General discussion and summary

Kuldeep Kumawat
General discussion and summary

Airway remodeling is a hallmark pathological feature of individuals with asthma and is associated with airway obstruction [1], airway hyperresponsiveness [2] and declining lung function in severe disease [3]. It is characterized by extensive structural changes in the airway wall which include airway smooth muscle (ASM) cell hypertrophy and hyperplasia, subepithelial fibrosis, mucus hypersecretion, neovascularization and increased and altered extracellular matrix (ECM) expression, leading to airway wall thickening [4]. Airway remodeling is a progressive condition [5] which cannot be targeted or resolved even with the most effective current therapies for asthma. Therefore, it is of utmost importance to completely understand the underlying molecular mechanisms that initiate, drive and maintain airway remodeling in asthma.

The internal milieu of asthmatic airways is highly heterogeneous due to the presence of a plethora of cytokines, chemokines and growth factors released by the inflammatory cells and structural components of the affected airways [6,7]. Transforming growth factor (TGF)-β is a pleiotropic mediator which functions via SMAD- and non-SMAD-dependent pathways [8-10] and is involved in diverse biological functions in the lungs, including immune cell regulation, cellular differentiation and proliferation and regulation of angiogenesis [11]. Enhanced abundance of TGF-β is found in the bronchoalveolar lavage (BAL) fluid and lungs of asthmatic subjects [12-14]. Interestingly, a single nucleotide polymorphism in human SMAD3 gene is associated with asthma in a large scale genomewide association study [15]. Of note, SMAD3-deficient mice are shown to be protected from allergen-induced airway remodeling [16] underlining a crucial role for TGF-β signaling in asthma pathology. While TGF-β abundance is contributed by almost all the structural and inflammatory cells, eosinophils constitute the major source of TGF-β in asthmatic lungs [17]. The TGF-β signaling is an integral part of asthma pathophysiology and both SMAD-dependent and – independent signaling is suggested to drive TGF-β effects in asthmatic airways [10,18]. Additionally, interaction of TGF-β with other pathways such as WNT signaling is associated with various physiological and pathological functions [10,19]. Studies, including those from our lab, have revealed a cross-talk between TGF-β and canonical WNT signaling effector β-catenin in various physiological and pathological processes. For instance, a recent study from our group has shown that TGF-β-induced inactivation of glycogen synthase kinase-3 (GSK-3) stabilizes β-catenin, leading to expression of ECM proteins by ASM cells [20]. Fibroblasts from COPD patients express more β-catenin than healthy subjects and are more responsive to TGF-β-induced effects such as fibronectin expression [21]. β-Catenin signaling and TGF-β/SMAD signaling induce pulmonary fibrosis and are associated with epithelial-mesenchymal transition (EMT) in alveolar epithelial cells [22]. Large body of literature largely implicates canonical WNT signaling in conjunction with TGF-β in various lung disorders including airway remodeling as we have discussed in Chapter 2. The contribution of noncanonical WNT signaling, however, has not been established until now. This thesis aims to explore the contribution of the noncanonical WNT ligand WNT-5A in the development of TGF-β-induced airway remodeling. A novel role for WNT-5A in ASM cells is described in Chapter 3 and Chapter 4 and the molecular mechanisms governing TGF-β-induced expression of WNT-5A are described in Chapter 5. Chapter 6 provides insight
Chapter 8

into the global regulation of WNT signaling family during development of asthma in a mouse model of chronic airway inflammation and Chapter 7 provides a comprehensive review about the signaling and function of WNT-5A in human health and disease.

**TGF-β-WNT-5A crosstalk in ASM: a novel axis regulating ECM expression**

WNT-5A has been implicated in a myriad of functions from flies to man spanning early embryonic to entire adult life (Chapter 7). While aberrant expression of WNT-5A has been implicated in several disorders such as malignant, inflammatory and fibrotic diseases (Chapter 7), its role in asthma pathobiology is unknown. A microarray analysis of endobronchial biopsies revealed that WNT-5A expression is highly correlated with the presence of high Th2 inflammation in asthmatics [23] suggesting a possible link of WNT-5A with asthma. Confirming that link, we identified increased WNT-5A mRNA and protein expression in primary ASM cells derived from asthmatics in comparison to healthy subjects (Chapter 3). Interestingly, TGF-β, which is highly expressed in asthmatic lungs, led to the induction of WNT-5A in an immortalized human ASM cell line (Chapter 3). Indeed, abnormalities in the ASM are key to the pathogenesis of asthma [24]. The ASM bundle is thickened in asthma and expresses more ECM and contractile proteins compared to healthy subjects [25-27]. Activation of TGF-β signaling induces expression of various ECM genes such as collagens, fibronectin, versican, plasminogen activator inhibitor-1 (PAI-1) by ASM cells [28], contributing to the proremodeling effects of TGF-β in the airways. The ECM profile of asthmatic airways differs from that of non-asthmatics with increased expression of specific collagens (I, III and V) and fibronectin among other ECM proteins in asthmatic airways [28,29]. Of note, siRNA-mediated knock-down of WNT-5A attenuated TGF-β-induced expression of collagen Iα1 and fibronectin (Chapter 3). Our study, thus, suggest that increased WNT-5A expression by TGF-β drives altered and enhanced ECM expression in ASM cells contributing to the airway remodeling (Chapter 3).

Increased abundance of WNT-5A in asthmatic ASM cells (Chapter 3) may result in enhanced downstream signaling driving ECM expression. WNT-5A, though predominantly noncanonical, can also evoke β-catenin signaling depending on the cell- and receptor-context. Indeed, analysis of lungs from a mouse model of allergen-induced chronic airway inflammation suggests increased β-catenin signaling [30] and β-catenin-dependent signaling can regulate various features of airway remodeling (Chapter 2). Does exaggerated WNT-5A abundance lead to increased β-catenin signaling in asthmatic lungs? We confirmed that WNT-5A doesn’t engage β-catenin signaling in ASM cells to drive ECM expression as the presence of Dickkopf-1, a canonical WNT signaling inhibitor, failed to reduce TGF-β-induced and WNT-5A-mediated ECM expression (Chapter 3). Similarly, WNT-5A knock-down didn’t alter TGF-β-stabilized β-catenin levels in ASM cells further confirming that β-catenin signaling is activated by TGF-β and not by WNT-5A. Instead of β-catenin-dependent signaling, WNT-5A activated c-Jun N-terminal kinase (JNK)- and Ca²⁺-dependent noncanonical WNT signaling to regulate TGF-β-induced ECM expression in ASM cells. This is in agreement with other reports implicating TGF-β-induced activation of Ca²⁺ and JNK signaling in the regulation of ECM protein expression [31,32]. We extend these observations by demonstrating that WNT-5A activates nuclear factor of activated T cells
(NFAT)c1, a calcium signaling-dependent transcription factor, and JNK in ASM cells. This provides novel functional insight into the role of noncanonical WNT-5A signaling in the regulatory mechanisms of ECM expression by ASM cells (Chapter 3).

WNT-5A can bind to a multitude of FZD and non-FZD receptors in various cell types to relay downstream effects (Chapter 7). We found that of the known WNT-5A receptors, TGF-β down regulates the expression of RYK, ROR2, FZD2, FZD4 and FZD5 whereas it up regulates FZD8 (Chapter 3). Of these receptors, FZD2 showed the highest basal expression levels followed by RYK. Notably, FZD8 was the WNT-5A receptor that showed highest induction in response to TGF-β. We demonstrate that FZD8 silencing decreased both collagen Iα1 and fibronectin whereas RYK knock-down only suppressed fibronectin expression in response to TGF-β. FZD2 knock-down, on the other hand, had no effect. Our data, thus, suggest a role for FZD8 and RYK in TGF-β-induced ECM expression which may function as WNT-5A receptors but their exact relationship needs to be determined (Chapter 3).

**Noncanonical WNTs partner with TGF-β: a link to airway remodeling**

Expanding the role for noncanonical WNT signaling in airway remodeling, we further identified a novel role for WNT-5A and WNT-11 in mediating TGF-β-induced α-smooth muscle actin (α-SMA) expression in ASM cells (Chapter 4).

TGF-β is a potent inducer of contractile proteins such as α-SMA, sm-MHC, Calponin and SM22 in ASM cells [33]. Moreover, WNT/β-catenin signaling in smooth muscle cells (SMC) is involved in various cellular functions involving differentiation and expression of contractile proteins. WNT-1 and WNT-3A activate β-catenin signaling inducing expression of proliferative regulator cyclin D1 in vascular SMCs [34,35] whereas WNT-7B-activated β-catenin signaling is required for proper development and differentiation of bronchial SMCs [30]. Similarly, TGF-β-activated β-catenin regulates pulmonary fibroblast differentiation and expression of α-SMA [21] whereas it partners with cAMP response element-binding protein (CREB)-binding protein (CREBBP or CBP) and regulates TGF-β-induced α-SMA expression in pulmonary epithelial cells [22]. Interestingly, WNT-3A augments TGF-β-SMAD2 signaling via β-catenin leading to the expression of α-SMA in mouse fibroblasts [36] further suggesting a crosstalk between these two pathways in the expression of contractile proteins.

We found that knock-down of either WNT-5A or WNT-11 attenuates TGF-β-induced α-SMA expression in ASM cells whereas stimulation with recombinant WNT-5A or WNT-11 is able to augment α-SMA expression (Chapter 4) demonstrating that WNT-5A and WNT-11 mediate TGF-β-induced α-SMA expression in ASM cells.

Increased expression of contractile apparatus is observed in asthma subjects [26]. Extending it to the noncanonical WNTs and ASM cells, we demonstrate that WNT-5A and WNT-11 can regulate contractile protein expression in ASM cells (Chapter 4). Both WNT-5A and WNT-11 are induced by TGF-β in ASM cells and we have earlier demonstrated that WNT-5A mediates TGF-β-induced ECM expression (Chapter 3). WNT-11 has earlier been shown to
regulate TGF-β-induced α-SMA expression in renal epithelial cells [37], we here confirmed the same in ASM cells and identify this novel function for WNT-5A in ASM cells.

Noncanonical WNT/Ca²⁺ and WNT/planar cell polarity signaling are involved in cytoskeletal reorganization and cell movements along with the transcriptional regulation of various genes. While there is no direct evidence implicating noncanonical WNT signaling in regulation of contractile proteins; NFAT and JNK, two of the mediators of noncanonical WNT signaling are known to regulate α-SMA expression. NFAT activation induces α-SMA expression in SMCs and inhibition of calcineurin-NFAT pathway attenuates it [38]. Similarly, JNK induces expression of α-SMA in response to mechanical strain [39] and arginine vasopressin [40] in vascular SMCs. Most importantly, the small GTPase RhoA, which mediates noncanonical WNT signaling and is also activated downstream of TGF-β signaling, is an integral part of SMC differentiation and expression of α-SMA [41]. We observed that WNT-5A and WNT-11 activate RhoA-dependent noncanonical WNT signaling in ASM cells. Recombinant WNT-5A and WNT-11 increased phosphorylation of myosin-binding subunit of myosin phosphatase 1 (MYPT1) indicating RhoA cascade activation in ASM cells (Chapter 4; data not shown). RhoA signaling has been linked to expression of contractile proteins including α-SMA in SMCs [42,43]. In line with that, pharmacological inhibition of RhoA signaling attenuated TGF-β-induced α-SMA expression in ASM cells (Chapter 4).

Noncanonical WNT signaling via small GTPases and the Ca²⁺ pathway regulates cytoskeletal remodeling and stress fiber formation to promote cell migration. Studies including those from our group have showed that inhibition of actin remodeling by latrunculin A or B inhibits contractile protein expression [42,44]. In Chapter 4, we demonstrate that WNT-5A and WNT-11 promote actin remodeling augmenting polymerized filamentous actin (F-actin) abundance with a concomitant decrease in monomeric globular actin (G-actin) in a RhoA-dependent manner. Moreover, inhibition of TGF-β-induced actin stress fiber formation by latrunculin A attenuates α-SMA expression suggesting that TGF-induced and noncanonical WNT-mediated actin dynamics are linked to the transcriptional control of α-SMA.

The smooth muscle cell (SMC)-specific genes essentially contain CArG box DNA elements [CC(A/T)₆GG] in their promoters which are regulated by serum response factor (SRF) in association with proliferative ternary complex factors or with myocardin family of transcription factors (myocardin and myocardin-related transcription factors) driving contractile program [45]. While myocardin is constitutively nuclear, myocardin-related transcription factors (MRTFs) remain associated with G-actin and stay primarily cytosolic. Actin remodeling depletes the G-actin pool leading to the release of MRTFs and their nuclear translocation where they associate with SRF and other transcriptional co-regulators to activate target gene transcription [46,47]. TGF-β has been shown to regulate the Rho-actin-MRTF axis [46,48-50], however, a direct link between noncanonical WNTs and MRTF-SRF signaling is undocumented. We show that TGF-β induces expression and nuclear translocation of MRTF-A in ASM cells where it drives TGF-β-induced α-SMA expression. Of note, inhibition of Rho kinase or knock-down of WNT-11 attenuates TGF-β-induced nuclear
localization of MRTF-A validating a RhoA-dependent and WNT ligand-mediated axis in MRTF-A nuclear shuttling. We provide the first evidence for the regulation of Rho kinase-actin-MRTF axis by noncanonical WNT ligands (Chapter 4).

The TGF-β-induced and noncanonical WNT-mediated regulation of Rho-actin-MRTF-A axis may have important implications in various processes in airway remodeling. For instance, as EMT is considered a contributing factor to the increased mesenchymal cell population in asthmatic airways, the TGF-β and WNT-5A, WNT-11 crosstalk might contribute to this process. Indeed, MRTF-A is a critical mediator of TGF-β-induced EMT [49,51] and epithelial-to-myofibroblast transition (EMyoT) [50]. Myofibroblasts are a rich source of ECM proteins and MRTF-A is a key mediator of myofibroblast activation and can regulate ECM expression. As demonstrated, MRTF-A induces collagen expression in lung fibroblasts [52]. Thus, the TGF-β-noncanonical WNTs axis can further potentiate ECM expression in airways via MRTF-A activation in fibroblasts and myofibroblasts whereas it might also serve as an additional mechanism of ECM expression by ASM cells. Also, it may promote myofibroblast differentiation augmenting the fibrotic features in asthmatic airways.

Bronchoconstriction is an important denominator in airway remodeling as inhibition of bronchoconstriction can ameliorate markers of airway remodeling in asthma [53]. Bronchoconstriction-activated RhoA-actin-MRTF-A signaling could be the underlying mechanism regulating markers of airway remodeling in asthma. Indeed, cellular mechanosensing and mechanotransduction in ASM cells can have profound implications on asthma [54]. Mechanical force activates RhoA-MRTF-A signaling and α-SMA expression [55] and inhibition of mechanotransduction by blocking the Rho-MRTF-A signaling or deficiency of MRTF-A ameliorates experimental pulmonary fibrosis in mice [56]. It is important to note that mechanical forces can activate TGF-β, probably via integrin signaling [44,54]. As such TGF-β could be an important target and mediator of force-induced processes. Similarly, mechanical forces can also be linked to the activation of canonical WNT signaling via YAP and TAZ which are regulated by mechanotransduction [57,58]. The effect of mechanotransduction on noncanonical WNT signaling, however, is not known. Considering the link between TGF-β and WNT-5A, WNT-11 expression, it is tempting to speculate a role for noncanonical WNT signaling in mechanical force-induced effects in airway remodeling. Thus, an intricate crosstalk between mechanical forces, TGF-β and noncanonical WNTs leading to actin-RhoA-MRTF-A axis activation can be envisaged that can modulate the pathophysiology of airway remodeling in asthma.

The evil axis in airway remodeling: Molecular mechanisms regulating TGF-β-induced expression of WNT-5A

In this thesis, Chapter 3 shows that WNT-5A expression is augmented in asthmatic primary ASM cells and is upregulated by TGF-β in immortalized ASM cells. Of note, we showed that WNT-5A plays important role in airway remodeling by regulating ECM expression and α-SMA expression in ASM cells (Chapter 3 and 4). Thus, regulation of WNT-5A by TGF-β is critical for our more defined understanding of the TGF-β-WNT-5A axis in airway remodeling.
In Chapter 5, we describe a signaling cascade consisting of TGF-β-activated kinase 1 (TAK1), β-catenin and Sp1 that regulates WNT-5A expression, thus providing an understanding of the mechanisms governing WNT-5A homeostasis. We demonstrate that TAK1 activity is required for WNT-5A expression in response to TGF-β stimulation and provide evidence for the involvement of β-catenin in this process, which, in turn, is regulated by TAK1 signaling. We further identify Sp1 as the transcription factor for WNT-5A in ASM cells and demonstrate its functional interaction with β-catenin. We show that Sp1 is recruited to the WNT-5A promoter in response to TGF-β, a phenomenon regulated by TAK1.

We also demonstrate that TAK1 mediates TGF-β-induced activation of p38 and JNK MAPKs and provide evidence for the direct involvement of p38 and JNK signaling in WNT-5A induction in ASM cells. MAPKs including p38 and JNK are downstream effectors of TAK1 in many cell types [59].

β-Catenin, the canonical WNT signaling effector, constitutes an important component in TGF-β signaling in ASM cells [10]. Our group has previously identified important physiological and functional roles for β-catenin in ASM cells such as regulation ECM expression, cell proliferation and contraction [20, 60-62]. In Chapter 5, we describe a previously unidentified role for β-catenin in WNT-5A induction. Silencing of β-catenin reduced TGF-β-induced WNT-5A induction. In addition to that, transient transfection of the degradation resistant S33Y-β-catenin mutant in ASM cells raised the basal WNT-5A protein abundance underlining the importance of β-catenin in WNT-5A induction. Remarkably, the presence of canonical WNT-3A also modestly augmented WNT-5A transcription, raising the possibility that β-catenin stabilization constitutes a primary phenomenon in WNT-5A expression in ASM cells.

In this thesis, we demonstrate that TGF-β-induced WNT-5A also mediates ECM production in ASM cells (Chapter 3) whereas another report from our group shows that β-catenin is required and sufficient for ECM production in ASM cells, even in the absence of TGF-β [20]. Noncanonical WNT-5A signaling has been shown to antagonize β-catenin-T-cell factor-Lymphoid enhancer-binding factor-1 (β-catenin-TCF-LEF) signaling via TAK1-nemo-like kinase (NLK) pathway in a cell- and receptor-specific manner [63,64] whereas studies in pancreatic cancer cells have demonstrated WNT-5A-mediated increase in β-catenin activation [65,66]. Of note, we observed that TGF-β-induced β-catenin stabilization is WNT-independent, as neither silencing of WNT-5A nor inhibition of WNT ligand secretion by IWP2 could alter TGF-β-induced β-catenin abundance in ASM cells (Chapter 3). How β-catenin and WNT-5A then regulate TGF-β-induced ECM expression? In Chapter 5, we address this intriguing issue as we identify an unanticipated but functional explanation validating β-catenin as an upstream mediator of WNT-5A induction in ASM cells. Thus, the WNT-independent TGF-β-induced β-catenin probably functions via WNT-5A, regulating ECM expression in ASM cells.

We also address the core transcriptional pathway involved in WNT-5A regulation in Chapter 5. The WNT-5A gene generates two very identical transcripts by utilization of alternative transcription start sites and the corresponding upstream sequences are termed as promoter A and B [67,68]. Both the promoters have comparable transcriptional potential;
their activity, however, is highly context dependent. However, WNT-5A promoter A has been suggested to be more active in human and murine fibroblasts as compared to promoter B [68]. CUTL1 [66], STAT3 [69], TBX1 [70] and NFκB [71,72] have previously been reported as transcription factors for WNT-5A in various cell types. Our in silico analysis of WNT-5A promoters revealed multiple putative transcription factor binding sites on both the promoters. Multiple Sp1 transcription factor binding sites appeared in the WNT-5A promoter screen. The presence of Mithramycin A, a selective inhibitor of Sp family of transcription factors [73-75], totally abrogated TGF-β-induced WNT-5A induction confirming a vital role for Sp1 in this process. Chromatin immunoprecipitation analysis further validated Sp1 occupancy of WNT-5A promoter which is enhanced by TGF-β via TAK1.

Both the β-catenin and Sp1 can associate with various transcription factors and co-activators in cell- and stimulus-dependent manner to mediate their cellular responses. However, the interaction between β-catenin and Sp1 has been shown to be counteractive and indirect. For instance, constitutive activation of WNT/β-catenin signaling in mouse brain represses Sp1 target gene expression via up regulation of Sp5, a Sp1 repressor protein [76]. On the other hand, Sp1 antagonizes β-catenin signaling by enhancing expression of E-cadherin which sequesters β-catenin to the membrane [77]. In Chapter 5, we report a previously undetected interaction between Sp1 and β-catenin in ASM cells which is further promoted by TGF-β suggesting a positive functional role in TGF-β cellular responses. TAK1 constitutes an integral part of cellular responses to genotoxic stress. TAK1 activity and expression has also been correlated with various cancers [78]. For instance, prolonged TAK1 activation participates in chronic inflammation and tumor progression in lung cancer [79] and has been shown to mediate TGF-β-induced breast cancer invasion and lung metastasis [80]. In ASM cells, TAK1 mediates ASM phenotype and cigarette smoke-induced inflammation. A study from our group has shown that TAK1 mediates PDGF induced activation of ERK1/2, leading to ASM cell proliferation and reduction in contractile proteins [81]. The authors, Pera et al (2012), also identified a pro-inflammatory role for TAK1 wherein it mediates cigarette smoke-induced release of interleukin (IL)-8 in ASM cells [82]. Interestingly, WNT-5A is a key player in pro-inflammatory responses in both the immune and non-immune cells (Chapter 7). For instance, WNT-5A is induced by LPS/interferon γ (IFNγ) in human macrophages where it mediates release of pro-inflammatory cytokines IL-8, IL-6, IL-1β and macrophage inflammatory protein-1β [83]. Similarly, WNT-5A induces macrophage activation and release of IL-8 and CXC chemokines in human monocytes [84]. Of note, WNT-5A also mediates pro-inflammatory responses in human aortic endothelial cells, a non-immune class of cells [85]. Our current findings correlating TAK1 activity and WNT-5A expression provide evidence for their close interaction to mediate pro-inflammatory reactions.

In this thesis we provide mechanistic insight intertwining TAK1, β-catenin and Sp1. Observations described in Chapter 5 suggest that TAK1 regulates TGF-β-induced WNT-5A expression by two simultaneous but linked mechanisms – 1) it augments expression of β-catenin which, in turn, partners with Sp1, perhaps, finalizing a transcriptional complex and
2) it promotes the binding of Sp1 transcriptional complex to WNT-5A promoter thereby allowing WNT-5A transcription.

**WNT pathway in Asthma: insights from a mouse model of allergen-induced chronic airway inflammation**

Activation of WNT signaling has been reported in several fibro-proliferative disorders. For example, there is increased expression of WNT signaling pathway genes and increased nuclear abundance of β-catenin within the fibrotic areas in fibroproliferative diseases of kidney, liver and bone [86-89]. Moreover, there is increased expression of WNT signaling pathway genes (WNT-1, WNT-7B, WNT-10B, FZD2, FZD3, β-catenin and LEF1) and induction of nuclear β-catenin expression in idiopathic pulmonary fibrosis patients [90,91], supporting a comprehensive role for WNT signaling genes in fibroproliferative disorders in the human body, including those of the lung.

Additionally, recent studies have provided some evidence about the possible involvement of WNT signaling in asthma. A study from our lab has reported key role for β-catenin in TGF-β-induced expression of ECM proteins in ASM cells implicating a cross-talk between TGF-β and canonical WNT signaling to regulate airway remodeling [20]. While **Chapter 2** discusses key roles for canonical WNT signaling effector β-catenin in airway remodeling, a direct link between canonical or noncanonical WNT signaling with asthma is not known. Recent studies indicate increased expression of WNT signaling pathway members in airway epithelium and endobronchial biopsies of asthma patients, including WNT-5A, although in those studies the cellular localization and functional roles of these WNT ligands were not determined [23,92]. In **Chapter 3**, we show that WNT-5A expression is increased in ASM cells derived from asthmatic patients in comparison to healthy subjects. WNT-5A is also overexpressed in fibroblasts of patients with usual interstitial pneumonia [93]. In these cells, WNT-5A regulates cell proliferation, resistance to cellular apoptosis and fibronectin protein expression. More importantly, we show that WNT-5A regulates ECM expression and contractile protein expression in ASM cells. Thus, higher expression of WNT-5A in asthmatic ASM cells indicates that WNT-5A may play important role in the remodeling of airways.

WNT signaling is a complex network comprising of large number of members from WNT ligands to their receptors, intracellular and extracellular mediators and modulators (**Chapter 1**). In view of the discussion in **Chapter 2** and the evidence presented in **Chapter 3 and 5** about crucial roles for WNT-5A and noncanonical WNT signaling in airway remodeling in asthma, we decided to expand our investigation to probe the alterations in the entire WNT pathway in asthma (**Chapter 6**) using a mouse model of allergen-induced chronic airway inflammation.

In **Chapter 6**, we present evidence for a wide range modulation of WNT signaling pathway components in lungs obtained from the mouse model of allergen-induced chronic airway inflammation. WNT ligands exhibit extensive up and downregulation in expression, with significant downregulation of WNT-7A, WNT-9A and WNT-10B in the OVA-challenged group in comparison to the saline-challenged control group. Similarly, Frizzled receptors and other components of WNT pathway examined also showed widespread modulation in
expression post-allergen challenge. Importantly, basal expression of WNT-5A was one of the highest in mice lungs with a trend towards upregulation post-allergen challenge. WNT-5A is a target of TGF-β as we observed in ASM cells (Chapter 3 and 5). However, in contrast to the effects of TGF-β in ASM cells, the lack of a specific TGF-β signature on WNT ligands and Frizzled receptor expression in the lungs obtained from mouse model could be attributed to the use of whole lungs instead of isolated compartments in this analysis. The effect of TGF-β on WNTs in ASM might be different from its effect in epithelial cells or fibroblasts.

WNT inhibitory factor 1 (WIF1) is an evolutionary conserved protein that can bind to and antagonize WNT signaling. WIF1 has been shown to bind to WNT-3A, -4, -5A, -7A, -9B and -11 via its WIF domain with varying affinities (WNT-5A>WNT-9B>WNT-11>WNT-4>WNT-7A>WNT-3A) [94]. WIF1 binds to WNT ligands via its WIF domain and tethers this WIF1-WNT complex in the extracellular matrix by its interaction with heparan sulphate proteoglycan (HSPG)-Glypican via EGF-like domains [95,96]. Indeed, glypicans can have modulatory effects on WNT signaling [97] and formation of this WNT-WIF1-Glypican complex is required for complete WNT antagonizing activity of WIF1 [95]. Chapter 6 demonstrates a significant decrease in the expression levels of WIF1 in the lungs of allergen-challenged group as compared to the saline-challenged group. This observation is particularly important as single nucleotide polymorphisms in WIF1 associate with lung function in two cohorts of asthma patients (14). Moreover, WIF1 is a target of bone morphogenetic protein 4 (BMP4)-SMAD1 signaling in developing lungs where it antagonizes WNT/β-catenin signaling and regulates lung morphogenesis by fine tuning WNT and BMP signaling. Abrogation of WIF1 expression leads to severe fetal lung abnormalities, in part, due to hyperactivation of WNT/β-catenin signaling [98]. Accordingly, a study has demonstrated downregulation of SMAD1 and WIF1 expression during the saccular stage of lung development in a mouse model of congenital diaphragmatic hernia, leading to the retardation of lung morphogenesis and appearance of hypoplastic lung [99]. Owing to the crucial role of WIF1 in WNT signaling regulation, suppression of WIF1 is often associated with malignancies such as prostate, breast, lung, and bladder cancer [100].

Chapter 6 demonstrates that TGF-β suppresses WIF1 expression in ASM cells. Since WIF1 can antagonize WNT-5A signaling, the downregulation of WIF1 by TGF-β could be an important mechanism for allowing maximum WNT-5A signaling to drive airway remodeling. Furthermore, low WIF1 expression would also permit enhanced WNT signaling in asthmatic lung irrespective of WNT ligand expression level due to loss of functional antagonism. It might also augment basal WNT signaling in the allergen-challenged lungs with detrimental outcomes. However, the functional relevance of WIF1 downregulation in asthma is not known and currently under investigation.

Epigenetic mechanisms might contribute to the TGF-β-induced downregulation of WIF1. Promoter hypermethylation is associated with WIF1 suppression in lung [101], breast cancer [102] and astrocytomas [103]. Moreover, a study has demonstrated that microRNA-374a can target WIF1 thereby limiting its expression in breast cancer cells [104]. The precise mechanism of TGF-β-induced downregulation of WIF1 needs further investigation.
Chapter 8

Thus, our investigation presented in Chapter 6 shows that the WNT signaling pathway is extensively modulated in asthmatic lungs with TGF-β targeting WNT antagonist WIF1 which might allow increased WNT signaling driving the pathological manifestations of asthma.

Future perspectives

WNT-5A is increased in various fibrotic, malignant and inflammatory disorders and has been shown to regulate cell survival, proliferation and migration. In this thesis, we identify novel roles for WNT-5A and demonstrate that it mediates expression of ECM proteins and contractile proteins in ASM cells. Increased WNT-5A abundance in the asthmatic ASM cells could be of wider significance, regulating various other feature of ASM cell biology and thus airway remodeling. ASM hyperplasia is considered to contribute to the increased ASM mass as observed in remodeled airways in asthmatics whereas migration of ASM has recently been proposed as another factor contributing to it. WNT-5A has been shown to regulate both the cell proliferation and migration. In addition, WNT-5A can also regulate the wide ranging immunomodulatory functions of ASM cells in asthmatic airways. Moreover, WNT-5A contributed by ASM cells could also act on other cells such as pulmonary fibroblasts, immune cells and airway epithelial cells modulating their functions and activities and augmenting proremodeling effects in asthmatic airways. Thus, work presented in this thesis highlights previously unidentified WNT-5A functions in ASM cells. Further studies are warranted to identify the comprehensive functions of WNT-5A in ASM cells as well as in other cellular compartments of the lungs to provide the much needed insight into the entire range of WNT-5A functions. Most importantly, in vivo studies using the WNT-5A transgenic or knock-out mouse models of allergen-induced chronic airway inflammation are needed to validate the regulatory role of WNT-5A in asthma.

Another important contribution of this thesis is to demonstrate extensive modulation of the entire WNT pathway in vivo using the mouse model of allergen-induced chronic airway inflammation. Of note, the downregulation of WIF1 in the lungs of allergen-challenged mice suggest a crucial role for WNT signaling in the pathophysiology. It is tempting to speculate that loss or low WIF1 may lead to augmented WNT signaling by various WNT ligands even at low expression levels. Interestingly, TGF-β suppresses WIF1 expression whereas augments WNT-5A and WNT-11 expression. Both WNT-5A and WNT-11 are targets for WIF1 antagonism and as we presented in this thesis, they can regulate key functions in airway remodeling. Further studies, therefore, are warranted for identifying the functional role of WIF1 in asthma pathophysiology. Above all, in vivo studies using the knock-out or transgenic mouse model would be valuable to ascertain the therapeutic value of WIF1 in asthma.

Furthermore, this thesis describes a novel signaling cascade comprising of TAK1, β-catenin and Sp1 in TGF-β-induced WNT-5A expression in ASM cells. We deduce the molecular pathway regulating WNT-5A expression which can have implications in various physiological and pathological situations involving WNT-5A. Moreover, our study also provides mechanistic insight into WNT-5A regulation which, perhaps, has a much wider applicability extending to other cell- and tissue-types and processes involving these factors. Interestingly, therapeutic tools targeting TAK1 [78] and Sp1 [105] are available whereas small...
molecule inhibitors for β-catenin [106] and WNT-5A [107] with therapeutic potential are fast emerging.

Studies presented in this thesis, thus, not only shed light on the novel regulatory mechanisms involved in airway remodeling in asthma but also provides multiple therapeutic targets which could be utilized to devise effective treatment strategies for wide array of diseases involving WNT-5A signaling.

**Main Conclusions**

In conclusions, this thesis identifies a novel role for WNT-5A in regulating airway remodeling in asthma and highlights the importance of WNT signaling in asthma pathobiology (Figure 1).

Following main conclusions could be derived from studies present in this thesis:

- Growth factor-activated β-catenin is an important mediator of features of airway remodeling in asthma and presents a promising therapeutic target (Chapter 2).
- TGF-β exerts wide modulatory effects on the expression of WNT ligands promoting the abundance of WNT-5A, WNT-5B and WNT-11 suggesting a shift to noncanonical WNT signaling in ASM cells by TGF-β (Chapter 3).
- WNT-5A is increased in abundance in primary ASM cells derived from asthmatic subjects suggesting a crucial pathological role for WNT-5A in asthma (Chapter 3).
- TGF-β induces WNT-5A expression in ASM cells where it mediates collagen Iα1 and fibronectin expression. WNT-5A engages noncanonical WNT signaling mediated by Ca²⁺-NFATc1 and JNK cascades to regulate ECM expression. FZD8 and RYK, the WNT-5A receptors, mediate these effects on ECM induction in ASM cells (Chapter 3).
- Noncanonical WNT-5A and WNT-11, both targets of TGF-β in ASM cells, regulate α-SMA expression. WNT-5A and WNT-11 engage a noncanonical RhoA-ROCK-dependent signaling leading to actin remodeling and nuclear translocation of MRTF-A which, in turn, regulates α-SMA expression in ASM cells (Chapter 4).
- TGF-β induces TAK1-dependent p38 and JNK activation leading to WNT-5A expression in ASM cells. Sp1 is the transcription factor for WNT-5A in ASM cells where it binds to the WNT-5A promoter in a TGF-β-induced and TAK1-dependent manner (Chapter 5).
- β-Catenin is an upstream regulator of WNT-5A expression in ASM cells and interacts with Sp1 to regulate WNT-5A expression (Chapter 5).
- The WNT signaling pathway shows extensive modulation in the lungs of a mouse model of chronic allergic airway inflammation. WIF1, a WNT antagonist, is downregulated suggesting that WNT pathway activation is a core mechanism underlying asthma pathogenesis. TGF-β represses WIF1 expression in ASM cells further supporting the crucial regulatory roles for TGF-β and WNT signaling in asthma pathophysiology (Chapter 6).
Taken together, the studies described in this thesis suggest that WNT-5A is a crucial mediator of airway remodeling in asthma. TGF-β-WNT-5A axis is a key regulator of this pathophysiology where WNT-5A mediates ECM and contractile protein expression in ASM cells. Furthermore, targeting WNT-5A expression by inhibiting its upstream regulators such as β-catenin, TAK1 or Sp1 ameliorate TGF-β-induced feature of airway remodeling.

Figure 1. Functional contribution of WNT-5A in TGF-β-induced airway smooth muscle cellular responses. (A) TGF-β binding to its receptors activates TAK1 which, in turn, activates MAPKs-JNK and p38 leading to the stabilization and increase in cytosolic abundance of β-catenin. Subsequently, β-catenin interacts with Sp1 to form a transcriptional complex which binds to the WNT-5A promoter and drives WNT-5A expression. (B) WNT-5A mediates TGF-β-induced ECM. WNT-5A signals through FZD8 receptor leading to the activation of JNK and Ca\(^{2+}\) signaling. Increased intracellular Ca\(^{2+}\) leads to nuclear translocation of NFATc1. Together JNK and Ca\(^{2+}/NFATc1\) signaling leads to expression of ECM genes. WNT-5A also regulates ECM expression, in part, via RYK by an unidentified mechanism. (C) WNT-5A mediates TGF-β-induced α-SMA expression. WNT-5A binding to FZD leads to the activation of RhoA-ROCK signaling which induces actin treadmilling thereby depleting G-actin pool and increasing F-actin. This leads to the release of MRTF-A from G-actin which translocate to the nucleus and activates α-SMA expression, presumably, in association with SMAD3 (not shown). In addition to WNT-5A, WNT-11 also mediates
TGF-β-induced α-SMA induction by same mechanism (not shown). TGFβR, TGF-β receptor I and II; TAK1, TGF-β activated kinase; JNK, c-Jun N-terminal kinase; Sp1, specificity protein 1; FZD, frizzled; NFATc1, nuclear factor of activated T cells c1; ECM, extracellular matrix; G-actin, globular actin; ROCK, Rho-associated kinase; MRTF-A, myocardin related transcription factor-A; α-SMA, α-smooth muscle actin
Chapter 8

References


83. Pereira C, Schaer DJ, Bachli EB, Kurrer MO, Schoedon G. (2008) Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for


