

## RESEARCH ARTICLE

# Generation of MiRNA sponge constructs targeting multiple MiRNAs

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**Abstract**

**Background:** MicroRNAs(miRNAs) are key regulators of gene expression in plants, animals and some viruses. Hence, alteration of miRNA levels in cells or tissues is common for miRNA studies. Loss-of-function of miRNA can be achieved using antisense oligonucleotides, sponges and gene knockout models.

**Methods:** Here, we showed an efficient, rational and economical way to construct multi-targeted miRNA sponges with desired copies. Four copies of miRNA sponge are used as “building-blocks”.

**Results:** These building-blocks, which can target same miRNA or different miRNAs, are linked together through ligation. Each time of ligation can double the number of sponge copies.

**Conclusions:** In this way, we constructed lentivirus vectors harboring sponges targeting miR-21, miR-31 and miR-155 and the combination of two miRNA sponges can inhibit cancer cell growth significantly.

**KEYWORDS**

cancer, miRNA, sponge

## 1 | INTRODUCTION

MicroRNAs (miRNAs), mediating translation through partially base pairing to complementary sequences in the 3' untranslated regions (3' UTR) of target mRNAs, involve in various physical processes or disease development.

*lin-4* and *let-7* (*let-7*), the first two discovered miRNAs, were originally found to regulate the division and differentiation of stem cells in *Caenorhabditis elegans*.<sup>1-3</sup> The finding that transgenic overexpression of miR-155 induces acute lymphoblastic leukemia/high-grade lymphoma in mice implied the tumorigenic roles of miRNAs.<sup>4</sup> Furthermore, miR-21<sup>LSL-Tetoff</sup> knock-in mouse model showed that overexpression of miR-21 causes the initiation and maintenance of tumor. Complete tumor regression after the withdrawal of miR-21

illustrated that the tumor addicts to miR-21 expression.<sup>5</sup> These findings suggested that tumor can be initiated by and addicted to aberrant expression of miRNA. The function of miRNA in cancerigenesis has subsequently been widely studied and the possibility of miRNAs to be used as biomarkers or therapeutic targets has also been discussed in numerous researches.<sup>6-8</sup>

Modulating miRNA levels in cells, tissues or even whole organisms is widely used to study the role of miRNAs in physiology or pathology processes. Loss function of miRNA can be achieved by chemically modified antisense oligonucleotides, small RNA zippers, miRNA-knockout mouse models, miRNA masks and miRNA sponges.<sup>7,9-12</sup> Oligonucleotides and small RNA zippers are more suitable for short-term than long-term experiments, due to DNA oligos degradation.<sup>13</sup> Combination therapy of antisense oligonucleotides targeting 2 or 3

Junjin Jie and Danni Liu contributed equally to this work.

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miRNAs has been reported but only in vitro.<sup>14,15</sup> Knockout mouse models are both time and cost-consuming work. Another challenge for miRNA knockout mouse model is certain miRNAs located inside protein coding genes and some have more than one copies in the genome.<sup>16</sup> Knockout of miRNAs inside protein coding genes not only silences miRNAs but also affects associated genes. The efficacy of miRNAs knockout which having more than one copies in the genome is relatively low. MiRNA sponges based on retroviral vector are able to knockdown miRNA in long-term experiments.<sup>12,16</sup> They can also block an entire miRNA seed family using one vector.<sup>12</sup>

The cloning strategy by high ratio ligation can be used for miRNA sponge constructs with more than 20 miRNA binding sites.<sup>16</sup> The procedure is convenient but the number of sponge copies is not accurate and the direction of inserted fragment is uncertain. This decreases the possibility to obtain desired miRNA sponge constructs. Here, we describe a new cloning method to construct miRNA sponge targeted to one or more miRNAs with accurate copy number of sponges based on lentiviral vector.

## 2 | MATERIALS AND METHODS

### 2.1 | Cloning of miRNA sponges

EGFP CDS is amplified by PCR using KOD-plus-neo kit (TOYOBO, Japan). Synthesized oligonucleotides (Sangon, China) are denatured, annealed and extended to form the DNA fragments with four miRNA sponges. Elongation of Oligonucleotides is conducted by Taq™ enzyme (Takara,). Restriction enzymes and T4 DNA ligase are bought from NEB. GenClean agarose gel DNA recovery kit (Generay,) is used to recycle the DNA fragments. Plasmids are validated by restriction enzyme digestion and direct sequencing (Invitrogen).

### 2.2 | Virus packing

The HEK-293T cell line which was purchased from the cell bank of Chinese Academy of Sciences (Shanghai) was cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (PAN Biotech, MA). Plasmids transfection is the same as described previously.<sup>17</sup> Lentivirus is collected, filtered and stored at -80°C 60h later. The titer of the miRNA sponge lentivirus was measured with a gradient virus infection of HEK-293T cells to obtain the most suitable concentration for infecting lung adenocarcinoma cells.

### 2.3 | RNA extraction and Reverse transcription

After washing the cells with PBS twice, total RNA was extracted from cultured cells with TRIzol (Thermo-scientific,) according to manufacturer's protocol. Total RNA was fully dissolved in DNase/RNase-free water. 1 µg total RNA was reversely transcribed to cDNA with the FastQuant RT Kit (Tiangen,).

### 2.4 | Quantitative real-time PCR

Quantitative polymerase chain reaction (qPCR) was performed by using the Bestar qPCR Master Mix (DBI Bioscience, German) in LightCycler 480 II system (Roche,). Primers were designed and synthesized by Sangon Biotech. GAPDH was used as control. The sequences of primers are listed in Table 1.

### 2.5 | Transfection of miRNA inhibitors

Cells were seeded in six-well plates one day before and used for transfection when they achieved ~70% confluent. 200pmol miRNA inhibitors (RiboBio,) were transfected into cells with X-tremeGENE™ HP (Merck KGaA,) according to the reagent protocol.

### 2.6 | Cell proliferation assays

For cell proliferation assay, the infected cells were seeded into 96-well plates at a density of 1000 cells per well. At 0, 24, 48, 72 and 96h after seeding, relative cell growth was measured by the cell titer 96 Aqueous one solution reagent (Promega,) according to the manufacturer's instructions. Briefly, each well was added with 20 ul solution and the plate was incubated at 37°C for 4 h. Absorbance at 490nm of each well was measured by using Thermo Fisher Multiskan FC (Thermo Fisher,).

### 2.7 | Western blot analysis

Protein lysates were extracted from cells by incubating with RIPA lysis and extraction buffer. Protein concentration was determined using the BCA method. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp,) and incubated overnight at 4°C with primary antibodies followed by blocking with skim milk power (5%, v/v). The primary antibodies included PDCD4 (1:1000, Anti-PDCD4 rabbit polyclonal antibody; Sangon Biotech BBI, CHINA), GAPDH (1:1000, rabbit pAb to GAPDH; Abcam,). Then, the membranes were incubated for 120 min at room temperature with the secondary antibody (1:2000, Sangon Biotech BBI,). Reacting bands were visualized using chemiluminescent HRP substrate (Millipore Corp,), and the density of the protein bands was semi-quantified using the software ImageJ.

### 2.8 | Statistical Analysis

All data are displayed as the mean±SD (standard deviation). Statistical comparisons between different groups or two groups were evaluated by one-way ANOVA test or t-test comparison. Statistical significance was determined with a *p*-value <0.05.

TABLE 1 Primers of quantitative real-time PCR

MiRNA	TARGET GENE	PRIMER
miR-155	SGK3-qPCR-F	CAGGAGTGAGTCTTACAG
	SGK3-qPCR-R	AATGGTGGTGAATCTTC
miR-155	RACK1-qPCR-F	GCCTTCTCCTTGACAACC
	RACK1-qPCR-R	AAGCGGACACAAGACACC
miR-155	E2F2-qPCR-F	AAGTGCATCAGAGTGGAT
	E2F2-qPCR-R	AGTGTACATACCGAGTCTTC
miR-31	ARID1A-qPCR-F	CCTGAAGAACTCGAACGGGA
	ARID1A-qPCR-R	CGGCTCCGTGAGGTTATTG
miR-31	RhoBTB1-qPCR-F	GGAGTGAAGGAGCCTGTGAG
	RhoBTB1-qPCR-R	TGCCAATGAACCCCTTACTC
miR-21	PDCD4-qPCR-F	GGCCTCAAGGAGTAAGACC
	PDCD4-qPCR-R	AGGGGTCTACATGGCAACTG
miR-21	FZD6-qPCR-F	CCCAGCACAAATGAAGATCAA
	FZD6-qPCR-R	ACATCTGCTGGAAGGTGGAC
miR-21	PTEN-qPCR-F	GACGAAGTGGTGAATGATATG
	PTEN-qPCR-R	GTGCCACTGGTCTATAATCC
GAPDH	GAPDH-qPCR-F	GCGACACCCACTCCTCCACCTTT
	GAPDH-qPCR-R	TGCTGTAGCCAAATTCGTTGTCATA
miR-21&miR-31	TIAM1-qPCR-F	AAGACGTAAGGATGATGATG
	TIAM1-qPCR-R	GACCCAAATGTCGCAGTCAG

### 3 | RESULTS

#### 3.1 | Design of miRNA sponges

In order to achieve long-term miRNA sponge expression, we first made a lentivirus vector harboring EGFP coding sequence with an extra *Bcl* I site at 3' end into the multiple cloning sites of pCDH-CMV-MCS-EF1-puro. As non-perfect pairing sponge sequence has better inhibition efficacy,<sup>12</sup> miRNA sponge sequence is complementary to the miRNA sequence with 3 nucleotides that are mismatched at position 9–11 from the 5' end of miRNA. Four sponge sequences are separated by a short "spacer", 8 nucleotides. A 5' *Eco* R I and a 3' *Bam* H I are added to the ends of the sequence to ease subcloning. The sequence is then divided into sense and anti-sense sequences for synthesis. They have 16 nucleotides overlap which can be used to anneal and elongate a full-length oligonucleotide duplex (Figure 1A).

#### 3.2 | Construction of multiple miRNA sponges

The pCDH-CMV-EGFP-EF1-puro vector was digested with *Eco* R I and *Bam* H I. Sense and antisense oligonucleotides were mixed, denatured, annealed and elongated and then also digested with *Eco* R I and *Bam* H I. Then, the oligonucleotide duplex with sticky ends was inserted into the vector to form pCMV-EGFP-4xsponge-EF1-puro. After the sequence was checked by direct sequencing, the 4xsponge

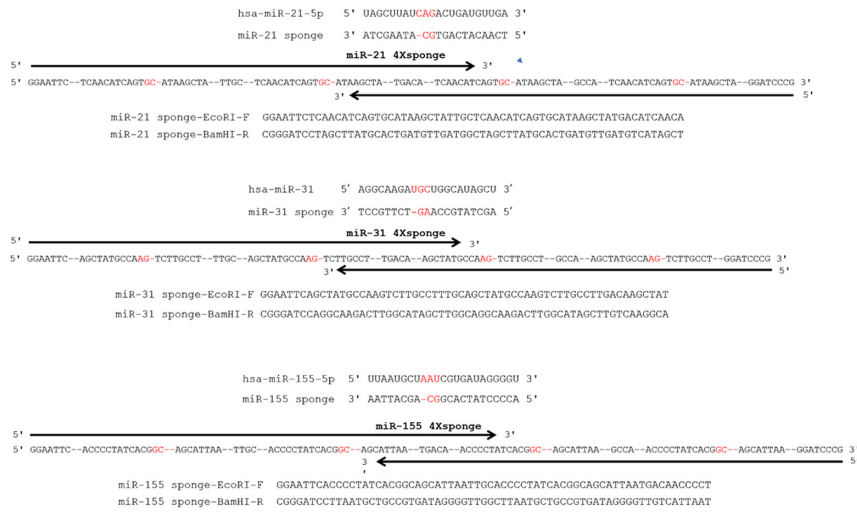
sequence was cut from it with *Bcl* I and *Sal* I and the backbone was digested with *Bam* H I and *Sal* I. Then, the 4xsponge sequence was ligated to the backbone to form pCMV-EGFP-8xsponge-EF1-puro (Figure 1B). Since *Bam* H I and *Bcl* I are isocandamers, the sponge sequence in the plasmids can be doubled through subcloning and sponge sequence targeting different miRNA can be set in one plasmid as designed.

MiR-21, miR-31 and miR-155 were reported as oncomiRs in several types of cancer. Sponges targeting to these miRNAs were constructed to test this method. pCMV-EGFP-16xmiR-21-sponge-puro, pCMV-EGFP-16xmiR-31-sponge-puro, pCMV-EGFP-16xmiR-155-sponge-puro, pCMV-EGFP-16xmiR-21&miR-31-sponge-puro and pCMV-EGFP-16xmiR-31&miR-155-sponge-puro have been constructed through subcloning. The plasmids were confirmed with double digestion with *Xba* I and *Sal* I (Figure 1C). These plasmids were then co-transfected with pCMV-dR8.2 and pCMV-VSVG into HEK-293T cells for lentivirus production.

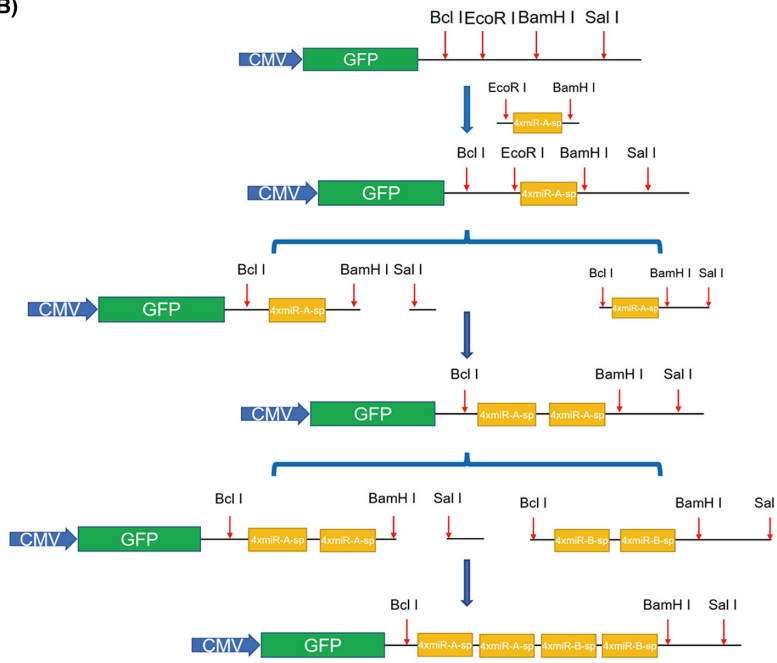
#### 3.3 | Validation of miRNA sponges' efficacy

In order to test the efficacy of these miRNA sponges and the combination of sponges targeting different miRNAs, we first test the expression levels of hsa-miR-21-5p, hsa-miR-31-5p and hsa-miR-155-5p in lung cancer cells lines and BEAS-2B cells which is derived from normal human bronchial epithelium. The expression levels of hsa-miR-31-5p and hsa-miR-155-5p in CRL-5810 cells are

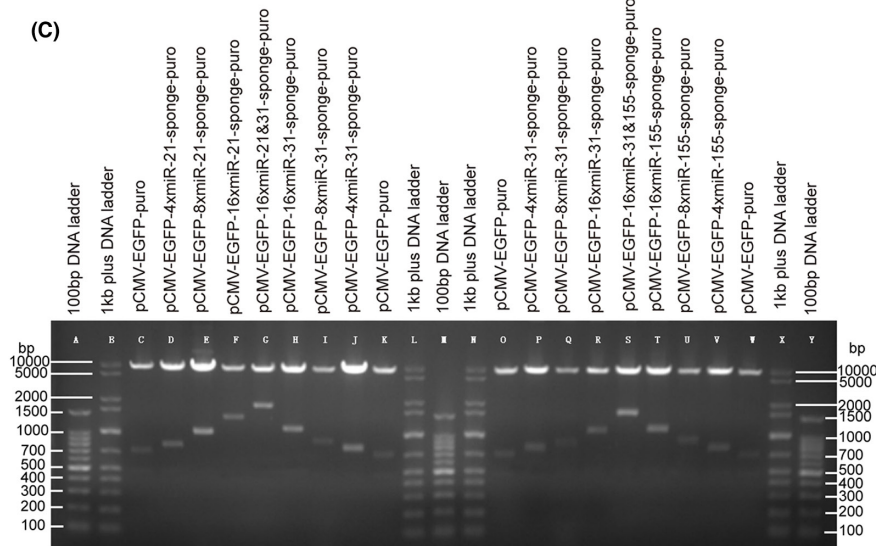
(A)



(B)



(C)



**FIGURE 1** Design of miRNA sponges and construction of multiple miRNA sponges. (A) Four sponge sequences are separated by a short “spacer”, eight nucleotides. A 5' *EcoRI* and a 3' *BamHI* are added to the ends of the sequence to ease subcloning. Sense and anti-sense sequences have 16 nucleotides overlap which can be used to anneal and elongate a full-length oligonucleotide duplex. The sequence of has-miR-21-5p, miR-21 sponge and sequence design of 4xmiR-21 sponge. The sequence of has-miR-31-5p, miR-31 sponge and sequence design of 4xmiR-31 sponge. The sequence of has-miR-155-5p, miR-155 sponge and sequence design of 4xmiR-155 sponge. (B) Schematic illustration shows the synthesis of multiple miRNA sponges. The pCDH-CMV-EGFP-EF1-puro vector was digested with *EcoRI* and *BamHI*. Sense and antisense oligonucleotides were digested with *EcoRI* and *BamHI*. Then, the oligonucleotide duplex with sticky ends was inserted into the vector to form pCMV-EGFP-4xsponge-EF1-puro. The 4xsponge sequence was cut from it with *BclI* and *SalI* and the backbone was digested with *BamHI* and *SalI*. Then, the 4xsponge sequence was ligated to the backbone to form pCMV-EGFP-8xsponge-EF1-puro. (C) DNA electrophoresis analysis of double digestion of constructed vectors. A, M, Y:100bp DNA ladder; B, L, N, X: 1 kb plus DNA ladder; C, K, O, W: pCDH-EGFP-puro; D: pCDH-EGFP-4xmiR-21-puro; E: pCDH-EGFP-8xmiR-21-puro; F: pCDH-EGFP-16xmiR-21-puro; G: pCDH-EGFP-16xmiR-21&16xmiR-31-puro; H, R: pCDH-EGFP-16xmiR-31-puro; I, Q: pCDH-EGFP-8xmiR-31-puro; J, P: pCDH-EGFP-4xmiR-31-puro; S: pCDH-EGFP-16xmiR-31&16xmiR-155-puro; T: pCDH-EGFP-16xmiR-155-puro; U: pCDH-EGFP-8xmiR-155-puro; V: pCDH-EGFP-4xmiR-155-puro

significantly higher than that in BEAS-2B. The levels of hsa-miR-21-5p and hsa-miR-31-5p in A549 are significantly higher than BEAS-2B (Figure 2A-C).

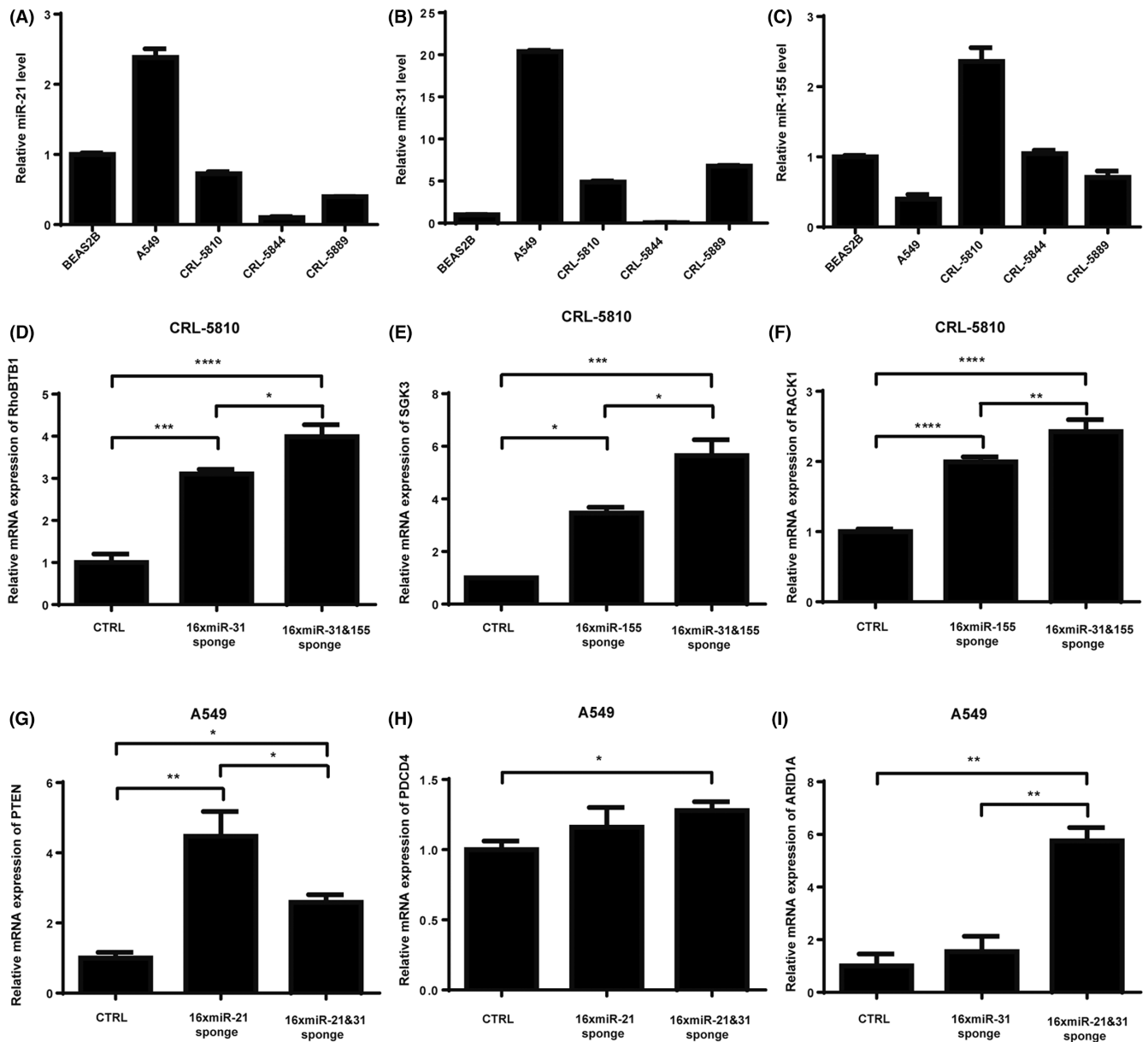
Next, miRNA sponges were used to decrease the levels of these miRNAs in CRL-5810 and A549 cells. CRL-5810 cells were infected with lentivirus expressing sponge sequences of miR-31 or miR-155 separately or both. After infection, cells were treated with puromycin for stable expression of sponge sequences. The levels of miR-31 and miR-155 did not change significantly in the cells expressing sponges. Perhaps, it is because the sponges tend to sequester miRNAs rather than induce degradation of them. Then, we tried to figure out the efficacy of miRNA sponges by measuring the expression levels of genes targeted by these miRNAs. MiR-31 has been reported that it can inhibit the expression of ARID1A, RhoBTB1, miR-155 can inhibit RACK1 and SGK3 and miR-21 can inhibit PDCD4 and PTEN.<sup>18-23</sup> We tested the mRNA levels of these genes. The results showed that SGK3 and RACK-1 mRNA levels are significantly increased in CRL-5810 cells with miR-155 sponges (Figure 2E-F). So does the mRNA of RhoBTB21 in CRL-5810 cells with miR-31 sponges (Figure 2D). PTEN and PDCD4 mRNA were increased in A549 cells with miR-21 sponges (Figure 2G-H). Plus, the relative protein levels of PDCD4 in A549 cells with 16xmiR-21 sponges and 16xmiR-21&16xmiR-31 sponges were also increased (Figure 3A). Hence, the miRNA sponges can inhibit the function of targeted miRNAs and increase the expression of miRNA target genes. TIAMA1 is co-targeted by miR-21 and miR-31.<sup>24</sup> The relative mRNA level of TIAM1 in A549 cells with 16xmiR-21&16xmiR-31 sponges was significantly higher compared to the single-targeted group and the control group (Figure 3B).

In order to test whether the inhibition of oncomiRs influences the cell growth, we tested cell growth of CRL-5810 cells infected with miR-31 or miR-155 sponges separately or both. The result demonstrated that sponges of miR-31 or miR-155 alone do not inhibit cell growth, whereas sponges of miR-31 and miR-155 together can inhibit cell growth (Figure 3C). Sponges of miR-21 and miR-31 together can inhibit cell growth, too (Figure 3E). The effect of sponges is more obvious compared with miRNA inhibitors (Figure 3D, F). These results indicate that this method can be used to check the function of miRNA sponge combinations.

## 4 | DISCUSSION

Here, we illustrated a method to establish desired miRNA sponge with accurate copy number of sponge sequence. There are several hints for successful cloning. In the construction process, if there is a small number of clones (<10) formed after ligating oligonucleotide duplex into the vector, duplex annealing, vector digestion, ligation and transformation process may have problem. A vector self-ligation control could be used to check whether the vector is prepared properly. There should be none or much fewer clones in self-ligation control. If there are many clones (>20) formed in the vector self-ligation control, a dephosphorylation reaction could be performed. To test the duplex preparation step, the DNA electrophoresis can be conducted after the digestion. The digestion mixture and a comparative oligonucleotides mixture without reaction could be compared; the digestion mixture would have a smear at about 100bp whereas oligonucleotides mixture does not have any smear. The transformation efficiency can be checked by transforming a circular plasmid. The efficiency should be higher than 10<sup>6</sup>cfu/ug DNA. If there are only a few clones (<10) formed during subcloning, the methylation of *BclI* could be the reason. The vector plasmid should be prepared from the *dam*<sup>-</sup> and *dcm*<sup>-</sup> competent cells.

If there is no obvious functional effect or expression change after sponge expression targeting a miRNA that has clear function or target gene, low binding ability of sponge sequence, a small quantity of sponge repeats or low infection efficiency can be the reason. To enhance binding ability, complete pairing is not recommended, 2-4 nucleotides mismatch would be better. Online software miRNAsong (<https://www2.med.muni.cz/histology/miRNAsong/index.php>) can also be used to optimize the sponge sequence. The final vector should have more than eight copies of sponge to achieve adequate effect. If the target miRNA is abundant in cell lines interested, at least 16 copies of sponge maybe needed and a strong promoter such as CMV, PGK and EF1 $\alpha$  can be used to increase the sponge expression. The infection efficiency can be checked by introducing a control lentivirus which only has EGFP CDS and no sponge. If the efficiency of control lentivirus is also low, the issue should be the process of virus packing. Low yield of lentivirus is usually caused by inappropriate transfection reagent or over-passaged

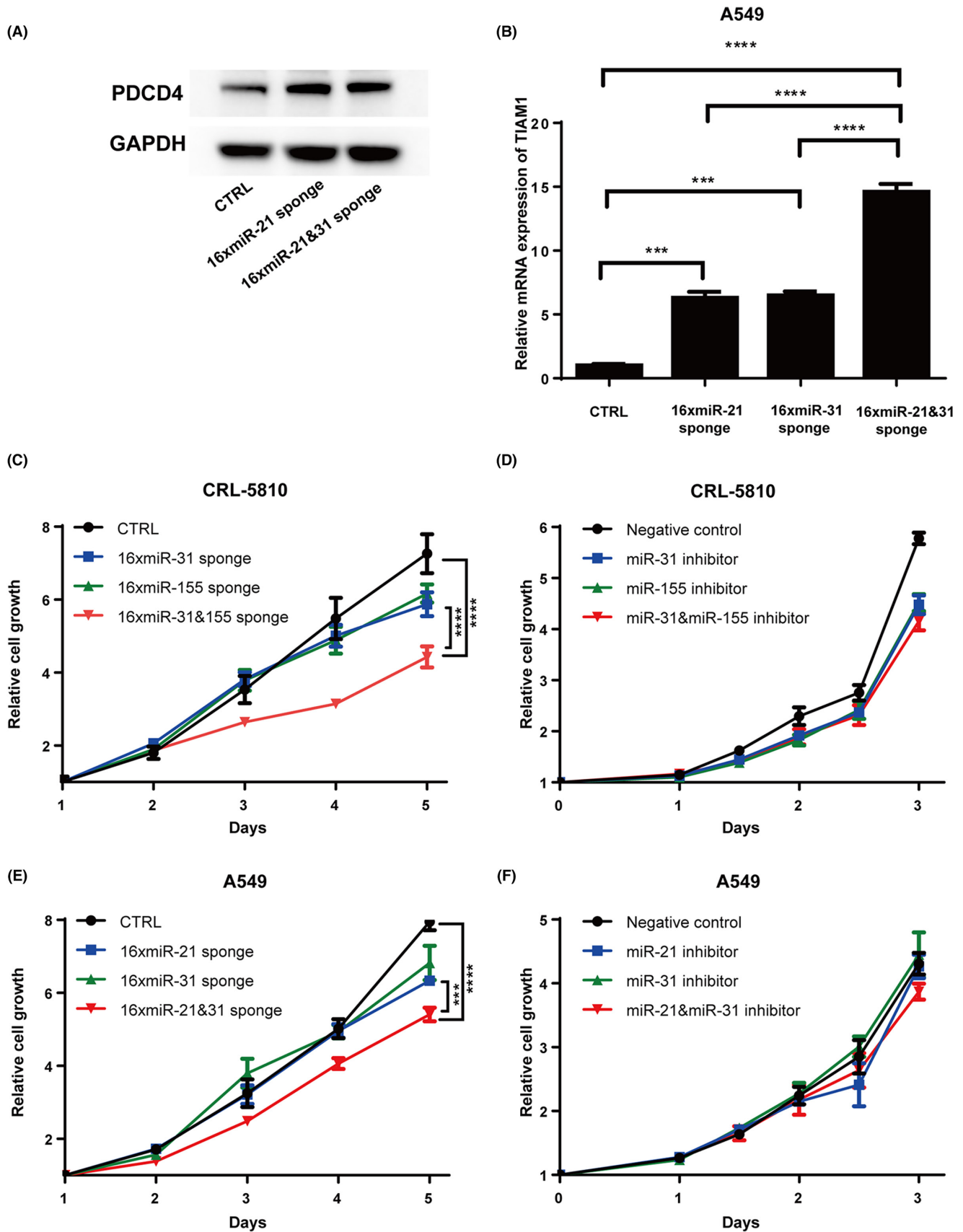


**FIGURE 2** Expression levels of hsa-miR-21-5p, hsa-miR-31-5p and hsa-miR-155-5p in lung cancer cells lines and BEAS-2B cells and the expression levels of genes targeted by hsa-miR-21-5p, hsa-miR-31-5p and hsa-miR-155-5p. (A) The relative expression levels of hsa-miR-21-5p in lung cancer cells lines and BEAS-2B cells which is derived from normal human bronchial epithelium. (B) The relative expression levels of hsa-miR-31-5p in lung cancer cells lines and BEAS-2B cells which is derived from normal human bronchial epithelium. (C) The relative expression levels of hsa-miR-155-5p in lung cancer cells lines and BEAS-2B cells which is derived from normal human bronchial epithelium. (D) The relative mRNA level of RhoBTB1 in CRL-5810 cells with 16xmiR-31 sponges and 16xmiR-31&16xmiR-155 sponges. (E) The relative mRNA level of SGK3 in CRL-5810 cells with 16xmiR-155 sponges and 16xmiR-31&16xmiR-155 sponges. (F) The relative mRNA level of RACK1 in CRL-5810 cells with 16xmiR-155 sponges and 16xmiR-31&16xmiR-155 sponges. (G) The relative mRNA level of PTEN in A549 cells with 16xmiR-21 sponges and 16xmiR-21&16xmiR-31 sponges. (H) The relative mRNA level of PDCD4 in A549 cells with 16xmiR-21 sponges and 16xmiR-21&16xmiR-31 sponges. (I) The relative mRNA level of ARID1A in A549 cells with 16xmiR-31 sponges and 16xmiR-21&16xmiR-31 sponges

HEK-293T cells. Transfection reagent with high efficacy and HEK-293T that has been passed less than 30 times can improve the virus production.

After cloning the first four copies of sponge into the lenti-virus vector, the number of sponge copy is doubled by subcloning each time. It is also convenient to combine sponges targeting

different miRNAs through one step of subcloning like "building blocks". In addition, different miRNA inhibitors may not be able to enter one cell at the same time and produce cumulative effects, while miRNA sponges linked in one vector targeting various miRNAs can be used to inhibit the function of multiple miRNAs in the same cell. The method described here will facilitate the process



**FIGURE 3** Cell growth of lung cancer cells infected with miRNA sponges or inhibitors. (A)The relative protein levels of PDCD4 in A549 cells with 16xmiR-21 sponges and 16xmiR-21&16xmiR-31 sponges. (B)The relative mRNA level of TIAM1 in A549 cells with 16xmiR-21 sponges , 16xmiR-31 sponges and 16xmiR-21&16xmiR-31 sponges. (C) The cell growth of CRL-5810 cells infected with miR-31 or miR-155 sponges separately or both. (D)The cell growth of CRL-5810 cells infected with miR-31 or miR-155 inhibitors separately or both. (E)The cell growth of A549 cells infected with miR-21 or miR-31 sponges separately or both. (F)The cell growth of A549 cells infected with miR-21 or miR-31 inhibitors separately or both

of sponge plasmid construction and make contribution to the research of miRNAs.

#### AUTHOR CONTRIBUTION

J.J.J.: Data curation, Project administration, Writing-original draft. D.N.L.: Investigation, Project administration, Validation. Y.F.W.: Data curation, Formal analysis, Software. Q.W.: Formal analysis, Validation. T.T.W.: Formal analysis, Software. R.F.: Conceptualization, funding acquisition, supervision.

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#### CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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