Plasticity of airway smooth muscle phenotype in airway remodeling
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Chapter 1

General Introduction
Airway smooth muscle and asthma

Asthma is an inflammatory airway disease characterised by exaggerated bronchoconstriction to neurotransmitters, inflammatory mediators and inhaled contractile stimuli [1-5]. Stimuli that are bronchoconstrictive for asthmatics, may even be hardly effective or ineffective in healthy individuals [6-8]. This airway hyperresponsiveness may in part be explained by increased shortening of the airway smooth muscle (ASM) layer in the airway wall, caused by the presence of inflammatory mediators that augment ASM contraction to other contractile agonists [4]. These inflammatory mediators can be released in the airways following the recruitment of inflammatory cells, but can also be released from structural cells, including ASM itself, providing a mechanism to adapt acutely to the pro-inflammatory environment.

Asthma is, however, chronic of nature. Chronically inflamed airways are subjective to structural changes (airway remodeling), which are thought to play an important role in the development of chronic airway hyperreactivity and decline of lung function. These include thickening of the basement membrane, subepithelial fibrosis, epithelial damage, increases in ASM mass, bronchial microvascular remodeling and mucus gland hypertrophy [9;10] (Figure 1.1).

Figure 1.1 Schematical cross-sections of a normal and a remodeled airway. Shown are increases in ASM mass, fibrosis of the subepithelial layer, mucus hyperplasia and bronchial microvascular remodeling, all characteristic for airway remodeling in asthma. (after Jeffery, [1]).
Due to airway remodeling, the elastic forces of the tissue surrounding the airway are diminished through uncoupling of ASM from its parenchymal recoil, which may contribute to exaggerated constriction [9;11]. In contrast, fibrosis of the subepithelial layer may stiffen the airway and protect against excessive airway narrowing [9]. The benefits of thickening of the airway wall are limited, however, as this will result in changes in airway diameter, sufficient to limit airway capacity to the extent seen in asthmatics [12]. In addition, myofibroblasts have been observed in the subepithelial layer in patients with chronic and severe asthma [13], which may contribute to constriction of the airway as a whole. The deposition of extracellular matrix proteins can be mediated by subepithelial fibroblasts, but recent reports suggest that ASM cells are also capable of producing matrix proteins such as fibronectin and collagen [14-16]. It is not completely clear whether matrix protein production by ASM represents a significant contribution to the subepithelial fibrosis seen in asthmatics. Nevertheless, the composition of matrix proteins in which the ASM is embedded may have a major impact on its contractile function [17;18].

Increased ASM mass may increase the force produced by bronchoconstrictor agents and therefore increase airway responsiveness to these stimuli. The physical obstruction of inward growing tissue may also contribute to changes in airway reactivity. Importantly, these changes in airway structure worsen with duration of disease, which could add to disease-induced chronic increase in severity of airway narrowing [19]. Studies in animal models of asthma have shown that the increase in ASM mass is most prominent in the larger airways and declines progressively towards the periphery [20]. Increased ASM mass may be explained in part by increases in cell number (hyperplasia), as confirmed in studies that determined changes in ASM cell number after repeated allergen challenge [21;22]. In asthmatics, however, both hyperplasia and increases in cell size (hypertrophy) have been noted [23]. The increase in ASM mass caused by either hypertrophy or hyperplasia is considered sufficient to comprise a major cause of exaggerated airway narrowing [24;25]. Thus, the increase in ASM in the central airways of allergen challenged rats was found to correlate with the increase in hyperreactivity to methacholine [20]. Although this would suggest a central role for ASM growth in chronic airway hyperreactivity, others have shown that the time-profile of the progressive increase in airway hyperreactivity does not match the time-profile of the increase in ASM mass [26]. It should also be noted that these studies do not take into account that ASM can adapt its phenotype, which may superimpose on changes in ASM mass.

**Airway smooth muscle phenotype**

Accommodating the elements that comprise the contractile machinery, has for a long time been considered the prominent function of ASM. However, recent findings have focused on plasticity in ASM function under pathophysiological conditions [27-30]. By changing its phenotype to hypercontractile, ASM shortens more rapidly [31;32], which may result in exaggerated airway narrowing [33]. *In vitro,* this
(hyper)contractile phenotype can be induced by growth arrest and is characterised by increases in contractile protein expression, such as smooth muscle-specific actin and myosin [34]. Also, muscarinic M₃ receptor expression and contraction regulatory protein expression (e.g. myosin light chain kinase, calponin) are known to increase under these conditions [34;35].

Nevertheless, it should be noted that the occurrence of a hypercontractile ASM phenotype in asthma is still subject of debate. In favour, isolated asthmatic ASM cells have been reported to contract more profound and more rapidly in vitro [36]. Furthermore, passive sensitization of human bronchi with atopic serum increases maximal contractility and agonist-sensitivity in vitro [37]. Interestingly, this effect is associated with serum IgE [38], suggesting a relationship between allergic sensitization and increased contractile responsiveness. Studies using a canine model of allergic sensitization have revealed similar effects after active sensitization [39;40]. This increase in contractility is accompanied by increases in MLCK expression [41], which has been demonstrated in sensitized human ASM as well [42]. However, although some have reported increased contractility or agonist-sensitivity of asthmatic ASM in vitro, a vast amount of reports on this subject suggest no major changes in contractility in vitro (cf. [43] for review). At the moment, it is therefore not certain whether hypercontractility to some extent is an artefact, caused by extremely favorable culturing conditions, or (patho)physiologically relevant indeed.

Paradoxically, switching to a less contractile phenotype may also be relevant in the pathophysiology of asthma and this may in part explain the controversy that exists about contractility changes in asthmatic ASM in vitro. As in other smooth muscle cell types, ASM is able to switch to a less contractile phenotype in a reversible fashion [27;29;44]. Switching to a less contractile phenotype generally occurs when smooth muscle cells, kept in culture, are exposed to high concentrations of fetal bovine serum. Under these conditions, ASM cells reduce their ability to contract due to diminished contractile protein expression [34]. In addition, M₃ receptor expression is strongly reduced [45]. Although a less contractile phenotype may seem favourable in airway diseases such as asthma, it is important to note that smooth muscle cells in culture proliferate faster and growth factor receptor expression is increased [46-49]. Also, organelles involved in synthesis and secretion such as the Golgi apparatus are increased under these conditions [50]. This has led to the hypothesis that smooth muscle cells reversibly switch between a contractile and a proliferative/secretory phenotype [46]. The latter state may contribute to the increase in ASM mass, seen in asthmatics. Indeed, cultured ASM cells obtained from asthmatics proliferate faster in response to the same mitogen and cannot be made quiescent by deprivation of fetal bovine serum [51].

Increases in ASM secretory function have also been postulated to contribute to airway inflammation and airway remodeling as ASM are potent producers of cytokines, chemokines and extracellular matrix proteins [52-56]. Moreover, passively sensitized ASM cells produce more extracellular matrix when compared to cells obtained from healthy controls and can therefore be considered hypersecretory [57].
Furthermore, the profile of extracellular matrix proteins produced by asthmatic ASM cells supports the induction of a hyperproliferative ASM phenotype [58]. Therefore, ASM phenotype switching may contribute to the pathophysiology of asthma by augmenting inflammation and proliferation during periods of allergen exposure and by augmenting contractile responses in the periods in between. This could increase ASM mass and contractile function in a cumulative fashion.

Recently, Moir et al. have shown an increase in ASM mass in bronchioli of repeatedly allergen challenged rats 24 h after the last allergen challenge which was accompanied by a reduction in contractility of the muscle, when corrected for changes in ASM cross-sectional area [59]. Conversely, 35 days after the last allergen challenge this increase in ASM mass was no longer present, whereas corrected ASM contractility was increased. This could indeed indicate switching of proliferative and contractile ASM phenotypes, although it should be noted that the observed contractile protein expression profiles do not completely match this hypothesis. Even 35 days after the last allergen challenge, reductions in contractile protein expression were observed, which is not easily explained. Nevertheless, this study provided evidence for the first time that phenotypic plasticity of ASM relates to airway remodeling in vivo.

In view of this potentially important role for phenotypic plasticity in the regulation of ASM function in asthma, insight into the mechanisms that control these processes is warranted. Multiple stimuli have been considered responsible. Mechanical strain for instance is known to increase contractile protein expression in cultured ASM cells and to trigger ASM cells into the cell cycle [60-63]. Strain may also change extracellular matrix composition as both cardiac fibroblasts and rabbit aortic smooth muscle cells produce collagen in response to mechanical forces [64;65]. It is uncertain however what role this strain-induced remodeling plays in the pathophysiology of asthma [63].

An altered composition of the extracellular matrix may cause altered contractile and proliferative characteristics of ASM. This is matrix protein-specific, as some (eg fibronectin, collagen type I) facilitate proliferation of cultured human ASM cells and induce proliferative marker protein expression such as the Ki67 nuclear antigen [17]. In agreement with the induction of a proliferative and less contractile phenotype by these matrix proteins, contractile markers such as calponin, smooth muscle specific myosin heavy chain and actin are reduced. In contrast, laminin as well as matrigel (a solubilized basement membrane matrix) can reduce proliferation of human ASM cells and increase contractile protein expression [17]. Similar results have been obtained with matrigel in vascular smooth muscle cells [66]. In view of the focus of this thesis, the possible role of growth factors and GPCR agonists in the regulation of ASM contractility and proliferation are of specific importance. Their potential role in airway remodeling and the signal transduction mechanisms involved will be discussed below.
Chapter 1

Peptide growth factors
As mentioned above, both ASM proliferation and the induction of a switch to the less contractile phenotype can be stimulated by treatment with fetal bovine serum. In contrast, deprivation of serum induces growth arrest and return to a contractile phenotype or even a hypercontractile phenotype. Peptide growth factors, which bind to receptors with intrinsic tyrosine kinase activity are major constituents of serum and are considered capable of inducing both ASM proliferation and ASM phenotype switching [30]. Furthermore, the >10 kD fraction (suggesting the presence of peptide growth factors) of dialysed broncho-alveolar lavage (BAL) fluid obtained from asthmatics, induces ASM proliferation and activates signaling pathways critical for proceeding the G1 phase of the cell cycle [67]. For this reason, it has been hypothesized that peptide growth factors are, at least in part, responsible for the increase in ASM mass in airway remodeling in asthma [29;68].

Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) have all been implicated in asthma based on increased immunoreactivity in lung tissue slices or protein expression in airway biopsies [69-72]. Although mitogenic for endothelial cells, the role of VEGF in ASM remodeling is not well characterised. EGF and bFGF, however, are considered mitogenic for ASM. The role of TGF-β in ASM mitogenesis is less certain, as it has been associated both with induction and inhibition of ASM proliferation. Rather, TGF-β is generally linked to the production of extracellular matrix (ECM) proteins and fibrosis (Table 1.1).

Interestingly, EGF induces contraction of guinea pig tracheal smooth muscle, presumably through the production of lipid mediators, such as leukotrienes, thromboxane A₂ and prostaglandins [76;100]. Similar actions of EGF and other growth factors have been noted in vascular smooth muscle [101-103]. This suggests the possibility that growth factor-induced smooth muscle contraction is a general physiological event and raises questions regarding the direct contribution of growth factor-induced ASM contraction to allergen induced bronchoconstriction. Despite of being of interest to vascular biologists as early as 1986 [102], growth factor-induced contraction of human ASM still remains to be established.

Insulin-like growth factor-1 (IGF-1) may also be relevant for asthma, although its levels have not been reported to be increased. The bioavailability of IGF-1 is negatively regulated through binding to IGF binding proteins (IGFBPs), the availability of which in turn is negatively regulated by IGFBP proteases. Together, these proteins constitute the IGF-axis [104]. In asthmatic airways, increased levels of the IGFBP protease MMP-1 have been demonstrated, which could increase the bioavailability of IGF-1 [105]. Although effects other than mitogenesis have not been studied in ASM [91;92], IGF-1 has profound effects on migration, phenotypic modulation and ECM production in vascular smooth muscle [104;106;107].
Table 1.1 Sources and ASM effects of growth factors.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Source</th>
<th>Effect on ASM</th>
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<tr>
<td>EGF</td>
<td>ASM</td>
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<td>bFGF</td>
<td>ASM</td>
<td>Proliferation</td>
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<td>ECM</td>
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<td>TGF-β</td>
<td>ASM</td>
<td>Proliferation (↑↓)</td>
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<td>Fibroblast ECM</td>
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<td>(IL-6, IL-8, IL-11, LIF)</td>
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<td>COX-2 expression ↑</td>
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<td>β-adrenergic responsiveness ↓</td>
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<td>PDGFR expression ↓</td>
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<tr>
<td>IGF-1</td>
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<td>Plasma</td>
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Platelet-derived growth factor (PDGF) is one of the most effective growth factors for ASM proliferation [92] and its effects are well characterised (Table 1.1). Although BAL fluid levels of PDGF are not increased in asthmatics when compared to healthy controls [108], increased PDGF and PDGF receptor expression have been reported in airway biopsies in asthma [109]. In addition, strongly synergistic mitogenic interactions with other peptide growth factors have been reported [99]. The dominant mitogenic signaling induced by PDGF may therefore still be relevant to asthma pathology whether or not expression levels of the growth factor are increased. Although fetal bovine serum is an established stimulus with regard to the induction of ASM phenotype switching, surprisingly, PDGF is the only purified growth factor directly associated with phenotype switching of ASM to a less contractile phenotype [17].

Less well studied growth factors putatively relevant to asthma and other airways diseases include connective tissue growth factor (CTGF), angiotensin II and insulin. Recently, CTGF was found to be expressed in human ASM cells in culture and in human ASM cells in tissue slices in situ. Interestingly, TGF-β induced a 70-fold increase in CTGF expression in asthmatic human ASM cells, compared to only 3-fold in healthy controls [56;97]. This mechanism may be very relevant to asthma, as CTGF is linked to ECM protein production by ASM cells [56].

Angiotensin II may also be of interest in view of increased plasma levels in acute severe asthma [110]. Moreover, intravenous administration of angiotensin II to mild asthmatics in concentrations similar to those endogenously present in severe asthmatics causes acute bronchoconstriction [98]. Also, antigen-induced airway hyperresponsiveness in guinea pigs has been found in part AT₁ receptor dependent [111]. Angiotensin II can also act as a hypertrophic growth factor in human ASM cells, presumably through the endogenous production of TGF-β [85].

A potential role for insulin as a mediator in asthma is still under investigation: a lower prevalence of asthma and atopy symptoms in patients with type I diabetes mellitus has been reported in epidemiological studies [112;113] and has been a topic of discussion for many years [114]. Also in animal models of experimental diabetes, allergen-induced airway inflammation and dysfunction of inhibitory neuronal M₂ autoreceptors can be strongly reduced [115-117]. Although this does not prove a direct role for insulin, it is considered mitogenic for ASM [99] and activates signaling pathways also activated by other growth factors [118]. It could therefore be involved in airway remodeling.

**Growth factor receptors**

Most growth factors couple to and signal through single membrane-spanning receptors with intrinsic tyrosine kinase activity. Upon binding of the receptor with a peptide growth factor, dimerization of receptor subunits occurs, which is required to induce cellular signaling [119]. The intrinsic kinase activity, which is localized
intracellularly, then allows the receptor subunits to cross-phosphorylate each other at tyrosine residues, which is referred to as receptor autophosphorylation. The phosphorylated tyrosine residues act as docking sites for other kinases, such as the non-receptor tyrosine kinase Src and phosphatidylinositol (PI) 3 kinase [119]. These kinases allow further downstream signaling as described below.

Platelet-derived growth factors signal through a similar, though slightly distinct mechanism. PDGF is not a single growth factor molecule but exists in multiple isoforms, the most studied being the A and B monomers. These form dimers, i.e. PDGF-AA, -AB or -BB. PDGF-AB is the most prevalent dimer and most commonly used in PDGF studies. Both the PDGF-A and -B isoforms are capable of coupling to receptors, which allows a single PDGF molecule to induce PDGF receptor dimerization [120]. The PDGF receptor also exists in two monomeric forms: α and β. The α monomer binds both the PDGF A and B isoforms, whereas the β monomer is selective for PDGF-B [121;122]. This causes PDGF-AB and PDGF-BB to be more effective as mitogens for ASM, when compared to PDGF-AA [123].

Receptors binding insulin and insulin-like growth factors can exist in two disulfide bond-linked single membrane spanning proteins even in the inactive state. Signaling through these receptors is different from the other growth factors, requiring tyrosine phosphorylation of insulin receptor substrate (IRS) proteins [104;118]. Phosphorylated IRS proteins can act as docking sites for non-receptor tyrosine kinases and PI 3-kinase which are involved in downstream signaling events. Although these signaling events seem comparable to those induced by the other growth factor receptors mentioned, cellular signaling is usually growth factor-specific. For instance, insulin treatment increases actin and myosin expression in chick gizzard smooth muscle cells, whereas PDGF decreases their expression [107;124]. It is not clear whether such differential signaling is relevant to ASM.

**Growth factors and signal transduction**

As mentioned above, receptor tyrosine kinases can activate PI 3-kinase upon docking at tyrosine residues. This is an important signaling mechanism in smooth muscle as it is associated with cell proliferation, differentiation, migration and contraction [94;106;107;125-127]. In ASM, PI 3-kinase is less well studied and, surprisingly enough, associated with proliferation and migration only [94;125;128]. Further studies have indicated that different classes of PI 3-kinase are expressed by ASM (IA, II and III), of which class IA PI 3-kinase may be involved in ASM proliferation [129].

PI 3-kinase phosphorylates phospho-inositides at the 3-position of the inositol ring, which leads to the formation of PI3P, PI(3,4)P₂ and PI(3,4,5)P₃. The PI(3,4,5)P₃ phospholipid appears to act as the most important of these second messengers [130]. These phospholipids can bind to and activate protein kinase B (PKB) either directly [131] or through activation of PIP₃ dependent protein kinase (PDK), which phosphorylates PKB [132]. PKB, in turn, is an upstream inhibitor of glycogen
synthase kinase 3 [133] and an activator of p70 S6 kinase [134]. Both activities are associated with transcriptional activation and protein synthesis leading to proliferation. PI 3-kinase activity may activate transcription and protein synthesis through other mechanisms as well: PI 3-kinase can activate the non-receptor tyrosine kinase Src directly [135], which in turn can activate other signaling pathways activated by receptor tyrosine kinases, such as the mitogen activated protein kinase (MAPK) pathway [136]. This PI 3-kinase mediated activation of the MAPK pathway has been shown to be important in the activation of MAPK by growth factors specifically at weakly mitogenic concentrations [137].

Figure 1.2 Signal transduction pathways activated by growth factors and interactions with GPCRs. Both RTKs and GPCRs are involved in a variety of signaling cascades, which are linked through complex signaling networks. Shown are pro-mitogenic interactions at the level of p42/p44 MAP kinase, PI 3-kinase and PKB.

Mitogen activated protein 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 [68]. Upon activation of receptor tyrosine kinases, an adaptor protein Shc binds phosphorylated tyrosine residues and recruits the nucleotide exchange factor Sos to the membrane which is involved in the activation of Ras, a monomeric G protein [119]. These events are followed by
the activation of a cascade of kinases that result in the activation of p42/p44 MAP kinase, also referred to as extracellular signalling regulated kinase (ERK) 1/2. p42/p44 MAP kinase is well studied and known to be involved in ASM proliferation, migration, cytokine and chemokine-production and contraction [95;138-140]. In addition, studies in vascular smooth muscle have shown its involvement in the regulation of smooth muscle phenotype [141]. Taken together, p42/p44 MAP kinase is considered a key signaling event in the regulation of smooth muscle function.

**G protein coupled receptor agonists**

Although receptor tyrosine kinases are potently and effectively coupled to signaling pathways involving PI 3-kinase and p42/p44 MAP kinase, G protein coupled receptors (GPCRs) are capable of regulating these pathways as well [142;143] (Figure 1.2). GPCRs are receptors with seven transmembrane spanning peptide chains and couple primarily to heterotrimeric G proteins. The subtype composition of the α, β and γ subunits which are associated to form the heterotrimeric G protein is critically important for the capacity of GPCRs to activate these mitogenic signaling pathways, as well as for the mechanisms involved.

Gs coupled receptors such as the β2 adrenoceptor and the PGE2 EP2 receptor, activate adenyl cyclase which increases the cytosolic cyclic AMP concentration [144;145]. By activating protein kinase A (PKA), cyclic AMP is capable of inhibiting p42/44 MAP kinase activity through inhibitory phosphorylation of Raf-1 [146]. Gs coupled receptors can also inhibit the expression of cell cycle regulatory proteins such as cyclin D1, resulting in diminished progression through the cell cycle [147]. On the other hand, cell cycle inhibitory proteins such as p21Cip1 and p27Kip1 are induced by cAMP [148]. Not surprisingly therefore, β2 agonists and PGE2 are anti-mitogenic for ASM [149-151] and can inhibit ASM migration [83]. In addition, cytokine and chemokine production by ASM cells in culture can be inhibited by β2 agonists and PGE2 [152-154].

Agonists acting on Gi and Gq coupled receptors on the other hand can favour airway remodeling. Thrombin for instance, which activates both Gi and Gq through activation of protease activated receptors (PARs) is highly mitogenic for ASM, which is dependent on both PI 3-kinase and p42/p44 MAP kinase [94;155]. In addition, thrombin induces ASM cytokine and growth factor production [156;157]. In contrast, muscarinic receptor agonists acting on both Gi coupled M2 and Gq coupled M3 receptors are usually ineffective or nearly ineffective as mitogens [99;158]. This is not an exception since GPCR agonists such as histamine, LTD4, endothelin-1, bradykinin and serotonin have all been described to be at most modestly mitogenic [14;92;159-163]. It is not yet fully clear what causes this discrepancy, since both Gi and Gq are capable of activating PI 3-kinase and p42/p44 MAP kinase. The βγ subunits released by the dissociation of Gi for instance are capable of directly activating PI 3-kinase, which in turn may activate p42/p44 MAP kinase through activation of receptor and non-receptor tyrosine kinases as described above [143] (Figure 1.2). Gq proteins which increase intracellular Ca2+ and activate protein kinase
C (PKC) may also activate these pathways either through transactivation of receptor tyrosine kinases by the Ca$^{2+}$ dependent protein tyrosine kinase Pyk2 or through PKC mediated phosphorylation and activation of Raf-1 [164-167] (Figure 1.2).

One possible explanation for why GPCR agonists are generally not mitogenic by themselves is that GPCR-induced PI 3-kinase or p42/p44 MAP kinase signaling is often too weak or too short-lived to induce ASM proliferation [92;155]. In support of this hypothesis, GPCR agonists can be aided by growth factors that act on receptor tyrosine kinases. When combined with EGF for instance, LTD$_4$, endothelin-1 and histamine are potent mitogens [14;159;162]. The mechanisms involved in this cross-talk, however, are largely unknown. Nevertheless, insight in this cross-talk is warranted since it may have significant pathophysiological implications. When synergistically interacting with growth factors that are increased under inflammatory conditions, GPCR agonists may be important as modulators of the increase in ASM mass in airway remodeling.

In addition to effects on ASM cell number, GPCR-induced effects on ASM phenotype could be envisaged as well. Studies on this subject are not available, however, let alone those describing interactions with growth factors. Isolated components of ASM phenotypic regulation on the other hand have been described, such as the regulation of smooth muscle specific gene expression (e.g. actin, myosin). This regulation appears to be dependent on the activation of the small monomeric G protein Rho, which causes activation and translocation of serum response factor (SRF) to the nucleus [168-170]. SRF acts as a transcription factor for smooth muscle specific genes [171]. Since GPCR agonists are capable of activating Rho and Rho-kinase [171], GPCR agonists may be involved in the regulation of SRF. In addition, Rho and Rho-kinase are involved in contraction, predominantly through calcium-independent mechanisms. Therefore activation of Rho-kinase may be an important regulatory mechanism both in the acute and long-term regulation of contraction.

**Aims of the studies**

Based on the above mentioned observations and mechanisms, GPCR agonists and peptide growth factors are potentially important in the regulation of airway remodeling. The purpose of this thesis is to gain insight in the regulation of ASM phenotype and proliferation by growth factors, GPCR agonists and combinations hereof, and to investigate the potential contribution of this cross-talk in airway remodeling. For this purpose we used cell culture, organ culture, as well as *ex vivo* approaches. This broad methodological approach allows both the investigation of cellular and molecular biological mechanisms in cell culture as well as the relevance of these mechanisms to more intact physiological systems.

The majority of the studies was conducted on bovine tracheal smooth muscle (BTSM), based on the consideration that human ASM is available occasionally from resection material from patients undergoing surgery for lung carcinoma and only in
very limited amounts. The specific use of BTSM is based on its well characterised physiology, which is representative for human tissue as regards the parameters of interest. Thus, ASM obtained from both species proliferates in response to growth factors, including PDGF, EGF and IGF-1, with similar concentration dependencies and similar mechanisms involved (i.e. p42/p44 MAP kinase and PI 3-kinase; compare [92;94;138] with [99;125;155]), although the magnitude of the proliferative effects may differ between the species, possibly because of subtle signaling differences [92;172]. Also, BTSM expresses GPCRs relevant for human airway physiology, e.g. G\textsubscript{i} coupled muscarinic M\textsubscript{2} and G\textsubscript{q} coupled muscarinic M\textsubscript{3}, histamine H\textsubscript{1} and bradykinin B\textsubscript{2} receptors [4;45;173-177].

The first part of the thesis focuses on the long-term regulation of ASM contractility through changes in smooth muscle phenotype. Chapter 2 describes the occurrence of these phenotype changes at the level of contractility in intact BTSM. Organ cultured strips were used in this study instead of cultured cells, since cell to cell and cell to matrix interactions are preserved in intact tissue. This may be very relevant as extracellular matrix has been described to regulate ASM phenotype [17]. Moreover, extracellular matrix may influence or even determine the responses of ASM to therapeutic intervention [178]. The organ culture model was evaluated using fetal bovine serum and a panel of purified growth factors to establish a potential relationship between the mitogenic strength of the growth factor and the change in contractility. In Chapter 3, these studies are extended to the induction of hypercontractility. Cultured canine tracheal smooth muscle cells are known to become hypercontractile upon prolonged exposure to serum-free medium containing insulin. The induction of hypercontractility by serum deprivation and insulin exposure were therefore compared in our organ culture model.

In view of potential effects of GPCR agonists on ASM phenotype, establishing the role of Rho-kinase in the regulation of contraction, phenotype and proliferation is an important consideration, since phenotypic parameters like actin and myosin have very recently been described to be regulated by Rho/Rho-kinase-dependent pathways. Chapter 4 addresses the involvement of Rho-kinase in proliferation and phenotype changes both in BTSM cells and organ cultured BTSM strips. In addition, the acute effects of Rho-kinase inhibition on both growth-factor and GPCR agonist induced contraction were measured in human bronchial smooth muscle, the results of which are described in Chapter 5. Finally, in Chapter 6, the involvement of GPCR agonists in the regulation of phenotype was investigated directly by long-term exposure of organ cultured BTSM strips to methacholine. Both contractility, proliferative capacity and contractile protein expression were used as parameters. The role of G\textsubscript{i} coupled M\textsubscript{2} receptors, G\textsubscript{q} coupled M\textsubscript{3} receptors and the signaling mechanisms involved (p42/p44 MAP kinase, PI 3-kinase, PKC) were determined using selective inhibitors. In addition, the possible interaction with PDGF-induced phenotype changes were studied.

The second part of the thesis involves the regulation of ASM proliferation by GPCR agonists and growth factors. The effects of the GPCR agonist methacholine and the
peptide growth factor PDGF on proliferation of BTSM cells were studied in Chapter 7, with particular focus on their interaction. This interaction was characterised and the role of $G_i$ coupled M$_2$ receptors and $G_q$ coupled M$_3$ receptors was determined in this study. The studies described in Chapter 8 further explore the mechanisms involved in the interaction of GPCR agonists and growth factors, using the GPCR agonist bradykinin and the peptide growth factor EGF. The involvement of p42/p44 MAP kinase was measured using selective inhibitors and measurement of p42/p44 MAP kinase activation by Western analysis using phosho-specific antibodies. In addition, the role of PKC isozymes was studied using subtype selective inhibitors.

Finally, the functional implications of interactions between growth factors and GPCR agonists for ASM phenotype changes and proliferation in vivo were studied in Chapter 9 using a guinea pig model for ongoing allergic asthma. Repeated allergen exposure-induced alterations in phenotype and ASM mass were studied by measurement of contractility, contractile protein expression, increases in cell number as well as morphometric analysis of total ASM mass. In addition, the involvement muscarinic receptors in the development of ASM remodeling was studied by treating the animals with the long-acting muscarinic antagonist tiotropium bromide.

References
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Chapter 1


