


Role of miR-185-5p as modulator of periostin synthesis and smooth muscle contraction in asthma

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Abstract

Asthma is a chronic respiratory disease produced by an aberrant immune response that originates with breathing difficulties and cough, through airway remodeling. The above pathophysiological events of asthma emerge the regulators of effectors, like epigenetics, which include microRNAs (miRNAs) who perform post-transcriptional regulation, controlling diverse pathways in respiratory diseases. The objective of the study was to determine how miR-185-5p regulates the secretion of periostin by airway structural cells, and smooth muscle cells contraction, both related to airway remodeling in asthma. We used miR-185-5p mimic and inhibitors in bronchial smooth muscle cells (BSMCs) and small airway epithelial cells (SAECs) from healthy subjects. Gene expression and protein levels of periostin (*POSTN*), CDC42, and RHOA were analyzed by RT-PCR and ELISA/Western blot, respectively. BSMC contractility was analyzed using cell-embedded collagen gels and measurement of intracellular calcium was performed using Fura-2. Additionally, miR-185-5p and periostin expression were evaluated in sputum from healthy and asthmatics. From these experiments, we observed that miR-185-5p modulation regulates periostin mRNA and protein in BSMCs and SAECs. A tendency for diminished miR-185-5p expression and higher periostin levels was seen in sputum cells from asthmatics compared to healthy, with an inverse correlation observed between *POSTN* and miR-185-5p. Inhibition of miR-185-5p produced higher BSMCs contraction induced by histamine. Calcium mobilization was not modified by miR-185-5p, showing that miR-185-5p role in BSMC contractility is performed by regulating CDC42 and RhoA pro-contractile factors instead. In conclusion, miR-185-5p is a modulator of periostin secretion by airway structural cells and of smooth muscle contraction, which can be related to asthma pathophysiology, and thus, might be a promising therapeutic target.

KEYWORDS

airway remodeling, asthma, miRNAs, physiopathology, sputum

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1 | INTRODUCTION

Asthma is a prevalent chronic disease characterized by a disruption in the physiological function of the airways causing breathing difficulties, cough, and respiratory distress (Erle & Sheppard, 2014). This pathology is orchestrated, by innate immune cells such as eosinophils, which damage the airways through the secretion of hazardous cytokines, enzymes, cationic proteins, and exosomes (Klion, 2017; Sastre et al., 2018). The effect of the exosomes on the receptor cell is carried out by their content, being it proteins, enzymes, or nucleic acids as microRNAs (miRNAs; Cañas et al., 2019; J. Zhang et al., 2015), which have been described as functional modulators in disease physiology (Y. Li et al., 2019; Sastre et al., 2017). miRNAs do not only perform roles in the pathophysiology of diseases but they can also be considered good biomarkers (J. Wang et al., 2016), that can be used to differentiate asthmatics from healthy subjects and other chronic respiratory diseases (Heffler et al., 2017; Hirai et al., 2020; Rodrigo-Muñoz et al., 2019, 2019).

The events that perform damage of the airways cause “airway remodeling,” characterized by alterations in the epithelium integrity, subepithelial fibrosis, and smooth muscle mass increase (Elias et al., 1999). Regarding epithelial repair and fibrosis, periostin is one of the molecules which are associated with the type 2 immune response in asthma, as it is secreted in response to cytokines like interleukin (IL)-4 and IL-13, inducing eosinophil recruitment (Izuhara et al., 2016), mucus secretion, and fibrosis (O'Dwyer & Moore, 2017).

Bronchial smooth muscle cells are also susceptible to hyperplasia and hypertrophy (Munakata, 2006), and they can secrete mediators of inflammation as cytokines or periostin, being active participators in airway remodeling (Makita et al., 2018). In asthma, the smooth muscle cell contraction is overactivated resulting in airway hyperresponsiveness (Sakai et al., 2017).

The objective of this study was to evaluate the function of miR-185-5p in asthma, as this miRNA was differentially expressed in the serum samples of asthmatics compared to healthy, it was associated with a more severe phenotype of asthma (characterized by late-onset asthma and more intensive care unit visits, and was a good biomarker for asthma diagnosis, respect to the healthy status (Rodrigo-Muñoz et al., 2019). To evaluate how miR-185-5p can be regulating the functions of the structural cells in asthma, we studied its effect over periostin expression and secretion in airway epithelial cells, smooth muscle cells with or without IL-13 (a periostin inductor; Makita et al., 2018), and in sputum, because this molecule is key in airway remodeling in asthma (O'Dwyer & Moore, 2017). Furthermore, we evaluated the role of this miRNA on the regulation of smooth muscle contraction which is one of the hallmarks of asthma disease, and it is performed by proteins such as RhoA and CDC42 (Chiba & Misawa, 2004; Sakai et al., 2017). With this study, we want to discover if this miRNA could be targeting two processes related to asthma at the same time, being both periostin secretion and smooth muscle contraction, opening new possibilities for therapeutic asthma targets.

2 | MATERIALS AND METHODS

2.1 | Subjects of study and sputum processing

Seven asthmatic subjects and four healthy controls from the Spanish Cohort MEGA Project (Muñoz et al., 2018) were recruited at Fundación Jiménez Díaz Hospital, following the GINA (Reddel et al., 2015) guidelines. Subjects signed informed consent. The study was conducted according to the Declaration of Helsinki principles and approved by the hospital's Ethical Committee.

Sputum induction was performed by inhaling hypertonic saline at increasing concentrations (Sastre et al., 2008) and it was mixed with a volume of dithiothreitol (DTT) 0.1% (Sigma-Aldrich, Merck KGaA) and incubated for 15 min at 37°C. DTT reaction was stopped with phosphate-buffered saline (PBS) Dulbecco 1× and filtered with 40 μM nylon mesh and then centrifuged at 1800 rpm for 10 min at 4°C. The supernatant was stored at -80°C. Cell viability was determined by the percentage of non-squamous living cells using Trypan blue solution (Thermo Fisher Scientific) from the total of non-squamous cells. Contamination was determined as the number of squamous cells from the total of cells. Cells were stored in QIAzol (Qiagen) at -80°C.

2.2 | Cell culture

Small airway epithelial cells (SAECs) and bronchial smooth muscle cells (BSMCs) from healthy subjects were purchased from Lonza. SAEC were cultured in Small Airway Epithelial Cell Growth Medium (Promocell) as described by the provider. BSMCs were cultured in Smooth Muscle Cell Growth Medium 2 (Promocell). Both media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained at 37°C in an atmosphere containing 5% CO₂. SAEC and BSMC were stimulated with IL-13 (20 ng/ml) to induce the expression and secretion of periostin and RhoA.

2.3 | MicroRNA mimics and inhibitors transfection

Hsa-miR-185-5p miRCURY LNA miRNA Mimic (50 nM) for over-expression experiments (MIMAT0000455: 5'UGGAGAGAAAGGCA-GUCCUGA) and miR-185-5p miRCURY LNA miRNA Inhibitor (100 nM) was purchased from Exiqon (Qiagen). Transfection of miRNA mimics was performed using TransIT-siQUEST[®] and TransIT-TKO[®] Transfection Reagent for SAECs and BSMCs, respectively. A TransIT-X2[®] Dynamic Delivery System was used for inhibitor transfection in SAECs and BSMCs. The efficiency of transfection reagents (Mirus Bio LLC) was determined using flow cytometry (BD *FacsCanto* II, BD Biosciences) by using fluorescent scrambled miRNA controls (Qiagen). The transfection mix was incubated for 15–30 min and then added to cells in 80% confluence for 24–72 h, being BSMC serum depleted for 24 h before transfection.

2.4 | RNA isolation from SAECs, BSMCs and sputum cells, reverse-transcription, and qPCR

RNA was purified from SAECs, BSMCs, or sputum cells by utilizing the phenol-chloroform technique from the QIAzol Lysis reagent (Qiagen). 500 ng of total RNA quantified by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), followed by semi-quantitative real-time PCR (qPCR) following manufacturer's guidelines on a 7500 Real-Time PCR System with TaqMan™ gene expression probes for *POSTN*, *RHOA*, *CDC42* and *GAPDH* and TaqMan™ Gene Expression MasterMix (Applied Biosystems). Alternatively, 100 ng of RNA were retro-transcribed using a miRCURY LNA™ RT Kit (Qiagen) followed by qPCR in a Light Cycler 96 thermocycler (Roche) using miRCURY LNA™ SYBR Green PCR Kit and hsa-miR-185-5p, miR-191-5p and U6, and UniSp6 probes (Qiagen). MiR-191-5p was used as house-keeping miRNA for BSMCs and U6 for SAECs/sputum. Relative gene expression was calculated using the Cycle Threshold (Ct) and the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001), where:

$$\begin{aligned}\Delta\Delta C_t &= \Delta C_{t_{Mimic/inhibitor}} - \Delta C_{t_{Scramble}} \text{ and } \Delta C_t \\ &= \Delta C_{t_{gene/miRNA}} - \Delta C_{t_{Housekeeping\ gene/miRNA}}\end{aligned}$$

2.5 | Periostin measurement by ELISA

Periostin levels were measured in the sputum, and in the supernatant of BSMCs and SAECs (for the assessment of the periostin secreted by these cells) by ELISA using the Human Periostin/OSF-2 DuoSet ELISA kit (R&D Systems) according to the manufacturer's recommendations. Samples were diluted at 1:50 or 1:100 in 1× PBS. Standards ranged from 62.5 to 4000 pg/ml. The experiment was read with a luminometer, TECAN-Infinite 200PRO (Tecan Trading AG) at 450 nm of absorbance with 570 nm as plate reference.

2.6 | Protein extraction, quantification, and evaluation by Western blot

Proteins were extracted in 1× RIPA Buffer (Sigma-Aldrich) with 1× Protease Inhibitor Cocktail (PIC) and 1× phenylmethylsulfonyl fluoride (PMSF) and thereafter quantified with Bradford protein reagent (Bio-Rad Laboratories GmbH, Munich, Germany) and resolved (10 µg) in Acrylamide/Bis-acrylamide gels followed by transference to polyvinylidene difluoride (PVDF) Amersham Hybond™-P membranes (GE Healthcare) plus 2 h blocking in 1× PBS/5% non-fat-dried milk/0.2% Tween-20 at room temperature. Overnight incubation with antibodies (Cell Signaling Technology) against β -actin (Cat# 4970, RRID:AB_2223172; 1:1000), CDC42 (Cat# 2466, RRID:AB_2078082; 1:500) and RHOA (Cat# 2117, RRID:AB_10693922; 1:500) in 1× PBS/0.5% non-fat-dried milk/Tween-20 0.2% was performed at 4°C and further incubation with the secondary antibody-HRP against rabbit (Millipore Cat# AP156P, RRID:AB_11213985; 1:1000) was

done for 2 h at room temperature. Immobilon® Crescendo Western HRP Substrate (Merck Millipore) was used, revealed in an Amersham Imager 600 (GE Healthcare). Data were curated using Quantity One 1-D software (RRID:SCR_014280; Bio-Rad).

2.7 | Collagen gel contraction assay

BSMC was transfected for 48 h with miR-185-5p mimics and inhibitors were trypsinized and resuspended (4×10^5 cells/ml) in a collagen gel solution as described by Kitamura et al. (2008). 500 µl of cell-collagen mix/well were deposited in 24-well plates and incubated 30 min at 37°C, 5% CO₂, and then incubated for 72 h with BSMCs culture medium. Then, the medium was replaced with Krebs–Henseleit buffer and the gel was detached using a 23-gauge needle. Histamine dihydrochloride >99% TLC (Sigma-Aldrich) 100 µM was added for 30 min, this dose was previously determined as optimal after dose–response curve (data not shown). Images were scanned (Canon MF620C Series, Canon) and measured using ImageJ software (RRID:SCR_003070). Gel contraction was calculated as gel size post-histamine/gel size pre-histamine, and relativized (%) against each scrambled miRNA control.

2.8 | Intracellular calcium measurement

BSMCs were transfected in 96-well plates for 72 h. The culture medium was replaced by HBSS buffer with 0.5 µM Fura-2AM (Sigma-Aldrich) probe in dimethyl sulfoxide (DMSO) and incubated for 30 min. The plate was read at 510 nm in an EnSPiRE Multimode Reader (Perkin-Elmer) exciting at 340 nm for Fura-2 and 380 nm as plate reference. Seven measurements were taken for each 9 time points, adding 10 µM ionomycin (Sigma-Aldrich) in DMSO after the third measurement. Fura-2 signal was calculated as a ratio of 340/380 for each time point.

2.9 | miRNA-gene target prediction tools/ algorithms

For miRNA target prediction, mirSystem (Lu et al., 2012) and miRDB (RRID:SCR_010848; Chen & Wang, 2020) were used.

2.10 | Statistical analysis

Results are expressed as mean \pm standard error of the mean (SEM). Data normality was calculated using the Shapiro–Wilk test. Groups comparisons were performed using unpaired, Mann–Whitney *U*-test for non-Gaussian samples. Kruskal–Wallis test followed by uncorrected Dunn tests were performed for multiple comparisons. Pearson correlation was used. A *p*-value of less than 0.05 was considered significant. Statistical tests and figures were performed using

GraphPad Prism 6 (GraphPad Prism, RRID:SCR_002798; GraphPad Software Inc.).

3 | RESULTS

3.1 | miR-185-5p targets POSTN in BSMCs and SAECs

miRNA mimics and inhibitors allow the regulation of miRNAs expression to evaluate their functions in cells. The efficiency of transfection ranged from 79.8% to 89.6% as determined by flow cytometry (data not shown). miR-185-5p mimics upregulated 20,000-fold its expression in BSMCs and 50,000 times in SAECs compared to the scramble control ($p < 0.05$; Figure 1a,b). Conversely miR-185-5p inhibitor decreased miR-185-5p expression

30 times less in BSMCs and 40 times less in SAEC ($p < 0.05$; Figure 1a,b).

Overexpression of miR-185-5p reduces *POSTN* ($2^{-\Delta\Delta Ct}$) expression in BSMCs (fold decrease of 0.68 ± 0.08 ; $p < 0.05$) and in SAECs (fold decrease 0.74 ± 0.12 ; $p < 0.05$; Figure 1c,d). When BSMCs were treated with IL-13 (20 ng/ml) after transfection, this reduction is also effective (0.48 ± 0.10 ; $p < 0.05$; Figure 1c), while IL-13 treatment similarly does not alter this reduction caused by miR-185-5p overexpression in SAECs, diminishing *POSTN* expression to 0.72 ± 0.13 ; $p < 0.05$ (Figure 1d). The sequestration of miR-185-5p with its inhibitor increased almost significantly the expression of *POSTN* (fold of 1.50 ± 0.57 ; $p = 0.05$; Figure 1c) in BSMCs; an increase which was maintained after IL-13 addition (fold of 1.13 ± 0.06 , $p < 0.05$; Figure 1c). In SAECs, inhibition of miR-185-5p causes a mild upregulation, which is nonsignificant (fold of 1.14 ± 0.09 ; $p > 0.05$; Figure 1d), being this increase almost significant in the SAECs treated

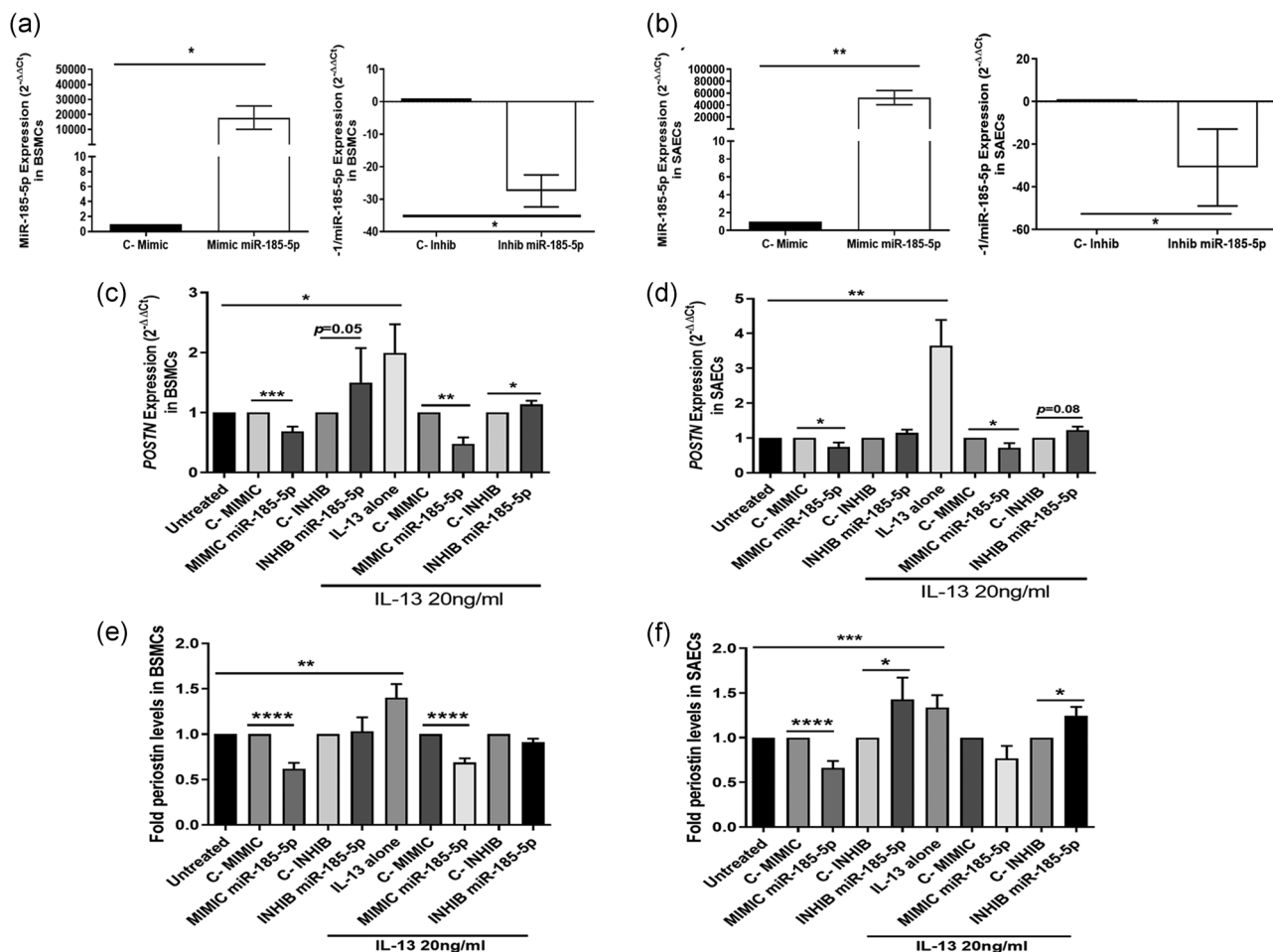


FIGURE 1 miRNA-gene target relationship between miR-185-5p and periostin in vitro. Relative expression ($2^{-\Delta\Delta Ct}$) of miR-185-5p in BSMC (a) and SAEC (b) after transfection with mimics or inhibitors for this miRNA ($n = 3-6$ experiments). Relative expression ($2^{-\Delta\Delta Ct}$) of *POSTN* mRNA in BSMC (c) and SAEC (d) after transfection with mimics or inhibitors for miR-185-5p, and with or without IL-13 (20 ng/ml) treatment after transfection ($n = 3-14$ experiments). Periostin levels (fold induction over its control) in BSMC (e) and SAEC (f) culture supernatant after transfection with mimics or inhibitors for miR-185-5p, and with or without IL-13 (20 ng/ml) treatment after transfection. $n = 5-17$ experiments. Mann-Whitney test was used for non-Gaussian samples group comparisons, analyzing miR-185-5p mimic versus c-mimic; miR-185-5p inhibitor versus c-inhibitor, and IL-13 alone treated cells versus untreated cells (a-f). BSMC, bronchial smooth muscle cell; SAEC, small airway epithelial cell. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

with IL-13 (fold of 1.22 ± 0.10 ; $p = 0.08$; Figure 1d), which might indicate that IL-13 treatment could enhance the effect of the inhibitor of miR-185-5p by augmenting *POSTN* expression in absence of miR-185-5p.

This reduction of gene expression is reflected in the secretion of periostin by BSMCs and SAECs. Upregulation of miR-185-5p reduced the supernatants' periostin by BSMCs at fold of 0.62 ± 0.07 ; $p < 0.05$; Figure 1e), and in the same way in the BSMCs treated with IL-13 (fold of 0.69 ± 0.05 ; $p < 0.05$; Figure 1e). Similarly, miR-185-5p mimic, decreased the levels of periostin in SAEC in a fold reduction of 0.66 ± 0.08 ($p < 0.05$; Figure 1f), a reduction that was effective but not significant in IL-13-treated SAECs (fold of 0.76 ± 0.05 ; $p > 0.05$; Figure 1f). Surprisingly, the inhibition of miR-185-5p neither did change the levels of secreted periostin (fold of 1.0 ± 0.15 ; $p > 0.05$; Figure 1e), nor in the presence of IL-13 (fold of 0.91 ± 0.04 ; $p > 0.05$; Figure 1e). In SAECs, on the other hand, miR-185-5p inhibition induced periostin secretion, with a fold increase of 1.43 ± 0.08 ($p < 0.05$; Figure 1f), which was also seen in the SAECs treated with IL-13, with a fold of 1.24 ± 0.1 ($p < 0.05$; Figure 1f). Hence, miR-185-5p modulation, especially its overexpression, changes periostin expression and secretion by airway cells.

3.2 | miR-185-5p inversely correlates with *POSTN* in sputum

Then, we analyzed miR-185-5p and periostin expression in the airways of asthmatic subjects and healthy controls from the Spanish MEGA Project (Muñoz et al., 2018). The clinical characteristics of the subjects, plus sputum cell counts, contamination, and viability are included in Table 1. Interestingly, miR-185-5p is mildly down-regulated in the sputum from asthmatics compared to controls (0.61 ± 0.21 vs. 1.16 ± 0.41 ; $p > 0.05$; Figure 2a), while *POSTN* expression is higher in the asthmatic's sputum cells compared to controls ($3 \times 10^{-3} \pm 0.7 \times 10^{-3}$ vs. $0.8 \times 10^{-3} \pm 0.4 \times 10^{-3}$; $p < 0.05$; Figure 2b). Indeed, the relative expression (ΔCt) of miR-185-5p and *POSTN* showed a significant and moderate-high inverse correlation of -0.69 (95% CI = -0.88 to -0.29 ; $p < 0.05$; Figure 2c). Periostin secreted to the airways was slightly higher in asthmatics (3.83 ± 1.99 ng/ml) compared to healthy (1.07 ± 0.36 ng/ml; $p > 0.05$; Figure 2d). The inverse correlation observed between miR-185-5p and *POSTN* accounts for the gene target-miRNA relationship in the airways in vivo.

3.3 | Inhibition of miR-185-5p augments the contractile capacity of BSMCs

Next, we sought if miR-185-5p regulates BSMCs contractility. miR-185-5p inhibition produced an enhancement in the contractile capacity of BSMCs in response to $100 \mu M$ histamine ($83.7 \pm 5.99\%$ in miR-185-5p inhibitor gel size vs. 100% in the control, $p < 0.05$; Figure 3a). In this process, histamine is indeed needed for the

TABLE 1 Clinical characteristics of the subjects of study

	Healthy (n = 6)	Asthmatics (n = 10)
Age (years) ^a	26.6 ± 1.5	42.2 ± 14.3*
Sex (female) (%)	5 (83.3)	2 (20.0)*
Atopy (%)	1 (16.6)	9 (90.0)*
BMI ^a	22.9 ± 1.0	26.1 ± 4.0
Smoking history		
Current (%)	1 (16.6)	3 (30.0)
Passive (%)	0 (0.00)	1 (14.3)
Nonsmoker (%)	5 (83.3)	5 (50.0)
FEV ₁ /FVC (%) ^a	83.5 ± 0.5	70.0 ± 10.5*
FeNO ^a	15.5 ± 6.8	65.7 ± 15.8*
Peripheral blood eosinophils (cells/ μ l) ^a	300 ± 200.0	371.4 ± 256.3
Inhaled corticosteroids and LABA (%)	0 (0.0)	9 (90.0)*
Squamous cell contamination ^a (%)	24.4 ± 25.5	24.7 ± 20.2
Cell viability ^a (%)	78.3 ± 19.6	70.0 ± 17.8
Sputum macrophages (%)	50.3 ± 13.2	42.1 ± 18.5
Sputum neutrophils (%)	40.4 ± 13.5	41.7 ± 14.2
Sputum lymphocytes (%)	8.2 ± 1.2	13.3 ± 6.6
Sputum eosinophils (%)	0.5 ± 0.8	3.6 ± 4.4

Abbreviations: FeNO, exhaled nitric oxide test; FEV1, forced expiratory volume in first second; FVC, forced vital capacity.

^aResults are shown as mean ± SD.

* $p < 0.05$.

depiction of the effect of miR-185-5p inhibition, as the cells transfected with miR-185-5p inhibitor do contract the gel differently ($105.4 \pm 7.0\%$; $p > 0.05$) compared to non-transfected cells in the absence of histamine, as observed in Figure 3a. No differences were seen for miR-185-5p overexpression respect to its control (100% vs. $104.0 \pm 7.69\%$; $p > 0.05$; Figure 3a).

3.4 | miR-185-5p contractile induction is not mediated through calcium waving

BSMC contractile behavior might be modulated by miR-185-5p intracellular calcium waving. First, we tested if histamine $100 \mu M$ was able to induce calcium mobilization, but it was not able to modulate it (data not shown), so we use ionomycin ($10 \mu M$) that increased intracellular calcium concentration in BSMCs (Figure 3b,c). Nevertheless, no differences at calcium levels were spotted with miR-185-5p modulation ($p > 0.05$; Figure 3b,c) for any of the studied time points, independently of the addition of ionomycin or not, compared to their respective controls. This

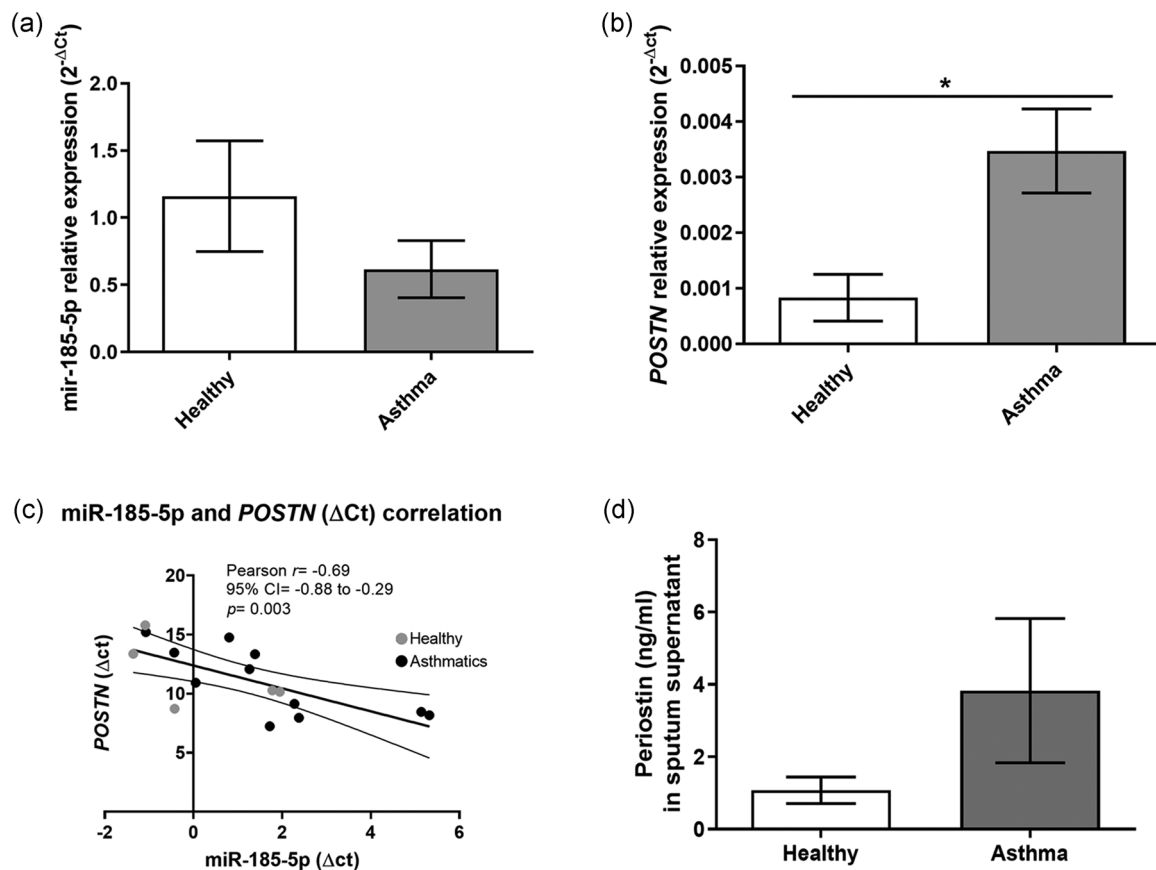


FIGURE 2 miRNA-gene target relationship between miR-185-5p and periostin is also present in vivo. Relative expression ($2^{-\Delta Ct}$) of miR-185-5p (a) and POSTN (b) in the sputum cells from asthmatic ($n = 11$) and healthy subjects ($n = 6$). (c) Pearson correlation between the relative expression (ΔCt) of miR-185-5p and POSTN mRNA. (d) Periostin levels in the sputum supernatant (ng/ml) obtained from healthy and asthmatics. Mann-Whitney test was used for non-Gaussian samples group comparisons (a, b, and d) and the Pearson r test was applied for correlation determination (c). * $p < 0.05$

result shows that neither in basal condition nor in the presence of higher intracellular calcium levels, the expression of miR-185-5p does not change the amount of Ca^{2+} ions inside the cell per se, although other mechanisms of calcium waving by miR-185-5p cannot be ruled out. Hence, the effect of BSMC contractile capacity change does not appear to be mediated by intracellular calcium regulation by this miRNA.

3.5 | Contractile proteins CDC42 and RHOA are regulated by miR-185-5p in BSMCs

RHOA and CDC42, which are involved in independent calcium smooth muscle contractility, are among the described targets of miR-185-5p (Chiba et al., 2010; Fediuk et al., 2014). miR-185-5p modulation at 48 h did not have any effect in RHOA mRNA expression (fold increase of 1.11 ± 0.24 in the miR-185-5p mimic vs. scrambled control; $p > 0.05$), neither for CDC42 (fold increase 1.02 ± 0.24 ; $p > 0.05$; Figure 4a). Inhibition of miR-185-5p produced a reduction in the expression of RHOA (fold decrease of 0.72 ± 0.04 ; $p < 0.05$) and CDC42 (fold decrease of 0.81 ± 0.26 ; $p > 0.05$), showing

unclear modulation of miR-185-5p over these two genes mRNA (Figure 4a).

CDC42 protein levels were increased (fold change of 1.35 ± 0.13 relative units) at 48 h after miR-185-5p inhibition ($p < 0.05$; Figure 4b), but not with the mimic (0.89 ± 0.07 ; $p > 0.05$; Figure 4b), differences between the miR-185-5p mimic being treated in respect to the miR-185-5p inhibitor transfected (0.89 ± 0.07 vs. 1.35 ± 0.13 ; $p < 0.05$). No change was observed at 48 h, in the levels of RhoA, neither with miR-185-5p inhibition (fold increase of 1.03 ± 0.23 vs. scrambled control; $p > 0.05$; Figure 4b), nor for miR-185-5p mimic (0.96 ± 0.11 ; Figure 4b). Surprisingly, at 72 h, a fold reduction of 0.88 ± 0.02 RhoA relative units was detected for miR-185-5p overexpression, and a mild increase (1.13 ± 0.05) was observed for miR-185-5p inhibition versus its control, the fold induction being different between the miR-185-5p overexpression status versus its inhibition ($p < 0.05$; Figure 4c). Only a tendency was observed at 72 h for miR-185-5p modulation over CDC42 protein (fold decrease of 0.96 ± 0.08 for upregulation; and 1.16 ± 0.29 for inhibition; $p > 0.05$; Figure 4c). The modification in the protein levels of CDC42 and RhoA induced by miR-185-5p may be relevant to explain the increase in the BSMCs contraction assays.

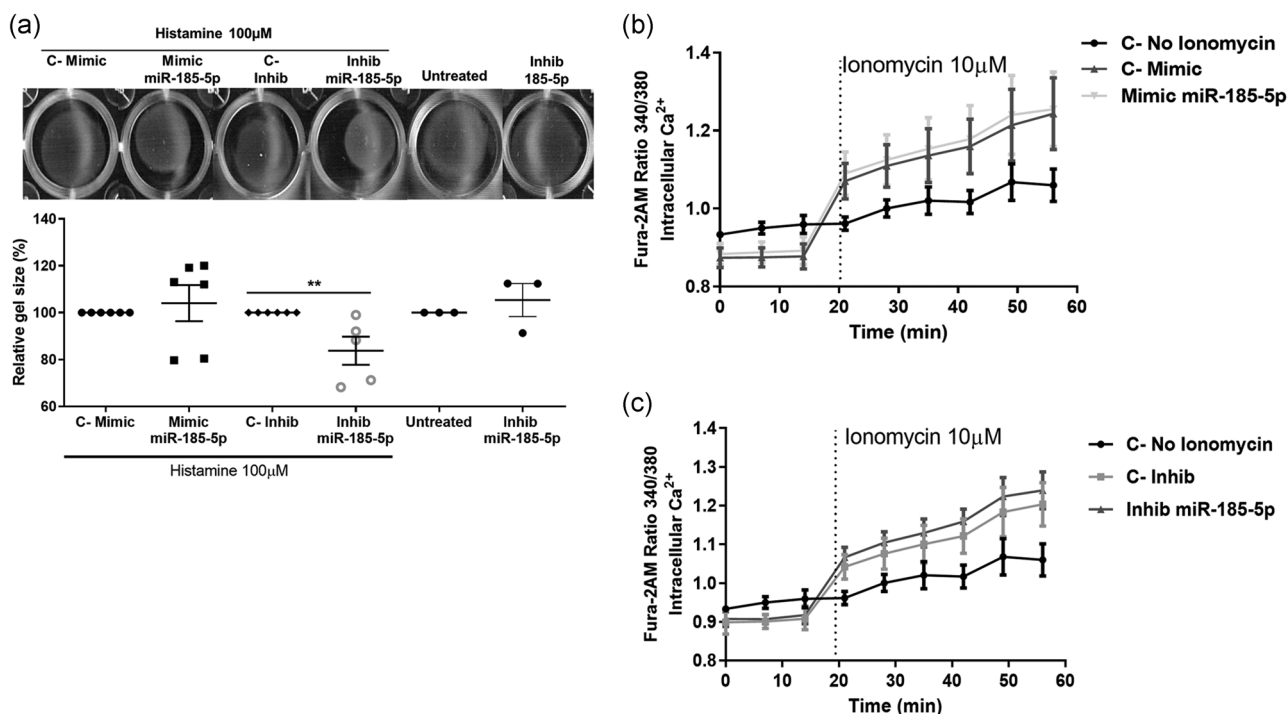


FIGURE 3 (a) Relative size collagen gels (%) embedded with transfected BSMC stimulated with 100 μM histamine ($n = 3-5$). Intracellular calcium mobilization measurement, pre- and post-addition of the ionomycin (1 μM) in BSMC, transfected with miR-185-5p mimic (b) or inhibitor (c) and scrambled controls. $n = 3$ experiments. Mann-Whitney test was used for non-Gaussian samples group comparisons of its sample to its respective control (a), unpaired t -test was applied for comparisons between miR-185-5p mimic or inhibitor versus its scrambled control for each time point (b, c). BSMC, bronchial smooth muscle cell. * $p < 0.05$; *** $p < 0.001$

4 | DISCUSSION

miRNAs are key molecules involved in asthma pathogenesis with additional functionality as disease biomarkers (Heffler et al., 2017; Sastre et al., 2017). This new research shows an inverse correlation between miR-185-5p and *POSTN* ex vivo in the sputum of asthmatic and healthy individuals. Functionally, miR-185-5p modifies airway structural cells' periostin secretion, and the contractile capacity of smooth muscle cells, both being important events in asthma symptoms development.

We previously described that some miRNAs, including miR-185-5p, were differentially expressed in blood eosinophils and serum from asthmatics compared to healthy controls, being biomarkers of disease (Rodrigo-Muñoz et al., 2019). To discover miR-185-5p role in asthma pathophysiology we used an overexpression-downregulation approach using artificial miRNA mimics and inhibitors in lung structural cells.

On the one hand, asthma is characterized by epithelial shedding, and subepithelial fibrosis, and on the other hand, asthma presents itself with increased smooth muscle contractile demeanor, being both related to airway remodeling (Al-Muhsen et al., 2011). Some cytokines are implicated in epithelial alteration and fibrosis (IL-13, transforming growth factor-beta [TGF β], matrix metalloproteinases), but in this study, we focused on periostin, a target for miR-185-5p previously described in cancer

cells utilizing luciferase reporter assays, qPCR and Western blot (Y. Wang et al., 2017).

Serum periostin levels have been proposed as a biomarker for anti-IL-13 therapy (Corren et al., 2011), and for Th2 immunity and eosinophils in the blood (Johansson et al., 2016) and in the airways (Jia et al., 2012). Nevertheless, the use of serum periostin as a biomarker for T2 endotype is still controversial (Pavlidis et al., 2019).

Functionally, this matricellular protein is secreted by airway epithelial cells, in response to IL-13. Periostin promotes keratinocytes proliferation and Th2 cytokines secretion (thymic stromal lymphopoietin [TSLP], tumor necrosis factor [TNF]- α , granulocyte-macrophage colony-stimulating factor [GM-CSF], and IL-1 α), while mice with a periostin knock out (*postn*^{-/-}), are not able to induce T2 immune responses (Masuoka et al., 2012). BSMCs are also able to secrete periostin in response to IL-13 via the JAK/STAT6, ERK1/2, and PI3K/Akt pathways (Makita et al., 2018). Periostin has a role in epithelial wound healing (Ontsuka et al., 2012), but, an uncontrolled periostin upregulation may have detrimental effects through enhancing subepithelial fibrosis, immune responses, and smooth muscle migration (G. Li et al., 2010).

Having this in mind, regulation of periostin secretion may be a therapeutic option. We observed that miR-185-5p upregulation was able to control the expression and secretion of periostin by airway epithelial and smooth muscle cells.

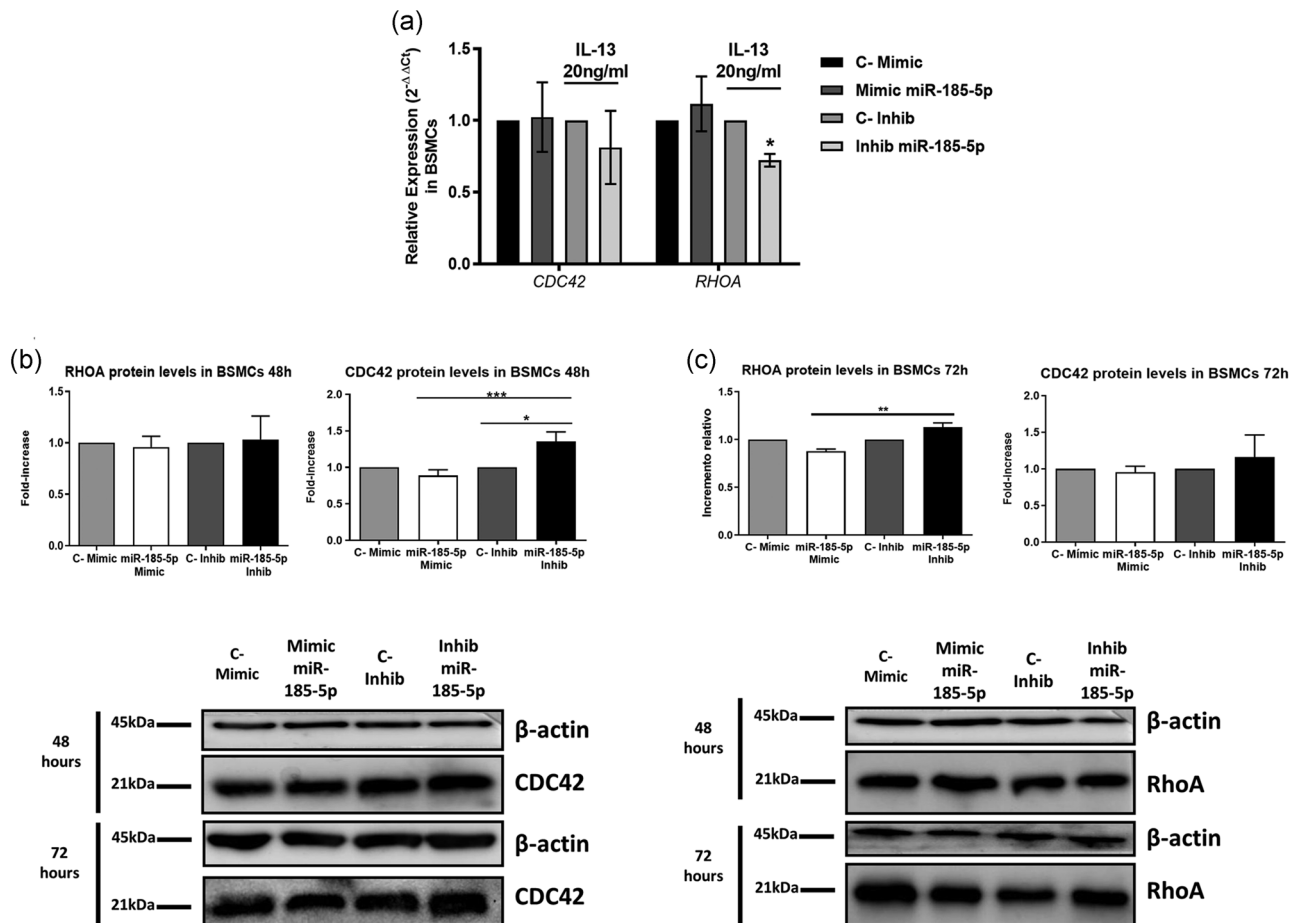


FIGURE 4 miRNA-gene target relationship between miR-185-5p and RhoA and CDC42 pro-contractile proteins. (a) Relative expression ($2^{-\Delta\Delta C_t}$) of RHOA and CDC42 mRNA in BSMC after transfection with mimic or inhibitor for miR-185-5p. Fold increase of RHOA and CDC42 protein levels at 48 h (b) and RhoA and CDC42 protein levels at 72 h (c) in BSMC after miR-185-5p mimic or inhibitor transfection, and its respective representative images of Western blots. $n = 3$ –5 experiments. Kruskal–Wallis test followed by uncorrected Dunn multiple comparison test was applied. BSMC, bronchial smooth muscle cell. * $p < 0.05$; ** $p < 0.01$

Furthermore, miR-185-5p and *POSTN* expression are moderately inversely correlated in sputum, being the levels of *POSTN* gene expression higher in asthmatic sputum, pointing out the nature of their miRNA-target relationship, relevant to asthma pathophysiology and control. Although the asthma population exceeds the healthy in age, this bias is not affecting the results obtained, as analyzed by correlation (Spearman $\rho > -0.4$ and < 0.4 ; $p > 0.05$).

Smooth muscle contraction increase is a hallmark of asthma disease, which can occur independently of inflammation (Crimi et al., 1998; McGrath et al., 2012), and is the main effector in airway narrowing, being a potential therapeutic target. Here we show that miR-185-5p inhibition increases the contractile capacity of primary BSMC in response to histamine, in a calcium-independent manner (Noble et al., 2014), being this miRNA important for physiologic contractile responses to histamine. Previous work showed that miR-185 targeted RhoA and CDC42 using luciferase-reporter assays, regulating both factors at protein levels, similarly to our results (M. Liu et al., 2011). Interestingly, these molecules also control smooth muscle contraction

(Fediuk et al., 2014; W. Zhang et al., 2012). More importantly, the regulation of smooth muscle contraction performed by RhoA and CDC42 is independent of intracellular calcium levels, a phenomenon known as calcium sensitization (Chiba & Misawa, 2004).

RhoA is activated by IL-13 and STAT6 signaling, and its inhibition reduces contraction potency (Chiba et al., 2009; Schaafsma et al., 2004), being this protein proposed as a possible therapeutic target itself (Chiba et al., 2010) and currently reduced by bronchodilators (C. Liu et al., 2006). We confirmed that both CDC42 and RhoA are targets for miR-185-5p in BSMC, being regulated at protein levels by this miRNA at different time points, so, the increase of contraction by inhibition of miR-185-5p may be due to enhanced RhoA and CDC42 protein levels. Nevertheless, it should be noted that miR-185-5p overexpression failed to reduce CDC42 protein levels, but instead, only miR-185-5p inhibition was able to modulate CDC42 levels. This absence of modulation might be due to the endogenous levels of this protein and their lifespan and degradation rate, as well as the measurement timing and kinetics, something that should be analyzed by further studies.

Both periostin and smooth muscle contraction are elements related to asthma development. Some miRNAs have been shown as potential regulators of periostin, like miR-599; and others for RhoA, like miR-133a (Chiba et al., 2009). Hence, miR-185-5p may be involved in the control of both components, being thus, a novel, alternative, and promising therapeutic target.

In conclusion, we show that miR-185-5p is reduced in the sputum from asthmatics not being able to control the expression of its targets periostin, CDC42, and RhoA, key factors of airway remodeling and muscle contraction. Artificial overexpression of miR-185-5p may account for a promising experimental therapy for controlling asthma symptoms by modulating multiple asthma pathophysiology nodes at the same time.

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CONFLICT OF INTERESTS

J.A.C. has received payment for a lecture by Astra Zeneca. JS reports having served as a consultant to ThermoFisher, MEDA, Novartis, Sanofi, Leti, Faes Farma, Mundipharma, and GSK; having been paid lecture fees by Novartis, GSK, Stallergenes, Leti, and Faes Farma; as well as having received grant support for research from ThermoFisher, Sanofi, and ALK. VdP reports having served as a consultant to Astra Zeneca and GSK; having been paid lecture fees by Astra Zeneca and GSK. Other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

José M. Rodrigo-Muñoz, Beatriz Sastre, and Victoria del Pozo conceived and designed research; José M. Rodrigo-Muñoz, José A. Cañas, Marta Gil-Martínez, and Raquel García Latorre performed experiments; José M. Rodrigo-Muñoz, José A. Cañas, Beatriz Sastre, and Marta Gil-Martínez analyzed data; José M. Rodrigo-Muñoz, José A. Cañas, Beatriz Sastre, Marta Gil-Martínez, Joaquín Sastre, and Victoria del Pozo interpreted results of experiments; José M. Rodrigo-Muñoz, José A. Cañas, Beatriz Sastre, and Victoria del Pozo prepared figures; José M. Rodrigo-Muñoz and Victoria del Pozo drafted manuscript; José M. Rodrigo-Muñoz, José A. Cañas, Beatriz Sastre, Marta Gil-Martínez, Raquel García Latorre, Joaquín Sastre, and Victoria del Pozo edited and revised manuscript; José M. Rodrigo-Muñoz, José A. Cañas, Beatriz Sastre, Marta Gil-Martínez, Raquel García Latorre, Joaquín Sastre, and Victoria del Pozo given final approval of the version to be published and agrees to be accountable for all aspects of the work related to its accuracy or integrity.

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