Disruption of connective tissue growth factor by short hairpin RNA inhibits collagen synthesis and extracellular matrix secretion in hepatic stellate cells

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Keywords
connective tissue growth factor – extracellular matrix – hepatic stellate cells – short hairpin RNA – transforming growth factor β1

Abstract
Purpose: Connective tissue growth factor (CTGF) plays a key role in the pathogenesis of liver fibrosis. This study aimed at investigating that the disruption of CTGF expression by short hairpin RNA (shRNA) could modulate the production of fibrosis-related components in hepatic stellate HSC-T6 cells.

Methods: Three plasmids expressing individual shRNA were constructed and transfected into HSC-T6 cells respectively. The levels of CTGF and transforming growth factor β1 (TGF-β1) expression were determined by reverse transcriptase-polymerase chain reaction and Western blot assays. Furthermore, the production of collagens and extracellular matrix (ECM) proteins, including type III procollagen (PC III), type IV collagen (collagen IV), laminin (LN) and hyaluronic acid (HA) in the CTGF-disrupted cells, were analysed by radioimmunoassay.

Results: Following transfection with the pEGFP-CTGFshRNA1, the expression of CTGF was disrupted in HSC-T6 cells. While the knockdown of CTGF expression failed to modulate the expression of TGF-β1, it did significantly reduce the production of PC III, collagen IV, LN and HA by HSC-T6 cells.

Conclusion: Our data suggest that shRNA-mediated disruption of CTGF expression can attenuate ECM synthesis. Potentially, our findings may aid in the design of a CTGF-based new therapy for treatment of hepatic fibrosis.

Hepatic fibrosis is a pathological process during which the liver cells respond to many chronic stimulators, such as chronic viral and parasitic infection, immunological attack, hereditary metal overload and toxicants (1). Hepatic stellate cells (HSC) are the principal fibrogenic cells in the liver and are involved in the process of hepatic fibrosis (2). Quiescent HSC, in response to chronic stimulation, activate and transform into proliferative, fibrogenic and contractile myofibroblasts (3), which are regulated by cytokines, remodelling of extracellular matrix (ECM) (4). Notably, higher levels of transforming growth factor β1 (TGF-β1) expression are associated with the fibrotic pathogenesis and TGF-β1 can recruit inflammatory cells and fibroblasts into the injured region, and stimulate the production of fibrogenic cytokines and ECM (5). However, disruption of TGF-β1 expression does not achieve favourable therapeutic effects on inhibiting the liver fibrosis, suggesting that other factors may overcome the function of TGF-β1 and mediate the cascade of fibrosis (6).

Connective tissue growth factor (CTGF) is a cysteine-rich protein with multiple functions, involved in the regulation of cell growth and remodelling of ECM (7). The expression of CTGF can be upregulated by TGF-β1 and can gradually increase during the process of liver fibrosis, chronic liver injury and inflammation. CTGF can trigger the transformation of quiescent HSC, positive feedback for the upregulation of CTGF expression. CTGF stimulates cell proliferation, adhesion and migration and the production of collagen, fibronectin, laminin and other ECMs (8). Wang et al. (9) found that CTGF-based siRNA effectively antagonized TGF-β-mediated upregulation of procollagen I and III mRNA transcripts in skin fibroblasts (10). Therefore, CTGF is a potential therapeutic target for the inhibition and prevention of fibrosis (7) and the disruption of CTGF expression may be an ideal strategy for the inhibition of liver fibrosis.

RNA interference (RNAi) is a potent and ubiquitous technique for silencing the expression of a specific
gene (10, 11). The short double-stranded RNA, also termed as small interfering RNAs (siRNAs), has been proven to be an effective agent for suppressing the expression of a specific gene (12, 13). RNAi-based gene silencing, in comparison with antisense oligonucleotides, shows greater potency and promising specificity. To date, RNAi has been a promising approach to achieve target-specific anticancer therapy (14). However, the preparation and delivery of siRNA is usually inconvenient and difficult, which becomes an obvious barrier to establishing siRNA-based anticancer therapy (15, 16). Notably, recent studies have revealed that delivery of plasmid DNA expressing short hairpin RNA (shRNA) is superior to siRNA because of its effective interference and convenient production.

In this study, we constructed three plasmids to express individual shRNAs, aimed at disrupting CTGF expression in HSC cells. We examined the efficacy of transfection with the plasmid on the expression of CTGF and determined whether knockdown of CTGF expression could modulate the expression of TGF-β1 in HSC cells. Furthermore, we investigated the impact of CTGF disruption on the production of fibrillar collagens (types I and III), procollagen III (PC III), collagen IV, laminin (LN) and hyaluronic acid (HA) by HSC-T6 cells. We discussed the implication of our findings.

Materials and methods

Materials

Monoclonal antibody against β-actin was from Abcam Inc. (Cambridge, UK). Antibodies against TGF-β1 or CTGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG or anti-goat IgG antibodies were from Cell Signaling Technology (Beverly, MA, USA) or Zhongshan Biotechnology (Beijing, China) respectively.

Construction of short hairpin RNA-expressing plasmids

The shRNA-expressing plasmids, pEGFP-CTGFshRNA1, 2 or 3 were derived from the pEGFP vector (Wuhan Genesil Biotechnology Co., Wuhan, China) and designed to express individual shRNA, driven by the human U6 promoter. One additional plasmid, pEGFP-HK, was constructed to express nonsense shRNA and used as the control. They contained three pairs of shRNA sequences targeting the CTGF gene and one pair of control shRNA sequence (Table 1). These plasmids were designed to transcribe a stem-loop-type RNA with loop sequences of TTC AAG ACG.

Cell culture

Rat hepatic stellate cell line (HSC-T6) was obtained from the Institute of Liver Disease, Shanghai University of Traditional Chinese Medicine (Shanghai, China). The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Sijiqing, Hangzhou, China) at 37 °C in a humidified atmosphere of 5% CO2 in air.

In vitro transfection

The HSC-T6 cells were incubated on culture plates. After overnight incubation, the cells were transfected

<p>| Table 1. The sequences of short hairpin RNA |</p>
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
<th>Targeting position</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGFshRNA1 Sense</td>
<td>5’-GAT CCG GGT CTC TTC TGC TTC TTC AAG ACG GAA GTC GCA GAA GAG ACC TTC TGG CTA GGA A-3’</td>
<td>450-470</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-AGC TTG TCG ACA AAA AAG GGT CTC TTC TGC TTC TTC CTG CTT GAA GAA GTC GCA GAA GAG ACC CGA 3’</td>
<td></td>
</tr>
<tr>
<td>CTGFshRNA2 Sense</td>
<td>5’-GAT CCG ACC TAC CGG GCT AAG TTC TTC AAG ACG GAA CTT AGC GTA GCC TTT TGT TGG CTA G-3’</td>
<td>1047-1067</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-AGC TTG TCG ACA AAA AAG ACC TAC CGG GCT AAG TTC CTG CTT GAA GAA CTT AGC CCA GGT CGA 3’</td>
<td></td>
</tr>
<tr>
<td>CTGFshRNA3 Sense</td>
<td>5’-GAT CCG CGA TGG CGA GAT CAT GAA ATT CAA GAC GTC TCC TCA TGA TCT CGC CAT GGT TTT TGG ACA A-3’</td>
<td>1142-1060</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-AGC TTG TCG ACA AAA AAC AAG CTA GCT GCC GAG ATC ATG AAA CGT CTT GTA TTT CAT CTC GCC ATC GCC 3’</td>
<td></td>
</tr>
<tr>
<td>Control shRNA Sense</td>
<td>5’-GAT CCG ACT TCA TAA GGC GCATG CTC TTC AAG ACG CAT GGC CCT TAT GAA GTC TTT TGG GAC A-3’</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>5’-AGC TTG TCG ACA AAA AAG ACT TCA TAA GGC GCATG CTC CCT GAA GCAA TGC GCC TTA GAC AGT CGA 3’</td>
<td></td>
</tr>
</tbody>
</table>
with one type of the plasmids using Metafectene (Biontex, Planegg, Germany), according to the manufacturer’s instructions. One day after transfection, the cells were analysed for the efficiency of transfection by fluorescence-activated cell sorter (FACS), gated on EGFP expression. Cells with more than 60% EGFP-positive were used for the following experiments.

**Semiquantitative reverse transcriptase-polymerase chain reaction**

The expression levels of the genes of interest were characterized by RT-PCR using the reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Takara, Tokyo, Japan), according to the manufacturer’s instruction. Briefly, total RNA was extracted from the cells that had been transfected with the plasmid expressing the target or control shRNA and reversely transcribed into cDNA, which was used as the template for the PCR. The specific primers for each gene were synthesized by Invitrogen Technologies (Shanghai, China) and are shown in Table 2. The PCR reactions were subjected to 94°C for 2 min, and then 28 cycles of 94°C 45 s, 57°C 45 s, and 72°C 45 s, followed by extension at 72°C for 10 min. The PCR products were separated by agarose-gel electrophoresis and the expression levels of CTGF, TGF-β1, collagen I or III were semiquantified by densitometric analysis. The relative levels of CTGF, TGF-β1, collagen I or III mRNA transcripts were determined and presented as the ratio of CTGF or TGF-β1 to GAPDH expression.

**Western blotting analysis**

The HSC-T6 cells were harvested 24 or 48 h after transfection, lysed in the cracking buffer [50 mM Tris-HCl (pH6.8), 100 mM DTT, 2% SDS, 0.1% bromphenol blue, 10% glycerol], and then boiled for 5–10 min. Equal amounts of cell lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto Hybond-C nitrocellulose membranes. The specific proteins on the membranes were probed with primary antibodies while the bound antibodies were detected by HRP-conjugated secondary antibodies. Immunoreactivity to the protein was visualized by exposure to an X-ray film using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Inc., Rockford, IL, USA), according to the manufacturer’s instructions. The levels of CTGF or TGF-β1 expression were semiquantified by densitometric analysis. The relative levels of CTGF or TGF-β1 expression were determined and presented as the ratio of CTGF or TGF-β1 to β-actin expression.

**Analysis of extracellular matrix secretion**

Following transfection, their supernatants were harvested 24 or 48 h later. The contents of PC III, collagen IV, LN or HA in the supernatants were analysed using their specific radioimmunoassay kits (Naval Institute of Biomedical Sciences, Shanghai, China), according to the manufacturer’s instructions.

**Statistics**

Data were expressed as mean ± SD. Differences were statistically evaluated by a student t-test using the spss11.8 software. A P-value of < 0.05 was considered to be statistically significant.

**Results**

Connective tissue growth factor short hairpin RNA effectively down-regulates the expression of connective tissue growth factor in hepatic stellate HSC-T6 cells

The HSC-T6 cells were transfected with pEGFP-CTGFshRNA1, p-EGFP-CTGFshRN2, pEGFP-

### Table 2. The sequences of specific primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences</th>
<th>Expected size (bp) of PCR products</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGF</td>
<td>Sense: 5'-CCT GAC CCA ACT ATG ATG C-3'</td>
<td>499</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-CCC TTA CTG CCT GGC TTT-3'</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Sense: 5'-CGG CAG CTG TAC ATT GAC TT-3'</td>
<td>271</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AGC GCA CGA TCA TGT TGG AC-3'</td>
<td></td>
</tr>
<tr>
<td>Collagen I</td>
<td>Sense: 5'-TGC CGT GAC CTC AAG ATG TG-3'</td>
<td>462</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-CAC AAG CGT GCT GTA GGT GA-3'</td>
<td></td>
</tr>
<tr>
<td>Collagen III</td>
<td>Sense: 5'-GAG CGG AGA ATA CGT GGT T-3'</td>
<td>529</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AAT GTC ATA GGG TGC GAT A-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5'-TCC CTC CAG ATT GTC AGC AA-3'</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AGA TCC ACA ACG GAT ACA TT-3'</td>
<td></td>
</tr>
</tbody>
</table>
CTGFshRNA3 or control pEGFP-HK. The cells, together with other control cells, were harvested 24 h later. The levels of CTGF mRNA transcripts and protein expression were determined by RT-PCR and Western blot assays. As shown in Figure 1a, the levels of CTGF mRNA transcripts in the cells transfected with pEGFP-CTGFshRNA1 or pEGFP-CTGFshRNA2 decreased by 80 or 65%, respectively, as compared with that in the cells transfected with control plasmid or unmanipulated cells. However, the levels of CTGF mRNA transcripts in the cells transfected with pEGFP-CTGFshRNA3 were similar to that of controls. In parallel, the interfering effects of different CTGFshRNAs on the expression of CTGF protein were observed by Western blot analysis (Fig. 1b and c). Therefore, the CTGFshRNA1 and CTGFshRNA2, but not the CTGFshRNA3, transcripts significantly inhibited the expression of CTGF in HSC-T6 cells. Based on its strongly interfering activity, the CTGFshRNA1 was further used for the following studies.

Effects of connective tissue growth factor by short hairpin RNA on the transforming growth factor-β1 expression in hepatic stellate HSC-T6 cells

To determine the effects of CTGFshRNA1 transcripts on the expression of TGF-β1, the levels of TGF-β1 mRNA transcripts or protein expression in the cells that had been transfected with, or without, the plasmid were characterized by RT-PCR or Western blot 24 or 48 h after transfection respectively. The levels of TGF-β1 mRNA transcripts in the cells transfected with pEGFP-CTGFshRNA1 were comparable with that in control cells, analysed at 24 or 48 h post-transfection (Fig. 2a and c). Similar patterns of TGF-β1 protein expressions were detected by Western blot assays (Fig. 2b and d). These data suggest that knockdown of CTGF expression by the CTGFshRNA transcripts failed to alter significantly the expression of TGF-β1 in HSC-T6 cells.

Effects of connective tissue growth factor by short hairpin RNA on collagen synthesis in hepatic stellate HSC-T6 cells

We next investigated the influence of CTGFshRNA-mediated disruption of CTGF expression on the expression of collagen in HSC-T6 cells. Twenty-four or 48 h after transfection, the cells were harvested and the levels of collagen I or III mRNA transcripts were determined by RT-PCR (Fig. 3a and c). Firstly, the levels of collagen I or III mRNA transcripts in the cells transfected with control plasmid were...
indistinguishable from that in unmanipulated controls, indicating that the control shRNA transcripts did not modulate the expression of collagen I and III in HSC-T6 cells (Fig. 3a and c). Secondly, the levels of collagen I or III mRNA transcripts in the cells that had been transfected with the pEGFP-CTGFshRNA1 were dramatically reduced as compared with that in control cells (Fig. 3a and c). Semiquantitative analysis of different transcripts revealed that the knockdown of CTGF expression by the CTGFshRNA1 inhibited the transcription of collagen I by 59 or 50% at 24 or 48 h post-transfection respectively (Fig. 3a and b). Similar inhibitory effects of the CTGFshRNA1 on the expression of collagen III were achieved in HSC-T6 cells 24 or 48 h after transfection. Therefore, CTGFshRNA-mediated disruption of CTGF expression significantly inhibited the synthesis of collagen I and III in liver stellate cells in vitro.

Effects of connective tissue growth factor by short hairpin RNA on extracellular matrix secretion by hepatic stellate HSC-T6 cells

To further explore the effects of CTGFshRNA transcripts on the ECM secretion by HSC-T6 cells, the levels of PC III, collagen IV, HA or LN in the supernatants were determined by a radioimmunoassay at 24 and 48 h post-transfection respectively (Table 3). The expression of control shRNA, as expected, did not modulate the production of ECM as the levels of PC III, collagen IV, HA or LN in the supernatants from the cells expressing control shRNA were similar to that from control cells. However, the knockdown of CTGF expression by the CTGFshRNA1 transcripts significantly reduced the secretion of ECM, evidenced by the fact that the levels of PC III, collagen IV, HA or LN in the supernatants from the cells transfected with the pEGFP-CTGFshRNA1 decreased by 50–72% for different ECMs tested. Thus, CTGFshRNA-mediated disruption of CTGF expression downregulated the production and secretion of ECM in liver stellate cells in vitro. Collectively, the knockdown of CTGF expression by CTGFshRNA considerably inhibited the synthesis of collagens, and the production and secretion of ECMs in hepatic stellate cells.

![Fig. 2. Effects of CTGFshRNA on the expression of TGF-β1 in HSC-T6 cells. HSC-T6 cells were transfected with pEGFP-CTGFshRNA1 or control pEGFP-HK and harvested 24 or 48 h after transfection. The levels of TGF-β1 mRNA transcripts or protein were analysed by RT-PCR or Western blot assays respectively. (a) RT-PCR analysis; (b) western blot analysis; (c) semiquantitative analysis of TGF-β1 mRNA; (d) semiquantitative analysis of TGF-β1 protein. The levels of TGF-β1 mRNA transcripts or protein were determined by densitometric analysis and expressed as the relative ratio of the levels of TGF-β1 expression to those of GAPDH or β-actin expression respectively. Data are representatives of three independent experiments. 1, pEGFP-CTGFshRNA1 transfected group; 2, pEGFP-HK transfected group; 3, lipofectamine-treated group; and 4, untreated group. CTGFshRNA, connective tissue growth factor by short hairpin RNA; HSC, hepatic stellate cells; RT-PCR, reverse transcriptase-polymerase chain reaction; TGF-β1, transforming growth factor β1.](image-url)
Discussion

Hepatic fibrosis occurs as a consequence of the transdifferentiation of HSC into myofibroblasts. Activated HSC proliferate, migrate and produce collagen and other ECM molecules (17). The HSC-T6 cell line applied in the current study is an immortalized rat liver stellate cell line and exists as activated fibroblast-like cells, which provides a useful tool to explore the mechanism underlying the hepatic fibrosis (18).

The increased levels of both CTGF and TGF-β1 expressions have been associated with the pathogenesis of many fibrosis disorders, including chronic viral infection-related liver fibrosis and cirrhosis. While high levels of TGF-β1 are mainly expressed at the early stage, a consistently high expression of CTGF is found during the pathogenic process of chronic fibrosis (19). Apparently, CTGF is a critical mediator, and indeed, the abnormal expression of CTGF has been considered to be a marker for the diagnosis of some fibrosis disorders. Given that the interference efficacies of plasmid-based RNAi reagents vary in diverse cell lines (20), we designed three pairs of candidate shRNAs aimed at disrupting the expression of CTGF in HSC cells in vitro. We found that although the CTGFshRNA1 transcripts effectively disrupted the expression of CTGF, it failed to modulate the expression of TGF-β1 in HSC-T6 cells, suggesting that CTGF did not feedback down-regulate the expression of TGF-β1. Apparently, CTGF-targeted shRNA may specifically suppress TGF-β1-induced CTGF-mediated fibrosis, but does not affect TGF-β1-related other signalling events. Therefore, the disruption of CTGF expression may be a more specific strategy to design therapy for hepatic fibrosis.

Interestingly, the different CTGFshRNAs we used showed varying levels of inhibitory effects in our experimental system. The CTGFshRNA1, which targeted the position 450–470 of the CTGF mRNA, showed a dramatically inhibitory effect on the expression of CTGF, the CTGFshRNA2 that targeted the position 1047–1067 displayed moderate levels of inhibition while the CTGFshRNA3 that specifically interacted with the position 1142–1060 failed to modulate the expression of CTGF significantly. The considerable inhibitory effects of the CTGFshRNA1 may be attributed to its high efficacy of transcription and unique conformational structure, effectively modulating the conformational structure of CTGF mRNA in a critical region for protein translation or increasing the degradation of CTGF mRNA by the CTGFshRNA1. However, the precise mechanisms underlying the variations of different CTGFshRNAs in
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inhibiting CTGF expression remain to be further investigated.

To further evaluate the effect of CTGFshRNA in liver fibrosis, we characterized the impact of CTGF disruption on the expression of ECM components, in particular, for the expression of major glycoproteins collagen I, III and cytokines that increase considerably and are mainly related in the cirrhotic liver (21). We found that the knockdown of CTGF by the CTGFshRNA considerably reduced the production of these types of collagens and multiple ECM proteins in HSC-T6 cells in vitro. These data demonstrated that the disruption of CTGF expression in HSC cells, similar to that of TGF-β, inhibits the production of ECM, potentially circumventing the process of liver fibrosis (22).

In spite of the tremendous efforts, there is no available siRNA-based therapeutic agent for the treatment of fibrosis in clinic. However, the RNAi is a very promising and effective approach in terms of the disruption of a specific gene expression and some of them have entered clinical trials for intervention of fibrosis. Although the development of RNAi-based therapy for intervention of fibrosis may be far from success, our findings provide new insights into understanding the mechanisms underlying the action of CTGF-based shRNA therapy for liver fibrosis.

In summary, we have successfully designed and validated a CTGF-specific shRNA, which effectively disrupted the expression of CTGF HSC-T6 cells in vitro. The knockdown of CTGF expression by the CTGFshRNA significantly reduced the production of collagens and multiple ECM proteins in HSC cells. Our results suggested that CTGF disruption by shRNA may be an efficient and more specific approach for therapy of liver fibrosis.

Table 3. Effects of CTGFshRNA on extracellular matrix secretion in HSC-T6 cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Groups</th>
<th>PC III (µg/L)</th>
<th>Collagen IV (µg/L)</th>
<th>HA (µg/L)</th>
<th>LN (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1</td>
<td>286.44 ± 20.34**</td>
<td>47.55 ± 13.14**</td>
<td>72.99 ± 17.79**</td>
<td>51.45 ± 14.1*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>462.31 ± 30.42</td>
<td>147.6 ± 32.4</td>
<td>198.01 ± 41.23</td>
<td>147.64 ± 31.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>468.77 ± 46.71</td>
<td>146.96 ± 20.54</td>
<td>189.7 ± 36.24</td>
<td>142.9 ± 24.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>487.69 ± 60.85</td>
<td>157.55 ± 31.2</td>
<td>215.48 ± 47.65</td>
<td>153.87 ± 40.3</td>
</tr>
<tr>
<td>48</td>
<td>1</td>
<td>376.22 ± 34.76*</td>
<td>67.27 ± 13.5**</td>
<td>175.70 ± 19.57**</td>
<td>69.39 ± 11.23**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>532.46 ± 64.35</td>
<td>177.35 ± 27.3</td>
<td>327.05 ± 46.23</td>
<td>148.40 ± 30.12</td>
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<tr>
<td></td>
<td>3</td>
<td>492.39 ± 49.74</td>
<td>154.20 ± 18.7</td>
<td>307.10 ± 31.58</td>
<td>139.3 ± 19.16</td>
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<tr>
<td></td>
<td>4</td>
<td>510.81 ± 54.53</td>
<td>164.98 ± 21.2</td>
<td>311.12 ± 39.45</td>
<td>126.08 ± 24.35</td>
</tr>
</tbody>
</table>

Group 1, pEGFP-CTGFshRNA1 transfected group; group 2, pEGFP-HK transfected group; group 3, lipofectamine treated group; group 4, untreated group. Data are expressed as mean ± SD from three independent experiments. Experimental and control groups were tested simultaneously.

*P < 0.05, or **P < 0.01 vs. controls.

References