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Hammerhead Ribozyme-Mediated Knockdown of mRNA for Fibrotic Growth Factors: Transforming Growth Factor-Beta 1 and Connective Tissue Growth Factor

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Abstract

Excessive scarring (fibrosis) is a major cause of pathologies in multiple tissues, including lung, liver, kidney, heart, cornea, and skin. The transforming growth factor- β (TGF- β) system has been shown to play a key role in regulating the formation of scar tissue throughout the body. Furthermore, connective tissue growth factor (CTGF) has been shown to mediate most of the fibrotic actions of TGF- β , including stimulation of synthesis of extracellular matrix and differentiation of fibroblasts into myofibroblasts. Currently, no approved drugs selectively and specifically regulate scar formation. Thus, there is a need for a drug that selectively targets the TGF- β cascade at the molecular level and has minimal off-target side effects. This chapter focuses on the design of hammerhead ribozymes, measurement of kinetic activity, and assessment of knockdown mRNAs of TGF- β and CTGF in cell cultures.

Keywords

Ribozymes; TGF- β ; CTGF; Scar formation; Transduction; Oligonucleotides

1. Introduction

Transforming growth factor-beta (TGF- β) and connective tissue growth factor (CTGF) play key roles in regulating scar formation in normal wound healing in tissues throughout the body (1, 2). Molecular analyses of pathological scars have found prolonged elevated levels of TGF- β and CTGF mRNAs and proteins, which has led to the hypothesis that fibrotic scars are a result of excessive activities of these two growth factor systems. In addition, CTGF has recently been shown to mediate most of the fibrotic activity of TGF- β , including stimulation of synthesis of extracellular matrix and differentiation of fibroblasts into myofibroblasts (3).

Currently, no approved drugs selectively and specifically regulate synthesis and/or action of TGF- β and CTGF systems. Neutralizing antibodies to TGF- β and inhibitors of TGF- β receptor kinase have been developed and evaluated in clinical trials, but none have received clearance from the Food and Drug Administration. Thus, there is a need for a drug that selectively targets the TGF- β cascade at the molecular level that produces minimal off-target side effects. Our approach has been to develop gene-specific, oligonucleotide-based drugs, specifically hammerhead ribozymes that cleave TGF- β and CTGF mRNA molecules.

This chapter gives a start to finish description of how to design, validate, and test in cell culture hammerhead ribozymes that target specific genes and could potentially be used as a

therapeutic agent. The first method describes the general steps to design a hammerhead ribozyme. This method is the same for the design of all hammerhead ribozymes. The second method describes how to validate a hammerhead ribozyme's ability to cleave its targeted mRNA substrate. The ribozyme cleavage time course study and the ribozyme multiturnover study can be done with minimal variation to assess the activity of different ribozymes. Two general approaches are typically used to deliver ribozymes to cells in culture: direct delivery of a chemically modified preformed ribozyme that resists enzymatic degradation by RNases and stable transfection with a plasmid expressing the ribozyme (4). Unprotected, nonchemically modified ribozymes have a half-life of seconds in the body and cell culture. Chemically protected ribozymes can be stabilized with a half-life that can be measured for several hours (5). To circumvent the short half-life of a ribozyme, construction of a plasmid that expresses the ribozymes allows for constitutive expression of the ribozyme. The third method describes the techniques used to make the plasmids containing either TGF- β 1 ribozyme or the CTGF ribozyme. The last three sections describe methods to test the efficiency of ribozymes in cell culture using different transfection techniques and four different ways to determine knockdown of the selected protein.

2. Materials

2.1. Ribozyme Design and Synthesis

Mfold program: <http://bioweb.pasteur.fr/seqanal/interfaces/mfold.html>.

2.2. Ribozyme Time-Course and Multiturnover Kinetics

1. Oligo deprotection and labeling with γ -[32 P]-dATP: Oligo-RNA (10 pmol/ μ L), 1 μ L RNasin (Promega; Madison, WI, USA), 1 μ L 0.1 M dithiothreitol (DTT), 3 μ L double-distilled water (ddH₂O), 1 μ L [γ 32 P]-dATP, 1 μ L 10 $\times$ PNK buffer, and 1 μ L T4 polynucleotide kinase (Roche Molecular Biochemicals; Indianapolis, IN, USA).
2. Phenol/chloroform/isoamyl alcohol extraction: A solution of the ratio of 25:24:1, respectively.
3. Sephadex G25 fine spin column (Roche Applied Science).
4. Ribozyme cleavage buffer: 40 mM Tris/HCl, pH 7.5, and 20 mM MgCl₂.
5. Ribozyme cleavage stop solution: 6 μ L of 90% formamide, 50 mM EDTA (pH 8.0), 0.05% xylene cyanol, and 0.05% bromophenol blue.
6. Polyacrylamide urea running buffer: 1 $\times$ Tris/borate/EDTA (TBE) (obtained by adding 200 mL of 5 $\times$ Novex® TBE Running Buffer to 800 mL of deionized water).

2.3. Plasmid Construction

1. Single-stranded synthetic DNA oligonucleotides encoding complementary sequences.
2. Restriction enzymes: *Nsi*I and *Hind*III.
3. Initial plasmid construct: pTR-UF21HP.

2.4. Analysis of Endogenous Target mRNA Knockdown by a Ribozyme

2.4.1. CTGF Ribozyme Analysis

Human Cell Culture and Transfection

1. Dulbecco's modified Eagle's medium (DMEM), Medium 199, Ham's F₁₂ nutrient mixture containing 1 mM NaHCO₃, and buffered with 25 mM HEPES at pH 7.4. The medium is supplemented with 10% heat-inactivated normal calf serum and 1× antibiotic-antimycotic (Gibco BRL).
2. 200 µg/mL geneticin (G418 Sulfate) dissolved in cell culture media.

Quantitative Reverse Transcription-Polymerase Chain Reaction

1. TRIzol reagent (Invitrogen, Gaithersburg, MD).
2. 1× TaqMan One-step reverse transcription-polymerase chain reaction (RT-PCR) Master Mix, 900 nM forward, 900 nM reverse primer, 2 µM fluorescent TaqMan probe, and RNA sample (CTGF mRNA standard or 500 ng of sample RNA) to a final volume of 25 µL per reaction.
3. TaqMan glyceraldehyde phosphate dehydrogenase (GAPDH) Control Kit (Applied Biosystems, Foster City, CA, USA).

CTGF Enzyme-Linked Immunosorbent Assay

1. Biotinylated and nonbiotinylated, affinity-purified goat polyclonal antibodies.
2. Blocking buffer: Phosphate-buffered saline (PBS)/0.02% sodium azide/1% bovine serum albumin.
3. Alkaline phosphatase-conjugated streptavidin (1.5 µg/mL, Zymed, South San Francisco, CA, USA).
4. Alkaline phosphatase substrate solution (1 mg/mL *p*-nitrophenyl phosphate).
5. Sodium carbonate/bicarbonate buffer/0.02% sodium azide, pH 9.6.

2.4.2. TGF-β1 Ribozyme Analysis

Mouse-Immortalized Cell Culture and Transfection

1. Equal parts Ham's F-12, Medium 199, and DMEM media, containing 20 mM HEPES, 1 mM NaHCO₃, 100 U/mL penicillin, and 100 µg/mL streptomycin, supplemented with 10% normal goat serum.
2. Hypoosmolar electroporation buffer (Eppendorf Scientific, Inc., Germany).
3. 200 µg/mL geneticin (G418 Sulfate) dissolved in cell culture media.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

1. TRIzol reagent.
2. Superscript™ First-strand synthesis system for RT-PCR (GIBCO BRL).

2.5. Analysis of Exogenous Synthetic Target Knockdown by a Ribozyme (TGF-β1): Human Embryonic Kidney298 Cell Culture and Dual Transfection

1. DMEM, with 4.5 g/L glucose and 1 g/L L-glutamine.
2. Turbofect reagent (Fermentas Inc.; Glen Burnie, MD, USA).
3. Quanti-Blue™ (InvivoGen, San Diego, CA, USA).

3. Methods

3.1. Ribozyme Design and Synthesis

All potential ribozyme cleavage sites within the human CTGF or TGF- β 1 cDNA sequences were initially identified based on the optimal G-U-C nucleotide sequence for hammerhead ribozymes (6). Potential cleavage sites were then evaluated for secondary folding structures around the G-U-C sequence using the theoretical lowest energy conformations calculated using the Mfold program (<http://bioweb.pasteur.fr/seqanal/interfaces/mfold.html>) (7). Only those sites for which the G-U-C sequence was in a single-stranded, nonbase-paired region were considered further. In addition, the nucleotide sequence of the 20mer centered on the G-U-C site were examined for the relative content of A and U bases, since previous studies had shown that flanking sequences with higher numbers of A and U bases tend to have more rapid release rate constants compared to flanking sequences that are rich in G-C content. The lengths of the 5' and 3' hybridization arms, which comprise the helical stems I and III of the ribozymes, were five and six nucleotides, respectively. The catalytic core structure was formed by the 21mer with the nucleotide sequence of CUGAUGAGGUCCUUCGGGACGAA (Fig. 1). Taken together, the hammerhead ribozyme sequence formed the 6-4-6-type helical stem structure which was shown to provide optimal in vitro kinetic values (8). Corresponding 33mer RNA hammerhead ribozymes and 12mer RNA targets were chemically synthesized with 2'-ACE protection. (Dharmacon Research Inc, USA).

3.2. Ribozyme Time-Course and Multiturnover Kinetics

1. Target oligo RNAs are deprotected according to the Dharmacon's deprotection protocol.

Centrifuge tubes briefly. Add 400 μ L of 2'-deprotection buffer to each tube of RNA. Add 800 μ L of 2'-deprotection buffer to oligos with homopolymer stretches of rA longer than 12 bases. Completely dissolve RNA pellet by pipetting up and down. Vortex for 10 s and centrifuge for 10 s. Incubate at 60°C for 30 min. Incubate at 60°C for 2 h for oligos with biotin modifications or homopolymer stretches of rA longer than 12 bases. Lyophilize or SpeedVac to dryness before use.
2. Label with γ -[32 P]-dATP using the following reaction: 2 μ L oligo-RNA (10 pmol/ μ L), 1 μ L RNasin (Promega; Madison, WI, USA), 1 μ L 0.1 M DTT, 3 μ L ddH₂O, 1 μ L [γ - 32 P]-dATP, 1 μ L 10 $\times$ PNK buffer, and 1 μ L T4 polynucleotide kinase (Roche Molecular Biochemicals; Indianapolis, IN, USA).
3. Incubate at 37°C for 30 min, and then dilute to 100 μ L with ddH₂O followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1).
4. Free nucleotides are removed by passing the aqueous layer on a Sephadex G25 fine spin column.
5. RNA is ethanol precipitated and resuspended in 100 μ L ddH₂O to a final concentration of 0.2 pmol/ μ L.
6. Ribozyme cleavage reactions are performed in the presence or absence of various concentrations of ribozyme and target RNA in a reaction mix (20 μ L) containing 40 mM Tris/HCl, pH 7.5, and 20 mM MgCl₂. Samples are incubated at 37°C and the reaction is initiated by addition of ribozyme to target RNA.
7. At the appropriate times, the reactions are arrested with the addition of a 6 μ L of 90% formamide, 50 mM EDTA (pH 8.0), 0.05% xylene cyanol, and 0.05% bromophenol blue.

8. Reaction products are separated on a 15–19% polyacrylamide gel containing 8 M urea which can resolve RNA from 20 to 800 bases. The gel is run according to Invitrogen specifications using 1× TBE running buffer. The gel is run at a constant voltage of 180 V for 50–85 min. The samples are quantitated by radioanalytic scanning (PhosphorImager; Molecular Dynamics, Durham, NC, USA).
9. In the time-course study, reaction mixtures include 10 pmol ribozyme and 100 pmol target RNA (containing 0.2 pmol γ -[^{32}P]-target). Reactions are stopped at 0.5, 1, 2, 3, 4, 5, 10, and 30 min, 1, 2, 3, and 15 h. In the multiturnover study, reactions are stopped at 1 min. Reactions include 0.015 pmol/ μL of ribozyme and increasing concentrations of target RNA (0.15–15 pmol/ μL) as shown in Table 1 (see Note 1) (Fig. 2).
10. Michaelis–Menton constant (K_m) and reaction rate at saturating substrate concentration (k_{cat}) are obtained using double-reciprocal plots of velocity versus substrate concentration. The concentrations of target RNA range from 0.15 to 15 pmol/ μL with a constant ribozyme RNA concentration of 0.015 pmol/ μL (Fig. 3).

3.3. Plasmid Construction

1. Ribozymes with the better kinetic properties for TGF- β 1 or CTGF are selected to test their efficiency in cells. The first step is to synthesize a plasmid that expresses the ribozyme. Single-stranded synthetic DNA oligonucleotides encoding complementary sequences of the ribozyme are chemically synthesized. A second pair of oligonucleotides is constructed which contains a single-nucleotide replacement, shown as the underlined nucleotides (C $\rightarrow$ G, G $\rightarrow$ C), to create an inactive ribozyme that would assess the general toxicity and antisense effect of the ribozyme.
2. The complementary oligonucleotides are annealed, producing *Nsi*I and *Hind*III restriction sites. The fragments are inserted into the pTR-UF-21HP vector, which has been linearized with *Hind*III and *Nsi*I restriction enzymes. Synthesis of the ribozyme is driven by the chicken β -actin promoter and CMV enhancer in this vector. The presence and correct orientation of the insert are verified by DNA sequencing. The pTR-UF21HP vector contains a hairpin ribozyme following the insert site that self-cleaves the mRNA when transcribed in the cell, yielding a relatively short 3' arm of hammerhead ribozyme that improves cleavage efficiency (7).

3.4. Analysis of Endogenous Target mRNA Knockdown by a Ribozyme

3.4.1. CTGF Ribozyme Analysis

Human Cell Culture and Transfection

1. Cultures of human newborn foreskin fibroblasts (ATCC; Manassas, VA, USA) are cultured in equal parts DMEM, Medium 199, Ham's F₁₂ nutrient mixture containing 1 mM NaHCO₃, and buffered with 25 mM HEPES at pH 7.4. The medium is supplemented with 10% heat-inactivated normal calf serum and 1× antibiotic–antimycotic (Gibco BRL). Exponentially growing cells are transfected with vector (pTR-UF21), inactive ribozyme plasmid (pTR-UF21-In), or active CTGF ribozyme plasmid (pTR-UF21-CHR745) using Lipofectamine reagent (Invitrogen Life Technologies; Carlsbad, CA, USA).

¹When setting up kinetic experiments, be sure to make reaction mix with the substrate, but wait to add the ribozyme. Also, bring the reaction mix containing the substrate to 37°C and then add the ribozyme. If you do not do this, your reaction will need to be heated up to 37°C and the catalytic activity will be reduced.

2. Since pTR-UF-21 contains a neomycin-resistance gene; cells that are stably transfected are selected with 200 $\mu\text{g/mL}$ geneticin (G418 Sulfate) added to the culture medium 48 h after transfection. After 7 days, selected cells are transferred to 48-well plates for evaluation of ribozyme effects.

Quantitative Reverse Transcription-Polymerase Chain Reaction

1. Confluent cultures of stably transfected fibroblasts in 48-well plates are held in serum-free medium for 48 h before RNA extraction (Qiagen RNeasy Kit; Valencia, CA, USA). Cells are stimulated for 24 h with 5 ng/mL human TGF- β 1 to stimulate CTGF expression. Total RNA is extracted using TRIzol reagent according to the manufacturer's protocol.
2. CTGF mRNA transcripts are detected using the TaqMan real-time quantitative RT-PCR procedure (9). A standard curve is generated using CTGF mRNA transcripts that are transcribed in vitro using T7 RNA polymerase from a plasmid containing CTGF cDNA. CTGF transcript is precipitated with ethanol and dissolved in diethylpyrocarbonate (DEPC)-treated water.
3. Reactions are assembled in a 96-well optical reaction plate. Each reaction contains 1 $\times$ TaqMan One-step RT-PCR Master Mix, 900 nM forward primer (5'-AGCCGCCTCTGCAT GGT-3'), 900 nM reverse primer (5'-CACTTCTTGCCCTTC TTAATGGTTCT-3'), 2 μM fluorescent TaqMan probe (5'-6FAM-TTCCAGGTCAGCTTCGCAAGGCCT-TAMRA-3'), and RNA sample (CTGF mRNA standard or 500 ng of sample RNA) to a final volume of 25 μL per reaction (see Note 2). The plate is analyzed on the ABI Prism 5700 Sequence Detection System (Applied Biosystem, Foster City, CA, USA), which simultaneously performs the RT-PCR and detects a fluorescence signal. A standard curve is generated using the transcribed CTGF mRNA samples (2.3×10^{-2} to 2.3×10^{-6} pmol). The level of GAPDH mRNA is also measured in each sample using the TaqMan GAPDH Control Kit (Applied Biosystems, Foster City, CA, USA), and the number of CTGF mRNA molecules in samples is expressed as pmol CTGF mRNA per pmol of GAPDH mRNA.
4. Levels of mRNA are expressed as mean $\pm$ standard error of six replicate samples for each condition, and ANOVA and Tukey's HSD post hoc test are used to assess statistical significance between times and groups (Fig. 4).

CTGF Enzyme-Linked Immunosorbent Assay

1. CTGF is measured in conditioned medium and in cytoplasmic extracts of serum-starved, cultured cells following stimulation for 24 h with 5 ng/mL human TGF- β 1 using a capture sandwich enzyme-linked immunosorbent assay (ELISA) with biotinylated and nonbiotinylated, affinity-purified goat polyclonal antibodies to human CTGF (10). A flat-bottom ELISA plate (Costar 96-well) is coated with 50 μL of goat antihuman CTGF antibody (which recognizes predominately epitopes in the N-terminal half of the CTGF molecule) at a concentration of 10 $\mu\text{g/mL}$ in PBS/0.02% sodium azide for 1 h at 37°C.
2. Wells are washed four times and incubated with 300 μL of blocking buffer (PBS/0.02% sodium azide/1% bovine serum albumin) for 1 h at room temperature (see Note 3).

²When performing PCR, always mix the reagents by lightly flicking and quickly centrifuge to bring the reagents to the bottom of the tube.

3. The wells are washed four times and 50 μ L of recombinant human CTGF protein (from 0.1 to 100 ng/mL) or sample is added and incubated at room temperature for 1 h.
4. After washing, 50 μ L of biotinylated goat antihuman CTGF (2 μ g/mL) is added and incubated at room temperature in the dark for 1 h, then washed, and 50 μ L of alkaline phosphatase-conjugated streptavidin is added and incubated at room temperature for 1 h.
5. The wells are washed again and incubated with 100 μ L of alkaline phosphatase substrate solution (1 mg/mL *p*-nitrophenyl phosphate in sodium carbonate/bicarbonate buffer/0.02% sodium azide, pH 9.6). Absorbance at 405 nm is measured using a microplate reader (Molecular Devices, Sunnyvale, CA).
6. CTGF levels are normalized for total protein content of samples using bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical, Rockford, IL, USA) and are expressed as ng/mg protein for six replicate samples for each condition. Sensitivity of the ELISA is 0.1 ng/mL with an intra-assay variability of 3%, which is similar to a previously published ELISA for CTGF (11).
7. Levels of protein are expressed as mean $\pm$ standard error of six replicate samples for each condition, and ANOVA and Tukey's HSD post hoc test are used to assess statistical significance between times and groups.

3.4.2. TGF- β 1 Ribozyme Analysis

Mouse-Immortalized Cell Culture and Transfection

1. CCL-1 cells (NCTC clone 929, ATCC, USA) are cultured with equal parts Ham's F-12, Medium 199, and DMEM media, containing 20 mM HEPES, 1 mM NaHCO₃, 100 U/mL penicillin, and 100 μ g/mL streptomycin, supplemented with 10% normal goat serum at 37°C.
2. Exponentially growing cells are trypsinized and resuspended in hypoosmolar electroporation buffer (Eppendorf Scientific, Inc., Germany). Cell density is adjusted to 10⁶ cells/mL. Cell suspensions are combined with pTR-UF-21-THRC576 or pTR-UF-21-THR576 (10 μ g/mL final concentration, in ddH₂O). 400 μ L of the mixture is placed in electroporation cuvettes (2-mm-gap width, Eppendorf). Following incubation on ice for 10 min, the suspensions are electroporated in a Multiporator (Eppendorf) at 1 pulse of 400 V for 50 μ s. Subsequently, the suspension is incubated in the cuvette for 5–10 min at room temperature. The suspension is then transferred from the cuvette into the culture medium and distributed into a 12-well plate.
3. Because pTR-UF-21 includes a gene for neomycin resistance, cells that are stably transfected are selected using Geneticin (G-418 Sulfate). 48 h after electroporation, G418 is added to the culture medium at a concentration of 600 μ g/mL, as determined by a series of concentration tests. After 7 days, the culture medium is changed and the G418 concentration is decreased to 200 μ g/mL to maintain selection. Cell monoclones are trypsinized and transferred to another plate to continue culturing in selecting culture medium (G418 200 μ g/mL). Nontransfected CCL-1 cells are used as a negative control. Before examining RNA and protein expression, confluent cells in 6-well plates are cultured with 2 mL serum-free medium containing 200 μ g/mL G418 for 48 h.

³When performing ELISA, cover the 96-well plate during the incubations. Also, when washing, gently tap the 96-well plate on a paper towel to remove all of the wash solution.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

1. Total RNA is extracted using TRIzol reagent according to the manufacturer's protocol. Cells are washed with PBS three times followed by addition of 1 mL TRIzol to each well to lyse the cells.
2. Concentration and purity of RNA are measured using spectrophotometry at 260 nm (GeneQuant; Amersham Pharmacia Biotech, Uppsala, Sweden). 260/280 nm ratios of all the samples should be 1.90 or greater.
3. Reverse transcription is performed using a first-strand synthesis kit (Superscript™ First-strand synthesis system for RT-PCR, GIBCO BRL) using oligodeoxythymidine primers. Relative cDNA levels are quantitated by co-amplification of the housekeeping gene beta-actin and TGFβ1 using PCR Master Mix (Promega). The primer sequences for beta-actin are as follows: forward, 5'-TGCGTGACATTAAGGAGAAG-3' and reverse 5'-GAAGGTAGTTTCGTGGATGC-3'. The primer sequences for TGFβ1 are forward primer 5'-GAAGCGCATCGAAGC ATCC-3' and reverse primer 5'-TTGGACAAC TGCTCCA CCTT-3'.
4. Since oligo-dT is used to perform the reverse transcription, the PCR products indicated intact RNA levels. Amplification is performed in a 50 μL reaction mixture using the following conditions: 1 μL of each primer (10 pmol/μL), 2 μL RT reaction product samples, 25 μL PCR master mix, and 19 μL ddH₂O. PCR amplifications are initiated at 94°C for 2 min followed by 28 sequential cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1.5 min. A final extension cycle at 72°C for 10 min is performed in a thermocycler (Twin blockTH System, San Diego, CA).
5. A video imaging and densitometry software system (Kodak digital science, Eastman Kodak Company) is used to quantify the relative band intensities of beta-actin and TGFβ1. TGFβ1 mRNA levels are then expressed relative to beta-actin (Fig. 5).

TGFβ-1 Enzyme-Linked Immunosorbent Assay: The protocol for the TGF-β1 ELISA is same as that previously described from the CTGF, except the antibodies used are specific for TGF-β1.

3.5. Analysis of Exogenous Synthetic Target Knockdown by a Ribozyme (TGF-β1)

3.5.1. Production of Secreted Alkaline Phosphatase Target Expression Plasmid

1. A secreted alkaline phosphatase (sAP) reporter gene driven by an hEF1-HTLV promoter is cloned into pBluescript and contains a multiple cloning site located upstream of the sAP reporter gene.
2. A target sequence, approximately 300 bp, containing the ribozyme target sequence is cloned into the multiple cloning site upstream of the sAP reporter gene.

3.5.2. Human Embryonic Kidney298 Cell Culture and Dual Transfection

1. DMEM, with 4.5 g/L glucose and 1 g/L L-glutamine: Medium is supplemented with 10% heat-inactivated normal calf serum and 1× antibiotic-antimycotic (Gibco BRL).
2. Exponentially growing cells are transfected with two plasmids, the first being the psAP Bluescript TGF-β1 target plasmid and the second being a plasmid that

expresses one of the following: green fluorescent protein (GFP), inactive TGF- β 1 ribozyme, and active TGF- β 1 ribozyme. Turbofect reagent (Fermentas Inc.; Glen Burnie, MD, USA) is used for the dual transfection following the manufacturer's protocol (see Note 4).

3. Forty-eight hours after transfection, concentration of sAP protein is assessed using Quanti-Blue™ (InvivoGen, San Diego, CA, USA). Ribozyme activity levels are expressed as relative sAP of GFP expression vector (Fig. 6).

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⁴From our experience, when doing a dual transfection using Turbofect, a ratio of 1:1 of plasmids gave the greatest expression of both plasmids.

Fig. 1.

Sequence and secondary structure of the synthetic RNAs and their targets. The *uppercase letters* represent the ribozyme RNA sequences, and the *lowercase letters* represent the target RNA sequences. *Roman numerals* label the helices. *Arrows* indicate the site of c1 eavage. (a) CTGF hammerhead ribozymes targeting nucleotide sequences at positions CHR 745 and CHR 859, (b) TGF- β 1 hammerhead ribozyme targeting nucleotide sequences at positions THR 576 and THR 1429.

Fig. 2.

Time-course studies of TGF-β ribozymes (Rz) cleaving target RNAs. Cleavage reactions were carried out at constant concentrations of 10 pmol ribozyme and 100 pmol target, and were stopped and analyzed at 0.5, 1, 2, 3, 4, 5, 10, and 30 min, 1, 2, 3, and 15 h. Both of TGF-β1 Rz 1 and TGF-β1 Rz 2 could cut their targets. Prolonged incubations caused a significant increase in cleaved product, although the reaction rates slowed markedly after 30 min. The TGF-β1 Rz 1 was slightly more active than TGF-β1 Rz 2, cleaving 62% compared with 43% at 10 min, and 94% compared with 82% at the end of incubation. Data was collected from three individual tests.

Multi-turnover Study

Fig. 3.

Multiturnover studies of TGF-β1 Rz 1 and TGF-β1 Rz 2. The concentration of ribozymes and targets is indicated in Table 1. The enzymatic reaction displayed reaction kinetics amenable to Michaelis–Menten analysis. TGF-β1 Rz 1: $K_m = 2.78 \mu M$, $k_{cat} = 74.1/\text{min}$. TGF-β1 Rz 2: $K_m = 12.50 \mu M$, $k_{cat} = 92.2/\text{min}$. Although TGF-β1 Rz 1 had lower V_{max} and k_{cat} than that of TGF-β1 Rz 2, the lower k_{cat} of this ribozyme is compensated by its lower K_m value. The k_{cat}/K_m of TGF-β1 Rz 1 and TGF-β1 Rz 2 were separate, $2.7 \times 10^7/\text{M}/\text{min}$ and $7.4 \times 10^6/\text{M}/\text{min}$. Thus, TGF-β1 Rz 1 is 3.6 times more efficient.

Fig. 4.

Effect of TGF ribozyme on expression in cell culture. **(a)** Effect of CTGF Rz 1 on CTGF mRNA expression in human dermal fibroblast cultures. CTGF mRNA was then measured using TaqMan quantitative RT-PCR and results were normalized to GAPDH mRNA. The level of CTGF mRNA expression in fibroblasts that were stably transfected with the plasmid expressing CTGF Rz1 was decreased by 55% ($p < 0.01$, $n = 6$) compared with nontransfected control fibroblasts. In contrast, transfection of fibroblasts with the empty expression vector pTR-UF21 or with a plasmid expressing the catalytically inactive ribozyme did not significantly alter the levels of CTGF mRNA from control cells. **(b)** Effect of CTGF Rz1 on CTGF protein expression in human dermal fibroblast cultures. CTGF protein was measured in cytoplasmic extracts and conditioned medium samples using CTGF “sandwich” ELISA and results were normalized for total protein concentration. The levels of CTGF protein measured in conditioned medium of detergent extracts of fibroblasts expressing CTGF Rz1 were reduced by 72 and 71%, respectively, compared with nontransfected control fibroblasts control groups ($p < 0.01$, $n = 6$).

Fig. 5.

(a) The effect of TGF-β1 Rz 1 reducing TGF-β1 expression was tested by intracontrol RT-PCR. Bands of 213 bps were the housekeeping gene beta-actin and bands at 1,014 bps were TGF-β1 expression in cells. Results showed that TGF-β1 expression in TGF-β1 Rz 1 transfected cells was significantly depressed by comparing with negative and inactive control groups; the decreasing rates are 16.2 and 12.1%, respectively. Semiquantitative study shows that in TGF-β1 Rz 1 transfected cells the TGF-β1 expression was significantly depressed compared with the control groups ($p < 0.01$, $n = 4$). (b) Testing the efficiency of TGF-β1 Rz 1 depressed the TGF-β1 protein expression in cytoplasm and culture medium supernatant. Protein expression was reduced by 59 and 37% in cytoplasm and conditioned medium, respectively. Compared with control groups, TGF-β1 Rz 1 depressed the TGF-β1 expression significantly, both in cytoplasm and culture medium supernatant ($p < 0.01$, $n = 4$).

Fig. 6.

The effect of TGF-β1 Rz 1 on exogenous synthetic target as reported by a secreted alkaline phosphatase gene. HEK293 cells were simultaneously transfected with two plasmids, one expressing the TGF-β1 ribozyme target sequence with secreted alkaline phosphatase reporter and the other a plasmid that expresses one of the following: GFP, inactive TGF-β1 ribozyme, or active TGF-β1 ribozyme. A statistically significant difference of 27% was found when comparing the GFP plasmid with the active TGF-β1 ribozyme plasmid.

Table 1

Multiturnover kinetics analysis

	1	2	3	4	5	6	7	8	9	10	11	12
Ribozyme	0	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015
Target	0.15	0.15	0.3	0.6	0.9	1.2	1.5	3.0	6.0	9.0	12	15