WNT and β-catenin signalling in airway smooth muscle: emerging concepts for asthma
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Revisiting asthma therapeutics: focus on wnt signal transduction

Review manuscript
Abstract

Asthma is a complex disease of the airways that develops as a consequence of both genetic and environmental factors. This interaction has highlighted genes important in early life, particularly those that control lung development, among which the WNT signalling pathway. Although aberrant WNT signalling is involved with a large array of human conditions, it has received little attention within the context of asthma. Yet it is highly relevant, driving events involved with inflammation, airway remodelling, and airway hyperresponsiveness. In this review, we revisit asthma therapeutics by answering the question: is WNT signalling a valid therapeutic target for asthma?

Keywords

Asthma, WNT, β-catenin, allergy, inflammation, airway remodelling, asthma therapy
Introduction

Asthma is a heterogeneous chronic inflammatory disease of the large and small airways. Over the last couple of decades we have come to consider asthma not as a single disease entity, but rather as a collection of different conditions with overlapping symptomatology, but diverse aetiologies. In most parts of the world, asthma prevalence is continuing to increase or remains stable and is considered one of the most common chronic disorders in the world. Asthma affects about 300 million people worldwide and is a huge burden on healthcare expenditure. A hallmark feature of asthma is airway hyperresponsiveness (AHR), defined as the exaggerated bronchoconstriction response to specific and non-specific stimuli. AHR results from a variable and persistent component, driven by either chronic inflammation or the progressive development of structural changes respectively. Structural changes, termed airway remodelling, encompass increased airway smooth muscle mass, mucous gland hypertrophy, bronchial microvascular remodelling, subepithelial fibrosis and epithelial changes including cell detachment and goblet cell hyperplasia. Although mortality rate has dropped significantly over the years with the regular use of inhaled glucocorticosteroids, 250,000 people still die from asthma annually and the global impact of asthma remains high. The prevalent mortality and morbidity is in part due to both poor adherence and response to corticosteroids in severe asthmatics and asthmatics who smoke, and in some cases patients experience no clinical effect at all. In addition, the effects of corticosteroids on airway remodelling remain controversial, and are hardly clinically significant for low doses. Bronchial thermoplasty has shown promise in decreasing smooth muscle mass in severe asthmatics for up to at least two years, and is associated with improved quality of life, reduced symptoms and number of exacerbations. However, the procedure is invasive and not without complications, and in some cases without clinical benefit. Thus, there is a clear need for new therapies for asthma that overcome the shortcomings of those that are currently available. In this review we discuss the evidence that supports the involvement of Wingless/Integrase-1 (WNT) signalling in asthma and we evaluate the WNT pathway as a potential therapeutic target.

WNT signalling

WNT signalling is an ancient pathway that dates back to the earliest metazo-
ans that started to develop a patterned body axis, and expanded dramatically as animals evolved into more complex organisms\textsuperscript{20}. In mammals there are 19 different WNT family members. They are critically involved in regulating embryogenesis and control diverse processes later in life, including cell proliferation, survival, migration, polarity, specification of cell fate and self-renewal in stem-cells\textsuperscript{21}. It is of no surprise that perturbation of the levels of WNT ligands, or altered activity of its downstream effectors, results in developmental defects and contributes to disease aetiology. Due to the large diversity of WNT signalling components, researchers have attempted to group individual WNT proteins into classes based on their intrinsic capabilities to activate the transcriptional regulator $\beta$-catenin. This resulted in WNTs being categorized as either canonical ($\beta$-catenin dependent), or non-canonical ($\beta$-catenin independent). However, the intrinsic properties of WNT ligands only cover part of the story, and in view of the increasing complexity of WNT signalling networks, it seems incongruous to refer to individual WNTs using this nomenclature. Throughout this review we will view WNTs within the context of the pathway that they are part of and will use the terms WNT/$\beta$-catenin and $\beta$-catenin-independent signalling accordingly.

WNT ligands are secreted proteins that are covalently modified by glycosylation and palmitoylation before entering the extracellular space. Palmitoylations render them hydrophobic and tethers them to cell membranes or their cognate receptors, known as Frizzled (FZD) receptors. They signal in an autocrine and paracrine fashion, mostly through a cell-bound manner\textsuperscript{23,24}. In the case of $\beta$-catenin dependent signalling, once secreted from their host cell, WNT ligands engage their cognate FZD receptors and the LRP5/6 transmembrane co-receptor, inducing complex formation between the two (figure 1). This results in a conformational change and enables phosphorylation of the cytoplasmic LRP tail, which inhibits glycogen synthase kinase 3 (GSK-3)\textsuperscript{25} and allows binding of the scaffold protein Axin. Conversely, when WNT ligands are absent, Axin forms a complex together with adenomatous polyposis coli (APC) and the constitutively active serine-threonine kinases Casein kinase (CK)-I$\alpha$ and GSK-3. This so-called destruction complex captures $\beta$-catenin and subjects it to sequential phosphorylation at serine 45 by CK-I$\alpha$, followed by phosphorylation at positions 41, 37 and 33 by GSK-3 at the
N-terminus, leading to its proteosomal degradation \textsuperscript{26,27}. WNT pathway activation results in recruitment of Axin to the phosphorylated tail of LRP. As a result, the destruction complex, while remaining intact, becomes saturated with the phosphorylated form of β-catenin. This results in newly synthesized β-catenin to accumulate and translocate to the nucleus independently from transporter receptors \textsuperscript{28} to facilitate gene transcription \textsuperscript{29}. Nuclear β-catenin governs transcriptional programs through association with a large array of transcription factors, among which the T-cell factor/Lymphoid enhancer-binding factor 1 (TCF/LEF1) family \textsuperscript{30}.

The β-catenin independent pathways are considerably more diverse in their intermediate effectors and final biological outcomes, among which orientation of cell division, planar cell polarity and convergent extension, and may include both transcriptional and non-transcriptional responses in the cell (figure 1) \textsuperscript{31}. The best characterized β-catenin independent WNT pathways are the planar cell polarity (PCP) pathway and the WNT/calcium pathway. Activation of PCP results in downstream events that involve activation of the small GTPases Rac-1, RhoA and Jun-N-terminal kinase (JNK).

Activation of these effectors can lead to changes in cytoskeletal structure or cell polarity, either directly or through transcriptional activation \textsuperscript{32}. PCP signalling generally does not require the presence of LRP5/6, but instead utilizes the co-receptors RAR-related orphan receptor (ROR), related to receptor tyrosine kinase (Ryk) and tyrosine-protein kinase-like 7 (PTK7) \textsuperscript{33}. WNT/calcium signalling involves the FZD-mediated activation of phospholipase C (PLC), which stimulates the production of diacylglycerol and inositol-1,4,5-triphosphate (Ins(1,4,5)P\textsubscript{3}) \textsuperscript{34}. Ins(1,4,5)P\textsubscript{3} triggers calcium release from intracellular stores and subsequent activation of calcium-dependent factors, such as calmodulin-dependent kinase II (CAMKII), calcineurin and certain isoforms of protein kinase C (PKC). These in turn act on the transcriptional regulator nuclear factor associated with T cells (NFAT) to promote gene transcription.

**Asthma genetics and epigenetics**

**Indications from GWA studies**

Asthma frequently expresses itself in early life and has a substantial heritable component \textsuperscript{35,36}, indicating a strong genetic contribution to disease susceptibility. Furthermore, suboptimal
foetal growth, maternal micronutrient deficiencies (e.g. vitamin E or vitamin D), and maternal smoking, are associated with impaired infant lung function and subsequent predisposition to develop asthma later in life, suggesting asthma develops as a consequence of the interaction of multiple environmental and genetic factors. Perinatal exposures may also drive remodelling upon birth. For example, maternal smoking during pregnancy induces airway remodelling in mice offspring, and these changes are associated with the differential expression of WNT pathway genes in neonates. This is in accordance with the observation that in many asthmatics airway remodelling develops in early life, even before asthma is officially diagnosed. Despite the large number of studies aimed to identify susceptibility loci, genome wide association studies (GWAS) of asthma have only yielded a handful of targets as strong asthma susceptibility genes, which only explain a small proportion of asthma heritability, with limited ability to predict overall disease risk. GWAS studies generally restrict to common single nucleotide polymorphisms (SNPs), but not rare or copy number variants, and positive hits require exceedingly small \( p \) values to declare significance, thus filtering out many potential true associations. In addition, the statistical models used in GWAS are simplistic and do not take into account models of interactions, such as gene-environment, which is highly relevant for asthma. A complex disease like asthma may therefore require a more sophisticated approach. Indeed, when incorporating gene interplay, WNT signalling was found to strongly associate with asthma risk, in particular Frizzled 3 (FZD3) and Frizzled 6 (FZD6). The importance of genotype-specific responses to environmental exposures suggests genes that control lung development may be especially relevant for asthma risk. Three large meta-analyses of genome wide association studies (GWAS) from individuals of European decent were recently published, and identified 28 loci that were associated with lung function. These studies prompted the question whether the same set of genes were implicated in chronic lung disease, such as asthma or chronic obstructive pulmonary disease (COPD). Two follow-up meta-analyses studies were performed by a single group to determine specifically whether the identified loci from these studies, associated with lung function in the general population, also determined lung function in individuals with asthma. They found that genetic
variants related to the *Family With Sequence Similarity 13 Member A* (*FAM13A*) gene associated with both lung function \(^{52-56}\) and asthma \(^{57-59}\). Interestingly, *FAM13A* has also consistently been linked with COPD \(^{60-70}\), even in never-smokers \(^{71}\). Importantly, *FAM13A* was recently found to regulate β-catenin stability, highlighting WNT signalling in asthma \(^{72}\). Although the function of *FAM13A* remains to be further investigated (see box 1) \(^{72,73}\), two splice variants have been identified in humans, (*FAM13A* isoform 1 (long variant) and isoform 2 (short variant) \(^{74}\)), expressed in mucosal cells, club cells, airway epithelial cells, alveolar cells and alveolar macrophages \(^{72}\). Further evidence in support of this view has come from a number of studies. In one study, from five selected WNT signalling pathway genes that were differentially expressed in human foetal pseudoglandular and canalicular stage lung tissue samples, two genes, *WNT-1-inducible-signaling pathway protein-1* (*WISP-1*) and *WNT inhibitory factor-1* (*WIF-1*), harboured polymorphisms in children diagnosed with mild to moderate persistent asthma (see box 1) \(^{75}\). This was later confirmed in asthmatics of Chinese decent \(^{76}\).

**Indications from epigenetic studies**

GWAS alone is unable to address whether SNPs are protective or whether they accelerate disease development, or even if the predicted gene is the key gene at that GWAS locus. Thus, focusing on epigenetic markers is a highly valuable tool to complement GWAS data. In one study, the β-catenin dependent genes *WNT-2*, *low density lipoprotein receptor-related protein 5* (*LRP5*), *adenomatous polyposis coli* (*APC*) and several other WNT genes were differentially methylated specifically in blood monocytes of patients with neutrophilic asthma, but not eosinophilic asthma \(^{77}\). Another study showed that differentially methylated regions corresponding to elevated expression of the *CTNNB1* (*encoding β-catenin*) and *AXIN-2* (a β-catenin target gene) genes in whole blood samples from children at the time of birth, was associated with increased risk for the child to develop late or persistent wheeze later in life \(^{78}\), which increased when mothers were exposed to high levels of stress. Contrary, at four years of age this association no longer remained, suggesting early exposures are critical in disease development.
**Indications from lung development**
The importance of β-catenin in driving lung developmental pathways has been demonstrated in numerous studies. Mice with β-catenin knocked-out at E14.5 in pulmonary epithelial cells (giving rise to airway and alveolar epithelial cells after birth) develop proximal lung tubules which differentiate normally. However, lungs fail to form peripheral airways and instead develop into proximal tubules, resulting in early death after birth \(^{79}\). In contrast, overexpression of β-catenin in CCSP-expressing Clara cells (which start to express CCSP approximately at E14.5) perturbs epithelial cell differentiation and causes goblet cell hyperplasia and air space enlargement \(^{80}\). In addition, constitutive expression of stabilized β-catenin prevents differentiation into secretory Clara cells and terminally differentiated ciliated cells, which is accompanied by a corresponding increase in functionally immature epithelial cells \(^{81}\). Also in the mesenchymal lineage is β-catenin important. Mesenchymal deletion of β-catenin impairs the amplification, but not differentiation of parabronchial smooth muscle progenitor cells as well differentiation into mature endothelial cells \(^{82}\), and several WNT ligands \(^{83–85}\) are essential for smooth muscle cell development in the airways.

An important area of study will be to further characterize the functional significance of genetic variants associated with WNT signalling and asthma risk, where genetic and environmental interactions is key to furthering our understanding of asthma. Although large-scale GWA studies incorporating interactions may prove challenging, a more flexible alternative to study global transcriptional and epigenetic responses to key exposures relevant for asthma may include *in vivo* and *in vitro* models. Of particular interest here is the FAM13A locus. How FAM13A regulates β-catenin is an important question to answer, both in adult life, but also during lung development. This will also help us understand how different SNPs within the FAM13A region relate to different diseases like asthma and COPD, that have both been associated with SNPs linked to the FAM13A gene \(^{60–70}\).

**WNT signalling in asthma: evidence from animal models**

Animal models, although lacking the genetic background that asthmatic individuals have, nonetheless provide a valuable tool to observe how disease development may occur, and to disentangle which factors are a cause
or determinant of the disease. Allergic asthma in mice is typically modelled by exposure to ovalbumin (OVA) in combination with aluminium hydroxide as an adjuvant to facilitate the early phase allergic response and skew inflammatory events in favour of T-helper type 2 (Th2) cells. Alternative allergens that are used include extracts of purified proteins from house dust mite, cockroach, ragweed, or fungi. In addition, occupational asthma can also be modelled and is usually accomplished by exposure to diisocyanates, the most commonly identified cause of occupational asthma. Protocols differ, but generally includes subcutaneous injection with liquid toluene diisocyanate (TDI) (sensitisation), followed by inhalation with TDI vapors (challenge). Although substantial differences have been noted, many features of diisocyanate asthma are similar to atopic asthma, including airway inflammation characterized by activated CD4+ T cells, eosinophils and mast cells, airway remodelling, and increased levels of IL-4 and IL-5.

Allergic asthma models have frequently been associated with a change in WNT signalling, although the direction seems to depend on the duration of the protocol and route of administration of the allergen. In acute OVA models (up to three days of challenge), β-catenin expression is generally reduced compared to control lungs, whereas in chronic OVA models (10 weeks or more) generally β-catenin expression is generally higher. For occupational asthma models the results are less clear. Balb/c mice sensitised to TDI for three weeks and then challenged for one week showed either reduced or mildly increased levels of total β-catenin, concomitant with increased levels of the non-phosphorylated form of β-catenin. Alternative, but less frequently used asthma models are also associated with changes in WNT/β-catenin signalling. Mice exposed to a mixture of benzene, toluene, xylene (collectively called BTX), and formaldehyde (FA) showed differential expression of several WNT-related microRNA’s, and Aspergillus fumigatus exposed mice exhibit elevated levels of Axin-2 in the ASM and epithelial layers. The initial reduction in β-catenin activity in the acute allergen model may reflect a physiological response to protect the host from excessive amounts of β-catenin. As ovalbumin exposure increases over time, this response may eventually lose ground as airway remodelling starts to develop, accompanied by increased activation of β-catenin. It is important to note that β-
Catenin is a pleiotropic gene and its activation requires tight regulation to coordinate cell behaviour. This translates into transient periods of activation, where both activation and diminution act in quick succession. As such, it is possible that β-catenin is activated in a wave-pattern in response to allergens, and failure to detect differences in β-catenin expression may be a result of 'missing the wave'. It should also be mentioned that some of the measured variables are not restricted to WNT signalling. For example, inactivation of GSK-3 through phosphorylation and its corresponding increase in β-catenin stability is achieved through WNT-independent factors, such as protein kinase B (PKB)/Akt, phospholipase C, or protein kinase A (PKA). These findings therefore may not reflect WNT-pathway activation. Finally, WNT pathway activation may not always be best determined by its expression. For example, studies with both animal models and asthmatic patient biopsies have shown decreased expression of the membrane-bound protein E-cadherin, resulting in disruption of barrier function. This observed reduction is paralleled by a decrease in junctional β-catenin, which may become active as it diffuses into the cytosol. These changes are maintained when epithelial cells are isolated and cultured in air liquid interface (ALI), suggesting these changes are intrinsic in nature.

**WNT signalling and inflammation in asthma**

Asthma is primarily considered a disease associated with activation of the adaptive immune response, most notably the Th2 cell-dependent promotion of IgE production and recruitment of mast cells. However, asthma is also characterized by innate immune responses that influence the activation and trafficking of dendritic cells, production of innate immune cytokines and priming of lymphoid cells. Both of these axes involve WNT signalling (figure 2).

**Evidence for β-catenin independent WNT signalling**

Evidence suggests a strong link between β-catenin independent WNT signalling and allergic inflammation. WNT-5A was recently implicated with asthma in peripheral blood mononuclear cells (PBMCs). PBMCs isolated from healthy individuals, treated with either interleukin-4 (IL-4) or interleukin-13 (IL-13) for 24 hours, and then processed for microarray analyses, showed increased expression of WNT-
5A for both IL-4 and IL-13. Accordingly, WNT-5A expression could be completely prevented by anti-IL-13 mAb. These findings were extended towards asthma patients in another study, where endobronchial biopsies from mild-to-moderate asthmatics, stratified into ‘Th2-high’ and Th2-low’ subphenotypes on the basis of a signature of three IL-13 inducible genes were analysed by whole genome microarray analyses. They reported that multiple WNT genes were positively correlated with the Th2-high signature. Moreover, WNT-5A expression was found to be increasingy expressed in PBMCs from asthmatics of Korean decent. These findings suggest a link between β-catenin independent WNT signalling and Th2-high asthma, or possibly between WNTs and allergy, which is generally considered to be a Th2-predominant response. A more recent paper has substantiated this idea, where bronchial airway epithelial brushings were screened for differentially expressed genes and then correlated to fractional exhaled nitric oxide (FeNO). They then used k-means clustering to partition the subset of genes that correlated with FeNO into five different asthma phenotypes, or subject clusters. One cluster was enriched with WNT pathway genes, among which WIF-1, WNT-5B and DKK-3. Of note, all of the patients in this cluster were atopic. Patients in this cluster had a normal FeNO, but the earliest age of asthma onset, longest disease duration, and a high disease severity and percentage of bronchoalveolar lavage (BAL) lymphocytes. Moreover, this cluster showed elevated levels of TNF-α signalling, which is known to drive expression of non-canonical WNT mediators.

Airway smooth muscle cells from mild-to-moderate asthmatics have also been shown to contain elevated levels of WNT-5A compared to healthy ASM. Apart from its role in regulating bronchomotor tone, the ASM is intimately involved in modulating airway inflammation. Collectively, these results strongly imply a role for β-catenin independent WNT signalling and inflammation, in particular allergic responses.

Evidence for WNT/β-catenin signalling

In blood samples, polymorphisms within the promoter region of CTNNB1 have been associated with either an increased or decreased risk to develop asthma, depending on whether these variants increased or decreased expression of β-catenin respectively. Furthermore, endobronchial biopsies from mild-to-moderate asthmatics...
showed that, \textit{WNT-3A} and \textit{WNT-10A} associated with Th2-high asthma\textsuperscript{103}. In support of this, the β-catenin destruction effector genes \textit{Axin-1}, \textit{APC} and \textit{GSK-3β} were all found to be decreased in PBMCs from Korean asthmatics\textsuperscript{104}.

Collectively, these results support the view that both axes of WNT signalling are elevated in asthmatic tissues and link to Th2-specific inflammation. These results are largely backed up by mechanistic and translational studies in animal models, although some discrepancies exist that will be further outlined below.

\textbf{Evidence from mechanistic studies on adaptive immunity}

WNT/β-catenin signalling is critically involved in T-cell development in the thymus\textsuperscript{110,111}, primarily through interaction of β-catenin with the transcription factor special AT-rich binding protein 1 (SATB1)\textsuperscript{112}, which was recently also shown to be associated with mucous hypersecretion\textsuperscript{113}. However, WNT/β-catenin has also been implicated in the Th2-mediated response that takes place after maturation in the thymus, specifically within the context of allergy. Transgenic mice producing WNT-1 in a tetracycline-based (tet-ON) manner, under control of the Clara cell secretory protein (CCSP) promoter, specific for Clara cells, and subjected to ovalbumin exposure to drive allergic asthma-like changes, show attenuated airway hyperresponsiveness (AHR), BAL eosinophilia and a reduction in mucus production\textsuperscript{114}. Overexpressed WNT-1 had no effect on systemic sensitization, as evidenced by unchanged OVA-specific IgE, IgG1, and IgG2b levels in serum. Treatment with the non-selective GSK-3 inhibitor lithium chloride could mimic these results, highlighting the role of β-catenin signalling in this response. In line with this, mice with a homozygous hypomorphic mutation at the Dickkopf-1 (DKK-1) allele, in which DKK-1 expression is reduced by approximately 90%, show amplified WNT/β-catenin signalling, accompanied by reduced levels of neutrophils, eosinophils and CD4+ T-cells in BAL fluid in response to allergen challenge with house dust mite\textsuperscript{115}. In another study, suppression of DKK-1 by a neutralizing antibody, or administration of WNT-3A, reduced neutrophil trafficking during acute inflammation\textsuperscript{116}. Moreover, inhibition of DKK-1 reduced the production of IL-4, IL-5, IL-10 and IL-13 in CD4+ T-cells, and suppressed IFN-γ expression under Th1-cell polarization conditions\textsuperscript{112,117}. WNT-10B was also recently implicated with Th2 activation\textsuperscript{118}. WNT-10 is
expressed in airway epithelium as well as in T-cells. Full body ablation of WNT-10 results in an increased Th2 predominant inflammatory response in an acute house dust mite mouse model. BAL fluid eosinophils were elevated as well as whole lung homogenate expression of IL-4 and IL-13 and infiltration of antigen-specific effector cells in the lungs, although there was no difference in the proportion of infiltrated T cells within the lungs of WNT-10B−/− mice. However, among the infiltrated cells was a higher number of effector cells, characterised as CD44high CD62Llow, suggesting antigen exposure is a requisite for the WNT-10B−/− state to take effect. In line with this, sorted T-cells from WNT-10B−/− mice exposed to IL-4 to drive Th2 polarization exhibited increased GATA-3 and IL-4 expression. Of note, these changes were absent under baseline conditions, and no differences were found in expression of T-bet (T-box transcription factor, expressed in CD4+ T cells committed to Th1 T-cell development). Moreover, WNT-10B−/− T-cells exposed to CD3/CD28 to drive clonal T-cell expansion through ligation of the T-cell receptor (TCR) showed increased proliferation. Other immune cells have been linked with WNT signalling as well, although not all of these studies have been tested within the context of an asthma or allergic inflammatory model. Isolated dendritic cells (DC) exposed to curcumin, a natural substance that increases β-catenin activity in these cells, prevents upregulation of the activation markers CD40 and CD68 induced by LPS. Curcumin also prevents lymphocyte proliferation following exposure to LPS in a mixed lymphocyte reaction assay, and reduces OVA-induced accumulation of inflammatory cells in the BAL fluid of mice. Furthermore, intestinal DCs deficient in β-catenin are compromised in their ability to produce retinaldehyde dehydrogenases (RALDH), an enzyme that is part of the conversion of vitamin A to retinoic acid. Failure to mount a RALDH response subsequently shifts Th polarization in favour of Th1 cells. Although retinoic acid production by DCs have been considered to be limited to gut-resident DCs only, other DC populations have recently been shown to also express RALDH, particularly lung-resident DCs that express RALDH-2. Survival of eosinophils has also been reported to require the nuclear presence of β-catenin, which can be triggered via IL-5 in a WNT-independent manner. Moreover, eosinophils from asthmatics can modulate the WNT secretory profile of cultured airway smooth muscle cells when adhered to. These changes
may subsequently affect how smooth muscle cells proliferate and maintain their ECM surroundings. Other cell types like mast cells or B-cells, although active WNT signalling is required for their proper differentiation, have thus far not been researched in an asthmatic or allergic setting 128,129.

**Evidence from mechanistic studies on innate immunity**

WNT/β-catenin signalling has also been demonstrated to regulate innate immune responses, primarily through interaction with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a transcription factor that drives the expression of multiple cytokines, chemokines and cell adhesion molecules that are involved in asthma pathophysiology. Its activation occurs mainly through interleukins, tumour necrosis factor (TNF) or is elicited by the activation of toll like receptors (TLRs) during a bacterial or viral-exacerbation. The usefulness of targeting NF-κB in asthma has already been demonstrated by the efficacy of glucocorticosteroids, which can be contributed in part to inhibition of NF-κB 130. β-catenin has been shown to interact with both the p65 131-133 and p50 131,134-136,132 subunit of NF-κB in various cell types, generally resulting in impaired DNA binding, transactivation activity and target gene expression mediated by NF-κB. GSK-3 is also required for NF-κB activation, presumably via degradation of β-catenin 137-139, although direct phosphorylation of NF-κB p65 by GSK-3 has also been proposed 140,141. Interestingly, another line of research has proposed a dependency of NF-κB for β-catenin. Increased β-catenin signalling in alveolar epithelial cells enhanced NF-κB signalling and transcriptional output *in vitro* 142. The nuclear co-factors CREB-binding protein (CBP) and E1A binding protein p300 (p300) have been shown to be required for β-catenin and NF-κB interaction 143,144. We have shown recently that in airway smooth muscle, inhibition of the β-catenin/CBP interaction could amplify NF-κB-mediated inflammation, whereas inhibition of the β-catenin/p300 interaction could attenuate it (author’s unpublished findings). Although detailed molecular events remain to be determined, these results suggest a molecular switch that directly controls NF-κB output, requiring the presence of β-catenin.

The interconnected nature of WNT/β-catenin signalling with both adaptive and innate immune responses complicate interpretation of genetic screening studies that have implicated β-catenin
signalling with asthma. On top of that, both inflammatory cascades interact with each other. Innate immune mechanisms are required for dendritic cell priming and can amplify a Th2 response to inhaled ovalbumin. Furthermore, commercially available ovalbumin is known to contain traces of lipopolysaccharides (LPS), which facilitates the priming of T-helper cells to inhaled ovalbumin. The degree of LPS exposure in conjunction with ovalbumin also determines the kind of T-cell response that is elicited (e.g. Th1 versus Th2). NF-κB p50−/− mice are completely devoid of airway inflammation when challenged with inhaled allergen in a murine model of asthma, and it has been shown that NF-κB is critical for Th2 differentiation through expression of GATA-3. To help facilitate the drug development process, future efforts aimed at identifying new WNT targets should extricate innate from adaptive immunity.

**WNT signalling and airway remodelling in asthma**

**Evidence for β-catenin independent WNT signalling**

WNT-5A is increasingly expressed in airway smooth muscle cells of mild- to moderate asthmatics compared to healthy individuals. A recent study from our laboratory has implicated WNT-5A with ASM contraction, where WNT-5A acts via autocrine signalling to promote actin polymerization in ASM cells. The increased presence of actin filaments increased maximum force generation in ASM cells without affecting sensitivity to histamine. Increased activity of WNT pathway activation and modulation of the actin cytoskeletal network could serve as an alternative model to explain airway hyperresponsiveness in asthma. In addition, WNT-5A is responsible for some of the actions mediated by TGF-β. In human airway smooth muscle, fibronectin and collagen synthesis following stimulation with TGF-β requires de novo synthesis of WNT-5A and subsequent activation of TGF-β-activated kinase 1 (TAK1) and specificity protein-1 (SP-1). We have further shown that some of the effects of WNT-5A by TGF-β require the release of actin binding proteins following formation of actin filaments. Of particular interest here is myocardin-related transcription factor A, which is released upon WNT stimulation and can drive expression of TGF-β target genes. It is worth noting here that individually, WNT-5A is unable to achieve the effects mediated by TGF-β, indicating cooperative signalling is a
The effects of TGF-β-WNT-MRTF-A are relevant for other aspects of airway remodelling as well. For example, MRTF-A is critically involved in the induction of TGF-β-mediated epithelial-mesenchymal-transition (EMT) and epithelial-to-myoﬁbroblast-transition. Myoﬁbroblasts are a rich source of ECM proteins, and MRTF-A is an important mediator of myoﬁbroblast activation and expression of ECM proteins. Inhibition of mechanotransduction by blocking the RhoA-MRTF-A axis attenuates experimental pulmonary ﬁbrosis in mice. In asthma, cross-regulation between TGF-β and WNT signalling may allow for the development of treatment strategies that can overcome the shortcomings of drugs that target TGF-β signalling more directly (which are associated with severe adverse effects). Going forward, it is essential that we study this level of integration in more detail, as the nature of this cross-talk can be overwhelmingly complex and context-dependent. Failure to recognize this level of integration will confound the development of effective therapeutic interventions in a complex disease like asthma.

### Evidence for WNT/β-catenin signalling

β-catenin is a critical regulator of airway remodelling, particularly in ASM and ﬁbroblasts. Both cells require active β-catenin signalling to promote cell growth and production of ECM proteins. Although the nature of WNT/β-catenin expression in animal models for asthma are controversial (see above), targeting this pathway may still be beneﬁcial in a therapeutic setting. OVA-exposed balb/c mice treated with siRNA targeted against β-catenin showed considerably reduced parameters of airway remodelling. Both deposition of newly synthesised collagen and expression of alpha smooth muscle actin (α-SMA) were attenuated following inhibition of β-catenin. Similarly, inhibition of the β-catenin/CBP interaction with the small-molecule ICG-001 was able to prevent airway smooth muscle thickness after repeated OVA challenge and showed a trend towards a decline in peribronchial collagen deposition. These results have been corroborated in a mouse model for occupational asthma. Moreover, inhibition of WISP-1 (an inducer of WNT/β-catenin signalling) by a neutralizing antibody attenuates OVA-induced ASM thickening in rats.
Chapter two

Figure 1. WNT signalling pathways. Simplified scheme showing the main WNT pathways. (A) WNT/β-catenin signalling. Under steady-state conditions, and in the absence of WNT ligands, glycogen synthase kinase 3 (GSK-3) phosphorylates β-catenin, which triggers its degradation. In the presence of extracellular WNT ligands, the destruction complex (comprising GSK-3, casein kinase-Iα (CK-Iα), Axin and adenomatosis polyposis coli (APC)) is recruited to the WNT-receptor complex and inactivated. This saturates the destruction complex and allows newly formed β-catenin to accumulate and translocate to the nucleus, where it activates the transcription of target genes under the control of T cell factor (TCF), among others. (B) β-catenin independent signalling with purple and blue labelled components depicting planar cell polarity (PCP) and WNT/Ca\(^{2+}\) signalling respectively. PCP signalling triggers activation of the small GTPases RhoA and Rac-1, which in turn activate Rho kinase (ROCK) and Jun-N-terminal kinase (JNK), leading to actin polymerization. This pathway is prominently involved in the regulation of cell polarity, cell motility, and airway smooth muscle contraction. The WNT/Ca\(^{2+}\) pathway activates Ca\(^{2+}\)- and calmodulin-dependent kinase II (CamKII), protein kinase C (PKC) and calcineurin (Cn). Calcineurin activates Ca\(^{2+}\)-sensitive transcription factors, among which nuclear factor of activated T cells (NFAT), which regulates the transcription of genes controlling cell fate and cell migration. DVL, Dishevelled, β-TrCP, beta-transducin repeat containing E3 ubiquitin protein ligase, ub, ubiquitin, LRP5/6, Low-density lipoprotein receptor-related protein 5/6.
Figure 2. Involvement of WNT signalling in asthmatic responses. Release of WNT ligands that engage in WNT/β-catenin signalling generally suppress adaptive immune responses on various levels. Trafficking of inflammatory cells into the alveolar space due to upregulation of adhesion molecules, proliferation of activated Th2 cells following antigenic exposure, and expression of Th2 cytokines are all inhibited upon activation of WNT/β-catenin signalling. Conversely, secreted negative regulators of WNT signalling (e.g. DKK) can undo this inhibition. Suppression of β-catenin signalling attenuates airway remodelling as well, examples being airway smooth muscle growth and synthesis of extracellular matrix proteins. β-catenin independent WNT signalling exerts diverse effects that in general are poorly described. Examples are modulation of airway smooth muscle contraction and activation of inflammatory responses. There is also a substantial amount of cross-regulation between β-catenin independent WNT signalling and other pathways, like TGF-β signalling that collectively drives airway remodelling.
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Figure 3. Targeting WNT signalling in asthma. When WNT pathway inhibition is to be achieved to treat asthma, it is important to consider the different levels of intervention, which can have profound impact on the final outcome of the treatment. Here, we categorise these levels in three compartments based on cellular architecture: 1) the extracellular environment, 2) the cytosolic environment, and 3) the nuclear compartment. Generally, designing compounds that act upstream may result in a lack of specificity, because of the interconnected nature of cell signalling and its numerous feedback loops. In this case, potential off-target effects may be expected. At the same time, this approach allows for a broader therapeutic reach, as upstream effectors (e.g. WNT ligands) are more likely to be shared by different cell types compared to downstream effectors. It also presents a more diverse platform for drug development; because cell permeability is not required, small molecules, monoclonal antibodies, recombinant proteins, and receptor constructs (e.g. fusion proteins) are all part of the drug repertoire. Conversely, designing compounds that act downstream in the cell allows for the inhibition of very specific signalling events, thus minimizing off-target effects. Maximum specificity may be achieved by inhibiting only a specific subset of protein-protein interactions, for example the interaction of β-catenin with CBP, but not P300. Targeted therapy in the nucleus presents more difficulties in the drug designing phase, however, as compounds have to cross several barriers before reaching their designated site, requiring them to be soluble and cell permeable, or encapsulated by a delivery vehicle. Additionally, the delivery and retainment of drugs inside the cell may be highly dependent on the presence and activity of ABC transporters that are expressed in the lungs, which may use pulmonary drugs as substrates.
A number of susceptibility genes have been associated with asthma risk, but for some it is not always clear how they affect cell behaviour. The function of FAM13A is not entirely clear, but studies have suggested a role in stabilizing levels of β-catenin through interaction with PP2A. In HEK293T and A549 cells FAM13A is phosphorylated at ser 322 by Akt, which increases its binding affinity with 14-3-3, leading to cytoplasmic sequestration of FAM13A 73. FAM13A bound to the B56 regulatory subunit of PP2A leads to dephosphorylation at ser 322, and promotes nuclear localization. FAM13A also interacts with Axin, but not GSK-3 and it has been suggested that FAM13A may regulate posttranslational modification(s) of Axin in the nucleus, leading to increased Axin turnover, which indirectly increases β-catenin stability 73. Another study was able to show that in 16HBE cells, overexpression of FAM13A resulted in increased phosphorylation (ser 33, 37, thr 41) and reduced levels of β-catenin 72. Similarly, depletion of FAM13A increased β-catenin stability and TOPFlash reporter activity. These different findings warrant further investigation. In light of this, it is worth noting that FAM13A contains a putative nuclear export signal (NES) sequence 73, as well as a bipartite nuclear localization signal (NLS) and two shorter Pat7 sequence motifs, which suggest nuclear presence and function of FAM13A 74. Isoform two is also associated with a RhoGAP domain, known to affect Rho family GTPases 74. Belonging to the family of secreted matricellular CCN proteins, WISP-1, along with other CCN family members, can interact with various receptors, including LRPs as part of WNT/β-catenin signalling 215. Its precise function however, remains poorly described. WISP-1 can interact with integrins through several integrin recognition sites 216–218. It may thus serve as a mediator of cell to matrix adhesion in a pleiotropic, cell-specific manner, with potential distinct functions depending on different cell-surface receptors in different cell types 219. Functionally, WISP-1 has been shown to drive proliferative and EMT responses in alveolar epithelial cells and increase the synthesis of ECM components in fibroblasts. Antibody-mediated inhibition of WISP-1 improved lung function in the bleomycin mouse model for pulmonary fibrosis 220. Both DKK-3 and WIF-1 are secreted negative regulators of WNT signal transduction 221. WIF-1 can directly bind and antagonize some WNT ligands. In addition, it contains a heparin sulphate binding site (membrane bound glycosaminoglycans, commonly covalently linked to heparin sulphate proteoglycans, thought to mediate localization of WNTs near the target cell surface 222), which is not necessary for WNT inhibition, but greatly facilitates it 221. Generally, inhibition of WIF-1 exacerbates WNT/β-catenin signalling, and its expression is commonly silenced in human lung cancer 223,224. DKK on the other hand inhibits WNT signalling by preventing WNT binding with LRPS/6 225. Interestingly, whereas WNT ligands typically bind to only one or two distinct structural domains within LRPS/6, DKK binds several, and can therefore potentially antagonize different WNT proteins at the same time 226. Similar to WIF-1, inhibition of DKK generally results in activation of WNT/β-catenin signalling, and its decreased expression is relevant in lung cancer. Its functional significance in relation to asthma has been described in more detail in the main body of text.
Current therapies and WNT signalling

According to the Global Initiative for Asthma (GINA), key points in asthma management are to achieve good symptom control, and to minimize future risk of exacerbations, fixed airflow limitation and side-effects of treatment, highlighting our lack of understanding in asthma aetiology and focus on symptomatic, rather than curative treatment. To date, there have been no clinical trials for asthma that involved modulation of the WNT signalling pathway. Current therapy is mainly based on (combinations of) inhaled corticosteroids, β₂-adrenergic receptor agonists, and leukotriene inhibitors. Some of these, most notably glucocorticoids, have been reported to elicit secondary effects on WNT signal transduction, mainly in off-target tissues. Mesenchymal cell commitment towards osteoblastic differentiation to promote bone formation requires endogenous glucocorticoids that signal through WNT pathways downstream. In line with this, osteoporosis, one of the most frequent side effects of long-term glucocorticoid therapy, is accompanied by inhibition of WNT/β-catenin signalling in osteoblasts. Glucocorticoids activate GSK-3, inhibit TCF/LEF, and increase the expression of WNT pathway inhibitors like DKK-1 and soluble Frizzled-related protein-1 (sFRP-1). GSK-3 can also phosphorylate the glucocorticoid receptor (GR), which facilitates its response to glucocorticoids. It would be interesting to assess the effects of glucocorticoids in different tissues that are more relevant for asthma pathophysiology, and to evaluate whether potential effects on WNT signalling activation are clinically significant. At the moment, there is no evidence that both short acting and long acting β₂-adrenergic receptor agonists can modulate WNT signalling in the lung. One study addressed the interaction of fenoterol with WNT pathway components in human bronchial rings, but these findings have thus far not been corroborated and require additional verification. Although it has been shown that cysteinyl leukotrienes can activate β-catenin signalling, primarily in a WNT-independent manner through activation of phosphatidylinositol 3-kinase (PI3K), there are no studies that have shown WNT pathway modulation by any of the currently available cysteinyl leukotriene-receptor antagonists montelukast, zafirlukast, and pranlukast. The same holds true for most other available treatment...
strategies, examples being immunoglobulin E (IgE) inhibition with omalizumab or cholinergic pathway inhibition with tiotropium. Of note, asthma treatment is moving towards personalized medicine and focus on asthma phenotypes and endotypes. Drug therapies previously deemed ineffective have gained renewed interest in light of these developments, one example being biologics targeted against Th2 cytokines. Due to the close involvement of WNT pathway components with innate and adaptive immunity, it would be interesting to re-evaluate these drugs based on their potential secondary and/or indirect effects on WNT signalling. In addition, several other drugs that are currently under trial might prove efficacious in terms of WNT signal modulation. For example, drugs that inhibit the prostaglandin (PG) D2 receptor subtype DP2 (also known as the chemoattractant homologous receptor expressed on Th2 cells (CRTh2)), important in Th2 and type 2 innate lymphoid cell (ILC2) function, but possibly also in airway remodelling, are now in clinical development for asthma. It is known that β-catenin functions downstream of the closely related eicosanoid PGE2 in a cyclic-AMP dependent manner in several malignant cell types, and it would be worthwhile to assess the effects of CRTh2 antagonists on β-catenin signalling.

Concluding remarks

More than thirty years after the discovery of what is possibly the oldest evolutionary conserved pathway in animals, and extensive research efforts to characterize this fundamental pathway, targeting WNT signalling in a clinical setting is still in its infancy. Despite intensive efforts to characterize this pathway in a disease setting, including asthma, unveiling a multitude of potential therapeutic points of intervention, there have been surprisingly little attempts to modulate WNT pathway components in clinical trials. This is not due to lack of available reagents that target the WNT pathway, which are increasingly being discovered and developed. Some of these compounds are currently being tested in clinical trials, of which most are within the scope of cancer treatment, but most other fields have so far lacked behind, including asthma. The majority of drugs tested in current trials target extracellular modulators of WNT signalling, including DKK-1, but also WNT ligands themselves, as well as WNT receptors. This is surprising, considering the widespread involve-
ment of WNT signalling in virtually every tissue within the human body, and is almost bound to have secondary effects in off-target tissues. Nonetheless, these studies will provide us critical clues into the safety profile of these WNT modulators, and whether or not they can be efficacious in a therapeutic (cancer) setting. They will also provide us with important information into whether full inhibition or activation of WNT signalling is the right approach for therapy. During drug screening approaches, the most potent drugs are usually selected for and tested in a clinical setting. However, full reduction of aberrant WNT signalling may not necessarily be the right approach. Because WNT signalling is intricately involved in tissue homeostasis, therapeutic targeting may require a more delicate approach, where WNT pathway activation needs to be brought back down to normal levels. In fact, the safety profile of WNT modulators currently in preclinical and clinical trials for cancer, among which PRI-724, LY209314, CWP232291, OMP-54F28, and OMP-18R5, show that many of these compounds share some of their adverse effects. Although the safety results from these studies showed good tolerability overall, almost all of these compounds associated with symptoms of nausea, diarrhea, and vomiting. While these side effects may raise concern into their general usability in the clinic, limiting systemic exposure by restricting their reach to the lungs through inhalation may largely overcome these issues. These results nonetheless highlight the difficulty that resides in developing safe and effective therapeutic compounds targeting this complex pathway.

The increasing interest in characterizing asthma phenotypes and endotypes, and the emerging concept that asthma may have a developmental basis, raises interesting thoughts in terms of future therapy. Identifying biomarkers for asthma development and susceptibility in early life could pave the way for treatment strategies that could alter disease progression entirely, possibly even halting or reversing it. In light of the genetic and developmental aspects of asthma, targeting the WNT pathway would be a primary candidate in this regard. Of particular interest would be WNT-associated therapies that affect airway remodelling in early life, as remodelling may already develop before the onset of asthma symptoms. Targeting WNT signalling in patients that already have asthma may also be beneficial. Of special interest here are compounds that target the selective inhibition of the interaction between β-
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catenin and co-factors like CBP or p300, as they only disrupt a very small subset of co-factor interactions, initiating a transcriptional program that potentially inhibits disease parameters, while leaving others intact. They also act significantly downstream in the cell, possibly preventing unwanted secondary effects. In order to improve the development of therapeutic targets downstream of WNT signalling, it is essential that we learn more about the nuclear actions of β-catenin, as this is currently an underappreciated topic. For example, CBP and p300 are paralogous genes that share a large degree of structural similarity, yet they are often ascribed opposing roles. How CBP and p300 can exert these seemingly bimodal functions remains to be determined. It has been suggested that the interaction between β-catenin and CBP or p300 results from competition, but this model seems too much of an oversimplification, as CBP and p300 are known to facilitate transcriptional output through a plethora of additional transcription factors. Additionally, differential phosphorylation of CBP or p300, or yet to be discovered binding partners may govern selectivity and binding with regulatory components. Both the ability of CBP/p300 to modulate chromatin and acetylate β-catenin or other proteins through their histone acetyltransferase (HAT) domain will be an important focus of study. Expanding our knowledge on these architectural elements will further our ability to design drug therapies that target a very selective range of transcriptional events involved in disease, without interfering with the crucial role of WNT signalling in tissue homeostasis. Five clinical trials are currently being conducted, all in cancer, using the small-molecule inhibitor PRI-724 (an enantiomer of ICG-001), which selectively targets the β-catenin/CBP interaction, with no effect on p300.

Type 2 inflammation can be efficiently suppressed in most patients with asthma with the regular use of inhaled glucocorticosteroids. Although Th2-high asthma is generally a corticosteroid-responsive endotype, a notable subgroup of patients with this endotype maintain symptoms and experience severe uncontrolled asthma in spite of regular use of steroids. Novel drug treatment of this group of steroid insensitive patients with severe asthma is highly warranted. Furthermore, some Th2-high asthmatics require high doses of inhaled steroids or oral steroids for maintenance therapy, and these patients are in need of alternatives to avoid excessive
adverse effects. The advent of more specific inhibitors, e.g. biologicals targeted against type 2 inflammation, has raised hope that these drugs will provide similar benefits to patients with asthma, while displaying less adverse effects. However, compared to glucocorticoids, these compounds have a more limited effect on airway function and asthma control, even when stratified for different asthma pheno- or endotypes. Thus, they have so far not been able to replace steroid therapy and are adjunctive at best. In addition, glucocorticoids have no noticeable effect on airway remodelling. This is where anti-WNT therapy may confer additional benefit, due to its combined effects on Th2 immunity, airway remodelling and muscle biology. The majority of trials using anti-DKK-1 antibody therapy in cancer are now complete, and a positive outcome of these studies will be important in furthering our understanding towards asthma therapy. In the next couple of years, these results and others should shed new light on whether or not we can use the WNT pathway as a therapeutic target in asthma.
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