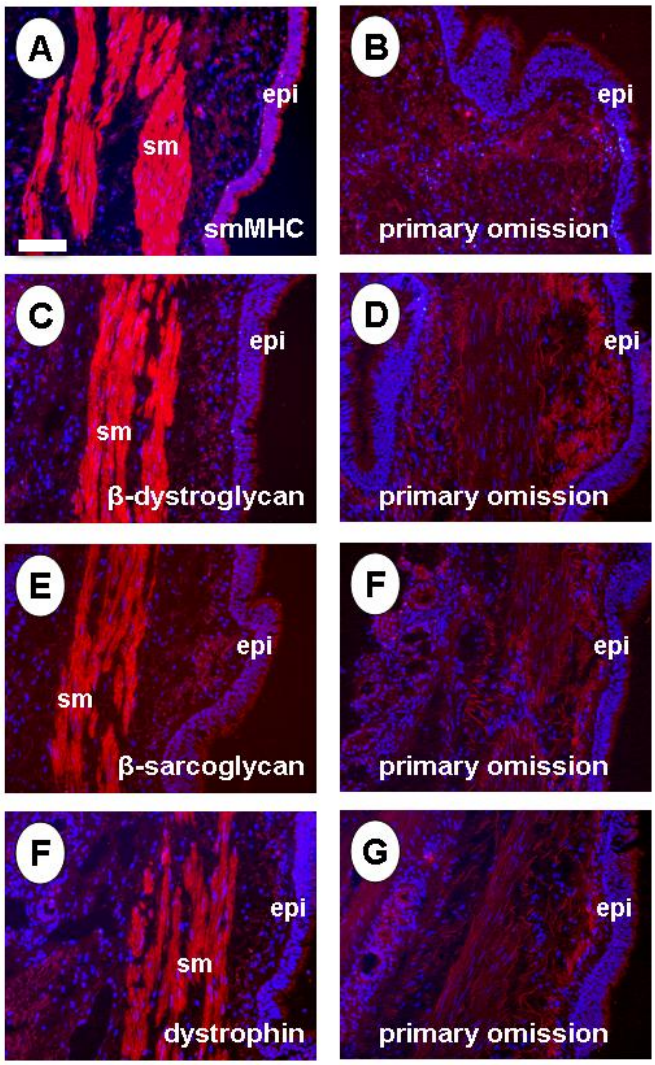


Figure 2. ***Expression of DGC subunits in human airway smooth muscle tissue.*** Segments of 2nd-to-4th generation main stem bronchi from healthy portions of human lung removed during lung resection surgery were cryosectioned serially into thin slices (5 microns) then subjected to immunostaining and fluorescence microscopy for **(A)** smMHC, **(C)** β-dystroglycan, (**E)** β-sarcoglycan, or **(G)** dystrophin. **B, D, F and H:** negative controls for adjacent panels in which primary antibodies were omitted and replaced with isotype-matched mouse immunoglobin. Nuclei were counterstained with Hoechst 33342 (10 μg/ml). The location of the airway smooth muscle layer (sm) and epithelium (epi) are indicated in primary antibody stained panels. Scale bar, 100μm.

Phenotype-dependent expression of the dystrophin-glycoprotein complex in cultured human ASM cells

Smooth muscle cells retain the capacity for reversible phenotype switching, which enables myocytes to exhibit contractile and synthetic/proliferative phenotypes *in vitro* and *in vivo*. Because our initial experiments exploring DGC expression were conducted in intact, contractile ASM tissues, we next used primary cultured and immortalized human ASM cells to investigate whether the expression of DGC subunits is phenotype dependent. Myocytes were

analyzed under conditions that promoted a proliferative phenotype (serum-fed, subconfluent cultures), or a contractile phenotype (4-day serum-free, confluent cultures) (Figure 3).

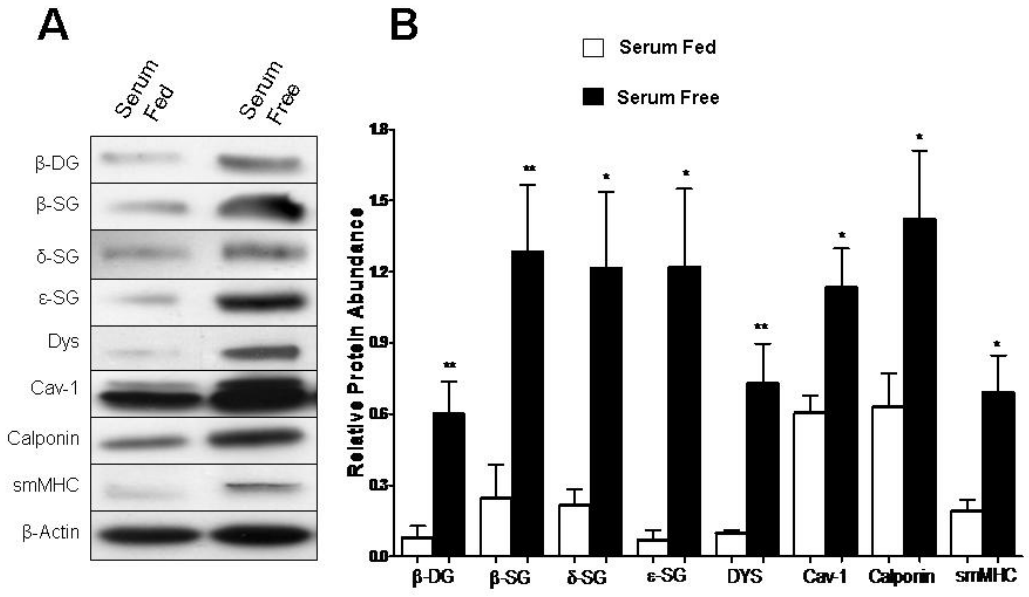


Figure 3. ***Characterization of phenotype-dependent expression of DGC subunits in human airway smooth muscle cells.*** **A:** representative Western blots from experiments carried out using four different hTERT human ASM cell lines. Matched lanes show results with lysates obtained from subconfluent, serum-fed cultures (serum fed, *day* 0) and confluent, contractile phenotype cultures subjected to 4-day serum deprivation (serum free, *day 4*). For all gels and subsequent densitometry β-actin was used to normalize for equal loading. **B:** results of densitometry analysis of proteins analyzed by Western blot in serum-fed and serum-free human ASM cell lines. Data are normalized for loading on each gel by comparing to β-actin, and are expressed as relative abundance. Data represent the means ± S.E. mean from at least 3 different human ASM cell lines. * $P<0.05$ and ** $P<0.001$ for serum fed, *day 0* vs. serum free, *day-4* (unpaired Student's *t*-test). DYS, dystrophin; Cav-1, caveolin-1.

Western blot analysis showed that DGC protein subunits, including β-DG, β-SG, δ-SG, ε-SG and dystrophin, were markedly increased after 4-day culture in serum-free conditions, which promotes phenotype maturation, as indicated by the concomitant accumulation of calponin, smMHC, and caveolin-1. Notably, densitometry confirmed that contractile phenotype marker proteins (calponin, caveolin-1, and smMHC) increased approximately two to three`

times in serum-deficient conditions, and we observed concomitant multi-fold accumulation of DGC subunits (Figure 3B). For example, we observed more than a 7-fold increase in β-DG ($p<0.01$) after 4-day serum deprivation compared to serum-fed proliferative phenotype cultures. The magnitude of this increase was mimicked by δ-SG ($p<0.05$), ε-SG ($p<0.05$) and dystrophin ($p<0.01$), and the level of β-SG was also increased by more than 5-fold ($p<0.05$) in cultures induced to a contractile phenotype. These results indicate that the abundance of DGC components is variable, and correlates with the dynamics of ASM cell phenotype expression *in vitro.*

Effects of maturation on human ASM cell morphology and phenotype

In canine and human ASM cells subjected to prolonged serum starvation, phenotype maturation occurs in a select subset of myocytes that become characteristically elongate, re-acquire responsiveness to contractile agonists, and accumulate abundant contractile marker proteins such as smMHC, calponin, and desmin [12, 16, 17]. Thus, we assessed whether accumulation of DGC subunits induced by serum deprivation was directly associated with the specific subset of human airway myocytes that undergo phenotype maturation. Using fluorescence immunocytochemistry, after 4-day serum deprivation we double-labeled primary cultured human tracheal smooth muscle cells with smMHC and β-DG, β-SG or dystrophin (Figure 4). Consistent with previous reports, myocytes exhibited phenotype heterogeneity, with 15-20% of ASM cells acquiring a contractile phenotype, as evidenced by a dramatic accumulation of smMHC. Notably, the maturation of individual human ASM cells to a contractile phenotype was uniquely associated with a dramatic increase in staining for DGC

subunits, including β-DG, β-SG and dystrophin, whereas little or no labeling for DGC proteins was evident in cells devoid of smMHC (Figure 4 D-F). In contrast to serum-deprived cultures, staining of very low intensity for smMHC, β-DG, β-SG and dystrophin was observed for all myocytes in serum-fed, subconfluent conditions (not shown). These results demonstrate, at the single-cell level, an association between the acquisition of a contractile phenotype and expression of DGC subunits.

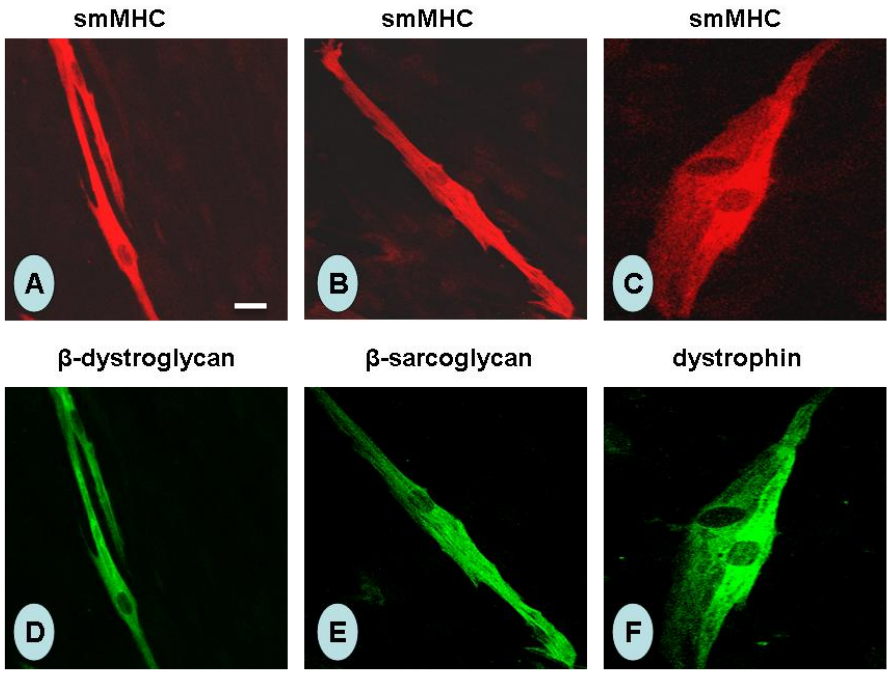
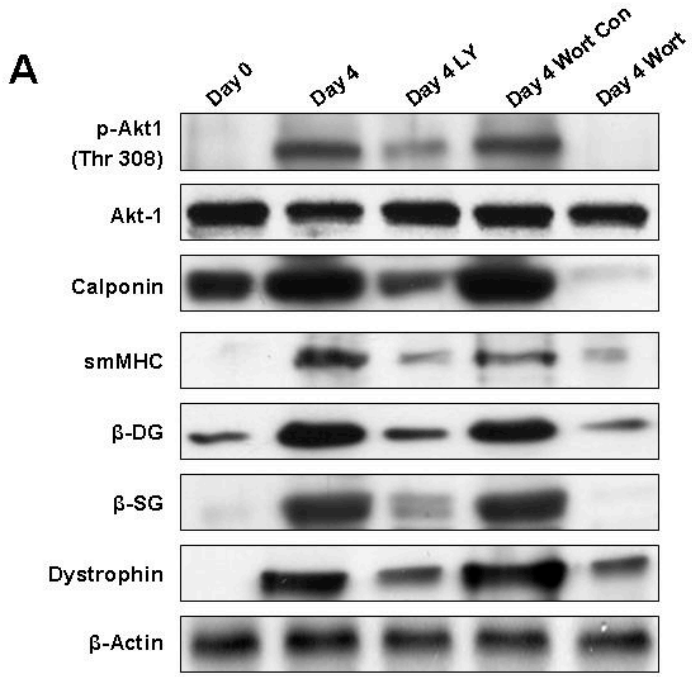


Figure 4. ***Coexpression of the DGC in human airway smooth muscle cells that acquire a contractile phenotype.*** Primary cultured human tracheal smooth muscle cells were grown to 90 % confluence on glass coverslips and subjected to 4-day culture in serum deficient conditions. Thereafter myocytes were fixed, and double labeled for (A-C) smMHC, and (D) β-dystroglycan, (E) β-sarcoglycan, or (F) dystrophin. Isotype-matched mouse IgG or rabbit antiserum was used for negative controls (not shown). Secondary antibodies conjugated with TxR or FITC were used to label smMHC (red) and DGC subunits (green) respectively. Images were obtained by confocal laser scanning microscopy. As a reference marker for matched fields (*A* and *D*, *B* and *E*, and *C* and *F*), arrows indicate the location of nuclei in individual cells. Images are representative of at least 3 different primary human tracheal myocyte cultures. Scale bar, 20 μm.

Effect of PI3K inhibitors on human ASM cell maturation and DGC expression

We next investigated whether accumulation of DGC proteins is merely coincident with ASM phenotype maturation in response to serum deprivation, or represents an integral component of the maturation process, making it a reliable marker for active acquisition of the contractile phenotype. Previous studies have demonstrated that signaling through the phosphatidylinositide-3-kinase (PI3K) pathway, including Akt1, p70S6 kinase, and mTOR, is required for ASM maturation, hypertrophy, and concomitant accumulation of contractile protein markers [12, 18].



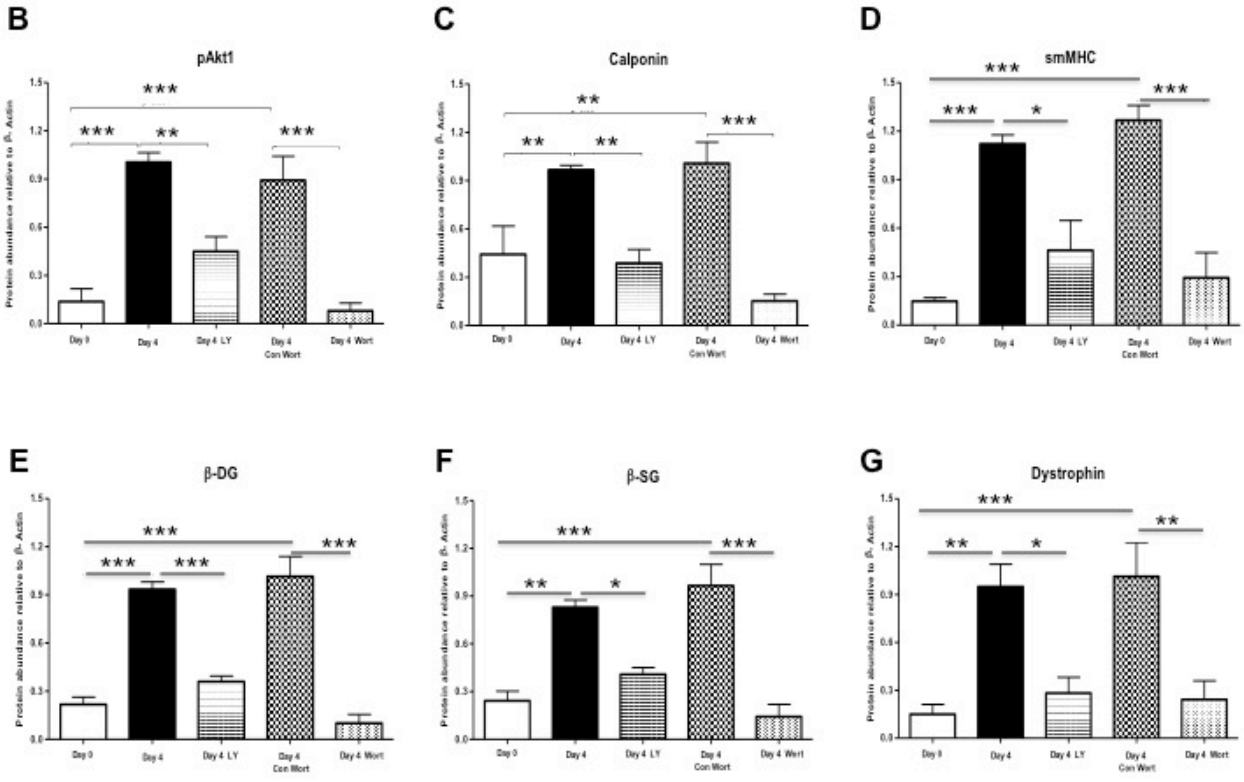


Figure 5. ***Inhibition of PI3K signaling prevents phenotype maturation and accumulation of DGC subunits in the human airway smooth muscle cells.*** For all panels, *day 0* represents protein lysates obtained from serum-fed, 90% confluent cultures, and *day 4* represents protein lysates obtained from confluent human ASM cell cultures after 4-day serum deprivation, with medium changed every 48 h. Beginning at day 0, some cultures were treated with LY-294002 (20 μM), and culture medium containing inhibitor was changed every 48 h thereafter (day 4 LY). Other cultures were treated with wortmannin (100 nM) beginning at day 0, but culture medium containing fresh inhibitor was replaced thrice daily for 4 days (day 4 Wort). Control cultures for wortmannin treatment had medium (without inhibitor) replaced 3 times daily for 4 days (day 4 Wort Con). **A:** representative Western blots typical of those obtained for 4 different human ASM cell lines. B: densitometry analysis of the effects of serum deprivation and PI3K inhibition on phospho-Thr308 Akt1. Data are normalized for β-actin loading and for total Akt1 (shown in **A**). Results of densitometric analyses showing effects of serum deprivation and PI3K inhibitors on relative protein abundance of calponin **(C)**, smMHC **(D)**, β-DG **(E)**, β-SG **(F)**, and dystrophin **(G)** are shown. For all histograms protein abundance was corrected for equal loading and normalized relative to β-actin abundance. Data shown represent means ± SE from experiments using 4 different hTERT ASM cell lines. Statistical comparisons shown were performed by 1-way ANOVA with Bonferroni's multiple comparison test for comparison between treatments, * $P<0.05$, ** $P<0.001$ and, *** $P<0.001$.

Thus, we performed experiments in which we blocked maturation of human hTERT ASM cells by treating serum-deprived cultures with pharmacological inhibitors of PI3K: LY294002 and wortmannin. To confirm that the addition of PI3K inhibitors to the culture media blocked downstream signaling we first assayed phosphorylation of Thr308 on Akt1 before (*day 0*) and after (*day-4*) serum deprivation (Fig. 5A). Consistent with previous studies, 4 days of serum deprivation in ITS-containing media resulted in elevated PI3K signaling, as revealed by a seven-fold increase in phospho-Thr308 Akt-1 ($p<0.001$). The increase in Akt1 phosphorylation was reduced by nearly 60% in cultures treated with 20μM LY-294002 ($p<0.01$), and was virtually abolished by treatment with 100nM wortmannin ($p<0.001$) (Fig. 5B). Of note, and consistent with previous studies, accumulation of the contractile phenotype markers smMHC and calponin was prevented by PI3K inhibition with LY-294002 ($p<0.05$) or wortmannin ($p<0.001$) (Fig. 5, C and D). Having confirmed that PI3K signaling is essential for hTERT ASM cell maturation *in vitro*, we next assessed the effects of PI3K inhibition on the accumulation of DGC subunits during serum-free culture. Importantly, whereas β-DG was increased by more than 4 fold ($p<0.001$) after 4-day serum deprivation, this was virtually abolished by LY-294002 ($p<0.001$) or wortmannin ($p<0.001$) (Fig. 5E). Similarly, the accumulation of β-SG and dystrophin that we saw associated with phenotype maturation of human ASM cell lines (Fig. 3) was also suppressed significantly by inhibition of PI3K (Fig. 5, F and G). Together, these results demonstrate that inhibition of PI3K signaling, which is essential for contractile phenotype expression by human ASM cells, is also required for the accumulation of DGC proteins.

Laminin competing peptide inhibits human ASM cell maturation and DGC expression

We recently showed [13, 17], using the laminin competing peptide YIGSR, that binding of endogenously expressed laminin to α_7-integrin subunits is required for maturation of primary cultured and human ASM cell lines. For these studies we used peptides based on the integrin-binding motif of laminin β1-chains to effectively inhibit phenotypic maturation of ASM cells *in vitro* [13]. In the present study we determined whether blocking laminin-mediated myocyte maturation with YIGSR also affected DGC protein accumulation during 4 days of serum-free culture. Consistent with our previous findings, treatment of hTERT ASM cells with YIGSR (1μM) prevented the accumulation of contractile phenotype marker proteins calponin ($p<0.01$), and smMHC ($p<0.001$) (Fig. 6, A–C). Similarly, YIGSR treatment prevented the accumulation of β-DG, β-SG and dystrophin (Fig. 6,D-F).

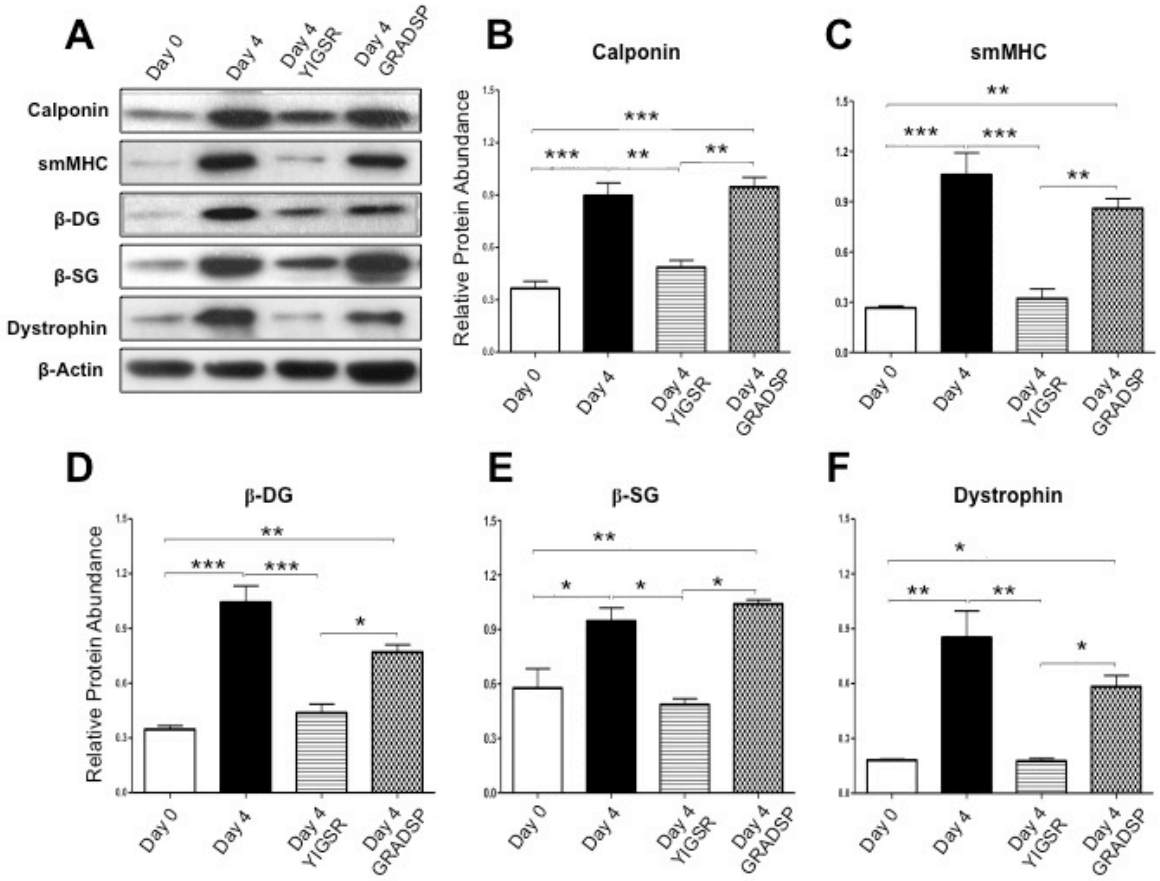


Figure 6. ***Inhibition of human ASM cell maturation and the accumulation of DGC subunits with the laminin competing peptide YIGSR.*** Human ASM cell lines from confluent cultures were replated at 100% confluence in DMEM containing 0.5% serum. Medium was changed to serum-free DMEM 16 h later (*day 0*) and was changed every 48 h thereafter. After 4 days in serum-free culture (*day 4*) protein lysates were prepared. In some cultures YIGSR (1 μM) or GRADSP (1 μM) was added at the *day 0* time point (fresh peptide was included when medium was changed thereafter) and protein lysates were prepared 4 days later (*day 4* YIGSR and *day 4* GRADSP, respectively). **A:** representative Western blots typical of those obtained for 4 different human ASM cell lines. Results of densitometric analyses showing effects of serum deprivation in the presence and absence of YIGSR or GRADSP on relative protein abundance of calponin **(B)**, smMHC **(C)**, β-DG **(D)**, β-SG **(E)**, and dystrophin **(F)** are also shown. For all histograms protein abundance was corrected for equal loading and normalized relative to β-actin abundance. Data shown represent means ± SE from experiments using 4 different hTERT ASM cell lines. The statistical comparisons shown were performed by 1-way ANOVA with Bonferroni's multiple comparison test for comparison between treatments, * $P<0.05$, ** $P<0.001$ and, *** $P<0.001$.

As a negative control some cultures were treated with the inactive peptide GRADSP, which was without effect on induction of smMHC, calponin, β-DG, β-SG and dystrophin (Fig. 6). These results demonstrate that the accumulation of DGC subunits in human ASM cells is directly associated with phenotype maturation. Moreover, expression of DGC proteins appears to be regulated by mechanisms that are essential determinants of the contractile phenotype.

Discussion

This study was undertaken to profile the proteins that comprise the DGC in human ASM cells, and to determine whether their expression correlates with phenotype switching. Our focus on the DGC stems from reports showing that expression of its subunits is developmentally regulated and required for skeletal muscle maturation and maintenance [19], and that animal models lacking sarcogylcan subunits exhibit altered arterial function that may be associated with changes in Ca^{2+} homeostasis [20, 21]. Our study is the first to provide systematic profiling of the DGC subunits expressed in human ASM tissue and cultured cells and to show the composition of the DGC is consistent with reports for other smooth muscles, because it includes α- and β-dystroglycan, dystrophin, sarcospan, and a sarcoglycan complex that includes β-, δ-, ε-, γ-, ζ-, and perhaps, α-SG. Our studies using cultured human ASM reveal that the expression of DGC protein subunits is dynamic, being lost or markedly reduced upon modulation to a proliferative phenotype, while they accumulate when individual cells re-acquire a contractile phenotype. The binding of laminin-2 to α_7-integrin is required for ASM phenotype maturation [13, 17]; our study revealed that blocking laminin-integrin binding prevented both phenotype maturation and the accumulation of DGC proteins, confirming that they are reliable markers for acquisition of the contractile phenotype in ASM cells. We also show for the first time that PI3K activity, which is critical for ASM maturation [12], is required for DGC protein accumulation. Collectively our results suggest that DGC protein expression is dynamically regulated by mechanisms that control ASM maturation. Moreover, we show that the DGC is abundant in contractile ASM, which suggests it could be associated with functional aspects of contraction in a manner similar to that seen for skeletal muscle.

DGC protein expression has been studied in vascular and visceral smooth muscle tissues, where the complex appears to include dystrophin, α- and β-DG, sarcospan, and sarcoglycans, including β-, δ-, and ε-SG [3-6]. There has been some debate about whether γ- or ζ-SG is the final member of the tetrameric sarcoglycan complex, however a recent report indicates that they are functionally indistinguishable, indicating that the distinction may be moot [22]. Our studies suggest a DGC composition for human ASM that is similar to other smooth muscles, with a core consisting of α- and β-DG, and β-, δ- and ε-SG. We demonstrate that mRNA for both γ- and ζ-SG is present in ASM, as well as for α-SG, a subunit that has been generally accepted to be skeletal muscle-specific [4]. However, our detection of α-SG is consistent with some recent immunohistochemical surveys of normal smooth muscle tissues, including ASM, that do suggest the presence of low levels of α-SG protein [3, 23]. We also observed mRNA for sarcospan, which is thought to stabilize sarcoglycan tetramers by forming a sarcospan-sarcoglycan complex within the DGC. Our RT-PCR analyses also indicate that utrophin, the autosomal homologue of dystrophin thought to support cellular architecture rather than a stress-bearing role like dystrophin, is expressed abundantly in ASM tissue. This is consistent with other reports indicating both utrophin and dystrophin are expressed in vascular smooth muscle [24]. A number of other DGC-associated proteins, such as the dystrobrevins, syntrophins, and actinin-associated LIM protein, are linked with intracellular signalling in skeletal muscle [2, 25, 26]; our studies did not evaluate the expression of these ancillary subunits in ASM, although future studies may be warranted. Collectively our study provides the first systematic assessment of DGC protein expression in human ASM, and supports a model in which a α- and β-DG may be

linked to the actin cytoskeleton by dystrophin or utrophin, and are associated with a sarcospan-stabilized, tetrameric sarcoglycan sub-complex that may exist in multiple heteromeric forms.

There are numerous reports linking the expression of the DGC with skeletal muscle differentiation, and myotube survival [27-29]. Nonetheless, before our present study, the association of the DGC with human ASM phenotype expression has not been investigated. We show here, and have described previously, that cell culture provides a useful tool for assessing mechanisms regulating phenotype plasticity of ASM cells, because they spontaneously modulate to a proliferative state in subconfluent, serum-fed conditions but can be induced to a mature, fully contractile phenotype by prolonged culture in insulin-supplemented serum-deficient conditions [12, 16, 30]. Importantly, on the basis of our Western blot analyses of serum-deprived human ASM cells, it appears that contractile ASM cells express a repertoire of DGC proteins that mirrors ASM tissue. Using fluorescent immunocytochemistry we saw that β-DG, β-SG and dystrophin accumulated exclusively in myocytes from serum-deprived cultures that acquired an elongate morphology marked by abundant smMHC. These observations provide first time, compelling evidence on a cell-by-cell basis for a direct association between the expression of DGC proteins and the acquisition of a contractile phenotype by human ASM cells.

We showed previously [13, 17] that α_7-integrin, which like α-DG is a receptor for extracellular laminin, is preferentially expressed by contractile phenotype ASM cells. This parallels studies in skeletal muscle showing that expression of both α_7-integrin and DGC proteins correlates with myogenesis [27]. The association of laminin-binding receptors with

ASM maturation is fully consistent with previous work showing endogenously expressed extracellular laminin promotes both the differentiation of ASM in the developing lung and the expression of a functional contractile phenotype by mature myocytes [13, 31]. In the present study we confirmed that blockade of ASM phenotype maturation with a laminin competing peptide, YIGSR, was sufficient to prohibit DGC protein accumulation. The YIGSR peptide corresponds to amino acids 929–933 of the β1 laminin chain [32], and it has been used frequently as an inhibitor of laminin binding *in vitro* and *in vivo* [13, 33-35]. Laminin is a trimer of α, β and γ polypeptide chains that possess a number of binding sites for integrins [36-38] and non-integrin receptor subtypes, including α-DG [1, 39, 40]. Because YIGSR mimics a motif in laminin β1 that selectively binds to integrins, our studies do not address the extent to which laminin binding to α-DG might directly regulate phenotype maturation of ASM cells. Interestingly, cross talk and linkages between integrins and the DGC have been reported and these appear to modulate downstream signaling [41]. There is also an emerging body of evidence that the DGC, in particular the cytoplasmic tail of β-DG, acts as a scaffold that contributes to signal transduction [2, 42]. This suggests that α-DG and the DGC could be a functional determinant in ASM phenotype switching. Furthermore, our findings suggest that the DGC could be functionally important during lung development and in other scenarios involving ASM phenotype maturation and growth, such as in asthma, which is marked by the accumulation of contractile phenotype ASM. Future studies that investigate these possibilities will be enlightening.

We [29] and others [37] have reported that PI3K–mediated signal transduction is critical for contractile phenotype maturation, and for myocyte elongation and hypertrophy of ASM cells. Similarly, in skeletal muscle, myotube hypertrophy and the accumulation of contractile proteins requires PI3K/Akt1/mTOR and PI3K/Akt1/GSK3 signal transduction pathways [43, 44]. Notably, PI3K signaling has been linked with laminin binding to the DGC and integrin subunits in skeletal and smooth muscle, respectively [45, 46]. Because our experiments with YIGSR peptide showed that laminin is required for myocyte maturation and DGC expression, we used two different chemical inhibitors of PI3K activity, LY-294002 and wortmannin, to test whether PI3K activity also modulates DGC expression. We observed that PI3K inhibition concomitantly prevented ASM contractile phenotype maturation and the accumulation of the DGC proteins dystrophin, β-DG and β-SG. To the best of our knowledge, these observations are the first to show that PI3K signaling regulates expression of DGC proteins. PI3K signaling cascades are complex and involve multiple downstream effectors that regulate proliferation, protein synthesis, cell survival, differentiation, and gene transcription [12, 44, 45]. Though in the context of our current studies it is not possible to discern whether PI3K mediates its effects directly or indirectly on DGC expression through transcription or protein translation-associated mechanisms, our studies do indicate that careful dissection of these mechanisms is warranted.

Because our data show that DGC proteins are abundant in intact ASM tissue and individual cultured myocytes of a contractile phenotype, a functional and/or structural role in differentiated myocytes is implicated. In patients with muscular dystrophy, defects in gastrointestinal smooth muscle function have been postulated to underpin frequent dysphasia,

vomiting, chronic constipation, and acute digestive dilatations [47, 48]. In dystrophic hamsters, DGC deficiency correlates with more pronounced loss of ASM mass and contractile responses with aging [7], an effect that could contribute to suppressed airway responsiveness *in vivo*. Although the functional role of the DGC in contractile smooth muscle cells has not been established, some studies provide clues in this area. North et al [10] reported that dystrophin is highly organized in contractile smooth muscle cells, segregating into longitudinal linear arrays in association with caveolae-rich plasma membrane domains. Interestingly, this more or less mimics skeletal muscle, in which the DGC is sequestered to costamers and provides a mechanical link between the ECM and the Z-disk [2]. In skeletal muscle the DGC appears to act as a molecular shock absorber during contraction and relaxation [1]. To date no studies have directly assessed the role of the DGC in mechanical load bearing in smooth muscle cells, though a recent report from Dye and colleagues [49] reveals that carotid arteries from *mdx* and δ-SG knock out mice exhibit decreased pressure-induced distensibitity with increased circumferential and axial stress. Most reports have suggested that disruption of the DGC in smooth muscle may be linked to changes in Ca^{2+} homeostasis. The absence of dystrophin in portal veins from *mdx* mice leads to reduced intercellular myocyte communication associated with stretch-induced myogenic contractile responses [50]. Notably, ectopic smooth muscle-specific expression of dystrophin can improve aberrant vasoregulation in *mdx* mice [51]. Morel et al [52] reported that decreased mechanical activity of duodenal smooth muscle in *mdx* mice is due to reduced type 2 ryanodine receptor expression that compromises sarcoplasmic reticulum calcium release. Interestingly, Cohn et al [20] showed that cardiac myopathy associated with coronary artery vasospasm in sarcoglycan knock out mouse models could be prevented by

verapamil, a vasodilatory Ca^{2+} channel blocker. The precise mechanism linking the DGC with Ca^{2+} handling in smooth muscle cells is not clear, however its association with caveolae [10] provides an interesting lead. In smooth muscle, including ASM, caveolae are sites where ion channels and Ca^{2+} binding proteins are sequestered, and caveolae are thought to be spatially aligned with the sarcoplasmic reticulum to facilitate receptor-mediated Ca^{2+} release [8, 9]. In fact, we recently confirmed that caveolae facilitate G-protein-coupled receptor mediated contraction and Ca^{2+} release in ASM muscle [53]. Future studies are needed to better ascertain the precise role of DGC in excitation-contraction coupling and mechanical force transmission in contractile smooth muscle cells and tissue.

In summary, our study characterized DGC subunit expression in human ASM tissue and demonstrates that the expression of DGC proteins is associated with, and dependent upon, phenotype switching between contractile and proliferative states in human ASM cells. Notably, we also demonstrate that DGC expression is subject to regulation by mechanisms involving laminin-integrin binding and the induction of PI3K signaling that are essential for ASM cell maturation. Our studies provide an important new platform for future studies investigating the direct role of the DGC, in particular laminin-2 binding to α-dystroglycan, in generating intracellular signaling cascades that support myocyte maturation and/or maintenance of a contractile phenotype. In addition, our studies provide compelling evidence to support future studies investigating the functional role of the DGC in contractile ASM, in particular in relation to mechanical load bearing and Ca^{2+} homeostasis.

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PS drafted and produced 100% of the experiments required for the realization of this manuscript

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Chapter 5

β-Dystroglycan Binds Caveolin-1 in Smooth Muscle: Functional Role in Caveolae Distribution and Ca^{2+} Release

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Abstract

The dystrophin-glycoprotein complex (DGC) links the extracellular matrix and actin cytoskeleton. Caveolae form membrane arrays on smooth muscle cells; we investigated the mechanism for this organization. Caveolin-1 and β-dystroglycan, the core transmembrane DGC subunit, co-localize in airway smooth muscle. Immunoprecipitation revealed the association of caveolin-1 with β-dystroglycan. Disruption of actin filaments disordered caveolae arrays, reduced association of β-dystroglycan and caveolin-1 to lipid rafts, and suppressed sensitivity and responsiveness of methacholine-induced intracellular Ca^{2+} release. We generated novel human airway smooth muscle cell lines expressing shRNA to stably silence β-dystroglycan expression. In these myocytes, caveolae arrays were disorganized, caveolae structural proteins caveolin-1 and PTRF/cavin were displaced, the signaling proteins PLCβ1 and $G_{\alpha q}$, which are required for receptor-mediated Ca^{2+} release, were absent from caveolae, and the sensitivity and responsiveness of methacholine-induced intracellular Ca^{2+} release, was diminished. These data reveal an interaction between caveolin-1 and β-dystroglycan and demonstrate that this association, in concert with anchoring to the actin cytoskeleton, underpins the spatial organization and functional role of caveolae in receptor-mediated Ca^{2+} release, which is an essential initiator step in smooth muscle contraction.

Introduction

Smooth muscle in hollow organs is subject to cellular deformation and mechanical stress during contraction and changes in transmural pressure. Mechanisms have evolved to

maintain contractile apparatus integrity and its association with the membrane and extracellular matrix so that force can be transmitted between cells, and to prevent plasma membrane damage [1, 2]. The dystrophin-glycoprotein complex (DGC) provides a strong mechanical link between the intracellular actin cytoskeleton and the extracellular matrix.

Mechanisms for the ordered distribution of caveolae in mature smooth muscle cells are not elucidated. In striated muscle, β-dystroglycan binds a putative WW-domain in caveolin-3 [5] and supports localization of T-tubules to costamers; however, there are no reports that confirm β-dystroglycan binds to endogenous caveolin-1 in smooth muscle. In smooth muscle the expression of DGC subunits is associated with myocyte maturation, suggesting a specific role in functionally contractile myocytes [6-10]. In these cells, dystrophin is localized to caveolae-rich arrays that are associated with Ca^{2+}-handling proteins and organelles [11-14]. There is evidence that the cytoplasmic tail of β-dystroglycan can serve as a scaffold for signaling proteins such as Grb2, nNOS and regulatory kinases [15-17], but no direct link to excitation-contraction coupling and Ca^{2+} handling in smooth muscle has been made.

In the current study, using human and canine airway smooth muscle cells and tissue, we tested the hypothesis that spatial distribution of caveolae in contractile smooth muscle is determined by the association of caveolin-1 with β-dystroglycan and this interaction supports a functional role for caveolae in facilitating G-protein coupled receptor (GPCR)-mediated Ca^{2+} mobilization. Our data demonstrate, for the first time, the direct interaction

between caveolin-1 and β-dystroglycan. In addition, we found that distribution of caveolae is determined by tethering to the actin cytoskeleton via caveolin-1 and the DGC, and the ordered distribution of caveolae in contractile smooth muscle cells is linked with the sequestration and function of proximal signaling proteins that mediate intracellular Ca^{2+} release.

Results

β-Dystroglycan co-fractionates and co-precipitates with caveolin-1.

We reported that DGC subunits are abundant in human airway smooth muscle tissue and cells [7]. Moreover, fluorescent microscopy has shown dystrophin and caveolin-1 exhibit overlapping immunolabeling in guinea pig taenia coli smooth muscle [12]. To determine whether DGC subunits and caveolae share cellular domains we used sucrose density gradient ultracentrifugation for sub-cellular fractionation of cells from human and canine airway smooth muscle tissue, and serum-deprived cultures of canine airway myocytes. DGC subunits (β-dystroglycan, β-sarcoglycan, γ-sarcoglycan and dystrophin) co-fractionate with high buoyant density caveolin-1-enriched microdomains (Fig. 1A,B).

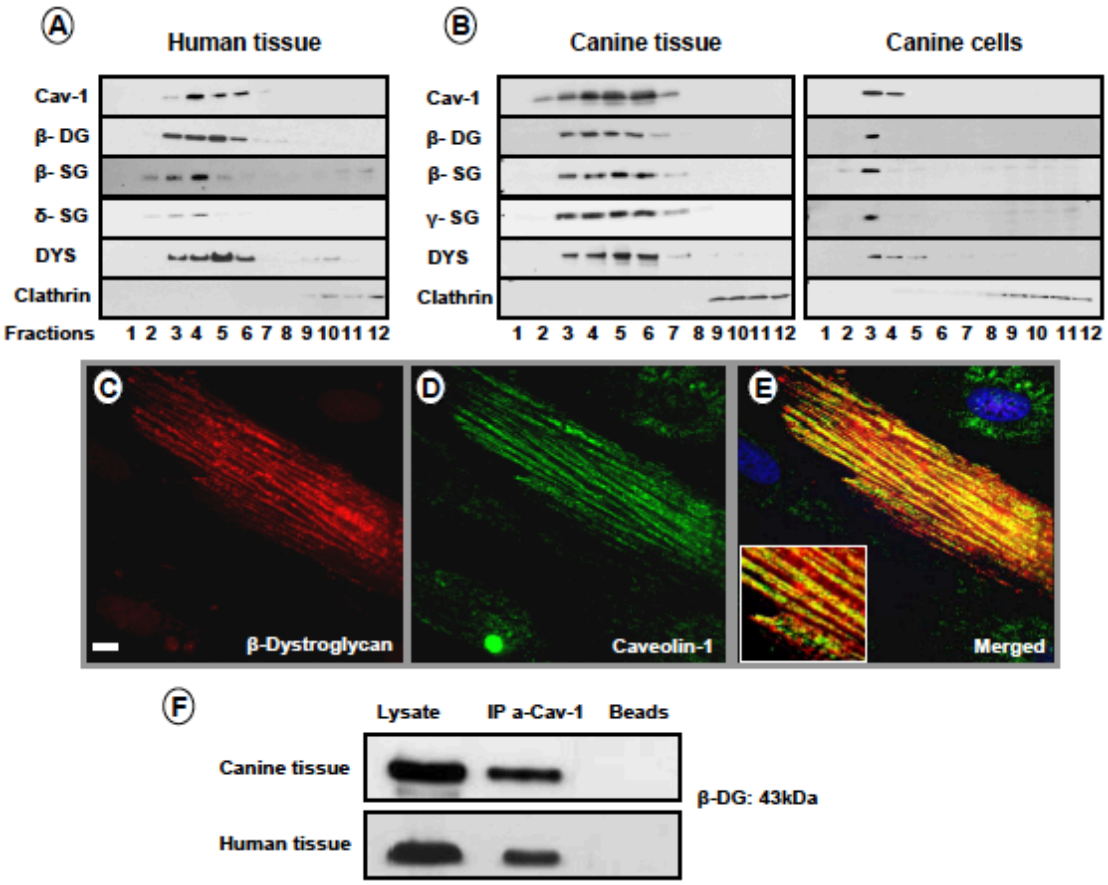


Figure 1. ***Association of dystrophin-glycoprotein complex subunits with caveolin-1.*** Human bronchial smooth muscle tissue **(A)**, canine airway smooth muscle tissue and serum-deprived canine airway smooth muscle cells **(B)** were lysed in carbonate buffer for isolation of caveolae rich fractions using sucrose density gradient centrifugation. After centrifugation, 1-ml fractions were collected (buoyant density *decreases* with increasing fraction numbers). Equal amounts of protein from each fraction were subjected to immunoblot analysis for the indicated proteins. (**C,E)** Serum-deprived (contractile) canine airway smooth muscle cells were fixed and double labeled for β-dystroglycan (red)(C) and caveolin-1 (green) (D). The corresponding merged image (E) with a higher magnification insert is also shown. Nuclei appear blue and are counterstained with Hoechst 33342 (10 μg/mL). Scale bar = 20 μm. **(F)** Lysates from canine and human airway smooth muscle tissue were used for immunoprecipitation (IP) with anti-caveolin-1 antibody (a-Cav-1) using protein G conjugated sepharose beads. Lane labeled "Beads" included sample but no antibody. Beads with immunoprecipitated proteins were used to perform protein blot analysis for β-dystroglycan.

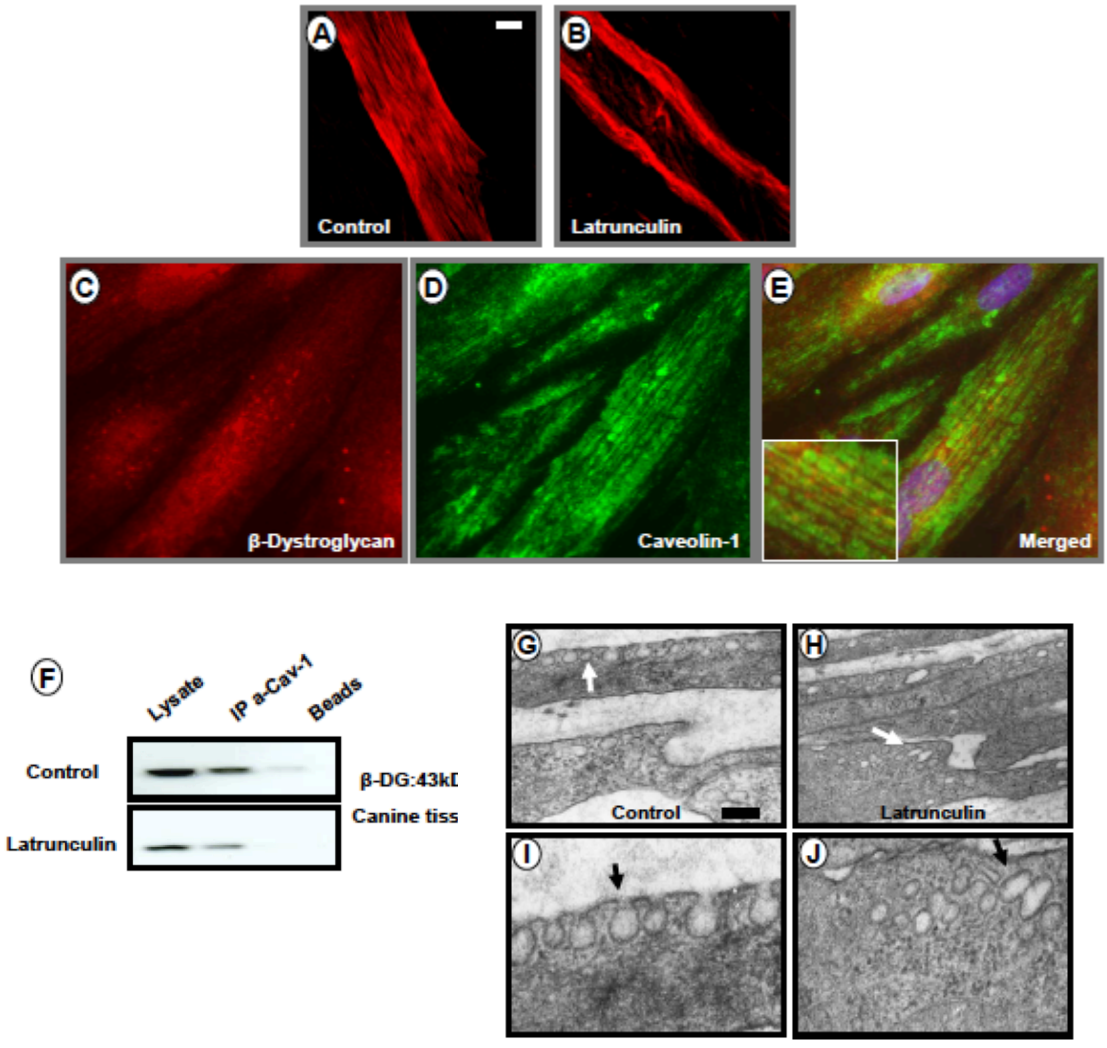
We next used double labeling of β-dystroglycan and caveolin-1 to assess their co-localization in individual elongate contractile smooth muscle cells in culture (Fig. 1C-E). The

proteins were organized into markedly overlapping longitudinal arrays. However, in striking contrast, cells that lacked β-dystroglycan (a feature of a short spindle shaped non-contractile myocyte subpopulation) did not form discrete linear arrays of caveolin-1 (Fig. 1E). To determine whether β-dystroglycan and caveolin-1 proteins interacted directly we performed immunoprecipitation, and found they could be readily co-precipitated from human and canine smooth muscle (Fig. 1F). Collectively, these data demonstrate caveolin-1 interacts with β-dystroglycan, and that this interaction may be critical to orchestrate the ordered distribution of caveolae in contractile smooth muscle.

The actin cytoskeleton underpins the ordered distribution of caveolae

As the association of caveolin-1 with β-dystroglycan suggests caveolae may be indirectly tethered to the intracellular actin cytoskeleton via dystrophin, we next assessed whether promoting loss of filamentous actin with latrunculin A affected co-localization and distribution of caveolin-1 and β-dystroglycan. Phalloidin labeling confirmed that filamentous actin was depleted by exposure to latrunculin A (Fig. 2A,B). Moreover, this was associated with the disruption of discrete linear arrays of β-dystroglycan and caveolin-1, resulting in far less dramatic visible co-localization of these proteins (compare Fig. 1C-E with Fig. 2C-E). Notably, co-immunoprecipitation from canine smooth muscle revealed that the association of β-dystroglycan with caveolin-1 was not directly affected by latrunculin A, suggesting that the actin cytoskeleton underpins the sequestration of these proteins to specific membrane microdomains, but is not involved in their binding *per se* (Fig. 2F). To characterize the ultrastructural consequences associated with disruption of the actin cytoskeleton, we next

used transmission electron microscopy. Individual myocytes in intact smooth muscle tissue exhibited characteristic arrays of membrane caveolae, whereas in latrunculin A-exposed specimens we observed greatly reduced numbers of caveolae invaginations, with the appearance of significant numbers of double membrane caveolae-like vesicles beneath the plasma membrane, suggesting that the loss of actin integrity promotes caveolae internalization (Fig. 2G-J).



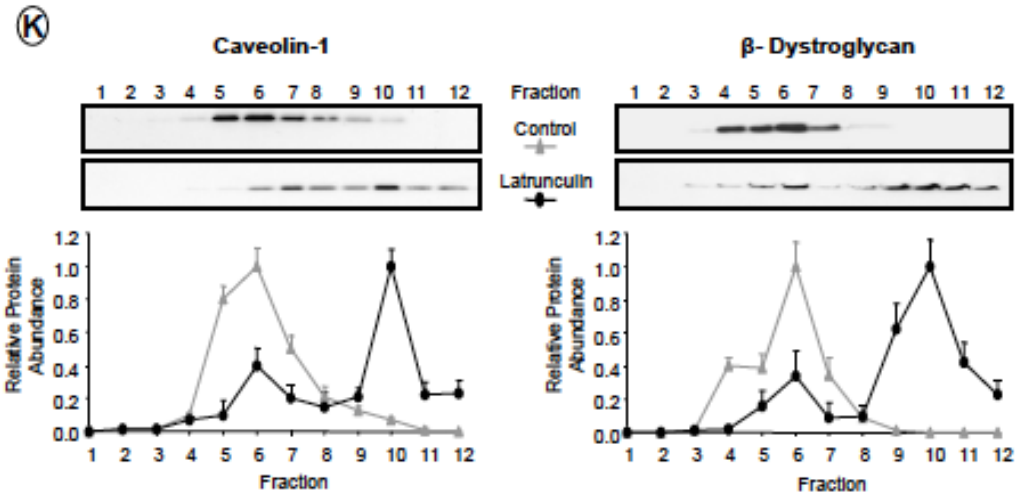


Figure 2. ***Effect of actin disruption on localization and distribution of caveolin-1 with β-dystroglycan.*** **(A,B)** Serum-deprived (contractile) canine airway smooth muscle cells were either incubated in HBSS or latrunculin A (1 µM, 1 h, 37° C), then labeled for filamentous actin using phalloidin-TxR (red). **(C-E)** Cells incubated with latrunculin A were fixed and double labeled for β-dystroglycan (red) and caveolin-1 (green). Nuclei were counterstained blue with Hoechst 33342 (10 µg/mL). The corresponding merged image with a higher magnification insert is shown in E. Scale bar: 20 µm. **(F)** Lysates from canine airway smooth muscle tissue (Control) and those pre-treated with latrunculin A were immunoprecipitated with anti-Cav-1 antibody using protein G conjugated sepharose beads. Lane labeled "Beads" included sample but no antibody. Immunoprecipitated proteins were subjected to protein blot analysis for β-dystroglycan. **(G-J)** Canine airway smooth muscle tissue was incubated in either HBSS (G,I) or latrunculin A (1 µM, 1 h, 37°C) (H,J), then fixed for transmission electron microscopy. Arrows in panels G and I indicate typical caveolae and linear arrays; whereas in panels H and J, arrows indicate internalized caveolae-like double membrane structures that appear with latrunculin A treatment. Scale bar: 100 nm. **(K)** Control and latrunculin A-treated canine airway smooth muscle tissue was homogenized and lysed in carbonate buffer for isolation of caveolae enriched by sucrose density gradient centrifugation. Equal amounts of protein from each fraction were subjected to protein blot analysis for caveolin-1 or β-dystroglycan. Results shown include three independent experiments performed on canine smooth muscle tissue.

This was confirmed using sucrose density cell fractionation, which revealed the abundance of both caveolin-1 and β-dystroglycan in cell fractions that typically harbor caveolae-enriched microdomains was dramatically reduced after actin disruption (Fig. 2K). Collectively these results support a role for the actin cytoskeleton, through its tethering to a

DGC-caveolin-1 complex, in establishing and maintaining discrete caveolae microdomains in smooth muscle cells.

Discrete organization of caveolae is required for receptor-mediated Ca^{2+} release

Through mechanisms involving the association of proximal signaling proteins with caveolin-1, caveolae modulate receptor-mediated Ca^{2+} release in the smooth muscle and other cell systems [11, 18-23]. We assessed whether this functional role is linked to the unique ordered distribution of caveolae in contractile smooth muscle cells. Primary cultured canine airway myocytes were loaded with the Ca^{2+}-sensitive dye, Fura-2, and we measured the effects of actin cytoskeleton disruption with latrunculin A on $G_{\alpha q}$-coupled muscarinic M3-receptor mediated Ca^{2+} release, which is the initiating step for contraction. Airway myocytes exhibited a dose-dependent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to the M3 receptor agonist methacholine (MCh), a profile that is reproducible in the same cells 1 hr later (Fig. 3 A,B). By contrast, peak response elicited by all concentrations of MCh was abrogated or dramatically reduced after incubation with latrunculin A (Fig. 3A,B).

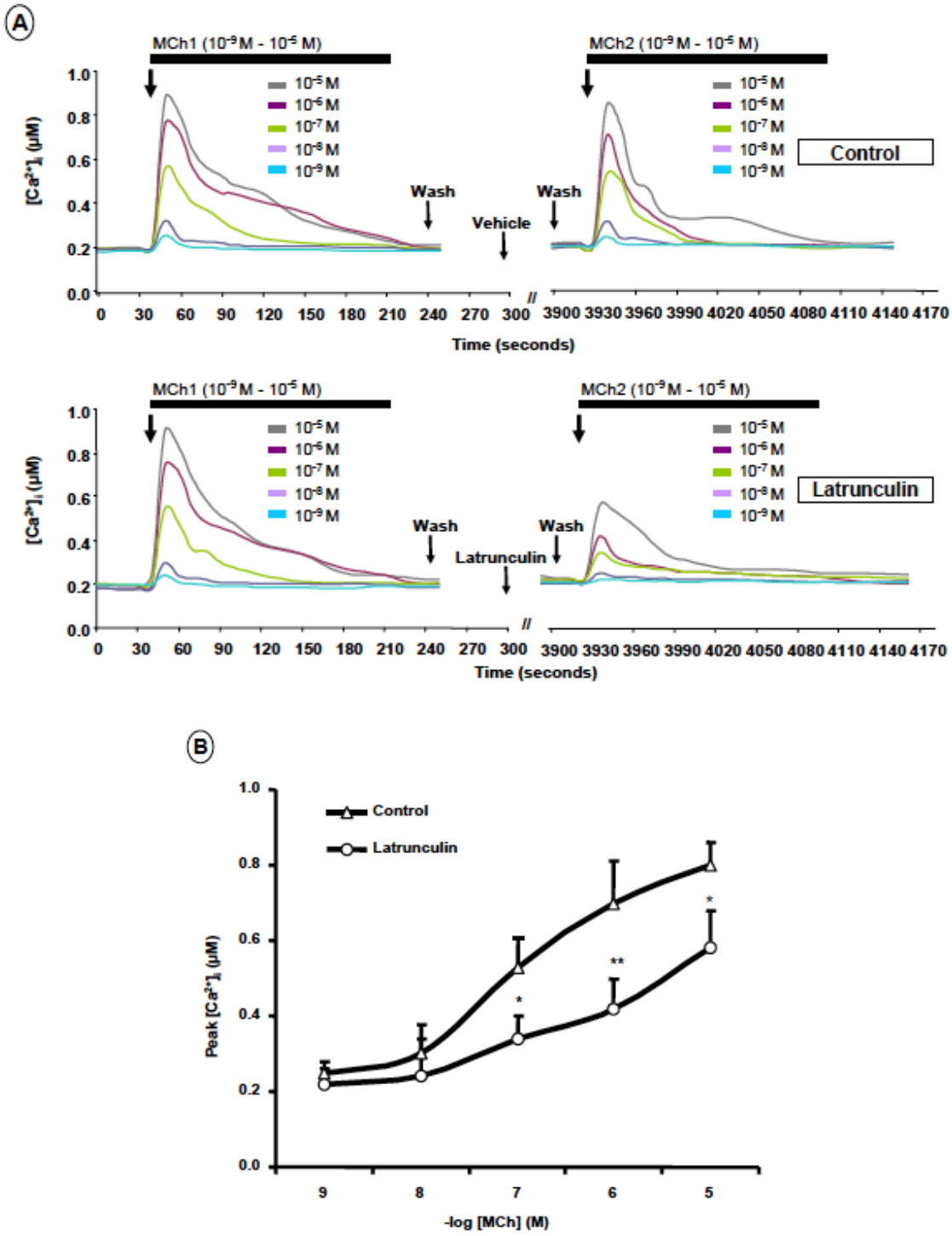


Figure 3. ***Actin disruption markedly alters receptor-mediated Ca^{2+} mobilization.*** **(A)** Representative tracings from experiments using Fura-2 loaded canine airway smooth muscle cells grown to confluence then serum-deprived in insulin-supplemented media for 7 days. Each tracing is the mean of 8-12 elongate cells identified in a single microscopic field. Cells were first stimulated with methacholine (MCh1, left panels) (10^{-9} to 10^{-5} M) and changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) recorded.

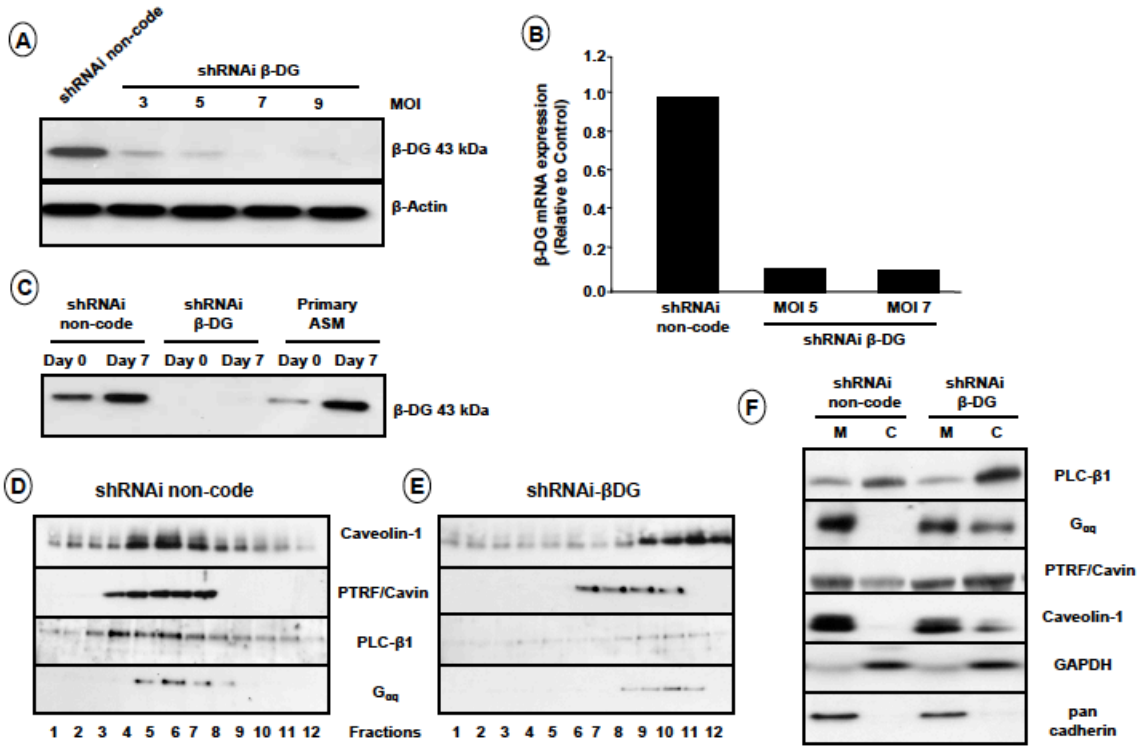
Thereafter, cells were incubated with either vehicle (top row) or latrunculin A (1 μM, 37°C) (bottom row) for 1 hour, and were subsequently treated with methacholine (MCh2) at the same concentration used for MCh1. Changes in $[Ca^{2+}]_i$ in response to MCh2 were recorded for the same cells monitored after MCh1. **(B)** Concentration-response curves for MCh2 in control and latrunculin A treated cells is plotted as peak $[Ca^{2+}]_i$. Curves are derived using individual data points that are the mean ± SEM of at least 30 cells in total (assayed in at least three different experiments). $*p < 0.05$, $**p < 0.01$, for control versus latrunculin A at a given MCh concentration.

Indeed, actin disruption significantly decreased sensitivity to MCh ($EC50_{Con}$ = 37nM ± 11nM: $EC50_{Lat}$ = 87nM ± 14nM; $p<0.05$), and actin disruption also reduced the maximum peak $[Ca^{2+}]_i$ induced with MCh (10^{-5}M) by 30% ($p<0.01$). These data demonstrate that the unique, highly ordered spatial distribution of caveolae underpinned by cytoskeletal actin has a central role in mediating GPCR -mediated mobilization of $[Ca^{2+}]_i$ in smooth muscle.

Association of β-dystroglycan with caveolin-1 is needed for modulation of $G_{\alpha q}$-coupled receptor signaling by caveolae

As our data indicate caveolae modulate intracellular $[Ca^{2+}]_i$ release and this capacity is linked to their actin-dependent spatial distribution on the sarcolemma, we next assessed whether direct interaction of caveolin-1 with β-dystroglycan was essential for caveolae integrity and regulation of GPCR excitation-contraction coupling. To address this, we prepared lines of primary human airway smooth muscle cells in which we stably silenced β-dystroglycan expression with lentivirus-delivered short-hairpin RNA (shRNA). shRNA interference using increasing lentiviral titers resulted in an initial 70% to 100% reduction of β-dystroglycan protein (Fig. 4A), and quantitative (real-time) PCR confirmed β-dystroglycan transcript was also reduced by 80-90% (Fig. 4B). Using puromycin selection, we generated

human airway smooth muscle cell lines with stably silenced β-dystroglycan in which the protein expression was abrogated even in cells subjected to prolonged serum deprivation, a condition that does promote the accumulation of β-dystroglycan in cell lines stably transduced with a non-coding β-dystroglycan refractory shRNA, and non-infected primary smooth muscle cells (Fig. 4C). To determine the impact of β-dystroglycan silencing on caveolae and caveolin-1 distribution we performed subcellular fractionation using sucrose density gradient centrifugation.



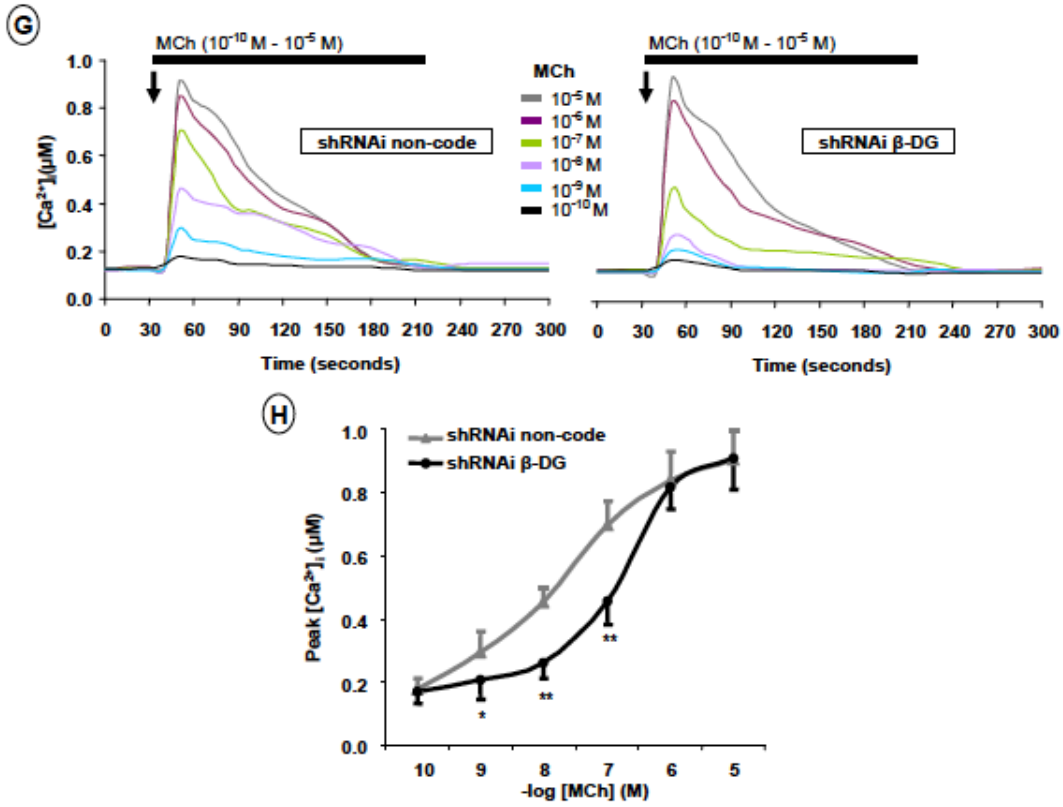


Figure 4. ***shRNA interference of* β*-dystroglycan expression alters distribution of caveolae proteins and signaling molecules.*** **(A)** Representative protein immunoblot probed for β-dystroglycan (β-DG) two days after human airway smooth muscle (ASM) cells were transduced with lentivurus carrying β-dystroglycan-specific (shRNAi β-DG) or non-coding β-dystroglycan refractory shRNA (shRNAi non-code). The MOI of lentivirus used is indicated for each sample. **(B)** Quantitative (real-time) RT-PCR for β-DG mRNA two days after human airway smooth muscle cells were transduced with lentivurus carrying shRNAi β-DG or a shRNAi non-code. Viral MOI used are indicated. **(C)** Representative protein immunoblot for β-dystroglycan in puromycin-selected human ASM cell lines. After cells were transduced with lentivirus they were subjected to 21 days of growth in the presence of puromycin, before passaging cells or experimentation. Before preparing lysates, shRNA-expressing cultures were grown to confluence (Day 0) and subjected to 7 days of serum deprivation (Day 7). Also shown are β-DG protein levels in extracts of non-infected primary human ASM cells (Primary ASM). **(D,E)** Human ASM cells expressing shRNAi non-code and shRNAi β-DG were grown to confluence, serum deprived for 7 days, then lysed in carbonate buffer for isolation of caveolae rich fractions by sucrose density gradient centrifugation. Buoyant density decreases with increasing fraction number. Equal amounts of protein from each fraction were subjected to protein blot analysis for the proteins indicated. **(F)** Isolated membrane (M) and cytosol (C) fractions from serum-deprived shRNAi non-code and shRNAi β-DG human ASM cell lines were assayed for the indicated proteins by immunoblotting. **(G)** Representative tracings showing changes in $[Ca^{2+}]_i$ in response to MCh (10^{-10} to 10^{-5} M) for Fura-2-loaded shRNAi non-code and shRNAi β-DG expressing human ASM cells. **(H)** Concentration-response curve for peak $[Ca^{2+}]_i$ in response to MCh for shRNAi non-code and shRNAi β-DG expressing human

ASM cell lines. Data at each concentration represent mean ± SEM from 35 cells measured in at least three different experiments. *$p < 0.05$, **$p < 0.01$.

Both caveolin-1 and PTRF (polymerase I and transcript release factor, also known as cavin-1), the structural protein with unique affinity for caveolin-1-sequestered lipid rafts and required for the invagination of caveolae microdomains [24, 25], were displaced from high buoyant density caveolae-rich fractions in β-dystroglycan silenced cells (Fig. 4D,E). These data indicate that β-dystroglycan is essential for the association of caveolin-1 with lipid rafts and for supporting caveolae formation and/or stability.

Muscarinic M3 receptors (M3R) are coupled to $G_{\alpha q,}$ which mediates the activation of PLCβ1 to generate secondary messengers that trigger the release of Ca^{2+} from intracellular sarcoplasmic reticulum stores [26]. We have shown that M3R can be associated with caveolae in airway smooth muscle [13], therefore we used sucrose gradient fractionation to compare the subcellular distribution of $G_{\alpha q}$ and PLCβ1 in control and β-dystroglycan-silenced human airway myocytes. Both PLCβ1 and $G_{\alpha q}$ were enriched in high buoyant density caveolin-1 enriched fractions of control smooth muscle cells. By contrast, mimicking our observation for caveolin-1 and PTRF/cavin, the absence of β-dystroglycan resulted in both proteins being displaced to less buoyant cell fractions (Fig. 4D,E). To further clarify the subcellular location of these signaling effectors, we compared their relative abundance in membrane and cytosolic fractions using protein blotting (Fig. 4F). In β-dystroglycan-deficient cells the relative abundance of PLCβ1 and $G_{\alpha q}$ in the cytosol was increased; a pattern that mirrored caveolin-1 and PTRF/cavin distribution. These data indicate that $G_{\alpha q}$ and PLCβ1 co-

localize with caveolin-1, but in the absence of β-dystroglycan, the stability of membrane caveolae is compromised resulting in the loss of these proteins from the sacrolemma.

We next investigated the functional consequences of β-dystroglycan silencing on GPCR-mediated mobilization of $[Ca^{2+}]_i$ in smooth muscle cells (Fig. 4G,H). Cells expressing non-coding control shRNA exhibited typical concentration-response characteristics. However, cells lacking β-dystroglycan exhibited significant suppression in peak $[Ca^{2+}]_i$ induced with lower concentrations of MCh. Indeed, in β-dystroglycan silenced myocytes there was significant reduction in the sensitivity to MCh ($EC50_{Control}$ = 50.3 ± 14nM *versus* $EC50_{shRNAi}$ = 220 ± 24nM; $p<0.01$). By contrast, and consistent with previous studies in which caveolin-1 expression was silenced [20], peak $[Ca^{2+}]_i$ induced with maximum concentrations of MCh were unaffected. Collectively, these data demonstrate that the requirement of β-dystroglycan for caveolae integrity is a critical determinant of the spatial profile of proximal signaling effectors, thereby having important consequences on the initiation of contraction by physiologically relevant agonists.

Discussion

DGC subunit expression is coupled to airway myocyte phenotype maturation *in vitro* and they are abundant in smooth muscle tissues [7]. Since previous studies show dystrophin co-localizes with caveolin-1 in specific membrane microdomains in contractile smooth muscle cells [12], we characterized the interaction of caveolin-1 with the DGC, which is tethered to the actin cytoskeleton, and investigated the functional significance of this

interaction. Our data show that endogenous caveolin-1 binds to β-dystroglycan, an association that had only been predicted before from work using recombinant peptides [5]. We also show that this interaction underpins a structural framework that involves filamentous actin and the DGC to support caveolae plasma membrane arrays. Importantly, our studies demonstrate that ordering of caveolae into stable cell membrane arrays is concomitant with sequestration of signaling effectors, including $G_{\alpha q}$ and PLCβ1, which transduce GPCR-mediated Ca^{2+} mobilization. Our study reveals a new level of regulation of receptor-mediated contraction of smooth muscle, thus are important for understanding function of hollow organs in health and disease.

The plasma membrane of contractile smooth muscle cells is highly ordered, consisting of repeating longitudinal rib-like arrays of caveolae and adherens junctions [27-31]. These domains are associated with disparate intracellular, membrane-associated and extracellular proteins; for example, adherens junctions are enriched in actin cross-linking proteins such as vinculin, transmembrane integrins, and fibronectin in the extracellular basal lamina [12, 27, 28]. North and colleagues [12] provided initial evidence that caveolae microdomains are marked by the presence of both caveolin-1 and dystrophin. This has contributed to models of the cytoskeletal organization in smooth muscle, but there has been no investigation assessing mechanisms for caveolin-1 and dystrophin co-localization, or of the functional relevance of this association. Our new data show that multiple subunits of the DGC, including β-dystroglycan, co-localize with caveolin-1 in membrane arrays in contractile myocytes. In striking contrast, in non-contractile cultured cells lacking endogenous β-

dystroglycan, caveolin-1 is expressed but is not organized into linear arrays (see Fig 1D). This suggests a need for the DGC in ordering of membrane caveolae domains, a conclusion supported by the fact that stable silencing of β-dystroglycan leads to disruption of caveolae linear arrays and loss of caveolin-1 and PTRF/cavin from lipid-raft rich membrane fractions. PTRF/Cavin is critical for caveolae formation [24], and its presence on the inside surface of caveolae stabilizes these structures, probably through interaction with the cytoskeleton [25]. Thus, β-dystroglycan is required for the organization of caveolae arrays through its interaction with caveolin-1, and this might impact at a more fundamental level on the association of PTRF/cavin and its role in forming and stabilizing caveolar structures. These observations are also important in light of our previous studies showing DGC expression is a feature of contractile phenotype myocytes [7], equipping them with the capacity to organize caveolae into arrays as a determinant of smooth muscle physiology.

We investigated the nature of the interaction between caveolin-1 and the DGC in smooth muscle. Caveolin-3 co-precipitates with β-dystroglycan in skeletal muscle, and a recombinant tagged peptide of a C-terminal WW domain from caveolin-3 (residues 34–129) appears to interact with a WW-binding motif from the cytoplasmic tail of β-dystroglycan [5]. In the same study a peptide harboring a putative WW domain in caveolin-1 (residues 61–156) was also used to co-precipitate a recombinant peptide encoding the β-dystroglycan WW binding domain. To the best of our knowledge, our new studies confirm for the first time in any tissue that endogenous caveolin-1 associates with native β-dystroglycan. WW domains are widely distributed among structural, regulatory, and signaling proteins, and are

named after two highly conserved tryptophan (W) residues spaced 20–22 amino acids apart in a semi-conserved motif of 38-40 residues [32, 33]. WW domains mediate protein-protein interactions by binding to peptide sequences containing proline-rich motifs, such as PPXY, as are found in WW-binding domain in β-dystroglycan (residues 884–895) [5, 34, 35]. Although our studies did not directly address whether the putative WW domain in caveolin-1 is the effector binding site for β-dystroglycan in smooth muscle cells, they do suggest a protein-protein interaction because caveolin-1 co-fractionates and co-immunoprecipitates with β-dystroglycan. This association was refractory to actin disruption as their interaction is not reliant on indirect association. Moreover, it was retained during disruption of caveolar structures. Thus we provide new evidence for an interaction between caveolin-1 and β-dystroglycan that appears to underpin the role of the DGC in orchestrating plasma membrane distribution of caveolae in contractile myocytes.

There is abundant evidence that the actin cytoskeleton has a key role in smooth muscle contraction, both as a principal component of a plastic contractile apparatus and cytoskeleton, and by effecting modulation of ion channels involved in Ca^{2+} mobilization [36-39]. The DGC interacts with and stabilizes actin filaments through a link involving dystrophin [40]. This led us to hypothesize that actin tethering is important for stabilization and organization of DGC and its role in orchestrating caveolae distribution via caveolin-1 on the sarcolemma. Latrunculin A is widely used to depolymerize actin as it binds to and prevents addition of G-actin monomers [41]. Our study demonstrates that disrupting actin filaments drastically changes the ultrastructure and molecular organization of membrane caveolae,

with significant changes in the localization of β-dystroglycan and caveolin-1 and loss of membrane caveolae arrays. Transmission electron microscopy also suggests that caveolar structures are internalized as a result of actin depolymerization. These observations are consistent with those of Mundy and colleagues [42] who showed actin dynamics are essential for location of caveolae to the cell membrane. A point that our study did not address is the role of microtubules in the organization caveolin-1 in caveolae and its interaction with DGC. This may be an important issue deserving future work as disruption of microtubules appears to decrease inward cycling of caveolae and increases the abundance of plasma membrane caveolin-1 and caveolae [42]. Nonetheless, our findings extend understanding, as they reveal that the DGC holds an important role in linking caveolae to a dynamic intracellular actin network.

We and others have shown in smooth muscle cells that depleting cholesterol or silencing of caveolin-1 expression disrupts the organization of membrane caveolae and alters functional responses as measured by contractile agonist-induced intracellular Ca^{2+} release and force generation [20, 21]. Our new data are consistent with these observations and those showing a role for the actin cytoskeleton in modulating Ca^{2+} responses in several other cell types [43-45], because induced changes in ultrastructure and the protein profile of caveolae were associated with a reduction in both sensitivity to MCh and in maximum peak $[Ca^{2+}]_i$. Trimeric G proteins are sequestered to caveolae though interaction of α-subunits with caveolin-1, and signaling machinery required to mobilize Ca^{2+} within smooth muscle cells is organized in caveolae [11, 20, 46-48]. Although PLC β1 is concentrated at the cell

membrane, it is also present throughout the cell, whereas $G_{\alpha q}$ is more-or-less membrane specific, where it stably associates with PLCβ1 to allow for rapid transmission of intracellular signals via GPCRs such as the M3 muscarinic receptor [49]. Recent evidence suggests caveolin-1 can play a regulator role, promoting dissociation of Gα subunits from βγ subunits to facilitate GPCR signal transduction [22]. Interaction between caveolin-1 and β-dystroglycan appears to be necessary for such a functional role, because in human airway smooth muscle cells with stably silenced β-dystroglycan, PLCβ1 and $G_{\alpha q}$ were lost from caveolae and accumulated in fractions that are typically devoid of caveolae. As this change mirrored that for PTRF/cavin and caveolin-1, it suggests that interaction of caveolae with structural proteins and the actin cytoskeleton supports sequestration of signaling molecules for Ca^{2+} mobilization, and enable a regulator role for caveolin-1.

The role of caveolin-1 in GPCR-mediated Ca^{2+} flux is also linked with the existence of an organelle triad involving caveolae, the sarcoplasmic reticulum and mitochondria that form nanospaces for localized signal transduction leading to induction of contraction [14, 50, 51]. Concomitant with loss of PLCβ1 and $G_{\alpha q}$ from caveolae membrane, silencing of β-dystroglycan disturbed the spatial distribution of caveolae. Studies using animal models that lack expression of DGC subunits indicate this deficiency contributes to altered Ca^{2+} homeostasis in smooth muscle [52, 53]. In our present study, silencing of β-dystroglycan reduced sensitivity for muscarinic M3 receptor-mediated $[Ca^{2+}]_i$ mobilization but had no effect on maximum peak $[Ca^{2+}]_i$. This is consistent with previous work, which showed that siRNA silencing of caveolin-1 results in reduced sensitivity to MCh without impacting peak

response [20]. The lack of effect of caveolin-1 depletion on peak $[Ca^{2+}]_i$ is due to existence of a significant M3 receptor reserve in airway smooth muscle. A similar mechanism is also likely also to be the root of our observations linked to β-dystroglycan silencing in the present study. In contrast to the effect of silencing of caveolin-1 or β-dystroglycan forced actin de-polymerization does suppress peak $[Ca^{2+}]_i$. This difference may be due to the fact that actin cytoskeleton affects the activity of various ion channels, including some that localize to caveolae, which are involved in Ca^{2+} mobilization, [36-39].

In summary, our study demonstrates that the unique membrane distribution of caveolae in contractile smooth muscle cells is facilitated through the direct interaction of caveolin-1 with β-dystroglycan and tethering of the DGC to the intracellular actin cytoskeleton network. Interaction of β-dystroglycan with caveolin-1 is required to organize membrane caveolae enriched in key GPCR effectors and caveolae stabilizing proteins. This appears to be critical for effective spatial organization of caveolae arrays with respect to Ca^{2+}-mobilizing effectors and machinery, thus revealing a new role for the actin cytoskeleton as a determinant of smooth muscle function. Collectively, our data suggest that interaction of caveolin-1, β-dystroglycan and the actin cytoskeleton is important for the structural and spatial distribution of membrane caveolae, and has a key role in $G_{\alpha q}$-and PLCβ-1 mediated Ca^{2+} mobilization in the cell.

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PS drafted and produced 100% of the experiments required for the realization of this manuscript

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Chapter 6

Mechanisms of intracellular calcium homeostasis by dystrophin glycoprotein complex

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This collaborative work is presented as a manuscript in preparation.

Abstract

The dystrophin glycoprotein complex (DGC) is localized to caveolae microdomains where it binds with caveolin-1. This interaction has been shown to play an important role in calcium mobilization in airway smooth muscle (ASM) but it is not clear whether it selectively affects release of specific pools of Ca^{2+}. We tested whether DGC disruption affects cytosolic Ca^{2+} release in the absence of extracellular Ca^{2+}. Disruption of DGC by latrunculin (causes actin depolymerization), silencing β-dystroglycan (disrupt binding of caveolin-1 with DGC), and cells lacking dystrophin from golden retriever muscular dystrophy (GRMD) we noted reduced Ca^{2+} release from the sarcoplasmic reticulum (SR). The sensitivity to methacholine (MCh) was found to be reduced in both latrunculin treated and β-dystroglycan silenced cells without affecting the GRMD ASM cells. Xestospongin-c (XeC) treatment significantly reduced the release of intracellular Ca^{2+} with actin disruption beyond basal Ca^{2+} release. While lack of β-dystroglycan and dystrophin exerted no additional effect on Ca^{2+}. We observed that DGC disruption significantly reduced the capacity of SR to sequester Ca^{2+} induced by thapsigargin (TpG: a SERCA inhibitor). Moreover, the ability to release Ca^{2+} from SR after DGC disruption was associated with increased expression of calnexin in these cells. These data indicate, disruption of DGC is associated with a concomitant suppression of receptor mediated Ca^{2+} release from SR *in vitro*, and altered expression of calnexin. We suggest that DGC is a key determinant of calcium release pathways in ASM cells.

Introduction

The dystrophin glycoprotein complex (DGC) is a multi-protein transmembrane complex providing an structural link to the extracellular matrix (ECM) and underlying actin cytoskeleton [1, 2]. We have shown that DGC is a marker of phenotype maturation in human airway smooth muscle (ASM) cells (Chapter 4) [3]. Higher expression of DGC in a contractile cell is functionally important in mobilizing intracellular Ca^{2+} to contractile agonists such as methacholine (MCh) (Chapter 5). We also demonstrated that β-dystroglycan, which serves as a central subunit of DGC, binds endogenous caveolin-1 in ASM tissue and thus provides further regulation in mobilizing intracellular Ca^{2+} in the ASM cell (Chapter 5) [4].

Excitation–contraction coupling in smooth muscle cells occurs by activating Ca^{2+} signaling. In addition Rho/Rho Kinase signaling can also modulate the contraction of smooth muscle cells through Ca^{2+} sensitization [5-7]. Membrane depolarization being a key element for the activation of many smooth muscle cells, much attention has been laid on the mechanisms responsible for depolarizing the membrane. However, there are other smooth muscle cells such as from airways where activation depends on the periodic release of Ca^{2+} from internal stores. The rhythmical contractions of ASM is driven by a cytosolic Ca^{2+} oscillator that is responsible for the periodic release of Ca^{2+} from the endoplasmic reticulum. The periodic pulses of Ca^{2+} often cause membrane depolarization, but this is not part of the primary activation mechanism but has a secondary role to synchronize and amplify the oscillatory mechanism [8-10]. Treatment of bronchial rings with cyclopiazonic acid results in only transient contractions, suggesting that a functional sarcoplasmic reticulum (SR) is required for sustained contraction [11]. Ca^{2+}release channels in the SR are gated by IP_3R RyR, both types of receptors occur in three isoforms and it

would appear that some diversity in smooth muscle Ca^{2+} signaling will occur via differential expression of these isoforms, as their properties differ. The expression of these receptors differs in various types of tissues [12-15].

Our data indicate that DGC has a role in mobilizing intracellular Ca^{2+} to muscarinic agonists through PLCβ1-Gα_q-IP_3 pathways. However the mechanism by which DGC affects the intracellular Ca^{2+} homeostasis in ASM cells is unclear. Thus we investigated whether DGC participates in mobilizing intracellular Ca^{2+} from SR by affecting SR Ca^{2+}-binding proteins independent of the extracellular calcium pool.

Results

Intact dystrophin-glycoprotein complex is required for intracellular Ca^{2+} mobilization

We showed in Chapter 5 [4] that Ca^{2+} mobilization induced by muscarinic agonists is dependent on the organization of DGC with in caveolae. Whether this phenomenon also exists in the true in the absence of an extracellular sources of Ca^{2+}. Herein we investigated this mechanism in cells lacking the organization of DGC using published strategies [4, 16, 17]. As shown in Fig. 1A, latrunculin A (widely used to depolymerize actin as it binds to and prevents addition of G-actin monomers [18]) to disrupt actin cytoskeleton in canine ASM which in turn disorganizes the DGC, by trafficking of key proteins from the caveolae thus preventing the crucial interaction of DGC with ECM.

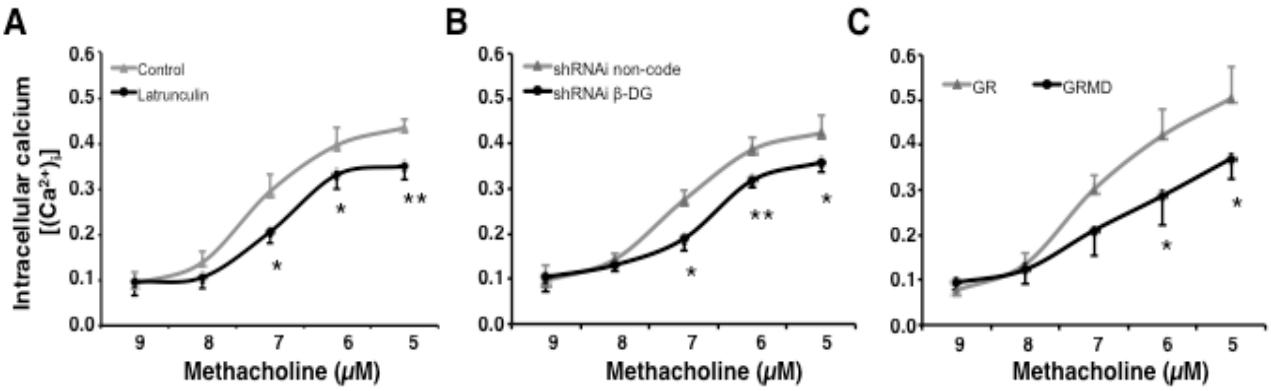

Figure 1. ***Integrity of DGC is important for intracellular Ca^{2+} mobilization.*** All the experiments were performed using Ca^{2+} free buffer to simulate conditions for the cells with out any extracellular source of Ca^{2+} **A:** Representative tracings from experiments using Fura-2 loaded canine airway smooth muscle cells grown to confluence then serum-deprived in insulin-supplemented media for 7 days. Cells were first stimulated with methacholine (MCh: 10^{-9} to 10^{-5} M) and changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) recorded. Thereafter, cells were incubated with latrunculin A (1 μM, 37°C) for 1 hour, and were subsequently treated with MCh at the same concentrations. Changes in $[Ca^{2+}]_i$ in response to MCh were recorded for the same cells monitored before adding latrunculin A. Concentration-response curves for MCh in control and latrunculin A treated cells is plotted as peak $[Ca^{2+}]_i$. Curves are derived using individual data points that are the mean ± SEM of at least 25 cells in total (assayed in at least three different experiments). **B:** Concentration-response curve for peak $[Ca^{2+}]_i$ in response to MCh for shRNAi non-code and shRNAi β-DG expressing human ASM cell lines. Data at each concentration represent mean ± SEM from 30 cells measured in at least three different experiments. **C:** Concentration-response curve for peak $[Ca^{2+}]_i$ in response to MCh for canine ASM cells obtained from healthy golden retriever (GR) and dystrophic golden retriever muscular dystrophy (GRMD) dog. Data at each concentration represent mean ± SEM from 35 cells measured in at least three different experiments. Statistical comparisons shown were performed by 1-way ANOVA with Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$ at a given MCh concentration.

The intracellular Ca^{2+} mobilization induced by muscarinic agonist methacholine (MCh) in absence of extracellular Ca^{2+} was greatly reduced at 100nM, 1μM and 10μM of MCh and there was a significant increase in the EC50 ($EC50_{con}$=84.30, ±5.70; $EC50_{lat}$=177.0, ±18.6) value in the latrunculin treated cells when compared to the controls (p<0.01, Table 1). Lentiviral mediated delivery of shRNA silenced the central subunit of DGC: β-dystroglycan in human ASM cells (Fig. 1B) [4]. We found significant reduction in the intracellular $[Ca^{2+}]$ induced by MCh in the absence

of extracellular Ca^{2+} at 100nM, 1μM and 10μM concentrations of MCh in cells lacking β-dystroglycan (shRNAi β-DG) when compared to control (shRNAi non-code) (Fig. 1B). Also as shown in table 1, there was a significant increase in the EC50 ($EC50_{shRNAi\ non\text{-}code}$=35.5, ±5.37; $EC50_{shRNAi\ \beta\text{-}DG}$=87.3, ±17) value in the β-DG silenced cells when compared to the control (p<0.05). We further used another approach using ASM cells from dystrophin deficient golden retriever muscular dystrophy dogs (GRMD) and healthy golden retriever (GR). We found that there was significant reduction in the intracellular Ca^{2+} mobilization induced by MCh at 1 and 10μM (Fig. 1C), but there was no difference in the EC 50 values between the two groups (Table 1). Collectively, these results indicate that the organization of DGC at the membrane with in caveolae is critical for normal Ca^{2+} homeostasis in the cell, in this case the release of Ca^{2+} from SR is compromised when the DGC is disrupted.

Table 1. EC 50 values of control *vs* DGC disrupted ASM cells.

	EC50 (nM)	SEM	t-test
Control Latrunculin	84.30 177.0	±5.70 ±18.6	p<0.01
shRNAi non-code shRNAi β-DG	35.5 87.3	±5.37 ±17.0	p<0.05
Healthy GR GRMD	58.3 62.3	±10.1 ±11.0	p>0.05

Reduction in Ca^{2+} mobilization is IP3 dependent

We have described earlier in chapter 5 [4] that the disruption of the DGC leads to the trafficking of key Ca^{2+} mobilizing molecules from caveolae microdomains to non-caveolae regions suggesting anclear role for Gαq-IP3-PLCβ1 pathways in this process. Here we used a chemical inhibitor, xestospongin-C (XeC), to block the IP3R. Fig. 2A-B shows the typical dose

response curve for XeC in presence of 0.1μM MCh. 1μM of XeC blocked over 50% intracellular Ca^{2+} from SR stores. Using a similar profile of cells described in Fig. 1 we measured the %Ca^{2+} release in the absence and presence of 1μM XeC in cells with ± latrunculin (Fig. 2C), ± β-dystroglycan (Fig. 2D) and ± dystrophin (Fig. 2E). The results clearly demonstrates that XeC by itself reduced the mobilization of intracellular Ca^{2+} in all three control ASM cells and there was no additional effect of XeC on Ca^{2+} release from SR when organization of DGC was affected both in cells lacking β-dystroglycan and dystrophin. Actin disruption with latrunculin resulted in significant reduction in Ca^{2+} when treated with XeC suggesting an important role of actin dynamics in this process.

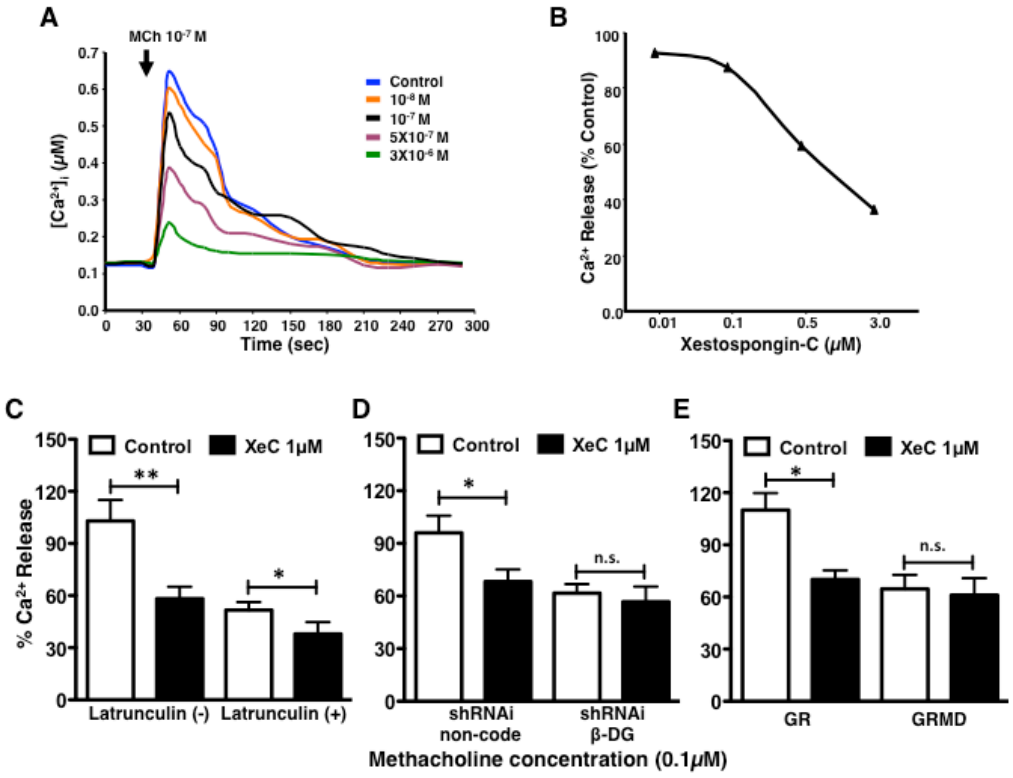

Figure 2. ***Ca^{2+} mobilization is mediated through IP3 receptor.*** All the experiments were performed using Ca^{2+} free buffer to simulate conditions for the cells with out any extracellular source of Ca^{2+} **A:** Representative tracings from experiments using Fura-2 loaded ASM cells grown to confluence then serum-deprived in insulin-supplemented media for 7 days. Cells were first incubated with Xestospongin-

C (XeC, 0.1nM–3.0μM) for 30 minutes and then stimulated with methacholine (MCh: 0.1μM) and changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) recorded. **B:** Graph showing %Ca^{2+} release at increasing concentrations of XeC. 1μM of XeC showing greater than 50% reduction in intracellular Ca^{2+} induced by 0.1μM MCh. **C:** Bar graph for peak $[Ca^{2+}]_i$ in response to MCh (0.1μM) in presence and absence of XeC in cells with ± latrunculin (1μM) in canine ASM cells. Results represent mean ± SEM from 30 cells measured in at least three different experiments **D:** Bar graph for peak $[Ca^{2+}]_i$ in response to MCh (0.1μM) in presence and absence of XeC in shRNAi non-code and shRNAi β-DG expressing human ASM cell lines. Data at each concentration represent mean ± SEM from 35 cells measured in at least three different experiments. **E:** Bar graph for peak $[Ca^{2+}]_i$ in response to MCh (0.1μM) in presence and absence of XeC in ASM cells obtained from healthy golden retriever (GR) and golden retriever muscular dystrophy (GRMD) animal. Data at each concentration represent mean ± SEM from 30 cells measured in at least three different experiments. Statistical comparisons shown were performed by 1-way ANOVA with Tukey's multiple comparison test, *$p < 0.05$, **$p < 0.01$.

Effect of store depletion on cytosolic calcium concentration

We showed that most of the receptor mediated cytosolic Ca^{2+} release induced by MCh came from SR and was IP3R dependent. We used thapsigargin (a non-competitive inhibitor of a class of enzymes known as sarco / endoplasmic reticulum Ca^{2+} ATPase (SERCA) which limits the ability of the cell to pump Ca^{2+} back in to SR), to raise cytosolic Ca^{2+} levels in ASM cells [19]. As shown in Fig. 3A-C, intracellular Ca^{2+} over time when cells were stimulated with 0.5μM thapsigargin (TpG) with their corresponding bar graphs. Our results demonstrate that when the organization of DGC was compromised using latrunculin (causes actin disruption: Fig, 3A), silencing β-dystroglycan (Fig. 3B), and in cells lacking dystrophin protein (Fig. 3C): the ability of the ASM cells to elevate cytosolic Ca^{2+} induced by TpG was significantly reduced. These results suggests the possible role of Ca^{2+} binding proteins in this process and also hints towards changes in the spatial distribution of SR with caveolae.

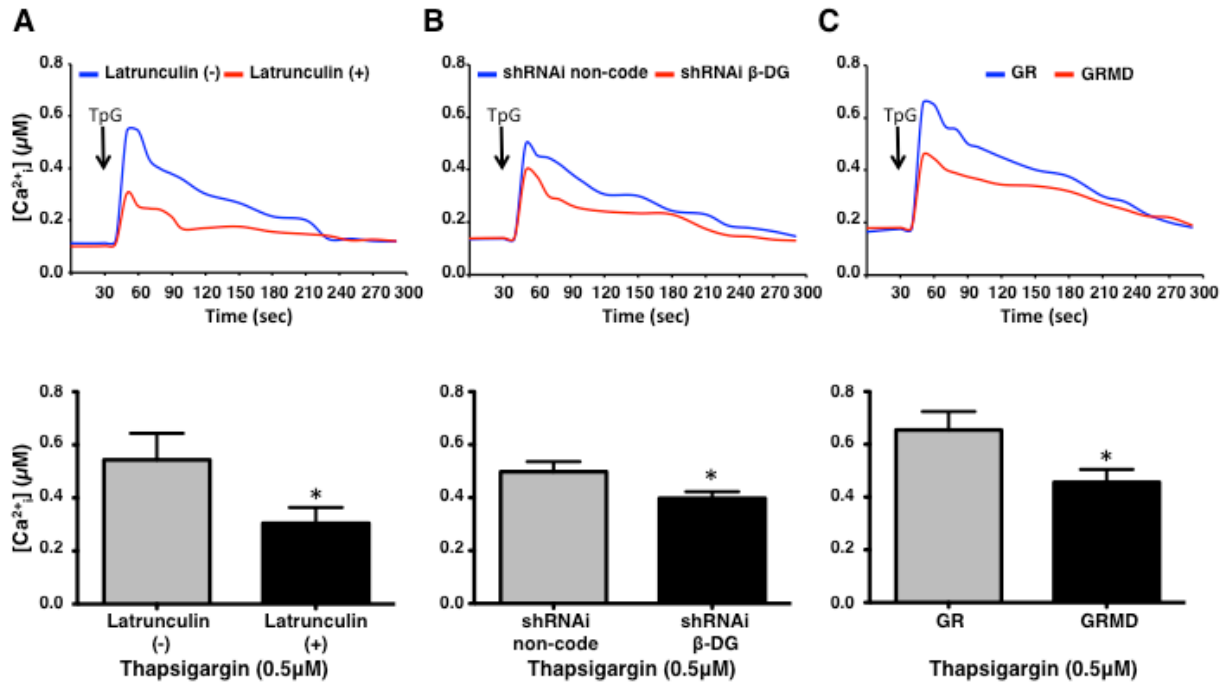

Figure 3. ***Reduced cytosolic Ca^{2+} in DGC disrupted ASM cells.*** All the experiments were performed using Ca^{2+} free buffer to simulate conditions for the cells with out any extracellular source of Ca^{2+} **A:** Representative tracings and the corresponding bar graph (plotted as peak $[Ca^{2+}]_i$) from experiments using Fura-2 loaded canine ASM cells grown to confluence then serum-deprived in insulin-supplemented media for 7 days. Cells were first stimulated with thapsigargin (TpG: 0.5μM) and changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) recorded. Thereafter, cells were incubated with latrunculin A (1 μM, 37°C) for 1 hour, and were subsequently treated with TpG at the same concentration. Changes in $[Ca^{2+}]_i$ in response to TpG were recorded for the same cells monitored before adding latrunculin A. Curves are derived using individual data points that are the mean ± SEM of at least 25 cells in total (assayed in at least three different experiments). **B:** Concentration-response curve and the corresponding bar graph for peak $[Ca^{2+}]_i$ in response to TpG (0.5μM) for shRNAi non-code and shRNAi β-DG expressing human ASM cell lines. Data at each concentration represent mean ± SEM from 30 cells measured in at least three different experiments. **C:** Concentration-response curve and the corresponding bar graph for peak $[Ca^{2+}]_i$ in response to TpG (0.5μM) for canine ASM cells obtained either from healthy golden retriever (GR) or golden retriever muscular dystrophy (GRMD) animal. Data at each concentration represent mean ± SEM from 35 cells measured in at least three different experiments. Statistical comparisons shown were performed by unpaired student's t-test, *$p < 0.05$.

Effect of DGC disruption on Ca^{2+} binding proteins

Calnexin (a Ca^{2+} binding protein) also binds misfolded proteins and prevents them from being exported from the endoplasmic reticulum to the Golgi apparatus, thereby targeting them for degradation. Here we investigated the effect of DGC disruption on the Ca^{2+} binding protein.

We found that in the ASM cell lysates obtained: i) after treating with latrunculin (to disrupt actin cytoskeleton), ii) silencing of β-dystroglycan, and iii) dystrophin deficient cells, the protein abundance of calnexin was significantly higher when compared to their respective controls (Fig. 4A-C).

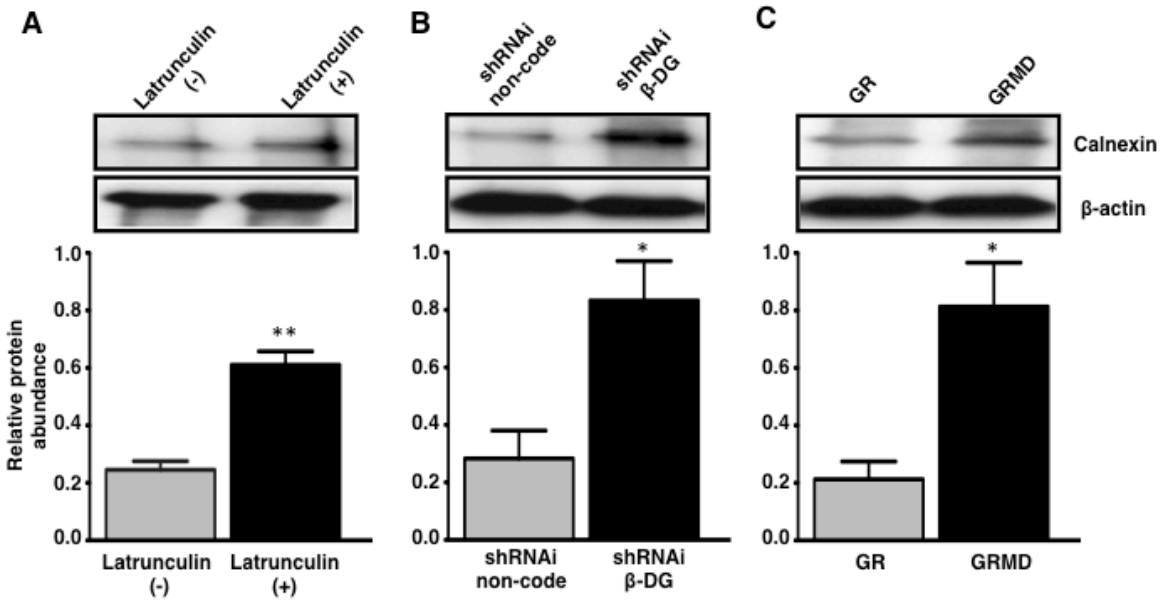

Figure 4. ***Disruption of DGC affects Ca^{2+} binding protein.*** **A:** Representative western and the corresponding bar graph from experiments using canine primary ASM cells grown to confluence then serum-deprived in insulin-supplemented media for 7 days. Thereafter, cells were incubated with latrunculin A (1 μM, 37°C) for 1 hour, lysed and probed for calnexin. **B:** Representative western and the corresponding bar graph for calnexin protein in human ASM cells carrying either shRNAi non-code or shRNAi β-DG expressing virus grown to confluence and serum-deprived for 7 days. **C:** Representative western and the corresponding bar graph for calnexin protein in primary ASM cells obtained from either healthy golden retriever (GR) or golden retriever muscular dystrophy (GRMD) animal. Data represents mean ± SEM from at least three different experiments. Statistical comparisons shown were performed by unpaired student's t-test, $*p < 0.05$, $**p < 0.01$.

These results indicate that the binding of calnexin with Ca^{2+} is affected and also hints at its possible role in protein degradation particularly caveolin-1 which is secreted through the Golgi pathway to form caveolae microdomains at the membrane. The intact caveolae facilitates receptor mediated Ca^{2+} mobilization in the ASM cells [4, 20].

Discussion

We have previously shown (Chapters 4 & 5) that the DGC is highly expressed in contractile ASM cells and a subunit of DGC binds caveolin-1 which facilitate receptor mediated Ca^{2+} release [3, 4]. We have also shown that disruption of this interaction using latrunculin or silencing of β-dystroglycan can lead to reduced Ca^{2+} release induced by contractile agonists [4]. Until now it was not clear whether the magnitude of this reduction in mobilization of intracellular Ca^{2+} was also affected at the sarcoplasmic level in the absence of extracellular source of Ca^{2+}. Here we investigated the effect of DGC disruption on the intracellular calcium pools. Our result shows that in absence of extracellular source of Ca^{2+} the magnitude of peak Ca^{2+} release induced by muscarinic agonist MCh was reduced by half which was significantly attenuated in the ASM cells having disrupted DGC. We also found that this Ca^{2+} release was sensitive to IP3R blocker XeC suggesting its release from SR. XeC had no additional effect in cells lacking dystrophin and β-DG but actin disruption markedly reduced Ca^{2+} release. Moreover, we found that SERCA inhibitor thapsigargin failed to raise the cytosolic Ca^{2+} in DGC disrupted ASM cells while expression of calnexin was found to be higher in DGC disrupted cells suggesting its possible role in this process.

It has been known for years now that caveolae microdomains plays a key role in Ca^{2+} homeostasis in ASM cells and harbor key signaling molecules required for effective Ca^{2+} mobilization induced by contractile agonists [20-24]. It has also been shown that DGC is localized to caveolae microdomains [25], where it is found to bind and interact with Caveolin-1, a principal caveolae protein [4, 26-28]. Caveolin-1 has been shown to facilitate Ca^{2+} mobilization

in the ASM cells as silencing caveolin-1 or reducing the caveolae using various approaches has been shown to reduce Ca^{2+} mobilization in the ASM cell [4, 20, 29]. However most of these studies have been performed using extracellular source of Ca^{2+}, which is a suboptimal approach for understanding Ca^{2+} changes in the cells. In the present study we investigated the mechanism by which DGC affects the intracellular Ca^{2+} release in ASM cells by eliminating the source of free extracellular Ca^{2+}. The magnitude of Ca^{2+} release by increasing concentrations of MCh was almost reduced to half (comparing to our previously published reports in chapter 5 [4]), highlighting the contribution of SR in releasing Ca^{2+} in the cell. We also found that disruption of DGC by using several approaches such as *i)* actin depolymerization by using latrunculin (which we have shown earlier to affect caveolae interaction with DGC along with global reduction in Ca^{2+} release induced by contractile agonist MCh [4, 18]), reduced the intracellular Ca^{2+} release when compared to latrunculin untreated ASM cells, *ii)* silencing β-dystroglycan (which is also shown to affect the interaction of caveolae with DGC and reduction in the Ca^{2+} sensitivity [4]), significantly reduced the release of intracellular Ca^{2+} when compared with the control shRNA cells, *iii)* using either dystrophin deficient (GRMD) or healthy (GR) ASM cells, we found reduction in the Ca^{2+} release induced by MCh without affecting the EC_{50}. These results demonstrate that the DGC not only affects the release of Ca^{2+} from extracellular source but also affects its release from intracellular stores such as SR.

As it is known that caveolin-1 and DGC works through the $G_{\alpha q}$-PLCβ1-IP3 pathway and it has also been shown that receptor mediated Ca^{2+} release is affected by acute silencing of either caveolin-1 or β-dystroglycan in ASM cells [4, 20, 21, 29]. We further investigated to confirm the

above findings and noted reduced Ca^{2+} mobilization from SR after DGC disruption in ASM cells. IP3R blocker XeC reduced the MCh induced intracellular Ca^{2+} from SR in all control ASM cells but it was without any additional effect in ASM cells having disrupted organization of DGC (in β-dystroglycan shRNA and dystrophin deficient cells). XeC had significant reduction in the Ca^{2+} release in ASM cells after actin disruption using latrunculin. These results highlight the greater complexity of actin dynamics in regulating Ca^{2+} release through other mechanisms within the cell. There is abundant evidence that the actin cytoskeleton has a key role in smooth muscle contraction, both as a principal component of a plastic contractile apparatus and cytoskeleton, and by effecting modulation of ion channels involved in Ca^{2+} mobilization [17, 30-32]. The DGC interacts with and stabilizes actin filaments through a link involving dystrophin [33]. We also know that actin tethering is important for stabilization and organization of the DGC and its role in orchestrating caveolae distribution via caveolin-1 on the sarcolemma [4].

We further investigated the effect of IP3 independent cytosolic Ca^{2+} release from intracellular stores by inhibition of the SERCA using TpG which raises the cytosolic concentration and blocks the refilling of SR [34]. TpG increased the cytosolic concentration by blocking SERCA in all control ASM cells (Fig. 3A-C) but there was a reduction in the ability of TpG to mobilize intracellular Ca^{2+} upon DGC disruption when compared to the respective controls. We found that unlike the IP3 mediated Ca^{2+} release, there was a uniformity by which TpG induced Ca^{2+} release between different approaches used to disrupt DGC. These results indicate that organization of DGC is crucial for effective functioning of SR and raised the possibility that various Ca^{2+}-binding proteins might be affected by disruption of the DGC. Thus our next goal

was to measure calnexin. It is a 90kDa integral protein of the SR with a large (50 kDa) N-terminal calcium-binding luminal domain, a single transmembrane helix and a short acidic cytoplasmic tail [35]. Calnexin is one of the chaperone molecules, which are characterized by their main function of assisting protein folding and quality control, ensuring that only properly folded and assembled proteins proceed further along the secretory pathway [36, 37]. Our result clearly shows that calnexin levels were higher when ASM cells were pretreated with latrunculin which disrupt the actin-cytoskeleton. Also when we silenced β-dystroglycan a key subunit of the DGC we found that calnexin abundance was higher in these cells. Similarly dystrophin absence also resulted in increased abundance for calnexin. Collectively, our findings show for the first time that DGC disruption is associated with increased calcium-binding protein calnexin. These findings have another implication as increased activity of calnexin could affect the secretary pathway of caveolin-1 through Golgi which is a key step for attaching to the inner leaflet of the plasmalemma to form caveolae. We have previously shown that DGC disruption either by silencing of β-dystroglycan or using latrunculin to prevent actin polymerization [4], caveolin-1 protein abundance is reduced in the caveolae microdomains and elevated in the cytosol. At this point we cannot say whether the increased cytosolic pool of caveolin-1 comes from the membrane or it is the result of increased activity of calnexin which could also prevent the secretary pathway in the cell.

In summary, we found that in various ASM cell systems having disrupted DGC we show that there is a reduction in receptor mediated Ca^{2+} mobilization induced by contractile agonist MCh which is mediated through IP3R on SR. Our results indicate that IP3-independent Ca^{2+}

release induced by SERCA inhibition is also dramatically reduced after DGC disruption suggesting the role of key proteins which bind Ca^{2+} in the SR. We show for the first time that DGC disruption leads to increased calnexin protein abundance in ASM cell. These findings have a functional significance as Ca^{2+} is a key step for ASM contraction *in vivo*.

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PS drafted and produced 100% of the experiments required for the realization of this manuscript

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Chapter 7

Key Role of Dystrophin in Airway Smooth Muscle Phenotype, Contraction and Lung Function

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This collaborative work is presented as a manuscript in preparation.

Abstract

Dystrophin links the transmembrane dystrophin-glycoprotein complex (DGC) to the actin cytoskeleton. We have shown that DGC subunits are markers for airway smooth muscle (ASM) phenotype maturation and together with caveolin-1, play an important role in calcium homeostasis in airway smooth muscle. We tested if dystrophin affects phenotype maturation, tracheal contraction and lung physiology. We used dystrophin deficient Golden Retriever dogs (GRMD) and *mdx* mice *vs* healthy control animals in our approach. Immunoblotting confirmed dystrophin increased in expression in control ASM cultures with serum deprivation, was undetectable in GRMD cultures. In serum deprived GRMD ASM cultures, expression of contractile phenotype markers smooth muscle myosin heavy chain (smMHC) and calponin were reduced 39 ±7% and 35 ±6% ($p<0.05$) compared ASM cells from controls. β-dystroglycan (β-DG) and caveolin-1 were also reduced 2-to-4 fold in GRMD ASM cells. Immunocytochemistry revealed reduced stress fibers and smMHC positive cells in GRMD ASM cells, when compared to control. Immunoblot analysis of Akt1, GSK3β and mTOR phosphorylation revealed that downstream PI3K signaling, which is essential for phenotype maturation, was suppressed in GRMD ASM cultures. Tracheal rings from *mdx* mice showed significant reduction in the isometric contraction to methacholine (MCh) ($EC_{50wild\text{-}type}$= 0.519μM ±0.098; EC_{50mdx}=2.48μM ±0.16) when compared to genetic control BL10ScSnJ mice (wild-type). Lung function studies using a small animal ventilator revealed a strong trend in reduction of the airway resistance in *mdx* mice with increasing doses of MCh when compared to the controls (~25% reduction at 50mg/ml MCh; $p<0.05$), while, there was no change in other lung parameters. These data show that the lack of dystrophin is associated

with a concomitant suppression of ASM cell phenotype maturation *in vitro*, ASM contraction *ex vivo* and lung function *in vivo*, indicating that a linkage between the DGC and the actin cytoskeleton via dystrophin is a determinant of the phenotype and functional properties of ASM.

Introduction

Dystrophin is a large gene (2.2 Mb) gene composed of 79 primary exons plus 6 alternate first exons. The gene encodes a 427 kDa protein that has an N-terminal actin binding domain, a large central rod domain, a cysteine-rich region, and a C-terminal domain The central rod domain contains 24 spectrin-like repeats, 4 hinge regions, and a central actin-binding domain [1, 2]. Dystrophin is an intracellular protein member of dystrophin-glycoprotein complex (DGC) that links the extracellular matrix (ECM) to the underlying actin cytoskeleton [3]. In adult skeletal muscles dystrophin is located at the sarcolemma in connection with the costameric lattice at Z- and M-lines of peripheral sarcomeres [4]. It is also present in smooth muscle where it provides a strong link between ECM and actin cytoskeleton. Further, it is distributed in an alternate position with the adherens junction protein vinculin and colocalize with the lipid raft protein caveolin-1 in a rib like manner-arranged parallel to the long axis of the cell [5-7]

Lack of dystrophin results in a progressive skeletal muscle wasting disease called Duchenne muscular dystrophy (DMD) [8, 9]. DMD muscle fibers are fragile and leaky [10, 11]. This permeability is made worse by mechanical stress and can be assessed in

experiments using Evans blue dye uptake in dystrophic animals [12, 13]. The skeletal muscle fibers begin to degenerate with a prominent inflammatory response followed by severe muscle weakness, many of the skeletal muscle fibers are replaced by fibrosis and adipose cells. It is also associated with smooth muscle abnormalities that affect digestion [14, 15].

Two animal models of DMD which are extensively used to study the pathophysiology and muscle function are **i)** *mdx* mice: caused by a nonsense mutation in exon 23 of the dystrophin gene C57BL10 mice leading to a loss of dystrophin protein expression [16, 17], and **ii)** Golden retriever muscular dystrophy (GRMD): caused by a point mutation in canine dystrophin gene resulting in loss of dystrophin transcript and the protein [18]. Dystrophin is thought to transfer lateral forces from the sarcomeres, to the extracellular matrix, and ultimately, to the tendon [19, 20]. The absence of dystrophin leads to profound reductions in the DGC at the sarcolemma [21]. The lack of structural support at the sarcolemma leaves muscles more susceptible to contraction-induced injury [22-26]. The muscles are atrophic, weaker, and more susceptible to contraction-induced injury [27, 28].

Loss in skeletal and cardiac muscle function in a dystrophin deficient animal has been studied and well described but studies in airway smooth muscle *per se* are lacking. We hypothesize that dystrophin has a key role in airway smooth muscle phenotype *in vitro* and also participates in ASM contraction and determination of airway function *in vivo.* We used two dystrophic animal models (GRMD and *mdx*) to study the effect of dystrophin on ASM phenotype and function. Our results showed that dystrophin expression is associated with

development of a contractile phenotype in ASM cells, while lack of dystrophin is associated with reduced contractile protein markers and reduced signaling required for accumulation of contractile proteins in the cell. Moreover, the functional data showed that dystrophin is needed to maintain ASM tone as loss of dystrophin caused reduction in tracheal sensitivity to contractile agonist methacholine (MCh). Airway mechanics data showed a clear trend in associating dystrophin with maintenance of lung function in intact animal. Taken together, we found that dystrophin is a key determinant of ASM phenotype and function.

Results

Dystrophin is absent in GRMD ASM cell cultures

ASM cells in culture have the unique capacity to acquire a long-elongated phenotype when grown in the absence of serum in ITS supplemented media [7, 29, 30]. ASM cells cultured from dystrophic (GRMD) and normal (GR) animals were subjected to serum deprivation for 7 days and were stained for dystrophin (in green) using fluorescence labeled antibodies (Fig. 1A,B). GRMD tracheal ASM cells showed no staining for dystrophin confirming that animals were dystrophic (Fig. 1B); while, the cells from control animal showed positive staining for dystrophin. To further confirm these results immunoblotting was performed on lysates harvested from ASM cells at day 0 (proliferative phenotype) and day 7 (contractile phenotype) (Fig. 1C). It further confirmed that ASM cells from GRMD animals lacked dystrophin.

ASM cells cultures from dystrophic and normal animals are morphologically similar

As shown in Fig 1, ASM cells grown in absence of serum with ITS become elongated after 7 days.

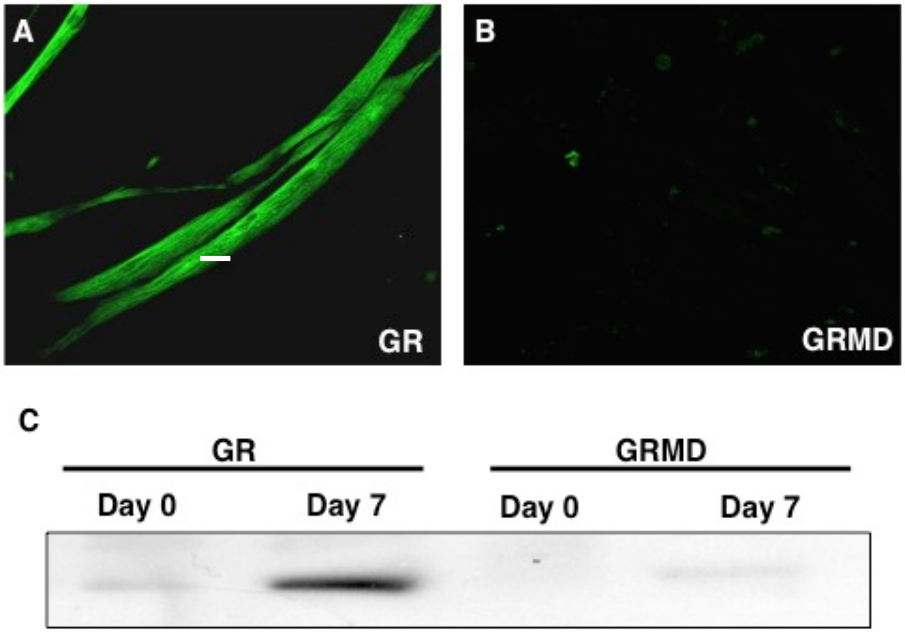

Figure 1. ***Dystrophin is absent in dystrophic ASM cells.*** Primary tracheal smooth muscle cells were grown to confluence from golden retriever (GR) **(A**) and golden retriever muscular dystrophy (GRMD) **(B)** animals and were serum starved in F12+ITS(1%) media to induce a contractile phenotype in culture. Cells were then stained for dystrophin (in green) and images were taken using a confocal laser scanning microscopy. Scale bar, 100μm. Images are representative of at least 3 different primary myocyte cultures Similarly western blotting was done for dystrophin **(C)** on primary tracheal smooth muscle cells from these animals at day 0 (proliferative phenotype) and day 7 (contractile phenotype).

ASM cells from dystrophic (GRMD) and normal (GR) animals were grown to confluence in DMEM supplemented with FBS (Day 0, Fig. 2A,C). Then serum was withdrawn and cells were subjected to a F12 media supplemented with ITS which promotes the contractile phenotype in culture (Day 7, Fig. 2B,D). The phase contrast pictures suggest that the morphology of cells lacking dystrophin was not different from the ones having functional dystrophin protein. These data suggest that dystrophin does not affect the morphology of the ASM cells in culture.

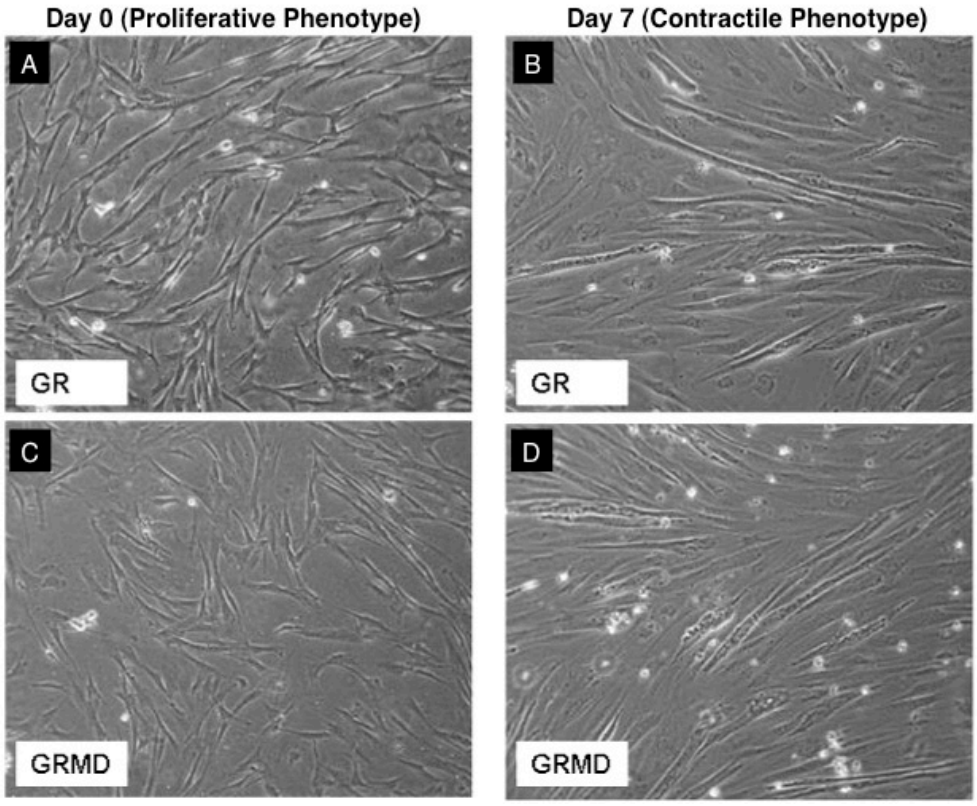

Figure 2. ***Dystrophin does not affect the morphology of the ASM cells.*** Primary tracheal smooth muscle cells were grown to confluence from golden retriever (GR) and golden retriever muscular dystrophy (GRMD) animals and then were serum starved in F12+ITS(1%) containing media to induce a contractile phenotype in culture. Phase contrast pictures were taken at day 0 (proliferative phenotype) for GR **(A)** & GRMD **(C)** and later at day 7 (contractile phenotype) for GR **(B)** & GRMD **(D)**. Images are representative of at least 3 different primary myocyte cultures.

Effect of dystrophin on airway smooth muscle cell phenotype

In canine and human ASM cells subjected to prolonged serum starvation, phenotype maturation occurs in a select subset of myocytes that become characteristically elongate, reacquire responsiveness to contractile agonists, and accumulate abundant contractile marker proteins such as smMHC, calponin, desmin and also forms a network of stress fibers [7, 29, 31, 32]. Thus, we assessed whether accumulation of these markers and stress fiber formation induced by serum deprivation was directly associated with dystrophin.

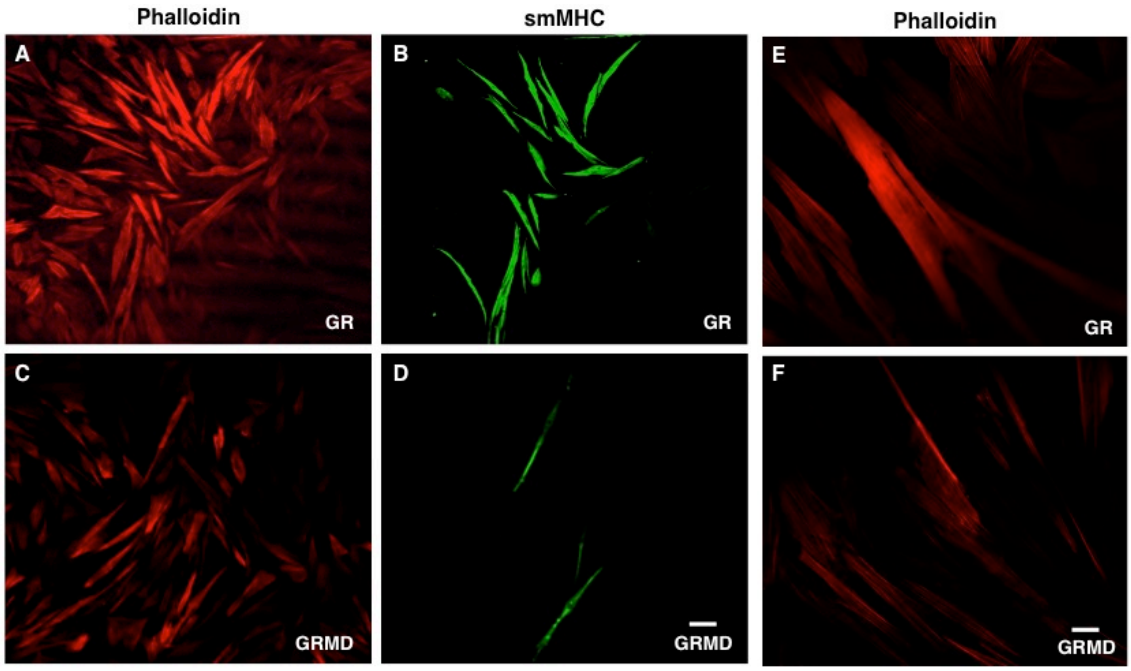

Figure 3. ***Effect of dystrophin on stress fibers and contractile marker myosin heavy chain.*** Primary cultured tracheal smooth muscle cells from (GR & GRMD) animals were grown to confluence on glass coverslips and subjected to 7 day culture in serum deficient conditions. Thereafter myocytes were fixed, and double labeled for **(A,C)** phalloidin, and **(B,D)** smMHC. Isotype-matched mouse IgG or rabbit antiserum was used for negative controls (not shown). Antibodies conjugated with TxR or FITC were used to label actin filaments (red) and smMHC (green) respectively. Images were obtained by confocal laser scanning microscope. Higher magnification of Fig. A & C for stress fibers are shown in panel E and F showing fluorescent phalloidin (red) marking actin filaments in GR **(E)** and GRMD **(F).** Images are representative of at least 3 different primary human tracheal myocyte cultures. Scale bars, 100 μm (A-D) and 20μm (E-F).

Using fluorescence immunocytochemistry, after 7-day serum deprivation we double-labeled primary cultured ASM cells (GRMD and GR) with smMHC and phalloidin (Figure 3). Consistent with previous reports, myocytes exhibited phenotype heterogeneity, with 15-20% of ASM cells acquiring a contractile phenotype, as evidenced by a dramatic accumulation of smMHC. Notably, the maturation of individual ASM cells to a contractile phenotype was uniquely associated with a dramatic increase in staining for smMHC and actin in cells having dystrophin (Fig. 3 A,B), whereas there was little labeling for smMHC and actin in cells devoid

of dystrophin (Fig. 3 C,D). Moreover, at higher magnification, the stress fiber formation was dramatically reduced in cells lacking dystrophin (Fig 3F) when compared to the control (Fig. 3E); suggesting that dystrophin is partly responsible for the dynamics of actin network in the cell. These results demonstrate, at the single-cell level, an association between the acquisition of a contractile phenotype and expression of dystrophin.

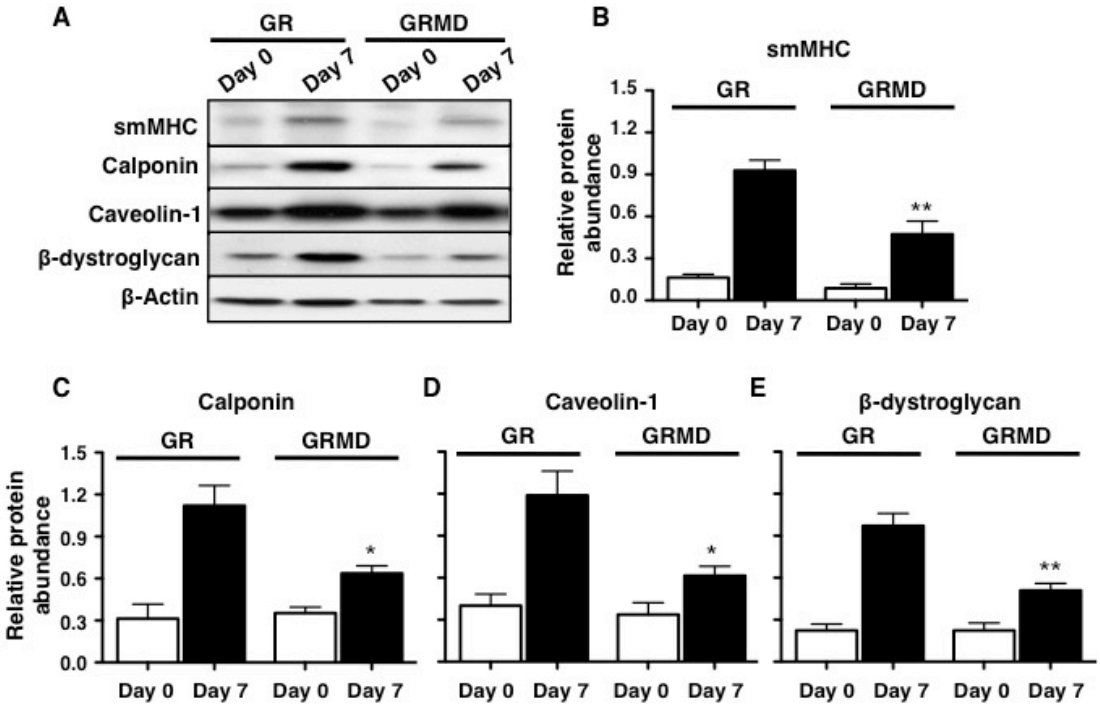

Figure 4. ***Loss of dystrophin reduces contractile protein markers.*** For all panels, *day 0* represents protein lysates obtained from serum-fed confluent cultures, and *day 7* represents protein lysates obtained from confluent primary tracheal smooth muscle cell cultures (obtained from GR & GRMD animals) after 7-day serum deprivation, with medium changed every 48 h. **A:** representative Western blots typical of those obtained for 3 different cell culture experiments. **B:** densitometry analysis of the effects of serum deprivation on smMHC **(B)**, calponin **(C)**, Caveolin-1 **(D)** and β-dystroglycan **(E)** in GR and GRMD tracheal smooth muscle cells are shown. For all histograms protein abundance was corrected for equal loading and normalized relative to β-actin abundance. Data shown represent means ± SE from experiments using 3 different primary tracheal smooth muscle cells. Statistical comparisons shown were performed by 1-way ANOVA with Tukey's multiple comparison test. **P* < 0.05, ***P* < 0.01, for GR day 7 versus GRMD day 7.

Loss of dystrophin reduced expression of airway smooth muscle contractile phenotype markers

Since we showed that expression of DGC is associated with phenotype maturation of ASM cells *in vitro* [7], here we investigated whether loss of dystrophin affects the accumulation of contractile phenotype markers. ASM cells from both control (GR) and dystrophic (GRMD) animals were grown to confluence and subjected to a serum deprivation protocol for seven days. As it can be seen in the panel for western blotting (Fig. 4A) and quantification of individual proteins in Fig. 4B-E; the loss of dystrophin in GRMD cell cultures was associated with significant reduction in markers of contractile phenotype namely smMHC and calponin when compared to cells having dystrophin (GR) (Fig. 4B,C). Furthermore, there was also a significant reduction in the protein abundance of caveolin-1 and β-dystroglycan (proteins recently shown to be associated with contractile phenotype [7]). These results clearly suggest that dystrophin has a role in expression of contractile phenotype markers in ASM cells.

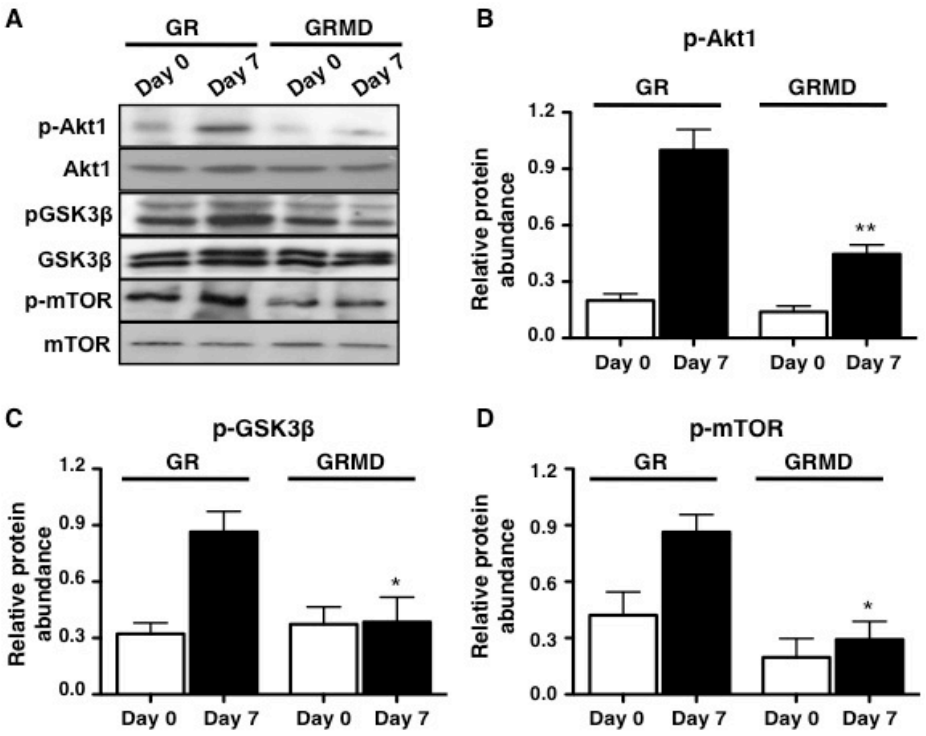

Figure 5. ***Loss of dystrophin reduces induction of PI3K-signaling.*** For all panels, *day 0* represents protein lysates obtained from serum-fed confluent cultures, and *day 7* represents protein lysates obtained from confluent primary tracheal smooth muscle cell cultures (obtained from GR & GRMD animals) after 7-day serum deprivation, with medium changed every 48 h. **A:** representative Western blots typical of those obtained for 3 different cell culture experiments. **B:** densitometry analysis of the effects of serum deprivation on p-Akt1 **(B)**, p-GSK3β **(C)** and p-mTOR **(D)** in GR and GRMD tracheal smooth muscle cells are shown. For all histograms protein abundance was corrected for equal loading and normalized relative to β-actin abundance. Data shown represent means ± SE from experiments using 3 different primary tracheal smooth muscle cells. Statistical comparisons shown were performed by 1-way ANOVA with Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, for GR day 7 versus GRMD day 7.

Dystrophin affects PI3K signaling in airway smooth muscle cells

Previous studies have demonstrated that signaling through the phosphatidylinositide-3-kinase (PI3K) pathway, including Akt1, p70S6 kinase, and mTOR, is required for ASM maturation, hypertrophy, and concomitant accumulation of contractile protein markers [7, 31, 33]. Here we investigated whether dystrophin affects PI3K signaling in ASM cells. Similarly to the previous figure, ASM cells were subjected to serum deprivation

protocol for 7 days and lysates were collected at day 0 and day 7. As seen in the panel for westerns (Fig. 5A), loss of dystrophin was associated with the reduction of activation of key signaling proteins namely p-Akt-1, p-GSK3β and p-mTOR. Densitometric analysis showed that the reduction in the phosphorylation of these signaling proteins in cells lacking dystrophin (GRMD) was significant when compared to the ones having normal dystrophin expression (GR) (Fig. 5B-D). These results clearly demonstrate that dystrophin is associated with the expression of contractile phenotype and also regulate the key signaling machinery required for this process to occur *in vitro*.

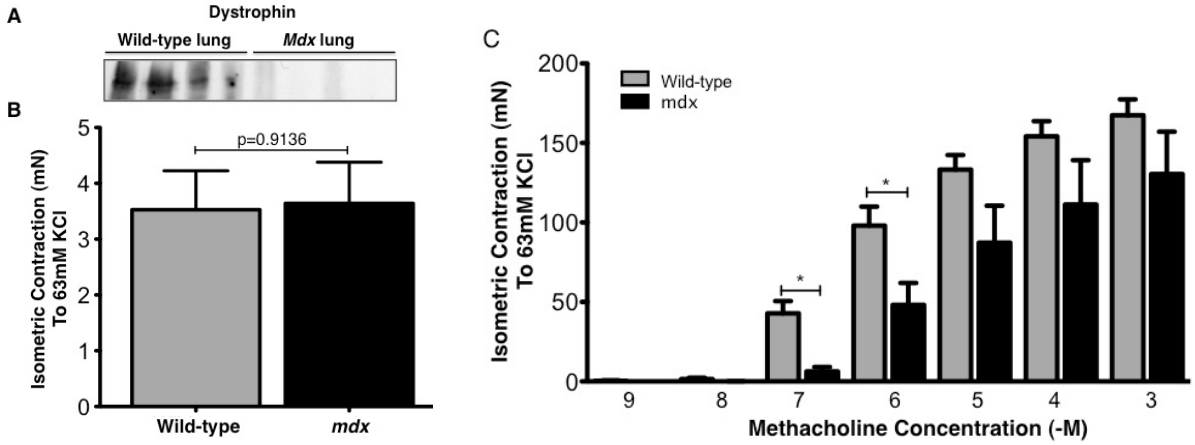

Figure 6. ***Loss of dystrophin reduces tracheal contractility.*** **A:** representative western blots typical of those obtained for 4 different *mdx* mice lungs showing dystrophin protein is absent in *mdx* mice. **B:** tracheal rings from *mdx* and wild-type mice were isolated and sliced to obtain 4 ~equal-sized segments containing 3-4 cartilage rings, isometric force responses were measured with 63 mM KCl substituted with K-H solution **(B)**. Tracheal rings from at least 4-6 mice were used to obtain the above results. Comparing the 2 groups, unpaired t-test indicated a *p-value of* 0.9136; n.s.. **C:** tracheal rings from *mdx* and wild-type mice were isolated and equilibrated for 90-120 min with intermittent (~20 min) instillation of 63mM KCl-substituted K-H to obtain a resting tension at ~0.6 mN. Concentration response studies with methacholine demonstrated significantly reduced responsiveness of *mdx* preparations at lower concentrations compared to wild-type **(C)**. Statistical comparisons shown were performed by 1-way ANOVA with Tukey's multiple comparison test. **P* < 0.05, for *mdx* versus wild-type.

Role of dystrophin in airway smooth muscle contraction

To further assess the role of dystrophin in determining the ASM contraction *ex vivo*, we employed mice having a spontaneous mutation in dystrophin gene (*mdx* mice), thus lacking functional dystrophin protein. As shown in Fig. 6A the lung homogenates from *mdx* mice showed no dystrophin when compared to the genetic controls BL10 (wild-type) mice. We then studied the isometric contraction of tracheal rings obtained from these mice. There was no change in the development of basal isometric force induced by KCl in *mdx* and wild-type mice (Fig. 6B). The cumulative dose-response-curve to muscarinic (M3) receptor agonist methacholine (MCh) in *mdx* and wild-type trachea showed that there was a significant reduction of isometric force at lower concentration of MCh (0.1μM and 1.0μM) (Fig. 6C), while there was a trend in reduction in force at higher concentration of MCh but it was not statistical significant. The sensitivity to MCh was reduced significantly in *mdx* mice trachea as evident by increase in the EC_{50} values ($EC_{50wild\text{-}type}$= 0.519μM ±0.098 as compared to EC_{50mdx}=2.48μM ±0.16) of *mdx* mice.

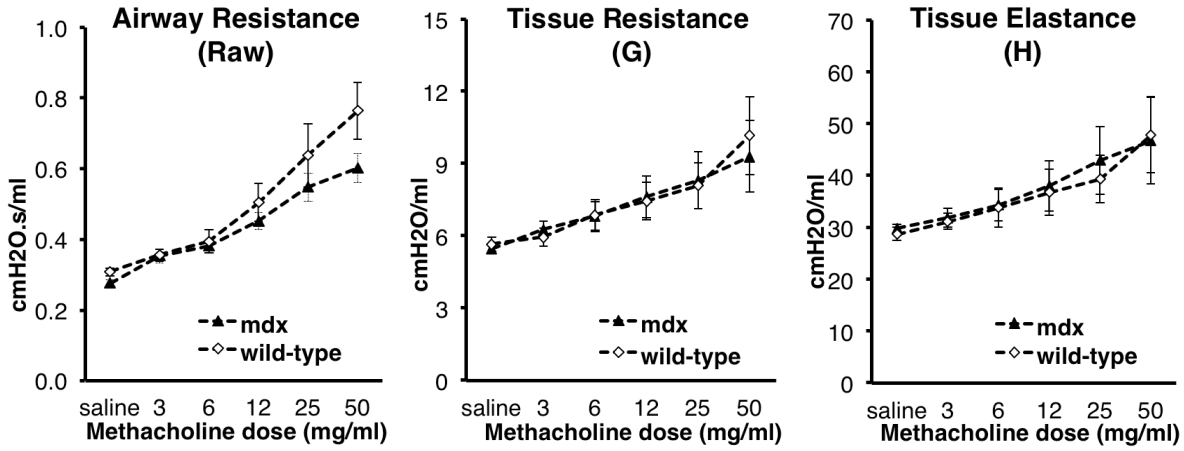

Figure 7. ***Effect of dystrophin on lung function.*** Wild-type or *mdx* mice tracheas were dissected and connected to a flexiVent small animal ventilator (Scireq Inc. Montreal, PQ). Mice were ventilated with a tidal volume of 10 ml/kg body weight, 150/minute. Mice were then subjected to an increased dose of nebulized methacholine (MCh) challenge protocol to assess characteristics of respiratory mechanics. For MCh challenge, ~ 30μL of saline containing from 0-50 mg/ml MCh was delivered over

10 seconds using an in-line ultrasonic nebulizer. By fitting respiratory mechanical input impedance (*Zrs*) to the constant phase model, flexiVent software calculated conducting airway resistance (Raw) **(A)**, peripheral tissue & airway resistance (G) **(B)**, tissue elastance or stiffness (H) **(C)**; each parameter was normalized according to body weight. Values for each parameter were calculated as the mean of all 20 perturbation cycles performed after each MCh challenge. Statistical comparisons shown were performed by 1-way ANOVA with Tukey's multiple comparison test. $*P < 0.05$, was considered significant for *mdx* versus wild-type. Data shown is the ±mean of 9-10 *mdx* and wild-type mice.

Role of dystrophin on lung physiology

Finally to determine the physiological role of dystrophin we used *mdx* and wild-type mice and performed lung function using a small animal ventilator (flexi vent). Airway resistance (R), tissue resistance (G) and tissue elastance (H) was determined using increasing doses of aerosolized MCh. There was a strong trend in reduction of airway resistance in *mdx* mice with increasing doses of MCh (nearing significance at the highest dose of MCh) when compared to wild-type mice (Fig. 7A); while there was no difference in tissue resistance (Fig. 7B) and elastance (Fig. 7C) between the two mice strains. The peak airway resistance (at 50mg/ml MCh) was reduced by ~25% in *mdx* mice when compared to the wild-type. These results point towards the role of dystrophin in determining lung physiology and airway responsiveness.

Discussion

We earlier showed that DGC subunits are abundantly expressed in a contractile ASM cells and tissue, and their expression is associated and dependent upon phenotype switching *in vitro* [7]. We recently showed that β-dystroglycan - a central subunit of DGC, interacts with caveolin-1 in ASM cells and tissue, and this interaction is important for mobilization of intracellular calcium by contractile agonists [34]. It is well known that caveolae

microdomains in ASM cell harbor key signaling proteins required for mobilization of intracellular calcium while its principal protein caveolin-1 plays an important role in modulating ASM phenotype and function [35-40]. Moreover, in smooth muscle, dystrophin colocalizes with caveolin-1 and occupies complementary distribution with adheren junctions [5]. Thus, we investigated its role in ASM phenotype and function. Our data indicate that expression of dystrophin expression is associated with a contractile phenotype in ASM cells, and its absence is associated with suppression of phenotype maturation. Notably, loss of dystrophin reduced the activation of the PI3K-Akt-mTOR signaling pathway, which is required for accumulation of contractile protein markers. Our studies demonstrate that dystrophin has an important role in ASM contraction and our data provide a first insight to its role in lung physiology.

The plasma membrane of contractile smooth muscle cells is highly ordered, consisting of repeating longitudinal rib-like arrays of caveolae microdomains and adherens junctions [6, 41-44]. North and colleagues [5] provided initial evidence that caveolae microdomains are marked by the presence of both caveolin-1 and dystrophin. This has contributed to the understanding of the organization of cytoskeleton in smooth muscle but there has been no investigation assessing the exact role of dystrophin in a contractile ASM cell, both in terms of phenotype and function in ASM cell. It has been shown earlier that dystrophin along with other DGC subunits is a marker of contractile phenotype in ASM [7], but our new data using dystrophic animals show that dystrophin loss is associated with the concomitant reduction of contractile protein markers upon phenotype switching. Our

studies are the first to describe the role of dystrophin loss in phenotype maturation of ASM cells. We have recently shown that actin cytoskeleton plays an important in calcium homeostasis; and dystrophin through β-dystroglycan provides a link between ECM and underlying actin cytoskeleton [34]. Our studies with dystrophin deficient ASM cells demonstrate that dystrophin is crucial for the formation of stress fibers in the cell as loss of dystrophin also resulted in significant reduction in the stress fibers formation.

It has been shown that PI3K-signaling is critical for contractile phenotype maturation, and for myocyte elongation and hypertrophy of ASM cells [31, 33]. This pathway is also important in skeletal muscle, because myotube hypertrophy and the accumulation of contractile proteins require PI3K-Akt1-mTOR and PI3K-Akt1-GSK3 signal transduction pathways [45, 46]. We previously showed that accumulation of dystrophin along with other DGC subunits is regulated by mechanisms such as laminin-integrin binding and induction of PI3K-signaling in ASM cells [7]. We investigated whether loss of dystrophin affects the PI3K signaling in a contractile myocyte. Our results show that loss of dystrophin (in dystrophic animals) resulted in suppression of PI3K and its downstream signaling. These results clearly suggest that in a contractile cell dystrophin is required for induction of PI3K-signaling, which is an essential pathway, required for ASM cells to undergo phenotype maturation.

It is also known that DGC complex plays an important role in stabilizing skeletal muscle as it provides support to the muscle during repeated contraction and relaxation [47]. Studies are lacking that have directly assessed the role of the DGC or dystrophin *per se* in

mechanical load bearing in smooth muscle cells, though a recent report from Dye and colleagues [48] reveals that carotid arteries from *mdx* and δ-sarcoglycan knock out mice exhibit decreased pressure-induced distensibitity with increased circumferential and axial stress. The absence of dystrophin in portal veins from *mdx* mice leads to reduce stretch-induced myogenic contractile responses [49]. Notably, ectopic smooth muscle-specific expression of dystrophin can improve aberrant vasoregulation in *mdx* mice [50]. Morel et al [51] reported that decreased mechanical activity of duodenal smooth muscle in *mdx* mice is due to reduced type 2 ryanodine receptor expression that compromises sarcoplasmic reticulum calcium release. Cohn et al [52] showed that cardiac myopathy associated with coronary artery vasospasm in sarcoglycan knock out mouse models could be prevented by verapamil, a general Ca^{2+} channel blocker with vasodilatory properties. Collectively these studies lead us to investigate the role of dystrophin in tracheal smooth muscle contraction in *mdx* mice. Our results clearly indicate that dystrophin plays an important role in maintaining tracheal smooth muscle sensitivity to contractile agonists as loss of dystrophin in *mdx* mice resulted in reduction of sensitivity to methacholine (MCh). However the maximal contraction of trachea was unaffected showing the physiological relevance at lower concentration of MCh.

A great deal of what is known about dystrophin structure-function has come from studies of a variety of dystrophin-deficient animals [17, 18], but by far the most prolific model has been the *mdx* mouse, first described in 1984 by Bulfield et al [16]. The skeletal muscles of *mdx* mice show a marked susceptibility to lengthening contraction-mediated

force decrements [53-56] (26– 32). Contractile dysfunctions are also evident in cardiac muscles of the *mdx* mouse [57, 58]. These observations suggest that dystrophin is important in excitation-contraction coupling of skeletal and cardiac muscles and lead us to investigate its role in lung physiology. Our results with *mdx* mice are suggestive of the fact that dystrophin might be important in determining lung function. Because the reduction in airway resistance in *mdx* mice did not achieve a statistical significance but showed a strong trend with increasing doses of MCh (the airway resistance was reduced by a quarter in *mdx* mice when compared to wild-type). Thus, we at this point cannot state that presence of dystrophin is a key determinant of lung function. Further studies are warranted in this direction to follow the mice at an older age, as it is known that dystrophic phenotype is progressive in nature.

In summary, our study investigated the role of dystrophin in phenotype maturation of ASM cells and also looked in to the mechanism on which dystrophin can affect the key signaling required for development of a contractile phenotype *in vitro*. Our tracheal smooth muscle contraction using *mdx* mice clearly showed that dystrophin is required for maintaining normal tone to muscarinic agonist *ex vivo.* On the contrary, these findings failed to translate completely in an intact animal where we saw a trend in the decline of the lung function in the *mdx* mice suggesting that further careful studies are warranted to elucidate the mechanisms of airway smooth muscle contraction and lung function using other pharmacological approaches.

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PS drafted and produced 100% of the experiments required for the realization of this manuscript.

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Chapter 8

Epithelium-dependent modulation of responsiveness of airways from caveolin-1 knockout mice is mediated through cyclooxygenase-2 and 5-lipoxygenase

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Abstract

Acute silencing of caveolin-1 alters receptor-mediated airway smooth muscle contraction. Cyclooxygenase-2 a key enzyme in prostaglandin biosyntheis colocalizes with caveolin-1, which inhibits cyclooxygenase-2 degradation. Here we tested the hypothesis that lack of caveolin-1 modulates epithelium mediated control of tracheal contraction through a mechanism associated with altered arachidonic acid cascade signaling due to changes in cyclooxygenase-2 abundance and activity. Using tracheal rings from caveolin-1 knockout (KO) and wild type mice (B6129SF2/J), we measured isometric contraction to methacholine and used PCR, immunoblotting, and immunohistology to monitor expression of pertinent proteins. Tracheal rings from caveolin-1 KO and wild type mice exhibited similar responses, but the cyclooxygenase-2 inhibitor, indomethacin, increased caveolin-1 KO responses to methacholine. Treatment with the phospholipase A_2 inhibitor, eicosatetraynoic acid, to prevent formation of substrates for both cyclooxygenase-2 and 5-lipooxygenase had no effect on wild type or caveolin-1 KO tissues. Indomethacin-mediated hyperreactivity was however normalized by the LTD_4 receptor antagonist (Montelukast) and 5-lipoxygenase inhibitor (Zileuton). Notably, the potentiating impact of Indomethacin on caveolin-1 KO responses to methacholine was also completely blocked by epithelial denudation. Immunoprecipitation, immunoblotting and qPCR showed that cyclooxygenase-2 binds caveolin-1 in wild type lungs. Also, abundance of both cyclooxygenase-2 and 5-lipooxygenase was significantly higher in caveolin-1 KO tracheas and lungs, but cyclooxygenase-1 was similar to wild type tracheas. Notably, in tracheas, epithelium removal quenched strain-related differences in cyclooxygenase-2 and 5-lipoxygenase abundance. Selective indomethacin induced hypercontractility of caveolin-1 KO tracheas is

linked to changes in epithelial control underpinned by increased expression of cyclooxygenase-2 and 5-lipoxygenase.

Introduction

Caveolae are flask-shaped invaginations of the plasma membrane in variable numbers in different cell types, being most prominent in vascular endothelial cells, adipocytes, fibroblasts, epithelial cells and smooth muscle cells. They segregate receptors and signaling intermediates and form a micro-environment where local kinases and phosphatases can modify downstream signaling events and cell responses [1-3]. Caveolin-1 (Cav-1), the primary structural protein of caveolae plays a key role in orchestrating activation of pathways that underpin cell proliferation, migration, and contraction [4, 5]. In smooth muscle caveolae are enriched in Ca^{2+}-handling and binding proteins, and trimeric G-proteins [6-9]. This is functionally important, as acute disruption of plasma membrane caveolae suppresses G-protein-coupled receptor (GPCR) mediated contraction in airway smooth muscle (ASM) [10-12]. ASM contraction in situ is regulated both by intrinsic cellular pathways and by mediators released from neighboring cells, such as the airway epithelium. Thus, despite a clear intrinsic role for caveolins in regulating isolated ASM cell contraction, there remains a need for systematic assessment of mechanisms that integrate contraction of intact multicellular airways from Cav-1 knock out (KO) mice.

Arachidonic acid metabolites play an important role in cellular physiology, and their aberrant biosynthesis via cyclooxygenase (COX)-2 or 5-lipoxygenase (LO) pathways has been linked with worsened allergic and inflammatory diseases [13-15]. The increased contractile function of ASM as seen in chronic airway diseases can be regulated by the airway epithelium,

which is a rich source of lipid mediators that regulate ASM tone and contractility [16-19]. A number of groups have shown that epithelium-associated changes in COX-2 activity and levels of endogenous PGE2 underpin changes in airway reactivity and bronchial inflammation in experimental models of asthma [20-22]. Recent research also suggests a role for Cav-1 in chronic lung diseases such as asthma, COPD and pulmonary fibrosis [23-25]. Moreover, allergen-naïve Cav-1 KO mice exhibit an altered lung phenotype that likely affects airway physiology [26]. Interestingly, though reduced Cav-1 expression in whole lung has been demonstrated in allergen challenged mice [27], the opposite effect has recently been reported for ASM upon allergen challenge of guinea pigs [23]. COX-2 enzyme is colocalized with Cav-1 in caveolae, and binding of COX-2 with Cav-1 targets the former for degradation via ER-associated mechanisms [28, 29]. These studies indicate a novel function for Cav-1 in controlling both COX-2 expression and activity.

On this basis we used Cav-1 KO mice to test the hypothesis that lack of Cav-1 modulates epithelium mediated control of tracheal contraction through a mechanism assocaited with altered arachidonic acid cascade signaling due to changes in COX-2 abundance and activity. We found that methacholine (MCh) responses of tracheal preparations from Cav-1 KO mice were highly sensitive to pharmacological COX-2 manipulation, an effect that could be reversed using 5-LO pathway inhibitors, but that was not observed in rings from wild-type mice. We also found that removal of the epithelium ameliorated COX-2 sensitivity in Cav-1 KO preparations, and that COX-2 and 5-LO abundance was markedly increased in the airway epithelia of Cav-1 KO mice. These data suggest that in the epithelium Cav-l regulates key enzymes associated with the

arachidonic acid cascade, and that this acts as a significant determinant of agonist-induced constriction of intact airways.

Results

Inhibiting COX-2 selectively induces hyperreactivity in tracheas from Cav-1 KO mice

In the absence of indomethacin (INDO), tracheal rings from Cav-1 KO mice and the wild type controls demonstrated statistically equivalent responses to MCh (Fig. 1A). However, in the presence of INDO (3 µM), tracheal rings from Cav-1 KO mice developed hyperreactivity to MCh whereas responses of tracheal rings from wild type mice were unaffected (Fig. 1A). In contrast to the selective effects of INDO on Cav-1 KO tissues, treatment with either cysteinyl leukotreince receptor (CysLTR1) antagonist Montelukast (10 µM) or the 5-LO inhibitor, Zileuton (10 µM) alone was without effect on MCh responses of tracheal rings, regardless of mouse strain (Fig. 1B and 1C).

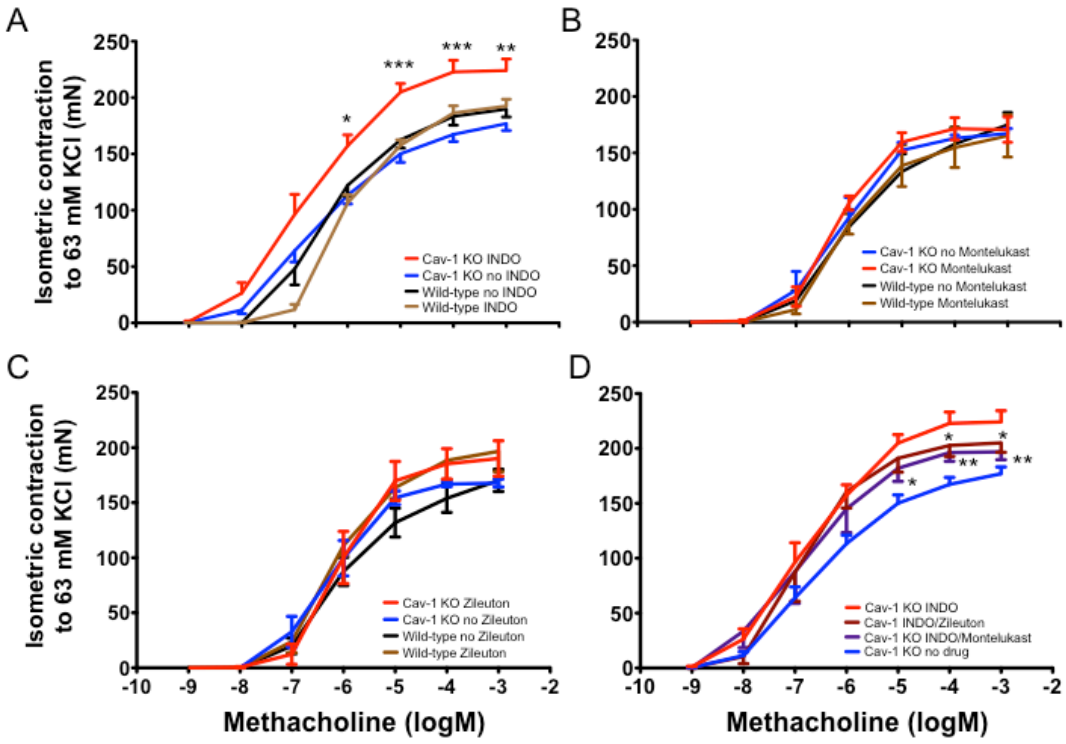

Figure 1. ***MCh-induced isometric force produced by Cav-1 KO and wild type tracheal rings in presence of epithelium.*** Trachea from Cav-1 KO and wild type mice were isolated and sliced to obtain 4 ~equal-sized segments containing 3-4 cartilage rings. Tissues were equilibrated for 90-120 min with intermittent (~20 min) instillation of 63mM KCl-substituted K-H to obtain a stable resting tension at ~0.6 mN. Isometric force was measured with increasing cumulative concentrations of MCh (10^{-9} M to 10^{-3} M). (**A**) Some tissues were incubated with INDO (DMSO used as vehicle) (n = 12 rings from 6 Cav-1 KO and n = 9 rings from 5 wild type mice) while others received DMSO alone (n = 21 rings from 13 Cav-1 KO and n = 31 rings from 17 wild type mice). With INDO treatment, significantly greater responsiveness of Cav-1 KO preparations occurred between 10^{-6} M – 10^{-3} M MCh compared to INDO-free Cav-1 KO tissues *(*One-way analysis of variance, **p*<0.05; ***p*<0.01; ****p*<0.001*).* (**B**) Tracheal rings were incubated for 30 min with Montelukast *(*10 μM*)* alone prior to performing MCh concentration response studies. No significant differences were seen between Cav-1 KO and wild type groups *(*n = 8 rings from 4 mice; One-way ANOVA). (**C**) Tracheal rings were incubated for 30 min with Zileuton *(*10 μM*)* alone prior to performing MCh concentration response studies. No significant differences were seen between Cav-1 KO and wild type groups *(n* = 7 rings form 4 mice). (**D**) Tracheal rings were incubated for 30 min with INDO in the presence or absence of Montelukast or *Zileuton (*10 μM*) prior* to performing MCh concentration response studies. Compared to INDO treated Cav-1 KO rings, significantly decreased responses were observed between Cav-1 KO groups co-treated with Montelukast (n = 8 rings from 8 mice) or Zileuton (n = 4 rings from 4 mice) (One-way analysis of variance, **p*<0.05; ***p*<0.01).

To further establish the role of arachidonic acid signaling as a determinant of tracheal responses we treated preparations with eicosatetraynoic acid (ETYA) (10 μM), an upstream inhibitor of the phospholipase A_2 pathway to deny formation of both COX-2 and 5-LO metabolites; ETYA was without effect on basal tone or MCh responsiveness in tissue from both mouse strains (not shown), suggesting that the effects of INDO on Cav-1 KO tissue may be the result of a unique disruption of intrinsic balance between COX-2 and 5-LO arms of the cascade in airways that lack Cav-1. To assess this possibility, in Cav1 KO tracheal rings treated with INDO we also added Montelukast (10 μM) or Zileuton (10 μM): both co-treatments significantly reversed INDO-induced hyperresponsiveness (Fig. 1D). These data suggest that COX-2 inhibition in Cav-1 KO, but not wild type, tissues, may shunt a functionally significant fraction of PLA_2 metabolites through the 5-LO pathway to promote contractile responses.

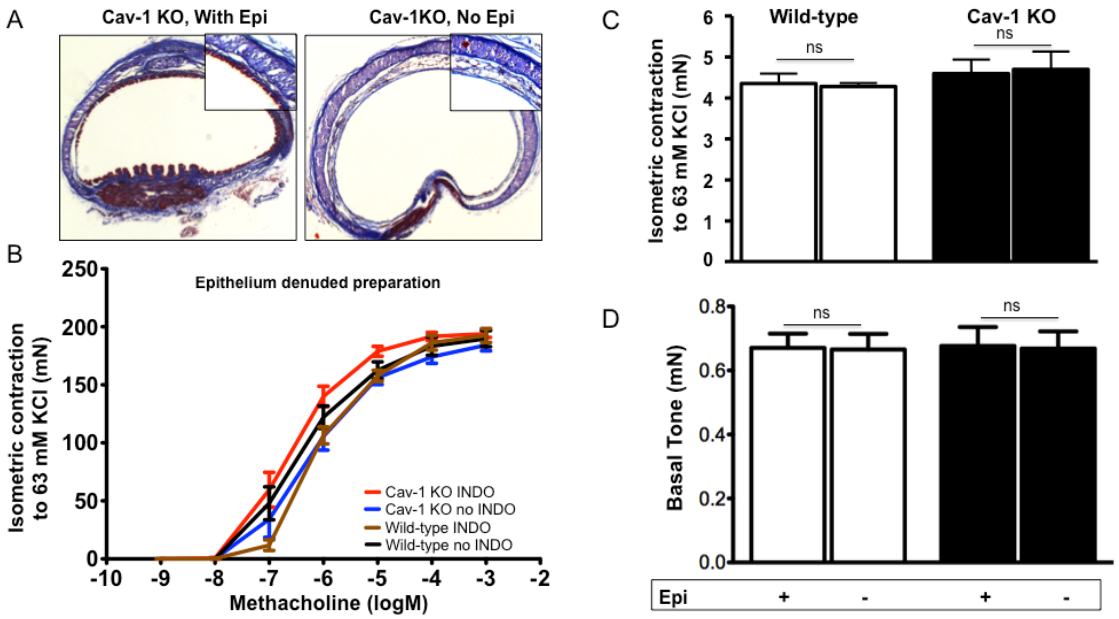

Figure 2. ***Epithelium denudation and KCl induced isometric force in tracheal rings from Cav-1 KO and wild type mice***. The epithelium of excised tracheal segments was removed by threading with surgical silk then rolling the rings three revolutions on a wetted paper towel. (**A**) Mason's Trichrome staining of randomly selected tracheal preparations showing result of epithelial denudation. Tracheal specimens from Cav-1 KO mice are shown. (**B**) Isometric MCh concentration response studies were performed using epithelium denuded tracheal segments from Cav-1 KO (9 rings from 6 mice) and wild type (9-12 rings from 5-7 animals) mice. After equilibration, rings were incubated for 30 min with either: DMSO or INDO then isometric force was measured with increasing concentration of MCh (10^{-9} M to 10^{-3} M). Results shown are the mean ± SEM., INDO had minimal effects on responsiveness to MCh (One-way anova, p>0.05). (**C**) Receptor independet force generating capacity of tracheal rings with (+) and without (-) epithelium (epi) from wild type and Cav-1 KO mice was assessed based on isometric force measured after 63 mM KCl substituted with K-H solution treatment. For each gropup, 5-7 tracheal rings from at least 3-4 mice were studied. There were no significant differences between the groups (One-way anova, *p*>0.05). (**D**) Histogram comparing basal tone in tracheal rings from Cav-1 KO and wild type mice with (+) and without (-) epithelium. For each gropup, 5-7 tracheal rings from at least 3-4 mice were studied. There were no significant differences between the groups (One-way anova, *p*>0.05).

INDO-induced MCh hyperreactivity in Cav-1 KO airways is epithelium dependent

As arachadonic acid signaling is prominent in epitheial cells, tracheal rings from Cav-1 KO and wild type mice were denuded of epithelium (Fig. 2A) and effects on responses to MCh in the absence and presence of INDO measured (Fig. 2B). MCh responsiveness in the absence of epithelium was indistinguishable between strains and was not different from epithelium-intact

preparations. In contrast to our studies with epithelium-intact preparations, the addition of INDO had no affect on MCh responses of Cav-1 KO preparations. These data suggest hyperreactivity unmasked by COX-2 inhibitor in Cav-1 KO tissues is likely the result of the absence of caveolin-1 from epithelial cells, but not a change in contractile capacity of the ASM *per se*. Consistent with this conclusion, we found that KCl-induced isometric force generated by epithelium-intact or -denuded isolated tracheal rings from each strain showed no statistical difference in maximal response to KCl (Fig. 2C). Moreover, there was no strain dependent difference in the baseline tone of epithelium-intact or-denuded tracheal rings (Fig. 2D).

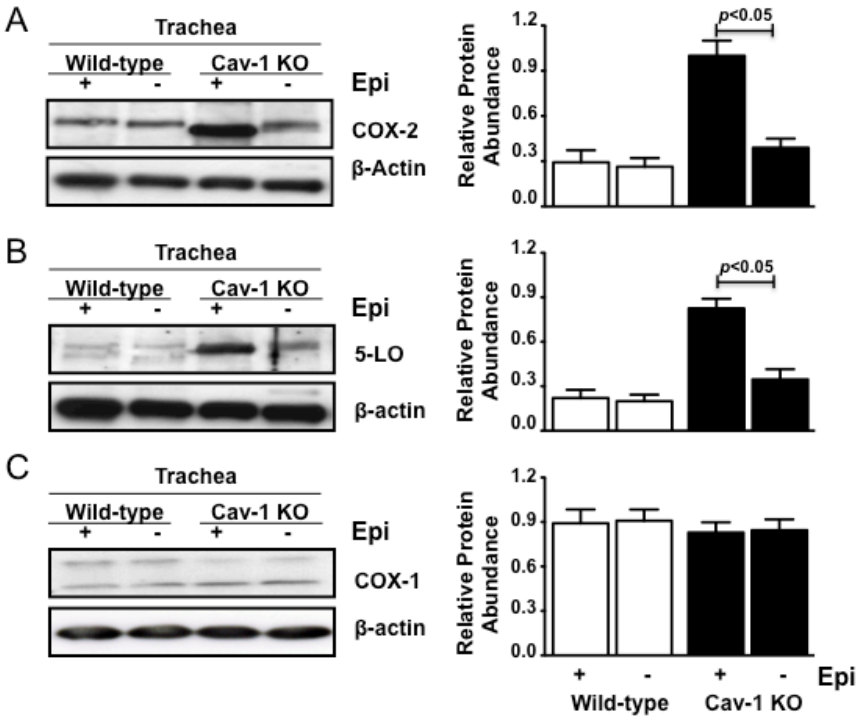

Figure 3. ***Increased expression of COX-2 and 5-LO in tracheas from Cav-1 KO mice.*** (**A**) Representative protein immunoblot and corresponding densitomtry analysis (right column) for COX-2, (**B**) 5-LO, and (**C**) COX-1 in lysates of tracheas with (+) and without (-) epithelium (epi) from Cav-1 KO and wild type mice. Densitometry results represent replicates of scans of samples from 12-16 tracheal rings pooled from 3-4 mice from each strain (One-way analysis of variance, *p<0.05; **p<0.01).

Increased COX-2 and 5-LO expression in Cav-1 KO airways and lungs

We estimated the protein abundance of COX-2, 5-LO and COX-1 in isolated tracheas of Cav-1 KO and wild type mice by immunoblotting. Significantly higher COX-2 and 5-LO protein expression were evident in protein lysates of whole tracheas from Cav-1 KO mice compared to wild type (Fig. 3A and 3B), however, there was no difference in COX-1 abundance (Fig. 3C). We also assessed COX-2, 5-LO and COX-1 abundance in lysates from tracheas that had been denuded of epithelium: for wild type mouse tissues, epithelial removal had little impact on COX-2 or 5-LO abundance. However, in striking contrast, in epithelium denuded Cav-1 KO tracheas we measured a significantly reduced abundance of both proteins that became similar to that of epithelium-denuded or -intact wild type tissues (Fig. 3A and 3B). COX-1 abundance was unchanged in the epithelium-denuded tracheal rings in both strains (Fig. 3C).

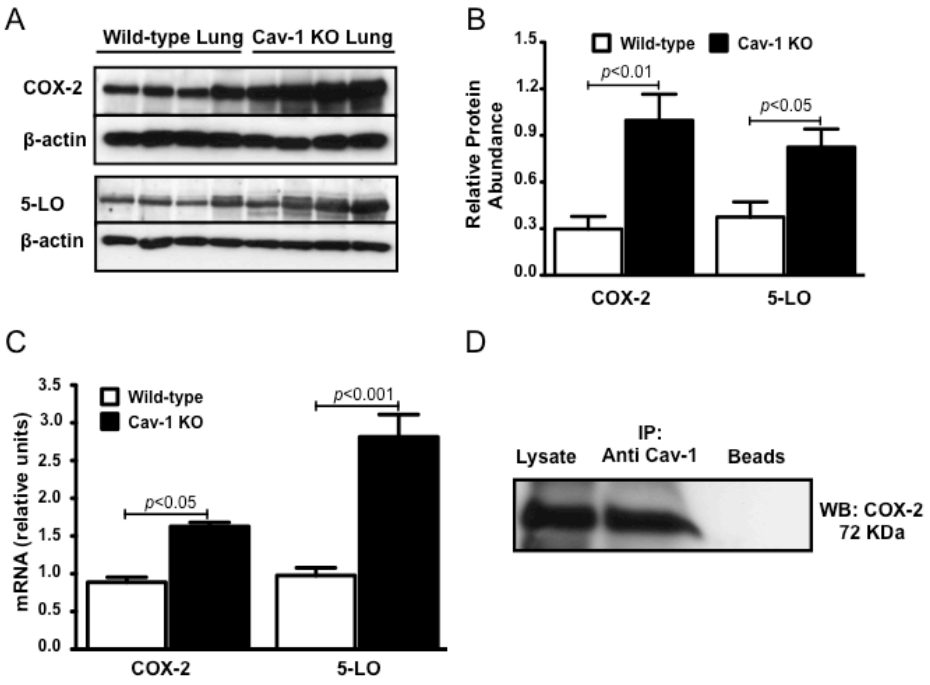

Figure 4. ***Increased expression of COX-2 and 5-LO in lungs from Cav-1 KO mice.*** (**A**) Representative western blots and **(B)** corresponding densitometric analyses showing COX-2 and 5-LO abundance in whole lung lysates from Cav-1 KO and wild type mice (n=4-5 for each group). (**C**) Results of quantitative RT-PCR for COX-2 and 5-LO mRNA in lysates from lungs of Cav-1 KO and wild type mice, 18SRNA was used as an internal control (n=4-5 for each group). **(D)** Western blot showing COX-2 abundance after immunoprecipitation (IP) with Cav-1 antibody and protein-G-conjugated Sepharose beads from lung

homogenates of wild type mice. The lane labeled 'Beads' included sample but no antibody (One-way Anova).

Table I. List of primers used for COX-2 and 5-LO in qPCR.

Gene	Forward Primer	Reverse Primer
COX-2 (PES-2)	TTGCTGTACAAGCAGTGGCAAAGG	ACAGGAGAGAAGGAAATGGCTGA
5-LO	CCATTGCCATCCAGCTCAACCAAA	TGTCTGAGGTGTTTGGTATCGCCA
18sRNA	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC

To determine whether differences in COX-2 and 5-LO expression we measured in Cav-1 KO tracheas was also evident in the lungs, we performed immunoblotting using lung lysates and found COX-2 and 5-LO protein to be significantly higher compared to wild type tissue (Fig. 4A and 4B). As Cav-1 is linked to regulation of COX-2 trascription in cancer cell lines [30], we also measured COX-2 and 5-LO mRNA using qPCR. Indeed, COX-2 and 5-LO mRNA levels in Cav-1 KO lungs were increased by 70% and 3-fold respectively compared to wild type tissue (Fig. 4C). As there is published evidence for COX-2 and Cav-1 binding [29, 31] and this is linked to COX-2 half-life [28, 30], using wild type mouse lungs we also performed immunoprecipitation of Cav-1 with subsequent immunoblotting for COX-2 (Fig. 4D). This analysis confirmed that COX-2 does associate with Cav-1 in murine lung cells.

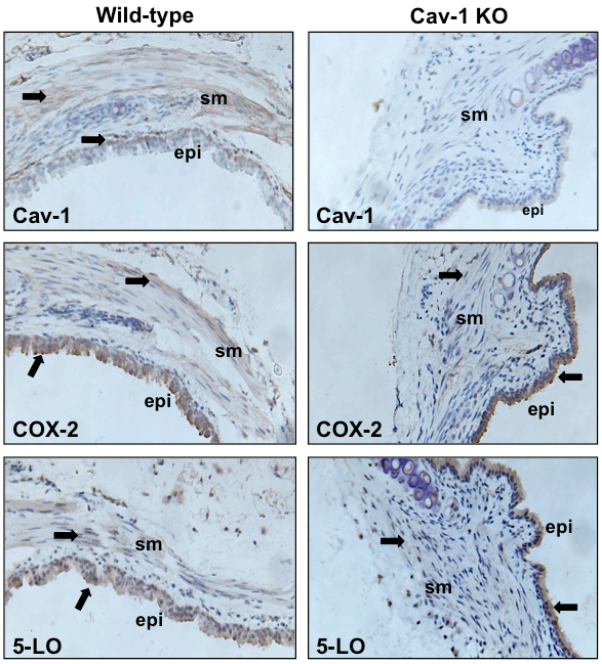

Figure 5. ***Cellular expression profile of Cav-1, COX-2 and 5-LO in airways from wild type and Cav-1 KO mice.*** Respresentative immunohistological sections showing staining (brown color highlighted with arrows) for Cav-1, COX-2 and 5-LO are shown. Legend: sm - airway smooth muscle layer; epi - epithelium.

As these data suggest that elevated COX-2 and 5-LO abundance in the respiratory system of Cav-1 deficient mice may be principally associated with accumulation in the airway epithelia, we next assessed the distribtion of COX-2 and 5-LO in resident airway cells by immunohistology of tracheas from Cav-1 KO and wild type mice (Fig. 5). This analysis demonstrated the epithelium to be the primary compartment in which COX-2 and 5-LO are expressed, though low levels of 5-LO and COX-2 staining were also evident in smooth muscle cells.

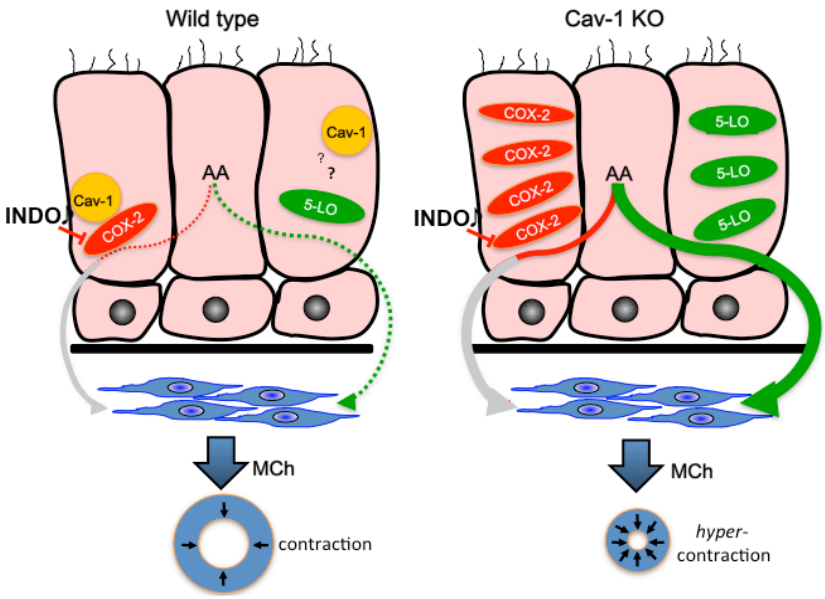

Figure 6. ***Schematic showing changes in epithelium-linked regulation of airway contractility in wild type and Cav-1 KO tissues.*** In wild type airways (left), relatively low leves of COX-2 and 5-LO are expressed in the epithelium (pink cells) due to presence of Cav-1. This may be due to direct interaction of COX-2 with Cav-1, however, whether 5-LO interacts with Cav-1 is unkown (question mark). Inhibition of the COX-2 pathway (hatched red line) in wild type airways with INDO suppresses the generation of prostaglandins (illustrated by grey line). This likely promotes shunting of arachidonic acid (AA) metabolites via the 5-LO arm of the cascade (hatched green arrow); however, this imbalnce is not sufficient to affect contraction of airway smooth muscle (blue cells, blue ring below represents an inact airway) in response to methacholine (MCh). In Cav-1 KO airways (right), lack of caveolin-1 leads to accumulation (and increased activity) of both COX-2 and 5-LO in airway epithelial cells. Inhibition of the COX-2 pathway (red line) in Cav-1 KO tissues with INDO suppresses the COX-2 pathway (illustrated by grey arrow) leading to shunting of AA metabolites via the 5-LO branch of the cascade (thick green arrow). Due to the elevated level of 5-LO sufficient levels of cysteinyl leukotrienes are synthesized and released to promote hypercontractility of the underlying airway smooth muscle (blue), an effect that is evident by excessive contraction in response to MCh (depicted with blue ring at bottom of the panel).

Discussion

Cav-1 KO mice exhibit lung remodeling and vascular defects, suggesting a broad impact on respiratory system physiology and function [26, 32, 33]. Nonetheless, prior to our study, the contractility of intact airways from Cav-1 KO mice had not been assessed. To our knowledge, our findings that COX-2 and 5-LO expression are increased in the lungs of Cav-1 KO mice have not been reported previously. Our functional findings indicate that this appears to provide a

platform for enhanced control of airway responsiveness by the airway epithelium. We found that pharmacological suppression of COX-2 with INDO produced hyperreactivity in Cav-1 KO airways (this despite a lack of difference in intrinsic contractile capacity of tracheal smooth muscle from wild type mice). Notably, the effects of COX-2 inhibition in Cav-1 deficient airways correlated with increased expression of COX-2 more or less selectively in the airway epithelium. We also noted 5-LO to be elevated in the tracheal epithelium and that COX-2 inhibition-induced hyperreactivity of tracheal rings was sensitive to inhibition of 5-LO signaling events. Based on long standing literature, this functional effect seems most likely to be the result of shunting of PLA_2 products through the 5-LO axis to produce contractile cysteinyl leukotrienes when COX-2 is blocked [34, 35]. Thus, our study indicates that caveolin-1 is involved with regulation of multiple biological functions that are of disparate importance in individual cells, and as a collective, through cellular cross talk underpins regulation of contractile responses of the airways.

It was unexpected that we observed that tracheal smooth muscle from Cav-1 KO mice exhibited normal contractile responses to methacholine (in the absence of INDO). Published data from us and others using *in vitro* and *ex vivo* models with acute molecular and pharmacological interventions show Cav-1 plays an important role in supporting agonist-induced Ca^{2+} mobilization and associated contraction of isolated human ASM [6, 10-12, 36, 37]. Thus, our new findings that indicate contractile function is not compromised in caveolin-1 deficient tracheal rings is at odds with evidence showing caveolae facilitate contraction. Importantly, prior studies have all used molecular and pharmacological interventions to induce acute, transient inhibition or silencing of Cav-1. Thus, our current findings using tissues from

mice with a constitutive Cav-1 null phenotype, suggest that compensatory mechanisms, for example augmentation of Ca^{2+}-independent pathways involving RhoA or PKC that have been linked to caveolae [38, 39], may be altered in Cav-1 KO airway smooth muscle. The data herein do not provide direct insight to such mechanisms, but do indicate that studies are needed to dissect the interplay between caveolin-1 regulated Ca^{2+}-dependent and -independent pathways in airway smooth muscle cells.

Cyclooxygenase is the key enzyme in prostanoid biosynthesis. One isozyme, COX-1, is expressed constitutively, whereas COX-2 is inducible in inflammatory conditions including lung cancer, and chronic airway diseases such as asthma and COPD [14, 15, 20-22, 40-43]. COX-2 is expressed in abundance by airway epithelial cells, equipping them with the capacity to synthesize a wide profile of prostanoids [44-47]. Prostaglandins can affect a variety of responses, including both the suppression of contractility and provocation of smooth muscle relaxation [48-50]. Several studies show that COX-2 can form a complex with Cav-1 and localize to caveolae [29, 31]. Interestingly, association of COX-2 with Cav-1 facilitates its degradation [28], Cav-1 can down regulate β-catenin-Tcf/Lef–dependent transcription of COX-2 [30], and it modulates post-transcriptional mechanisms such as augmented proteosomal degradation [51, 52], that regulate protein stabilty. Caveolin-1 can also regulate COX-2 activity, suppress PGE_2 synthesis, and modulate transport of PGE_2-containing vesicles to the plasma membrane for release [30, 53]. These observations are of relevance, as we observed that COX-2 and 5-LO abundance is markedly increased in Cav-1 KO lung and trachea, and that COX-2 associates with caveolin-1 in wild type lungs. Thus, COX-2 expression and protein half-life are likely increased in Cav-1 KO mice due to the absence of Cav-1 supported degradation; an effect most evident in

airway epithelial cells because removal of epithelium normalizes COX-2 and 5-LO levels in Cav-1 KO tracheal lysates. This also correlates with our data showing that INDO-mediated suppression of COX-2 activity selectively promotes hyperreactivity in Cav-1 KO tracheas. Thus, it appears that caveolin-1 plays a key role in determining the extent to which epithelium-dependent modulation of airway contraction is manifested.

Our data show that wild type murine airways are refractory to the effects of INDO, indicating that absence of caveolin-1 in KO mice likely contributes to de-inhibition of pathways that can impart control of airway responsiveness to the epithelium. Our experiments do not allow precise identification of these pathways, but revealing that Cav-1 is a regulator of these pathways is of relevance. For instance, smooth muscle contractility in chronic airway diseases is regulated by the epithelium, a rich source of lipid mediators and other types of contracting and relaxing factors [16-19, 54, 55]. In asthma, aberrant biosynthesis of lipid mediators by COX-2 or 5-LO has been linked with changes in airway reactivity and bronchial inflammation [13-15, 20-22]. In bovine tracheal preparations contraction is refractory to epithelial denudation [56], whereas prostanoids are released in abundance in the guinea pig airway [42]. Thus, our finding that caveolin-1 modulates expression and activity of COX-2 and 5-LO in the airway epithelium indicates that Cav-1 needs to be considered for potential contributon to mechanisms that lead to changes in bronchial contstriction in pathophysiological conditions, and for physiological dispartity between species and different airways.

One of the novel findings of our study is that 5-LO abundance is elevated, chiefly in the airway epithelium, in mice lacking Cav-1. However, unlike for COX-2, to our knowledge there

are no reports that indicate caveolin-1 can interact with, or is linked to pathways that regulate expression of 5-LO. Thus, the mechanism for increased 5-LO in Cav-1 KO lungs is not clear, and further investigation is warranted. We do show that inhibition of 5-LO or blockage of the cysteinyl leukotriene receptor 1 (CysLTR1), which is expressed by ASM cells [57], is sufficient to reverse INDO-induced hyperreactivity in tracheal rings with intact epithelium from Cav-1 KO mice. However, treatment of tracheal rings with Montelukast or Zileuton alone had no effect on contraction of tissues from Cav-1 KO or wild type mice. These findings reveal that 5-LO signaling only contributes to enhanced contraction of Cav-1 KO tissues when COX-2 is inhibited. Thus, as depicted schmatically in Figure 6, the fact that COX-2 and 5-LO are both increased in Cav-1 KO tissues appears to be the key prediposing factor for INDO-induced hyperreactivity. Indeed, both epithelial COX-2 and 5-LO are expressed by wild type tissues, albeit at much lower levels than in Cav-1 KO mice, but we observed no change in airway contraction with INDO treatment. Collectively, this supports a paradigm for Cav-1 as a determinant of the level of control of the airway epithelium on airway responsivness through effects on expression and activity of both COX-2 and 5-LO. Consistent with this are our observations that epithelial denudation was not sufficient to unmask tracheal smooth muscle hyperreactivity in Cav-1 KO preparations, a result that would otherwise have been expected if elevated epithelial COX-2 activity alone was at the root of INDO-induced hyperreactivity in caveolin-1 deficient airways. As depicted in Figure 6, based on established paradigms [35, 58], it seems likely that 5-LO product synthesis, and subsequent binding to CysLTR1, is associated with shunting of PLA_2 products through the 5-LO pathway when COX-2 is inhibited in Cav-1 KO airways. This likey also occurs in wild type airways, but our functional evidence indicates this is not sufficient to alter airway contractilty,

probably because of the much lower levels of COX-2 and 5-LO in the wild type airway epithelium. One caveat to our intepretation is that our studies do not preclude the possibility of direct negative regulation of leukotriene-induced contraction by prostaglandins, a mechanism that will require future experiments using selected leukotrienes, prostanoids and receptor antagonists in combination to decipher.

In summary, our findings suggest that differences in caveolin-1 expression can regulate the degree to which the airway epithelium controls bronchoconstriction, in part through effects on expression and activity of both epithelial COX-2, which drives the synthesis of prostaglandins such as PGE_2, and 5-LO, which mediates synthesis of cysteinyl leukotrienes. Moreover, our findings suggest that the ability of airway smooth muscle to contract in response to GPCR ligands (i.e. MCh) is unchanged in Cav-1 KO mice, a result that differs from a body of evidence showing acute transient silencing of caveolin-1 supresses Ca^{2+} mobilization and contraction. Our new findings suggests that alternate mechanisms to maintain contractility may be enhanced with long term caveolin-1 depletion. This study also reveals that caveolin-1 and caveolae offer complex control of airway responsiveness via effects in different cell types that lead to alterations in both intrinsic cellular responses and intercellular communication.

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PS drafted and produced 100% of the experiments required for the realization of this manuscript

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Chapter 9

Altered airway smooth muscle contraction and lung function in δ-sarcoglycan knock out mice.

Pawan Sharma, Aruni Jha, Sujata Basu and Andrew J. Halayko

This collaborative work is presented as a manuscript in preparation.

Abstract

It has been shown that the dystrophin-glycoprotein complex along with caveolin-1 plays an important role in mobilization of intracellular calcium in airway smooth muscle (ASM). DGC is also an integral part of caveolae microdomains and it has ben shown that the DGC (through β-dystroglycan) interacts with caveolin-1 and participates in keeping the caveolar structures at the membrane in *vitro*. It is also known that the sarcoglycan complex provides stability to the dystroglycan complex by binding of δ-sarcoglycan to β-dystroglycan. The role of the sarcoglycan complex in ASM contraction and lung physiology in not clearly understood. We tested the hypothesis that δ-sarcoglycan through its interaction with β-dystroglycan is a determinant of ASM contraction *ex vivo* and airway mechanics *in vivo.* Using δ-SG KO mice we measured isometric contraction induced by contractile agonist methacholine (MCh) and compared it with the wild-type mice. We also measured airway mechanics using a small animal ventilator to study the role of δ-SG on airway physiology. We used immunoblotting to measure pertinent DGC and caveolae proteins in the trachea of δ-SG KO and wild-type mice. Lastly, we performed transmission electron microscopy to look at the ultrastructural changes after loss of δ-SG protein in the tracheal smooth muscle tissue. Our results show that there was a significant reduction in the MCh induced isometric contraction (100μM and 1mM) in δ-SG KO mice (aged 17-19 weeks) when compared to the age-matched wild-type, while there was no change at 8-10 weeks of age. The lung function data showed significant reduction in the airway resistance at the highest dose of MCh (50mg/ml) in δ-SG KO mice (8-10 weeks of age), while other parameters of lung mechanics were unchanged. The changes in contraction and lung function correlated with decreased caveolin-1 and β-dystroglycan proteins (key units of caveolae and

DGC). Also, ultrastructural studies found that the caveolae microdomains at the cell membrane were completely lost in the δ-SG KO mice at 17-19 weeks of age (no change at 8-10 weeks of age) showing the effect on reduced contractile response is correlated with effects on caveolae. Collectively, these results indicate the importance of DGC in lung physiology and the contractile property of the ASM.

Introduction

The Dystrophin-glycoprotein complex (DGC) confers a structural link between laminin-2 in the extracellular matrix and the F-actin cytoskeleton [1, 2] and also protects muscle cells from contraction-induced damage [3]. The DGC is composed of multiple transmembrane, cytoplasmic, and extracellular protein subunits (described in chapter 1) [1, 2]. The sarcoglycan (SG) complex in the DGC comprises hexameric proteins namely α-SG, β-SG, δ-SG, ε-SG, ζ-SG and γ-SG [4, 5]. The presence of these proteins varies from one muscle to another: smooth muscle includes β-SG, δ-SG, ε-SG and ζ-or γ-SG [6-8]. The SG complex closely associates with dystroglycan and is thought to stabilize the interactions of dystroglycan with the extracellular matrix and with dystrophin and its associated proteins [9]. Mutations in the genes encoding sarcoglycans can produce cardiomyopathy and muscular dystrophy which primarily affects heart and skeletal muscles [10]. Its role in vascular smooth muscle function is emerging as recent research indicates that the loss of the sarcoglycan complex may lead to reduced vascular patency arising from the loss of vascular smooth muscle function [11]. Recent reports also suggest its role in vascular smooth muscle function especially in vascular development and remodeling processes [12, 13].

Inspite of its emerging role in vascular smooth muscle biology nothing is known about the functional role of sarcoglycans in airway smooth muscle (ASM). In this study we hypothesized that δ-SG (a key SG complex sub-unit) plays a key role in determining ASM function and also plays a role in determining lung mechanics. To answer this we used δ-SG knock out (KO) mice to perform isometric tracheal contraction studies *ex vivo*, we also determined lung function using a small animal ventilator to assess the airway mechanics in the KO mice *in vivo*. We also looked at the ultrastructural changes occurring in the airway smooth muscle using transmission electron microscopy (TEM) in the δ-SG KO mice and compared to the wild-type mice. Our results suggest that the sarcoglycan complex plays an important role in determining airway smooth muscle contraction in the aged mice, though there was a trend in reducing the contraction in young δ-SG mice. The lung function data indicate a strong trend towards declining lung function with increasing concentrations of methacholine (MCh) (statistical significance attained at the highest concentration of MCh). The TEM data demonstrated that there was no effect on the membrane caveolae in the tracheal smooth muscle in young δ-SG KO mice but there was complete loss of caveolae in the aged δ-SG mice when compared to the wild-types. Moreover, there was a reduction in the protein abundance of caveolin-1 and β-dystroglycan in the tracheal smooth muscle tissue obtained from the δ-SG KO mice.

Results

Genotyping of mice colony to identify the δ-SG KO mice

The genotyping was performed using EZ Fast Tail PCR Genotyping Kit from EZ BioResearch, Saint Louis, MO, USA to identify the δ-SG KO, wild-type and the heterozygous mice in each litter. As shown in Fig. 1, the δ-SG was identified by presence of only a mutant allele (587 bp CCAGCTCATTCCTCCCACTCA). The wild-type was identified by having only a wild-type allele (398 bp: TGCTCCTGTGGTGGGAATACTG), while the heterozygous mice had two alleles: common (398 bp: TGCTCAGGCTAATGCCACATTG) and a mutant one.

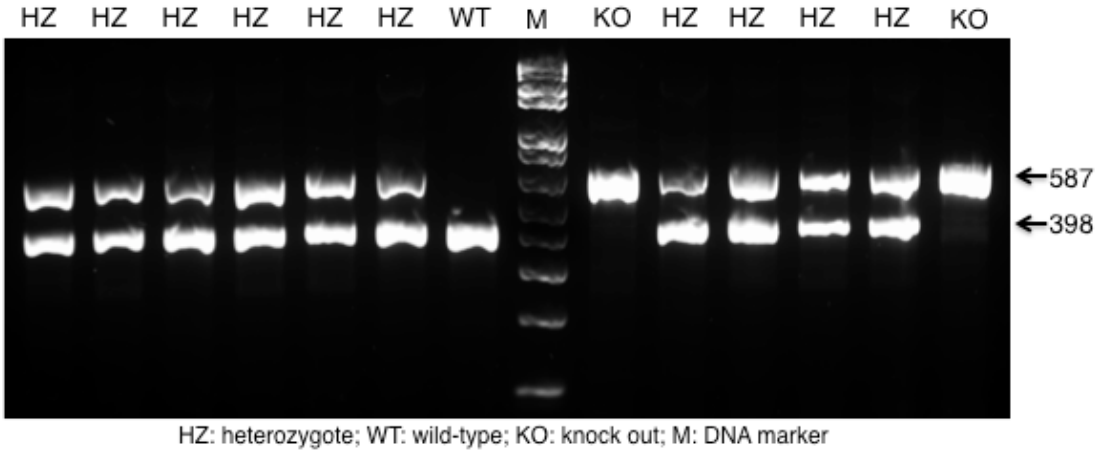

Figure 1. ***Genotyping for δ-SG KO mice colony.*** Tail clips were obtained from the litters and genomic DNA was isolated using a kit from EZ BioResearch. DNA was then amplified by PCR using primer sets, which recognized target regions in the DNA for individual mice. The agarose gel shown here is the typical example by which we identified the animals. The heterozygotes had two bands; the KO had only the mutant band while the wild-type had only one band as represented in the figure.

Effect of δ-SG loss on airway smooth muscle contraction

We have previously shown that DGC plays an important role in mobilizing intracellular calcium *in vitro* and lack of dystrophin in an *mdx* mice showed reduced tracheal contraction *ex vivo* (chapter 7) [14]. Here we investigated the effect of sarcoglycan protein subunit: δ-SG, which binds to β-dystroglycan and stabilizes the interaction of the DGC with the EC matrix and the underlying cytoskeleton. We first used relatively young δ-SG KO mice (8-10 weeks) to study

the effect of a contractile agonist on tracheal responsiveness (Fig. 2A-B). Our results showed that there was no difference in the KCl induced isometric contraction between δ-SG KO and the wild-type tracheas (p>0.05). We further did a concentration response curve with muscarinic agonist methacholine (MCh) and found no significant differences in the contractile responses of the tracheal rings obtained either from KO or wild-type mice. However, there was a trend in reduction of tracheal contractility in δ-SG KO mice at higher concentrations of MCh.

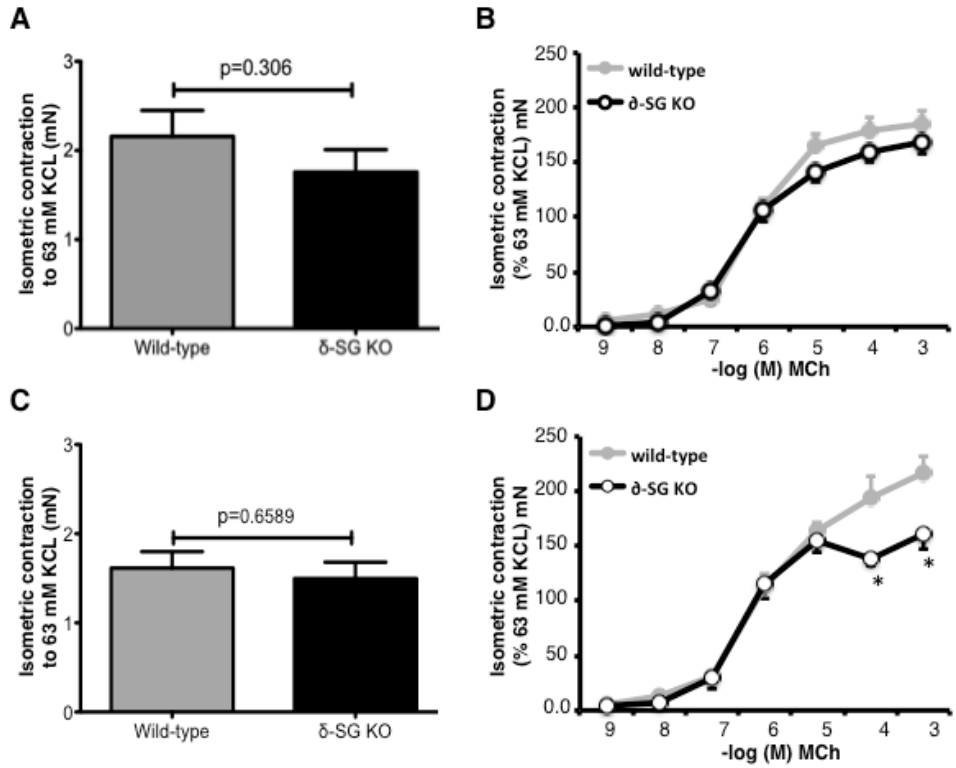

Figure 2. ***Determination of tracheal contraction in aged δ-SG KO mice***. Trachea from δ-SG KO and wild-type mice (either at 8-10 or 17-19 weeks of age) were isolated and sliced to obtain 4 ~equal-sized segments containing 3-4 cartilage rings. Tissues were equilibrated for 90-120 min with intermittent (~20 min) instillation of 63mM KCl-substituted K-H to obtain a stable resting tension at ~0.6 mN. (**A**) In 8-10 weeks old mice, receptor independent force-generating capacity of tracheal rings from wild type and δ-SG KO mice was assessed based on isometric force measured after 63 mM KCl substituted with K-H solution treatment. For each group, 14 tracheal rings from at least 6-8 mice were studied. There were no significant differences between the groups (t-test, p>0.05). (**B**) In 8-10 weeks old mice, isometric force was measured with increasing cumulative concentrations of MCh (10^{-9} M to 10^{-3} M). Compared to wild-type tracheal rings from δ-SG KO mice showed no differences in contractile response to MCh (n = 14 rings from 6-8 mice; One-way analysis of variance, p>0.05). (**C**) In 17-19 weeks old mice, receptor

independent force-generating capacity of tracheal rings from wild type and δ-SG KO mice was assessed based on isometric force measured after 63 mM KCl substituted with K-H solution treatment. For each group, 6 tracheal rings from at least 3-4 mice were studied. There were no significant differences between the groups (t-test, $p>0.05$). (**D**) In tracheal rings from 17-19 weeks old mice isometric force was measured with increasing cumulative concentrations of MCh (10^{-9} M to 10^{-3} M). Compared to wild-type tracheal rings from δ-SG KO mice showed significant differences in contractile response to MCh at 10^{-4} and 10^{-3} M MCh (n = 6 rings from 3-4 mice) when compared to wild-type mice (One-way analysis of variance, $*p<0.05$).

Because we found no differences in the tracheal responsiveness of δ-SG KO and wild-type animals we planned to perform these experiments in aged mice (17-19 weeks of age). Typically the muscle and cardiac pathology starts developing in δ-SG KO after 8-10 weeks of age and both skeletal and cardiac muscle deficits can also be recorded at this time point. In the tracheal rings obtained from δ-SG and the wild-type mice at 17-19 weeks of age (Fig 2C-D), we found that there was no change in the KCl induced tracheal contraction ($p>0.05$), but we found that the tracheal rings from δ-SG showed reduced responsiveness at the highest concentrations of methacholine (100μM and 1mM; $p<0.05$) without changing the EC50.

Effect of δ-SG loss on lung physiology

We used a small animal ventilator to study the airway mechanics in δ-SG KO mice at 8-10 weeks of age (Fig. 3A-C). The δ-SG KO showed reduced airway resistance only at the highest concentration of MCh (50mg/ml; $p<0.05$), while there was no change in the EC50 to MCh in the KO mice when compared to the wild-type mice. Also we found no significant changes in the tissue resistance and tissue elastance in the δ-SG KO mice when compared to the wild-type animals. These results hint that there is some contribution of δ-SG in determining the lung physiology.

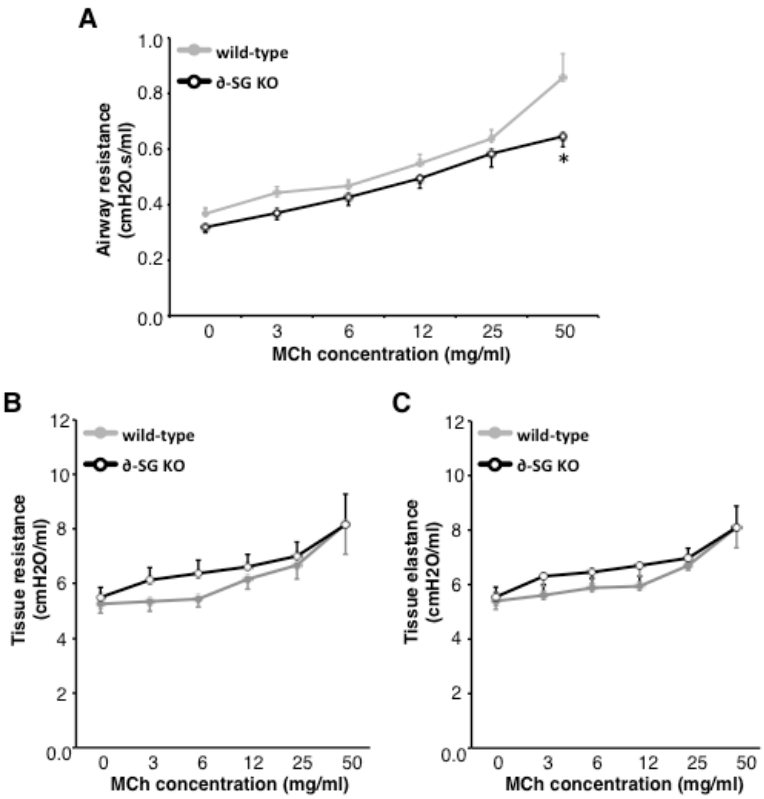

Figure 3. ***Determination of lung mechanics in δ-SG KO mice.*** Wild-type or δ-SG KO mice tracheas were dissected and connected to a flexiVent small animal ventilator (Scireq Inc. Montreal, PQ). Mice were ventilated with a tidal volume of 10 ml/kg body weight, 150/minute. Mice were then subjected to an increased dose of nebulized methacholine (MCh) challenge protocol to assess characteristics of respiratory mechanics. For MCh challenge, ~ 30μL of saline containing from 0-50 mg/ml MCh was delivered over 10 seconds using an in-line ultrasonic nebulizer. By fitting respiratory mechanical input impedance (*Zrs*) to the constant phase model, flexiVent software calculated conducting airway resistance (Raw) **(A)**, peripheral tissue & airway resistance (G) **(B)**, tissue elastance or stiffness (H) **(C)**; each parameter was normalized according to body weight. Values for each parameter were calculated as the mean of all 20 perturbation cycles performed after each MCh challenge. Statistical comparisons shown were performed by 1-way ANOVA with Tukey's multiple comparison test. **P* < 0.05, was considered significant for δ-SG KO versus wild-type. Data shown above is the mean ± sem of 7-9 animals used for both wild-type and δ-SG KO.

Effect of δ-SG loss on caveolae and DGC organization

Because our results showed that there are differences in the contractility of tracheal smooth muscle in δ-SG KO mice we performed protein analysis for β-dystroglycan and caveolin-1 which has been shown to regulate intracellular calcium mobilization in ASM [14].

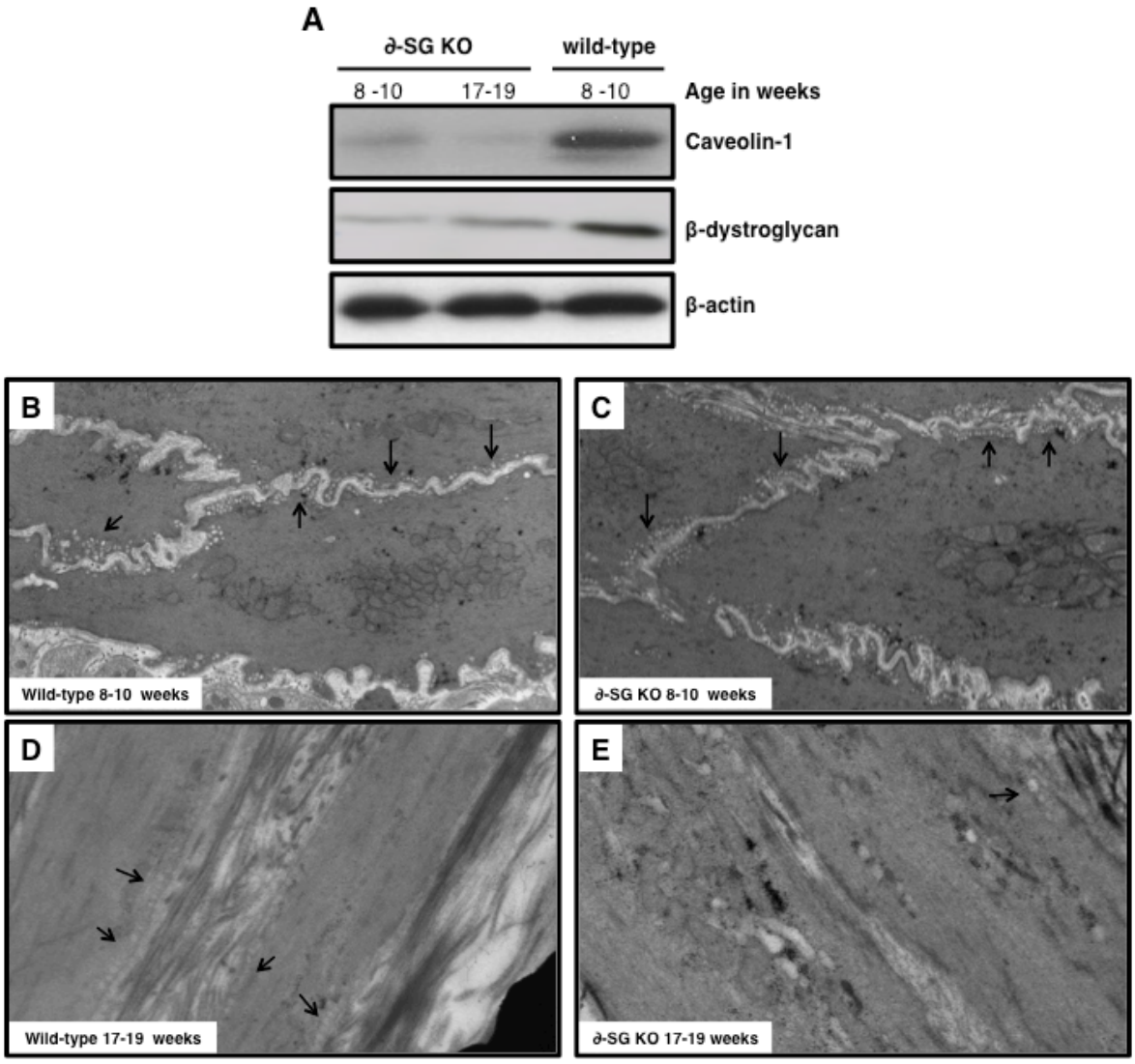

Figure 4. ***Organization of DGC and caveolae in δ-SG KO mice.*** (**A**) Tracheal lysates enriched in ASM tissue were prepared from either δ-SG KO or wild-type mice. Representative western showing caveolin-1, β-dystroglycan in 8-10 or 17-19 weeks old δ-SG mice and 8-10 weeks old wild-type mice. The δ-SG KO showed reduction in caveolin-1 and β-dystroglycan protein abundance when compared to the wild-type. Tracheal rings from atleast 4-5 mice were pooled together in each group while β-actin was used a loading control for the western. **(B-E)** Tracheas from δ-SG KO and wild-type mice at 8-10 weeks of age (B, C) or at 17-19 weeks of age (D, E) were fixed for transmission electron microscopy. Arrows in panels B, C and D indicate typical caveolae-linear arrays, whereas in panels E, arrows indicate lack of caveolae-like double membrane structures at the membrane. Scale 11000 X (Fig. B &C), 15600 X (Fig. D & E).

Our results using tracheal smooth muscle lysate from δ-SG KO mice showed almost complete loss of both β-dystroglycan and caveolin-1 in the tracheas when compared to wild-type mice (Fig. 4A). As the DGC facilitates intracellular calcium mobilization along with caveolae,

we performed transmission electron microscopy in the trachea to see the ultrastructural changes taking place at the caveolae level both in young and aged δ-SG KO mice (Fig. 4B-E). Ultrastructure studies demonstrated that in young (8-10 weeks) δ-SG KO and wild-type mice there was a linear arrangement of caveolae on the plasma membrane of tracheal smooth muscle tissue (Fig. 4B-C). Indeed, this linear arrangement of caveolae almost diminished in the aged (17-19 weeks) δ-SG KO mice when compared to the age-matched wild-type animal (Fig. 4D-E). These results clearly indicate that DGC proteins are an important part of the caveolae microdomains in the ASM cells and also show that loss of DGC subunit could alter the organization of DGC and caveolae which eventually translates into loss of contractile function in the δ-SG KO mice.

Discussion

In previous chapters we have discussed the role of DGC subunits β-dystroglycan and dystrophin in modulating intracellular calcium *in vitro* and ASM contraction *ex vivo.* In previous Chapters we have also shown an *in vivo* role of the DGC by using dystrophin KO mice (*mdx* mice). To further understand and extend our knowledge on the functional role of DGC in the airways we used δ-SG KO mice and assessed MCh responsiveness of the trachea and also evaluated airway mechanics in intact animals. Our results demonstrate for the first time that in δ-SG KO mice, the responsiveness of the airways is age dependent as we found greater reduction in the tracheal contraction in the older animals when compared to their wild-type counterparts. We also found that the airway mechanics in δ-SG KO at 8-10 weeks of age was not greatly different than wild-types as we found a significant reduction in the airway resistance

only at the highest concentration of MCh. The functional deficit in the δ-SG KO mice correlated with a net reduction of the DGC subunits and caveolae protein β-dystroglycan and caveolin-1 in the trachea. The most striking finding was the loss of caveolae microdomains on the plasma membrane in tracheal smooth muscle tissue of δ-SG KO at 17-19 weeks of age while there was no change at 8-10 weeks of age when compared to wild-type mice. Together, these findings suggest that DGC plays an important role in the organization of caveolar structures on the ASM cell membrane, which underpins the functional responsiveness of ASM to various contractile agonists.

δ-SG is a central protein member of the hexameric sarcoglycan complex, which is a part of the DGC and stabilizes the dystroglycan complex by associating directly with its β-subunit [1, 2, 10, 15]. The sarcoglycan complex composition varies from one muscle-type to another, in ASM the sarcoglycan complex consists of β-SG, δ-SG, ε-SG and ζ-or γ-SG [1, 2, 4, 5, 10, 16]. The SG complex plays a key role in skeletal and cardiac muscles as loss of any of these causes several forms of muscular dystrophy and cardiomyopathy [9, 17-21]. The functional significance of the SG complex is well appreciated in skeletal and cardiac muscles but studies in ASM are lacking. In our study we used well-established δ-SG KO mice colony from Jackson's Labs, USA, mice were bred in-house to obtain the δ-SG KO mice, which was assessed by genotyping. We used the wild-type littermates and the δ-SG KO mice to study the contraction of ASM and assess the airway mechanics using a thin-wire myograph and a small animal ventilator.

To our knowledge our study is the first to report on ASM contraction and lung function in δ-SG KO mice. Although the functional role of the DGC in contractile smooth muscle cells has not been established, some studies provide clues in this area. In dystrophic hamsters, DGC deficiency correlates with more pronounced loss of ASM mass and contractile responses with aging [22], an effect that could contribute to suppressed airway responsiveness in vivo. Though previous reports in vascular smooth muscle shows that the arteries from *mdx* and δ-SG KO mice showed decreased distensibilities to load suggesting that loss of DGC proteins may induce adaptive biomechanical changes that can maintain overall wall mechanics in response to normal loads [23]. Most reports have suggested that disruption of the DGC in smooth muscle may be linked to changes in Ca^{2+} homeostasis [24]. The absence of dystrophin in portal veins from *mdx* mice results in reduced intercellular myocyte communication associated with stretch-induced myogenic contractile responses [25]. Morel et al [26] reported that decreased mechanical activity of duodenal smooth muscle in *mdx* mice is due to reduced type 2-ryanodine receptor expression that compromises sarcoplasmic reticulum calcium release. Cohn et al [17] showed that cardiac myopathy associated with coronary artery vasospasm in SG KO mouse models could be prevented by verapamil, a vasodilatory Ca^{2+} channel blocker. Collectively, all these studies clearly demonstrate that DGC participates in maintaining smooth muscle function particularly in the vessels. It creates a need to investigate the function of DGC in lung physiology especially the role SG complex in determining airway physiology. Our new findings using δ-SG KO mice on ASM demonstrate that δ-SG plays a similar role as in vascular smooth muscle. Our studies also indicate that δ-SG is essential in organization of the DGC and caveolae at the membrane, which provides a structural link between extracellular matrix and actin

cytoskeleton in ASM cells. The organization of caveolae along with DGC and its link with actin plays a determining role in modulating contractile properties of ASM.

Our lung function data using δ-SG mice demonstrates that the DGC has a role in determining lung physiology. Our result shows that in mice lacking δ-SG there was a reduction in the airway resistance at the highest concentration of MCh. One limitation our study is that we did not go beyond 50 mg/ml of MCh to assess the lung function. There are several studies in the literature where they have used more than 50mg/ml of MCh to assess the lung function in mice [27, 28]. We speculate that the reduction in lung function could be greater at higher doses of MCh. But our *ex vivo* and *in vivo* data is quite convincing to state that there are alterations in the lung physiology of δ-SG KO mice which partially come from the altered responsiveness of ASM to contractile agonists.

The DGC is a multi-protein complex, which is a part of caveolae microdomains at the cell membrane. The DGC along with caveolae facilitates intracellular calcium mobilization induced by contractile agonist in ASM cells (chapter 5, 6) [14]. The functional deficit in δ-SG KO correlates quite well with our protein data and ultrastructural studies on tracheal samples obtained from δ-SG KO and wild-type mice. Our western data show that in δ-SG KO mice there was a reduction in the protein abundance for both caveolin-1 and β-dystroglycan, which are key constituents of caveolae and the DGC. We further investigated whether reduction in caveolin-1 and DGC proteins was translating to changes in the caveolae microdomains at the cell membrane. Using TEM we found that in the tracheal tissue sections, the caveolae

microdomains were linearly oriented across the plasma membrane both in δ-SG KO and wild-type mice at 8-10 weeks of age. In the mice aged 17-19 weeks, we found that there was a disruption of linear caveolae structures in the δ-SG KO mice but there was no change in the caveolae structures of wild-type mice. These studies support the previous findings that the DGC is a key determinant of maintaining caveolae microdomains at the cell membrane as disruption in the DGC proteins either through latrunculin (indirectly by preventing actin polymerization) or by silencing β-dystroglycan (a key subunit of DGC) leads to both reduction in caveolae proteins and increases the internalization of caveolae structures in the cell [14]. In summary, our functional data using δ-SG KO mice indicate that the DGC plays an important role in contractile properties of ASM and is also a key determinant of airway physiology *in vivo*.

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PS drafted and produced 100% of the experiments required for the realization of this manuscript

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Chapter 10

GENERAL SUMMARY

Contractile myocytes exist in smooth muscle tissues throughout the body and are chiefly designed to stiffen, shorten, or relax in response to chemical and mechanical signals. It speaks volumes for how far ASM research lags behind skeletal muscle research in understanding the contraction of ASM and its function. In terms of subcellular and molecular level characterization, much remains to be done. *Although deficits in knowledge of structure and function for ASM exist, windows of opportunity are opening that may allow ASM research to be conducted at the level where we can understand the role of new proteins and molecules regulating the phenotype and its function* in vivo*.* It is known that expression of the DGC and its subunits are developmentally regulated and required for skeletal muscle maturation and maintenance [1]. Moreover animal models lacking the sarcogylcan subunits exhibit vascular deficits associated with changes in Ca^{2+} homeostasis [2, 3]. The current work investigated the expression and functional significance of a group of structural proteins called dystrophin glycoprotein complex (DGC) along with caveolae in ASM, using human bronchial and tracheal, canine tracheal, and mice airways to elucidate their role in ASM phenotype and lung physiology. Our project broadly examined the role of the DGC proteins in organizing membrane caveolae through its link with actin cytoskeleton and has investigated its role on receptor-mediated cell signaling leading to ASM contraction *in vitro*, *ex vivo*, and *in vivo*.

Expression of DGC in ASM is associated with contractile phenotype

Our studies in chapter 4 [4] were undertaken to profile and characterize the proteins that comprise the DGC in human ASM cells, and to determine whether their expression correlates with phenotype switching. Our study is the first to provide systematic profiling of the DGC subunits expressed in human ASM tissue and cultured cells and shows the composition of the DGC is consistent with reports for other smooth muscles, because it includes α- and β-dystroglycan, dystrophin, sarcospan, and a sarcoglycan complex that includes β-, δ-, ε-, γ-, ζ-, and perhaps, α-SG.

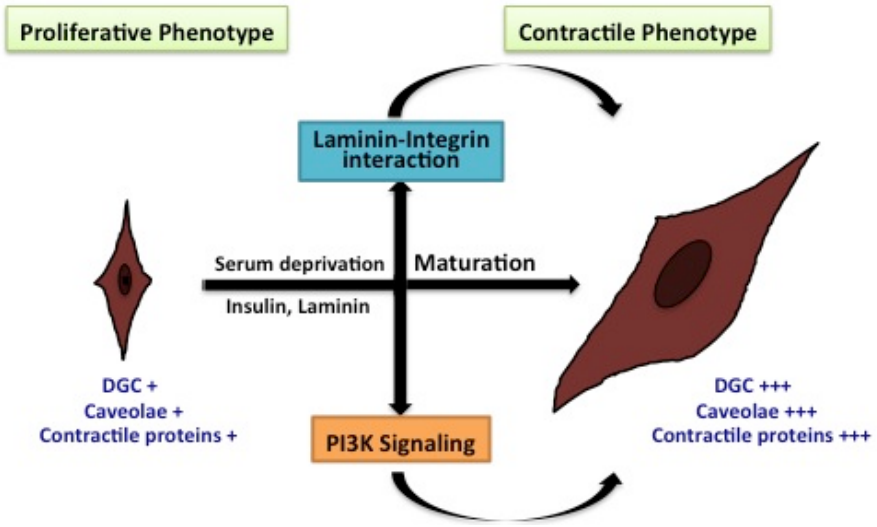

Figure 1. **DGC, Caveolae and Phenotype Maturation**. In culture ASM cells exists as proliferative phenotype characterized by reduced contractile apparatus, few caveolae, and reduced DGC proteins. The acquisition of a contractile phenotype as a result of phenotype maturation is characterized by abundant contractile proteins in the cell along with concomitant expression of DGC proteins and caveolae. The expression of DGC is dependent upon laminin-integrin interaction and PI3K signaling in the mature myocytes.

As shown in Fig. 1, our studies using cultured human ASM reveal that the expression of the DGC protein subunits is dynamic, being lost or markedly reduced upon modulation to a proliferative phenotype, while they accumulate when individual cells re-acquire a contractile phenotype. The binding of laminin-2 to α_7-integrin is required

for ASM phenotype maturation [5, 6]; our study revealed that blocking laminin-integrin binding prevented both phenotype maturation and the accumulation of DGC proteins, confirming that they are reliable markers for acquisition of the contractile phenotype in ASM cells. We also show for the first time that PI3K activity, which is critical for ASM maturation [7], is required for DGC protein accumulation. Collectively our results suggest that DGC protein expression is dynamically regulated by mechanisms that control ASM maturation. Moreover, we show that the DGC is abundant in contractile ASM, which suggests it could be associated with functional aspects of contraction in a manner similar to that seen for skeletal muscle. Our studies in this chapter provide an important new platform for future studies investigating the functional role of the DGC in contractile ASM, in particular in relation to caveolae and its role in Ca^{2+} homeostasis.

Interaction of DGC with caveolin-1 is important for intracellular Ca^{2+} mobilization

North et al has shown that dystrophin co-localizes with caveolin-1 in specific membrane microdomains in contractile smooth muscle cells [12]. We further investigated the interaction of caveolin-1 with the DGC, which is tethered to the actin cytoskeleton, and studied the functional significance of this interaction (described in chapter 5 [8]). As shown in Fig. 2, our data showed for the first time that endogenous caveolin-1 binds to β-dystroglycan, an association that had only been predicted before from work using recombinant peptides [9]. Furthermore we showed that this interaction underpins a structural framework that involves filamentous actin and the DGC to support caveolae plasma membrane arrays. Importantly, our studies demonstrated that

ordering of caveolae into stable cell membrane arrays is concomitant with sequestration of signaling effectors, including Gαq and PLCβ1, which transduce GPCR-mediated Ca^{2+} mobilization. Thus, the studies outlined in chapter 5 [8] revealed a new level by which receptor-mediated contraction of smooth muscle can be regulated. These processes are of prime importance in understanding function of hollow organs in health and disease.

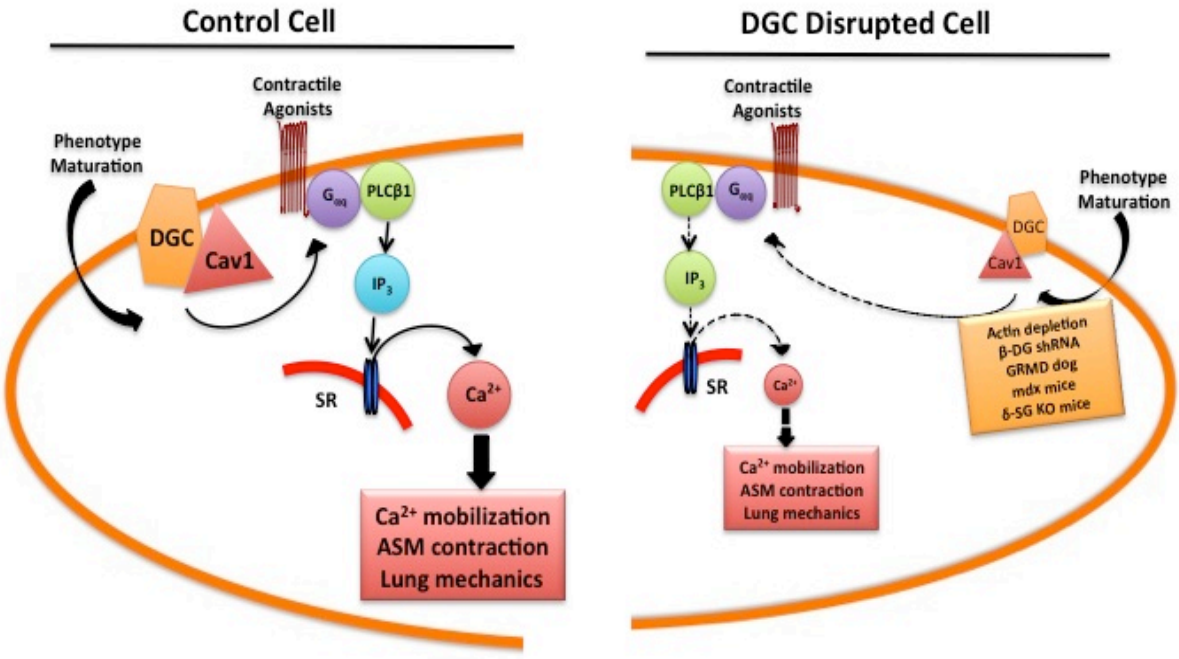

Figure 2. DGC and Caveolae: Functional Significance. In ASM cell, phenotype maturation leads to accumulation of contractile protein markers along with increased accumulation of Cav1 and DGC subunits. In control cells (left panel): Cav-1 and DGC proteins (through β-DG) interact with each other resulting in formation of linear arrays on the cell membrane. These organized arrays are in close proximity to GPCRs, calcium handing proteins and SR. This spatial organization of DGC and Cav-1 within caveolae allows optimization of receptor mediated calcium release by contractile agonist, which ultimately determines ASM phenotype and function in vitro and in vivo. In another situation when the caveolar organization is disrupted (right panel) using various approaches such as latrunculin A (actin sequestering agent), silencing β-DG, and using various animal models lacking functional DGC proteins (∂-SG KO and dystrophin KO (mdx) mice), the phenotype maturation leads to reduced expression of contractile protein markers, along with Cav-1, DGC subunits and calcium handing units (Gαq, PLCβ1) in caveolae microdomains. This disruption of the organizational structure of DGC results in trafficking of key signaling molecules from caveolar spaces creating a spatial separation between them leading to ineffective receptor mediated signaling by contractile agonist (represented by dashed arrows) resulting in reduced

functional responses of ASM in vitro and in vivo. (ASM, airway smooth muscle; DGC, dystrophin glycoprotein complex; Cav-1, caveolin-1; β-DG, β-dystroglycan; GPCR, g-protein coupled receptors; PLC β 1, phospholipase β 1; IP3, inositol 1,4,5-trisphosphate; ∂ -SG, ∂ -sarcoglycan; KO, knock out).

Our findings in chapter 5 [8] led us to study the mechanism by which the DGC affects intracellular Ca^{2+} homeostasis in ASM cells. Thus, in chapter 6 we investigated the mechanism by which DGC mobilizes intracellular Ca^{2+} from SR by affecting the SR Ca^{2+}-binding proteins, independent of extracellular calcium pool. Using various ASM cell systems having disrupted DGC we showed that there is a reduction in receptor mediated Ca^{2+} mobilization induced by contractile agonist MCh. Our results also demonstrate that IP_3-independent Ca^{2+} release induced by SERCA inhibition is also dramatically reduced after DGC disruption suggesting the role of key proteins which binds Ca^{2+} in the SR. We show for the first time that DGC disruption leads to increased calnexin protein abundance in ASM cell. These findings have a functional significance, as there is reduced Ca^{2+} release after DGC disruption, which is a key step for ASM contraction *in vivo* as shown in the schematic Fig. 2.

Expression of dystrophin is a key determinant of contractile phenotype and lung physiology

Loss in skeletal and cardiac muscle function in a dystrophin deficient animal has been studied and described very well but studies are lacking in smooth muscle *per se.* In this study we hypothesize that dystrophin has a key role in airway smooth muscle phenotype *in vitro* and also participates in ASM contraction and determination of lung

function *in vivo.* We used two dystrophic animal models (GRMD and *mdx*) to study the effect of dystrophin on ASM phenotype and function. Our results showed that dystrophin expression is associated with development of a contractile phenotype in ASM cell, while loss of dystrophin was associated with reduced contractile protein markers and reduced signaling required for accumulation of contractile proteins in the cell. Moreover, the functional data showed that dystrophin is needed to maintain ASM tone as loss of dystrophin caused reduction in tracheal sensitivity to the contractile agonist MCh. The lung function data showed a strong trend in associating dystrophin with maintenance of lung function in intact animals. Taken together, we found that dystrophin is a key determinant of ASM phenotype and function.

Our study investigated the role of dystrophin in phenotype maturation of ASM cells and also looked into the mechanism by which dystrophin can affect the key signaling required for development of a contractile phenotype *in vitro*. Tracheal smooth muscle contraction in *mdx* mice clearly showed that dystrophin is required for maintaining normal tone to muscarinic agonist *ex vivo.* On the contrary, these findings failed to translate completely in an intact animal but we got some interesting lead. A more in-depth and careful dissection of the data is required to plan and perform future studies which will elucidate the mechanisms by which dystrophin affects ASM contraction and lung function.

Novel mechanisms regulating contraction in Cav-1 KO mice

ASM contraction *in situ* is regulated both by intrinsic cellular pathways and by mediators released from neighboring cells, such as the airway epithelium. Thus, despite a clear intrinsic role for caveolins in regulating isolated ASM cell contraction, there remains a need for systematic assessment of mechanisms that integrate contraction of intact multicellular airways from Cav-1 KO mice. Arachidonic acid metabolites play an important role in cellular physiology, and their aberrant biosynthesis via COX-2 or 5-LO pathways has been linked with worsened allergic and inflammatory diseases [10-12]. The increased contractile function of ASM as seen in chronic airway diseases can be regulated by the airway epithelium, which is a rich source of lipid mediators that regulate ASM tone and contractility [13-16]. We found that MCh responses of tracheal preparations from Cav-1 KO mice were highly sensitive to pharmacological COX-2 manipulation, an effect that could be reversed using 5-LO pathway inhibitors, but that was not observed in rings from wild-type mice. We also report that removal of the epithelium ameliorated COX-2 sensitivity in Cav-1 KO preparations, and that COX-2 and 5-LO abundance was markedly increased in the airway epithelia of Cav-1 KO mice. These data suggest that in the epithelium Cav-1 regulates key enzymes associated with the arachidonic acid cascade, and that this acts as a significant determinant of agonist-induced constriction of intact airways. Moreover, our findings indicate that the ability of ASM to contract in response to GPCR ligands (i.e. MCh) is unchanged in Cav-1 KO mice, a result that differs from a body of evidence showing acute transient silencing of Cav-1 suppresses Ca^{2+} mobilization and contraction. Thus, our new findings suggest that alternate mechanisms to maintain contractility may be enhanced with long-term

caveolin-1 depletion. This study also reveals that Cav-1 and caveolae offer complex control of airway responsiveness *via* effects in different cell types that lead to alterations in both intrinsic cellular responses and intercellular communication.

SG complex plays an important role in airway physiology

Inspite of its emerging role in vascular smooth muscle biology nothing is known about the functional role of sarcoglycans in ASM. In this study we used δ-SG KO mice to perform isometric tracheal contraction studies *ex vivo,* we also determined lung function using a small animal ventilator to assess the airway mechanics in the KO mice *in vivo*. We also looked at the ultrastructural changes occurring in the airway smooth muscle using transmission electron microscopy in the δ-SG KO mice and compared to the wild-type mice. Our results suggest that sarcoglycan complex plays an important role in determining tracheal smooth muscle contraction and the airway physiology in aged ð-SG KO mice through organization of caveolae. These studies supports the previous findings that DGC is a key determinant of maintaining caveolae microdomains at the cell membrane as disruption in the DGC proteins either through latrunculin (indirectly by preventing actin polymerization) or by silencing β-dystroglycan (a key subunit of DGC) leads to both reduction in caveolae proteins and increased the internalization of caveolae structures in the cell [8]. In summary, our functional data using δ-SG KO mice suggests that the DGC plays an important role in contractile properties of ASM and is also a key determinant of airway physiology *in vivo*.

Significance

This project broadly examined the role of DGC and its interaction with Cav-1 on M_3R mediated cell signaling that leads to ASM contraction *in vitro*, *ex vivo*, and *in vivo*. This is important, because inhibitors of GPCRs can prevent allergen-induced airway hyperresponsiveness. Since most current relief therapies for asthma target GPCRs expressed by ASM, better understanding of how caveolae modulate agonist-induced ASM cell responses could be important in developing new and more effective therapies for the disease.

Future directions

The work outlined in the thesis is the first systematic characterization of the DGC subunits in the ASM cells and tissues. Moreover we have broadly investigated the role of the DGC in determining the functional properties of ASM *in vitro*, *ex vivo* and *in vivo*. Outlined below are the areas of research where the work of the current project could be taken further to ask direct yet mechanistic questions.

DGC and cell signaling and survival

The role of DGC as a molecular shock absorber and its structural role in various cell types substantiate its importance in providing a physical link between actin and the extracellular matrix (ECM) [17, 18]. Although the mechanical role of the DGC is important, many lines of evidence now point to a significant contribution by the DGC as a receiver and transducer of signals [19]. Emerging evidence suggests that the DGC has a

more active function in the transduction of a variety of signaling pathways through ERK-MAP kinase pathway [20], which might modulate cell response to integrin engagement on laminin. This possibility needs to be tested in ASM cells. Further research investigating the role of DGC (especially the β-dystroglycan) in these signaling pathways will not only provide greater insight into a number of fundamental processes governing ASM phenotype but will also help us to understand its function in pathophysiological conditions such as asthma and COPD where ASM plays an important role.

Loss of the DGC subunits not only disrupts any physical link but also the protective role that this might confer to muscle cell. Previous findings showed role of DGC in skeletal muscle cell survival [21], however, its role in ASM cell survival is unclear. This is important as the interaction of DGC with laminin is a key determinant of ASM phenotype (Chapter 4 [4]) and perturbation of this link could lead to the disruption of survival signaling in muscle cells. As the DGC-laminin-integrin signaling operates through PI3K signaling (a key cell survival pathway in the cell), future research investigating its role in ASM cell survival is warranted.

DGC in mechanotransduction

The DGC maintains the mechanical stability of the muscle fiber membrane during muscle contraction and relaxation [22]. Since *in vivo* ASM cells are always subjected to the mechanical forces, the aberrant regulation of this pathway in response to mechanical stretch could be involved in the muscle could be involved in the muscle

pathogenesis [23]. The elucidation of mechanosignaling pathways (using an artificial stretching system such as Flexcell) in ASM cells with respect to the organization of DGC and caveolae could provide a detailed understanding of mechanistic link between DGC and regulation of mechanosignaling. The physiological role of mechanosignaling can be translated to *in vivo* setting using mice lacking DGC subunits. The airway mechanics results described in the thesis could be better correlated when airway mechanics is measured in a group of KO mice (mdx and ð-SG) after mechanical ventilation. These studies will provide initial lead on the contribution of contraction-induced damage on airway physiology.

DGC in allergen-induced airway remodeling and hyperresponsiveness

Recent research suggests a role for caveolin-1 in chronic lung diseases such as asthma, COPD and pulmonary fibrosis [25-27]. Moreover, allergen-naïve caveolin-1 KO mice exhibit an altered lung phenotype that likely affects airway physiology [28]. The physiological effects of caveolin-1 could be partly regulated by the organization of DGC subunits in the caveolae. This hypothesis needs to be tested in DGC subunits KO mice (mdx and ð-SG) in a model of asthma using house dust mite as an allergen. The impact DGC and caveolae in airway biology and function, and in allergen-induced airway inflammation and remodeling is unknown. Thus these studies will tell us whether DGC along with caveolae modulate responsiveness of the airway to allergens.

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