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Original Article

MicroRNA-26a promotes epithelial mesenchymal transition in pleural mesothelial cells

Najmunnisa Nasreen[1,2], Bhagyalaxmi Sukka-Ganesh[1,2], Judith A Johnson[3], Ruxana T Sadikot[4], Steven A Sahn[5], Kamal A Mohammed[1,2]

[1]North Florida/South Georgia Veterans Health System, Gainesville, FL; [2]Division of Pulmonary and Critical Care Medicine, Department of Medicine; [3]Emerging Pathogens Institute, University of Florida, Gainesville, FL; [4]Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Emory University School of Medicine, Atlanta, GA; [5]Division of Pulmonary and Critical Care Medicine, Medical University of South Carolina, Charleston, SC

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Abstract: Pleural space is lined with a dynamic monolayer of cells, the Pleural Mesothelial Cells (PMCs). Pleural inflammation/injury often leads to the development of pleural fibrosis. Pleural fibrosis is characterized by the formation of sub-pleural foci of myofibroblasts that contribute to the exuberant pleural fibrosis. Emerging evidence indicate that microRNA-26a (miR-26a) play a key role in the process of fibrosis. In this study we have determined the role of miR- 26a in PMC Epithelial Mesenchymal Transition (EMT). We hypothesized that PMCs when stimulated with TGF-β1 undergo EMT targeting E-cadherin and this transformation of PMC to myofibroblasts is dependent on miR-26a and smad-2 signaling. The expression of EMT markers such as fibroblast specific protein-1 (FSP-1) and collagen type I, E-cadherin, α-Smooth muscle actin (α-SMA), Vimentin, and Cytokeratin-8 were analyzed in PMC treated with TGF-β1 or transfected with miR-26a. TGF-β1 significantly up-regulated miR-26a expression in PMC, and transfection of miR-26a enhanced α-SMA and collagen type-1 expression while the E-cadherin expression was decreased. In addition, luciferase reporter assay was performed to confirm E-cadherin as a target of miR-26a in PMCs. Furthermore in PMC, silencing miR-26a using anti-sense 2'-O-methyl oligo attenuated α-SMA and enhanced E-cadherin in response to TGF-β1. Consistent with the hypothesis, TGF-β1 increased miR-26a expression in PMC, whereas knocking down of miR-26a attenuated smad-2 phosphorylation overtime in response to TGF-β1. Taken together, these data indicate that miR-26a regulates the process of EMT in PMC which is mediated by TGF-β1 and smad-2 signaling.

Keywords: Pleural mesothelial cells, miR-26a, TGF-β1, E-Cadherin, α-SMA, epithelial-mesenchymal transition, pleural fibrosis, extra cellular matrix-proteins, Smad-2

Introduction

Pleural inflammation/injury often leads to the development of pleural fibrosis. Pleural fibrosis is characterized by the formation of sub-pleural foci of myofibroblasts that contribute to the exuberant pleural fibrosis. The pleural mesothelium is a metabolically active monolayer of cells, covering the chest wall and lungs. During pleural inflammation, PMC are exposed to high levels of cytokines, chemokines and growth factors including TGF-β1 [1-3]. Many studies reported that TGF-β1 is a master switch for the induction of fibrosis by promoting EMT in various organs including the lung [4-7]. Under stress conditions PMC attaining plasticity due to the loss of polarity and mesothelial markers. Consequently, PMC acquire spindle shape morphology by cytoskeleton reorganization and expression of Fibroblast Specific Protein (FSP-1), a mesenchymal marker. The newly formed fibroblasts called myofibroblasts that constitutively express FSP-1, a specific marker for EMT. In many tissues myofibroblasts are prominent components of fibrosis including the pleura [8-10]. In the presence of TGF-β1 transformed PMC express myofibroblast proteins like FSP-1 and α-SMA, suggesting the conversion of PMC to myofibroblasts. The transformed PMC lose the expression of E-cadherin and cytokeratin, adherens junctional protein and acquire mesenchymal markers [11, 12].

In normal cells E-cadherin, maintains the cellular integrity, besides this highly conserved gene can play a major role in malignant cell transfor-

mation, especially in tumor development and progression. The suppression of E-cadherin expression is regarded as one of the important events responsible for dysfunction in cell-cell adhesion. Thus, loss of function of E-cadherin correlates with increased invasiveness and metastasis of tumors [13]. E-Cadherin can be targeted by miRs specifically miR-200 and miR-9 [14]. In addition, zinc finger E-box-binding proteins (ZEB)-1 and ZEB-2, which are E-cadherin transcriptional repressors are target of miR-200 family responsible for protecting tubular epithelial cells from mesenchymal transition. The present data demonstrates the associating role of miR-26a with TGF-β mediated changes in E-cadherin expression. MiR-26a constitutes a class of unknown regulatory molecules that have the capacity to modulate the expression levels of cell adhesion molecules like E-cadherin. MiR-26a complementarily binds to E-cadherin in the 3'UTR region and induces transcriptional repression of E-cadherin. However, if miR-26a induced repression of E-cadherin promotes EMT in PMC is not known.

MicroRNAs (miRs) are 22 nucleotide non-coding sequences known to regulate gene expression and aberrant expression of miRNA have been linked to various diseases; however, their role in pulmonary fibrosis is now emerging. MiR exert a biologically important effect on post-transcriptional gene expression [15], and control the expression of a large number of genes by translational repression and destabilizing the target mRNAs [16]. MiRs have been associated in multiple biological processes, including development and disease [17-19]. There is increasing evidence that miRNAs are key regulators of genes involved in fibrosis of organs, such as the heart [20, 21], liver [22] and kidney [23]. The role of miRNA let-7d in regulating EMT has been reported indicating its protective role for pulmonary fibrosis [24]. Furthermore, miRNA are involved in many forms of tissue fibrosis [25]. In cardiac fibrosis, miR-21, miR-29, miR-30, miR-133 expression and regulation have been reported [21, 26, 27]. The current study was designed to analyze the role of miR-26a in PMC undergoing EMT. MiR-26a has been identified as a tumor-related regulator in several cancers, but its pathophysiological properties and correlation in the development of fibrotic lung disease remains unclear.

Several studies have reported the role of TGF-β1 in EMT involving smad signaling [28, 29]. Smads are intracellular proteins that play a critical role for transmitting the TGF-β1 induced signals from the cell surfaceto the nucleus to promote transcription of target genes [30, 31]. Earlier studies from our lab reported the role of smad-2 in PMC EMTprocess [11]. In the present study, we explore the role of miR-26a in the progression of PMC-EMT and lung fibrosis. We have demonstrated *in vitro* that the PMC transfected with miR-26a undergo transition from epithelial phenotype to mesenchymal phenotype (EMT) and this transition of PMC to myofibroblasts is dependent on TGF-β1 and smad-2 signaling.

Materials and methods

PMC culture

Pleural mesothelial cells Met-5A (CRL-9444, ATCC®- Manassas, VA), purchased from American Type Culture Collection. PMCs were sub cultured in medium 199 as reported earlier [11]. In brief, the PMCs were cultured in medium containing 10% FBS (Atlanta Biologicals), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were plated in 100-mm culture dish (Corning Costar) and incubated at 37°C in 5% CO_2-95% air. The cells were trypsinized upon confluency and seeded into culture dish or chamber slides as needed for various experiments. The cells from one lot have been used for all the experiments.

Phase-contrast microscopy

PMC phenotypic changes were determined by phase-contrast microscopy. PMC in cultures were transfected with miR-26a or control-miR, purchased from Ambion®, Inc. The morphological changes were visualized by phase-contrast microscopy for 24 hr, 48 hr and 72 hr. Images were collected with Inverted Microscope (Nikon).

Western blot analysis for EMT markers

PMC were plated in 60 mm culture dish (Costar) and treated with recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ) upon confluence. TGF-β1 at a final concentration of 5 ng/ml was used. PMC cultured without TGF-β1 were considered as controls. PMC about 70% confluency

in cultures were transfected with miR-26a or control-miR. Total cell lysates were prepared as reported previously [32]. Bicinchoninic acid (BCA) method (Pierce, Rockford, IL) was used to measure the total protein. 20 μg/lane of protein were loaded. Proteins in the samples were separated in denaturing sodium dodecyl sulfate (SDS)-4-15% polyacrylamide gels (Bio-Rad), and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA) in transfer buffer at 4°C overnight. Nonspecific binding to the membrane was blocked overnight at 4°C with 5% nonfat milk. The primary antibodies used were: anti-cytokeratin-8 (Ck-8; Sigma), anti-vimentin (Sigma), anti-E-cadherin (BD Transduction Labs), anti-α-SMA (R&D Systems), anti-smad-2, phospho-smad-2, (Cell Signaling) at a concentration of 1-2 μg/ml for 1 hour at room temperature. After washing, the membranes were incubated with the secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody, Amersham) at a dilution of 1:2,000 for 1 hour. The target proteins were detected by enhanced chemiluminescence (ECL, Amersham Bioscience, Piscataway, NJ).

Quantitative RT-PCR analysis

Total RNA from cultured PMC was isolated as reported earlier [32]. In brief the RNA at a concentration of 100 ng/mL were reverse transcribed into complementary DNA (cDNA). Ten microliters of diluted cDNA product were mixed with 25 μl of SYBR Green JumpStart Taq-ReadyMix, 0.5 μl of internal reference dye, and 2.0 μl of specific oligonucleotide primers obtained from QIAGEN were added to a total volume of 50 μl for quantification of the real time polymerase chain reaction (PCR). Applied Biosystems 7500 Real Time PCR System was used. Data analysis was carried out by using the ABI sequence-detection software using relative quantification. The threshold cycle (Ct), which was defined as the cycle at which PCR amplification reaches a significant value was expressed as the mean value. The relative expression of messenger RNA (mRNA) was calculated by using the ΔCt method (where ΔCt was the value obtained by subtracting the Ct value of the house keeping gene β-actin mRNA from the Ct value of the target mRNA). The amount of the target relative to β-actin mRNA was expressed as 2-(ΔCt).

MicroRNA target prediction

Computer-based programs were used to predict microRNAs that potentially bind E-cadherin. Using “E-cadherin” as a search term, we queried EMBL-EBI, MicroCosm targets. We confirmed the miR-26a target binding by performing Luciferase assay.

Luciferase reporter assay

The luciferase reporter vectors (pEZX-MT01) were purchased fromGeneCopia™. Each luciferase reporter construct, including Luc+miR-26a, Luc+E-cadherin 3’UTR and negative control vectors was co-transfected into PMC in 6-well plate using Lipofectamin-2000 (Invitrogen, CA). After incubation for 18 hours, the cells were transferred into 96-well plate and Firefly and renillaluciferase activities were determined after 24 hours, using Luc-Pair™ miR Luciferase Assay kits (GeneCopia™) according to the manufacturer’s instructions.

Small interfering RNA transfection

PMC were grown to 70% confluence in 60 mm culture dishes, and transient transfection was performed with specific stealth small interfering RNA (siRNA) against smad-2 or control siRNA overnight using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer’s protocol. RNAi designer software program was used to select the specificsiRNA sequences for smad-2 and control siRNA and synthesized from Invitrogen as reported earlier [11]. The smad-2 (accession number BC01-4840) target sequences used: sense CAG UGG GAU ACA ACA GGC CUU UAC A, antisense UGU AAA GGC CUG UUG UAU CCC ACU G; siRNAcontrol: sense CAG UGG GAU ACA ACA GGC CUU UAC A, antisense UGU AAA GGC CUG UUG UAU CCC ACU G. The most effective siRNA concentration for smad-2 silencingwas determined to be (300 nM). The transfection medium was changed with culture medium containing 5% FBS for 24 h before harvesting the cells for various assays. TGF-β1 at a final concentration of 5 ng/ml was added to the cell cultures in serum free medium (SFM) or cells were cultured in SFM without TGF-β1 (control). The cells were harvested at different time points for further-experiments.

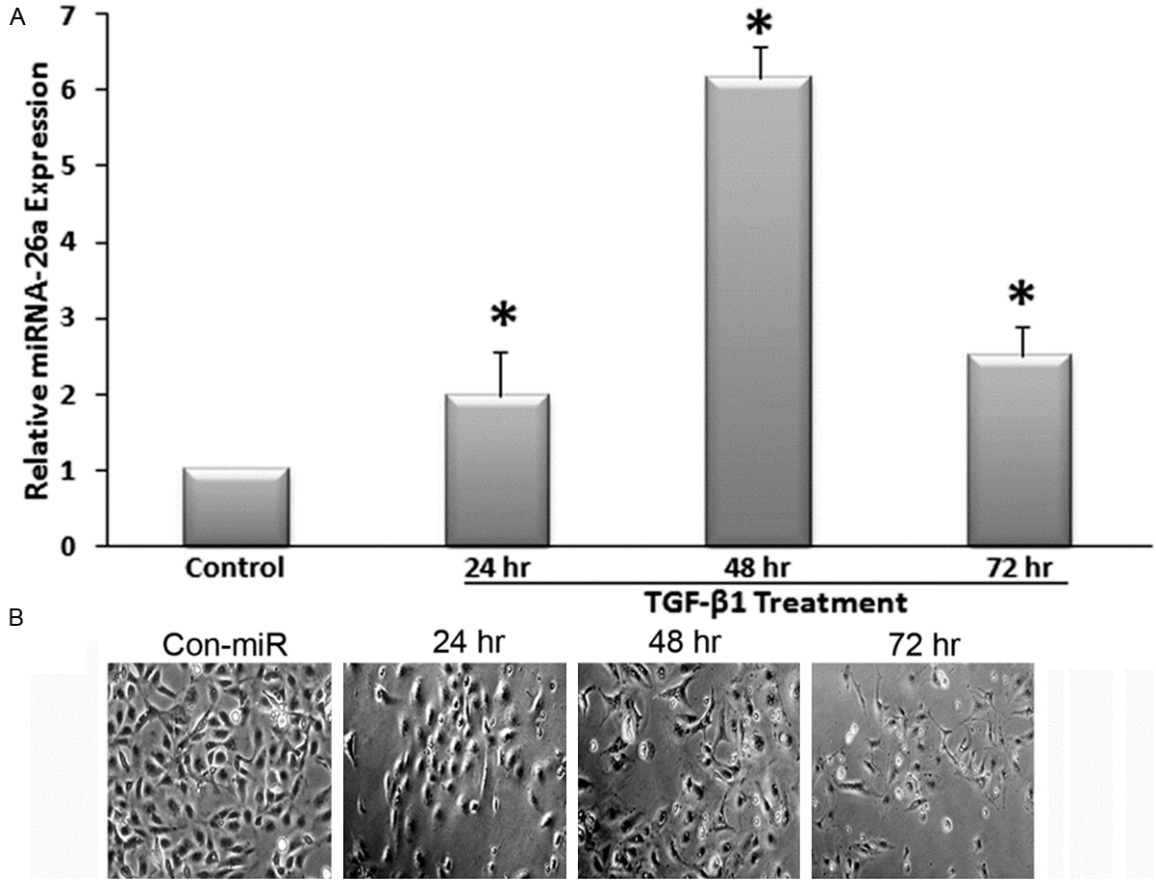

Figure 1. TGF-β1 induces miR-26a expression in PMC. A. Expression of miR-26a over time. PMC treated with TGF-β1 for 48 hr show significantly increased expression of miR-26a when compared to control. Data presented is the mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared to control. B. MiR-26a transfection induces mesenchymal phenotype morphology in PMC. PMC were transfected with miR-26a or control-miR (control), changes in morphology (epithelial to myofibroblast-like phenotype) were evaluated by using phase-contrast microscope.

Immunofluorescence microscopy

EMT markers expression in PMC was analyzed by immunofluorescence staining using Zeiss LSM 510 (Axiovert 100M), [Zeiss, Thornwood, NY] as reported previously [11]. In brief, the cells were cultured to confluence on glass coverslips, after respective treatments, they were fixed in 4% paraformaldehyde. The cells were permeabilized with 1.2% Triton X-100 for 5 min, rinsed three times, incubated with 2% bovine serum albumin (BSA for 1 hour, and then stained for the expression markers with primary antibodies: mouse anti-vimentin (Sigma), mouse anti-E-cadherin (BD Transduction Labs, Franklin Lakes, NJ), mouse anti-α-SMA (R&D Systems), and mouse anti-collagen type-I (Sigma) at 1:150 dilution and respective secondary antibody IgG conjugated with FITC (Zymax, San Francisco, CA or Molecular Probes, Eugene, OR). Cells were co-stained with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) to visualize nuclei. The stained cells we re mounted with mounting medium (DakoCytomation, Carpentaria, CA) and viewed by immunofluorescence microscopy.

Statistical analysis

SigmaStat 3.5 (SYSTAT Software, San Jose, CA) was used for data analyses. Results are expressed as means ± SEM. All values were derived from at least three independent experiments. Student's *t*-test was used for the comparison of control and treatment groups. Statistical analyses for comparison over the time course were performed with repeated-measure analysis of variance (ANOVA) and post hoc

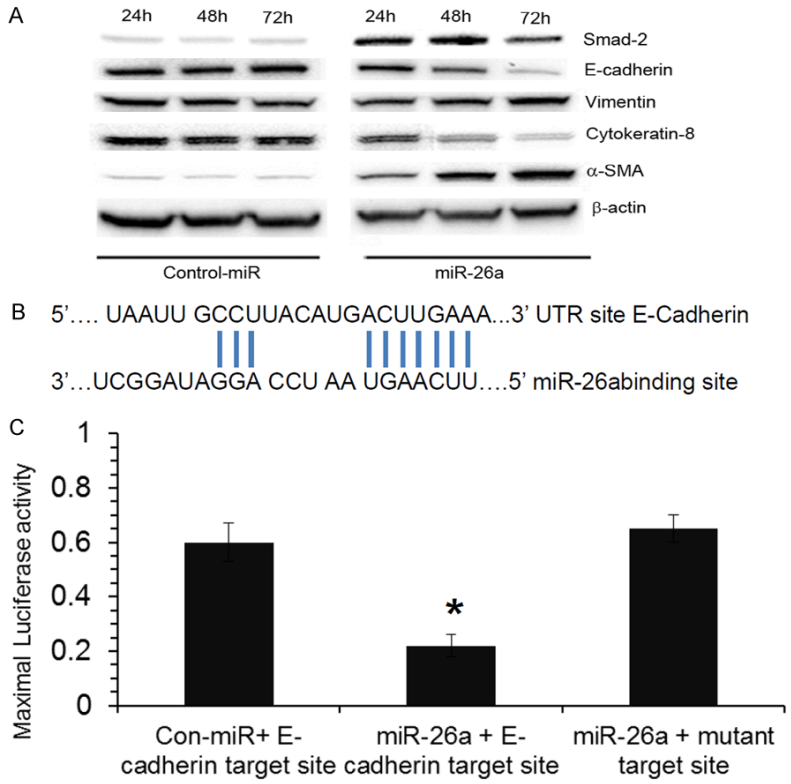

Figure 2. Determination of EMT markers in PMC. A. PMC was transfected with miR-26a or Control-miRas indicated. Total cell lysates were prepared and immuno-blotted with anti-bodies specific to EMT markers. Expression of α-SMA, Cytokeratin-8, Vimentin, E-cadherin, and Smad-2 were determined. β-actin was detected to ensure equal loading of protein for each sample. Data is the representative of 3 separate experiments; B. The predicted binding site of miR-26a and 3'UTR region of E-cadherin by The TARGETSCAN miRanda site; C. Luciferase assay shows miR-26a targets E-cadherin. PMC were transfected with E-cadherin Luciferase reporter plasmid and miR-26a plasmid or mutant miR plasmid (GeneCopoeia, Rockville, MD) as indicated. The cells were transferred to a 96-well plate 18 h after transfection and cultured for another 24 hours. Both firefly Luciferase and Renilla Luciferase activities were measured. Firefly Luciferase activity was then normalized with Renilla Luciferase activities in the same well.

Bonferroni/Dunn correction. Differences were considered significant if P values were < 0.05.

Results

TGF-β1 induces miR-26a expression in PMC

In earlier studies we reported that TGF-β1 induces EMT in PMC [11]. To access whether the EMT induced by TGF-β1 is regulated by miR-26a in PMC, we treated the PMC with TGF-β1 and determined the expression of miR-26a. TGF-β1 induced miR-26a expression over time in PMC. **Figure 1A** indicates that when compared to controls, PMC treated with TGF-β1 showed enhanced expression of miR-26a in a time-dependent manner with a six fold increases in the expression at 48 hr treatment. Because TGF-β1 plays a crucial role in the pathogenesis of lung fibrosis [33, 34], the induction of miR-26a by TGF-β1 may have a potential role in the development of pathological features of lung fibrosis.

MiR-26a induces mesenchymal phenotype transformation in PMC

To determine if miR-26a transfection induces mesenchymal phenotype in PMC, *in vitro* PMC were transfected with miR-26a, and morphological changes were recorded after 24, 48 and 72 hours. PMC transfected with control miR (control) showed a typical polygonal and cobblestone morphology in monolayer (**Figure 1B**). PMC when transfected with miR-26a showed phenotypic changes after 24 hr. Remarkable phenotypic changes were observed at 48 hr and 72 hr of miR-26a treatment. As compared to control, miR-26a treated PMC showed spindle shaped, elongated morphology characteristic of fibroblasts, suggesting that PMC attained mesenchymal phenotype. PMC transfected with control-miR did not show any changes in morphology.

MiR-26a induces cytoskeletal changes in PMC

To determine the role of miR-26a in the development of EMT, the expression of epithelial and mesenchymal markers were evaluated. PMC were transfected with miR-26a for 24, 48 and 72 hr or control miR. The PMC transfected with-miR-26a showed down-regulation of epithelial markers and that was accompanied by up regulation of mesenchymal markers. MiR-26a transfection did not affect the expression of vimentin; the expression of vimentin remained unchanged in miR-26a transfected PMC as

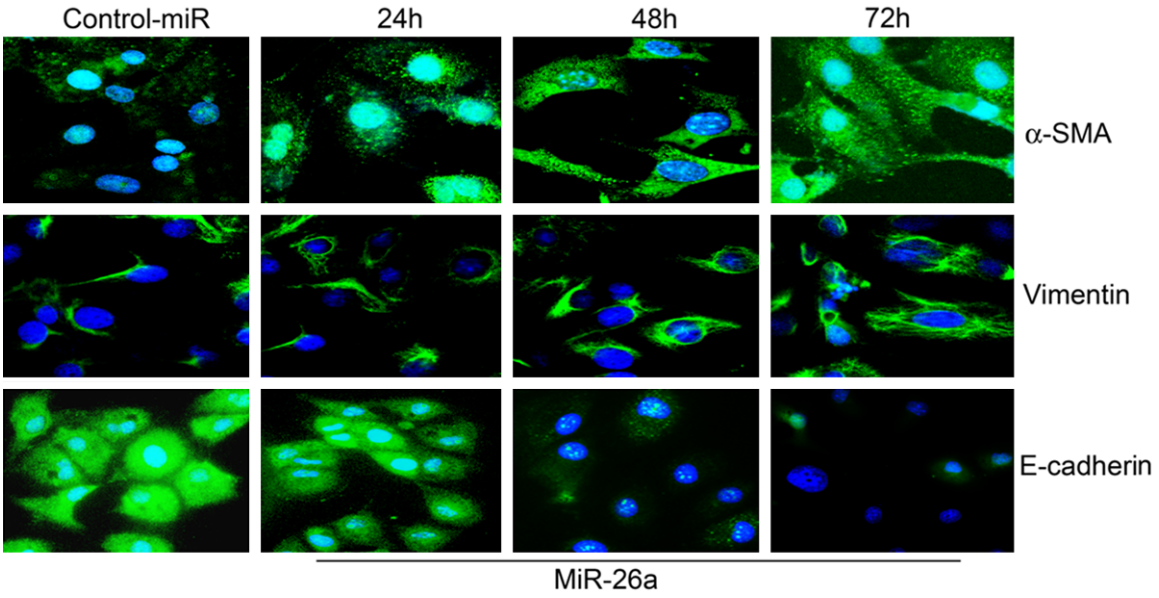

Figure 3. MiR-26a induces EMT in PMC. Immunofluorescence staining for EMT markers (α-SMA, Vimentin, and E-cadherin was evaluated in PMC transfected with miR-26a and control-miR. For the nuclear staining 4',6-Diamidino-2-phenylindole (DAPI, blue) was used (blue); Green fluorochrome (FITC) was used for respective target proteins. Scale bars, 40 μm.

compared to control miR (**Figure 2**). Whereas, the expression of Ck-8 decreased after 48 hr of miR-26a transfection when compared to controls. Epithelial marker E-cadherin the adheren junctional protein is expressed by PMC, and maintains the cellular integrity in PMC. E-cadherin down regulationin PMC marks the onset of EMT. In the presence of miR-26a, the expression E-cadherins was significantly decreased in miR-26a treated PMC as compared with control PMC. To confirm that the miR-26a binds to E-cadherin in 3'UTR region, we performed a luciferase reporter assay. The alignment of miR-26a with the 3'UTR inserts shown in **Figure 2B**. When the PMC were co-transfected with miR-26a and Luc+3' UTR E-cadherin vector the expression of luciferase was strongly decreased. In contrast, suppression of luciferase activity was almost abolished when the PMC cells were co transfected with miRNA negative control vector and Luc+3' UTR E-cadherin vector **Figure 2C**. These data indicate that the 3' UTR E-cadherin is critical for the direct and specific binding of miR-26a to E-cadherin mRNA and, miR-26a directly inhibits the expression of E-cadherin by binding to its target sequence.

In addition the immunofluorescence staining for vimentin, E-cadherin and α-SMA were evaluated in miR-26a and control miR treated PMC (**Figure 3**). The expression of vimentin did not show any remarkable changes in both miR-26a and control-miR treated PMC. Expression of E-cadherin was strong in control-miR treated PMC while miR-26a treated PMC showed significant down regulation of E-cadherin expression. In addition the mesenchymal marker α-SMA showed an increased expression over time when compared to control miR treated PMC. Furthermore, smad-2 expression was increased in PMC transfected with miR-26a for 48 hours when compared to controls. However, at 72 hours a decrease in the expression of smad-2 was noticed (**Figure 2**).

MiR-26a induces FSP-1 and collgen-1 expression in PMC

In orderto determine if miR-26a is involved in the activation of mesenchymal marker FSP-1 that caused phenotype changes in PMC, we examined the expression of FSP-1 in PMC. When PMC were transfected with miR-26a over time for 24 hr, 48 hr, and 72 hr, a significantly increased expression of FSP-1, about 12 fold was noted after 48 hr of miR-26a transfection when compared to control miR (**Figure 4A**). This data suggest that miR-26a induced expression of FSP-1 may be responsible for the phenotype

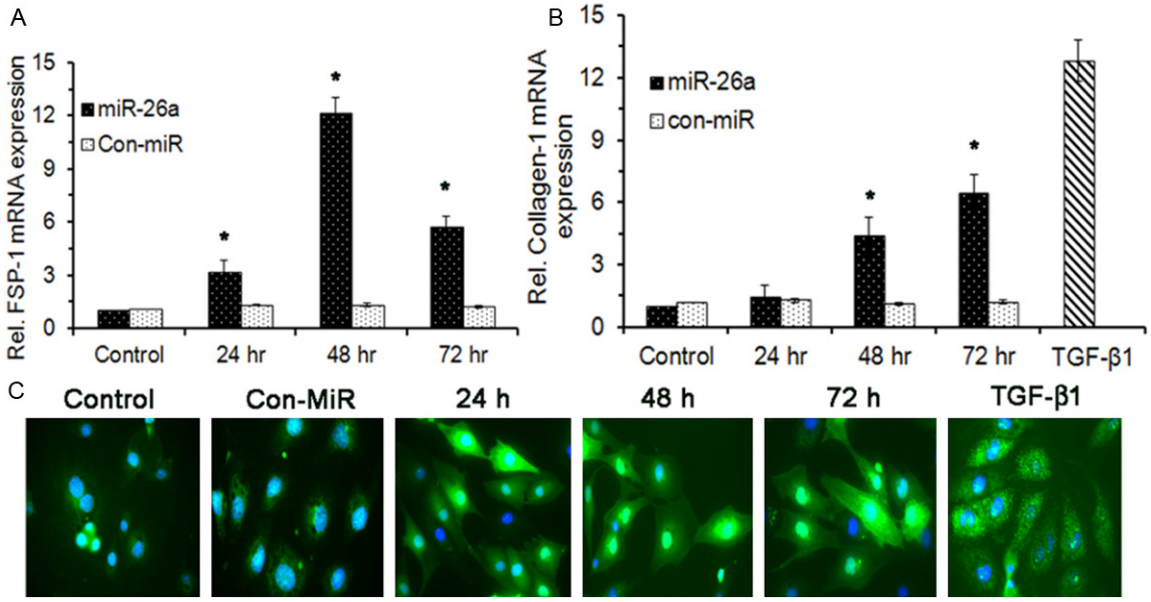

Figure 4. MiR-26a induces fibroblast-specific protein-1 (FSP-1) expression in PMC. A. Expression of FSP-1 over time. Data presented is the mean ± SEM of three independent experiments. *P < 0.05 compared to control-miR. B. MiR-26a induces collagen type-1 expression in PMC. Collagen type-I mRNA expression levels was evaluated by quantitative RT-PCR. Data presented is the mean ± SEM of three independent experiments. *P < 0.05 compared to control-miR. C. Immunofluorescence staining for collagen type I (green). MiR-26a transfected PMC showed up-regulated collagen expression over time; untreated PMC (0 h) did not show any collagen deposition. TGF-β1 was used as a positive control. Scale bar, 20 μm.

change of PMC to fibroblast like morphology and EMT. To determine if miR-26a is involved in the activation of extracellular matrix protein that caused phenotype changes in PMC, we also examined the expression of collagen type 1 in PMC transfected with miR-26a. A significantly increased synthesis of collagen type 1 was noted after 24 hr of miR-26a transfection-when compared to control miR (**Figure 4B**). The immunofluorescence staining of collagen type 1 clearly detected the collagen fibrils in the cytoplasmic region of PMC transfected with miR-26a at both 48 hr and 72 hr respectively when compared to control miR (**Figure 4C**). TGF-β1 was used as a positive control.

Silencing smad-2 signaling blocks miR-26a expression in PMC

To confirm whether miR-26a mediated EMT in PMC involves smad-2, siRNA for smad-2 were used to silence the expression of smad-2 gene in PMC. A significant knockdown of miR-26a expression was observed in PMC transfected with siRNA-smad-2 followed by TGF-β1 treatment when compared to control siRNA and only TGF-β1 treatment (**Figure 5B**). These results suggest that the mesenchymal changes induced by miR-26a are regulated by smad-2 signaling in PMC. In addition, the effect of siRNA smad-2 on miR-26a transfected PMC was confirmed by immunofluorescence staining. In miR-26a transfected PMC the expression of E-cadherin was significantly restored after the knock-down of smad-2 when compared with control siRNA and PMC alone (**Figure 5A**, **5B**). Also, α-SMA staining was less intense in PMC after the knockdown of smad-2 in the presence of miR-26a (**Figure 5A**). Furthermore, the cellular morphology of miR-26a-transfected PMC's returned from a spindle shape to a more cuboidal/cobblestone shape after the knock-down of smad-2 (**Figure 5A**, phase). However, the spindle shaped morphology was maintained in control siRNA transfected PMC suggesting the role of smad-2 signaling in EMT of PMC.

MiR-26a induced by TGF-β1 controls the pro-fibrogenic events in PMC

Studies were made to understand the role of miR-26a in the presence of TGF-β1 in PMC (**Figure 6A**, **6B**). TGF-β1 up-regulated miR-26a expression in a time-dependent manner in PMC, suggesting the potential involvement of

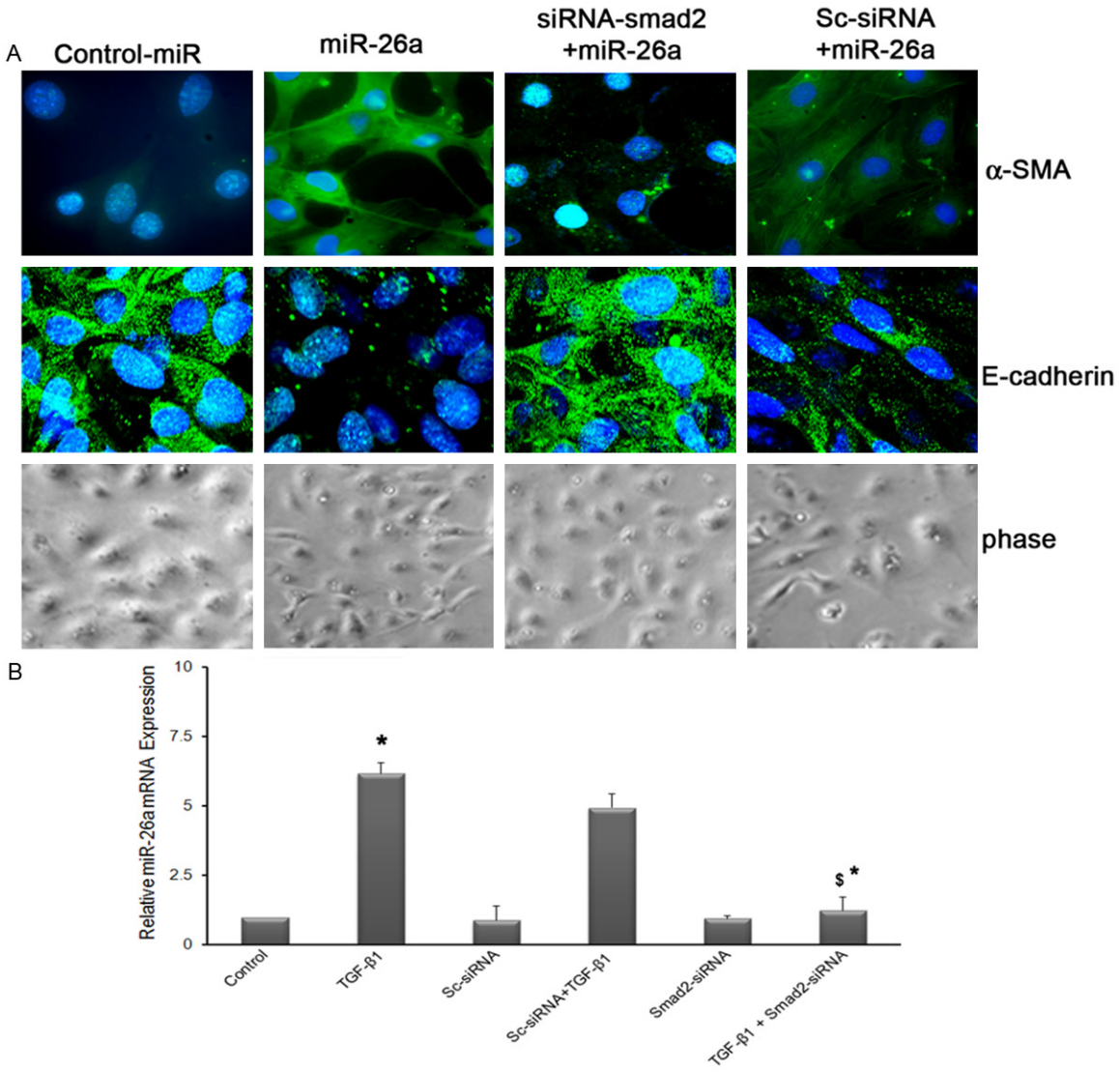

Figure 5. TGF-β1 induced miR-26a regulates the pro-fibrogenic activities in PMC. A. PMC was transfected with control miRNA or miR-26a or siRNA-smad2 or sc-siRNA. The expression of α-SMA, E-cadherin, was determined by immunofluorescence staining and a phase contrast microscopy. B. The mRNA expression of miR-26a was evaluated by quantitative RT-PCR. Results are representative data of 3 separate experiments. Data presented is the mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared to control, $^{\$}P < 0.05$ siRNA-smad2+ TGF-β1 compared to sc-siRNA +TGF-β1. Scale bar, 40 μm.

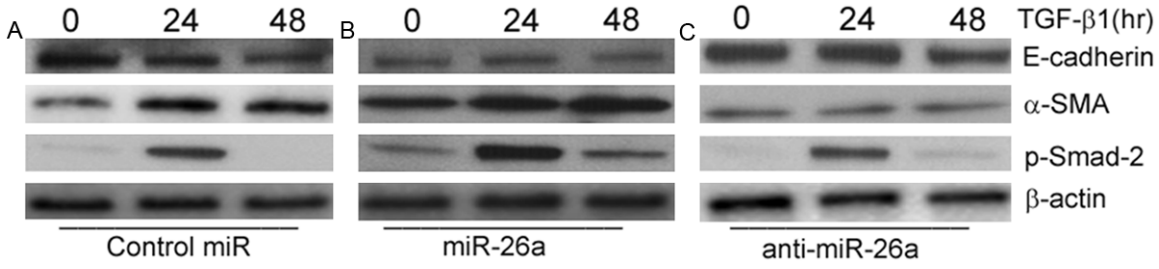

Figure 6. MiR-26a regulates EMT marker expression in PMC. A. PMC transfected with control-miR and treated with TGF-β1; B. PMC transfected with miR-26a and treated with TGF-β1; C. PMC transfected with anti-sense miR oligo for miR-26a and treated with TGF-β1 for indicated period of time. E-cadherin, α-SMA, phospho-Smad2 protein levels, were determined by Western blotting. β-actin was detected to ensure equal loading of protein for each sample. This is a representative of 3 similar observations noticed in separate experiments.

miR-26a in TGF-β1-related signaling events. Increasing miR-26a levels by transfection of miR-26a enhanced TGF-β1 induced translation of α-SMA with decreased E-cadherin expression in PMC (**Figure 6B**). Whereas knocking down of miR-26a by anti-sense oligo for miR-26a attenuated α-SMA and restored E-cadherin in response to TGF-β1 treatment (**Figure 6C**). Taken together, these results indicate that the anti-fibrotic effects of miR-26a antisense oligo may be mediated through the regulation of TGF-β1 related signaling events. Consistent with this hypothesis, the present findings support the potential role of miR- 26a in the regulation of EMT process in PMC.

Discussion

The present investigation demonstrate that PMC respond to miR-26a transfection by transforming into myofibroblasts *in vitro*, indicating that PMCs have the capacity to initiate and modulate the expression of epithelial markers into mesenchymal markers to undergo EMT and this is regulated by miR-26a. We found that TGF-β1 treated PMC cells up-regulates miR-26a expression inducing mesenchymal phenotype transformation in PMC. Little is known about how miR-26a expression is regulated by TGF-β1. We found increased phosphorylation of smad-2 in miR-26a transfected and TGF-β1 activated PMC. Phospho-smad-2 exerts its role in the nucleus by binding to different gene promoters and activating or inhibiting their transcription. The evidence presented in **Figure 6**, strongly suggested that increasing miR-26a levels enhanced, whereas knocking down miR-26a attenuated smad-2 phosphorylation overtime in response to stimulation of TGF-β1. In addition, silencing smad-2 expression by siRNA and followed by TGF-β1 treatment down-regulated the expression of miR-26a in PMC. These observations suggest the potential role of miR-26a in TGF-β1 mediated smad-2 signaling in PMC. Furthermore, it's evident from our results, **Figures 3**, **4** and **6** that up-regulation of miR-26a in PMC suppresses the TGF-β1-induced activation of downstream target gene, i.e., Ck-8, E-cadherin and up regulatesthe expression of mesenchymal markers α-SMA, and collagen-I. In miR-26a transfected PMC cobblestone morphology was lost. Furthermore, the adherens junction protein E-cadherin that is known to have a significant role in pleural permeability, and cellular movements has been suppressed in miR-26a transfected PMC that causes loss of cell-cell contact and promotes EMT in PMC.

Smad-dependent and independent signaling is involved in the commencement of EMT. Previous studies on TGF-β1 signaling pathway reported Smad3 as a significant mediator, having dual functionality, suppresses tumor growth in initial stages of cancer, but also playing an important part of promoting EMT [35-38]. Smad3 is reported to promote the morphological changes that characterize EMT through the transcriptional regulation of target genes in fibrosis of the eye and kidney [38, 39]. In addition, the double knock-out of smad-2 and smad-3 gene in mouse hepatocytes revealed the significant role of smad-3 for EMT [40]. In contrast, increased MMP-2 and α-SMA were dependent on both smad-2 and smad-3 signaling [41].

The studies of miRNA involvement in pulmonary fibrosis are now emerging. The role of let-7, miR-21, and miR-29 has been associated with pulmonary fibrosis [24, 42, 43]. In vitro and in vivo inhibition of let -7 in epithelial cells significantly increased the expression of mesenchymal specific genes, such as vimentin, α-SMA, indicating its role in EMT and fibrosis [24]. Another study on renal fibrosis reported the role of miR-200 family in the progression of renal fibrosis [14]. Taken together, these studies indicated the regulatory role of miRs in the development of fibrosis. In the current study, we report that miR-26a mediate the EMT process in PMC by modulating the expression of target genes associated with EMT. However, controversial reports are available on role on miR-26a. In lung cancer cells miR-26a enhances the metastatic potential via AKT signaling pathway [44]. In glioma, miR-26a promotes gliomagenesis in vivo [45]. Whereas, anti-fibrotic effects of mir-26a have been reported in idiopathic pulmonary fibrosis [46], and cardiac fibrosis [47]. The differential responses of miR-26a may be due to the different cell types and tissues and warrants further studies.

TGF-β had its influence on the regulatory role of smad-2 in transcribing miR-26a and expression of EMT markers. The study shows smad-2 regulates the transcription of miR-26a, which in turn is a repressor of E-cadherin at posttranscrip-

tional level in a TGF-β-dependent manner. We determined that by employing the computational Target Scan Human 5.2, the predicted miR-26a target as E-cadherin gene, we understand that miR-26a bind to the predicted target site of E-cadherin and modulate EMT in a TGF-β1-dependent manner. The present data suggest that E-cadherin in PMC maintains cellular integrity; any change in the expression levels of these adherent junctional proteins may result in transformation of cellular morphology and remodeling of the pleural mesothelial monolayer through the process of EMT. During pathological disorders the aberrant expression of miR-26a in pleural space may affect PMC to be transformed into myofibroblasts and the transformed PMC may possibly travel to the deeper layers of the lung parenchyma, facilitating the progression of fibrosis.

In conclusion, the significance of the present study is the identification of role of miR-26a in the regulation of TGF-β1, the important mediator of EMT process. Here we demonstrate the role of miR-26a via TGF-β1 and smad-2-mediated EMT in PMC. The implication of the present work is that miR-26a transfected PMC undergo the process of EMT via expression of mesenchymal markers FSP-1 and α-SMA, repression of E-cadherin, increased transcription factor smad-2 expression. In addition, silencing siRNA-smad-2 significantly blocked the expression of miR-26a. Furthermore, inhibitor of miR-26a blocked α-SMA and attenuated smad-2 phosphorylation. Taken together, our study clearly signifies that miR-26a plays a critical role in pleural fibrosis through its capability to regulate array of events during the process of EMT.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kamal A Mohammed, North Florida/South Georgia Veterans Health System, Gainesville, FL. E-mail: mkamal@medicine.ufl.edu

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