

Tetrandrine induces microRNA differential expression in human hypertrophic scar fibroblasts *in vitro*

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ABSTRACT. MicroRNAs (miRNAs) have recently been shown to play a role in normal wound healing process. miRNAs may be linked to pathologic wound healing and closely related to the formation of hypertrophic scars. This study aimed to explore the effects of tetrandrine on the miRNA expression profile in human hypertrophic scar fibroblasts (HSFs) *in vitro*. HSFs were randomly divided into two groups: the tetrandrine treatment group and the control group. The experimental and control groups were collected and analyzed by miRNA array after a 48-h culture. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to confirm the array results. The targets of differentially expressed miRNA were functionally annotated using bioinformatic approaches. miRNA microarray analysis identified 193 differentially expressed miRNAs and the expression of 186 miRNAs in the experimental group decreased while that of 7 miRNAs increased compared to the control group. The most significantly

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downregulated miRNA was hsa-miR-1246, and hsa-miR-27b had the highest expression level. Significant differentially expressed miRNAs were predicted to be related to several important signaling pathways related to scar wound healing. The differential miRNA expression identified in this study provides the experimental basis for further understanding the antifibrosis effect of tetrandrine.

Key words: Fibroblasts; Hypertrophic scars; MicroRNA; Expression profile; Tetrandrine

INTRODUCTION

Hypertrophic scars result from an abnormal fibrous wound healing process and represent a connective tissue response to trauma, inflammation, surgery, or burns. It is believed that proliferation of fibroblasts plays an important role in hypertrophic scar formation and is involved in reepithelialization, extracellular matrix (ECM) deposition, neovascularization, and ECM remodeling (van der Veer et al., 2009). Both experimental and clinical researchers have shown that tetrandrine, a bisbenzylisoquinoline alkaloid isolated from the root of *Stephania tetrandra*, exerted anti-inflammatory properties, attenuated ECM deposition, and exhibited antifibrogenic activity against fibroblasts (Reist et al., 1993; Huang and Hong, 1998; Oh and Lee, 2003). Our previous studies have provided evidence that tetrandrine significantly inhibits proliferation of hypertrophic scar fibroblasts (HSFs) and decreases the expression of DNA (Liu et al., 2001). However, the underlying mechanism remains unclear.

MicroRNAs (miRNAs) are a new class of regulatory noncoding single-stranded RNAs (19-22 nucleotides), which can suppress the expression of protein-coding genes by targeting the 3'-untranslated region (UTR) of messenger RNAs (mRNAs) and play a key role in cellular growth and differentiation, as well as in disease development (Bartel, 2004; Kloosterman and Plasterk, 2006; Zhao and Srivastava, 2007). It is predicted that up to 30% of human genes are regulated by miRNAs. In addition, each miRNA can target several hundred mRNA 3'-UTRs, making miRNAs a large family of 'regulatory' molecules (Aberdam et al., 2008). However, the anti-fibrosis metabolism mechanism of tetrandrine and whether it is related to miRNAs remains unclear. In this study, we tested the effects of tetrandrine on mRNA expression in HSFs and analyzed putative targets of differentially expressed miRNAs using bioinformatic approaches.

MATERIAL AND METHODS

Fibroblast isolation and cell culture

HSFs were established as a primary cell line from hypertrophic scar tissue obtained from severe burn patients who underwent orthopedic surgery at the Department of Plastic and Reconstructive Surgery of the First Affiliated Hospital of Nanchang University, China. Written informed consent was obtained according to the rules and regulations set by the Ethics Committee of the First Affiliated Hospital of Nanchang University.

Hypertrophic scar tissue was cut into 0.5- to 1-mm³ pieces using a pair of scissors, and the epidermis and dermis were isolated by digestion with 0.25% Trypsin + EDTA (Gibco, USA) at 4°C

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for 10-12 h. The pieces were then placed in 25-cm² cell culture flasks (Corning, USA), and 5 mL culture medium containing Dulbecco's modified Eagle's medium (DMEM) with 100 U/mL penicillin and 100 U/mL streptomycin (Solarbio) and 10% fetal bovine serum (HyClone, USA) was added. The flasks were maintained at 37°C in air containing 5% CO_2 . The culture medium was changed (5 mL) every 5 days. HSFs grew to fusion for 14 days and were then subcultured into 25-mm² culture flasks. Experiments were performed with early passage cells (4-6) (Russell and Witt, 1976).

Drug treatment and morphological observation of cultured fibroblasts

HSFs (1 x 10⁵) seeded on 6-well plates were subjected to different treatments: a control group consisting of fibroblasts cultured with DMEM only and a test group where 5 mg/mL tetrandrine (molecular formula $C_{_{38}}H_{_{42}}N_{_2}O_{_6}$, Yingtao, China) was added to the culture medium based on our previous results indicating that the inhibition ratio of HSFs was 50.72% at 5 mg/mL tetrandrine (Zunwen et al., 2012). After adding the tetrandrine, morphological changes in the HSFs were observed with an inverted microscope, and photos were taken every 24 h.

Sample harvest

After 72 h, the test and control group cells were digested and made into single-cell suspensions. Then, the cells were centrifuged at 1000 rpm for 5 min and the supernatant removed.

Total RNA extraction and purification

RNA was isolated using TRIZOL and then quantitated using a spectrophotometer. The total RNA quality was examined using formaldehyde denaturing gel. Total RNA from the cells was isolated using the mirVana[™] miRNA Isolation Kit (Applied Biosystems) following the manufacturer protocol.

miRNA microarray analysis

We used 100 ng miRNA to perform the *in situ* oligonucleotide microarray. Fluorescent miRNA was labeled using the miRNA Complete Labeling and Hyb Kit (Agilent, USA) according to the manufacturer protocol. The hybridization was carried out for 20 h at 55°C in a rotating hybridization oven according to the instructions. After hybridization, slides were washed and then scanned by AgilentHD_miRNA. The Feature Extraction (v10.7) software was required for analysis of the images and extraction of scan data. Then, the GeneSpring software was used for data normalization, and the criterium using for differentially expressed miRNAs analysis was an absolute fold-change of at least more than 1-fold.

RT-PCR for miRNA expression analysis

To validate the miRNA microarray data, the significantly upregulated and downregulated miRNAs were selected and analyzed by RT-PCR assays, which were carried out using a 7900 HT Fast RealTime PCR system (Applied Biosystems). The miRNA-specific primers for hsa-miR-125b, hsa-miR-27b, and internal control U6 were purchased from Invitrogen (USA). Expression levels of each mature miRNA were evaluated using the comparative threshold cycle (Ct) method and

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normalized to those of U6 small nuclear RNA for each condition (calculated using the comparative Ct method and analyzed by $2^{-\Delta\Delta Ct}$; Schmittgen and Livak, 2008).

Prediction of mRNA target

Three commonly used databases including miR, (http://www.microrna.org/microrna/home. do), PicTar (http://www.pictar.org/), and TargetScan (http://www.targetscan.org/) were used to obtain predicted gene targets for significantly differentially expressed miRNAs. The genes identified using at least two of the methods were considered potential target genes regulated by a given miRNA. Predicted target genes in combination with miRNA and whole-genome microarray data were used to visualize possible biological miRNA/mRNA processes correlating to HSF growth and/or differentiation. Then, the predicted target genes underwent enrichment analysis of cell signaling pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Hua et al., 2009).

RESULTS

Morphological changes in HSFs

HSFs cultured for 48 h exhibited a long spindle-shaped structure with larger cell bodies. The cytoplasms of the fibroblasts were rich and showed multiple-angle shapes, which grew two or three different synaptic lengths (Figure 1A). Compared to the control cells, the number of HSFs treated with tetrandrine decreased, the shape of cells became smaller and round, and spindles became shorter or disappeared (Figure 1B).



Figure 1. Morphological changes of hypertrophic scar fibroblasts (HSFs) observed under inverted microscope (magnification 200T). HSFs were subjected to different treatment: control group (A), tetrandrine group (B). It represents the morphological changes of HSFs for 48 h.

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Tetrandrine and miRNA profile in HSFs

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Bas-mik-S2 Bas-mik-S2 bas-mik-S2 39.016 39.089 bas-mik-S2 33.74667 419.162 bas-mik-S2 33.74667 419.162 bas-mik-S30 135.9667 419.162 bas-mik-101 11.2223 33.74667 bas-mik-1038 15.0 434.11072 1303.179 bas-mik-750 67.0917 250.1685 250.00 bas-mik-757 67.0917 250.1685 250.00 bas-mik-892b 57.324 163.1691 153.984 bas-mik-892b 57.324 163.1691 153.986 bas-mik-762 273.9874 766.546 153.986 bas-mik-762 273.9874 766.5601 153.986 bas-mik-762 273.9874 766.5601 153.986 bas-mik-762 101.8523 275.8135 155.95 bas-mik-763 218.59781 590.577 153.94821 bas-mik-755 55.2021 147.10541 153.94821 bas-mik-737 4.20893 11.246 153.94821 </td <td>3.0943468</td>	3.0943468
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Nisa-mik-424 133.966/ 419.182 Nisa-mik-101 11.2223 3.3.744667 Nisa-mik-1308, v15.0 434.11072 130.3179 Nisa-mik-300 13.2306 3.8.25769 Nisa-mik-575 87.0917 250.1585 Nisa-mik-844 77.8722 223.0759 Nisa-mik-826.50, v15.0 199.30449 568.25476 Nisa-mik-826.50, v15.0 199.30449 568.25476 Nisa-mik-826.20, v15.0 199.30449 768.546 Nisa-mik-826.21 273.9874 768.546 Nisa-mik-826 112.953866 312.659 Nisa-mik-826 112.953866 312.659 Nisa-mik-828 190.9077 292.881 Nisa-mik-828 190.9077 292.881 Nisa-mik-828 109.0077 292.881 Nisa-mik-828 109.0077 292.881 Nisa-mik-829 12.8696 33.34922 Nisa-mik-820 12.8686 52.8124 Nisa-mik-823 12.8686 52.8124 Nisa-mik-823 13.34922	2.981648
Bas-miR-306 1303 179 Bas-miR-306 13 2306 38 25769 Bas-miR-306 38 25769 Bas-miR-306 38 25769 Bas-miR-306 38 25769 Bas-miR-844 77 8722 Disa-miR-865-30 v15.0 199 30449 Sea-miR-802.5 57 324 Bas-miR-822.5 57 324 Bas-miR-762 273 9874 Pisa-miR-762 273 9874 Bas-miR-763 112 953866 Bas-miR-763 12 953866 Bas-miR-763 12 953866 Bas-miR-763 12 853866 Bas-miR-771 500 577 Bas-miR-782 16 90 0787 Bas-miR-755 5 5 2021 Bas-miR-755 5 2021 Bas-miR-752 19 8854 Bas-miR-753 4 28093 Bas-miR-753 3 4222 Bas-miR-754 2 48699 Bas-miR-755 5 3 471508 Bas-miR-755 3 471508 Bas-miR-752 9 6 22861 Bas-miR-752 9 6 228777 <t< td=""><td>2.9751027</td></t<>	2.9751027
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Bisa-mik-8/15 8// 0.91/ 2.50, 1985 misa-mik-8494 77, 8722 223,0759 misa-mik-846-30, v15.0 199, 30449 568, 25476 misa-mik-8205 57, 324 163, 1691 misa-mik-8205 57, 324 163, 1691 misa-mik-826 114, 9884 114, 9884 misa-mik-762 273, 9974 768, 546 misa-mik-762 1273, 9974 768, 546 misa-mik-762 1273, 9974 768, 546 misa-mik-762 1273, 9974 768, 546 misa-mik-762 147, 10541 198, 56601 misa-mik-733* 172, 4642 196, 56601 misa-mik-730 222, 881 199, 577 misa-mik-750 132, 4821 198, 5660 misa-mik-751 142, 10541 158, 342, 1054 misa-mik-752 19, 8854 52, 8124 misa-mik-752 19, 8854 52, 8124 misa-mik-752 14, 20033 11, 2446 misa-mik-762 19, 62, 2814 168, 4124 misa-mik-763 14, 1508	2.7904298
hsa-miR-896-30 199.30449 566.25476 hsa-miR-8263 163.1691 hsa-miR-218 40.8108 114.9884 hsa-miR-228 273.9674 768.546 hsa-miR-23a* 112.953866 312.059 hsa-miR-23a* 16.78239 45.90055 hsa-miR-23a* 16.78239 45.90055 hsa-miR-31 72.4642 196.56601 hsa-miR-320c 101.8523 275.8135 hsa-miR-328 109.0787 292.881 hsa-miR-626 5.20003 13.94821 hsa-miR-626 5.20003 13.94821 hsa-miR-82a 12.8690 33.34922 hsa-miR-82a 12.8690 33.4922 hsa-miR-82a 12.8690 33.4922 hsa-miR-737 4.20035	2.7718544 2.7644079
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Isa-mik-210 40.000 114.300+ Isa-mik-220 273.9674 768.546 Isa-mik-22a* 112.95386 312.659 Isa-mik-22a* 112.85386 312.659 Isa-mik-22a* 112.85386 312.659 Isa-mik-22a* 112.8523 275.6135 Isa-mik-22b 101.8523 275.8135 Isa-mik-320c 101.8523 275.8135 Isa-mik-62B 52.0003 13.94821 Isa-mik-62C 52.021 147.10541 Isa-mik-755 55.2021 147.10541 Isa-mik-82a 12.6899 33.34922 Isa-mik-752 19.854 52.8124 Isa-mik-753 4.28093 11.2446 Isa-mik-722 96.22861 90.1275 Isa-mik-737 248.725 19.5277 Isa-mik-747 7.44844 18.4124 Isa-mik-747 7.44844 18.4124 Isa-mik-747 2.25.704 54.7146 Isa-mik-747 2.24.750 54.751 Isa-mik-747 2.25.704 </td <td>2.7468398</td>	2.7468398
hsa-miR-26a 112.953896 312.659 hsa-miR-26a 16.76229 45.90035 hsa-miR-31 72.4642 196.66601 hsa-miR-320c 101.8523 275.8135 hsa-miR-320c 101.8523 275.8135 hsa-miR-328 208.1 500.577 hsa-miR-455 50.577 292.881 hsa-miR-155 55.2021 14.710641 hsa-miR-155 55.2021 147.10641 hsa-miR-152 19.8654 52.8124 hsa-miR-152 19.8654 52.8124 hsa-miR-202 96.22661 246.725 hsa-miR-373* 4.20033 11.2446 hsa-miR-362 96.22661 246.725 hsa-miR-3202 96.22661 246.725 hsa-miR-347* 7.44844 18.4124 hsa-miR-342-3p 10.5277 26.3865 hsa-miR-342-3p 13.4891 32.79506 hsa-miR-342-3p 13.4891 32.79506 hsa-miR-151 14.131 14.131 hsa-miR-320a 69.05	2.706893
hsa-mik-23a* 16,78239 45,90035 hsa-mik-23a* 17,2,6442 196,56601 hsa-mik-320c 101,8523 275,8135 hsa-mik-383 218,57781 590,577 hsa-mik-628 109,0787 222,881 hsa-mik-626 5,20603 13,94821 hsa-mik-622 19,8854 52,814 hsa-mik-622 19,8854 52,814 hsa-mik-755 55,2021 147,10541 hsa-mik-762 12,8899 33,34922 hsa-mik-792 12,8893 11,2446 hsa-mik-795 3,4,1608 90,1275 hsa-mik-795 3,4,1608 90,1275 hsa-mik-795 10,5277 26,3865 hsa-mik-795 10,5277 26,3865 hsa-mik-847 2007,053 16,5139 hsa-mik-847 2007,053 16,514 hsa-mik-847 2007,053 16,539 hsa-mik-1250 57,519 163,539 hsa-mik-1260 77,47,474 345,15 hsa-mik-1260 77,47,414 <td>2.6711693</td>	2.6711693
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hsa-miR-838 218.59781 590.577 hsa-miR-838 109.0787 292.881 hsa-miR-862 5.20603 13.94821 hsa-miR-862 13.94821 147.10541 hsa-miR-862 12.8699 33.34922 hsa-miR-873* 4.28093 11.2446 hsa-miR-873* 4.28093 11.2446 hsa-miR-822 96.22861 26.725 hsa-miR-823 10.5277 26.3865 hsa-miR-842-3p 10.5277 26.3865 hsa-miR-842-3p 13.4891 32.79506 hsa-miR-842-3p 13.4891 32.79506 hsa-miR-845 14.131 14.8124 hsa-miR-845 14.131 14.131 hsa-miR-845 14.131 14.131 hsa-miR-825 5.87581 14.131 hsa-miR-1915 144.2714 345.15 hsa-miR-1920 77.47149 183.04642 hsa-miR-1935 14.2529 31.1877 hsa-miR-1945 14.7519 63.03497 hsa-miR-137 52.1592 <td>2.6132224</td>	2.6132224
Issa-IIIR-1200 109.07/ 242.681 Issa-IIIR-1200 13.94621 1 Issa-IIIR-125 5.20603 13.94621 Issa-IIIR-155 55.2021 147.10541 Issa-IIIR-152 19.8854 52.8124 Issa-IIIR-152 12.8854 52.8124 Issa-IIIR-73° 4.28033 11.2446 Issa-IIIR-73° 4.28033 11.2446 Issa-IIIR-73° 4.28033 11.2446 Issa-IIIR-73° 4.28033 11.2446 Issa-IIIR-73° 26.3865 1.05277 Issa-IIIR-73° 7.44544 18.4124 Issa-IIIR-730 13.4891 32.279506 Issa-IIIR-730 13.4891 32.279506 Issa-IIIR-71 832.487 2007.053 Issa-IIIR-71 832.487 2007.053 Issa-IIIR-720 5.87581 14.131 Issa-IIIR-720 5.87581 14.131 Issa-IIIR-720 6.9.0542 163.539 Issa-IIIR-720 7.747149 183.04642 Issa-IIIR-720 <td>2.607129</td>	2.607129
hsa-miR-155 55 2021 147.10541 hsa-miR-152 19.8854 52.8124 hsa-miR-02a 12.8690 33.34922 hsa-miR-373* 4.28093 11.2446 hsa-miR-155 34.71608 90.1275 hsa-miR-159 34.71608 90.1275 hsa-miR-373* 4.28093 11.2446 hsa-miR-150 34.71608 90.1275 hsa-miR-320 96.22661 246.725 hsa-miR-342-3p 10.5277 26.3865 hsa-miR-151-3p 13.4891 32.79506 hsa-miR-320a 66.777 26.3865 hsa-miR-320a 58.7861 14.131 hsa-miR-320a 69.0642 163.539 hsa-miR-320a 69.0642 163.539 hsa-miR-1280 77.47149 13.04642 hsa-miR-1281 13.2529 31.1877 hsa-miR-137 52.1592 119.804944 hsa-miR-137 52.1592 28.2029 hsa-miR-137 52.1592 28.2029 hsa-miR-140 19.806196	2.591094
hsa-miR-152 19.8854 52.8124 hsa-miR-152 12.6899 33.34922 hsa-miR-92a 12.6899 33.34922 hsa-miR-92a 12.6899 33.34922 hsa-miR-92a 12.6899 33.34922 hsa-miR-95 34.71508 90.1275 hsa-miR-95 34.71508 90.1275 hsa-miR-920 96.22861 246.725 hsa-miR-920 10.5277 26.3865 hsa-miR-947 7.44844 18.4124 hsa-miR-909 22.57304 54.7166 hsa-miR-909 22.57304 54.7146 hsa-miR-909 22.57304 54.7146 hsa-miR-909 22.57304 54.7146 hsa-miR-909 58.7561 14.131 hsa-miR-1320a 69.0542 163.539 hsa-miR-1320a 69.0542	2.5716083
Bas-miR-372* 1.246 Bas-miR-372* 4.28093 11.2446 Bas-miR-375* 4.28093 90.1275 Bas-miR-185 34.71508 90.1275 Bas-miR-342-30 10.5277 26.3865 Bas-miR-342-30 10.5277 26.3865 Bas-miR-342-30 10.5277 26.3865 Bas-miR-342-30 13.4891 32.79506 Bas-miR-39b 22.57034 64.7146 Bas-miR-39b 22.57034 64.7146 Bas-miR-325p 5.87681 14.131 Bas-miR-320a 69.0542 163.539 Bas-miR-320a 69.0542 163.539 Bas-miR-1260 77.47149 183.04642 Bas-miR-1281 13.2529 31.1877 Bas-miR-1281 13.2529 31.1877 Bas-miR-128 4.76358 11.1013 Bas-miR-128 11.903444 18 Bas-miR-137 52.1592 19.804944 Bas-miR-137 52.1592 28.292 Bas-miR-146 599.672 131.3466 <td>2.5629108</td>	2.5629108
hsa-miR-195 34.71508 90.1275 hsa-miR-302 96.22861 246.725 hsa-miR-307 7.44844 18.4124 nsa-miR-307 7.44844 18.4124 nsa-miR-497 8.32.487 2007.063 nsa-miR-495 8.32.487 2007.063 nsa-miR-1915 144.2714 345.15 nsa-miR-1915 144.2714 345.15 nsa-miR-1200 77.47149 183.04642 nsa-miR-1200 77.47149 183.04642 nsa-miR-128 4.76559 11.1013 nsa-miR-128 13.2529 31.1877 nsa-miR-128 13.2652 28.3029 nsa-miR-128 14.7555 19.804944 nsa-miR-137 52.1592 21.8029 nsa-miR-137 12.55625 28.3029 nsa-miR-199b-5p 351.6671 <td>2.5347652</td>	2.5347652
Bisa-mik-2d2 96.22661 246.225 Bisa-mik-2d2-3p 10.5277 26.3865 Bisa-mik-342-3p 7.44844 18.4124 Bisa-mik-151-3p 13.4891 32.79506 Bisa-mik-151-3p 13.4287 2007.053 Bisa-mik-151 14.2214 345.15 Bisa-mik-1260 77.47149 183.04642 Bisa-mik-1260 77.47149 183.04642 Bisa-mik-1260 77.47149 163.034397 Bisa-mik-1260 77.5519 63.034397 Bisa-mik-127 25.1592 119.004944 Bisa-mik-137 52.1592 28.2029 Bisa-mik-137 52.1592 28.029 Bisa-mik-140 14.902.39 15.8671 Bisa-mik-16 599.572 13.13.446 1	2.505365
hsa-miR-497 7.44844 18.4124 hsa-miR-167-3p 13.4891 32.79506 hsa-miR-167-3p 25.704 54.7146 hsa-miR-423-5p 22.57204 54.7146 hsa-miR-167 832.487 2007.063 hsa-miR-423-5p 5.87561 14.131 hsa-miR-423.5p 5.87581 14.131 hsa-miR-320a 69.0542 163.539 hsa-miR-1260 77.47149 183.04642 hsa-miR-1281 13.2529 31.1877 hsa-miR-128 4.76358 11.1013 hsa-miR-137 52.1592 63.04397 hsa-miR-137 52.1592 28.3029 hsa-miR-137 52.1592 28.3029 hsa-miR-137 52.1592 28.3029 hsa-miR-137 52.1592 28.3029 hsa-miR-14671 4902.39 119.804944 hsa-miR-137 52.1592 28.3029 hsa-miR-137 52.16871 78.60196 hsa-miR-14671 4902.39 118.3046 hsa-miR-160 59	2.4186897
Insa-miR-151-3p 13.4891 32.279506 Insa-miR-151-3p 63.77146 63.77146 Insa-miR-39b 22.57304 64.7146 Insa-miR-39b 22.57304 64.7146 Insa-miR-39b 22.57304 64.7146 Insa-miR-325p 58.7581 14.131 Insa-miR-1915 144.2714 345.15 Insa-miR-320a 69.0542 163.559 Insa-miR-320a 69.0542 163.559 Insa-miR-320a 13.2529 31.1877 Insa-miR-381 13.2529 31.1877 Insa-miR-126 4.76358 11.1013 Insa-miR-127 52.1592 63.04397 Insa-miR-137 52.1592 28.3029 Insa-miR-137 52.1592 28.3029 Insa-miR-1374b 12.55625 28.3029 Insa-miR-146 59.9572 28.402.30 Insa-miR-16 59.9572 131.346 Insa-miR-128-5 89.6197 138.446 Insa-miR-128-5 86.1997 138.4135 Insa-miR-124-5p<	2.3854864
Bas-let 7i B32.487 2007.053 Bas-let 7i 832.487 2007.053 Bas-miR-423.5p 5.87581 14.131 Bas-miR-1915 144.2714 345.15 Bas-miR-520a 69.0542 163.539 Bas-miR-520a 69.0542 163.539 Bas-miR-520a 69.0542 163.539 Bas-miR-520a 69.0542 118.04642 Bas-miR-520a 13.2529 31.1877 Bas-miR-501-5p 27.15319 63.034397 Bas-miR-501-5p 27.15319 63.034397 Bas-miR-501-5p 27.15319 63.034397 Bas-miR-501-5p 27.15319 63.04397 Bas-miR-501-5p 27.15319 63.04397 Bas-miR-501-5p 27.1531 78.069494 Bas-miR-502 28.3029 119.809494 Bas-miR-137 52.1592 219.4671 Bas-miR-137 52.029 19.809196 Bas-miR-1905-5p 351.8671 4902.39 Bas-miR-16 599.572 1313.446 Bas-miR-26	2.3461585
hsa-miR-423-5p 5.87581 14.131 hsa-miR-423-5p 5.87581 14.131 hsa-miR-1915 144.2714 334515 hsa-miR-1200 69.0542 163.539 hsa-miR-1200 77.47149 183.04642 hsa-miR-1200 13.1877 10.11013 hsa-miR-120 63.03497 63.03497 hsa-miR-137 52.1592 28.3029 hsa-miR-1374b 12.55825 28.3029 hsa-miR-199b-5p 351.8671 78.69196 hsa-miR-199b-5p 2194.671 4902.39 hsa-miR-16 599.572 1313.446 hsa-miR-120-5b 88.1997 188.4135 hsa-miR-120-5b 35.1823 76.369 hsa-miR-120-60 86.1997 188.4135	2.3265529
Issamili-320a Issamili-320a 345.15 Issamili-320a 69.0542 163.539 Issamili-320a 69.0542 163.539 Issamili-320a 13.2529 31.1877 Issamili-321 13.2529 31.1877 Issamili-325 63.04842 163.359 Issamili-325 31.1877 13.2529 Issamili-325 63.04397 13.3464 Issamili-137 52.1592 119.809494 Issamili-137 52.1592 28.3029 Issamili-1374b 12.55825 28.3029 Issamili-160 599.572 131.3446 Issamili-161 599.572 131.3446 Issamili-126b 86.1997 184.4135 Issamili-1264 699.572 13.1446 Issamili-1264 599.572 13.146 Issamili-1264 99.572 13.13.446 Issamili-1264 69.973 16.8435 Issamili-1264 59.51223 76.869 Issamili-1264-50 39.1823 76.869	2.3207967
hsa-miR-1260 77.47149 183.04642 hsa-miR-1260 77.47149 183.04642 hsa-miR-381 13.2529 31.1877 hsa-miR-381 13.2529 31.1877 hsa-miR-501-5p 27.15319 63.034397 hsa-miR-374b 12.55825 119.09494 hsa-miR-374b 12.55825 28.3029 hsa-miR-374b 12.55825 28.3029 hsa-miR-1990-5p 351.8671 789.66196 hsa-miR-16 599.572 1313.446 hsa-miR-16 599.572 1313.446 hsa-miR-122b 28.1997 188.4135 hsa-miR-1220 35.1823 76.369 hsa-miR-1224-5p 35.1823 76.369	2.2854054
nsa-mik-xsin 13.2529 31.1877 nsa-mik-xsin 13.2529 31.1877 hsa-mik-xsin 4.76358 11.1013 hsa-mik-28 4.76358 11.1013 hsa-mik-137 52.1592 119.80494 hsa-mik-137 52.1592 119.80494 hsa-mik-137 52.1592 119.80494 hsa-mik-137 52.1592 28.3029 hsa-mik-137 52.1592 28.3029 hsa-mik-137 52.1592 28.3029 hsa-mik-137 12.55625 28.3029 hsa-mik-125b 219.4.671 4902.39 hsa-mik-125b 219.4.671 4902.39 hsa-mik-126b 86.1997 188.4135 hsa-mik-126b 86.1997 188.4135 hsa-mik-124.5p 35.1823 76.369 hsa-mik-124.5p 35.1823 76.369 hsa-mik-124.5p 9.0707 67.86842	2.2800863
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hsa-miR-196a 276.8318 594.26404	2.0715487
hsa-mIR-572 9.68648 20.7287 beam beam beam beam beam beam beam beam	2.0650852
Josefilication Josefilication 113.0001 Josefilication 36.456 76.92737	2.0363092
hsa-let-7e 375.427 789.65796	2.0297637
19a-mIR-125a-5p 75.8074 158.9961	2.0239823
5.16288 10.8205	2.0224042
hsa-miR-151-5p 99.916306 209.0674	2.0224943
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Table 2. Upregulated miRNAs in the test group relative to the control group.							
miRNA-ID	Raw experimental signal values (test group)	Raw experimental signal values (control group)	Absolute fold-change (test vs control)				
hsa-miR-27b	246.7309	171.95459	1.4868867				
hsa-miR-29b-1*	45.064598	32.8065	1.4234551				
hsa-miR-193a-3p	35.012	28.9008	1.2553804				
hsa-miR-493*	18.6228	16.9013	1.1418079				
hsa-miR-100	1330.14	1253.593	1.0995347				
hsa-miR-27a	1042.83	1010.114	1.0698209				
hsa-miR-29h	1373 474	1334 3251	1.0666622				

Differential miRNA expression between the test group and the control group

To distinguish the differentially expressed miRNAs, we examined the expression of miRNAs in the test and control groups using an miRNA microarray. Only miRNAs with changes of at least 1-fold were included. By this criterion, 193 miRNAs were identified, among which 186 were downregulated (Table 1 shows miRNAs whose expression exceeded 2-fold) and 7 were upregulated (Table 2) in the test group compared to that in the control group.

Validation of the microarray data by RT-PCR

To validate the results from the miRNA microarray, we further employed RT-PCR to measure the abundance of the miRNA, including downregulated hsa-miRNA-125b and upregulated hsa-miRNA-27b. The 2^{-ΔΔCt} values showed that hsa-miR-27b (1.92>1.0) was upregulated, while hsa-miR-125b (0.74<1.0) was downregulated. The RT-PCR data indicated that the transcriptional level of hsa-miRNA-125b and hsa-miRNA-27b coincided perfectly with the microarray results.

Putative targets of miRNAs and functional analysis by bioinformatics

All differentially expressed miRNAs were subjected to target gene prediction. Each miRNA potentially regulates many targets. To decrease the total number of false-positive targets, targets predicted by both methods were considered putative candidates. After carefully analyzing the putative targets, we identified targets for each miRNA. To elucidate the target pathways of miRNAs, KEGG pathway analysis was employed to clarify the biological significance of these potential target pathways, helping us to further understand the biological processes and corresponding metabolic networks regulated by potential miRNAs. We found that many targets played significant roles in several signaling pathways that play important roles in wound repair including vascular endothelial growth factor (VEGF), apoptosis, and the cell cycle. Table 3 shows part of the putative targets of hsa-miR-125b and hsa-miR-27b.

Table 3. Putativ	ve targets of hsa-mi	iR-125b and hsa-miR-27b.	
miRNA-ID	Targets	Gene name	
hsa-miR-125b	STARD13	Star-related lipid transfer (START) domain containing 13	
	COL4A3	Collagen, type IV, alpha 3	
	FGFR2	Fibroblast growth factor receptor 2	
	BCL2L12	BCL2-like 12	
	TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4	
	ATP10D	Atpase, Class V, type 10D	
hsa-miR-27b	VEGFC	Vascular endothelial growth factor C	
	APAF1	Apoptotic protease activating factor	
	COL19A1	Collagen, type XIX, alpha 1	
	GDF8	Growth differentiation factor 8	
	EGFR	Epidermal growth factor receptor	

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DISCUSSION

Hypertrophic scar formation occurs when the equilibrium between positive and negative cytokines stimulated by inflammation and the synthesis and metabolism of ECM is broken (van der Veer et al., 2009). The abnormal biological behavior of fibroblasts plays a critical role in the scar formation process. Tetrandrine is a bisbenzylisoquinoline alkaloid derived from S. tetrandra. Previous pharmacological and clinical studies have shown that tetrandrine possesses antiinflammatory, anti-proliferative, immunosuppressive, and antitumor activities (Dong et al., 1997; Lai et al., 1998; Xie et al., 2002; Yoo et al., 2002; Lee et al., 2002; Kuo and Lin, 2003; Wang et al., 2004). In recent years, it was discovered that tetrandrine can inhibit proliferation of human Tenon's capsule fibroblasts and differentiation of osteoclasts (Takahashi et al., 2012; Li et al., 2012). In our previous studies, we showed that tetrandrine inhibited proliferation of HSFs and synthesis of collagen and DNA (Liu et al., 2001). However, it is still unclear how tetrandrine inhibits HSFs. MicroRNAs are known to play critical roles in development, cell proliferation, and other fundamental cellular processes (Bartel, 2004; Zhao and Srivastava, 2007). Our previous studies have confirmed distinct differences in miRNA expression between human hyperplastic scar tissue and normal skin, which may be closely correlated to the formation, development, and evolution of hyperplastic scarring (Ning et al., 2012). Furthermore, Wilmink et al. (2010) have shown that dermal fibroblasts differentially express 123 miRNAs when exposed to hyperthermia using an miRNA microarray. In this study, we identified 193 potential miRNAs that were differentially expressed between the tetrandrine test group and the control group with 186 miRNAs downregulated and 7 upregulated.

Some miRNAs identified in this study have been shown to play important roles in some cellular mechanisms. Gutierrez et al. (2011) demonstrated that thyrotroph embryonic factor is downregulated by miR-125b through activation of p53 and this novel regulation pathway helps determine the actin distribution and the shape of fibroblasts. Moreover hsa-miR-125b was observed to regulate cell proliferation and differentiation in both breast cancer and ovarian cancer cell lines and plays an important role in osteoblastic differentiation (lorio et al., 2005; Mizuno et al., 2008; Guan et al., 2011). Hsa-miR-125a-3p and hsa-miR-125a-5p were found to affect the migration and invasion of lung cancer cells, while hsa-miR-99a was found to affect the differentiation of keratinocytes (Jiang et al., 2010; Lerman et al., 2011). Recent evidence has shown that hsa-miR-155 may promote cell proliferation by regulating its target genes and can regulate the expression of the angiotensin II type 1 receptor in primary human lung fibroblasts (Kong et al., 2010; Martin et al., 2013). While upregulated in the test group, hsa-miR-29b has been found to inhibit the expression of collagen 1 protein in skin fibroblasts by the way of classic mRNA transcriptional regulation in vitro (Wang et al., 2011). The results of the study by Crist et al. (2009) showed that overexpression of an miR-27b transgene in Pax3-positive cells in the embryo leads to downregulation of Pax3, resulting in interference with progenitor cell migration and in premature differentiation. Furthermore, miR-27b inhibitors were transfected into cultures of adult muscle satellite cells that normally express miR-27b at the onset of differentiation, which resulted in continuing Pax3 expression leading to more proliferation and a delay in the onset of differentiation.

To obtain a better understanding of the functional significance of miRNA, it was important to identify and validate the miRNA targets. In this study, hundreds of target genes were predicted, including several key mediators of cellular signaling pathways and some ECM proteins. Among them, we analyzed the putative targets of hsa-miR-125b and hsa-miR-27b, which were validated by RT-PCR. According to the results, these targets were involved in several signaling pathways

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such as VEGF, the cell cycle, and apoptosis. The BCL2 protein played a key role in apoptosis, cell migration, proliferation, and differentiation (Cory and Adams, 2002). COL4 was closely related with collagen formation, and fibroblast growth factor was important for fibroblast growth (van der Veer et al., 2009). VEGF, the target gene of hsa-miR-27b, was not only an important growth factor for promoting endothelial cell proliferation but also played an important role in protein synthesis and the formation of granulation tissue in the inflammatory and wound repair proliferative phases (Ferrara et al., 2003). Moreover, the target genes of hsa-miR-27b including *COL19A1*, *GDF8*, and *EGFR* were also related to collagen formation, cell differentiation, and epidermal growth. GDF8 has been proven to be a pro-fibrogenic factor that promotes fibroblast proliferation and ECM synthesis in tendons and ligaments (van der Veer et al., 2009; Fulzele et al., 2010). miRNAs might contribute to the anti-fibrosis properties of tetrandrine by regulating its targets and influencing multiple-signaling pathways.

As a traditional Chinese medicine, tetrandrine has been used for the treatment of arthritis, arrhythmia, hypertension, inflammation, and silicosis for years in the clinic and as a therapeutic (Fang and Fang, 1996; Pang and Hoult, 1997; Shen et al., 2001). However, tetrandrine has not been utilized as a clinical treatment for hypertrophic scarring because its mechanism is unknown. This study has identified the differentially expressed miRNAs in HSFs treated with tetrandrine through genomic profiling, and the putative targets of these altered miRNAs are most likely involved in multiple signaling pathways that have previously been shown to be implicated in cellular growth. Hence, certain miRNAs might contribute to the anti-fibrosis of tetrandrine in HSFs through the signaling pathways of these targets.

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