Small antisense RNA to cyclin D1 generated by pre-tRNA splicing inhibits growth of human hepatoma cells

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Abstract **Introns are present in some human pre-tRNAs. They are spliced out during the maturation processes of pre-tRNAs in a way that is irrelevant to their specific nucleotide sequences. This unique characteristic of tRNA splicing can be used for generation of small antisense RNAs by replacing the intron sequences with corresponding antisense sequences. In this work, the intron sequence of human pre-tRNA^{tyr} gene was replaced with a 20 bp antisense sequence targeted to the 5′ coding region of cyclin D1, a molecule that was over-expressed in many malignant proliferating cells. Under the control of U6 SnRNA promoter to further enhance transcription efficiency of the modified pre-tRNA^{tyr} gene and subsequent antisense generation, the antisense RNA exhibited obvious suppression of cyclin D1 expression in H22 hepatoma cells. The growth of H22-transplanted tumors in mice was significantly inhibited when treated with naked plasmid DNA harboring the cyclin D1 antisense RNA generating cassette. Such tumor growth inhibition might be due to apoptosis caused by reduced cyclin D1 expression as revealed by immunohistochemical analysis of tumor samples.**

Keywords: tRNA splicing; Antisense; Cyclin D1; Hepatocellular carcinoma

1. Introduction

In eukaryotes, introns of tRNA precursors are small and invariably interrupt the anticodon loop 1 base 3′ to the anticodon. During the maturation processes of tRNA precursors, which are best understood in yeasts, introns are removed by a stepwise action of three enzymes: an endonuclease that removes the intervening sequence, a ligase that joins the two half molecules leaving a 2′ phosphate at the splice junction, and a phosphotransferase that transfers the 2′ phosphate to NAD [1–3]. The endonuclease recognizes splice sites by measuring the distance from the mature domain to the splice sites. That means the specificity of cutting at both splice sites is determined solely by the length of anticodon stem. Replacement of the anticodon stem with equal length of poly(U) yielded anticipated normal splicing [4]. However, the intron itself is not completely passive in the recognition process. A base pair formed between the pyrimidine at position 32 and a purine in the intron was reported to be necessary to position the 3′ splice site [5]. Whether a specific artificial intron in the place of the native intron can be removed correctly has to be determined experimentally. The endonuclease associates with membranes in yeast [6]. Among its four subunits (Sen54p, Sen2p, Sen34p and Sen15p), the Sen2p subunit contains a potential transmembrane sequence. It was believed that endonuclease located in the nuclear membrane through anchoring of Sen2p [7]. So splicing of tRNA was speculated to occur near the inner nuclear membrane and might be coupled to tRNA export [3]. However, a recent study showed that the majority of Sen2p, Sen54p and the endonuclease activity are not localized in the nucleus, but on the mitochondrial surface. The endonuclease is peripherally associated with the cytosolic surface of the outer mitochondrial membrane. Thus, it was proposed that tRNA splicing mainly occurs on the mitochondrial surface in yeast [8]. Nevertheless, the human counterpart of yeast endonuclease complex was located in the nucleus [9], which, together with the fact that human intron-containing tRNA^{tyr} gene can be spliced correctly by Hela cell nuclear extract [10], implies that human pre-tRNA splicing occurs in nucleus. The fate of excised introns is rarely described, but they exhibited good stability in yeast, which make them easily detectable in Northern blot analyses [11]. As described above, splicing of pre-tRNAs exhibited special properties that the specific intron sequences have little effect on correct splicing and the removed introns are stable, which make it possible to utilize pre-tRNA splicing machineries to generate small antisense RNA for gene silencing. By replacing the 20 bp human intron of tRNA^{tyr} gene with an equal length of antisense sequence targeted at the 5′ coding region of cyclin D1, we constructed a set of plasmids for intracellular generation of antisense RNA. The antisense generated this way could decrease cyclin D1 expression and thus inhibit the growth of hepatoma cells, implying that pre-tRNA splicing could be utilized in antisense technology.

2. Materials and methods

2.1. Plasmids construction

A chemically synthesized DNA (Fig. 1A) was inserted into the *Kpn*I/*Xba*I sites of plasmid pUC18, yielding plasmid pUT. Two chemically synthesized oligonucleotides Ccnd1a (5′-TCGCAGCACAGGAGCTGGT-3′) and Ccnd1b (5′-GATCACCAGCTCCTGTGCTGCGA-3′) were annealed, generating the flanking sites of *Stu*I and *Bam*HI. The resulting double-strand oligonucleotide was inserted into

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Fig. 1. Synthetic DNA sequences. Capital letters in bold indicate sequences from human $tRNA^{tyr}$ gene (A and B). The intron sequence of $tRNA^{tyr}$ (plain capital letters in B) was substituted with a 10 bp oligonucleotide (plain capital letters in A), generating two restriction sites (*Stu*I and *Bam*HI) to ease insertion of any antisenses, not limited to cyclin D1 as in this work. Letters in lower case represent the promoter sequence of U6 SnRNA gene (A), which enhances the transcription efficiency of subsequent pre-tRNA. The U6 SnRNA promoter and the tRNA gene were spaced by two copies of *tet* operator 2 sequence that serve as the binding sites for two molecules of the Tet repressor protein, so as to block the transcription of pre-tRNA from both U6 SnRNA and tRNA promoters unless with tetracycline inducement.

the corresponding *Stu*I/*Bam*HI sites of pUT, yielding pUT-ccnd1. This plasmid was used for generation of antisense RNA (5′-UCGCAGCACAGGAGCUGGUG-3′) to human cyclin D1 (corresponding to the 5′ coding region) by pre-tRNA splicing. As a positive control of splicing, native $tRNA^{tyr}$ gene was synthesized chemically (Fig. 1B) and inserted into the *Sal*I/*Xba*I sites of pUT and pUC18, yielding pUT-tyr and pUC-tyr, respectively. To create stable cell lines in which generation of antisense RNA can be regulated by tetracycline, two plasmids, pTRUT-ccnd1 and pTRUT-tyr, were created by inserting the blunted *Kpn*I/*Xba*I fragments of pUT-ccnd1 and pUT-tyr into the blunted *Xho*I site of plasmid pcDNA6/TR (Invitrogen), respectively.

2.2. In vitro transcription and splicing of pre-tRNAs using Hela nuclear extracts

Hela nuclear extracts, containing all the components for transcription and splicing of pre-tRNAs, were prepared according to Lee et al. [12] and Dignam et al. [13] with slight modification. In brief, Hela cells of about 80% confluence were trypsinized and washed twice with PBS. The cells were suspended with one cell package volume of cold Buffer A (10 mM HEPES–KOH, pH 7.9, at 4 °C; 1.5 mM $MgCl_2$; 10 mM KCl; and 0.5 mM DTT) and were let swell on ice for 15 min. Then, the cells were passed quickly through a Chinese standard 5# needle (inner diameter 0.24 mm) for 10 times. Nucleus was collected by centrifugation at $12\,000 \times g$ for 30 s, then resuspended with 2/3 original cell package volume of cold Buffer C (20 mM HEPES–KOH, pH 7.9, at 4 °C; 25% glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT; and 0.5 mM PMSF), and incubated on ice for 30 min with shaking. After centrifugation at $12\,000 \times g$ for 2 min, the supernatants were dialyzed against Buffer D (20 mM HEPES–KOH, pH 7.9, at 4 °C; 20% glycerol; 0.1 M KCl; 0.2 mM EDTA; 0.5 mM DTT; and 0.5 mM PMSF) at 4 °C for 2 h. The extracts were kept in liquid nitrogen until use. For in vitro transcription and splicing, 5 μl of Buffer D, 1 μg of DNA template, 1.5 μl of 0.1 M $MgCl_2$, 0.5 μl of RNasin, 7.5 μl of Hela nuclear extract, 1 μl of 10 mM ATP/CTP/GTP each, 1 μl of 0.2 mM UTP and 0.01 mCi of [α-^{32}P]UTP were mixed well, and incubated at 30 °C for 1 h. The samples were applied to 16% denatured polyacrylamide gel electrophoresis (PAGE) with 8 M urea and directly applied to autoradiography after electrophoresis.

2.3. Establishment of antisense generating H22 cell lines regulated by tetracycline

Plasmids pTRUT-ccnd1 and pTRUT-tyr were transfected into human hepatoma H22 cells (provided by Dr. Chen of PLA General Hospital, China) with Lipofectamine 2000 reagent (Invitrogen). Stable blasticidin resistant clones were screened out according to Invitrogen's instructions. Antisense RNA was induced for 24 h with 1μg/ml of tetracycline added to the culture medium and then cyclin D1 expression was analyzed by Western blot.

2.4. Western blot analysis of cyclin D1 expression

The stably transfected H22 cells with or without antisense RNA induction by tetracycline were collected by trypsinization, washed twice with PBS, then resuspended in cold lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.5% sodium deoxycholate; 0.1% SDS; 1% NP-40; and 0.5 mM PMSF) at exactly 10^7 cells/ml and incubated at 4 °C for 30 min. After brief centrifugation, 20 μl of the supernatant was applied to 12% reduced SDS–PAGE, then electrotransferred overnight onto PVDF membrane (Millipore, Bedford, MA) at 40 V. The bands were visualized by a primary anti-cyclin D1 monoclonal antibody (cat# sc-8396, Santa Cruz Biotechnology) and an AP-conjugated goat-anti-mouse IgG (Promega) as the secondary antibody. The membrane was developed by Western Blue® substrate (Promega).

2.5. Proliferation assay of H22 cells

Equal number of each stable blasticidin resistant cell line was seeded into 48-well plate and cultured with or without 1 μg/ml tetracycline for antisense RNA induction. After 2 days of induction, medium was changed to 1 mg/ml MTT dissolved in serum-free medium. The cells were incubated at 37 °C for 4 h and then MTT solution was discarded. The dye synthesized by active live cells was dissolved in 300 μl of isopropanol and optical absorbance at 560 nm was measured.

2.6. Plasmid purification for gene therapy

Escherichia coli DH5α containing plasmid pUT-ccnd1 was cultured in a 10 L Bioflo V fermenter (NBS, New Jersey). Plasmid was extracted from the bacteria by conventional alkaline lysis procedure and then purified by strong anion exchange chromatography using Q-Sepharose XL and SOURCE 15Q absorbents (Amersham Biosciences, Uppsala, Sweden) according to the protocols provided by the manufacturer.

2.7. Animal experiments

Four-week-old female KM mice were inoculated s.c. in the right thigh with 50 μl ascites of H22 hepatoma cells. When the tumor grew to about 200 mm^3 in size at day 5, 50 μg of purified plasmid pUT-ccnd1 in 50 μl TE buffer or TE only control buffer was injected directly into the tumors every other day. The length and width of tumor nodules were measured with a caliper, and tumor volume was estimated using the following formula:

$$\text{Volume} = (\text{Length} \times \text{Width})^{3/2} \times \pi/6.$$

2.8. In situ detection of cyclin D1 expression and apoptosis by immunohistochemistry

The tumor samples were fixed and kept in 4% formaldehyde. Before use, the samples were dehydrated with increasing concentration of ethanol solution and xylene and embedded in paraffin. The samples were sliced into about 4 μm sections and placed on clean glass slides. The slides were baked at 80 °C for 30 min to enhance the attachment of sample sections onto the glass slides. For cyclin D1 detection, the sections were hydrated, and probed with an anti-cyclin D1 monoclonal antibody as mentioned above, and then a biotinized anti-mouse antibody and SABC complex (streptavidin–biotin-peroxidase, Boster Biotech, Wuhan, China) in sequence. Labeled cells were visualized by chromagen 3′,3′-diaminobenzidine, slightly conterstained with hematoxylin solution, and mounted with Clearmount™ (Zymed, South San Francisco, CA). To detect apoptosis, the sections were labeled with DIG at the end of fragmented DNA, which was characteristic of apoptosis, by labeling mixture (containing DIG-labeled dUTP, terminal deoxynucleotidyl transferase and reaction buffer, Boster Biotech) at 37 °C for 2 h, and then probed with a biotinized anti-DIG antibody and SABC. Labeled cells were visualized, strongly counterstained and mounted as mentioned above.

3. Results

3.1. Efficient small antisense RNA generation in vitro

Hela cell extract contains all the necessary components for pre-tRNA transcription and splicing. The tRNA gene contains a class three promoter within the gene itself. However, such a promoter is relatively weaker than the promoter for U6 SnRNA, which transcribes about 400 000 copies of U6 SnRNA out of only a few copies of functional U6 gene per cell [14]. In order to enhance pre-tRNA transcription for antisense generation, the promoter of U6 SnRNA was put ahead of tRNA gene. Fig. 2 shows that with U6 SnRNA promoter, tRNA transcription in pUT-tyr and pUT-ccnd1 was considerably higher than pUC-tyr, which contains only $tRNA^{tyr}$ gene. It also proved that the 20 bp antisense RNA insert was correctly spliced out as predicted.

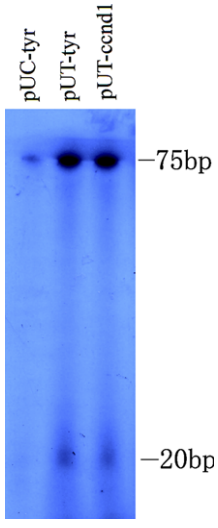

Fig. 2. In vitro transcription and splicing of pre-tRNA. With U6 SnRNA promoter, pre-tRNAs in pUT-tyr and pUT-ccnd1 were transcribed more efficiently than pUC-tyr control. The 20 bp anti-cyclin D1 insert replacing the intron sequence of $tRNA^{tyr}$ gene was correctly spliced out (pUT-ccnd1) as well as the native intron of $tRNA^{tyr}$ gene (pUT-tyr).

3.2. Cyclin D1 expression in H22 hepatoma cells decreased due to antisense generation

It was attempted to create a stable cell line harboring antisense RNA generating plasmid to test the efficacy of cyclin D1 inhibition by antisense RNA. But H22 hepatoma cells grow poorly in RPMI1640/10% FBS medium and transfection of cyclin D1 antisense RNA generating plasmids into the cells resulted in cell death. To overcome this problem, an inducible strategy was designed. When transfected into H22 cells, plasmid pTRUT-ccnd1 will express a Tet repressor protein. The repressor can bind to the two copies of *tet* operator 2 ($TetO_2$) sequence located between U6 SnRNA promoter and tRNA gene, which had been integrated into the same plasmid, and thus block tRNA transcription and subsequent splicing. Such blocking can be released by tetracycline, which binds to Tet repressor, changes its conformation and releases it from $TetO_2$. So addition of tetracycline into culture medium can induce antisense RNA production. Plasmid pTRUT-ccnd1 and control plasmid pTRUT-tyr with native $tRNA^{tyr}$ gene were transfected into H22 cells, and stable blasticidin-resistant clones were screened out. Each of such stable clones and H22 negative control clone were cultured until 90% confluence, and then tetracycline was added to the medium to 1 μg/ml final concentration for induction of antisense RNA. Cyclin D1 expression was analyzed 24 h later. Fig. 3 shows that antisense RNA against cyclin D1 generated this way inhibited cyclin D1 expression efficiently.

3.3. Antisense RNA slowed down proliferation of H22 hepatoma cells

Overexpression of Cyclin D1 is observed in many carcinoma cells and is a key molecule for cell cycle progression. The expression of cyclin D1 is highly relevant to the proliferation ability of mammalian cells. Cyclin D1 expression in H22 cells was significantly reduced by antisense RNA as proved above. Such inhibition of cyclin D1 reduced the proliferation ability of H22 cells, as shown in Fig. 4.

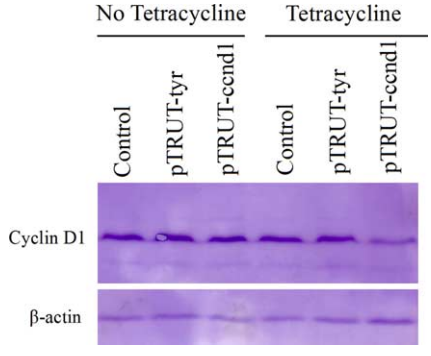

Fig. 3. Western blot analysis of cyclin D1 expression. Stable blasticidin-resistant H22 cell lines were created by transfection with pTRUT-tyr or pTRUT-ccnd1. Both of the two cell lines together with un-transfected H22 control cells were induced with tetracycline for 24 h. Then, cyclin D1 expression was analyzed by Western blot. β-Actin expression was also analyzed as a control. In the figure, cyclin D1 expression was significantly reduced by cyclin D1 antisense RNA. Whereas intron of pre-$tRNA^{tyr}$ generated in the same way had no effect on cyclin D1 expression.

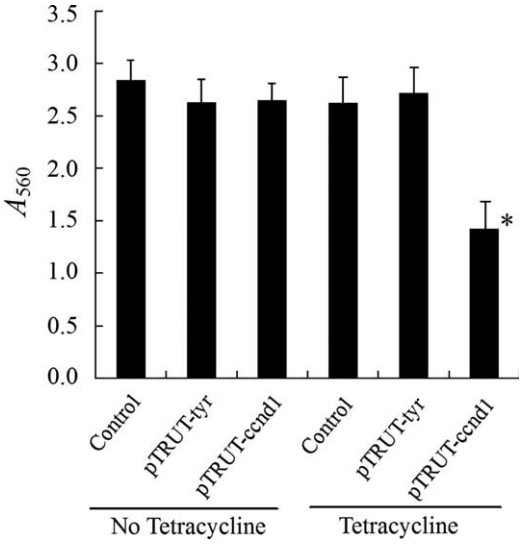

Fig. 4. Cell proliferation assay by MTT method. H22 cells were stained by MTT. Proliferation of the cells was significantly ($P < 0.05$) reduced by the antisense RNA. The bars represent the standard deviations calculated from three repeated experiments.

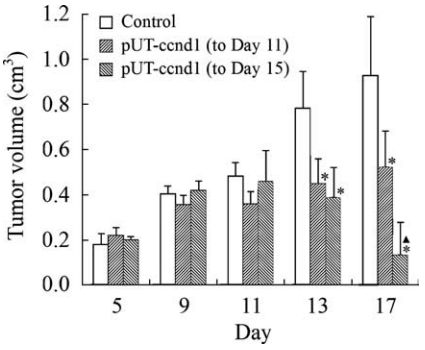

Fig. 5. Effect of plasmid administration on tumor growth. Four days after inoculation of H22 cells (Day 5), pUT-ccnd1 plasmid was injected into the s.c. tumor xenografts at multiple sites until day 11 or day 15. Tumor volumes were determined by bidimensional caliper measurements and were presented as the mean tumor volume ($n = 8$). The bars stand for standard deviation. (*) Significant ($P < 0.05$) volume difference between plasmid treated tumors and TE treated control tumors. (▲) Significant ($P < 0.05$) difference between short-term plasmid treated tumors (to day 11) and long-term treated tumors (to day 15).

3.4. *Growth of preformed hepatomas in mice inhibited by antisense RNA*

H22 hepatoma cells had been passaged in KM mouse for many generations and became well adapted to KM mouse. Ascites containing H22 cells was inoculated s.c. into the right thigh of KM mouse at day 1, then tumor nodules grew rapidly to about 200 mm^3 at day 5. Afterwards, the mice were randomly divided into three groups. Mice in group I were injected with 50 μl of TE buffer directly into the tumor at multiple sites every other day as a control. Mice in group II were injected with 50 μg of pUT-ccnd1 in 50 μl TE buffer every other day until day 11, then with TE buffer. Mice in group III were injected continuously with 50 μg of pUT-cnnd1 in 50 μl TE buffer every other day until day 15. Fig. 5 shows that the growth of transplanted tumors was apparently inhibited by the antisense RNA to cyclin D1 generated by tRNA splicing. Such

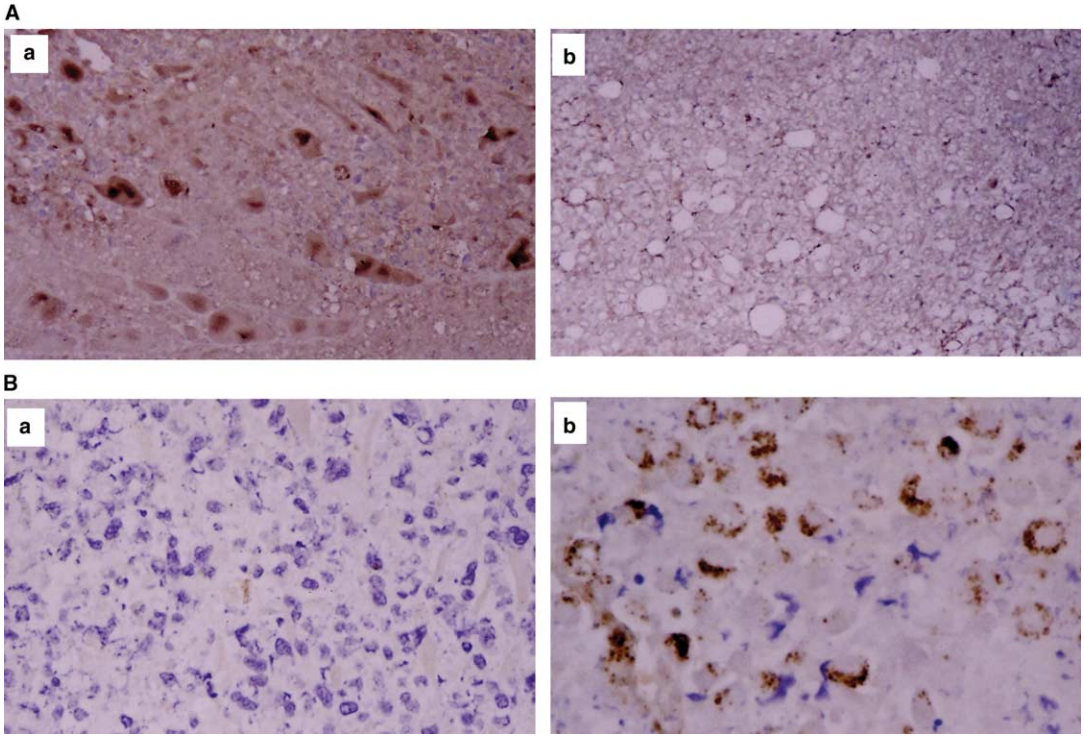

Fig. 6. Immunohistochemical analysis of cyclin D1 expression and apoptosis in s.c. tumor xenografts with and without plasmid pUT-ccnd1 injection. Preformed tumor xenografts were harvested at day 17 after 6 times of plasmid injection. Cyclin D1 expression (A) and apoptosis (B) were analyzed as described in Section 2. (a) s.c. tumors treated with TE only; (b) s.c. tumors treated with plasmid pUT-ccnd1.

inhibition was more effective by continuous application of pUT-ccnd1 than application for only limited days.

3.5. Reduced cyclin D1 expression and apoptosis detected in transplanted H22 hepatoma cells

Immunohistochemistry was performed to confirm inhibition of cyclin D1 expression by antisense RNA in preformed s.c. tumors and to detect apoptosis caused by such inhibition of cyclin D1 expression. Cyclin D1 was expressed in untreated tumors in a homogeneous way (Fig. 6A(a)). However, in plasmid treated tumors its expression was significantly inhibited (Fig. 6A(b)) at multiple sites, which may correspond to the nearby regions of injection sites. Apoptosis was detected in plasmid treated tumors (Fig. 6B(b)), but not in untreated tumors (Fig. 6B(a)).

4. Discussion

Amplification or overexpression of cyclin D1 is frequently observed in various human carcinomas. It occurs in about 60% of breast cancers, 40% of colorectal cancers, 40% of squamous carcinomas of the head and neck, 30% of hepatocellular carcinoma and 20% of prostate cancers [15,16]. Overexpression of cyclin D1 in hepatocellular carcinoma was also described elsewhere [17,18]. Cyclin D1 knockout mice exhibited resistance to breast cancers induced by the *neu* and *ras* oncogene [19]. Inhibition of cyclin D1 by antisense technology has been described to lead to loss of tumorigenicity, increased chemosensitivity or apoptosis in many cancer cells, including hepatocellular [20], squamous [21,22], lung [23,24], pancreatic [25,26], gastric [27], ovarian [28], colon [29] and esophageal [30] carcinomas. Therefore, cyclin D1 was selected in this study as the target of antisense RNA produced by pre-tRNA splicing to test the feasibility of this system.

Antisense technology acts by interacting with specific target sequences on mRNAs or their precursors through Watson–Crick pairing, and inhibits translation or splicing of mRNAs, thus inhibits expression of corresponding proteins. In contrast to conventional pharmacological antagonists for inhibiting the function of specific proteins, antisense technology is more attractive due to its high specificity for their targets and its ease in designing an antisense compound, which requires the information on only a small portion of nucleic acid sequence encoding a given protein even without knowing the function of that protein [31]. Antisense technology includes antisense oligodeoxynucleotides (ODN), antisense RNA and ribozymes. Antisense ODNs have been most extensively studied and were widely used in gene inhibition, especially in cancer therapy [32,33]. Antisense ODNs have the advantages that they can be synthesized commercially and easily standardized. However, bioavailability and half-life in vivo have always been a challenge for ODNs due to poor permeability into the cells and degradation by nucleases. Chemical modifications or analogs can be used to improve bioavailability, but side effects may arise such as toxicity and poor specificity. In contrast, antisense RNA transcribed intracellular from a vector would have a longer duration of action after a single administration. In addition, using tissue specific promoters can render antisense RNA with tissue specificity. Most of the gene delivery vectors for gene therapy can be used for antisense RNA administration. The only difference is changing the mRNA generating cassette to an antisense RNA producing cassette [31]. In most published papers, antisense RNA generation is confined to class II promoters [34]. These resulting antisense RNAs would be 5′ capped and 3′ tailed, which may decrease the efficiency in pairing with target mRNAs. In spite of the fact that natural regulatory antisense RNAs are transcribed exclusively by RNA polymerase II in human cells [35,36], their regulatory effects might not be fulfilled by tight pairing. Thus, RNA polymerase III promoters may be preferable in antisense RNA transcription. However, RNA polymerase III locates in the nucleus, and it is highly probable that the antisense RNAs they transcribed could not be exported efficiently into the cytosol, because they might lack the special exporting structure as in U6 SnRNA or 7SK RNA. So integrating the antisense RNA into tRNA and utilizing its exporting machinery could be a good choice. By replacing the anticodon loop of $tRNA^{pro}$ with an antisense sequence targeted at *tat* of HIV, Biasolo et al. [37] showed that the 20 bp antisense RNA embedded in $tRNA^{pro}$ could resist HIV infection. In this study, however, we replaced the intron, not the anticodon, of pre-$tRNA^{tyr}$ with an equal length of anti-cyclin D1 sequence. The antisense RNA could have been exported into the cytosol as efficiently as native pre-$tRNA^{tyr}$, and released into the cytosol without irrelevant 5′ and 3′ adjacent tRNA sequences to interfere with the base pairing with corresponding target, and thus was believed to be more efficient than those described by Biasolo et al. [37] in target gene inhibition.

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