WNT signaling pathway is altered in a mouse model of chronic allergic airway inflammation

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Abstract

Asthma is a chronic obstructive disease of the airways characterized by bronchial hyperresponsiveness, airway inflammation and remodeling. The underlying mechanisms that initiate, drive and maintain asthma are not completely understood. We have recently reported increased abundance of the WNT ligand, WNT-5A, in asthmatic airway smooth muscle (ASM) cells and have identified its novel role in TGF-β-induced extracellular matrix (ECM) expression. The WNT pathway is composed of a multitude of signaling components and participates in a myriad of biological functions in health and disease including pulmonary disorders. We have undertaken a comprehensive analysis of expression of WNT signaling members using an animal model of allergen-induced chronic inflammation to identify potential mediators of asthma pathophysiology. We here show a wide ranging modulation of most of the WNT ligands, Frizzled (FZD) receptors and various intracellular and extracellular mediators and modulators in the lungs of mice after ovalbumin (OVA) exposure. Notably, WNT-7A, -9A and -10B and FZD4 and FZD6 were significantly downregulated along with a modest but significant reduction in β-catenin mRNA levels post-OVA challenge. Of note, WIF1, a WNT signaling antagonist, showed strong downregulation in OVA-challenged lungs as compared to saline-challenged controls. Moreover, TGF-β reduced WIF1 mRNA in ASM cells. Owing to its antagonistic effect on WNT signaling, WIF1 downregulation may have key effects. In conclusion, allergen-induced modulation of the WNT signaling pathway with suppression of WIF1 may indicate a significant pathological event.

Introduction

Asthma is a heterogeneous chronic obstructive disease of the airways inflicting approximately 300 million people worldwide and imposing a substantial burden on the patients and healthcare system [1]. It is characterized by the presence of chronic inflammation, bronchial hyperresponsiveness and extensive structural changes in the airways, termed as airway remodeling [1,2].

Exposure to inhaled stimuli such as allergens or respiratory viruses triggers an exaggerated response in asthmatic airways inducing airway constriction leading to episodes of breathlessness and wheezing. Asthma can be effectively managed by β2 adrenoreceptor-agonists and/or corticosteroids in most patients providing substantial relief from the episodic breathlessness. However, despite the most effective current therapies, a subset of asthma patients remain poorly controlled even at the highest doses of the asthma medication [3,4]. Asthma is a complex 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 [1,5]. However, the factors and mechanisms that govern the initiation, progression and maintenance of asthma remain poorly understood hampering the efforts to develop new and more effective therapeutic strategies targeting asthma.

Airway remodeling is a key pathological feature of individuals with asthma and is associated with airway obstruction [6] and bronchial hyperresponsiveness [7]. It is characterized by
extensive structural changes in the airway wall which include airway smooth muscle (ASM) cell hypertrophy and hyperplasia, subepithelial fibrosis, mucus hypersecretion, neovascularization and increased and altered extracellular matrix (ECM) expression, leading to airway wall thickening [8]. 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 [8]. Similarly, the thickness of the remodeled airway wall also correlates with the severity of the disease [8-11].

WNT signaling is an important developmental pathway which plays critical roles in embryonic morphogenesis and regulates biological functions in post-natal life. The term WNT is derived from a combination of two homologues genes integrase 1 (int1) and wingless (wg) [12]. The Int1 gene was first identified as a locus for the integration of mouse mammary tumor virus DNA leading to its activation and involvement in the development of virally-induced breast tumors in mice [12,13]. Wg, which was identified for its role in development of wing tissue in Drosophila and regulation of larval segment polarity, was later found to be a homologue of Int1 [12]. The WNT signaling family has grown multifold since then both in the number of its members and its 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 [14]. 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 noncanonical WNT signaling.

Accumulating evidence point to a key role of WNT signaling pathway in asthma pathology. A study has highlighted an indirect link between the expression of various WNT ligands such as WNT-5A, -3A and 10A and the presence of Th2 inflammation in asthmatic subjects [15]. Moreover, we have recently identified significantly increased abundance of WNT-5A mRNA and protein in the airway smooth muscle (ASM) cells of asthma patients as compared to the healthy subjects (Chapter 3). We demonstrated that TGF-β, a growth factor implicated extensively in airway remodeling and asthma pathobiology [16], induces WNT-5A expression in ASM cells where it mediates ECM expression, thus, providing a possible link between WNT signaling and features of asthma pathobiology (Chapter 3).

WNT is an inherently complex pathway comprised of large number of members. In view of the crucial roles assigned to some of its members in lung development and diseases including asthma, we evaluated WNT signaling pathway expression patterns in lungs during asthma using a mouse model of allergen-induced chronic airway inflammation. Here, we present evidence of extensive modulation of WNT signaling components in ovalbumin (OVA)-challenged lungs and demonstrate that WNT inhibitory factor 1 (WIF1), a WNT signaling antagonist, was significantly attenuated on OVA exposure.
Material and Methods

**Reagents** - Recombinant human TGF-β, recombinant human WIF1 and mouse anti-WIF1 antibody were from R&D systems (Abingdon, UK). Human WIF1 siRNA and mouse anti-GAPDH, mouse anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-total β-catenin antibody was from BD Biosciences (San Jose, CA, USA) and mouse anti-active β-catenin antibody (clone 8E7) was obtained from Millipore (Amsterdam, the Netherlands). HRP-conjugated goat anti-mouse antibody and human Interleukin-1β (IL-1β) was obtained from Sigma (St. Louis, MO, USA) and Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody was procured from Jackson ImmunoResearch Europe (Suffolk, UK). Non-targeting siRNA was procured from Qiagen (Venlo, The Netherlands) and rabbit- anti-α-smooth muscle-actin and rabbit anti-WIF1 antibody were obtained from Abcam (Cambridge, UK). X-tremeGENE siRNA and X-tremeGENE DNA HP transfection reagents were purchased from Roche Applied Science (Mannheim, Germany). All other chemicals were of analytical grade.

**Animals** - Female BALB/c mice about 8-12 weeks old, were procured from Charles River Laboratories (Leiden, the Netherlands). Animals were housed under a conventional 12-hour light/dark cycle and received food and water *ad libitum*. All animal studies were conducted according to the national guidelines and were approved by the University of Groningen Committee for Animal Experimentation (no. 5912A).

**Animal Model** - Animals were sensitized to OVA (Sigma-Aldrich, Zwijndrecht, the Netherlands) on Days 1, 14, and 21 by intraperitoneal administration of 10 µg OVA emulsified in 1.5 mg aluminum hydroxide (Aluminject; Pierce Chemical, Etten-Leur, The Netherlands) in a final volume of 200 µl in PBS. Subsequently, mice were divided into two groups (n=10-12) and challenged with either saline or OVA aerosols (1% in PBS) for 20 minutes twice weekly on consecutive days for 4 weeks (Figure 1). The aerosol was delivered to a Perspex exposure chamber (9 liter) by a DeVilbiss nebulizer (type 646; De Vilbiss, Somerset, PA) driven by an airflow of 40 L/min, providing an aerosol with an output of 0.33 ml/min, as described previously (19).

![Figure 1. Animal model of allergen-induced chronic inflammation. Female BALB/c mice (n=10 per group) were sensitized to ovalbumin (OVA) on Day 1, 14 and 21. Animals were subsequently challenged with saline or OVA aerosols twice weekly for 4 weeks as described in the material and methods section. 24 hour following the last challenge, animals were sacrificed and lungs were harvested for further analysis.](image-url)
Chapter 6

**Tissue collection**- 24 hours following the last challenge, animals were anesthetized with ~2% (v/v) Isoflurane in oxygen and sacrificed by exsanguination. Right lung was inflated with 50% Tissue-Tek in PBS (Sakura Finetek, Alphen aan de Rijn, the Netherlands) and lungs were subsequently harvested. The smallest lower and the upper right lung lobes were separately snap-frozen for mRNA analysis, the two middle right lung lobes were snap frozen for immunohistochemistry. Uninflated left lung was snap frozen for protein analysis.

**Immunohistochemistry**- Morphometric analyses were performed on the transverse tissue cryosections of 5µM thickness. Eosinophils were visualized by staining cryosections with diaminobenzidine (SigmaAldrich, Zwijndrecht, the Netherlands) for cyanidexresistant endogenous peroxidase activity. The number of eosinophils around the airways was counted and expressed as number of cells per mm basement membrane. Cryosections were stained for α-smooth muscle-actin using a rabbit anti-α-smooth muscle-actin antibody (Abcam, Cambridge, UK) which was visualized by staining with horseradish-peroxidase-linked secondary antibody and diaminobenzidine (Sigma-Aldrich, Zwijndrecht, the Netherlands). Airways within sections were digitally photographed and α-smooth muscle-actin around the airway was quantified using Image J (National Institute of Health). The surface of positively stained tissue was expressed as mm² per mm² basement membrane.

**Immunofluorescence**- For immunofluorescence, cryosections were air-dried, fixed in acetone for 10 minutes and blocked with a mix of 2% donkey serum and 1% BSA for 1 hour. WIF1 was detected using rabbit anti-WIF1 antibody (Abcam, Cambridge, UK) and FITC-conjugated donkey anti-rabbit antibody. β-Catenin was visualized by mouse anti-β-catenin antibody using mouse-on-mouse (MOM) elite peroxidase kit (Vector Laboratories, Burlingame, USA) as per manufacture’s instruction with minor modifications. Briefly, following the blocking with a mix of 2% donkey serum and 1% BSA as mentioned above, cryosections were further incubated with the mouse Ig blocking reagent for 1 hour and with MOM diluent for 5 minutes. Subsequently, cryosections were incubated with anti-β-catenin antibody for 30 minutes and with a secondary rabbit anti-mouse IgG for another 30 minutes. β-Catenin was visualized by using a tertiary FITC-conjugated donkey anti-rabbit antibody. Nuclei were stained with Hoechst diluted in ddH2O. After staining, cryosections were mounted in ProLong Gold Antifade reagent (Life Technologies, Bleiswijk, the Netherlands). Immunofluorescence was analyzed using Olympus AX70 microscope.

**Cell culture**- Immortalized human airway smooth muscle cell lines (ASM) cells and human bronchial epithelial cell lines (HBE) were used for in vitro experiments. Cell lines were maintained and used for experiments as described previously ([Chapter 3](#) and [17]).

**RNA isolation and real time PCR**- Lung homogenates were prepared by pulverizing tissues under liquid nitrogen. Total RNA was extracted from the lung homogenates and cell lines using the Nucleospin RNAII kit (Macherey-Nagel, Duren, Germany) as per manufacturer’s instructions. Equal amounts of total RNA were reverse transcribed using the
Reverse Transcription System (Promega, Madison, USA). 1 µl of 1:2 diluted cDNA was subjected to real-time PCR using FastStart Universal SYBR Green Master (Rox) from Roche Applied Science (Mannheim, Germany) in an Illumina Eco Personal QPCR System (Westburg, Leusden, the Netherlands). Real time PCR was performed with denaturation at 94°C for 30 seconds, annealing at the mentioned temperatures (Table 1) for 30 seconds and extension at 72°C for 30 seconds for 40 cycles followed by 10 minutes at 72°C. Data was analyzed using the comparative cycle threshold (Cq: amplification cycle number) method. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA (ΔCq). Where appropriate, relative differences were determined using the equation 2^(-ΔΔCq). Primers used for PCR amplification are listed in Table 1.

**Western Blotting**- Lung homogenates were prepared by pulverizing tissues under the liquid nitrogen with subsequent sonication in SDS lysis buffer (62.5 mM Tris, 2% w/v SDS, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM β-glycerophosphate, pH 6.8). The hTERT and HBE cells were also lysed in SDS lysis buffer supplemented with protease inhibitors.

Protein concentration was determined using Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were separated by electrophoresis, electro-transferred to nitrocellulose membranes and analyzed for proteins of interest using specific primary and HRP-conjugated secondary antibodies. Westerns were subsequently visualized using the G-box gel documentation system (Syngene, Cambridge, UK) using enhanced chemiluminescence reagents and were quantified by densitometry using Genetools software where necessary.

**siRNA transfection**- ASM cells were grown to ~90% confluence in 6-well cluster plates and transfected with 200 pmol of WIF1-specific in serum and antibiotic free DMEM with X-tremeGENE siRNA transfection reagent (Roche). Control transfections were performed using a non-targeting control siRNA (Qiagen). After 6 hours of transfection, medium was replaced with DMEM supplemented with antibiotics and ITS for a period of 42 hours before TGF-β stimulation.

**DNA transfection**- ASM cells or HBE cells were grown to ~90% confluence in 6-well or 24-well cluster plates and were subsequently transfected with 1 µg of Myc-DDK-tagged human WIF1 plasmid (Myc-WIF1) (Origene, Rockville, MD, USA) in serum and antibiotic free medium using X-tremeGENE HP DNA transfection reagent. 1 µg of Green Fluorescent Protein (GFP) expression vector was transfected as control. After 6 hours of transfection, medium was replaced with DMEM or MEM supplemented with antibiotics and 10% (v/v) fetal bovine serum (FBS) for 18 hours. Cells were then serum-deprived for 24 hours before stimulation(s).

**IL-8 ELISA**- ASM cells were stimulated for 24 hours with IL-1β. Supernatants were collected and IL-8 concentrations were determined by ELISA as per manufacturer's instructions (Sanquin, the Netherlands).
Table 1: Primers used for WNT pathway gene expression analysis

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<th>Gene</th>
<th>Forward 5’ → 3’</th>
<th>Reverse 5’ → 3’</th>
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<tr>
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<tr>
<td>WNT-3</td>
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<tr>
<td>WNT-3A</td>
<td>GTGCAACCTGCAAAGTAGGA</td>
<td>TCTCCGCCCCAGTCAAGAA</td>
</tr>
<tr>
<td>WNT-4</td>
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</tr>
<tr>
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<tr>
<td>WNT-6</td>
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</tr>
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<td>WNT-16</td>
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<td>CTNNBP1 (ICAT)</td>
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<td>TCCCTCTTCATGCTGACAC</td>
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<td>Axin2</td>
<td>GCTGAGAGGATGCGATCAGAGC</td>
<td>GGCAGAAGCTGCTAGATTT</td>
</tr>
<tr>
<td>Wisp1</td>
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</tr>
<tr>
<td>18S</td>
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<td>CCTCAAATGAGATACCTG</td>
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**Data Analysis** Values reported for all data are represented as mean ± SEM. The statistical significance of differences between means was determined on log transformed data by Student’s t-test or by 1-way ANOVA, followed by Student-Newman Keuls multiple comparisons test. Differences were considered to be statistically significant when p<0.05.

**Results**

**Inflammation and remodeling in the mouse model of allergen-induced chronic airway inflammation.** Persistent chronic inflammation and airway remodeling are important characteristics of asthma. We assessed these parameters in our animal model to ascertain the presence of asthma-like characteristics. OVA treatment induced eosinophilia as observed by a massive increase in the number of eosinophils in the lungs of OVA-challenged mice underlining the presence of inflammation in the animal model (Figure 2A). Similarly, a profound increase in the α-smooth muscle-actin positive area surrounding the airways was observed in OVA-challenged mice in comparison to saline-challenged mice underlining increased ASM mass (Figure 2B). Taken together, our data shows the presence of inflammation and airway remodeling post-OVA challenge validating our animal model used in this study.

![Figure 2. Morphometric analysis of the lungs.](image)

Animals were treated as described in Figure 1. Lungs were harvested 24 hours after the last challenge. (A) Eosinophils were analysed using DAB, counted and expressed as number per mm of basal membrane (n=8 each group). Quantification and representative image is shown here. (B) α-Smooth muscle-actin is stained using specific antibody to determine airway smooth muscle mass. Positively stained area is quantified and expressed as per mm² of basement membrane (n=8 per group). Data represent mean ±SEM. **p< 0.01, ***p<0.001 compared to saline-challenged control mice; 2-tailed Student’s t test for unpaired observations. Magnification 200X.
Extensive modulation of WNT signaling pathway by allergen challenge.

Having validated our animal model, we next screened the lung homogenates for mRNA expression of various components of the WNT signaling pathway. OVA challenge led to a wide ranging modulation in gene expression of WNT ligands. WNT-1, -3, -3A, -5A, -5B, -7B, -9B, -10A, -11 and -16 showed varying degree of up or downregulation following OVA challenge but the observed changes failed to achieve statistical significance. On the other hand, WNT-7A, -9A and -10B were significantly downregulated upon OVA exposure in comparison to the saline-challenged group (Figure 3A, 3B). Similarly, FZD4 and FZD6 expression were significantly downregulated by OVA exposure whereas other FZD receptors remained either unaltered or showed minimum modulation (Figure 3C, 3D). The canonical WNT signaling mediator-β-catenin, showed modest but significant reduction following OVA challenge whereas Axin2, a member of the β-catenin destruction complex, remained unchanged (Figure 3E). The expression of secreted FZD related protein (SFRPs) and Dickkopf1 (DKK1) which function as extracellular inhibitors of WNT signaling, SFRP1 showed a trend of increased expression upon OVA challenge whereas SFRP2 and DKK1 didn’t change (Figure 3F). Similarly, the intracellular inhibitor of β-catenin-mediated transcription- inhibitor of β-catenin and TCF (ICAT), also remained unaltered. One of the most important observations came from the analysis of WIF1 which showed a significant decline in response to OVA challenge (Figure 3F). WIF1 is an extracellular WNT inhibitor which antagonizes WNT signaling by binding to various WNT ligands.

Since the reduction in WIF1 was among the most significant changes, we further expanded the mRNA observations at protein level. We performed immunofluorescence microscopy on lung cryosections using WIF1-specific antibody which indicated a reduction in WIF1 abundance in the OVA-challenged airways as compared to saline-challenged group (Figure 3G).

Thus, our data suggest a widespread modulation of WNT signaling pathway components in the lungs of OVA-challenged animals with a significant decline in expression of the WNT antagonist WIF1 among others.

Functional significance of WIF1 downregulation in asthma. We directed our focus on WIF1 which was significantly downregulated by OVA-challenge. We hypothesized that reduced abundance of WIF1 releases its target WNT ligands from inhibition and allows enhanced intracellular WNT signaling, irrespective of their expression levels. To this aim, we first analyzed the activation of β-catenin signaling. As revealed by immunofluorescence staining, β-catenin shows a clear localization to the lateral membrane of airway epithelial cells in the saline-challenged group, in line with its established role as a component of adherens junctions (Figure 4A). Interestingly, OVA challenge completely altered the membrane localization of β-catenin in the airway epithelial cells with the lateral staining completely lost and replaced by a less clear apical staining pattern (Figure 4A). Of note, we also failed to detect the nuclear localization of β-catenin, a marker of active β-catenin signaling, post-OVA challenge. We also performed western blot analysis to detect any changes in β-catenin signaling. As shown in Figure 4B, there was no difference in abundance
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**A**

WNT Ligand

Saline

Ova

Relative mRNA expression (fold of respective saline)

Frizzled receptors

1 2 3 4 5 6 7 8 9 10

**B**

WNT Ligand

Saline

Ova

Relative mRNA expression (fold of WNT-9A)

Frizzled receptors

1 2 3 4 5 6 7 8 9 10

**C**

Saline

Ova

Relative mRNA expression (fold of FZD 5)

Frizzled receptors

1 2 3 4 5 6 7 8 9 10

**D**

Saline

Ova

Relative mRNA expression

Frizzled receptors

1 2 3 4 5 6 7 8 9 10
Interestingly, the nonphospho β-catenin which represents the active fraction of β-catenin showed a modest increase in the lung homogenates of OVA-challenged group as revealed by quantification of the westerns. Though the data indicate a possible activation of β-catenin signaling, they remain inconclusive due to lack of any statistical significance in the effects.

Figure 3. Expression analysis of WNT family members. Animals were treated as described in Figure 1. Lungs were harvested 24 hours after the last challenge. Total RNA was isolated from whole lung homogenates and mRNA expression was determined using qRT-PCR. Expression of genes is represented as Cq-values corrected for 18S rRNA. WNT ligands mRNA abundance as fold of respective saline-challenged control (A) or relative to WNT-9A (B). FZD receptor mRNA expression as fold of respective saline-challenged control mice (C) or relative to FDZ5 abundance (D). (E, F) Expression of various WNT pathway components relative to saline-challenged control mice as depicted in the respective panels. Data represent mean ±SEM; n=10 each group. *p<0.05, **p<0.01, ***p<0.001 compared to respective saline-challenged control mice; 2-tailed Student's t test for unpaired observations. (G) Animals were treated as described in Figure 1. Lungs were harvested 24 hours after the last challenge. Cryosections were prepared and immunofluorescence microscopy was performed as described in material and methods. Representative image showing WIF1 staining in saline- and OVA-challenged mice. Magnification 40X.
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Figure 4. Effect of OVA exposure on β-catenin protein levels. (A) Animals were treated as described in Figure 1. Lungs were harvested 24 hours after the last challenge. Cryosections were prepared and immunofluorescence microscopy was performed as described in material and methods. Representative image showing β-catenin staining in saline- and OVA-challenged mice. Magnification 40X. (B) Total and active β-catenin expression levels were analysed in whole lung extracts by western blotting. Equal protein loading was verified by analysis of β-actin. Abundance of total and active β-catenin bands is quantified and expressed as percentage of saline-challenged controls. Data represent mean ±SEM; n=4 each group.

We expanded our investigation in vitro using ASM and HBE cell lines to identify which compartments of lungs are contributing to the observed reduction in WIF1 expression in OVA-challenged group. TGF-β is the most prominent cytokine which is increased in asthmatic airways and regulates various aspects of asthma pathophysiology [18-21]. Interestingly, TGF-β significantly reduced WIF1 mRNA expression in ASM cells but not in HBE cells underlining the cell-dependent effects (Figure 5A, 5B). Additionally, HBE cells were treated with house dust mite extract (HDM), a common allergen, which also failed to alter WIF1 mRNA levels (Figure 5B).
Our previous study has shown that TGF-β induces expression of WNT-5A in ASM cells where it mediates expression of TGF-β-induced ECM proteins (Chapter 3). As WIF1 can antagonize WNT-5A and as observed, TGF-β decreases WIF1 expression in ASM cells (Figure 5A), we investigated the possible role of WIF1 in TGF-β-induced ECM expression. We observed that the presence of recombinant WIF1 had no effect on TGF-β-induced collagen 1α1 and fibronectin expression in ASM cells (Figure 6A). Similarly, exogenous WIF1 failed to affect other key TGF-β responses in ASM cells such as upregulation of WNT-5A and reduction of SMAD3 expression (Figure 6B, 6C). An increase in WNT-5A message in ASM cells appears only after 12 hours of TGF-β stimulation (Chapter 3), so we decided to coincide WIF1 treatment with the time of TGF-β-induced WNT-5A expression. ASM cells were first stimulated with TGF-β for 12 hours following which recombinant WIF1 was added and cells were further incubated for 12 hours. However, we failed to observe any effect of exogenous recombinant WIF1 on TGF-β-induced expression of collagen and fibronectin (Figure 6D). Considering that complete absence of serum might have some effect on the stability or function of WIF1 directly or indirectly, we decided to tweak our experimental conditions. Instead of serum-free media, stimulations were carried out using a medium supplemented with 0.5% serum and ASM cells treated with or without TGF-β and/ or recombinant WIF1. However, presence of exogenous WIF1 didn’t change the expression of TGF-β-induced ECM (Figure 6E).

Using a loss-of-function approach, we observed that knock-down of WIF1 using specific siRNA had no effect on collagen 1α1 or fibronectin expression in response to TGF-β in ASM cells (Figure 7A). In a reciprocal approach, we overexpressed WIF1 in ASM cells and evaluated expression of TGF-β-induced ECM proteins. Following transfection with Myc-
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WIF1, the WIF1 message showed ~26000 fold increase at basal level (Figure 7B). However, similar to knock-down, WIF1 overexpression failed to alter collagen or fibronectin expression in response to TGF-β (Figure 7C).

WNT signaling has recently been linked to Th2 inflammation in asthma subjects [15]. So, we investigated whether WIF1 participates in the inflammatory responses in ASM cells. IL-1β induces IL-8 secretion in ASM cells [22,23]. However, WIF1 overexpression had no effect on IL-1β-induced IL-8 release in ASM cells (Figure 8).

Taken together, our comprehensive analysis revealed that WIF1 had no effect on TGF-β-induced ECM expression and doesn’t participate in regulating IL-1β-induced inflammatory responses in ASM cells.

Discussion

In the current study, we evaluated alterations in the expression of WNT pathway components using an animal model of allergen-induced chronic airway inflammation. We demonstrate a broad modulation of WNT family gene expression levels post-OVA challenge. Most importantly, we identified a significant decrease in WIF1 in OVA-challenged lungs compared to the saline-challenged controls. Moreover, functional studies using gain- and loss-of-function approaches revealed that WIF1 doesn’t participate in TGF-β-induced ECM production or IL-1β-induced IL-8 release in ASM cells.

Aberrant WNT signaling activation has been reported in several fibroproliferative disorders. For instance, increased expression of WNT signaling pathway genes and enhanced nuclear abundance of β-catenin is observed in fibroproliferative diseases of kidney, liver and bone [24-27]. Similarly, expression of several WNT signaling pathway genes such as WNT-1, WNT-7B, WNT-10B, FZD2, FZD3, β-catenin and LEF1 and nuclear localization of β-catenin are augmented in idiopathic pulmonary fibrosis patients [28,29], supporting a comprehensive role for WNT signaling in fibroproliferative disorders, including those of the lung. We have recently shown that WNT-5A expression is increased in ASM cells derived from asthmatic patients in comparison to healthy subjects. WNT-5A is also overexpressed in fibroblasts of patients with usual interstitial pneumonia [30]. Here, we present evidence for a wide range modulation of WNT signaling pathway in asthmatic lungs obtained from the mouse model of allergen-induced chronic airway inflammation. WNT ligands exhibit extensive up and downregulation in expression, with significant downregulation of WNT-7A, WNT-9A and WNT-10B in OVA-challenged group in comparison to the saline-challenged control group. Similarly, FZD receptors and other components of WNT pathway examined also showed widespread modulation in expression post-allergen challenge. Importantly, basal expression of WNT-5A was one of the highest in mice lungs with a trend towards upregulation post-allergen challenge. WNT-5A along with WNT-5B and WNT-11 and FZD8 are targets of TGF-β in ASM cells as we reported previously. However, lack of a specific TGF-β signature on WNT ligands and FZD receptor expression in the lungs obtained from mouse model could be attributed to the use of whole lungs instead of isolated compartments in this analysis. The effect of TGF-β on WNTs in ASM might be different from its effect in epithelial cells or fibroblasts.
Figure 6. Effects of exogenous WIF1 on TGF-β cellular responses. (A-C) ASM cells were stimulated with TGF-β (2 ng/ml) in the presence or absence of recombinant WIF1 (3 µg/ml) for 24 hours. Expression of collagen 1α1, fibronectin and PAI1 mRNA (A), WNT-5A mRNA (B) and SMAD3 mRNA (C) was determined by qRT-PCR, corrected for 18S rRNA and expressed relative to the untreated control. Data represent mean ±SEM of 3-7 independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to untreated control; 1-way ANOVA followed by Newman-Keuls multiple comparisons test. (D) ASM cells were stimulated with TGF-β (2 ng/ml) for 12 hours following which recombinant WIF1 (3 µg/ml) was added and cells were further incubated for 12 hours. Expression of collagen 1α1, fibronectin mRNA was determined by qRT-PCR, corrected for 18S rRNA and expressed relative to the untreated control. Data represent mean ±SD of 2
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Independent experiments. (E) ASM cells were stimulated with TGF-β (2 ng/ml) in the presence or absence of recombinant WIF1 (3 µg/ml) for 24 hours. DMEM supplemented with 0.5% FBS is used for all treatment conditions. Expression of collagen Iα1, fibronectin mRNA was determined by qRT-PCR, corrected for 18S rRNA and expressed relative to the untreated control. Data represent mean ±SD of 2 independent experiments.

![Bar chart](image)

**Figure 7. Effects of WIF1 loss- or gain-of-function in ECM production in ASM cells.** (A) ASM cells were transfected with WIF1-specific siRNA or a negative (control) siRNA as control. Subsequently, cells were stimulated with TGF-β (2 ng/ml) for 24 hours. Expression of collagen Iα1 and fibronectin mRNA was analyzed by qRT-PCR and expressed relative to negative siRNA-transfected, untreated control. Data represent mean ±SD of 2 independent experiments. (B, C) ASM cells were transfected with Myc-WIF or GFP as control. Subsequently, cells were stimulated with TGF-β (2 ng/ml) for 24 hours. Expression of WIF1 mRNA (B) and collagen Iα1 and fibronectin mRNA (C) was analyzed by qRT-PCR and expressed relative to GFP-transfected, untreated control. Data represent mean ±SD of 2 independent experiments.

Earlier studies have provided some evidence about the possible involvement of WNT signaling in asthma. Airway remodeling is a critical feature of the asthmatic airway which involves extensive structural changes in the affected airway [1,2]. These changes include ASM hypertrophy and hyperplasia, epithelial damage, basement membrane thickening and altered and augmented ECM deposition [1,2]. A study from our lab showed that β-catenin mediates TGF-β-induced expression of ECM expression in ASM cell, implicating cross-talk between TGF-β and canonical WNT signaling mediator to regulate airway remodeling [31].
TGF-β is a pleiotropic cytokine and highly increased in asthmatic lung tissue and broncho-alveolar lavage (BAL) fluid and functions as master regulator of airway remodelling in asthma [16,19-21]. While accumulating evidence have key roles for β-catenin in airway remodeling (Chapter 2), a direct link between canonical or noncanonical WNT signaling with asthma is not known. Increased expression of WNT signaling pathway members in airway epithelium and endobronchial biopsies of asthma patients, including WNT-5A, have been reported although the cellular localization and functional roles of these WNT ligands were not determined [15,32]. Here, we demonstrate a direct link with WNT signaling family modulation using our animal model.

Furthermore, we demonstrate a significant decrease in WIF1 mRNA levels in the lungs of OVA-challenged mice as compared to saline-challenged mice. WIF1 is an evolutionary conserved protein that can bind to and antagonize WNT signaling. WIF1 has been shown to bind to WNT-3A, -4, -5A, -7A, -9B and -11 via its WIF domain with varying affinities (WNT-5A > WNT-9B > WNT-11 > WNT-4 > WNT-7A > WNT-3A) [33]. WIF1 binds to WNT ligands via its WIF domain and tethers this WIF1-WNT complex in the ECM by its interaction with the heperan sulphate proteoglycan glypican via epidermal growth factor-like domains [34]. Indeed, glypicans can have modulatory effects on WNT signaling [35] and formation of this WNT-WIF1-glypican complex is required for complete WNT antagonizing activity of WIF1 [34]. Of note, the observed suppression of WIF1 is particularly important as single nucleotide polymorphisms in WIF1 associate with lung function in two cohorts of asthma patients [14]. Moreover, WIF1 is a target of bone morphogenetic protein 4 (BMP4)-SMAD1 signaling in developing lungs where it antagonizes WNT/β-catenin signaling and regulates lung morphogenesis by fine tuning the WNT and BMP signaling. Abrogation of WIF1 expression leads to severe fetal lung abnormalities, in part, due to hyperactivation of WNT/β-catenin signaling [36]. In line with this, a study has demonstrated downregulation of SMAD1 and WIF1 expression during the saccular stage of lung development in mouse model of congenital diaphragmatic hernia, leading to the retardation of lung morphogenesis and appearance of hypoplastic lung [37]. Owing to the crucial role of WIF1 in WNT signaling

Figure 8. Functional analysis of WIF1 in inflammation in ASM cells. ASM cells were transfected with Myc-WIF or GFP as control. Subsequently, cells were stimulated with IL-1β (1 ng/ml) for 24 hours. IL-8 abundance was measured in cell supernatants by ELISA. Data represent mean ±SEM of 3 independent experiments. ***p<0.001 compared to GFP transfected, untreated control; 1-way ANOVA followed by Newman-Keuls multiple comparisons test.
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regulation, suppression of WIF1 is often associated with malignancies such as prostate, breast, lung, and bladder cancer [38]. Thus, WIF1 downregulation could lead to disruption of lung homeostasis both during embryogenic and adult life.

Lungs are highly specialized organ composed of many different compartments. Epithelium and ASM represent important structural and functional units of the lung. Epithelium constitutes the barrier between airways and the external environment and also serves as the first line of defense. ASM cells, on the other hand, provide mechanical properties to the airways and maintain bronchial tone. Both epithelium and ASM cells can respond to a range of growth factors and other substances present in the lung and as such actively participate, not only in maintenance of lung homeostasis but also in respiratory disorders such as asthma. In fact, anomalies in epithelial and ASM bundle are prominent features of a remodeled airway in asthma [1,2,39]. We attempted to identify the lung compartment(s) contributing to the reduction WIF1 abundance in OVA-challenged lung. We here demonstrate that TGF-β suppresses WIF1 expression in ASM cells but not in HBE cells. Since epithelial cells are exposed to external environmental allergens, we treated HBE cells with HDM extract, a common aeroallergen. HDM extract alone or in combination with TGF-β failed to convincingly reduce WIF1 mRNA levels in HBE cells (Figure 3B; data not shown) suggesting a highly context dependent regulation of WIF1 expression. Moreover, basal abundance of WIF1 transcripts is much higher in ASM cells as compared to HBE cells. In view of these observations, reduced expression of WIF1, as seen in OVA-challenged lungs, might be attributed to the ASM cells.

We have recently demonstrated that WNT-5A regulates TGF-β-induced ECM expression in ASM cells (Chapter 3). We hypothesized that downregulation of WIF1 by TGF-β in ASM cells could be an important mechanism for allowing maximum WNT-5A signaling to drive ECM expression. However, the presence of exogenous WIF1 didn’t affect TGF-β-induced ECM expression in ASM cells. Similarly, loss of WIF1 using siRNA or overexpression of WIF1 didn’t show substantial effects on TGF-β-induced ECM expression negating our preliminary hypothesis about the role of WIF1 in TGF-β responses. However, the observations from WIF1 loss-of-function and gain-of-function investigation should be viewed with caution owing to insufficient replicates for statistical analysis. Further experiments are warranted to determine the functional significance of WIF1 downregulation in ASM cells.

Epigenetic mechanisms and posttranscriptional mechanisms can regulate WIF1 expression levels. DNA methylation is an epigenetic modification that renders target gene inactive. Promoter hypermethylation is associated with WIF1 suppression in lung [40], breast cancer [41] and astrocytomas [42]. Further, a study demonstrated that miRNA-374a can target WIF1 transcripts therby limiting its expression in breast cancer cells [43]. Interestingly TGF-β has been shown to induce expression of DNA methyltransferases (DNMTs), enzymes involved in DNA methylation, in prostate cancer cells to suppress its own signaling [44]. On the contrary, TGF-β downregulates DNMTs in cardiac fibroblasts thereby reducing collagen type I promoter methylation and allowing its subsequent expression [45] highlighting the context-dependent regulation of DNA methylation by TGF-β. How TGF-β induces WIF1
suppression in ASM cells and whether any epigenetic mechanisms are involved or not needs further investigation.

Taken together, the data presented in current study establish that the WNT signaling pathway is extensively modulated in response to allergen challenge. Of note, downregulation of WIF1 by TGF-β may represent a detrimental event which may allow increased WNT signaling in the lungs driving asthma pathological manifestations. The inference, however, be taken with caution as more experiments are needed to substantiate the findings.

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Chapter 6


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