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Motility, Survival and Proliferation

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Abstract

Airway smooth muscle has classically been of interest for its contractile response linked to bronchoconstriction. However, terminally differentiated smooth muscle cells are phenotypically plastic and have multifunctional capacity for proliferation, cellular hypertrophy, migration, and the synthesis of extracellular matrix and inflammatory mediators. These latter properties of airway smooth muscle are important in airway remodeling which is a structural alteration that compounds the impact of contractile responses on limiting airway conductance. In this overview we describe the important signaling components and the functional evidence supporting a view of smooth muscle cells at the core of fibroproliferative remodeling of hollow organs. Signal transduction components and events are summarized that control the basic cellular processes of proliferation, cell survival, apoptosis and cellular migration. We delineate known intracellular control mechanisms and suggest future areas of interest to pursue to more fully understand factors that regulate normal myocyte function and airway remodeling in obstructive lung diseases.

Keywords

Airway smooth muscle; Apoptosis; Cell proliferation; Chemotaxis; Hypertrophy

Introduction

The classical role of smooth muscle cells in the surrounding muscle layer of hollow organs is to regulate dynamic changes in lumen caliber and wall stiffness. Appreciation of the multifunctional behavior of smooth muscle cells in physiology and pathophysiology has steadily increased since initial insight provided by Wissler's work on large elastic arteries [1]. In addition to contraction, terminally differentiated smooth muscle cells are also capable of reversibly adopting capacity to express and secrete cytokines, chemokines and extracellular matrix proteins, to proliferate, and to migrate. This has led to current paradigms that place smooth muscle cells at the core of fibroproliferative remodeling of hollow organs

Supplementary Information

None

in diseases of the vasculature (atherosclerosis and hypertension) and the airways (asthma and chronic obstructive disease).

Remodeling of the airways involves thickening of the bronchial and bronchiolar walls due to multiple events involving multiple cell types. There is epithelial cell denudation, mucus gland hyperplasia, increased smooth muscle mass, thickening of the *lamina reticularis* and accumulation of sub-epithelial extracellular matrix (ECM), increased numbers of sub-mucosal myofibroblasts, increased vascularization, and development of a chronically healing epithelium [2, 3]. Evidence points to progressive structural change in the airway wall due to rounds of inflammation-driven wound healing as a fundamental component for development of fixed airway narrowing [4, 5]. A significant component of irreversible airway hyperresponsiveness in long-standing asthma excludes the inflammatory response, suggesting that fibro-proliferative changes associated with mesenchymal cell populations in bronchial wall may underpin fixed airway dysfunction [2, 6]. Local inflammation is complex as it is manifest both by recruited leukocytes and mast cells, but also by the intrinsic capacity of airway myocytes to express and release cytokines, chemokines and other pro-inflammatory molecules [7]. Thus airway smooth muscle (ASM) thickening results from a collective of biological signals that induce several trophic myocyte responses.

Though ASM has classically been of interest for its contractile response linked to bronchoconstriction, terminally differentiated smooth muscle cells are phenotypically plastic and have multifunctional capacity for proliferation, cellular hypertrophy, migration, and the synthesis of extracellular matrix and inflammatory mediators [8–11]. It is this property of ASM that positions it as an effector of airway remodeling which is a structural alteration that itself compounds the impact of contractile responses on limiting airway conductance. Understanding airway smooth muscle-associated cellular mechanisms that contribute to airway remodeling is of great relevance for several reasons. First, though remodeling and thickening consists of multiple structural changes, the increased mass of contractile ASM is the most significant causal feature for airway hyperactivity and excessive narrowing that reduces airflow [12–14]. Second, airway remodeling is characterized by increased numbers of myofibroblasts in the submucosal compartment. Their accumulation after allergen challenge is rapid, thus there is growing belief that migration of airway myocytes from the adjacent smooth muscle layer feeds this response [15, 16]. Last, in the preceding decade there has been growing interest in research aimed at developing new therapeutics that target airway smooth muscle to treat asthma [17–19].

As an overarching paradigm for this chapter, Figure 1 provides a schematic model for cellular mechanisms, including migration, proliferation, hypertrophy and apoptosis, which likely support a primary affective and effective role in airway remodeling and hyperresponsiveness linked with obstructive lung disease. This chapter provides an overview of current understanding of ASM proliferation, hypertrophy, apoptosis and migration. Moreover, we delineate intracellular control mechanisms and the repertoire of biological factors that engage them in regulating myocyte function.

Airway Smooth Muscle Proliferation

Airway smooth muscle cells can respond to a variety of mitogenic cues that promote traversing the Gap 1 (G1), S, Gap 2 (G2) and M(itosis) phases of the cell cycle. As an early response to mitogen stimulation, from a quiescent G0 state myocytes enter the G1 phase of the cell cycle coincident with increased expression of specific D-cyclins, such as cyclin D1 [20, 21]. Initially, progression through the G1 phase depends on the binding of one or several D-type cyclins (D1, D2, and/or D3) to existing cyclin dependent kinases (CDK4 and -6), forming active complexes that subsequently activate cyclin E/CDK2. This leads to increased phosphorylation of retinoblastoma protein (Rb), which in turn dissociates from an elongation factor E2F/Rb complex. E2F/Rb is otherwise bound to E2F responsive genes, effectively halting their transcription and creating a cell cycle block; the release of E2F permits the transcription of various genes, including DNA polymerase, essential for effective transit of cells through G1 and into S phase. G1/S transition represents a restriction point (R) past which DNA will be synthesized (S phase), cells will increase in size and synthesize microtubules (G2) and eventually undergo mitosis [22, 23].

This whole process is of course tightly regulated. The activity of CDKs and their effects on cell cycle progression can be negatively regulated by CDK inhibitors during the G1/S transition. In this regard, two principal families of genes have been identified based on their structure and specific CDK targets: 1) the Cip/Kip family (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}), which interfere with cell cycle in the G1 phase by inactivating cyclin D-, E- and A dependent kinases [22]; and, 2) the INK4/ARF family (Inhibitor of Kinase 4/Alternative Reading Frame; p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}), which negatively affect the catalytic subunits of CDK4 and -6 and as such prevent interaction with cyclin D1 [22].

During the cell cycle, cells will go through a number of 'checkpoints' to ensure that each phase of the cycle has been accurately completed before entering the next one; at each point the cell is screened for DNA integrity, and requires a collective of effective temporal mitogen stimulation. The first cycle checkpoint occurs at the end of the G1 phase, just before entering into S phase, where it is typically decided whether the cell should proceed, enter a resting/repair stage, or exit the cycle via apoptosis. At this checkpoint DNA damage is monitored through a process involving the tumor suppressor protein, p53, which has capacity to arrest cycling of G1 cells by activating transcription of p21^{Cip1}, leading to subsequent CDK inhibition [24]. Depending on the severity of DNA damage, p53 can either activate DNA repair proteins enabling the cell to eventually continue cell cycle or, in cases of irreparable DNA damage, induce apoptosis [24]. A second checkpoint is located at the end of the G2 phase and regulates initiation of M phase. This checkpoint is sub-served by a complex of cyclin B/CDK1 complex (referred to as MPF, maturation promoting factor), which is responsible for essential phosphorylation events in a number of proteins required for mitosis [25]. A third checkpoint (the mitotic spindle checkpoint) occurs during metaphase when chromosomes have aligned at the mitotic plate and are under bipolar tension from the spindle apparatus. The appropriate tension created by this bipolar attachment is necessary to initiate progression to anaphase during which individual chromosomes are segregated and pulled toward opposite poles. Thereafter, cytokinesis proceeds and the original cell spawns

two daughter cells that can then continue through G1 phase and another cell cycle, or be diverted to a quiescent state in G0 [24].

Factors controlling airway smooth muscle cell proliferation

Airway smooth muscle proliferation can be affected by at least three groups of mitogens: polypeptide growth factors, G-protein coupled receptor (GPCR) agonists and pro-inflammatory cytokines [20] (Table 1). In addition, extracellular matrix proteins are important regulators of mitogen-induced proliferation [26, 27]. In asthma, excessive accumulation of (contractile) smooth muscle has frequently been described in central and small airways [28–30], and is typically associated with myocyte hyperplasia and hypertrophy. Thus, increased ASM mass may, in part, be due to cellular proliferation driven by growth factors, inflammatory mediators and neurotransmitters [31, 32].

Polypeptide Growth Factors—Polypeptide growth factors induce proliferation by activating receptors with intrinsic protein tyrosine kinase (RTK) activity and are among the most effective inducers of ASM proliferation. This group of mitogens includes for instance basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and insulin, which have all been shown to induce ASM cell proliferation (Table 1). Several RTK growth factors, including EGF, PDGF, and IGF-1, have been implicated in asthma pathogenesis based on either increased immunoreactivity of the growth factor, bioavailability, and/or receptor expression [33–36]. Importantly, some combinations of these growth factors (e.g. EGF + PDGF, insulin + PDGF) can produce synergistic proliferative responses in airway myocytes [37–41]. A number of RTKs, for example the PDGF and EGF receptors, are located in caveolae in the plasma membrane, where they associate with caveolin-1 [42]. This may represent a mechanism for additive or synergistic effects of mitogens. For instance PDGF and EGF receptors uncouple from caveolin-1 in response to mitogen stimulation and thus activated, traffic to peripheral caveolae-free membrane sites, where p42/44 MAPK activation can take place [42, 43].

G-protein Coupled Receptor Agonists—Contractile agonists, such as acetylcholine and cysteinyl leukotrienes, acting via G-protein coupled receptors (GPCRs) have been associated with increased ASM thickening in asthma (Table 1) and in animal models of asthma [44–47]. However, stimulation of muscarinic receptors or cysteinyl leukotriene receptors alone is not sufficient induce ASM cell proliferation. Rather, these GPCR agonists exert profound promitogenic effects in the presence of a peptide growth factor, manifest as a synergistic increase in the proliferative response induced by the growth factor in isolation [31, 48, 49]. In addition to muscarinic M₃ and CysLT1 receptor agonists, it has become apparent that these effects are also observed for a number of other contractile agonists, including histamine, bradykinin and thrombin [48, 50–52]. The synergistic effects of contractile agonists on growth factor-induced proliferation are principally mediated through receptors that are coupled to turmeric G-proteins of the G_q subfamily [31, 48, 52]. In addition to G_q-protein coupled receptors, several agonists (e.g. thromboxane, thrombin and lysophosphatidic acid) that mediate effects via G_i-coupled GPCRs also have synergistic effects on growth factor-induced ASM proliferation [51, 53, 54]. Notably, the intracellular

mechanisms for this effect differs from that of G_q -coupled receptors, as these agonists do not necessarily require interaction with RTKs. Specific details on this issue are discussed in a subsequent section describing molecular mechanisms for proliferation.

In contrast to G_q and G_i -coupled receptor agonists, various G_s -protein coupled receptor agonists, including PGE_2 and β_2 -receptor agonists, inhibit ASM cell proliferation [55–57]. These effects appear to rely on the potency of these agonists to induce prolonged cAMP production and subsequent PKA activation [58, 59].

Pro-inflammatory Cytokines—The involvement of pro-inflammatory cytokines, such as TNF, IL-6 and IL-1 β in ASM cell proliferation is controversial. Several reports suggest modest proliferative effects [60, 61], whereas others demonstrate no effects or even growth inhibition (Table 1) [62, 63]. It has become apparent that for IL-6, IL-1 β and TNF that these paradoxical findings might be explained by cytokine-induced production of anti-proliferative mediators such as the cyclooxygenase-2 product prostaglandin E2 or IFN β , which exert an autocrine effect on the ASM cells [40, 64, 65]. Most of the cytokines of interest exert their effects on gene regulation through cell surface glycoprotein complexes, comprising 2 to 4 receptor chains that couple to several non receptor tyrosine kinases, such as Src family proteins and components of the MAPK and JAK/STAT cascades [20] (Figure 2). The balance between parallel and functionally opposing signaling pathways and unique phenotype of the cell population are ultimately the determinants of the effects of cytokines on ASM proliferation.

Extracellular Matrix Proteins—Several extracellular matrix (ECM) proteins have emerged as regulators of growth factor-induced ASM cell proliferation (Table 1). Cells cultured on monomeric collagen I or fibronectin matrices progress towards a more proliferative phenotype, as evidenced by an augmented basal proliferative response [26, 66] and an augmented mitogenic response towards either RTK or GPCR ligands [26, 27, 66, 67]. Conversely, when cultured on a laminin or laminin-rich Matrigel substrate, growth factor-induced proliferation is markedly suppressed [26, 27, 68]. These observations could be of significant relevance to airway wall remodeling and asthma pathogenesis, as both the quantity and the composition of the ECM is altered in the airways of chronic asthmatics. Deposition of collagen IV and elastin is decreased in the airway wall of asthmatic patients, whereas collagen I, III, V, fibronectin, tenascin, hyaluran, versican and laminin α_2/β_2 chains are increased [69–72]. Importantly, changes in matrix-composition directly surrounding ASM cells have also been reported: collagen I, hyaluronan and versican increased in patients with asthma [73, 74]. Human ASM cells also secrete ECM proteins in response to asthmatic sera [75] suggesting a cellular source for ECM deposition in airways and implicating a novel mechanism in which ASM cells may modulate autocrine proliferative responses.

ECM proteins interact with smooth muscle cells through integrins, which are heterodimeric glycoproteins consisting of membrane-spanning, non-covalently associated, α and β subunits [76]. Enhancement of growth factor-induced proliferation of ASM cells on a collagen I or fibronectin matrix is dependent on activation of $\alpha_2\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, of which $\alpha_5\beta_1$ has emerged as a crucial signaling integrin for proliferation both in

healthy and asthmatic ASM cells [67]. Laminin most likely exerts its anti-proliferative effects through the $\alpha 7\beta 1$ integrin [77].

Molecular Signaling Pathways in Airway Smooth Muscle Cell Proliferation

Major pathways described below are shown schematically in Figure 2.

MAP Kinases—The mitogen-activated protein (MAP) kinases are a superfamily of serine/threonine directed protein kinases involved in transcriptional regulation in response to a variety of extracellular stimuli, including growth factors [78], thereby being responsible for intracellular transmission of extracellular trophic signals. MAP kinases share a common activation mechanism which involves the phosphorylation of tyrosine and threonine residues in a Thr-X-Tyr (TXY) motif positioned in their activation loop. Based on the identity of the residue between the threonine and tyrosine, the MAP kinase superfamily can be divided into three main groups: ERKs (Thr-Glu-Tyr); Jun amino terminal kinases (JNKs) (Thr-Pro-Tyr); and p38s (Thr-Gly-Tyr). Each MAP kinase is activated by successive activation of a MAP kinase kinase kinase and a MAP kinase kinase. Activation of the ERK pathway constitutes an important regulator of cell cycle entry and G1 progression, and is required for DNA synthesis and proliferation in an extensive variety of mammalian cell systems, including bovine, rat and human ASM [63, 79–81]. The traditional path to ERK activation is comprised of the growth factor receptor binding protein Grb2, the nucleotide exchange factor Son of sevenless (Sos), the monomeric 21 kDa GTPase Ras, the 74 kDa cytosolic serine/threonine kinase Raf-1, and the 45 kDa dual function kinase MAP kinase/ERK kinase kinase (MEK)-1. Grb2 is found in a stable complex with the nucleotide exchange factor Sos. Docking of Grb2 to a receptor tyrosine kinase causes Sos to bind to and activate Ras. Ras then escorts Raf-1 to the cell membrane, resulting in Raf-1 activation [82]. Raf-1 phosphorylates MEK1 on two serine residues, Ser²¹⁸ and Ser²²² [83]. MEK1 phosphorylates tyrosine and threonine residues in the ERK activation loop. Induction of the Ras/Raf1/MEK/ERK1/2 pathway has emerged to be a key pathway in the transcriptional activation of the cyclin D1 promoter, cyclin D1 activity and protein expression [20, 84, 85].

It has been suggested that p21Ras can act as a point of convergence for mitogenic signals induced by different receptor-operated mechanisms [20, 86]. Activation of p21Ras results not only in its binding to Raf-1 but also phosphoinositide 3-kinase (PI-3-kinase) (the latter effect is described in the next section) (Figure 2). Notably, the mechanistic difference between the pro-proliferative effects of G_i and G_q coupled receptors may be explained by the differential involvement of the p42/44 MAPK cascade. Thus, G_i, *but not* G_q, activates p21Ras in ASM cells [87]. For example, Ras/Raf/MEK/p42/p44MAPK signaling is involved in the mitogenic effects of the G_i-protein coupled receptor agonists thromboxane A₂, thrombin and lysophosphatidic acid [88–91]. G_i mediates p42/p44 MAPK activation via its $\beta\gamma$ -receptor subunits, which have been shown to increase p21Ras activation through an augmented tyrosine phosphorylation of Shc leading to an increased functional association between Shc, Grb2 and SOS [92, 93] (Figure 2). Along with p42/44 MAPK, p38 MAPK has emerged as a regulator of ASM cell proliferation [94, 95]. However, the involvement of p38MAPK appears to be stimulus dependent, as it is not involved in TGF β 1-induced proliferation of human ASM cells [96].

Ras dependent PI3-kinase pathways—Activation of RTKs results in the intracellular phosphorylation of receptor tyrosine residues (receptor autophosphorylation), which serve as docking sites for other kinases, including Src and phosphatidylinositol 3 kinase (PI-3-kinase), and mediates p21Ras activation through the guanine nucleotide exchange factor SOS [97]. PI-3-kinase has emerged as a key signaling molecule of proliferation and cellular hypertrophy of ASM [98–100] (Figure 2). Three distinct classes of PI-3-kinase, specifically IA, II and III, have been identified in ASM, of which class IA is primarily involved in cell proliferation, being required for both RTK and GPCR mitogen effects [101]. PI-3-kinase regulates cell function by phosphorylating phosphoinositides (PIP) at the 3-position of the inositol ring. This results in PI3P, PIP₂ and PIP₃ formation, of which the latter appears to be the most important of these second messengers [102, 103]. Subsequent recruitment of phosphoinositide dependent kinase1 (PDK1) to the cell membrane results in Akt1 activation, which acts as an inhibitor of the constitutively active glycogen synthase kinase 3 (GSK-3) and an activator mTOR and p70 S6 kinase [99, 104–106]. These activities are important for transcriptional activation and protein translation leading to ASM cell proliferation and hypertrophy [106, 107].

A portion of the synergizing effects of GPCRs on growth factor-induced proliferation can be explained by augmented PI3-kinase activity. Together with a peptide growth factor the $\beta\gamma$ -subunit derived from a G_q-coupled receptor can synergistically stimulate PI3-kinase, Akt and p70 S6kinase [48, 51, 52, 107], resulting in increased proliferation.

RTK-induced PI3 kinase activity also results in phosphorylation of the non-RTK Src; activation is required for ASM cell proliferation [108], and therefore represents an important pathway by which PI-kinase modulates mitogenesis. Another route through which PI3-kinase affects ASM cell cycle progression is through Rho family GTPases [109]. Indeed, PI3-kinase-dependent activation of Rac1 and Cdc42, but not RhoA, and subsequent induction of cyclin D1 promoter activity has been demonstrated in ASM; importantly, this effect appeared to be independent of ERK1/2, suggesting parallel pathways in the induction of cyclin D1 [110, 111].

Protein Kinase C—In addition to potential synergistic activation of p42/44 MAPK and/or PI3-kinase pathways, as described above, signaling that involves protein kinase C (PKC) can be important in the synergistic effects of GPCRs on RTK-mediated ASM proliferation [50, 107, 112] (Figure 2). It has been postulated that the synergistic effects of PKC activation are mediated through inhibition of GSK-3 β . In its unphosphorylated form, GSK-3 β is constitutively active and negatively regulates several pro-mitogenic transcription factors and cell cycle regulatory proteins in quiescent cells [113]. Thus far, the involvement of this pathway has been elucidated for muscarinic receptor-mediated synergism only [107]; however, PKC-dependency has also been demonstrated for other G_q-coupled receptor agonists, including bradykinin and endothelin [50, 112]. This indicates that PKC activity, and likely subsequent GSK-3 β inhibition, could represent a general pathway in GPCR-mediated synergism of RTK-induced ASM proliferation.

Reactive Oxygen Species—In parallel with the activation of MAPKs and PI-3-kinase, RTKs can activate a signaling cascade involving the small G protein Rac1, which constitutes

part of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex that produces reactive oxygen species (such as H_2O_2 and O_2^-). Induction of this pathway is linked to cyclin D1 promoter activity and ASM cell proliferation, likely via the involvement of NF- κ B [111, 114, 115]. In addition, a role for Janus kinase 2 (JAK2) and signal transducer and activator of transcription-3 (STAT3) in response to reactive oxygen species that are generated by PDGF stimulation appears to be an important regulatory pathway in the expression of c-myc and cyclin D1, and subsequent DNA-synthesis [116]. In line with these findings, inhibition of p22-and p67phox, subunits of NADPH oxidase, prevents mitogen-induced cyclin D1 promoter activity [109] and DNA synthesis [114] in ASM. Moreover, a role for the nonphagocyte NADPH oxidase catalytic homolog Nox4 in the regulation of TGF β 1-induced mitosis is evident, as silencing of this molecule prevents TGF β 1-induced phosphorylation of Rb and 4E-BP-1 that is essential for ASM cell proliferation [117]. Collectively, these findings implicate an important role for reactive oxygen species in the promotion of growth factor-induced ASM cell proliferation.

Rho - Rho kinase signaling—In airway smooth muscle, the Rho-Rho kinase signaling pathway has emerged as an important regulator of many cellular functions [109]. In ASM cell proliferation its involvement is somewhat controversial, with some studies suggesting a rather limited role for the pathway in PDGF and EGF-induced proliferation [89, 118]. In contrast, other studies with human ASM cells suggest a key role for RhoA and Rho kinase, as prevention of RhoA activation and/or pharmacological inhibition of Rho kinase prevent proliferation induced by fetal bovine serum (FBS) [119]. Furthermore, the proliferative response of human ASM cells to the G-protein coupled receptor agonist lysophosphatidic acid (LPA) alone and its strong synergism with EGF can be markedly diminished by Rho inhibition [89]. Parallel effects of Rho kinase inhibition on LPA, LPA/EGF and FBS-induced proliferation likely relates to the fact that LPA is a major component of FBS. The difference in Rho/Rho kinase dependency between FBS and individual RTK mitogens may also be explained by the observation that PDGF induced proliferation relies more on Rac and Cdc42 mediated pathways [110], whereas FBS-induced proliferation of human ASM cells appears independent of Rac and Cdc42 mediated signaling [119]. Thus, Rho/Rho kinase signaling may regulate proliferation of ASM cells; however the level of activation and relative contribution of this pathway is stimulus-dependent.

Integrin-mediated signaling in airway smooth muscle cell proliferation—

Integrins mediate signals in response to ECM protein stimulation through (auto)phosphorylation of a number of signaling molecules, including the nonreceptor cytoplasmic tyrosine kinases, focal adhesion kinase (FAK) and c-Src. These kinases subsequently activate other effector proteins, like PI3-kinase, p38 MAPK and ERK 1/2, which are, as described previously, associated with growth factor-induced proliferation [120, 121]. However, the exact mechanisms by which ECM proteins modulate ASM cell proliferation distinct from growth factor-induced signaling are still elusive and might very well be species and stimulus dependent. For instance, in human lung carcinoma cells fibronectin has been shown to affect proliferation by reducing expression of the cell cycle inhibitory protein p21^{Cip1} in an ERK 1/2- and Rho-kinase-dependent fashion [122]. In

contrast, in bovine ASM cells proliferation induced by PDGF has been shown to be dependent on ERK 1/2, p38 MAPK and PI3-kinase, but not Rho-kinase [118].

Regulation of Airway Smooth Muscle Hypertrophy

In asthma, excessive accumulation of contractile smooth muscle in central and small airways is associated not only with myocyte hyperplasia, but also smooth muscle cell hypertrophy [28–30]. With respect to myocyte hypertrophy it is clear that the process requires co-ordinated and selective protein synthesis that supports accumulation of contractile proteins. Therefore it is important to understand the signaling pathways that regulate hypertrophic ASM cell growth.

In cell culture, the levels of contractile protein markers vary depending upon cell confluence and the exogenous stimuli provided by the media. Plating ASM cells at low density in the presence of FBS represses expression of contractile proteins such as α -actin, myosin light chain kinase (MLCK), and smooth muscle myosin heavy chain (smMHC) [123–126]. Conversely, long term serum deprivation of confluent myocyte cultures promotes accumulation of contractile proteins and induces the formation of large contractile myocytes [98, 127, 128]. Interestingly the transcriptional activity of contractile protein genes actually peaks whilst myocytes are undergoing proliferation and nearing confluence; this becomes dramatically reduced at confluence when mRNA levels of contractile markers such as SM22 and smMHC reach a maximum and is sustained thereafter during prolonged serum deprivation [98, 127]. During this period of reduced transcription, contractile proteins do, however, accumulate greatly as cells acquire an enlarged, contractile phenotype morphology. Collectively this suggests that accumulation of smooth muscle proteins associated with myocyte enlargement is regulated by critical post-transcriptional mechanisms. Post transcriptional regulation of ASM contractile protein expression is consistent with studies of hypertrophy in other systems including cardiac and skeletal muscles, and vascular smooth muscle [129–135].

The study of smooth muscle cell hypertrophy led to the development of novel cell lines and interventions that enable repression of cell cycle transit and promote myocyte growth. As described in a previous section, the proliferation of eukaryotic cells is tightly regulated through a balance of positive and negative regulatory proteins that exert their effects during the first gap phase (G1) of the cell cycle [23, 136]. Transit through the cell cycle requires accumulation of G1 cyclins that leads to activation of CDKs and phosphorylation of downstream targets that ultimately allows entry into the S phase. The activity of G1 cyclin kinases is modulated by several key proteins, including p21^{CIP1}, p16^{INK4}, and p27^{Kip1} [137–140]. Based on this paradigm, adenovirus mediated over expression of cell cycle inhibitors p27^{Kip1} and p21^{Cip1} has been used as an experimental means of inducing cellular hypertrophy [141]. For cultured human ASM cells, transformation using temperature sensitive simian virus 40 large tumor antigen to induce p21^{Cip1/Waf1} p53^{Kip2} expression has been shown to invoke cell cycle arrest in mid-G₁ with concomitant accumulation of contractile proteins and an increase in cell size [28, 142]. With cell cycle blockade, serum induced cell division is prevented however hypertrophic growth appears to continue as contractile protein abundance increases (without affecting mRNA levels). [143] These

observations further support the concept that hypertrophic protein accumulation in ASM is regulated in a post-transcriptional manner, likely being under control of effectors that modulate protein translation. This paradigm is consistent with Woodruff and colleagues [30] who reported increased sm- α -actin protein (without any change in mRNA) in airway biopsies from mild asthmatics.

Factors Affecting Airway Smooth Muscle Hypertrophy

Cellular hypertrophy is largely mediated by signaling through peptide growth factors: insulin-like growth factor (IGF)-1 and growth hormone (GH), the latter acting predominantly via increased production of IGF-1 [144]. Although levels of interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF) are increased in the bronchoalveolar lavage fluid derived from asthmatics [145], whether these cytokines stimulate ASM growth in vitro remains controversial. IL-1 β and IL-6 stimulate hyperplasia and hypertrophy of cultured guinea pig ASM cells [61]. Endothelin-1, which is secreted by the epithelium and is elevated in lung lavage fluid from asthmatics [146–148], is also a potent inducer of hypertrophy human ASM cells that is marked by accumulation of contractile phenotype marker proteins such as smMHC, calponin, and α -SMA [149]. When IGF-1, insulin, and other growth factors bind to their membrane tyrosine kinase receptors, a 110-kDa lipid kinase, phosphatidylinositol-3 kinase class IA (also referred to as p110 α) is activated [150]. Accumulated data suggest that PI-3-kinase signaling is a critical underpinning for hypertrophy. Gene knockout of p110 α is lethal at E9.5–E10.5 in mice (showing a severe proliferative defect) [151]. Indeed, a central role of the p110 α pathway in IGF-1 - induced growth and hypertrophy has been demonstrated in different cell systems [98, 150, 152–155]. The details of this signaling cascade are provided in a subsequent section and is outlined in Figure 3.

The extracellular matrix appears to affect the full functional repertoire of smooth muscle cells. Asthmatic airways smooth muscle cells in culture produce increased amounts and an altered composition of extracellular matrix proteins [75, 156]. Airway remodeling is characterized by the deposition of extracellular matrix (ECM) proteins in the airways [71, 157]. ECM proteins (collagen I, III, and V; fibronectin; tenascin; hyaluronan; versican; and laminin 2/ β 2) are increased in profusion in asthmatic airways [69, 71, 158, 159]. Seeding ASM onto fibronectin or collagen type-1 promotes a proliferative phenotype, whereas laminin-rich matrices promote retention or maturation of a contractile phenotype [27, 160, 161]. Moreover, endogenously expressed laminin-2, which is required for myocyte maturation and hypertrophy [161], is increased in the asthmatic airway. Notably, the ability of laminin-2 to promote maturation and support hypertrophy of human airways smooth muscle cells is mediated selectively via a α 7 β 1 integrin heterodimer [160]. Thus an intrinsic autocrine mechanism appears to exist wherein myocytes can express both an ECM element (laminin-2) and requisite receptor (α 7 β 1) to support accumulation of contractile proteins and hypertrophic growth. Given the association of laminin accumulation with airway remodeling, the expression of this glycoprotein and its receptors may be a central intrinsic mechanism regulating ASM hypertrophy in the adult airway.

Signal Transduction Pathways That Regulate ASM Hypertrophy

Pathways discussed in detail below are shown schematically in Figure 3.

PI-3-kinase—Insulin or IGF-I have been proposed to regulate developmental and physiological growth of the cells. Ligand binding to the IGF-I receptor activates PI-3-kinase of the Ia IA subgroup; p110 α , which phosphorylates the membrane phospholipid phosphatidylinositol 4,5 biphosphate at the 3' position of the inositol ring [150]. Through this mechanism PI 3-kinases thus recruit effector proteins containing PI(3,4,5)P₃-binding pleckstrin homology (PH) domains to the plasma membrane [162]. These include Akt (also called PKB), a 57-kD serine/threonine kinase encoded by three genes, and 3-phosphoinositide-dependent protein kinase-1 (PDK1) [163]. This enforced co-localization of Akt and PDK1 causes the latter to phosphorylate the former at its Thr308 residue, a necessary step in for Akt activation [164]. Full Akt1 activation requires membrane localization and phosphorylation at its Thr308 and Ser473 residues. Phosphorylation of Ser473 on Akt is proposed to be mediated by PDK-2, which appears to be identical to the so-called mTOR complex 2 appears (described below) [165, 166].

Downstream targets of PI(3) kinase and Akt that are associated with promoting protein synthesis and accumulation include glycogen synthase kinase-3 β (GSK-3 β), p70S6 kinase (p70S6K), and PHAS-1/4E-BP [150, 167] (Figure 3). Akt1 phosphorylates and inhibits GSK-3 β resulting in downstream de-inhibition of the translation initiator eIF2 [105, 168, 169]. Akt1 can also phosphorylate, and in part activate, the rapamycin-sensitive threonine/serine kinase, mammalian target of rapamycin (mTOR), a 290-kD protein similar in structure to phosphoinositide kinases, that can be effectively inhibited by the immunosuppressor compound rapamycin when the latter is bound to intracellular FK506-binding protein. Of relevance to a role in cellular hypertrophy, mTOR has downstream targets that include the mitogen- and amino acid-sensitive serine/threonine kinase, p70S6K, and the translation repressor PHAS-1/4E-BP1 [170–172]. mTOR mediated phosphorylation of PHAS-1/4E-BP1 releases the latter from binding to the protein translation mediator, eukaryotic initiation factor 4E (eIF4E), thereby increasing the availability of eIF4E to form an active complex with eIF4F and promote translation of specific sets of mRNA transcripts [170].

Activation of p70S6K activation regulates efficiency of protein translation by phosphorylating of the 40S ribosomal protein S6 [170, 173], and is required for PI-3-kinase mediated differentiation and hypertrophy of skeletal myotubes [150, 152], angiotensin II-induced vascular smooth muscle hypertrophy [174], and autocrine loop mediated ASM cell maturation and hypertrophy [98, 128]. Phosphorylation of ribosomal S6 protein increases translation of mRNAs with 5' TOP tracts, many of which are involved in mRNA-translation-like elongation factors and ribosomal proteins. Though the principal site required for mTOR dependent activation of p70S6K is Thr389, which resides in a region between catalytic and auto-inhibitory domains [175], full activation of p70S6K is achieved through hierarchical phosphorylation of seven Ser/Thr sites targeted by mTOR, PDK1, and other PI(3) kinase-dependent kinases [176]. As phosphorylation of p70S6K is sensitive to inhibition by both rapamycin and chemical inhibitors of PI-3-kinase, researchers often place PI 3-kinase, mTOR, and S6 kinase into a linear signaling pathway. Such a linear scheme is too simplistic, however, as a rapamycin-resistant mutant of S6 kinase is still sensitive to inhibition by the PI-3-kinase inhibitor wortmanin [177], indicating that mTOR and PI-3-kinase signals to p70S6K can be dissociated. Indeed, S6 kinase may also be phosphorylated

by PDK-1 [178, 179], thus providing a mechanism for mTOR-independent, PI-3-kinase-dependent activation. Similarly, mTOR-independent mechanisms of 4E-BP1 phosphorylation may exist. Recent evidence shows that class IA PI-3-kinases may function as 4E-BP1 kinases [180], and ERK can reportedly also phosphorylate 4E-BP1 [181].

Recent studies have elucidated the role of two signaling molecules that link Akt and mTOR in the regulation of cell size. PI-3-kinase may positively regulate cell size via the successive: activation of Akt, inactivation of TSC2, activation of Rheb, and activation of mTOR [182–185]. It is now known that mTOR exists in two distinct multi-protein complexes, one rapamycin-sensitive (mTOR complex 1) and one rapamycin-insensitive (mTOR complex 2) [186]. mTOR complex 1 includes mTOR and Raptor; mTOR complex 2 is comprised of mTOR-Rictor and mammalian stress-activated protein kinase interacting protein. Furthermore, as noted above, mTOR complex 2 appears to be identical to the proposed Akt kinase, PDK-2, which phosphorylates serine 473 on Akt [165, 166]. Thus, Akt acts as both an upstream activator of mTOR complex 1, and is a target for activation by mTOR via mTOR complex 2 to permit high-level PIK/Akt signaling [187].

GSK-3 β —GSK-3 β is a constitutively active serine/threonine kinase that phosphorylates multiple substrates including eIF2B ϵ , cyclin D1, and p21 [168, 188–190]. Phosphorylation by Akt inactivates GSK-3 β , leading to dephosphorylation and the activation of eIF2B, as well as a general enhancement of ribosomal 43S pre-initiation complex formation [168]. GSK-3 β also negatively regulates transcription factors involved in muscle-specific gene expression, including nuclear factors of activated T cells (NFAT), GATA4, and β -catenin [191–195] suggesting a critical role in ASM growth. The phosphorylation of GSK-3 β by Akt indicates that PI-3-kinase may regulate mRNA translation via three distinct mechanisms (see Figure 3): (i) regulation of cap-dependent mRNAs via activation of the Akt/TSC2/Rheb/mTOR/4E-BP1 pathway, (ii) regulation of 5' TOP tract-containing mRNAs via activation of p70S6K (through either mTOR or PDK-1), and (c) a general enhancement of translation initiation via activation of the Akt/GSK-3 β /eIF2B pathway. Recent studies also implicate regulation of GSK-3 β as a key downstream mechanism for the effects of integrin-mediated effects of extracellular matrix proteins on cell growth; this involving the signaling intermediate, integrin linked kinase (ILK) [196–198].

Despite human studies indicating the presence of ASM hypertrophy and increased contractile protein expression in asthma, little information is available concerning the signaling intermediates and translation initiation factors involved. In confluent serum-deprived canine tracheal myocyte cultures, PI-3-kinase and p70S6K activities are increased five and two days after serum deprivation, respectively, and immunohistochemical studies show selective phosphorylation of Akt and p70S6K in elongated cells expressing smMHC five to seven days after serum deprivation [98]. LY294002 and rapamycin blocked S6 kinase phosphorylation and phenotypic change, implying that PI-3-kinase, mTOR, and p70S6K are responsible for contractile protein accumulation and myocyte hypertrophy. Recently it has been shown by Deng et al [199] that inhibition of GSK-3 β (which activates eIF2B) contributes to ASM hypertrophy *in vitro* and *in vivo*. More strongly in a mouse model of allergic asthma it has been shown that phosphorylation and inactivation of GSK-3 β is

associated with ASM hypertrophy [200] while p70S6K alone is responsible for the myocyte enlargement, without changing the contractile protein expression *in vitro* [201].

Rho GTPases—Rho kinase signaling plays an important role in regulation of smooth muscle gene transcription, which promotes serum response factor (SRF) nuclear localization and increased cytoplasmic actin filaments [202–204]. The ability of the Rho/Rho kinase pathway to promote actin polymerization leads to a loss of globular actin (G-actin), which results in the release of the SRF co-activator MAL, a G-actin binding protein [205]. Thus, SRF, a central regulator of smooth muscle-restricted gene transcription, is under tight control by the Rho/Rho kinase pathway [8, 206]. Rho/Rho kinase activation is regulated by receptor tyrosine kinases and GPCRs through the action of Rho-specific guanine exchange factors (RhoGEFs). Ligand binding to muscarinic M₃ receptors coupled to Gαq can induce RhoA activation, likely via p63RhoGEF, and promotes Rho kinase dependent actin polymerization leading to SRF translocation and the induction of smooth muscle specific gene expression. Insulin induced expression of contractile phenotype markers and the induction of a functionally hypercontractile phenotype also requires the Rho/Rho kinase pathway, though the GEFs involved have not yet been identified [41, 207]. It indicates that Rho/Rho kinase signaling plays an important role in the transcription of genes that encode mRNA required for synthesis and accumulation of contractile proteins in hypertrophic ASM.

Protein Kinase C—Protein kinase C (PKC) is a superfamily that includes three classes of isoenzymes. So-called, conventional isoforms (α, β1, β2 and γ) are activated by calcium, phorbol esters and phosphatidylserine; novel isoforms (δ, ε, ι, θ and μ) are calcium-insensitive and activated by phorbol esters and phosphatidylserine; and, atypical isoforms (ζ, τ/λ) are calcium and phorbol ester-insensitive and activated by phosphatidylserine. PKC α, β1, β2, δ, and ζ, but not γ or ι, are expressed in bovine tracheal myocytes [208], whereas PKC α, β1, β2, δ, ε, θ, ι, ζ, τ and μ have each been identified in human tracheal myocytes [209]. There are several studies suggesting the role of PKC's in ASM proliferation [210–214]. Their role in hypertrophy is not entirely clear but data from other systems indicate that over expression of these select PKC isoforms can induce cardiac hypertrophy in transgenic mice [215]. Moreover, activation of PKC isoenzymes via GPCRs has been linked to GSK-3β phosphorylation, suggesting this class of enzymes could play a permissive role in protein translation via eIF2B [107]. On this basis, future focus on the role of PKCs in airway myocyte hypertrophy appears to be warranted.

Regulation of Airway Smooth Muscle Apoptosis

Tissue development and homeostasis is subject to rounds of cell division and differentiation, but of equal importance is the duration of cell survival and the capacity to orchestrate self-termination to cull infected, damaged, and unwanted cells. Such programmed cell death, dubbed apoptosis, follows specific patterns and includes shrinkage of the cell, margination of chromatin, and nuclear fragmentation [216–218]. Apoptosis occurs in response to environmental or developmental signals, cellular stresses and specific cell death signals. This self-inflicted death involves a number of evolutionarily conserved biochemical pathways that have been intensively studied for over two decades [219].

In mammals, programmed cell death can be initiated by two major pathways: (i) the extrinsic pathway, which can be triggered by ligation of death receptors and subsequent caspase 8 activation; and, (ii) the intrinsic pathway, which is initiated by cellular stress followed by activation of caspase 9 (Figure 4). Each of these pathways converges to a common execution phase that requires the activation of caspases-3 or -7 from their inactive zymogen form to their processed, active form [218, 220–222]. The apical activators (caspase-8 and -9) have a primary specificity for cleavage at Asp297 located in a region that delineates the large and small subunits of active caspases-3 and -7 [218]. Apoptotic cell death is centrally controlled by both caspase activation cascades and/or mitochondrial membrane permeabilization (MMP), processes that are inextricably linked [223–225]. Indeed, MMP itself stimulates caspase activation through the release of several caspase-activating proteins, in particular cytochrome *c* [222, 224], and caspase activation of proteins such as truncated Bid, Bad, Bcl-X_L triggers MMP [226–228]. MMP manifests at the level of the outer membrane, which allows for the release of cytochrome *c*, as well as at the level of the inner membrane as a loss of the mitochondrial transmembrane potential (ψ_m) [223, 224, 229].

Airway Smooth Muscle Apoptosis and Asthma

Asthma, particularly if severe and/or of long duration, is accompanied by increased ASM mass due both to myocyte hyperplasia and hypertrophy [28–30, 230]. The possible mechanisms of myocyte proliferation to ASM remodeling have been discussed above, but it needs to be pointed out that there is not a compelling accumulation of data from animal models and human specimens that confirm a place for proliferation as the primary underpinning of remodeling. Indeed more recent work suggest that apoptosis may be of equal importance to proliferation in determining the extent of airway remodeling in animal models of asthma [231–233]. In another study using rats, reduced ASM apoptosis was shown to contribute to the airway remodeling process [234]. Furthermore, dexamethasone was shown to induce myocyte apoptosis possibly by increasing pro-apoptotic Bax expression and the decreasing anti-apoptotic Bcl-2 expression [234]. Using a rat model for emphysema, it has also been confirmed that Fas and FasL participate in apoptosis of myocytes in small airways [235]. Interestingly, injection of the Chinese herbal remedy, shenmai, modulated Fas and FasL protein expression and reduced ASM cell apoptosis, likely associated with inhibitory effects on TNF and inflammation [235].

It has been shown that Fas (CD95 – the receptor for FasL) is expressed by ASM tissue in vivo and on the surface of cultured human airway myocytes in vitro [236]. Moreover, cross-linking of surface Fas induces apoptosis in a significant number of cultured myocytes, an effect that is *a*) potentiated by stimulation with TNF- α , which upregulates surface Fas expression, and *b*) reduced by prolonged serum deprivation, which, in the absence of TNF- α treatment, reduces surface Fas expression. This effect could be very important considering that even a small sustained level of apoptosis might have a significant impact on smooth muscle accumulation within intact asthmatic airways because the proliferative index of ASM appears is low even in the presence of substantial airway inflammation [237].

ECM protein alterations are a characteristic feature of asthmatic airway remodeling [238, 239]. These changes include modification such as collagen I, III, and V

increase, changes in glycoproteins (fibronectin and tenascin), and alterations in deposition of various proteoglycans (PG) (versican, biglycan, and decorin) [238–243]. It has been reported that culturing cells on different ECM matrices can variably affect ASM number, with laminin in particular imparting a pro-survival response [239, 244]. Culture on decorin resulted in a persistent decrease in cell number via its effects on both proliferation and apoptosis [239], therefore the anti-proliferative and/or proapoptotic effect of decorin could serve to limit the growth of ASM beyond its usual compartment.

The endothelins (ETs) are a family of three isopeptides, acting through two G protein-coupled receptors, ET_A and ET_B. ET-1 in particular elevates smooth muscle tone [245] and causes a marked potentiation of cholinergic nerve-evoked contraction of ASM [246]. ET-1 expression is increased in asthma and is primarily released from the bronchial epithelium [146–148]. Bronchial smooth muscle cells highly express the ET_B receptor which represents about 82–88% of the total ET receptor population [247]. ET-1 is a potent inducer of hypertrophy human ASM cells and at the same time increases the contractile potential of these cells by increasing expression of sm-MHC, calponin, and α -SMA [149]. ET-1-induced-ASM survival has been causally linked with apoptosis inhibition [149], and is a concomitant mechanism leading to increased size and synthetic activity of these cells in primary cell culture [149].

Cigarette smoke has long been considered as a major causative factor for chronic obstructive pulmonary disease (COPD) [248, 249]. A number of mechanisms have been suggested for the pathogenesis of COPD, including disproportionate activities of proteases and antiproteases [250], influx of inflammatory cells into the lung, and oxidative stress [251]. In addition to these mechanisms, gathering evidence suggests that apoptosis may play a significant role in clinical and experimental COPD pathogenesis [249, 252, 253]. It has been reported that cigarette smoke extract (CSE) could induce oxidative stress and apoptosis in ASM cells through activation of both the mitochondrial pathway and death receptor pathway [249]. Neutrophilia is a common feature of smoking induced inflammation and of severe asthma and these cells are a rich source of elastases in the human lung [254]. The degradation of ECM by neutrophil elastases is believed to contribute to decreased airway stability [255]. Neutrophils can also induce apoptosis in ASM, for example, detachment-induced apoptosis (defined as anoikis) [218] with characteristic caspase-3 cleavage [256]. Neutrophil-induced myocyte apoptosis appears to result from the proteolytic activity of proteins released by neutrophils as concomitant fibronectin degradation occurs, and the serine protease inhibitor, α 1-antitrypsin, has a protective effect [256].

Most recently it has been reported that simvastatin, an inhibitor of HMG-CoA reductase which is the proximal rate-limiting enzyme in cholesterol biosynthesis [257], can induce apoptosis in primary cultured human airways smooth muscle cells [222]. This effect involves a novel p53-dependent pathway with selective release of mitochondrial protein, Smac and Omi, which inactivate so-called inhibitor of apoptosis protein (eg. XIAP), allowing for cytochrome c independent activation of caspase-9. The pro-apoptosis effects of simvastatin is mainly initiated by depletion of the intracellular pool of cholesterol intermediates called isoprenoids [farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)], which are essential for membrane anchoring and activation of small Rho GTPase

proteins [222]. This finding suggests there may be means for development of future new asthma therapy to target ASM hyperplasia in asthma.

Airway Smooth Muscle Cell Migration

Airway smooth muscle elongation and smooth muscle differentiation in lung development

Thickening of the airway smooth muscle layer in diseased airways is thought to involve migration of airway smooth muscle cells that recapitulates events of embryonic development. During embryogenesis formation of smooth-muscle containing hollow organs is thought to include migration and reorientation of smooth muscle cells. Cell migration is a common process in formation of blood vessels, the airways and the gastrointestinal system. During lung development migration and differentiation of ASM precursor cells is orchestrated by autocrine and paracrine factors as well as cell-matrix interactions that promote maturation of the airway wall [258, 259]. The molecular and cellular remodeling that occurs during smooth muscle migration may contribute to lung development by mediating elongation of mesenchymal progenitor cells. In the developing airways mesenchymal progenitors differentiate into elongated cells that express smooth muscle-restricted contractile proteins. Elongation is required for differentiation and is associated with activation of the p38 MAP kinase pathway [260], which is known to mediate cell migration [261]. Cell elongation and expression of differentiation marker proteins during development appears to be a mechanical signaling phenomenon because soluble signals that drive smooth muscle differentiation in culture (TGF β 1 and retinoic acid) had no effect on upregulation of smooth muscle marker protein expression [260]. The role of smooth muscle migration in airway development appears to be to more of an effect on cell elongation and orientation rather than a long range chemotactic migration of progenitor cells that occurs in the developing vasculature. In the mouse, the shape change of smooth muscle progenitor cells and ultimately smooth muscle cell differentiation depends critically on expression of laminin 1 and laminin 2 [260, 262]. Both laminins 1 and 2 can ligate integrin α 7, and integrin α 7 is a protein known to promote vascular and ASM differentiation [263–265]. Disrupting critical changes in cell shape by knocking down laminin 1 and laminin 2 expression results in bronchial smooth muscle hypoplasia [260, 262]. Schuger and colleagues have suggested that mechanical forces in the developing lung that are transmitted through integrin-laminin interactions are critical for upregulation of serum-response factor expression and expression of smooth muscle-restricted genes in differentiated ASM [258]. The hypothesized mechanical signals are integrated with epithelium-derived soluble signals including FGF10, BMP4 and components of the Wnt/catenin and hedgehog signaling families. The combined effect of mechanical signals and biochemical signaling is to drive mesenchymal precursor cells to an elongated, differentiated smooth muscle phenotype. Because cellular processes underlying tube formation are highly conserved from *Drosophila* to humans [266], it seems reasonable to infer an important role of smooth muscle migration in airway development. However, there are no definitive lineage marker studies of the source of new ASM cells in vivo during lung development. The key question is what percentage of new muscle originates from existing smooth muscle vs. progenitor cells migrating from the surrounding mesenchyme that deposit in the airway wall?

Smooth muscle cell migration and airways remodeling

As discussed above, hyperplasia can result from increased proliferation and diminished apoptosis. In addition, increased cell number could be a result of migration of new cells into the airway wall. There is evidence for two sources of migrating cells in the airways, the lung parenchyma and the blood. Evidence for parenchymal cells as a source of new smooth muscle comes from a structural study of lung biopsies from asthmatics in which lung myofibroblasts were found to migrate in response to allergen challenge [15]. An important question is whether the myofibroblasts are resident cells or are derived from circulating fibrocytes, which are CD34+, collagen I+, α smooth muscle actin+ progenitor cells. These cells are thought to differentiate to myofibroblasts, to contribute to subepithelial fibrosis and possibly to become contractile cells in ASM bundles [35, 267, 268]. A central question for studies of ASM cell migration is whether tube formation during development, wall thickening and epithelial-mesenchymal transformation all require smooth muscle cell migration. An argument can be made for cell migration during tube formation based on analogous events in vascular development. Some interesting questions that need to be tested critically are whether differentiated smooth muscle cells originating in the muscularis migrate in response to cues such as inflammation or lung injury, and does this recapitulate events that occurred during development [269]. Recent evidence for cell migration in remodeling of asthmatic airways is more consistent with immigration of blood-borne fibrocytes [35, 267]. Fibrocytes are present in increased numbers in the lamina propria in patients with asthma [267], the number of fibrocytes in the muscularis increases after allergen challenge [15], and migration of fibrocytes is enhanced by coculture with differentiated ASM cells. While the recent data are quite provocative it remains to be proven by lineage marking approaches that the migrating cells contribute to subepithelial fibrosis, differentiate to contractile airway smooth muscle cells or both. Another important question is whether some fibrocytes remain in the muscle layer as a population of progenitor cells that can be activated to proliferate then differentiate to smooth muscle cells. These are important questions because increased ASM mass and myofibroblast numbers are thought to be major determinants of fixed airway obstruction that is unresponsive to corticosteroid and bronchodilator therapy [28]. Another unresolved issue is what initiates influx of fibrocytes and differentiation of myofibroblasts to smooth muscle cells. As described below many growth factors (eg. PDGF) and proinflammatory signaling proteins (eg. interleukins) stimulate ASM cell migration. Many recent studies of ASM cell migration have focused on the molecular mechanisms that transduce pro-growth and pro-inflammatory signals to cell motion. The following sections summarize conserved features of migration of motile cells, the known promigratory and antimigratory signals affecting ASM migration and some of the key signal transduction pathways that underlie cell migration in ASM and other cell types.

Cellular processes and molecular structures necessary for migration

Cell migration begins with stimulation of receptors that trigger cytoskeletal remodeling and repositioning of organelles as illustrated in Figure 5. There are many receptor systems that sense promigratory stimuli, but we will limit the discussion of these events to the three major classes of receptors involved in cell migration: G protein coupled receptors (GPCR), receptor tyrosine kinases (RTK) and matrix adhesive proteins (integrins). One of the earliest events following receptor ligation and signal transduction is polymerization of actin at the

leading edge of a motile cell. This is a fundamentally important process that extends the edge of the cell in the direction of the stimulus during chemotaxis (Figure 5A). For the leading edge of the cell to stick to the substrate and affect forward motion focal contacts must assemble just behind the leading edge (Figure 5B). Myosin II motors bind actin filaments in the body of the cell to generate traction force that moves the cell forward. Myosin I motors at the leading edge are thought to control cortical stiffness and membrane tension. Simultaneously the actin and microtubule cytoskeletal systems remodel and focal contacts at the rear of the cell detach to allow the body of the cell to follow the leading edge towards the stimulus. The nucleus, mitochondria, golgi and endoplasmic reticulum are tethered by adaptor proteins and motors to the cytoskeleton. One role of myosin II motors is to move cellular organelles along with the remodeling cytoskeleton. Depending on the experimental approach cells in vitro will move about randomly in the absence of a chemical gradient (chemokinesis), or move directionally as they follow concentration gradients of soluble attractants (chemotaxis). They may also follow paths of varying matrix adhesiveness and concentrations of bound chemical attractants (haptotaxis), which is a major mechanism of organ formation during embryonic development. A common goal of cell migration studies is to establish the sufficiency and necessity of particular chemicals and signal transduction pathways in migration. Another common goal is to define the cellular machinery necessary for cell movement, and to determine if the function of the machinery is compromised in disease. Studies over the past ten years in ASM muscle have illustrated several important characteristics of migration relevant to airway development and airway remodeling in asthma. The remainder of the chapter summarizes extrinsic molecules that modify ASM migration and the signaling pathways involved in controlling migration. For a more general overview of cell migration and protocols for assaying wound healing and chemotactic migration there are several elegant reviews published by members of the Cell Migration Consortium (www.cellmigration.org). The reader is also referred to previous reviews of smooth muscle cell migration that provide references to methods used in studies of ASM cell migration [270–272].

Conserved biochemical processes known to occur in migrating cells are illustrated in Figure 5. The figure summarizes literature from both nonmuscle and muscle cell motility studies [273, 274]. We will summarize the consensus for how migration occurs in many cell types and then highlight the known and unknown features of ASM migration. In all migrating cells actin polymerization and depolymerization is required. There are numerous actin-associated proteins that coordinate polymerization and depolymerization with some of the best defined proteins being illustrated in the inset Figure 5A. Some of the earliest events in chemotactic cell migration are receptor activation, changes in cell Ca^{2+} signaling, production of phosphatidyl inositol bis phosphate (PIP₂), and activation of monomeric and trimeric G proteins (Figure 6). Each of these proximal signal transduction events can activate multiple signaling cascades. It is impossible to represent all the known signaling mechanisms in a simple schematic, so Figure 6 was designed to make the point that signaling occurs at multiple levels via parallel signaling pathways converging on actin polymerization and myosin II motors, both of which are necessary for traction forces required for cell migration. We will focus on signaling events triggered by platelet-derived growth factor (PDGF) in this chapter because it plays a critical role in smooth muscle cell

migration. However, the reader should be aware that numerous promigratory stimuli have been described for ASM (Table 2), and that each stimulus acts via some of the same signaling pathways as well as stimulus-specific pathways not shown in Figure 6. With these limitations in mind we focus on PDGF family members to illustrate the principles of smooth muscle cell migration. PDGF is thoroughly studied in smooth muscle cell migration, and is thought to play a critical role in tube formation during vascular development as well as wound healing in response to injury and inflammation. The β isoform of PDGF receptor (PDGFR- β) is coupled via PI3-K and phospholipase C γ which elicits changes in myoplasmic calcium, hydrolysis of PIP2 and activation of MAP kinases [261, 275]. These signaling intermediates act together with the small G proteins (Rac and Cdc42) to initiate nucleation of F-actin. Nucleation is promoted in several ways: de novo at the minus (pointed) end, uncapping of plus (barbed) ends by dissociation of actin capping proteins, or by forming new branches (Figure 5A). Nucleation and branching are promoted by proteins of the ARP2/3 complex, profilin and the formins (mDia 1 and 2). The net effect of these proteins is to increase polymerization at the plus ends of existing actin filaments. Profilin is bound to membrane phospholipids in the absence of promigratory stimuli. In the presence of stimuli that activate phospholipases plasma membrane PIP2 levels decrease which releases sequestered profilin. Profilin then enhances adenine nucleotide exchange on G-actin and drives actin polymerization. The formins are activated by binding monomeric G proteins - mDia1 is activated by RhoA, and mDia2 is activated by Cdc42 and Rac. Small G proteins also promote filament branching by activating WASP-family verprolin-homologous protein (WAVE) complex and Wiskott-Aldrich syndrome protein (WASP) respectively. WAVE and WASP proteins activate components of the ARP2/3 complex to increase the number of nucleation sites and the number of sites for branching of filamentous actin. Increased F-actin nucleation, polymerization and branching are all necessary for formation of filopodia and the lamellipodium leading to extension of leading edge of a migrating cell (cross hatched area of the cell in Figure 5). In addition to nucleation and branching, actin filaments must be severed and depolymerized in order to produce effective migration. Actin severing is mediated by several protein including gelsolin and cofilin. Gelsolin is activated by both increased Ca²⁺ concentrations and by PIP2 (Figure 5A). The number of actin nucleation sites increases when gelsolin is released from the plus end of actin filaments. Migration depends critically on filament growth at the plus ends and filament shrinkage at the minus end. The dynamic behavior of actin filaments is greatly enhanced by cofilin, which promotes depolymerization at the minus end and severs actin filaments thus increasing nucleation sites (Figure 5A). The net effect of all the processes just described is to generate propulsive force at the leading edge of the cell extending filopodia and the lamellipodium towards the stimulus [276]. During the initial stages of lamellipodial extension focal contacts must form between the cell membrane and the extracellular matrix in order for cells to move (Figure 5B). Focal contacts are critically important adhesive structures that are dynamic in a motile cell, forming rapidly, maturing and eventually disassembling at the rear of the cell thus releasing tail of the cell from the matrix.

Focal contacts in airway smooth muscle cells and tissues

The protein composition of the focal contact “adhesome” and the function of focal contacts to sense the biochemical and physical environment surrounding a motile cell has been

reviewed recently [277, 278]. Geiger and colleagues divided the components and functions of focal contacts into a signaling module, an actin-linking module and an actin-polymerizing module (see Figures 5A and 5B). In this section we will focus on the components of focal contacts that have been described in cultured ASM cells and intact smooth muscle tissue. Several components of the actin linking module have been described including paxillin [279, 280], vinculin [281] and talin [279]. Elements of the signaling module have also been described in ASM including focal adhesion kinase (FAK) [279, 280], Src [108, 282, 283], PI3-kinase [284], Ca^{2+} and phospholipase C [285] and several MAP kinases (see below). Signaling module proteins catalyze a variety of reactions, including phospholipid metabolism, protein phosphorylation and dephosphorylation, and increased cell Ca^{2+} , all of which contribute to dynamic formation and degradation of focal contacts during migration. Phosphorylation of focal contact components including FAK, paxillin and talin has been shown in ASM tissue during contraction [280, 286] and following strain of cultured cells [279]. In migrating ASM cells FAK is phosphorylated and degraded during urokinase-stimulated migration [287]. Carlin et al. (2005) also found Src trafficked to the cell membrane during urokinase-induced migration consistent with Src being phosphorylated and activated during ASM cell migration [108, 283]. Components of the signaling module are critical for catalyzing phosphorylation and dephosphorylation events that promote both formation and turnover of the nascent focal contacts at the leading edge.

In addition to an important role for protein phosphorylation there is also a requirement for proteolysis of focal contact proteins by metalloproteinases. Turnover of mature focal contracts is due in part to proteolysis occurring at the trailing edge. In migrating ASM cells, as in many other cell types, upregulation of MMPs 1, 2 and 3 increases during migration. The necessity for MMP activity was demonstrated clearly by the fact that both tissue inhibitors of metalloproteinases (TIMPs) and chemical protease inhibitors reduced or completely blocked AMS cell migration [288, 289]. Protease inhibitors block migration in part because stable focal contacts at the rear of the cell must eventually disassemble for the cell to move forward.

There are some interesting unaddressed questions about the spatial and temporal features of proteins in nascent and mature focal contacts in ASM. It is not clear which components are most sensitive to inflammation, which are altered by mechanical strain during tidal breathing or how the focal adhesion composition and spatial distribution changes as a function of the differentiation state of ASM cells. We assume that many components of focal contacts are similar to those of migrating nonmuscle cells, and evidence to date has largely confirmed this assumption. However, identifying unique protein components of the airway smooth muscle “adhesome” and its constituent modules (signaling, actin binding and polymerization) is important for identifying novel targets for inhibiting or reversing airway remodeling.

Mechanics of cell migration

The primary sources of force generation during for cell migration are actin polymerization promoting protrusion of the leading edge and force generated by myosin motors. Myosin II motors produce traction force that is transmitted to the matrix through the focal contacts

(Figure 5B). Smooth muscle myosin II is a phosphoprotein that is activated by Ca^{2+} -calmodulin activation of myosin light chain kinase (MLCK). Activated myosin II binds to actin filaments that contain tropomyosins and caldesmon (predominantly I-caldesmon in cultured ASM cells). Phosphorylation of myosin regulatory light chains increases actomyosin ATPase activity and crossbridge cycling, thus generating traction force. In addition to the canonical Ca^{2+} -calmodulin-MLCK activation pathway there may also be an important Ca^{2+} -independent activation of myosin II mediated by RhoA activation of Rho kinases (ROCKs). Rho A and Rho kinases are well known inhibitors of myosin light chain phosphatase via phosphorylation of the myosin binding subunit of the phosphatase [290]. Activation of Rho A and ROCKs increases phosphorylation of myosin II in ASM tissues and in a variety of cultured smooth muscle cells, fibroblasts and cancer cell lines (reviewed by [291, 292]). In addition there is some evidence for direct phosphorylation of myosin regulatory light chains by ROCK in 3T3 fibroblasts [293], but there is no direct evidence yet for this reaction in ASM. Details of myosin II filament assembly and distribution during ASM migration are mostly undefined.

One interesting question that remains unexplored is how myosin motors respond to the physical nature of the matrix. This is potentially important given that there are dynamic changes in matrix composition and stiffness of the lung parenchyma during development and disease. Ingber and coworkers showed that decreasing stiffness of an artificial fibronectin matrix reduced myosin light chain phosphorylation in cultured pulmonary artery smooth muscle cells [294]. In addition, inhibition of myosin ATPase with 2,3-butanedione 2-monoxime reduced myosin light chain phosphorylation, suggesting that traction forces are necessary for proper function of the Ca^{2+} -calmodulin-MLCK signaling pathway. In the same study, disrupting microtubules with nocodazole increased myosin phosphorylation. The authors suggested that decreased adhesiveness, decreased matrix stiffness and reduced force from myosin II motors all reduced the prestress on the cytoskeleton. Reduced prestress then inhibited myosin phosphorylation, possibly by altering proper assembly of the enzymes and other proteins regulating myosin phosphorylation. Ingber et al. have proposed a model where myosin II generates traction force on the matrix, the matrix modifies myosin phosphorylation rate and level, and therefore the activity of actomyosin as a function of matrix stiffness. If this is true several interesting questions arise related to airway remodeling in inflammatory lung diseases. Does inflammation enhance migration of fibrocytes, myofibroblasts or existing smooth muscle cells through increased contractile tone and thus increased prestress? Do anti-inflammatory drugs reverse or inhibit such an effect? Does a decrease in elastic modulus of the lung parenchyma influence migration of these cells in asthmatic airways? Would reversing the changes in parenchymal mechanics prevent immigration of fibrocytes and their subsequent differentiation to myofibroblasts and smooth muscle cells? While there are no direct studies of both matrix composition and migration in asthma, the promigratory influences of collagen, elastin and laminin on ASM cell migration in vitro is consistent with the hypothesis that matrix composition and matrix mechanics could be a key regulator of ASM cell migration in vivo [283, 295]. Further studies of migration of fibrocytes and myofibroblasts on matrices that mimic the remodeled asthmatic airway are warranted.

Microtubules and cell migration

Actin polymerization/depolymerization and focal contact remodeling have justifiably been the focus of many studies of cell migration. However, it is clear that microtubules must also remodel during migration, and that this critical process is not as well defined as remodeling of the actin cytoskeleton. In stationary cells such as ASM embedded in extracellular matrix of the airway walls the microtubule organizing center (MTOC) and the nucleus are centered in the cell. However, during migration the nucleus is relocated toward the trailing edge of the cell. One important mediator of this relocation is Cdc42 regulation of myotonic dystrophy kinase-related Cdc42 binding kinase (MRCK) [296]. Gomes et al. (2005) found that nuclear relocation required phosphorylation myosin II by MRCK. Whether a similar event occurs in ASM cells is unknown, but there is some evidence that dynamic instability of microtubules is required during migration of vascular smooth muscle cells. Paclitaxel (Taxol), which stabilizes microtubules by binding the sides of the tubulin polymer, blocks VSM cell migration [297]. Microtubules clearly affect the degree of prestress in the ASM cytoskeleton and influence traction forces in cultured cells [298, 299], but it is not known to what extent dynamic instability is necessary during ASM migration. In nonmuscle cells dynamic instability of microtubules is important for disassembly of stable focal contacts at the rear of migrating cells, which is required for disengagement of the trailing edge from the matrix [300]. At this time there is only indirect evidence to infer a signaling pathway that would promote dynamic instability in ASM. A study of urokinase-stimulated ASM cell motility showed that urokinase induces ASM migration via a pathway including PI3-kinase [301]. Studies in vascular smooth muscle cell migration indicate urokinase also activates Akt and glycogen synthase kinase 3 β (GSK3 β) [302]. GSK3 β interacts with adenomatous polyposis coli (APC), which is known to regulate cell polarity by interacting with the plus end of microtubules [303]. Whether this signaling model functions in ASM migration is unclear, but it is likely that a functionally analogous system is required for microtubule remodeling during detachment and translocation of the rear of a migrating ASM cell. Blocking detachment of the trailing edge of the cell might in theory be beneficial for blocking remodeling events in the asthmatic airway. Inhibition of Akt and GSK3 β signaling might have the appealing feature of reducing hypertrophy as well as reducing immigration of new cells to the muscle layers of diseased airways (see discussion of Akt and GSK3 β signaling above).

Soluble and solid state signals that modulate migration

There are many chemically and structurally diverse molecules that enhance or inhibit ASM cell migration (Table 2). Many are soluble signaling molecules, but some are components of the extracellular matrix that are presented to ASM cells as solid-state signals. One of the first soluble promigratory molecules used to stimulate ASM migration in vitro was PDGF [261]. Subsequent studies have identified biogenic amines, lipids, growth factors, cytokines and chemokines as soluble modulators of ASM migration. Many of the promigratory compounds are autocrine or paracrine signaling molecules that are secreted at elevated levels in diseased airways. The earliest studies of solid-state signals that promote migration were by Schuger and colleagues who showed that laminin β 1 chain as necessary for migration of smooth muscle cells from mouse lung explants [262]. Later in vitro studies described the promigratory effects of collagens, fibronectin, laminins and matrix

metalloproteinases, and antimigratory effects of tissue inhibitors of metalloproteinases and chemical protease inhibitors [283, 288, 289]. Although it is clear that matrix composition changes in the asthmatic airway and that matrix expression by cultured ASM cells changes upon exposure to proinflammatory agents [73, 295], it is not known whether matrix composition alters migration in vivo in the lungs of asthmatic humans or in experimental asthma in mice. Whether a given signaling molecule or pathway is necessary for ASM migration could be tested in knockout and transgenic mouse models using lineage marking strategies similar to those used to define the source of vascular smooth muscle cells in atherosclerotic plaques (reviewed by [304]) and the necessity for PDGF signaling in blood vessel development [305].

Signaling cascades

Multiple highly conserved signal transduction cascades are activated during cell migration. The pathways studied most frequently in both nonmuscle and smooth muscle cell migration are illustrated in Figure 6. In this simplified scheme signal transduction events are shown as cascades beginning with receptor activation. We focus the illustration on three fundamentally important types of receptors: receptor tyrosine kinases (RTK), G protein coupled receptors (GPCR), and integrins, which are each known to promote cell migration. Coupling of early activation to G proteins is common to many promigratory stimuli. Both small G proteins (RhoA, Rac, Cdc42) and trimeric G-proteins are known to participate in promigratory signaling depending of the stimulant used in the experiment. Activated G proteins, Ca^{2+} , and changes in phospholipids including PIP2 and IP3 activate protein kinase cascades that include PI3-kinase, Ca^{2+} -dependent protein kinases, Rho-activated protein kinases (ROCK) and mitogen-activated protein kinases (MAPK) (Figure 6). The substrates for the various protein kinases include other protein kinases (MAPKAP kinase and LIM kinase) as well as proteins that interact with or regulate actin filament formation (HSP27, cofilin, myosin II). The monomeric G proteins (RhoA, rac and cdc42) also frequently regulate proteins that influence F-actin formation (mDia1, WAVE, WASP, ARP2/3). The more distal effector proteins in this scheme (blue ellipses in Figure 6) regulate two critical cellular processes: actin polymerization and activation of myosin II. The proteins required for actin polymerization and coupling of F-actin to the cell membrane integrins are illustrated in Figures 5A and 5B. Stimuli that increase myoplasmic Ca^{2+} oscillations or mean Ca^{2+} concentration in a cell activate MLCK which phosphorylates the regulatory light chains of myosin II, which is the protein that generates traction forces necessary to move the cell. Some of these pathways have been described in some detail in airway smooth muscle (Src, ERK, p38 MAPK, PI-3kinase), but other aspects of signaling are less well defined or undefined in ASM migration (Rac, RhoA, ROCK, LIM kinase, cofilin, ARP2/3). The reader is referred to a previous review for more details of signal transduction pathways known to participate in ASM migration [270]. Better understanding of signal transduction processes necessary for ASM migration is significant because migration is a fundamental process in lung development, and migration is presumed to be altered by lung diseases possibly contributing to airway wall thickening in asthmatics.

Modulation of ASM cell migration by drugs

One of the exciting aspects of studies of ASM cell migration is that biochemical processes mediating migration might be novel therapeutic targets for preventing or reversing airway remodeling in asthma. This is frequently cited as a rationale for exploring novel aspects of ASM migration. Identifying novel drug targets has also been a driving force in studies of vascular smooth muscle cell migration. In fact a number of cardiovascular drugs have beneficial effects in reducing atherogenesis and promoting recovery from vascular injury in part by reducing vascular smooth muscle proliferation and cell migration (reviewed by [270]). A clear proof of principle comes from the effects of statins, rapamycin and taxol, which all reduce proliferation, inhibit cell migration and reduce vascular wall remodeling [297, 306, 307]. In the case of statins, the therapeutic goal is to inhibit cholesterol synthesis to reduce serum LDL levels. There may also be a secondary benefit resulting from inhibiting mevalonate synthesis and isoprenylation of small G proteins. As discussed above, statins have been found to reduce airway hyperreactivity in a mouse asthma model [263], reduce cell proliferation [119] and to enhance apoptosis [217]. The pleiotropic effects are very likely due to disrupting signaling via small G proteins that participate in multiple biochemical processes including cell migration (see Figs. 5 and 6). Extended, low dose therapy with statins may well inhibit or reverse pathological airway remodeling by multiple mechanisms including reduced migration of ASM cells, fibrocytes or myofibroblasts. Whether such an effect occurs in the airways is an interesting question that has been raised in recent reviews of ASM as a target for novel asthma therapies [308, 309].

Several established drugs used to treat asthma also have significant antimigratory effects (Table 2). Corticosteroids and β -adrenergic agonists are mainstays of combination therapy for long term asthma control. Both classes of drugs as well as other drugs acting via cAMP have antimigratory effects. This suggests the hypothesis that combination of corticosteroids and long acting β -agonists might act by a combination of reducing ASM proliferation, preventing matrix remodeling and reducing ASM cell migration. Although this is a provocative hypothesis, there is no in vivo animal or human clinical data that critically tests this notion.

Other potential drug targets that should inhibit ASM cell migration include MAP kinases, and Rho kinases. P38 MAP kinases have been targeted for development of drugs to treat inflammatory diseases since the mid-1990s, but development has been limited by toxicity of first generation inhibitors. The advent of second generation inhibitors increases the possibility that expression of numerous contractile, proinflammatory and promigratory signaling proteins might be reduced by blocking p38 MAP kinase signaling [310–312]. In theory, inhibiting p38 MAP kinases could reduce expression of the extracellular signals for cell migration (eg. PDGF, IL1 β and IL8) as well as block migration directly [261]. Evidence from asthma models is consistent with this hypothesis [311], but preclinical studies in animal models of asthma models and studies in humans using newer inhibitors are needed to critically test this strategy [310].

Rho kinases (ROCK1 and ROCK2) are also potential antimigratory target proteins. It is clear that Rho kinase inhibitors can inhibit ASM cell migration [313], and a Rho kinase inhibitor (Fasudil) has been tested in humans to reduce cerebral vasospasm and treat angina

pectoris. The latter effects are due to vasodilation. Rho kinase inhibitors are also effective bronchodilators in mouse models of asthma [314, 315], but there is no published evidence of clinical benefit to humans with asthma. In addition, there are no data demonstrating a significant effect of Rho kinase inhibition on cell migration in vivo and airway wall remodeling. Nevertheless, Rho kinase inhibitors and p38 MAP kinase inhibitors are mechanistically appealing for modifying multiple aspects of the cell biology of airway dysfunction including smooth muscle contraction, proliferation and cell migration. Off-target effects are the major limitations of protein kinase inhibitors. However, new generation drugs with enhanced selectivity combined with local delivery to the lungs might address this problem and thereby expand the tools available to the pulmonary physician for long term therapy of asthma.

Conclusion

Rapid progress has been made in the past decade in studies of key processes underlying airway remodeling. Based on clinical studies and animal models of asthma it is clear that both airway smooth muscle hyperplasia and hypertrophy occurs. To increase cell number and size in the airway wall cells it is assumed that cells proliferate, survive for longer time periods and increase expression of proteins. In addition it is possible that some immigration of progenitor cells from beyond the muscularis occurs in diseased airways as well as shape changes in resident cells that differentiate to contractile smooth muscle. These notions stimulated a host of studies of biochemical pathways that control the fundamental processes of proliferation, apoptosis and cell migration. Many of the key stimuli, receptors and transduction pathways are conserved molecules known to participate in remodeling of the vasculature and in tumorigenesis. Developing novel drugs or novel uses of existing drugs to modify organ remodeling is one of the compelling reasons for studying many of pathways described. While a comprehensive view of some pathways and processes is emerging there are still basic and applied science questions remaining. Some outstanding basic science questions include the degree to which ASM cells proliferate in vivo in diseased lungs, the source of migrating cells and the potential for novel features of translational control of protein expression and cell survival to be discovered. The latter point is particularly important for developing organ-selective drugs targeting pathways unique in airway remodeling. In addition, even if organ or cell specificity is not possible it is possible that known drugs being tested or used currently in cancer chemotherapy and cardiovascular medicine can be delivered in a lung-restricted manner to alter airway remodeling. Broad-based, multidisciplinary approaches employing cell, animal and human studies will be required to integrate the basic molecular and cell signaling studies into an effective translational strategy for developing novel therapy of obstructive lung diseases.

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Figure 1. Schematic representation of the role of airway smooth muscle cell proliferation, cellular hypertrophy, apoptosis and migration in development of airway remodeling in asthma
 A key local driving force for airway remodeling are cytokines, chemokines, and growth factors released by the epithelium that act on the underlying airway wall (myo)fibroblasts and airway smooth muscle cells. Airway smooth muscle and fibroblasts also release trophic and pro-fibrotic factors that contribute to local inflammation and tissue repair. Central to the initiation and modulation of inflammation, tissue damage and repair is recruitment of active inflammatory cells including Th-2 and Th-1 polarized lymphocytes, eosinophils, neutrophils and mast cells.

Figure 2. Schematic representation of key signaling mechanisms associated with control of airway smooth muscle cell proliferation

See text for details and Table 1 for list of factors that control activation of these pathways.

Figure 3. Schematic representation of key signaling mechanisms associated with control of hypertrophic cell growth

See text for details.

Figure 4. Simplified schematic representation of essential pathways for caspase-dependent apoptotic cell death

Apoptosis is triggered by internal cellular stress (intrinsic pathway) or extracellular signals (extrinsic pathway) that mediate effects via the binding of ligands (eg. Fas, TNFR1, DR5) to cell surface death receptors. Extrinsic pathways directly activate executioner caspases (caspase-3) through initiator caspases (eg. caspase-8 and -9) ultimately leading to cell death. In intrinsic pathways, death signals are conducted through mitochondria, increasing permeability that leads to the release of cytochrome c. Cytosolic cytochrome c binds Apaf-1 to activate the apoptosome and caspase-9 which ultimately leads to downstream activation of executioner caspase-3 [218].

Figure 5. Schematic model illustrating the prominent features of a migrating cell

The leading edge of the cell is represented by the cross hatched region on the right. **Inset A:** The actin polymerization module located at the leading edge is a site of rapid actin polymerization, depolymerization and filament branching. Actin nucleating proteins (mDia1, mDia2, VASP) promote filament formation at the plus (barbed) end. G-actin monomers are added by the action of profilin. Actin filaments are severed by gelsolin and depolymerized by cofilin. Actin branching is regulated by small G proteins acting on WAVE, WASP and proteins of the ARP2/3 complex. The stiffness of the actin gel and traction forces on the matrix are controlled in part myosin II motor proteins that are regulated by activation of multiple kinases (MLCK, PAK, ROCK) and myosin light chain phosphatase (MLCP). **Inset B:** Signaling and actin attachment modules in the leading edge promote formation of nascent focal contacts (red bars) that rapidly assemble to transiently attach the cell to the matrix. Actin attachment components include integrins, adaptor proteins (talin, vinculin, tensin, paxillin). Signaling module components control assembly and maturation of the focal contact. These include regulatory proteins (Src, CAS, FAK) and proteins controlling actomyosin assembly and myosin II activation and (MLCK, PAK, MLCP and ROCK). As the cell migrates, nascent focal contacts mature and move towards the rear of cell. Focal contacts at the rear of the cell (red bars on the left) are disassembled as the cell advances. Disassembly requires the action of multiprotein complexes that depend on microtubules (gray filaments) emanating from the microtubule organizing center (MTOC). Reprinted from Gerthoffer ^[270] by permission of the American Thoracic Society.

Figure 6. Signaling pathways that regulate actin polymerization and myosin II motors in smooth muscle cell migration

Activation of G protein coupled receptors (GPCR) and receptor tyrosine kinases (RTK) initiates activation of parallel signaling cascades that culminate in actin filament remodeling, changes matrix adhesiveness and regulation of myosin II motors that generate traction force. Immediate post-receptor events include activation of trimeric G proteins, Src family tyrosine kinases, phospholipase C (PLC) and PIP₂, PI3-kinases (PI3-K) and increased Ca²⁺. Multiple small G proteins (RhoA, Rac, Cdc42) and calmodulin (CaM) then activate downstream targets that are shown here in darker shades of red. Some targets are effector proteins that regulate actin polymerization including the formins (mDia1 and mDia2), WAVE and WASP and the ARP2/3 complex. Other targets include members of the MAP kinase family (p38 MAPK and ERK), Rho kinases (ROCK) and p21-activated protein kinases (PAK). The signaling kinases phosphorylate other protein kinases (MAPKAPK, LIMK) or phosphatases (MLCP) to regulate effector proteins (dark blue ovals) that control actin polymerization and traction forces generated by myosin II. Most of the schematic is organized as sets of parallel linear signaling cascades, which is an oversimplification for the sake of clarity. Pathway convergence and crosstalk are known to occur between the pathways shown. Regulation of MLCK is a good example where both positive and negative inputs are integrated to determine the level of myosin II regulatory light chain phosphorylation and traction force. Reprinted from Gerthoffer²⁷¹ by permission of the American Heart Association.

Table 1

Primary factors affecting airway smooth muscle cell proliferation in culture.

Class	Pro-proliferative	Anti-proliferative	References
Receptor tyrosine kinases	PDGF (A, B, C), IGF-1, bFGF, EGF, NGF, insulin		[37, 39, 41, 316–321]
G protein coupled receptors	Histamine, thromboxane A ₂ , endothelin-1, α -adrenergic agonists, cysteinyl leukotrienes, thrombin, tryptase, substance P, sphingosine phosphate, lysophosphatidic acid, muscarinic M ₃ receptor agonists, 5-hydroxytryptamine, urotensin II, ATP, UTP, bradykinin	PGE ₂ , β -adrenergic agonists, VIP, sphingosine, atrial natriuretic peptide	[31, 42, 48, 49, 54–57, 318, 322–333]
Cytokines	IL-1 β , TNF- α , TGF- β 1, IL-6	IL-4, TNF- α , TGF- β 1, IFN γ , IFN β	[40, 60, 61, 65, 316, 334–339]
Matrix proteins	Fibronectin, collagen I, vitronectin	Laminin, chondroitin sulphate	[26, 27, 67]

Table 2

Summary of agents that modulate ASM cell migration.

PROMIGRATORY AGENTS	ANTIMIGRATORY AGENTS
Growth Factors and Cytokines bFGF ^[340] , CXCL10/CXCR3 ^[267] , CC Chemokine ligand 19 (CCL19) ^[341] , IL-1 β ^[261] , IL-8 ^[285] , Leukotriene B4 ^[342] , Leukotriene E4 ^[343] , PDGF ^[261, 344, 345] , TGF β 1 ^[261]	β-adrenergic Agonists and the PKA pathway Dibutyl cAMP ^[313] , Formeterol ^[301] , Forskolin ^[340] , Cilomolast ^[340] , Salmeterol ^[340] , Theophylline ^[313]
Extracellular Matrix Collagens I, III, V ^[343] , Fibronectin ^[343] , Integrins α 5, α V ^[343] , Laminin ^[343] , MMP-3 ^[346]	Immunomodulating Drugs Fluticasone ^[340] , Pyrimidine synthesis inhibitor, FK778 ^[347] , Sirolimus ^[347]
Other Promigratory Agents Cyclodextrin ^[287] , Lysophosphatidic acid ^[313] , Thrombin ^[289] , Urokinase plasminogen activator ^[348]	Protease Inhibitors 4-(2-Aminoethyl) benzenesulfonylfluoride HCl (AEBSF) ^[289] , Ilomastat ^[289] , Prinomastat ^[288] , TIMPs 1–4 ^[289]
	Protein Kinase and Phosphatase Inhibitors LY294002 ^[343, 349] , PP1 ^[283] , PD98059 ^[261] , SB203580 ^[261, 343] , U-0126 ^[301, 349] , Vanadate ^[287] , Y27632 ^[313, 343]
	Other Antimigratory Agents Pertussis toxin ^[301] , Prostaglandin E2 ^[343] , Retinoic acid ^[349] , SB649146 (SP-1 inverse agonist) ^[345]