

MicroRNA-responsive ON-OFF hybrid mRNA switch for precise protein expression control

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Messenger RNA (mRNA) technology is gaining attention for its potential in developing next-generation therapeutics. However, developing effective protein expression control technologies to regulate mRNA translation, particularly in a cell type- and tissue-specific manner, remains challenging. To achieve enhanced precision in translational regulation, we developed a microRNA (miRNA)-sensing hybrid mRNA switch, integrating an ON-type switch, which activates translation by detecting specific miRNAs expressed in target cells, into an OFF-type switch, which represses translation by sensing another miRNA in non-target cells. We first investigated effective module sequences after the poly(A)-tail for the ON-type mRNA switch to ensure appropriate translational activation in response to target cell-specific miRNAs. Moreover, the hybrid mRNA switch with a dual miRNA-sensing system reduced expression leakage in non-target cells and enhanced ON/OFF selectivity *in vitro* human cell cultures and *in vivo* mouse model. This ON-OFF hybrid mRNA switch technology allows for the selective expression of target proteins in designated cells and holds promise for the next generation of mRNA therapeutics.

INTRODUCTION

The application of synthetic messenger RNA (mRNA) for therapeutic purposes poses virtually no risk of insertional mutagenesis and allows for the expression of any protein of interest within host cells. This advantage has driven its application in vaccine development, rebuilding of cellular functions, and cell engineering.^{1–3} However, even in the context of mRNA vaccine applications, the importance of restricting the site of protein expression has been proposed.⁴ In cancer and autoimmune disease therapies as well, suppression of off-target expression is considered essential for the development of new therapeutic strategies.^{5–8} Therefore, developing mRNA technologies capable of precisely controlling protein expression is crucial.

Recently, ON-type mRNA switches—a single-component mRNA technology that induces expression in response to intrinsic cellular biomolecules by incorporating synthetic *cis*-elements such as modified internal ribosome entry site and adenosine deaminases acting on RNA (ADAR)-recognized sensor sequences—have been reported to induce protein expression in response to intracellular RNA (e.g.,

mRNA or small RNA).^{9–13} Some of these approaches have demonstrated functionality under cultured cell conditions with *in vitro* transcribed RNA.^{6,10} The development of such a “smart mRNA,” capable of detecting target molecules and executing protein expression only within target cells, is expected to advance mRNA therapeutics.^{14,15} However, there have been few *in vivo* demonstrations of ON-type mRNAs that selectively activate translation by sensing endogenous molecules using synthetic mRNA.

We have developed a synthetic mRNA device that utilizes intracellular microRNA (miRNA) activity to repress (miRNA-OFF switch) or facilitate translation (miRNA-ON switch).^{16,17} Given miRNA activity varies according to tissues and cell types,¹⁸ it is possible to induce protein expression in target cells while repressing it in non-target cells using target cell type-specific miRNAs. The miRNA-ON switch has previously been designed by inserting miRNA target sequences (fully complementary to target miRNA) followed by “extra sequences” with over 495 nucleotides (nt) or 30× CAG repetitive sequences added to the 3′ end of the poly(A) tail, making it possible to reduce protein expression in the absence of the target miRNA (Figures 1A and 1B). In contrast, in the presence of target miRNA, it binds to complementary miRNA target sequences and likely induces mRNA cleavage via the slicer activity of the AGO2 protein.¹⁹ Thus, target miRNAs remove the extra sequence that otherwise suppresses protein expression, resulting in the derepression and induction of protein expression. With cell type-specific miRNAs identifiable through small RNA sequencing²⁰ and miRNA-sensing reporter libraries,^{21,22} this *cis*-regulatory ON system integrated into mRNA format is promising and versatile because of design simplicity (target-specific miRNAs can be easily changed by swapping the miRNA target site) (Figure S1). However, it remains unclear whether the miRNA-ON switch could regulate protein expression by sensing endogenous miRNAs expressed in a target cell type-specific manner *in vivo*.

In this study, we first investigated how extra sequences affect the performance of the miRNA-ON switch and found that short U-rich

Received 5 December 2024; accepted 11 June 2025;
<https://doi.org/10.1016/j.omtn.2025.102609>.

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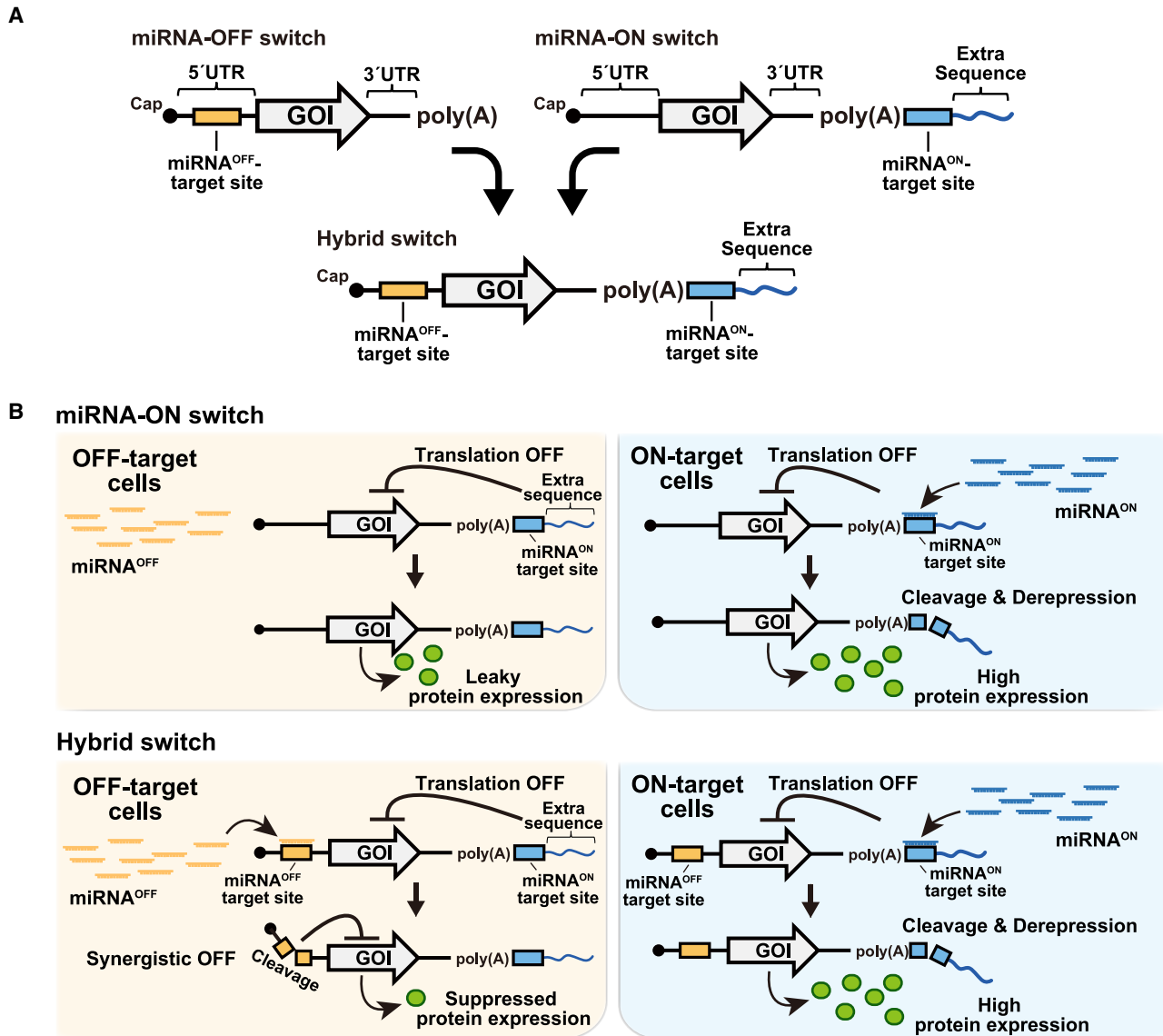


Figure 1. Schematic illustration of miRNA-responsive mRNA switches

(A) Schematic representation of the synthetic mRNA switch constructions. OFF switch contains a miRNA-binding site in the 5' UTR. ON switch includes a target miRNA-binding site immediately downstream of the poly(A) tail, followed by an extra sequence. The hybrid switch combines OFF and ON switches in a single mRNA. (B) Schematic illustration of miRNA-ON switch and hybrid switch. For the miRNA-ON switch, in off-target cells, translation is suppressed by the extra sequence positioned downstream of the poly(A) tail, but some leaky protein expression may still occur. In on-target cells, miRNA binds to the target site, leading to cleavage of the extra sequence and derepression of translation, resulting in activated protein expression. The hybrid switch enhances this system by integrating miRNA-responsive elements in both 5' and 3' ends. In off-target cells, the extra sequence suppresses translation, while miRNA-mediated cleavage in the 5' UTR provides additional suppression.

sequences (4–32 nt) after the poly(A) tail are useful for miRNA-ON switch design. Next, we designed a new “ON-OFF hybrid” mRNA switch (hybrid switch), a single mRNA containing both ON and OFF switch-responsive sequences (Graphical abstract and Figure 1). Importantly, a hybrid switch—with a single-component design and simple programmability—can simultaneously sense the activities of two different miRNAs between distinct cell types to sup-

press off-target expression in non-target cells while activating protein expression in target cells. We successfully demonstrated that the designed miRNA-ON and ON-OFF hybrid switches exhibit protein expression control by sensing endogenous miRNAs in both *in vitro* human cell cultures and an *in vivo* mouse model, providing a new mRNA tool for precise expression control for future mRNA therapeutic applications.

Table 1. Target miRNAs and extra sequences evaluated

Target miRNA evaluated	Extra sequence name	RNA sequence
miR-206/miR-21-5p	UUUAx1	UUUA
miR-206/miR-21-5p	UUUAx2	UUUAUUUA
miR-206/miR-21-5p	UUUAx4	UUUAUUUAUUUAUUUA
miR-206/miR-21-5p	UUUAx8	UUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUA
miR-206/miR-21-5p	CCCAx1	CCCA
miR-206/miR-21-5p	CCCAx2	CCCACCCA
miR-206/miR-21-5p	CCCAx4	CCCACCCACCCACCCA
miR-206/miR-21-5p	CCCAx8	CCCACCCACCCACCCACCCACCCACCCACCCA
miR-206/miR-21-5p	GGGAx1	GGGA
miR-206/miR-21-5p	GGGAx2	GGGAGGGA
miR-206/miR-21-5p	GGGAx4	GGGAGGGAGGGAGGGA
miR-206/miR-21-5p	GGGAx8	GGGAGGGAGGGAGGGAGGGAGGGAGGGAGGGGA
miR-206/miR-302a-5p/miR-21-5p	UUUAx6	UUUAUUUAUUUAUUUAUUUAUUUA
miR-206/miR-302a-5p/miR-21-5p	UUUAx6-U6	UUUAUUUAUUUAUUUAUUUAUUUAUUUUUU
miR-206	UUUAx6-CAGCAG	UUUAUUUAUUUAUUUAUUUAUUUACAGCAG
miR-206	UUUCx6	UUUCUUUCUUUCUUUCUUUCUUUCUUUC
miR-206	UUUCx6-U6	UUUCUUUCUUUCUUUCUUUCUUUCUUUCUUUUUU
miR-206	UUUCx6-CAGCAG	UUUCUUUCUUUCUUUCUUUCUUUCUUUCUCCAGCAG
miR-206	UUUGx6	UUUGUUUGUUUGUUUGUUUGUUUG
miR-206	UUUGx6-U6	UUUGUUUGUUUGUUUGUUUGUUUGUUUUUUU
miR-206	UUUGx6-CAGCAG	UUUGUUUGUUUGUUUGUUUGUUUGUUCAGCAG
miR-206	CAGx10	CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
miR-206	CAGx8-U6	CAGCAGCAGCAGCAGCAGCAGCAGCAGUUUUUU

miRNAs evaluated for targeting the miRNA-binding site inserted immediately downstream of the poly(A) tail, along with the extra sequence added to the 3' end in the experiment shown in [Figure 2](#).

RESULTS

Effects of extra sequences after the poly(A) tail on ON switch design

For miRNA-ON switch designs, extra sequences are employed as a critical regulatory module for suppressing protein expression in the OFF state. (The OFF state indicates the switch condition where the target miRNA is absent, thus suppressing protein expression due to extra sequences. In contrast, the ON state refers to the condition in which the presence of the target miRNA induces protein expression.) In our previous report, sequences with lengths of 495–1,250 nt and a sequence containing 30 repeats of CAG (126 nt) were used as extra sequences.¹⁶ However, the impact of the length and composition of extra sequences on the ON switch remains largely unknown. Thus, we first investigated various patterns of extra sequences and their effects on ON-switch performance.

We constructed enhanced green fluorescent protein (EGFP)-coding ON switches responsive to miR-206 with extra sequences of different nucleotide compositions and varying lengths and evaluated them via translational reporter assays (Table 1). We co-transfected various

ON switches and reference mRNA encoding iRFP670, and the ON/OFF ratio was analyzed by flow cytometry in the presence or absence of a target miRNA (miRNA-206 mimic),²³ with the relative EGFP fluorescent level (EGFP/iRFP670) as the quantifiable output (Figures 2A and 2B). Because the extra sequences used in our previous study were long (126–1,250 nt), we investigated whether shorter extra sequences (less than 32 nt) can be used to design efficient ON switches. We first chose a repeating UUUA sequence due to its known role in destabilizing mRNA when inserted into the 3' UTR²⁴ as a candidate for establishing the OFF state in the miRNA-ON switch when present as the extra sequence after the poly(A) tail. For comparison, additional extra sequences with different nucleotide compositions were prepared by substituting U with C or G (CCCA or GGGA). Since our study aimed to develop mRNA switches suitable for therapeutic applications, we primarily evaluated N1-methyl-pseudouridine (m1Ψ)-modified mRNAs because of their superior protein production efficiency.^{25–27} Accordingly, unless otherwise specified, “UUUA” denotes the m1Ψ-m1Ψ-m1Ψ-A sequence. Interestingly, one repeat sequence is sufficient for establishing the OFF state and induced miRNA-dependent protein expression (Figures 2A, 2B, and S2), functioning as an ON switch.

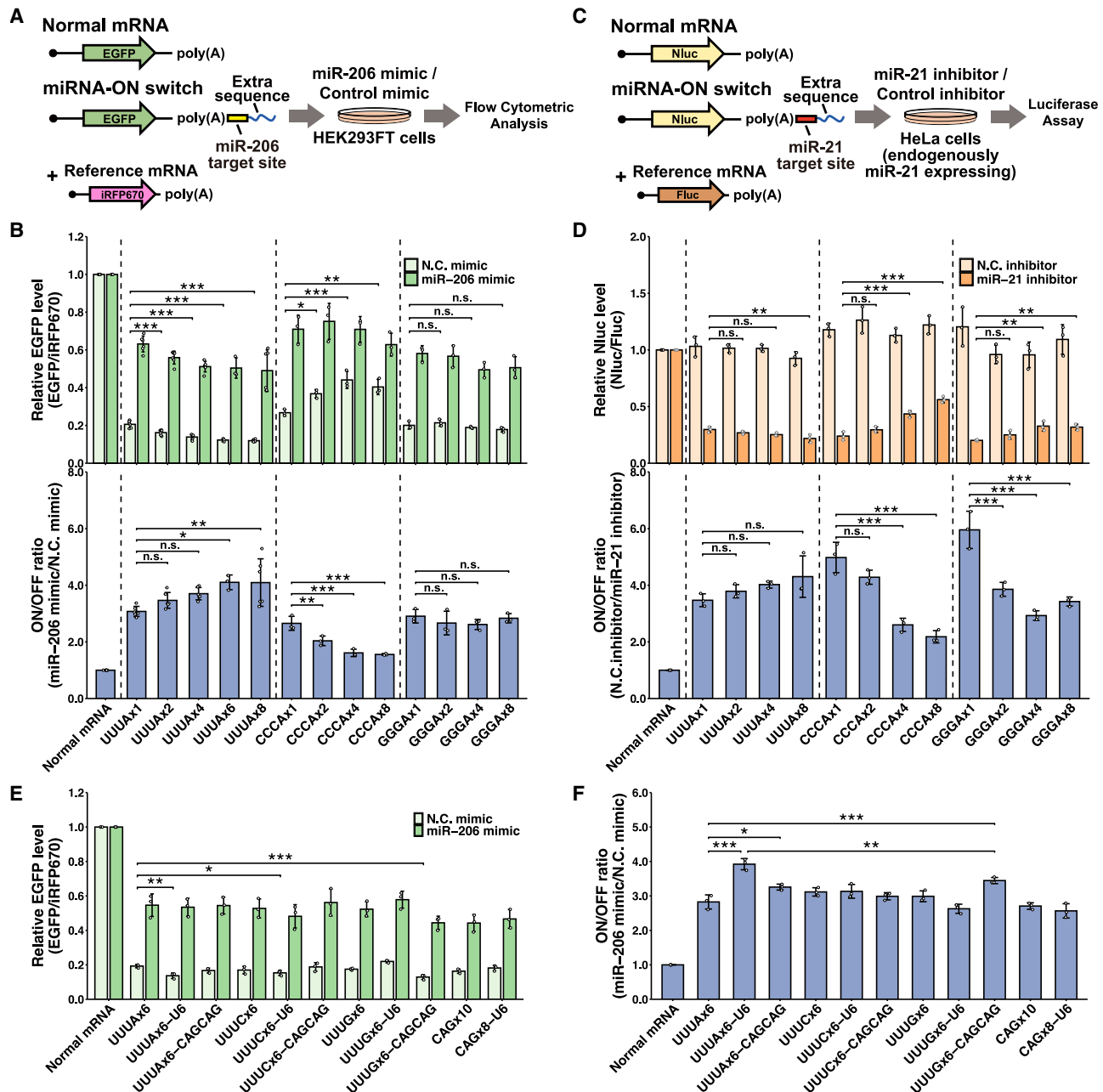


Figure 2. Effects of extra sequences on switch activity

(A) Schematic of the experiment for miRNA-ON switch encoding fluorescent proteins: Normal mRNA and miRNA-ON switches with EGFP coding sequences were transfected into 293FT cells with miR-206 or negative control (N.C.) mimics. iRFP670 mRNA was co-transfected, with iRFP670 expression measured as a reference. (B) Relative EGFP/iRFP670 levels to normal mRNA (top) and ON/OFF ratios (bottom) for each extra sequence. Data points represent biological replicates ($n = 6$ for normal mRNA and UUUAx1, 2, 4, 8 switches, $n = 3$ for other switches). Mean $\pm$ SD are shown. Statistical significance was tested by comparing the 1-repeat extra sequence with corresponding repeats using one-way ANOVA and Dunnett's test. (C) Schematic of the experiment for different combinations of target miRNA, ORF, and cell type: normal mRNA and miRNA-ON switches with Nluc coding sequences were transfected into HeLa cells with miR-21-5p or N.C. inhibitors. Fluc mRNA was co-transfected, with Fluc activity measured as a reference. (D) Relative Nluc/Fluc levels to normal mRNA (top) and ON/OFF ratios (bottom) for each extra sequence. Data points represent biological replicates ($n = 3$). Mean $\pm$ SD are shown. Statistical significance was tested by comparing the 1-repeat extra sequence with corresponding repeats using one-way ANOVA and

(legend continued on next page)

The observed ON/OFF ratios of single repeat-containing ON switches (UUUA: 3.1-fold, CCCA: 2.7-fold, and GGGA: 2.9-fold) were comparable to the ON switches we previously developed (approximately 2- to 3-fold) with longer extra sequences,¹⁶ therefore indicating that the shorter extra sequence is sufficient to design ON switches.

Expression behavior varied across the three types of sequences: while UUUA and CCCA showed a decreasing and increasing OFF state with increased repeats, respectively, GGGA showed no length-dependent changes. The decreased expression of UUUA repeats in OFF state led to a higher ON/OFF fold-change compared to other nucleotide compositions (Figures 2B and S2). To further examine the effects of these repetitive sequences, we next targeted a different miRNA (miR-21-5p), endogenously expressed in HeLa cells,¹⁶ and evaluated another coding sequence (luciferase) as a reporter. We investigated relative Nluc levels with or without a miR-21 inhibitor, which blocks endogenous miR-21 activity (Figures 2C and 2D). Similar to the EGFP reporter assay, UUUA and CCCA showed a repeat length-dependent decrease and increase in OFF state leakage, respectively, with a corresponding increase and decrease in the ON/OFF ratio (Figure 2D). In this assay, single-repeat CCCA and GGGA showed higher ratios than UUUA. However, while the UUUA sequence consistently exhibited high performance across various conditions involving different miRNA target sites, coding sequences, and cell types, the effect of CCCA/GGGA appeared to be context-dependent (Figures S3A–S3C). In addition, evaluation of mRNA switches synthesized with unmodified nucleotides revealed that U-rich sequences reduced leaky expression in a repeat length-dependent manner regardless of uridine modification. However, under unmodified conditions, the ON/OFF performance of U-rich sequences was lower than that observed with their m¹Ψ-modified counterparts (Figure S3D). Thus, the UUUA repeat sequence is an attractive module for ensuring optimal ON-switch performance because of its consistent superiority in enhancing ON/OFF ratios across different contexts.

Next, we investigated whether additional sequences after UUUA repeats could further enhance ON-switch performance. We evaluated the effects of a 3' terminal oligo(U) sequence (U6-tract), known to promote mRNA degradation through decapping and exonuclease mechanisms,²⁸ as it may further reduce leaky expression in the OFF state. Indeed, U6-tract insertion after the UUUA repeat in the miR-206-responsive mRNA switch reduced the level of OFF-state compared with a UUUA repeat-only sequence and significantly increased the ON/OFF ratio in HEK293FT cells (Figures 2E, 2F, and S4A). This effect was further confirmed using different cell lines (HeLa cells and hiPSCs) and different target miRNAs (miR-21-5p and miR-302a-5p), which

are selectively expressed endogenously in the target cell type (Figures S4B–S4E).^{29,30} Additionally, we tested other sequences (e.g., CAGCAG) after UUUA repeats or UUUC/G repeats instead of UUUA repeats and further performed a direct comparison with the previously used long extra sequences.¹⁶ UUUAx6-CAGCAG and UUUGx6-CAGCAG also showed improved fold change, but UUUAx6-U6 exhibited the highest performance among them (Figures 2E, 2F, S4A, and S4F). Based on these data, we chose the UUUAx6-U6 sequence as an extra sequence to design the ON switch for further study.

ON-OFF hybrid switch to reduce off-target expression and enhance ON/OFF ratio

We have previously developed the miRNA-responsive mRNA OFF switch, which includes miRNA binding sites inserted into the 5' UTR, causing efficient translational suppression in response to the target miRNA.^{29,31,32} Herein, we aimed to develop a single-molecular, ON-OFF hybrid switch (hybrid switch) by combining the OFF-switch module with the ON switch (Figures 1A and 3A). The ON-switch module in the hybrid switch contains a miRNA binding site responsive to miRNAs (miRNA^{ON}) expressed in on-target cells, along with an optimized extra sequence (UUUAx6-U6) downstream of the poly(A) tail, while also integrating an additional miRNA binding site in the 5' UTR, designed to target miRNAs (miRNA^{OFF}) expressed in off-target cells, where protein expression should be suppressed (Figure 1). We expected that dual regulation of protein expression by two different miRNAs has the potential for added precision and enhanced switch activity between target and non-target cell types.

We evaluated the effectiveness of the hybrid switch by examining the switch response to several miRNAs with differential expression between hiPSCs and HeLa cells.³⁰ To selectively activate translation in hiPSCs but repress it in HeLa cells, we designed RNA switches targeting the respective endogenous miRNAs (miR-302a-5p: highly expressed in hiPSCs; let-7b-5p, miR-27a-3p, and miR-98-5p: expressed in HeLa cells) (Figure 3A). We first analyzed the response of the simple OFF switch to distinguish these cell lines using flow cytometry and fluorescence microscopy. As expected, these OFF switches targeting miRNA^{OFF}s expressed in HeLa cells repressed translation in HeLa cells while maintaining normal translation levels in hiPSCs (Figures 3B and 3C). The ON/OFF ratios varied among switches, from 1.6- (miR-98-5p) to 5.2-fold (miR-27a-3p), which correlated with the expression of each miRNA in HeLa cells (let-7b-5p: 7255.405 reads per million mapped reads (RPM), miR-27a-3p: 9662.722 RPM, and miR-98-5p: 3008.752 RPM, derived from DIANA-miTED³³), and the suppressive effect was weaker for miR-98-5p, which was expressed at a lower level. We observed leaky EGFP expression from the tested OFF switches in HeLa cells

Dunnett's test. (E) Relative EGFP/IRFP670 levels of ON switches, including U-rich or CAG-repeats extra sequence, to normal mRNA. Data points represent biological replicates ($n = 3$). Mean $\pm$ SD are shown. Statistical significance was tested by comparing UUUAx6 with other extra sequences using one-way ANOVA and Dunnett's test. Only significance levels of $p < 0.05$ were indicated in the graph. (F) ON/OFF ratios for each extra sequence listed in Figure 2E. Data points represent three biological replicates ($n = 3$). Mean $\pm$ SD are shown. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test, comparing either UUUAx6 or UUUAx6-U6 with other extra sequences. Significance levels: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. n.s. indicates no significance.

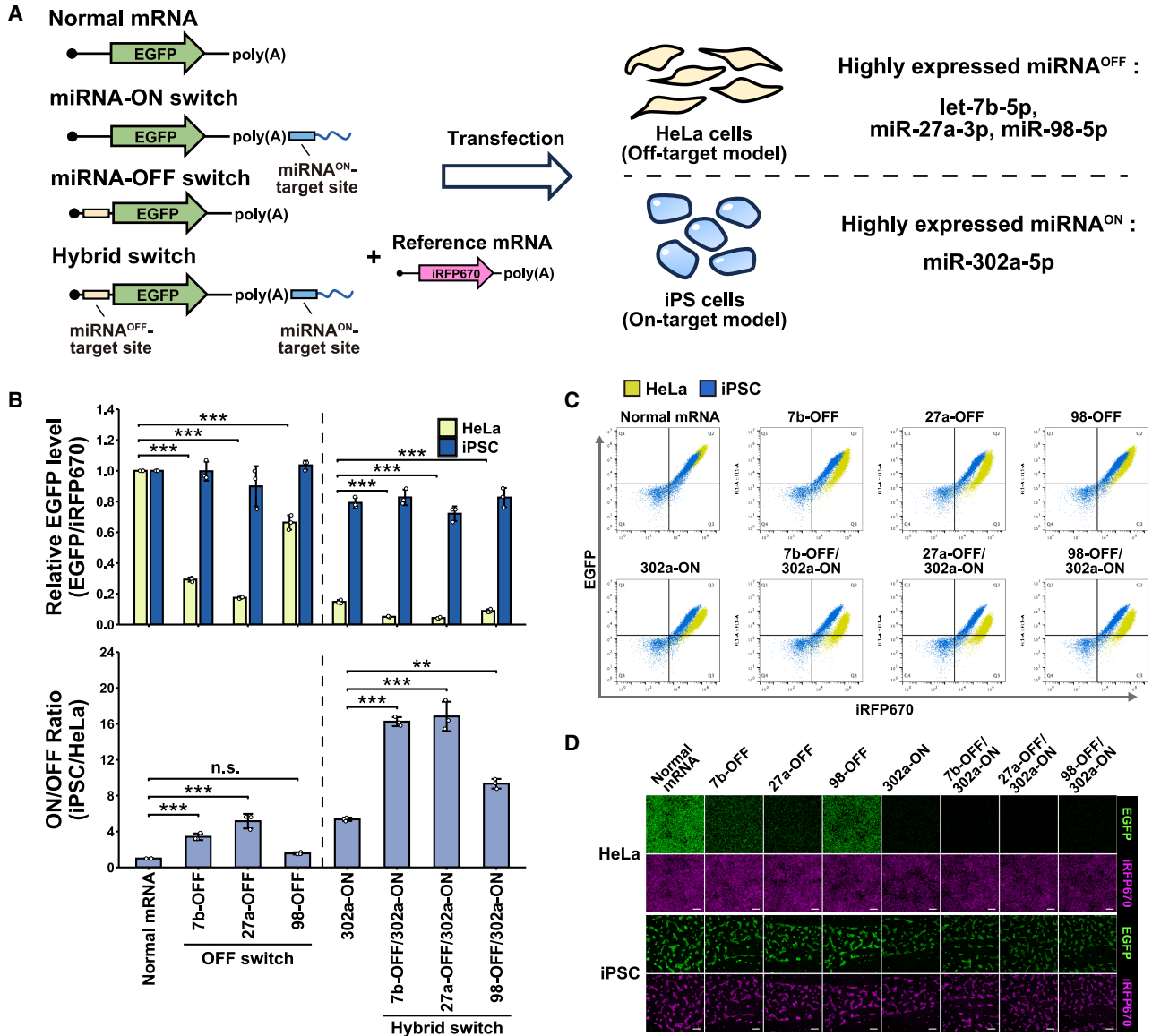


Figure 3. Hybrid strategy integrating ON and OFF switches

(A) Schematic of the experiment. Normal mRNA, miRNA-ON switch, miRNA-OFF switches, and hybrid switches were transfected into both HeLa cells and hiPSCs. Normal mRNA encoding iRFP670 was co-transfected as a reference. miRNA^{OFF} refers to miRNAs highly expressed in model off-target cells, HeLa cells, while miRNA^{ON} refers to miRNAs highly expressed in model on-target cells, hiPSCs. (B) Relative EGFP/iRFP670 levels to normal mRNA (top) and ON/OFF ratios (bottom) for normal mRNA, OFF switches, ON switch, and hybrid switches. Normal mRNA encoding iRFP670 was co-transfected as a reference. Data points represent three biological replicates ($n = 3$). Mean $\pm$ SD are shown. Statistical significance was tested by comparing mRNAs without a target site on the 5' UTR with corresponding switches transfected into HeLa cells (OFF state) using one-way ANOVA followed by Dunnett's test. (C) Scatterplots generated from flow cytometric analyses in Figure 3B. (D) Representative fluorescence images. The images demonstrate that ON switches and each hybrid switch corresponding to highly expressed miRNAs in HeLa cells suppressed EGFP expression. Significance levels: ** $p < 0.01$ and *** $p < 0.001$. n.s. indicates no significance. Scale bars, 500 μ m.

(Figure 3D), and it was difficult to distinguish two cell types clearly with miR-98-responsive OFF switch (Figures 3B and 3C). We also tested the performance of miR-302a-5p (hiPSC-high miRNA^{ON})-sensing ON switch and confirmed that it activated translation in hiPSCs but repressed it in HeLa cells (ON/OFF ratio: 5.4-fold, Figures 3B–3D), even though we observed some leaky EGFP expres-

sion in HeLa cells (Figure 3D). Herein, we designed the hybrid switch containing both OFF- and ON-switch regulatory elements in a single mRNA. Interestingly, when the miR-302a-ON switch was fused with each miRNA^{OFF}-sensing OFF switch, we observed the suppression of leaky expression in HeLa cells, and a marked increase in ON/OFF fold change, with values of 16.3, 16.8, and 9.3 for let-7b-5p,

miR-27a-3p, and miR-98-5p, respectively (Figures 3B–3D). Importantly, while the simple miR-98-responsive OFF switch showed weak responsiveness, the hybrid miR-98-responsive switch demonstrated significantly improved responsiveness. Although the hybrid system reduced the ON-state level only slightly, by approximately 1.2-fold compared to the simple OFF switch, it significantly reduced the OFF-state level by 4- to 7-fold (Figure S5A). So far, we have compared the activity of the hybrid switch between HeLa cells (“miRNA^{OFF} (high-expression: high)/miRNA^{ON} (low-expression: low)”) and hiPSCs (“miRNA^{OFF} (low)/miRNA^{ON} (high)”). To further evaluate the system, we tested switch activity under conditions mimicking “miRNA^{OFF} (high)/miRNA^{ON} (high)” or “miRNA^{OFF} (low)/miRNA^{ON} (low)” by using miR-302a mimic and inhibitor. Under these conditions, the hybrid switch specifically induced protein expression in the target context (“miRNA^{OFF} (low)/miRNA^{ON} (high)”) and exhibited the lowest leakage under the “miRNA^{OFF} (high)/miRNA^{ON} (low)” condition, highlighting its ON/OFF dual-module property (Figure S5B). Thus, key characteristics of the hybrid system to highlight include improvements to the ON/OFF ratio due to the presence of the extra sequence, which intensifies the OFF state compared to OFF and ON switches developed separately, even in cases of weak miRNA^{OFF} activity (as seen with miR-98-5p). This switch is a highly efficient single-molecular device that integrates two distinct miRNA switches, proving to be a robust tool for effectively suppressing leakage in off-target cells.

Tissue-selective protein expression *in vivo* using ON and hybrid switches

miRNA-sensing OFF switches (insertion of a miRNA target site into the 3' UTR) have been shown to function *in vivo* previously;⁵ however, miRNA-sensing ON switch functionality has never been demonstrated *in vivo*. Given that we have succeeded in developing miRNA-responsive ON switches that can recognize target miRNAs and induce protein expression under cultured cell conditions, we hypothesize that by appropriately selecting tissue- and cell type-specific miRNAs, miRNA-ON switches could control protein expression between target and non-target cell types *in vivo*.

To evaluate the *in vivo* function of miRNA switches, we constructed mRNAs encoding firefly luciferase (Fluc) and examined their function by injecting miRNA switches into mice. Upon intramuscular administration, normal mRNA without miRNA target sequence expressed Fluc in both the liver and muscles (Figures 4A–4C). Next, we evaluated the effects of muscle-selective protein expression from the switch (Figure 4A). We designed an ON switch responsive to miR-1a-3p (1a-ON), highly expressed in limb muscles (Figure S6A).³⁴ The miR-1a-responsive ON switch preferentially induced Fluc expression in muscles rather than in the liver compared with normal mRNA; however, there was leaky expression in the liver from the miR-1a-ON switch (Figures 4B, 4C, and S6B). For mRNA-based therapy, it is important to eliminate off-target expression in non-target tissues. Thus, we hypothesized that further suppression of leakage in the liver could improve the ON/OFF ratio of target (muscle) to non-target (liver) tissues. For this aim, we utilized miR-122-

5p, known for its high activity in the liver (Figure S6A),⁵ as an input for the OFF switch.^{35,36} We found that a hybrid switch combining miR-122-OFF and miR-1a-ON responsiveness shows marked effectiveness *in vivo*, significantly cutting liver expression and successfully improving the ON/OFF ratio compared to the miR-1a-ON switch alone. In this experiment, the simple miR-122-OFF switch achieved similar suppressed expression in the liver while providing a higher ON/OFF ratio. This is probably due to the marked difference in miR-122-5p levels between liver and muscle cells (Figure S6A). However, hybrid switches detecting different miRNAs could be useful because it is not always possible to find a single miRNA that can clearly distinguish target cell types to differentiate between cells and tissues *in vivo*. Altogether, this is the first demonstration that mRNA ON-OFF hybrid switches can function *in vivo* by recognizing two distinct miRNA activities in different tissues to selectively repress or activate protein expression in each cell type (liver-OFF and muscle-ON).

To further demonstrate the applicability of ON and hybrid switches, we employed an alternative approach to controlling protein expression in different tissues. Intravenous injection is a commonly used method for mRNA delivery, and when delivered using technologies such as lipid nanoparticles, mRNA is frequently distributed not only to the liver but also to the spleen.^{37–39} Therefore, we evaluated the efficacy of our switches in targeting these organs following intravenous injection. In the ON switch designed to respond to miR-122-5p, Fluc was more preferentially expressed in the liver than spleen (Figures 4D–4F and S6C), indicating that miR-122-ON switch primarily activates translation in the liver (liver-ON and spleen-OFF). Although this ON switch exhibited a protein expression pattern responsive to miR-122-5p, its ON/OFF performance was weaker compared to the ON switches evaluated *in vitro* experiments. However, when the same mRNA was evaluated *in vitro* HeLa cells using miR-122 mimic, it showed reasonable switch activity (Figure S6D), suggesting that the weaker ON switch performance observed *in vivo* is likely attributable to factors specific to the *in vivo* environment. Next, using the simple OFF switch, we aimed to reduce leaky expression in the spleen by utilizing miR-142-3p, known for high activity in cells constituting the spleen (Figure S6A).⁵ In contrast to the miR-122-OFF switch (Figure 4C), the simple miR-142-responsive OFF switch failed to suppress leaky expression adequately in the spleen (Figure 4E). In this case, although the simple ON and OFF switches each responded to their respective target miRNAs, their individual performance was not sufficient. Given the possibility that the hybrid system could enhance overall performance under such conditions, we evaluated a hybrid switch combining a miR-142-OFF module with a miR-122-ON module. As expected, the hybrid switch (miR-142-OFF and miR-122-ON) successfully achieved the highest ON/OFF ratio compared to tested mRNAs (Figures 4E and 4F).

Taken together, we demonstrated that miRNA-ON and hybrid switches respond to tissue-specific endogenous miRNAs appropriately, and the hybrid switch represents a useful strategy to enhance ON/OFF selectivity.

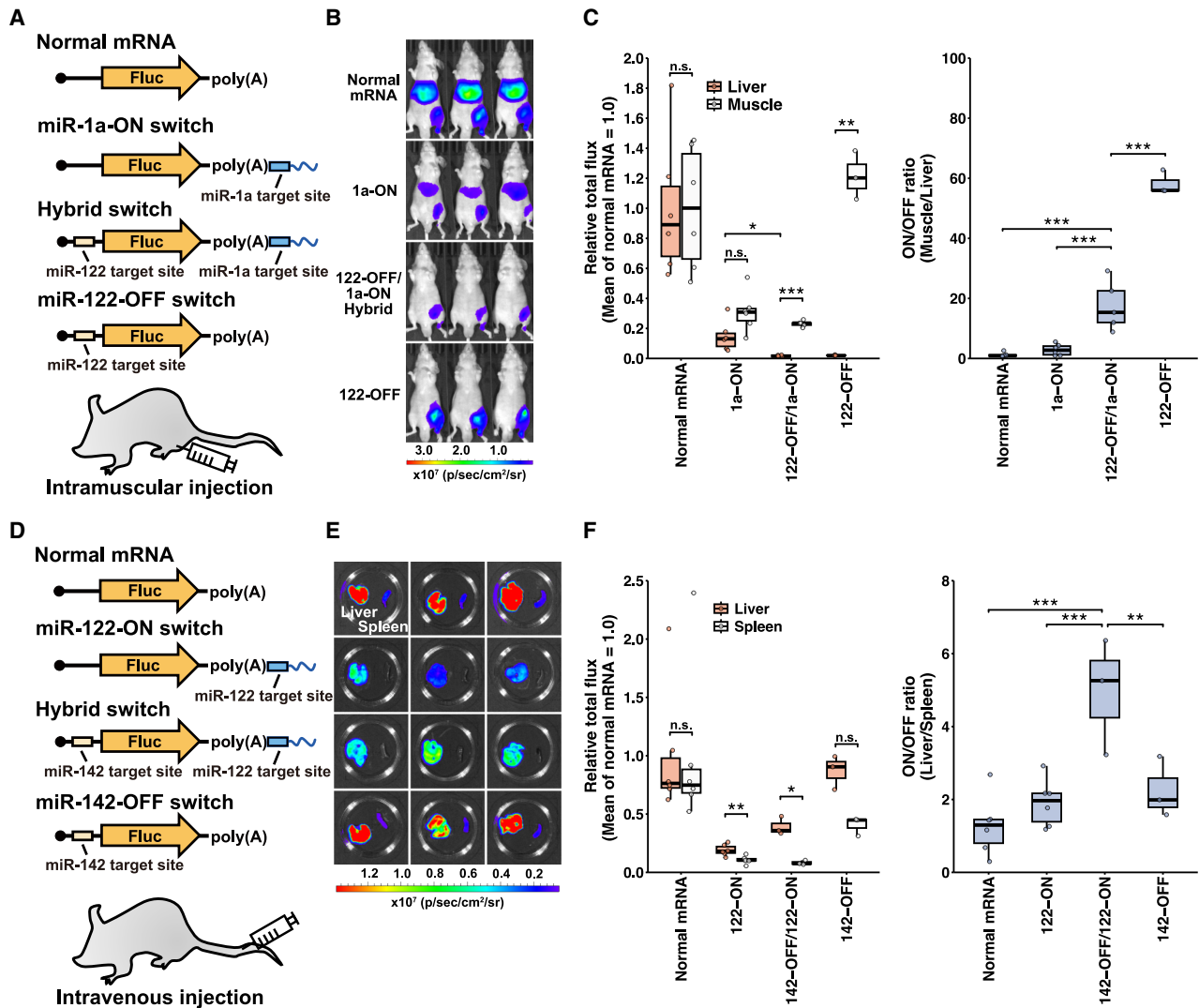


Figure 4. In vivo evaluation of miRNA-ON and hybrid switches

(A) Schematic of the experiment for intramuscular injection. (B) Representative images showing bioluminescence signals from mice following intramuscular injections of mRNA switches. (C) Relative total flux of Fluc signal normalized to the mean of normal mRNA (left) and ON/OFF ratios (right) for each mRNA switch. Data points represent biological replicates ($n = 6$ for normal mRNA and miR-1a-ON switch, $n = 5$ for miR-122-OFF/miR-1a-ON hybrid switch, and $n = 3$ for miR-122-OFF switch). The boxplot shows the median and interquartile range, with whiskers representing data dispersion. Statistical significance was assessed by a two-tailed paired t test comparing liver and muscle, and by an unpaired two-tailed t test comparing miR-1a-ON and hybrid switches in the liver. Values shown for the miR-1a-ON switch indicate p values. For the ON/OFF ratio, statistical significance was tested by comparing the hybrid switch with other mRNAs using one-way ANOVA followed by Dunnett's test. (D) Schematic of the experiment for intravenous injection. (E) Representative bioluminescence images of excised liver and spleen from mice injected intravenously with mRNA switches, corresponding to those described in Figure 4D. IIS images of three independent replicates, each administered with the corresponding mRNA, are shown. (F) Relative total flux of Fluc signal normalized to the mean of normal mRNA (left) and ON/OFF ratios (right) for each RNA switch. Data points represent biological replicates ($n = 6$ for normal mRNA and miR-122-ON switch, $n = 3$ for miR-142-OFF/miR-122-ON hybrid switch and miR-142-OFF switch). The boxplot shows the median and interquartile range, with whiskers representing data dispersion. Statistical significance was assessed by comparing the liver and spleen using two-tailed paired t test. Values shown for the miR-142-OFF switch indicate p values. For the ON/OFF ratio, statistical significance was tested by comparing the hybrid switch with other mRNAs using one-way ANOVA followed by Dunnett's test. Significance levels: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. n.s. indicates no significance.

DISCUSSION

In this study, we demonstrated that the miRNA-ON switches incorporating a new *cis*-regulatory module respond to specific miRNAs expressed in target tissues (limb muscles and the liver) and regulate

protein expression following intramuscular and intravenous mRNA administration in mice (Figure 4). To the best of our knowledge, this is the first example of ON-type mRNA switches capable of discerning and appropriately responding to endogenous miRNAs *in vivo*.

Furthermore, the newly designed hybrid switch, which contains both OFF (5' UTR) and ON (after poly(A) tail) elements in one mRNA, enables sensing of two different miRNAs to effectively suppress undesired expression in off-target cells and tissues while facilitating protein expression in target cells, thus highlighting its potential application in mRNA therapy. We have previously constructed RNA-only circuits by transfecting multiple mRNA components.^{23,40} Compared to those studies, hybrid switches, composed of a single mRNA containing both OFF- and ON-regulatory elements, reduce the number of mRNAs necessary for sensing different miRNAs and are therefore useful for designing future mRNA drugs.

The key feature of the miRNA-ON switch is the extra sequence at the 3' end after the poly(A) tail, which is essential for repressing protein expression from synthetic mRNA. We investigated the effects of different types of extra sequences on ON-switch activity, gaining insights into the impact of sequence length and pattern. Notably, many extra sequences tested in this study established an OFF state regardless of nucleotide lengths and composition (Figure 2). In addition, we found the UUUA-repeat sequence is sufficient to design ON switches with decent performance. While the underlying mechanisms responsible for the reproducible reduction in the OFF state associated with an increase in UUUA repeats remain elusive, RNA-binding proteins interacting with AU-rich elements may be implicated.⁴¹ Alternatively, the U-rich sequence might hybridize with the poly(A) tail, potentially hindering the binding of PABPC1, a key factor in translation.⁴² Also, we observed that the same extra sequence can exhibit variable performance depending on the specific context (Figures 2B, 2D, and S3A–S3C; CCCAx1 and GGGAx1). However, the underlying factors responsible for these context-dependent differences remain incompletely understood. Adding to this complexity, although the 5' half of the miRNA target site remains downstream of the poly(A) tail after miRNA-mediated cleavage (Figure 1B), the impact of this residual sequence on ON-switch activity has not been systematically examined. Our evaluation of extra sequences revealed that U-rich sequences are relatively effective at repressing protein expression. Therefore, the presence of such motifs in the 5' half of a miRNA target site may reduce ON-state expression. A broader evaluation of target miRNA suitability will likely be needed in future work.

The hybrid switch integrated with both ON and OFF switches is a readily available single-RNA molecule device representing an effective approach for achieving selective expression in target cells and tissues by sensing two different miRNA activities. When aiming to express a desired protein in specific cells, large-scale expression profiling analyses for identifying a trigger miRNA with high activity specific to target cells can often reveal miRNAs with high activity in non-target cells.²⁹ Thus, OFF switches leveraging such miRNAs are highly accessible, and the combination of ON and OFF switches is straightforward to control protein expression in target and non-target cell types. Indeed, we showed two types of hybrid switches (miR-122-OFF/miR-1a-ON and miR-142-OFF/miR-122-ON) to demonstrate muscle-ON/liver-OFF

(Figures 4A–4C) and liver-ON/spleen-OFF (Figures 4D–4F) expression, indicating that the hybrid switch is versatile in terms of miRNA and tissue selectivity. In our hypothesis, the OFF switch employs a stringent protein suppression mechanism wherein the miRNA binding site inserted in the 5' UTR is cleaved by the slicer activity of miRNA-AGO2, resulting in the mRNA containing the coding sequence being separated from the cap and translation being blocked. Our results suggest that the translational suppression activity driven by the extra sequence of the ON switch acts synergistically with the translational shutoff mechanism of the OFF switch, resulting in a bivalent suppression mechanism in the hybrid switch that amplifies the OFF state.

In this study, we found that the expression levels of both the ON and hybrid switches in the ON state were lower than those of normal mRNA under *in vivo* conditions (Figures 4C and 4F). Since the *in vivo* function of the ON switch represents a key finding of this study, elucidating and resolving the cause of its reduced ON-state expression remains a critical issue. While the hybrid strategy can improve the ON/OFF fold change in cases where the OFF switch alone provides insufficient repression, reduced ON-state expression may limit the applicability of this approach in certain contexts. Therefore, careful selection of target miRNAs and switch modules will likely be required depending on the intended application.

We also observed that the ON-state expression level of the 142-OFF/122-ON hybrid switch was approximately twice that of the 122-ON switch alone (Figure 4F). Although the underlying mechanism is not yet fully understood, this observation suggests that additional sequence elements may influence expression levels in certain contexts. One possible explanation is that molecular interactions involving the 5' UTR and the extra sequence may contribute to increased basal expression in the hybrid construct. Importantly, the hybrid design maintains its functional benefit by providing additional OFF activity through the second module, resulting in an improved ON/OFF ratio compared to the ON switch alone (Figure 4F). Further investigation into the molecular effects of extra sequence elements will be important for better understanding switch behavior and for informing future design strategies.

In conclusion, this study demonstrates the potential of the miRNA-ON switch and hybrid switch systems in the precise regulation of protein expression *in vitro* and *in vivo*, which is expected to make contributions to the rapidly evolving field of next-generation mRNA therapeutics for controlling protein expression in a cell state-specific-manner through advanced RNA engineering.

MATERIALS AND METHODS

Cell lines

HEK293FT (Thermo Fisher Scientific, no. R70007) was cultured in Dulbecco's modified Eagle's medium (DMEM) 4.5 g/L glucose (Nacalai Tesque, no. 08459-64) supplemented with 10% fetal bovine serum (FBS) (Biosera, no. FB-1285/500), 0.1 mM MEM non-essential amino acids (Thermo Fisher Scientific, no. 11140-050), 2 mM

L-glutamine (Thermo Fisher Scientific, no. 25030-081), and 1 mM sodium pyruvate (Sigma-Aldrich, no. S8636). HeLa (ATCC, no. CCL-2) cells were cultured in DMEM 4.5 g/L glucose (Nacalai Tesque, no. 08459-64) supplemented with 10% FBS (Biocera, no. FB-1285/500) and 1 mM sodium pyruvate. A human induced pluripotent stem cell (hiPSC) line (201B7) was cultured in StemFit AK02N (Ajinomoto, no. AK02) on laminin-511 E8 (iMatrix-511 silk, Matrixome, no. 892021) using a feeder-free culture system.⁴³ All cell lines were cultured at 37°C with 5% CO₂.

Construction of plasmids and *in vitro* transcription (IVT) template

To prepare template plasmids for mRNA switches, all PCRs were performed by KOD One PCR Master Mix (TOYOBO, no. KMM-101). After the template was digested by DpnI (TOYOBO, no. DPN-101) at 37°C for 30 min, PCR products were purified by the Monarch PCR & DNA Cleanup Kit (New England Biolabs, no. T1030). PCR products were cloned into PCR-linearized pUC19 vector by Gibson assembly using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, no. E2621) and transformed to DynaCompetent Cells JetGiga *E. coli* DH5 α (BioDynamics Laboratory, no. DS230). After culturing *E. coli* at 37°C for 16 h with 3 mL LB/ampicillin (Amp) media, plasmids were purified by the PureYield Plasmid Miniprep System (Promega, no. A1223). piRFP670-N1 was gift from V. Verkhusa (Addgene plasmid #45457; <http://n2t.net/addgene:45457>; RRID:Addgene_45457).⁴⁴ All plasmid sequences were checked by commercial Sanger sequencing (Eurofins Genomics or Azenta Life Sciences).

Template DNAs for IVT were generated by PCR using the KOD One PCR Master Mix (TOYOBO, KMM-101) from template plasmids. Oligo DNAs used for PCR were purchased from Eurofins Genomics. After removing template plasmids by DpnI treatment at 37°C for 30 min, all PCR products were purified by the Monarch PCR & DNA Cleanup Kit (New England Biolabs, no. T1030). For miRNA-ON switch IVT templates, a second PCR was performed on purified PCR products to add the extra sequence and was purified similarly. For let-7b-5p, miR-27a-3p, and miR-98-5p responsive OFF or hybrid switches, template DNAs were generated using two types of forward primers: the first forward primer without a T7 promoter and the second forward primer containing a T7 promoter. For the Ex495-ON switch, the extra sequence was separately amplified by PCR from the template plasmid (pGEM-TAP) using the extra forward and extra reverse primers. This PCR product was then fused with the first construct containing the EGFP coding sequence through fusion PCR. All IVT templates were confirmed by agarose gel electrophoresis of the PCR products to verify the presence of appropriately sized amplicons. Primer sequences and plasmids are listed in (Table S1), with the construction information listed in (Table S2).

mRNA synthesis

All mRNAs were synthesized using the MEGAScript T7 Transcription Kit (Thermo Fisher Scientific, no. AM1334) with 6 mM ATP, 6 mM CTP, 6 mM GTP, 6 mM N1-methylpseudo-UTP (m1 Ψ) (TriLink Bio-

Technologies, no. N-1081) and 6 mM CleanCap Reagent AG (TriLink Biotechnologies, no. N-7113-10). Reaction mixtures were incubated at 37°C for 4 h and further incubated at 37°C for 30 min in the presence of TURBO DNase (Thermo Fisher Scientific) to remove the template DNA. RNA products were purified with the Monarch RNA Cleanup Kit (New England Biolabs, no. T2040) according to the manufacturer's protocol and subjected to antarctic phosphatase (New England Biolabs, no. M0289) treatment at 37°C for 15 min. Finally, mRNA products were purified using the Monarch RNA Cleanup Kit. The amounts of product mRNAs were determined by NanoDrop2000 (Thermo Fisher Scientific), and mRNAs were stored at -30°C until use.

mRNA transfection

Cells were seeded onto 24-well plates at 5×10^4 cells/well (HEK293FT and hiPSC) or 1×10^5 cells/well (HeLa) the day before transfection. All tested mRNAs were transfected into cells using Lipofectamine MessengerMAX (Thermo Fisher Scientific, no. LMRNA015) according to the manufacturer's protocol. For miRNA-responsive analysis, mRNAs were co-transfected with synthetic miRNA mimics or inhibitors (Thermo Fisher Scientific). For fluorescent analyses, a reference iRFP670 mRNA was co-transfected with all EGFP-encoding mRNAs. For luciferase assays, a reference Fluc mRNA was co-transfected with all Nluc-encoding mRNAs. The transfection conditions are listed in (Table S3).

Fluorescence imaging

Before flow cytometry measurements, cell images were captured using the APEXVIEW APX100-HCU (EVIDENT). The following channels were used to capture fluorescent images: GFP channel (Excitation 470 nm/Emission 508 nm) for EGFP and Cy5 channel (Excitation 625 nm/Emission 670 nm) for iRFP670. Captured images were analyzed using ImageJ (NIH).

Flow cytometric analysis

After 24 h of mRNA transfection, cells were washed with PBS (Nacalai Tesque, no. 4249-24), treated with 0.25% Trypsin-EDTA (Thermo Fisher Scientific, no. 25200072) (HEK293FT and HeLa cells) or Accumax (Nacalai tesque, no. 17087-54) (hiPSCs) and incubated at 37°C with 5% CO₂ for 5 min. The extra sequence analyses of HEK293FT and HeLa cells were performed using a CytoFLEX S flow cytometer (Beckman Coulter) with an emission filter at 525/40 nm and 660/10 nm, and for hiPSCs using an Accuri C6 (BD Bioscience) flow cytometer with an emission filter at 533/30 nm and 675/25 nm. The hybrid switch analysis was performed using Accuri C6 (BD Bioscience) flow cytometer with an emission filter at 533/30 nm and 675/25 nm or a CytoFLEX S (Beckman Coulter) with emission filters at 525/40 nm and 660/10 nm. All acquired data were analyzed using FlowJo 10.6.2 (BD Biosciences). iRFP670 intensities from viable cell populations were used to gate for the positively transfected cell population. For normalized EGFP expression, EGFP intensities from positively transfected cell populations were normalized by iRFP670 intensities.

Luciferase assay

Luciferase assays were conducted using the Nano-Glo Dual-Luciferase Reporter Assay System (Promega, no. N1610) following the manufacturer's instructions. Cells were harvested and lysed at 24 h after transfection. NanoLuc (Nluc) and Firefly luciferase (Fluc) activities were measured using a GloMax Navigator Microplate Luminometer (Promega). Relative luciferase levels were calculated by normalizing Nluc activity to Fluc activity in each sample for the Nluc ON switch assay, and Fluc activity to Nluc activity for the Fluc ON switch assay.

In vivo bioluminescence assay

All animal studies were conducted in compliance with ethical regulations in Kyoto University and were approved by the Center for iPS Cell Research and Application, Kyoto University (approval no. 20-140-12). BALB/cSlc-nu/nu mice (male, 7–8 weeks old) were used for intramuscular injection of Fluc-encoding mRNAs, while C57BL/6NcrSlc (male, 7–8 weeks old) (Japan SLC, Inc.) mice were used for tail vein injections. Mice were maintained under specific pathogen-free conditions with a 12-h/12-h light/dark cycle and food and water *ad libitum*. For mRNA delivery, InvivoFectamine 3.0 reagent (Thermo Fisher Scientific, no. IVF3005) was used according to the manufacturer's protocol. In brief, for intramuscular injections, an aqueous solution of mRNA (1 µg/µL, 50 µL) was mixed with an equal volume of complexation buffer. Then, 100 µL of 3.0 reagent was added, and the sample mixture was incubated at 50°C for 30 min. The mRNA solution was administered to mice via intramuscular injection at a dose of 10 µg in 40 µL. For intravenous injections, an aqueous solution of mRNA (0.8 µg/µL, 42 µL) was mixed with an equal volume of complexation buffer. Then, 84 µL of 3.0 reagent was added, and the sample mixture was incubated at 50°C for 30 min. Samples were diluted 5-fold with PBS and administered via tail vein injection at a dose of 10 µg in 250 µL. At 6 h after mRNA injection, mice were anesthetized with isoflurane and injected with 3 mg of D-luciferin (Promega, no. P1043) diluted in PBS, 6 min before organ excision for liver and spleen comparisons or 8 min before whole-body imaging of skeletal muscles and livers. Then, the mice or organs were transferred to an imaging chamber in an IVIS Imaging System (PerkinElmer), and the Fluc signal was measured.

RT-qPCR analysis

For RT-qPCR targeting endogenous miRNAs, tissues were homogenized in TRIzol Reagent (Thermo Fisher Scientific, no. 15596018), and total RNA was then treated with DNase and purified using the Direct-zol RNA Kit (Zymo Research, no. R2050). To synthesize first-strand cDNA, 800 ng of total RNA was subjected to reverse transcription (RT) using the Mir-X miRNA First-Strand Synthesis Kit (Takara, no. 638313). The resulting cDNA was analyzed by qPCR using SYBR Green qPCR Master Mix (Thermo Fisher Scientific, no. A66732) on a QuantStudio 3 Real-Time PCR Systems (Applied Biosystems), with miRNA-specific primers and the primer set provided in the RT kit. The qPCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 25 s, and a melting curve program. Each qPCR reaction was run in duplicate. Expression

levels were quantified using standard curves generated from serially diluted cDNA samples and normalized to U6 snRNA as an endogenous control. Primers used for qPCR are listed in Table S4.

Quantification and statistical analyses

Statistical details of each experiment can be found in the figure legends. All statistical analyses were performed using R 4.2.1 and RStudio.

DATA AVAILABILITY

All data, plasmids, and mRNAs used in this study are available upon request.

ACKNOWLEDGMENTS

We thank Shigetoshi Kameda (Kyoto University) for providing the Fluc plasmids and discussing the results. We also thank Dr. Tatsuyuki Yoshii and Dr. Shunsuke Kawasaki (Kyoto University) for evaluating and discussing the results; Dr. Kelvin K. Hui (Kyoto University) for proofreading the manuscript; Hiromi Takemoto, Yoshiko Ogawa, and Hiroki Ono (Kyoto University) for the administrative support; and all donors to the iPS Cell Research Fund. This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (22KJ1892 and JP20H05626); the Japan Agency for Medical Research and Development (AMED) (23bm1323001h0001 and 23bm1123040h0001); the Japan Science and Technology Agency (JST) CREST (JPMJCR23B3); and the iPS Cell Research Fund from Center for iPS Cell Research and Application, Kyoto University.

AUTHOR CONTRIBUTIONS

K.M. performed all the experiments. K.M., Y.F., and H.S. designed experimental strategies and wrote the paper.

DECLARATION OF INTERESTS

Y.F. and H.S. are the investigators of a record listed on the patent application (PCT/JP2017/023513, filed by Kyoto University on 27 June 2017) related to the design of RNA-ON switch. Y.F. and H.S. possess shares of aceRNA Technologies Ltd. and are external directors of aceRNA Technologies Ltd.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2025.102609>.

REFERENCES

- Kowalski, P.S., Rudra, A., Miao, L., and Anderson, D.G. (2019). Delivering the messenger: Advances in technologies for therapeutic mRNA delivery. *Mol. Ther.* 27, 710–728.
- Qin, S., Tang, X., Chen, Y., Chen, K., Fan, N., Xiao, W., Zheng, Q., Li, G., Teng, Y., Wu, M., and Song, X. (2022). mRNA-based therapeutics: powerful and versatile tools to combat diseases. *Signal Transduct. Target. Ther.* 7, 166.
- Parhiz, H., Atochina-Vasserman, E.N., and Weissman, D. (2024). mRNA-based therapeutics: looking beyond COVID-19 vaccines. *Lancet* 403, 1192–1204.
- Brook, B., Duval, V., Barman, S., Speciner, L., Sweitzer, C., Khanmohammed, A., Menon, M., Foster, K., Ghosh, P., Abedi, K., et al. (2024). Adjuvantation of a SARS-CoV-2 mRNA vaccine with controlled tissue-specific expression of an mRNA encoding IL-12p70. *Sci. Transl. Med.* 16, eadm8451.
- Jain, R., Frederick, J.P., Huang, E.Y., Burke, K.E., Mauger, D.M., Andrianova, E.A., Farlow, S.J., Siddiqui, S., Pimentel, J., Cheung-Ong, K., et al. (2018). MicroRNAs Enable mRNA Therapeutics to Selectively Program Cancer Cells to Self-Destruct. *Nucleic Acid Ther.* 28, 285–296.
- Teng, X., Zhao, X., Dai, Y., Zhang, X., Zhang, Q., Wu, Y., Hu, D., and Li, J. (2024). ClickRNA-PROTAC for tumor-selective protein degradation and targeted cancer therapy. *J. Am. Chem. Soc.* 146, 27382–27391. <https://doi.org/10.1021/jacs.4c06402>.

7. Schlöder, J., Shahneh, F., Schneider, F.-J., and Wieschendorf, B. (2022). Boosting regulatory T cell function for the treatment of autoimmune diseases - That's only half the battle. *Front. Immunol.* *13*, 973813.
8. Gao, X. (2025). Empower the age of smart mRNA medicine: Programmable RNA sensor and molecular tools refine therapeutic payload production. *Science* *388*, 40–41.
9. Kaseniit, K.E., Katz, N., Kolber, N.S., Call, C.C., Wengier, D.L., Cody, W.B., Sattely, E.S., and Gao, X.J. (2022). Modular, programmable RNA sensing using ADAR editing in living cells. *Nat. Biotechnol.* *41*, 482–487.
10. Jiang, K., Koob, J., Chen, X.D., Krajewski, R.N., Zhang, Y., Volf, V., Zhou, W., Sgrizzi, S.R., Villiger, L., Gootenberg, J.S., et al. (2022). Programmable eukaryotic protein synthesis with RNA sensors by harnessing ADAR. *Nat. Biotechnol.* *41*, 698–707.
11. Qian, Y., Li, J., Zhao, S., Matthews, E.A., Adoff, M., Zhong, W., An, X., Yeo, M., Park, C., Yang, X., et al. (2022). Programmable RNA sensing for cell monitoring and manipulation. *Nature* *610*, 713–721.
12. Ning, H., Liu, G., Li, L., Liu, Q., Huang, H., and Xie, Z. (2023). Rational design of microRNA-responsive switch for programmable translational control in mammalian cells. *Nat. Commun.* *14*, 7193.
13. Zhao, E.M., Mao, A.S., de Puig, H., Zhang, K., Tipples, N.D., Tan, X., Ran, F.A., Han, L., Nguyen, P.Q., Chory, E.J., et al. (2022). RNA-responsive elements for eukaryotic translational control. *Nat. Biotechnol.* *40*, 539–545.
14. Andries, O., Kitada, T., Bodner, K., Sanders, N.N., and Weiss, R. (2015). Synthetic biology devices and circuits for RNA-based “smart vaccines”: a propositional review. *Expert Rev. Vaccines* *14*, 313–331.
15. Zhang, J., and Salaita, K. (2021). Smart nucleic acids as future therapeutics. *Trends Biotechnol.* *39*, 1289–1307.
16. Fujita, Y., Hirose, M., Hayashi, K., Hatani, T., Yoshida, Y., Yamamoto, T., and Saito, H. (2022). A versatile and robust cell purification system with an RNA-only circuit composed of microRNA-responsive ON and OFF switches. *Sci. Adv.* *8*, eabj1793.
17. Abe, I., Ohno, H., Mochizuki, M., Hayashi, K., and Saito, H. (2024). Split RNA switch: Programmable and precise control of gene expression by ensemble of pre- and post-translational regulation. Preprint at bioRxiv. <https://doi.org/10.1101/2024.09.25.614879>.
18. Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., and Benenson, Y. (2011). Multi-input RNAi-based logic circuit for identification of specific cancer cells. *Science* *333*, 1307–1311.
19. Pratt, A.J., and MacRae, I.J. (2009). The RNA-induced silencing complex: a versatile gene-silencing machine. *J. Biol. Chem.* *284*, 17897–17901.
20. Kim, H., Kim, J., Kim, K., Chang, H., You, K., and Kim, V.N. (2019). Bias-minimized quantification of microRNA reveals widespread alternative processing and 3' end modification. *Nucleic Acids Res.* *47*, 2630–2640.
21. Chen, S.J., Hashimoto, K., Fujio, K., Hayashi, K., Paul, S.K., Yuzuriha, A., Qiu, W.-Y., Nakamura, E., Kanashiro, M.A., Kabata, M., et al. (2024). A let-7 microRNA-RALB axis links the immune properties of iPSC-derived megakaryocytes with platelet producibility. *Nat. Commun.* *15*, 2588.
22. Mullokandov, G., Baccarini, A., Ruzo, A., Jayaprakash, A.D., Tung, N., Israelow, B., Evans, M.J., Sachidanandam, R., and Brown, B.D. (2012). High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries. *Nat. Methods* *9*, 840–846.
23. Matsuura, S., Ono, H., Kawasaki, S., Kuang, Y., Fujita, Y., and Saito, H. (2018). Synthetic RNA-based logic computation in mammalian cells. *Nat. Commun.* *9*, 4847.
24. Zubiaga, A.M., Belasco, J.G., and Greenberg, M.E. (1995). The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol. Cell Biol.* *15*, 2219–2230.
25. Andries, O., McCafferty, S., De Smedt, S.C., Weiss, R., Sanders, N.N., and Kitada, T. (2015). N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J. Control. Release* *217*, 337–344.
26. Metkar, M., Pepin, C.S., and Moore, M.J. (2024). Tailor made: the art of therapeutic mRNA design. *Nat. Rev. Drug Discov.* *23*, 67–83.
27. Li, B., Luo, X., and Dong, Y. (2016). Effects of chemically modified messenger RNA on protein expression. *Bioconjug. Chem.* *27*, 849–853.
28. Song, M.-G., and Kiledjian, M. (2007). 3' Terminal oligo U-tract-mediated stimulation of decapping. *RNA* *13*, 2356–2365.
29. Miki, K., Endo, K., Takahashi, S., Funakoshi, S., Takei, I., Katayama, S., Toyoda, T., Kotaka, M., Takaki, T., Umeda, M., et al. (2015). Efficient detection and purification of cell populations using synthetic MicroRNA switches. *Cell Stem Cell* *16*, 699–711.
30. Parr, C.J.C., Katayama, S., Miki, K., Kuang, Y., Yoshida, Y., Morizane, A., Takahashi, J., Yamanaka, S., and Saito, H. (2016). MicroRNA-302 switch to identify and eliminate undifferentiated human pluripotent stem cells. *Sci. Rep.* *6*, 32532.
31. Hirose, M., Fujita, Y., Parr, C.J.C., Hayashi, K., Kashida, S., Hotta, A., Woltjen, K., and Saito, H. (2017). Cell-type-specific genome editing with a microRNA-responsive CRISPR-Cas9 switch. *Nucleic Acids Res.* *45*, e118.
32. Endo, K., Hayashi, K., and Saito, H. (2019). Numerical operations in living cells by programmable RNA devices. *Sci. Adv.* *5*, eaax0835.
33. Kavakiotis, I., Alexiou, A., Tastsoglou, S., Vlachos, I.S., and Hatzigeorgiou, A.G. (2022). DIANA-miTED: a microRNA tissue expression database. *Nucleic Acids Res.* *50*, D1055–D1061.
34. Rishik, S., Hirsch, P., Grandke, F., Fehlmann, T., and Keller, A. (2025). miRNATissueAtlas 2025: an update to the uniformly processed and annotated human and mouse non-coding RNA tissue atlas. *Nucleic Acids Res.* *53*, D129–D137.
35. Hewitt, S.L., Bai, A., Bailey, D., Ichikawa, K., Zielinski, J., Karp, R., Apte, A., Arnold, K., Zacharek, S.J., Iliou, M.S., et al. (2019). Durable anticancer immunity from intratumoral administration of IL-23, IL-36γ, and OX40L mRNAs. *Sci. Transl. Med.* *11*, eaat9143. <https://doi.org/10.1126/scitranslmed.aat9143>.
36. Domenger, C., and Grimm, D. (2019). Next-generation AAV vectors-do not judge a virus (only) by its cover. *Hum. Mol. Genet.* *28*, R3–R14.
37. Melamed, J.R., Hajj, K.A., Chaudhary, N., Strelkova, D., Arral, M.L., Pardi, N., Alameh, M.-G., Miller, J.B., Farbiak, L., Siegwart, D.J., et al. (2022). Lipid nanoparticle chemistry determines how nucleoside base modifications alter mRNA delivery. *J. Control. Release* *341*, 206–214.
38. Cheng, Q., Wei, T., Farbiak, L., Johnson, L.T., Dilliard, S.A., and Siegwart, D.J. (2020). Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing. *Nat. Nanotechnol.* *15*, 313–320.
39. Tanaka, H., Hagiwara, S., Shirane, D., Yamakawa, T., Sato, Y., Matsumoto, C., Ishizaki, K., Hishinuma, M., Chida, K., Sasaki, K., et al. (2023). Ready-to-Use-Type Lyophilized Lipid Nanoparticle Formulation for the Postencapsulation of Messenger RNA. *ACS Nano* *17*, 2588–2601.
40. Wroblewska, L., Kitada, T., Endo, K., Siciliano, V., Stillo, B., Saito, H., and Weiss, R. (2015). Mammalian synthetic circuits with RNA binding proteins for RNA-only delivery. *Nat. Biotechnol.* *33*, 839–841.
41. Otsuka, H., Fukao, A., Funakami, Y., Duncan, K.E., and Fujiwara, T. (2019). Emerging evidence of translational control by AU-rich element-binding proteins. *Front. Genet.* *10*, 332.
42. Passmore, L.A., and Collier, J. (2022). Roles of mRNA poly(A) tails in regulation of eukaryotic gene expression. *Nat. Rev. Mol. Cell Biol.* *23*, 93–106.
43. Nakagawa, M., Taniguchi, Y., Senda, S., Takizawa, N., Ichisaka, T., Asano, K., Morizane, A., Doi, D., Takahashi, J., Nishizawa, M., et al. (2014). A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. *Sci. Rep.* *4*, 3594.
44. Shcherbakova, D.M., and Verkhusha, V.V. (2013). Near-infrared fluorescent proteins for multicolor in vivo imaging. *Nat. Methods* *10*, 751–754.

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Supplemental information

MicroRNA-responsive ON-OFF hybrid mRNA switch for precise protein expression control

Kaito Masaki, Yoshihiko Fujita, and Hirohide Saito

Table S1. List of primer and plasmid sequences

This table is provided as a separate Excel file (Table S1.xlsx)

Table S2. IVT Template List with Associated Primers and Plasmids

This table is provided as a separate Excel file (Table S2.xlsx)

Annotation of abbreviations used in the table: IVTt indicates the IVT template; T7pro represents the T7 promoter; the number followed by 'A' denotes the number of adenosine residues; and 'ts' refers to the target site.

Table S3. List of transfection conditions

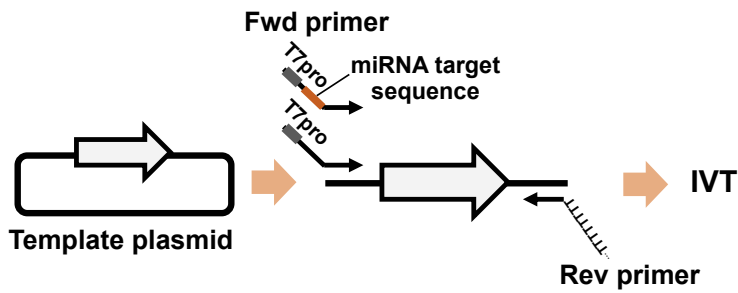
All experiments were performed in 24-well plates.

Figure	Transfected-mRNA (/well)	miRNA mimic / inhibitor (/well)
2B, 2E, 2F, S2, S3A, S3D, S4A, S4F	100 ng Normal mRNA or ON switches 100 ng Reference mRNA	0.5 pmol Negative control mimic or miR-206 mimic
S3B	100 ng Normal mRNA or ON switches 100 ng Reference mRNA	0.5 pmol Negative control mimic or miR-21 mimic
2D	10 ng Normal mRNA or ON switches 100 ng Reference mRNA	2 pmol Negative control inhibitor or miR-21-5p inhibitor
S3C	10 ng Normal mRNA or ON switches 100 ng Reference mRNA	0.5 pmol Negative control mimic or miR-206 mimic
S4B, S4C	100 ng Normal mRNA or ON switches 100 ng Reference mRNA	2 pmol Negative control inhibitor or miR-302a-5p inhibitor
S4D, S4E	100 ng Normal mRNA or ON switches 100 ng Reference mRNA	2 pmol Negative control inhibitor or miR-21-5p inhibitor
3B-D, S5A	100 ng Normal mRNA or ON switches 100 ng Reference mRNA	-
S5B	100 ng Normal mRNA or ON switches 100 ng Reference mRNA	0.5 pmol Negative control mimic or miR-302a-5p mimic 2 pmol Negative control inhibitor or miR-302a-5p inhibitor
S6D	100 ng Normal mRNA or ON switches 100 ng Reference mRNA	0.5 pmol Negative control mimic or miR-122-5p mimic

Table S4. List of primers for RT-qPCR

Target	Primers (5'-3')
qPCR primers for miRNA	
mmu-miR-122-5p - fwd	TGGAGTGTGACAATGGTGTTTG
mmu-miR-1a-3p - fwd	TGGAATGTAAAGAAGTATGTAT
mmu-miR-142-3p - fwd	TGTAGTGTTTCCTACTTTATGGA

Normal mRNA and miRNA-OFF switch



miR-ON switch and Hybrid switch

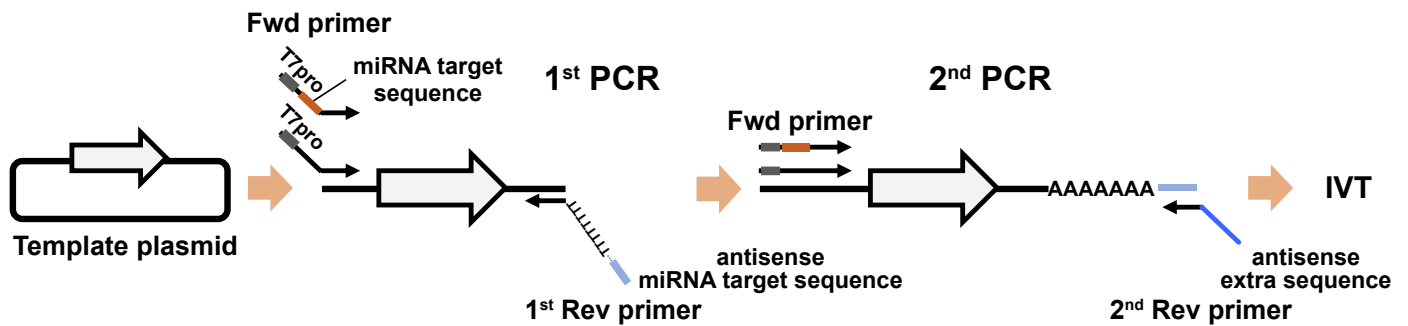


Figure S1. Procedure for constructing normal mRNA and miRNA switches

The miRNA-ON switch was generated through two independent rounds of PCR, using a first reverse primer with an antisense sequence complementary to the target miRNA from the template plasmid and a second reverse primer containing an extra sequence. The resulting PCR product was used as a template for *in vitro* transcription (IVT).

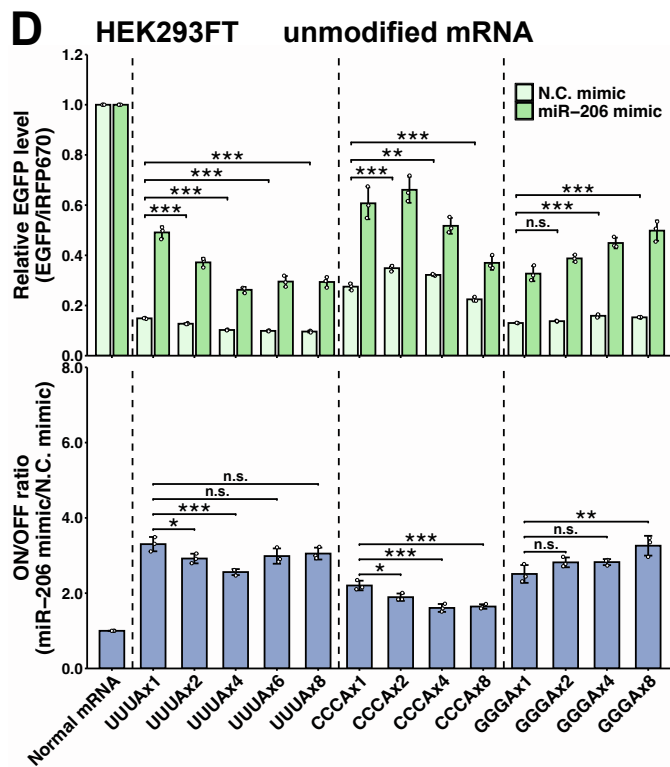
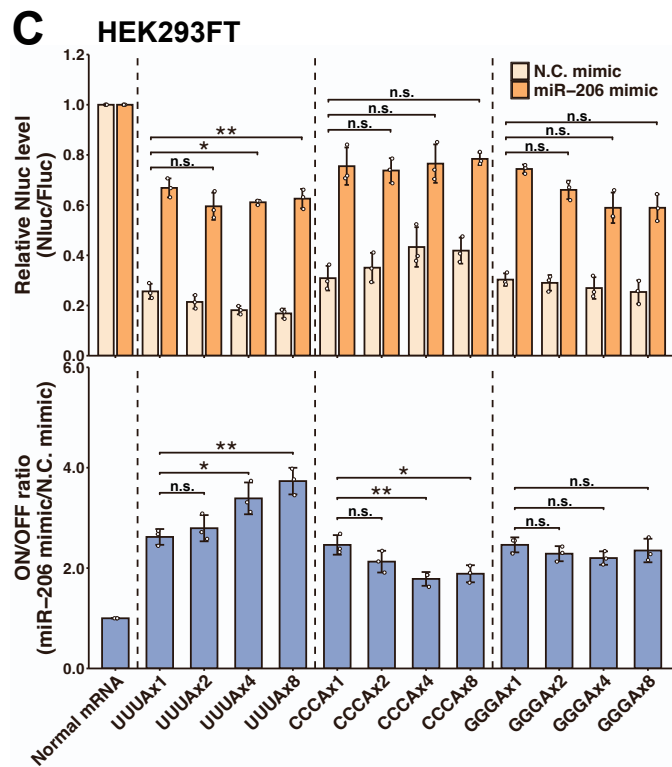
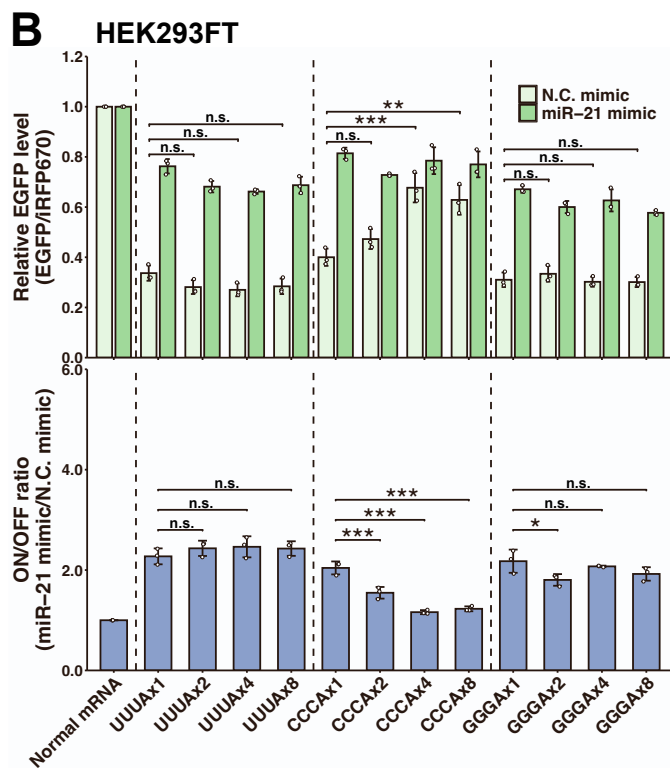
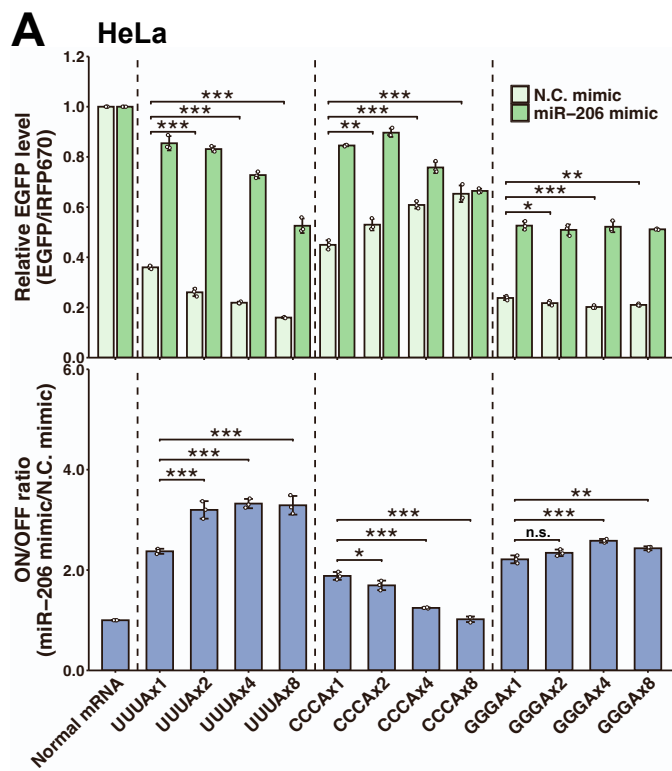


Figure S3. ON switch performance across different experimental conditions

(A, B) Relative EGFP/iRFP670 levels to normal mRNA (Top) and ON/OFF ratios (Bottom) for each extra sequence in HeLa (A) and HEK293FT (B) cells. (C) Relative Nluc/Fluc levels to normal mRNA (Top) and ON/OFF ratios (Bottom) for each extra sequence in HEK293FT. (D) Relative EGFP/iRFP670 levels of unmodified mRNAs compared to normal mRNA (Top), and ON/OFF ratios (Bottom) for each extra sequence in HEK293FT cells. Data points represent biological replicates ($n = 3$). Means $\pm$ SD are shown. Statistical significance was tested by comparing the 1-repeat extra sequence with corresponding repeats using one-way ANOVA and Dunnett's test. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. n.s. indicates no significance.

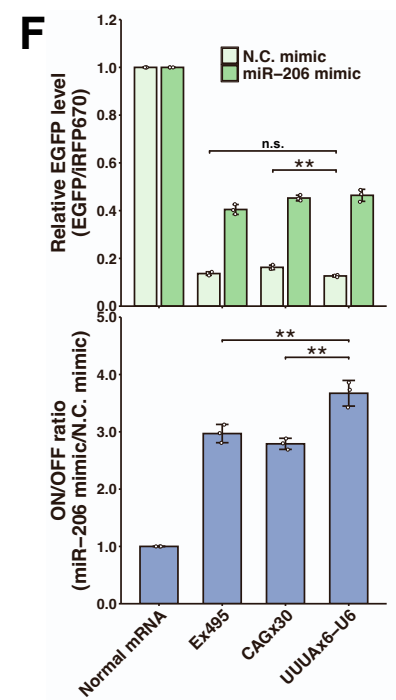
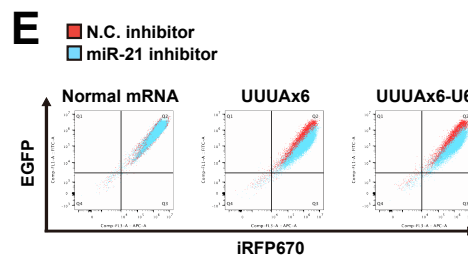
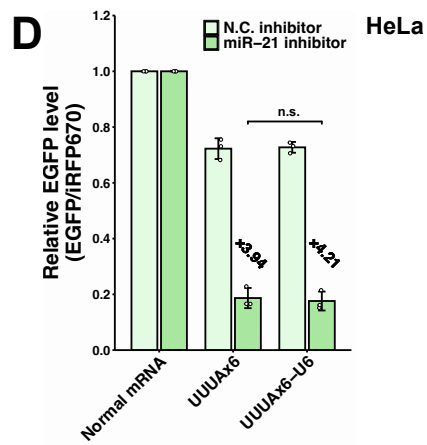
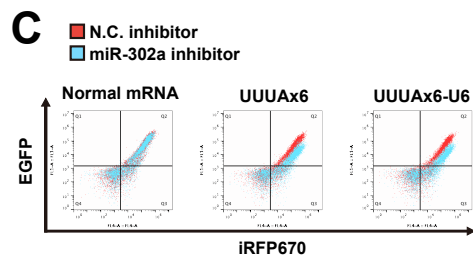
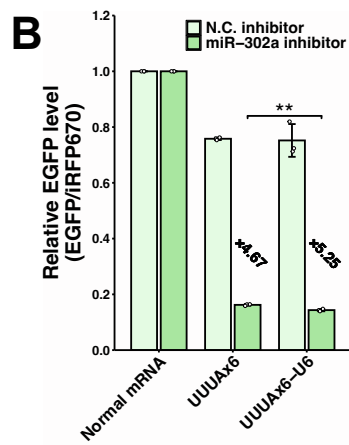
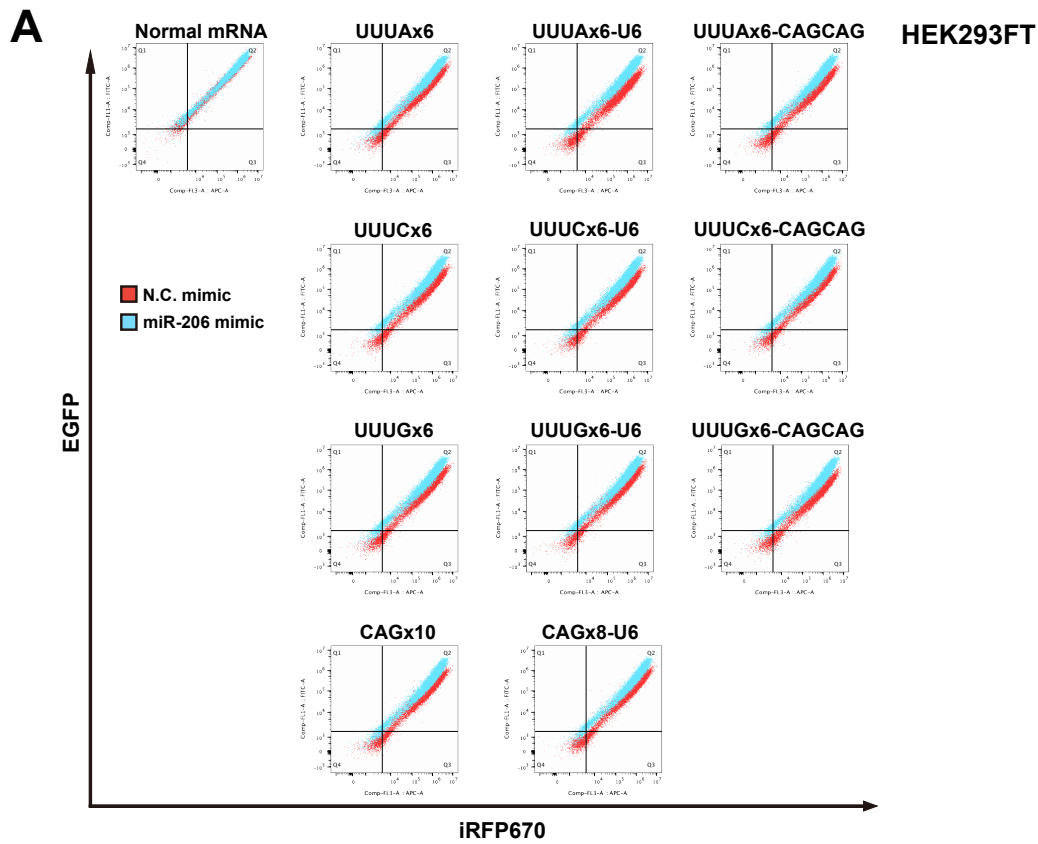
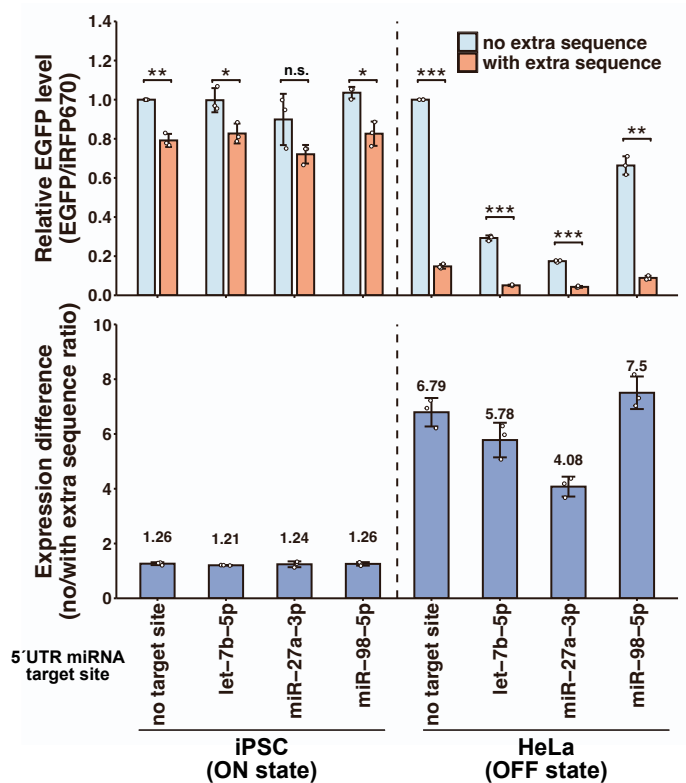
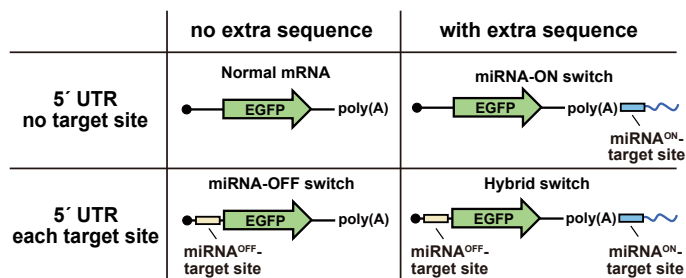


Figure S4. Effect of 3'-terminal U6-tract on ON switch activity

(A) Scatter plots generated from flow cytometric analyses in Figure 2E. (B) Relative EGFP/iRFP670 levels to normal mRNA for miR-302a-ON switches transfected into hiPSCs with miRNA inhibitors. Data points represent three biological replicates ($n = 3$). Means $\pm$ SD are shown. Statistical significance was assessed by comparing UUUAx6 with UUUAx6-U6 using unpaired two-tailed t-test. (C) Scatter plots generated from flow cytometric analyses in Supplementary Figure S4B. (D) Relative EGFP/iRFP670 levels to normal mRNA for miR-21-ON switches transfected into HeLa cells with miRNA inhibitors. Data points represent three biological replicates ($n = 3$). Means $\pm$ SD are shown. Statistical significance was assessed by comparing UUUAx6 with UUUAx6-U6 using unpaired two-tailed t-test. (E) Scatter plots generated from flow cytometric analyses in Supplementary Figure S4D. Values starting with "x" represent the ON/OFF ratio. (F) Relative EGFP/iRFP670 levels to normal mRNA for miR-206-ON switches transfected into HEK293FT cells with miRNA mimics. Data points represent three biological replicates ($n = 3$). Means $\pm$ SD are shown. Statistical significance was assessed by comparing UUUAx6-U6 with other extra sequences using one-way ANOVA and Dunnett's test. Significance levels: ** $p < 0.01$. n.s. indicates no significance.

A



B

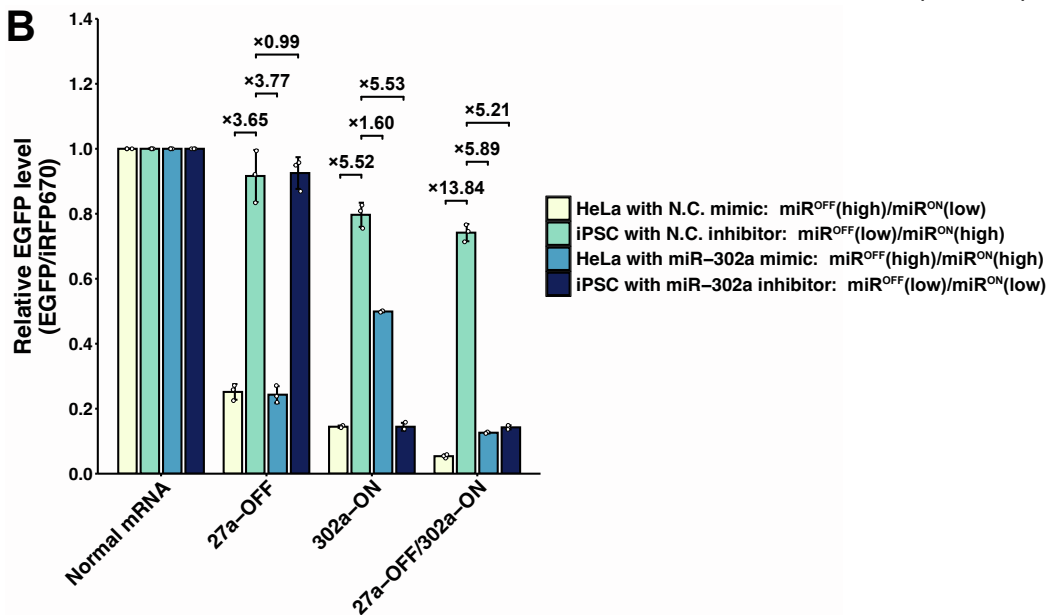


Figure S5. Hybrid strategy integrating ON and OFF switches

(A) Relative EGFP/iRFP670 levels to normal mRNA (i.e., no 5' target site and no extra sequence) (Top) and downregulated levels in the presence of the extra sequence (Bottom) for RNA switches in hiPSCs and HeLa cells, as shown in a modified version of Figure 3B. Values in the graph represent the mean. Data points represent three biological replicates ($n = 3$). Means $\pm$ SD are shown. Statistical significance was tested by comparing mRNAs with and without an extra sequence using unpaired two-tailed t-test. (B) Relative EGFP/iRFP670 levels to normal mRNA under different combinations of miRNA^{OFF} and miRNA^{ON} conditions, modeled by transfecting N.C. mimic or miR-302a mimic into HeLa cells, and N.C. inhibitor or miR-302a inhibitor into iPSCs. Normal mRNA encoding iRFP670 was co-transfected as a reference. Data points represent three biological replicates ($n = 3$). Means $\pm$ SD are shown. Statistical significance was tested by comparing constructs under the "miRNA^{OFF} (low)/miRNA^{ON} (high)" condition with those under other miRNA activity conditions, using one-way ANOVA followed by Dunnett's test. Significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s. indicates no significance.

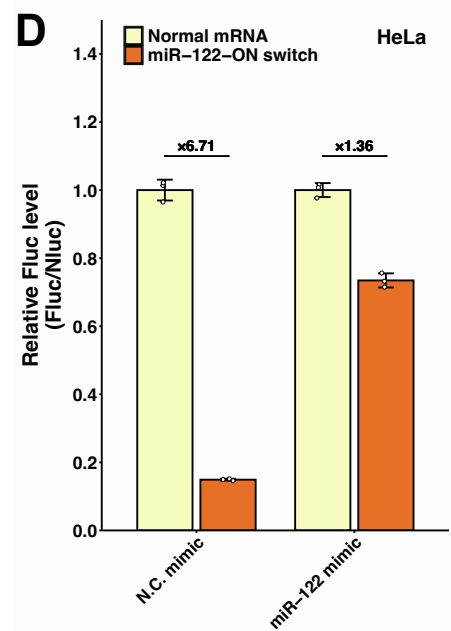
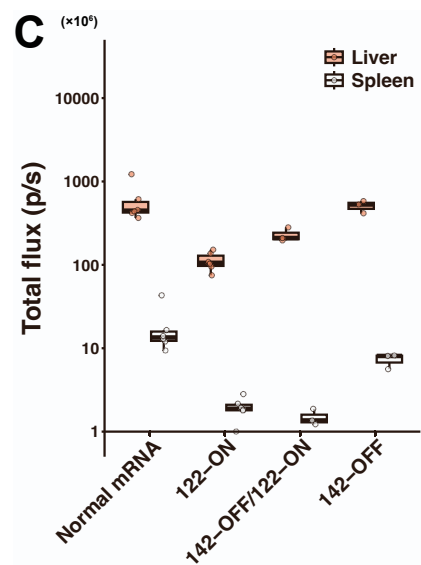
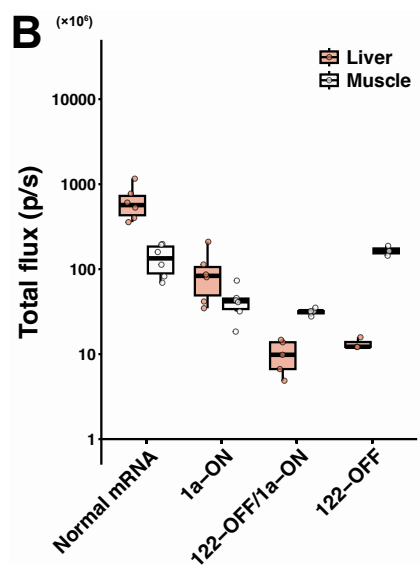
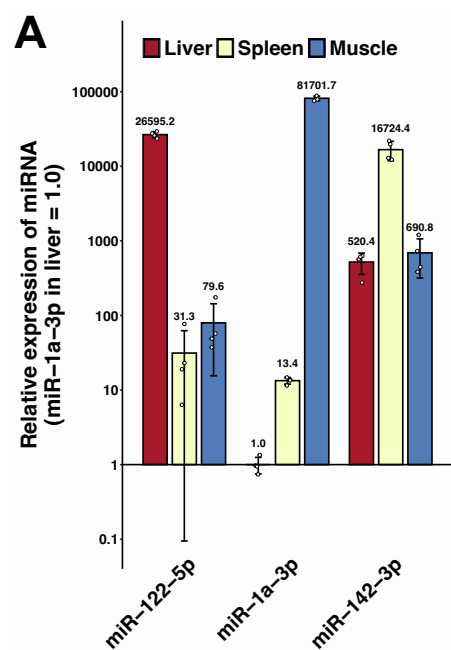


Figure S6. Tissue miRNAs and *in vivo*-related switch evaluation

(A) Endogenous miRNA levels, as measured by RT-qPCR. Data are normalized to the U6 snRNA internal control. Data points represent four biological replicates ($n = 4$). (B) Raw values of total flux corresponding to Figure 4C. Data points represent biological replicates ($n = 6$ for normal mRNA and miR-1a-ON switch, $n = 5$ for miR-122-OFF/miR-1a-ON hybrid switch, and $n = 3$ for miR-122-OFF switch). (C) Raw values of total flux corresponding to Figure 4F. Data points represent biological replicates ($n = 6$ for normal mRNA and miR-122-ON switch, $n = 3$ for miR-142-OFF/miR-122-ON hybrid switch and miR-142-OFF switch). The box plot shows the median and interquartile range, with whiskers representing data dispersion. (D) Relative Fluc/Nluc levels to normal mRNA of the miR-122-responsive ON switch in HeLa cells with N.C. mimic or miR-122 mimic. Data points represent biological replicates ($n = 3$). Means $\pm$ SD are shown.