Int J Clin Exp Pathol 2017;10(5):5366-5372
www.ijcep.com /ISSN:1936-2625/IJCEP0048672

Original Article

The role of miR-203a in hypertrophic scars and its mechanism

Wenya Han, Daojiang Yu, Tianlan Zhao

Department of Plastic Surgery and Cosmetology, Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, China

Received January 11, 2017; Accepted March 6, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: Studies have indicated that miR-203a was down-regulated in Hypertrophic scar (HS) tissues, however, the exact role of miR-203a in HS remains unclear. The aim of this study was to investigate the role of miR-203a in HS, and explore its underlying mechanisms. We fist confirmed the down-regulation of miR-203a in human hypertrophic scar fibroblasts (hHSFs) by using qRT-PCR, and the results were consistent with the previous study. TargetScan was applied to predict the target genes of miR-203a, and the prediction was verified by Dual-luciferase reporter assay. To investigate the role of miR-203a in HS, a stable miR-203a-over-expression cell line was established using miR-203a mimics. CCK-8, cell cycle assay and cell apoptosis assay were preformed to investigate the proliferation, cell cycle and apoptosis of hHSFs. Additionally, mRNA and protein levels of related genes were measured by qRT-PCR and western blot analysis respectively. Our results suggested that HOXD3 is a target gene of miR-203a, and it can be repressed by miR-203a. miR-203a could inhibit the proliferation, block transition of the G2/M phase, increase the apoptosis of hHSFs, decrease the expression of cell cycle and apoptosis-related proteins, EGFR, p-AKT, CCNB1, CDK1, Bax and Bcl-2. In conclusion, miR-203a inhibited the proliferation and induced apoptosis of hHSFs by targeting HOXD3. miR-203a may be a useful target for HS management.

Keywords: Hypertrophic scar, miR-203a, HOXD3, proliferation, apoptosis

Introduction

Excessive scars, which may be caused by burns, lacerations, surgery, and vaccinations, physically and psychologically affect a patient's quality of life, by causing pain, pruritus and contractures. Hypertrophic scars (HS) is a dermal fibroproliferative disorder caused by anomalous wound healing after skin injury. HS is characterized by excessive extracellular matrix accumulation and fibroblasts overgrowth [1]. Millions of people suffer this disease every year [2]. Many studies on HS formation have been performed for decades, and great improvements have been made on the therapeutic strategies of HS treatment in recent years [3]. However, the complex mechanisms of HS formation are still poorly understood, and most therapeutic means remain clinically unsatisfactory. Therefore, it is crucial and urgent to find new and effective strategy to prevent HS.

MicroRNAs (miRNAs), a class of short (~22 nt), non-coding, single stranded and highly conserved RNAs, negatively regulate gene expression in many cellular events, including proliferation, apoptosis, and differentiation by targeting the 3'UTR of target genes [4-7]. Dysregulation of miRNAs is known to be involved in various pathophysiologic processes, including the normal wound healing process. Emerging evidences suggested that miRNAs are involved in pathologic wound healing and closely related to HS formation. For example, miR-21 has been found to control HS fibroblast cell growth though regulating hTERT expression via the PTEN/PI3K/AKT pathway [8]. miR-200b can inhibit cell proliferation and promote apoptosis of human hypertrophic scar fibroblasts, and may be a useful target for hypertrophic scarring management [9]. miR-138 plays important roles in regulating human hypertrophic scar fibroblasts proliferation and movement [10]. miR-10a and miR-181c were reported to have critical roles in HS pathogenesis via the regulation of collagen type I [11]. Gras et al. suggested that miR-145 is a promising therapeutic target to prevent or reduce HS of the skin [12].

Table 1. Primer sequence for PCR

Sequence (5'-3')
miR-203a-Forward: 5'-ATCCAGTGCGTGTCGTG-3'
miR-203a-Reverse: 5'-TGCTGTGAAATGTTTAGGA-3'
HOXD3-Forward: 5'-TCAAGAAAACACACACATACATAATTG-3'
HOXD3-Reverse: 5'-TGCTGAATCCTGAGAGAGCTG-3'
U6-Forward: 5'-TGCGGGTGCTCGCTTCGGCAGC-3'
U6-Reverse: 5'CCAGTGCAGGGTCCGAGGT3'
GAPDH-Forward: 5'CTTTGGTATCGTGGAAGGACTC3'
GAPDH-Reverse: 5'GTAGAGGCAGGGATGATGTTCT3'

Previous study suggested that compared with the matched normal skin tissues, miR-203a was down-regulated in hypertrophic scar tissues [9]. To the best of our knowledge, the exact functional role of miR-203a remains unclear. Thus, the present study aimed to investigate the role of miR-203a in HS formation and explore its underlying mechanisms.

Materials and methods

Cell culture

The human embryonic skin fibroblasts, CCC-ESF-1 (ESF), and human hypertrophic scar fibroblasts (hHSFs) were obtained from Bioleaf Corporation (Shanghai, China). Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin solution in 5% CO_2 humidified atmosphere at 37°C.

QRT-PCR

Total RNA was extracted from cells by using TRIzol (Invitrogen) in line with the manufacturer protocols. cDNAs were generated by using RevertAid First Strand cDNA synthesis Kit (Fermentas, Pittsburgh, PA, USA). Real-time PCR was performed using SYBR Green qRCR Mix (TOYOBO, Osaka, Japan) in a Thermal Cycler Dice Real-time system. U6 (for miRNA) or GAPDH (for mRNA) was used as an internal control. The primer sequences were listed in **Table 1**. All tests were performed in triple.

Western blot analysis

Total cell protein was extracted from cells by using RIPA lysis buffer (Auragene, Changsha, China). The BCA protein assay kit (Beyotime, China) was performed protein quantification. Protein samples were resolved by SDS-PAGE and then transferred onto nitrocellulose membrane (Millipore, Bedford, MA, USA). Membranes were incubated with primary antibodies at 4°C overnight. After wishing with TBST solution for at least 3 times, the membranes were then incubated with second antibodies (CST, USA; dilution ratio, 1:5000) at room temperature for 4 h. An ECL kit (Applygen, Beijing, China) was carried out to detect the protein bands. The primary antibodies, HOXD3, EGFR, p-AKT, CCNB1, CDK1, Bax, Bcl-2 and β-actin (Cell Signaling Technology, Beverly, MA) were diluted 1:1000 respectively.

Cell transfection

hHSFs were seeded into 6-well plates the day before transfection, then miR-203a mimics (mimic group) or its negative control (NC group) was transfected into hHSFs using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's protocols. Cells without any treatments were considered as the control group (Control group). Fresh culture medium was replaced after incubating for 4 hours. 48 h after transfection, following experiments analysis was carried out.

Dual-luciferase reporter assay

To investigate whether miR-203a targets the 3'-UTRs of HOXD3, the vectors named HOXD3-3'UTR-WT and HOXD3-3'UTR-MUT with wild-type and mutated 3'UTR of HOXD3 mRNA were established. 293T cells were seeded in 24-well plates and then co-transfected with HOXD3-3'UTR-WT or HOXD3-3'UTR-MUT and miR-203a or its negative control (hsamiR-NC) vector using Lipofectamine 2000 in line with the manufacturer's instructions. Luciferase activity was assessed with the Dual-Luciferase Reporter Assay Kit (Promega, USA) according to the manufacturer's protocols. All tests were performed at least 3 times.

Cell counting kit-8 assay

CCK-8 (Solarbio, Beijing, China) was used to determine the effects of miR-203a on cell proliferation. In short, 48 hours after transfection, hHSFs were seeded at 5×10^3 cells per well in a 96-well plate. 10 μl CCK-8 solution was added into each well. After incubating for 4 h at 37°C, absorbance was detected at 450 nm. Tests were repeated three times.

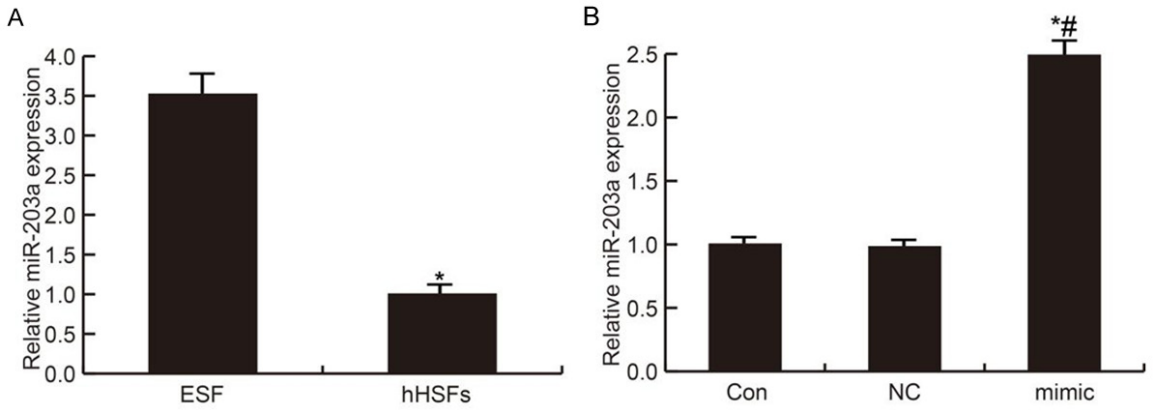

Figure 1. miR-203a expression level. miR-203a expression was detected by qRT-PCR. A: Relative expression level of miR-203 in human embryonic skin fibroblasts (ESF) and human hypertrophic scar fibroblasts (hHSFs); B: Relative expression level of miR-203 in hHSFs. Con: control group; NC: negative control group; mimic: hHSFs transfected with miR-203a mimics group. *, $P < 0.05$ vs. Con; #, $P < 0.05$ vs. NC.

Cell cycle analysis

At 48 h post transfection, hHSFs were collected, fixed with 70% ethanol, centrifuged (1500 rpm for 5 min), and then incubated with 0.1 mg/mL RNase A and 0.05 mg/mL propidium iodide (PI) for 30 min at 4°C. Flow cytometry was applied to analyze the cell cycle. Tests were repeated three times.

Apoptosis assay

48 h after transfection, the apoptosis of hHSFs was determined, and the apoptosis detection kit (BD Biosciences, NJ, USA) was performed. Briefly, hHSFs were transfected with miR-203a mimics or its negative control respectively, and 48 hours after transfection, the cells were incubated with Annexin V and propidium iodide for 20 min at room temperature. The cells were analyzed by flow cytometry. Tests were repeated three times.

Statistical analysis

Unpaired Student's t test was performed for data analysis. Data were displayed as the means ± SD. A value of $P < 0.05$ was considered significant.

Results

miR-203a is down-regulated in hHSFs

We first confirmed the expression level of miR-203a in human embryonic skin fibroblasts (ESF) and human hypertrophic scar fibroblasts (hHSFs) by using QRT-PCR. And we found that compared with the ESF, miR-203 level was significantly decreased in hHSFs (**Figure 1A**). This data is consistent with previous study.

To explore the role of miR-203a in hypertrophic scaring, miR-203a was over-expressed by using miR-203a mimics. 48 h after transfection, the higher expression of miR-203a in hHSFs was revealed by qRT-PCR (**Figure 1B**).

miR-203a targets HOXD3

To investigate the mechanism of miR-203a functions in hypertrophic scaring, we first studied the target genes of miR-203a. We first predicted the target genes of miR-203a by using TargetScan (**Figure 2A**), and then luciferase reporter gene assay was carried out to verify our prediction (**Figure 2B**). The results suggested that miR-203a binded to the HOXD3 gene at the 3'-UTR nucleotide site, indicating that miR-203a directly targets HOXD3.

To explore the regulation of miR-203a to HOXD3 in hHSFs, 48 h after transfection, QRT-PCR (**Figure 2D**) and western blotting (**Figure 2C**) were applied respectively. Compared to the control and negative control, HOXD3 level notably decreased in the miR-203a mimic group. Taken together, the data indicated that HOXD3 is a target gene of miR-203a and can be negatively regulated by miR-203a.

miR-203a represses the proliferation of hHSFs

To investigate the effect of miR-203a on hHSFs proliferation, CCK-8 assay was per-

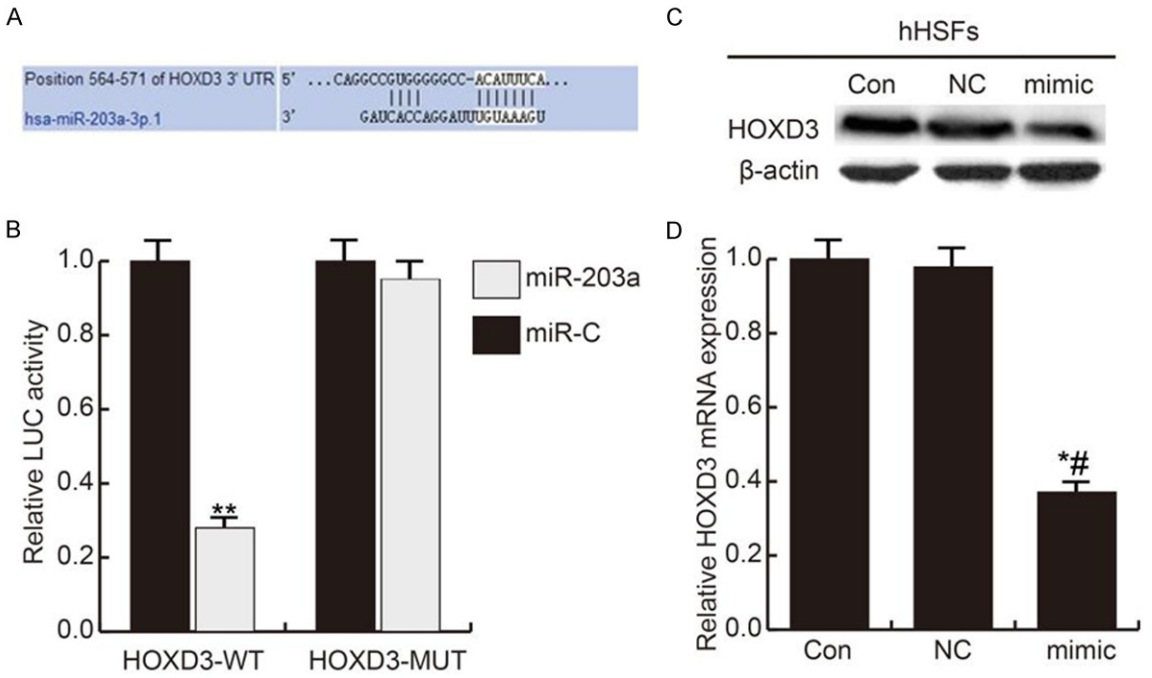

Figure 2. miR-203a targets HOXD3. A: Interaction between miR-203a and 3'UTR of HOXD3 was predicted using TargetScan; B: Luciferase activity of a reporter containing a wild-type HOXD3 3'UTR or a mutant HOXD3 3'UTR are shown in the bar graph (**P < 0.01 vs. control). Here, "HOXD3 3'UTR-MUT" indicates the HOXD3 3'UTR with a mutation in the miR-203a binding site. UTR, untranslated region. C: Protein level of HOXD3 in hHSFs; D: mRNA level of HOXD3 in hHSFs. Con: control group; NC: negative control group; mimic: hHSFs transfected with miR-203a mimics group. *, P < 0.05 vs. Con; #, P < 0.05 vs. NC.

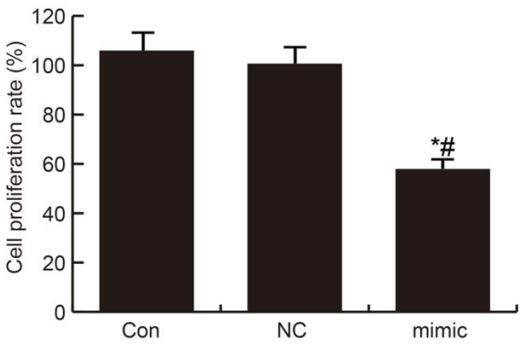

Figure 3. miR-203a inhibits hHSFs cell proliferation. 48 h after hHSFs cells were transfected with miR-203a mimics or its negative control, CCK-8 assay was used to detect cell proliferation. Con: control group; NC: negative control group; mimic: hHSFs transfected with miR-203a mimics group. *, P < 0.05 vs. Con; #, P < 0.05 vs. NC.

formed. As shown in **Figure 3**, compared with the control and negatively control group, the cell proliferation ability was significantly decreased in hHSFs transfected with miR-203 mimics. The data indicated that miR-203a could inhibit hHSFs proliferation.

miR-203a represses the proliferation of hHSFs though G2/M arrest

To further investigate the mechanisms by which miR-203a inhibit hHSFs proliferation, cell cycle assay was applied. The results showed that a marked accumulation of hHSFs in G2/M phase was observed in miR-203a mimic group, suggesting that miR-203a induced G2/M phase arrest in hHSFs (**Figure 4A**).

In addition, the expression levels of the cell cycle regulators were determined. We found that EGFR, p-AKT, CCNB1, and CDK1 were down-regulated in hHSFs in miR-203a mimic group (**Figure 4B**). Thus, miR-203a could repress the proliferation of hHSFs though G2/M arrest by regulating the expression of the cell cycle regulators.

miR-203a induces hHSFs apoptosis

We also examined the effect of miR-203a on cell apoptosis of hHSFs. As shown in **Figure 5A** and **5B**, up-regulation of miR-203a promoted the cell apoptosis, which is consistent with the

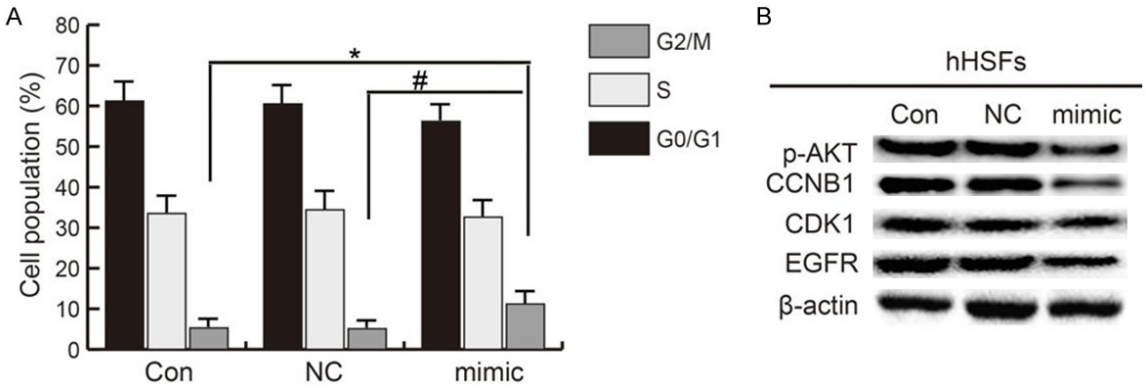

Figure 4. miR-203a induces hHSFs cell arrested in G2/M phase. 48 h after hHSFs cells were transfected with miR-203a mimics or its negative control, cell cycle assay was carried out to analyze cell cycle. A: Data analysis of cell cycle; B: Protein level of cell cycle related proteins was detected by western blotting. Con: control group; NC: negative control group; mimic: hHSFs transfected with miR-203a mimics group. *, $P < 0.05$ vs. Con; #, $P < 0.05$ vs. NC.

results of CCK-8. In addition, cell apoptosis related proteins were determined, and we found that up-regulation of miR-203a resulted in increased Bax expression and decreased Bcl-2 expression (**Figure 5C**). All these results demonstrate that miR-203a induces the apoptosis of hHSFs through altering Bax/Bcl2 protein ratio.

Discussion

MiRNAs have been found to play important roles in regulating the proliferation and apoptosis of a variety of cells, but studies on their roles in HS are still in its infancy [13]. miR-203a acts as a tumor suppressor/promoter in various cancers including gastric cardiac adenocarcinoma (GCA), hepatocellular carcinoma and renal cell carcinoma, by its regulation of the proliferation, apoptosis, invasion, and migration of cancer cells [14-16]. miR-203a was found to be down-regulated in gastric cardiac adenocarcinoma tissues, and reactivation of miR-203a may have therapeutic potential for GCA patients [14]. MiR-203a has been recognized as a potential tumor suppressor because it represses HOXD3 expression in hepatocellular carcinoma cells [15]. Moreover, in renal cell carcinoma, it was found that inhibition of miR-203a could inhibit renal cell carcinoma cell proliferation and migration, arrest them in G1 phase, and promote apoptosis [16], indicating its tumor promoter role in renal cell carcinoma progress. MiR-203a has been found to be down-regulated in hypertrophic scar tissues [9]; however, the exact role of miR-203a in hypertrophic scar formation remains unknown.

In our present study, we found that miR-203a was significantly decreased in human hypertrophic scar fibroblasts compared to that of the human embryonic skin fibroblasts. And we found that HOXD3 is a target gene of miR-203a, and it can be negatively regulated by miR-203a in hHSFs. HOXD3 is a third paralogous member of the HOXD gene family, which play important roles in cancer pathogenesis via affecting tumorigenesis and cancer cell biology through differentiation, apoptosis, receptor signaling, and other unknown mechanisms [17-19]. HOXD3 has been found to play critical roles in the regulation of immigration or invasion-related gene expression [20, 21]. A recent study indicated that HOXD3 inhibits cell proliferation and promotes apoptosis through the EGFR-MAPK/AKT pathway in hepatocellular carcinoma [15].

We further investigated the role of miR-203a in HS formation, and CCK-8, cell cycle assay and cell apoptosis assay were preformed. By transfecting miR-203a mimics into hHSFs, we found that miR-203a could repress the proliferation of hHSFs though G2/M arrest and induce apoptosis in hHSFs. To further investigate the underlying mechanisms of the regulation of cell cycle and cell apoptosis by miR-203a, the expression level of cell cycle and apoptosis-related proteins, EGFR, p-AKT, CCNB1, CDK1, Bax and Bcl-2 were determined in our present study. The findings suggested that EGFR, p-AKT,

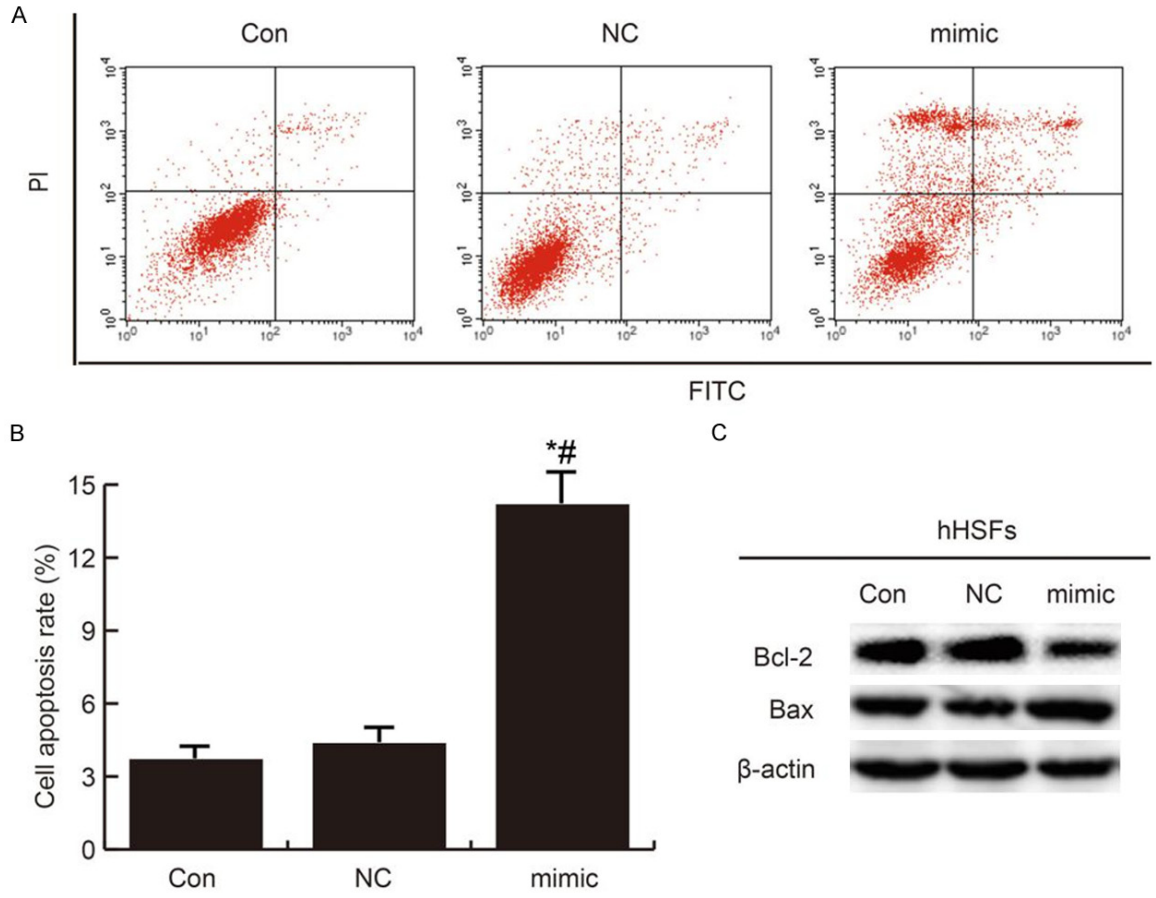

Figure 5. miR-203a promotes hHSFs cell apoptosis. 48 h after hHSFs cells were transfected with miR-203a mimics or its negative control, cell apoptosis assay was performed to analyze cell apoptosis. A: Cell apoptosis was detected by FCM; B: Data analysis of cell apoptosis rate; C: Protein level of cell apoptosis related proteins was detected by western blotting. Con: control group; NC: negative control group; mimic: hHSFs transfected with miR-203a mimics group. *, $P < 0.05$ vs. Con; #, $P < 0.05$ vs. NC.

CCNB1, and CDK1 were down-regulated in hHSFs transfected with miR-203a mimics, and the ratio of Bax/Bcl-2 significantly increased.

Taken together, our study demonstrates that miR-203a was down-regulated significantly in hypertrophic scars. miR-203a inhibited hHSFs cell proliferation and induced apoptosis by directly targeting HOXD3 though EGFR/AKT pathway. Over-expression of miR-203a may be a potential clinical strategy for hypertrophic scars treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Tianlan Zhao, Department of Plastic Surgery and Cosmetology, Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Suzhou 215007, Jiangsu Province, China. Tel: 0512-67783506; E-mail: zhaotl2016@sina.com

References

[1] Gauglitz GG, Korting HC, Pavicic T, Ruzicka T, Jeschke MG. Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. Mol Med 2011; 17: 113-125.

[2] Bayat A, McGrouther DA. Clinical management of skin scarring. Skinmed 2005; 4: 165-173.

[3] Meaume S, Le Pillouer-Prost A, Richert B, Roseeuw D, Vadoud J. Management of scars: updated practical guidelines and use of silicones. Eur J Dermatol 2014; 24: 435-443.

[4] Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.

[5] Soifer HS, Rossi JJ, Saetrom P. MicroRNAs in disease and potential therapeutic applications. Mol Ther 2007; 15: 2070-2079.

[6] Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010; 11: 597-610.
[7] O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol 2010; 10: 111-122.
[8] Zhu HY, Li C, Bai WD, Su LL, Liu JQ, Li Y, Shi JH, Cai WX, Bai XZ, Jia YH, Zhao B, Wu X, Li J, Hu DH. MicroRNA-21 regulates hTERT via PTEN in hypertrophic scar fibroblasts. PLoS One 2014; 9: e97114.
[9] Li P, He QY, Luo CQ. Overexpression of miR-200b inhibits the cell proliferation and promotes apoptosis of human hypertrophic scar fibroblasts in vitro. J Dermatol 2014; 41: 903-911.
[10] Xiao YY, Fan PJ, Lei SR, Qi M, Yang XH. MiR-138/peroxisome proliferator-activated receptor β signaling regulates human hypertrophic scar fibroblast proliferation and movement in vitro. J Dermatol 2015; 42: 485-495.
[11] Li C, Zhu HY, Bai WD, Su LL, Liu JQ, Cai WX, Zhao B, Gao JX, Han SC, Li J, Hu DH. MiR-10a and miR-181c regulate collagen type I generation in hypertrophic scars by targeting PAI-1 and uPA. FEBS Lett 2015; 589: 380-389.
[12] Gras C, Ratuszny D, Hadamitzky C, Zhang H, Blasczyk R, Figueiredo C. miR-145 Contributes to hypertrophic scarring of the skin by inducing Myofibroblast activity. Mol Med 2015; 21: 296-304.
[13] Gammell P. MicroRNAs: recently discovered key regulators of proliferation and apoptosis in animal cells: identification of miRNAs regulating growth and survival. Cytotechnology 2007; 53: 55-63.
[14] Liu W, Dong Z, Liang J, Guo X, Guo Y, Shen S, Kuang G, Guo W. Downregulation of potential tumor suppressor miR-203a by promoter methylation contributes to the invasiveness of gastric cardia adenocarcinoma. Cancer Invest 2016; 34: 506-516.
[15] Wang L, Sun H, Wang X, Hou N, Zhao L, Tong D, He K, Yang Y, Song T, Yang J, Huang C. EGR1 mediates miR-203a suppress the hepatocellular carcinoma cells progression by targeting HOXD3 through EGFR signaling pathway. Oncotarget 2016; 7: 45302-45316.
[16] Hu G, Lai P, Liu M, Xu L, Guo Z, Liu H, Li W, Wang G, Yao X, Zheng J, Xu Y. miR-203a regulates proliferation, migration, and apoptosis by targeting glycogen synthase kinase-3β in human renal cell carcinoma. Tumour Biol 2014; 35: 11443-11453.
[17] Darda L, Hakami F, Morgan R, Murdoch C, Lambert DW, Hunter KD. The role of HOXB9 and miR-196a in head and neck squamous cell carcinoma. PLoS One 2015; 10: e0122285.
[18] Gonzalez-Herrera A, Salgado-Bernabe M, Velazquez-Velazquez C, Salcedo-Vargas M, Andrade-Manzano A, Avila-Moreno F, Pina-Sanchez P. Increased expression of HOXB2 and HOXB13 proteins is associated with HPV infection and cervical cancer progression. Asian Pac J Cancer Prev 2015; 16: 1349-1353.
[19] Shah N, Sukumar S. The Hox genes and their roles in oncogenesis. Nat Rev Cancer 2010; 10: 361-371.
[20] Ohta H, Hamada J, Tada M, Aoyama T, Furuuchi K, Takahashi Y, Totsuka Y, Moriuchi T. HOXD3-overexpression increases integrin alpha v beta 3 expression and deprives E-cadherin while it enhances cell motility in A549 cells. Clin Exp Metastasis 2006; 23: 381-390.
[21] Okubo Y, Hamada J, Takahashi Y, Tada M, Tsutsumida A, Furuuchi K, Aoyama T, Sugihara T, Moriuchi T. Transduction of HOXD3-antisense into human melanoma cells results in decreased invasive and motile activities. Clin Exp Metastasis 2002; 19: 503-511.