MicroRNA Dysregulation in Pulmonary Arteries from Chronic Obstructive Pulmonary Disease Relationships with Vascular Remodeling

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Abstract

Pulmonary vascular remodeling is an angiogenic-related process involving changes in smooth muscle cell (SMC) homeostasis, which is frequently observed in chronic obstructive pulmonary disease (COPD). MicroRNAs (miRNAs) are small, noncoding RNAs that regulate mRNA expression levels of many genes, leading to the manifestation of cell identity and specific cellular phenotypes. Here, we evaluate the miRNA expression profiles of pulmonary arteries (PAs) of patients with COPD and its relationship with the regulation of SMC phenotypic change. miRNA expression profiles from PAs of 12 patients with COPD, 9 smokers with normal lung function (SK), and 7 nonsmokers (NS) were analyzed using TaqMan Low-Density Arrays. In patients with COPD, expression levels of miR-98, miR-139-5p, miR-146b-5p, and miR-451 were upregulated, as compared with NS. In contrast, miR-197, miR-204, miR-485-3p, and miR-627

were downregulated. miRNA-197 expression correlated with both airflow obstruction and PA intimal enlargement. In an *in vitro* model of SMC differentiation, miR-197 expression was associated with an SMC contractile phenotype. miR-197 inhibition blocked the acquisition of contractile markers in SMCs and promoted a proliferative/migratory phenotype measured by both cell cycle analysis and wound-healing assay. Using luciferase assays, Western blot, and quantitative PCR, we confirmed that miR-197 targets the transcription factor E2F1. In PAs from patients with COPD, levels of E2F1 were increased as compared with NS. In PAs of patients with COPD, remodeling of the vessel wall is associated with downregulation of miR-197, which regulates SMC phenotype. The effect of miR-197 on PAs might be mediated, at least in part, by the key proliferative factor, E2F1.

Keywords: vascular remodeling; smooth muscle cell phenotypic switch; microRNAs; pulmonary artery; COPD

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Clinical Relevance

The molecular mechanisms underlying pulmonary vascular changes in chronic obstructive pulmonary disease (COPD) are not fully understood. MicroRNAs (miRNAs) are small, noncoding RNAs that regulate mRNA expression levels modulating cell phenotypes. There is no information on the potential role of miRNA dysregulation in the development of pulmonary vascular remodeling, which is frequently observed in COPD. In pulmonary artery specimens from patients with COPD, we found alteration of miRNA expression, including the downregulation of miR-197. miR-197 expression correlated with the severity of both pulmonary vascular remodeling and airflow obstruction. *In vitro*, miR-197 regulates smooth muscle cell proliferation by targeting, among others, the proliferative transcription factor E2F1. The current findings provide new clues for a better understanding of the mechanisms underlying pulmonary vascular disease in COPD.

Pulmonary vascular remodeling is a characteristic feature of chronic obstructive pulmonary disease (COPD) and is considered the principal determinant of pulmonary hypertension (PH) (1, 2). Intimal proliferation of dedifferentiated vascular smooth muscle cells (SMCs) is the main cellular contributor to pulmonary vessel remodeling (intimal hyperplasia) in COPD (3). Other cell populations might also contribute to the enlargement of the intima (4–6). The molecular mechanisms underlying pulmonary vascular cell dysfunction, including SMC proliferation, in this clinical setting are poorly understood.

MicroRNAs (miRNAs) are an evolutionarily conserved 21-nucleotide (nt) class of small, noncoding RNAs involved in a number of cellular processes. miRNAs bring the RNA-induced silencing complex to a specific target mRNA by binding to complementary sites within the 3' untranslated regions (UTRs) and promoting its translational repression and/or degradation (7).

Recent studies show that miRNAs modulate the cell fate of both SMCs and

endothelial cells (ECs) in vessel remodeling (8–10). Specifically, miR-143/145 regulates SMC differentiation and is necessary to maintain a contractile phenotype (11). Its expression is reduced in plexiform lesions of pulmonary arterial hypertension (PAH) (12), and its downregulation promotes the proliferation of neointimal cells after vascular injury, whereas restoration of miR-145 expression reverses intimal growth (13). miR-204 is also downregulated in plexiform lesions (12), explanted lungs (14), and plasma of patients with PAH (15). miR-204 acts as a negative regulator of SMC proliferation (14). By contrast, miR-21 and miR-126 are upregulated in the plexiform lesions of PAH (12) and in experimental models of PH (11), whereas the expression of miR-17-92 in human pulmonary artery (PA) SMCs is downregulated in PAH (16, 17). miR-21 has been shown to regulate both SMC proliferation and differentiation (18). miR-126 induces SMC differentiation, and miR-17-92 maintains a differentiated SMC phenotype (16). Alteration of the miRNA expression profile has also been found in lungs of rats and in human airway epithelium exposed to cigarette smoke (16, 19), as well as in patients with COPD (20, 21), underscoring the potential role of these molecules in the regulation of pulmonary vascular remodeling in these conditions.

miRNA expression is tissue specific, and every cell type contains specific miRNA profiles that help to establish and maintain distinctive gene expression signatures. We hypothesized that miRNAs might contribute to the pathogenesis of pulmonary vascular remodeling in COPD, specifically by modulating SMC phenotype. Accordingly, the present study aimed to identify miRNAs that mediate cell proliferation in PAs obtained from lung tissue samples of patients with COPD and control subjects.

Methods

Detailed descriptions of methods are provided in the data supplement.

Patient Characteristics

PA segments from surgical lung resection patients, collected over 2 years, with localized lung neoplasms were evaluated. A total of 12 patients were diagnosed with COPD, 9 patients were current smokers

with normal lung function (SK), and the remaining 7 patients were nonsmokers with normal lung function (NS) (Table 1). The study was approved by the Ethics Committee of the Hospital Clinic, Barcelona, Spain.

miRNA Expression in PAs

RNA isolation of PAs was performed using Trizol (Invitrogen). The retrotranscription was performed using 10 ng of total RNA using the Taqman miRNA reverse transcription kit and megaplex pools of RT primers (Applied Biosystems) according to the manufacturer's instructions. Taqman low-density array human miRNA cards A and B set v3.0 (Applied Biosystems) was used to analyze expression of 381 miRNAs.

Cell Differentiation Experiments

We studied a cellular model of SMC differentiation triggered by cell-to-cell contact and cell confluency, as previously described (22).

Immunofluorescence

SMC differentiation was assessed by immunofluorescence, as previously described (22, 23). The primary antibodies used were directed against α -SMA (1/750) and calponin (1/75; DAKO Cytomation). An antibody against antigen Ki-67 (1/50; Novocastra) was used to measure cell proliferation.

Gene Expression Analysis

To study mRNA expression, RNA isolation followed by real-time RT-PCR was performed. miRNA expression was analyzed by Northern blot, and protein expression was evaluated using Western blot.

Functional *In Vitro* Assays in SMC Cultures

These studies included analysis of cell migration by wound-healing assays, the use of 2'-O-methyl antisense oligonucleotides chemically synthesized (Pierce) to inhibit miRNA, and luciferase assays to demonstrate miRNA-197 target binding.

Study Design

miRNA expression of PAs from patients was analyzed. Limma and a gene network analysis to evaluate the miRNA signature and its associated interactome were performed. Among the dysregulated miRNAs, miR-98, miR-451, and miR-197 correlated with spirometric measurements

Table 1. Characteristics of the Patients Enrolled in this Study

	Nonsmokers (n = 7)	Smokers (n = 9)	COPD (n = 12)
Age, yr	63 ± 11	64 ± 10	67 ± 9
Male sex, n (%)	4 (50)	8 (88) [‡]	13 (100)*
Weight, kg	64 ± 7	73 ± 8	70 ± 8
Height, cm	161 ± 8	164 ± 8	169 ± 5
BMI	25 ± 3	28 ± 4	25 ± 2
FEV ₁ % predicted	100 ± 6	90 ± 22	64 ± 13* [†]
FVC % predicted	96 ± 7	93 ± 12	84 ± 13*
FEV ₁ /FVC % predicted	77 ± 7	70 ± 15	55 ± 8* [†]
Smoking history, pack/year	5 ± 8	59 ± 27 [‡]	77 ± 29*
DL _{CO} % predicted	89 ± 20	75 ± 31	66 ± 11*
PaO ₂ , mm Hg	88 ± 15	81 ± 14	79 ± 16

Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; NS = nonsmokers; PaO₂ = arterial oxygen pressure; SK = smokers.

All values are reported as mean (±SEM).

*P < 0.05 NS versus COPD.

[†]P < 0.05 SK versus COPD.

[‡]P < 0.05 NS versus SK.

and vascular remodeling, but only miR-197, which is related to cell proliferation in other cell systems, has not previously been studied in SMCs or intima hyperplasia. By using bioinformatics inference, we found that miR-197 targets cell proliferation-related genes. Next, miR-197 function was evaluated in an *in vitro* model of SMC differentiation. Finally, to find new insights into the mechanism by which miR-197 regulates proliferation, expression of the plausible target, E2F1, was analyzed in SMCs after miRNA inhibition with antisense 2'-O-methylated RNA oligos and in PAs.

Statistical Analysis

All values are reported as mean (±SEM). Measurements were performed in duplicate, and at least three independent experiments were performed for each set of conditions. Two-group comparisons were analyzed using the two-tailed paired Student's *t* test for dependent samples (paired measurements for one set of items) or the Mann-Whitney rank sum test for non-normally distributed data. Group comparisons were performed using one-way ANOVA. *Post hoc* pairwise comparisons were made using the Student-Newman-Keuls test for normally distributed variables or the Kruskal-Wallis and Dunn tests for non-normally distributed variables. For all procedures, *P* values less than 0.05 were considered statistically significant.

Results

Differential Expression of miRNAs in PAs

We analyzed the expression of 381 miRNAs in PA homogenates isolated from COPD, SK, and NS lung tissue samples using Taqman low-density arrays. After differential expression analysis, obtained by applying *limma* to the miRNA expression data, we found that patients with COPD showed upregulation of miR-98, miR-139-5p, miR-146b-5p, and miR-451, and downregulation of miR-197, miR-204, miR-485-3p, and miR-627 when compared with NS (Table 2 and Figure 1; see Table E1 and Figures E1 and E2 in the data supplement). Interestingly, PAs from SK displayed a similar miRNA expression pattern as in COPD, although they did not achieve statistical significance (Table 2, Figure 1, and Figure E2).

Deregulated miRNAs in COPD Target a Number of Genes Related to Proliferation

Each miRNA has a large number of targets according to the TargetScan database. We focused on those targets with functions in cell proliferation and mapped them to the comprehensive human interactome (24), which represents a network of all ascertainable protein-protein interactions in a cell. As a result, an miRNA-regulatory network in COPD describing the dysfunctional cell proliferation module was constructed (Figure 2).

Correlation of miRNA Expression with COPD Severity and Vascular Remodeling

Among the differentially expressed miRNAs, the expression levels of miR-139 and miR-485 correlated with the severity of airflow obstruction, assessed by the percent of predicted value of the forced expiratory volume in 1 second (Figure E3). The expression of miR-197 was significantly correlated with the severity of airflow obstruction (the lower the miRNA expression, the lower the forced expiratory volume in 1 second) and inversely correlated to the vascular remodeling degree, assessed by the thickness of the intimal layer (the lower the miRNA expression, the greater the intimal thickness) (Figure 3A). Expression of miR-98 and miR-451 also correlated with airflow obstruction and intimal thickness (Figures 3B and 3C).

Expression of miR-197 Increases during SMC Differentiation

Among all miRNAs that correlated with pulmonary function parameters, the contribution of miR-197 was consistent, and has not been previously evaluated in processes involving vascular remodeling. miR-197 function has been associated with the regulation of cell cycle-related genes, and has been shown to modulate SMC proliferation in leiomyoma (25, 26). Using an *in vitro* model of SMC differentiation induced by cell-to-cell contact (4), we performed Northern blot analysis at different stages of differentiation—Day 0 (D0) (proliferative cells), D2 (confluent cells), and D6 (differentiated/contractile cells)—and observed that miR-197 expression was increased in differentiated SMCs with contractile phenotype (Figure 4A).

In SMCs isolated from PAs of the studied subjects, Northern blot analysis showed reduced expression of miR-197 in SMCs from patients with COPD and SK as compared with NS (Figure 4B).

Effect of miR-197 Inhibition on SMC Differentiation and Proliferation

Transfection of SMCs with a 2'-O-methylated antisense (AS) RNA against miR-197 (AS-miR-197) and a scrambled AS (AS-miR-CTL) at D0 of differentiation was used to inhibit the expression of miR-197 (Figure 5A). miR-197 inhibition in SMCs blunted the expression of the SMC differentiation markers, myocardin, calponin, and sm22-α, after induction of

Table 2. MicroRNAs with Altered Expression in Pulmonary Arteries from Patients

	COPD versus NS		COPD versus SK		SK versus NS	
	FC	P Value	FC	P Value	FC	P Value
hsa-miR-485-3p	−3.38	0.0064	−1.35	0.2671	−2.50	0.0697
hsa-miR-197	−3.18	0.0117	−1.90	0.1095	−1.67	0.3271
hsa-miR-139-5p	1.95	0.0173	1.69	0.0353	2.06	0.0636
hsa-miR-146b-5p	2.84	0.0178	2.73	0.0015	1.51	0.2000
hsa-miR-451	5.27	0.0263	1.63	0.4802	3.24	0.1967
hsa-miR-627	−2.27	0.0382	−1.27	0.3910	−1.79	0.1946
hsa-miR-204	−1.70	0.0385	1.05	0.8668	−1.78	0.1376
hsa-miR-98	2.37	0.0413	1.81	0.0851	1.31	0.2600

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; FC = fold change; NS = nonsmokers; SK = smokers.

Table showing the dysregulated microRNAs in pulmonary arteries from subjects with COPD, SKs, and NSs.

differentiation by cell-to-cell contact (22), as assessed by quantitative RT-PCR (Figure 5B). Analysis of both α -SMA and calponin by immunofluorescence showed a marked decrease of actin and calponin fiber formation in AS-miR-197-transfected cells as compared with control cells (Figure 5B). In addition, miR-197 inhibition induced the proliferation of SMCs, as measured by increased expression of Ki67 at RNA and protein levels (Figure 5C). In agreement with these results, proliferation and migration capacities analyzed by a wound-healing assay were significantly increased in SMCs after transfection with AS-miR-197 (Figure 5D).

miR-197 Targets the Transcription Factor, E2F1

Cotransfection of E2F1 3' UTR coupled to a luciferase report vector with the AS-miR-197 in SMCs (Figure 6A) showed increased luciferase expression compared with cells that received the scrambled control miRNA inhibitor. In addition, upregulation of E2F1 RNA was observed after miR-197 inhibition in differentiated SMCs (Figure 6B). These results confirm that miR-197 targets mRNA encoding the transcription factor, E2F1. According to these results, E2F1 expression followed an inverse pattern to that of miR-197 during SMC differentiation (Figure 6C).

Expression of E2F1 Is Increased in PAs from Patients with COPD

Representative microphotographs of immunohistochemical stains for both the endothelial marker, CD31 (Figure 7A) and α -SMA (Figure 7B) of PAs used in this study are shown in Figure 7. Enlargement of the vessel wall is apparent in PAs from patients with COPD (Figure 7B). Such an enlargement is mainly due to the proliferation of α -SMA-expressing cells (Figure 7B).

To investigate whether or not the expression of E2F1 might be dysregulated in PAs from patients with COPD, we analyzed the expression of E2F1 by immunohistochemistry (Figures 7D and 7E) and Western Blot (Figure 7F). The expression of E2F1 in the COPD and the SK groups was upregulated, as compared with the NS, mirroring the downregulation of miR-197 in COPD PAs.

Discussion

The present study demonstrates the involvement of miRNAs in COPD-related vascular remodeling. Our work shows that from the 381 studied miRNAs, only 2% of miRNAs were differentially expressed in PAs of patients with COPD compared with NS. By applying Limma analysis, we identified eight deregulated miRNAs in COPD. From these miRNAs, miR-98, miR-139-5p, miR-146b-5p, and miR-451 were upregulated, and miR-197, miR-204, miR-485-3p, and miR-627 were downregulated. Interestingly, expression of miRNAs in PAs from SK was similar to that of patients with COPD, although they did not achieve significance, indicating that smoking without airflow obstruction is an intermediate phenotype between NS and COPD, as it has been already suggested in other studies (27, 28).

The gene ontology annotation analysis of all of these miRNAs using the TargetScan database identified mainly genes involved in cell proliferation, suggesting that vascular remodeling in COPD is associated with changes in miRNAs that control cell proliferation. By mapping these targets, we constructed an miRNA-regulatory network, which represents a cell proliferation functional module in COPD as compared with NS. This approach allowed us to identify the miRNAs and their canonical targets associated with multiple pathogenic

Figure 1. MicroRNA (miRNA) expression in patients with chronic obstructive pulmonary disease (COPD). Graphic representing the fold change of the top eight deregulated miRNAs. * $P < 0.05$. NS = nonsmokers; SK = smokers.

Figure 2. A miRNA-regulatory network in COPD. The green nodes represent miRNAs, and the blue nodes represent miRNA targets. All the target genes have the annotation “cell proliferation.”

pathways central to SMC proliferation in COPD.

We documented a downregulation of miR-204 in PAs of patients with COPD. In addition, a decreased expression of miR-204 was observed in both proliferative SMCs and in PA-derived SMCs from patients with COPD (Figure E4). Supporting this finding, reduced expression of miR-204 in SMCs from PAs has been reported in patients with PAH as well as in rodent experimental models of PAH (14). Downregulation of miR-204 in dedifferentiated SMCs suggests that miR-204 has a potential role in regulating SMC differentiation. Interestingly, miR-204 targets the transcription factor Slug in cancer cells, regulating changes in cell phenotype (29, 30). In this regard, we have recently shown

that Slug modulates the SMC-proliferative phenotype, and that its expression is increased in both highly remodeled human PAs and in lungs of mice with severe PH (22). These results indicate that miR-204 might regulate SMC phenotype through suppressing Slug expression, as has been previously suggested (16).

In PAs of patients with COPD, we also observed upregulation of miR-451, as has been shown in experimental models of PH and in humans with PAH (11). The transient loss of miR-451 is protective in the development of PAH (34). Other dysregulated miRNAs in this study were miR-146b, which is known to promote SMC proliferation (31), and miR-98, which mediates endothelial inflammation in atherosclerosis (32). Finally, miR-197, miR-

485, and miR-627 are broadly involved in cancer cell proliferation (26, 31, 33). We hypothesized that deregulation of miRNAs might promote cell proliferation and contribute to intimal hyperplasia in PAs in COPD.

Among dysregulated miRNAs, the degree of vascular remodeling and the severity of airflow obstruction were associated with the upregulation of both miR-98 and miR-451 and the downregulation of miR-197. The miRNA that displayed both the strongest correlation and the highest consistency in relation to parameters of disease severity was miR-197. Interestingly, miR-197 has been found to be downregulated in leiomyoma, promoting SMC proliferation (26, 34), and eventually it might also be involved in other disorders,

Figure 3. Correlation analysis of miRNA expression with both forced expiratory volume in 1 second (FEV_1) and intimal enlargement. (A) Negative correlation between miR-197 expression (calculated by the $\Delta\Delta$ threshold cycles [ddct]) and FEV_1 ; $P < 0.05$ by Pearson (left panel). Positive correlation between miR-197 expression (ddct) and % intima with respect to total area $P < 0.05$ by Spearman analysis (right panel). (B) Positive correlation between miR-451 expression (ddct) and FEV_1 $P < 0.05$ by Pearson (left panel) and negative correlation between miR-451 expression (ddct) and % intima with respect to total area $P < 0.05$ by Spearman (right panel). (C) Positive correlation between miR-98 expression (ddct) and FEV_1 $P < 0.05$ by Pearson (left panel) and negative correlation between miR-98 expression (ddct) and % intima with respect to total area $P < 0.05$ by Spearman (right panel).

by promoting SMC proliferation. Accordingly, we further analyzed the role of miR-197 in modulating the proliferative phenotype of PA SMCs using an *in vitro* model of SMC differentiation. Our results show that an increased expression of miR-197 was associated with an SMC-differentiated/contractile phenotype, whereas its inhibition downregulated mature markers of SMCs in differentiated cells. Moreover, functional studies

performed after transfection of SMCs with AS-miR-197 revealed that this miRNA regulates SMC phenotype denoted by higher rates of proliferation/migration. Cultured SMCs obtained from explanted PAs of patients with COPD also showed low levels of miR-197 that was associated with a less differentiated phenotype.

Available data indicate that miRNA-197 binds to the 3' UTR of E2F1 (35). This is important, because E2F1 is an

oncoprotein that regulates many cellular processes, including cell proliferation (36). E2F1 participates in both cell cycle progression and apoptosis, depending on which pathway is activated (37, 38). E2F1 induces S-phase entry, activating pro-survival and proliferative genes, such as cyclins, p53, or c-myc, and many S-phase genes (39, 40), whereas activation of the regulators, p53, p73, or Bcl2, induces apoptosis. In our study with PA SMCs, using a luciferase report assay and quantitative PCR, we validated that E2F1 is a target of miR-197. E2F1 expression was high in proliferative/dedifferentiated SMCs in accordance with oncogene function, and decreased during the acquisition of a contractile phenotype, following an inverse pattern to that of miR-197 expression. Importantly, we found that E2F1 was upregulated, paralleling the decreased expression of miR-197, in PAs from patients with COPD. The localization of E2F1 protein in remodeled arteries corresponds to sites of SMC proliferation in the vessel wall. These results agree with previous studies showing that E2F1 and its targets regulate SMC proliferation and vessel remodeling (41, 42). In addition, E2F1 is expressed in the endothelium. Further studies are needed to analyze which cell population of the enlarged artery wall contribute to the downregulation of miR-197. Our results indicate that SMCs derived from smokers and patients with COPD exhibit decreased miR-197 expression. As miR-197 is also expressed in ECs, future studies should analyze the expression of this miRNA in ECs derived from patients with COPD.

By using network analysis, we documented that miR-197 binds canonically with at least 19 different factors related to cell proliferation. From these factors, we confirmed the increased expression of insulin growth factor-1 after miR-197 inhibition (Figure E4), a known factor involved in both pulmonary vascular remodeling (43) and stimulation of SMC proliferation (44). This result suggests that insulin growth factor-1 might also be a target of miR-197, but more studies are needed to validate it.

Further studies are needed to unravel the mechanisms by which miR-197 is downregulated in COPD-associated vascular remodeling. Cigarette smoke products might alter miR-197 expression. Nicotine induces signal transducer and

Figure 4. Analysis of miR-197 expression in smooth muscle cell (SMC) phenotypic change and in SMCs from patients. (A) Representative Northern blot showing an increase of miR-197 in differentiated (Day 6 [D6]) as compared with proliferative (D0) SMCs. (B) Representative Northern blot showing the downregulation of miR-197 in SMCs derived from pulmonary arteries of smokers with normal lung function (SK; $n = 3$) and patients with COPD ($n = 3$) with respect to control patients (NS) ($n = 3$). U6 = noncoding small nuclear RNA 6. Day 2 (D2) confluent cells. nt = nucleotide.

activator of transcription 3 (STAT3) activation in both smooth muscle and inflammatory cells (45). STAT3 promotes the downregulation of miR-204 in SMCs by epigenetic mechanisms (46). Interestingly, in hepatocellular carcinoma cells, activation of the IL-6/STAT3 pathway induces the downregulation of miR-197, which, in turn,

targets STAT3 mRNA to promote cancer progression (31). However, smoking, as an intermediate condition (30), is not enough to explain COPD alteration. Hypoxia might also promote miR-197 downregulation. In this respect, IL-6 is increased in cells with both a hypoxic environment and a more proliferative/dedifferentiated SMC phenotype

in PH (47). The mechanism of miRNA-197 downregulation might also involve DNA hypermethylation, which is known to induce the downregulation of tumor suppressor miRNAs in cancer (48), and has also been described in vascular remodeling (46).

The present study has some limitations. First, despite the fact that PAs were isolated from a distant area, away of the solid tumor, we cannot exclude some effects of tumor factors on miRNA expression. Considering this, we performed an additional network analysis discriminating different cancer types, specifically, squamous versus nonsquamous cancers. In this analysis, miR-197 did not show any difference among cancer samples (data not shown). Second, due to the difficulties in obtaining adequate lung tissue from patients with COPD and control subjects, the small sample size represents another limitation of this study. Third, we were unable to correlate the

Figure 5. Inhibition of miR-197 abrogates SMC differentiation. (A) Northern blot analysis of miR-197 after 48 hours of transfection with antisense (AS)-miR-197 and a scrambled control (AS-miR-CTL) in SMCs. AS-miR-197 blunted the expression of miR-197 in SMCs. (B) Transfection of AS-miR-197 induces SMC phenotype switching, as is shown by the concomitant decrease of the SMC markers myoCD, sm22- α , and calponin analyzed by real-time qPCR and by the decrease of actin and calponin fibers assessed by immunofluorescence. Scale bars: 50 μ m. (C) miR-197 inhibition promoted an increase in the expression of the marker of proliferation Ki67 at both RNA (left panel) and protein levels (right panel). Scale bars: 50 μ m. (D) Scratch analysis shows an increased migration rate of SMCs transfected with AS-miR-197 with respect to the scrambled control, correlating well with SMC dedifferentiation. * $P < 0.05$ by paired test. All experiments were performed at least three times in duplicate. Data with error bars represent mean ($\pm$ SEM).

Figure 6. The E2F transcription factor 1 (E2F1) is a target of miR-197. (A) Luciferase assay shows an increase in luciferase units after miR-197 inhibition, corroborating that E2F1 is an miR-197 target. (B) E2F1 expression decreases along SMC differentiation performed by PCR. (C) miR-197 inhibition promotes the increase of E2F1 expression analyzed by real-time qPCR. * $P < 0.05$ by paired test. All experiments were performed at least three times in duplicate, except the luciferase assay, which was performed two times in triplicates. Data with error bars represent mean ($\pm$ SEM).

documented miRNA profile with the status of SMC differentiation *in vivo* at the moment of miRNA analysis, due to limitations in obtaining good quality and adequate quantity

of PA tissue and the impossibility to *in vivo* cell track the SMC lineage.

In summary, we observed downregulation of miR-197 in PAs from

smokers and patients with COPD. The expression of this miRNA was inversely related to the degree of vascular remodeling and the severity of airflow obstruction.

Figure 7. The transcription factor E2F1 is increased in pulmonary arteries (PAs) from patients with COPD. Representative microphotographs of immunostaining of PA sections from nonsmokers (NS; $n = 3$), smokers (SK; $n = 5$), and patients with COPD ($n = 6$). (A) CD31 (B) and smooth muscle α -actin immunohistochemistry of PAs showing increased expression of α -actin in the media and intima of patients with COPD. E2F1 immunohistochemistry (C and D) and immunodetection (E) in PAs displays an increase in COPD and smokers with normal lung function (SK) as compared with controls (NS). * $P < 0.05$ by one-way ANOVA. Scale bars: 50 μm . Data with error bars represent mean ($\pm$ SEM).

E2F1, a transcription factor targeted by miR-197, was upregulated in PAs from smokers and patients with COPD. Taken together, our studies support the view that miR-197 downregulation induces a SMC-proliferative phenotype, at least in part by releasing the suppression of the E2F1 transcription factor, which, in turn, regulates cell cycle entry. Accordingly, miR-197 should be considered a contributing

player among other dysregulated miRNAs in this condition. The combination of gene expression dysregulation leads to dysfunctional SMCs in COPD, promoting the “vascular remodeling phenotype” (49). The identification of miRNAs involved in cell proliferation associated with pulmonary vascular remodeling in COPD opens a new view in its pathogenesis and, eventually, its therapeutic approach. ■

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