SiRNA inhibits CFs Proliferation and reverses Cardiac Fibrosis

Huiyu Yang

Xi’an medical college, Xi’an 710021, Shaanxi, China

Abstract

To investigate that Ets-1 specific siRNA can reduce Ets-1 mediated overexpression of CTGF and PAI-1 by gene silencing. Thereby inhibiting the proliferation of CFs and the excessive secretion of ECM, and reversing the progression of cardiac fibrosis. The Ets-1 gene of CFs cells was silenced using Ets-1 siRNA; The expression levels of Ets-1 mRNA and protein in each group were determined by Real-time PCR and western blot. Ets-1 specific siRNA could effectively silence Ets-1 gene, reduce the synthesis of Ets-1 protein, and reduce the expression of target genes PAI-1 and CTGF. This has a certain inhibitory effect on the proliferation and secretion of ECM in CFs cells. In this experiment, we used the siRNA method to accelerate the degradation of Ets-1mRAN, which enabled the gene silencing of Ets-1, and the downstream target gene products were also reduced. Along with this change, the ability of CFs to proliferate and secrete collagen is significantly reduced. This may provide some help for us to further understand the molecular mechanism of AngII-induced CFs proliferation and to find new ways to treat cardiac fibrosis.

Key words: SiRNA, Ets-1, Curcumin, Fibrosis.

1. Introduction

SiRNA (small interfering RNA), also known as short interfering RNA or silencing RNA. siRNA is a double-stranded RNA molecule, generally composed of 20-25 ribonucleotides, with broad biological effects. Often, siRNA is primarily involved in the physiological process of RNA interference, ie it can regulate a specific gene. In addition, siRNA also plays an important role in the body against viral infection and regulation of a certain gene structure. siRNA was first discovered by David Baulcombe's research team at the Sainsbury Laboratory in England. Subsequently, the team wrote the findings into a paper titled "Study on a Specific MicroRNA in Plant Post-transcriptional Gene Silencing" and published in the journal Science [1]. Subsequently, in 2001, Thomas Tuschl wrote in "Nature" that synthetic siRNA can also produce RNA interference in mammalian cells cultured in vitro[2]. As a result, a research climax of siRNA and RNA interference has been triggered in the field of biological research and drug development. Pre-study found that curcumin can effectively inhibit the overexpression of Ets-1 mRNA and protein in CF cells induced by AngII; The antiproliferative effect of curcumin is achieved by down-regulation of the expression of the Ets-1 gene. In this study, Ets-1 siRNA was used to perform gene silencing of Ets-1, showing anti-proliferative effects.
2. Experiment and Method

1.1 Identification of 3 Candidate SIRAN Transfection Efficiency

(1) Experimental grouping
Divided into 6 groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Group Ets-1siRNA1</th>
<th>Ets-1siRNA1 sequence</th>
<th>Detection Indicator</th>
<th>siRNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td>Ets-1siRNA2</td>
<td>5'-GCU UCA UCA CAG AGU CCU ATT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5'-UAG GAC UCU GUG AUG AAG CTG -3'</td>
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<table>
<thead>
<tr>
<th>Group</th>
<th>Group Ets-1siRNA2</th>
<th>Ets-1siRNA2 sequence</th>
<th>Detection Indicator</th>
<th>siRNA sequence</th>
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<tr>
<td>Group B</td>
<td></td>
<td></td>
<td>Ets-1siRNA2</td>
<td>5'-GCU UCG ACU CCG AGG ACU ATT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5'-UAG UCC UCG GAG UCG AAG CTG-3'</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Group Ets-1siRNA3</th>
<th>Ets-1siRNA3 sequence</th>
<th>Detection Indicator</th>
<th>siRNA sequence</th>
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<tbody>
<tr>
<td>Group D</td>
<td>Negative control</td>
<td></td>
<td>Ets-1siRNA2</td>
<td>5'-CGG AUU ACU UCA UUA GCU ATT -3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5'-UAG CUA AUG AAG UAA UCC GAG -3'</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Group E</th>
<th>Empty transfection control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group F</td>
<td>Fluorescent group</td>
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</table>

(2) Transfection of CFs Cells
The CFs cultured for 3 passages were inoculated into a 6-well plate, and the number of cells per well was about $1 \times 10^6$, and when the cells were fused to 30 to 50%, transfection was prepared. Before transfection, replace the cell culture medium with serum-free DMEM medium and overnight. The next morning, three Ets-1 specific siRNAs, negative control siRNAs, and FAM fluorescently labeled siRNAs were each mixed in 50 ml of serum-free DMEM medium and allowed to stand. Then, 5 μl of Lipofectamine 2000 was taken and mixed with 50 μl of serum-free DMEM medium to stand still. After 5 minutes, each tube of siRNA was mixed with Lipofectamine 2000, gently mixed with a pipette, and allowed to stand at room temperature for about 20 minutes. Add 5 tubes of siRNA and Lipofectamine 2000 to 5 wells, shake gently, and add only 5 μL of Lipofectamine 2000 and 50 mL of serum-free DMEM medium as control. Put in 5% CO$_2$, 37 °C. In the incubator. Since the operation involves siRNA containing a FAM fluorophore label, it is necessary to protect from light in all the steps. After 6 hours, the cells were removed and the wells containing the FAM fluorescently labeled siRNA were observed under a fluorescence microscope. The FAM fluorophore is a green fluorophore with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Successfully transfected CFs showed a green fluorescence distribution within the cell outline. Subsequently, the serum-free DMEM medium was changed to DMEM medium containing 15% FBS, and placed in a 5% CO2 incubator at 37 ° C. After 24 hours, extract the mRNA of each well[3].

(3) Real-time RT-PCR Reaction
The reaction is carried out in a dedicated 8 tube
Primer design: According to the accounting sequence of rat Ets-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in GenBank, primer design was performed using Oligo 7.36 Demo, synthesized by Nanjing Jinsite Biological Co., Ltd[4].

<table>
<thead>
<tr>
<th>Ets-1 Primer sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection Indicator</td>
<td></td>
</tr>
<tr>
<td>Ets-1</td>
<td>justice 5'-TGA TGT TGA GGG CTG TGA ATG AG-3'</td>
</tr>
</tbody>
</table>
The reaction system was configured according to the following reaction composition:

cDNA template 2 μl
Justice primer 1 μl
Antisense primer 1 μl
2× TransStart Green qPCR SuperMix 25 μl
DyeⅢ 1 μl
ddH2O 20 μl

The real-time PCR reaction was carried out as follows:

95°C 5 min
95°C 15 sec
55°C 15 sec 45 cycles
72°C 30 sec

(4) Real-time RT-PCR Reaction Analysis
Each group of samples was analyzed using Bio-Rad 5 software, and the relative expression of GAPDH in each group was obtained, and a histogram was drawn, as shown in Figure 1.

![Figure 1](image)

A: Ets-1siRAN1 group; B: Ets-1siRAN2 group; C: Ets-1siRAN3 group; D: negative control group; E: empty transfection group

Figure 1. Relative expression level of Ets-1 in each group

It can be clearly shown from the figure that Ets-1 siRNA2 has the best inhibitory effect. Therefore, all subsequent experiments were carried out using Ets-1 siRNA2. The sequence is as follows:

Ets-1 siRNA2 sequence

<table>
<thead>
<tr>
<th>Detection Indicator</th>
<th>siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ets-1siRNA2</td>
<td>5’-GCU UCG ACU CCG AGG ACU ATT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-UAG UCC UCG GAG UCG AAG CTG-3’</td>
</tr>
</tbody>
</table>

3. Results

After 72 hours of transfer of Ets-1 specific siRNA into CFs cells, the results observed under a normal microscope were as follows: There was no significant change in the siRNA+AngII group compared with the control group. The number of cells in the AngII group increased significantly, the cell volume increased, into a polygonal or flaming shape, and the cells were closely connected, showing a relatively strong growth trend [5], as shown in Figure 2.
The morphology of the cells in the siRNA+AngII group (×200) was observed under ordinary light microscope as follows: The number of cells is small, the density is low, and the cells are fusiform; Clear outline and less peripheral protrusions; The cells are normal in volume and loosely connected. The number of cells in the AngII group (×200) increased significantly and the density was larger. The cells have many peripheral protrusions, which can be flaming and polygonal; The intercellular connections are tight and the results show a strong growth trend.

![Cell morphology of siRNA+AngII group (×200)](image1)

![Cell morphology of the AngII group (×200)](image2)

**Figure 2-1.** Cell morphology of siRNA+AngII group (×200) observed under ordinary light microscope: fewer cells, lower density, fusiform cells; clear outline, less peripheral protrusions; normal cell volume, more connected loose;

**Figure 2-2.** The cell morphology of the AngII group (×200) is significantly increased, and the density is larger. The cells have more peripheral protrusions, which can be flaming and polygonal. The cells are closely connected and show a strong growth trend. Comparison of cell morphology of Ets-1 Ets-1 specific siRNA after transfection into CFs cells

**3.1. Western-Blot grayscale Analysis Results**

After scanning the bottom plate, the results of Western-blot were analyzed using Gel-pro 32 grayscale analysis software. Using the gray scale of the control group as a reference value, the relative IOD value is calculated according to the following formula:

\[
\text{Relative IOD value} = \frac{\text{IOD values of the internal parameters of each group}}{\text{IOD values of the corresponding bands of the three proteins in each group}}
\]

(1)

The results showed that there was no significant difference in the expression of Ets-1, PAI-1 and CTGF between siRNA+AngII group and 24h after AngII stimulation (P>0.05). The expression of three proteins in AngII group increased significantly (P<0.05). Compared with AngII group, the expression of Ets-1, PAI-1 and CTGF protein in siRNA+AngII group was significantly decreased (P<0.05).

![Western-blot results](image3)

**Figure 3.** Expression of PAI-1, Ets-1, CTGF protein in three groups of cells
4. Conclusions

The biological effects of siRNA are as follows:

1. Multiple double-stranded RNAs are cleaved by dicer in the cytoplasm into siRNA fragments of different lengths, but limited to 20-25 ribonucleotides[6];
2. The newly generated siRNA is in line with the specific protein composition in the cytoplasm to prevent rapid degradation, and is called RNA induced silencing complex (RISC);
3. siRNA self-structural changes, the helical structure is released, RISC is called activation state[7][8];
4. The activated RISC binds to the corresponding mRNA and degrades it, completing the gene silencing function.

Therefore, in the cytological study, the search for the most suitable silencing siRNA sequence is called the key step affecting the success of the experiment [9][10]. As previously studied, inhibition of the expression and regulation of Ets-1 can significantly reduce the degree of fibrosis. According to this, after pretreatment with curcumin, the proliferation of CFs cells induced by AngII and the regulation of Ets-1 expression. To further illustrate the relationship between the two, we used the Ets-1 specific siRNA to silence the Ets-1 gene, and obtained similar experimental results with curcumin. These results further demonstrate that Ets-1 specific siRNA can reduce Ets-1 mediated CTGF and PAI-1 protein over-synthesis by gene silencing, inhibit CFs proliferation and ECM over-secretion, thereby reversing cardiac fibrosis progression.

References