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MiR-29 Mediates TGFβ1-Induced Extracellular Matrix Synthesis Through Activation of PI3K-AKT Pathway in Human Lung Fibroblasts

Tao Yang,[1,2] Ying Liang,[1] Qinlu Lin,[1] Junwen Liu,[3] Feijun Luo,[1] Xinhua Li,[4] Hui Zhou,[1,2] Sheng Zhuang,[1] and Hongliang Zhang[5]*

[1] *National Engineering Laboratory for Rice and By-product Deep Processing, Central South University of Forestry and Technology, Changsha 410004, Hunan Province, People's Republic of China*

[2] *College of Chemistry and Bioengineering, Changsha University of Science and Technology, Changsha, Hunan 410076, People's Republic of China*

[3] *Department of Histology and Embryology, School of Basic Medical Sciences, Central South University, Changsha 410013, Hunan Province, People's Republic of China*

[4] *Department of Gastroenterology, Xiangya Hospital, Central South University, Changsha 410008, Hunan Province, People's Republic of China*

[5] *Emergency Department, the Second Xiangya Hospital, Central South University, Changsha 410011, Hunan Province, People's Republic of China*

ABSTRACT

TGFβ1 is very important in the synthesis and degradation of extracellular matrix, and also in the mediation of human lung fibroblasts proliferation, and miR-29 plays an important role in this process. To explore the interactions of miR-29 family members and TGFβ1, the effects of transforming growth factor TGFβ1 on the expression of miR-29 and whether miR-29 is involved in pro-survival signaling pathways mediated by TGFβ1 were examined in human lung fibroblasts. Treatment of the human embryonic lung fibroblast cell line IMR90 with TGFβ1 caused a decrease in expression of miR-29a/b/c by real-time PCR analysis. TGFβ1 stimulation increased cell proliferation, colony formation and up-regulated expression of COL1A1; transfecting with miR-29a/b/c mimics reverse TGFβ1-induced phenotype changes in IMR90 cells. Western blot analyses showed that TGFβ1 treatment unchanged total protein expression levels of PI3K or AKT, but the expression levels of p-PI3K, p-AKT, and COL1A1 were increased; and miR-19a/b/c mimics interfering blocked phosphorylation of PI3K or AKT and decreased expression of COL1A1 after TGFβ1 treatment. The results indicate that TGFβ1 beta uses the PI3k-Akt pathway in these embryonic fibroblasts and miR29 blocks this activation pathway. It indicates a novel biological function of the PI3K-Akt pathway in IMR90. Elevated expression of miR-29 may play an important role in the pathogenesis of diseases related to fibrogenic reactions in human lung fibroblasts. J. Cell. Biochem. 114: 1336–1342, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: MIR-29; LUNG FIBROSIS; TGFβ1; PI3K-AKT

The extracellular matrix (ECM) is a dynamic microenvironment that contributes to idiopathic pulmonary fibrosis (IPF), via activation of profibrotic pathways and matrix metalloproteinases that enhance collagenase activity [Tomasek et al., 2002; Thannickal et al., 2004]. Fibroblasts and myofibroblasts play essential roles in the pathological process by secreting growth factors, as well as excessive collagens, and other matrix proteins [Bouzeghrane et al., 2005].

Tao Yang, Ying Liang, Quinlu Lin, and Junwen Liu contributed equally to this work.

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*Correspondence to: Hongliang Zhang, Emergency Department, the Second Xiangya Hospital, Central South University, Changsha 410011, Hunan Province, People's Republic of China. E-mail: zhlzzj@163.com

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Transforming growth factor TGFβ1 is well known to induce the phenotypic transformation of fibroblasts to myofibroblasts in the lungs and is thought to play a significant role in fibrosis [Campbell and Katwa, 1997; Uhal et al., 2007]. It has been shown to be an important mediator of lung fibrosis and can induce differentiation of pulmonary fibroblasts into myofibroblasts characterized by α-smooth muscle actin expression and active synthesis of ECM proteins [Lee et al., 2006; Cutroneo et al., 2007].

miRNAs are small regulatory RNAs, which control a large number of gene expressions by translational suppression and destabilization of target mRNAs [Bartel, 2009]. miRNAs have been implicated in multiple biological processes, including development and disease[-Calin and Croce, 2006; Van Rooij and Olson, 2007; Taft et al., 2010]. Increasing evidences showed that miRNAs are key regulators of genes involved in fibrosis in organs, such as heart [Thum et al., 2008; Van Rooij et al., 2008], kidney [Chung et al., 2010], and liver [Chu and Friedman, 2008]. Two recent reports have demonstrated that let-7, miR-21 and miR-29 are involved in pulmonary fibrosis by regulating EMT and TGFβ1 signaling activity, respectively [Pandit et al., 2010; Cushing et al., 2011].

In addition to these preliminary studies, the molecular mechanism of miR-29-mediated phenotype changes has not yet been elucidated. We speculated that miR-29 may act as an important pathogenic modulator in the matrix-remodeling processes. To test this hypothesis, we investigated the effect of TGFβ1 on expression of miR-29 in IMR-90 cell. Then, we analyzed whether TGFβ1 induces IMR-90 cell proliferation through miR-29. We subsequently investigated whether miR-29 affects pro-survival signaling pathways to mediate COL1A1 (an important member of ECM proteins) expression in IMR-90 cell, and finally results in phenotype alterations mediated by TGFβ1.

MATERIALS AND METHODS

REAGENTS

Human recombinant TGF-β1 was purchased from PeproTech. MiR-29 mimics, miR-29 inhibitor, and real-time PCR primers were synthesized by Auragene Bioscience.

CELL CULTURE

Human primary pulmonary cell line IMR-90 was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C with 5% CO_2 in DMEM containing 10% FBS.

TOTAL RNA EXTRACTION AND REAL-TIME PCR

Total RNA from IMR-90 cell was isolated using the Qiazol reagent as instructed (Qiagen, USA). Total RNA yields were determined by A260 measurement using the ND-1000 NanoDrop spectrophotometer (NanoDrop, Wilmington) and RNA integrity was assessed by microcapillary electrophoresis (2100 BioAnalyser, Agilent Technolologies, Waldbronn, Germany). MiR-29 was quantified by a two-step real-time PCR using the miScript-Reverse Transcription Kit and the miRNA-SYBR Green PCR Kit (Qiagen). The first step includes total RNA polyadenylation and reverse transcription, followed by real-time PCR. Primers used for cDNA synthesis and real-time PCR were selected and purchased from the GeneGlobe Search Center (Qiagen). Two nanograms of all samples were used and processed in triplicate in agreement with the supplier's guidelines. Cellular miRNA levels were normalized using RNU6 as reference RNA.

WESTERN BLOTTING

Total protein concentrations were determined using the BCA protein assay (Auragene Biosciences, Changsha, China). Equal amounts of protein (10 mg) were resolved on 4–12% SDS–polyacrylamid gels (Auragene Biosciences), electrotransferred to Hybond ECL nitrocellulose membranes (Auragene Biosciences) and immunostained with the anti-collagen I, from Abzoom biolabs (Abzoom Biolabs, Dallas), or with anti-PI3K, anti-AKT, anti-phospho-PI3K, anti-phospho-AKT antibodies from Abzoom Biolabs, or anti-actin antibodies from Abzoom Biolabs.

MTT ASSAY

MTT assay was performed to assess the effect of miR-29 and TGFβ1 (10 ng/ml) on cell proliferation. Cells (8×10^3 cells/well) were plated in a 96-well plate and maintained in RPMI-1640 supplemented with 10% FBS. At 24, 48, 72, and 96 h after seeding, culture medium was removed, cells were treated with 20 μl sterile MTT dye (5 mg/ml, Sigma, USA) for 4 h at 37°C, and then 200 μl of DMSO was added and thoroughly mixed for 30 min. Spectrometric absorbance at wavelength of 570 nm was measured on a microplate reader. Cells in three wells of each group were quantified in each experiment.

SOFT AGAR ASSAY

Non-transformed IMR-90 cells or miR-29-IMR-90 cells treated with TGFβ1 (10 ng/ml) were mixed into 0.5 ml of 0.35% agar containing growth medium and layered over a base of 0.5% agar to prevent anchorage-dependent cell growth. Once this layer was solidified, it was overlaid with 1 ml of normal growth medium, which was replaced every 2 days for 14 days. A colony is defined as a cell aggregate larger than 100 μm. Pictures were taken and visible colonies were counted after 14 and 28 days.

STATISTICAL ANALYSIS

All values in this study were presented as mean + SD. Statistical analysis was performed based on a Student's *t*-test at the significance level of $P < 0.05$. Spearman's correlation analysis was used to determine the correlation of the expression levels of miR-29, cell proliferation, and colonies, using SPSS software. All experiments were repeated at least three times.

RESULTS

TGFβ1 TREATMENT DECREASE EXPRESSION OF MIR-29 IN IMR-90 CELL

TGFβ1 has been shown to play a dominant role in the production and deposition of in the lung and induces a transformation of fibroblasts [Border and Noble, 1994]. We first examined the effect of TGFβ1 treatment on the expression of three miR-29 family members in IMR-90 cell. As shown in Figure 1, the expression of miR-29a/b/c was significantly decreased after the cells were treated with 10 ng/ml TGFβ1, and it can maintain for 2 days the lower expression levels of miR-29 for 2 days after treatment.

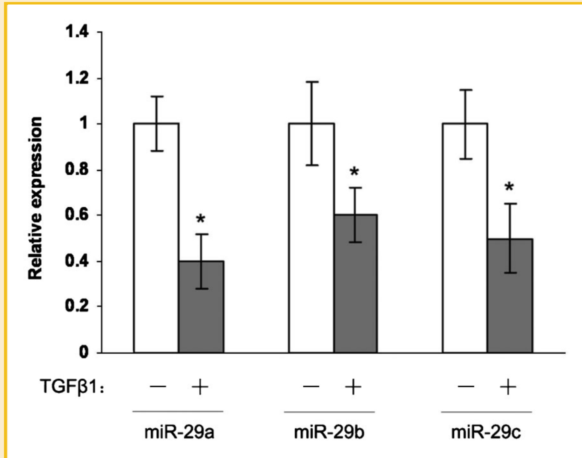

Fig. 1. Real-time PCR analysis indicates that all three miR-29 family members are down-regulated in IMR-90 after treatment with TGFβ1 for 48 h. $^{*}P<0.05$ compared with untreated IMR-90.

MIR-29 INHIBITS TGFβ1-INDUCED PROLIFERATION OF IMR-90 CELL

As previously reported [Fuentes-Calvo et al., 2012], TGFβ1 played a role in inducing ECM synthesis and promotes cell proliferation. The results of the previous section showed that the expression of miR-29 was reduced after TGFβ1 treatment. We examined the role of miR-29 on I TGFβ1-induced MR-90 cell proliferation by MTT assay. As shown in Figure 2, after 1, 2, 3, 4 days of seeding, up-regulating expression of miR-29 decreased the number of the cells and is a negative regulator. It suggests that miR-29 blocks TGFβ1 pathway and inhibits TGFβ1-induced proliferation of IMR-90 cell.

MIR-29 DECREASES COLONY FORMATION OF IMR-90 CELL AFTER TGFβ1 TREATMENT

Colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. To better understand the role of miR-29 on the TGFβ1-induced IMR-90 proliferation, we treated cells with TGFβ1 and miR-29 mimics. Overexpression of miR-29 greatly reduced IMR-90 cell proliferation, and the number

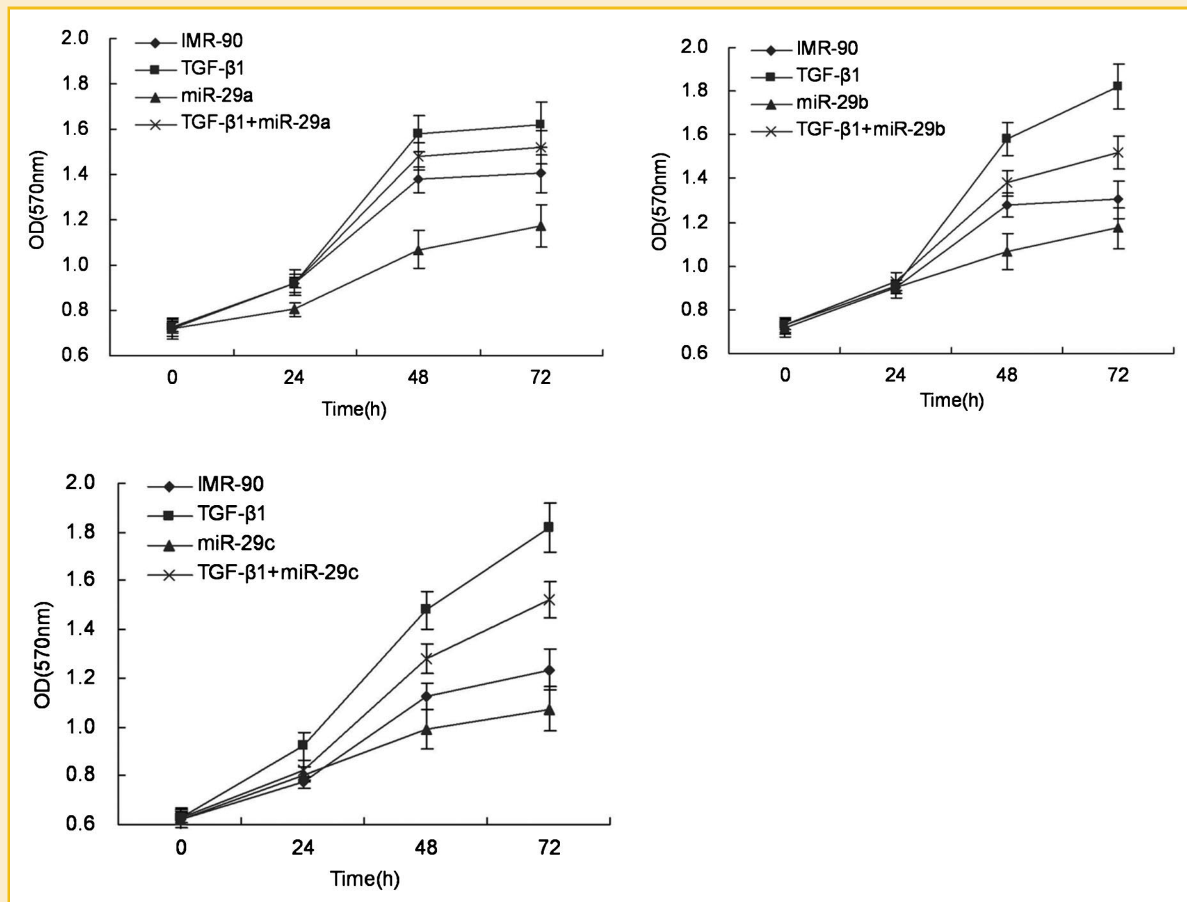

Fig. 2. miR-29 inhibited TGFβ1-induced proliferation of IMR-90 cells. IMR-90 cells treated with miR-29a/b/c mimics, TGFβ1 or both of them. IMR-90 cells were seeded in 96-well plates and grew for 24 h, 48 h, 72 h, or 96 h monitored by MTT assay. (IMR-90; TGFβ1; miR-29a/b/c; miR-29a/b/c+ TGFβ1).

of colonies formed in cells treating with miR-29 mimics was only 30% of the value in the control group (TGFβ1 treatment only; Fig. 3). A possible explanation for the decreased colony formation of IMR-90 cell lies in overexpressing miR-29 mediating in the process of TGFβ1-induced ECM synthesis and promoting IMR-90 cell proliferation.

MIR-29 MEDIATES TGFβ1-INDUCED ACTIVATION OF PI3K-AKT PATHWAY

To determine whether miR-29 mediates TGFβ1-induced activation of PI3K-Akt pathway, control and overexpressing miR-29 IMR-90 cells were treated with TGFβ1 and PI3K/Akt protein expression levels were analyzed by Western blotting in the cell lysates. Overexpressing miR-29 inhibited TGFβ1-induced phosphorylation of PI3K-AKT, and non-phosphorylated Akt and PI3K protein levels remained constant in each sample (Fig. 4).

MIR-29 MEDIATES EXPRESSION COL1A1 THROUGH DOWN-REGULATION OF PI3K-AKT PHOSPHORYLATION

To determine whether miR29 reduced the expression of COL1A1 involving in PI3K-Akt phosphorylation, cells treated with or without LY294002 (a PI3K-Akt phosphorylation inhibitor), and cells were cultured in the presence of miR-29 inhibitor examined by Western blot analysis. Our data showed that LY294002 inhibited phosphorylation of PI3K, which indirectly inhibited Akt activation. As shown in Figure 5, up-regulated expression levels of COL1A1 were detected from the lysates of cells treated with miR-29 inhibitor and decreased the expression with LY294002 compared with control cells. These

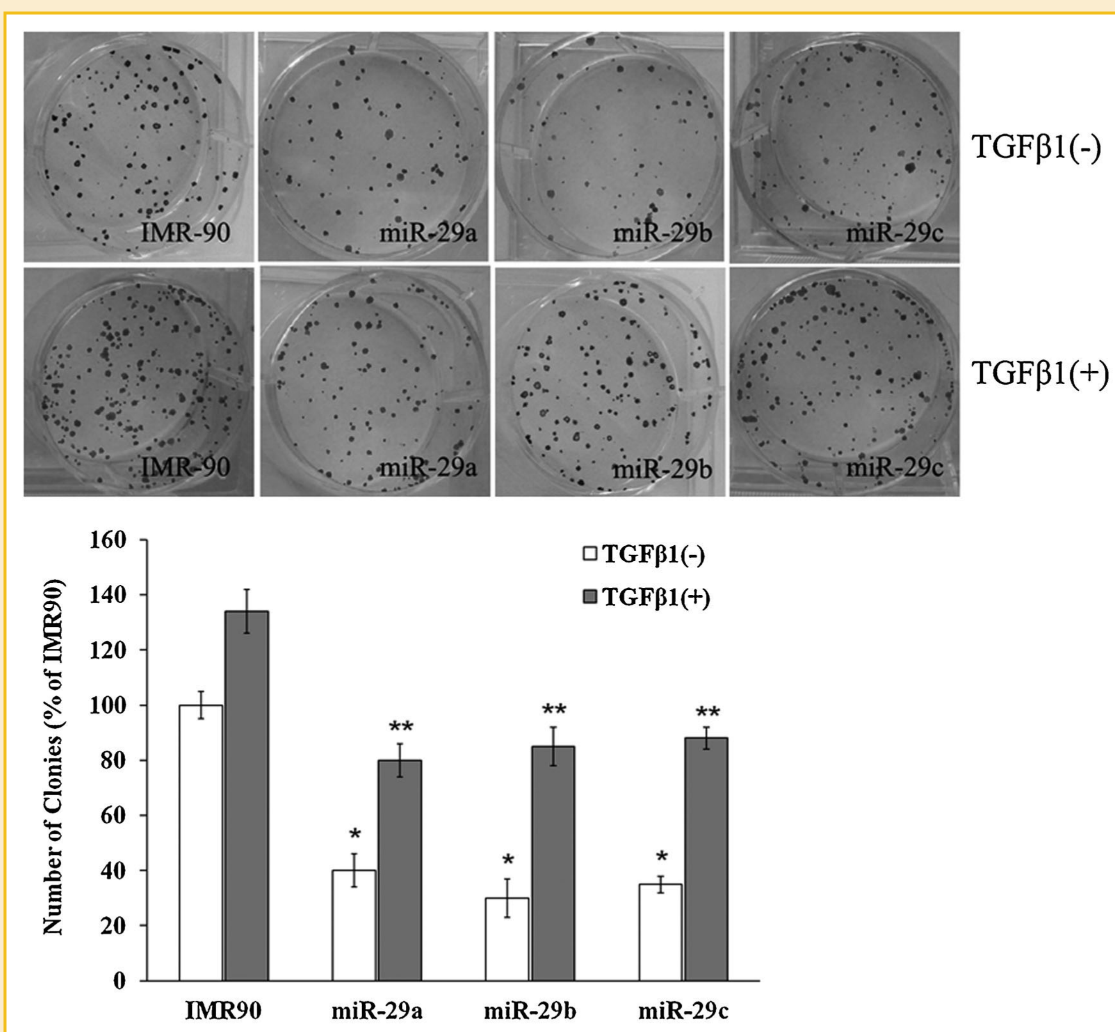

Fig. 3. Overexpression of miR-29 inhibited TGFβ1-induced colony formation of IMR-90 cells. IMR-90 cells were treated with miR-29a/b/c mimics, TGFβ1 or both of them, and IMR-90 cells were seeded in soft agar as described in Materials and Methods. A: Cells were treated as indicated and were cultured for 14 days, then stained with crystal violet, and photographed. B: Numbers of colonies are presented as the percentage of colonies obtained relative to IMR90. *,**, $P < 0.05$ compared with untreated IMR-90. (IMR-90; TGFβ1; miR-29a/b/c; miR-29a/b/c+ TGFβ1).

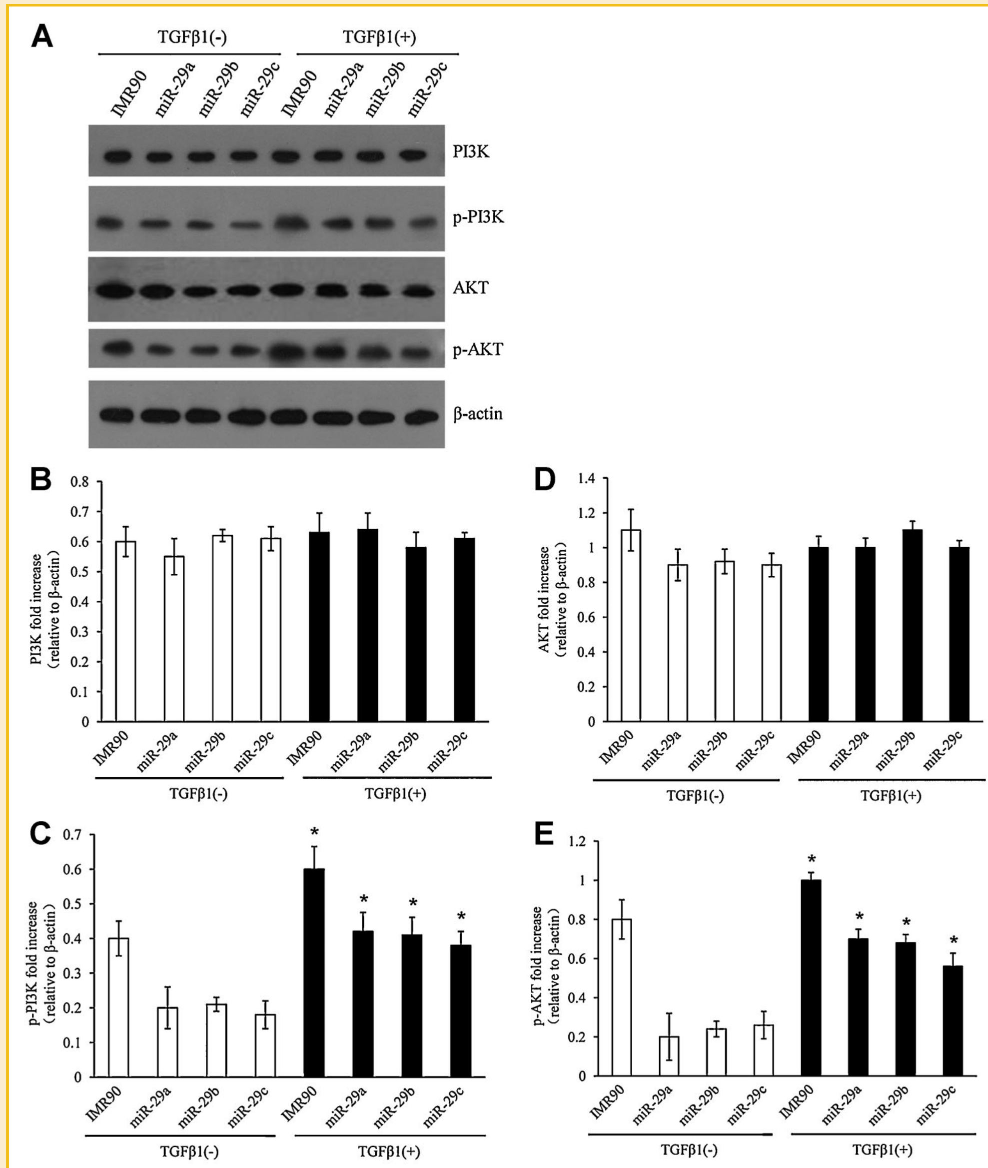

Fig. 4. miR-29 mediated TGFβ1-induced phosphorylation of PI3K and AKT in IMR-90 cells. A: IMR-90 cells were cultured in DMEM medium supplemented with 10% FBS and were transfected with 50 nM miR-29a/b/c precursors, or a negative control, which had a scrambled sequence (control) using Lipofectamine 2000. After 6 h, the medium was changed to DMEM containing 0.1% FBS with or without 2 ng/ml TGFβ1, and continually culture for another 24 h. Effect of miR-29a/b/c precursors on the protein expression of PI3K, phospho-PI3K, AKT, phospho-AKT and β-actin in the presence (+) or absence (−) of TGFβ1. Results are representative of those in three repeated experiments. B: Band intensities were quantitated by Image-Pro Plus. The intensities of the bands corresponding to PI3K were compared to those corresponding to β-actin. Results shown are mean ± SD values from three independent experiments. C: The intensities of the bands corresponding to p-PI3K were compared to those corresponding to β-actin. D: The intensities of the bands corresponding to AKT were compared to those corresponding to β-actin. E: The intensities of the bands corresponding to p-AKT were compared to those corresponding to β-actin. The asterisk indicates significant difference when compared to no TGFβ1 treated cells ($P < 0.05$).

results indicate that miR-29 decreases the expression COL1A1 through down-regulation of PI3K-Akt phosphorylation.

DISCUSSION

TGFβ1 has been shown to be a key modulator in the synthesis and degradation of ECM, and also in the mediation of human lung fibroblasts proliferation [Chen et al., 1999; Saika et al., 2001]. Likewise, miR-29 has been shown to be an important player in this process of down-regulating several extracellular proteins, including collagens [Van Rooij et al., 2008]. Also, miR-29 occupies an important role in integrating functionally connected pathways involved in pulmonary fibrotic disease through its ability to regulate multiple important signaling events involved in fibrogenesis [Cushing et al., 2011]. Thus, it may represent an attractive therapeutic target for pulmonary fibrosis.

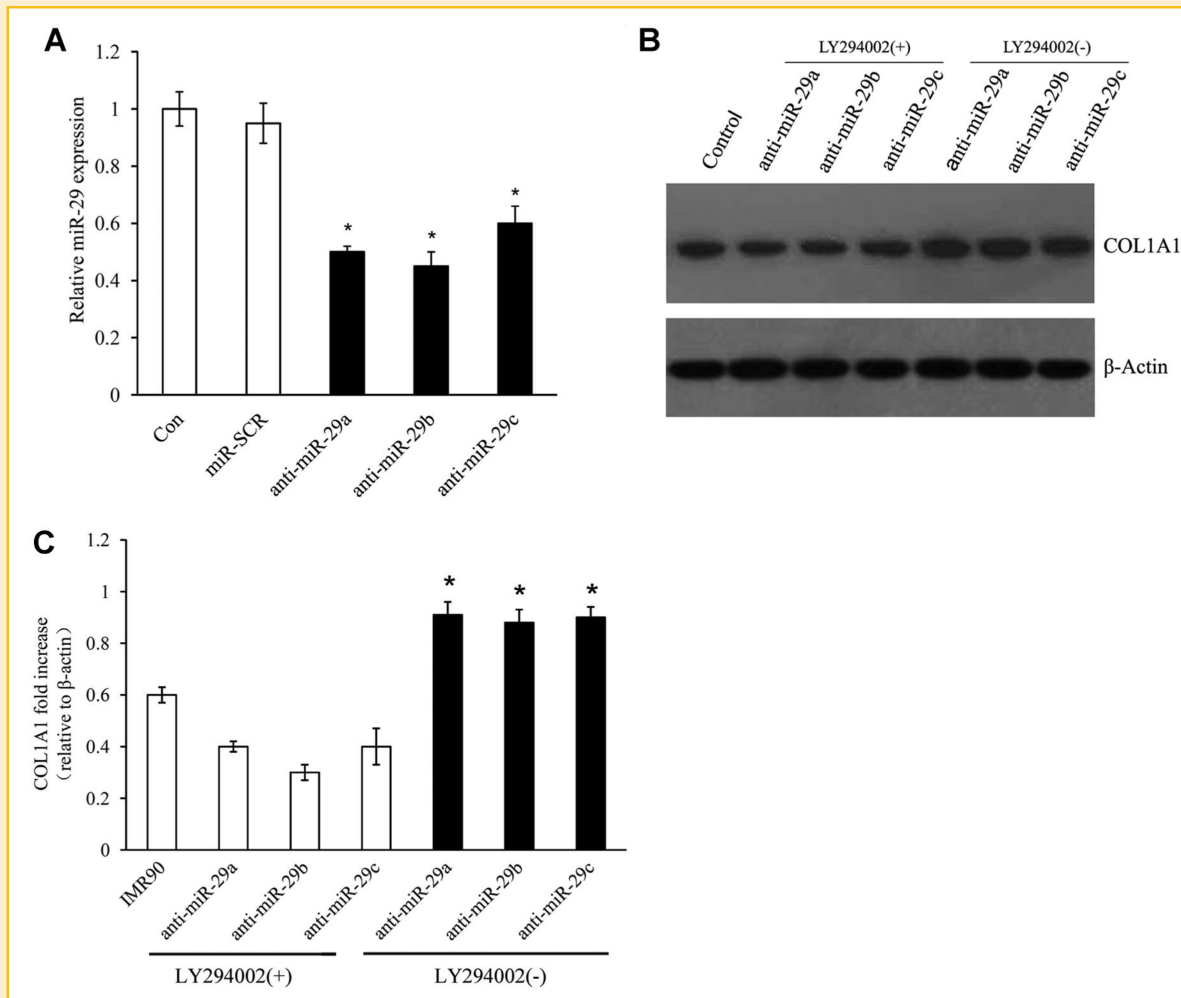

Fig. 5. miR-29 mediated expression of COL1A1 through PI3K/AKT signaling pathway in IMR-90 cells. A: IMR-90 cells were cultured in DMEM medium supplemented with 10% FBS and were transfected with 50 nM anti-miR-29a/b/c precursors, or a negative control, which had a scrambled sequence (control) using Lipofectamine 2000. Transfection of anti-miR-29a/b/c into IMR-90 suppressed miR-29a/b/c expression by real-time PCR. The asterisk indicates significant difference when compared to miR-SCR treated cells. ($P < 0.05$) (B) After 6 h, the medium was changed into DMEM containing 0.1% FBS with or without 10 ng/ml LY294002, and the culture was continued for another 24 h. Then, lysed, and analyzed by Western blot to determine expression levels of COL1A1. Results are representative of those in three repeated experiments. C: The intensities of the bands corresponding to COL1A1 were compared to those corresponding to β-actin. The asterisk indicates significant difference when compared to LY294002 treated cells ($P < 0.05$).

Several prior studies have demonstrated that the expression and activation of the miR-29 is tightly coupled to the effects of TGFβ1. For example, TGFβ1 has been reported to decrease expression of miR-29 in various cells including human liver fibroblasts, human epidermal keratinocytes, human cardiac fibroblasts, and skeletal muscle cells. Similar observations have been made with fibroblasts after treatment with TGFβ1 [Chu and Friedman, 2008; Thum et al., 2008; Van Rooij et al., 2008; Chung et al., 2010; Pandit et al., 2010]. We postulate that the decreased expression of miR-29 by TGFβ1 may lead to aberrant augmentation of ECM in human lung fibroblasts and play an important role in the formation of pulmonary fibrosis. A model to account for the role of miR-29 as a regulator of fibrosis is shown in Figure 6.

Because TGFβ1 is a central pathological mediator of lung fibrosis [Cutroneo et al., 2007; Moore and Hogaboam, 2008]. In this study, we demonstrated that the expression of all three miR-29 family members were repressed by TGFβ1 in IMR90 cell. Overexpressing miR-29 significantly ablated TGFβ1-induced proliferation of IMR90 cells. Soft agar assay on the same set of samples demonstrated similar results to the data by the MTT assay. Taken together, it suggests miR-29 is required for TGFβ1-induced cell proliferation and miR-29 regulates fibrosis by modulating TGFβ1 signaling pathway.

To understand the mechanism of miR-29 actions in regulating cell proliferation, we further investigated the signal transduction pathways involved in TGFβ1 reduced expression of miR-29. We found that TGFβ1 stimulated expression of COL1A1 and phosphorylation of PI3K and Akt. PI3K inhibitor reduced TGFβ1-induced the expression of COL1A1. On the contrary, transfecting with miR-29 mimics decreased the TGFβ1 induced expression of COL1A1. Our results showed that miR-29 reduced expression of COL1A1 and repressed phosphorylation of PI3K and Akt. It matches the previous

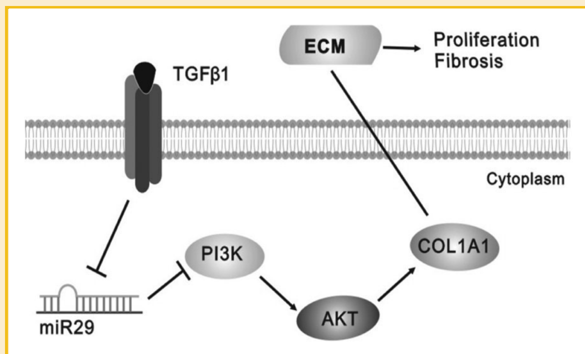

Fig. 6. A model for the role of miR-29 in IMR-90 cells. TGFβ1 down-regulated the expression of miR-29 and consequently up-regulated the phosphrylation of PI3K and AKT in IMR-90 cells. Moreover, miR-29 reduced the expression of COL1A1 (one of the ECM members) through inhibiting the phosphrylation of PI3K-AKT pathway. The process plays an important role in the proliferation of IMR-90 cells and the formation of pulmonary fibrosis.

report that miR-29 suppresses the p85γ regulatory subunit of PI3K [Park et al., 2009]. Thus, miR-29 mediates TGFβ1-induced ECM synthesis through PI3K-AKT pathway in human lung fibroblasts.

TGFβ1 could reduce expression of miR-29, and it did not mean TGFβ1 directly regulating miR-29 expression. Although miR-29 mediated ECM synthesis through PI3K-AKT pathway, it may not be the only pathway causing human lung fibroblasts proliferation due to miR-29. For example, Smad, MAPK, and Wnt pathways are also related to the process. Thus, deregulated expression of miR-29 could arise from aberrations in multiple critical signaling pathways involved in pulmonary fibrosis. Further studies are needed to investigate the signaling pathway intermediating between ligand-bound TGFβ1 and miR-29, and other signaling pathways of miR-29 mediating ECM synthesis.

In conclusion, TGFβ1 activated the synthesis of ECM by PI3K-Akt pathway, and this activation was essential for the expression of miR-29 in human lung fibroblasts. Our results indicate a novel biological function of the PI3K-Akt pathway in the proliferation of human lung fibroblasts. Elevated expression of miR-29 may play an important role in the pathogenesis of diseases related to fibrogenic reactions in human lung fibroblasts. These results also raise the possibility that specific inhibitors of the PI3K-Akt cascade may have beneficial effects in preventing pathogenic fibrosis in human lung fibroblasts.

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