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• BASIC RESEARCH •

Preparation and identification of anti-transforming growth factor β1 U1 small nuclear RNA chimeric ribozyme *in vitro*

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Abstract

AIM: To study the preparation and cleavage activity of anti-transforming growth factor (TGF)β1 U1 small nuclear (sn) RNA chimeric hammerhead ribozymes *in vitro*.

METHODS: TGFβ1 partial gene fragment was cloned into T-vector at the downstream of T7 promoter. ^{32}p-labeled TGFβ1 partial transcripts as target RNA were transcribed *in vitro* and purified by denaturing polyacrylamide gel electrophoresis (PAGE). Anti-TGFβ1 ribozymes were designed by computer, then synthetic ribozyme fragments were cloned into the U1 ribozyme vector pZeoU1EcoSpe containing U1 snRNA promoter/enhancer and terminator. ^{32}p-labeled U1 snRNA chimeric ribozyme transcripts were gel-purified, incubated with target-RNAs at different conditions and autoradiographed after running denaturing PAGE.

RESULTS: Active U1snRNA chimeric ribozyme (U1Rz803) had the best cleavage activity at 50 ℃; at 37 ℃, it was active, K_m=34.48 nmol/L, K_{cat}=0.14 min^{-1}; while the point mutant ribozyme U1Rz803$_m$ had no cleavage activity, so these indicated the design of U1Rz803 was correct.

CONCLUSION: U1Rz803 prepared in this study possessed the perfect specific catalytic cleavage activity. These results indicate U1 snRNA chimeric ribozyme U1Rz803 may suppress the expression of TGFβ1 *in vivo*, therefore it may provide a new avenue for the treatment of liver fibrosis in the future.

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INTRODUCTION

The incidence of liver cirrhosis is still high all over the world, especially in China[1-6]. Cirrhotic livers are characterized by extensive fibrosis throughout the entire hepatic parenchyma[7-12]. Many factors inducing liver injury and inflammation will lead to chronic liver disease, and hepatic fibrosis[13-22].

TGFβ1 is an important cytokine in the regulation of the production, degradation, accumulation of extracellular matrix proteins, and that it may play a pivotal role in the fibroproliferative changes that follow tissue damage in many vital organs and tissues, including liver, lung, kidney, skin, heart, and arterial walls[7,23-27]. In the past decade dramatic advances have been made in the understanding of cellular and molecular mechanisms underlying liver fibrogenesis, it is thought that TGFβ1 is of crucial importance in rat hepatic fibrosis *in vivo*[7, 28-34]. Inhibition of TGFβ can not only prevent liver fibrosis, but also preserve organ function[30]. So TGFβ1 has been thought to be an ideal target molecule to prevent the progression of liver fibrosis.

Ribozymes are a class of small catalytic RNA molecules that recognize specific substrate RNA molecules by their complementary nucleotide sequence, cleavaging the substrate RNA as an endoribonuclease at enzymatic rates[35-38]. In the last years ribozyme-mediated inhibition of gene expression in intact cells have been tested many times, but some of them were largely unsuccessful[39-42]. Factors that contributed to ribozyme efficacy in transfected cell are expression level, stability against rapid degradation, correct folding for exposure to target, and subcellular localization of ribozyme and target. U1 snRNA is a highly expressed stable small RNA (164 nucleotides) involved in both splicesome and catalytic processing during pre-mRNA splicing. U1small nuclear RNA expression cassette can provide an excellent vehicle for ribozyme delivery and expression in intact cell because of stability, nuclear localization, highly efficient expression[43-45].

Because TGFβ1 plays a crucial role in liver fibrosis, in this study we designed ribozymes directed against TGFβ1 by computer, then cloned them into U1 snRNA chimeric ribozyme vector, it had been proven that it could cleave target RNA efficiently *in vitro* through the cleavage reaction, so it indicated that it might suppress intracellular TGFβ1 expression, which would provide a new avenue in treatment of liver fibrosis.

MATERIALS AND METHODS

Materials

HSC-T6 cell line is a kind gift from Dr. Scott L. Friedman (Dept of Medicine and Division of Liver Diseases, Mount Sinai School of Medicine). pZeoU1EcoSpe was provided by Dr. Harry C. Dietz (Department of Pediatrics, Medicine and Molecular Biology & Genetics, Johns Hopkins University School of Medicine). pGEM-T vector kit, transcription kit were purchased from Promega Company. Trizol kit, DMEM were purchased from Gibco BRL Company. The PCR primers and ribozyme fragments were synthesized in the Beckman oligo-1 000 DNA synthesizer. Zeocin was purchased from Invitrogen Company. Newborn calf serum was purchased from Hyclone Company. RT-PCR kit, RNase inhibitor, restriction endonucleases, and T4 DNA ligase were purchased from Takara Company. α ^{32}P UTP was purchased from Beijing Ya-Hui Company.

Methods

Construction of target RNA *in vitro* Total RNA was extracted using Trizol Kit (GIBCO BRL) from cultured HSC-T6 cell, an immortalized rat hepatic stellate cells line (HSC), exhibited an activated phenotype[46,47]. The upstream primer P1 (5' -GAATTCATTCAGGACTATCAC CTACC-3') in the untranslated region and the downstream primer P2 (5' -AAGCTTTTCTGGT AGAGTTCTACGTG -3' ;) in the open reading frame were selected to amplify a 651-base pair fragment corresponding to bases 279 to 930 of the rat TGF beta 1[48]. The extracted RNA was reversely transcribed and polymerase chain reaction (PCR) amplified using a pair of primers in one step reverse transcriptase (RT) PCR kit. The PCR products were analyzed and purified on 1 % (w/v) agarose gels. Purified PCR products were ligated into pGEM-T vector. DNA sequencing results showed that the PCR-amplified fragments were cloned into the molecular cloning sites of pGEM-T vector at the downstream of T7 promoter as pTGFβ1. Target RNA was prepared through *in vitro* transcription of PCR-amplified products of pTGFβ1, which contained T7 promoter at the upstream of upper primer. The sequence of the primers for transcription was GAATTC***TAATACGACTCACTATAGGG***AGGCGGA-CTACTACGCCAA and TTCTGGTAGAG TTCTACGTG; ***TAATACGACTCACTATAG GG*** represents T7 promoter. Then PCR product was analyzed and purified by 1 % (w/v) agarose gels electrophoresis as the template for transcription. *In vitro* transcription was carried out at 37 ℃ for 90 min in a 40 μL final volume containing 40 mmol/L of Tris· HCl (pH 7.5), 5 mmol/L of DTT, 2 mmol/L of spermidine, 8 mmol/L of $MgCl_2$, 0.25 mmol/L of ATP, GTP,CTP, 0.05 mmol/L of UTP, 20 μCi alpha ^{32}p-UTP, 80 U T_7 RNA polymerase and 2 μg purified PCR product. Target RNA was purified by 6 % denaturing gel electrophoresis through cutting off the autoradiograph bands and soaking in NES (0.5 mol/L NH_4Ac, 0.1 mol/L EDTA, 0.1 % SDS pH 5.4) at 42 ℃ overnight. the products were precipitated by ethanol, washed twice by 75 % ethanol, dissolved in DEPC H_2O and reserved under -20 ℃.

Construction of recombinant plasmid for ribozyme pZeoU1EcoSpe contained the pZeoSV plasmid DNA modified by excising the SV40 promoter, SV40 polyadenylation site, and polylinker at the *Bam*HI sites. In constructing the pZeoU1 EcoSpe, a U1 snRNA expression cassette in pUC13[49, 50] was excised with *BamH*I digestion and ligated into the *BamH*I sites of the modified pZeoSV. Two rounds of site-directed mutagenesis were then performed to change 4 nt flanking the Sm protein binding site of U1 snRNA, creating unique *EcoRI* and *SpeI* restriction sites. The 5' -flanking region of the inserted U1 snRNA expression cassette possessed a promoter/enhancer comparable in strength with the SV40 early promoter[51]. The ribozymes for TGFβ1 were designed according to the computer software pcFOLD combiled by professor Zuker (Canadian Academy of Science). The homologous possibility with the gene of rat was excluded by consulting with RNA sequence of rat cells from NCBI Genbank. The enclosed vector pZeoU1EcoSpe was cut by *EcoRI* and *SpeI* restriction enzymes and purified by 1 % (w/v) agarose gels electrophoresis. The synthesized oligonucleotides of ribozyme were mixed with equal molar amounts together, then were cloned into the *Eco*RI/*Spe*I sites of pZeoU1EcoSpe to create pU1Rz803. pZeoU1EcoSpe and the reconstruction could be confirmed by DNA sequencing (Figure 1). The oligonucleotides of Rz803 were 5' AATTACATATATACT (***G/A***)ATGAGTCCGTGAGGACGAAACTGTGT3' and 5' CTAGACACAGTTTCGTCCTCACGGACTCAT(***C/T***) AGTATATATGT3' ; ***G*** and **C** for activated ribozyme, ***A*** and **T** for inactivated ribozyme.

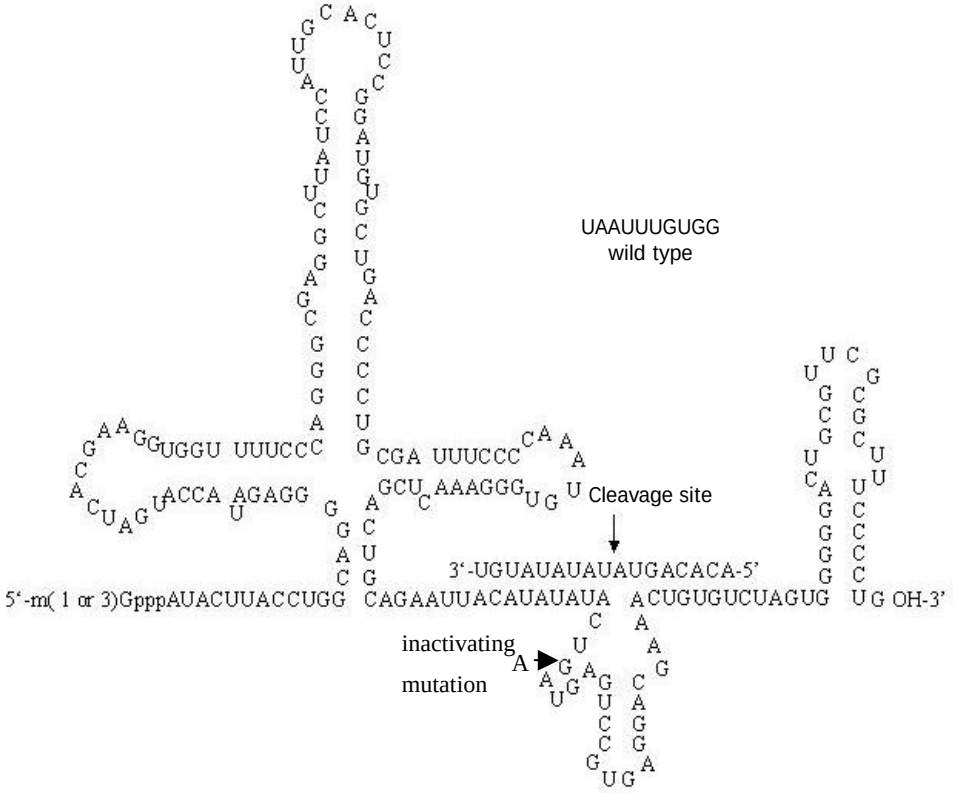

Figure 1 Sequence and predicted structure of U1 snRNA chimeric ribozymes. arrows represent cleavage site and inactivating mutation.

Preparation of ribozymes *in vitro* The templates used for transcription of U1 snRNA chimeric ribozymes were obtained by PCR amplification of pU1Rz803. The primers used for transcription were as follows: upstream primer: 5'-GAATTC***TAATACGACTCACTATAGGG*** GATACTTACC TGGCAGGGGA-3'; downstream primer: 5'-CAGGGGAAAGCGCGAACGCA-3'; ***TAATACGA CTCACTATAGGG*** represented T7 promoter. The purification of PCR products was the same as that of the template for target RNA. *In vitro* transcription and purification of ribozyme were done as described above.

***In vitro* cleavage reaction of U1Rz803 and U1Rz803$_m$** U1Rz803, U1Rz803$_m$ and target RNA were quantified by measuring their radioactivate cpm in 1 μL solution. The cleavage reaction was carried out in 5 μL solution containing 50 mmol/L Tris· HCl (pH7.5), 20 mmol/L $MgCl_2$. The molar ratio between ribozyme and target RNA could be estimated according to the cpm value combined with the U number in their RNAs. The initial experiment was: (I) Ribozyme (R): Substrate (S)=1:1(mol/L) ratio, 37 ℃, 120 min; (II) the condition as (I), R:S=1:5 (mol/L) ratio, 37 ℃, 120 min; (III) the condition as (I), U1Rz803 was incubated with target RNA at different temperatures and at different times. 1 μL loading buffer (0.25 % Bromophenol Blue, 0.25 % Xylene cyanol FF, 20 mmol/L EDTA and saturated Urea) was added to stop the reaction. The result could be analyzed after running a 6 % denaturing polyacylamide gel electrophoresis. The cleavage efficiency [CE] was calculated from Bq values of the bands of substrate (S) and products (P) which were cut off from denaturing PAGE. CE=[P/(P+S)]· 100 %.

Kinetics studies of the reaction The procedure was described by Uhlenbeck[52]. The Michaelis constant (K_m) and K_{cat} were determined for the ribozyme by performing multiple turnover kinetics experiments. The volume of kinetics reaction is 15 μL. Ribozyme concentration was held constant at 5 nmol/L and substrate concentrations ranged from 10 nmol/l to 160 nmol/L. The cleavage reaction was done in the same buffer as described above at 37 ℃ for 20 minutes. The results were analyzed as above. K_m and K_{cat} were calculated by Lineweaver-Burke method (double- riciprocal plot).

RESULTS

Identification of transcription of target RNA and ribozyme

The length of target RNA transcribed from PCR-amplified template should be 220 nt. In this study the ribozymes were embedded into U1 snRNA, but stem-loop structures of U1 snRNA were maintained. Therefore, the transcripts of PCR-amplified template included U1 snRNA and ribozyme, the transcripts of U1snRNA chimeric ribozyme should be 205 nt These results (Figure 2) were inconsistent with our design and proven to be correct.

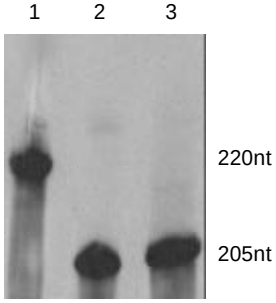

Figure 2 *In vitro* transcripts of target RNA and U1snRNA chimeric ribozymes. 1: transcript of target RNA (220 nt), 2: transcripts of U1Rz803(205 nt), 3: transcripts of U1Rz803$_m$ (205 nt).

In vitro cleavage reaction of U1Rz803 and U1Rz803$_m$

The cleavage result showed that U1Rz803 was capable of cleaving target RNA *in vitro*. It could cleave target RNA (220 nt) efficiently and exactly to produce two fragment 93 nt/127 nt. while U1Rz803$_m$ showed no *in vitro* cleavage efficacy after 120 min (Figure 3), even at Rz:S=5:1 (data not shown). At a 1:1 U1Rz803-to-S molar ratio, the cleavage efficiency (CE) was calculated under the condition of 37 ℃ and 120-minute reaction time, CE=51.36 %. At a 1:5 U1Rz803-to-S molar ratio, CE=27.81 %. This result indicated that the cleavage efficiency increased with increase of ribozyme concentration. The temperature and time would affect the cleavage efficiency.

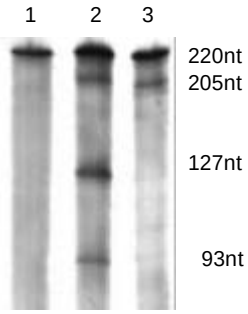

Figure 3 Cleavage of U1Rz803 and U1Rz803$_m$ *in vitro*. 1: Target RNA, 2: target RNA incubated with U1Rz803, 3: target RNA incubated with U1Rz803$_m$. The ribozymes (205 nt) were shown in this figure because the transcripts were incorporated into alpha ^{32}p-UTP in the preparation of ribozymes.

Cleavage activity of U1Rz803 in vitro

Temperature course When the ratio of U1Rz803 to target RNA was 1:1(molar ratio), the reaction mixture were incubated at different temperature for 90 minutes. The optimal temperature was 50 ℃, the cleavage efficacy increased at higher temperatures ranging from 0 ℃ to 50 ℃, but when the temperature was above 50 ℃, the cleavage efficacy decreased because the combination of ribozyme and target RNA was weakened (Figure 4).

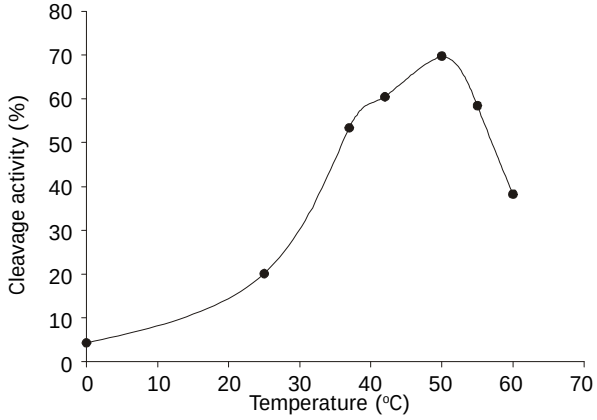

Figure 4 Temperature curve of the cleavage reactions of U1Rz803 prepared *in vitro*.

Time course The cleavage mixture (Rz:substrate=1:5 mol· L^{-1}) were incubated at 37 ℃ for different times, it was shown that the reaction product increased with increase in incubation time and it was linear within 60 min, CE_{max}=27.81% (Figure 5).

The kinetics of cleavage reaction Under the condition of 37 ℃ and 20-minute reaction time the cleavage efficiency was calculated at Rz:S=1:2, 1:4,1:8, 1:16 and 1:32 (mol/L)ratio. K_m and K_{cat} were obtained by the Lineweaver-Burke method (Figure 6) K_m=34.48 nmol/L, K_{cat}=0.14 min^{-1}.

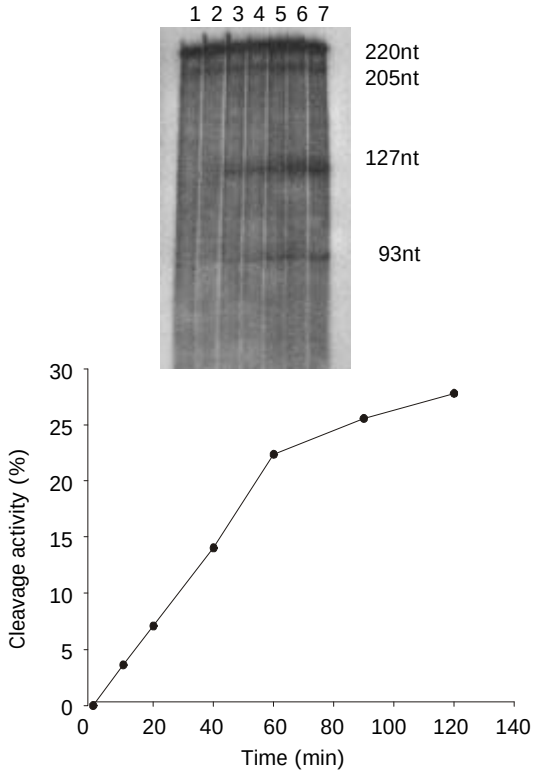

Figure 5 Time course. (A) Specific cleavage of target RNA by U1Rz803 *in vitro* at 37 ℃ for different times. Lane 1: substrate control; lane 2 : incubated for 10 min; lane 3: 20 min; lane 4: 40 min; lane 5: 60 min; lane 6: 90 min; lane 7: 120 min. (B) Time curse of cleavage reactions of U1Rz803 prepared *in vitro.*

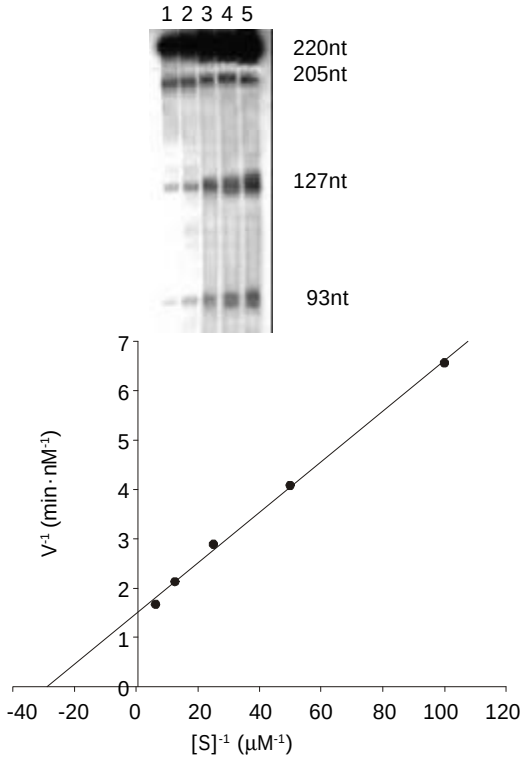

Figure 6 The kinetic of cleavage reaction for U1Rz803. (A) Specific cleavage of target RNA by U1Rz803 for the kinetic of U1Rz803 *in vitro.* U1Rz803 concentration was 5 nmol·L^{-1}, substrate concentration was 10, 20, 40, 80, 160 nmol·L^{-1} from left to right. (B) Lineweaver-Burk kinetic plots of cleavage reaction for U1Rz803 *in vitro.* U1Rz803 concentration was 5 nmol·L^{-1}, substrate concentration was 160, 80, 40, 20, 10 nmo·L^{-1} for each dot from left to right. Reactions were performed at 37 ℃ for 20 minutes.

DISCUSSION

Hepatic fibrosis is a common response to chronic liver injury from many causes, including alcohol, persistent viral infection and hereditary metal overload. To date, reversing the causative agent is the only effective therapy to stop or even reverse the liver fibrosis, but the efficacy is limited. Therefore, the development of effective antifibrotic therapies represents a challenge for modern hepatology. With the knowledge on molecular mechanism underlying pathological fibrosis expanding, there are many antifibrotic therapies based on sound biological mechanisms have been carried out. Ueki *et al* injected a mix of a haemagglutinating virus of Japan (HVJ) liposomes and a plasmid containing the complementary DNA for human hepatocyte growth factor (HGF) into the gluteus muscle of rats treated with dimethylnitrosamine (DMN), a model of persistent liver fibrosis, that could produce the resolution of fibrosis in the cirrhotic liver[32], but tumorigenicity found in transgenic mice overexpressing HGF[53] and repetitive *in vivo* transfection are two disadvantages. Qi and Nakamura *et al* prevent liver fibrosis from blockade of TGF beta signal by adenovirus-mediated local expression of a dominant negative type II TGF-beta receptor in the liver of rats treated with DMN, this intervention not only suppressed fibrosis, but also facilitated hepatocyte regeneration, however prolonged period of blocking TGF beta signal could result in unfavorable consequences, such as the inflammation and tissue necrosis[30,33]. Because TGFβ1 plays a crucial role in liver fibrosis and no report on anti-TGFβ1 ribozyme-mediated cleavage of target RNA for the treatment of liver fibrosis has been published, in this study we designed ribozyme targeting against TGFβ1 and cloned the ribozyme genes into U1 ribozyme vector, prepared U1 snRNA chimeric ribozymes and identified the cleavage activity of ribozymes *in vitro*.

In the previous study on cleavage activity of U1 snRNA chimeric ribozyme *in vitro*, ribozymes were prepared through the transcription of synthesized ribozyme genes containing T7/SP6 promoter, the transcripts only included ribozyme[43]; but ribozyme structure induced by the secondary structure of long flanking sequences would affect ribozyme' s turnover ratio and/or binding activity as the results of less accurate hybridization and less cleavage. In this study we prepared ribozyme by the PCR-amplified templates. The transcripts included U1 snRNA and ribozyme. Compared with the previous study, it may reflect the cleavage activity of U1Rz803 more accurately *in vivo*. From our study, we found that the cleavage activity of U1sn RNA chimeric ribozyme was inferior to that of non-modified ribozyme, the result was not shown in this paper. Trimethylguanosine 5' cap, stable stem-loop structures at both end, high GC content of 3' loop in the structure of U1 snRNA confer resistance to exonucleases *in vivo*[54]. The hypermethylation of the 5' cap structure and Sm binding site enable U1 snRNA to accumulate in the nucleoplasm, these make U1 snRNA an effective vector for efficient expression and delivery of ribozyme in the nuclear compartment *in vivo*. The transcripts of ribozyme labeled by isotope not only provided convenience for us to isolate ribozyme by cutting off autoradiograph bands, but the transcripts were qualified more accurately than non-labeled transcripts of ribozymes. In this study K_m and K_{cat} of U1Rz803 were measured at 37 ℃, not at optimal temperature. Because the ribozyme is used *in vivo* and the temperature *in vivo* is constant at 37 ℃, these results may reflect cleavage activity of U1Rz803 in physiological condition.

Ribozymes have all the properties of antisense RNA with the additional feature of catalytic cleavage. To separate antisense from cleavage effect, we created inactive ribozymes by substituting an essential nucleotide of the catalytic core with

an inactive one. The cleavage reaction revealed that U1Rz803 possessed the perfect cleavage activity, while U1Rz803$_m$ possessed no catalytic activity. It can be used as control to exclude antisense effect of ribozyme *in vivo* in order that it is proven that the activity of U1Rz803 is due to catalytic cleavage *in vivo*. The kinetics of U1Rz803 showed that U1Rz803 possessed perfect specific ability of cleaving the TGFβ1 transcripts *in vitro*. These results made U1Rz803 to be worthy of being studied in intact cell and be developed as a nucleic acid drug in the future. However the *in vitro* result cannot completely reflect *in vivo* performance. The secondary and tertiary structure formed by the total TGF beta 1 mRNA transcript in the cell, the subcellular compartment which the ribozyme and target are located in, degradation of ribozyme, the complexes which are formed by ribozyme and ribonucleoprotein within cell and gene delivery system affect expression of ribozyme and cleavage activity of ribozyme. So *in vivo* effect of the ribozyme should be investigated as soon as possible. Experimental analysis of activity of the anti-TGF beta1 ribozyme in HSC-T6 cell is in progress.

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