***miR-142-3p* is associated with aberrant Wingless/Integrase I (WNT) signaling during airway remodeling in asthma**

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ABSTRACT

Background: Asthma is characterized by a chronic inflammation and remodeling of the airways. While inflammation can be controlled, therapeutic options to revert remodeling do not exist. Thus, there is a large and unmet need to understand the underlying molecular mechanisms in order to develop novel therapies.

Objective: we previously identified a pivotal role for *miR-142-3p* in regulating airway smooth muscle precursor (ASM) cell proliferation during lung development by fine-tuning the Wingless/Integrase I (WNT) signaling. Thus, we here aimed to investigate the relevance of this interaction in asthma.

Methods: We performed qRT-PCR and immune-staining in a murine model for ovalbumin-induced allergic airway inflammation and in bronchial biopsies from patients with asthma and isolated primary fibroblasts thereof.

Results: *miR-142-3p* was increased in hyper-proliferative regions of lung in murine and human asthma, while this miRNA was excluded from regions with differentiated ASM cells. Increases in *miR-142-3p* were associated with a decrease of its known target *Adenomatous polyposis coli* (*Apc*). Further, we observed a differential expression of *miR-142-3p* in bronchial biopsies from patients with early or late onset severe asthma, which coincided with a differential WNT signature.

Conclusion: Our data suggest that *miR-142-3p* is involved in regulating the balance between proliferation and differentiation of ASM cells in asthma, possibly via controlling WNT signaling. Thus, this miRNA might be an interesting target to prevent airway smooth muscle hyper-proliferation in asthma.

INTRODUCTION

Western countries have experienced a large rise in chronic inflammatory diseases such as type 1 diabetes, inflammatory bowel disease but also asthma (2) over the last decades, and asthma currently affects around 235 million people worldwide (25). Available treatments can only relieve symptoms, thus asthma represents a huge burden for health care systems. Accordingly, there is a large need to develop novel therapeutic approaches.

Asthma is characterized by chronic inflammation, hyper-responsiveness and remodeling of the airways. The latter being particularly important as it may already develop in childhood (19) but usually does not respond to therapy. Hallmarks of airway remodeling include goblet cell metaplasia, basement membrane thickening and hyper-proliferation of airway smooth muscle (ASM) cells around the airways (reviewed in (10)).

The molecular mechanisms driving airway remodeling in asthma are far from being understood, but there are first hints that microRNAs (miRNAs) might be critically involved (20). These short, non-coding RNAs can post-transcriptionally regulate target gene expression, and thus represent a dynamic regulatory fine-tuning of pathways (7). miRNAs also have been shown to be involved in asthma (reviewed in (15).

In previous work, we identified dysregulated miRNAs in lung tissue of mice with allergic airway inflammation (AAI), and *miR-142-3p* was one of the most highly up-regulated miRNAs (4). *miR-142-3p* has been previously described for fine-tuning the balance between proliferation and differentiation of ASM progenitor cells during lung

development (6), due to targeting *Adenomatous polyposis coli* (*Apc*), a negative regulator of the Wingless/Integrase-1 (WNT) pathway (12).

Therefore, we hypothesized that *miR-142-3p* is involved in controlling proliferation and differentiation of ASM cells during asthma pathogenesis by fine-tuning WNT signaling. Accordingly, we investigated this in lung tissue of a murine AAI model and bronchial biopsies from human patients with mild or severe asthma.

MATERIAL AND METHODS

Animals

Female Balb/c J mice (Charles River, Sulzfeld, Germany) were housed in individually ventilated cages with *ad libitum* access to a standard pellet diet and water. All experiments were conducted under German federal guidelines for the use and care of laboratory animals and were approved by the Government of the District of Upper Bavaria (AZ 55.2-1-54-2531-49-07).

Induction of allergic airway inflammation in mice

Allergic airway inflammation (AAI) was induced as previously described (4). Briefly, mice were sensitized six times intra-peritoneally with 1 µg ovalbumin (OVA) in alum (or PBS in alum) and challenged with 1 % OVA aerosol on day 70 and 71 after first sensitization. AAI was verified by confirmation of OVA-specific IgE, inflammatory (eosinophilic) cell infiltration and goblet cell metaplasia (4).

RNA extraction

Total RNA including small RNAs was isolated by using the microRNeasy micro kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions and quantified and analysed for purity (presence of protein of phenol) by a NanoDrop® ND-1000 (Nanodrop Technologies, Erlangen, Germany). Absence of RNA degradation was evaluated by denaturing gel electrophoresis for murine RNA samples and by checking for equal expression of housekeeping genes between the samples for both human and murine RNA.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR and respective data analysis was performed as previously described (5). We used a TaqMan real-time PCR system (Life technologies, Carlsbad, CA, USA) for analyzing miRNAs and specific WNT pathway primers (Qiagen, Venlo, The Netherlands) on the LightCycler 480 (Roche, Mannheim, Germany). Data was normalized to small control RNA *U6* (Life technologies) for miRNA and expression differences were calculated with the ΔΔCt method (16).

Immunofluorescence (IF) and in-situ Hybridization

IF and In situ hybridizations were performed as previously described (6). Briefly, tissues were fixed in 4% PFA, embedded in paraffin, and sectioned on poly-L-lysine coated slides. Antibodies against APC (Abcam, Cambridge, UK), KI67 (Novabios, Limena, Italy) and ACTA2 (Sigma Aldrich, Hamburg, Germany) were used at 1:200 dilutions. All IF data was analyzed by trained investigators who did not know the identity of the samples. All adjustments for background and thresholds were performed identically for all images prior to quantification.

Human samples

We included subjects with mild (n=5) and severe (n=16) asthma, while severe were stratified for early (<18 years old; n=6) and late onset (>18 years old; n=10). All patients were identified and treated according to the GINA and ERS/ATS criteria (Global Initiative for Asthma (GINA) (9)), while early and late onset did not differ in the dose of inhaled corticosteroids (unpaired t-test). Fiberoptic bronchoscopy was performed as previously described (17). Biopsy samples were either embedded in Tissue Tek II OCT (Miles Scientific) or directly frozen at liquid nitrogen. Primary

cultures of asthmatic bronchial fibroblasts were obtained by enzymatic digestion of bronchial biopsy specimens and characterized as previously described (18).

Ethical statements for human samples

The local ethics committee (San Luigi Hospital: protocols 1759/2008-14871/2009) approved the study, which conformed to the Declaration of Helsinki; written informed consent was obtained from each subject.

Statistical Analysis

All results have been evaluated by using student's t-test with *$p<0.05$.

RESULTS

Increase of *miR-142-3p* expression in hyper-proliferative regions of the murine lung

To determine a possible association between *miR-142-3p* and ASM cell proliferation or differentiation during asthma pathogenesis, we first evaluated its expression in a murine model for acute OVA-induced AAI (Fig. 1A).

We observed a significant up-regulation of *miR-142-3p* in murine lung homogenate of OVA/OVA treated animals versus PBS/OVA controls (Fig. 1B). A subsequent *in situ* hybridization of lung tissue sections combined with a co-staining with KI67 revealed that *miR-142-3p* was increased in hyper-proliferative regions in the lung (Fig. 1C-F). On the other hand Apc, the previously reported target for *miR-142-3p* (6), was excluded from those regions (Fig. 1K-N), while a WNT target gene, Myc, was increased in animals with AAI (Fig1 G-J).

Activation of WNT signaling in experimental asthma

As *miR-142-3p* has been shown to regulate WNT signaling, we next analyzed the expression of 20 prominent WNT pathway genes in lung homogenate by qRT-PCR. Overall, we observed an activation of WNT signaling in AAI (OVA/OVA) compared to controls (Fig. 1O). In particular, we found a significant increase of *Wnt5a*, *Myc*, *Cyclin D1* (*Ccnd1), E1A-associated protein 300 (Ep300), Frizzled (Fzd5), and RuvB Like AAA ATPase (Ruvbl1)* but a decrease of *Wnt11, Wnt16, Wnt2b, Apc*, and *Axin2*.

Inverse regulation of *miR-142-3p* and *APC* in human asthma

To translate our findings to human asthma, we investigated bronchial biopsies of patients with mild and severe asthma (Table 1) or primary fibroblasts isolated from those.

Staining biopsies with alpha-smooth muscle actin (ACTA2) to determine fully differentiated ASM cells, revealed more $ACTA2^{+}$ cells in severe asthma patients ((Fig. 2A-D) compared to mild ones. *miR-142-3p* expression was decreased in severe asthma (Fig. 2E) and seemed to be inversely correlated with ACTA2 expression and was not co-expressed in the same cell type (Fig. 2A-D). Further, when we cultured primary fibroblasts isolated from bronchial biopsies, cells were more $KI67^{+}$ when derived from mild asthma patients (Fig. 2F-H), but less APC^{+} (Fig.2 I-K) compared to cells from severe asthmatics, suggesting a higher proliferative potential.

In order to investigate possible different molecular endotypes within severe asthma patients, we stratified severe asthma patients by an early asthma onset (age < 18) or a late asthma onset (age > 18). Patients with late onset asthma showed increased levels of *miR-142-3p* in bronchial biopsies (Fig 2L) compared to early onset patients.

WNT signaling is differentially activated in early versus late onset severe asthma

As our previous data suggested a role for *miR-142-3p* in the regulation of WNT signaling, we next performed an extensive qRT-PCR-based expression analysis of 78 WNT associated genes in bronchial biopsies of patients with early or late onset severe asthma. The factors *Frizzled 8 (FZD8), MYC, JUN, Segment polarity protein dishevelled homolog (DVL1), F-box/WD repeat-containing protein 4 (FBXW4), Matrix Metalloprotease (MMP7), Ras Homolog Family Member U (RHOU), WNT inhibitory factor 1 (WIF1), WNT16, WNT3* and *WNT9A* were found to be increased in late onset severe asthma compared to early onset (Fig. 3). The WNT ligands *WNT5a*

and *WNT6*, and Dickkopf-related protein 1 (DKK1) were significantly higher patients with early onset severe asthma.

DISCUSSION

This study demonstrates an association between *miR-142-3p*, WNT signaling and hyper-proliferative regions in the lung in murine AAI and patients with asthma. Furthermore, we observed for the first time that several WNT-associated factors are differentially expressed in patients with early or late onset severe asthma.

miR-142-3p has already been described in differentiation of stem cells, or cardiomyocytes, in hematopoiesis, immune tolerance or lung cancer (reviewed in (22)). Previously, we found that *miR-142-3p* regulates ASM cell proliferation and differentiation by fine-tuning WNT and FGF signaling during lung mesenchyme development. Briefly, specific targeting of *Apc* by *miR-142-3p* in murine embryonic lungs lead to activation of WNT signaling and enhanced proliferation of ASM progenitor cells. Upon a *miR-142-3p* knock-down, progenitor cells differentiated into ASM cells due to negative regulation of WNT signaling by Apc (6). Here we now show first hints that fine-tuning of ASM differentiation by *miR-142-3p* might be implicated in asthma, a disease characterized by hyper-proliferation of ASM cells, leading to airway obstruction. We here report that *miR-142-3p* is mainly expressed in hyper-proliferative regions in the murine AAI lung, while its target Apc was excluded from those. In line with this, APC was significantly decreased in highly proliferating $KI67^+$ primary human bronchial fibroblasts of patients with mild asthma, while *miR-142-3p* levels were increased in whole biopsies of those patients compared to severe asthmatics. IF of human bronchial biopsies revealed an increased presence of $ACTA2^+$ terminally differentiated ASM cells, but a lack of *miR-142-3p* in those regions. Altogether, these data suggest that *miR-142-3p* might be involved in the early initiation of ASM remodeling in asthma by suppressing WNT signaling to initiate the proliferation of ASM progenitor cells. It is intriguing to speculate that in later phases

of asthma pathogenesis, possibly due to a loss of *miR-142-3p*, these cells differentiate into ASM cells and contribute to pathology. Therefore, *miR-142-3p* might represent an interesting target for approaches to prevent airway remodeling.

The role of *miR-142-3p* in asthma is presumably due to the simultaneous regulation of several relevant targets, however we here focused on WNT signaling, as this pathway is a) critical for regulating the balance between proliferation and differentiation of ASM progenitor cells (6) and b) has been implicated with asthma pathogenesis in several studies before (3, 8, 21, 23). WNT signaling plays a pivotal role in organism development and is in general activated upon binding of a WNT ligand to receptors, such as *Frizzled (FZD),* leading to context-dependent transcription of targets genes (reviewed in (1)).

We here validated the *miR-142-3p*-mediated *Apc* downregulation in proliferative cells of the lung, but expanded this to asthma pathogenesis. APC is a negative regulator of ß-catenin (CTNNB1) that induces, upon binding in combination with AXIN and GSK3B, its ubiquitination and subsequent degradation, thereby preventing CTNNB1 nuclear translocation and transcription of target genes (1). We observed an increase of typical targets for ß-catenin driven gene expression (canonical WNT signaling) such as *Ccnd1*, *Myc* and *Ep300* in lung homogenate of murine OVA-induced AAI. Our finding of a significant increase in *Wnt5a* expression in mice with AAI compared to healthy controls complements previous studies, and suggests that next to canonical, also ß-catenin independent, non-canonical WNT signaling is involved in asthma pathogenesis. In humans, *WNT5a* expression has been associated with a Th2 signature in airways (8) and is increased in ASM cells of patients with mild to moderate asthma. Further, a siRNA-mediated knock-down of *WNT5a* reduced the deposition of extracellular matrix by ASM cells (13). Thus, non-canonical WNT signaling via

235 *WNT5a* might be implicated in the early development of airway remodeling in
236 asthma. Whether this is also (indirectly) regulated by *miR-142-3p* needs to be
237 assessed in future studies.

238 Stratifying in early or late onset of severe asthma enabled us to identify different
239 'molecular disease endotypes'. We observed higher *miR-142-3p* levels in patients
240 with a late onset. Due to ethical limitations we could not compare the expression to
241 healthy controls, as we here used very precious samples from fiberoptic bronchial
242 biopsies. Further, miR-142-3p is almost not expressed in airway epithelial cells, thus
243 hampering comparisons of our study to others using bronchial brushings.
244 Nonetheless, miR-142-3p has been recently reported to be increased in cell-free
245 sputum of patients with severe asthma compared to healthy controls and levels
246 correlated with FEV1/FVC ratios (14). Despite this limitation, we identified
247 significant differences in 14 genes associated with WNT signaling in early vs late
248 onset severe asthma. In particular, *WNT5a* was significantly increased in patients with
249 early onset asthma, while ß-catenin dependent, canonical, target genes such as *JUN*,
250 *MYC* and *MMP7* were higher patients with a late onset severe asthma. Thereby, early
251 onset is often associated with an allergic, Th2-prone asthma, which is reflected in our
252 data as all patients were atopic, while late onset asthma is generally "non-allergic-
253 eosinophilic type 2" or "non type 2" including neutrophilic asthma (24). Of note,
254 MMP7 secretion of airway epithelium has already been shown to promote AAI in
255 mice (11). The observed increase in *WIF-1* expression in late onset severe asthma, is
256 in accordance to a previous study showing this to be associated with FVC and
257 FEV_1/FEV in asthmatic patients (21).

258 In summary, our extensive qRT-PCR based screening approach revealed alterations in
259 several factors of canonical and non-canonical WNT signaling in early onset vs late

onset severe asthma. As we here could only assess a small number of patients per group, the results need to be validated in larger studies in the future, which should be combined with an unbiased transcriptomics approach in order to in depth characterize distinct molecular endotypes and pave the way for precision medicine approaches.

Finally, our data suggest an involvement of *miR-142-3p* in regulating the balance between proliferation and differentiation of ASM cells in the pathogenesis of asthma. This study complements previous work on this topic, and we propose *miR-142-3p* as interesting molecular target to prevent early ASM hyper-proliferation in asthma.

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DISCLOSURES

The authors declare that there are no relevant conflicts of interest in relation to this article.

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FIGURES LEGENDS

Figure 1: (A) Treatment scheme (B) qRT-PCR of *miR-142-3p* in murine lung homogenate. (C-N) In situ hybridisation for miR-142-3p (green) combined with IF for KI67 (red) (C-F), MYC (red) (G-J) and APC (K-N). Scale bars: G, I: 75 µm; C, E, K, M: 25 µm, H, J 8 µm. (O) qRT-PCR of WNT signaling genes in lung homogenate displayed as relative expression of OVA/OVA vs PBS/OVA. All n=5 animals/group, *p<0.05.

Figure 2: (A-D) In situ hybridization for *miR-142-3p* (green) and IF for ACTA2 (red) in bronchial biopsies of mild (A, B) vs severe asthmatics (C, D). Scale bar A, B 75 µm; C, D, F-J: 25 µm. Dashed boxes indicate magnification area for C & D. Representative images of n=3 per group. (E) qRT-PCR for *miR-142-3p* in frozen bronchial biopsies from mild (n=4) and severe (n=8) asthmatics. (F-K) IF of primary bronchial fibroblasts for KI67 (red) (F-H), and APC (red) (I-K) with respective quantification (H, K) mean ± SD (n=3, n=4), *p<0.05. Scale bar=25 µm; (L) qRT-PCR for *miR-142-3p* in bronchial biopsies of early (n=5) vs late (n=4) onset severe asthmatics. mean ± SD, *p<0.05.

Figure 3: qRT-PCR for 78 WNT associated genes in bronchial biopsies from early (n=5) and late onset (n=4) severe asthma patients. mean ± SD; *p<0.05

TABLES

Table 1: Patient demographics

	All	Mild	Severe	Severe early onset	Severe late onset
	n=21	N=5	n=16	n=6	n=10
Sex (F/M)	11/10	4/1	7/9	3/3	4/6
Atopy (y/n)	10/11	3/2	7/9	6/0	1/9
Smoke (y/ex/no)	2/9/10	1/2/2	1/8/7	0/2/4	1/5/4
Age	58±9	51,8±6	60±10	56±11	62±9
BM I	25±3	23,5±4	26±3	24,7±2,5	27±3,5
FEV1(%pred)	72±19	92±11	66±16*	65±16	66±17
FVC (%pred)	75±23	92±11	67±21**	78±21	61±19
FEV1 post (ml)	279±112	267±80	282±119	243±48	305±145
FeNO	29±25	35±30	27,5±20	28±26	28±17
Exacerbation	2±2	0,5±0,5	2,4±1,9*	2,3±2,1	2,4±1,9
Beclomethasone HFA	436±269	150±50	526±245*	603±328	480±185
Blood eosinophils (cells/µl)	258±200	316±233	240±194	186±164	294±213
Bronchial eosinophils(cell/mm^2)	42,8±30	36±24	45±33	45±26	45±38
Blood neutrophils (cells/µl)	4365±2221	3406±671	4665±2461	3723±871	4722±2621
Bronchial neutrophils(cell/mm^2)	70,9±39	34±29	82±36*	88±28	79±40

Data are expressed as mean+/- SD