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Regulatory mechanism of miR-29 over TGF-β1 and COL1 in scar cells

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Abstract. – OBJECTIVE: To study the regulatory mechanism of miR-29 over TGF-β1 and COL1 in scar cells.

PATIENTS AND METHODS: 5 clinical cases of hypertrophic scar (HS) skin and adjacent normal skin tissues were separated into fibroblast for primary culture and subculture before being observed morphologically and standard HE staining under an ordinary optical microscope. RT-PCR method was applied to test the expression level of miR-29, TGF-β1, and COL1 mRNA. ELISA method was applied to test the expression level of extracellular matrix COL1, fibronectin (FN) and α-SMA. The miR-29 overexpression vector was built and transfected *in vitro*. RT-PCR method was applied to test related genes and ELISA method was applied to test the expression level of the extracellular matrix.

RESULTS: The color of karyon and cytoplasm of normal fibroblast were both light red, with little ECM. The color of karyon of scar fibroblast was blue. The cytoplasm was red of different degrees, with relatively much ECM, in deep blue color. Compared with that in the normal fibroblast group, the miR-29 mRNA in fibroblast in the scar group significantly decreased ($p<0.05$). The TGF-β1 and COL1 mRNA significantly increased ($p<0.05$). The COL1, FN and α-SMA level were significantly higher ($p<0.05$) than that in the normal group. These mRNAs levels in miR-29 overexpression group were lower than scar group but higher than the normal group.

CONCLUSIONS: The expression of miR-29 which regulates the expression of TGF-β1 and COL1 and increases the level of ECM significantly decreases in scar cells. This one suggests a mechanism of the formation of the scars through TGF-β1 and COL1.

Key Words:
miR-29, Scar cells, TGF-β1, COL1, Fibronectin protein, α-SMA.

Introduction

Hyperplastic scar (HS) is a common complication during the healing process of skin trauma, with the occurrence rate as high as 4-16%, which seriously affect the functional recovery and appearance of the tissue[1]. The collagen of synthesis and the imbalance of degradation are the primary cause of HS formation[2]. The gene chip test shows[3], the miR molecule expression in normal skin and HS tissues shows significant differences. Among them, 13 kinds of miR molecules have low expression and 92 kinds high expression. The target genes these miR molecules regulate are related to the cell proliferation and apoptosis, movement and period, metabolism and synthesis and other functions[4]. The miR-29 shows the most significant difference, with the change multiplied by 15.0 times, which manifests the obvious down-regulation[5]. Three bioinformatics software, PicTar, Target Scan, and miRanda, have been used for the analysis and the results are as follows: TGF-β1 and COL1 are both effective target genes of miR-29. Therefore, we put forward the mechanism: the continuously low-level expression of miR-29 loses its effective regulation over the expression of TGF-β1and COL1, causing the excessive deposition of ECM which is mainly collagen, and finally lead to the formation of HS. Partial application of miR-29 is expected to curb the over fibrosis process and can be the new target spot for the prevention and treatment of HS.

Patients and Methods

The Separation and Cultivation of Fibroblast

This study was approved by the Ethics Committee of The Third Military Medical University.

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Signed written informed consents were obtained from all participants before the study. 5 clinical cases of hypertrophic scar skin and adjacent normal skin tissues were acquired from patients aged from 34 to 62. Among them, there were 2 cases of burn, 2 cases of scald, and 1 case of a cut wound. 3 scars were on the limbs, 1 on the chest, and 1 on the abdomen. The POSAS evaluation standards have been fulfilled, and the informed consent rights of the patients have been obtained. Within one hour after getting the tissue sample, primary culture of the cells was carried out on the clean bench (Medical Apparatus and Instruments Company, Shanghai, China). The main procedures include: 0.1 M phosphate buffered saline (PBS) washing, tissue cutting, 0.1% I type collagenase solution (Worthington Biochemical Co., Lakewood, NJ, USA), vibration and digestion at 37°C (normal tissue 90 min, HS tissue 180 min). LG-DMEM culture solution (Cell Culture Basics - Gibco®, Grand Island, NY, USA) which contains isopycnic 10% fetal bovine serum (FBS; HyClone™ Laboratories, Logan, UT, USA) to terminate the digestion, 1200 r/min centrifuge for 10 minutes. Dispose of the supernatant liquid and get the high-density tissue cell clusters. Suspend them with high glucose solution (Cell Culture Basics - Gibco®, Grand Island, NY, USA). Inoculate in culture dishes of 100 mm diameter at the density of $4\times104/cm^2$. Cultivate them normally under 37°C 5% CO_2 100% humidity. Change the solution for the first time after 24 hours. Afterwards, change the solution every two days until the cells to become monolayer cells. When the cells are 80% blended, they were sub-cultivate in the proportion of 1:3.

The main equipment includes CO_2 incubator (US Pharma, Thermo Fisher, Waltham, MA, USA), microscale adjustable transfer pipette (Eppendorf, Hamburg, Germany), vibrator (Medical Apparatus and Instruments, Shanghai, China), and ordinary centrifuge (Biofuge Company).

Routine HE Staining

Carry out the morphological observation and standard HE staining of the collagenous fiber under an ordinary optical microscope (Olympus, Tokyo, Japan).

RT-PCR Method to Test the Expression Level of miR-29, TGF-β1 and COL1 mRNA

Trizol reagent method (Invitrogen, Carlsbad, CA, USA) for the extraction of total RNA; ultraviolet spectrophotometer (Beijing Liuyi Instruments Factory, China) for the test of RNA concentration; inverse transcription kit (Invitrogen, Carlsbad, CA, USA) to synthesize the cDNA; primer design (Shanghai Bioengineering Technology Co., Ltd., China); and PCR reaction. miR-29 (F):5'-CGGGTACCGGTCCTTTCTAG GTT-3', (R):5'-CGGAATT CAAAAATGTGGGC-3',253bp; TGF-β1(F): 5'-CTGCTACCGCTGCTGTGGCTACTG-3', (R):5'-CGGTCGCGGGTGCTGTTGT-3',227bp; COL1(F): 5'-GAAGGCACTCCCACCATC-3', (R):5'-AGCCCCTCTTCTCATACAG-3',209bp; Internal reference GAPDH(F): 5'-GCACCGTCAAGGCTGAGAAC-3', (R):5'-TGGTGAAGACGCCAGTGGA-3', 203b. The reaction system is Takara SYBR Premix Ex Taq 10 μl+ upstream and downstream primer each 0.510 μl+cDNA template 1 μl, add water until the total volume reaches 20 μl. The reaction conditions were 94°C 2 min, 94°C 40s, 60°C 40s, and 72°C 1 min. There are 30 cycles under 72°C 5 min. The result is shown in the form of relative expression amount of the target genes and internal reference genes. The $2^{-\Delta\Delta Ct}$ method has been applied.

Main reagent and equipment: TaKaRa RNA PCR Kit (Shiga, Otsu, Japan), ABI 7500 fluorescent quantitative PCR detector (Applied Biosystems, Foster City, CA, USA).

ELISA Method to Test the Expression Level of ECM COL1, FN and α-SMA

The kit was bought from Beijing Zhongshan Jinqiao Biology Co., Ltd. The microplate reader was bought from US Sigma Company (St Louis, MO, USA). The instructions should be strictly followed.

miR-29 Overexpression Vector Establishment in Vitro

Refer to the above miR-29 primer sequence, and respectively add AgoI and EcoRI enzyme locus (TaKaRa Company, Dalian, China) at the 5' end. PCR reaction condition: 94°C 2 min, 94°C 30s, 56°C 30s, 72°C 1 min, 35 cycles in total, 72°C 10 min. Apply double enzyme digestion for the PCR products after purification and pTA2 vector (Shanghai Jikai Company, China). After gel recovery of the enzyme digestion products, use the T4 ligase at 1:1 mole ratio (TaKaRa Company, Dalian, China) to link, transform, and planking culture. Choose 10 clones and send them to Shanghai Yingjun Company, China, for sequencing. Clone the miR-29 target gene to adenovirus framework vector pAdtrack, transform BJ5183 bacteria recombination, get pAdeasy-Adtrack-miR-29

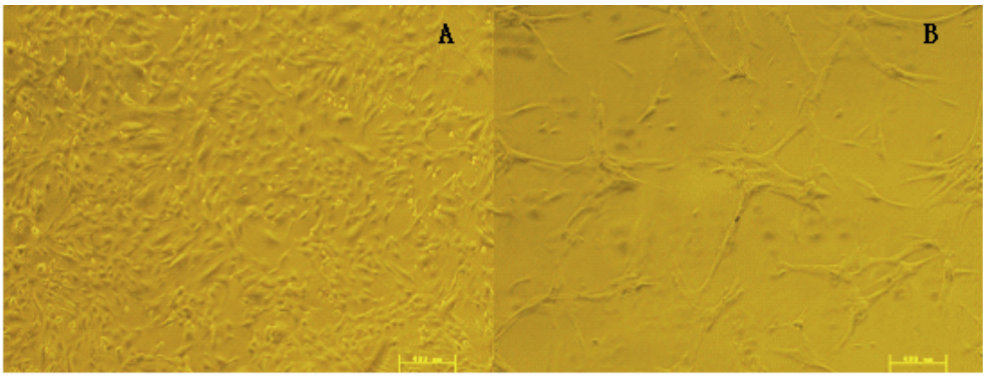

Figure 1. Morphological observation under microscope. 200X ***A,*** for the normal fibroblasts, ***B,*** for the scar fibroblasts.

adenovirus recombinant plasmid and is identified by Pac I enzyme digestion. Transfect 293 cell to package virus. After repeatedly amplify the virus for 5 times, apply the plaque test method to test the virus titer. Add the supernatant liquid of the virus and polybrene infection-accelerating reagent (5 μg/ml) together into the fibroblast culture medium for infection. Use the BD FACS Aria Cell Sorter (BD Biosciences, San Jose, CA, USA) to select cells with positive GFP expression. Culture them in the Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% FBS. Specific RT primer and TaqMan probe have been used to quantitative detection of overexpression of miR-29 fibroblast. Apply standard Trizol method for RNA extraction, cDNA combination and qPCR experiment, based on the instructions of TaqMan® MicroRNA Reverse Transcription Kit (ABI, Foster City, CA, USA) and TaqMan® MicroRNA Assays (ABI, Foster City, CA, USA). Analyze the relative differences of miR-29 expression through 2 $^{-\Delta\Delta Ct}$ method.

Main reagent: Escherichia coli competence DH-5α (Beijing Tiangen Company, China), transfection reagent Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA).

Statistical Analysis

The SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The quantitative data are presented in the form of the mean ± standard deviation (SD). Apply single factor ANOVA analysis for a comparison among groups. The LSD method was applied for a comparison between the two objects. $p<0.05$ is considered as statistically significant.

Results

Morphological Observation and HE Staining

The amount of normal fibroblasts was large. They were in the shape of short sticks and closely connected to each other in relatively regular forms. The quantity of scar fibroblasts was small. They were in the shape of long fusiform or irregular forms and loosely connected and in disorder. The color of karyon and cytoplasm of normal fibroblast were both light red, with little extracellular matrix (ECM). The color of karyon of scar fibroblast was blue. The cytoplasm was red of different degrees, with relatively much ECM, in deep blue color (Figures 1 and 2).

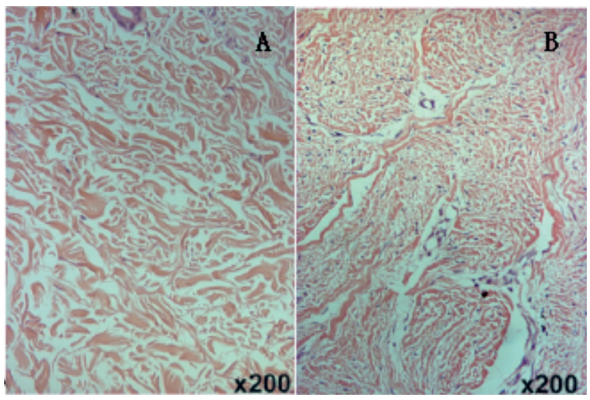

Figure 2. HE staining. ***A***, for the normal fibroblasts, ***B,*** for the scar fibroblasts.

Test for miR-29 Overexpression

The result of lentiviral vector sequencing of miR-29 overexpression shows that the inserted segment is completely the same as the target se-

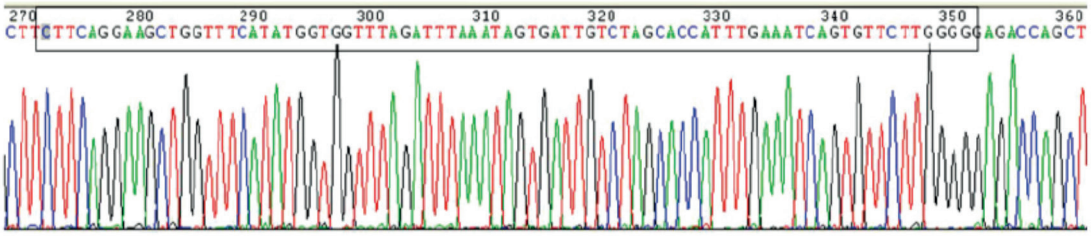

Figure 3. pTA2- miR-29 sequencing results.

quence (Figure 3). After being transfected with the fibroblasts, the miR-29 overexpression vector shows green fluorescence under the inverted fluorescence microscope (Figure 4).

Test Results with RT-PCR Method

Compared with the normal fibroblasts group, the miR-29 mRNA of the scar fibroblasts group significantly decreased, while the level of TGF-β1 and COL1 mRNA significantly increased. The overexpression miR-29 group was in the middle. The differences among groups were statistically significant ($p<0.05$).

Test Results with ELISA Method

The COL1, FN and α-SMA level of the scar fibroblasts group were significantly higher than that in the normal group. The above mRNAs levels in miR-29 overexpression group were lower

Table I. Test results with RT-PCR method.

Group	miR-29	TGF-β1	COL1
normal fibroblasts group	1.0000±0.0153	0.1203±0.0241	0.1365±0.0528
scar fibroblasts group	0.4093±0.0427	0.6548±0.0527	0.7210±0.0965
overexpression miR-29 group	0.8654±0.0632	0.3206±0.0426	0.3527±0.0876
F	10.326	15.234	14.208
p	<0.001	<0.001	<0.001

Table II. Test results with ELISA method.

Group	COL1	FN	α-SMA
normal fibroblasts group	0.13±0.05	0.12±0.04	0.10±0.03
scar fibroblasts group	0.57±0.12	0.62±0.15	0.71±0.16
overexpression miR-29 group	0.29±0.08	0.30±0.07	0.28±0.05
F	7.528	8.203	8.634
p	<0.001	<0.001	<0.001

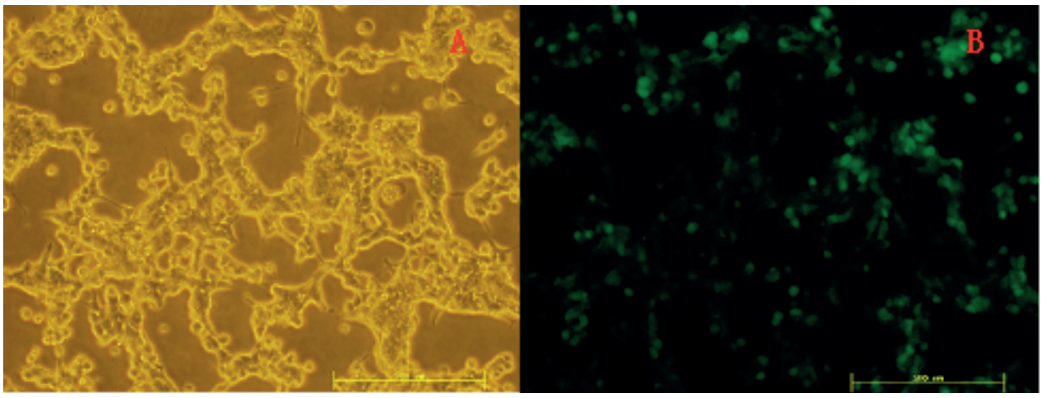

Figure 4. Observation of fibroblasts after transfection of overexpression under the microscope. Scale 200X. ***A,*** for the normal fibroblasts, ***B,*** for the scar fibroblasts

than scar group but higher than the normal group. The differences among groups were statistically significant ($p<0.05$) (Table II).

Discussion

Transforming Growth factor-β (TGF-β) is a type of conservative cell factor, which plays a very important role in embryonic development, cell cycle, cell differentiation, repair in trauma and inflammation reaction, etc., especially in the tissue recovery and fibrosis degeneration, during which process TGF-β is a key medium[7]. It exists widely in various types of normal and transformed cells. For the fibroblasts, it mainly stimulates the cell division and differentiation[8]. The formation of HS is a complicated pathological process. Its occurrence and development are jointly decided by the abnormal expression of multiple genes in the pathological microenvironment. These genes include ECM type genes, cytoskeletal protein, genes related to cell cycles, cancer and anti-cancer genes, apoptosis-related genes, and cell-metabolism-related genes[9]. However, the direct cause for the formation of HS is an excessive synthesis of ECM which is mainly made up of collagen under the stimulation of various fibrosis-accelerating factors[10]. Among all the factors, TGF-βis an important known fibrosis-accelerating factor, which is proved by both *in vivo* and *in vitro* experiments[11,12]. The high expression TGF-β1 in HS tissue, on the one hand, can stimulate the proliferation of fibroblasts and curb its apoptosis process, accelerate the synthesis of ECM, etc., on the other hand, it can curb the degradation effect of matrix metalloproteinases-2 (MMP-2) on collagen. As a result, excessive deposition of ECM will finally accelerate the formation of HS.

Among all miRNAs regulated by TGF-β, miR-21, miR-200 and miR-29 are related to the tissue fibrosis[13]. Among them, miR-21 strengthens the TGF-β signal through the positive feedback loop, and consequently lead to fibrosis[14]. On the contrary, miR-200 and miR-29 respectively curb EMT and the deposition of ECM to prevent the presence of fibrosis[15]. We found that the miR-29 mRNA level in scar fibroblasts significantly decreased by about 60%; the TGF-β1 and COL1 mRNA significantly increased. The level of COL1, FN and α-SMA significantly increased; the overexpression miR-29 can up-regulate miR-29 mRNA, reduce TGF-β1 and COL1 mRNA and lower the COL1, FN andα-SMA level. Further test and analysis of cell proliferation and apoptosis condition can highlight whether miR-29 can curb the growth of scar fibroblasts. Through *in vivo* animal models and clinical experiments, we will confirm whether the miR-29 interference can be the effective target spot for curing HS.

Conclusions

The miR-29 expression in scar cells significantly down-regulates. Adjusting and regulating the expression of TGF-β1 and COL1 can increase the level of ECM, which may be the formation mechanism of scars.

Acknowledgments

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Conflict of Interest

The authors declare no conflicts of interest.

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