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Original Research Article

Programming anti-ribozymes to sense trigger RNAs for modulating gene expression in mammalian cells

Wenhui Zhang [a,b,1], Shi Zhao [b,1], Mengyuan Wang [b], Chunbo Lou [a], Yanhui Xiang [a,*], Qiong Wu [b,**]

[a] Center for Cell and Gene Circuit Design, CAS Key Laboratory of Quantitative Engineering Biology, Shenzhen Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, 518055, China
[b] State Key Laboratory of Green Biomanufacturing, MOE Key Lab. Bioinformatics, Center for Synthetic and Systems Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China

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ABSTRACT

Synthetic RNA-based switches provide distinctive merits in modulating gene expression. Simple and flexible RNA-based switches are crucial for advancing the field of gene regulation, paving the way for innovative tools that can sense and manipulate cellular processes. In this research, we have developed programmable ribozymes that are capable of suppressing gene expression in response to specific, endogenously expressed trigger RNAs. We engineer ribozymes by introducing upstream antisense sequences (anti-ribozymes) to inhibit the self-cleaving activity of the hammerhead ribozyme and open the expression of the target gene. The trigger RNA is designed to recognize and bind to complementary sequences within the anti-ribozymes, thereby inhibiting their ability to direct protein synthesis. The anti-ribozyme performance is optimized by regulating the essential sequence modules that play a crucial role in determining the specificity and efficiency of the anti-ribozyme's interaction with its trigger RNA. By applying this switch mechanism to various ribozyme designs, we have shown that it is possible to achieve control over gene expression across a wide range of trigger RNAs. By exploiting these programmable anti-ribozymes, we aim to create a powerful tool for controlling gene expression in mammalian cells, which could have important implications for basic research, disease diagnosis, and therapeutic interventions.

1. Introduction

The regulation of gene expression has indeed been a cornerstone of synthetic biology research. The development of more sophisticated and versatile gene regulatory tools will likely play a central role in driving innovation and progress in engineering functional biological systems. Commonly used protein-based systems at the transcription level often require complex expression cassettes, which can involve multiple genes and regulatory elements [1–3]. These cassettes can be difficult to design, assemble, and optimize, and they may also be prone to unwanted interactions and side effects. On the other hand, RNA-based switches function primarily through RNA sequence base-pairing interactions or allosteric structures. They can be designed and engineered using relatively simple and well-established principles of RNA biochemistry and structure, allowing for the creation of synthetic gene circuits and complex regulatory networks [4–6]. This flexibility and modularity make RNA-based switches an attractive choice for synthetic biology applications, where precise control over gene expression is crucial. With the development from basic research to gene therapy and other areas, the demand for adaptable and programmable RNA switches that can be used in a variety of applications is indeed growing. Designing a flexible RNA-based switch with strong programmability has become a new requirement.

Previous attempts have been made to design intracellular RNA-based switches. By incorporating aptamers into RNA-based switches, researchers have created switches that respond to a wide range of ligands, including metal ions [7,8], chemical molecules [9,10], and even specific RNA structures [11]. While these switches have shown great potential

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* Corresponding author.
** Corresponding author.
E-mail addresses: yh.xiang@siat.ac.cn (Y. Xiang), wuqiong@mails.tsinghua.edu.cn (Q. Wu).
[1] The authors contributed equally to this study.

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for regulating gene expression, there are several limitations associated with their use. The limited availability of ligands [12–16] and the potential safety risks associated with their use [17] are important challenges that need to be addressed. Moreover, the manual addition or removal of ligands to activate or deactivate RNA switches is a rigid process that is not compatible with internal input signals. Researchers are also exploring alternative approaches to gene regulation that do not rely on ligands. James J. Collins and his colleagues reported eukaryotic toehold switches (eToeholds) as modular riboregulators that could control translation in response to specific trigger RNAs (trRNAs) [18]. However, the robustness of the eToehold system requires the introduction of internal ribosome entry sites (IRESs) and exogenous polymerase systems into the expression cassette. Other tools such as microRNAs [19–21] and CRISPR-based synthetic transcription factors [22–24] have also provided researchers with a range of options for regulating gene expression but remain significantly under development, with ongoing efforts to enhance their practicality [25–27]. Overall, it's still urgent to continue to develop new RNA-based switches that can provide greater precision, efficiency, and versatility.

Ribozymes with self-cleaving activities are valuable tools for precisely regulating gene expression. These unique RNA sequences have the capability to catalyze their own cleavage reactions, making them highly useful in various biotechnological and therapeutic applications [28–31]. To overcome current challenges associated with the addition of effector molecules such as small molecule induction [32,33] and short oligonucleotide supplements [34,35], we explore new approaches for regulating ribozyme switches in response to endogenous expression signals. Here, we propose the anti-ribozyme switch that can recognize and bind to a specific endogenous trigger RNA, which would then trigger a conformational change in the ribozyme switch and allow for control over gene expression. We utilize the well-characterized satellite RNA of tobacco ringspot virus (sTRSV) hammerhead ribozyme [29] as a basis for developing an inhibitory structure that can interfere with its self-cleaving activity, and then design an anti-sTRSV that can communicate with specific trigger RNA sequences to rescue this activity. The anti-sTRSV switch depends on base-pairing interactions to turn off and turn on the ribozyme self-cleaving activity, which can meet the high affinity and specificity requirements of effector molecules. Trigger RNA sequences are expressed intracellularly based on DNA transfection that can supply effector molecules more flexibly. Essential functional modules of the anti-sTRSV switch focus on engineering the ribozyme itself without the need for additional modified elements, which has reduced the complexity and cost of the system, while still allowing for effective regulation of intracellular RNA-based gene expression.

Altogether, we developed the anti-ribozyme switch to precisely regulate gene expression in response to endogenous trigger RNAs expressed in mammalian cells. To assess the inhibitory effect on sTRSV's self-cleaving activity, we introduced various lengths and forms of complementary sequences upstream of the ribozyme. Through further design of the interacting RNA sequences, we achieved up to 4-fold increase in trigger-RNA-induced inhibition of the controlled gene. Lastly, we assessed and confirmed the effect of the switch on various ribozymes to evaluate the programmability of this platform. Our results demonstrate the feasibility of the anti-ribozyme switch and its potential for further customization and programming. The anti-ribozyme switch, with its ability to regulate gene expression in response to in-cell trigger RNAs, holds great promise from basic research to therapeutic development.

2. Materials and methods

2.1. Plasmid construction

All plasmids relevant to the switches used in this study are listed in the supplementary data. These plasmids were constructed using standard molecular cloning methods [36]. PCR fragments were purified using a Gel Extraction Kit (Omega). Ligation products were produced by Gibson assemble mix (New England Biolabs, NEB) or T4 DNA ligase reaction mix (NEB) under appropriate incubation temperature and time. The reaction products were transformed into *E. coli* competent cells Trans10 (TransGen Biotech). The plasmids were then purified using the extraction kit (Qiagen) and the concentration was detected by Nanodrop (Life technology). All primers were synthesized by RuiBiotech. All the sequences of the constructed plasmids were verified by Tsingke Biotechnology.

The plasmid construction workflow for anti-ribozymes and trigger RNAs included the following major experimental phases. The CMV promoter-polyA cassette with essential plasmid elements was PCR-amplified from laboratory-stored parental plasmids using high-fidelity DNA polymerase. Synthesized green fluorescent protein (Citrine) or blue fluorescent protein (TagBFP) gene fragments were directionally cloned into the linearized backbone through Gibson assembly. Hammerhead ribozyme variants and their complementary anti-ribozyme sequences were introduced through overlap-extension PCR followed by T4 DNA ligase-mediated blunt-end ligation. Trigger RNA cassettes were PCR-amplified from anti-ribozyme-containing plasmids and subsequently integrated into destination vectors using Gibson assembly.

2.2. Cell culture and transfection

The mainly used HEK293T cells were cultured in Dulbecco's Modified Eagle Media (DMEM), high glucose (Hyclone), supplemented with 10 % FBS (Gibco) and 1 % Penicillin-streptomycin solution (Cytiva). Cells were kept in a humid incubator (Thermo) at 37 °C and 5 % CO_2. When the cell density reached more than 90 %, the cells were trypsinized (0.25 % Typsin-EDTA, Gibco), collected, and transferred to a fresh medium. Lipo8000 Transfection Reagent (Beyotime Biotechnology) was mainly used for cell transfection. The day prior to transfection, HEK293T cells were seeded in 12-well plates at 2×10^5 cells/well. Upon reaching 70–80 % confluency, the cells were ready for transfection following the manufacturer's protocol. For transfection using Lipo8000, each well received 1 μg of plasmid DNA, which was first diluted in 50 μL of serum-free DMEM. This was then mixed with 1.6 μL of Lipo8000 per 1 μg of plasmid. The mixture was carefully added dropwise to the cells, followed by gentle swirling of the plate to ensure even distribution. At 12 h post-transfection, the medium was replaced with fresh complete DMEM. After an incubation period of approximately 48 h, the cells were analyzed using fluorescence imaging and flow cytometry techniques.

2.3. Fluorescence imaging

After transfection for 48 h, the cells expressing fluorescent proteins were captured and imaged under the microscope at 100 × magnification using the EVOS M7000 system (Invitrogen). The images for the same batch of cell samples were taken under the same exposure time.

2.4. Flow cytometry Measurement (FCM)

Forty-eight hours post-transfection, the cells were collected for FCM. The cells to be analyzed were trypsinized and harvested in around 200 μL fresh DMEM medium. Then the cells were transferred to sterile 96-well plates and kept on ice. Citrine, red fluorescent protein (mCherry), and TagBFP fluorescence were detected using an LSRFortessa flow cytometer (BD Biosciences) equipped with a 96-well HTS injector. For each sample, 100000 cells were harvested and analyzed. The fluorescence of non-treated cells was detected to measure the background fluorescence. All reported data was collected from three replicate samples.

2.5. Data analysis

Data analysis was performed using the paired, two-tailed Student's *t*-

test. The results represent the mean ± standard deviation (SD) from three replicate cell samples. Differences between tested samples were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results and conclusion

3.1. Design of the anti-ribozyme switch for modulating gene expression

To target and respond to specific RNA molecules within cells, we utilized hammerhead ribozymes as the signaling medium. Hammerhead ribozymes, a class of small, self-cleaving ribozymes, exhibit rapid response, high efficiency, and straightforward design, rendering them ideal for programming and targeting various RNA sequences. The gene expression reporter cassette consisted of a cytomegalovirus (CMV) promoter driving the expression of the fluorescent protein Citrine, with the hammerhead ribozyme module incorporated between the 3′ untranslated region (3′UTR) and the transcription termination signal (Fig. S1). Upon insertion of the self-cleaving ribozyme, the mRNA transcript was terminated without the poly(A) tail, leading to its subsequent degradation and a corresponding reduction in reporter protein levels. To enable the ribozyme switch to detect the expression state of specific RNA targets, we introduced competing sequences upstream of the ribozyme, which blocked its self-cleaving activity. As a result of the competing sequences blocking the ribozyme's self-cleaving activity, the reporter gene was activated in the absence of a trigger RNA (abbreviated as TA in figures). To investigate the recognition and displacement between the trigger RNA and the blocking sequences, we determined the essential modules of the blocking sequences based on a study by Yin et al. on toehold switches [37]. The blocking sequence can be divided into three main parts: complementary sequences that disrupt the structure of the ribozyme, extension sequences that mediate RNA strand displacement, and a stem-loop structure that assists in toehold formation.

To recover the self-cleaving activity of the anti-ribozyme, we designed a trigger RNA sequence that pairs with the blocking sequence to disrupt the inhibitory structure of the ribozyme. We chose to express this trigger RNA sequence as a fusion with TagBFP mRNA. In the presence of trigger RNAs, the occupied sequences of the ribozyme were released, allowing the self-cleaving activity to be restored and thereby regulating reporter gene expression. Overall, the ribozyme switch relies on two key components: the anti-ribozyme module and the trigger RNA. As illustrated in Fig. 1, we expected to develop a novel RNA-based gene ON/OFF switch that is functional in mammalian cells.

3.2. Characterization of the anti-sTRSV switch

Before verifying the sensitivity of the designed ribozyme switch to specific RNA, it is crucial to first confirm the inhibitory effect of the anti-ribozyme module on the self-cleaving activity of the ribozyme. By using sTRSV as a test case, we integrated sTRSV into the gene expression cassette and observed a significant reduction in Citrine expression level (~17.3-fold) compared to the inactive control sTRSV ContI (Fig. S1). Based on the sTRSV sequence, we have designed three components for the anti-ribozyme sequence (Fig. 2a): a complementary sequence to directly inhibit sTRSV activity, a stem-loop sequence to stabilize the structure of the complementary sequence, and an extension sequence to facilitate RNA chain displacement. To design the anti-ribozyme sequence that effectively inhibits sTRSV activity, we first used the mfold web server [38] to predict the RNA secondary structure of sTRSV. We next engineered complementary sequence inserts spanning variable regions of the sTRSV 5′ terminus. By varying the length of the complementary sequences, we were able to assess the impact of sequence length on the inhibitory effect of the anti-ribozyme module (Fig. 2b). As illustrated in Fig. 2b and c, Citrine expression levels in cells were significantly restored when the length of the complementary sequence reached 2-base pairs (bp). As the length of the paired sequence increased, the structure of sTRSV was further disrupted, resulting in a more pronounced increase in Citrine expression. We discovered that a 9-bp base-pairing interaction resulted in approximately 80 % recovery of Citrine expression compared to the sTRSV ContI control. However, further extension of the complementary sequence did not significantly enhance the fluorescence intensity.

To investigate the potential impact of the stem-loop structure inserted between the complementary sequence and sTRSV on the secondary structure of sTRSV and its interactions with trigger RNAs, we tested the number of base pairs in the inserted stem structure. We found that anti-sTRSV constructs with longer stem sequences exhibited varying effects on Citrine expression, but a 9-bp complementary sequence combined with a 3-bp pairing sequence within the stem structure consistently maintained the optimal recovery effect (Fig. S2). Therefore,

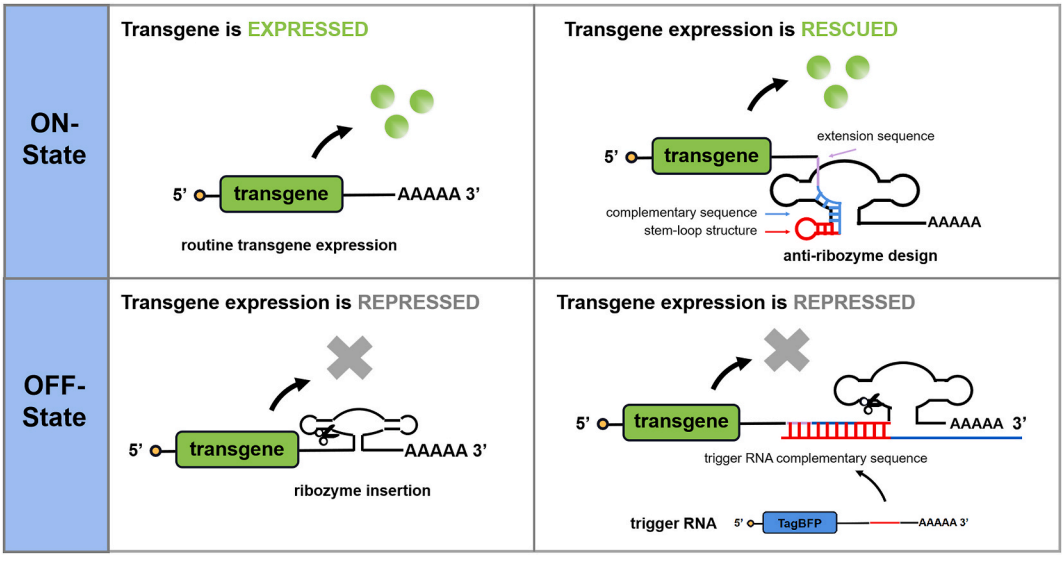

Fig. 1. Design of a programmable anti-ribozyme sensing system The trigger RNA sequence expressed *in vivo* can be specifically recognized and bound by our designed anti-ribozyme sequence. When the endogenous trigger RNA is not expressed, the secondary structure formed by the anti-ribozyme sequence and the hammerhead ribozyme inhibits the active site of the hammerhead ribozyme. However, when the trigger RNA is sensed, the secondary structure formed by the anti-ribozyme is unlocked, resulting in the transition of gene expression from an on state to an off state.

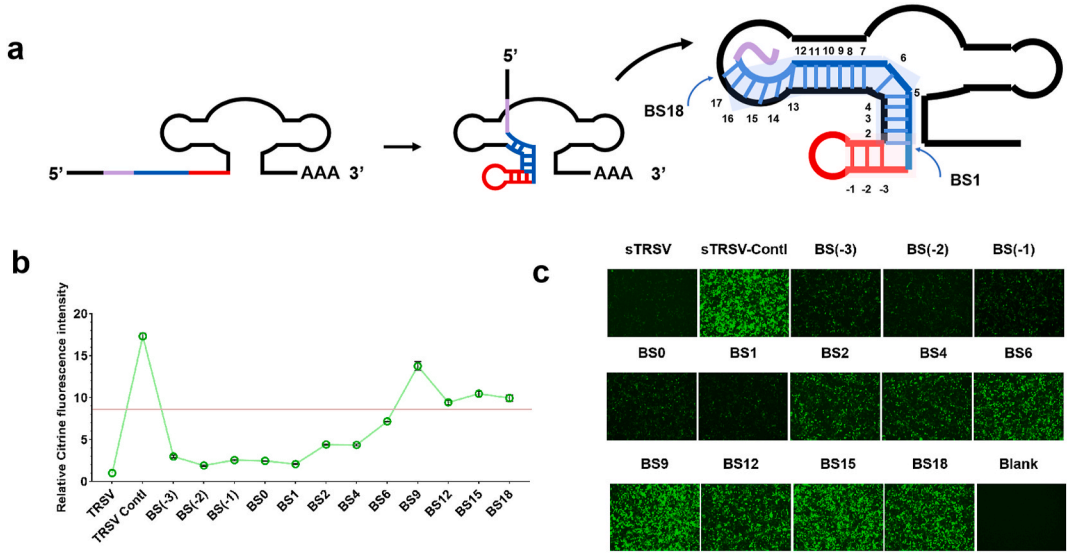

Fig. 2. Anti-sTRSV design and characterization. (a) The design of the anti-ribozyme blocking sequence. The anti-ribozyme blocking sequence can primarily be divided into mainly three parts: complementary sequences to disrupt the structure of the ribozyme (blue), extension sequences mediated RNA strand displacement (purple) and stem-loop structure assisting in toehold formation (red). When the anti-ribozyme sequence is inserted upstream of the ribozyme in a genetic construct, it can form an inhibitory structure that restores the expression of a reporter gene. (b) Comparison of Citrine expression controlled by different designs of anti-sTRSV. The introduction of different lengths of complementary sequences into anti-sTRSV designs can bring about varying changes in the structure of sTRSV and, consequently, affect the expression of the reporter protein Citrine. The relative fluorescence intensity is calculated as the ratio of the Citrine fluorescence intensity in the presence of the anti-sTRSV to the Citrine fluorescence intensity in the presence of sTRSV alone. "BS with number" represents the anti-ribozyme blocking sequence that contains a different number of base pairs in its complementary sequence. (mean ± SD, $N = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test) (c) Fluorescence microscope images of Citrine expression with different inhibitory sequences inserted upstream of sTRSV.

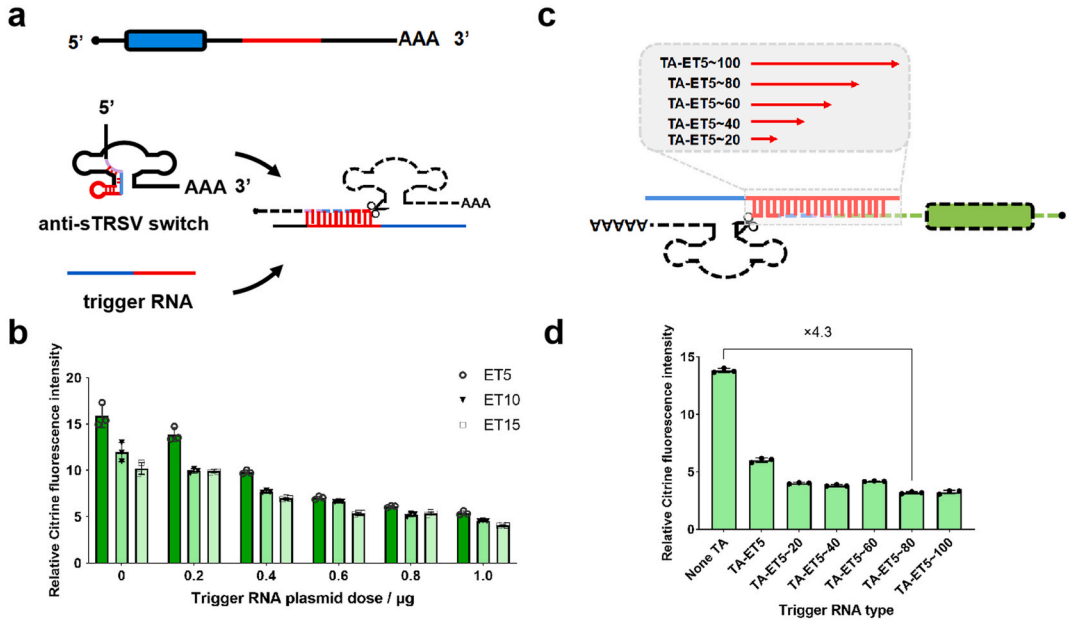

Fig. 3. Trigger RNA design and characterization for sensing anti-sTRSV sequences. (a) Schematic illustration of the trigger RNA annealing with anti-sTRSV switch. The trigger RNA contains sequences complementary to anti-sTRSV and has a specific hairpin structure inserted at its 5′ end to increase its stability and separate it from flanking sequences. The trigger RNA can specifically target the blocking sequence in the anti-sTRSV switch and remove the inhibitory structure by RNA annealing. (b) The dose-dependent inhibition of reporter protein expression by the trigger RNA. The effect of an anti-sTRSV switch, which contains 5-bp, 10-bp, and 15-bp extension sequences, was examined in response to varying doses of trigger RNAs using flow cytometry (FCM). The relative fluorescence intensity was the ratio of Citrine fluorescence intensity with different kinds of anti-sTRSV to with sTRSV. "ET with number" denotes engineered anti-ribozyme blocking sequences comprising a conserved 9-bp complementary core paired with variable-length terminal extensions (5-, 10-, or 15-bp configurations). (mean ± SD, $N = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test). (c) Schematic illustration of varying complementary sequence extension lengths. A range of annealing lengths, spanning from 20 bp to 80 bp, were meticulously designed for the reporter gene cassette of trigger RNAs to optimize the switching efficiency. The "TA-ET5" designation signifies a specific trigger RNA that harbors a complementary sequence to the anti-ribozyme's blocking sequence. The "TA-ET5~X" nomenclature, where "X" denotes a numerical suffix, represents annealing sequences that extend to various lengths. (d) Longer complementary sequences enhance trigger RNA responsiveness. Citrine expression was detected by FCM analysis. The relative fluorescence intensity was the ratio of Citrine fluorescence intensity with anti-sTRSV switch to with sTRSV. (mean ± SD, $N = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test).

we concluded that the inserted stem-loop structure did not require further extension.

We also selected various lengths of extension sequences that facilitated RNA strand displacement. The result indicated that the recovery effect of anti-sTRSV on the expression of the reporter protein diminished as the length of the terminal extension sequence increased (Fig. S3). We hypothesized that an excessively long terminal extension sequence might interfere with the binding of the complementary sequence to sTRSV, thereby disrupting the inhibition of sTRSV activity. The annealing effect of trigger RNAs should be considered when selecting extension sequences.

These results demonstrated that anti-sTRSV was successfully designed to modify the activity of the ribozyme by incorporating specific sequences. Previous studies have provided various clues for engineering a ribozyme that incorporates a competing-sequence-dependent module [33,39–41], which may offer important new insights into perfecting the design of switches.

3.3. Anti-sTRSV switch for detection of trigger RNAs

After observing the impact of different inhibitory sequences on sTRSV and Citrine expression, the next step was to test the restorative effect of the trigger RNA on ribozyme activity. In the experiment described, the trigger RNA sequences were genetically linked to the TagBFP gene and constitutively expressed downstream (Fig. 3a). The goal was to use a trigger RNA to specifically trigger the blocking sequence in the anti-sTRSV switch and remove the inhibitory structure by RNA annealing (Fig. 3a). The cleavage of the mRNA substrate is expected to be activated in response to the trigger RNA signal. Thus, the presence of endogenously expressed targets could enable us to achieve our goal of obtaining a dynamic switch for fine-tuning gene expression.

We investigated the effect of trigger gene expression, integrated with an anti-sTRSV switch, under varying doses of trigger-expressing constructs. HEK293T cells were co-transfected with reporter plasmids and trigger-expressing plasmids, which contained complementary sequences to the regulatory elements or not. As expected, the result showed that Citrine expression decreased in a dose-dependent manner with the presence of trigger RNAs. Specifically, we observed over two-fold decrease in Citrine expression in the presence of high-dose trigger RNAs (Fig. 3b). We also evaluated the response effect of anti-sTRSV using different extension sequences (Fig. 3b). Elongating the extension sequences did not result in a significant fold change in Citrine expression, so we continued using the primary 5-bp-extension sequence (ET5).

To further enhance the effect of trigger RNAs on the reporter construct, we modified the annealing length of the trigger RNAs (Fig. 3c). As the length of the complementary binding sequence to the reporter gene was increased, we observed a corresponding increase in the inhibitory effect of trigger RNAs on Citrine expression, reaching a maximum of ~4.3-fold change when the complementary sequence was extended to 80-bp (Fig. 3d).

We then investigated whether varying the copy number of trigger RNA complementary sequences could influence the switch effect. When the copy number was varied from 1 to 8, we observed no significant difference in Citrine expression (Fig. S4). We hypothesized that different copies of trigger RNA sequences might interfere with each other, potentially affecting the interactions between trigger RNAs and the switch. Although we inserted spacer sequences and hairpin structures to minimize this interference, further rational design of sequences and structures is still needed to improve the system. To assess the synergistic effect of the switch, we incorporated multiple anti-sTRSV switches into the reporter gene expression construct (Fig. S5a). We observed that the insertion of two copies of anti-sTRSV switches attenuated the recovery of Citrine expression (Fig. S5b). Additionally, our results revealed that multiple copies of anti-sTRSV switches had no significant effect on the modulation of reporter protein expression controlled by trigger RNAs (Fig. S5c).

In summary, our results demonstrated that the anti-sTRSV switch has the capability to sense endogenously expressed trigger RNAs and modulate gene expression. By regulating RNA annealing, the switch can achieve a response effect of more than 4-fold. Our exploration of different approaches to optimizing the functional modules of the switch indicates that there is still significant room for improvement in terms of its sensitivity.

3.4. Programming multiplex anti-ribozyme switches

To assess the feasibility of designing a switch for specific RNA sequences, we selected several distinct types of ribozymes (Fig. 4a, S6), namely LtsvJ, PlmJ, PlmvJ, and SltJ [42], all of which are classified under the category of hammerhead ribozymes. The secondary structures of these ribozymes, including sTRSV, along with their corresponding sequences, are depicted in Fig. S7. The self-cleaving activity of these selected ribozymes was evaluated in HEK293T cells, and our findings revealed that all of them exhibited an inhibitory effect on reporter protein expression, indicating their potential utility in designing anti-ribozyme switches (Fig. 4b). Based on the observed performance of the anti-sTRSV switch, we introduced varying lengths of blocking sequences upstream of the selected ribozymes (LtsvJ, PlmJ, PlmvJ, and SltJ) to further modulate their activity. Our findings indicate that the majority of the ribozymes tested could be effectively inactivated by the introduction of 9-bp complementary sequences upstream of their cleavage sites (Fig. 4c). However, in the case of the ribozyme ChmJ, we observed strong self-cleaving activity, which required a longer complementary sequence to effectively disrupt the ribozyme's normal structure and inactivate its activity. For the remaining ribozymes, we did not observe any significant difference in the recovery of Citrine expression when the complementary sequences were extended (Fig. S8).

After identifying the crucial components of these ribozyme switches, we aimed to assess the regulatory impact on the reporter gene in response to trigger RNAs (Fig. 4d and e). In the presence of trigger RNAs specific to each ribozyme switch, we observed a dose-dependent decrease in Citrine expression (Fig. S9). We observed approximately a 3-fold change in Citrine expression for each ribozyme switch construct (Fig. 4e). This result suggests that the incorporation of a blocking sequence into a ribozyme to detect a trigger RNA can be utilized across various types of ribozymes.

3.5. Conclusion

We designed the ribozyme to work in conjunction with deactivating blocking sequences and activating trigger RNAs, thereby enabling dynamic regulation of gene expression. Rather than inserting an aptamer that recognizes effector molecules, we fused complementary sequences and specific structures together to create a functional module that blocks ribozyme activity and senses the trigger RNA. To assess the performance of anti-sTRSV, we initially characterized it by designing various lengths of complementary sequences, stem-loop structures, and extension sequences. With the blocking sequence inhibiting its self-cleaving activity, the anti-sTRSV was able to achieve up to 80 % recovery of reporter protein expression. Throughout the modulation process, the blocking sequences played a crucial role in achieving a wide dynamic range of gene regulation levels, potentially offering novel insights for controlling ribozyme activity. Additionally, the anti-sTRSV switch demonstrated the ability to detect endogenously expressed trigger RNAs in HEK293T cells, achieving a more than 4-fold change in reporter protein ON/OFF expression through optimization of the trigger RNAs. Lastly, we showcased the possibility of programming various types of ribozymes to fulfill the switching function, with the majority of them capable of attaining approximately a 3-fold change in reporter protein expression.

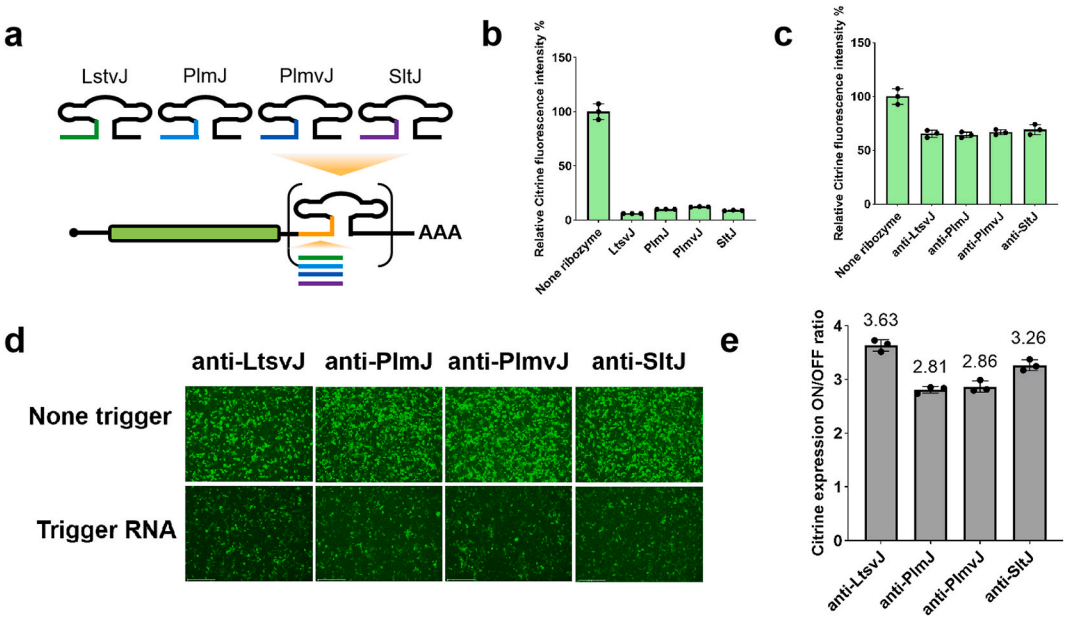

Fig. 4. Detection of multiplex anti-ribozymes. (a) Schematic diagram for programming of multiple anti-ribozyme switches. We designed anti-ribozymes and their corresponding trigger RNAs, analogous to those of sTRSV, for several hammerhead ribozymes, including LtsvJ, PlmJ, PlmvJ, and SltJ. Subsequently, we conducted a series of experiments to investigate their effects. (b) Influence of various ribozymes on reporter protein expression. We selected several types of ribozymes, including LtsvJ, PlmJ, PlmvJ, and SltJ, and inserted them downstream of the reporter gene. The fluorescence intensity was then measured using FCM. (c) Detection of anti-ribozymes possessing 9-bp complementary sequences. 9-bp blocking sequence was inserted upstream of selected ribozymes (LtsvJ, PlmJ, PlmvJ, and SltJ) to modulate their activity. "Anti-ribozyme" denotes this switch design specifically tailored for these ribozymes. The fluorescence intensity was then measured using FCM. (d, e) Various anti-ribozyme switches could be designed and engineered to respond specifically to trigger RNAs. Citrine expression levels were measured for various anti-ribozyme switches in response to differing trigger RNA-expressing plasmids. The fluorescence intensity was examined and analyzed by FCM. The relative fluorescence intensity (%) was the ratio of Citrine fluorescence intensity with trigger RNAs to with no trigger. Citrine expression controlled by trigger RNAs at high doses was imaged (d) and the ON/OFF ration was analyzed (e). (mean ± SD, $N = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test).

4. Discussion

RNA switches have found extensive application in regulating gene expression and cellular metabolism, with a notable focus on developing novel RNA switches tailored for diverse application scenarios. Although significant advancements have been achieved in designing RNA switches across various aspects, crucial challenges persist in creating switches that can respond to specific trigger RNAs expressed within cells. In summary, we have developed a novel RNA-based switch that can be custom-engineered to respond to trigger RNAs and precisely modulate gene expression. Our experimental framework focuses on functional validation within the native cellular environment [33,35]. The observed anti-ribozyme-based regulation across multiple assays conclusively demonstrates ribozyme-mediated control. While the current study focuses on *in vivo* functional validation, we acknowledge the necessity of complementary *in vitro* characterization. A systematic kinetic characterization of ribozyme cleavage efficiency will be conducted under precisely controlled *in vitro* conditions, employing *in vitro* cleavage reactions coupled with high-resolution denaturing polyacrylamide gel electrophoresis (PAGE) to quantitatively evaluate the performance of the anti-ribozyme switch [43–45]. These experiments will establish quantitative structure-activity relationships to further elucidate the mechanism of RNA-triggered regulation.

This anti-ribozyme switch carries out its regulatory function without needing complex transcription factors and can be orthogonal to other techniques, making it a promising tool for developing new synthetic tools and therapeutic strategies. In some cases, even slight changes in gene expression can have major biological impacts, depending on context and gene type. We aim to use this simple RNA switch to precisely control gene expression in various cell environments, including modulating cancer antigens for immune response [46], intervening in cellular aging [47], and dynamically regulating gene therapy [48]. This enables more accurate cellular responses to stimuli.

Furthermore, the functional modules that make up the anti-ribozyme switch can be rationally designed, including the complementary sequences, the toehold structures that aid in regulation and sensing, and the trigger RNAs. By inserting specific sequences into the blocking module, we can utilize the switch to detect target input RNA signals, such as those relevant to cellular states. The trigger-expressing cassette can also be engineered to achieve dynamic, rather than constitutive, expression by combining it with certain cascade synthetic approaches. Through testing the switch effect on various types of ribozymes, we discovered that random sequences may be recognized by trigger RNAs, resulting in varying degrees of reduction in reporter protein expression. This significant characteristic suggests a promising potential for developing programmable ribozyme-based switches, although the regulatory effects still require further improvement. We anticipate that future anti-ribozyme switches will be designed to respond to arbitrary trigger RNA sequences with specific modifications, enabling their development into programmable RNA-based devices akin to the CRISPR-Cas system. When combined with improvements in specificity and sensitivity, anti-ribozyme switches hold significant potential for expanding the range of RNA-based devices used to modulate gene expression and control cellular functions.

CRediT authorship contribution statement

Wenhui Zhang: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Shi Zhao:** Writing – review & editing, Formal analysis, Data curation. **Mengyuan Wang:** Data curation. **Chunbo Lou:** Writing – review & editing, Supervision, Conceptualization. **Yanhui Xiang:** Writing – review & editing, Supervision, Conceptualization. **Qiong Wu:** Writing – review & editing, Supervision, Conceptualization.

Ethical approval

The article mentioned does not incorporate any research or studies that involve human participants or animals.

Consent for publication

There is agreement among all authors regarding the content and its suitability for publication.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2025.03.011.

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