Department of Emergency, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

MicroRNA-33a deficiency inhibits proliferation and fibrosis through inactivation of TGF-β/Smad pathway in human cardiac fibroblasts

Bo Yu#, Wei Li#, Fen Ai, Zhen Chen*

Received April 7, 2017, accepted April 14, 2017

**Corresponding author: Zhen Chen, Department of Emergency, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science & Technology, No. 16, Gusaoshu Road, Jianghan District, Wuhan 430014, Hubei Province, China*
chenzhen113@126.com
#These authors contributed equally to the work.

Pharmazie 72: 456–460 (2017) *doi: 10.1691/ph.2017.7561*

Cardiac fibroblasts (CFBs) play pivotal roles in myocardial fibrosis, which is the leading cause of arrhythmia. This study was aimed to investigate the modulation of microRNA (miR)-33a on proliferation, apoptosis and fibrosis of human CFBs. CFBs were respectively transfected with miR-control, miR-33a mimic or miR-33a inhibitor, followed by induction of transforming growth factor-β (TGF-β). Non-treated CFBs acted as control. Cell viability, apoptosis, and fibrosis which reflected by expressions of Col-I, Col-III and α-smooth muscle actin (α-SMA) were evaluated by CCK-8 assay, flow cytometry, qRT-PCR and Western blot analysis. Finally, key kinases involved in the TGF-β/Smad pathway were evaluated by Western blot analysis. TGF-β enhanced CFB viability, and expression levels of Col-I, Col-III and α-SMA in CFBs ($P < 0.01$ or $P < 0.001$). The increased CFB proliferation, and upregulation of Col-I, Col-III and α-SMA were all further enhanced by miR-33a mimic ($P < 0.05$ or $P < 0.001$), whereas reversed by miR-33a inhibitor ($P < 0.05$, $P < 0.01$ or $P < 0.001$). The CFB apoptosis was remarkably promoted by miR-33a inhibitor ($P < 0.001$). Results of signaling pathway showed that phosphorylated levels of Smad-2 and Smad-3 were both upregulated by TGF-β ($P < 0.001$). The upregulated phosphorylations were further improved by miR-33 mimic ($P < 0.05$) while reversed by miR-33a inhibitor ($P < 0.05$ or $P < 0.001$). miR-33a deficiency inhibits proliferation and fibrosis of CFBs while promotes CFB apoptosis by inactivation of TGF-β/Smad pathway.

1. Introduction

Cardiovascular disease (CVD) is the most common cause of death, which causes two times as many deaths as cancer in Europe (Townsend et al. 2015). Sudden death, mainly caused by cardiac arrhythmias accounts for nearly half of the deaths from CVD (Huikuri et al. 2002). Characterized by accumulation of fibrillary collagen in the heart, cardiac fibrosis contributes to the progression of arrhythmias and might finally lead to death (Du et al. 2009). In myocardium, there are multiple cell types, including cardiomyocytes, cardiac fibroblasts (CFBs), endothelial cells and smooth muscle cells (Coulombe et al. 2014). Among these cells, CFBs account for the largest cell population (Camelliti et al. 2005). In response to risk factors including volume/pressure overload, cardiac injury, genetic susceptibility and age, CFBs are activated and differentiated into myofibroblasts (myoFBs), which is rarely found in healthy myocardium but appears after cardiac injury (Baum and Duffy 2011; Petrov et al. 2002). The process is accompanied by a massive production of extracellular matrix (ECM), resulting in cardiac fibrosis (Chan et al. 2010). CFBs are most conspicuously linked to heart disease (Gourdie et al. 2016; Iwata et al. 2011).

MicroRNAs (miRNAs) are a kind of small non-coding RNAs which function through inhibition of target gene expression or degradation of mRNAs (Qin et al. 2016). Large numbers of investigations have demonstrated that miRNA (miR)-125b (Nagpal et al. 2016), miR-133a (Chen et al. 2014), miR-101a (Zhao et al. 2015) are involved in cardiac fibrosis. The encoding region of miR-33a is embedded in the sterol-response-element-binding protein gene (SREBP2), and inhibition of miR-33a is reported to raise plasma high-density lipoprotein and lower very low-density lipoprotein for the treatment of CVD (Rayner et al. 2011). Huang et al. (2014) once reported the miR-33a could be a marker for liver fibrosis. Moreover, the level of miR-33a was significantly increased in liver and serum with a progressing process of hepatic fibrosis (Huang et al. 2015). Thus, we hypothesized the miR-33a might be a potential modulator of cardiac fibrosis. However, there are few related investigations focusing on the role of miR-33a in modulation of CFBs, and the underlying mechanism remains unclear.

The cost of heart diseases linked to fibrosis is staggering. Thus, it becomes urgent at both clinical and economic cases for development of novel and effective therapeutic approaches for cardiac disease linked to fibrosis (Gourdie et al. 2016). Transforming growth factor β (TGF-β) plays a crucial role in cardiac fibrosis by activation of CFBs and production of collagen (Annes et al. 2003). A recent study has reported that TGF-β is a potential therapeutic target for cardiac fibrosis (Leask 2010). As a consequence, in your study, we chose cardiac fibroblasts to deeply investigate the modulation of miR-33a in TGF-β induced proliferation and fibrosis of CFBs. The underlying molecular and cellular mechanisms were also explored.

2. Investigations and results

2.1. TGF-β promotes cell proliferation and fibrosis in CFBs

To investigate the influence of TGF-β, CFBs were assigned into two groups - control group, in which cells were cultured without treatment; and TGF-β group, in which cells were cultured with 10 ng/mL of TGF-β. After stimulation for 48 h, cell viability and mRNA

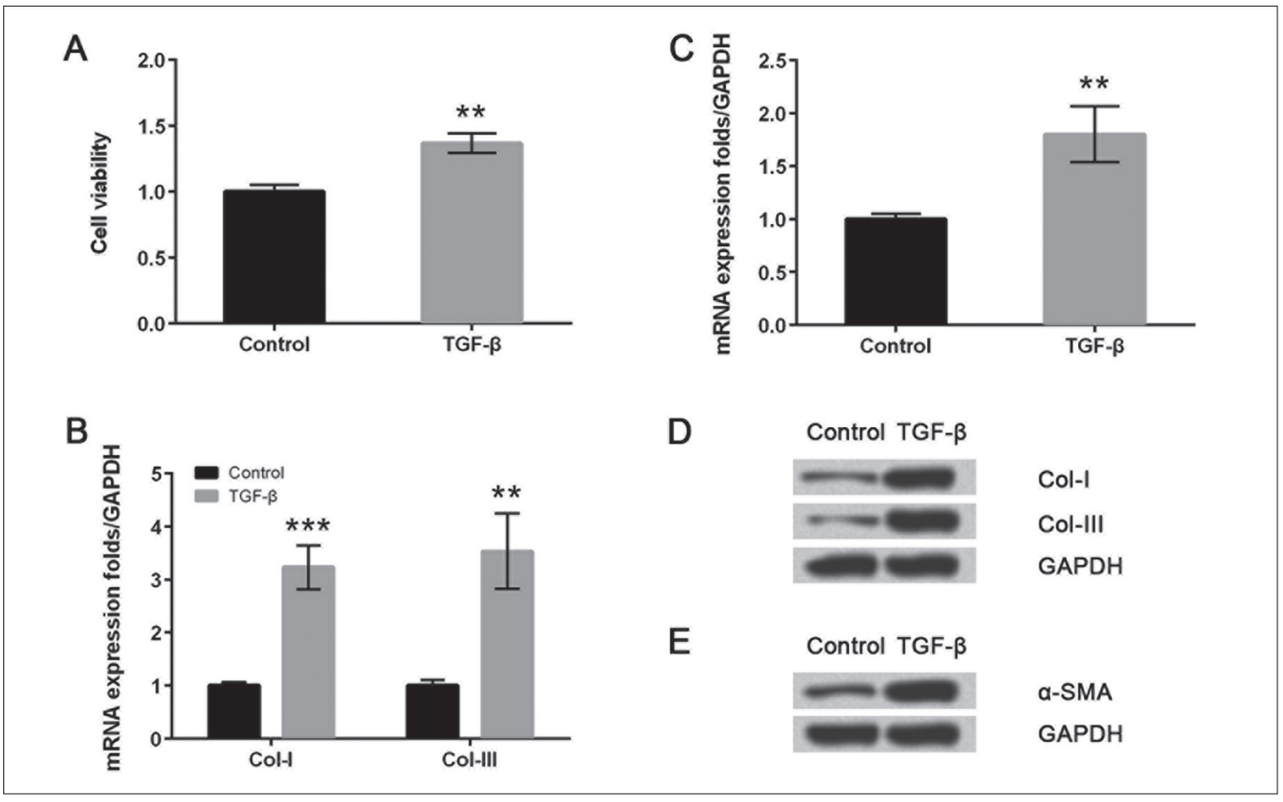

Fig. 1: The effect of TGF-β on viability and fibrosis of human CFBs. A, cell viability; B, mRNA expression levels of Col-I and Col-III; C, mRNA expression level of α-SMA; D, protein expression levels of Col-I and Col-III; E, protein expression level of α-SMA. Cells were divided into two groups-control group, cells in which cultured without treatment; TGF-β group, cells in which cultured with TGF-β. Data presented were the mean of at least three independent experiments. Error bars indicate SD. **, $P < 0.01$; ***, $P < 0.001$. TGF-β, transforming growth factor-β; CFB, cardiac fibroblast; Col-I, collagen I; Col-III, collagen III; α-SMA, α-smooth muscle actin.

expression levels of Col-I, Col-III and α-SMA were respectively assessed by CCK-8 assay and qRT-PCR. In Fig. 1A, cell viability was significantly improved by TGF-β when compared to control ($P < 0.01$). As shown in Fig. 1B-1E, both mRNA and protein expression levels of Col-I, Col-III and α-SMA were remarkably upregulated by TGF-β when compared to control ($P < 0.01$ or $P < 0.001$). Thus, we demonstrated that TGF-β could promote proliferation and fibrosis of CFBs.

2.2. miR-33a deficiency inhibits TGF-β induced proliferation of CFBs

In order to explore the functional role of miR-33a in CFB proliferation, CFBs were firstly transfected with miR-control, miR-33a mimic or miR-33a inhibitor. Forty-eight hours later, the cells were induced by 10 ng/mL TGF-β and cell viability was determined by CCK-8 assay at 1, 2, 3 and 4 d after induction. Cells cultured without any treatment acted as control. In Fig. 2, viability of CFBs in TGF-β group was obviously increased compared with control group at 2-4 days ($P < 0.01$). When compared with TGF-β+miR-control group, viability of cells in TGF-β+miR-33a inhibitor group was remarkably decreased at 2-4 days after induction ($P < 0.001$). Oppositely, viability of cells in TGF-β+miR-33a mimic group was remarkably increased at 3-4 days after induction ($P < 0.001$). Thus, we indicated miR-33a deficiency inhibited TGF-β induced proliferation of CFBs.

2.3. miR-33a deficiency promotes apoptosis of CFBs

After transfection and stimulation, CFBs were assigned into five groups as described above. Cell apoptosis was assessed by flow cytometry. Data in Fig. 3 showed a significant enhancement of apoptotic cells in TGF-β+miR-33a inhibitor group in comparison with TGF-β+miR-control group ($P < 0.001$). The results suggested that miR-33a deficiency promoted CFB apoptosis.

2.4. miR-33a deficiency suppresses TGF-β induced fibrosis of CFBs

After transfection and stimulation, CFBs were assigned into five groups as described above. mRNA and protein expressions were respectively assessed by qRT-PCR or Western blot analysis. In Fig. 4A-4D, both mRNA and protein expression levels of Col-I, Col-III and α-SMA were significantly upregulated by TGF-β in comparison with control group ($P < 0.001$). The upregulation was

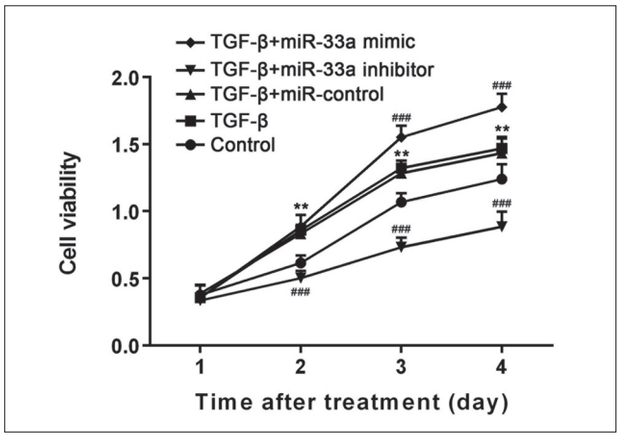

Fig. 2: The effect of miR-33a on proliferation of human CFBs. After transfection with miR-control, miR-33a mimic or miR-33a inhibitor and stimulation with TGF-β, cells were divided into five groups. Cells without any treatment served as control. Cell proliferation was measured by CCK-8 assay. Data presented were the mean of three independent experiments. Error bars indicated SD. * indicates significant difference compared with control group. **, $P < 0.01$; # indicates significant difference compared with TGF-β+miR-control group. ###, $P < 0.001$. TGF-β, transforming growth factor-β; CFB, cardiac fibroblast.

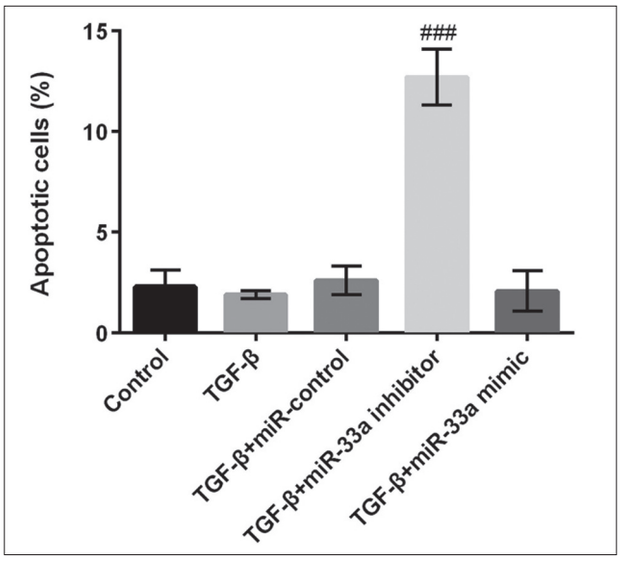

Fig. 3: The effect of miR-33a on apoptosis of human CFBs. After transfection with miR-control, miR-33a mimic or miR-33a inhibitor and stimulation with TGF-β, cells were divided into five groups. Cells without any treatment served as control. Cells without any treatment and cells stimulated with TGF-β served as controls. Cell apoptosis was measured by flow cytometry. Data presented were the mean of three independent experiments. Error bars indicated SD. # indicates significant difference compared with TGF-β+miR-control group. ###, $P < 0.001$. TGF-β, transforming growth factor-β; CFB, cardiac fibroblast.

further enhanced by miR-33a mimic, resulting in a significant increase in comparison with TGF-β+miR-control group ($P < 0.05$). However, the upregulation was obviously reduced by miR-33a inhibitor, resulting in a significant decrease in comparison with TGF-β+miR-control group ($P < 0.05$ or $P < 0.01$). Therefore, we concluded that miR-33a deficiency suppressed TGF-β induced fibrosis of CFBs.

2.5. miR-33a deficiency inactivated the TGF-β/Smad signaling pathway

The expressions of key kinases involving in TGF-β/Smad signaling pathway were evaluated by Western blot analysis. The CFBs were also assigned into five groups as described above after transfection and stimulation. In Fig. 5A and 5B, the phosphorylated levels of Smad-2 and Smad-3 were both upregulated by TGF-β simulation compared to control ($P < 0.001$). Moreover, the upregulation was further improved by miR-33a mimic, leading to a significant increase in comparison with TGF-β+miR-control group ($P < 0.05$ or $P < 0.01$). However, the upregulation was obviously reduced by miR-33a inhibitor, leading to a significant decrease in comparison with TGF-β+miR-control group ($P < 0.05$ or $P < 0.001$).

3. Discussion

Cardiac fibrosis is one of the leading causes of cardiac arrhythmia, thus, deep investigations on mechanism of myocardial fibrosis are essential for the treatment of cardiac disease. In our present study, the first step was to validate the promotion of TGF-β on proliferation and fibrosis of CFBs. Then, we used TGF-β to induce fibrosis in CFBs and explored the regulation of miR-33a in this process. The results suggested that deficiency of miR-33a significantly impaired CFB proliferation, induced CFB apoptosis and downregulated expressions of Col-I, Col-III and α-SMA. In addition, miR-33a deficiency obviously inactivated the TGF-β/Smad signaling pathway.

The CFBs are commonly stay quiescently in healthy heart, whereas upon stimulation, these cells proliferate and differentiate into activated myoFBs (Samuel et al. 2011). Myocardial fibrosis induced by cardiac pressure overload is reported to result from CFB proliferation (Lucas et al. 2009). In the remodeling heart, the composition of ECM changes to facilitate proliferation of CFBs. Moreover, Lexow et al. (2013) also implied that cardiac fibrosis is characterized by excessive proliferation of CFBs. In our present study, the proliferation of CFBs was significantly enhanced by TGF-β, which greatly coincided with previous studies, indicating the promotion of TGF-β on CFB fibrosis. Simultaneously, the promotion of cell proliferation induced by TGF-β is obviously repressed by miR-33a deficiency while further enhanced by

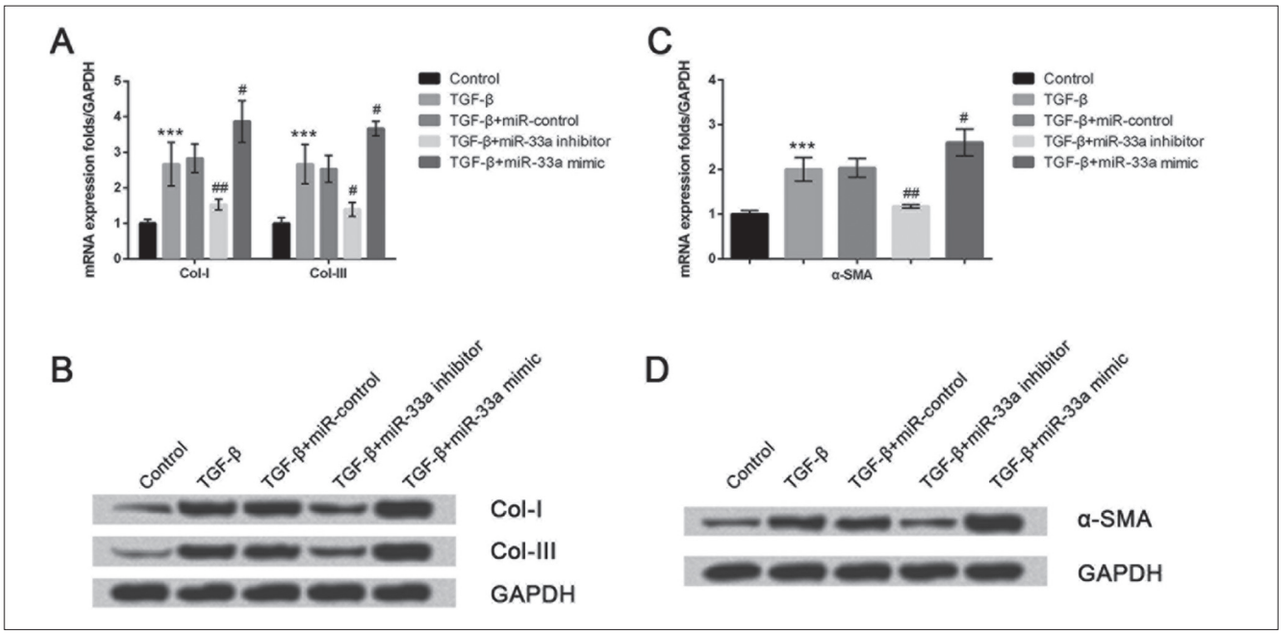

Fig. 4: The effect of miR-33a on fibrosis of human CFBs. A, mRNA expression levels of Col-I and Col-III; B, protein expression levels of Col-I and Col-III; C, mRNA expression level of α-SMA; D, protein expression level of α-SMA. After transfection with miR-control, miR-33a mimic or miR-33a inhibitor and stimulation with TGF-β, cells were divided into five groups. Cells without any treatment served as control. mRNA and protein expression levels were respectively evaluated by qRT-PCR and Western blot analysis. Data presented were the mean of three independent experiments. Error bars indicated SD. * indicates significant difference compared with control group. ***, $P < 0.001$; # indicates significant difference compared with TGF-β+miR-control group. #, $P < 0.05$; ##, $P < 0.01$. miR-33a, microRNA-33a; TGF-β, transforming growth factor-β; CFB, cardiac fibroblast, qRT-PCR, quantitative reverse transcription PCR.

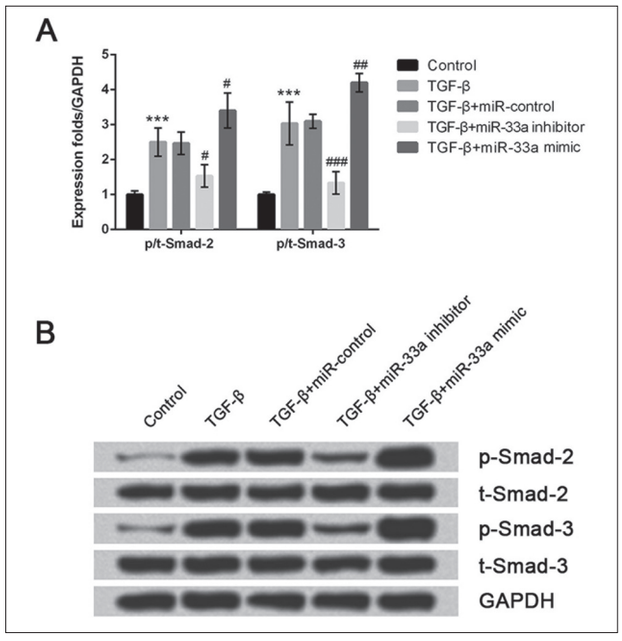

Fig. 5: The effect of miR-33a on TGF/Smad signaling pathway. After transfection with miR-control, miR-33a mimic or miR-33a inhibitor and stimulation with TGF-β, CFBs were divided into five groups. Cells without any treatment served as control. Protein expression levels were evaluated by Western blot analysis. The band intensity was estimated by Image Lab™ Software. The phosphorylation rate was expressed as the relative intensity of phosphorylated kinases/kinases and the final results were normalized by GAPDH. Data presented were the mean of three independent experiments. Error bars indicated SD. * indicates significant difference compared with control group. ***, $P < 0.001$; # indicates significant difference compared with TGF-β+miR-control group. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$. miR-33a, microRNA-33a; TGF-β, transforming growth factor-β; CFB, cardiac fibroblast; p-Smad-2, phosphorylated Smad-2; p-Smad-3, phosphorylated Smad-3.

miR-33a overexpression, indicating that miR-33a deficiency might inhibit CFB fibrosis by inhibition of cell proliferation. Besides, the results of cell apoptosis also illustrated the promotion of miR-33a deficiency on CFB apoptosis. While for cancer cells, miR-33a was reported to suppress proliferation of non-small-cell lung carcinoma (NSCLC) cells (Du et al. 2017), breast cancer cells (Zhang et al. 2015) and melanoma cells (Zhou et al. 2015). The possible reason might be miR-33a targeting different genes in different cell types.

Multiple smooth muscle cell markers which are not expressed in quiescent CFBs appear in myoFBs, including α-SMA. When the CFBs are activated by hormones or cytokines, such as TGF-β, the CFBs are termed myoFBs due to the high expression of contractile α-SMA, which constitute contractile microfilaments (Gabbiani 2003). Thus, α-SMA is considered as hallmarks of myoFBs. Meantime, production of Col-I and Col-III is significantly increased in cardiac fibrosis, making the upregulated expression of Col-I and Col-III becomes another hallmarks of myoFBs (Cleutjens et al. 1995; Kong et al. 2014). Taken together, we selected the expression levels of Col-I, Col-III and α-SMA to evaluate the fibrosis of CFBs. In our study, TGF-β significantly up-regulated the expression of these three hallmarks, indicating the fibrotic effect of TGF-β which is in line with previous studies. Multiple comparisons between TGF-β+miR-control and TGF-β+miR-33a mimic or inhibitor illustrated the TGF-β induced fibrosis of CFBs was further enhanced by miR-33a mimic, whereas the fibrosis was reversed by miR-33a inhibitor. In other words, miR-33a deficiency might effectively repress differentiation of CFBs into myoFBs. A previous study also suggested a profibrotic effect of miR-33a in hepatic stellate cells (Li et al. 2014).

As the important mediator for fibrogenesis, TGF-β is reported to mainly function via the TGF-β/Smad signaling pathway. To begin with, TGF-β binds to TβRII on the cell membrane, and then the TβRI-kinase was activated. After that, the Smad-2 and Smad-3 were both phosphorylated by TβRI-kinase. Then, activated Smad-2 and Smad-3 combined with Smad-4, translocate into the nucleus and thereby regulate the transcription of large numbers of target genes (Lan and Chung 2012; Tang and Lan 2014). In our study, the phosphorylated levels of Smad-2 and Smad-3 were both improved by TGF-β, which agreed with previous results. Multiple comparisons between TGF-β+miR-control and TGF-β+miR-33a mimic or inhibitor showed that miR-33 mimic could further increase TGF-β induced activation of Smad-2 and Smad-3, while miR-33 knockdown could reverse the effect of TGF-β. That is miR-33a deficiency could inactivate the TGF-β/Smad signaling pathway. Interestingly, Bai et al. (2015) showed that loureirin B inhibited fibroblast proliferation and extracellular matrix deposition in a hypertrophic scar via the TGF-β/Smad pathway). Chen et al. (2015) also implied that GW26-e1586 AMPK attenuated proliferation of cardiac fibroblast via regulating TGF-β1/Smad pathways. Therefore, we drew the conclusion that miR-33a deficiency might inhibit proliferation and fibrosis of CFBs through inactivation of the TGF-β/Smad signaling pathway.

In conclusion, we preliminarily explored the modulation of miR-33a in proliferation, apoptosis and fibrosis of CFBs. The results not only provide a theoretical basis for myocardial fibrosis but also suggest novel therapeutic strategies for arrhythmia. More experiments are needed to investigate on the target genes of miR-33a in CFBs.

4. Experimental

4.1. Cell culture and treatment

Human CFBs were purchased from Cell Bank of Tongpai Biotechnology Co., Ltd. (Shanghai, China). The CFBs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum (FBS; both from Gibco, Rockville, MD, USA). Exponentially growing CFBs were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The TGF-β (R&D Systems, Wiesbaden, Germany) treatment was performed through stimulation with a 10 ng/mL.

4.2. miRNA transfection

The miR-33a mimic, miR-33a inhibitor and non-targeting control of miR-33a (miR NC) were all purchased from GenePharma Co. (Shanghai, China). These miRs were transfected into cells with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of supplier.

4.3. CCK-8 assay

The viability of human CFBs was performed using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA). In brief, CFB cells were seeded in 96-well plates with a density of 5×10^3 cells/well. After treatment (transfection or simulation), the WST-8 with a final concentration of 10% was added into each well and then the cells were maintained at 37 °C for 1 h. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

4.4. Apoptosis assay

Annexin V-FITC/PI apoptosis detection kit (Biosea Biotechnology, Beijing, China) was used to access cell apoptosis. In brief, after transfection for 48 h, the CFB cells were stimulated with TGF-β. Then, floating and adherent cells were combined and washed by pre-cold phosphatebuffered saline (PBS). Resuspended with binding buffer, the cells with an amount of 1×10^5 cells were stained with 10 μL of FITC-conjugated annexin V and 5 μL of propidium iodide (PI) following the supplier's instructions. Subsequently, cell apoptosis was detected by an FACS can (Beckman Coulter, Fullerton, CA, USA) and analyzed by FlowJo software (Tree Star, San Carlos, CA, USA).

4.5. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen) and DNaseI (Promega, Madison, WI, USA). Then, Multiscribe RT kit (Applied Biosystems) and random hexamers or oligo(dT) were used for reverse transcription, the procedure of which were 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C. The final quantitative PCR was performed by QuantiFast SYBR green RT-PCR kit (Qiagen, Hilden, Germany) in line with the protocol of supplier. Primers as shown in the Table were designed and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The mRNA expression was calculated by using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). GAPDH acted as an internal control.

4.6. Western blot analysis

The proteins of CFB cells were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors (Roche, Indianapolis,

Table: Primers sequences

Gene	Primer sequence
Col-I	forward 5'-TCTAGACATGTTCAGCTTTGTGGAC-3'
	reverse 5'-TCTGTACGCAGGTGATTGGTG-3'
Col- III	forward 5'-CCACGGAAACACTGGTGGAC-3'
	reverse 5'-GCCAGCTGCACATCAAGGAC-3'
α-SMA	forward 5'-GACAATGGCTCTGGGCTCTGTAA-3'
	reverse 5'-TGTGCTTCGTCACCCACGTA-3'
GAPDH	forward 5'-TCAACGACCACTTTGTCAAGCTCA-3'
	reverse 5'-GCTGGTGGTCCAGGGGTCTTACT-3'

Col-I, collagen I; Col-III, collagen III; α-SMA, α-smooth muscle actin

IN, USA). After quantitation by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), equal amounts of proteins were loading onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Primary antibodies against Collagen I (Col-I, ab34710), Collagen III (Col-III, ab137768), α-smooth muscle actin (α-SMA, ab5694), phosphorylated Smad-2 (p-Smad-2, ab184557), Smad-2 (ab33875), phosphorylated Smad-3 (p-Smad-3, ab138659), Smad-3 (ab40854) and GAPDH (ab128915) (all from Abcam, Cambridge, UK) were incubated with the membrane at 4 °C overnight. Thereafter, the membranes were washed and incubated with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing again, the membranes were sent into a Bio-Rad ChemiDoc™ XRS system, and then 200 µL of Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Hercules, CA, USA). The phosphorylated level referred to a ratio between the phosphorylated and the respective total forms.

4.7. Statistical analysis

All experiments were repeated three times. The results were presented as the mean ± standard deviation (SD). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). The *P*-values were calculated using the one-way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni correction for comparison between three groups. In addition, two-tailed unpaired *t*-test was employed to calculate the *P*-values for comparison between two groups. A *P*-value of <0.05 was considered to indicate a statistically significant result.

Acknowledgement: The work was not supported by any funding agency.

Conflict of interests: The authors declared no conflict of interests.

References

Annes JP, Munger JS, Rifkin DB (2003) Making sense of latent TGFbeta activation. J Cell Sci 116: 217-224.

Bai X, He T, Liu J, Wang Y, Fan L, Tao K, Shi J, Tang C, Su L, Hu D (2015) Loureirin B inhibits fibroblast proliferation and extracellular matrix deposition in hypertrophic scar via TGF-β/Smad pathway. Exp Dermatol 24: 355–360.

Baum J, Duffy HS (2011) Fibroblasts and myofibroblasts: what are we talking about? J Cardiovasc Pharmacol 57: 376-379.

Camelliti, Borg TK, PeterKohl (2005) Structural and functional characterisation of cardiac fibroblasts. Cardiovasc Res 65: 40-51.

Chan CK, Rolle MPS, Braun KR, Van Biber BP, Laflamme MA, Murry CE, Wight TN (2010) Differentiation of cardiomyocytes from human embryonic stem cells is accompanied by changes in the extracellular matrix production of versican and hyaluronan. J Cell Biochem 111: 585–596.

Chen S, Puthanveetil P, Feng B, Matkovich SJ, Ii GWD, Chakrabarti S (2014) Cardiac miR-133a overexpression prevents early cardiac fibrosis in diabetes. J Cell Mol Med 18: 415-421.

Chen Y, Lin C, Zhong Y, Lu G, Ma Y, Huang H (2015) GW26-e1586 AMPK attenuates proliferation of cardiac fibroblast via regulating TGF-β1/Smad pathways. J Am Coll Cardiol 66: C74-C74.

Cleutjens JP, Verluyten MJ, Smiths JF, Daemen MJ (1995) Collagen remodeling after myocardial infarction in the rat heart. Am J Pathol 147: 325-338.

Coulombe KLK, Bajpai VK, Andreadis ST, Murry CE (2014) Heart regeneration with engineered myocardial tissue. Biomed Engin 16: 1-28.

Du XJ, Xu Q, Lekgabe E, Gao XM, Kiriazis H, Moore XL, Dart AM, Tregear GW, Bathgate RA, Samuel CS (2009) Reversal of cardiac fibrosis and related dysfunction by relaxin. Annals of the New York Academy of Sciences 1160: 278–284.

Du M, Zhang Y, Mao Y, Mou J, Zhao J, Xue Q, Wang D, Huang J, Gao S, Gao Y (2017) MiR-33a suppresses proliferation of NSCLC cells via targeting METTL3 mRNA. Biochem Biophys Res Comm 482: 582-589.

Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. J Pathol 200: 500-503.

Gourdie RG, Dimmeler S, Kohl P (2016) Novel therapeutic strategies targeting fibroblasts and fibrosis in heart disease. Nature Rev Drug Discov 15: 620-638.

Huang CF (2014) miR-33a, an important marker and putative therapeutic target in chronic HBV-induced fibrosis. RNA Dis 1: 10-14800/rd.14416.

Huang CF, Sun CC, Zhao F, Zhang YD, Li DJ (2015) miR-33a levels in hepatic and serum after chronic HBV-induced fibrosis. J Gastroenterol 50: 480-490.

Huikuri HV, Castellanos A, Myerburg RJ (2002) Sudden death due to cardiac arrhythmias. New Engl J Med 346: 1473-1482.

Iwata M, Cowling RT, Yeo SJ, Greenberg B (2011) Targeting the ACE2–Ang-(1–7) pathway in cardiac fibroblasts to treat cardiac remodeling and heart failure. J Mol Cell Cardiol 51: 542-547.

Kong P, Christia P, Frangogiannis NG (2014) The pathogenesis of cardiac fibrosis. Cell Mol Life Sci 71: 549-574.

Lan HY, Chung AC (2012) TGF-β/Smad signaling in kidney disease. Sem Nephrol 32: 236-243.

Leask A (2010) Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. Circulation Res 106: 1675-1680.

Lexow J, Poggioli T, Sarathchandra P, Santini MP, Rosenthal N. (2013) Cardiac fibrosis in mice expressing an inducible myocardial-specific Cre driver. Dis Model Mech 6: 1470-1476.

Li ZJ, Ouyang PH, Han XP (2014) Profibrotic effect of miR-33a with Akt activation in hepatic stellate cells. Cell Signal 26: 141-148.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

Lucas JA, Zhang Y, Li P, Gong K, Miller AP, Hassan E, Hage F, Xing D, Wells B, Oparil S (2009) Inhibition of transforming growth factor-beta signaling induces left ventricular dilation and dysfunction in the pressure-overloaded heart. Am J Physiol Heart Circ Physiol 298: H424-432.

Nagpal V, Rai R, Place AT, Murphy SB, Verma SK, Ghosh AK, Vaughan DE (2016) MiR-125b is critical for fibroblast-to-myofibroblast transition and cardiac fibrosis. Circulation 133: A12623-A12623.

Petrov V, Fagard R, Lijnen P (2002) Stimulation of collagen production by TGF-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 39: 258-263.

Qiu Y, Yu H, Shi X, Xu K, Tang Q, Liang B, Hu S, Bao Y, Xu J, Cai J, Peng W, Cao Q, Yin P (2016) microRNA-497 inhibits invasion and metastasis of colorectal cancer cells by targeting vascular endothelial growth factor-A. Cell Prolif 49: 69–78.

Rayner KJ, Esau CC, Hussain FN, Mcdaniel AL, Marshall SM, van Gils JM, Ray TD, Sheedy FJ, Goedeke L, Liu X (2011) Inhibition of miR-33a/b in non-human primates raises plasma HDL and reduces VLDL triglycerides. Nature 478: 404-407.

Samuel CS, Unemori EN, Mookerjee I, Bathgate RA, Layfield SL, Mak J, Tregear GW, Du XJ (2011) Relaxin modulates cardiac fibroblast proliferation, differentiation, and collagen production and reverses cardiac fibrosis in vivo. Endocrinology 145: 4125-4133.

Tang PM-K, Lan H-Y (2014) MicroRNAs in TGF-β/Smad-mediated tissue fibrosis. Curr Pathobiol Rep 2: 235-243.

Townsend N, Nichols M, Scarborough P, Rayner M (2015) Cardiovascular disease in Europe--epidemiological update 2015. Eur Heart J 34: 3028-3034.

Zhang C, Zhang Y, Ding W, Lin Y, Huang Z, Luo Q (2015) MiR-33a suppresses breast cancer cell proliferation and metastasis by targeting ADAM9 and ROS1. Protein Cell 6: 881-889.

Zhao X, Wang K, Liao Y, Zeng Q, Li Y, Hu F, Liu Y, Meng K, Qian C, Zhang Q (2015) MicroRNA-101a inhibits cardiac fibrosis induced by hypoxia via targeting TGFβRI on cardiac fibroblasts. Cell Physiol Biochem 35: 213-226.

Zhou J, Xu D, Xie H, Tang J, Liu R, Li J, Wang S, Chen X, Su J, Zhou X (2015) miR-33a functions as a tumor suppressor in melanoma by targeting HIF-1. Cancer Biol Ther 16: 846-855.