Comparative study of microRNA profiling in keloid fibroblast and annotation of differential expressed microRNAs

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Keloids are tumor-like skin scars that grow as a result of the aberrant healing of skin injuries, with no effective treatment. The molecular mechanism underlying keloid pathogenesis is still largely unknown. In this study, we compared microRNA (miRNA) expression profiles between keloid-derived fibroblasts and normal fibroblasts (including fetal and adult dermal fibroblasts) by miRNA microarray analysis. We found that the miRNA profiles in keloid-derived fibroblasts are different with those in normal fibroblasts. Nine miRNAs were differentially expressed, six of which were significantly up-regulated in keloid fibroblasts (KFs), including miR-152, miR-23b-3p, miR-31-5p, miR-320c, miR-30a-5p, and hsv1-miR-H7, and three of which were significantly down-regulated, including miR-4328, miR-145-5p, and miR-143-3p. Functional annotations of differentially expressed miRNA targets revealed that they were enriched in several signaling pathways important for scar wound healing. In conclusion, we demonstrate that the miRNA expression profile is altered in KFs compared with in fetal and adult dermal fibroblasts, and the expression profile may provide a useful clue for exploring the pathogenesis of keloids. miRNAs might partially contribute to the etiology of keloids by affecting several signaling pathways relevant to scar wound healing.

Keywords microrna; fibroblasts; keloid; fetal dermis; adult dermis

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Introduction

Keloid disease represents a benign dermal fibroproliferative tumor that occurs due to an abnormal wound-healing process after skin injury. They are characterized by the over-production of extracellular matrix (ECM), and the scar tissue progressively invades the surrounding normal skin and beyond the original wound area [1–3]. The altered expression and regulation of various ECM components by keloid fibroblasts (KFs), such as collagen, fibronectin, elastin, proteoglycans, and matrix-directed protease and protease inhibitors, have been implicated in keloid fibrosis [4–8]. Keloids are the result of unbalanced cellular dynamics caused by overabundant fibroblast proliferation and the lack of fibroblast apoptosis [9]. Although excess deposition of ECM components, such as collagen [10,11], by fibroblasts is responsible for keloids, the etiology and mechanism are still poorly understood. Although keloids are benign dermal tumors, their management is one of the most challenging clinical problems. Keloids do not regress over time, and surgical excision alone results in a high rate of recurrence. Various conservative therapies have been attempted, but definite and effective treatment has not yet been established [12,13].

MicroRNAs (miRNA) are short non-coding RNAs that play critical roles in many important biological processes, such as cell growth, proliferation, differentiation, and apoptosis [14]. To date, hundreds of miRNAs have been identified to be dysregulated in various diseased tissues [15], but only a fraction of them have been functionally characterized. Recently, some miRNAs have been reported to participate in fibrosis and ECM metabolism, and miRNAs play a crucial role in the formation and growth of keloids [16,17]. As a new therapeutic approach for fibrotic disorders, several miRNA gene therapies have been attempted. In particular, antagonizing endogenously up-regulated miRNAs using antisense strands has been proposed [18]. Van Rooij et al. [19] have shown that inhibiting miR-29 using a cholesterol-conjugated antisense strand increased collagen expression in...
the liver, kidney, and heart of mice. The miR-29 family members (miR-29a, miR-29b, and miR-29c) directly regulate the translation of various ECM mRNAs, such as the collagen superfamily [16,17]. They are also implicated in fibroblasts in cardiac fibrosis [19], stellate cells in hepatic fibrosis [20,21], and dermal fibroblasts in systemic sclerosis [22]. Another research group has reported that miR-21 expression is selectively increased in fibroblasts in failing hearts and controls interstitial fibrosis and cardiac hypertrophy [23]. The inhibition of miR-21 prevented interstitial fibrosis and cardiac hypertrophy in a mouse model of heart infarction [24]. Thus, miRNAs play a number of roles in fibrosis and have attracted attention as a new target for gene therapy. The essential roles of miRNAs in skin development and pathophysiology have been taken into consideration. However, the functional roles of miRNAs in the pathogenesis of keloids remain largely unknown.

We hypothesized that different expression patterns of miRNAs might contribute to the formation and growth of keloids. To test this hypothesis, we performed comprehensive miRNA profiling and a comparative miRNA analysis between KFs and adult and fetal dermal fibroblasts. Bioinformatics analysis of the putative targets of these miRNAs proved that several signal-transducing pathways modulated by miRNAs may contribute to the pathogenesis of keloids, which helps to elucidate the etiology of keloids and reveal therapeutic targets. This is the unique report of differentially transcribed miRNAs in keloid samples compared with fetal and adult dermal fibroblasts.

Materials and Methods

Primary fibroblast cell culture
A primary culture of dermal fibroblasts was established as previously described [25]. Explants were maintained in Dulbecco’s Modified Eagle’s Medium (Gibico, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% (w/v) penicillin/streptomycin in a 5% CO₂ humidified atmosphere at 37°C. Fibroblasts obtained during the first culture for 2 weeks (at passage 0) were used in this study, except as indicated otherwise.

Tissue samples
Keloids and the corresponding normal skin tissues, and fetal dermal specimens were collected from the Sixth People’s Hospital, Shanghai Jiao Tong University, in accordance with human subject guidelines approved by the Scientific and Ethical Committee of Shanghai Jiao Tong University. All patients’ diagnosis of keloids were confirmed by histology tests. No patients received any treatment before the surgical procedure. Keloid tissue and normal tissue were carefully excised, then 4-mm punch biopsies were taken from every sample. All of these samples were collected by experienced surgeons.

Western blot analysis
Tissues were harvested and homogenized in ice-cold sodium dodecyl sulfate (SDS) lysis buffer. Total cell lysates were collected, and equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk powder for 1 h, washed for 1 h in TBS, and incubated with anti-collagen-I antibody (Abcam, Cambridge, USA) at 4°C for 3 h. Then the membranes were washed with 1 × TBS/Tween 20 buffer for three times (5 min each time) and incubated with horseradish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody (Abcam) for 1 h. The membranes were developed with the enhanced plus chemiluminescence assay kit (Pierce, Rockford, USA) according to the manufacturer’s instructions.

miRNA microarray expression profiling and data analysis
Microarrays were produced using an locked nucleic acid (LNA)-based oligonucleotide probe library (miRCURY LNA array ready to spot v.7.1; Exiqon, Woburn, USA). Oligonucleotides were spotted in duplicate in phosphate buffer on CodeLink microarray glass slides (GE Health Care, Bethesda, USA) using a VersArray ChipWriter Pro system (Bio-Rad, Hercules, USA) as previously described [26]. In total, 2 μg of sample RNA was directly labeled with Hy3 using the miRCURY LNA array labeling kit (Exiqon). As a reference, we used a pool of RNA extracted from bladder, prostate, and colon tumors. For each experiment, we labeled 2 μg of the reference RNA with Hy5 using the LNA array labeling kit (Exiqon). Hybridization and washing of the microarray slides were performed as recommended by Exiqon. Scanning was performed using a ScanArray 4000 scanner (GSI Lumonics, PerkinElmer, Waltham, USA). After scanning the microarrays, we used TIGR Spotfinder 2.23 software to generate raw intensity data, which were LOWESS (global) normalized using TIGR MIDAS 2.19 software [27]. Average log 2 ratios were calculated from the normalized data based on the two measurements of each miRNA. The microarray analysis was performed using three arrays per miRNA sample, and the results were averaged.

Hierarchical cluster analysis
For hierarchical cluster analysis, human miRNA probes on the array were median-centered and normalized. Samples were then clustered hierarchically using Cluster 2.0. To evaluate the robustness of the formed clusters, a clustering analysis was performed using three different similarity
metrics (correlation-centered, correlation uncentered, and Spearman’s rank) and two different clustering algorithms (average linkage clustering and complete linkage clustering). Treeview 2.0 was used for visualization. The cluster dendrograms shown in this article were produced using the correlation-centered metric and average linkage clustering algorithm.

Quantitative reverse transcription-polymerase chain reaction
Total RNA was extracted from the three types of fibroblasts using Trizol total RNA isolation reagent (Invitrogen, Carlsbad, USA) as per the manufacturer’s protocol. cDNA was synthesized from total RNA using gene-specific primers of eight different mature miRNAs. Quantitative polymerase chain reaction (qPCR) assay primers were as follows: hsa-miR-152: 5'–UCAGUGCAUGACAGAUCUUGG-3'; hsa-miR-23b-3p: 5'–AUCACAUGCCAGGAUACC-3'; hsa-miR-30a-5p: 5'–UGUAAACAUCCUCGACUGGAAG-3'; hsa-miR-145-5p: 5'–GUCAGUUUUCCAGAAUCCCU-3'; hsa-miR-31-5p: 5'–AGGCAAGUUGGCGCAUGGCU-3'; hsa-miR-143-3p: 5'–UGAGAUGAAGCUGUAGCUC-3'; hsa-miR-4328: 5'–CCAGUUUUCCAGGAUU-3'; hsa-miR-320c: 5'–AAAAGCUGGUUGAGAGGGU-3'. Quantitative reverse transcription-PCR (qRT-PCR) was performed using an Applied Biosystems 7500 Sequence detection system (Applied Biosystems, Carlsbad, USA). Default threshold settings were used to determine the threshold cycle data. The relative amount of each miRNA was calculated using the comparative threshold (Ct) method with \( \Delta Ct = Ct (\text{miRNA}) - Ct (U6) \). The relative quantification of miRNA expression was calculated with the \( 2^{-\Delta Ct} \) method.

Prediction of miRNA targets and categorization of signaling pathways composed of miRNA targets
Three publicly available databases, TargetScan Human 5.1 (http://www.targetscan.org/), DIANA-microT v3.0 (http://www.diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi), and MicroCosm Targets version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), were used for miRNA target gene prediction. The predicted gene was considered to be a putative target candidate when it was predicted by all three databases. Predicted target genes in combination with miRNA and whole-genome microarray data were used to visualize possible biological miRNA/mRNA processes correlating to keloid development and/or progression. The bioinformatic annotations of all putative miRNA targets, including categorization of biological processes and signaling pathways, were performed using the online DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/). A modified Fisher’s exact test resulting in a \( P \) value was performed to determine whether the proportions of genes falling into each category were due to random chance.

Statistical analysis
All experiments were carried out in triplicate. miRNA microarray data are expressed as the mean \( \pm \) standard deviation of at least three independent experiments. Differences between miRNA expression levels among two groups were evaluated using analysis of variance (ANOVA) (Version 18.0, SPSS, Inc., Chicago, USA). \( P < 0.05 \) was considered significant difference.

Results
Differential expression of collagen-1 in primary fibroblasts
Three types of fibroblasts were obtained from human keloid, fetal, and adult dermal tissues. To identify the different characteristics of keloid, protein expression of collagen-1 was examined by western blot using specific antibody. As a result, there was no obvious difference in the collagen-1 expression between the fetal and normal adult dermal-derived fibroblasts (Fig. 1). However, the expression of collagen-1 was remarkably increased in keloid-derived fibroblasts. These results are consistent with previous studies reporting that excess ECM components, such as collagen, were deposited by fibroblasts in keloids [10,11], suggesting that fibroblasts might function as a downstream signal regulator involved in the etiology of keloid.

Expression profile of miRNAs in KFs
To identify miRNAs that were specifically regulated in KFs, we performed a comprehensive analysis of miRNA expression in KFs and fetal and adult dermal fibroblasts using miRNA expression microarrays. A total of 1200 miRNAs were included in the miRNA microarray, which represents all of the human miRNAs known to date. Each of the three samples of KFs and fetal and adult dermal fibroblasts was examined for changes in miRNA expression (Fig. 2). A total of nine miRNAs were identified to be differentially expressed in KFs, fetal and adult dermal fibroblasts (ANOVA, \( P < 0.05 \)). Of these, hsa-1-miR-H7 is a viral miRNA, which is expressed by herpes simplex virus-1(HSV-1) and varicella-zoster virus (VZV) in latently infected human neurons in vivo, and is likely to play key roles in lifestyle of HSV-1 or VZV. Therefore, we only analyzed the other eight miRNAs in this study, which might be involved in the generation of keloid. Compared with adult dermal fibroblasts, five miRNAs were over-expressed and three were under-expressed in KFs and fetal fibroblasts (Table 1). Among these miRNAs, miRNA-145-5p, miR-4328, and miR-143-3p showed the largest fold change (miR-145-5p: 9.79 folds, miR-4328: 7.61 folds, and miR-143-3p: 0.32 fold in KFs compared with adult dermal fibroblasts). Results also showed that the expression profile of miRNAs in KFs was similar to that of fetal fibroblast, suggesting a transition state to naive cells in KF.
Validation of miRNA expression by qRT-PCR

To validate the microarray results, qRT-PCR was performed with eight miRNAs that exhibited more than 2-fold change in expression (Table 2). In agreement with the microarray results, miR-4328, miR-145-5p, and miR-143-3p were down-regulated, whereas miR-31-5p, miR-23b-3p, miR-30a-5p, miR-320c, and miR-152 were up-regulated in the KFs compared with adult dermal fibroblasts and fetal fibroblasts, and these differences were statistically significant (Table 2). Overall, the microarray data were considered sufficient to warrant further analyses in a clinical setting.
Putative targets of miRNAs and functional analysis by bioinformatics

We performed computational predictions of target genes for seven differentially expressed miRNAs. For hsa-miR-4328 is a relatively new member in the miRNAs family, related research has just started with new reports emerging, so we did not perform its computational predictions. Each miRNA of the seven miRNAs potentially regulates many targets. To decrease the total number of false-positive targets, the targets were considered as putative candidates if they were identified by three programs. A total of 839 potential targets were predicted, and the number of potential targets of each miRNA varied from 9 to 97. To elucidate target pathways of miRNAs, we carried out gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses, which helped to further elucidate the biological process and corresponding metabolic network regulated by potential miRNAs. The DAVID platform was used to identify GO and KEGG signaling pathways for the putative targets of miRNAs. We found that many targets played significant roles in several important pathways which are closely associated with keloids, including transforming growth factor-β (TGF-β) family and mitogen-activated protein kinase (MAPK) pathways, apoptosis, and the cell cycle (Table 3).

**Discussion**

Previous studies have shown that a small subset of miRNAs may define keloid entities better than microarray expression data from thousands of miRNAs [28]. In the present study, we characterized, for the first time, a 9-miRNA signature that can differentiate between KFs and normal adult fibroblasts. The

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**Table 2 Validation of miRNA microarray results using qRT-PCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Fetal dermal</th>
<th>Adult dermal</th>
<th>Keloid</th>
<th>ANOVA P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-4328</td>
<td>4.01 ± 0.22</td>
<td>6.87 ± 0.79</td>
<td>8.89 ± 0.52</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-152</td>
<td>9.68 ± 0.34</td>
<td>8.33 ± 0.35</td>
<td>7.31 ± 0.41</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-23b-3p</td>
<td>4.28 ± 0.45</td>
<td>3.76 ± 0.16</td>
<td>3.26 ± 0.14</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-145-5p</td>
<td>3.08 ± 0.31</td>
<td>5.75 ± 0.32</td>
<td>3.90 ± 0.29</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-31-5p</td>
<td>8.53 ± 0.39</td>
<td>6.37 ± 0.35</td>
<td>6.31 ± 0.51</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-320c</td>
<td>9.58 ± 0.26</td>
<td>8.23 ± 0.25</td>
<td>6.89 ± 0.30</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-143-3p</td>
<td>2.76 ± 0.16</td>
<td>3.69 ± 0.74</td>
<td>3.88 ± 0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>hsa-miR-30a-5p</td>
<td>9.58 ± 0.44</td>
<td>10.66 ± 0.57</td>
<td>6.34 ± 0.56</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Expression levels of miRNAs in keloid and normal and fetal dermal fibroblasts. The data are presented as ΔCt of relative threshold cycles, indicating fold changes over control primer U6 (ΔCt = Ct (miRNA) − Ct (U6)). Low values indicate high levels of miRNA.

**Table 3 Composition of the miRNA signature in keloid fibroblasts**

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Expression level</th>
<th>Putative targetsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-152</td>
<td>High</td>
<td>GNS, PSMD1, STT3A, PPAP2C, INHBB, TGFB2, MRAS, TGFB2, RPS6KA4, MAP3K4, DUSP1, SOS1, RASGRP1, SOS2, CACNA1E, IKKB, GADD45A, CHUK, MAP2K5</td>
</tr>
<tr>
<td>hsa-miR-23b-3p</td>
<td>High</td>
<td>FUT9, ST8SIA1, B3GNT1, FUT4, BCL2, MAPK11, APAF1, NEFL, PRPH2, ATG12, MAP3K1, MAPK11, IL12B, CHUK, AZI2</td>
</tr>
<tr>
<td>hsa-miR-145-5p</td>
<td>Low</td>
<td>RAD23B, ZBTB33, SOX11, SSH2, AKAP12, SMC6, RNF207, GLCE, TBC1D15, ARL11, SLC1A2, CLK4, RN2, WDR43, CLINT1, FNDC3A, SLC1A2, BIRC2</td>
</tr>
<tr>
<td>hsa-miR-31-5p</td>
<td>High</td>
<td>CACNG4, MAPKAP2K2, RASA1, PIK3C2A, OXSR1, MAPKAP2K2, PRKCE</td>
</tr>
<tr>
<td>hsa-miR-320c</td>
<td>High</td>
<td>MARK1, HUNK, PDIK1L, ULK1, MAP3K2, RIOK3, CDC42BPA, ROR2, NRK, MASTL, BMPR1A, PCDH6a, CDH20, PCDH2a, PCDHA5, PCDHA10, CDH2, PCDHAC2, PCDH19, PCDHAC1, ITK, STK38, WNK1, CLK1</td>
</tr>
<tr>
<td>hsa-miR-143-3p</td>
<td>Low</td>
<td>CD44, IAPP, BCL2, PLA2G1B, PKD2, TNKS, TLR2, CD28</td>
</tr>
<tr>
<td>hsa-miR-30a-5p</td>
<td>High</td>
<td>EGFR, CACNA2D1, TGFB1, TP53, PPP3R1, FG13, HSPA1A, HSPA1B, TGFB1, AKT1, BDNF, JUN, PPP3CA, FGF2, MAP3K12, PPP5C, CDKN1A, PPM1D, CDKN2A, TNFRSF10B, CDK6, THBS1, NOS1, GRIA2, BCL2</td>
</tr>
</tbody>
</table>

The putative targets were selected based on functional aspects (DAVID Database) and described gene associations (GENIG).

*a*Putative target genes identified from Mirbase and TargetScan using DAVID database and GENIG software.
Dysregulated miRNA expression may occur via a number of mechanisms, such as gene copy gain or loss [37], germline mutation of precursor miRNA molecules [38], promoter methylation [39], aberrant miRNA processing due to altered expression of the miRNA biogenesis machinery [40], or transcription factors [41]. The dysregulation of miRNAs has also been identified in fibroproliferative diseases of the skin including scleroderma and keloids. miR-29 is down-regulated in scleroderma biopsy specimens, explanted fibroblasts, and bleomycin-induced skin fibrosis [22]. Independently, the down-regulation of miR-29 is confirmed along with miR-145 and the up-regulation of miR-21[42]. miR-29 targets collagen [22,42] and is shown to be down-regulated in response to TGF-β1 and platelet derivative growth factor. miR-196a is down-regulated in explanted KFs and directly targets transcripts of collagens genes (COL1A1 and COL3A1) [34].

In our study, of these nine miRNAs identified in KFs, many are known to be associated with the cell cycle, differentiation, and cancer. Some miRNAs identified in this study have been shown to play important roles in biological procedures of cellular mechanisms [43]. For example, miR-152, miR-23, miR-30a, and miR-31 show increased levels in tissue samples diagnosed with lung cancer [44], while the expression levels of miRNA-320c are correlated with aging, and miRNA-320c can regulate human cartilage metabolism [45]. Recent evidence has shown that miR-152 is required for proper cell cycle progression and plays critical roles in the S-phase and G2/M-phase cell cycle progression of diploid fibroblasts. The inhibition of miR-152 resulted in decreased cell proliferation and eventually a reduced number of cells. In this study, we found that miR-152 was over-expressed in KFs. Further experiments are needed to determine the function of miR-152 in the formation of keloids.

In summary, the expression profiles of miRNAs in KFs and normal fibroblasts were distinct. As each individual miRNA could have hundreds of mRNA targets, miRNAs that are differentially regulated in KFs may have a variety of functions in keloid pathogenesis. The mechanisms of the reduced expression of miRNAs remain to be elucidated. However, miRNAs could be attractive therapeutic targets, as attempts have been made to test the topical administration of RNA-based drugs [46,47].

**Funding**

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