

Modulating miRNA binding sites within circRNA for enhanced translation efficiency

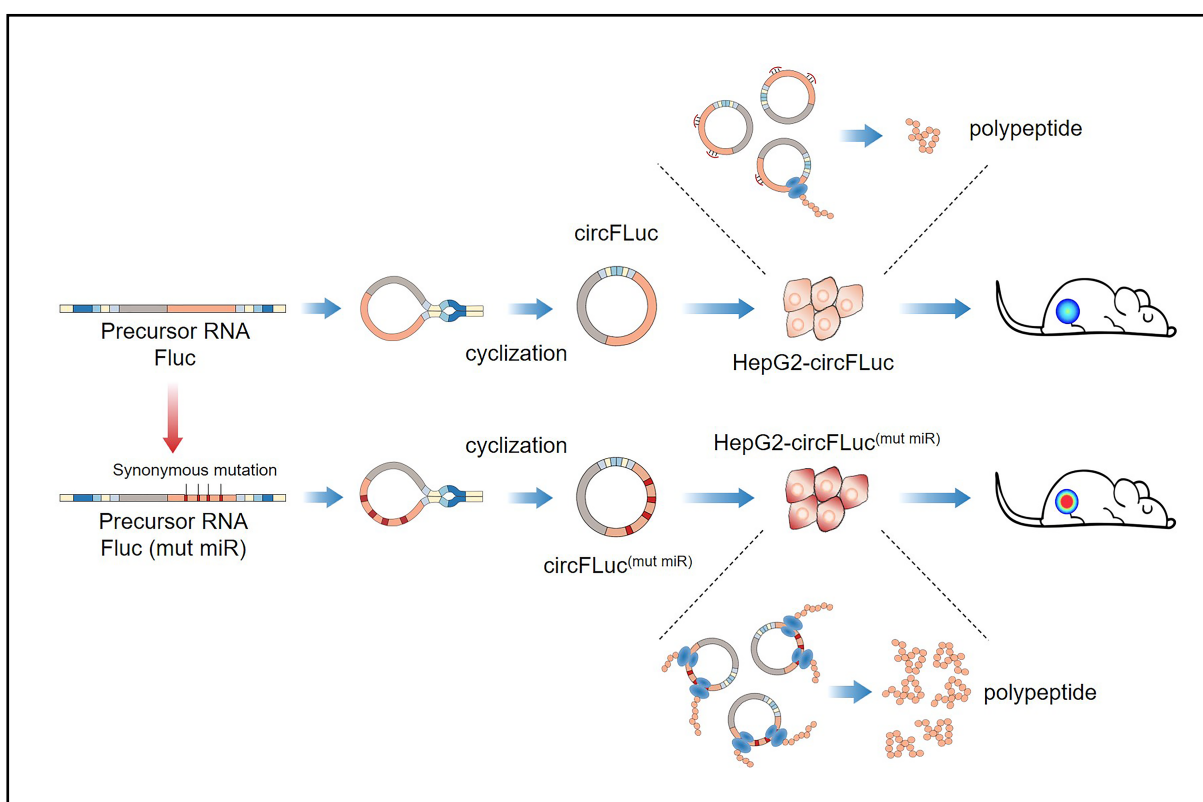
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Graphical abstract



Modulating miRNA binding sites within circRNAs can effectively enhance their translation efficiency both in vitro and in vivo.

Public summary

- A circular RNA capable of expressing firefly luciferase and renilla luciferase was constructed.
- Binding sites for miRNAs within the circular RNA decrease its translation efficiency without affecting its stability.
- Synonymous mutations in the miRNA binding sites can enhance the translation efficiency of the circular RNA without affecting its stability.
- In vivo experiments using nude mice and cellular-level experiments both demonstrate that reducing specific miRNA binding sites can improve the translation efficiency and expression duration of the circular RNA.

Modulating miRNA binding sites within circRNA for enhanced translation efficiency

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Supporting Information

Abstract: Circular RNAs (circRNAs) are covalently closed circular RNAs, and some of them preserve translation potency. However, modulation of circRNA translation efficiency and its applications need to be explored. In this study, RNAs containing the translation initiation element CVB3 IRES and the coding sequence of luciferase protein were transcribed and circularized in vitro by T7 RNA polymerase and an optimized permuted intron–exon (PIE) splicing strategy. The circularized RNAs were then transfected and translated into active luciferase in the cultured cells. Insertion of miRNA binding sites at the flanking region of the luciferase coding sequence significantly reduced the translation efficiency of the circRNAs. Mutations of the miRNA binding sites in the firefly luciferase coding sequence led to increased translation efficiency of synthetic circRNAs in cells. We also proved that mutations of the binding sites of specific miRNAs also enhanced the translation efficiency of synthetic circRNAs. Further in vivo experiments via bioluminescence imaging showed that synonymous mutation of the miRNA binding sites promoted synthetic circRNA translation in nude mice. This study demonstrates that the modulation of miRNA binding sites affects the translation efficiency of synthetic circRNAs in vitro and in vivo, which could be used as versatile tools for future applications in clinical imaging.

Keywords: circRNA; circRNA translation; in vitro circularization; miRNA; circRNA-based application

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1 Introduction

Circular RNAs (circRNAs) are covalently closed single-stranded RNA molecules generated by back-splicing or other RNA circularizing mechanisms^[1–3]. Due to the lack of free 5' and 3' termini, circRNAs are resistant to several RNA turnover mechanisms and have longer half-lives than linear mRNA^[4]. Although most circRNAs found in nature are non-coding^[5–7], it has been demonstrated that some endogenous circRNAs in humans and fruit flies, as well as synthetic circRNAs, can encode polypeptides^[8,9], some of which are functional. Several circRNA-based applications^[10,11], which were generated by in vitro synthesizing and circularizing long transcripts into circRNAs, were developed based on the distinct conformation, stability, and immunogenicity characteristics of circRNAs.

Translation of circRNAs relies on cap-independent mechanisms, such as internal ribosome entry sites (IRESs)^[12], to initiate translation. Taking advantage of this characteristic, researchers have artificially designed and synthesized circRNAs containing internal ribosome entry sites (IRESs) and coding sequences, enabling them to express functional proteins and serve as protein substitutions^[10,11]. Due to the immense application potential of coding circRNAs, there is an increasing exploration of factors regulating the translation of synthesized circRNAs. These investigations include vector topology, 5' and 3' untranslated regions, internal ribosome entry

sites, and synthetic aptamers that recruit translation initiation machinery^[13–15]. These studies have further enhanced the translation efficiency of synthesized circRNAs. Although considerable advancements have been made in in vitro circRNA translation, improving the efficiency of synthetic circRNA translation in vivo remains critical for their potential applications, and optimization of circRNA in vivo translation has yet to be thoroughly dissected.

The main objective of this study was to investigate the factors influencing the translation of in vitro synthesized circRNAs. Considering that circRNAs can act as miRNA sponges and regulate downstream target genes by sequestering specific miRNAs through complementary binding^[16–19], miRNAs can also interact with circRNAs and recruit Ago2 protein to cleave circRNAs^[20]. Furthermore, miRNAs themselves constitute a vast regulatory network that plays a crucial role in intracellular and intercellular regulation^[21–24], and the expression profiles of miRNAs in different human tissues and the miRNA-circRNA binding mechanism are well known^[1,23–26]. Therefore, this study primarily investigates whether miRNA binding to circRNAs can affect the translation efficiency of circRNAs.

The efficient translation of circRNAs in vivo has led to a need for efficient methods to generate circRNAs in vitro via various methods of circularization, including the PIE system^[27,28], chemical ligation^[29–31], and enzymatic ligation^[32,33],

with circularization via the PIE (permuted intron–exon) system being the most widely used strategy. The PIE system is a naturally occurring class I intron self-splicing system that can synthesize long circular RNAs both in vivo and in vitro. After optimization of its intron sequence, this system can circularize RNA sequences up to 5000 nt in length^[34]. Currently, in vitro synthesized mRNA has broad applications in vaccines, medicine, and even for preventing and alleviating COVID-19^[35]. However, increasing stability and reducing immunogenicity are ongoing challenges in mRNA vaccine research. It is necessary to establish better circRNA therapeutics based on circRNA in vitro circularization and translation^[36, 37], thereby improving the overall efficacy of exogenous RNA in various applications^[38].

In this study, we synthesized and circularized linear RNA transcripts in vitro through a PIE system with high circularizing efficiency^[34]. The CVB3 IRES was used to initiate circRNA translation, and translation efficiency was assessed by luciferase assay. We further observed that the existence of miRNA binding sites within the synthetic circRNAs led to decreased circRNA translation, and the introduction of synonymous mutations to these miRNA binding sites increased circRNA translation. In vivo experiments on mice demonstrated that transfection of in vitro synthetic circRNAs with synonymous mutations of the miRNA binding sites resulted in increased bioluminescence, which suggested potential applications of circRNA-based therapeutics by modulating miRNA binding sites in the synthetic circRNAs.

2 Materials and methods

2.1 Animals and cell culture

The animal experiments were approved by the Animal Care and Use Committee of USTC (USTCACUC23030123007). Five-week-old female BALB/c nude mice were maintained under specific pathogen-free conditions with individually ventilated cages and a 12-h light-dark cycle with ad libitum access to food and water. Human cell lines, including HEK293T and HepG2 cells, were purchased from the American Type Culture Collection (ATCC, <http://www.atcc.org>). All human cells were cultured with DMEM under standard conditions including 10% FBS and 1% penicillin/streptomycin at 37 °C under 5% CO₂. Cells were tested for mycoplasma by DAPI staining to ensure the absence of contamination.

2.2 Expression profile of miRNAs

The miRNA expression profile in human tissues was derived from the miRNA Tissue Expression Database^[39] (DIANA-miTED database). The miRNA expression (RPM) data from healthy human tissues were normalized using the Z-score method ($Z = (X - \mu) / \sigma$, where X = raw score, μ = sample mean, σ = sample standard deviation), and a heatmap was generated with human tissue organs as the horizontal axis and miRNAs as the vertical axis. The aim was to visualize the expression levels of miRNAs in different human tissues and organs.

2.3 Construction of plasmids

For plasmids used for cyclization in vitro, protein-coding,

group I self-splicing intron, and IRES sequences were chemically synthesized (GENERAL BIOL) and cloned and inserted into a PCR-linearized plasmid vector (PGEM-T) containing a T7 RNA polymerase promoter by homologous recombination using a ClonExpress™ MutiS One Step Cloning Kit (Vazyme, C113). Spacer regions, homology arms, and other minor alterations were added via PCR. Primers for this and the following methods can be found in the supporting information Table S1.

2.4 RNA design, synthesis, and purification

CircRNA precursors were synthesized by runoff in vitro transcription from a linearized plasmid DNA template using a T7 High Yield RNA Synthesis Kit (YEASEN, 10623ES60), and reactions were treated with DNase I (Thermo Fisher Scientific, EN0521) for 15 min. After DNase treatment, RNA was purified by TRIzol Reagent (Invitrogen, 15596026). For circRNAs, RNA was then heated to 70 °C for 5 min and immediately placed on ice for 3 min. GTP was added to a final concentration of 2 mmol/L along with a buffer including magnesium (50 mmol/L Tris-HCl, 10 mmol/L MgCl₂, 1 mmol/L DTT, pH 7.5), and then reactions were heated at 55 °C for 10 min. RNA was then column purified using an RNA Clean & Concentrator™-5 kit (Zymo Research, R1015). In some cases, circRNAs were digested with RNase R: 20 µg of RNA was diluted in water (86 µL final volume) and then heated at 70 °C for 3 min and cooled on ice for 2 min. Then, 10 U RNase R and 10 µL of 10 × RNase R buffer (Epicenter, RNR07250) were added, and the reaction was incubated at 37 °C for 30 min. RNase R-digested RNA was column purified.

2.5 Cell transfection

For plasmid transfection of HEK293T cells, transfections were conducted with Lipofectamine 2000 (Invitrogen, 11668-019) according to the manufacturer's protocols. Cells were harvested for further analysis or downstream experiments 48 h posttransfection. For circRNA and miRNA mimic transfection of HEK293T or HepG2 cells, transfections were conducted with Lipofectamine MessengerMax (Invitrogen, LM-RNA008) according to the manufacturer's protocols. Cells were harvested for further analysis or downstream experiments 24 h posttransfection.

2.6 RT-qPCR of miRNAs and circRNAs

Cell total RNA was extracted with TRIzol (Invitrogen, 15596026), and complementary DNA (cDNA) of circRNAs was prepared using an ABScript II cDNA First-Strand Kit (ABclonal, RK20400) according to the manufacturer's protocol. For reverse transcription of miRNAs, miRNA-specific RT primers were used in addition to the abovementioned kit. Quantitative real-time PCR was performed with Genius 2X SYBR Green Fast qPCR Mix (ABclonal, RK21204) on an ABI QuantStudio3 system followed by 40 amplification cycles according to standard procedures. All primers used are listed in Table S1.

2.7 Cell proliferation assay

Transfected cells were seeded into a 96-well plate at a density of 2000 cells per well ($n=5$). The plate was incubated at 37 °C in a CO₂ incubator. After culturing for 1, 2, 3, 4, and 5

d, the cells were subjected to detection. For the detection, 10 μ L of CCK-8 reagent (BIOMIKY, MK001B) was added to each well, followed by an additional 3-hour incubation. Absorbance at 450 nm was measured, and statistical analysis was conducted.

2.8 Luminescence assays

For luminescence assays, cells were harvested 24 h posttransfection. To detect luminescence from firefly luciferase and renilla luciferase, cell lysate was transferred to a flat-bottomed white-walled plate (Corning, 3917). Then, 100 μ L of firefly luciferase reagent including stabilizer (YEASEN, 11402ES60) was added to each sample, and luminescence was measured on a SpectraMax iD5 reader (Molecular Devices) after 10 s. After that, 100 μ L of Renilla luciferase reagent was added and tested in the same manner.

2.9 Tumor xenografts and in vivo imaging

When HepG2 cells reached 60%–70% confluency, circRNAs were transfected into the cells. Twenty-four hours later, the desired number of cells was collected and suspended in a mixture of serum-free medium and Matrigel (BD Biosciences, 356234) at a 1 : 1 ratio. The cell suspension was kept on ice and injected into BALB/c female nude mice within 20 min. BALB/c female nude mice were inoculated subcutaneously with HepG2 cells (10^6). One group of mice ($n=6$) received HepG2 cells transfected with circFLuc^(mut miR) as the experimental group, another group of mice ($n=6$) received HepG2 cells transfected with circFLuc as the control group, and the final group of mice ($n=3$) received empty HepG2 cells as the negative control group. Two days after injection, D-luciferin potassium salt (150 mg/kg) (YEASEN, 40902ES02) was injected intraperitoneally, and the mice were subjected to in vivo imaging using the Xenogen IVIS Lumina System (Caliper Life Sciences). The experimental results were analyzed using Living Image 3.1 software (Caliper Life Sciences). The mice were then observed and analyzed every two days until luminescence was no longer detectable.

2.10 Statistical analysis

GraphPad Prism 9 software was used for all statistical analyses. Data from all experiments are presented as the mean $\pm$ SD, which were replicated at least three times. Student's two-tailed *t*-test was used to assess the statistical significance of the difference.

3 Results and discussion

3.1 In vitro synthesis of circRNAs with translation potential

To generate circRNAs with translation potential in vitro, we first synthesized circRNA linear precursors containing the coxsackievirus B3 internal ribosome entry site (CVB3 IRES) using the Permuted Intron–Exon (PIE) splicing method^[34], which includes a firefly luciferase (FLuc) coding sequence, two designed spacer sequences, two short regions corresponding to PIE structure exonic segments, and the 3' and 5' intron segments of the Anabaena pretrRNA group I intron arranged by run-off transcription^[27] (Fig. 1a). In the presence of

guanosine triphosphate (GTP) and Mg^{2+} , these precursor RNA molecules underwent double transesterification reactions, which are characteristic of group I-catalyzed introns. Due to the fusion of exons, the region between the two intron segments was then excised, resulting in a covalently linked circular RNA (circFLuc)^[27] (Fig. 1a). In the same way, circularization of the precursor RNA molecule, which includes a Renilla luciferase (RLuc) coding sequence, was achieved, resulting in circRLuc (Fig. 1b). To confirm the successful circularization of circFLuc and circRLuc, we assessed the RNAs after treatment with RNase R, an exonuclease that specifically digests linear RNAs but not circRNAs^[40], and observed enrichment of the presumed circularized RNA band (Fig. 1c). Subsequent Sanger sequencing detecting the junction of circRNAs further confirmed that the in vitro transcribed linear RNA was circularized (Fig. 1d). To investigate whether the synthetic circRNAs containing Fluc or RLuc coding sequences were translatable, we transfected circFLuc and circRLuc into HEK293T cells and conducted a luciferase assay, which showed that both circFLuc and circRLuc exhibited stable and significant luciferase signals compared to the control (Fig. 1e). The luciferase activity of circRLuc was observed to be higher than that of circFLuc, partially contributing to the fact that renilla luciferase is relatively shorter than firefly luciferase and only requires coelenterazine and oxygen, while firefly luciferase uses luciferin in the presence of oxygen, ATP and magnesium to produce light^[41, 42]. Overall, we obtained translatable circRNAs with stable luciferase expression from synthetic circRNAs generated by linear precursors via an optimized circularization method.

3.2 Insertion of miRNA binding sites reduced the translation efficiency of circRNAs

We asked whether introducing miRNA binding sites in the synthetic circRNA sequence affects circRNA translation efficiency. We examined the expression profiles of three miRNAs, miR-21-5p, miR-101-3p, and miR-192-5p, which were highly expressed in several healthy human tissues^[39] and 293T cells (Fig. 2a and b). Therefore, we chose these three miRNAs for further experiments.

We inserted binding sites of miR-21-5p, miR-101-3p, and miR-192-5p into the upstream and downstream sequences of firefly luciferase to generate circFLuc^(miR) through in vitro circularization (Fig. 2c), with circFLuc and circRLuc prepared as controls. RNase R digestion also showed successful enrichment of circFLuc^(miR) (Fig. 2d). We transfected circFLuc^(miR), as well as circFLuc and circRLuc as controls, into HEK293T cells and conducted a luciferase assay, which showed that all three synthetic circRNAs exhibited significant luciferase signals compared to the blank control (Fig. 2e). Notably, the luciferase expression of circFLuc^(miR) with inserted miRNA binding sites was reduced by 60% compared to circFLuc and circRLuc, suggesting that the insertion of miRNA binding sites in the flanking coding sequence affected circRNA translation.

To further validate this hypothesis, we then investigated whether the changes in circRNA levels, instead of miRNA binding, affected circRNA translation efficiency. We transfected the synthetic circRNAs into HEK293T cells to assess

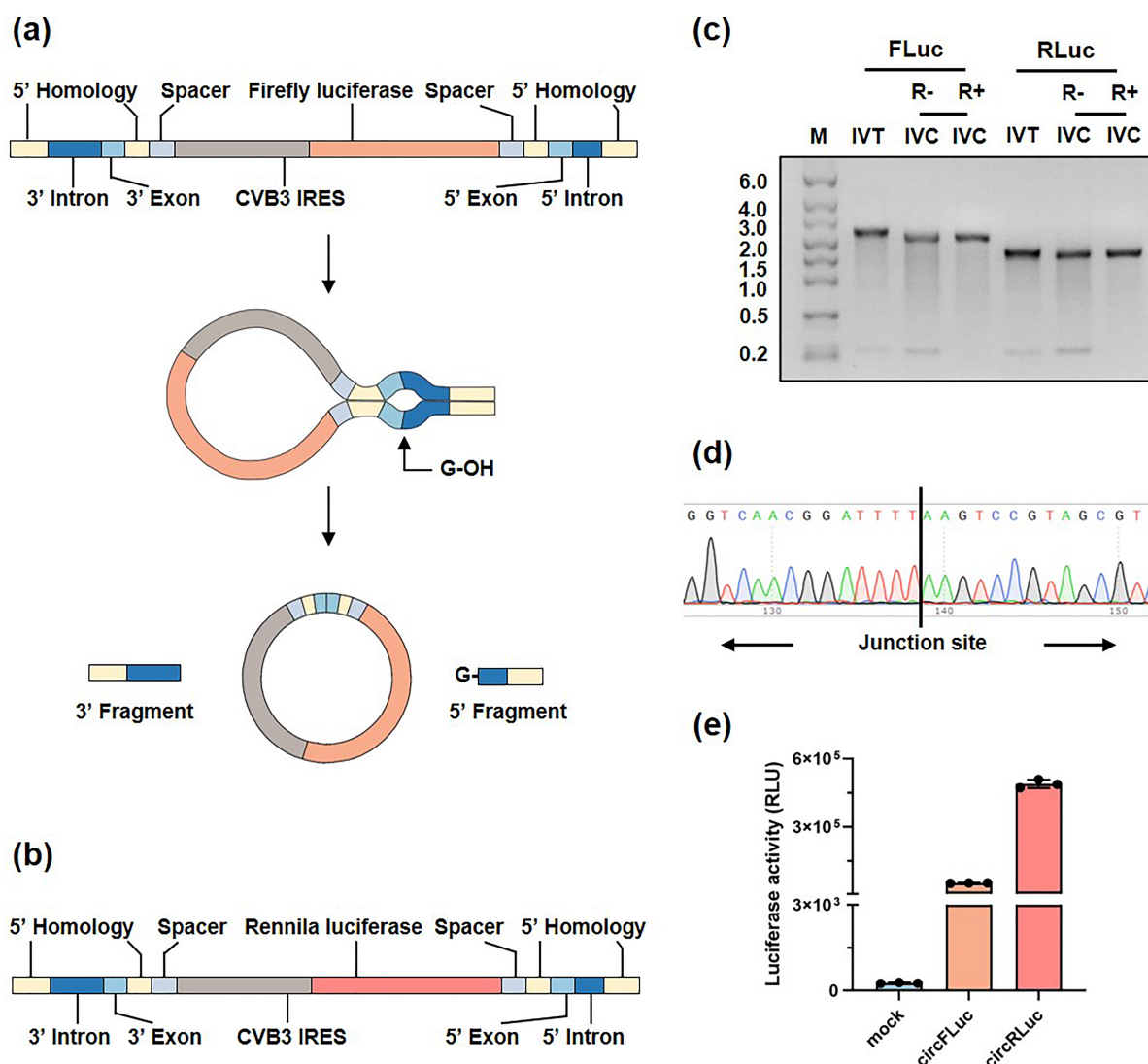


Fig. 1. Protein expression initiated by circRNAs. (a, b) Schematic representation of the elements required for circFLuc (a) and circRLuc (b) production through the PIE system. (c) Agarose gel showing linear RNA after in vitro transcription (IVT) and untreated and treated in vitro cyclization (IVC) RNA products with RNase R. (d) Sanger sequencing chromatogram for the junction site of the reverse-transcribed circFLuc and circRLuc samples. (e) Luciferase activity (RLU) of circFLuc and circRLuc in HEK293T cells. Statistical analysis of luciferase activity was performed at 24 h posttransfection. FLuc represents firefly luciferase. RLuc represents Renilla luciferase. RLU, relative light unit.

the translation efficiency at the indicated times, in which circFLuc^(miR) and circFLuc were transfected at a 1 : 1 ratio with circRLuc. Cells were collected at 6 h, 12 h, 24 h, and 48 h after transfection and subjected to luciferase activity measurements. The results showed that luciferase activity generated by circFLuc^(miR) with inserted miRNA binding sites was significantly lower than that of the control group at all four time points (Fig. 2f). The greatest reduction was observed at 24 h, with the circFLuc^(miR) group exhibiting only one-fifth of the control group's activity (Fig. 2f). However, the levels of circRNAs exhibited no significant difference at all four time points in both groups and remained relatively steady (Fig. 2g). We speculate that it is the miRNA binding sites within circRNAs that attract the corresponding miRNAs, thereby affecting their own translation.

Finally, to confirm that our experimental results were attributed to the inserted miRNA binding sites rather than the

sequence itself, we performed the same experiment using the mouse fibroblast 3T3 cell line, which exhibited low expression levels of miR-21-5p, miR-101-3p, and miR-192-5p. The results showed that in 3T3 cells, the luciferase activity expressed by circFLuc^(miR) containing miRNA binding sites was approximately half of the control group (Fig. S1a and b), whereas in the experimental results obtained from 293T cells, this difference was even more significant, approximately one-fourth (Fig. 2f). These findings provide evidence for the role of miRNA binding sites within circRNAs.

Overall, we concluded that the insertion of miRNA binding sites at the flanking region of the coding sequence could reduce the translation efficiency of circRNAs without affecting their circRNA levels in cultured cells.

3.3 Synonymous mutations in miRNA binding sites enhanced the translation efficiency of circRNAs

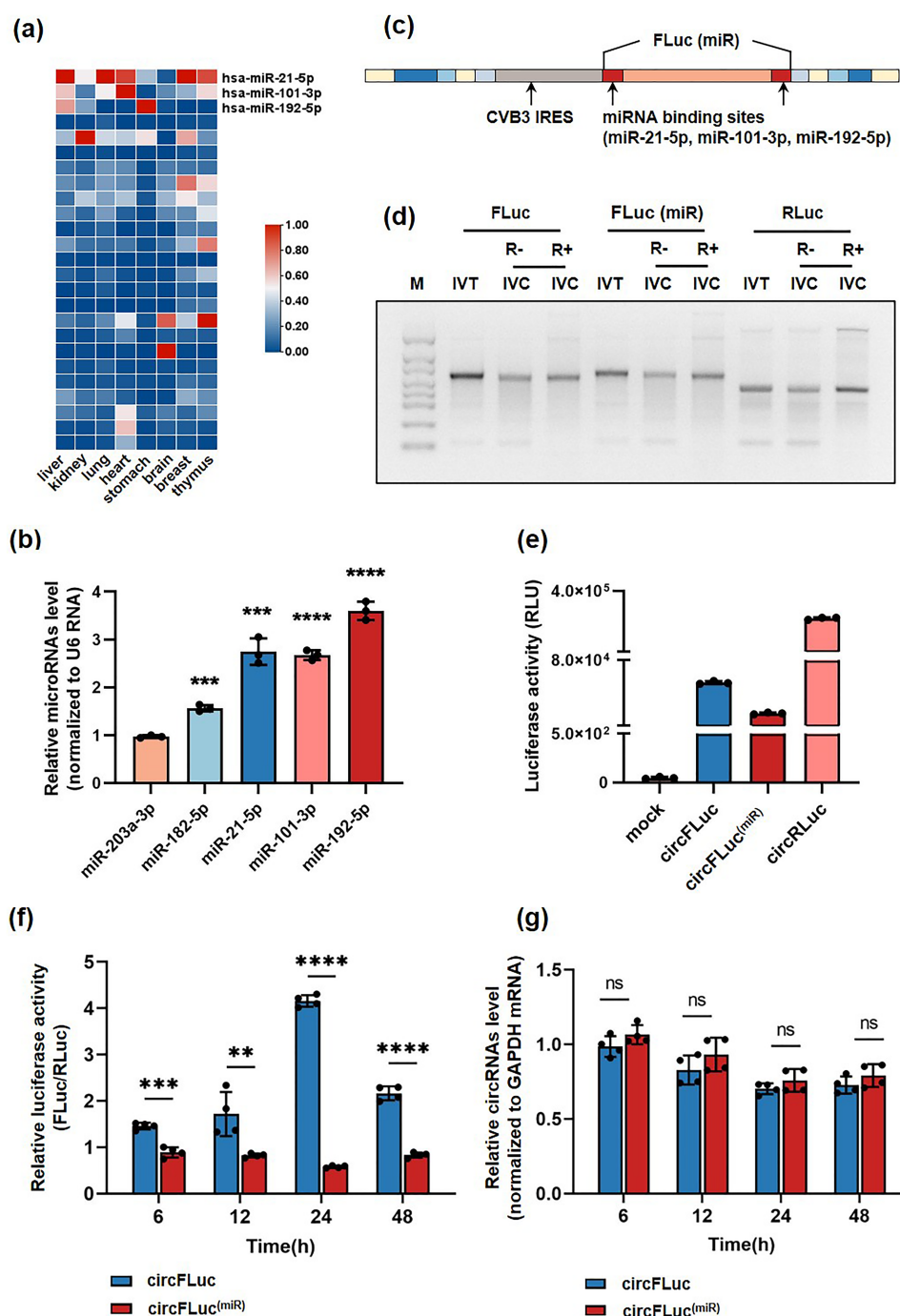


Fig. 2. Translation efficacy of circFLuc(mir) and circFLuc. (a) Heatmap displaying the expression profile of miRNAs in human healthy tissues. Data were normalized using the Z-score method. (b) Relative expression levels of microRNAs in the database were determined by RT-qPCR, with U6 RNA serving as an internal control ($n = 3$). (c) Schematic diagram of the circularized sequences after the addition of miRNA binding sites (miR-21-5p, miR-101-3p, miR-192-5p). (d) Agarose gel showing linear RNA after in vitro transcription (IVT) and untreated and treated in vitro cyclization (IVC) RNA products with RNase R. (e) Luciferase activity (RLU) of circFLuc(mir), circFLuc, and circRLuc in HEK293T cells. Activity was measured at 24 h posttransfection. (f) Relative luciferase activity of 293T cells transfected with circFLuc(mir) and circFLuc, with cotransfected circRLuc serving as an internal control, and relative firefly luciferase activity normalized to RLuc ($n = 4$). (g) Relative circRNA levels in HEK293T cells transfected with circFLuc(mir) and circFLuc, normalized to GAPDH mRNA and circRLuc ($n = 4$). Statistical analyses were performed at different time points. RLU, relative light unit. ns represents no significant difference, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To further investigate the effect of miRNA binding on circular RNA translation efficiency, we introduced synonymous mutations in the firefly luciferase coding sequence of circRNAs. We predicted the binding sites of 85 miRNAs within

the FLuc sequence and selected them for synonymous mutations based on the following principles (Table S1). Without changing the encoded amino acids, we synonymously replaced the original bases, such as A to T and C to G,

as much as possible to avoid potential binding of the corresponding miRNAs. A firefly luciferase sequence (termed FLuc^(mut)) with significantly fewer miRNA binding sites (Table S1) was obtained and used to construct the above constructs for circularization (Fig. 3a). The circularized form of FLuc^(mut) (termed circFLuc^(mut)) was assessed by gel electrophoresis and was enriched after RNase R treatment

(Fig. 3b). CircFLuc and circRLuc were used as control groups and internal references, respectively (Fig. 3b). We transfected circFLuc^(mut) into HEK293T cells to assess the circRNA translation efficiency in the presence of compromised miRNA binding via luciferase assay. The results showed that circFLuc^(mut) with synonymous mutations of miRNA binding sites, circFLuc and circRLuc stably expressed luciferase compared

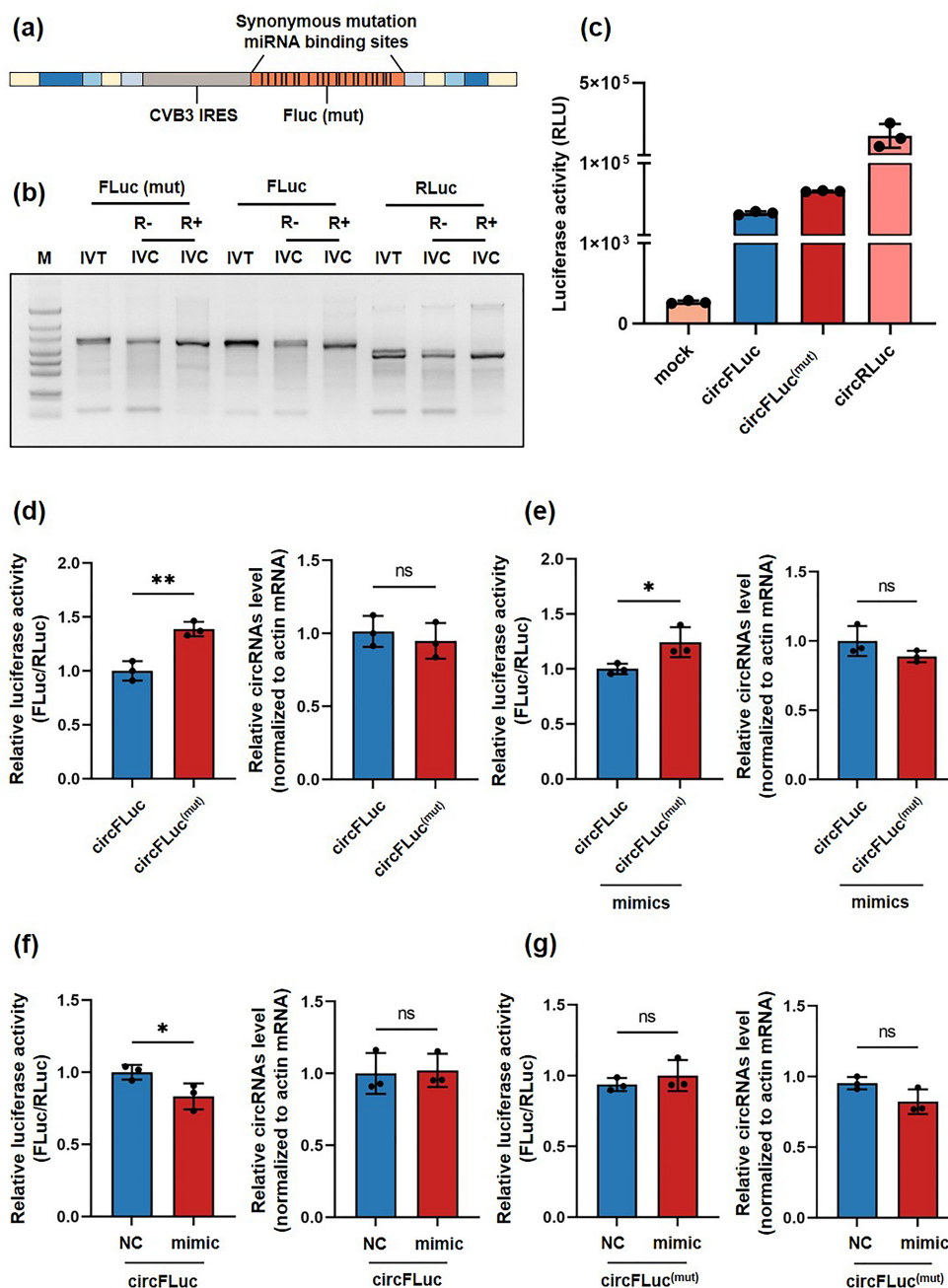


Fig. 3. Translation efficacy of circFLuc^(mut) and circFLuc. (a) Schematic illustration of circularized sequences with synonymous mutations introduced in miRNA binding sites. (b) Agarose gel showing linear RNA after in vitro transcription (IVT) and untreated and treated in vitro cyclization (IVC) RNA products with RNase R. (c) Luciferase activity (RLU) of circFLuc^(mut), circFLuc and circRLuc in HEK293T cells at 24 h posttransfection. (d–g) Left panel shows the relative luciferase activity of HEK293T cells transfected with circFLuc^(mut) or circFLuc ($n = 3$). CircRLuc was used as an internal control for cotransfection, while miR-129-5p and miR-362-5p were overexpressed through mimics (mimic). The nonoverexpressing group was transfected with NC-mimics as a control (NC). The relative luciferase activity was standardized using RLuc activity. The right panel shows the relative expression level of circRNAs in transfected cells ($n = 3$) normalized to actin mRNA and circRLuc. Statistical analysis was performed at 24 h posttransfection. RLU, relative light unit. ns, not significant, * $p < 0.05$, ** $p < 0.01$.

to the mock control (Fig. 3c), and circFLuc^(mut) had significantly higher luciferase activity compared to circFLuc (Fig. 3d (left)). No significant changes in circRNA levels were observed between circFLuc^(mut) and circFLuc, while luciferase activity was increased in circFLuc^(mut) (Fig. 3d (right)).

We noticed that miR-129-5p and miR-362-5p each have two binding sites in the firefly luciferase coding sequence according to our prediction, and they have inhibitory effects on cancers. To further validate the impacts of synonymous mutations of miRNA binding sites on circRNA translation efficiency, we designed and utilized mature miRNA mimics of miR-129-5p and miR-362-5p to overexpress the miRNAs. miRNA mimics were transfected into HEK293T cells, and the overexpression of miR-129-5p and miR-362-5p was evaluated at both 24 h and 48 h posttransfection (Fig. S2a). To eliminate the possibility that mimics would affect the cell proliferation status, the proliferation status of cells transfected with miRNA mimics was examined, which showed no significant difference in the growth rate between the cells transfected with miRNA mimics and the control group transfected with NC mimics (Fig. S2b).

We hypothesized that synonymous mutations in the firefly luciferase coding sequence could avoid the binding of miRNAs to their target sites, resulting in increased translation efficiency of circFLuc^(mut) in cells. To further verify this hypothesis, we conducted experiments with the following strategies. First, circFLuc^(mut) and circFLuc were transfected with miRNA mimics. Cotransfection of miRNA mimics did not affect the circFLuc^(mut) translation efficiency, which was still higher than that of circFLuc (Fig. 3e and Fig. S2c). Cotransfection of miRNA mimics with circFLuc enhanced the reduction of circRNA translation efficiency, with circRNA levels unchanged (Fig. 3f). Cotransfection of miRNA mimics with circFLuc^(mut) had no effects on the translation efficiency or circRNA levels (Fig. 3g). All groups were normalized to circRLuc. Taken together, synonymous mutations in miRNA binding sites can enhance the translation efficiency of circRNAs in cells without altering their RNA levels. Cotransfection of miRNA mimics reduced the translation efficiency of circRNAs with miRNA binding sites, while no changes were observed in circRNAs with synonymous mutations. This finding indicates complementary binding between overexpressed miRNAs and circRNAs harboring their respective binding sites, leading to an impact on translation efficiency, which further confirmed that modulation of miRNA binding sites within circularizing sequences affected the translation efficiency of circRNAs.

3.4 Synonymous mutation of specific miRNA binding sites enhances the translation efficiency of circRNAs

MiR-129-5p and miR-362-5p mimics were used to overexpress the miRNAs, leading to a reduction in the translation efficiency of circFLuc. To investigate the effect of miRNA binding on circRNA translation under physiological conditions, we selected the precursor miR-129-5p and miR-362-5p sequences, as well as the flanking sequences of 300 bp on each side, to construct miRNA overexpression plasmids, which undergo natural processing and maturation in cultured cells. The miRNA plasmids were transfected into HEK293T

cells, and the overexpression of miR-129-5p and miR-362-5p was validated at 24 h, 36 h, and 48 h posttransfection (Fig. S3a). The proliferation status of cells transfected with miRNA overexpression plasmids was examined, and the results showed no significant difference in the growth rate between the cells transfected with miRNA overexpression plasmids and the control group transfected with empty vectors (Fig. S3b).

To further investigate whether synonymous mutation of specific miRNA binding sites affected circRNA translation, we designed a circularized firefly luciferase coding sequence with mutated binding sites for miR-129-5p and miR-362-5p (termed FLuc^(mut miR)) (Fig. 4a). The circularized form of FLuc (mut miR) (termed circFLuc^(mut miR)) was assessed by gel electrophoresis and was enriched after RNase R treatment. circFLuc and circRLuc were used as control groups and internal references, respectively (Fig. 4b). We transfected circFLuc^(mut miR) into HEK293T cells to assess the circRNA translation efficiency via luciferase assay. The results showed that circFLuc^(mut miR) with synonymous mutations of miR-129-5p and miR-362-5p binding sites, circFLuc, and circRLuc stably expressed luciferase compared to the mock control (Fig. 4c). In addition, circFLuc^(mut miR) had significantly higher luciferase expression compared to circFLuc (Fig. 4d (left)), without affecting the circRNA levels (Fig. 4d (right)). Of note, synonymous mutations of miR-129-5p and miR-362-5p binding sites exhibited comparable or even higher effects with synonymous mutations of all miRNA binding sites (Fig. 3c and d), suggesting that the modulation of specific miRNA binding could also exert the same effect on circRNA translation efficiency.

We next assessed the effects of plasmid overexpression of miR-129-5p and miR-362-5p on circRNA translation. We found that circFLuc^(mut miR), which had mutated binding sites for miR-129-5p and miR-362-5p, exhibited a more than twofold increase in luciferase expression compared to circFLuc (Fig. 4d). The same results were also observed upon overexpression of miR-129-5p and miR-362-5p in circFLuc and circFLuc^(mut miR) (Fig. 4e and Fig. S3c). However, overexpression of miR-129-5p and miR-362-5p in circFLuc significantly reduced luciferase expression (Fig. 4f), while overexpression of these miRNAs in circFLuc^(mut miR) did not affect circRNA translation efficiency, further indicating that miRNA binding to circFLuc affected circRNA translation and that mutations of the binding sites avoided these effects. No significant differences were observed in the levels of circRNAs from all groups, indicating that the circRNA levels in cells did not affect circRNA translation. Collectively, synonymous mutation of miR-129-5p and miR-362-5p binding sites can also enhance the translation efficiency of synthetic circRNAs in cultured cells.

3.5 Mutations of miRNA binding sites enhance the in vivo translation efficiency of circRNAs

We showed that synonymous mutations of miRNA binding sites enhanced the translation efficiency of synthetic circRNAs in cultured cells (Figs. 3 and 4). To further investigate the in vivo effect of circRNA translation and to explore the potential biomedical applications, we conducted in vivo

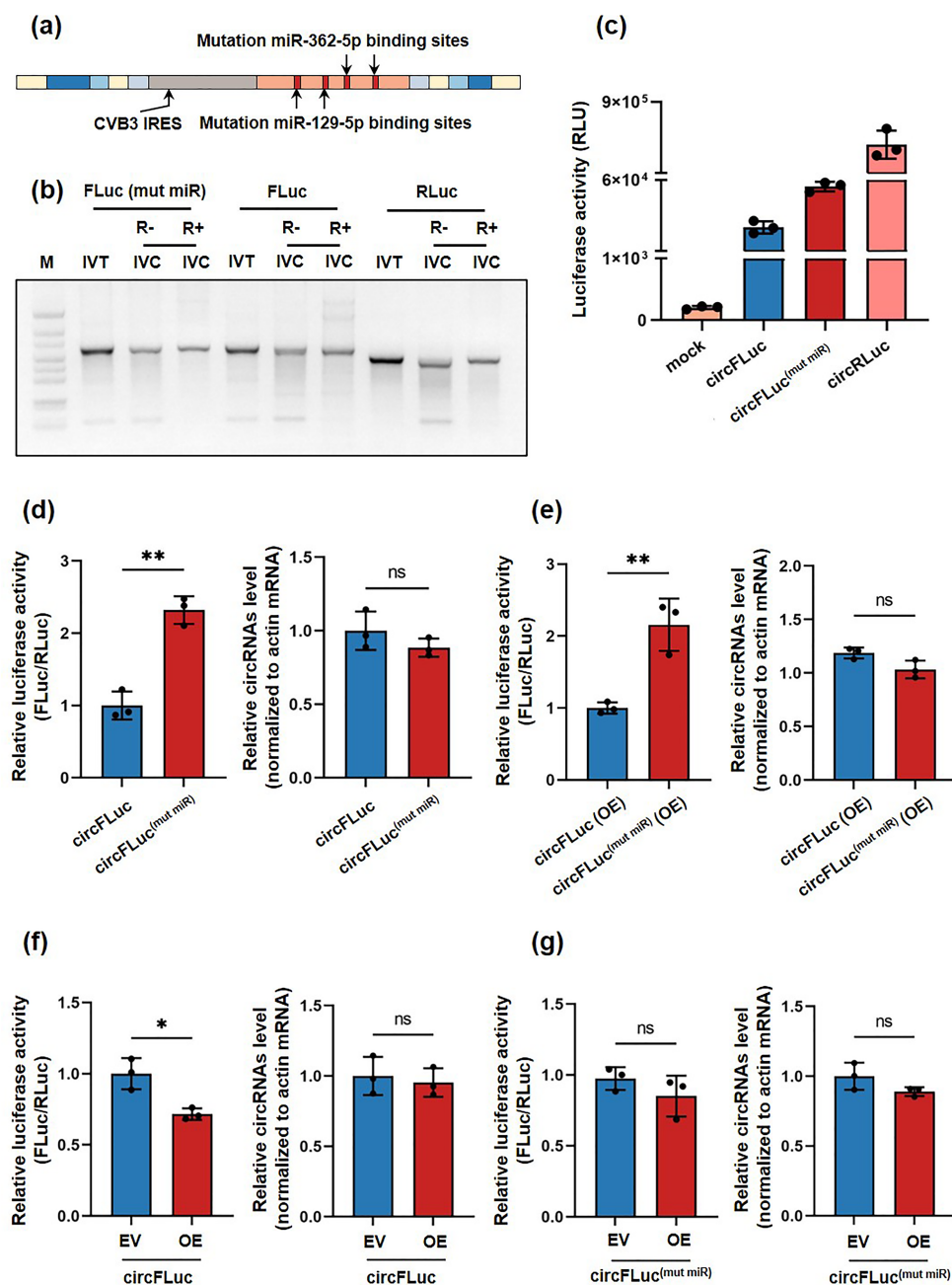


Fig. 4. Translation efficacy of circFLuc^(mut miR) and circFLuc. (a) Schematic diagram of the circularized sequence with synonymous mutations in the miR-129-5p and miR-362-5p binding sites. (b) Agarose gel showing linear RNA after in vitro transcription (IVT) and untreated and treated in vitro cyclization (IVC) RNA products with RNase R. (c) Luciferase activity (RLU) of circFLuc^(mut miR), circFLuc, and circRLuc in HEK293T cells, with detection of luciferase activity at 24 h posttransfection. (d–g) The left panel shows the relative luciferase activity of HEK293T cells ($n = 3$) transfected with circFLuc^(mut miR) or circFLuc. CircRLuc was used as an internal control for cotransfection, while miR-129-5p and miR-362-5p were overexpressed through plasmids (OE). The nonoverexpressing group was transfected with an empty plasmid backbone as a control (EV). The relative luciferase activity was standardized using RLuc activity. The right panel shows that the relative circRNA level was standardized using actin mRNA and circRLuc. Statistical analysis was performed at 24 h posttransfection. RLU, relative light unit. ns, no significance, * $p < 0.05$, ** $p < 0.01$.

experiments using circFLuc^(mut miR) in nude mice using bioluminescence imaging. circRNAs were transfected into HepG2 cells, and the cells were then injected subcutaneously into nude mice. Live bioluminescence imaging was used to observe and measure luciferase activity in vivo at the indicated times (Fig. 5a).

To test whether the cells transfected with translatable circRNAs were suitable for subsequent bioimaging experiments

in vivo, we first transfected circFLuc and circFLuc^(mut miR) into HepG2 cells and collected them for luminescence imaging after 24 h (Fig. 5b). Compared to the mock control, cells transfected with circFLuc or circFLuc^(mut miR) showed significant luciferase activity (bioluminescence imaging signal). Moreover, luciferase activity increased with cell number and was positively correlated with the number of cells (r^2 of circFLuc group and circFLuc^(mut miR) group were 0.9965 and

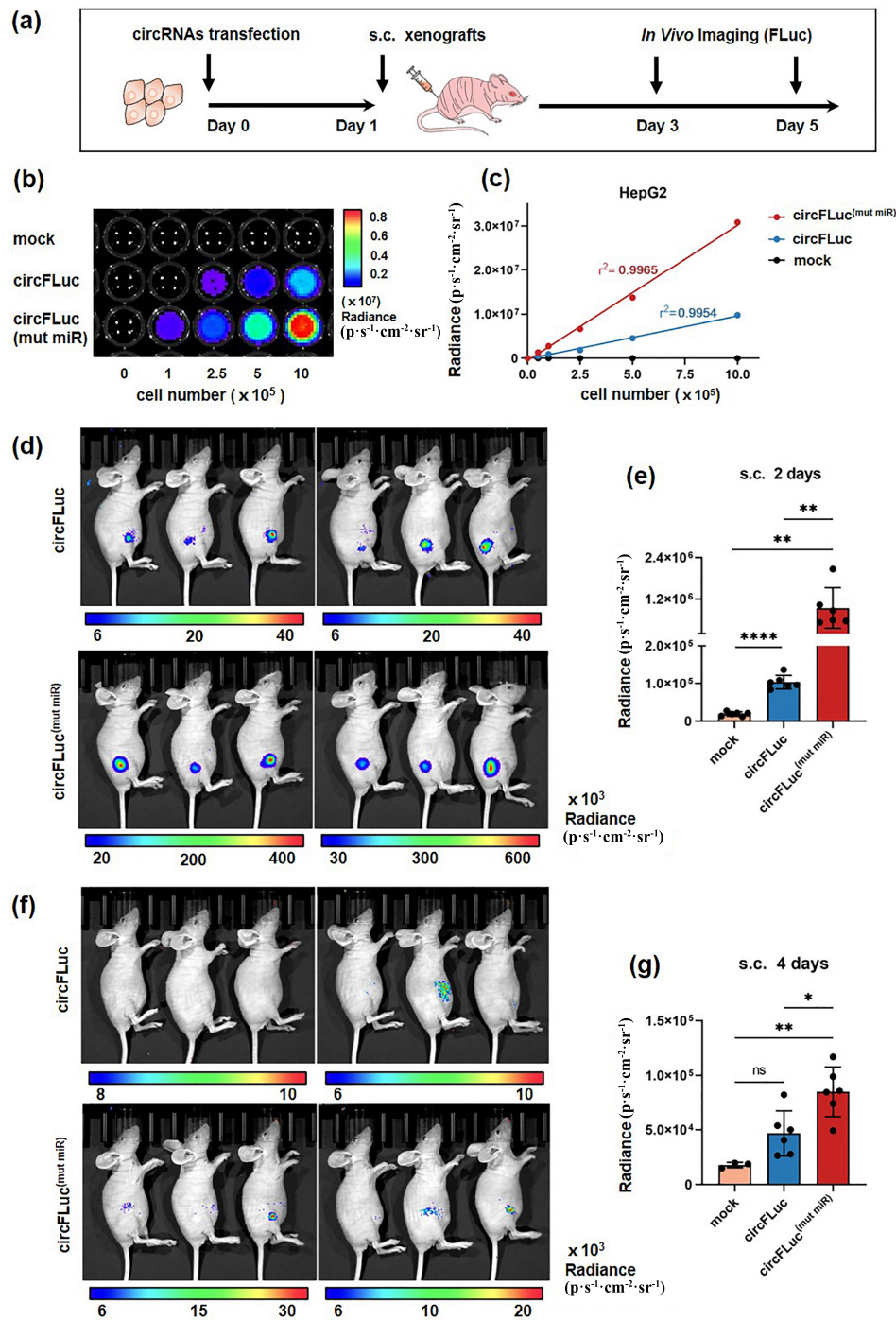


Fig. 5. In vivo translation efficiency of circFLuc^(mut miR) and circFLuc. (a) Schematic diagram of the in vivo experiment in nude mice. (b) Bioluminescence imaging of HepG2 cells (top), HepG2-circFLuc cells (middle), and HepG2-circFLuc^(mut miR) cells (bottom) was performed. Luciferase activity was detected 24 h after transfection of different cell numbers. Images show color-coded maps of photon flux superimposed on black-and-white photographs of the assay plates. (c) The statistical results of (b) show that the bioluminescence values of circFLuc^(mut miR) and circFLuc are linearly correlated with the number of cells, with r^2 values of 0.9965 and 0.9954, respectively. Luminescence values in the mock group did not change with increasing cell number. (d) In vivo bioluminescence image of mice subcutaneously injected with HepG2-circFLuc (top) and HepG2-circFLuc^(mut miR) (bottom) and measured for luciferase activity 2 d post-injection ($n = 6$ per group, 10^6 cells per mouse). (e) Statistical analysis of the in vivo luminescence values from (d). (f) In vivo bioluminescence image of mice subcutaneously injected with HepG2-circFLuc (top) and HepG2-circFLuc^(mut miR) (bottom) and measured for luciferase expression 4 d post-injection ($n = 6$ per group, 10^6 cells per mouse). (g) Statistical analysis of the in vivo luminescence values from (f). ns, no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

0.9954) (Fig. 5c). Consistent with the results of the cell experiments, the translation efficiency of circFLuc^(mut miR), as

revealed by luciferase activity, was three times higher than that of circFLuc with the same cell number (Fig. 5c).

To investigate whether the regulation of circRNA translation via the modulation of miRNA binding sites could be used in vivo as a biomedical imaging tool, we conducted in vivo imaging experiments using 5-week-old female BALB/c nude mice. Compared to the mock group, both the circFLuc and circFLuc^(mut miR) groups showed significant luciferase activity two days after cell injection, in which luciferase activity in the circFLuc^(mut miR) group was significantly higher than that in the circFLuc group (Fig. 5d and Fig. S4 (left)). Despite being weaker, luciferase signals could also be detected four days after cell injection, and luciferase activity in the circFLuc^(mut miR) group was consistently higher than that in the circFLuc group (Fig. 5e and Fig. S4 (right)).

Taken together, circRNAs with translation potential could be translated in vivo and visualized through bioimaging. Modulation of specific miRNA binding sites may affect the translation efficiency of circRNAs in vivo, which could be used as a therapeutic indicator or drug.

4 Conclusions

In our study, we utilized firefly luciferase as a coding protein marker and viral CVB3 IRES as a translation initiator for in vitro and in vivo translation of synthetic circRNAs. We confirmed that translation efficiency could be reduced by introducing miRNA binding sites, and synonymous mutations of the miRNA binding sites enhanced the translation efficiency. We also validated that synonymous mutations of specific miRNAs could exert a comparable effect. In vivo experiments revealed that the translation efficiency of circRNAs with mutations of miRNA binding sites was markedly higher, as much as 8 times, than that of the control group. We

conclude that synonymous mutations in miRNA binding sites of circRNA coding sequences can effectively enhance their translation efficiency both in vitro and in vivo, providing a promising strategy for future investigations and applications of circRNAs with coding potential (Fig. 6).

In vitro circularization of translatable circRNAs from linear precursors is a prerequisite step for subsequent investigations on circRNA translation. We utilized the widely used PIE system, a highly efficient self-cleavage system optimized for intron sequences, for our downstream circularization, with the longest sequence used in this study being as long as 2.6 kb^[34]. This system facilitates the circularization of long RNA sequences under certain conditions after optimizing the intron sequences and adding auxiliary elements such as homologous arms. Previous studies have successfully circularized Gaussia luciferase-encoding RNA with this method and efficiently expressed it in mouse visceral tissues^[43]. More optimizations and even novel strategies for circRNA circularization should be further investigated in the future.

IRES uses stable and dynamic RNA structures to recruit ribosomes driving protein synthesis and is crucial for translation initiation. Viral IRESs, such as the viral CVB3 IRES, are popular in current mechanistic studies and RNA translation applications^[44]. We hence used the viral CVB3 IRES to initiate circRNA translation, and subsequent experiments confirmed that the CVB3 IRES could effectively initiate circRNA translation at both the cellular and animal levels. Since most of the recognized IRESs have higher activity in initiating translation, other viral IRESs for circRNA translation initiation can also be assessed in the future, including type I IRESs with higher translation initiation activity^[45] or IRES-like short sequences with translation initiation functions that

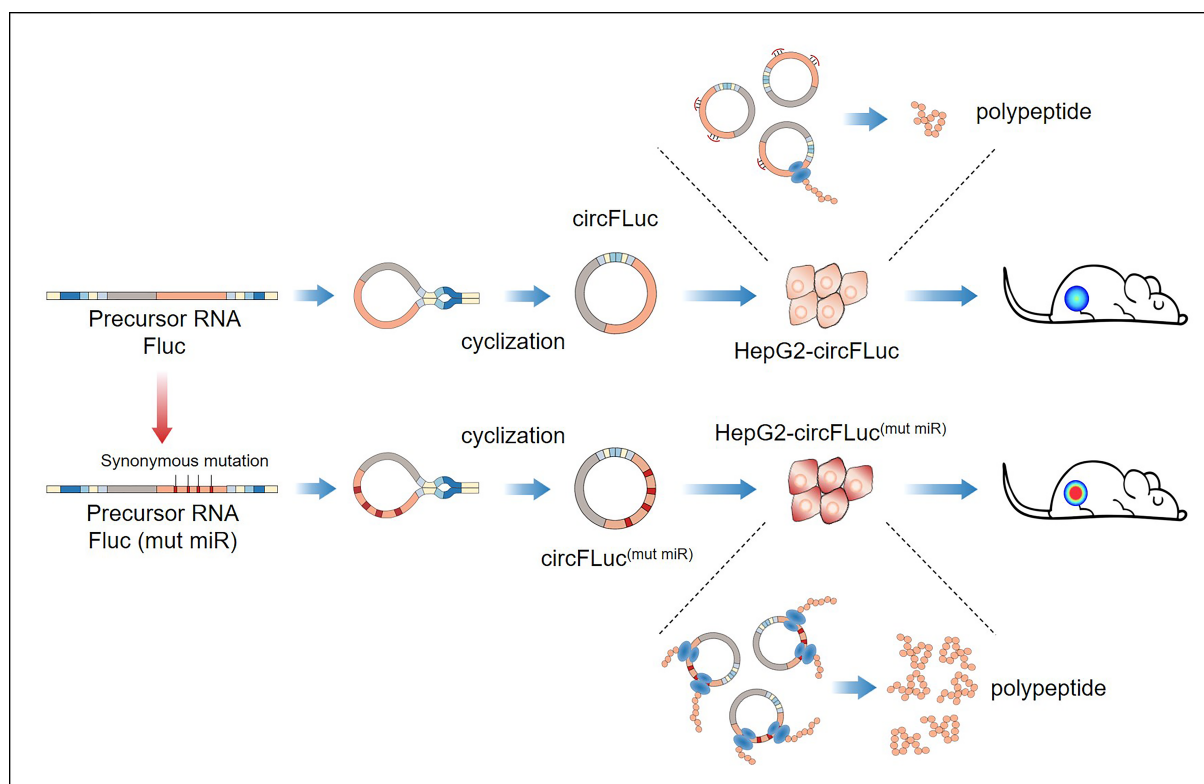


Fig. 6. A simplified summary of this study, in vitro synthesis of circRNA and related changes in miRNA binding sites and their roles in vitro and in vivo.

are more favorable for in vitro circularization^[46]. In addition, circRNA translation is multifaceted and complicated and is regulated by various factors. For instance, complementarity to 18S rRNA and structured RNA elements located on the IRES are critical for driving endogenous circRNA translation^[13]; different lengths of exons are also factors for circRNA translation^[47], which can also be considered in the future optimization of circRNA translation.

The synonymous mutations of miRNA binding sites in the circRNA sequence to enhance the translation efficiency of circRNA are a highlight of our study. It is well known that circRNAs can act as miRNA sponges by binding miRNAs via complementarity binding to affect downstream pathways^[16–19]. MiRNAs binding to circRNAs can also initiate Ago2 protein cleavage of circular antisense RNA^[20] to exert biological effects. Therefore, we propose that miRNAs binding to circRNAs may affect circRNA translation. miRNAs that are enriched in tissues were selected, and insertion of these miRNA binding sites at the flanking region of the coding sequence could reduce the translation efficiency of circRNAs. Moreover, synonymous mutations of miRNA binding sites improved the translation efficiency of circRNAs. Furthermore, we found that both in vitro and in vivo, synonymous mutation of miR-129-5p and miR-362-5p binding sites can enhance the translation efficiency of synthetic circRNAs, with an even greater impact (Figs. 4 and 5), which indicates that modulation of miRNA binding sites, at least certain critical ones, may affect the translation efficiency of circRNAs.

In vivo translation of circRNA encoding luciferase and the modulation of translation efficiency via miRNA binding demonstrated a potentially druggable use in clinical imaging and therapy. We observed that mutating miRNA binding sites could enhance the translation efficiency of circRNA in vivo. CircRNAs with coding potential have been widely applied. For instance, circRNA vaccines encoding the Spike protein receptor-binding domain against SARS-CoV-2 have been developed^[11]; circRNAs with unmodified IRES, delivered via nanoparticles, have a longer translation duration than linear counterparts in cells and mouse adipose tissue^[34,43]. In addition, circRNAs encoding antigens encapsulated in LNPs trigger strong innate and adaptive immune activation and display excellent antitumor efficacy in multiple mouse tumor models^[48], which all provide novel prospects for translatable circRNA applications^[11,49]. Unlike previous studies, we directly injected cells transfected with circRNA into mice subcutaneously, enabling the translation of circFLuc and circFLuc^(mut miR) in vivo. The combination of bioluminescence imaging visually compared the translation efficiency of luciferase. Further investigations are required to improve the circRNA duration time, increase circRNA stability in vivo and search for the miRNA binding sites that exert the best effect on circRNA translation. Clinical use via modulation of functional miRNA binding to the circRNAs either by visualizing the translation efficiency through bioluminescence imaging as prognostic indicators or regulating the downstream pathways as drugs may also expand the potential of translatable circRNAs.

In summary, we obtained synthetic circRNAs with translation potential using an optimized in vitro circularization

method and enhanced the translation efficiency of circRNAs through synonymous mutations of miRNA binding sites in the coding sequences in vitro and in vivo, which might be used as versatile tools in future clinical imaging for both clinical prognosis and diagnosis.

Supporting information

The supporting information for this article can be found online at <https://doi.org/10.52396/JUSTC-2023-0048>. The supporting information includes 4 figures and 1 table which lists the sequences and primers used in this study.

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Conflict of interest

The authors declare that they have no conflict of interest.

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