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Fisiopatología

Pathobiology of airway smooth muscle remodeling

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El mecanismo primario de asma y EBPOC involucra una excesiva contracción de las vías aéreas (VA), cuya severidad se encuentra en relación a inflamación crónica. Evidencia reciente sugiere que las células de músculo liso de las vías aéreas (MLVA) poseen elevada plasticidad celular que puede contribuir en inflamación, resultando en su engrosamiento mediante hiperplasia y/o hipertrofia. La interacción MLVA-microambiente tisular es la base para hiperreactividad y remodelado tisular, con contribuciones importantes de virus y mediadores químicos, especialmente acetilcolina. Esta revisión abarca la fisiopatología del remodelado del MLVA en relación a fenotipos graves de enfermedades inflamatorias bronquiales. Un análisis *in silico* de hibridación entre secuencias de ARN humano y virales fue realizado, obteniendo datos para apoyar una hipótesis de 'hit and run'. Como una propuesta de integración, se resumen los últimos hallazgos moleculares con una perspectiva que ayude al establecimiento de fundamentos para investigaciones futuras y la comprensión de las vías de señalización que regulan la biología del MLVA.

Palabras Claves:Asma, EPOC; musculo liso bronquial; receptores muscarínicos; virus ARN

Title

Patobiología del remodelado de las vías aéreas

Abstract

The primary mechanism of morbidity and mortality in asthma and COPD is excessive airway narrowing, which severity is based on chronic inflammation. New evidence suggests airway smooth muscle (ASM) cells show extraordinary cellular plasticity that may contribute to airway inflammation, ensuing ASM thickening by either hyperplasia and/or hypertrophy. Tissue microenvironment-ASM interaction is a complex crosstalk that supports hyperresponsiveness and tissue remodeling, with major contributions of viruses and chemical mediators, especially acetylcholine. This review addresses the ASM pathology in relation with severe phenotypes of airway inflammatory diseases. An *in silico* analysis of hybridization between human and viral RNA strands was performed, obtaining data to support a 'hit and run' hypothesis. As an integrative proposal, we summarized the last molecular findings in this field with a perspective that helps to set the stage for future research toward understanding the signaling pathways regulating ASM biology.

Key Word

Asthma; COPD; airway smooth muscle; muscarinic receptors; RNA virus

Introduction

Asthma and chronic obstructive pulmonary disease (COPD) comprise chronic inflammatory disorders characterized by airway hyperresponsiveness and airflow obstruction that can fluctuate over time. They have an increasing economic burden, not to mention their associated disabilities and fatal outcomes. Despite of outnumbered research, we still have not completely understood the big picture of their natural history. Quite a lot of evidence arising from epidemiological, clinical, pathological, and molecular studies, have only provided a short view of the pathobiological mechanisms generating those diseases. The development of better functional assessment techniques along with a wider availability of new biomarkers allowed to recognize that inflammatory airway diseases, especially asthma, involve multiple subphenotypes that differ in clinical severity, histopathology, response to therapy, and long-term outcome. In consequence, heterogeneous groups have been identified, which are likely originated from a unique genetic background/ environment combination.

Severe phenotypes of airway diseases that course with airway hyperreactivity, implicate airflow obstruction that is either irreversible or only partially reversible. Their severe nature apparently is due to longstanding inflammation in the airways. In this setting, injury cyclicity, age, genetic factors, and previous tissue history induce structural changes; a phenomenon commonly coined as 'airway remodeling'. Airway remodeling is assumed to result in severe phenotypes. However, several clinical and animal studies indicate that the relationship between inflammation, remodeling, and hyperresponsiveness is complex, and still not completely understood. Considering that airway hyperreactivity results in an abnormal airway tone, smooth muscle thickening is thought as the main substrate of abnormal airway mechanics. This has been deeply explored; hence, considerable data is available. In this review, we gathered the last experimental findings in this field to formulate a model of airway smooth muscle (ASM) remodeling that fits into the natural history of common airway diseases, mainly focusing on molecular mechanisms.

I. Overview of airway smooth muscle remodeling

Growing evidence supports various pathophysiological mechanisms of airway diseases, including structural changes seen on severe asthma, COPD, chronic bronchitis, cystic fibrosis, and bronchiectasis[1-4]. Thus, airway remodeling has been defined by modifications in the composition, amount, and organization of local cells, including epithelium, glands, blood vessels, extracellular matrix (ECM), and smooth muscle (see Fig. 1).

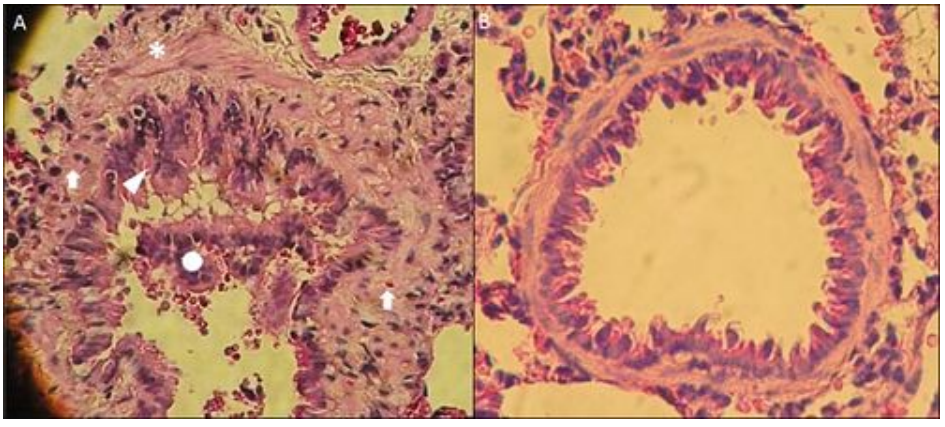

Figure 1. Histopathology of small airways in OVA-sensitized Rats. A bronchiole from OVA-sensitized rat(A) in comparison to a normal bronchiole from saline-nebulized rat(B). Tissue shows remodeling features, such as: epithelial hyperplasia (arrowhead), ASM thickening (asterisk), lymphocytic/eosinophilic inflammation (arrows), and luminal exudate (circle), compare with the normal airway. Lung samples were extracted from rats after a protocol previously described(66). Magnification 200X, Hematoxylin-eosin staining.

Interaction of genetic and environmental factors evolve into assorted outcomes after injury. Many models[5] and clinical studies have shown that symptoms and functional findings are caused by three interconnected factors: 1) chronic inflammation, 2) airway hyperresponsiveness (AHR), and 3) tissue remodeling. Notwithstanding, a major concern in this field is that there is no clear chronologic and quantitative relationships. A current perspective considers that unbalanced immune responses to external factors, such as allergens in asthma, sets up a harmful microenvironment of cyclic injury and repair, which leads to abnormal structure and function[6, 7]. Furthermore, a recent study suggests that chronic mechanical stress resulting from bronchoconstriction *per se* may also lead to remodeling without inflammation[8]. Despite of

airway remodeling could be disorder-specific, the airway structure may play a common role to airway narrowing and airflow limitation carrying out poorly reversible airway obstruction.

Evidence of ASM thickening in Asthma and COPD

Asthma and COPD are well-differentiated clinical entities with some overlapping syndromes in the middle[3, 9]. They share some features, especially at pathological level, including: epithelial hyperplasia and dysfunction, subepithelial fibrosis, increased myofibroblasts, increased vascularization, abnormal neurite branching, and dense ASM layers[2, 7, 10]; highlighting that a greater basement membrane thickness as well as smooth muscle hyperplasia are commonest seen on asthma[10, 11]. Their deepness frequently wedges with clinical expression and severity[12, 13]. A study that evaluated bronchial wall thickness by high resolution computed tomography in mild-to-moderate asthma and COPD revealed the airway diameter and thickness were similar[14], but asthma still has received more attention respect to ASM remodeling. Increased ASM mass could be attributable to hyperplasia, hypertrophy or both. Under physiological conditions, ASM located in the central and peripheral airways are bands that wrap up around the airways in a helical pattern. Its thickness, relative to the diameter of the airway lumen, increases towards the periphery, but in absolute terms, the amount is less in the peripheral airways[15]. A morphometric study indicates that the bronchial smooth muscle mass of patients suffering of fatal asthma was twice than non-asthmatics[16]. A major concern of this proposal is the ECM volume was not measured. To solve it, a recent study showed ASM hypertrophy in the large airways in both nonfatal and fatal asthma, but hyperplasia was only seen in large and small airways in fatal cases. Both groups were associated with an absolute increase in ECM[17]. Some degree of airway wall thickening was regularly detected in asthmatics of all severities with predominance in severe cases[18]. The occurrence of remodeling does not seem to depend on the inflammatory response subtype; since, airway structure does not differ between asthmatics with eosinophilia and those without[19]. Contrasting results debate the importance of ASM remodeling because it is not always found in asthma, hence, no differences in averageof smooth musclecell cross-sectional area[20].

There is less evidence supporting ASM thickening in COPD than asthma. Obliteration and fibrosis of the alveolar wall, mucous gland hypertrophy, and goblet cell hyperplasia are well-known pathological features of COPD[21]. Increased ASM thickness has been found as compared to control, but lesser than asthmatics[15]. Functional implications have been shown, as it correlates with the airway obstruction degree[22]. Additionally, ASM mass and adventitia increased together by 50% in severe COPD affecting the small airway physiology[23]. Biopsy studies from large airways reported no increase in ASM; moreover, smooth muscle protein isoforms were not increased, but there was a slight increment in myosin light chain kinase (MLCK) without changing the myosin light chain phosphorylation[24]. Conflicting results showed that remodeling may occur in the central airways by greater ECM protein deposition and increased ASM[25].

This data points out that asthma and COPD could progress with variable degree of ASM remodeling, but no direct evidence has been obtained supporting reversibility. A murine model of asthma suggests that after allergen cessation, the goblet hyperplasia and collagen deposition resolved first and then lymphocytic infiltration along with ASM thickening[26]. This brings up an open question whether in human diseases a complete removal of the tissue hazard can be accompanied by spontaneous resolution of airway remodeling.

Inflammatory Microenvironment Orchestrates ASM Remodeling

Airway remodeling is associated with longstanding inflammation. Interleukin (IL)-1β, IL-6, and Tumor Necrosis Factor-α (TNF-α) as well as growth factors such as Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), Insulin-like Growth Factor (IGF), and Transforming Growth Factor-β (TGF-β), have pleiotropic effects; however, specific immune responses portray distinctive pathologic features[7, 27]. T cell reactions cover a wide spectrum of divergent cytokine networks. In asthma, for example, clinical phenotypes match inflammatory profiles: I-type hypersensitivity reaction (IgE-dependent), Th2 predominant inflammation, and non-Th2 associated response[28]. However, a common feature seen in all cases is the ASM thickening (see Fig. 2).

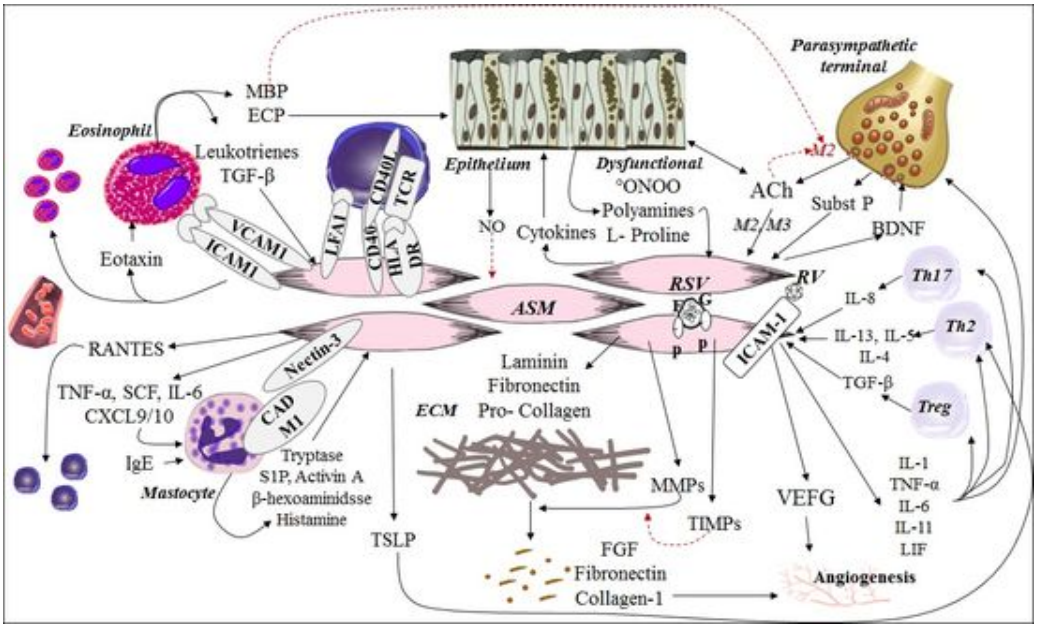

Figure 2. Crosstalk between ASM with cellular and non-cellular components of the airways during inflammation. (See the text for explanation).

Eosinophils are the most prominent inflammatory cells in the airways of asthmatics[21]. In the course of hypersensitivity reactions, eosinophils localize in close relation to ASM. For instance, small airways contain eosinophils in their outer portion (between ASM and alveolar attachment), whereas in the large airways they are present predominantly in their inner portion (between ASM and basement membrane)[29]. This eosinophil-ASM relationship can enhance cell proliferation by cysteinyl leukotriene secretion[30]. Eosinophil homing in the airways depends on Th2 cytokines and eotaxin. Adhesion to ASM is mediated by cell adhesion molecules (ICAM-1, VCAM-1) that are constitutively expressed and also upregulated. ASM cells (ASMC)-derived cytokines could promote eosinophil differentiation, perpetuating the burden of eosinophils into ASM bundles[31].

In a similar way, mastocyte infiltration is prominent in ASM bundles[32]. Mastocyte migration and adhesion potentiate tissue remodeling because histamine, tryptase, activin A, sphingosine 1-phosphate (S1P), β- hexosaminidase, and TNF-α, stimulate many ASMC functions[33]. Despite these mediators can stimulate cell proliferation, mast cell seems not to be relevant for neither proliferation or survival[34]. On the other hand, mastocyte can induce the thymic stromal lymphopoietin (TSLP) in ASM that is highly determinant of Th2 polarization[35]. Mast cell placement, proliferation and survival into the ASM could occur through allergen-independent mechanisms[36].

Neutrophilic inflammation also occurs in severe asthma and COPD. Even though, clinical phenotypes that course with predominant eosinophilic inflammation lead to ASM thickening in both large and small airways, the neutrophil infiltration is almost restricted to concurrent small airway remodeling[37]. A histopathological study of children with fatal untreated respiratory syncytial virus (RSV) infection showed vascular leakage and neutrophil recruitment into the submuscular layer, smooth muscle, and airway epithelium, resembling fatal cases of obstructive diseases[38]. IL-8 has been implicated, and it seems to be secreted by Th17 cells[39]. Nowadays, it is known that Th9, Th22, and Th25 cells also modulate countless aspects of airway immunity[40]. Nonetheless, the most significant cytokine expression in asthma includes IL-4, IL-9, IL-13, eotaxin and RANTES. This profile correspond to an upregulated Th2 reaction[41]. $CD4^+$ T cells transfer from ovalbumin (OVA)-sensitized rats to non-sensitized rats (adoptive lymphocyte transfer), showed that an specific subset of Th2 cells drove airway remodeling in non-sensitized rats after few OVA challenges[42]. However, evidence from animal models and humans indicate that Th2 hypothesis is an incomplete explanation for asthma pathogenesis, as allergic and nonallergic types are pathologically indistinguishable[43]. It has also been reported that airway epithelial cells in asthmatics upregulate the EGF receptor (EGFR) expression, a receptor tyrosine kinase (RTK), even in absence of significant eosinophilic inflammation[44]. A recent study demonstrated subepithelial fibrosis in severe asthmatics without evidence of Th2 inflammation[45]. Depletion of $CD4^+$ cells, previous to chronic OVA challenge, significantly reduced peribronchial inflammation but did not completely reverse ASM thickening[46]. Although Th2 cytokines have pro-remodeling actions *in vitro*, controversial results have been found as IL-5 and IL-13 do not increase ASMC proliferation, but they induce phenotypic switching[47, 48]; and IL-4 inhibits ASMC replication[49]. These studies suggest that remodeling can also occur independently of Th2 inflammation and other factors are needed.

COPD is also accompanied by airway inflammation that is different from asthma, but ASM

remodeling still occur[21]. Chronic inflammation induced by chronic cigarette smoking consists of neutrophil, macrophage, B cell, and $CD8^+$ T cell recruitment, and it worsens as disease severity increases[50]. T-lymphocytes and macrophages are the predominant cells, being $CD8^+$ T-lymphocyte infiltration the most remarkable feature in both large and small airways, and there is also absence of significant eosinophilic inflammation[51]. Although, $Foxp3^+$ regulatory T cells play a role in fibrogenesis, there is no predominant T $CD4^+$ subset. This supports the concept that cyclic events of cytokine and growth factor surges could be the main drivers regardless of the etiology and immune polarization.

An intricate network underlies the ASM and its surroundings, not only immune cells but also neural parasympathetic endings, mesenchymal cells, ECM, and epithelium (see Fig.2). For example, ASM activation by proinflammatory cytokines and substance P can induce the brain-derived neurotrophic factor (BDNF) expression for spatial coordination of neuronal branching[52], and vascular endothelial growth factor (VEGF) for control of angiogenesis to assure adequate perfusion, the latter have an important repercussion on vascular leakage and vasogenic edema during fatal asthma[53]. Neuronal development also would coordinate spatial distribution of ASM, because substance P induces both migration and proliferation[54]. Nevertheless, the airway epithelium could have a greater contribution due to its plasticity and inflammatory properties. Dysfunctional epithelial cells release growth factors, as well as, acetylcholine (ACh) and leukotrienes that could contribute to ASM growth, ECM deposition, and angiogenesis[55]. Moreover, the epithelium is an important source of nitric oxide (NO) in the airways, which has relaxing and other anti-remodeling effects. Physiological NO is produced by constitutively expressed neuronal and endothelial NO synthase (n-,e-NOS)[56]. However, cytokines increase inducible NOS (iNOS) and arginase expression. A greater iNOS/ arginase activity decreases L-arginine bioavailability, which generates an uncoupled iNOS that not only synthases NO, but it also produces superoxide and peroxynitrite. These molecules are capable of causing cellular toxicity and promoting AHR[57]. Functional consequences of increased arginase are reinforced by L-arginine transport blockage with eosinophil-derived polycations. L-Ornithine, a product of urea cycle, is a precursor of polyamines and L-proline, both involved in cell proliferation, collagen synthesis and chromatin remodeling[58]. This exemplifies how noncontractile ASM functions are modulated by many conditions; therefore, the commonest experimental approaches based on univariate analysis can under- or overestimate their contribution on smooth muscle processes.

ASMC are multifunctional

The relevance of ASMCs in pulmonary diseases has been recognized since the last century. The consensus until a few years ago was to consider them just as effectors. However, far from their abilities to contract and relax, ASMCs proliferate, migrate, secrete chemokines/cytokines, and express surface receptors for cell adhesion and leukocyte activation, having a crucial role in airway dysfunction[59]. A concept of plasticity emerged when those functions were associated with specific circumstances and required wide adjustment in gene expression[60, 61]. ASM hypertrophy and/or hyperplasia involve not only outer cell influences, but also ASMC reactions with paracrine/autocrine properties[7, 62]. Quite a lot of molecules could coordinate this loop, such as: growth factors, cytokines, chemokines, ECM molecules, G protein-coupled receptor (GPCR) agonists, natriuretic peptides (NPs), NO, and others[63-66].

Several *in vitro* synthetic functions have been shown. Also, ASM in mild asthmatics has constitutive staining for RANTES[67]. Further cytokines secreted by ASM include IL-1β and IL-6 family cytokines, such as leukemia inhibitory factor (LIF) and IL-11[68]. These have deeper effects on recruitment, proliferation, and differentiation of eosinophils, mastocytes, T cells, and B cells, establishing a bidirectional regulatory network. Mainly, a $CD4^+$ T cell- myocyte crosstalk through direct contact has shown to be determinant of ASM remodeling[42]. Airway homing of T cells is CCL5 or RANTES-guided, which is released by ASMCs. Likewise, strong adhesion between these two cell types has also been described[69]. Remarkably, even though ASMCs are not usually thought as antigen-presenting cells, evidence supports the expression of major histocompatibility complex class (MHC) II molecules making them capable of antigen presentation. Moreover, ASMCs express the cell adhesion molecules (CAMs)/costimulatory molecules, CD40, CD40L, CD80, CD86, ICAM-1 (CD54), VCAM-1 and LFA-1 (CD11a/CD18)[70]. The CD44-dependent T cell adhesion to ASMCs is not only significant to exchange inflammatory signals, but also to induce ASM hyperplasia through RTK activation[71]. This cooperative signaling mediates proasthmatic-like changes in ASM responsiveness, and denotes a potential mechanism of remodeling.

In the airway, a net of collagenous and noncollagenous proteins influences cellular behaviors. ECM components include collagens, fibronectin, members of the matrix metalloproteinase (MMP) family, as well as their inhibitors (TIMP)[59]. After serum stimulation, ASMCs were found to generate elastin, laminin-β1,-2, and -γ1, thrombospondin, collagen-I-V, and decorin[72]. In addition to promoting ECM deposition, ASMCs are capable to affect its degradation. Human ASMCs release progelatinase A (MMP-2 precursor) and, after TNF-α stimulation, gelatinase B

(MMP-9)[73]. MMP production suggests that ASM contributes to ECM turnover, and subsequently the airway remodeling, because inhibition of the autocrine-derived MMP-2 has antiproliferative effects on ASMC culture[74]. Therefore, ECM degradation could be essential for ASM phenotypic modulation, being degradation of the pericellular collagen fibrils a requirement to allow cell division[75]. Serum levels of TIMP-1 and MMP-9 are raised in both asthma and COPD, supporting a straight relationship between clinical expression and tissue remodeling. The MMP-9/TIMP-1 ratio and periostin levels could be consider biomarkers of active disease[76]. Cyclic inflammation/ repair simultaneously occur to cyclic ECM degradation and deposition, which could be a critical phase in ASM thickening.

Crosstalk between ASM Remodeling and Hyperresponsiveness

Airway narrowing and abnormal muscle relaxation are the hallmarks of asthma, COPD, and bronchitis. Multiple mechanisms have been proposed to explain the AHR, like increased vagal tone, cytokine-potentiated increment of free intracellular calcium, increased MLCK activity, and activation of the procontractile Rho kinase pathway[77]. All of them have in common that could hasten the shortening velocity. Therefore, even though remodeling can be triggered by hypersensitivity reactions, infections, environmental pollutants, and developmental abnormalities, AHR could be just generated by unbalanced responses to contractile vs relaxing factors[78]. The role of ASM remodeling as a substrate of AHR was uncertain because functional abnormalities can be seen without changes in the bronchial smooth muscle mass[79]. However, increasingly data supports a role in severe AHR phenotypes, and irreversible or partially reversible airflow obstruction[43]. The structure determines both passive tone and active responses to agonist stimulation. ASM remodeling involves phenotypic changes that enhance its thickening, and during this process a decline in force induced by repetitive length changes is seen, but then it rapidly adapts and recovers its ability to generate force. In this way, higher passive stiffness could contribute to increased AHR by attenuating the extent of ASM length fluctuations during tidal breathing, i.e., ASMCs adapt by assuming a shorter resting length while retaining its ability to generate force[27]. For that reason, after induced bronchoconstriction, deep inspiration causes airways of asthmatic individuals to dilate transiently.

Expression of immunomodulatory molecules by ASMCs can delay inflammation resolution and lead to aberrant healing, which is a potential mechanism of AHR[78]. The change in the ASMC population compromises an increase of synthetic properties, which can modulate the contractile mass. Particularly, if it is considered that the whole ASM is coupled by gap junctions, and the calcium dynamic differs between ASMC phenotypes. Propagation of wave-like calcium currents from modulated ASMC to contractile ASM would hypothetically affect not only contractile functions but also noncontractile activities, as discussed in following sections. Other noncontractile elements, including excessive ECM content, may lead to nonreversible airway obstruction by reducing airway distensibility[80]. Whether increased ASM supports abnormal reactions to agonists or makes the airway stiffer, it definitely provides an exceptional substrate for AHR in a framework of progression and severity, at least for asthma. In fatal asthma, airway wall thickness is increased around 50-230%, while in nonfatal asthma it ranges from 25 to 150%, most studies pointing out hyperplasia over hypertrophy as the predominant mechanism[11-13, 43].

ASMC plasticity: origins and phenotypes

The ASM thickening has been studied in many animals and human models, wherein ASMC cultures have provided some ideas about pathways underlying the origin of hyperplasia and hypertrophy. Once ASMC populations were characterized *in vivo* and *in vitro*, heterogeneous subgroups with distinctive phenotypes were identified. A wide range of functions depend on culture conditions[60]. Manipulating such environments allowed comprehension of the rules for phenotypic transition[61, 81, 82]. Hence, ASMC could be sorted into three categories: 1) contractile (c-ASMC), 2) synthetic/proliferative (s/p-ASMC), and 3) hypercontractile (h-ASMC) (see Table 1). Also, a few switching routes have been described, where *modulation* means a shift from contractile to synthetic/proliferative, and *maturation* is the inverse transition. Turning into hypercontractile is also possible, and some authors have speculated about its irreversibility; however, *in vitro* ASMCs can tolerate cyclic phenotypic adjustments. An important aspect is that modulation and maturation exemplify an adaptation model to tissue microenvironment fluctuations, events that could take place *in vivo* and drive critical phases of airway remodeling. Accordingly, transition from native c-ASMC to s/p-ASMC would be the initial step, then, replication of s/p-ASMC would warrant smooth muscle hyperplasia, and finally aberrant differentiation from either s/p-ASMC or c-ASMC to h-ASMC would cause muscle hypertrophy. How these phenotypical modifications fit in the natural history of airway diseases is a matter of debate.

Phenotypic Markers

Smooth muscle has typical features in primary cultures (see Table 1, Fig. 3). A long cellular body, central nucleus, few granulations, and cytoplasmic inclusions (3A), a confluent monolayer with “hill and valley” aspect (**3C**), and shrunk reaction to contractile agonists define smooth muscle cells[83]. However, each ASMC subpopulation has specific characteristics. For example, the synthetic/proliferative phenotype has satellite flattened shape with multiple extensions (3B), a high number of organelles for protein and lipid synthesis, abundant mitochondria, a higher proliferative response, decreased contractile proteins, shutdown of responses to contractile agonists, and secretion of growth factors, collagen, cytokines, bradykinin, and eotaxin[81,84]. Furthermore, modulated ASMCs show increased protein expression of fetal and non-muscle isoforms. Synthetic and proliferative functions do not correspond to different traits. Indeed, the cell distribution with synthetic activities could vary between 20 to 60%, and almost half of replicating ASMCs produce cytokines. Also, secretion can be done by non-replicating cells[81]. On the other hand, the contractile phenotype is associated with a decreased number of synthetic organelles, a stronger response to contractile agonists, increased expression of contractile and structural proteins, and an increased M_3/M_2 muscarinic receptor expression rate[84-86]. Other markers including Ca^{2+} profiles[87], miRNA expression[88], and transcription factors expression[89] have been useful for phenotype distinction.

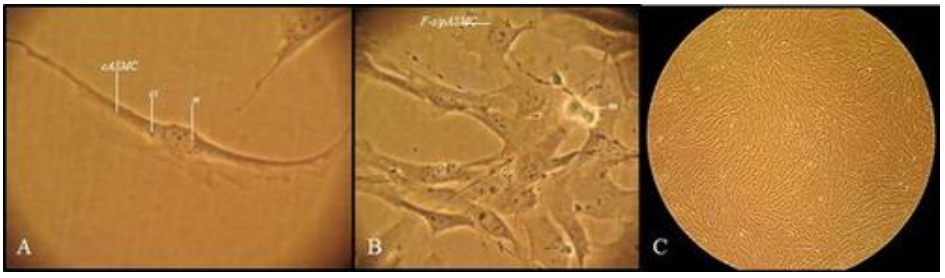

Figure 3. Primary cultured of Rat ASMCs. Cells were obtained by enzymatic digestion of rat trachea and cultured in supplemented medium as previously described(122) . (A) Contractile phenotype, (ci) cytoplasmic inclusions, (n) cell nucleus. (B) When the cell population underwent to growth some cells adopted a myofibroblast-like morphology (F-s/pASMC), (m) mitosis. (C) Cell confluence of 80-90%, cell population readopted contractile morphology with a hill and valley array. Magnification 400X A, B, 100X C.

Table 1. Characterization and Biomarkers of ASMC Phenotypes

Phenotype	Contractile	Synthetic/ Proliferative	Hypercontractile
Function	Regulation of airway resistance	Immunomodulation ECM Turnover Chemotaxis ASM hyperplasia AHR	ASM hypertrophy AHR
Features in primary culture			
Induction	Maturation -High density -Serum starvation	Modulation - Low density - Mitogens	Cyclic exposure to: - Mitogens, TGF-β
Proliferative rate	Slow	Fast	--
Morphology			
Shape	Elongated-spindle	Satellite Flattened	Elongated-spindle
Myofilaments	+	+/-	+++
Contractile proteins/cell size ratio	↔	↓	↔
Mitochondria	+	+++	++
SR/ Golgi apparatus	+	+++	++
Caveolae	+ (> 150.000)	-	+++
Gap junctions	+++	+	+++
Biomechanics			
V_o/V_{max}	Normal	↓	↑↑↑
Contribution to airway resistance	Reversible (normal contraction-relaxation)	-- (affect dynamics of contractile mass)	↑↑↑: ↓ relaxation
Response to agonists	+	+/-	+++: AHR
Biomarkers			
Contractile Proteins			
sm-α-actin	++	+++	++
sm-MHC	++	+/-	++
Structural Proteins			
non-sm-actin (β/γ)	+/-	+++	++
non-sm-MHC	+/-	+++	?
vimentin	+	+++	?
smoothelin-A	++	+/-	?
SM-22	++	+/-	?
Regulatory Proteins			
MLCK	++	+/-	++++
***h*-Caldesmon**	++	+/-	?
***l*-Caldesmon**	+/-	++	?
Calponin	++	+/-	?
Caveolin	+++	+/-	?
Desmin	++	+/-	?
PKC (α/βI/βII)	+/-	+++	?
CD44	+/-	+++	?
M_3AchR	++	+/-	+++
ET_A receptor	++	+++	?
ET_B receptor	++	+/-	?
Distrofin	++	+/-	?
Integrin $\alpha_7\beta_1$	++	+/-	?
Cathepsin k	+	++	?
FSP	++	+	?
AKR1C3	-	-	-
Calcium Dynamics	Sparks	Waves	Sparks

++: expressed; +++: highly expressed; ++++: very highly expressed; +/-: poorly expressed; V_o: velocity of shortening; V_{max}: maximum shortening capacity. ?: unknown, ↑: increased, ↓: decreased, *h*: heavy form, *l*: light form, FSP: fibroblast surface protein, AKR1C3: Aldo-keto reductase 1C3 (specific marker of mesenchymal cells)

Evidence of *in vivo* Plasticity

Several findings support *in vivo* occurrence of ASMC plasticity, especially in asthma. Plasticity is a universal property of primary ASMC cultures derived from both healthy and diseased humans, and healthy and sensitized animals. Immunohistochemistry to identify contractile proteins is highly variable as well, which can reflect a broad heterogeneity of myocytes in the normal airway that is maintained in cell culture, as demonstrated by a divergent proliferative capacity[90]. If functional features are compared, healthy or control vs asthmatic or sensitized groups, significant differences can be found. ASMCs from asthmatics or sensitized animals show more proliferative and synthetic capabilities than their physiologic counterparts, findings that are preserved despite tissue dissolution follow by cell culture[62, 66, 89, 91-93]. Abnormal ASMCs could not only resemble s/p-ASMCs or arise from c-ASMCs, but also have distinctive features such as: abnormal protein synthesis[94], expression of odd transcription factors isoforms with a lack of response to glucocorticoids[91], increased mitochondrial biogenesis and activity[93], abnormal calcium dynamics[92], increased CysLTR-1 leukotriene receptor expression[55], increased activity of promitogenic pathways[95], and declined of antiproliferative pathways[66]. In consequence, an increased ASM mass may be explained by *intrinsic* alterations in pathological ASMCs that facilitate their proliferative and secretory activities. Asthmatic ASM produces more proinflammatory, proangiogenic, and proremodeling factors, including eotaxin, VEGF, and connective tissue growth factor (CTGF), and fewer antimitogenic factors, such as E2- type prostaglandin (PGE_2)[59]. These would reflect deeper differences in cell populations that constitute the ASM under pathological settings, and they would likely

originate from comparable modulation and maturation events on native ASMCs.

Many questions arise from the alterations observed on asthmatic or sensitized cultured ASMCs. Based on the plasticity phenomena, any transformation of *in vivo* ASMC phenotype which persistence depends on tissue microenvironment should not be seen *in vitro* because the phenotype will adjust to culture conditions. Although, cited studies do not precise whether those pathologic features are irreversible along culture passages, persistence of functional abnormalities after tissue fragmentation and culturing suggests that these cells underwent through a dysfunctional route of phenotypic modulation, which could be at least partially irreversible. In view of that, epigenetic mechanisms could be a suitable explanation to such phenotypic switch. For example, eotaxin hypersecretion by ASMCs has been related to histone H4 lysine 5 and lysine 12 acetylation at the eotaxin promoter induced by TNF-α[96]. Other synthetic activities, such as VEGF hypersecretion, were due to a loss of a repression complex, in which a differential histone H3 lysine 9 methylation modulating Sp1 and RNA polymerase II binding to the VEGF promoter was implicated[97]. Binding of serum response factor (SRF), a transcription factor that controls phenotypic stability, to DNA is associated with post-transcriptional histone modifications including di-methylation of lysine residues 4 and 79 on histone H3, acetylation of lysine 9 on histone 3 and acetylation of histone H4. Histone deacetylases (HDACs) have also been implicated in regulating smooth muscle replication because the HDAC inhibitor TSA can prevent cell proliferation[88]. In *in vivo*, the valproic acid (HDAC inhibitor) did not affect inflammation induced by OVA challenges, but notably reduced the airway thickening including the ASM with blunting of AHR[98]. In addition to HDAC modifications, DNA methylation can generate a specific long-term *signature*. For example, expression of IL-13 in the airways ensued significant changes in methylation of 177 genes, most of which were associated with a Th2 signature over resident cells[99]. Using methylated DNAimmunoprecipitation-next generation sequencing (MeDIP-seq), it was determined that airway remodeling and AHR in house- dust- or mite-sensitized rats are related to specific methylation patterns at several TGF-β signaling-related genes[100], explaining the longevity of abnormal pro-fibrotic responses of local cells during inflammation and phenotypic persistence after tissue extraction. Unfortunately, there is currently no direct evidence of epigenetic regulation of ASMC proliferation. Moreover, whether or not DNA methylation or histone acetylation can influence phenotypic switching have to be determined as well. The contribution of miRNAs will be discussed in following sections.

Potential Sources of ASMCs

The *in vivo* source of ASMCs under pathological conditions is unclear. ASM may originate from increased proliferation or prolong survival of preexisting smooth muscle with proliferative and/or contractile phenotype; however they could also arise from other cell lines that could migrate into the bundles and then differentiate into ASMCs (see Fig.4). Remarkably, other airway cells may undergo to phenotypic modulation that is characterized by α-sm-actin expression and development of organelles for synthetic functions. Accordingly, mesenchyme such as fibroblasts may generate myofibroblasts, whose classical phenotypic markers are indistinguishable from s/p-ASMCs[101]. This fact allowed researchers to postulate a spectrum of mesenchymal plasticity (fibroblasts ↔ myofibroblasts ↔ ASMCs)[102]. However, it does not undermine experimental findings obtained with *in vitro* systems, as a high proportion (~60%) of primary airway mesenchymal cultures truly correspond to primary smooth muscle[60].

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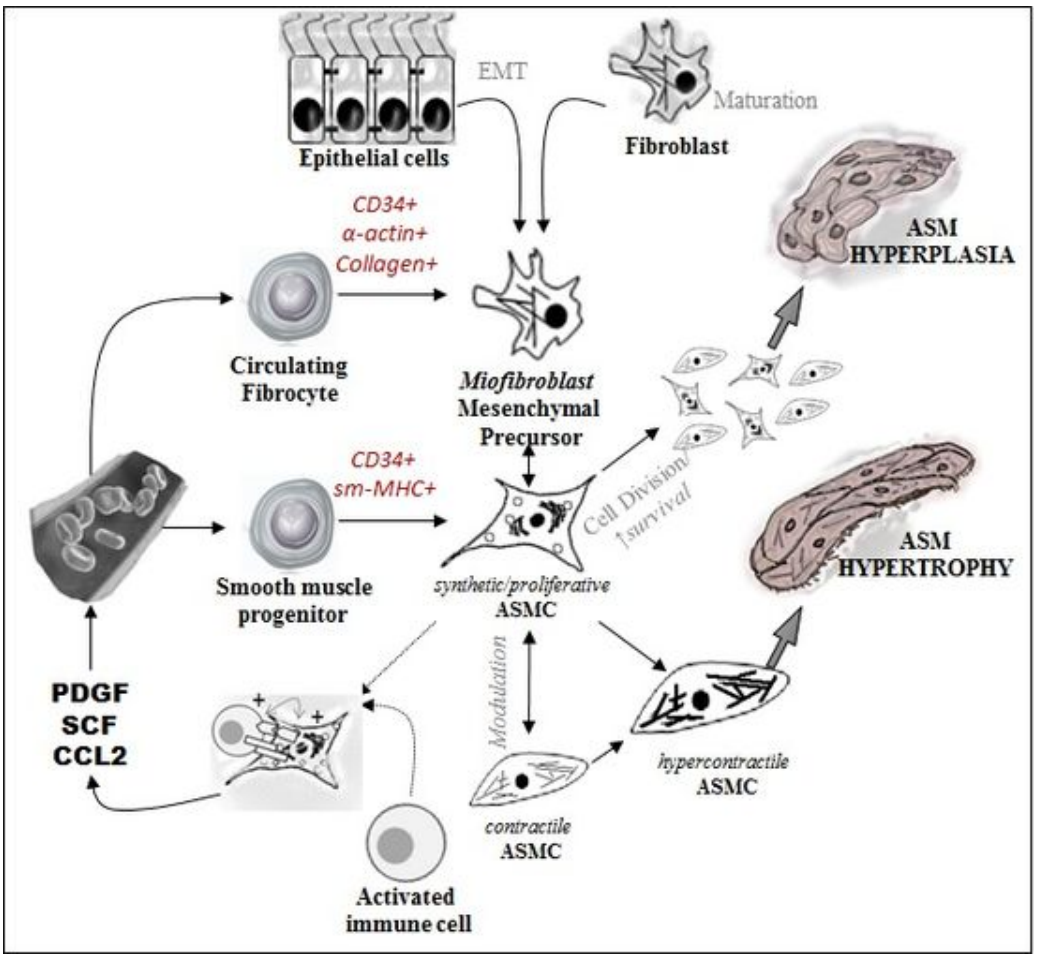


Figure 4. Potential sources of ASMC precursors in the origin of ASM thickening. (See the text for explanation).

Potential progenitors also include true multipotent mesenchymal progenitors and stem cells, either located within the airway or derived from peripheral blood. For example, $CD34^+$-$CCR7^+$-Collagen 1^+-sm-α-$actin^+$ circulating fibrocytes can migrate towards ASM bundles during inflammatory challenges, and they were unresponsive to the apoptotic effects of glucocorticoids in culture[103]. Fibrocyte migration is directed by the ASM-derived PDGF and CCL2, and at that point its co-locating induces proinflammatory activities in ASMCs[104, 105]. A rare population of $CD34^+$-sm-MHC^+ peripheral mononuclear cells (known as smooth muscle progenitors) has been identified by flow cytometry in OVA-sensitized mice. A similar population seems to generate the smooth muscle in atherosclerosis; however, the study did not precise whether stem cell homing occurred into ASM bundles[106].

The airway epithelium can turn into mesenchymal cells through the epithelial-mesenchymal transition (EMT) route, which has been considered as another source of ASMCs[107], but a linage-tracing study suggests that it may just be a consequence of culture conditions and could not occur *in vivo*[108]. In asthma, epithelial cells show fragileness due to downregulation of cell adhesion molecules, which makes EMT more likely[109]. EMT is initiated by extracellular signals, such as collagen or hyaluronic acids, and by growth factors like TGF-β and EGF[110]. This process is modulated by bone morphogenesis proteins, and allergen exposure, which amplifies and accelerates it[111]. Hormones have also been associated, since vitamin D attenuates TGF-β-induced expression of EMT markers[112]. Epithelial and mesenchymal cells express both type-1 and type-3 muscarinic receptors (M_1, M_3)[113]. TGF-β-induced EMT was abolished by muscarinic receptor (mAChR) antagonists and enhanced by acetylcholinesterase (AChE) inhibitors[114]. A positive feedback loop of autocrine and paracrine production of non-neuronal acetylcholine (ACh) and TGF-β orchestrates EMT during chronic inflammation, being a likely source of ASM.

Additionally, an increased number of fibrocytes was observed in the ASM bundles from asthmatics of all severities[115]. However, this study failed to show any link with the lung function, and this location could not be considered abnormal as fibrocytes are normal constituents of ASM bundles under physiological conditions[116]. An increased in mesenchymal cells would be nonspecific and occur in parallel to other cellular changes ongoing in the ASM bundles. Although, many airway cell lines can follow similar modulation pathways as native ASMCs, we focus here on how the behavior and responses of c-ASMC vs s/p-ASMC can

explain many abnormal structural and functional features seen on airway diseases.

Acetylcholine: more than bronchoconstriction

The parasympathetic network penetrates deeply the airway wall and regulates bronchoconstriction. ACh is the predominant parasympathetic neurotransmitter. Although, ASM express both cholinergic receptors, nicotinic receptors (nAChRs) and mAChRs, the cholinergic effects seem to be mediated by muscarinic activation[117]. In asthma, cutting the parasympathetic supply of the airways (vagotomy) prevents an increased smooth muscle contraction[118], and AHR induced by persistent parasympathetic activation has been shown[119]. The striking role of ACh in airway remodeling was highly commented in a recent article[8], where authors proposed methacholine-induced bronchoconstriction as the main driver of epithelial hyperplasia and subepithelial collagen deposition, independently of inflammation, as chronic stretch and mechanotransduction pathways are well-known mechanisms of muscle differentiation. However, the cited study failed to conclusively demonstrate that only mechanical stress was responsible of all histopathological changes, because they did not assess the non-neuronal and non-contractile effects of cholinergic networks, a non-asthmatic group was not included (taking into account that inflammatory signatures on resident cells change their responses), and ASM layers were not assessed. Nonetheless, despite the limitations, the contribution of ACh in airway remodeling was uncovered.

Two ACh sources have been identified: 1. neural, along parasympathetic fibers from vagal nerve, and 2. non-neural, from airway epithelium and immune cells. Both are implicated in increased ASM thickening in asthma[120], which was prevented by M_3-specific antagonists such as tiotropium bromide[121]. Also, M_2 is expressed and its downstream signaling has been related to modulation of some ASMC functions, either promotion or inhibition[122]. ACh, either neuronal or non-neuronal, regulates inflammatory cell responses that may explain the anticholinergic benefitsin asthma and COPD[123]. Collectively, these findings are revealing new therapeutic targets, therefore, we chose the cholinergic signaling pathway to be explored deeply in this review as a prototype of GPCR agonism.

Muscarinic Receptor Signaling

The mAChR family consist of five receptor subtypes that belong to GPCRs. Mammal airways including humans express M_1, M_2, and M_3 subtypes; more precisely, epithelial cells (M_1–M_4), pulmonary vessel endothelial cells (M_1–M_5), mesenchymal cells, such as smooth muscle fibers (M_2, M_3) and fibroblasts (M_2> M_1> M_3> M_4), and lung-infiltrating immune cells, such as mononuclear leukocytes (M_1–M_5) [124]. The M_4 mRNA and protein have been reported in rabbit bronchiolar ASM, but not from humans. Although, pharmacological ligand binding studies showed a mixed population M_2:M_3 in a 4:1 ratio, respectively, a functional dominance of M_3 appears to mediate muscarinic effects under physiological circumstances[125]. This predominance could be a consequence of receptor compartmentalization, facilitating or inhibiting signal transduction depending on accessibility to specific transducers or kinetic regulation. M_3-induced bronchoconstriction is mainly mediated by a caveolae-dependent system. M_2 contribution was mainly uncover in $M_3^{-/-}$ knockout mice with cellular caveolae dissolution. Location of M_2 and M_3 in caveolae is dependent on Caveolin-1 (Cav-1) and Caveolin-3 (Cav-3) expression, respectively[126]. Many processes involve coupling of mAChRs to their cellular effector systems, via heterotrimeric G proteins. These are composed of one α-, β- and γ-subunit and transduction signals depends on both the α-subunit and βγ-subunit. In ASMCs, muscarinic activation is classically considered as the main signal for muscle contraction. However, there is increasing evidence indicating that alterations in its downstream signaling pathways might be responsible for ASM remodeling and AHR[127].

Muscarinic signaling can be divided in pathways for muscle contraction and those with non-contractile effects. In this way, the G_q-coupled M_3 activates phospholipase C (PLC), causing hydrolytic conversion of phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5-trisphosphate ($InsP_3$) and sn-1,2-diacylglycerol (DAG). InsP3 is involved in Ca^{2+} mobilization from intracellular stores, which generates a rapid and transient increase for muscle contraction, while generated DAG activates PKC with subsequent triggering of mitogen-activated protein kinase (MAPK) signaling for non-contractile effects. Also, MAPK cascade can be activated through direct phosphorylation of Raf-1, independently of PKC[117]. β-arrestins mediate homologous receptor desensitization and endocytosis via clathrin-coated pits of agonist-activated GPCRs. The third intracellular loop (i3) of M_3 is required for β-arrestin recruitment after homodimerization (M_3/M_3) and heterodimerization (M_2/M_3). Those macrocomplexes not only induce downregulation of mAChRs, but also act as scaffolds for components of the MAPK cascade, facilitating its activation[128]. This is a third mechanism of M_3 signal transduction for cell growth and differentiation.

As a counterpart, $G_{i/o}$-coupled M_2 contributes to muscle contraction either affecting adenylyl cyclase in an inhibitory manner, or directly enhancing potassium and non-selective ion channels opening, and they both depend on the released βγ dimer. Additionally, M_2 modulates the relaxant effects of atrial natriuretic peptide (ANP), as it suppresses the ANP-induced activation of a membrane-spanning guanylyl cyclase via a pertussis toxin (PTX)-sensitive G protein[129]. Since $G_{i/o}$ is involved in muscarinic-induced actin reorganization, RhoA/Rho-kinase signaling pathway has been related to Ras and phosphoinositide 3-Kinase (PI3-K) activation, contributing to growth factors effects[120].

The complexity of non-canonical muscarinic signaling is illustrated by the ambiguity of their downstream pathways. The cGMP/PKG pathway is a well-documented mediator of muscle relaxation, and it has also anti-remodeling effects. Molecular evidence suggests it could depend on which compartment is activated and signal pattern. Some cascades activated by mAChRs are linked to second messengers such as cGMP by activation of two distinctive guanylyl cyclases. Muscarinic activation of tracheal ASM fragments is associated with contraction, but it also involves the generation of two cGMP signals, at 20-s and 60-s[130]. These signals seem to be essential in reaching the contractile effects of several muscarinic agonists and might be relevant in ASM remodeling. The proposed model[131] for this novel pathway emphasized that 20-s cGMP signal is linked to $G_{i/o}$ coupled M_2 activation, inducing a massive and transient $\alpha_1\beta_1$-NO-soluble guanylyl cyclase (sGC) translocation from cytoplasm to plasma membranes, whereas, the 60-s cGMP signal is associated with a natriuretic peptide receptor (NPR)-GC-B, activated by a G_{q16}-coupled M_3 sensitive to mastoparan. Those signals regulate the muscarinic signal transduction efficacy in response to agonists through phosphorylation events. In this way, M_3 phosphorylation by PKG-II may correlate to changes in the receptor affinity to agonists and antagonists. It has been proposed that cGMP induces PKG phosphorylation of the i3 loop that confers a potential feedback mechanism to terminate the cGMP-dependent muscarinic signal transduction cascades at the sarcolemma[132]. Since phosphorylation of the same loop by others serine/threonine kinases downregulates the receptor by endocytosis and G protein uncoupling, indirect observations suggest that PKG-II phosphorylation of M_3 induces a mAChR dimer formation. Homodimer formation stabilizes or 'freezes' the M_3 population, in a refractory state to agonist activation, and prone to antagonist binding[132]. We speculate in caveolae systems a higher density M_3 receptor population would support positive cooperativity through homodimer/heterodimer formation, which could enhance the signal transduction for MAPK activation and subsequent altered gene expression. Thus, cGMP produced in response to muscarinic agonists could be involved in growth promotion instead of classical cell arrest. Relevantly, we demonstrated that this pathway is still functional in sensitized ASMCs, while cGMP cascade induced by NPs and NO was downregulated[131]. A wide-spectrum of outcomes can result from diverse experimental designs. This complicates our understanding of the cholinergic signaling in human airway diseases. A brief scheme that will be discussed herein, includes effects related to remodeling promotion: 1. c-ASMC modulation, 2. s/p-ASMC proliferation synergism, and 3. synergism on h-ASMC induction, and alternatively possible actions for remodeling prevention like decreased s/p-ASMC proliferation. Although, *in vivo* relevance keeps hypothetic, this provide a coherent and useful picture for planning future research.

Modulation vs Maturation

Signaling Pathways Associated with Maturation

Phenotypic switching is regulated by growth factors, GPCR agonists, ECM molecules, and other mediators found in the bronchoalveolar lavage (BAL) of patients with asthma or COPD[133,134]. The contractile phenotype induction is attainable after exposure to either TGF-β, insulin, or laminin[135-138]. Also, maturation can be supported by lacking of mitogens; in primary ASMC cultures (see Fig.3C), high cell confluence lead to cell cycle arrest by cell contact[82, 86]. sm-specific protein expression is enhanced by RhoA/Rho Kinase and/or PI3-K activation. RhoA/Rho kinase promotes actin polymerization as a downstream effector of either GPCR- or RTK-associated pathways (see Fig.5). Subsequent phosphorylation events, RhoK→ phospholipase D (PLD) → LIMK-1→ cofilin could be responsible for G-actin polymerization into F-actin[139]. A decrement in globular actin level releases some proteins into the cytosol, like the transcriptional coactivator megakaryocytic acute leukemia/ megakaryoblastic leukemia-1 (MAL/MKL-1), which can be trafficked into the cell nucleus and make a macrocomplex with both myocardin (other coactivator) and SRF, a transcription factor. As a whole, they bind gene promoters to increase sm-specific gene expression[140, 141]. TGF-β can amplify this pathway for hypercontractile phenotype induction. Type-1 and type-2 TGF-β receptor activation induce phosphorylation and nuclear translocation of Smad proteins that bind to SRF, building up a macrocomplex similar to MAL/MKL-1/myocardin/SRF, with subsequent gene expression modifications[142].

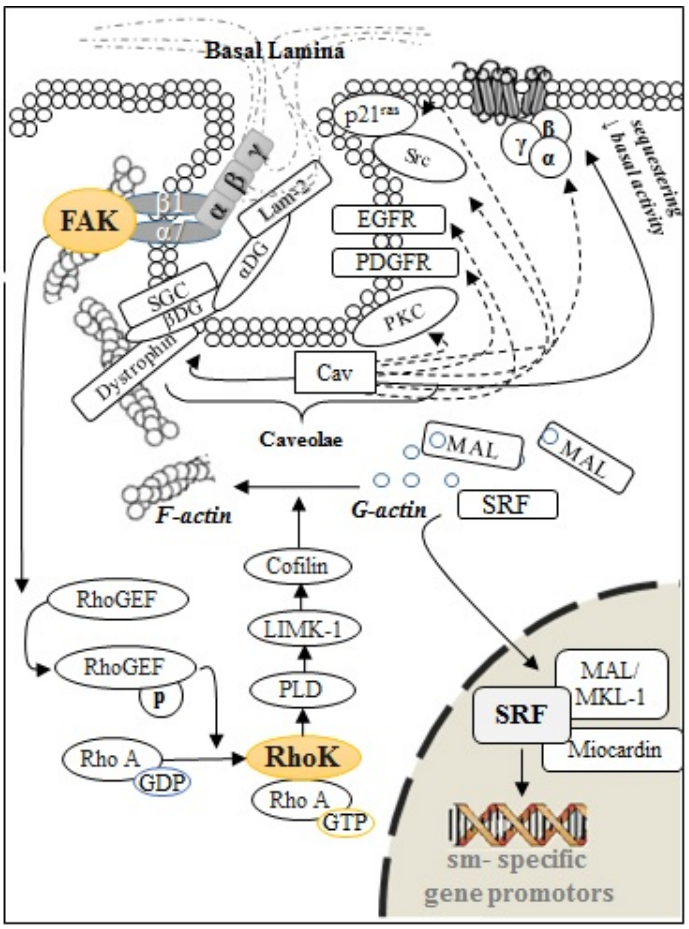


Figure 5. Signaling pathways involve in preserving the ASM in a differentiated state.

The Akt/PI3-Kpathway also affect sm-specific gene expression through the transcription factor FOXO-4[143, 144]. Unphosphorylated FOXO-4 binds myocardin and inhibits its association with SRF. Hence, myocardin is released once PI3-K phosphorylates FOXO-4. However, the reach of this pathway goes far beyond transcriptional regulation. Signaling pathways that converge to ribosomal regulation are needed to complete ASMC maturation, which include effectors such as PI3-K, Akt-1, mammalian target of rapamycin (mTOR), and p70 ribosomal S6 kinase ($p70^{S6K}$). Pharmacologic inhibition of PI3-K and mTOR are enough to prevent $p70^{S6K}$ activation and sm-specific protein accumulation[143]. Moreover, activated mTOR can phosphorylate eIF4 binding protein-1 (4E-BP1), releasing and increasing the eukaryotic initiation factor-4 (eIF4) activity[145], followed by contractile protein accumulation (see **Fig. 6B**).

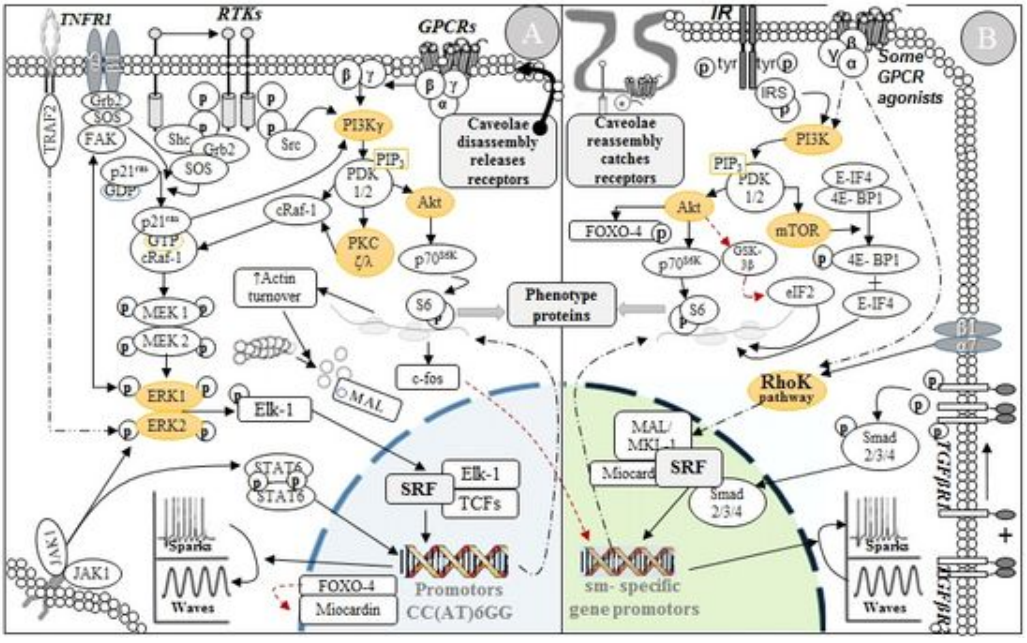


Figure 6. Signaling pathways involve in the control of ASM phenotypes. (A) Modulation, (B) Maturation.

Persistence of the c-ASMC phenotype is highly dependent on caveolae membrane system and

cav-1 expression (see Fig. 5)[146]. These flask-shaped structures are classically considered as special compartments for signaling regulation. Cav-1 is located inside those microdomains, being responsible for their formation and maintenance. Interestingly, cav-1 leads to reduced basal activity and sequestration of several receptors and signal transducers related to synthetic/proliferative phenotype induction, such as: PKC, PDGFR, EGFR, Src, and p21ras[147]. Proteins such as α-subunit of G proteins, Rho family members, adenylate cyclase isoforms, 7TM receptors, and others with binding domains to glycosyl- phosphatidylinositol are regulated as well. Caveolae act on behalf of cell adhesion by linking the actin cytoskeleton with the basal lamina, facilitated by laminin-2/$\alpha_7\beta_1$ integrin interaction. The triggered downstream pathway activates a guanine nucleotide exchange factor, RhoGEF, leading to Rho Kinase activation[147]. For this reason, it is possible that mitogen stimulation is not enough to ignite ASMC modulation and cell division, thus caveolae disassembly would be a strict requirement[148].

Laminin is a trimer that bind integrins and non-integrin receptor subtypes, including the dystrophin-glycoprotein complex (DGC). PI3-K inhibition prevents both ASMC maturation and accumulation of DGC proteins, β- and α-DG[149]. Preferential expression of DGC in c-ASMC is related to a tighter regulation of reactions to contractile agonists by caveolae system. There is also evidence suggesting that DGC, through β-DG, influences signal transduction by scaffolding properties and interactions with cav-1[150]. Additionally, basal activity and low grade stimulation of some RTKs, such as the insulin receptor (PI3-K pathway) and GPCRs (Rho Kinase pathway), could have a role in contractile phenotype conservation, especially in confluent cells. These observations acquire more relevance considering that the ASM mass is likely composed of c-ASMC under physiological conditions. In the disease-setting, ECM component dissolution by MMPs affect the ASMC attachment, shutting down the Rho kinase activity. Unconstrained ASMCs are more susceptible to paracrine influences. Thus, high levels of cytokines and growth factors, and subsequent anomalous repair due to TGF-β, might match with a continuous and dynamic process of phenotypical modulation (contractile → synthetic/ proliferative → hypercontractile/ fibrotic), explaining the ASM thickening and dysfunction.

Signaling Pathways Associated with Modulation

Transition to a synthetic/proliferative phenotype is enhanced by mitogens such as PDGF, EGF, IGF, fibronectin, collagen-I and -II, bradykinin, GPCR agonists, cigarette smoke extract, lipopolysaccharide (LPS), and reactive oxide species (ROS)[151, 152]. Modulation is quickly reached when *in vitro* ASMCs are not confluent under mitogenic influences (see Fig. 3B), especially fetal bovine serum (FBS) and fetal calf serum (FCS)[86, 122]. Associated pathways converge to increase c-fos (coactivator) expression, which paradoxically needs a prior SRF activation in order to alter gene expression[144]. Nevertheless, this apparent duality could be due to a wide variety of transcriptional coactivators that assertively induce selective gene transcription. Contrasting the SRF-myocardin complex effect, SRF cooperativity with ternary complex factors (TCF) such as Elk-1 affects gene promotors with a CArG ($CC(AT)_6GG$) sequence, inducing cell proliferation instead of maturation. In this way, c-fos upregulation is key to halt contractile gene expression[153]. Furthermore, this coactivator Elk-1 is phosphorylated and activated by the extracellular signal- regulated kinase (ERK)-1 and -2[154]. These kinases along with p38, c-Jun N- terminal kinase (JNK), janus kinases (JAKs), and transcriptional factors, like NFκB and AP-1, could participate in signal delivery to increase synthetic and proliferative activities[154]. Protein synthesis associated with modulation is also favored following S6 ribosomal subunit phosphorylation[138]. Increased cytoskeleton metabolism with actin polymerization blockade generates G-actin accumulation that avoids nuclear translocation of MAL/MKL-1. Also, it has been described that *in vitro* both maturation and modulation are reversible.

ASMC modulation is improved by Th2 cytokines. A mix with TGF-β and leukotriene D4, triggers the expression of 29 transcription factors[155]. IL-13 is relevant to control the expression of aroung 300 locus. Its receptor, the IL-13Rα1/IL-4Rα complex, mediates the phosphorylation of STAT-6, triggering MAPKs for phenotypic modulation[156]. IL-13 can also affect calcium dynamics by upregulation of sarcolipin, which is a transmembrane protein placed at the sarcoplasmic reticulum (SR) that inhibits the sarco/endoplasmic reticulum Ca^{2+}- ATPase (SERCA) activity[157]. Expression of calcium regulatory proteins changes with modulation, therefore, a decrease in voltage-dependent calcium channels, ryanodine receptors, and SERCA2 levels translate into a calcium dynamics dominated by wave-like propagations. This kind of flow contributes to MAPK pathway activation. Ca^{2+} waves also affect the conformational stability of cis elements in 5'untranslated regions (UTRs) of mRNAs and interactions between translational components, regulating protein synthesis[158]. IL-13 signaling is under control of the type-1 suppressor of cytokine signaling (SOCS-1), a protein with chaperone properties. SOCS1 expression is decreased in asthmatic ASMCs and its inactivation raises synthetic activities when exposure to Th2 cytokines[159]. In summary, diverse signaling pathways are responsible for driving phenotypic modulation of ASMCs (see Fig. 6A).

Muscarinic Activation leads to ASMC modulation

ASM thickening is attainable by persistent muscarinic stimulation[127]. Both pathways, $G_{i/0}$ coupled M_2 and G_q coupled M_3, could generate the activation of MAPK, Rho- kinase, and PI3-K signaling[117]. Moreover, shifting from a synthetic-proliferative to a contractile phenotype is accompanied by a decrease in M_2 and a parallel increase in M_3 expression[160]. Those observations inquire whether or not cholinergic stimulation may affect phenotypic switching. Accordingly, long-term incubation of rabbit ASMCs with ACh or carbachol (CCh) induced a switch towards s/p-ASMC[161]. Prolonged treatment of bovine ASM strips with the methacholine also diminished contractile protein expression[162]. Transition to a synthetic-proliferative phenotype is characterized by M_3 downregulation and blunted contractile responsiveness to cholinergic stimulation[161]. In cited studies, signaling pathways were not evaluated, but considering that cholinergic-induced mitogenesis is related to MAPK activation, it is possible that muscarinic activation allows the nuclear translocation of Elk-1, affecting gene expression linked to the phenotype transition.

Role of non-coding RNAs on Phenotypic Stability

The miRNAs are small noncoding RNAs that have an outstanding participation in gene expression regulation. It makes them excellent candidates to control cell plasticity. Multiple mechanisms are involved in miRNA synthesis and gene regulation, as it was previously described[163]. Shortly, mature miRNA is part of the active RNA- induced silencing complex (RISC) that mediates miRNA/mRNA interaction in a specific fashion. This interaction mostly occurs in the 3'UTR by partial complementarity, thus, miRNAs inhibit elongation during translation, or destabilize mRNA promoting its degradation. In smooth muscle biology, multiple miRNAs regulate cell differentiation and proliferation, under physiological and pathological conditions, especially in vascular smooth muscle, although little is known about ASM (see **Table 2**)[164].

Table 2. Functions of non-coding RNAs in ASM

	RNA (Contribution/ Effect)
Phenotypic Stability	miR-25 (↑modulation) miR-10a (↑maturation, keeps steady state, stem cell differentiation to smooth muscle) miR-26a(↓proliferation, hypercontractile induction) miR-143, miR-145(↑ accumulation contractile proteins)
Inflammatory Response	let7, miR133a(↑RhoA, AHR induction), miR-143 (modulates cell migration), miR-145 (cytoskeletal remodeling, affects podosome formation), miR146a (see table 4), miR 155 (see table 4), miR-708 (negative feedback to TNF-α)
Cell Division	miR-25 (↑proliferation), miR221 (↑proliferation), miR-371-5p, miR-718, miR-1181, miR-1207-5p, miR-1915, miR-3663-3p, LINC00882-002, LINC00883-005, BCYRN1, RP11-46A10.4
Airway Remodeling	miR-16, miR-25(↓matrix protein synthesis), miR26a (airway remodeling by chronic stretch, ↓GSK-3β), miR-133a (↓SRF), miR-146a
Innervation	miR-206 (neuronal branching)

Around 11 miRNAs are upregulated in cytokine-exposed ASMCs. Particularly, miR-25 is significantly modulated after prolonged OVA-challenge[165]. Some cytokines through the ↑miR-25/↓KLF-4 system promote ASMC modulation[166]. The transcription factor kruppel-like factor-4 (KLF-4) represses sm-specific gene expression by recruiting histone H4 deacetylase activity to smooth muscle cell genes, thereby blocking SRF association with methylated histones and CArG box chromatin. Next-generation sequencing identified miR-10a as the most abundant miRNA expressed in primary human ASMCs, accounting for more than 20% of all small RNAs. miR-10a directly suppresses PI3KCA expression and its overexpression reduces ASMC proliferation[167]. TNF-α-induced expression of miR-708 in asthmatic ASMCs is greater than in non-asthmatic. miR-708 decreased JNK, MAPK and Akt phosphorylation and increased MAPK phosphatase-1 (MKP-1) and phosphatase and tension homolog (PTEN)expression. It constitutes a negative feedback for TNF-α signaling downregulation[168]. miR-133a levels were decreased in human ASMCs, along with upregulated RhoA expression during AHR. Those findings were replicated after treatment with IL-13[169]. Sonic hedgehog signaling blocks miR-206 expression to increase the release of BDNF by ASMCs, coordinating branch innervation[52].

A recent study explored the RNA expression profile in cultured ASM[170]. Remarkably, over 200 miRNAs were detected including: miR-371-5p, miR-718, miR-1181, miR-1207-5p, miR-1915, and miR-3663-3p. These miRNAs had been previously related to aberrant proliferation in other cells. Paradoxically, predicted targets cut down gene expression of proteins that are known for remodeling promotion. They also detected a specific long non-coding RNA (lncRNA) profile. lncRNAs have recently emerged as epigenetic tools for gene expression regulation. They can regulate miRNAs as target site decoys, can also directly bind to transcription factors and participate in assembly of chromatin-modifying complexes as structural components and recruiters of genomic targets[171]. Stimulated human ASMCs expressed 29 lncRNAs, and some of them were previously identified as cell proliferation regulators. Relevantly, an increase in LINC00882-002 and LINC00883-005, and a decrease in BCYRN1 and RP11-46A10.4, could explain why despite of specific miRNAs, a target mRNA transcript is still translated. These lncRNAs could act as 'sponges' for the miRNAs-1207, -150, -940, and -371, blocking the RISC association with the translational machinery. The analysis is more complex if it is considered that a variable expression is seen in each cell cycle phase. In summary, phenotypic switching encompasses responses exquisitely coordinated by multiple signaling pathways, orchestrating gene expression not only at a promotor level, but also involving specific changes in the RNA metabolism.

Regulation of ASMC proliferation: a mechanism of hyperplasia

ASMC proliferation in culture increases after exposure to BAL fluids from asthmatic patients recently exposed to allergens[133]. Also, myocytes grow faster than those from non-asthmatics, so they display an 'intrinsic' abnormality that is independent of culture conditions[62]. Although, it is believed that c-ASMCs have an apparatus for cell division, *in vitro* cell population arise from s/p-ASMC expansion due to its greater replicative rate[84]. Consequently, ASMC proliferation and perhaps ASM hyperplasia may require modulation as a prerequisite. For that reason, the following signaling pathways are thought to be dominant in the synthetic/proliferative phenotype.

Mitogenic Pathways

In terms of 'stimuli' for ASM proliferation, mitogens can be sorted into five major categories: 1. RTKs, polypeptides such as growth factors, PDGF, IGF, EGF, VEGF, BDNF and basic fibroblastic growth factor (FGF), 2. GPCR agonists, like ACh, α-thrombin, endothelin-1, serotonin, leukotriene D4, and thromboxanes, 3. Cytokines, including thymic stromal lymphopoietin (TSLP), Th17 family and proinflammatory cytokines, which signaling pathway is generally associated with glycoprotein receptors, 4. ECM proteins, collagen I or fibronectin matrix, dependent on activation of $\alpha_2\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, via the nonreceptor cytoplasmic tyrosine kinases, focal adhesion kinase (FAK) and c-Src; and 5. Immunoglobulin IgE, through FcεRI-spleen tyrosine kinase (Syk) pathway[172, 173]. Moreover, other molecules secreted by inflammatory cells or leaked from plasma have been related[6]. Endocrinopathies have been shown to worsen asthma symptoms; hormones can modulate airway structure. For example, T_4 and T_3 synergistically promote a proliferative activity by $\alpha_V\beta_3$ activation, explaining why hypothyroidism improves whereas hyperthyroidism deteriorates asthma control[174]. Recently, leptin was shown to raise ASMC proliferation[175], considering that insulin can induce hypercontractile differentiation (see next section), both hormones can contribute to ASM thickening and airway obstruction seen on obesity. The $G_0 \rightarrow G_1$ transition and overcoming the cell cycle checkpoints are mediated by activation of at least three systems with high convergence level: MAPK pathway, atypical PKC isoforms, and PI3-K pathway. The contribution of each pathway depends on the agonist and the experimental design. A summary is shown in Table 3.

Table 3. Effects of Chemical Mediators on ASMC functions

Mediator	Receptor	Signaling	Source	Effect		
				C	P	M
EGF	EGFR, ErbB-2	PI3-K, MAPK, atypical PKC	Ep, P	n	+	+
PDGF	PDGFRα-PDGFRβ	PI3-K, MAPK, atypical PKC	P, Mθ, ASMC, Ep	n	+	+
FGF	FGFR-1, -2	PI3-K, MAPK, atypical PKC	ECM, Mθ, ASMC	n	+	+
TGFβ	TGFβR-1, -2	Smad, MAPK	Mθ, P, ECM, T	H	+	+
Tryptase	PAR-2:	PLC/IP_3/Ca^{2+}, MAPK	Mast	+	+	+
S1P	S1PR- 1,-3,-5,-6	PLC/IP_3/Ca^{2+}, MAPK	Mast	+	+	+
β-hexosaminidase	Mannose receptors	PLC/IP_3/Ca^{2+}, MAPK	Mast	?	+	?
ACh***	$G_{i/o}$ coupled M_2	MAPK, ↓AMPc/(-) K_{Ca}, cGMP pathway	Ep, Neuronal	+	+	?
	G_q coupled M_3	PKC, MAPK, PLC/IP_3/Ca^{2+}, cGMP pathway		+	+	+
Histamine	H_1, TLR3	PLC/IP_3/Ca^{2+}, MAPK	Mast	+	+	?
	H_2	cAMP pathway		-	n	?
α-thrombin	PAR-1	PI3-K, MAPK	Pl	+	+	n
Endothelin-1	ET_A	PLC/IP_3/Ca^{2+} MAPK, JAK/STAT	P, Mθ, Pl, Ep	+	+	+
	ET_B			+	n	?
Tachykinins	NK-1	PLC/IP_3/Ca^{2+}, MAPK	Neuronal, Ep	+	+	+
Serotonin	5-HT_{2A}	K_{Ca}, PI3-K, MAPK	P, Pl	+/-	+	?
TxA_2	TPr	PLC/IP_3/Ca^{2+}, MAPK	P, Mθ	+	+	?
LTD_4	CysLT1-R	PLC/IP_3/Ca^{2+}, PI3-K, MAPK	Mθ	+	+	+
α- Agonists	α1,2	PLC/IP_3/Ca^{2+}, MAPK?	Endocrine	+	+	?
β-Agonists	β2	cAMPc pathway	Endocrine	-	-*	-
PGE_2	EP2,4	cAMP pathway	Ep, ASMC, Mθ	-	-	-
Angiotensin II	AT1R	PLC/IP_3/Ca^{2+}	Endocrine	+	?	?
IL- 1β	IL-1 Receptor type1	MAPK pathway	T, Mθ, ASMC, Ep	H	+	?
IL- 4	IL-4Rα	K_{Ca}, STAT6, MAPK	T	-	-	?
IL- 6	IL-6R, gp130	STAT3/ MAPK	T, Mθ, ASMC, Ep	?	+	?
IL-13	IL-13Rα1, IL-4Rα	CD38/cADPR, STAT6, MAPK	T	H	+	?
TNF- α	TNFR1	NFκB, MAPK	T, Mθ, ASMC, Ep	H	+	?
IFN- γ	IFNGR	(-) Cdk	T	H	-	?
Fibronectin	Integrin $β_1$	FAK, MAPK	ECM	+	+	+**
Collagen type I	Integrin $β_1$	FAK, MAPK	ECM	+	+	+
ROS	--	RhoK, MAPK	Ep, ASMC	+	+	?
CNP	GC-B	cGMP pathway	Ep	-	-	?
ANP	GC-A	cGMPc pathway	Ep	-	-	?
NO	sGC	cGMP pathway	Ep, ASMC, NANC	-	-	?

* S- albuterol can stimulate ASMC proliferation by the platelet activated factor (PAF) receptor. **Improve cell migration induced by growth factors and LTD_4. ***CCh can supress *in vitro* rat ASMC proliferation. +: stimulate, -: inhibit, n: none, H: hyperresponsiveness to agonists, ?: unknown, C: contraction, P: proliferation, M: migration, Ep: epithelial cells, P: platelets, Mθ: Macrophages, ECM: Extracellular matrix, T: T- lymphocyte, Pl: plasma, Mast: mastocytes.

Inhibitory Pathways

Several molecules have a counterregulatory role on ASMC proliferation, like PGE_2[176], 15-deoxy-$D^{12,14}$-prostaglandin J_2[177], IL-4[49], $β_2$- adrenergic agonists[178], NO, and NPs[65, 179]. Some of them can lead to cell arrest after increasing second messengers, either cAMP or cGMP. Although, $β_2$-agonists have anti-proliferative effects on cultured ASMCs, it seem to be less relevant *in vivo* due to quick desensitization when used as a therapeutic intervention.

The NP family includes three peptides: ANP, B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). In the lungs, ANP is expressed predominantly by epithelium and ASM[180]. Its receptor is a particulate guanylyl cyclase, NPR-A or GC-A, which converts GTP to cGMP. cGMP pathway effectors include cGMP-regulated ionic channels, PKG-I and -II, and phosphodiesterases (PDE)[181]. On the other hand, CNP effects are transduced by B-type particulate guanylyl cyclase (NPR-B or GC-B), which contains an intrinsic domain with GTPase activity similar to NPR-A. NPs have antiproliferative effects on ASMCs[65]. The most likely mechanisms includes: endothelin-1 inhibition, increased MKP-1 levels and activity, Raf-1 phosphorylation at Ser43 by PKG, and MAPK pathway blocking[182].

The approach to NO effects should be made carefully. Under physiological circumstances, NO is released by non-cholinergic non-adrenergic fibers and airway epithelium, generating fM to pM fractions. In this range, overall effects include relaxation and reduced proliferation[179]. Cell cycle inhibition is mediated by the cGMP/PKG pathway, shutting down MAPKs[183]. Also, there is expression of inhibitory proteins ($p21^{Cip1/Waf1}$, $p16^{INK4a}$) and repression of A-, D1-, and E-cyclin promoters[184]. NO donor effects are reversed by ODQ, therefore, cGMP production is due to sGC enzyme activity[66]. We recently found (unpublished data) that constitutive sGC activity decreased rat ASMC proliferation, likely reflecting basal NO synthesis by nNOS in ASMCs[185]. However, paradoxically, exhaled NO is increased in asthmatics. Accordingly, high levels of iNOS enzyme are expressed by asthmatic and sensitized-animals[56]. It is possible that excessive NO production induces downregulation of a highly expressed sGC in the lung, which may occur in asthma. ROS like $ONOO^-$ could also impair the sGC-associated prosthetic group, making it unresponsive to NO[186]. In fact, basal and stimulated cGMP production is reduced in ASMCs from OVA-sensitized rats[66]. In a murine asthma model, a substantial decrease of around 60-80% of sGC subunit mRNA and protein levels was detected[187]. These findings confirm that airway remodeling can be promoted by downregulation of antiproliferative

pathways.

Emerging data suggests influences of non-cGMP/cAMP-dependent pathways could contribute to ASMC proliferation inhibition. For example, cav-1 phosphorylation induced by growth factors can blunt its antiproliferative effects and constitutive inhibition over ERKs[148]. Peroxisome proliferator activated receptor-γ (PPAR-γ) gene is highly expressed in ASMCs, and interaction with its ligands induce cell cycle repression and synergism on anti-remodeling effects of β_2-agonists[188]. Furthermore, a rat model of vitamin D deficiency showed an increased ASM mass and AHR[189]. Calcitriol, but not dexamethasone, inhibited PDGF-induced ASM DNA synthesis concentration, which was mediated by blocking of retinoblastoma protein (Rb) phosphorylation and checkpoint kinase-1[190]. Additionally, some cytokines could have a counterregulatory role. For example, Rb hyperphosphorylation after mitogen stimulation is suppressed by IFN-γ[191]. IL-4 suppresses unstimulated and PDGF-stimulated ASMC proliferation[192]. Consequently, cytokines have diverse roles in airway remodeling.

Synergism and Inhibition by Muscarinic Agonists: Cholinergic Duality

GPCR agonists have been described as promotors of ASM thickening, being ACh the prototype. Although, it has been postulated that mAChRs mediate its remodeling effects, a recent study suggests that activation of nAChRs by nicotine enhanced rat ASMC proliferation via Akt pathway[193]. Previous reports failed to show any nAChR-mediated effect on ASMC proliferation, which could be a consequence of species and agonist-dependence. The relevance of cholinergic systems was reinforced since tiotropium bromide, a M_3-specific antagonist[117], prevented many *in vivo* aspects of OVA-induced remodeling[121]. A OVA-sensitized knockout mice model recently showed that both ASM and pulmonary vascular smooth muscle mass were 30-100% lower in $M_3^{-/-}$ mice, whereas $M_1^{-/-}$ and $M_2^{-/-}$ responded similarly to the wild-type[194]. Allergen-induced inflammation was similar in all knockout mice, suggesting that pro-remodeling effects of ACh are independent of inflammation, and may involve only bronchoconstriction-induced remodeling, as it was previously suggested[8].

In vitro studies emphasize that muscarinic agonists are not strong stimuli for ASMC proliferation, hence the mitogenic effect is only observed in combination with growth factors[64, 152]. However, CCh alone was shown to increase rat ASMC replication; although, the effect was smaller than obtained with growth factors or TNF-α[122]. In human and bovine ASMC a sustained activation is required to enhance cell division. It is possible that the threshold to overcome the arresting signals varies among models, as we have found ERK-1 and ERK-2 phosphorylation in response to CCh, without assistance of other mitogens (unpublished data). Accordingly, other study showed an increased [^{3}H]thymidine incorporation in rabbit ASMCs, serum starved for 24 h before muscarinic agonists addition[161]. These results suggest that the muscarinic effect depends on ASMC phenotype, as the length of serum starvation can influence gene protein expression. On the other hand, G_q protein-coupled M_3 pathway could be the preferential mAChR in c-ASMC, whereas $G_{i/o}$ protein coupled M_2 pathway in s/p-ASMC[117].

In vivo, it is more likely that muscarinic agonists act synergistically to growth factor. Muscarinic synergism in human and bovine ASMCs is mainly blocked by the M_3-specific antagonists 4-DAMP and DAU-5884[160]. However, we showed that CCh-induced proliferation was attenuated by AF-DX-116 over 4-DAMP, which suggests M_2 as the mediator of cholinergic synergism in a rat ASMC proliferation model [122]. In this study, M_2 basal activity was partially required to reach the maximum proliferative effect of FBS. This receptor is coupled to a PTX-sensitive $G_{i/o}$ protein. Other study showed that PTX antagonized the mitogenic effects on human ASMC proliferation[195]. Curiously, dissociation of heterotrimeric G-proteins induced by growth factors has been reported in other cell lines, through either direct interaction with growth factor receptor (EGF→$G_{i/o}$, G_s; PDGF→$G_{i/o}$) or by means of an indirect way (PDGF/EDG1→$G_{i/o}$), but these findings need to be confirmed[196]. PDGF-induced Src and PI3-K activation in bovine ASMCs is partially dependent on PTX-sensitive G-proteins[197]. It is possible that $G_{i/o}$ protein activation makes more efficient the signal transduction of growth factors. Supporting this statement, methacholine potentiated TGF-β-induced proliferation, which was reduced by PTX, tiotropium and the M_2 antagonist gallamine, but not DAU5884[198].

Surprisingly, we recently found that CCh can repress rat ASMC proliferation[122]. This anti-mitogenic effect was reversed, in a dose-dependent manner, by AF-DX-116 (M_2-specific), more efficiently than 4-DAMP (M_3-specific). The inhibitory effect was observed only at maximum concentration of mitogens like FBS, EGF and TNF-α; otherwise, the overall effect was promotion of cell division. The fact that ASMC proliferation inhibition was only observed at high doses of mitogens leads to inquire whether inhibition is triggered by a mitogenic-related pathway, which is concurrently associated with facilitation of anti-proliferative pathways (e.g. ↑$[cGMP]_{int}$) or over-activation (e.g. p38 or JNK) that triggers cell death[154]. Cell proliferation inhibition by muscarinic agonists has been reported in ovarian cells[199], and cancer cells[200], involving the

M_3 activation and $p21^{Cip1/Waf1}$ expression. Likewise, duality on cell proliferation has also been reported[201].

ASMC proliferation inhibition by mAChRs may be the result of two signaling pathways: 1) cGMP/PKG cascade[202-204], and/or 2) MAPK pathway: p38 and JNK[205]. Increased cGMP production by muscarinic agonists was previously discussed. Recently, a $G_{i/o}$ coupled M_2-dependent process was described, which increased NO-sGC activity in bovine ASM and was NO-independent[130, 204]. Similarly, M_3/G_q-protein complex has been associated with NPR-GC-B activation[202, 203], which could activate PKG with subsequent cell cycle arrest by $p21^{Cip1/Waf1}$[199]. However, cGMP production by mAChRs might be on behalf of receptor cooperativity and growth signal transduction. The role of muscarinic-induced cGMP pathway in ASMC needs to be clarified and is under intense research in our laboratory. Thus, some additional experimental findings (unpublished data) support the anti-proliferative response to CCh: 1. ODQ, a selective inhibitor of NO-sGC, cannot reverse cell proliferation inhibition by CCh, and 2. CCh potentiated antiproliferative effects of CNP (NPR-GC-B activator). In any case, the growth factor concentration determinate the final outcome of muscarinic activation, as low levels allow mitogenic actions and high levels downregulates mitogenic pathways or facilitates antiproliferative signaling after muscarinic activation. Supporting this idea, MKP-1 expression can be upregulated or repressed by ERK and p38 activation, respectively[206].

It is a highly complex matter to understand *in vivo* molecular relevance of muscarinic activation. Signal transduction comprises divergence and convergence with others pathways at several network points[207]. An approach of system biology would help define the conditions that lead towards one or other outcome after receptor activation[208]. However, reported findings point out ACh and mAChR activation as pro-remodeling through stimulation of ASMC proliferation, EMT synergism, phenotypic modulation, and tissue fibrosis. The novel finding of ASMC proliferation inhibition highlights the possibility that counterregulatory pathways are still operational under inflammatory microenvironments. To support this statement, in a chronic asthma model, administration of tiotropium bromide significantly reduced ASM thickening and peribronchial collagen deposition[209]. Interestingly, these effects matched with a decreased M_3 and increased M_2 expression in the lungs. Moreover, M_3 and M_2 expression tend to increase and decrease, respectively, with age. These differences were related to remodeling markers, which were significantly higher in older OVA-sensitized mice than in the young OVA group[210]. A differential regulation of mAChRs might induce a regression of airway remodeling in chronic asthma, which could be mediated by facilitation of M_2-induced antiproliferative pathways. Identification of its downstream effectors is essential in order to recognize new potential therapeutic targets, by which airway remodeling could be reduced, if not reversed.

Is ASM Hyperplasia due to Decreased Apoptosis?

A major issue to be addressed is whether or not ASM hyperplasia derived from increased proliferation or decreased apoptosis. Although, it is commonly assumed that both processes occur under similar circumstances, their regulation is significantly different since specific pathways mediate each outcome. This uncertainty can be underlined by the TNF-α effects. Increased activity of the three MAPK effectors has been reported in TNF-α-exposed ASMCs. Thus, ERKs were related to cell proliferation, p38 with increased synthetic activities, and JNK with cell apoptosis[211]. We showed TNF-α increases ASMC proliferation, but at the same time decreased cell viability[122]. Human ASMC apoptosis is potentiated by TNF-α with concomitant Fas upregulation, a component of the TNF-α receptor. Binding to inflammatory cell-derived Fas-Ligand (FasL) greatly induces ASMC apoptosis[212], exemplifying how a stimulus that promotes cell division can induce apoptosis, and not always an increased survival.

There is much less information on ASM apoptosis. ECM components, such as fibronectin, laminin-1 and collagens I and IV, provide strong survival signals to ASMCs[213]. Also, proteinases derived from inflammatory cells degrade ECM proteins, including fibronectin, and promote apoptosis[214].A study of T cell-mediated airway remodeling, showed that the ASM thickening was due to both decreased apoptosis and increased proliferation[42]. Reduced apoptotic rates have been reported following exposure to various mediators, such as: Th17 cytokines[215], chemokines[216], cardiotropin-1 (CT-1)[217], ET-1[218] , and increased calcium influx induced by vanilliod transient receptor potential channel (TRPV)1[219]. Signaling involved in maturation also provides survival signals in the context of ECM preservation. In this setting, Increased G_{12}/G_{13}-protein expression may contribute to Rho activation, subsequent actin polymerization, and dysregulation of calcium homeostatic systems[220]. Translocation of RhoA into plasmatic membrane is associated with cell cycle arrest and transcription factor sequestration. This brings up a controversial aspect of ASMC biology for *in vivo* circumstances. Several pathways could increase ASM thickening just preventing that ASMCs move to G_1 phase because the contractile phenotype represent a quiescent cell state less susceptible to cell death than the synthetic-proliferative ones. If ASMC precursors are stem cells, progressive recruitment of c-ASMCs along with an increased survival of native ASMCs can support the ASM hyperplasia under pathological circumstances. The data dominating this field mainly derived

from *in vitro* studies. For this reason little is known about the normal ASM renewal. It is essential to determine if the total ASM mass is preserved by a steady cell number that undergo into apoptosis and cell division, or if it merely relies on maintaining the entire ASM in G_0 phase.

'Intrinsic' Dysregulation of ASMCs in Asthma

Human ASMCs from asthmatics and sensitized animals differ from its physiologic counterparts at many functional levels. Amongst ASMC studies, abnormal C/EBP and SERCA expression, and mitochondrial alterations are the most significant findings that clearly show their difference. Most researchers have mentioned these abnormalities as the main drivers of ASM hyperplasia in asthma. Supportive evidence will be described next, even though, their potential causes are still under extensive research.

C/EBP compromises a transcription factor family involved in cell differentiation and proliferation[221]. Among the six isoforms, C/EBP-α is almost absent in asthmatic ASMCs, causing a decreased α/β ratio. This alteration is specific to this cell type, since lymphocytes have normal levels[89, 91]. C/EBP-α generates cell-cycle arrest through cyclin and cyclin kinase inhibition, and also indirectly by means of $p21^{Waf1/Cip1}$. Moreover, its deficiency in asthmatics might explain the poor action of glucocorticoids in ASMC proliferation reduction, as restoring C/EBP-α levels brought back GR-mediated inhibition. The mRNA encoding C/EBP-α (CEBPA) levels are essentially normal, indicating that this disorder relies on translation rather than transcription. There are two C/EBP-α isoforms with antagonistic effects, a full-length (p42) isoform and a truncated (p30) isoform. p42 mediates the antimitogenic effects, while p30 lacks this inhibitory function and has been associated with hyperplasia instead. Also, the p42/p30 ratio is changed in asthmatic ASMCs[89, 94]. Remarkably, considering that both p42 and p30 isoforms are decreased in asthmatics, a dysregulation on 3'UTR by miRNAs should be considered.

It is currently widely known that calcium regulates a broad range of cellular functions, from cell contraction to growth and differentiation[222]. Calcium kinetic in asthmatic ASMCs is quite different due to partial absence of SERCA2 in SR membranes. SERCA2 mRNA is reduced in moderate but not mild asthma. This alteration jeopardizes the normal SR replenishment, but its main consequence is fluctuations in the spatial Ca^{2+}kinetic, from transient and spark calcium signaling to wave-like increment of cytosolic Ca^{2+}[92, 223]. Calcium waves can activate the TRPV4 Ca^{2+} sparklet microdomains and subsequently the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, calcineurin, which dephosphorylates proteins like the transcription factor NFAT. Dephosphorylated NFAT is translocated into the nucleus in order to bind to specific target promoter elements[224]. CaM kinase IIδ activation by Ca^{2+}/CaM complexes that colocalize with TRPV channels, links calcium signaling to MAPK pathway. Thus, wider Ca^{2+} signals activate different pathways for cell proliferation. Store-operated calcium channels entry (SOCE) occurs in response to SR Ca^{2+}store depletion.Stromal interaction molecule (STIM)-1 is a SR Ca^{2+}sensor that translocates into the SR underneath the plasma membrane upon SR Ca^{2+}store depletion, where it interacts with Orai1, the molecular component of the store-operated calcium channels (SOCC), and triggers SOCE.This supports ASMC proliferation, as SOCE is upregulated during cell division accompanied by a mild increase in STIM1 and a significant raise in Orai1 expression[225]. It is possible that lacking of SERCA2 potentiates these molecular events. Similar to C/EBP-α abnormalities, decreased SERCA2 is also cell-type specific, since other smooth muscle tissues did not show any pathology in asthmatics. It is important to quote that using RNAi technology to target SERCA2 expression in ASMCs from healthy donors reproduce, and in some cases even worsen the calcium dynamics[92].

Another pathologic feature is an increased number of mitochondria, which also seems to be disease-specific. Improved mitochondrial biogenesis is limited to ASMC, compared to endothelial and epithelial cells, making it cell-type specific. It has been found a straight relationship between disease length, FEV_1/FVC ratio, and the ASMC mitochondrial mass. The mitochondrial mass may not be due to adaptation to metabolic dysfunction because oxidative phosphorylation was efficiently coupled[93]. On the other hand, treatment of normal ASMCs with TNF-α and IL-13 induces asynchrony between agonist-induced $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{mito}$ transients. Additionally, disruption of the mitochondrion-SR coupling leads to ROS formation, mitochondrial fragmentation and increased cell proliferation[226]. Such event is not seen spontaneously on asthmatic ASMCs, but it can be a potential etiologic mechanism of increased mitochondrial biogenesis. The mitochondrial alteration could be linked to C/EBP-α deficiency, because approximately 76 known human mitochondrial genes contain a potential C/EBP binding site[227]. Furthermore, promoter activation for mitochondrial biogenesis, such as PPARγ coactivator-1α (PGC-1α), mitochondrial transcription factor A (mtTFA), and nuclear respiratory factor (NRF-1), could be calcium-dependent via Ca^{2+}/CaM/CaM Kinase, linking the abnormal calcium signaling to the abnormal mitochondrial biogenesis[93].

As a summary, a disorder in protein synthesis may be responsible of the intrinsic alterations observed in ASMCs from patients suffering severe asthma. Aforementioned experimental findings suggest that translation dysfunction affecting gene expression is essential for

phenotypic modulation and increased proliferation. Epigenetic mechanisms can be involved. Recently, miRNA expression in relation to abnormal cell division was studied. ASMC proliferation is greater in asthmatic than healthy subjects. Promitogenic effects were linked to a miR-221–dependent downregulation of $p27^{kip1}$and $p21^{WAF1}$[228].Interestingly, we performed a target prediction analysis of both 3p- and 5p- miR-221 arms using the online database miRDB[229] and taking a score >70 as significant (results in **Table 4**). The results suggest additional mRNA targets of miR-221 that could be relevant in ASM remodeling. In a study, the miR-155 and miR-146a expression have positive and negative correlation, respectively, with COX-2 expression in asthmatic ASMCs after cytokine challenge[230, 231]. Induced miR-146a expression might reflect that asthmatic ASMCs still have some potential anti-inflammatory targets, despite of deeply phenotypic changes. However, the ability of miR-146a to repress translation also relies on the absent of specific lncRNAs, which were not evaluated. Downregulation of COX-2 and IL-1β by miR-146a provides a good candidate for RNAi technology employment in asthma treatment, but the authors[230, 231] did not consider that some potential targets of miR-146a could worsen several aspects of airway remodeling, as we predicted by miRDB (see Table 4). More studies are needed to elucidate the pathogenic mechanisms of abnormal ASMCs.

Table 4. Target Prediction of human (hsa) miR-221, -155, and -146a, and hypothetic role in ASM remodeling

Gene	*Seed Location*	*Score*	*Potential Consequence*
hsa-miR-221			
5p-arm sequence: *5'ACCUGGCAUACAAUGUAGAUUU-3'* Target: *3'GGACCGT-5'*			
CASP2	228	84	↓Caspase →↓Apoptosis
GUCD1	1929	83	cGMP Pathway downregulation →↑ Cell Proliferation
ITGB1	175	80	↓Integrin β_1 subunit→(-) Maturation
3p-arm sequence: *5'AGCUACAUUGUCUGCUGGGUUUC-3'* Target: *3'CGATGTA-5'*			
MEGF9	736	92	↓Laminin signaling→ (-)Maturation
EF3J	62	84	↓eIF3 expression→ Translation dysfunction
TIMP3	2453	81	↑ECM degradation→(+)Modulation, migration
GUCY1A2	760	70	cGMP Pathway downregulation →↑ Cell Proliferation
hsa-miR-155			
5p-arm sequence: *5' UUAAUGCUAAUCGUGAUAGGGGU-3'* Target: *3'ATTACGA-5'*			
TP53INP1	475	97	↓TP53→ ↑Cell proliferation
RCN2	605	96	↓Reticulocalbin-2, affect Ca^{2+} dynamics
SOCS5	1540, 2062	95	Sensibilization to IL-4 signaling
3p-arm sequence: *5' CUCCUACAUAUUAGCAUUAACA-3'* Target: *3'AGGATGT-5'*			
PRKAR2B	1611	96	cAMP pathway downregulation
ISPD	121	93	↓Isoprenylation→ Signaling Dysregulation
CREB3L2	1439,5396	89	cAMP pathway downregulation
hsa-miR-146a			
5p-arm sequence: *5' UUAAUGCUAAUCGUGAUAGGGGU-3'* Target: *3'CTCTTGA-5'*			
EIF4G2	256	98	↓eIF4 expression→ Translation dysfunction
CREBL2	456	90	cAMP pathway downregulation
PRKAA2	3943, 6246	79	cAMP pathway downregulation
5p-arm nucleotide sequence: *5' CCUCUGAAAUUCAGUUCUUCAG-3'* Target: *3'GAGACTT-5'*			
ATG13	802	97	Dysregulation of autophagy
HMGCR	145, 691, 1173	96	↓Isoprenylation/ affects growth signaling
EIF4E3	2119, 5082	88	↓eIF4E expression/ Translation dysfunction

The target prediction was performed as follow: seed sequences were defined by complementarity and likelihood to hybridize, according to high free energy of the reaction. The most likely sequence of 7 nucleotides, in both 5'arm and 3'arm of the corresponding stem- loop miRNA, were analyzed. Annealing with 3'UTR of human mRNA (coding strand obtained from NCBI gene index) was performed by the algorithm defined in the prediction tool MirTargetV3, available at http://mirdb.org/miRDB/index.html. We take as significant every score> 70, although only scores >80 provide high confidence and <60 are very unlikely. Also, targets of the 5' arm are most likely to be include in the RISC complex. Among all mRNA tested, we chose those thought to be expressed by ASMCs, having a functional coherence. This data requires experimental validation.

Hypercontractile phenotype: a mechanism of hypertrophy

ASM hypertrophy (increased cell size and protein content) is an important feature of severe asthma. Contractile protein accumulation during culturing ASMCs for maturation can be prominent. However, histopathological studies did not found a significant increment in the immunostaining intensity for sm-α-actin, and sm-MHC, relative to enlarged ASMC size[12]. Thus, a greater muscle mass could be attributable to an increased cell size and a normal amount of proteins, instead of disproportionate increase in protein expression respect to cell size. Other studies support stronger immunostaining for several contractile proteins[11], but they failed to consider the total amount of protein in relation to the fiber bundle size. The cell dimension estimation is challenging because lacking of precise cell boundaries and distinction from ECM components. Taking into account this statement, protein hyperaccumulation does not seem to be a mechanism of hypertrophy during *in vivo* circumstances. In its place, increased individual cell size along with hyperplasia would contribute to a greater ASM mass. On the other hand, hypercontractility as a potential source of AHR could not be secondary to increased responsive capacity by a greater absolute amount of the contractile apparatus; instead,

regulatory pathways of muscle contraction could be deregulated, as increased MLCK expression in asthmatics could determine a higher actin-myosin crossbridge formation. In any case, abnormal protein expression is likely a determinant of ASM hypertrophy.

Signaling Pathways for ASM hypertrophy

Protein accumulation is important to achieve any phenotypic change, and h-ASMC induction involves similar pathways (see Fig. 6B). Therefore, PKB/Akt (or its upstream activator, PI3-K) activation is required for cultured ASMC hypertrophy and proportionately contractile protein accumulation (SM22, sm-α-actin, and sm-MHC relative to β- actin)[232]. Peculiarly, even though Akt signaling enhanced protein translation, mRNA encoding SM22 and sm-α-actin were reduced. Opposing effects on gene transcription and translation could prevent hyperaccumulation of contractile proteins, which also suggests that the hypercontractile phenotype transition is a highly regulated process. Similarities with maturation pathways and a great susceptibility of quiescent cultured ASMCs to switch toward a hypertrophic phenotype support this idea[233]. Pharmacological inhibition of PI3-K or mTOR (upstream activator of $p70^{S6K}$) blocked accumulation of contractile proteins during serum-free culture, suggesting that signaling through the PI3-K/Akt/mTOR/$p70^{S6K}$pathway is the most important pathway for protein accumulation. Also, prolonged activation of Akt1 alone can cause hypertrophy without affecting the glycogen synthase kinase-3β (GSK-3β)[143, 232]. Treatment with TGF-β, endothelin- 1, and CT-1 increased $p70^{S6K}$ phosphorylation in human ASMCs. In this model, contrasting with the previous statement, specific siRNA against $p70^{S6K}$ and its substrate S6 only prevented cell enlargement[234], but not contractile protein accumulation and cell shortening. However, shortening capacity was measured in unloaded ASMCs after KCl stimulation, which can underestimate the active tension developed, especially if they are comparing two cell populations with variable length size. Considering that $p70^{S6K}$ has other molecular targets besides S6, protein accumulation could depend on simultaneous activation of other Akt substrates.

Many translational mechanisms, including phosphorylation of eIF4 binding protein-1 (4E-BP) and GSK-3β, have additive roles during hypertrophy.Phosphorylated 4E-BP-1 releases eIF4E, allowing ribosomal assembly and initiation of protein translation during TGF-β signaling[145]. On the other hand, the factor eIF2 is a multimer that binds $tRNA^{Met}$ and conforms the eIF2-GTP-Met-$tRNA^{met}i$ complex. Binding of eIF2 to GTP is regulated by the eIF2B activity, a guanine nucleotide exchange factor that is phosphorylated and inhibited by GSK-3β, to repress protein synthesis in ASMCs[235]. In an OVA-sensitized mouse model, a lower GSK-3β activity was detected in lung homogenates, which correlated with both ASM hyperplasia and hypertrophy[236]. GSK-3β has repressive effects on phenotypic transition and proliferation of ASMCs, acting as a master control of cell stability preservation. Thus, GSK-3β inhibition could lead to an increment in cyclin D1 and α-actin mRNA, as well as the transactivation of NFAT, SRF, c-Myc, c-Jun, and myocardin. However, not all of them occur at the same time, due to highly signaling compartmentalization and involvement in divergent pathways. In the context of CT-1-induced hypertrophy, Akt and other kinases can phosphorylate and block GSK-3β preserving eIF2B in its active form (dephosphorylated). Although, TGF-β augments cell size and contractile protein synthesis through several distinct mechanisms, phosphorylation of GSK-3β is not essential to increase cell size, but could have a relevant role in derepressing the transactivation of NFAT and SRF[235].

Other mediators can also increase protein synthesis and contribute to h-ASMC induction. For example, Angiotensin II can induce human ASMC hypertrophy through MAPK activation and upregulation of erg-1, c-fos, and c-Jun[237]. Endothelin-1, a GPCR agonist, is detected in BAL fluid of asthmatics and it increases α-actin, SM22, and calponin expression in an ERK- and JAK1/STAT3–dependent manner[218]. CT-1, a member of the IL-6 superfamily, is secreted following stimulation with either TGF-β or IL-4, and also after serum deprivation. CT-1 not only increases some contractile proteins, but also qualitatively alters the SM22 distribution by the MAPK pathway[217]. Although, mTOR/$p70^{S6K}$ inhibition with rapamycin inhibited IFNs and EGF-induced protein synthesis, IFN-β and IFN-γ increased miR143/145 expression and sm-α-actin accumulation with little effect on ASMC size. In contrast, EGF increased ASMC size but had little effect on miR143/145 expression[238].Other reports showed muscarinic stimulation augmented functional TGF-β effects in human ASMCs[137]. Besides chemical mediators, physical forces elicit signals that promote a hypertrophic phenotype. Chronic stretch of human ASMCs inhibited cell proliferation through miR-26a up-regulation and promoted hypertrophy. Enforced miR-26 expression had similar effects in the absence of stretch. Promoter analysis suggests that C/EBP-α directly binds to the miR-26 promoter and increases its expression. They also identified GSK-3β as a target gene ofmiR-26a[239]. Hypertrophic induction requires downregulation of desmin expression, an intracellular load-bearing protein, which influences airway compliance and contractile responsiveness. The ERK/Egr-1/miR-26a/GSK-3β pathway can suppress almost 90% of desmin expression[240]. Thus, multiple mechanisms can support the acquisition of an h-ASMC phenotype.

Viral infection induces ASM remodeling

The association between respiratory viral infections and asthma has been known for more than 30 years. Some epidemiological data have postulated a winter viral infection as a causal factor for asthma development[241]. Considering the wide-clinical spectrum of asthma phenotypes, and the implications in other airway diseases, the role of respiratory viruses seems to be more linked to recurrence and exacerbations. In this way, viruses were detected in the respiratory secretion of 80 to 85% school children who were experiencing asthma exacerbations, and 90% of infants at their first three years of life[242]. Similarly, studies of adults admitted to emergency room with asthma exacerbations revealed a viral infection prevalence of 76%[243]. Initially, RSV was the most commonly detected virus in respiratory secretions, but after the PCR development, rhinovirus (RV) has become the most common virus in asthma exacerbation. However, it is still believed that both viruses play equally role in asthma pathogenesis[244, 245]. Moreover, studies have not confirmed whether this relationship is causal. There is currently a common paradigmatic thought that asthma exacerbations are mostly triggered by viruses. If viral infection have some direct role in ASM remodeling and phenotypic switching, two important inquiries must be addressed. First, respiratory viruses should have the ability to infect ASMCs. Second, the infecting viruses should persist in ASMCs long enough to induce chronic alterations.

RSV and RV infect ASMCs

Regarding virus infectivity, viral capsid proteins and miRNA profile could determine its tropism. A study demonstrated the presence of RSV RNA in bronchial epithelial cells and ASM, but no virus was found in lymphoid tissue, brain, spleen or bone marrow[246]. After RSV infection and interaction with respiratory mucosal surface, the viral G protein mediates attachment and the F protein induces viral envelope fusion with the plasma membrane of host cells, which results in internalization of the virus[247]. The ability of RSV to infect ASMCs was confirmed, and it was also associated with β_2-agonist desensitization[248]. Additionally, there are findings that highlight the role of ICAM-1 for RV-host interaction. ICAM-1 had been previously identified as a receptor ligand of β_2 integrin, and several reports showed it can interact with a RV capsid protein as well. Attachment of RV (90% of serotypes) to ICAM-1 initiates the incursion into host cells with insertion of the virus genome[249]. Additionally, ICAM-1 expression is induced in ASMCs by remodeling-associated cytokines like IL-1β, IL-6, and IL-8, which would enhance RV spreading along ASM bundles[250]. However in a mice model, RV elicited proasthmatic changes in ASMCs independently of viral infection, where virus-ICAM-1 interactions induced both an increased contractile response and an attenuated β_2-adrenoceptor-mediated ASM relaxation[251]. These findings confirm that other virus-cell host interactions exist, in addition to ordinary intracellular infections.

IFNs, Viral Persistence and ASM Remodeling

The persistence of viral infections depends on the virus serotype, the host (genetic susceptibility, age) and environmental (allergen exposure, season) factors. At the beginning, infection occurs by inhalation and spreading to the lower respiratory tract. Infection is firstly restricted to airway epithelium, which induces the release of mediators that drive subsequent immune and physiological responses[252]. Thus, RSV avoids immune clearance by inducing skewed Th-2 cell responses, antagonizing antiviral cytokines, mimicking chemokines, inhibiting cell apoptosis, and infecting immune-privileged cells such as pulmonary neurons. It can also escape from eradication through antigenic drift[253]. RV- or RSV-infected mice were reported to have proasthmatic airway changes. RSV infection was still detected in lungs for more than 150 days after intranasal inoculation[254]. Guinea pigs inoculated with human RSV, showed histologic features of acute bronchiolitis, and chronic persistence of viral antigens was associated with long standing AHR for >5 weeks[255]. In four independent studies, timing of RV infection was essential for developing proasthmatic changes. Virus inoculation in the first week of life induced long-term AHR and increased airway resistance that were consistent with peribronchial inflammation, elevated TNF-α, Th2 cytokines expression, and increased mRNA and protein levels of TGF-β. These mice showed a greater response to OVA-induced allergic inflammation and subsequent RV infections than infected mice at an older age[256-259]. This information, in association with an epidemiological study about the relationship between children's age and virus incidence during winter as a risk factor for developing asthma[241], suggest that age is an important factor for viral modulation of host cells in human disease.

The interaction of IFNs with ASM has been shown to increase inflammation through the production of an outnumber of inflammatory mediators, to impair steroid function via the dominant-negative activity of GRβ isoform, and to regulate AHR via changes in ASM contractility through calcium regulatory proteins, including CD38[260]. Although, IFNs play a role in ASM hyperresponsiveness, it is not clear whether its production is enough to allow viral clearance in asthma, which would also allow long-term replication and chronic inflammation. Accordingly, impaired TLR3-mediated IFN-β and -λ production by asthmatic epithelial cells

increases the susceptibility to both viral infection and allergic sensitization[261]. A Th2-skewed eosinophilic airway is a weaker immune response towards superimposed RV infection, as induction of anti-viral cytokines IFN-α, IFN-γ and IL-12 is depressed[262]. Reduced primary IFN production by airway epithelium enables some viruses to replicate, leading to cytotoxicity, release of inflammatory products and enhanced viral shedding. When asthmatic epithelial cells are damage by RV infection, these cells generate an increased amount of the pro-Th2 cytokine TSLP[263], which stimulates dendritic cells and increases allergic inflammation. Enhanced autophagic pathways by IL-13 could support RV spreading, as exogenous IFN-β and IFN-λ restore inhibition of autophagy and reduce RV replication[264]. IFN-γ has well-known antiviral actions that promote an efficient virus elimination. After RV exposure, peripheral blood mononuclear cells from asthmatic subjects produced significantly lower levels of IFN-γ and IL-12, and higher levels of IL-10, compared to normal subjects[265]. Also, they exhibited reduced expression of intracellular signaling molecules, including interferon regulatory factors (IRF1, IRF7), NF-κB family members (p50, p52, p65 and IκKα) and STAT1, along with reduced responsiveness to TLR7/TLR8 activation[266].These findings suggest that in asthmatic patients, as a result of impaired IFN production and failure of IFN-independent anti-viral mechanisms, respiratory viruses should be able to escape easily from immune clearance.

As it was previously mentioned, ASMCs constitutively express MHC-I and may express MHC-II after interacting with activated T cells. Additionally, a bidirectional communication ASMC↔T cells was demonstrated[70]. An abnormal crosstalk between ASMCs and T cells would be responsible of viral persistence in ASM. Correspondingly, $CD8^+$ T cells have potential inhibitory effects on airway remodeling[267]. In the context of virus-infected ASMCs that are exposed to a microenvironment characterized by low IFNs, high Th2-cytokines and mitogens, a deficient antiviral activity may be due to virus-induced decreased MHC-I expression[268]. Following RV infection, airway levels of MHC-I related chains are upregulated, and there is a positive correlation between sputum MHC-I B chain levels and the percentage of bronchoalveolar natural killer cells in healthy subjects, but not in asthmatic patients[269]. dsRNA, a RV replication intermediate, increases TLR3- and RLR-mediated expression of RIG-I, MDA5, IFN-β, and IFN-λ1 mRNA by ASMCs[270]. As regarding epithelial cells, it is possible that this ASMC response is abnormal in asthma and it is likely related to tissue remodeling. For example, synthetic dsRNA enhanced ASM mass and ASMC proliferation in a rat model of bronchial asthma[271]. Interestingly, in contrast to most viruses, some RNA viruses like RV use the autophagic pathway to promote their own replication[272]. The autophagosome provides membranous support for viral RNA replication complexes, possibly enabling the nonlytic release of cytoplasmic content, including progeny virions from infected cells. Autophagy can be triggered in smooth muscle cells by growth factors, cytokines and changes in the redox potential, which helps to remove contractile proteins, but also could hasten the transition to s/p-ASMC and increase cell survival under conditions associated with an increased oxidative stress in airway diseases[273]. This process is regulated by the enzyme geranylgeranyl transferase-1 (GGT1), which is involved in the posttranslational prenylation ofsignalingproteins, such as small GTPases. Pharmacologic inhibition of GGT1 induces simultaneously p53-dependent protein expression and autophagy in ASMCs, whereas inhibition of autophagic proteins stimulates apoptosis[274]. Thus, ASMCs are suitable candidates to perform as a viral reservoir, and such viral infection could lead to increased resistance to proapoptotic pathway activation and improved synthetic functions that instead of enhancing the control of viral infection, they could contribute to viral persistence and airway remodeling.

Hypothetical viral non-coding RNA targets support a 'hit and run' strategy

The hit-and-run hypothesis was proposed once researchers found that virus-induced AHR and tissue remodeling persisted even after viral clearance[275]. In this mice model, interferon deficiency has not any effect on the final outcome, and abnormalities were related to allergen-challenge responses at long-term. Discussed evidence supports that viral infection contributes to ASM remodeling. Therefore, viruses could have a causal role in some asthma phenotypes, in which case the infection should deliver a specific signature. Inflammation comprises cytokine and growth factors that *per se* coordinate a phenotypic transition. However, viral genomes contain specific sequences that could affect the cell phenotype regardless of the inflammatory status. Viral RNA induce a change in the miRNA profile that is expressed by resident cells[276]. Also, viral non-coding RNA has been implicated in cell transformation[277], and viral miRNAs have been predicted[278]. Interactions between RV RNA and host RNA were suggested because RV RNA co-immunoprecipitated with the argonaute-2 protein, which is an important mediator of miRNA effects. Thus, host miRNA could bind to viral RNA during infection[279].

To explore whether RSV or RV RNA sequences affect the ASMC phenotype, we took advantage of an *in silico* approach. We assumed that viral RNA could be processed in order to obtain either viral-miRNA or –lncRNA. Those products could disturb the host cell transcriptome, as virus-derived miRNA downregulates cellular mRNA and virus-derived lncRNA traps cellular miRNA, inducing gene expression for phenotype switching. For assessment of virus-derived lncRNA, we chose matching miRNAs involved in synthetic and proliferative responses of ASMCs. Then,

hybridization with the RSV and RV-C RNA strands was predicted using the tool RNAhybrid. On the other hand, targets of putative RSV and RV miRNAs (available at the Vir-Mir database) were predicted using the RNAhybrid service. The results are shown in the Table 5. Remarkably, this analysis indicates novel interactions of RNA viruses with ASMCs restricted to RNA metabolism that need experimental and functional validation.

Table 5. Annealing prediction of human miRNA with RSV and RV genomes/antigenomes, and target prediction of putative viral miRNAs.

hsa miR	RSV genome		RV- C genome		RSV miRNA	RV-B miRNA
	- ssRNA	*Antigenome*	*+ssRNA*	*Antigenome*	*3'p arm targets*	*3'p arm targets*
-25, 5p	-22.0 (8760)	-23.0 (7212)	-24.4 (696)	-24.1 (1737)	**AACSL (0.008)**	BRCA2 (0.03)
3p	-22.2 (4572)	-22.8 (3245)	-21.8 (87)	-24.6 (4445)	C1orf187 (0.02)	HSPC047 (0.01)
-10a, 5p	-21.9 (5987)	-23.4 (11741)	-20.0 (5591)	*-25.4 (5749)*	**DFFA (0.02)**	ASL (0.007)
3p	-21.7 (10052)	-21.3 (2543)	-21.8 (219)	-22.7 (5362)	EXTL3 (0.02)	LOC439968 (0.0006)
-221, 5p	-20.1 (7783)	-21.1 (5005)	-24.0 (985)	-20.4 (255)	SPTB (0.004)	LOC439971 (0.03)
3p	-23.4 (7962)	-22.8 (14207)	*-26.6 (2083)*	-24.8 (5680)	HSB1L (0.02)	LOC439962 (0.03)
-26a, 5p	-19.8 (412)	-24.2 (12161)	-24.0 (6035)	-20.4 (2892)		
-155, 5p	-23.6 (4306)	-22.9 (14634)	-19.8 (3115)	-22.6 (3718)		
3p	-18.4 (6276)	-20.8 (8984)	-22.9 (5077)	-20.5 (4289)		
-206, 3p	-20.1 (3981)	-21.7 (2083)	-21.4 (6023)	-22.4 (3260)		
-133a,5p	-18.8 (11026)	-19.9 (2135)	-20.2 (2580)	-21.1 (1591)		
3p	-23.9 (1776)	-23.1 (5384)	-22.5 (6060)	*-25.0 (102)*		
-146a,5p	-23.0 (3919)	-23.6 (5355)	-20.4 (439)	-20.9 (5471)	*5'p arm targets*	*5'p arm targets*
3p	-17.7 (11135)	*-25.0 (4859)*	-19.5 (798)	-17.1 (6442)		
-146b,5p	-20.8 (4876)	-22.5 (5358)	-22.8 (439)	-20.1 (3333)	FLJ34969 (0.02)	**HLA-G (0.007)**
3p	-22.0 (6903)	*-29.5 (4859)*	*-28.2 (1387)*	*-28.7 (3818)*	C9orf75 (0.001)	PNCK (0.02)
-145, 5p	-20.5 (8386)	-23.5 (12165)	*-26.8 (5333)*	-22.6 (2147)	C1orf117 (0.002)	**SLC26A9 (0.01)**
3p	-18.5 (6789)	-20.7 (11574)	-23.3 (6040)	-24.4 (5043)	LOC400680 (0.03)	**DLC1 (0.04)**
-143, 5p	*-29.4 (3393)*	*-26.7 (3943)*	*-27.6 (5236)*	*-26.8 (102)*	LOC440568 (0.003)	**SH3GLB2 (0.008)**
3p	-21.0 (7690)	-23.1 (7506)	-23.6 (5400)	-21.9 (1576)	LOC388107 (0.00005)	STK11 (0.006)
-150, 5p	-21.4 (10720)	*-28.3 (4002)*	*-25.3 (4595)*	-24.4 (1131)	**IMP-2 (0.01)**	NUDT16 (0.04)
3p	*-25.2 (6020)*	*-25.9 (591)*	*-26.8 (3023)*	*-29.7 (1382)*	C5orf14 (0.006)	BRF1 (0.01)
-371, 5p	-21.2 (9764)	*-28.2 (10865)*	*-25.22 (2766)*	-22.2 (2222)	**PRKA1A (0.003)**	**DES (0.006)**
-1207,5p	*-28.3 (5380)*	*-29.0 (10719)*	*-27.0 (6594)*	*-28.1 (1559)*	**CUGBP2 (0.07)**	**CTNNBIP1 (0.03)**
-718, 3p	*-30.6 (1836)*	*-28.1 (7594)*	*-28.7 (1776)*	*-26.6 (5552)*	**MBD3 (0.01)**	**SCOTIN (0.01)**

On the left, selected RV (gene bank ID: JN815251.1) and RSV (gene bank ID: KP856967.1) RNA sequences are serotypes associated with severe respiratory illness, (available at: http://www.ncbi.nlm.nih.gov/nuccore). Antigenomes (complementary strands) were determined at: http://www.bioinformatics.org/sms/rev_comp.html. Annealing with human miRNAs, both 5'- and 3' p arms, was performed with the prediction tool RNAhybrid (max bulge loop length= 1, max internal loop length= 1), available at http://bibiserv.techfak.uni-bielefeld.de/mahybrid. The minimum free energy (mfe) of hybridization for the best potential binding site per target is shown, in parenthesis the seed location. Usually, any mfe<-25kcal/mol is significant, a real match is more likely while this number is lesser. Hybridization scores and *P*-value cannot be determined in this assay. *On the right*, viral miRNA sequences were previously predicted at http://alk.ibms.sinica.edu.tw/: RV-B 5'p arm GCCAUUCAGCAUAACACACAACAGCG, 3'p arm UUCUUGUGUUGUUAUUGUUGAAUUCC, RSV 5'p arm GUUAGGUGUUGGAUCUGCAAUCGCC, and 3'p arm GUAUCUAAGGUCCUGCACCUAGAAGG. Host targets were obtained with the RNAhybrid service (helix constraint 4-9, mfe percentage 75%, at: http://alk.ibms.sinica.edu.tw/cgi-bin/RNAhybrid/RNAhybrid.cgi), in parenthesis the *P*- value, targets that could have a relevant role in ASMCs are in bold. This data requires experimental validation.

ASM remodeling as a therapeutic target: experimental evidences

Our current failure to treat some phenotypes of severe asthma is a reflection from our poor understanding of its underlying etiology. Classic interventions are directed to exacerbation management and prevention, including mainly bronchodilators and steroids to keep most patients away from flares, but in many cases they are ineffective as shown by clinical trials. Prospective placebo-controlled studies have not shown long-term beneficial effect of steroid treatment for RSV bronchiolitis and subsequent wheezing or asthma[243]. Doubling the dose of inhaled steroids in moderate to severe asthmatics was ineffective in two unrelated studies[280, 281]. Moreover, in adults with persistent asthma even optimal therapy is only able to reduce the frequency of exacerbations by around 40%[282]. In school-age children, moderate doses of inhaled steroids are completely ineffective at reducing exacerbation frequency, duration and severity of wheezing episodes associated with viral infection[283]. Furthermore, 5-day course of oral steroids at the onset of exacerbations in preschool children was ineffective at reducing the duration or exacerbation severity, even in children with systemic eosinophilia[284]. Not to mention the detrimental effects of glucocorticoids, seen on patients who had fatal asthma, in addition to the side effects by a prolonged therapy[285]. The reasons of failure could be secondary to an acquired insensitivity to glucocorticoid actions, which could prevent these drugs from blocking many events that conduce to airway remodeling. A review of the potential mechanisms has been recently published[286]. It is beyond the objectives of this review to analyze all the current recommended asthma and COPD therapies, for which there are available guidelines[287, 288], or to underestimate the corroborated benefits of steroid treatment in the vast majority of asthmatic patients. However, recognizing that common interventions could not affect final outcomes is crucial to find better therapeutic targets. If novel therapies for airway remodeling are developed, it is possible that steroids might be sent apart from the primary treatment of asthma, and COPD progression could be effectively delayed. In this section, we explore some experimental evidence that supports such potential interventions.

Long-Acting Muscarinic Receptor Antagonists

The long-acting muscarinic antagonists (LAMAs), aclidinium, glycopyrronium and tiotropium,

bind to human M_1-M_5 receptors in a concentration-dependent manner, but the highest selectivity is for M_3, followed by M_2[289]. Blockage of mAChRs has anti-inflammatory and anti-remodeling properties, although, most studies include only tiotropium in their protocols. For example, the anti-inflammatory activity associated with tiotropium on cigarette smoke-induced pulmonary inflammation in mice was related to a dose-dependent reduction of leukotriene-B4, IL-6, chemokines and TNF-α, and also a decreased cell numbers in BAL[290]. In the same way, eosinophil recruitment and AHR in a guinea-pig model of asthma were inhibited by vagal blockade-independent mechanisms[291]. The mechanism of tiotropium was linked to inhibition of TGF-β-induced MAPK signaling and a decreased MMP expression[292]. LAMAs would further affect cell plasticity, as it was demonstrated that aclidinium can inhibit the CCh-, TGF-β-, and cigarette-induced transition from human fibroblast to myofibroblast[293]. Tiotropium also significantly inhibited ASM thickening and Th2 cytokine production by human peripheral blood mononuclear cells in a murine model[294]. The anti-remodeling effect also include decreased sm-MHC expression and decreased isometric relaxation of tracheal strips that were previously exposed to repeated allergen challenge[121]. From the clinical standpoint, in COPD, all three drugs produced significant FEV_1 improvement,but only glycopyrronium reduced dyspnea. In severe asthma, only tiotropium has been tested, and it has demonstrated to raise $FEV_1$and decline the risk of exacerbations[289].

Emerging Therapies: Statins, Macrolides, Endothelin Antagonists, Calcium Channel Antagonists and PPARγ Agonists

Statins, through inhibition of 3-hydroxy-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), reduce the synthesis of groups needed for protein isoprenylation, farnesylation and geranylgeranylation, influencing cell signaling. The inhibitory effect ofsimvastatinon FBS-inducedRhoAactivation is antagonized by geranylgeranyl pyrophosphate, but not by farnesyl pyrophosphate. These isoprenoids are required for prenylation of the small G proteinsRhoAand Ras, and it was shown that inhibition ofASMC proliferationbysimvastatinwas due to prevention of geranylgeranylation ofRhoA, but not by farnesylation of Ras[295]. Moreover, lovastatin, isoprenylation inhibitors, or other pharmacological approaches for preventing localization of RhoA in the membrane localization should be considered as a preventiveantiviraltherapyfor selected groups with high risk for severe RSV disease[296]. However, statins are known to decreased cell survival, impacting on signaling that also contributes to bring cells under molecular stress. Autophagy, especially macroautophagy, was discussed as a potential mechanism of phenotypic modulation. Considering that some RNA viruses take advantage of the double-membrane vacuoles, potential improvement of RV replication could be an undesirable effect due to autophagic induction in response to statins[274]. Affecting some pathways associated with maturation can also reinforce ASMC modulation. This may explain why despite the well-stablished anti-inflammatory and pleiotropic effects of statins, clinical trials still failed to show any improvement of inflammatory and functional outcomes in patients with severe asthma[297].

Macrolides are antibiotics that have been widely used in the treatment infectious diseases. Additionally, immunomodulatory and anti-inflammatory effects have been shown in relation to a suppression of goblet cell hyperplasia and cytokine secretion by regulating the activation of a MAPK/NF-κB pathway[298]. Experimental evidence supports anti-remodeling actions of macrolides, e.g., roxithromycin inhibited ASMC proliferation in a dose-manner dependent. This effect was dependent on the loss of the mitochondrial membrane potential, cytoplasmic accumulation of Cyt c, caspase activation and increasing of $p27^{Kip1}$expression[299]. The same researcher group showed that roxithromycin decreased bronchial wall thickness andASM layer in OVA-sensitized rats, and also downregulated ERK1/2 and upregulated caveolin-1 expression[300].Long-term therapy may improve some functional parameters without affecting clinical outcomes, as showed by a recent metanalysis[301].

Endothelin-1 induces bronchoconstriction, mediates eosinophil recruitment during allergic inflammation, and contributes to airway remodeling by inducing fibroblast and ASMC differentiation and proliferation[302]. Despite of *in vitro* results obtained with endothelin receptor antagonists, such as sitaxsentan or bosentan, a recent small clinical trial did not demonstrate any improvement of functional tests or symptoms in poorly controlled asthma when compared to placebo[303]. On the other hand, calcium channel blockers were classically tested as bronchodilators, but after recent findings of pro-remodeling effects of altered calcium signals, long-term blockade has been proposed. Therefore, gallopamil administration reduces the mitochondrial mass and subsequent ASMC proliferation[304]. A recent doubled-blind randomized clinical trial showed that this calcium channel blocker decreased ASM thickness after 1 year of treatment. Although, there was no immediate clinical improvement during the treatment phase, a significant reduction in asthma exacerbations related to ASM mass reduction was seen during the follow-up[305]. In the same way, the PPAR-γ ligands, rosiglitazone and pioglitazone, have shown to regulate noncontractile and contractile functions of *in vitro* ASMCs, including decreased in proliferation and synthetic activities by increasing heme oxigenase-1 activity, and β_2-AR expression that could reduce AHR[306]. However, its benefits in obstructive airway diseases remains to be tested in humans.

Biologic Therapy

Current management of autoimmune diseases and cancer, is based on blocking specific molecular targets through inhibitors and monoclonal antibodies. Unquestionable evidence has been obtained with anti-IgE (omalizumab) for treatment of severe asthma associated with high IgE levels, being included in GINA guidelines. Similar medications have been considered on behalf of their efficacy in chronic inflammatory diseases. A large list, including anti-IL-5 (mepolizumab, reslizumab, enralizaumab), anti-TNF-α (etanercept), anti-IL-4 (pascolizumab, nuvance), anti-IL-4/13 (pitrakinra), anti-IL-9, anti-CD25 (daclizumab), anti-VCAM-1 and anti-TSLP, are now under clinical trials, and some recent publications have shown controversial results[307, 308]. Based on animal models, blocking those pathways results in a reduced airway remodeling via decreased eosinophil, monocyte and T cell recruitment. However, decreasing inflammation was not always correlated with anti-remodeling effects. Clinical studies have not shown consistent results regarding improvement of FEV_1, symptom control, and decreased use of short-acting β_2-agonists. A common finding is that the number of exacerbations tends to decrease in the higher-steroid dose groups, without significant clinical efficacy. Whether or not these results were related to prevention of remodeling is unknown, because histopathological assessment were not included in their protocols.

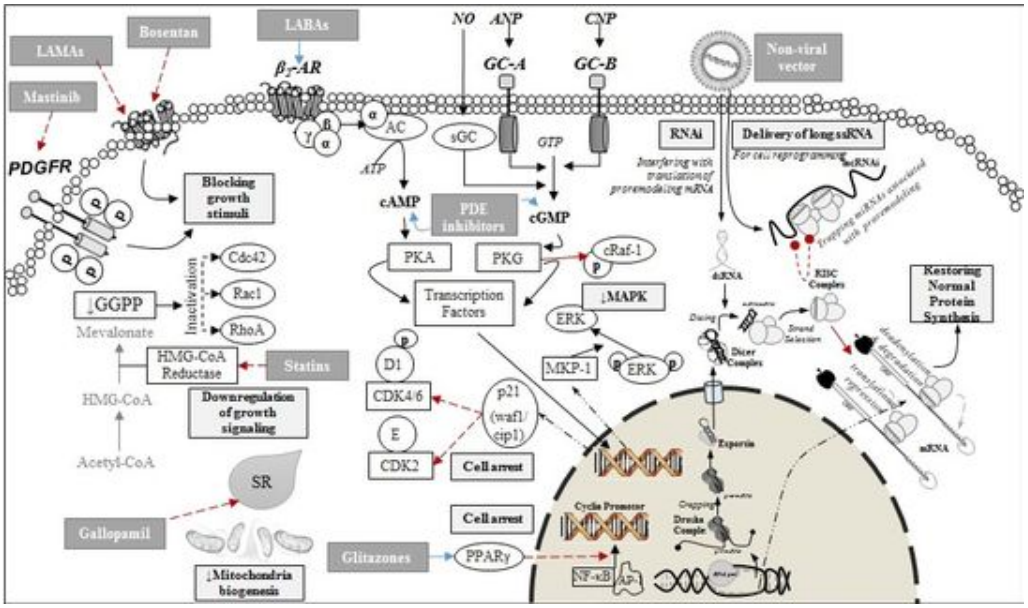
A specific approach is attained with the c-kit/PDGF receptor tyrosine kinase inhibitor mastinib. RTK inhibition by imatinib mesylate decreases collagen deposition, eosinophil infiltration, and ASM thickening in a murine model[309]. Mastinib improved the asthma control score and number of exacerbations[310]. However, no significant improvement in lung function was observed. More randomized clinical trials are needed to precise what biologic therapy is suitable for specific subgroups. New drugs that target specific pathways, such as: antiproteases for modulation of ECM deposition, NFκB inhibitors, PI3K inhibitors, chemokine receptor antagonists, and even old drugs with anti-inflammatory properties, like thalidomide, increase the spectrum of therapeutic interventions[311], but no experimental and clinical research focusing on ASM remodeling have been addressed.

Bronchial Thermoplasty

Reduction of dense ASM using physical forces, like radiofrequency energy, has shown promising results. Bronchial thermoplasty is a FDA-approved bronchoscopy procedure for patients with severe asthma, which delivers high thermal energy to the airway wall to heat and reduce the amount of its cellular components. Although, airways swell on immediate heat administration, this blanching and erythema usually resolved within 1 week, and no long-term adverse effects were noted. In essence, epithelial, blood vessel and nerve injury are follow by tissue regeneration, however for unknown reasons, ASM has demonstrated almost no capacity of regeneration after this procedure, being replaced by connective tissue, instead. Increased airway distensibility, decreased bronchomotor tone both at baseline and in response to increasing doses of methacholine suggest that the AHR reduction correlated well with the degree of ASM reduction, supporting a role of ASM remodeling in humans[312].

Conclusion

ASM thickening is a consistent finding in airway remodeling that most likely contribute to the AHR and irreversible or partially reversible airflow obstruction seen on airway diseases, especially those with severe symptoms. Chronic inflammation is a major mechanism of structural transformation occurring at all airway layers. Nevertheless, ASM can also be generated by means of non-inflammatory pathways that would explain the lack of clinical correlation between inflammation and AHR. It remains unclear if ASM thickness depends on resident smooth muscle plasticity, mesenchymal expansion, myofibroblast migration, or stem cell differentiation, all with considerable evidence suggesting a role in this process. A complex molecular network between heterogeneous ASM bundles, containing the contractile, synthetic/proliferative, and hypercontractile phenotypes, and its tissue microenvironment determine the ASM function and outcome after injury, which real contribution can only be estimated by a system biology approach. Cytokines, growth factors, ACh, and viruses seem to have major influences in the genesis of ASM hyperplasia and hypertrophy, particularly, muscarinic signaling has potent effects on the ASMC metabolism regardless of its phenotypic status. Therefore, muscarinic receptors activation catalyzes processes for remodeling modulation, besides its well-known contractile effects, hence, it is an attractive target for long-term pharmacologic blockage by LAMAs. Further understanding of these mediators and the interaction immune cell-smooth muscle at a epigenetic level could help to identify accurately the pathobiologic mechanisms of abnormal ASM functions and thickening, providing so, specific targets to develop future treatments (see **Fig. 7**) in the advent of gene therapy and nanotechnology[313].



Disclosure Statement

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

Authors' contributions

C.A.F.A., M.J.A., I.L.B and R.F.R. participated in selection and reviewing of cited literature. C.A.F.A. aimed and wrote the preliminary version of this manuscript, designed tables, performed art-work of figures, and performed the *in silico* analysis for miRNA target and RNA hybridization prediction. F.A.P.U. did the staining technique for figure 1. C.A.F.A., R.F.R., and F.A.P.U. implemented cell culture techniques for figure 3. C.A.F.A, R.F.R, F.A.P.U, R.G.A, M.J.A, I.L.B read, drafted, discussed and approved the final version of this manuscript.

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