Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

Allergy

ORIGINAL ARTICLE AIRWAY DISEASES

MicroRNA-19a enhances proliferation of bronchial epithelial cells by targeting *TGFβR2* gene in severe asthma

I. Haj-Salem[1], R. Fakhfakh[1], J.-C. Bérubé[1], E. Jacques[1], S. Plante[1], M. J. Simard[2], Y. Bossé[1] & J. Chakir[1]

[1]Centre de recherche, Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval; [2]St-Patrick Research Group in Basic Oncology, Hôtel-Dieu de Québec (Centre Hospitalier Universitaire de Québec), Laval University, Cancer Research Center, Quebec City, Québec, Canada

To cite this article: Haj-Salem I, Fakhfakh R, Bérubé J-C, Jacques E, Plante S, Simard MJ, Bossé Y, Chakir J. MicroRNA-19a enhances proliferation of bronchial epithelial cells by targeting *TGFβR2* gene in severe asthma. *Allergy* 2015; **70**: 212–219.

Keywords
epithelial cells; microRNA; severe asthma; TGF-β receptor 2.

Correspondence
Dr. Jamila Chakir, Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie du Québec, 2725 Chemin Sainte-Foy, Québec G1V 4G5, Canada.
Tel.: 418-656-4760
Fax: 418-656-4509
E-mail: jamila.chakir@fmed.ulaval.ca

Accepted for publication 24 November 2014

DOI:10.1111/all.12551

Edited by: Hans-Uwe Simon

Abstract

Background: Allergic asthma is characterized by inflammation and airway remodeling. Bronchial epithelium is considered a key player in coordinating airway wall remodeling. In mild asthma, the epithelium is damaged and fails to proliferate and to repair, whereas in severe asthma, the epithelium is highly proliferative and thicker. This may be due to different regulatory mechanisms. The purpose of our study was to determine the role of miRNAs in regulating proliferation of bronchial epithelial cells obtained from severe asthmatic subjects in comparison with cells obtained from mild asthmatics and healthy controls.

Methods: Human bronchial epithelial cells (BEC) were isolated by bronchoscopy from bronchial biopsies of healthy donors and patients with mild and severe asthma. MiRNA expression was evaluated using the TaqMan low-density arrays and qRT-PCR. Transfection studies of bronchial epithelial cells were performed to determine the target genes. Cell proliferation was evaluated by BrdU incorporation test.

Results: MiR-19a was upregulated in epithelia of severe asthmatic subjects compared with cells from mild asthmatics and healthy controls. Functional studies based on luciferase reporter and Western blot assays suggest that miR-19a enhances cell proliferation of BEC in severe asthma through targeting TGF-β receptor 2 mRNA. Moreover, repressed expression of miR-19a increased SMAD3 phosphorylation through TGF-β receptor 2 signaling and abrogated BEC proliferation.

Conclusion: Our study uncovers a new regulatory pathway involving miR-19a that is critical to the severe phenotype of asthma and indicates that downregulating miR-19a expression could be explored as a potential new therapy to modulate epithelium repair in asthma.

Asthma is a complex respiratory disease characterized by chronic inflammation and remodeling of airway tissues (1). Bronchial epithelium is considered a key player in coordinating airway wall remodeling (2). Airway remodeling comprises a number of structural modifications including epithelial change, smooth muscle hypertrophy, and subepithelial fibrosis (3). These features have been correlated with the decrease in lung function as asthma severity progresses. In mild asthma, the epithelium is damaged and fails to proliferate and to repair (4). In contrast, in severe asthma, the epithelium was reported to be highly proliferative, which contributes to its thickness (5). However, the mechanism and exact process of this difference are still poorly understood.

Recently, the research focus has shifted away from immune basis of asthma to earlier events in disease progression such as the deregulation of genes. A substantial portion of

Abbreviations
ABEC, Asthmatic bronchial epithelial cells; NBEC, Normal bronchial epithelial cells; SABEC, Severe asthmatic bronchial epithelial cells; TGFβR2, Transforming growth factor beta receptor 2; TGF-β, Transforming growth factor beta; TLDA, TaqMan low-density array.

post-transcriptional gene regulation is controlled by microRNA (miRNA) networks. Hence, an alteration in the expression of miRNAs is emerging as a significant contributing factor to human respiratory disease. MiRNAs are small noncoding RNAs. The main mechanism of miRNA action is the post-transcriptional regulation via RNA interference, generally by binding to the 3′ untranslated region (UTR) of target mRNAs in a specific manner and either triggering their degradation or inhibiting translation (6–8).

MiRNAs are involved in a wide spectrum of biological processes including development (9), cell proliferation (10), apoptosis (11), tumorigenesis (12) and the immune response (13). Changes in the expression of several miRNAs have been shown to be associated with the development and/or improvement in animal model of asthma (14). In human asthma, miRNAs studies are limited. Williams et al. (15). looked at miRNA expression in freshly isolated airway tissue obtained by endobronchial biopsy from normal and mild asthmatic subjects. This study revealed cell-type-specific miRNA profiles but could not find an association between specific patterns of miRNA expression and asthma. Other studies identified 66 miRNAs that were differentially expressed by epithelial cells obtained from asthmatic and healthy subjects (16). A decreased expression of the miR-34/449 family members in mild and moderate asthma has been reported (17). This miR family plays a critical regulatory role in airway ciliated cells differentiation by direct repression of NOTCH1/DLL1 pathway (18). These studies indicate that structural changes observed in the airway epithelium in asthma may be related to deregulation of specific microRNAs. Among the functional modifications observed in the airway epithelium is the perturbation of cell proliferation and repair (5, 19). Therefore, the aim of this study was to identify miRNAs that regulate proliferation of bronchial epithelial cells obtained from severe asthmatic subjects in comparison with cells obtained from mild asthmatics and healthy controls.

Materials and methods

Subjects

Asthmatic subjects fulfilling the American Thoracic Society criteria for asthma (20) were recruited from the Asthma Clinic at the Institut Universitaire de Pneumologie et de Cardiologie de Québec. Subject characteristics are summarized in Table 1. Mild asthmatic subjects were atopic with at least one positive response to common allergens on allergy skin prick tests. They were nonsmokers and used only an inhaled β_2-agonist on demand and did not use inhaled or systemic corticosteroids (CS). In the month preceding the study, none of the subjects reported a respiratory infection or an increase in asthma symptoms. Severe asthmatics have severe asthma according to the ATS refractory asthma definition (21). Subjects were on continuous treatment of high doses of inhaled CS (>1000 mg of fluticasone propionate or the equivalent in combination with a long-acting β_2-agonist. They were nonsmokers, and their asthma was stable with no exacerbation for the last 4 months. Healthy subjects were nonatopic and nonsmokers with no history of asthma or atopy or any respiratory diseases. The study was approved by the Institut Universitaire de Cardiologie et de Pneumologie (Québec, Canada) ethics committee, and all subjects signed an informed consent form.

Bronchial epithelial cell culture

Biopsy specimens were collected from healthy controls ($N = 9$), mild asthmatic subjects ($N = 9$) and severe asthmatic subjects ($N = 6$) by flexible bronchoscopy. Epithelial cells were isolated and cultured as we previously described (22). All cells were used at passage 2 to 3.

Proliferation

Bronchial epithelial cells (BEC) proliferation assays were performed as we previously reported (19). Cells (10 000 cells/well) were seeded in 96-well tissue culture plates. Twenty-three hours before harvesting cells, bromodeoxyuridine (BrDU) was added at a final concentration of 10 μM. Cell proliferation was measured using a cell proliferation colorimetric ELISA commercial kit (Roche, Laval, QC, Canada) following the manufacturer's instructions. The viability and the adherence of the cells were controlled.

miRNA expression

MicroRNAs were extracted using short RNA enrichment method of the miRVana miRNA isolation kit (Cat. No.

Table 1 Clinical characteristics of healthy controls and asthmatic subjects

	Healthy control subjects	Subjects with mild asthma	Subjects with severe asthma
Age (years)	30.7 ± 9.9	23.3 ± 3.6	58.2 ± 10.6
Sex, M:F (%F)	5M:4F	8F	4M:2F
FEV1 (% predicted)	102.9 ± 0.62%	98.7 ± 13.8%	64.7 ± 11.79%
Methacholine PC20 (mg)	122.4 ± 63.3	1.8 ± 2.4	ND
Blood eosinophils (%)	3.9 ± 3.9	3.7 ± 1.7	5.12 ± 2.47
Sputum eosinophils (%)	ND	ND	54.5 ± 27.8
Atopic (%)	–	100	83.3
ICS dose (μg)	–	–	1229.2 ± 450.1

ND: not determined.

AM1560, Life Technologies, Burlington, ON, Canada) according to the manufacturer's instructions. MiRNA expression was performed using the 384-well microfluidic TaqMan low-density array based on stem-loop real-time PCR-based TaqMan miRNA expression assay following the manufacturer's instructions (TaqMan human microRNA card v2.0, Applied Biosystems, Life Technologies). The threshold cycle (CT) values over 30 were defined as undetectable. Validation of selected miRNAs was assessed by individual TaqMan qRT-PCRs (Applied Biosystems). RT reactions were performed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers. The expression level of miRNA was normalized to U6 snRNA and calculated using the ΔΔCT method. Patients with severe asthma were treated with high doses of inhaled CS. To exclude the possibility that this treatment may be responsible for the upregulation of miR19-a observed in severe asthmatic cells, we treated epithelial cells from steroid naive asthmatics with mometasone furoate (Merck Frost) at 10^{-9} and 10^{-11} M for 24 h, and we evaluated miR-19a expression.

Effect of miR-19a on cell proliferation

To assess the effect of miR-19a mimic and inhibitor on cell proliferation, normal (NBEC) and severe asthmatic (SABEC) epithelial cells were transfected with 10 nM of miR-19a mimic, inhibitor or scramble negative control miRNA using Lipofectamine RNAiMAX. After 48 h, bromodeoxyuridine (BrdU) was added at a final concentration of 10 mM. Cell proliferation was measured.

Transfection

Epithelial cells were plated at a density of 2×10^5 cells in six-well plates. Cells were transfected with 30 nM scramble negative control, 30 nM miRNA-19a mimic or 30 nM miRNA-19a inhibitor (Ambion Applied Biosystem, Life Technologies) using lipofectamine RNAiMAX (Invitrogen, Life Technologies) and Opti-MEM® I Reduced Serum Medium (Gibco, Life Technologies) for 48 h. Optimization of transfection experiments was performed using the pre-miR hsa-miR-1 miRNA precursor as a positive control that is well known to downregulate a validated target *PTK9* gene at the mRNA level.

miR-19a prediction targets

TargetScan 6.0 human, miRDB, miRanda and ingenuity pathway analysis were used to identify predicted miR-19a targets. mRNA was considered targets if selected miR was predicted with high probability to interact with their 3′-UTR. mRNA targets were then compared to genes and pathways associated with human cell cycle to evaluate potential miRNA regulation of cell proliferation.

RT-PCR

Total RNA was extracted using the illustra RNAspin Mini Kit following the manufacturer's protocol (GE Healthcare, Pittsburgh, PA, USA). TGFβR2 (158 bp) mRNA was amplified at an annealing temperature of 60°C and using the following primers: TGFβR2 (F):5′-CAGAAATCCTGCATGAGC-3′ and TGFβR2 (R): 5′-GCAGCATCTTCCAGAATAAAG-3′; mRNA was normalized to that of the corresponding housekeeping gene GAPDH as previously described (23). Effect of corticosteroid on TGFβR2 expression was evaluated on NBEC and ABEC treated for 24 h with mometasone furoate (Merck Frost) at 10^{-9} M.

Luciferase reporter assay

Reporter plasmid pMiR-TGFβR2 containing the 3′UTR derived from the human transforming growth factor receptor 2 gene was designed by Origene. The 3′UTR-TGFβR2 was cloned immediately downstream of the firefly luciferase reporter. pMiR-Target served as an empty vector control. For transfection experiments, BEAS-2B cells were plated at 150 000 cells per well in a six well plate and transfected with 30 nM of miR-19a mimic (Ambion) or scramble miR (Ambion) using Lipofectamine RNAiMAX. The next day, cells were transfected with 2.0 μg of pMir-TGFβR2 firefly luciferase reporter plasmid and 1.0 μg of Renilla luciferase expression plasmid pRL-SV40 (Promega, Madison, WI, USA) with Lipofectamine 2000 (Invitrogen). Cells were then analyzed using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Each sample was prepared in duplicate, and the entire experiment was repeated three times. Firefly luciferase activity from pMiR-Target and pMiR-TGFβR2 3′UTR was normalized to Renilla luciferase expression from plasmid pRL.SV40 (Promega), which served as a transfection control. Luciferase activity was evaluated by the BioTek™ Microplate Reader (BioTek, Winooski, VT, USA).

Western blots

Fourty-eight hours after transfection with mimic or inhibitor miR-19a, cells were placed in serum-free medium for 4 h and then stimulated with TGFβ at 5 ng/ml for 30 min. Cells were then detached using cell scrapers, and pellets were resuspended in lysis buffer containing phosphatase inhibitor cocktail 2 (Sigma Aldrich, Oakville, ON, Canada) and phosphatase inhibitor cocktail set IV (Calbiochem). Western blots were performed as previously described (19).

The following antibodies and dilutions were used: the anti-TGFβ-RII (1:100) was from Santa Cruz biotechnology (Santa Cruz, CA); the anti-Smad3 (1:1000) was from Abcam (Cambridge, MA) and the anti-phosphorylated Smad3 (1:1000) was from Cell Signaling Technology (Danvers, MA). Anti-β-actin (1:10000) was from Sigma Chemicals. Peroxidase-conjugated secondary antibodies (1:2000) were from Amersham Biosciences. Densitometry analyses were performed using densitometric analysis software (Genesnap, Syngene, MD).

Statistical analysis

All experiments in this study were performed at least three times. To compare groups and conditions, the data were

analyzed using a crossed-nested design. A posteriori multiple comparisons were performed using Tukey–Kramer test. Mean values ± SEM of quantitative variables were used as representative measures. All reported P values were two-sided and were declared significant at 0.05 levels.

Results

Increased epithelial cell proliferation rate in severe asthma

Figure 1A shows the percentage of cell proliferation of bronchial epithelia cells from healthy controls (NBEC), mild asthmatics (ABEC) and severe asthmatics (SABEC). Epithelial cells from severe asthmatics show a significant higher rate of proliferation compared to controls (192.5 ± 21.03%, $P < 0.0001$). In mild asthmatics, a significant decrease in the proliferative rate was observed (68.8 ± 9.16% compared to controls, $P = 0.0026$), which confirm our previous published data (19). Histological sections from bronchial biopsies of controls, mild asthma and severe asthma show disorganization of the epithelium in mild asthma and thickening in severe asthma (Fig. 1B).

miR-19a is upregulated in bronchial epithelial cells of patients with severe asthma

Analysis of microarray showed difference in miRNA expression between groups (Table S1 in supporting information). miR-19a is the only miRNA that differentiates severe asthma from mild asthma and controls. Hence, qRT-PCR confirmation showed that miR-19a level was upregulated in bronchial epithelial cells of patients with severe asthma (1.9-fold induction; $P = 0.05$ and $P = 0.008$) compared to cells from controls and mild asthmatics, respectively (Fig. 2). No significant difference in miR-19a expression was found between controls and mild asthmatic subjects. Corticosteroid did not affect miR-19a expression in epithelial cells of severe asthmatics, which exclude the possibility that change in miR-19a is due to corticosteroid treatment received by these patients (Fig. S1A in supporting information).

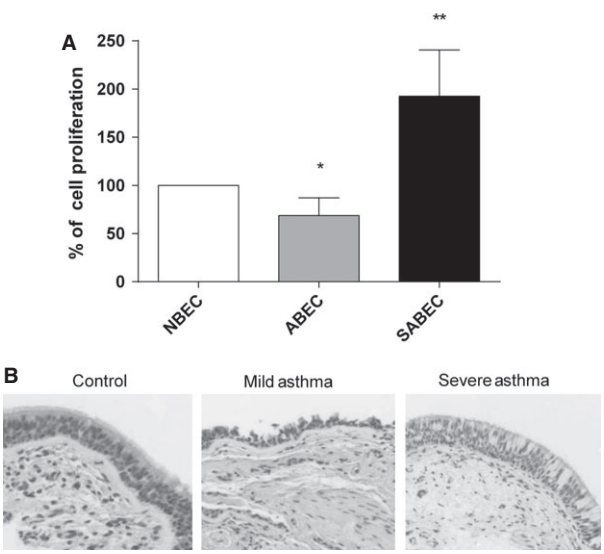

Figure 1 Increased epithelial thickness and cell proliferation rate in subjects with severe asthma. (A) Baseline proliferation of bronchial epithelial cells from normal (NEBC; $n = 9$), mild (ABEC; $n = 9$) and severe (SABEC; $n = 6$) asthmatic subjects. $*P < 0.003$, $**P < 0.0001$. (B) Endobronchial biopsies were obtained from subjects with severe or mild asthma and normal subjects and subjected to hematoxylin–eosin staining. Representative photomicrographs (magnification 40×) are demonstrated for each subject.

miR-19a promotes bronchial epithelial cell proliferation

Transfection was performed in bronchial epithelial cells from controls and severe asthmatics with mimic and inhibitor miR-19a. Figure 3 indicates that baseline proliferation of

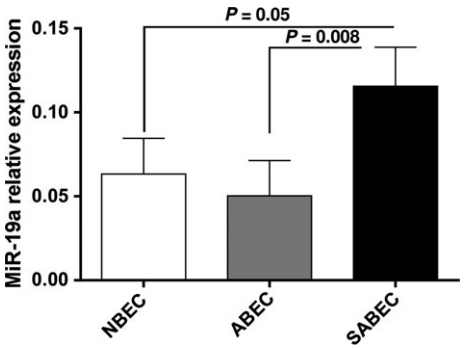

Figure 2 miR-19a is upregulated in BECs of patients with severe asthma. qRT-PCR validation of TLDA data showing increased miR-19a expression in patients with severe asthma (SABEC, $n = 6$) compared to normal subjects (NBEC; $n = 9$) and patients with mild asthma (ABEC; $n = 9$). Results are reported as level of miR-19a relative to the control RNA U6. Data are expressed as means ± SEM. $P < 0.05$.

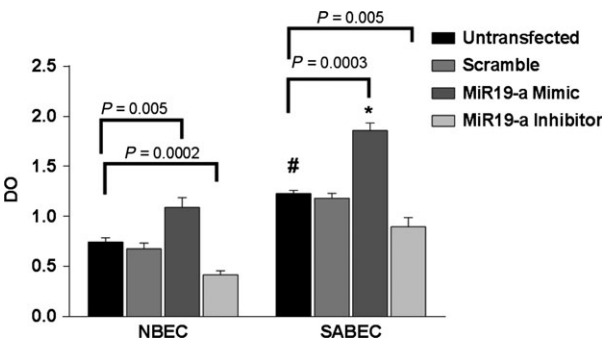

Figure 3 miR-19a promotes bronchial epithelial cell proliferation. Effect of miR-19a modulation on cell proliferation; epithelial cells obtained from healthy severe asthmatic subjects were transfected with 10 nM of miR-19a mimic or miR-19a inhibitor, and proliferation was evaluated by bromodeoxyuridine (BrdU) incorporation. $^{\#}P < 0.00001$; $*P = 0.00012$.

cells from severe asthmatics showed a significant higher rate of proliferation compared to healthy subjects ($P < 0.0001$). Transfection with miR-19a mimic showed an increase in proliferation rate compared to nontransfected cells (mean DO was 0.7458 *vs* 1.091 for NBEC; $P = 0.0054$ and 1.229 *vs* 1.8582 for SABEC; $P = 0.0003$). This rate was significantly higher in BECs from severe asthmatics compared to cells from healthy subjects ($P = 0.00012$). Inhibition of miR-19a in bronchial epithelial cells derived from healthy as well as severe asthmatic subjects significantly decreased their proliferation (mean DO was 0.7458 *vs* 0.4182 for NBEC; $P = 0.0002$ and 1.229 *vs* 0.816 for SABEC; $P = 0.0057$).

TGFβR2 is a target of miR-19a in BECs

To identify a miR-19a putative target gene that might be involved in BECs proliferation, we conducted an *in silico* analysis. A 7mer-A1 site located 265–271 bp downstream of the stop codon of TGFβR2 was predicted by the TargetScan, miRDB and miRanda programs to match with the seed sequence of miR-19a, whereas two other potential binding sites at position 563–587 and 1212–1233 downstream of the stop codon of TGFβR2 were predicted only by miRanda program. Site 1 is predicted to have better accessibility than sites 2 and 3 based on miRanda outputs. Transfection and qRT-PCR analysis were conducted to determine the mechanism by which miR-19a inhibited TGF-β receptor 2 mRNA. Figure 4A indicates that TGFβR2 gene expression at baseline is significantly lower in BEC from severe asthmatics compared to BEC from controls ($P = 0.05$). Transfection of BEC from controls with a miR-19a mimic induced a significant decrease in TGF-β receptor 2 mRNA expression levels (Fig. 4A), while silencing of miR-19a using miR-19a inhibitor in BEC from severe asthmatics resulted in an increase of TGFβR2 gene expression, indicating that miR-19a regulates TGFβR2 gene expression (Fig. 4A). Western blots revealed that the protein level of TGFβR2 was markedly reduced in the cells overexpressing miR-19a compared to the nontransfected cells ($33.55 \pm 4.98\%$ *vs* $100 \pm 15.62\%$; $P = 0.0279$), and silencing of miR-19a resulted in an increase of TGF-β receptor 2 protein level ($76.94 \pm 3.22\%$ *vs* $27.4 \pm 0.67\%$; $P = 0.0021$) (Fig. 4B). In addition, corticosteroid treatment did not affect TGFβR2 gene expression (Fig. S1B in supporting information).

Effect of miR-19a modulation on SMAD3 phosphorylation

We assessed whether or not miR-19a modulation altered the expression of Smad pathway proteins after TGF-β1 treatment. As shown in Fig. 4C, the baseline expression of the functional phosphorylated form of SMAD3 is lower in patients with severe asthma than in normal healthy subjects. MiR-19a modulation did not affect the expression of SMAD3. However, the overexpression of miR-19a in NBECs reduced significantly the level of the phosphorylated form of SMAD3 ($18.76 \pm 3.37\%$ *vs* $100 \pm 2.04\%$; $P = 0.0011$) while repression of miR-19a in SABECs increased phospho-SMAD3 levels ($71 \pm 6.28\%$ *vs* $16.53 \pm 1.31\%$; $P = 0.0067$) (Fig. 4C).

3′UTR of TGFβR2 interacts directly with miR-19a

To verify whether there is interaction between the predicted binding sites for miR-19a within TGFβR2 mRNA, we co-transfected the luciferase reporter plasmid containing the 3′UTR of TGFβR2 (pMiR-TGFβR2) and either miR-19a mimic or scramble miR in BEAS-2B cells. In the presence of miR-19a mimic, we observed a significant decrease of 79 ± 6, 96% of relative luciferase activity compared with cells transfected with the scramble miR using as negative control (Fig. 5).

Discussion

In the present study, we showed that miR-19a is specifically increased in airway epithelial cells obtained from severe asthmatic subjects compared to mild asthmatic and healthy subjects. MiR-19a increased proliferation in severe asthma through the downregulation of TGF-β receptor 2. We showed that proliferation rate of epithelial cells from severe asthmatics is higher than in controls and mild asthmatics. This *in vitro* observation is in agreement with data reported in bronchial biopsies by other groups (24, 25). Cohen et al. proposed that epithelium dysregulation in severe asthma is due to greater epithelial proliferation occurring rather than apoptosis, resulting in a thicker remodeled epithelium. However, the key factors leading to this heightened epithelial proliferation in severe asthma are unclear (5). In the present study, we showed that miR-19a is a regulator of proliferation in cells from severe asthma. miR-19a belongs to the cluster mir-17~92 that plays a key role in the control of cell cycle and cell death (26, 27). Essential roles for the miR17~92 cluster have been established in normal development of heart and lung (28). Indeed, miR-17~92-deficient mice exhibit severe developmental defects of the lung and the heart, which caused death shortly after birth (29). MiR-17~92 null embryos showed severely hypoplastic lungs. Furthermore, specific overexpression of the miR-17~92 cluster in embryonic lung epithelium increased proliferation and inhibited differentiation of lung epithelial progenitor cells (30). Taken together, those phenotypes provide an interesting link between the physiological functions of miR-19a, a member of this cluster, and its potential proliferative effect in bronchial epithelium of patients with severe asthma.

Our data showed that introduction of a miRNA-19a mimic promoted cell proliferation, suggesting that miRNA-19a could be involved in regulating cell survival and proliferation. Interestingly, repression of miR-19a in bronchial epithelial cells transfected with miR-19a inhibitor decreased proliferation, suggesting that miR-19a was able to reduce DNA synthesis detected in BrdU assay and arrest bronchial cell under S phase of the cell cycle. In particular, inhibition of cell cycle progression can be associated with increased expression of genes that block cell cycle and decreased expression of genes needed for progression through cell cycle phases. Biological functions of miR-19a in regulating bronchial epithelium proliferation are not known. In our previous studies, we showed that TGF-β1 signaling is one of the main regulators of

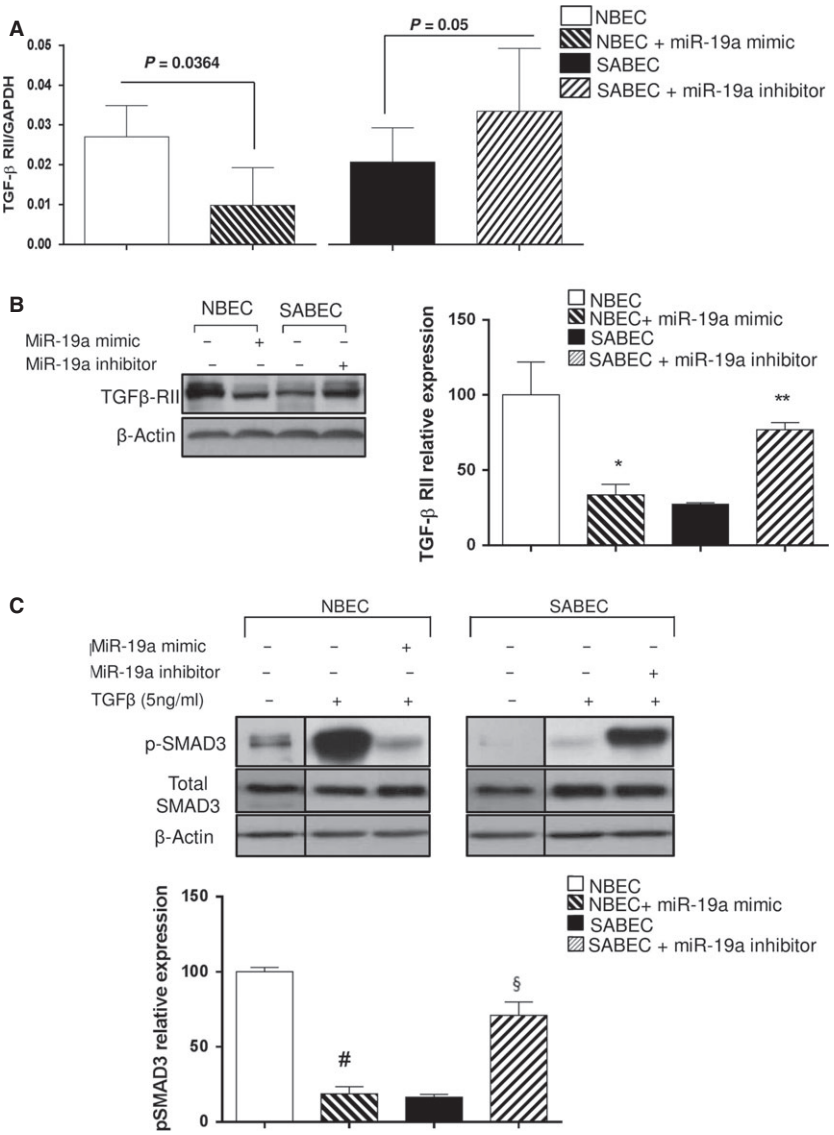

Figure 4 Effect of miR-19a on TGFβR2 mRNA and protein expression in bronchial epithelial cells. (A) RT-PCR analyses of TGFβR2 gene expression by epithelial cells from normal subjects (NBEC) before and after transfection with 30 nM of miR-19a mimic (at left) and from subjects with severe asthma (SABEC) before and after transfection with 30 nM of miR-19a inhibitor (at right). (B) Representative Western blots (at left) and densitometric analyses (at right) for TGF-β receptor-II expression by epithelial cells from normal subjects (NBEC) and from subjects with severe asthma (SABEC) before and after transfection with miR-19a mimic and inhibitor, respectively. The experiment was repeated in four different cell lines, and results were normalized with β-actin. Statistical analysis was performed by Student's *t*-test. $*P = 0.0279$, $**P = 0.00219$. (C) Representative Western blots and densitometric analyses for total and phosphorylated (pSMAD3) forms of SMAD3 expression in epithelial cells from normal subjects (NBEC) and from subjects with severe asthma (SABEC). The experiment was repeated in four different cell lines, and results were normalized with β-actin at baseline level and after transfection with miR-19a mimic and inhibitor and stimulation with TGFβ at 5 ng/ml. Statistical analysis was performed by Student's *t*-test. $^{\#}P = 0.0011$, $^{\S}P = 0.0067$.

bronchial epithelial cell proliferation in patients with asthma (4, 19). Our validation studies based on luciferase assays, Western blot and quantitative real-time PCR analyses clearly showed that miR-19a regulates the expression of the *TGFβR2* gene through one of the three potential seed regions located in its 3′UTR. This finding is consistent with what has been reported by Mestdagh and coworkers, indicating that the cluster 17~92 is a potent inhibitor of TGF-β signaling through targeting TGF-β receptor 2, SMAD2 and SMAD4 (31).

After TGF-β1 binding to the TGF-β receptor 2, the phosphorylated TGF-β receptor 1 recruits and phosphorylates

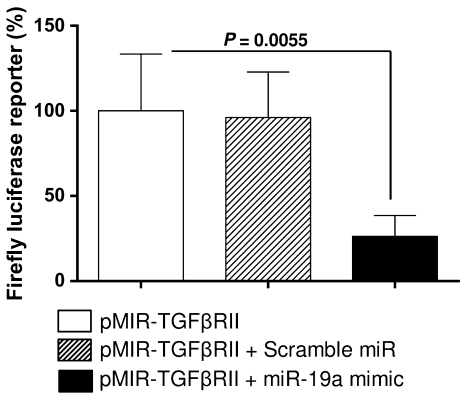

Figure 5 miR-19a targets TGFβRII 3′UTR: Dual luciferase assay was performed on BEAS-2B cells transfected with pMIR-TGFβR2 containing the 3′UTR region of TGFβR2. Reporter vector was co-transfected with a miR-19a mimic or scramble miR.

receptor-regulated Smad proteins (Smad2/3 complex). Thus, we evaluated the functional phosphorylated form of SMAD3 after miR-19a modulation and TGF-β1 treatment. Our data showed a reduced level of the phosphorylated activated form of SMAD3 after miR-19a overexpression whereas miR-19a repression increased the phospho-SMAD3 level. Therefore, a downregulation of TGF-β receptor 2 and phosphorylated form of SMAD3, as shown in the epithelium of our asthmatic subjects, indicates cell cycling or proliferation.

MiR-19a has been reported to regulate growth, adhesion and apoptosis by acting on Bim (32) and Pten (33) genes. Qin et al. (34) have shown that miR-19a is an early responsive gene that mediates the antiproliferative effect of laminar shear stress by directly targeting cyclin D. It was reported that miR-19a inhibition led to growth arrest and survival in cancer cells (31, 35). These reported data indicate that miR19a may function as either a progrowth and prosurvival gene depending on the types of stimulation and tissues.

Few studies have demonstrated the association between the abnormal expression profile of miRNAs and asthma. MiRNA expression in bronchial biopsies from normal and mild asthmatic subjects (15) revealed cell-type-specific miRNA profiles but could not find an association between specific patterns of miRNAs expression and asthma. MiRNA profiling of epithelial cells obtained by brushing from controls and mild asthmatic subjects showed difference in miRNA that regulates inflammation. MiR-203 was highly downregulated in asthmatics (16). The study by Solberg et al. showed a decreased expression of the miR-34/449 family members in epithelial cells obtained by brushing from steroid naive and steroid using asthmatics (17). This miR family plays a critical regulatory role in airway ciliated cell differentiation by the direct repression of NOTCH1/DLL1 pathway (18). They also showed that treatment with corticosteroids has a limited effect on miRNA expression. This is in agreement with our data showing that treatment of cells with corticosteroid has no significant effect on miR-19a expression. Consequently, the observed increase in miR-19a may reflect a specific feature of epithelial cells of severe asthmatic subjects rather than a consequence of corticosteroid treatments. In this regard, Williams et al. showed that treatment of asthmatic subjects with inhaled corticosteroids for 4 weeks has no significant effect on miRNA profile in the airways.

In conclusion, our study describes alteration of miR-19a in bronchial epithelial cells obtained from severe asthma compared to cells from mild asthma and controls. Moreover, we showed that miR-19a is involved in high proliferation rate of severe epithelial cells by targeting TGF-β receptor 2 mRNA. This pathway may be explored to understand functional and phenotypic changes of airway epithelium in severe asthma.

Acknowledgments

We would like to thank Dr. Michel Laviolette and Dr. Julie Milot for performing bronchoscopy with bronchial biopsies, Dr Sebastien Bonnet for his input, the 'Banque de Tissus du Réseau en Santé Respiratoire du FRSQ, site Institut Universitaire de Cardiologie et de Pneumologie de Québec' and Serge Simard for statistical analysis.

Author contributions

I.H.S. performed research, analyzed data and wrote the paper; R.F. performed TLDA assay; J.C.B. analyzed TLDA data; E.J. analyzed data and revised critically the manuscript; S.P. prepared cell cultures; S.J.M. provided essential materials for TLDA assay; Y.B. revised critically the manuscript; and J.C. designed the research, analyzed and discussed the results and commented on the manuscript.

Funding

The study was supported by the Canadian Institutes of Health Research (CIHR) and Fondation JD Béguin de l'IUCPQ. Dr. R Fakhfakh was the recipient of RSR/CIHR training program fellowship.

Conflicts of interest

All other authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Probeset information for TLDA data showing significantly different miRNAs.

Figure S1. (A) MiR-19a relative expression after mometasone furoate treatment of asthmatic bronchial epithelial cells at 10^{-9} and 10^{-11} M; (B) TGFβR2 expression after mometasone furoate treatment of normal (NBEC) and asthmatic (ABEC) bronchial epithelial cells at 10^{-9} M.

References

1. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;**161**:1720–1745.
2. Holgate ST, Lackie P, Wilson S, Roche W, Davies D. Bronchial epithelium as a key regulator of airway allergen sensitization and remodeling in asthma. *Am J Respir Crit Care Med* 2000;**162**:S113–S117.
3. Busse W, Elias J, Sheppard D, Banks-Schlegel S. Airway remodeling and repair. *Am J Respir Crit Care Med* 1999;**160**:1035–1042.
4. Semlali A, Jacques E, Rouabhia M, Milot J, Laviolette M, Chakir J. Regulation of epithelial cell proliferation by bronchial fibroblasts obtained from mild asthmatic subjects. *Allergy* 2010;**65**:1438–1445.
5. Cohen L, E X, Tarsi J, Ramkumar T, Horiuchi TK, Cochran R et al. Epithelial cell proliferation contributes to airway remodeling in severe asthma. *Am J Respir Crit Care Med* 2007;**176**:138–145.
6. Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol* 2007;**17**:118–126.
7. Liu J. Control of protein synthesis and mRNA degradation by microRNAs. *Curr Opin Cell Biol* 2008;**20**:214–221.
8. Chekulaeva M, Filipowicz W. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol* 2009;**21**:452–460.
9. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE et al. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 2000;**403**:901–906.
10. Brennecke J, Cohen SM. Towards a complete description of the microRNA complement of animal genomes. *Genome Biol* 2003;**4**:228.
11. Baehrecke EH. miRNAs: micro managers of programmed cell death. *Curr Biol* 2003;**13**:R473–R475.
12. Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res* 2003;**1**:882–891.
13. Schickel R, Boyerinas B, Park SM, Peter ME. MicroRNAs: key players in the immune system, differentiation, tumorigenesis and cell death. *Oncogene* 2008;**27**:5959–5974.
14. Garbacki N, Di Valentin E, Huynh-Thu VA, Geurts P, Irrthum A, Crahay C et al. MicroRNAs profiling in murine models of acute and chronic asthma: a relationship with mRNAs targets. *PLoS ONE* 2011;**6**:e16509.
15. Williams AE, Larner-Svensson H, Perry MM, Campbell GA, Herrick SE, Adcock IM et al. MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy. *PLoS ONE* 2009;**4**:e5889.
16. Jardim MJ, Dailey L, Silbajoris R, Diaz-Sanchez D. Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene. *Am J Respir Cell Mol Biol* 2012;**47**:536–542.
17. Solberg OD, Ostrin EJ, Love MI, Peng JC, Bhakta NR, Hou L et al. Airway epithelial miRNA expression is altered in asthma. *Am J Respir Crit Care Med* 2012;**186**:965–974.
18. Marcet B, Chevalier B, Luxardi G, Coraux C, Zaragosi LE, Cibois M et al. Control of vertebrate multiciliogenesis by miR-449 through direct repression of the Delta/Notch pathway. *Nat Cell Biol* 2011;**13**:693–699.
19. Semlali A, Jacques E, Plante S, Biardel S, Milot J, Laviolette M et al. TGF-beta suppresses EGF-induced MAPK signaling and proliferation in asthmatic epithelial cells. *Am J Respir Cell Mol Biol* 2008;**38**:202–208.
20. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. *Am Rev Respir Dis* 1987;**136**:225–244.
21. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. *Am J Respir Crit Care Med* 2000;**162**:2341–2351.
22. Chakir J, Dubé J, Laviolette M, Goulet F, Germain L, Auger F et al. Isolation and characterization of human airway fibroblasts in culture. *Methods Mol Med* 2000;**44**:53–65.
23. Bergeron C, Page N, Joubert P, Barbeau B, Hamid Q, Chakir J. Regulation of procollagen I (alpha1) by interleukin-4 in human bronchial fibroblasts: a possible role in airway remodelling in asthma. *Clin Exp Allergy* 2003;**33**:1389–1397.
24. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;**167**:1360–1368.
25. Vignola AM, Chiappara G, Siena L, Bruno A, Gagliardo R, Merendino AM et al. Proliferation and activation of bronchial epithelial cells in corticosteroid-dependent asthma. *J Allergy Clin Immunol* 2001;**108**:738–746.
26. Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S et al. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res* 2005;**65**:9628–9632.
27. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006;**103**:2257–2261.
28. Mendell JT. miRiad roles for the miR-17-92 cluster in development and disease. *Cell* 2008;**133**:217–222.
29. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* 2008;**132**:875–886.
30. Lu Y, Thomson JM, Wong HY, Hammond SM, Hogan BL. Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Dev Biol* 2007;**310**:442–453.
31. Mestdagh P, Bostrom AK, Impens F, Fredlund E, Van PG, De AP et al. The miR-17-92 microRNA cluster regulates multiple components of the TGF-beta pathway in neuroblastoma. *Mol Cell* 2010;**40**:762–773.
32. Takakura S, Mitsutake N, Nakashima M, Namba H, Saenko VA, Rogounovitch TI et al. Oncogenic role of miR-17-92 cluster in anaplastic thyroid cancer cells. *Cancer Sci* 2008;**99**:1147–1154.
33. Cloonan N, Brown MK, Steptoe AL, Wani S, Chan WL, Forrest AR et al. The miR-17-5p microRNA is a key regulator of the G1/S phase cell cycle transition. *Genome Biol* 2008;**9**:R127.
34. Qin X, Wang X, Wang Y, Tang Z, Cui Q, Xi J et al. MicroRNA-19a mediates the suppressive effect of laminar flow on cyclin D1 expression in human umbilical vein endothelial cells. *Proc Natl Acad Sci USA* 2010;**107**:3240–3244.
35. Cheng AM, Byrom MW, Shelton J, Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* 2005;**33**:1290–1297.