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Conditional RNA Interference Mediated by Allosteric Ribozyme

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RNA interference (RNAi) has emerged as a powerful tool that enables specific gene silencing in cell culture and multicellular organisms.[1] However, constitutive knock-down of many genes (e.g., by siRNAs) may result in undesirable outcomes, especially in biomedical applications such as gene therapy. Therefore, spatial and/or temporal regulation of RNAi is of significant importance for basic research as well as practical applications of RNAi.

At present, two major conditional RNAi strategies are available.[2] Chemically inducible expression systems (e.g., Tet-On and Tet-Off systems) based on engineered transcription factors and modified promoters are used to transcribe short-hairpin RNAs (shRNAs) to achieve conditional RNAi. Alternatively, irreversible induction of RNAi can be accomplished by Cre-mediated DNA recombination at loxP sites. Both approaches, however, rely on exogenous proteins to be coexpressed. These proteins may be toxic to the cells or the host animal, may induce immunogenic reactions, and may significantly add to the genetic size and complexity of the system. Moreover, the variety of the chemical inducers is severely limited by the availability of the engineered transcription factors.

We sought to overcome these limitations in the protein-based conditional RNAi strategies by a multipartite RNA architecture that employs an RNA aptamer as the chemical sensor. Allosteric hammerhead ribozymes (aptazymes) incorporating an RNA aptamer at the stem III was chosen as the molecular sensor and transducer for the following reasons. First, numerous allosteric hammerhead ribozymes incorporating different aptamers have been engineered via *in vitro* selection,[3] demonstrating the remarkable flexibility of the ribozyme. Second, several groups have cleverly adapted allosteric hammerhead ribozymes to regulate gene expression in bacteria[4] and in yeast[5] (but not in mammalian cells).

Our design strategy is illustrated in Figure 1A. The RNA transcript comprises three parts: (1) a structural analogue of primary miRNA (pri-miRNA) responsible for RNAi; (2) an allosteric hammerhead ribozyme with an embedded theophylline aptamer;[6] (3) an inhibitory strand that forms a stable stem by hybridizing with the 5′ end of the pri-miRNA analogue. In this study, we adapted the theophylline-responsive ribozyme reported by Wieland and Hartig that was used to regulate gene expression in *Escherichia coli*[4a] as well as variants of their ribozyme. The RNA transcript does not induce RNAi in the absence of theophylline because the RNA cannot be processed by the ribonuclease Drosha which requires long single-stranded regions flanking the short hairpin structure in its substrates.[7] Addition of theophylline was expected to induce ribozyme self-cleavage, resulting in the exposure of the 5′ single-stranded region originally masked by the inhibitory strand. The pre-miRNA analogue produced by Drosha would be subsequently transported to the cytoplasm. The RNA would be further processed by Dicer to yield the short duplex RNA and would ultimately induce RNAi. The sequences of the aptazymes used in this study are shown in Figure 1B, and the full RNA sequence and structure are shown in Figure S1 (Supporting Information).

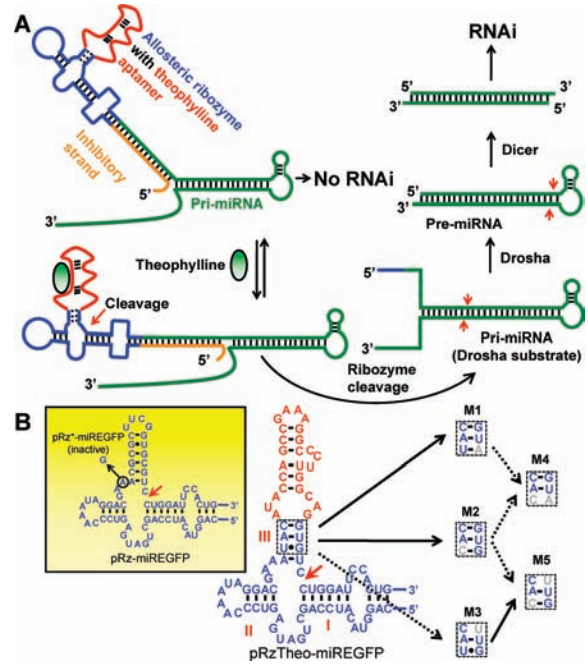

Figure 1. Design strategy for conditional RNAi mediated by allosteric ribozyme. (A) A structural analogue to pri-miRNA (green) is fused to the allosteric hammerhead ribozyme (ribozyme: blue, theophylline aptamer: red) via the 5′ terminus. The inhibitory strand shown in orange is attached to the 5′ end of the ribozyme which is designed to form a stable stem with the 5′ single-stranded portion of the pri-miRNA module. The ribozyme is activated upon binding theophylline which yields the pri-miRNA analogue that can be processed by Drosha. After being transported to the cytoplasm, the pre-miRNA is digested by Dicer, and RNAi is induced. See Supporting Information for additional discussion on the putative mechanism. (B) Sequence of the theophylline-activated hammerhead ribozyme[4a] embedded in the RNA transcripts expressed from the pRzTheo-miREGFP plasmid vector and its variants (M1−M5) studied. The variants differ in the stem III that connects the aptamer and the ribozyme. The solid arrows represent single mutations that stabilize the stem, while the broken arrows represent single mutations that destabilize the stem. Mutated bases are shown in gray. pRz-miREGFP expresses the construct containing a constitutively active ribozyme in which the aptamer is replaced with a stable stem loop (yellow box). The adenine at the catalytic core shown in the circle was replaced with guanine in pRz*-miREGFP which is known to inactivate the ribozyme (negative control).

Plasmids that express the multipartite RNA illustrated in Figure 1 with the pri-miRNA sequence targeting enhanced green fluorescent protein (EGFP) were constructed. The plasmids were cotransfected with pEGFP-N1 and pDsRed-N1 which express the autofluorescent proteins EGFP and DsRed, respectively, into HEK293 cells and were cultured in the absence and presence of theophylline. EGFP fluorescence was significantly suppressed in the cells transfected with pRzTheo-miREGFP (with the theophylline-activated ribozyme) only in the presence of theophylline (Figure 2A, B), indicating that RNAi was induced by theophylline. In

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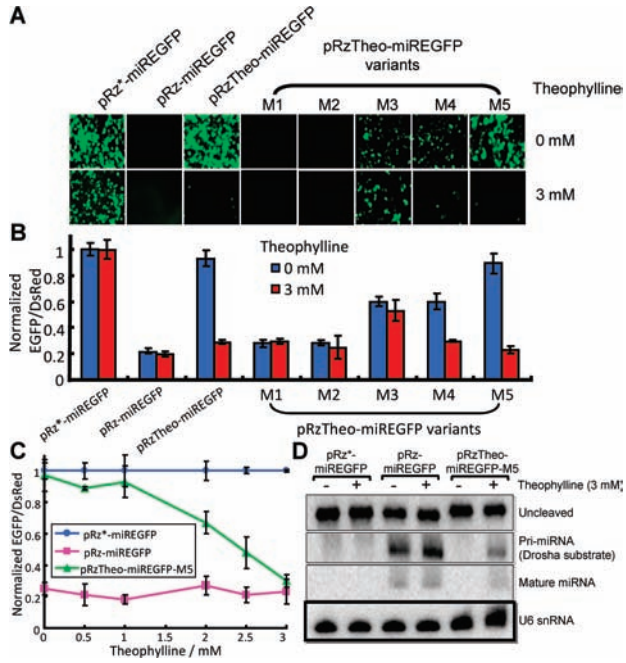

Figure 2. Gene silencing by the engineered RNA constructs. (A) EGFP expression in HEK293 cells cultured in the absence and presence (3 mM) of theophylline, visualized under a fluorescence microscope. (B) Normalized fluorescence intensities of the cells cotransfected with the RNA expression plasmids, pEGFP-N1 and pDsRed1-N1. Fluorescence was measured 48 h after transfection. EGFP/DsRed ratios measured were normalized by the EGFP/DsRed ratio of the negative control (pRz*-miREGFP). (C) Dose-dependent EGFP expression measured in cells expressing the M5 variant and the controls. (D) Detection of the RNA species by Northern blot using a DNA probe that is complementary to the predicted mature miRNA species. Expression of U6 snRNA was detected in a separate gel to confirm the loading levels. The data shown in (B) and (C) are averages of triplicate transfections, and the error bars represent standard deviations. See Supporting Information for the experimental procedures and additional control experiments (Figure S2).

contrast, EGFP fluorescence of the cells expressing the RNA constructs without the aptamer (pRz*-miREGFP, pRz-miREGFP) was not affected by theophylline (Figure 2A, B).

Wieland and Hartig suggested that the stability of stem III, which connects the aptamer and the ribozyme, is the critical determinant of the allosteric regulation of the ribozyme activity.[4a] Consistent with this view, the variants with single mutations that stabilize the stem (M1 and M2) silenced EGFP even without theophylline, whereas a destabilizing mutation (M3) resulted in an intermediate phenotype (Figure 2A, B). M4, which contains a destabilizing mutation with respect to M1 and M2, partially recovered the allosteric response. Another variant M5 that combines the two mutations in M2 and M3 exhibited a theophylline response similar to that of the original construct (pRzTheo-miREGFP) (Figure 2A, B). Dose-dependent suppression of EGFP expression was observed with the variant M5 (Figure 2C).

To detect the RNA species illustrated in Figure 1A, Northern blot was performed using the cellular RNAs extracted from the transfected cells. Using a probe that is complementary to the predicted mature miRNA sequence, we detected the intact RNA transcript as the major species under all conditions (Figure 2D, uncleaved). However, the product of the ribozyme self-cleavage (pri-miRNA) and the mature miRNA were detected only in the positive control (pRz-miREGFP), and pRzTheo-miREGFP-M5, in the presence of theophylline (Figure 2D).

The present work is the first to demonstrate *activation* of RNAi by an aptamer ligand. Previously, we have reported modulation of RNAi by incorporating the theophylline aptamer in the loop region of the shRNA to *inhibit* RNAi in the presence of theophylline by interfering with Dicer processing,[8a] which we later applied to regulate an endogenous gene.[8b] These results marked the first examples of using RNA aptamer−small molecule interaction to modulate RNAi. More recently, the Smolke group employed a similar design with an aptamer in the shRNA loop to inhibit RNAi by the aptamer ligand.[8c] Incorporation of an aptamer in the loop, however, suffers limitations due to the substrate requirements of Dicer which makes the design inflexible.[8] In our new RNA architecture, the aptazyme that functions as the molecular sensor is functionally uncoupled from the RNAi effector (pri-miRNA) which makes the design highly modular and extensible. Consequently, the design should enable one to take advantage of the extensive work reported on allosteric ribozymes.[3−5] Another advantage of the RNA-based conditional RNAi is that it does not require an engineered promoter in contrast with the strategies based on engineered transcription factors. Thus, the compact self-contained transcript can be expressed from any promoter, including endogenous tissue- and tumor-specific promoters.

In summary, we present an efficient and highly modular conditional RNAi strategy that does not require engineered proteins. The simple design and the potential compatibility with a large number of known RNA aptamers[9] should greatly facilitate the efforts to manipulate mammalian gene expression in response to a variety of endogenous and exogenous chemical triggers.

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Supporting Information Available: Detailed description of the experimental procedures and materials used. This material is available free of charge via the Internet at http://pubs.acs.org.

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