Biochemical and Biophysical Research Communications 454 (2014) 512–517

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-26a modulates transforming growth factor beta-1-induced proliferation in human fetal lung fibroblasts

Xiaoou Li [a,b,1], Lian Liu [a], Yongchun Shen [a,b], Tao Wang [a,b], Lei Chen [a,b], Dan Xu [a,b], Fuqiang Wen [a,b,*]

[a] Division of Pulmonary Diseases, State Key Laboratory of Biotherapy of China, West China Hospital, West China School of Medicine, Sichuan University, Chengdu, Sichuan, China
[b] Department of Respiratory Medicine, West China Hospital, West China School of Medicine, Sichuan University, Chengdu, Sichuan, China

ARTICLE INFO

Article history:
Received 17 October 2014
Available online 27 October 2014

Keywords:
MicroRNAs
Lung fibroblasts
Proliferation
Cell cycle
Transforming growth factor beta one
Transforming growth factor beta receptors I

ABSTRACT

MicroRNA-26a is a newly discovered microRNA that has a strong anti-tumorigenic capacity and is capable of suppressing cell proliferation and activating tumor-specific apoptosis. However, whether miR-26a can inhibit the over-growth of lung fibroblasts remains unclear. The relationship between miR-26a and lung fibrosis was explored in the current study. We first investigated the effect of miR-26a on the proliferative activity of human lung fibroblasts with or without TGF-beta1 treatment. We found that the inhibition of endogenous miR-26a promoted proliferation and restoration of mature miR-26a inhibited the proliferation of human lung fibroblasts. We also examined that miR-26a can block the G1/S phase transition via directly targeting 3′-UTR of CCND2, degrading mRNA and decreasing protein expression of Cyclin D2. Furthermore, we showed that miR-26a mediated a TGF-beta 2-TGF-beta 1 feedback loop and inhibited TGF-beta R I activation. In addition, the overexpression of miR-26a also significantly suppressed the TGF-beta 1-interacting-CTGF–collagen fibrotic pathway. In summary, our studies indicated an essential role of miR-26a in the anti-fibrotic mechanism in TGF-beta1-induced proliferation in human lung fibroblasts, by directly targeting Cyclin D2, regulating TGF-beta R I as well as TGF-beta 2, and suggested the therapeutic potential of miR-26a in ameliorating lung fibrosis.

1. Introduction

Lung fibrosis is a disease that can be caused by abnormal repair after lung injury, chronic inflammation in the peribronchial space or idiopathic pulmonary fibrosis (IPF) [1,2]. Lung fibrosis is characterized by the irreversible proliferation of fibroblasts, which leads to a poor prognosis [3]. Additionally, activated lung fibroblasts secrete TGF beta family, which are believed to mediate pulmonary fibrogenesis by regulating cell proliferation, also inducing the expression of other pro-fibrotic factors such as CTGF, and promoting the deposition of ECM [4,5].

MicroRNAs (miRNAs) are a class of small non-coding conserved single-strand RNAs that are 21–26 nucleotides in length [6]. miRNAs function via RNA-induced silencing complexes (RISCs), which base-pair with complementary sequences in the 3′ untranslated regions (3′UTRs) of target genes in an imprecise way, leading to the degradation or repression of a target mRNA [7,8]. miRNAs have been found to be involved in multiple physiological and pathological processes, including organ development, cell proliferation and inflammation [9–11].

let-7 and miR-26 families have been found that are important for lung development, and these factors are enriched in adult human and mouse lung tissue [12]. Decreased expression of let-7 leads to poor survival, and the restoration of let-7 acts as a potent anti-fibrotic mediator by inhibiting cell growth [13]. Similar to the let-7 families, highly conserved small non-coding RNAs in the miR-26 family has the potential to modulate the anti-fibrotic process as well. Recent studies have confirmed that in cardiac fibrosis, the overexpression of mature miR-26a plays a dominant inhibitory role by interacting with the 3′UTR of the important pro-fibrotic genes-connective tissue growth factor (CTGF) and collagen type I, and prevents NF-kB activity in a negative feedback loop [14]. Furthermore, miR-26a is also identified to perform the anti-tumorigenic ability to suppress cell proliferation and to activate tumor-specific apoptosis in nasopharyngeal carcinoma and liver cancer [15,16].

However, the role of miR-26a in lung fibrosis remains unclear. In this study, we revealed the mechanism by which miR-26a prevents the proliferation of lung fibroblasts. We demonstrated that

* Corresponding author at: Division of Pulmonary Diseases, State Key Laboratory of Biotherapy of China, West China Hospital, West China School of Medicine, Sichuan University, Chengdu, Sichuan, China.
E-mail address: wenfuqiang.scu@gmail.com (F. Wen).
[1] Current address: Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA.

http://dx.doi.org/10.1016/j.bbrc.2014.10.106
0006-291X/

miR-26a modulated a TGF-beta 1-dependent pathway. Our data suggested that miR-26a can suppress cell growth via directly targeting Cyclin D2, inducing a G1 phase arrest. On the other hand, inhibition of endogenous miR-26a promoted the proliferation via increasing percentage of S phase of lung fibroblast. In addition, we showed that this process is through inhibiting TGF-beta 2 involved feedback loop to reduce the production of endogenous TGF-beta 1. Moreover, we first found that its indispensable receptors, TGF-beta R I, is also regulated by miR-26a. These findings reveal that miR-26a may act as an important modulator in lung fibrogenesis and is a potential therapeutic factor for the treatment of lung fibrosis.

2. Materials and methods

2.1. Cell culture

HFL-1 human primary fetal lung fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37 °C in a 5% CO_2 environment in DMEM (Invitrogen, Life Technologies, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (penicillin–streptomycin, Invitrogen).

2.2. Reagents and antibodies

Recombinant human TGF-beta 1 was purchased from PeproTech. Anti-TGF-beta R I (Santa Cruz Biotechnology, CA) and anti-TGF-beta 2 (Boster Biotech, China) primary antibodies were used for Western blotting. Anti-Cyclin D1, anti-Cyclin D2 primary antibodies were obtained from Cell Signaling Technology (Danvers, MA).

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated from lung fibroblasts using the EZNA total RNA Kit I purchased from Omega Bio-tek (Norcross, GA) and genomic DNA were removed during RNA processing. RNA was then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The amplification of the cDNA was performed in a 10 μl reaction system, processing 40 cycles including an initial denaturation at 95 °C for 10 min, 95 °C for 15 s and 60 °C for 30 s. The primers for qRT-PCR were synthesized by Invitrogen, and all the sequences of primers in the study are listed in Table S1.

2.4. Transfection

Synthesized negative control, miR-26a mimics, miR-26a inhibitor were purchased from RiboBio (Guangzhou, China). Fibroblasts were cultured in a 96-well or 6-well plate 24 h before transfection. Transfection was carried out according to the manufacturer's protocol using Lipofectamine 2000 (Invitrogen, CA) with or without 2.5 ng/ml TGF-beta 1.

Cell proliferation was determined by WST-8 formazan dye colorimetric assays using Cell Counting Kit-8 (Sigma–Aldrich, China), following the manufacturers' protocol. The absorbance value was measured at 450 nm using a microplate reader.

2.5. Flow cytometry

For cell cycle analysis, HFL-1 cells were treated as described for the cell proliferation assays. The cells were centrifuged at 800 rpm for 8 min, collected, washed twice with PBS and fixed with 70% ice-cold ethanol overnight at 4 °C. The ethanol was then removed, and the cells were incubated in 50 μg/ml propidium iodide (PI)-staining solution and RNase A for 30 min. The cell cycle distribution was determined with FACS using a flow cytometer.

2.6. Western blotting analysis

The cells were lysed and centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatants were collected. The protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Western blotting was performed as previously described [17]. The density of the bands was quantified using ImageJ software and normalized against GAPDH.

2.7. Construction of 3′ untranslated region reporter

The 3′-UTR of the human CCND2 gene was amplified by PCR from human genomic DNA. Primers for CCND2 3′-UTR were as follows: CCND2-F: 5′-GCGACTCGAGCTATACTTCTCCTTTGGACATGG AA-3′, CCND2-R: 5′-AATGCGGCCGCAAAATGAGAGACCCTTAGATTG TTTC-3′. The forward primer contains an XhoI restriction site, and the reverse primer includes a NotI restriction site. The miR-26a binding sites were mutated with the TaKaRa MutanBEST Kit. The PCR product were excised with XhoI and NotI to clone to pmiR-RB-REPORT™ vector (Guangzhou RuiBio Corp., Guangzhou, China) downstream from coding region of Renilla luciferase gene. The cloning was confirmed by sequencing.

2.8. Luciferase reporter assays

In each well, cells were transfected with 100 ng of wild type or mutant 3′-UTR plasmid alone or combination with 50 nM of either control or miR-26a mimic using lipofectamine 2000 (Invitrogen, Carlsbad, CA). 48 h after transfection, luciferase assay was performed using Dual-luciferase Reporter Assay System (Promega) according to the manufacture's instruction. The data recorded on the luminometer were analyzed by dividing renilla luciferase activity with that of firefly luciferase.

2.9. Statistical analysis

Data were presented as the mean ± SEM. All statistical analysis was conducted using SPSS (SPSS 17.0, Chicago, IL). The data were analyzed using 2-tailed, unpaired Student *t*-tests or one-way analysis of variance (ANOVA) followed by the LSD Test as the post hoc test. Differences between groups were considered significant if $p < 0.05$.

3. Results

3.1. miR-26a inhibits the TGF-beta1-induced growth of primary human lung fibroblasts

Firstly, HFL-1 cells were transfected with miR-26a inhibitor at 20 nM, 50 nM or 100 nM. 100 nM miR-26a inhibitor promoted the proliferation of HFL-1 at 48 h post transfection, showing a physiological function of endogenous miR-26a (Fig. 1A). To investigate the anti-fibrotic potential of miR-26a, we next evaluated the inhibition on proliferation activity of primary human lung fibroblasts. Briefly, HFL-1 fibroblasts were transiently transfected with 20 nM, 50 nM or 100 nM miR-26a or control miR. miR-26a significantly inhibited the baseline level of HFL-1 cells growth in a dose-dependent manner: 100 nM miR-26a showed inhibition effects at 24 h and both 50 nM and 100 nM miR-26a can suppresses HFL-1 cells growth at 48 h (Fig. 1B). Since TGF-beta 1 is the most potent protein to activate cell growth, we then confirmed

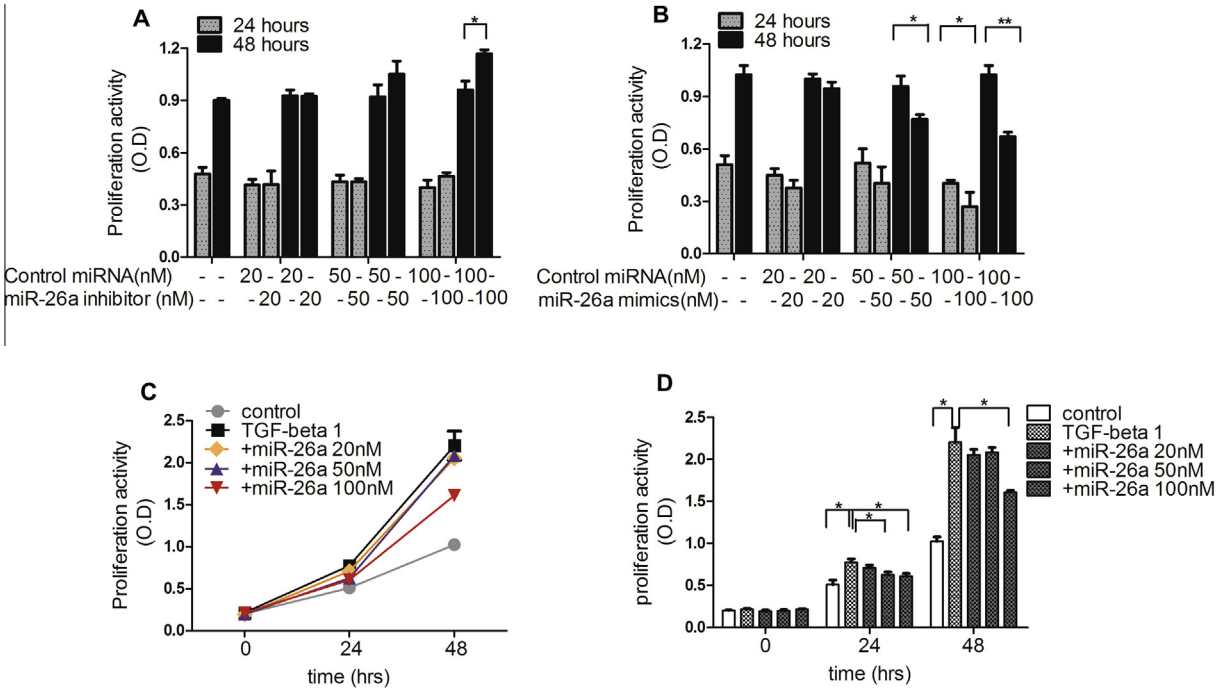

Fig. 1. miR-26a inhibits the TGF-beta 1-induced overgrowth of human lung fibroblasts. (A) miR-26a inhibitor suppressed endogenous miR-26a and promoted cell growth. HFL-1 cells were transfected with control miRNA or a miRNA inhibitor at 20 nM, 50 nM, 100 nM. At different time points (24 h and 48 h) after transfection, the cells were incubated with CCK8 solution for 1–4 h. The absorbance of the reactions was read at 450 nm. (B) miR-26a decreases human fetal lung fibroblasts (HFL-1) proliferation at baseline levels. HFL-1 cells were transfected with either a control miRNA or a miRNA-26a at 20 nM, 50 nM, 100 nM. The proliferation activity was measured as previously described. (C) Human lung fibroblasts were pretreated with different doses of miRNA-26a by transfection, and 2.5 ng/ml TGF-beta 1 was added to the medium to stimulate cell growth. Proliferation was measured after 24 and 48 h. The data were quantified in (D). (D) miR-26a inhibits TGF-beta 1 induced proliferation in a dose dependent manner. The experiment was repeated four times. The error bars represent the standard error. $^{*}p < 0.05$, $^{**}p < 0.01$.

whether miR-26a still perform anti-proliferation function with TGF-beta 1 stimulation. The data showed the effect of TGF-beta 1 was suppressed by 50 nM and 100 nM miR-26a (Fig. 1C and D). The results indicated that miR-26a inhibitor suppressed endogenous miR-26a effect and induced proliferation of lung fibroblasts. Furthermore, miR-26a can inhibit human lung fibroblast growth at baseline and with TGF-beta 1 treatment.

3.2. miR-26a regulated the G1/S phase transition via directly targeting TGF-beta 1 activated CCND2

We then examined how miR-26a can inhibit the proliferation of lung fibroblasts. To address this issue, we measured the cell cycle distribution of the HFL-1. Interestingly, we found that inhibition of endogenous miR-26a significantly decreased the percentage of G1 phase and slightly increased percentage of G2/M and S phase when compared to control group (Fig. 2A). Conversely, the overexpression of miR-26a shifted the cell cycle distribution from S phase to G1 phase, with a reduced percentage of G2/M phase cells compared to negative control (Fig. 2B).

Because the G1/S-phase was regulated by miR-26a inhibitor and blocked by mature miR-26a restoration, the related essential cell cycle regulators may be involved in the process. Thus we investigated the relationship between cell cycle-related genes and miR-26a. The cells were transiently transfected with miR-26a in the presence or absence of TGF-beta 1. The mRNA expressions of the Cyclin family (CCND2 and CCND1) were determined. We found that the mRNA level of CCND2 (Cyclin D2) was down-regulated significantly by miR-26a, and was lower than baseline with or without TGF-beta 1 stimulation (Fig. 3C). However, miR-26a seemed to promote the transcription of CCND1 (Cyclin D1) mRNA (Fig. 3C). Correspondingly, according to the bioinformatic software (miRBASE and TargetScan algorithms), CCND2 but not CCND1 was predicted to be the essential target of miR-26a. As shown in Fig. 2D, miR-26a and its putative target site on 3′-UTR of CCND2 showed highly complementary sequence. Then we constructed reporter plasmids containing wild type or mutant of 3′-UTR of CCND2 and co-transfected the plasmids with or without miR-26a into HEK 293 cells. 48 h after transfection, luciferase activity was measured. Binding site mutant prevented endogenous miR-26a binding to 3′-UTR of CCND2 and showed increased luciferase activity. Furthermore, overexpression of miR-26a only inhibited the luciferase activity of wild type but not mutant 3′-UTR of CCND2 reporter gene. The results supported that the 3′-UTR of CCND2 transcript is the direct target of miR-26a (Fig. 2E). We also measured the protein level of Cyclin D2 and Cyclin D1 with miR-26a incubation. miR-26a significantly down-regulated TGF-beta 1-induced protein expression of Cyclin D2 and Cyclin D1 (Fig. 2F–H). Since there is no binding site of matured miR-26a on 3′-UTR of CCND1, there may be a post-translational regulation involved in the process. The above findings indicated that miR-26a can inhibited the transition from G1 to S phase in human lung fibroblast by directly targeting 3′-UTR of CCND2, inducing degradation of mRNA of CCND2 and down-regulation of protein level of Cyclin D2.

3.3. miR-26a down-regulates indispensable receptor TGF-beta receptor type I and TGF-beta 2 in TGF-beta 1 induced proliferative pathway

TGF-beta 1 is known to play a strong pro-fibrotic role via combination with TGF-beta receptors in fibrogenesis in lung disease. But the TGF-beta precursor protein is inactive until it binds to specific surface receptors recognized as TGF-beta receptor I, II, and III (TGFBRI, TGFBRII, TGFBRIII). According to above results, we

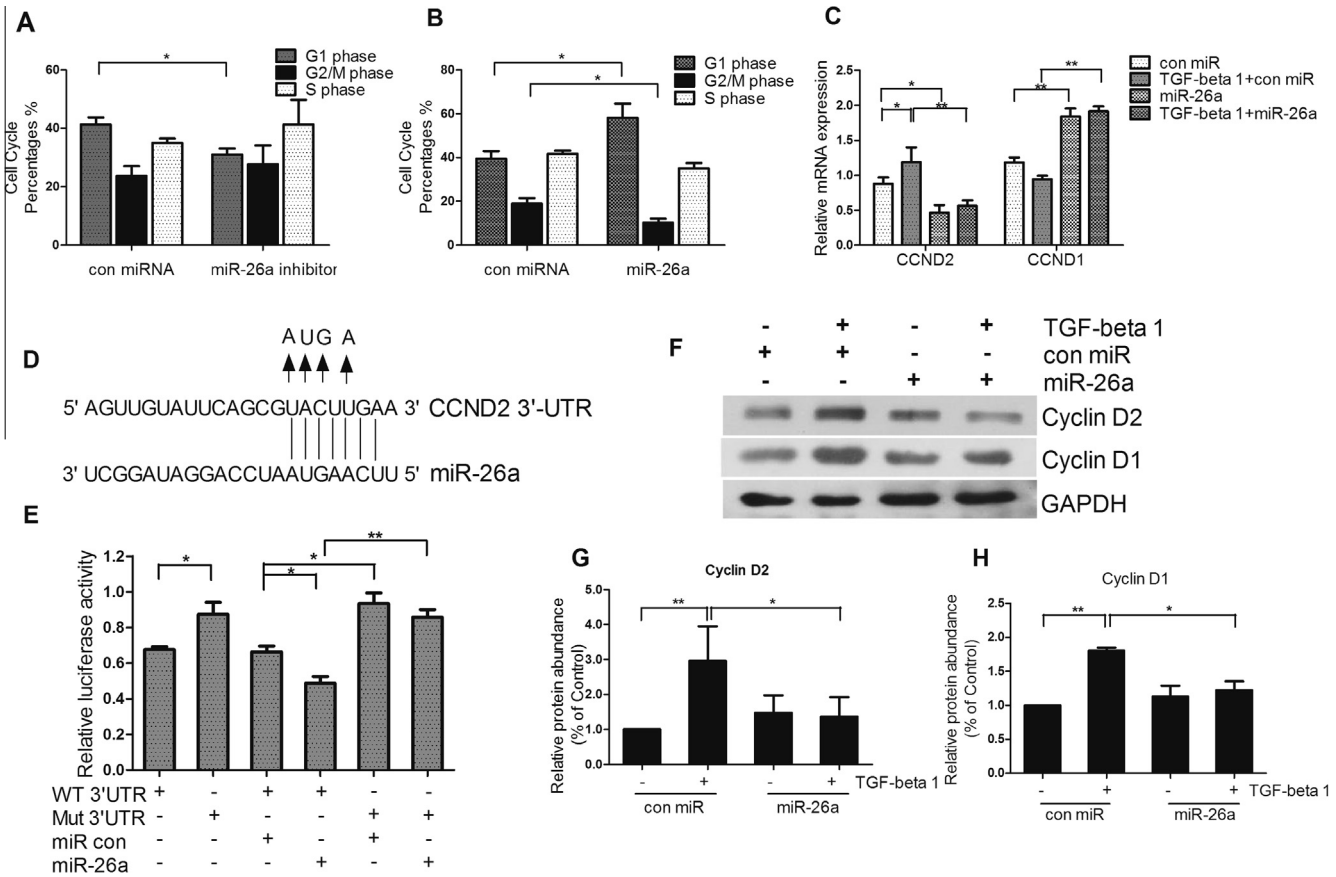

Fig. 2. miR-26a modulates the fibroblast cell cycle and the TGF-beta 1-induced expression of the Cyclin family. (A) Human lung fibroblasts were cultured and transfected with miRNA-26a inhibitor as previously described. After 48 h, the cells were collected, fixed, and stained with PI. The cell cycle distribution was determined using a flow cytometer. (B) Human lung fibroblasts were cultured and transfected with miRNA-26a as previously described. The cell cycle distribution was determined using a flow cytometer. (C) The cells were treated with control miR or miR-26a and then with or without 2.5 ng/ml TGF-beta 1 stimulation. The mRNA expression of CCND2 and CCND1 were detected and calculated by normalizing to B2M. (D) CCND2 is the direct target of miR-26a. Putative miR-26a binding site in 3′-UTR of CCND2 and nucleotide changes of binding site mutants were displayed. (E) HEK 293 cells were transiently co-transfected with CCND2 3′-UTR reporter plasmid with or without miR-26a. The ratio of renilla luciferase (reporter) to firefly luciferase (control) in relative luminescence unit was normalized. (F) HFL-1 cells were cultured as previously described. The samples were harvested, and protein was isolated. The expression level of Cyclin D2 and Cyclin D1 and GAPDH were detected by Western blotting. (G, H) The density of the bands corresponding to the above proteins were quantified and normalized against GAPDH. The experiment was repeated six times. The error bars represent the standard error. $^{*}p < 0.05$, $^{**}p < 0.01$.

presume there three specific receptors may participate in TGF-beta 1 induced proliferation. We investigated whether miR-26a regulate TGF-beta receptors. Interestingly, TGFBRI (Fig. 3A–C), but not TGFBRII and TGFBRIII (Fig. 3D and E), was down-regulated at both mRNA and protein level by enhanced miR-26a expression under TGF-beta 1 stimulation .The above results showed that inhibition of miR-26a on TGF-beta 1-induced proliferation may go through regulation of TGF-beta receptor I.

We previously showed that TGF-beta 2 is involved in TGF-beta 1 production in a feedback loop [18]. The reduction of TGF-beta 2 may down-regulate TGF-beta 1 levels. Therefore, in this study, we also evaluated effect of miR-26a on TGF-beta 2 expression. Our data showed that miR-26a dramatically suppresses TGF-beta 2 expressions at the transcriptional and protein levels (Fig. 3F–H). Here we indicate that TGF-beta 2 involved feedback loop is weakened by miR-26a.

3.4. Enhanced miR-26a mediates TGF-beta 1-induced pro-fibrotic factors and ECM deposition

In addition to TGF-beta 1, several essential factors also contribute to lung fibrosis. Recent studies addressed the roles of collagen, CTGF and CD44 in regulating ECM and EMT production. We then tested whether the expression of miR-26a can influence the transcription of these genes. We observed that collagen mRNA increased in cells treated with TGF-beta 1, and can be prevented by restoring miR-26a (Fig. 4A). Compared to control, CTGF mRNA expression was dramatically reduced in the miR-26a group (Fig. 4B), but miR-26a had no effect on CD44 mRNA levels (Fig. 4C). To conclude, these results showed that miR-26a may mediate TGF-beta 1-induced ECM deposition as well.

4. Discussion

More than 1800 miRNAs encoded by the human genome have been identified from miRNA registration database (mirbase.org). Accumulating evidence shows that various miRNAs are abnormally expressed in fibrosis [19]. In particular, a number of miRNAs and families, including let-7d, miR-200 and miR-29 families perform anti-fibrotic effects by regulating epithelial–mesenchymal transition (EMT), degrading ECM and inhibiting the growth of fibroblasts/myofibroblasts [20–22]. For example, miR-29, a downstream element of TGF-beta 1/smad3, can reduce TGF-beta 1 and CTGF expression as well as Smad3 signaling and prevent bleomycin-induced pulmonary fibrosis [20] .These findings reveal the essential role of miRNAs in the modulation of fibrosis.

miRNA-26a was first discovered based on its participation in tumorigenesis. Decreased miR-26a expression leads to a poor

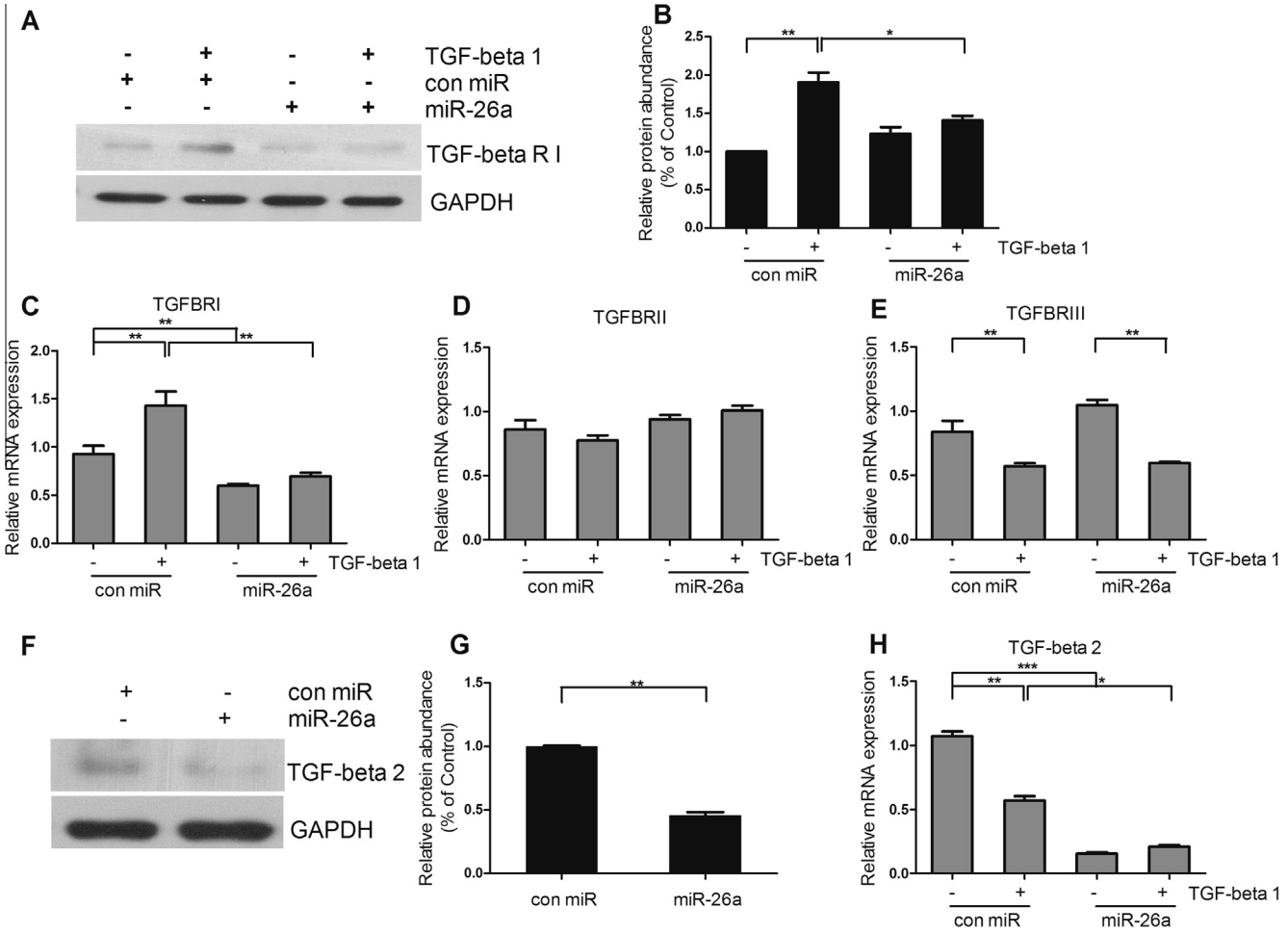

Fig. 3. The expressions of TGF-beta receptors I and TGF-beta 2 were down-regulated by miR-26a. (A) HFL-1 cells were treated with control miR or miR-26a with or without 2.5 ng/ml TGF-beta 1 stimulation. Cell extracts were analyzed using Western blotting with specific TGF-beta receptor I and GAPDH antibodies. (B) Bands density were quantified and normalized against GAPDH. (C–E) mRNA expression of TGFBRI, TGFBRII and TGFBRIII were detected by qRT-PCR and normalized to B2M as housekeep gene. (F) HFL-1 cells were treated with control miR or miR-26a for 48 h. Cell extracts were analyzed using Western blotting with specific TGF-beta 2 and GAPDH antibodies. (G) Bands density were quantified and normalized against GAPDH. (H) mRNA expression of TGF-beta 2 was detected using qRT-PCR and normalized to B2M. The experiment was repeated six times. The error bars represent the standard error. $^{*}p < 0.05$, $^{**}p < 0.01$.

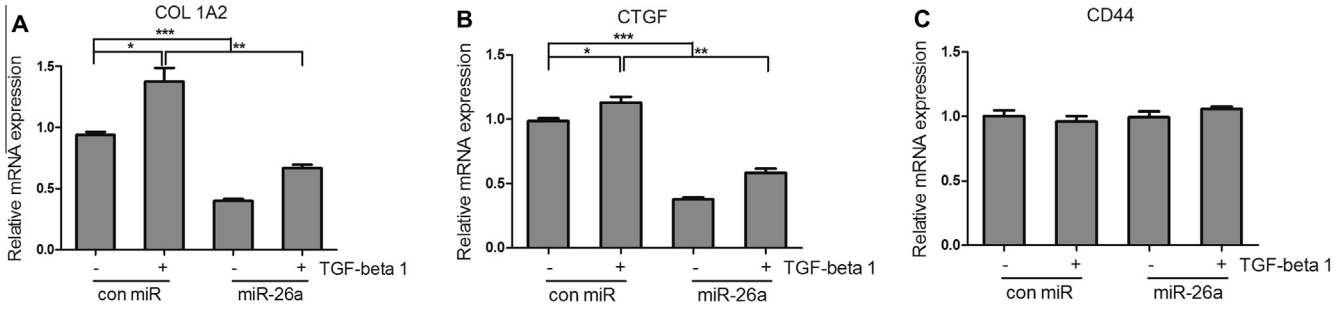

Fig. 4. Enhanced miR-26a mediates pro-fibrotic factors and ECM related genes. Human lung fibroblasts were cultured in six-well plates one day before transfection. Then miR-26a was transfected into the cells, and the cells were treated with or without 2.5 ng/ml TGF-beta 1. The cells were collected and RNA was extracted. The mRNA expressions of COL 1A2 (A), CTGF (B) and CD44 (C) were detected using qRT-PCR and normalized to B2M. The experiment was repeated six times. The error bars represent the standard error. $^{*}p < 0.05$, $^{**}p < 0.01$.

prognosis in osteosarcoma patients [23]. miRNA-26a expression levels are lower in human nasopharyngeal carcinoma (NPC) specimens, and its overexpression significantly reduces cell-cycle related enhancer of EZH2 and Cyclin D2 [16]. Recent studies have reported the strong ability of miRNA-26a to inhibit cell proliferation, migration and invasion, but whether miR-26a is involved in the growth of lung fibroblasts remains mysterious.

Therefore, in present study, we explored the relationship between miR-26a and lung fibrosis. Based on the suppressive role of miRNA-26a on cell proliferation in various cancer cell lines, we first evaluated cell growth and cell cycle transitions in primary lung fibroblasts transfected with mature miR-26a or miR-26a inhibitor. Our miR-26a inhibitor result demonstrated the physiological role of endogenous miR-26a in mediating cell growth of

lung fibroblast. Conversely, miR-26a inhibits HFL-1 cell growth by shifting a high S-phase population into G1-phase. A recent study also showed miR-26a blocked the G1/S phase in adenocarcinoma cell lines and induced apoptosis [24]. To unravel the molecular mechanism underlying this effect, we examined the expression of essential mediators that are related to cell cycle under TGF-beta 1 stimulation. In our study Cyclin D2 was strongly suppressed by miRNA-26a at the post-transcriptional and protein levels. Furthermore, we confirmed that miR-26a degraded mRNA of CCND2 via directly targeting 3′-UTR of CCND2 mRNA. Our findings suggested that inhibiting effect of miR-26a on proliferation of lung fibroblast depends on down-regulation of Cyclin D family. Consistent findings have also been reported recently [25].

TGF-beta superfamily is a class of dimeric polypeptide growth factor, that is mainly produced by activated fibroblasts, myofibroblasts and epithelial cells [18,26]. Excessive TGF-beta 1 secretion is the predominate regulator of fibrogenesis in a spectrum of diseases including lung fibrosis. It is well known that TGF-beta 1 precursor protein cannot perform multiple functions until binding to indispensable receptors [27]. A recent study noted that miR-21 can attenuate the TGF-beta-activated Smad2/3 pathway in prostate cancer by targeting TGFBRII [28], and this finding drove us to wonder whether miR-26a regulate TGF-beta receptors. Here we showed miR-26a inhibits TGFBRI expression, but does not affect TGFBRII and TGFBRIII. Our previous research demonstrated that TGF-beta 2 induced the release of abundant TGF-beta 1 in human lung fibroblasts, leading to a higher TGF-beta 2 concentration, forming a positive feedback loop in an autocrine or paracrine manner, and contributing to fibrotic progression [18]. Interestingly, in this study, we found that increased miR-26a inhibited TGF-beta 2 and down-regulated its expression. Our results imply that the inhibition of this feedback loop by miR-26a may reduce endogenous TGF-beta 1 levels. Although we found that miR-26a significantly inhibited TGF-beta receptor I and TGF-beta 2 participated TGF-beta 1 pathway, there is no predicted miR-26a binding site in conserved 3′-UTR sequence of either TGFBRI or TGF-beta 2. Therefore, we thought miR-26a may regulate TGFBRI and TGF-beta 2 through other indirectly mechanism. To conclude, we think miR-26a not only suppress TGF-beta 1–TGF beta receptor I–Cyclin D pathway, but also regulate TGF-beta 1 production via TGF-beta 2 involved feedback loop, and therefore participating in anti-fibrotic progression (Fig. S1).

Another hallmark of TGF-beta 1-induced lung fibrosis is the aberrant accumulations of extracellular matrix, predominantly collagens [4]. TGF-beta 1 usually interacts with CTGF to promote collagen production. We found that increasing miR-26a prevented TGF-beta 1-induced collagen deposition and CTGF expression. These results suggested miR-26a may also carry out its anti-fibrotic activity by inhibiting the TGF-beta-1-CTGF-collagen pathway. Correspondingly, miR-26a has been shown that can directly targets 3′-UTR of CTGF and collagen I and is involved in an Ang II-mediated negative feedback loop of NF-kB in cardiac fibrosis [14]. In addition, we also measured the essential EMT-related gene CD44. However, miR-26a had no effect on the expression of CD44.

In summary, our studies suggest that miR-26a may have anti-fibrotic properties in the inhibition of proliferation of lung fibroblasts by regulating TGF-beta 1 induced cell cycle and the TGF-beta 2, indicating the therapeutic potential of miR-26a in ameliorating lung fibrosis.

Acknowledgments

This study was supported by grants 31171103 and 8123001 from the National Natural Science Foundation of China to Dr. F.Q. Wen. The authors acknowledge the support of the members of the lab. The authors also acknowledge to Dr. F. Guo for comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.106.

References

[1] M. Kolb, P.J. Margetts, D.C. Anthony, et al., Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis, J. Clin. Invest. 107 (2001) 1529–1536.
[2] T.J. Gross, G.W. Hunninghake, Idiopathic pulmonary fibrosis, N. Engl. J. Med. 345 (2001) 517–525.
[3] A.L. Katzenstein, J.L. Myers, Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification, Am. J. Respir. Crit. Care Med. 157 (1998) 1301–1315.
[4] A. Leask, D.J. Abraham, TGF-beta signaling and the fibrotic response, FASEB J. 18 (2004) 816–827.
[5] T.J. Broekelmann, A.H. Limper, T.V. Colby, et al., Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 6642–6646.
[6] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[7] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233.
[8] J. Krol, I. Loedige, W. Filipowicz, The widespread regulation of microRNA biogenesis, function and decay, Nat. Rev. Genet. 11 (2010) 597–610.
[9] D. Sayed, M. Abdellatif, MicroRNAs in development and disease, Physiol. Rev. 91 (2011) 827–887.
[10] J.F. Chen, E.M. Mandel, J.M. Thomson, et al., The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation, Nat. Genet. 38 (2006) 228–233.
[11] M.M. Perry, S.A. Moschos, A.E. Williams, et al., Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells, J. Immunol. 180 (2008) 5689–5698.
[12] A.E. Williams, S.A. Moschos, M.M. Perry, et al., Maternally imprinted microRNAs are differentially expressed during mouse and human lung development, Dev. Dyn. 236 (2007) 572–580.
[13] X. Hu, J. Guo, L. Zheng, et al., The heterochronic microRNA let-7 inhibits cell motility by regulating the genes in the actin cytoskeleton pathway in breast cancer, Mol. Cancer Res. 11 (2013) 240–250.
[14] C. Wei, I.K. Kim, S. Kumar, et al., NF-kappaB mediated miR-26a regulation in cardiac fibrosis, J. Cell. Physiol. 228 (2013) 1433–1442.
[15] X. Yang, L. Liang, X.F. Zhang, et al., MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6–Stat3 pathway, Hepatology 58 (2013) 158–170.
[16] J. Lu, M.L. He, L. Wang, et al., MiR-26a inhibits cell growth and tumorigenesis of nasopharyngeal carcinoma through repression of EZH2, Cancer Res. 71 (2011) 225–233.
[17] T. Yang, F. Luo, Y. Shen, et al., Quercetin attenuates airway inflammation and mucus production induced by cigarette smoke in rats, Int. Immunopharmacol. 13 (2012) 73–81.
[18] F.Q. Wen, T. Kohyama, C.M. Skold, et al., Glucocorticoids modulate TGF-beta production by human fetal lung fibroblasts, Inflammation 27 (2003) 9–19.
[19] K.V. Pandit, J. Milosevic, N. Kaminski, MicroRNAs in idiopathic pulmonary fibrosis, Transl. Res. 157 (2011) 191–199.
[20] J. Xiao, X.M. Meng, X.R. Huang, et al., MiR-29 inhibits bleomycin-induced pulmonary fibrosis in mice, Mol. Ther. 20 (2012) 1251–1260.
[21] S. Yang, S. Banerjee, A. de Freitas, et al., Participation of miR-200 in pulmonary fibrosis, Am. J. Pathol. 180 (2012) 484–493.
[22] K.V. Pandit, D. Corcoran, H. Yousef, et al., Inhibition and role of let-7d in idiopathic pulmonary fibrosis, Am. J. Respir. Crit. Care Med. 182 (2010) 220–229.
[23] Q.C. Song, Z.B. Shi, Y.T. Zhang, et al., Downregulation of microRNA-26a is associated with metastatic potential and the poor prognosis of osteosarcoma patients, Oncol. Rep. 31 (2014) 1263–1270.
[24] X. Dang, A. Ma, L. Yang, et al., MicroRNA-26a regulates tumorigenic properties of EZH2 in human lung carcinoma cells, Cancer Genet. 205 (2012) 113–123.
[25] J. Kota, R.R. Chivukula, K.A. O'Donnell, et al., Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model, Cell 137 (2009) 1005–1017.
[26] N. Khalil, R.N. O'Connor, K.C. Flanders, et al., TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study, Am. J. Respir. Cell Mol. Biol. 14 (1996) 131–138.
[27] J. Massague, TGFbeta signalling in context, Nat. Rev. Mol. Cell Biol. 13 (2012) 616–630.
[28] S. Mishra, J.J. Deng, P.S. Gowda, et al., Androgen receptor and microRNA-21 axis downregulates transforming growth factor beta receptor II (TGFBR2) expression in prostate cancer, Oncogene 33 (2014) 4097–4106.