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Conditional and Target-Specific Transgene Induction through RNA Replacement Using an Allosteric Trans-Splicing Ribozyme

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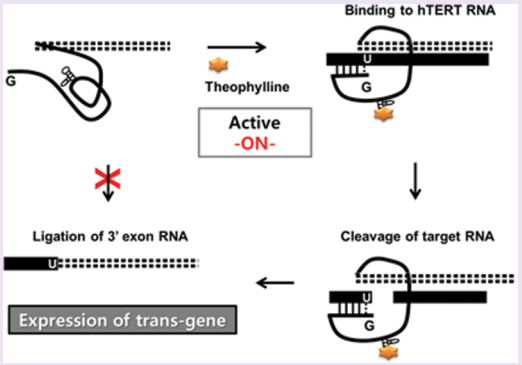
Supporting Information

ABSTRACT: Gene therapeutic approaches are needed that can simultaneously induce the well-controlled expression of therapeutic genes and suppress the expression of disease-causing genes for maximization of their efficacy. To address this challenge, we designed an allosteric ribozyme that comprises a *Tetrahymena* group I-based trans-splicing ribozyme as an active domain for RNA replacement, a small molecule-specific RNA aptamer as a sensor domain, and a communication module as an active transfer domain. The effectiveness of this approach was assessed by constructing various ribozymes in combination with a theophylline-binding aptamer to identify an allosteric ribozyme, which is controlled by theophylline both *in vitro* and in cells. Moreover, we constructed adenoviral vectors encoding the ribozymes and validated allosteric regulation of trans-gene expression via theophylline-dependent RNA replacement in target RNA-expressing cells. Results demonstrate that an allosteric trans-splicing ribozyme is an applicable RNA-based framework for engineering external ligand-controlled gene expression regulatory systems that exhibit adjustable regulation, design modularity, and target specificity.



Gene therapy technologies have intensively been developed as new approaches to treat incurable human diseases and as a means of elucidating the molecular causes and factors of diseases. Nevertheless, many issues have yet to be overcome for the realization of gene therapy. Gene therapy for the treatment of genetic diseases is usually devised by transferring a normal gene into a patient's cells to restore proper function of a mutant gene.[1] Theoretically, in order to achieve therapeutic effects through this approach, a desirable and controllable amount of the normal gene product should be produced *in vivo*. Because of limitations on the size of transgene that can be packaged into viral particles for delivery, nearly all gene therapies transfer the desired gene in the form of cDNA under the control of a chosen promoter or the gene's own promoter. This approach excludes other genetic elements that may be critical for regulated expression of the transferred gene and might thus fail to maximize the desired effect for treatment of the disease. In addition, components used for gene expression could undesirably activate other kinds of promoters and inadvertently change chromatin structure to increase the expression of other unwanted genes (e.g., proto-oncogenes) in target cells. Moreover, transfer of the normal gene does not affect the level of mutant gene product in a patient's cells. If the mutant gene product has a dominant negative effect, a therapeutic effect may not be achieved by use of aforementioned conventional approaches. Therefore, there exists a demand for gene therapy systems that can achieve inducible, adjustable expression of normal genes while simultaneously suppressing expression of mutant genes.

RNA is highly useful for the regulation of gene expression not only inherently but also artificially because of its conformational flexibility and functional versatility. For example, metabolite-sensing riboswitches are known to control gene expression in most bacteria, plants, and fungi.[2,3] These riboswitches consist solely of RNA, and they induce or repress gene expression through changes in conformation that occur upon ligand binding. Mechanisms of riboswitches rely on modulation of translation, transcription termination, or RNA self-cleavage through the direct interaction of specific intracellular metabolites with the mRNA. These mechanisms could be applied to develop artificially controllable riboswitches.[4−7] However, such systems possess some practical and theoretical limitations, including target specificity and efficacy.

The *Tetrahymena* group I intron-based trans-splicing ribozyme has been suggested as a new gene therapeutic regimen to specifically target and cleave a substrate RNA and trans-ligate a 3′-exon of the ribozyme onto the downstream U nucleotide of the cleaved target RNA, both *in vitro* and in cells.[8−10] The trans-splicing ribozyme has been used for therapeutic applications, such as in the treatment of human genetic, infectious, or malignant diseases.[11−15] Previously, we

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validated that a trans-splicing ribozyme is a potent anticancer agent, acting through selective targeting and RNA replacement of transcripts predominantly expressed in cancer cells. We developed a trans-splicing ribozyme targeting human telomerase reverse transcriptase (hTERT) RNA and demonstrated that this hTERT-specific ribozyme could efficiently induce suicide gene activity via a target-specific trans-splicing reaction not only in cells but also in tumor xenograft models when treated with prodrugs.[16−19]

Group I intron structures have been analyzed through high-resolution structural analysis, using both X-ray crystallography and NMR spectroscopy.[20] Understanding of this structure could provide information for the rational design of allosteric RNA molecules.[21] For example, artificial riboswitches based on allosteric ribozymes could be developed through the insertion of an RNA aptamer,[22,23] which can be selected to bind specifically to a target ligand by using *in vitro* selection technology.[21] Aptamers have emerged as viable alternatives to antibody-based clinical agents due to their great specificity, high affinity, chemical manufacturability, pharmaceutical amenability, low immnugenecity, etc.

In this study, in order to establish an exogenously controllable system based on the group I ribozyme, we designed allosteric ribozymes containing a small molecule-specific RNA aptamer as a regulatory site, and a communication module sequence linking the aptamer and the ribozyme (Figure 1). These allosteric trans-splicing ribozymes could be used as a

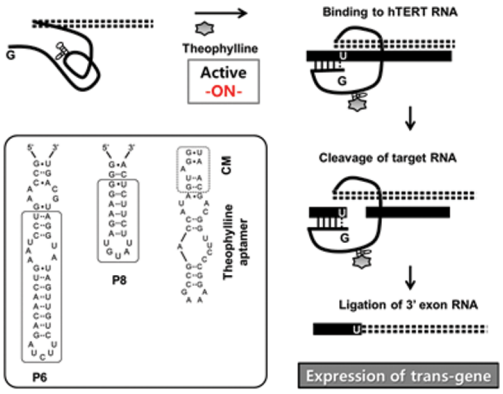

Figure 1. Scheme for the control of RNA replacement by an allosteric trans-splicing ribozyme and proposed secondary structures and sequences of P6 and P8 of the ribozyme. The ribozyme was modified by replacing P6, P8, or P6 and P8 (boxed portions) with a communication module (CM) and theophylline-binding aptamer (6T, 8T, and 6T8T, respectively).

system that is able to target certain disease-specific RNAs, providing the ability to artificially control RNA replacement in therapeutic targets by using exogenous small molecules to activate the function of the ribozyme.

In order to engineer an allosteric group I-based trans-splicing ribozyme whose activity is modulated by the binding of an effector molecule to an aptamer domain, which is located apart from the active site, it is first necessary to determine which residues and structures in the original ribozyme contribute to its catalytic function. The *Tetrahymena* group I ribozyme is a large, multidomain catalyst that exhibits considerable complexity in the folding pathway required to achieve its active conformation.[20,24,25] Previously, we showed that modification of P6, P8, or both produced catalysts that exhibit allosteric affects.[26,27] Here, we used this framework to rationally design hTERT-specific allosteric trans-splicing ribozymes. We similarly fused a theophylline-specific aptamer sequence into the core regions of the trans-splicing ribozyme via a communication module to make these separate RNA folding domains interdependent (Figure 1). Then, we identified the most optimal allosteric trans-splicing ribozyme that showed high trans-gene expression activity upon binding to theophylline.

To evaluate ligand-dependent ribozyme activity, we constructed various hTERT-specific allosteric trans-splicing ribozymes that contained the communication module and theophylline-specific aptamer in place of P6, P8, or both. Detailed description of the ribozyme constructs was illustrated in Supplementary Figure S1. We performed *in vitro* trans-splicing reactions with these ribozymes and hTERT RNA in the presence of theophylline, caffeine, or PBS, and compared ribozyme activities with those by unmodified hTERT-targeting trans-splicing ribozyme, W-P9[16] (data not shown). Most ribozymes could not distinguish the difference between theophylline and caffeine. However, the W-P9 6T8T and ΔP9 6T ribozymes produced trans-splicing product specifically dependent on theophylline, but not caffeine or PBS, indicating target hTERT RNA specificity with ligand inducible activity of the ribozyme.

We then examined and compared trans-gene expression in the presence or absence of ligands in an hTERT positive HEK293 cell line using luciferase reporter assays. W-P9 6T8T, ΔP9 6T, and ΔP9 8T ribozymes without antisense sequence allosterically induced reporter activity in the presence of theophylline (data not shown). These allosteric ribozymes were subsequently modified to contain antisense sequence to hTERT RNA to increase the efficacy of trans-splicing activity in cells. Finally, AS325 W-P9 6T8T was found to be regulated most specifically by theophylline, not by caffeine, using a luciferase reporter assay in the HEK293 cell line (Figure 2).

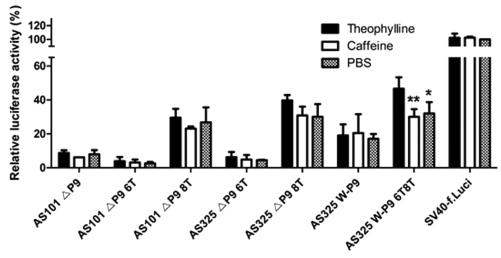

Figure 2. Regulation of trans-splicing ribozyme activity by theophylline in cells. Luciferase reporter assays were performed for cells grown in the presence of indicated ligands. Expressed values represent means and standard deviations of three independent determinations. Student's *t* test for paired data was used for statistical comparison between theophylline and caffeine or PBS: $*P < 0.05$ and $**P < 0.01$.

Sequence analysis of the trans-splicing product confirmed the accuracy of trans-splicing activity in HEK293 cells (data not shown). Constructs that exhibited the greatest allosteric regulation were not the same both *in vitro* and in cells. Moreover, allosteric regulation of trans-splicing ribozymes was changed through modification to increase efficacy, such as the inclusion of an hTERT antisense sequence of 101 bp or 325 bp in the ribozyme. However, W-P9 6T8T exhibited the ability to be regulated by theophylline in both assays. We confirmed that the catalytic activity of W-P9 6T8T was allosterically regulated by ligand binding, while maintaining target specificity and

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efficacy of the original W-P9 hTERT-targeting trans-splicing ribozyme.

To evaluate ligand-dependent trans-gene expression by the modified hTERT-targeting trans-splicing ribozyme, we constructed an adenoviral vector encoding the hTERT-specific W-P9 6T8T allosteric trans-splicing ribozyme under control of a strong, constitutive CMV promoter (Ad-W-P9 6T8T; Figure 3a). Adenovirus encoding hTERT-specific trans-splicing ribozyme without an aptamer domain was used as a positive control for transgene expression (Ad-W-P9). Adenovirus encoding the lacZ gene under control of the CMV promoter (Ad-LacZ) was used as a negative control. Adenovirus encoding herpes simplex thymidine kinase (HSVtk) under control of the CMV promoter (Ad-TK) was used as a positive control for suicide gene activity with ganciclovir (GCV) and to determine the efficacy of RNA replacement by hTERT-specific ribozymes. Cytotoxicity was tested under the following conditions: (1) treatment with various amounts of adenovirus (MOI) at fixed concentration of GCV in the absence or presence of theophylline or caffeine; (2) treatment with various concentrations of theophylline or caffeine at fixed MOI of adenovirus and concentration of GCV. In addition, cytotoxicity was compared in two cell lines including hTERT(+) HT-29 cancer cells and hTERT(−) IMR-90 cells. Notably, Ad-W-P9 6T8T induced cytotoxicity in HT-29 cells in a theophylline dose-dependent manner but had little effect in cells treated with caffeine (Figure 3b). However, Ad-W-P9 infected HT-29 cells treated with ligand in the same manner induced cell death in the presence of either theophylline or caffeine (Figure 3b). As expected, the Ad-TK was cytotoxic in both cell lines independent of hTERT RNA expression, whereas Ad-W-P9 and Ad-W-P9 6T8T specifically affected hTERT(+) HT-29 cells and had negligible effect on hTERT(−) IMR-90 cells even at high concentration (Figure 3b,c).

Expression of trans-spliced RNA and target hTERT RNA in HT-29 cells was evaluated using RT-PCR analysis (Figure 4a). In cells infected with Ad-W-P9 6T8T, trans-spliced product was generated and hTERT RNA was decreased more significantly with theophylline treatment. In addition, we compared the levels of trans-spliced RNA in Ad-W-P9 6T8T-infected HT-29 cells treated with different ligands using quantitative RT-PCR

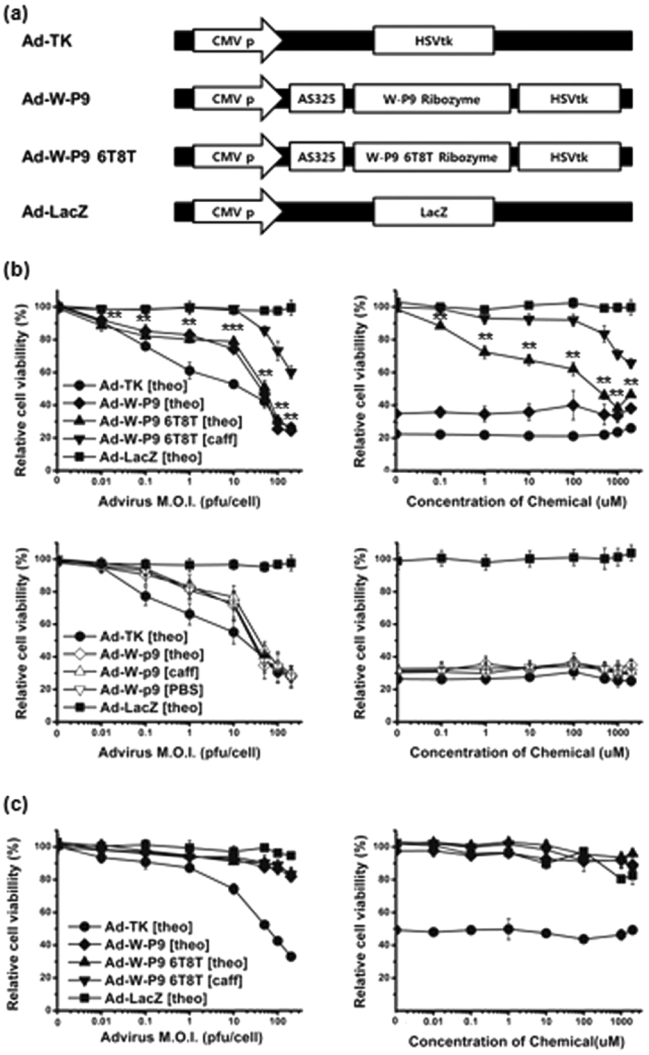

Figure 3. Theophylline-dependent cytotoxicity of the adenovirus encoding hTERT-targeting allosteric trans-splicing ribozyme. (a) Schematic diagram of recombinant adenoviral vectors. (b) HT-29 cells expressing hTERT were infected with each adenoviral vector at various MOI (left panel; Chemical 100 μM, GCV 10 μM) in the absence or presence of indicated chemical at a various concentrations (right panel; symbols are the same as left; Adenovirus 50 M.O.I., GCV 10 μM). (c) hTERT negative IMR-90 cells were subjected to the same procedure as in panel b (left panel; Chemical 100 μM, GCV 50 μM/ right panel; Adenovirus 50 M.O.I., GCV 50 μM). Cell viability was determined using an MTS assay. Results are presented as the mean ± standard deviation for triplicate experiments. Student's t test for paired data was used for statistical comparison between theophylline and caffeine: $^{**}P < 0.01$ and $^{***}P < 0.0001$.

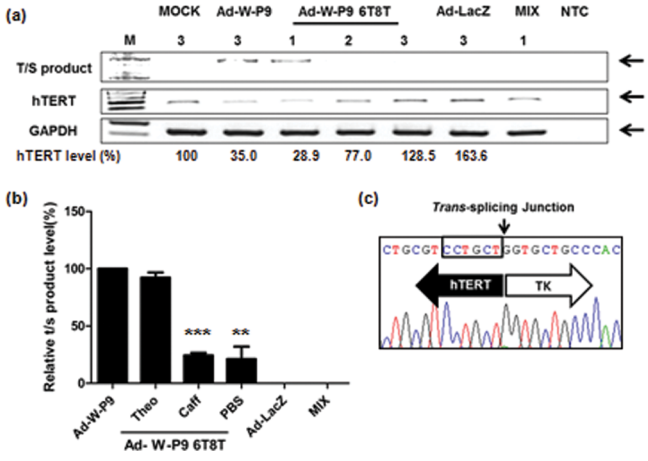

Figure 4. Regulated induction of trans-splicing activity by theophylline. (a) Trans-spliced (T/S) and hTERT RNA expression patterns upon treatment with theophylline (1), caffeine (2), or PBS (3) in HT-29 cells infected with indicated adenovirus (Ad-W-P9, Ad-W-P9 6T8T, or Ad-LacZ) were analyzed using RT-PCR. Endogenous GAPDH RNA was amplified as an internal control. Level of hTERT RNA was determined and expressed as a percentage of RNA level of mock-infected cells. IMR-90 cells infected with Ad-W-P9 were mixed with mock-infected HT-29 cells (mix). NTC denotes nontemplate control. (b) Analysis of relative trans-splicing RNA expression levels by qRT-PCR in theophylline-, caffeine-, or PBS-treated HT-29 cells infected with indicated adenovirus. The averages are represented with the standard deviation for three different experiments. Student's t test for paired data was used for statistical comparison between theophylline and caffeine or PBS: $^{**}P < 0.01$ and $^{***}P < 0.0001$. (c) Representative sequence analysis of trans-spliced product generated from HT-29 cells infected with Ad-W-P9 6T8T.

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(Figure 4b). The amount of the trans-splicing product was increased more than 4 fold in cells treated with theophylline compared to those treated with caffeine or PBS. Moreover, trans-splicing activity of the Ad-W-P9 6T8T construct was almost equal to the activity of Ad-W-P9. Sequence analysis of the trans-splicing product confirmed target specificity in hTERT positive cells (Figure 4c).

In this study, we describe a new class of rationally designed allosteric ribozymes based on a target-specific trans-splicing group I ribozyme that can be regulated by the externally provided ligand, theophylline. The design involved substitution of both P6 and P8 in the hTERT-targeting trans-splicing ribozyme with theophylline-specific RNA aptamers as sensor domains, which were joined through a communication module. In the absence of theophylline, the allosteric ribozyme remains largely in the inactive form, but can be switched into the active conformation in the presence of the effector, resulting in the induction of catalytic activity. The activated allosteric ribozyme subsequently cleaves the target RNA and trans-ligates a 3′-exon of the ribozyme onto the cleaved RNA specifically in cells expressing hTERT to induce transgene activity.

The allosteric trans-splicing ribozyme can be used as a means to artificially control the expression of therapeutic target genes, specifically in cells expressing target RNA, using exogenous small molecules to activate ribozyme function. RNA replacement or RNA repair using such trans-splicing ribozymes can provide regulated expression of the transgene and simultaneously reduce expression of the disease-causing gene. Therefore, transgene expression can be finely controlled in a specific tissue and time, dependent on both intracellular target RNA expression and exogenous small molecule activator. Moreover, with such allosteric ribozymes, innovative concepts for target-specific and reversible gene therapy technologies or for engineering genetic control elements can be developed. The target recognition sequence and 3′-exon of the ribozyme can be modularly replaced. Moreover, the aptamer region can conceivably be replaced with another aptamer that specifically binds extra- or intracellular ligands. Such ribozyme could therefore be useful to monitor and/or modulate any cell fate using specific endogenous or exogenous cues.

METHODS

Design of the Ribozymes. Expression vector that encodes for hTERT-specific trans-splicing ribozyme, W-P9, under the control of cytomegalovirus (CMV) promoter was constructed as previously described.[16,17] W-P9 directed at +21 uridine residue on hTERT contains an extended internal guide sequence, such as an extension of the P1 helix, an additional 6-nt long P10 helix. W-P9 ribozyme was modified by the replacement of P6, P8, or both with a communication module (CM)-theophylline specific aptamer.

The modified hTERT-specific trans-splicing ribozymes were generated by the PCR amplification of self-splicing ribozyme templates with a theophylline aptamer and CM sequence[26,27] using the 5′ PCR primer (5′-GGGGAATTCTAATACGACTCACTATAGGCAGG-AAAAGTTATCAGGCA-3′) and the 3′ PCR primer (5′-CGAGT-ACTCCAAAACTAATCAA-3′). cDNA of a 3′-exon encoding for either Luciferase or herpes simplex virus thymidine kinase (HSVtk) genes was inserted into the *Nru*I/*Xba*I sites downstream of the modified group I intron expression construct. The ΔP9 ribozyme set (deletion of 71 nt encompassing 350−421, which is not conserved among group I introns, has no effect on core catalytic activity of the intron[28]) was also constructed in the same procedure. AS101 or AS325 ribozyme sets were created via the insertion of 101- or 325-nt long antisense sequence against the downstream region (+30 to +130 or +30 to +354 residue, respectively) of the targeted residue of the hTERT RNA into the *Hind*III site of ribozyme constructs.[16]

Assessment of Trans-Splicing Ribozyme Activity *in Vitro* and in Cells. Detailed procedures of the experiments are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

In vitro trans-splicing reaction, cell cultures, verification of specificity and efficiency of trans-splicing ribozyme in cell lines, and schematic diagram of allosteric trans-splicing ribozyme constructs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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