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General introduction

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1.1 Asthma

Asthma is a heterogeneous chronic obstructive disease of the airways inflicting approximately 300 million people worldwide and imposing a substantial burden on patients and the healthcare system [1]. A precise definition and diagnosis of asthma is still unclear, mainly due to the existence of a number of different asthma phenotypes such as allergic, non-allergic, nocturnal and occupational asthma. Often, these phenotypes co-exist and act synergistically in patients albeit with different underlying mechanisms. The Global Initiative for Asthma (GINA) has defined asthma as "a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation causes an associated increase in the airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing particularly at night or in early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment" [2].

Asthma is a multifaceted manifestation of (epi)genetic and environmental factors that contribute to the evolution of the disease from childhood and often actively regulate the course of disease and its management in later stages. Allergic asthma, in particular, frequently starts in early childhood and can be identified by the allergen-specific symptoms with a positive skin prick test and presence of allergen-specific serum IgE along with other respiratory symptoms such as wheezing and cough [3].

Exposure to the inhaled stimuli such as allergens or respiratory viruses triggers exaggerated response in asthmatic airways inducing airway constriction leading to episodes of breathlessness and wheezing. Asthma can be effectively managed in mild asthma patients using short-acting β₂ adrenoreceptor agonists alone (e.g. Albuterol) and if needed using a corticosteroid (e.g. Fluticasone, Budesonide), with or without a long-acting β₂ adrenoreceptor agonist (e.g. Salmeterol, Formoterol) thereby providing substantial relief from the episodic breathlessness. However, despite the most effective current therapies, a subset of severe asthma patients remain poorly controlled even at the highest doses of asthma medication [4,5]. Increasing our understanding of asthma pathophysiology and contribution of its various components to the disease severity and management by current therapies would help develop new drugs that target the patient subsets more effectively.

1.2 Pathophysiology

Asthma is characterized by the presence of chronic airway inflammation, airway hyperresponsiveness (AHR), reversible airflow obstruction, extensive structural changes in the airways termed as airway remodeling and decline in lung function in severe disease [6]. The internal milieu of asthmatic airways is highly heterogeneous due to presence of plethora of cytokines, chemokines and growth factors released by the inflammatory cells and structural components of the affected airways [7,8]. Thus, asthma has an intricate multicomponent pathophysiology. Some of the key features are discussed here.


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1.2.1 Airway Inflammation

Chronic inflammation in asthmatic airways is characterized by the presence of activated allergen-specific type 2 T helper (Th2) cells, eosinophils, mononuclear cells such as lymphocytes and macrophages and IgE production [6,9,10]. Bronchial provocation by inhaled allergens leads to an asthmatic response in patients which is divided into two phases—early and late asthmatic reaction. The early reaction, as the name suggests, is an immediate response to the allergen, driven by local mast cell activation, release of mediators and prompt decrease in bronchial airflow constituting as an acute asthmatic attack. The early phase is followed by a more severe late asthmatic reaction which is driven by the infiltration of inflammatory cells with persistent decline in bronchial airflow for prolonged periods [10]. Many of these inflammatory mediators also worsen development of AHR in asthmatics.

The onset of the chronic inflammation is believed to result from inappropriate Th2 responses to common environmental agents and is maintained in later stages by a complex interplay of the host immune and structural components. A plethora of cytokines and chemokines have been implicated in asthmatic inflammation [8,11]. Most abundant among them are Th2-derived cytokines such as Interleukin (IL)-4, -5, -9, -13 and -25. IL-4 and IL-13 are crucial for driving IgE production whereas IL-5 drives eosinophilic inflammation. IL-9 and IL-13 are involved in AHR [12].

Type 2 polarization of CD4+ T-helper cells is influenced by two prominent cytokines - IL-33 and thymic stromal lymphopoietin (TSLP) which are contributed primarily by airway structural cells [13-17]. Increased expression of IL-33 has been shown in endobronchial biopsies and bronchoalveolar lavage (BAL) fluid of asthmatics [13,14]. Further, deficiency of the IL-33 receptor - ST2 impedes the development of Th2 responses as demonstrated in a mouse model of pulmonary granuloma [18] whereas blocking of IL-33 using a soluble receptor (ST2) isoform attenuates the release of Th2 cytokines from splenocytes obtained from allergen-challenged mice [19] underlining the importance of IL-33 in Th2 polarization in allergic airway inflammation. Similarly, TSLP abundance is increased in asthma [17,20,21] and a single nucleotide polymorphism in the TSLP gene has been associated with asthma susceptibility [22,23] underlining its vital role in asthma pathophysiology. Lung epithelium-specific transgenic expression of TSLP under the surfactant protein C (Sp-C) driver induces airway inflammation and AHR with production of Th2-specific cytokines and increased serum IgE levels [24]. In line with its role in asthma, deficiency of the TSLP receptor protects against the development of allergic airway disease [24,25], primarily due to an impaired Th2 response against inhaled allergens [25].

In addition to the type 2 polarized T helper cells, other subsets of T cells have recently been identified as new players in asthma pathology. Th17 is a subset of CD4+ T cells, primarily derived in response to IL-23 but can also be generated by TGF-β and IL-6 stimulation [26,27]. Th17 cells release IL-17 which can recruit neutrophils either directly by IL-8 production or indirectly via release of colony stimulating factors and other peptides [26]. Neutrophilic inflammation has been linked with fixed airflow obstruction in severe asthma [28], sudden-onset fatal asthma [29], occupational [30] and nocturnal asthma [31].
Additionally, IL-17 can also promote release of profibrotic cytokines such as TGF-β, IL-11 and IL-6 from eosinophils [32] and fibroblasts [33] aggravating airway remodeling.

Regulatory T cells (Tregs) have recently been associated with asthma pathology [12]. Reduced number of Tregs have been reported in the BAL fluid from children with asthma as compared to healthy subjects [12,34]. Interestingly, the percentage of Tregs in BAL correlated positively with forced expiratory volume in one second (FEV1) in asthmatic children underlining a crucial role for Tregs in asthma pathobiology [34]. Tregs are a subset of CD4+ T cells which also express CD25. These CD4+ CD25+ T cells do not proliferate and produce cytokines. Of note, Tregs suppress the proliferation and inflammatory response by other T cells including Th2 cells [12]. In line with that, Tregs have been shown to suppress established AHR and airway inflammation in animal models of allergic airway disease [35]. Thus, reduced number of Tregs would lead to augmentation of T cell-driven inflammation in asthma.

1.2.2 Airway Remodeling

Airway remodeling is a hallmark pathological feature of individuals with asthma and is associated with airway obstruction [37], AHR [38] and declining lung function in severe disease [39]. The tissue repair response that is activated during remodeling is normally associated with lung development and response to tissue injury, where it is appropriate and regulated. However, aberrant airway remodeling, as observed in chronic airway diseases such as asthma and chronic obstructive pulmonary disease, is pathological and has detrimental consequences for the patient. Persistent airway remodeling along with chronic inflammation leads to compromised lung function [40]. Airway remodeling is associated with the severity of disease. For instance, in fatal asthma, the entire airway tree is massively remodeled whereas in non-fatal asthma, remodeling is less prominent and afflicts mainly small airways [40]. Similarly, the thickness of the remodeled airway wall also correlates with the severity of the disease [40-43]. Airway remodeling 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 [40] (Figure 1).

1.2.3 Reticular Basement Membrane Thickening

Reticular basement membrane (RBM) thickening is a predominant feature in asthma and contributes to the airway wall thickening mainly by deposition of extracellular matrix proteins such as collagen I, III and IV and laminins [40]. Endobronchial examinations have revealed a tremendous increase in the thickness of the collagen layer below the airway epithelium. Contrasting reports exist about the impact of RBM thickening on airway function in asthma. While studies have suggested a correlation between airway distensibility and RBM thickening, other studies have found asthmatics without RBM thickening and non-asthmatics with RBM thickening [40]. The significance of this event in airway remodeling and asthma needs further investigation.
Figure 1. Airway remodeling in asthma. (A) Schematic representation of cross-sectional view of the healthy and asthmatic airways. Asthmatic airways show features of airway remodeling such as airway wall fibrosis, airway smooth muscle thickening, increased vasculature, epithelial thickening and increased presence of mucus in airway lumen. (B) Major biological events involved in airway remodeling. In asthmatic patients, exposure to various inhaled triggers such as viruses, allergens and environmental triggers leads to damaged epithelium with goblet cell hyperplasia, inflammation, airway smooth muscle (ASM) hypertrophy and hypertrrophy, fibroblast activation and altered extracellular (ECM) composition. (Image B is taken from Prakash YS (2013) [36].)
1.2.4 Vasculature

Neovascularization and expansion of existing airway vasculature is widely observed in airway remodeling. Morphometric analysis of postmortem lung tissues and endobronchial biopsies from asthmatics have shown multifold increase in total number of vessels and in the vascular area of the airways in comparison to healthy subjects and correlated to asthma severity [44]. Increased airway vasculature and associated hyperpermeability are believed to contribute to the clinical manifestations of asthma as both these alterations could increase tissue swelling thereby decreasing the airway dispensability and increasing the airway narrowing. Increased vasculature could also promote chronic inflammation, provide increased access of various pathological mediators to the airway components, thus, supporting airway remodeling. In addition, it could assist rapid and increased availability of various medications to the airways and fast-track the clearance of spasmogens supporting disease management but it could also promote rapid clearance of medication thereby hampering the therapeutic intervention [44].

1.2.5 Airway mesenchymal cells

Hypertrophy and hyperplasia of airway mesenchymal cells is an important feature of airway remodeling. Myofibroblasts and ASM cells constitute an important source of various cytokines and growth factors in airways and can contribute to the asthma pathophysiology [45-47]

1.2.5.1 Myofibroblasts

Myofibroblasts are specialized cells derived from the differentiation of either fibroblasts or smooth muscle cells and possess features of both the fibroblast and myocyte lineage and are basically associated with repair processes. Increased myofibroblast population is suggested in the asthmatic airways, particularly in the submucosa [45,48]. The source of myofibroblasts in asthmatic airways remains unclear but various growth factors such as TGF-β can induce differentiation of fibroblasts into myofibroblasts and hence, could contribute to the observed myofibroblast population in asthma [49,50]. In addition, myofibroblasts could be derived from circulating fibrocytes or from a less defined precursor already present in the asthmatic airways such as epithelial cells as they appear quickly in the airways post allergen challenge [47,51,52]. Myofibroblasts are a rich source of ECM proteins and as such can contribute to the fibrotic component of airway remodeling.

1.2.5.2 Airway smooth muscle cells

Increased ASM mass is a predominant feature of airway remodeling in asthma and correlates with the severity of disease [48,53,54]. Both hypertrophy and hyperplasia can contribute to the increased volume of ASM in asthmatics, although the relative contribution of individual processes remains unclear [53]. While a study observed two different phenotypes on analysis of ASM bundles in fatal asthma- one with predominant hyperplasia and another with predominant hypertrophy [53,55], another found only hyperplasia and no hypertrophy in the ASM layer of asthmatics [54]. Hyperplasia is believed to be a major source of increased
ASM mass in asthmatics. This could be, in part, due to increased proliferation or increased survival of existing ASM. Indeed, asthmatic ASM show increased proliferation in comparison to healthy ASM cells in vitro [56]. Similarly, ASM bundle in endobronchial biopsies from severe asthma patients show higher population of proliferative cells in comparison to the moderately asthmatic and healthy non-asthmatic subjects, providing an evidence for hyperplastic ASM cells in vivo [57]. Interestingly, migration of fibroblasts and myofibroblasts may also lead to the increased thickening of ASM bundle as demonstrated by the induced migration of mesenchymal cells towards the ASM bundle in response to chemotactic agents such as platelet derived growth factor (PDGF) and CC chemokine ligand 19 released by ASM and mast cells [58,59]. In addition, new ASM cells can also be derived from differentiation of mesenchymal stem cell (MSCs). Tissue-resident MSCs are a common feature of many tissues where they play critical roles in repair and regeneration. The role of lung resident MSCs, however, is unclear. Increased abundance of MSCs has been demonstrated in the lungs of mouse model of chronic airway inflammatory disease [60]. Interestingly, MSCs have been shown to attain a myofibroblast phenotype, however, a direct evidence for their contribution to ASM hyperplasia is absent [61].

Mesenchymal cells can also arise from the epithelial cells and fibroblasts [62] and may contribute to the increased ASM mass. Epithelial cells can transdifferentiate to lose adhesion and attain mesenchymal characteristics like the presence of vimentin and α-smooth muscle actin (α-SMA), enhanced motility and production of ECM, in a process known as epithelial-to-mesenchymal transition (EMT). TGF-β is considered as a master inducer of EMT in various organs including lung epithelial cells [63]. A study demonstrated that TGF-β can induce extensive EMT throughout the bronchial epithelium derived from the asthmatic subjects as compared to rather localized EMT specific to basal cells in non-asthmatic epithelium [64]. Another study provided in vivo evidence for EMT in a mouse model of allergic airway inflammation where intranasal administration of house dust mite (HDM) extract led to loss of epithelial markers and gain of mesenchymal markers in airway epithelial cells and their subsequent migration to the subepithelial regions [52]. While cellular transdifferentiation can generate mesenchymal cells, the relative contribution of such processes in increased ASM mass in airway remodeling requires further investigation.

Mathematical modeling studies have suggested that increased ASM mass is one of the key functional manifestations of airway remodeling in the asthmatic airways leading to airflow obstruction, assuming the force generated by the ASM bundle is proportional to its mass. Studies have indicated that asthmatic ASM cells contract with greater velocity and maximum shortening capacity in comparison to the healthy ASM cells [46,65,66]. This difference could be, in part, attributed to the increased abundance of various contractile proteins such as smooth muscle-nyosin light-chain kinase (sm-MLCK), smooth muscle-specific SM22 and smooth muscle-nyosin heavy chain (sm-MHC) in asthmatic ASM cells in addition to increased ASM mass [46]. Interestingly, bronchial thermoplasty has been shown to improve asthma control in patients [67] which might be attributed to the reduced ASM mass following this treatment. However, bronchial thermoplasty might also damage the neural circuits in the treated area. The relative contribution of damaged innervation and reduced ASM mass to these beneficial effects of thermoplasty remains unclear. Thus, multiple
mechanisms could contribute to the increase in ASM mass in asthmatics which can have tremendous implications for the pathophysiology of asthma.

Primarily involved in the maintenance and regulation of bronchial tone due to their contractile properties, ASM cells have emerged as a major source of proinflammatory and proremodeling factors [46,68,69]. For instance, ASM cells in culture release RANTES (regulated upon activation normal T-cell expressed and secreted) in response to tumor necrosis factor-α (TNF-α) [70] and eotaxin when stimulated with IL-1β, TNF-α or platelet-activating factor (PAF) [71-74]. Both RANTES and eotaxin are potent eosinophil chemoattractant contributing to the airway inflammation in asthma [75]. Similarly, stimulation of ASM cells with IL-1β, TNF-α and interferon γ (IFNγ) induces the release of granulocyte-macrophage colony-stimulating factor (GM-CSF) [76] and prostaglandin E2 (PGE_2) in culture [77]. Whereas PGE_2 is believed to have protective effects in allergen-induced airway responses and airway inflammation in asthma [78,79], GM-CSF promotes survival of eosinophils [80]. ASM cells are also an important source of Th2-polarizing cytokines TSLP [16,17] and IL-33 [13]. In addition, ASM cells can contribute plethora of other proinflammatory mediators such as IL-1β, IL-6, leukemia inhibitory factor and IL-8 thereby actively participating in the chronic airway inflammation in asthma [68]. Further, ASM cells are a rich source of various ECM proteins and matrix metalloproteases contributing to airway remodeling [46].

1.2.6 Extracellular Matrix

ECM is an intricate network of macromolecules composed of a variety of proteoglycans and fibrous proteins produced and deposited locally by various mesenchymal cells in the airway including fibroblasts and airway smooth muscle cells [81,82]. Altered and enhanced ECM protein deposition within and surrounding the smooth muscle bundle has been observed in asthmatic airways and contributes to the pathology [46,83,84]. Asthmatic airways show increased deposition of collagen (I, II and V), fibronectin, hyaluronan, biglycan, versican, tenascin and laminin α2/β2 whereas abundance of collagen IV, elastin and decorin is decreased [46,83]. Alterations in ECM composition in asthmatic airways denote the disruption of ECM homeostasis and may modify the mechanical and functional properties of embedded structural components such as airway smooth muscle cells [85-87].

1.3 TGF-β: key regulator of airway remodeling

TGF-β is a pleiotropic mediator involved in many biological functions in the lungs, including the regulation of inflammatory cells, the differentiation and proliferation of resident structural cells and regulation of angiogenesis [88]. Enhanced abundance of TGF-β is found in BAL fluid and lungs of asthmatic subjects [89-91]. While TGF-β is contributed by almost all the structural and inflammatory cells, eosinophils constitute the major source of TGF-β in asthmatic lungs [92]. Preformed and newly synthesized TGF-β can activate several pathways leading to both transcriptional and post-transcriptional regulation of factors that are involved in airway remodeling (Figure 2). Therefore, understanding TGF-β biology and mechanisms associated with its cellular effects is an important step in elucidating the
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pathophysiology of airway remodeling as well as for the development of novel therapeutic approaches.

A. The SMAD-dependent TGF-β signaling pathway

B. The SMAD-independent TGF-β signaling pathway
### 1.3.1 TGF-β Signaling

The TGF-β family comprises of three structurally related isoforms (β₁, β₂ and β₃) which share high sequence homology and have non-redundant but sometimes overlapping functions [94,95]. TGF-β is the most extensively studied isoform in airway remodeling and other diseases. Each isoform is synthesized as a large precursor molecule from its mRNA containing a signal peptide with a mature form of TGF-β at the C-terminal. On proteolytic cleavage of the signal peptide, mature TGF-β is secreted as an inactive homodimer bound to latency-associated peptide (LAP). The release of TGF-β from this inactive complex is a tightly regulated process and can be catalyzed via several mechanisms such as transient changes in pH, cleavage by proteases (integrin αβ₆, MMP-2 and -9, plasmin and calpains) or conformational rearrangements (thrombospondin). Active TGF-β exerts its cellular effects via a receptor complex comprised of three structurally related transmembrane serine/threonine kinase proteins TβRI, TβRII and TβRIII. The TGF-β homodimer binds to the receptor concluding a tetrameric complex along with TβRI and TβRII. TβRI phosphorylates TβRII in its cytosolic domain leading to its activation. The phosphorylated TβRI, in turn, initiates the intracellular signaling cascades by interacting with and phosphorylating SMAD proteins SMAD2 and 3. Phosphorylated SMAD2/3 form a heteromeric complex with SMAD4 and translocate to the nucleus where they bind to the SMAD-binding elements in the TGF-β-target promoters via their DNA-binding domains.

In addition to the activation of SMAD-dependent canonical signaling, TGF-β/TβR complex can also activate multiple SMAD-independent signaling cascades including MAPKs like...

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**Figure 2. The TGF-β signaling pathway.** Diagrammatic representation of the TGF-β signaling pathway. Binding of dimeric TGF-β ligand leads to the formation of a ternary heteromeric membrane complex composed of TGF-β and TGF-β receptors TβRI and TβRII with subsequent phosphorylation of TβRI by TβRII. TGF-β may activate SMAD-dependent or –independent signaling cascades downstream of receptor activation. (A) SMAD-dependent pathway. The activated receptor complex phosphorylates R-SMADs SMAD2 and SMAD3 which, in turn, form heteromeric complex with co-SMAD SMAD4. The R-SMAD-SMAD4 complex translocates to the nucleus and associates with the genomic SMAD-binding element (SBE) in a sequence-specific manner. Additionally, the R-SMAD–co-SMAD complex interacts with other transcription factors that can bind to distinct sequences adjacent to the SBE and allows for high-affinity binding to the SBE elements. SKI and SNO (also known as SKIL) are nuclear antagonists of SMADs. SMAD7, an inhibitory SMAD, antagonizes TGF-β signaling at the receptor level by inducing the degradation of TβRI and/ or by inhibiting phosphorylation of R-SMADs or it can inhibit the formation of the R-SMAD–co-SMAD complex. In addition to regulating transcription, TGF-β signaling can also participate in microRNA (miRNA) biogenesis by mediating the processing of primary miRNA into precursor miRNA in the nucleus in an R-SMAD-dependent and co-SMAD-independent process. 'mG' and 'AAAAA' represent 5′ capping and 3′ polyadenylation of mRNAs, respectively. (B) SMAD-independent pathway. The activated TGF-β-TβRI-TβRII complex transmits downstream signaling via activation of various pathways as depicted in the figure. (Images shown in panel A and B are taken from Akhurst and Hata (2012) [93])
extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal Kinase (JNK) and p38, TGF-β-activated kinase 1 (TAK1) and WNT/β-catenin signaling in various cell types including ASM cells [96,97]. Rapid activation of ERK by TGF-β has been observed in epithelial cells, breast cancer cells, airway smooth muscle cells and fibroblasts [97,98]. Autophosphorylation or Src-dependent phosphorylation of TβR-II allows the docking of Shc to the membrane which in turn recruits Grb2/Sos complex leading to Ras/MEK1/ERK cascade activation. Another mechanism is the tyrosine phosphorylation of TβR-I and activation of ShcA recruiting Grb2/Sos complex to the membrane and leading to the Ras-MAPK cascade activation [97].

In addition to ERK, TGF-β can also activate the p38 and JNK pathway in various cell types [97]. The activation of p38 and JNK is mediated by a complex TNF receptor-associated factor6 (TRAF6)-TAK1 cascade where it constitutes a key signaling event in TGF-β-induced apoptosis [99,100]. TAK1, first identified as a mitogen-activated kinase kinase kinase (MAP3K7) activated by TGF-β, is a critical regulator in inflammatory, immune and stress response signaling. TAK1 constitutes an integral part of IL1, TLR and TNF signaling, activating NFκB and MAPK pathways [101,102]. In TGF-β signaling, TAK1 interacts with TβR-I and is required for JNK and p38 activation. Mechanistically, TGF-β signaling activates the recruitment and subsequent Lysine 63-linked polyubiquitination of TRAF6. Polyubiquitinated TRAF6 interacts with and recruits TAK1 to the TβRI complex. Subsequently, TRAF6 ubiquitinates TAK1 at Lysine 34 triggering its activation which, in turn, leads to p38 and JNK activation [100] (Figure 2B).

1.3.2 TGF-β in airway remodeling

TGF-β exerts extensive immunomodulatory and profibrotic effects on the constituents of airways contributing to airway remodeling in asthma [103]. The key TGF-β-regulated features of airway remodeling are discussed below.

1.3.2.1 Epithelial Shedding

Damage and loss of epithelial integrity is observed in airway remodeling. Repeated allergen exposures and ensuing chronic inflammation in asthmatic airways may keep epithelium under duress. The presence of TGF-β in such circumstances can initiate p38 signaling leading to apoptosis and epithelial damage [104].

In addition to direct effects, TGF-β can also potentiate apoptosis by other pathways, for instance, TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is expressed by many structural and inflammatory cells in asthmatics such as fibroblasts, epithelial, endothelial cells, eosinophils and macrophages [105]. It is a pro-apoptotic protein and can induce both the extrinsic and intrinsic cell death pathways in cells expressing its receptors- TRAIL-RI and TRAIL-RII. TGF-β induces expression of TRAIL in epithelial cells and TRAIL can induce TGF-β expression suggesting a vicious apoptotic cycle in epithelial cells leading to extensive damage and airway remodeling [105].
1.3.2.2 Subepithelial Fibrosis

Enhanced ECM deposition under the airway epithelium is present in airway remodeling. TGF-β is a highly potent inducer of ECM proteins, mainly from cells of mesenchymal lineage which also crowd the subepithelial space in asthmatics. TGF-β induces proliferation and activation of fibroblasts [106,107] and increases their survival by anti-apoptotic mechanisms [108-110]. In addition, TGF-β promotes their differentiation into more active myofibroblasts. TGF-β can induce pulmonary fibroblast activation and myofibroblast differentiation by several mechanisms. For instance, it can limit the antagonistic cAMP response element-binding protein (CREB) phosphorylation via glycogen synthase kinase (GSK)-3 [111] or activate β-catenin signaling [107] leading to these effects. TGF-β can also induce the release of growth factors such as fibroblast growth factor-2 (FGF-2) and connective tissue growth factor (CTGF) [86], both of which can induce mesenchymal cell proliferation and the release of angiogenic mediators. In addition, CTGF can also induce ECM production, cell adhesion and migration [86]. Together with fibroblasts, myofibroblasts produce and deposit ECM proteins such as collagen, fibronectin and proteoglycans leading to the subepithelial fibrosis.

1.3.2.3 Airway smooth muscle cell remodeling

One of the most important features of ASM remodeling attributed to TGF-β is ECM production. TGF-β can induce expression of various ECM components like fibronectin, perlecan, collagen (I, II, III, IV, V), versican, elastin, CTGF and laminin (α1, β1, β2 and γ2) by ASM cells. It can also promote expression of MMPs and TIMPs (20, 21) by ASM cells thereby influencing ECM turnover. Altered composition of ECM modulates the mechanical properties of ASM bundle influencing the stiffness as well as transfer of force between the ASM bundle and surrounding tissues [46].

Additionally, TGF-β promotes ASM hyperplasia and survival by direct or indirect mechanisms. TGF-β induces activation of MAPKs ERK, p38 and JNK in ASM cells leading to cell proliferation [98] whereas it promotes cell survival via a p38/PI3K signaling axis [110]. ECM proteins induced by TGF-β such as fibronectin, collagen and CTGF also have promitogenic and prosurvival effects on ASM cells [46]. The molecular mechanisms behind these effects are still not clearly understood but widely believed to be mediated via integrins. Indeed, a study has shown that administration of an integrin-binding peptide RGDS, which inhibits fibronectin, collagen and laminins binding to integrins, prevented allergen-induced ASM hyperplasia [112,113].

Another feature of ASM remodeling in increased expression of contractile apparatus in asthmatic ASM cells. TGF-β is a potent inducer of contractile proteins such as α-smooth muscle actin, sm-MHC, calponin and SM22 in ASM cells [114]. These smooth muscle cell (SMC) -specific genes essentially contain CArG box DNA elements [CC(A/T)6GG] in their promoters which serve as binding sites for serum response factor (SRF)- a ubiquitously expressed transcription factor [115]. SRF can activate both the proliferative and contractile gene programs via CArG boxes. Interestingly, SRF selectively associates with SMC-specific gene promoters in SMCs whereas it activates growth-related genes in both the SMCs and...
The specificity to SRF function is conferred by sets of specific transcriptional coregulators in a tissue- and stimulus-specific manner. The myocardin family of transcription factors, which includes myocardin and myocardin-related transcription factors –A and –B (MRTF-A and MRTF-B) is a principle binding partner of SRF for regulation of SMC-specific gene expression program. In smooth muscle cells, myocardin partners with SRF and binds to the CArG box elements regulating SMC-specific genes. On the other hand, presence of serum or a growth factor such as PDGF promotes association of SRF with ternary complex factor family members such as Elk-1, SAP-1 and SAP-2, thereby displacing myocardin from SRF and attenuating SMC-specific gene expression [117]. While expression of myocardin is restricted to cardiac- and smooth muscle-specific lineages, MRTFs are more ubiquitously expressed. MRTFs possess an N-terminal RPEL domain which can interact with monomeric globular actin (G-actin) resulting in their retention in the cytoplasm. At the same time, the association with G-actin also makes MRTFs sensitive to the cellular actin dynamics. Myocardin, on the other hand, has poor interaction with G-actin, and thus, remains predominantly nuclear and insensitive to actin dynamics [118,119]. Induction of actin polymerization by various stimuli such as TGF-β and mechanical stress depletes the monomeric G-actin pool by its progressive incorporation into the filamentous actin (F-actin) stress fibers in a process known as actin treadmilling. This releases MRTFs from G-actin which subsequently translocate to the nucleus and partner with SRF concluding a transcriptional complex that binds to the CArG box elements and initiate smooth muscle-specific gene transcription [118,119]. Interestingly, mere nuclear translocation of MRTFs from cytosol is not sufficient for transcriptional activation as they can be efficiently sequestered by the nuclear G-actin pool. Thus, a substantial depletion of both the cytosolic and nuclear G-actin pool is required for complete MRTF activity [118,119].

RhoA plays a critical regulatory role in this actin-MRTF axis, mainly as an upstream activator and regulator of actin treadmilling.

TGF-β has been shown to induce differentiation of fibroblasts into myofibroblasts in the MRTF-dependent manner [120,121]. In pulmonary fibroblasts, TGF-β induces myofibroblastic differentiation by activation and nuclear translocation of SRF. Similarly, TGF-β induces RhoA-dependent SRF-MRTF activation and binding to the CArG element and subsequent contractile gene expression in renal epithelial cells during EMT [122,123]. In addition, SMAD3 and the canonical WNT signaling effector β-catenin, both downstream effectors of TGF-β in ASM cells, can also modulate MRTF transcriptional activity via direct and indirect mechanisms [123-125].

While a direct evidence of TGF-β-induced RhoA-SRF-MRTF axis activation in ASM cells is still awaited, considering the evidence discussed above, it is plausible that TGF-β regulates contractile gene expression program in ASM cells via MRTFs thereby promoting airway remodeling.

**1.3.2.4 Vasculature**

TGF-β is a potent inducer of proangiogenic vascular endothelial growth factor (VEGF) via GSK-3β and SMAD signaling [126]. Exaggerated VEGF abundance is present in the lung tissue, BAL fluid and induced sputum of asthma patients [127-129]. TGF-β also possesses...
direct angiogenic properties. While VEGF opposes endothelial cell apoptosis, it mediates TGF-β-induced apoptosis. Interestingly, the angiogenic properties of TGF-β are dependent on VEGF-mediated apoptosis as inhibition of VEGF blocks TGF-β induced apoptosis and angiogenesis [130]. In addition, TGF-β also augments production of plasminogen activator inhibitor-1 (PAI-1), a vascular remodeling factor, via SMAD and WNT/β-catenin pathway [131-133]. These studies shed light on a possible link between TGF-β and bronchial vascular remodeling in asthma, direct evidence, however, is still unclear.

1.4 WNT signaling

WNT signaling is a key pathway involved in various aspects of embryonic morphogenesis, maintenance of adult tissue homeostasis, repair and regeneration and stem cell renewal [134-138]. WNT is a broad multicomponent signaling pathway and is highly conserved among species, with varied number of WNT members. Investigating the WNT signaling pathway in the context of airway remodeling in asthma could be of importance as 1] loss of various components of WNT signaling leads to abnormalities in lung development including complete agenesis, underscoring its importance in lung biology; and 2] aberrant activation of WNT signaling has been associated with a myriad of human pathologies including cancer, inflammatory disorders and fibrosis [139]. Of note, fibrosis and inflammation are key components of asthma pathophysiology.

The term WNT is derived from a combination of two homologues genes integrase 1 (int1) and wingless (wg) [137]. Int1 gene was first identified for its activation by integration of mouse mammary tumor virus DNA and involvement in the development of virally-induced breast tumors in mice. Wg, which was identified for its role in the development of wing tissue in Drosophila and regulation of larval segment polarity, was later found to be a homologue of Int1 [137]. The WNT signaling family has grown multifold since then both in the number of its members and complexity.

In humans, the WNT family is comprised of 19 WNT ligands, 10 Frizzled (FZD) receptors, low-density lipoprotein receptor-related protein (LRP) 5/6 coreceptors, several non-frizzled receptors such as RYK, ROR2, PTK7 along with intracellular mediators, several extracellular and intracellular antagonists and a range of modulators [139]. These WNT ligands can function through signaling mechanisms broadly categorized on the basis of the requirement of an intracellular mediator-β-catenin. The β-catenin-dependent WNT signaling pathway is termed as canonical WNT signaling whereas all the WNT ligands activated signaling cascades functioning independent of β-catenin are collectively described as the non-canonical WNT signaling.

1.4.1 WNT ligands

WNT ligands are secreted, cysteine-rich, lipid modified and heavily glycosylated proteins which act as autocrine and paracrine signaling cues and elicit myriad of cellular responses. Structurally, WNT ligands are composed of ~350 amino acids and contain a signal sequence for secretion along with a highly conserved distribution of 22 cysteine residues [140]. Before secretion, WNT ligands undergo posttranslational modifications in the endoplasmic
reticulum by oligosaccharyltransferase complex (OST) and Porcupine, which adds oligosaccharide moieties via N-glycosylation to the WNT peptide. WNT ligands are, then, secreted by a poorly understood mechanism involving Wntless/Evenness-interrupted (Wls/Evi), into the extracellular space where they remain localized in the vicinity of the cell surface, adhered to various ECM proteins such as perlecan, syndecan, glypican and biglycan [139,140]. The significance of extensive modifications on WNT ligands is not completely understood but may have a role in their secretion and function. For instance, N-linked glycosylation at Asparagine 87 and 29, Serine 209- and Cysteine 77-linked palmitoylation of murine WNT-3A are required for its secretion and palmitoylation at Cysteine 77 is also crucial for its signaling activity [141,142]. Importantly, these modifications could also be a major contributor to the hydrophobicity and poor solubility of WNT ligands which, in turn, govern the limited diffusion of WNT ligands observed in the aqueous extracellular space. Poor diffusion allows the formation of a concentration gradient of WNT ligands with highest density near the secretory cell surface and may define their autocrine and paracrine nature of signaling activity. However, WNT ligands have also been suggested to signal long-range assisted by carrier proteins (flotillin-2, lipoprotein particles) or conformational changes (multimerization of WNT ligands) that allows shielding of the hydrophobic motifs and increases their solubility and diffusion [139,140].

1.4.2 WNT receptors

WNT ligands signal through membrane-bound receptors, most common are the seven-pass transmembrane FZD receptors and the single-pass LRP coreceptors. FZD receptors contain an extracellular N-terminal cysteine-rich domain (CRD), a seven-pass transmembrane domain and a short cytosolic C-terminal tail. WNT ligands bind to FZD receptors via their CRD whereas cytosolic domain which is composed of PDZ-binding domain is required for the intracellular signaling where it facilitates interaction of various signaling mediators such as DVL and heterotrimeric G-proteins in a poorly understood process [143]. Due to the presence of the seven transmembrane domain, FZD receptors are listed as a novel family of G-protein coupled receptor (GPCR) by the International Union of Pharmacology as class FZD [144]. While the association of G-proteins with FZDs have been addressed in many studies using biochemical approached, a direct evidence for the contribution of WNT-induced association of FZD-G-proteins in the WNT signaling and its physiological relevance remains unclear [145].

LRP5 and LRP6 are highly homologous single-pass transmembrane proteins basically involved in receptor-mediated endocytosis of lipoproteins and protein ligands [146]. LRPCs are composed of ~1600 amino acid with an N-terminal signal peptide, four tandem β-propeller (hp) domains each connected by an epidermal growth factor (EGF)-like domain followed by three low-density lipoprotein-type A repeats, a transmembrane domain and a C-terminal intracellular signaling domain [147]. The β-propeller domains, hp1 and hp3, provide the binding sites for WNT ligands and other LRP interaction partners which are also suggested to have some degree of specificity. For instance, WNT-1, -2 and -6 bind to hp1 domain, WNT-3 and -3A bind to hp3 whereas Dickkopf-1 (DKK-1) binds to both. In WNT signaling, they function as coreceptors and form a membrane complex with FZD receptors.
and WNT ligands [147]. Of note, LRP5 and 6 are required for β-catenin-dependent canonical WNT signaling [148].

### 1.4.3 Alternative ligands and receptors

In addition, Noggin and R-spondins also participate in WNT signaling as secreted ligands and can signal through FZD receptors alone or in combination with leucine-rich repeat-containing G-protein coupled receptor 4/5 (LGR4/5) receptors leading to WNT pathway activation [149].

Ryk is a mammalian ortholog of Drosophila derailed and functions as an alternative receptor in WNT signaling. It is a receptor tyrosine kinase type I receptor and has been shown to play key roles in axonal guidance and pattern formation [150]. It contains an extracellular WNT inhibitory factor (WIF) domain but lacks the CRD domain, a characteristic of WNT receptors. Ryk can bind to WNT-1, -3A and -5A and can also function as coreceptor by its association with FZD receptors such as FZD8 [150-152]. Ryk can participate in both the canonical and noncanonical WNT signaling pathways depending on the receptor- and cell-context. Similarly, other receptor tyrosine kinase type I receptors- ROR1 [153], ROR2 [154] and PTK7 [155-157] also function as alternative WNT receptors.

### 1.4.4 WNT modulators and mediators

Several extracellular and intracellular modulators of WNT signaling have been described that play key roles in fine tuning and regulation of WNT signaling.

#### 1.4.4.1 Secreted Frizzled related proteins

Secreted Frizzled related proteins (sFRPs) are secreted glycoproteins and contain an N-terminal CRD domain resembling the WNT-binding CRD of FZD receptors and a C-terminal containing a hydrophilic heparin-binding region. sFRPs can bind to WNT ligands preventing their interaction with the FZD receptors and/or bind directly to FZD receptors preventing the assembly of an active complex thereby antagonizing the downstream WNT signaling [139]. Contrary to their antagonistic function, sFRP1 and 2 have recently been shown to positively regulate canonical WNT/β-catenin signaling and assist in WNT ligands gradient formation [158]. sFRPs, thus, modulate WNT signaling positively and negatively in a context-dependent manner.

#### 1.4.4.2 WNT Inhibitory Factor 1

WNT inhibitory factor 1 (WIF1) is an evolutionary conserved protein that can bind to WNT ligands and antagonize WNT signaling. Considered as a member of sFRP class of proteins, it lacks CRD domain but contains a highly conserved N-terminal WIF domain (WD) as found in Ryk receptors, five EGF-like repeats as found in LRP5s and a hydrophilic C-terminal domain. 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) [159]. The crystal structure of the WIF domain of WIF1 shows a binding pocket for the acyl
groups present on WNT ligands providing an interaction surface. In addition, the EGF-like domains, particularly, EGFII-V are suggested to constitute a heparan sulfate proteoglycan (HSPG)-binding site [160]. Thus, WIF1 binds to WNT ligands via its WIF domain and tethers this WIF1-WNT complex in the extracellular matrix by its interaction with HSPG-glypican via EGF-like domains [161]. Glypicans can have modulatory effects on WNT signaling [162]. Interestingly, formation of this WNT ligand-WIF1-glypican complex is required for complete WNT antagonizing activity of WIF1 [161]. Thus, WIF1 can antagonize WNT signaling by interfering with the formation of a functional WNT/FZD complex. A role of additional mechanisms of WIF1, however, cannot be ruled out. Interestingly, owing to the crucial role of WIF1 in WNT signaling regulation, suppression of WIF1 is often associated with malignancies [163].

1.4.4.3 Dickkopf

Dickkopf (DKK) proteins are another class of extracellular WNT modulators associated with regulation of β-catenin-dependent canonical WNT signaling. Four members of the DKK family are currently known – DKK-1-4, with DKK-1, -3 and -4 having antagonistic function whereas DKK-2 can be inhibitory or stimulatory depending on cellular context [139]. DKK proteins do not interact with WNT ligands but inhibit WNT signaling by their interaction with LRP5/6 coreceptors interfering with the formation of a WNT-FZD-LRP5/6 complex [139]. Kremen1 and Kremen2 are type-I transmembrane proteins and are recently identified as high-affinity receptors for DKK-1. DKK-1 interacts with LRP6 and Kremen2 forming a ternary complex that leads to the endocytosis and membrane depletion of LRP6, thereby, inhibiting canonical WNT signaling [164].

1.4.4.4 β-catenin

β-Catenin is remarkable in its ability to perform dual cellular functions. As a membrane-bound protein, it constitutes a key component of adherens junctions where it interacts with the cadherins and connects them to the cytoskeleton [165]. In addition to its role in adherens junctions, β-catenin is the central mediator of canonical WNT signaling where it functions as the transcriptional co-activator for WNT-responsive genes [165].

The ability of β-catenin to perform dual functions is conferred by its structural composition [165]. β-Catenin consists of an N-terminal domain, a central region of twelve Armadillo repeats followed by a C-terminal domain and a helix located between the last Armadillo repeat and a C-terminal domain. β-Catenin binds to the adherens junction protein-cadherin via its central domain, a region also shared by some critical interaction partners involved in its transcriptional co-activator function such as adenomatous polyposis coli (APC) and T-cell factor (TCF)/lymphoid enhancer factor (LEF) [139,165]. Thus, the mutually exclusive binding of β-catenin interaction partners involved in the adhesion and transcription functions confers the versatility in β-catenin functions.

The membrane-bound pool represents the predominant cellular fraction of β-catenin where it is present as part of the adherens junctions. Adherens junctions are cell-cell adhesion complexes that contribute to the polarity and integrity of epithelium [166]. Cadherins, the
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core component of adherens junctions, are single-pass transmembrane glycoproteins which interact with cadherins of the adjacent cells by a Ca\(^{2+}\)-dependent homophilic association [166]. The intracellular cytoplasmic tail of cadherins assembles as complex comprised of the catenins- p120-catenin and β-catenin. β-Catenin links the cadherins to the α-catenin, which in turn, links with the actin cytoskeleton. In addition, β-catenin also protects cadherins from proteasomal degradation, probably by masking a PEST sequence, as disassembly of the adherens junction complex leads to the degradation of E-cadherin [165,167-170].

β-Catenin can be released from the cadherin complex by various mechanisms and can contribute to its transcriptional pool. Cleavage of cadherins by metalloprotease ADAM10 has been shown to disrupt cell junctions and release β-catenin which translocates to the nucleus and targets gene transcription [171,172]. Thus, loss of cadherins under various physiological or pathological processes [173-176] may increase the transcriptional pool of β-catenin. Precise regulation of the membrane-bound pool of β-catenin is still unclear and is a matter of intense investigation. However, a range of phosphomodifications of β-catenin have been described that modulate its affinity for the cadherin complex and regulate its structural and signaling functions. For instance, phosphorylation at Serine 684, 686 and 692 by casein kinase (CK) 2 and GSK-3β tremendously increases β-catenin-cadherin interaction whereas phosphorylation at Tyrosine 142 by Fyn, Fer or c-Met attenuates α-catenin-β-catenin interaction thereby impairing its structural function [165]. Phosphorylation of β-catenin by JNK at various points such as Serine 31, 191, 605 and Threonine 41 also negatively regulates cadherin-β-catenin interaction and promotes its nuclear translocation [165,177]. Similarly, phosphorylation of β-catenin at Serine 552 by EGF receptor-activated AKT kinase leads to its dissociation from the cadherin complex and translocation to the nucleus and augmentation of its transcriptional activity [178]. Tyrosine 654 phosphorylation of β-catenin by EGF receptor or c-Src also impairs β-catenin binding to cadherins. An additional phosphorylation at Serine 675 by protein kinase A (PKA) is suggested to be required for complete augmentation of transcriptional function of Tyrosine 654-phosphorylated β-catenin, presumably by recruiting various co-activators such as CREB-binding protein (CREBBP or CBP) and TATA-binding protein (TBP) [165]. Thus, various mechanisms exist that promote the transcriptional role of β-catenin at the expense of its structural function, however, it might not be a universal scenario and β-catenin released from adherens junctions could also participate in functions other than transcription.

While the β-catenin released from the membrane contributes to the free cytosolic pool of β-catenin, it is believed to be predominantly maintained by the newly synthesized nascent β-catenin. The cytosolic abundance of this pool is tightly controlled by a multiprotein destruction complex which is an integral part of canonical WNT signaling. The multiprotein destruction complex is comprised of scaffold proteins Axin and APC and kinases GSK-3β and CK1α. Under the steady state conditions, free β-catenin is captured by Axin and APC and phosphorylated by CK1α at Serine 45, priming it for subsequent phosphorylation by GSK-3β at Threonine 41, Serine 37 and Serine 33. The phosphorylated β-catenin is ubiquitinated by E3 ligase Jade1 or more predominantly, by E3 ubiquitin ligase complex Skp-Cullin-F-box protein/β-transducin repeat-containing protein (SCF\(^{\beta\text{-TRCP}}\) and degraded by 26S
proteasome [139,165]. Thus, free β-catenin is constantly degraded thereby maintaining its low cytosolic levels.

The presence of canonical WNT ligands leads to the inhibition of destruction complex by poorly understood mechanisms. This allows β-catenin to evade the phosphorylation and subsequent degradation thereby increasing the cytosolic levels of β-catenin. The free cytosolic β-catenin, then, translocates to the nucleus. Inside the nucleus, β-catenin binds to the TCF/LEF and activates target gene transcription.

In addition to WNTs, several other growth factors can also stabilize β-catenin and activate β-catenin-dependent processes. GSK-3 mediated phosphorylation is a key event in β-catenin degradation. GSK-3, encoded by two isoforms-α and β, is a constitutively active kinase and is predominantly regulated by an inactivating phosphorylation at Serine 21 at GSK-3α and Serine 9 for GSK-3β [179]. Canonical WNT signaling, however, may not utilize phosphoinactivation of GSK-3 for β-catenin stabilization [180,181] but engages alternative strategies such as changing the compartmentalization of GSK-3 [182] or its dissociation from the destruction complex to separate GSK-3 activity on β-catenin [183,184], thus, rescuing β-catenin. Several growth factors, on the other hand, can inactivate GSK-3 by inhibitory phosphorylation and, in turn, stabilize β-catenin. For instance, PDGF and fetal bovine serum (FBS) stabilize β-catenin and promote its nuclear localization via GSK-3β inactivation in airway smooth muscle cells [185]. Similarly, protein kinase C (PKC) mediates inactivation of GSK-3β in bronchial epithelial cells and activates β-catenin signaling in an in vitro model of tissue injury [186]. A study has proposed an interesting mechanism for non-WNT growth factor-mediated rescue of β-catenin from the destruction complex. PDGF stimulation of colon cancer cell lines led to activation of c-Abl which phosphorylated a RNA helicase p68 at Tyrosine 593. The phosphorylated p68 blocked GSK-3β mediated phosphorylation of β-catenin by displacing it from the Axin complex and activated the TCF/LEF-dependent gene transcription [187]. Although GSK-3 can target a plethora of cellular proteins, it is suggested to modify β-catenin only in the destruction complex in association with Axin and APC. It is, thus, tempting to speculate that non-WNT growth factors may inactivate destruction complex-associated GSK-3 along with other cellular pools of GSK-3 leading to β-catenin rescue.

1.4.5 Canonical WNT signaling

Stabilization of cytosolic β-catenin is the key process in canonical WNT signaling, although the sequence of events is unclear. In a widely-accepted model of canonical WNT signaling (Figure 3), binding of WNT ligands to the FZD receptor and LRP5/6 co-receptor leads to the formation of a heteromeric ternary membrane complex. This leads to the polymerization of dishevelled (DVL) proteins and their recruitment to the ternary complex. DVL, in turn, recruits Axin and GSK-3β to the receptor complex. GSK-3β and CK1γ both phosphorylate the cytosolic tail of LRP coreceptors in their Pro-Pro-Ser-Pro repeats resulting in a multifold increase in the affinity of Axin for LRP. Subsequently, Axin is recruited to the phosphorylated LRP cytosolic tail leading to the depletion of free cellular pool of Axin. Axin, being present in limited amounts, is a rate limiting factor for β-catenin phosphorylation by destruction
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Figure 3. Canonical WNT signaling pathway. In the absence of WNT ligands, a destruction complex comprising axin, GSK-3β, CK1 and APC captures cytosolic β-catenin and phosphorylates it sequentially via CK1 and GSK-3β activity. The phosphorylated β-catenin is degraded by the ubiquitin-proteasome system. Activation of the FZD and LRP5/6 receptor complex by extracellular WNT ligands leads to sequestration of the β-catenin destruction complex to the membrane receptor complex, primarily mediated by the scaffold protein DVL. This prevents phosphorylation of β-catenin by GSK-3β and CK1 and subsequent proteasomal degradation culminating into the accumulation of cytosolic β-catenin. Unphosphorylated and stabilized β-catenin translocates to the nucleus and activates β-catenin-dependent gene transcription. APC, Adenomatous polyposis coli; CK1, Casein kinase 1; DVL, dishevelled; GSK-3β, Glycogen synthase kinase-3β; FZD, Frizzled; LEF, Lymphoid enhancer factor; LRP5/6, Lipoprotein receptor-related protein 5/6; TCF, T-cell factor. (Schematic is taken from Chapter 2)

complex and can modulate rapid assembly and disassembly of the complex. This sequestration of Axin at the membrane by LRP5 leads to the disassembly of the destruction complex leading to an increase in the non-phosphorylated cytosolic pool of β-catenin [188].

While membrane sequestration of Axin and GSK-3 is a prevalent model, several alternative mechanisms of WNT-dependent β-catenin activation have also been reported. A study has
suggested that WNT ligands stimulation leads to LRP5/6 and Axin mediated sequestration of GSK-3 into multivesicular bodies. This change in GSK-3 compartmentalization effectively segregates β-catenin from GSK-3 leading to its accumulation [182]. In another study, authors have shown that DVL can bind directly to the Axin, leading to disruption of its interaction with GSK-3 which may lead to the disassembly of destruction complex [183,189]. Alternatively, GSK-3 interacting proteins such as GSK-3-binding protein (GBP)/frequently rearranged in advanced T-cell lymphomas 1 (FRAT-1), may compete with GSK-3 for its Axin-binding domain leading to the dissociation of GSK-3 from Axin and destruction complex. This process is facilitated by DVL which can also bind to GBP and FRAT-1 [139,184,190,191]. Another recent model proposed WNT ligand-induced monoubiquitination of GSK-3β leading to its association with β-TRCP which renders both the GSK-3β and β-TRCP unavailable to interact with and ubiquitinate β-catenin [192].

Furthermore, a recent study has shown that WNT ligand stimulation doesn’t lead to disassembly of the destruction complex or recruitment of individual Axin and GSK-3 to the membrane, nor does it lead to inhibition or any alteration in the activity of destruction complex constituents [193]. The authors have shown that β-catenin is not only phosphorylated but also ubiquitinated by β-TRCP inside the destruction complex. WNT ligand stimulation leads to dissociation of β-TRCP from the destruction complex and recruitment of the entire destruction complex to the WNT-FZD-LRP ternary complex at the membrane. In the absence of β-TRCP, the destruction complex-associated β-catenin cannot be ubiquitinated and hence can’t be removed leading to saturation of the destruction complex by phospho-β-catenin [193]. As such, no nascent β-catenin interacts with the destruction complex leading to accumulation of β-catenin in the cytosol and its nuclear translocation [193].

Interestingly, Yes-associated protein/transcriptional coactivator with PDZ-binding motif (YAP/TAZ) have been shown to mediate association of β-TRCP with the destruction complex [194]. YAP and TAZ are transcriptional cofactors whose nuclear shuttling is primarily believed to be controlled by cell density sensing Hippo signaling [195] and mechanical stress sensing pathways [196]. In a novel mechanism, YAP and TAZ have been shown to be an integral part of the canonical WNT signaling. YAP and TAZ associate with Axin and reside in the destruction complex in the absence of WNT ligands [194] where they recruit β-TRCP to the destruction complex which degrades phosphorylated β-catenin. In the presence of WNT ligands, the destruction complex is recruited to the phosphorylated intracellular domain of LRP5/6 via Axin. As both LRP5/6 and YAP/TAZ compete for the same domain of Axin, YAP/TAZ dissociate from the destruction complex which also dislodges β-TRCP from the destruction complex [194]. Thus, impaired destruction complex-β-TRCP association stabilizes cytosolic β-catenin.

Once stabilized, β-catenin can readily translocate to the nucleus by an unidentified mechanism as it lacks nuclear localization sequence (NLS) and also seem to be independent of importin-β and Ran-GTPase, the common nuclear transporters [165]. Inside the nucleus, β-catenin partners with various primary and auxiliary proteins including transcription factors, histone modification proteins and chromatin remodeling complexes, to relay the
effects of its inducers [165,197]. The most common binding partners for β-catenin in canonical WNT signaling are TCF/LEF transcription factors. In the absence of WNT signaling, TCF/LEF occupy the WNT responsive elements (WREs) in association with transducin-like enhancer of split (TLE)/Groucho where it suppresses the gene transcription. Binding of β-catenin displaces TLE/Groucho from TCF/LEF allowing the transcriptional activation of WNT responsive genes [165]. While TCF/LEF occupy central Armadillo repeats of β-catenin, it recruits several cofactors via its N-terminal and C-terminal domains such as BCL9, Parafibromin, MED12, Histone acetyltransferases (eg. CBP/p300), chromatin remodeling complexes (eg. BRG1) and histone methyltransferases (eg. MLL complexes, COMPASS) that modulate the gene transcription [197].

1.4.6 Noncanonical WNT signaling

In addition to the β-catenin dependent canonical WNT signaling, WNT ligands can activate multiple signaling cascades broadly classified as noncanonical WNT signaling [198,199] (Figure 4). Noncanonical WNT signaling is essentially independent of both β-catenin and LRP5/6 coreceptors but utilizes FZD and alternative receptors. Additionally, a different set of intracellular mediators such as Ca$^{2+}$-dependent factors, MAPKs and small GTPases are employed to relay cellular effects of noncanonical WNT ligands. The major cellular effects of noncanonical WNT signaling are transcriptional activation of target genes, reorganization of cytoskeleton and cell movement.

1.4.6.1 WNT/planar cell polarity (WNT/PCP)

Cell polarity is an important feature of living organisms whether unicellular or multicellular [201]. It arises by an asymmetrical distribution and organization of cell contents such as cell membrane, intracellular organelles and cytoskeleton. Cell polarity is involved in almost all aspects of eukaryotic life from cell movement and migration to asymmetric cell divisions and organization of a well-structured metazoan body. For instance, polarization of cells is absolutely required in gastrulation to generate germ layers, in the development of tissues such as neurons and epithelium or polarized structures such as limbs and in determining the proximal-distal (P-D) and anterio-posterior (A-P) axes of the body. In adult life, cell polarity is also critical for the directional migration of motile cells such as fibroblasts and immune cells, required for repair and regeneration and is intrinsic to the maintenance of tissue architecture and integrity. In malignant diseases, cells modulate their polarity to migrate during metastasis. One of the major underlying mechanisms of cell polarity is directional organization of the cytoskeleton which can be affected by a multitude of factors [201]. Generation and maintenance of cell polarity is a tightly regulated process and is associated with the noncanonical WNT signaling.

Most of the WNT/PCP pathway is characterized in Drosophila with the identification of various homologues proteins in vertebrates. The core WNT/PCP pathway is comprised of FZD, Van Gogh (Vangl1 and 2 in vertebrates), flamingo, prickle and DVL [198]. Binding of noncanonical WNT ligands to FZD receptors may activate the heterotrimeric G-proteins which, in turn, leads to the recruitment and phosphorylation of DVL [198,199]. Alternatively, noncanonical WNT ligands such as WNT-5A can bind to ROR2 and Vangl2
leading to the formation of a ternary signaling complex- WNT-5A-ROR2-Vangl2 recruiting DVL via Vangl2 [202]. Activated DVL, in turn, can engage multiple pathways leading to actin cytoskeleton reorganization and/or transcriptional responses. DVL associates with the DVL-associated activator of morphogenesis 1 (Daam1) and RhoA and subsequently activates RhoA [203]. Activated RhoA, in turn, leads to the activation of its downstream kinase- Rho-associated coiled-coil containing protein kinase 1 (ROCK1) which regulates actin cytoskeleton remodeling. Additionally, activated Daam1 interacts with profilin, an actin binding protein, and initiates actin polymerization and cytoskeletal remodeling [204].

In another pathway, interaction of DVL with Rac1 leads to the activation of JNK kinase [205]. It has also been suggested that DVL can directly interact with JNK via its DEP domain and

![Diagram of WNT/PCP pathway and WNT/Ca²⁺ pathway]

**A. WNT/PCP pathway**

**B. WNT/Ca²⁺ pathway**
activate it independently of Rac1 [206]. The requirement of small GTPases in DVL mediated JNK activation seems to be cell- and stimulus-dependent. JNK, in turn, may regulate actin remodeling [207] or induces activation of multiple downstream signaling cascades including activation of a transcription factor - c-Jun [208]. In addition, RhoA can also activate JNK signaling in the WNT/PCP pathway as demonstrated in Xenopus convergent extension movements, the key morphogenetic movements wherein tissue narrows down along one direction and elongates in the perpendicular direction thereby shaping the body axis [209].

The noncanonical WNT ligand WNT-11 stabilizes XRNF185, a Xenopus homologue of human RING finger protein RNF185 and induces its interaction with DVL [210]. This, in turn, facilitates XRNF185 interaction with paxillin [210]. Paxillin is a focal adhesion complex protein and participates in actin cytoskeleton remodeling and cell motility. XRNF185-paxillin interaction results in increased paxillin ubiquitination and subsequent degradation leading to enhanced paxillin turnover and actin cytoskeleton remodeling [210].

### 1.4.6.2 WNT/\Ca^{2+}\textsuperscript{2+} pathway

Ca\textsuperscript{2+}-dependent signaling is another key noncanonical WNT pathway. Binding of WNT ligands leads to the recruitment of DVL to the FZD receptor, presumably via G-proteins, which in turn, may activate phospholipase C (PLC) leading to generation of diacylglycerol (DAG) and inositol trisphosphate (IP\textsubscript{3}). IP\textsubscript{3} leads to the release of Ca\textsuperscript{2+} from intracellular stores and rise in Ca\textsuperscript{2+} concentrations whereas DAG activates PKC [211].

**Figure 4. Noncanonical WNT signaling pathways.** (A) WNT/planar cell polarity (PCP) pathway. Binding of WNT ligand to Fz receptor leads to the activation of DVL and its subsequent recruitment to the receptor. Additionally, AP2 and βarr2 may be recruited leading to the receptor internalization. DVL activates multiple pathways leading to the cytoskeletal remodeling and transcriptional regulation as shown in the figure. FZD-induced membrane recruitment of DVL is regulated by various kinases such as PAR1, PKCS8 and CK1ε. In addition, Glypican, Syndican, PAPC, RTK7, DVL-binding proteins such as Div and Inv also regulate WNT/PCP signaling. (B) WNT/\Ca^{2+}\textsuperscript{2+} pathway. Binding of WNT ligand to Fz receptor leads the activation of DVL which promotes generation of DAG and IP\textsubscript{3}. IP\textsubscript{3} induces intracellular Ca\textsuperscript{2+}. DVL also activates PDE6 leading to decline in intracellular cGMP levels and subsequent inhibition of PKG activating Ca\textsuperscript{2+} release. Ca\textsuperscript{2+} activates CaMKII-TAK1-NLK cascade inhibiting β-catenin-TCF signaling. DAG and Ca\textsuperscript{2+} also activate PKC which activates Cdc42 and regulates actin cytoskeletal remodeling. Additionally, Ca\textsuperscript{2+} activates the phosphatase CAN leading to activation and nuclear translocation of NFAT. DVL, dishevelled; Fz, Frizzled; βarr2, β-arrestin 2; AP2, adaptor protein complex 2; Daam1, DVL-associated activator of morphogenesis; ROCK, Rho-associated coiled-coil containing protein kinase; MRLC, myosin regulatory light chain; PXN, paxillin; XRNF185, Xenopus ring finger protein 185; CapZIP, CapZ interacting protein; Dub, duboraya; PAPC, paraxial protocadherin; RTK7, receptor tyro sine kinase 7; TK, tyrosine kinase; PAR1, partitioning-defective 1; PKCS8, protein kinase C8; CK1ε, casein kinase 1ε; Inv, inversin; Div, diversin; PLC, phospholipase C; PIP\textsubscript{2}, Phosphatidylinositol 4,5-bisphosphate; IP\textsubscript{3}, inositol trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; Cdc42, cell division cycle 42 protein; PDE6, phosphodiesterase 6; PKG, protein kinase G; CaMKII, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase 2; TAK1, TGF-β-activated kinase 1; NLK, nemo-like kinase; β-catenin; TCF, T-cell factor; CAN, calcineurin; NF-AT, nuclear factor of activated T cells; G, G protein αf3 subunits (Schematics are adapted from Semenov et al (2007) [200])
In addition, WNT ligands activate cGMP phosphodiesterase 6 (PDE6) which leads to the depletion of cGMP and inhibition of protein kinase G (PKG) \[212-214\]. PKG is a negative regulator of Ca\(^{2+}\) mobilization. Thus, inhibition of PKG leads to a rise in intracellular Ca\(^{2+}\) concentrations. Alternatively, heterotrimeric G-proteins can activate PDE6 in a DVL-independent manner via p38 MAPK stimulation \[215\]. While these studies mention a role for G-proteins in WNT/Ca\(^{2+}\) signaling, direct evidence supporting the physiological relevance of WNT-induced FZD-G-protein interaction is still awaited \[145\].

High intracellular Ca\(^{2+}\) activates protein kinase C (PKC), calmodulin-dependent kinase II (CaMKII) and calcineurin-NFAT signaling. Activation of PKC by Ca\(^{2+}\) and/or DAG leads to actin cytoskeleton remodeling, probably, via small GTPases \[216-218\]. Further, increased Ca\(^{2+}\) leads to activation of calmodulin which in turn activates a protein phosphatase calcineurin. Activated calcineurin dephosphorylates NFAT transcription factors which translocate to the nucleus and activate gene transcription \[219\].

In addition, calmodulin also activates CaMKII which can antagonize the canonical WNT signaling pathway. CaMKII activates TAK1 which, in turn, stimulates nemo-like kinase (NLK) activation. NLK phosphorylates TCF/LEF transcription factor which prevents their interaction with β-catenin thereby inhibiting canonical WNT signaling pathway \[220,221\].

### 1.4.6.3 Other noncanonical WNT signaling pathways

In addition to the WNT/PCP and WNT/Ca\(^{2+}\) pathways, several other signaling cascades are activated by WNT ligands in a β-catenin independent manner. For instance, WNT ligands, in particular WNT-5A, can regulate the cell polarity complex in developing neurons. WNT-5A leads to association of DVL with members of polarity complex- partitioning-defective (PAR) 3, PAR6 and atypical PKC (aPKC) which inhibits the activity of PAR1 leading to inhibition of microtubule organization thereby regulating axonal guidance \[222\].

Noncanonical WNT signaling activates CREB in various systems. WNT-5A binding to FZD3 leads to rise in cAMP concentrations which, in turn, activate protein kinase A (PKA) \[223\]. PKA directly phosphorylates and activates CREB. In addition, it also phosphorylates a dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), an antimigratory protein \[223\]. Activated DARPP-32 inhibits PP1, a protein phosphatase, and potentiates WNT-5A-PKA mediates CREB phosphorylation. Also, DARPP-32 suppresses Cdc42 inhibiting filopodia formation \[223\]. During mammalian myogenesis, WNT ligands WNT-1 and -7A activate G-protein dependent increase in cAMP levels leading to PKA activation in mouse presomitic mesoderm. PKA, in turn, phosphorylates CREB leading to activation of myogenic transcriptional factors such as Pax3, MyoD and Myf5 \[224\]. In addition, WNT ligands can activate p38, JNK, ERK and NFkB signaling in β-catenin independent manner expanding the array of noncanonical WNT signaling pathways \[225\].
1.5 WNT-5A: a noncanonical WNT ligand (adapted from chapter 7)

1.5.1 WNT-5A

WNT-5A is one of the most studied WNT ligands associated predominantly with the noncanonical WNT signaling [226]. WNT-5A is highly conserved among species and plays key roles in embryonic development and post-natal homeostatic processes. Homozygous WNT-5A knock-out mice show perinatal lethality, primarily due to respiratory failure, and present extensive developmental abnormalities. It is involved in lung [227], heart [228] and mammary gland morphogenesis [229] and regulates stem cell renewal [230,231] and tissue regeneration [232]. In addition, it has also been associated with a myriad of pathological conditions such as cancer, fibrosis, inflammation and neurodegeneration.

1.5.2 WNT-5A gene

WNT-5A cDNA was first isolated from mice fetal tissues [233] followed by isolation and sequencing from human cells [234]. The human WNT-5A gene is located on chromosome 3p14-p21. The WNT-5A gene generates two very identical transcripts by utilization of alternative transcription start sites. The corresponding upstream sequences are termed as promoter A and B [235] and their products as WNT-5A-L and WNT-5A-S, respectively [236]. Both the promoters have comparable transcriptional potential; their activity, however, is highly context-dependent. WNT-5A promoter A has been suggested to be more active in human and murine fibroblasts compared to promoter B [237]. Both the isoforms have similar biochemical properties such as stability, hydrophobicity and signaling activity [236]. While the significance of individual WNT-5A isoforms is not completely understood, a recent study showed that they might have different functions [236]. When ectopically expressed, WNT-5A-L inhibited proliferation of various cancer cells lines whereas WNT-5A-S lead to stimulation of growth [236].

**Figure 5. Human WNT-5A promoter.** Schematic representation of various transcription factor binding sites on WNT-5A promoter A as derived from in silico analysis.

1.5.3 WNT-5A transcription

WNT-5A is a transcriptional target of an array of cytokines and growth factors. CUTL1 [238], STAT3 [239], TBX1 [240] and NFκB [241,242] have been reported as transcription factors for WNT-5A in various cell types. TGF-β has been shown to induce WNT-5A expression in pancreatic cancer cells [238]. Similarly, pro-inflammatory cytokines-IL-1β [242], TNF-α [241], LPS/IFNγ [243], IL-6 family members- leukemia inhibitory factor and cardiotropin-
1 [244], high extracellular Ca\(^{2+}\) concentration [245] all augment whereas amino acid limitation [246] represses WNT-5A expression in various cell types. A schematic representation of WNT-5A promoter A is presented in Figure 5 showing the predicted transcription factor binding sites (Figure 5).

Numerous AU-rich motifs are present in the 3′-untranslated region of WNT-5A mRNA which is about ~2.5-fold longer than the coding region in humans and evolutionarily conserved among species [247]. AU-rich element binding proteins (ARE-binding proteins) associate with the AREs and tightly regulate their stability by posttranscriptional mechanisms. HuR,
a member of the embryonic lethal abnormal vision (ELAV)-like family of ARE-binding proteins, binds to the 3'-UTR AREs in WNT-5A mRNA and suppresses its translation [247].

1.5.4 WNT-5A protein

WNT-5A-L and WNT-5A-S, composed of 380 and 365 amino acids respectively, are heavily glycosylated and lipid modified proteins. Each isoform consists of an N-terminal hydrophobic signal sequence, a conserved asparagine-linked oligosaccharide consensus sequence and a highly conserved distribution of 22 cysteine residues [234] (Figure 6). The cleavage of N-terminal signal sequence is predicted to generate mature proteins containing either 343 or 338 amino acids [236]. However, N-terminal sequencing of mature WNT-5A isoforms revealed that WNT-5A-L is cleaved after 43rd amino acid whereas WNT-5A-S has much longer signal sequence with cleavage after 46th amino acid, generating 337 and 319 amino acid containing mature proteins, respectively (Figure) [236]. Interestingly, mouse WNT-5A which is ~99% homologues to the human WNT-5A generates same mature protein as human WNT-5A-S [234,248]. Asparagine 114, 120, 312 and 326 have been identified as the N-linked glycosylation sites whereas a palmitoylation has been identified at Cysteine 104. The palmitoylation of WNT-5A is necessary for its binding to FZD5 and signaling activity but not required for its secretion [249,250]. In contrast, glycosylation of WNT-5A is required for its secretion but dispensable for its signaling activity [249].

1.5.5 WNT-5A: receptors and signaling

WNT-5A binding to receptors activates various β-catenin-independent noncanonical WNT signaling cascades, however, it can also activate canonical WNT signaling depending on the cell and receptor context.

WNT-5A can signal through multiple receptors and according to current understanding FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, RYK, ROR2 and CD146 may function as WNT-5A receptors [245,248,251-261].

WNT-5A has been shown to bind to FZD2 inducing intracellular Ca\textsuperscript{2+} release and PKC activation in Xenopus [262] and zebrafish embryos [263] and WNT-5A-FZD2-induced Ca\textsuperscript{2+} spikes in neurons are also implicated in traumatic brain injury [264]. WNT-5A binds to FZD2 in a ROR1- or ROR2-dependent manner and recruits DVL and β-arrestin to FZD2 leading to the clathrin-mediated internalization of FZD2 [251]. Internalization of FZD2 is

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**Figure 6. Human WNT-5A protein.** (A) A comparative analysis of amino acid sequences of WNT-5A-L and WNT-5A-S isoforms. Grey highlighted area represents N-terminal signal sequence in respective protein. Bold arrows mark the N-terminal of mature protein of respective isoform post-signal sequence cleavage. The amino acids marked in red-bold represent posttranslational modification sites on protein backbone. Number represents the respective position of the amino acid from the first N-terminal amino acid. The protein sequences are taken from NCBI; NP_001243034.1 (WNT-5A-L) and NP_003383.2 (WNT-5A-S). (B) Diagrammatic representation of WNT-5A-L protein. N-terminal signal sequence is represented by blank box. (ԕ) represents palmitoylation and (ؼ) represents N-linked glycosylation on the protein backbone. The respective amino acids locations are marked above the modification sites.
essential for WNT-5A-induced Rac activation [251]. WNT-5A also induces clathrin-mediated internalization of FZD4 [265] in a PKC- and β-arrrestin-dependent process and that of ROR2 in a PKC-dependent manner [258]. Binding of WNT-5A to FZD5 [249] also leads to its internalization but the functional relevance is unknown. Internalization of receptors is considered as a critical step in WNT signaling and a reflection of active signaling. Although the exact mechanisms underlying the functional significance of receptor internalization are not clear, it is believed to facilitate intracellular signaling activation by recruitment of scaffolding proteins such as β-arrrestin and may also facilitate the termination of signaling and receptor recycling [266].

WNT-5A binding to FZD7 activates pro-survival PI3K/AKT cascade in human melanoma cells which can account for the resistance of these cells to BRAF inhibitors [259]. Similarly, WNT-5A can activate the PI3K/AKT cascade via FZD3 in human dermal fibroblasts and promotes integrin-mediated adhesion of these cells [252]. In contrast, WNT-5A-activated PI3K/AKT signaling induces migration in human osteosarcoma cells [267]. Similarly, WNT-5A induces migration in gastric cancer cells by activating the PI3K/AKT pathway which phosphorylates and inactivates GSK-3β and activates Rac leading to cytoskeleton remodeling [268]. Indeed, cytoskeletal reorganization and cell migration are major cellular effects of WNT-5A signaling.

WNT-5A-FZD6 interaction is suggested to regulate cell fate in hair-follicles [261] whereas WNT-5A-FZD5 signaling plays critical role in tuberculosis immunology regulating the immune responses by antigen presenting cells and activated T cells in response to mycobacterium infection [253].

WNT-5A binding to an adhesion molecule CD146 leads to the recruitment of DVL2 to the complex and activation of downstream noncanonical JNK signaling cascade [256]. CD146 has been linked to cell migration via RhoA-dependent cytoskeletal rearrangements [269]. In line with that, WNT-5A-CD146 axis regulates polarity and migration of cells [256].

ROR2 is a key receptor for WNT-5A-induced effects during development as demonstrated by remarkable phenotypic resemblance between the ROR2 knock-out and WNT-5A knock-out mice [270]. Multiple mechanisms have been suggested to explain the close functional relationship between WNT-5A and ROR2. WNT-5A interacts with ROR2 and Vangl2 to form a ternary complex leading to the CK1δ-induced phosphorylation of Vangl2 which serves to relay the gradient effects of WNT-5A thereby regulating WNT-5A-induced planar cell polarity and embryonic morphogenesis [271]. WNT-5A associates with FZD7 in the presence of ROR2 to form a complex required for DVL polymerization and activation of Rac-dependent noncanonical WNT signaling [260]. WNT-5A activates ERK1/2 in intestinal epithelial cells via ROR2 [272], whereas it activates JNK-mediated c-Jun transcriptional activity to induce production of receptor activator of nuclear factor-κB (RANK), a regulator of osteoclast differentiation and activation, in osteoclast precursor cells via ROR2 [273].

WNT-5A activates intracellular Ca^{2+} release to fine tune neuronal growth by axonal outgrowth and repulsion. WNT-5A signals via Ryk leading to Ca^{2+} release from stores through IP_{3} receptors as well as Ca^{2+} influx through transient receptor potential (TRP)
channels inducing axonal outgrowth. On the other hand, simultaneous association of WNT-5A with Ryk and FZD releases Ca\(^{2+}\) from TRP channels without involvement of IP\(_3\) receptors and induces axonal repulsion [274]. WNT-5A also forms a ternary complex with Ryk and Vangl2 to relay the WNT/PCP effects [275] whereas WNT-5A-Ryk signaling is required for inhibition of reactive oxygen species (ROS) production and maintenance of hematopoietic stem cell quiescence [151]. WNT-5A engages ROR2 to activate JNK signaling and regulates convergent extension movements [226] and human dental papilla cell migration [276] whereas it induces assembly of DVL-apKc and polarity complex (PAR3 and PAR6) to regulate neuronal differentiation and polarity [222,277].

Noncanonical WNT ligands counteract WNT/β-catenin signaling by preventing β-catenin and TCF/LEF interaction. Interestingly, WNT-5A can inhibit or activate WNT/β-catenin signaling depending on the receptor- and cell-context. Indeed, a study has shown that WNT-5A can both activate and inhibit canonical WNT signaling during mouse embryonic development [278]. The WNT-5A-activated CaMKII-TAK1-NLK cascade has been implicated in WNT/β-catenin suppression [220]. In addition, WNT-5A inhibits WNT-3A-induced β-catenin signaling via ROR2 and CD146 [248,256]. In hematopoietic stem cells, WNT-5A inhibits β-catenin signaling, probably via suppression of ROS production [151]. Similarly, WNT-5A inhibits β-catenin signaling by promoting degradation of β-catenin through an alternative E3 ubiquitin ligase complex comprised of siah2-APC-Ebi [279]. Purified WNT-5A, on the other hand, can activate β-catenin-dependent transcription in the presence of FZD4 and LRP5 [248]. Similarly, osteoblast-lineage cells from WNT-5A knockout mice show reduced WNT/β-catenin signaling and WNT-5A pre-treatment potentiated the WNT/β-catenin signaling in bone marrow stromal cells via upregulation of LRP5 and LRP6 expression [280].

### 1.5.6 WNT-5A: functions

#### 1.5.6.1 Embryogenesis:

WNT-5A has been identified for its key involvement in defining the body outgrowths in addition to many other specific features. WNT-5A expression is most abundant during early embryonic developmental stages between 10-14 day post conception [233,281]. Importantly, homozygous WNT-5A knock-out mouse embryos show perinatal lethality underlining its vital role in embryogenesis. During development, regions undergoing extensive outgrowth like limbs, tail and facial structures exhibit prominent WNT-5A expression where it is present in a graded fashion with highest abundance at the tips of these structures and lowest in the proximal areas [233,281]. WNT-5A knockout leads to severe malformations in the outgrowth structures, a shortened A-P axis and severely compromised P-D axis. These malformations could be traced back to the underlying axial skeleton which exhibited a shortened vertebral column due to smaller vertebrae size and absence of caudal vertebrae [281]. The phenotype apparently originates from the critical role of WNT-5A as a mitogen required for the proliferation of the mesodermal progenitors early in embryonic development. The mesodermal stem cells which arise early in development can continue to develop in the primitive streak even in the absence of WNT-5A but lack the ability to divide and give rise to the progeny [281]. Impaired self-renewal capacity leads to progressive
depletion of the stock of these stem cells resulting in insufficient numbers of cells to develop the distal skeleton and leading to the absence of related structures [281].

Similar to WNT-5A knock-out mice, WNT-5A transgenic mice show perinatal lethality when WNT-5A is induced early in development exhibiting severe deformities resembling the WNT-5A knock-out phenotype [282]. Overexpression of WNT-5A induced malformations of limbs, tail and facial structures. Underdeveloped limb skeletal elements, reduced number of tail vertebrae and shortened upper and lower jaw bones constituted the mutant phenotype. Interestingly, overexpression of WNT-5A in later embryonic stages and in adult animals was well-tolerated with no visible phenotype [282]. This study highlights a critical window during embryonic development when WNT-5A activity is most required [282].

Further studies have looked into the organ-specific developmental roles of WNT-5A and have identified a crucial role for distal morphogenesis of internal organs. For instance, WNT-5A knock-out mice fail to develop the genital tubercle [281] and have intestinal deformities [283]. Prominent WNT-5A expression is observed in the gut mesenchyme during intestinal morphogenesis which persists throughout the development of the small intestine [281,284]. In line with that, WNT-5A knock-out mice show severe malformations in the small intestine with drastically reduced length and the presence of a secondary cavity. In addition, the mutants present an imperforated anus [283]. Interestingly, overexpression of WNT-5A during embryonic development also leads to gut malformations resembling the WNT-5A knock-out phenotype. Specifically, WNT-5A overexpression caused shortening of the small and large intestine, caecum and stomach and also presents anal imperforation [282]. Of note, both the loss and overexpression of WNT-5A doesn’t interfere with the intestinal differentiation or cell fate decisions. The underlying mechanisms that lead to the malformations observed in WNT-5A transgenic mice are not clear yet. However, the observation that overexpression of WNT-5A leads to the downregulation of ROR2 in intestine [282] could reveal the reason behind the similarities in both the WNT-5A overexpressed and WNT-5A knock-out phenotypes. ROR2 is a receptor for WNT-5A and ROR2 knock-out mice show a phenotype resembling that of WNT-5A knock-out [270]. Therefore, increased expression of WNT-5A which leads to the downregulation of ROR2 could present a similar phenotype as ROR2 knock-out. Although the downstream WNT-5A signaling after overexpression remained intact, it is tempting to speculate that ROR2-dependent WNT-5A signaling is crucial for the embryonic development and that the loss of ROR2 in WNT-5A transgenic mice underlies the similarity with the WNT-5A knock-out phenotype.

Lungs are complex organs with extensive branching, a large number of different types of specialized cells and distinct P-D polarity. WNT-5A, as a major determinant of P-D polarity, is prominently expressed in the embryonic lungs [227,233] where it is localized in both the mesenchymal and epithelial compartments. WNT5A signaling is most enhanced at the tip and around the branching epithelium [227]. In later stages, WNT-5A is predominantly localized to the lung epithelium and attains a typical P-D gradient with most expression in the distal branching epithelium and almost no presence in the proximal regions [227]. Analysis of lungs obtained from WNT-5A knock-out mice revealed extensive developmental
malformations. The trachea was truncated with reduced number of cartilages [227]. The branching morphogenesis of WNT-5A knock-out lungs was compromised as revealed by increased number and overexpanded terminal airways. Also, the intersaccular walls were thick and hypercellular indicating failed maturation of lungs in WNT-5A knock-out embryos. Further analysis revealed that loss of WNT-5A didn’t interfere with cell differentiation but led to hyperproliferation resulting in intersaccular septum thickening and disrupted vasculature [227]. Interestingly, WNT-5A knock-out lungs presented increased expression of sonic hedgehog/patched (SHH/PTC), FGF and bone morphogenetic protein 4 (BMP4) indicating the molecular mechanisms involved in the observed WNT-5A knock-out phenotype [227]. Notably, lungs of WNT-5A knock-out mice show resemblance with the FGF-10 knock-out [285], SHH knock-out [286,287], SHH transgenic [288] and BMP4 transgenic [289] lung phenotype, which underlines the interactive network of WNT-5A, FGF-10, SHH/PTC and BMP4 in lung development. Lung-specific WNT-5A transgenic expression also disrupts lung morphogenesis as demonstrated by dilated terminal airways, loss of branching and smaller size of the lungs [290]. Interestingly, supporting a role for WNT-5A in regulating other signaling cascades, WNT-5A overexpression repressed SHH/PTC expression and distribution in the lung epithelium whereas it augmented FGF-10 abundance in the mesenchyme [290]. While FGF-10 expression is increased, WNT-5A overexpression severely impairs the ability of epithelium to respond to FGF-10 [290]. Thus, WNT-5A fine-tunes the developmental signaling underlying the epithelial-mesenchyme communication which is required for proper lung morphogenesis [290].

1.5.6.2 Migration

Cell migration requires acquisition of new asymmetry and polarity along with reorganization of the cytoskeleton and breaking and/or reprocessing cell-cell and cell-substrate adhesions. As such, the WNT/PCP and WNT/Ca^{2+} pathways have been linked with migration of cells. WNT-5A-activated noncanonical WNT signaling pathways have been associated with the convergent extension movements. Several studies have elucidated the significance and molecular mechanisms of WNT-5A-induced cell migration. For instance, a study has identified the WNT-5A-ROR2 axis in regulating cell motility. WNT-5A interacts with ROR2 and induces its association with filamin A, an actin binding protein, which, in turn, leads to formation of filopodia [291]. Filopodia are actin based structures projecting at the leading edge of migrating cells and are important in formation of focal adhesions attaching to the substrate and facilitating directional cell movement [292]. WNT-5A-induced ROR2-Filamin A association activates aPKC which in turn activates JNK. Activated JNK may mediate cell migration by microtubule organizing center (MTOC) reorientation and actin remodeling via phosphorylation and activation of CapZ-interacting protein (CapZIP) [293]. In addition, JNK can also phosphorylate paxillin regulating focal adhesion complexes [294,295] and modulating cell motility in response to WNT-5A. In another mechanism, WNT-5A induces cell migration via Daple (DVL-associating protein with a high frequency of leucine residues)-mediated Rac activation [296]. Daple interacts with DVL in response to WNT-5A and facilitates its interaction with aPKC consequently inducing Rac activation. This leads to cytoskeletal reorganization promoting lamellipodia formation and cell migration [296].
addition to aPKC, WNT-5A can also employ Rab35 to activate Rac in a DVL-dependent manner and induce cell migration [297].

Besides noncanonical WNT signaling, WNT-5A can also activate β-catenin-dependent signaling to promote cell migration. In melanoma cells, WNT-5A activates small GTPase ADP-ribosylation factor 6 (ARF6) via FZD4-LRP6 binding. ARF6 released membrane-bound β-catenin from N-cadherin increasing its cytosolic abundance and triggering β-catenin-dependent transcriptional program that induces invasion and metastasis [257].

1.5.6.3 Stem cell differentiation and regeneration

Owing to its property of regulating cell polarity, cell movement and cell proliferation along with antagonistic effects on WNT/β-catenin signaling, WNT-5A may play a critical role in modulating cell fate determination and differentiation of stem cells.

Hematopoietic stem cells exhibit a shift from canonical to noncanonical WNT signaling with ageing where high levels of WNT-5A are present in the aged cells [231]. Interestingly, treatment of young hematopoietic stem cells with WNT-5A induced age-related changes such as ageing-associated stem-cell apolarity, reduced regenerative capacity and an ageing-like myeloid–lymphoid differentiation shift via activation of small Rho GTPase Cdc42 [231]. On the other hand, reduction of WNT-5A expression in aged hematopoietic stem cells leads to their functional rejuvenation [231].

Similarly, WNT-5A is also critical in mesenchymal stem cell (MSC) biology. MSCs can differentiate into multiple cell types such as adipocytes and osteocytes. The presence of WNT-5A in human bone marrow MSCs inhibits adipogenesis and promotes osteoblastogenesis by inhibition of peroxisome proliferator-activated receptors γ (PPARγ) activation via a CaMKII-TAK1-TAK1-binding protein2 (TAB2)-NLK signaling axis and simultaneous induction of runt-related transcription factor (RUNX) expression [298].

In line with its role in morphogenesis and stem cell differentiation, WNT-5A has recently been shown to be involved in tissue repair and regeneration after injury. A study demonstrated robust induction of WNT-5A-positive mesenchymal cells following an intestinal injury which are specifically localized in the wound bed [232]. The presence of WNT-5A provided a demarcation of the regenerating proliferative area via potentiation of TGF-β signaling. This allowed a fine-tuning of regeneration and proper wound healing [232]. Increased amount of WNT-5A is observed in lung tissue from mouse model of acute respiratory distress syndrome (ARDS) which could be the repair response of damaged lungs to resolve the injury [299]. Indeed, WNT-5A can promote the survival of bone marrow-derived MSCs following an oxidative-stress injury and can induce their differentiation into the type II alveolar epithelial cells (ATII) via activation of JNK and PKC signaling [299].

1.6 WNT signaling in pulmonary diseases

Aberrant WNT signaling has been linked to a myriad of pathological conditions including fibroproliferative, malignant and inflammatory disorders [137]. A growing body of literature
implicates WNT signaling in pulmonary fibrosis but the underlying mechanisms are not well understood. Increased expression of WNT signaling pathway genes (WNT-1, WNT-7B, WNT-10B, FZD2, FZD3, β-catenin and LEF1) have been detected in lung biopsies from idiopathic pulmonary fibrosis (IPF) patients [301] whereas elevated expression of WNT-3A, LRP5 and LRP6 have been reported in peripheral blood monocytes isolated from IPF patients and has been linked with disease progression [302]. Immunohistochemistry analysis revealed an increased activation of WNT/β-catenin signaling in the bronchial and alveolar epithelium of IPF lung sections [301] whereas increases immunoreactivity to WNT-7B is observed in the IPF lung tissues which was localized in the regions of active fibrotic changes [303]. Similarly, in lung biopsies from IPF/UIP patients there was an increase in nuclear β-catenin, a hallmark of active β-catenin signaling, associated with the regions of proliferative bronchiolar lesions and fibroblast foci [304]. Interestingly, increased expression of matrilysin/MMP7 [304], a target of WNT/β-catenin signaling, overlapped with the regions of high nuclear β-catenin [305]. Genetic ablation of matrilysin/MMP7 confers protection against bleomycin-induced lung injury [306] supporting an important but detrimental functional role of WNT/β-catenin signaling in pulmonary fibrosis. Increased nuclear β-catenin is also observed in fibroblasts from different fibrotic conditions including IPF and systemic sclerosis pulmonary fibrosis patients [307,308]. Whilst these studies identify a detrimental role for WNT/β-catenin signaling in pulmonary fibrosis, alveolar epithelial cell-specific genetic ablation of β-catenin augmented cell death and impaired the repair response post-lung injury suggesting a protective role for β-catenin [309] as accelerated apoptosis of alveolar epithelial cells contributes to the progression of pulmonary fibrosis [310]. This observation by Tanjore et al (2013) highlights an important role for WNT/β-catenin signaling in alveolar cells where it regulates the repair process after lung injury [309]. Perhaps, the strong increase in WNT/β-catenin signaling could signal a repair response of the lung in response to an insult. β-Catenin, thus, may serve different roles in different structural compartments of the lung. The differential roles could be attributed to the downstream interaction partners which associate with β-catenin concluding the transcriptional complex and activating target gene transcription. Indeed, preventing β-catenin-CBP interaction rescues mice from bleomycin-induced lung injury [311] whereas inhibition of β-catenin-p300 interaction worsens lung epithelial repair during inflammation [312] underlining a detrimental role for β-catenin-CBP and a protective role for β-catenin-p300 interaction. These observations reveal that fine-tuning of β-catenin-mediated responses by its interaction partners can channel β-catenin signaling into a positive or negative outcome.

In addition to β-catenin, other components of WNT signaling are also implicated in pulmonary fibrosis. Global knockout of LRP5, the canonical WNT signaling coreceptor, protects against pulmonary fibrosis by decreased β-catenin signaling which, in turn, reduced the expression of TGF-β [302].

The profibrotic cytokine, TGF-β, also suppresses the expression of a WNT antagonist- DKK-1, thereby facilitating increased WNT/β-catenin signaling suggesting a causal link between altered WNT signaling and development of fibrosis [307]. WNT-5A, a non-canonical WNT ligand, is also highly upregulated in fibroblasts isolated from IPF patients and regulates cell
proliferation, survival and expression of fibronectin [313]. Furthermore, in a murine model of mechanical ventilation-induced pulmonary fibrosis, WNT-5A expression is increased considerably and contributes to lung injury and fibrosis [314]. As mentioned in the earlier sections, disrupted ECM homeostasis is an important denominator of fibrosis. Increasing evidence suggests a potential regulatory role of WNT signaling in ECM expression and deposition. TGFβ and WNT signaling can crosstalk at multiple levels playing a crucial role in fibrotic disorders [96]. Interestingly, TGF-β exerts a wide modulatory effect on WNT ligand and receptor expression in airway smooth muscle cells and β-catenin is required for TGFβ-induced ECM expression in airway smooth muscle cells [315].

Interactions between the WNT signaling pathway and the ECM are not unidirectional, as the ECM can also influence WNT signaling. For instance, mechanical stretch, which is partly defined by the composition and extent of the ECM, can lower the expression of the WNT antagonist DKK-1 in a dose-dependent manner, thereby activating WNT/β-catenin signaling [316]. Although the phenomenon is not confirmed in lungs, decreased abundance of DKK-1 in the fibrotic lungs could be explained by progressive stiffening of the disease afflicted organ.

Altered expression of microRNAs (miRNAs) has been associated with pulmonary fibrosis in clinical and experimental studies [317,318]. miRNAs are small noncoding RNA molecules of ~22 nucleotides which can repress expression of protein-coding genes by blocking the translation and/or promoting the degradation of specific target mRNAs [319]. WNT signaling and miRNAs can cross-regulate each other at multiple levels. Interestingly, abnormal expression of some of the miRNAs in pulmonary fibrosis can also be linked to WNT signaling modulation [318]. For instance, let-7d expression localizes to alveolar epithelium and is significantly downregulated in lung explants from IPF patients, an effect which is linked to TGF-β. Inhibition of let-7d in animal model induces features of severe lung fibrosis [320]. Interestingly, WNT/β-catenin signaling suppresses expression of let-7 family miRNAs [321]. miRNA-21 expression is increased in the lungs and serum from IPF patient [322,323] and can be induced by WNT/β-catenin signaling [324]. High levels of miRNA-21 can also be linked to enhanced WNT pathway activation as miRNA-21 targets and suppresses DKK-2 expression, a WNT antagonist, thereby promoting WNT/β-catenin signaling [325]. WNT-1 inducible signaling pathway protein 1 (WISP1) or CCN4, a target of WNT/β-catenin signaling pathway, is a cysteine rich, secreted matricellular protein of CCN family and regulates cell proliferation, survival and differentiation [326,327]. WISP1 has been linked to lung fibrosis in both experimental and human IPF. Considerable upregulation of WISP1 is found in the lung biopsies from IPF patients where its expression is localized to the hyperplastic, proliferating ATII cells in close proximity to epithelial lesions and fibroblast foci [328]. Similarly, marked augmentation in WISP1 expression, both at the gene and protein level, is also observed in the lungs obtained from mice subjected to bleomycin-induced lung fibrosis [328]. Interestingly, WISP1 expression in bleomycin-treated lungs is also primarily localized to the ATII cells [328]. In line with its prohypertrophic role, treatment with exogenous recombinant WISP1 led to a strong proliferative effect on the primary ATII and lung epithelial cell line-A549 cells [328]. Interestingly, recombinant WISP1 induced myofibroblast differentiation and ECM expression in mouse and human lung
fibroblasts [328] whereas orthotracheal administration of neutralizing anti-WISP1 antibodies attenuated bleomycin-induced lung fibrosis and partially restored normal lung function [328]. These observations underline a critical role for WISP1 in WNT/β-catenin-induced pulmonary fibrosis.

Based on the insights from current literature, WNT signaling homeostasis clearly emerges as a major determinant of healthy and diseased conditions. Our understanding about the mechanisms and direct consequences of WNT signaling activation in fibrotic pulmonary disorders such as airway remodeling is poor. Further studies are warranted to understand the role of WNT signaling in airway remodeling and maintenance of its homeostasis in order to achieve our ultimate goal of utilizing WNT signaling modulation for treating fibrosis and other ailments involving deregulation of this developmental pathway.

**1.6.1 WNT signaling: therapeutic potential**

WNT signaling and its target genes are linked to proliferation, survival, matrix protein expression, inflammatory responses, stemness and differentiation. With the ever evolving understanding about its role in disease, WNT signaling is fast emerging as a promising therapeutic target. Many small molecule inhibitors and modulators are available and many more are under development for targeting WNT signaling pathway at different levels in the cascade [139]. As discussed in previous sections, β-catenin partners with various proteins to channel the effects of its activators, many strategies target the specific interaction of β-catenin with its downstream partners. For instance, small molecules like PKF115-584 are available to prevent β-catenin/TCF/LEF transcriptional assembly whereas ICG-001 and IQ-1 can inhibit association of β-catenin with transcriptional coactivators -CBP and p300, respectively [311,312]. Both CK1α and Axin facilitate β-catenin degradation. Pyrvinium, a small molecule inhibitor, can promote β-catenin degradation by activating CK1α whereas XAV939, an inhibitor of axin destabilizing kinase tankyrase, does the same by promoting Axin stability. In addition, small molecule inhibitors also exist for targeting DVL and WNT ligand secretion and availability of recombinant WNT antagonists like DKK and sFRPs further add to the resources [139].

**1.7 TGF-β and WNT signaling crosstalk: potential implications in airway remodeling** (adapted from Yeganeh et al (2013) [96])

Both TGF-β and WNT signaling regulate a myriad of processes including development, cell fate determination, and cellular differentiation and regeneration across the phyla from Drosophila to mammals [136,329]. Studies have implicated extensive cross-talk between TGF-β and WNT signaling pathways at various levels in their multi-component signaling system, from early development to post-natal tissue homeostasis (Figure 7). For instance, in Xenopus, both TGF-β and WNT signaling play important role in establishing the Spemann’s organizer mediated by association of SMAD4 with β-catenin in a LEF1/TCF-dependent manner [330]. Another study demonstrated that SMAD3 physically interacts with high mobility group (HMG) domain of LEF1 inducing transcriptional activation of twin and contributing to the patterning of embryo [331]. Other studies have also shown the TGF-β-dependent and independent association of SMAD proteins with β-catenin and LEF1/TCF in...
human and other mammalian cell systems [126,332-334]. Furthermore, TGF-β and various growth factors have been shown to activate β-catenin signaling through GSK-3β inactivation [107,126,315,335]. Observations suggest that TGF-β/WNT pathway cross-talk is highly context dependent. For instance, β-catenin is not required and doesn’t affect TGF-β-induced expression of PAI-1 in ASM cells and pulmonary fibroblasts [107,315], whereas in renal epithelial cells PAI-1 is a target of β-catenin-dependent WNT signaling [132]. Also, it is interesting to note that TGF-β-activated β-catenin doesn’t drive expression of canonical WNT target genes [107] but contributes to the activation of specific TGF-β target genes, probably those with LEF1/TCF- and / or SMAD-binding sites in their promoters. These differential effects of TGF-β/β-catenin axis can be attributed to the association of β-catenin with various nuclear partners in a stimuli- and cell-dependent manner.

![Diagram](image.png)

Fig. 7. TGF-β-WNT signaling crosstalk in airway smooth muscle cells. β-Catenin is the key effector of canonical WNT signaling wherein WNT ligand-induced GSK3 inhibition leads to protection of β-catenin from proteasomal degradation. TGF-β also prevents degradation of β-catenin by inactivating GSK3, which in combination with ERK1/2 mediated transcriptional upregulation of β-catenin, leads to the cytosolic accumulation of this transcriptional co-activator. Subsequently, non-phosphorylated transcriptionally active β-catenin contributes to the TGF-β-induced cellular effects in ASM cells. In addition, TGF-β may also induce expression of WNT ligands, however, their role in TGF-β signaling is not known (Taken from Yeganeh et al (2013) [96]).

ASM cells show remarkable phenotypic plasticity and accumulating evidences suggest a central role for TGF-β-WNT pathway cross-talk in regulating various processes governing structural and functional features of ASM cells. It has been shown that TGF-β increases abundance of β-catenin in ASM cells by transcriptional up-regulation [185,315]. TGF-β can also activate β-catenin signaling via inactivation of GSK-3β and rescuing it from proteasomal degradation leading to subsequent increase in active β-catenin in ASM cells [315]. Besides
inactivating GSK-3β, TGF-β signaling can protect β-catenin from degradation using SMAD3 [336]; however, this observation is not yet confirmed in ASM cells. Components of TGF-β and WNT signaling have been shown to be mutual targets of each other. For instance, WNT signaling can induce expression of TGF-β [337] whereas TGF-β can modulate expression of β-catenin in ASM cells [185]. The contribution of autocrine WNT production and signaling to TGF-β-induced β-catenin signaling is not yet established in ASM. Nonetheless, β-catenin signaling can regulate various aspects of ASM plasticity and contributes actively to TGF-β responses in ASM cells [338]. Interestingly, stabilization of β-catenin is both required and sufficient to induce ECM protein production in ASM cells [315]. This finding is important as deregulated ECM homeostasis is linked to airway remodeling in chronic lung diseases [46]. Accordingly, a study in smooth muscle cells linked TGF-β-induced inactivation of GSK-3β to increased cell size and expression of contractile proteins [339]. Indeed, hypertrophy and hypercontractility of ASM cells are features of airway remodeling [46]. Deng et al (2008) did not show any evidence concerning activation or involvement of β-catenin [339]; however it has been shown that β-catenin contributes to smooth muscle cell contractility. As demonstrated by Jansen et al (2010), β-catenin is required for force generation in bovine tracheal smooth muscle (BTSM) strips [340]. This effect is attributed to the β-catenin mediated stabilization of cell-cell adhesions [340]. A change in the expression of contractile proteins was not observed in this study, but other studies have implicated β-catenin-dependent contractile protein expression in fibroblasts [107,337]. In addition to contributing to force generation, β-catenin has also been shown to regulate proliferation of ASM cells. Nunes et al (2008) showed that growth factors induce inactivation of GSK-3β and subsequent increase in nuclear β-catenin levels, an effect which was required for the increase in DNA synthesis as cell proliferation was attenuated by β-catenin siRNA [341].

Further studies are therefore required for delineating the cross-regulatory mechanisms by which TGF-β and WNT pathways regulate ASM phenotype and functions. Also, the functional role of autocrine WNT secretion in response to TGF-β needs to be investigated further. A role for TGF-β-WNT signaling networks in hypercontractility, hypertrophy and hyperplasia of ASM cells along with their contribution to enhanced and altered deposition of ECM proteins emerges strongly. Hence, a crucial role of TGF-β-WNT cross-talk in airway remodeling can be envisaged.

1.8 Scope of the thesis

As discussed above, WNT signaling is a major player in lung morphogenesis and has been linked to several respiratory diseases with a large body of literature supporting the role of canonical WNT signaling mediator-β-catenin in various fibrotic disorders including those afflicting lungs. A study from our group has also reported a novel role for β-catenin in TGF-β-induced ECM production by airway smooth muscle [315]. Additional work from our lab has revealed the importance of β-catenin signaling in fibroblasts [107]. Nonetheless, the role of noncanonical WNT signaling is not known in airway remodeling. The primary aim of this thesis is to investigate the role of noncanonical WNT signaling in the initiation of cellular responses associated with airway remodeling. To this aim, in vitro experiments are performed using cultured ASM cell lines and human primary bronchial smooth muscle cell
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to identify the role of noncanonical WNT signaling in various aspects of airway remodeling. Underlying molecular mechanisms are dissected by employing pharmacological inhibitors and gene-specific knock-down strategies.

Chapter 2 provides a review of current understanding of the functional significance of β-catenin in airway remodeling in asthma and its therapeutic potential. A detailed account is provided about the contribution of β-catenin in various aspects of airway remodeling and the current strategies which can possibly target β-catenin signaling to counter airway remodeling.

Chapter 3 identifies WNT-5A, a noncanonical WNT ligand, as a novel player in airway remodeling. A comprehensive analysis of WNT signaling pathway alteration by TGF-β is performed in ASM cells. siRNA-mediated gene silencing was used to probe the specific roles of WNT-5A and for identification of its receptors. WNT-5A-activated downstream signaling was identified using recombinant protein antagonists and pharmacological inhibitors for JNK and Ca²⁺ signaling.

In Chapter 4, we assessed the role of noncanonical WNT ligands in TGF-β-induced expression of α-SMA, a key contractile protein of ASM cells. We identified a role of actin remodeling by observing the globular and filamentous actin fractions using specific binding dyes, specific pharmacological inhibitors such as latrunculin A and Y27632, and probed for the possible transcription mechanism linked to actin remodeling.

Chapter 5 describes the molecular mechanisms involved in TGF-β-induced WNT-5A expression and identifies Sp1 as a novel transcription factor for WNT-5A. We have dissected the signaling cascade employed by TGF-β in WNT-5A transcriptional upregulation using pharmacological inhibitors and siRNA mediated gene silencing. Furthermore, we did in silico analysis of WNT-5A promoter to predict candidate transcription factors and performed chromatin immunoprecipitation (ChIP) to identify the transcription factor.

The WNT signaling family is complex with large number of members with varied functions. Many of them are implicated in pulmonary diseases as discussed previously. Chapter 6 provides insights from a mouse model of chronic airway inflammation into modulation of WNT signaling in the lungs of an animal model of asthma. We performed a comprehensive gene expression analysis of WNT signaling family in whole lung extracts to identify novel candidates and mechanisms involved in asthma.

Chapter 7 reviews the current knowledge about WNT-5A signaling, functions and roles in various pathologies including pulmonary diseases.

Finally, in chapter 8, we summarize our results and discuss our findings providing a broader perspective and provide future perspectives.
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