WNT-5A and WNT-11 drive TGF-β-induced α-sm-actin expression in smooth muscle via Rho kinase-actin-MRTF-A signaling
Abstract

Airway smooth muscle (ASM) remodeling is a key feature in asthma which includes changes in smooth muscle specific gene and protein expression. The mechanisms governing ASM remodeling are poorly understood. We have recently demonstrated increased abundance of the noncanonical WNT ligand WNT-5A in ASM cells derived from asthmatics as compared to healthy subjects. Here, we studied the functional interaction between noncanonical WNT signaling and TGF-β in ASM cells and demonstrate that WNT-5A and WNT-11 are preferentially expressed in contractile myocytes and regulate α-sm-actin expression in ASM cells. Knock-down of WNT-5A or WNT-11 attenuated TGF-β-induced α-sm-actin expression in ASM cells and the presence of exogenous recombinant WNT-5A or WNT-11 augmented α-sm-actin levels in the absence of TGF-β. Further, we demonstrate that TGF-β-induced α-sm-actin expression is mediated by WNT-5A- and WNT-11-regulated induction of RhoA activation and subsequent actin cytoskeletal remodeling as pharmacological inhibition of either Rho kinase by Y27632 or actin remodeling by latrunculin A attenuated α-sm-actin induction. Moreover, we show that TGF-β upregulates the expression and induces Rho GTPase-dependent nuclear translocation of MRTF-A which, in turn, mediates α-sm-actin expression in ASM cells. Finally, we demonstrate that MRTF-A nuclear translocation is dependent on noncanonical WNT ligands. The present study, thus, demonstrates a WNT-5A- and WNT-11-dependent Rho kinase-actin-MRTF-A signaling axis that regulates the expression of α-sm-actin in ASM cells.

Introduction

Airway remodeling is a hallmark pathological feature of individuals with asthma and is associated with airway obstruction [1], airway hyperresponsiveness [2] and declining lung function in severe disease [3]. It is characterized by extensive structural changes in the airway wall which include increased airway smooth muscle (ASM) mass, subepithelial fibrosis, mucus hypersecretion, neovascularization and increased and altered extracellular matrix (ECM) expression, contributing to airway wall thickening [4].

Increased ASM mass is a predominant feature of airway remodeling in asthma and correlates with the severity of disease [5-7]. ASM cells show a remarkable phenotypic plasticity where they can exist in a synthetic proliferative phenotype actively undergoing cell divisions and also releasing a number of inflammatory and other mediators and growth factors. In contrast, mature ASM cells have low proliferation index and high abundance of contractile apparatus like sm-MHC, SM-22 and α-smooth muscle-actin (α-sm-actin) [8-10]. Increased ASM mass and increased expression of contractile and contraction regulatory proteins are important features of the asthmatic airway [7,11].

WNT signaling regulates a myriad of functions from embryonic development extending throughout the lifespan of humans [12]. Intracellular WNT signaling is broadly classified into canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) branches. The activation of canonical WNT signaling results in the cytosolic accumulation of the transcriptional co-activator β-catenin which, in turn, translocates to the nucleus and activates WNT-target gene transcription in association with the T-cell factor/lymphoid.
enhancer-binding factor (TCF/LEF) transcription factors. Noncanonical WNT signaling, on the other hand, activates various signaling cascades independent of β-catenin and regulates cell polarity, cell movements, cytoskeletal reorganization and gene transcription. WNT/Ca\(^{2+}\) and WNT/planar cell polarity (PCP) pathways are the best characterized members of noncanonical WNT signaling. The WNT/Ca\(^{2+}\) signaling is transduced by activation of calcium-dependent signaling molecules, including protein kinase C (PKC), Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and nuclear factor of activated T-cell (NFAT), whereas the WNT/PCP pathway is relayed by RhoA or activation of c-Jun N-terminal Kinases (JNKs) via small Rho-GTPases [12].

We have recently reported high abundance of WNT-5A in asthmatic ASM cells as compared to healthy subjects and demonstrated that WNT-5A mediates TGF-β-induced ECM expression. Also, we have shown that noncanonical WNTs such as WNT-5A and WNT-11 are transcriptional targets of TGF-β in ASM cells (Chapter 3). TGF-β is a potent inducer of contractile proteins including α-sm-actin in ASM cells and regulates ASM maturation [13]. Whether noncanonical WNT signaling also participates in ASM maturation is unknown. However, noncanonical WNT signaling induces extensive actin cytoskeletal reorganization which can alter the G-actin/F-actin ratio [14,15]. In addition, WNT-11 has been shown to mediate TGF-β-induced α-sm-actin expression in renal epithelial cells [16].

Here, we investigated the role of WNT-5A and WNT-11 in TGF-β-induced α-sm-actin expression in ASM cells and demonstrate that the expression of these noncanonical WNT ligands increases during maturation. Furthermore, we show that WNT-5A and WNT-11 mediate TGF-β-induced actin remodeling and activate Rho kinase signaling in ASM cells. Finally, we demonstrate that WNT-5A and WNT-11 regulate myocardin-related transcription factor –A (MRTF-A) dynamics and mediate α-sm-actin expression.

Materials and Methods

**Reagents**- Recombinant human TGF-β, recombinant human WNT-5A and recombinant human WNT-11 were from R&D systems (Abingdon, UK). siRNA specific for human WNT-11 and human MRTF-A, rabbit anti-MRTF-A antibody, mouse anti-Lamin A/C antibody and mouse anti-GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-MYPT1 (Thr850) antibody was obtained from Cell Signaling Technology (Beverly, MA, USA) and anti-β-actin antibody, anti-α-sm-actin antibody, HRP-conjugated goat anti-mouse antibody and HRP-conjugated goat anti-rabbit antibody were obtained from Sigma (St. Louis, MO, USA). Alexa Fluor 488-conjugated Phalloidin, Alexa Fluor 594-conjugated DNAse I, Hoechst 33342 stain and ProLong Gold Antifade reagent were from Life Technologies (Bleiswijk, The Netherlands). Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit antibody and Cy3-conjugated donkey anti-mouse antibody were procured from Jackson Immunoresearch Europe (Suffolk, UK). Human non-specific control and human WNT-5A siRNAs were procured from Qiagen (Venlo, The Netherlands) and X-tremeGENE siRNA transfection reagent was purchased from Roche Applied Science (Mannheim, Germany). LL-Zn1640-2, Y-27632 dihydrochloride and latrunculin A were from Tocris (Bristol, UK). All other chemicals were of analytical grade.
**Cell culture-** Human ASM cell lines, immortalized by human telomerase reverse transcriptase (hTERT) and primary ASM cells were used for all the experiments. The primary cultured human ASM cells used to generate each hTERT immortalized cell line were prepared as described previously [17]. All procedures were approved by the Human Research Ethics Board (University of Manitoba). hTERT-ASM cell lines were maintained on uncoated plastic dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics (50 U/ml streptomycin, 50 µg/ml penicillin) and 10% (v/v) fetal bovine serum (FBS). For each experiment, hTERT-ASM cell lines (ASM cells) derived from two to three different donors were used for repeated measurements. Cells were serum-deprived in DMEM supplemented with antibiotics and ITS (5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium) before each experiment. When applied, inhibitors were added 30 min before the TGF-β stimulation.

Primary ASM tissue and cells were derived from trachea and mainstem bronchus obtained from healthy transplant donors. Selection criteria for healthy lung transplant donors are listed in the Eurotransplant guidelines and include the absence of primary lung disease such as asthma and COPD and no more than 20 pack years of smoking history. The ASM tissue was snap frozen after isolation and stored at -80°C till further use. The primary ASM cell cultures derived from the trachea and mainstem bronchus were prepared as described previously (Chapter 3) and were cultured on uncoated plastic dishes in DMEM supplemented with antibiotics, 1% MEM essential vitamins and 10% FBS.

**siRNA transfection-** ASM cells were grown to ~90% confluence in 6-well cluster plates and transfected with 200 pmol of specific siRNA in serum and antibiotic free DMEM with X-tremeGENE siRNA transfection reagent. Control transfections were performed using a non-targeting control siRNA. 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.

**RNA isolation and real-time PCR-** Total RNA was extracted using the Nucleospin RNAII kit (Macherey-Nagel, Duren, Germany) as per the manufacturer’s instructions. Equal amounts of total RNA were then reverse transcribed using the Reverse Transcription System (Promega, Madison, USA). 1 µl of 1:2 diluted cDNA was subjected to real-time PCR, which was performed with the Illumina Eco Personal QPCR System (Westburg, Leusden, the Netherlands) using FastStart Universal SYBR Green Master Mix from Roche Applied Science (Mannheim, Germany). Real time PCR was performed with denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds for 40 cycles followed by 10 minutes at 72°C. Real time PCR 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 (∆Cq). Relative differences were determined using the equation 2^(-∆∆Cq). Primers used to analyze gene expression are: α-sm-actin Fwd 5’- GACCCTGAAGTACCCGATAGAAC -3’ and Rev 5’- GGGCAACACGAAGCTCATTG -3’, MRTF-A Fwd 5’- GCCAGGTGAACTATCCCAAA -3’ and Rev 5’- CACAGAACCCTGGGACTCAT -3’ and 18S rRNA Fwd 5’- CGCCGCTAGAGGTGAAATTC -3’ and Rev 5’- TTTGCAAATGCTTTTCGCTC -3’.
Immunofluorescence- Phalloidin was used to stain F-actin and DNAse I was used to stain G-actin. ASM cells were cultured on coverslips or in Labtek 8-chamber slides. Post-stimulation, cells were washed in warm PBS and fixed in 4% paraformaldehyde (PFA) plus 4% sucrose in PBS for 15 min. Cells were then incubated with 0.3% Triton X-100 in PBS for 2 min and blocked in 5% BSA in PBS for 1 hour following which cells were incubated with Alexa Fluor 488-conjugated Phalloidin (1:500) plus Alexa Fluor 594-conjugated DNAse I (1:500) in 1% BSA for 1 hour. Nuclei were stained with Hoechst stain (1:10000) diluted in ddH2O. After staining, coverslips were mounted in ProLong Gold Antifade reagent. Immunofluorescence was analyzed using a Leica microscope. For MRTF-A and α-sm-actin immunofluorescence was done as described earlier [18] using specific antibodies (dilution 1:100) and donkey anti-rabbit FITC or donkey anti-mouse cy3 antibodies (dilution: 1:50).

Nuclear extract preparation- Nuclear extracts were prepared as described previously (Chapter 3).

Preparation of cell lysates- The whole cell extracts were either prepared as described previously (Chapter 3) using SDS lysis buffer or by direct lysis in 2X Laemmli loading buffer.

Western analysis- Protein samples were subjected to electrophoresis, transferred to nitrocellulose membranes, and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. Bands 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.

Data Analysis- Values reported for all data are represented as mean ± SEM. The statistical significance of differences between means was determined 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

WNT-5A and WNT-11 in contractile ASM cells. Noncanonical WNT signaling is implicated in cytoskeletal reorganization and cell movements. We first investigated whether there is any possible link between noncanonical WNT ligands and the contractile phenotype of ASM cells. We approached this question using human tracheal smooth muscle tissue, cultured hTERT-ASM cells and a previously reported model of contractile phenotype maturation in primary human ASM cells [19].

We cultured cells in serum-containing medium until 50% confluence and used these as the serum-fed group expressing a proliferative smooth muscle phenotype or until 100% confluence followed by a serum-deprivation for 7 days labeling these as the serum-starved group expressing a contractile phenotype [19]. Accordingly, we found that serum-starved primary human ASM cells showed increased expression of α-sm-actin in comparison to serum-fed cells (Figure 1A). Interestingly, expression of both WNT-5A and WNT-11 were also augmented significantly in serum-starved cells in comparison to serum-fed cells (Figure 1A).
ASM cells in culture tend to lose contractile characteristics and are less contractile than fresh ASM tissue. In line with this, expression of α-sm-actin was significantly higher in fresh tracheal smooth muscle tissue in comparison to cultured hTERT-ASM cells (Figure 1B). Corroborating the link between the contractile phenotype and expression of WNTs, mRNA abundance of WNT-11 was significantly higher in human tracheal smooth muscle tissue than in the hTERT-ASM cells in culture (Figure 1B). WNT-5A, however, showed similar expression levels in both the experimental samples (Figure 1B).

Our data, thus, suggest a link between contractile phenotype and the expression of WNT-5A and WNT-11 in ASM cells.

Figure 1. WNT-5A and WNT-11 expression in contractile ASM cells. (A) Primary human ASM cells were cultured with (proliferative phenotype) or without serum (contractile phenotype) as described in the materials and methods section. α-sm-actin, WNT-5A and WNT-11 mRNA were determined by qRT-PCR, corrected for 18S rRNA and expressed relative to the serum-fed culture condition. Data represent mean ± SEM of 5 independent experiments. *p<0.05, ***p<0.001 compared to serum-fed; 1-way ANOVA followed by Newman-Keuls multiple comparisons test. (B) α-sm-actin, WNT-5A and WNT-11 mRNA expression were determined in hTERT-ASM cells in culture and in freshly-isolated human ASM tissue by qRT-PCR and expressed as 18S rRNA corrected values. Note that data are expressed as threshold cycle (Cq) values with highest values representing lowest mRNA expression. Data represent mean ± SEM of 3-4 independent experiments. *p<0.05, ***p<0.001 compared to fresh ASM tissue; 1-tailed Student’s t test for unpaired observations.

WNT-5A and WNT-11 mediate TGF-β-induced expression of α-sm-actin. To investigate the link between the co-expression of α-sm-actin and WNT ligands, we first analyzed the effect of TGF-β on these genes in hTERT-ASM cells. TGF-β is known to induce the expression of α-sm-actin in various cell types including ASM cells [13]. We have previously shown that TGF-β induces time- and dose-dependent increase in WNT-5A in hTERT-ASM cells. We also confirmed expression kinetics of α-sm-actin and WNT-11 in hTERT-ASM cells and observed the time- and dose-dependent increases in the expression levels (Figure 2). We have earlier reported a similar time- and dose-dependent increase in WNT-5A expression by TGF-β (Chapter 3).
WNT-11 has been shown to mediate TGF-β-induced expression of α-sm-actin in renal epithelial cells [16]. We investigated whether WNT-5A and WNT-11 are involved in α-sm-actin expression in ASM cells. Indeed, siRNA-mediated knock-down of WNT-5A or WNT-11 significantly attenuated TGF-β-induced expression of α-sm-actin in hTERT-ASM cells (Figure 3A).

Having confirmed the requirement for WNT-5A and WNT-11 in TGF-β-induced α-sm-actin expression, we wondered whether WNT-5A and WNT-11 can induce α-sm-actin in ASM cells. We observed that recombinant WNT-5A and WNT-11 induce α-sm-actin in ASM cells (Figure 3B).

We have previously shown that TGF-β activated kinase 1 (TAK1) is required for the induction of WNT-5A in hTERT-ASM cells (Chapter 5). Accordingly, TAK1 inhibition by LL-Z1640-2 attenuated TGF-β-induced expression of α-sm-actin mRNA and protein (Figure 3C, D).

Altogether, our data shows that both WNT-5A and WNT-11 are required for and mediate TGF-β-induced α-sm-actin expression in ASM cells.

**TGF-β-induced actin cytoskeleton remodeling is required for α-sm-actin expression.** As both TGF-β and noncanonical WNT signaling can induce cytoskeleton reorganization, we hypothesized that actin remodeling is required for α-sm-actin induction in response to TGF-β in ASM cells. To test this hypothesis, we pretreated hTERT-ASM cells with an inhibitor of actin polymerization- latrunculin A which binds to and stabilizes the G-actin fraction of actin pool. The stabilized G-actin fails to incorporate into the polymerizing F-actin, preventing actin polymerization and thus inhibiting actin cytoskeletal remodeling. We performed immunofluorescence microscopy to visualize actin polymerization and stained F-actin using fluorescently-labelled phalloidin and G-actin using fluorescently-labelled DNAse I. The microscopy analysis revealed that TGF-β induces increase in actin polymerization as reflected by an increased staining of phalloidin and appearance of more filamentous structures in the hTERT-ASM cells (Figure 4A). In addition, a decrease in the G-actin pool can be observed (Figure 4A). In line with its role in actin polymerization, latrunculin A pretreatment abrogated TGF-β induction of phalloidin-stained F-actin filaments and increased DNAse I-stained G-actin (Figure 4A).

We next addressed the requirement of actin polymerization in α-sm-actin expression in hTERT-ASM cells. Interestingly, the presence of latrunculin A completely abrogated TGF-β-induced α-sm-actin expression in hTERT-ASM cells as demonstrated by western blotting (Figure 4B) and microscopy analysis (Figure 4C). The inhibition of actin polymerization, however, had no effect on the expression levels of the β-actin pool of cytoskeleton (data not shown). Collectively, our data suggest that TGF-β-induced actin cytoskeleton remodeling is essential for α-sm-actin expression in ASM cells.

**WNT-5A and WNT-11 mediate TGF-β-induced actin cytoskeleton remodeling via Rho kinase signaling.** Next, we investigated the role of WNT-5A and WNT-11 in the regulation of TGF-β-induced cytoskeletal remodeling in ASM cells using various strategies. TAK1 inhibition attenuates WNT-5A induction (Chapter 5) and accordingly, α-sm-actin...
WNT-5A and WNT-11 drive α-sm-actin expression via Rho kinase-MRTF-A signaling

Figure 2. Expression kinetics of α-sm-actin and WNT-11 in ASM cells. (A, E) hTERT-ASM cells were either left unstimulated or stimulated with TGF-β (2 ng/ml) for the indicated duration. α-sm-actin mRNA (A) and WNT-11 mRNA (E) expression was determined by qRT-PCR, corrected for 18S rRNA and expressed relative to untreated cultures. Data represent mean ± SEM of 4 independent experiments. *p<0.05, **p<0.01 compared to basal; 2-tailed Student’s t test for unpaired observations. (B) hTERT-ASM cells were treated as mentioned above. Whole cell extracts (WCE) were

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analysed by western anaylsis to determine α-sm-actin abundance. Expression of GAPDH was used to verify equal loading. (C, F) hTERT-ASM cells were either left unstimulated or stimulated with TGF-β at the indicated concentrations for 24 hours. α-sm-actin mRNA (C) and WNT-11 mRNA (F) expression was determined by qRT-PCR, corrected for 18S rRNA and expressed relative to untreated cultures. Data represent mean ± SEM of 4 independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared to basal; 2-tailed Student’s t test for unpaired observations. (D) hTERT-ASM cells were either left unstimulated or stimulated with TGF-β at indicated concentrations for 48 hours. WCE were analysed by western anaylsis to determine α-sm-actin abundance. Expression of GAPDH was used to verify equal loading.

induction by TGF-β (Figure 3B, C). In line with these observations, TAK1 inhibition also attenuated TGF-β-induced augmentation in F-actin appearance indicating a requirement of WNT-5A in this process (Figure 5A).

We next performed knock down of WNT-5A and WNT-11 and analyzed F-actin and G-actin abundance in response to TGF-β. Interestingly, the presence of either WNT-5A- or WNT-11-specific siRNA attenuated TGF-β-induced F-actin abundance confirming an upstream role for WNT-5A and WNT-11 in TGF-β-induced actin remodeling in hTERT-ASM cells (Figure 5B).

Rho kinase signaling has been intrinsically linked to both cytoskeletal remodeling and α-sm-actin expression. We confirmed the essential role of the Rho kinase cascade in TGF-β-induced actin remodeling. Microscopy analysis of F- and G-actin further confirmed this observation as the presence of Y27632 attenuated the TGF-β-induced increase in appearance of F-actin filaments (Figure 6A). Also, pharmacological inhibition of Rho kinase by Y27632 completely reversed the TGF-β-induced increase in F-actin/G-actin ratio (data not shown). Accordingly, Rho kinase inhibition also attenuated TGF-β-induced α-sm-actin expression (Figure 6B).

Noncanonical WNT signaling also activates RhoA as part of the planar cell polarity pathway. We next investigated whether WNT-5A and WNT-11 utilize the Rho signaling arm to mediate actin remodeling. Interestingly, the presence of exogenous WNT-5A or WNT-11 induced actin cytoskeletal remodeling as revealed by increase in the F-actin staining (Figure 6C). Of note, pharmacological inhibition of Rho kinase attenuated WNT-5A- and WNT-11-induced actin remodeling (Figure 6C). Our data thus shows that WNT-5A and WNT-11 mediate TGF-β-induced actin remodeling in ASM cells by activation of Rho kinase signaling.

WNT-5A- and WNT-11-mediated MRTF-A nuclear translocation is required for TGF-β-induced α-sm-actin expression. Our observations coupling actin remodeling to α-sm-actin expression intrigued us to investigate the underlying mechanistic link. MRTF-A is a transcription factor which is released from G-actin following actin polymerization and translocates to the nucleus. Earlier reports have also shown its role in smooth muscle-specific gene expression including α-sm-actin [20]. We found that TGF-β induced upregulation of MRTF-A at both the mRNA and protein levels in hTERT-ASM cells (Figure 7A, B). Interestingly, TGF-β also induced nuclear translocation of MRTF-A as demonstrated
Figure 3. WNT-5A and WNT-11 are required for TGF-β-induced α-sm-actin expression.

(A) hTERT-ASM cells were transfected with a negative control siRNA or WNT-5A-specific or WNT-11-specific siRNA. Subsequently, cells were stimulated with TGF-β (2 ng/ml) for 48 hours and WCE were analyzed for α-sm-actin protein abundance by western blotting. Equal loading was verified by analysis of GAPDH. Graph represents quantification of band intensities of α-sm-actin corrected for GAPDH as percentage of TGF-β-induced expression in control siRNA-transfected cells. Data represent mean ± SEM of 4-5 independent experiments. *p<0.05 compared to control siRNA-transfected, untreated cells, #p<0.05 compared to negative control siRNA-transfected, TGF-β-stimulated cells; 1-way ANOVA followed by Newman-Keuls multiple comparisons test.

(B) hTERT-ASM cells were either left untreated or stimulated with recombinant WNT-5A (100 ng/ml) or WNT-11 (100 ng/ml) for 24 hours. WCE were analysed for α-sm-actin protein abundance. GAPDH expression was used to verify equal loading. (C) hTERT-ASM cells were either left unstimulated or stimulated with TGF-β (2 ng/ml) in the presence or absence of LL-Z1640-2 at the indicated concentrations for 24 hours. α-sm-actin mRNA expression was determined by qRT-PCR, corrected for 18S rRNA and expressed relative to vehicle treated cultures. Data represent mean ± SEM of 4 independent experiments. ***p<0.001 compared to vehicle basal, ###p<0.001 compared to TGF-β-stimulated cells; 1-way ANOVA followed by Newman-Keuls multiple comparisons test.

(D) WCE from cells stimulated with TGF-β (2 ng/ml) in the presence or absence of LL-Z1640-2 (0.5 µM) were analyzed for α-sm-actin protein abundance. GAPDH expression was verified for equal loading.
by its increasing abundance in the nuclear lysate (Figure 7C) confirming a functional effect of TGF-β on MRTF-A.

To elucidate the requirement of MRTF-A in TGF-β-induced α-sm-actin expression, we performed knock down of MRTF-A using specific siRNA. While TGF-β induced MRTF-A expression in the presence of non-specific negative siRNA, its expression was reduced in the presence of MRTF-A-specific siRNA (Figure 7D) confirming an efficient knock down. Interestingly, MRTF-A knock down also abrogated TGF-β-induced α-sm-actin expression in hTERT-ASM cells (Figure 7D) confirming the requirement of MRTF-A in α-sm-actin induction.

We next sought to identify the upstream mediators and mechanisms of MRTF-A nuclear translocation. As WNT-5A and WNT-11 activate Rho-kinase signaling and mediated TGF-β-induced actin remodeling, the presence of Rho kinase inhibitor Y27632 attenuated TGF-β-induced MRTF-A nuclear translocation (Figure 7E). More importantly, knock down of WNT-11 completely abrogated TGF-β-induced nuclear translocation of MRTF-A confirming a role for noncanonical WNT ligands in this phenomenon (Figure 7F).

Altogether, our data suggest that WNT-5A and WNT-11 mediate TGF-β-induced nuclear translocation of MRTF-A via activation of a Rho kinase-actin polymerization pathway which is required for α-sm-actin induction in ASM cells.

Discussion

In the present study, we demonstrate a novel role for the noncanonical WNT ligands WNT-5A and WNT-11 in the regulation of TGF-β-induced α-sm-actin expression in ASM cells. We show that WNT-5A and WNT-11 induce Rho kinase-dependent actin remodeling in ASM cells which is required for TGF-β induction of α-sm-actin. Furthermore, TGF-β induces an increase in the abundance and nuclear translocation of MRTF-A which is regulated by noncanonical WNTs and is required for α-sm-actin expression in ASM cells.

TGF-β is a potent inducer of contractile proteins such as α-sm-actin, sm-MHC, calponin and SM22 in ASM cells [13]. Moreover, WNT/β-catenin signaling is involved in differentiation and the expression of contractile proteins. For example, WNT-7B-activated β-catenin signaling is required for proper development and differentiation of bronchial SMCs [21]. Similarly, TGF-β-activated β-catenin regulates pulmonary fibroblast differentiation and expression of α-sm-actin [18] whereas it partners with cAMP response element-binding protein (CREB)-binding protein (CREBBP or CBP) and regulates TGF-β-induced α-sm-actin expression in pulmonary alveolar epithelial cells [22]. Interestingly, WNT-3A augments TGF-β-SMAD2 signaling via β-catenin leading to the expression of α-sm-actin in mouse fibroblasts [23] further suggesting a crosstalk between these two pathways in the expression of contractile proteins. We found that knock-down of either WNT-5A or WNT-11 attenuates TGF-β-induced α-sm-actin expression and polymerization in ASM cells whereas stimulation with recombinant WNT-5A or WNT-11 is able to increase α-sm-actin expression demonstrating that WNT-5A and WNT-11 mediate TGF-β-induced α-sm-actin expression in ASM cells. Our data add to these previous observations as we demonstrate that noncanonical
WNT-5A and WNT-11 drive α-sm-actin expression via Rho kinase-actin-MRTF-A signaling

Figure 4. Actin remodeling is required for TGF-β-induced α-sm-actin expression. (A) hTERT-ASM cells were stimulated with TGF-β (2 ng/ml) in the presence or absence of latrunculin A (0.1 µM) for 48 hours. F-actin, G-actin and nuclear stainings were done as described in materials and methods section. Green=F-actin, Red=G-actin and Blue=Nucleus. Magnification 20X. (B) hTERT-ASM cells were stimulated with TGF-β (2 ng/ml) in the presence or absence of latrunculin A (0.1 µM). WCE were analysed for α-sm-actin protein abundance. GAPDH expression was used to verify equal loading. (C) hTERT-ASM cells were stimulated as described above. α-sm-actin (red) and nuclear (blue) staining were done as described in the materials and methods section and analysed by immunofluorescence microscopy. Magnification 20X.
Figure 5. WNT-5A and WNT-11 mediate TGF-β-induced actin remodeling. (A) hTERT-ASM cells were stimulated with TGF-β (2 ng/ml) in the presence or absence of LL-Z1640-2 (0.5 µM) for 48 hours. F-actin, G-actin and nuclear staining were done as described in the materials and methods section. Green=F-actin, Red=G-actin and Blue=Nucleus. Magnification 20X. (B) hTERT-ASM cells were transfected with a negative control siRNA or WNT-5A-specific or WNT-11-specific siRNA. Subsequently, cells were stimulated with TGF-β (2 ng/ml) for 48 hours and stained for F-actin, G-actin and nucleus and analyzed by immunofluorescence microscopy. Green=F-actin, Red=G-actin and Blue=Nucleus. Magnification 20X.
WNT signaling plays a key role in the regulation of TGF-β induced α-sm-actin expression in smooth muscle cells.

Noncanonical WNT/Ca²⁺ and WNT/planar cell polarity signaling are involved in cytoskeletal reorganization and cell movements along with the transcriptional regulation of various genes. While there is no direct evidence published implicating noncanonical WNT signaling in the regulation of contractile proteins; NFAT and JNK, two of the mediators of noncanonical WNT signaling are known to regulate α-sm-actin expression. NFAT activation induces α-sm-actin expression in SMCs and inhibition of the calcineurin-NFAT pathway attenuates it [24]. Similarly, JNK induces expression of α-sm-actin in response to mechanical strain [25] and arginine vasopressin [26] in vascular SMCs. Most importantly, the small GTPase RhoA, which mediates noncanonical WNT signaling and is also activated downstream of TGF-β signaling, is an integral part of SMC differentiation and expression of α-sm-actin [20]. Here, we show that WNT-5A and WNT-11 activate Rho kinase-dependent noncanonical WNT signaling in ASM cells. Recombinant WNT-5A and WNT-11 increased the phosphorylation of MYPT1 (data not shown) confirming Rho kinase cascade activation in ASM cells. Rho kinase signaling is known to regulate the expression of contractile proteins including α-sm-actin in SMCs [27,28]. In line with that, pharmacological inhibition of Rho kinase signaling attenuated TGF-β-induced α-sm-actin expression in ASM cells.
Figure 6. Rho kinase regulates TGF-β and WNT-5A/WNT-11-induced actin remodeling. (A-B) Rho kinase regulates actin cytoskeletal remodeling. (A) hTERT-ASM cells were stimulated with TGF-β (2 ng/ml) in the presence or absence of Y27632 (1.0 µM) for 48 hours. F-actin, G-actin and nucleus were stained and analyzed by immunofluorescence microscopy. Green=F-actin, Red=G-actin and Blue=Nucleus. Magnification 20X. (B) hTERT-ASM cells were treated as described in (A) and western analysis was performed to detect the abundance of α-sm-actin in WCE. Equal loading was verified by analyzing GAPDH expression. (C) hTERT-ASM cells were stimulated with recombinant WNT-5A (100 ng/ml) or WNT-11 (100 ng/ml) in the presence or absence of Y27632 (1.0 µM) for 2 hours. F-actin, G-actin and nuclear staining were done and analyzed by immunofluorescence microscopy. Green=F-actin, Red=G-actin and Blue=Nucleus. Magnification 20X.
WNT-5A and WNT-11 drive α-sm-actin expression via Rho kinases-actin-MRTF-A signaling

Figure 7. Role for MRTF-A in TGF-β and WNT-5A/WNT-11-induced α-sm-actin expression. (A) hTERT-ASM cells were stimulated with TGF-β (2 ng/ml) for 24 hours and MRTF-A mRNA expression was determined by qRT-PCR, corrected for 18S rRNA and expressed relative to the basal. Data represent mean ± SEM of 4 independent experiments. **p<0.01 compared to vehicle basal; 2-tailed Student’s t test for unpaired observations. (B) MRTF-A protein expression was determined by western blotting. Equal loading was verified by GAPDH analysis. (C) Nuclear translocation of MRTF-A. hTERT-ASM cells were stimulated with TGF-β (2 ng/ml) for 16 and 24 hours. Nuclear extracts were prepared and analyzed for the presence of MRTF-A by western blotting. Lamin A/C abundance was analysed to determine equal loading. (D) hTERT-ASM cells were transfected with MRTF-A-specific or negative control siRNA. Subsequently, cells were stimulated with TGF-β (2 ng/ml) for 48 hours, WCEs were prepared and western analysis was performed to determine the expression of MRTF-A and α-sm-actin. Equal loading was verified by GAPDH expression. (E) Nuclear extracts, prepared from the hTERT-ASM cells stimulated with TGF-β (2 ng/ml) in the presence or absence of Y27632 (1.0 µM) for 24 hours, were analysed for MRTF-A abundance by western blotting. Equal loading was verified by Lamin A/C expression. (F) hTERT-ASM cells were transfected with WNT-11-specific or negative control siRNA. Subsequently,
Noncanonical WNT signaling via small GTPases and the Ca\(^{2+}\) pathway regulates cytoskeletal remodeling and stress fiber formation to promote cell migration. Studies including those from our group have showed that inhibition of actin remodeling by latrunculin A or B inhibits contractile protein expression [27,29]. We demonstrate that WNT-5A and WNT-11 promote actin remodeling by augmenting polymerized filamentous actin (F-actin) abundance with a concomitant decrease in monomeric globular actin (G-actin) in a Rho kinase-dependent manner. Moreover, inhibition of TGF-\(\beta\)-induced actin stress fiber formation by latrunculin A attenuates \(\alpha\)-smooth actin expression suggesting that TGF-induced and noncanonical WNT-mediated actin dynamics are linked to the transcriptional control of \(\alpha\)-sm-actin.

The SMC-specific genes essentially contain CArG box DNA elements \([CC(A/T))_6 GG]\) in their promoters which are regulated by serum response factor (SRF) in association with proliferative ternary complex factors or with myocardin family of transcription factors (myocardin and myocardin-related transcription factors) driving contractile gene expression programs [30]. While myocardin is constitutively nuclear, myocardin-related transcription factors (MRTFs) remain associated with G-actin and stay primarily cytosolic. Actin remodeling depletes the G-actin pool leading to the release of MRTFs and their nuclear translocation where they associate with SRF and other transcriptional co-regulators to activate target gene transcription [31,32]. TGF-\(\beta\) has been shown to regulate the Rho-actin-MRTF axis [31,33-35], however, a direct link between noncanonical WNTs and MRTF signaling is undocumented. We show that TGF-\(\beta\) induces expression and nuclear translocation of MRTF-A in ASM cells where it drives TGF-\(\beta\)-induced \(\alpha\)-sm-actin expression. Of note, inhibition of Rho kinase or knock-down of WNT-11 attenuates TGF-\(\beta\)-induced nuclear localization of MRTF-A validating a Rho kinase-dependent and WNT ligand-mediated axis in MRTF-A nuclear shuttling. We provide the first evidence for the regulation of a Rho kinase-actin-MRTF axis by noncanonical WNT ligands.

The TGF-\(\beta\)-induced and noncanonical WNT-mediated regulation of a Rho kinase-actin-MRTF-A axis may have important implications in various processes in airway remodeling. Clearly, noncanonical WNT signaling could contribute to contractile protein expression by ASM. Further, as EMT is considered a contributing factor to the increased mesenchymal cell population in asthmatic airways, the TGF-\(\beta\) and WNT-5A or WNT-11 crosstalk might also contribute to this process. Indeed, MRTF-A is a critical mediator of TGF-\(\beta\)-induced EMT [34,36] and epithelial-to-myofibroblast transition (EMyoT) [35]. Myofibroblasts are a rich source of ECM proteins and MRTF-A is a key mediator of myofibroblast activation and can regulate ECM expression. As demonstrated, MRTF-A induces collagen expression in lung fibroblasts [37]. Thus, the TGF-\(\beta\)-noncanonical WNTs axis could also regulate ECM expression in the airways. In support, we have demonstrated earlier that WNT-5A mediates...
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TGF-β-induced ECM expression via noncanonical Ca²⁺-NFAT and JNK signaling further suggesting a role for noncanonical WNT signaling in airway remodeling.

In conclusion, we demonstrate that noncanonical WNT signaling via WNT-5A and WNT-11 plays an important role in contractile protein expression in smooth muscle. WNT-5A and WNT-11 are preferentially expressed in contractile myocytes and regulate TGF-β induced expression of sm-α-actin via Rho kinase mediated actin polymerization and MRTF-A nuclear translocation. These findings further support a role for noncanonical WNT signaling in airway smooth muscle remodeling, implying that targeting this pathway may be a therapeutic strategy worth pursuing.

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Chapter 4

References

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