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Supporting Information

of

Bidirectional modulation of microRNA with a clamp-like triplex switch for enhanced and programmed gene therapy

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Experimental Section

Chemicals

All the oligonucleotides used in this study were synthesized and HPLC-purified by Sangon Biotech (Shanghai, China). The sequences of oligonucleotides were listed in **Table S1**. Ammonium persulfate, Tris, glacial acetic acid, copper sulfate pentahydrate, dimethyl sulfoxide and spermine were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Antibody for SOCS1 (AF5378; 1:1000) was purchased from Affinity Biosciences group ltd (Changzhou, China). Antibodies for HMGA2 (20795-1-AP; 1:1000), β-actin (66009-1-Ig; 1:5000) and the HRP goat anti-rabbit IgG (SA00001-1; 1:5000) were purchased from Protein tech Co., Ltd (Wuhan, China). Trizol reagent was obtained from TAKARA Bio. Inc. (Dalian, China). Lipofectamine 3000 were purchased from Thermo Fisher Scientific Inc (Delaware, USA). Hoechst 33342 staining solution and PI/Annexin V-FITC apoptosis detection kit were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). MTT, 4% paraformaldehyde and crystal violet were obtained from Sangon Biotech (Shanghai, China). 10 mM Tris-buffer was prepared by adding 10 mM Tris, 10 mM $MgCl_2$, 1 mM spermine and 0.2 mM of $CuSO_4$ in ultrapure water and adjusted to pH 6.5 by a San-Xin MP511 miniature pH meter (Shanghai, China). Ultrapure water was obtained by the Direct-Q 8UV-R ultrapure water system (Dubuque, USA). All the reagents were of analytical grade. All the solutions and ultrapure water used were treated with DEPC and autoclaved to avoid degradation of RNA.

Preparation and Characterization of CTS

10 μL of Anti-155-E (1 μM) and 10 μL of let-7a mimic (1 μM) were first mixed in 80 μL of Tris-buffer (10 mM, pH 6.5, containing 10 mM $MgCl_2$, 1 mM spermine and 0.2 mM $CuSO_4$). Then the mixture was heated to 95 °C for 5 min and rapidly cooled to 4 °C, following by hybridization at 37 °C for 1.5 h to form CTS. Control probes, including Con_L-CTS, Con_D-CTS and Con_N-CTS were prepared in the similar way.

15% native polyacrylamide gel electrophoresis (Native-PAGE) was performed at 90 V in 1×TAMg buffer (20 mM Tris, 20 mM acetic and 10 mM $MgCl_2$) to verify the formation of CTS. After 200 min electrophoresis in ice bath, the gel was stained with SYBR Gold for 30 min, and then was analyzed with Tanon 2500R (China).

For fluorescence analysis, fluorescence spectra of Cy5 were monitored by a Quanta Master fluorescence spectrophotometer (E_x=620 nm, E_m=640-740 nm). The target-triggered disassembly of CTS was investigated after incubation with DNA-155 (molar ratio 1:1) at 37 °C for 2 h. To investigate the stability, fluorescence behavior of CTS (100 nM) in 10% fetal bovine serum was monitored at 37 °C for 80 min. The melting temperature (T_m) curves of CTS, hybrid of Anti-155-E and miR-155, Con_D-CTS and Con_N-CTS (100 nM) was recorded by a Quanta Master fluorescence spectrophotometer at 670 nm with an excitation wavelength of 620 nm.

Typically, 300 μL each sample (2 μM) was recorded by a MOS-500 circular dichroism (CD) spectrophotometer (Bio-Logic, France) in the range of 200~320 nm at room temperature. In each case, the background of the buffer solution was subtracted from the CD data and processed using Origin software.

Cell Culture

MCF-7 cells (human breast cancer cell line), MDA-MB-231 cells (Human breast cancer cell line) and MCF-10A cells (normal human mammary epithelial cell line) were obtained from the cell bank of the Central Laboratory of Xiangya Hospital (Changsha, China). MCF-7 cells were cultured in Dulbecco's modified Eagles medium with high glucose (DMEM, Thermo Fisher Scientific, USA) supplemented with 10% (v/v) Fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin. MDA-MB-231 cells were cultured in the complete RPMI 1640 cell culture medium (Thermo Fisher Scientific, USA) containing 10% FBS and 1% penicillin-streptomycin (100 μg/mL streptomycin and 100 U/mL penicillin). MCF-10A cells were cultured in Mammary Epithelia cell medium (Procell, China). All the cells were maintained at 37 ℃ in a 5% CO_2 humidified incubator (Thermo Fisher Scientific, USA).

Cytotoxicity of Lipofectamine 3000

Generally, 3×10^4 cells were firstly seeded and cultured in 96-well plates for 24 h. After washing with PBS, the cells were treated with 200 μL cell medium containing the different dose of Lipofectamine 3000 at 37℃ for 6 h. Then, the cell medium was removed, and 200 μL of fresh cell medium was replenished. After 72 h incubation, 15 μL MTT solution (5 mg/mL in PBS) was added into each well. After 4 h incubation, all the solution was removed, followed by addition of 150 μL

DMSO. The absorbance in 490 nm was measured using a Varioskan Flash Multimode Reader (Thermo Scientific, USA).

Intracellular Activation of CTS

miR-155 triggered disassembly of CTS in tumor cells was evaluated by laser scanning confocal microscopy (CLSM). Generally, 4×10^5 cells were firstly seeded and cultured in CLSM dishes for 24 h. After washing 3 times with PBS, triplex switches (100 nM) were transfected into the cells by using Lipofectamine 3000. Transfection was performed according to the manufacturer's instructions. After incubation at 37°C for 6 h in a 5% CO_2 humidified incubator (unless otherwise noted), Hoechst 33342 was added to label the cell nuclei for another 20 min at room temperature. Then, the cells were washed 3 times with PBS and then imaged by FV3000 confocal microscope (Olympus, Japan) with a 40× objective (zoom=2). Excitation wavelength and emission filters were described as follows. Hoechst 33342: E_x=405 nm, E_m=430-460 nm bandpass; Cy5: E_x=633nm, E_m=660 nm long-pass.

In Vitro Cytotoxicity Assessment

Cells were seeded and cultured in 96-well culture plates at 37℃ for 24 h with a density of 3×10^4 cells per well. Then, the cells were treated with CTS or control therapeutic (100 nM) at 37℃ for 6 h. After removing the medium and washed twice with PBS, each well was replenished with fresh medium (10% FBS) for further incubation. 48 h (unless otherwise noted) later, 15 μL sterile MTT stock solution (5 mg/mL in PBS) was added into each well. After 4 h incubation, all the solution was removed, followed by addition of 150 μL DMSO. The absorbance in 490 nm was measured using a Varioskan Flash Multimode Reader (Thermo Scientific, USA).

Cellular Migration and Survival Performance

For colony formation assay, 2×10^5 MCF-7 cells were firstly treated with different therapeutics formulations (100 nM) for 6 h at 37 °C, then cultured in 24-well plates at a density of 200 cells per well. About 2 weeks later, cells were fixed using 4% paraformaldehyde and stained with 0.05% crystal violet for 30 min. Cell colonies were washed three times with ultrapure water and methanol. Colonies were recorded via a light microscope.

For wound healing assay, cells (4×10^5/well) were seeded in CLSM dishes for 48 h at 37 °C. While the cell fusion degree was greater than 90%, the cells were treated with different therapeutics

formulations (100 nM) for 6 h, an artificial wound was created onto the monolayer using a sterile tip, after removing the medium and washed twice with PBS, each well was replenished with fresh medium (10% FBS) for further incubation. Then, after 0 h, 6 h, 12 h, 24 h, time-lapse photographing of the wound closures was performed by an Olympus light microscope (Tokyo, Japan).

Cell Apoptosis

Cells were cultured in the 6-well plates (4×10^5/well) at 37 °C for 24 h. After treatment with different therapeutic formulations (150 nM) for 6 h, the cells were washed twice with PBS and supplemented with culture medium for 48 h incubation. Then, the cells were collected for flow cytometric assay after staining by Annexin V-FITC and PI for 15 min. Flow cytometric assay was performed using A00-1-1102 Flow Cytometer/ (Beckman Coulter, United States).

RT-PCR Quantification

To quantify the relative expression of hmga2 and socs1, MCF-7 cells (4×10^5/well) were seeded in 6-well plates for 24 h, and then the cells were incubated with CTS or other different control therapeutic (150 nM) at 37℃ for 6 h. After removing the medium and washed twice with PBS, each well was replenished with 2 mL fresh medium (10% FBS) for further incubation. After 48 h, total cellular RNA was extracted from the cells using Trizol reagent according to the kit protocol. cDNA was prepared from 1000 μg of total RNA by using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). Real-time PCR was then performed with 2×SYBR Green qPCR Master Mix on a CFX96TOUCH Real-Time PCR system (BIO-RAD, USA). The reactions were processed under the following cycling steps: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 55 °C for 60 s, and 72 °C for 30 s. β-actin was used as an internal control. The corresponding forward primer and reverse primer were listed in **Table S1**.

Western Blot Analysis

Generally, cells were lysed in ice-cold RIPA buffer (Solarbio, China) supplemented with protease inhibitor mixture after undergoing different treatment as described in the RT-PCR assay. The lysates were placed on ice for 30 min and then centrifuged for 15 min with the speed of 14000 rpm at 4°C to remove cell debris. Afterward, the lysates were determined by the BCA protein assay reagent. Total cellular proteins were resolved on a 15% SDS-PAGE and transferred to a PVDF membrane.

After blocking with 5% skimmed milk in TBS containing 0.1% Tween-20 (TBST), antibodies against the HMGA2 and SOCS1 were added, respectively, for 12 h incubation at 4 °C. The membrane was washed three times with TBST, followed by incubation with horseradish peroxidase-conjugated goat anti-Rabbit IgG secondary antibody. After three times washing, proteins in membrane were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA) and analyzed with chemiluminescence detection system.

Supplementary Tables

Table S1. All of the oligonucleotides and triplex switches used in this work[a]

Oligomer or Triplex switch		Sequence (5'-3')
CTS	Anti-155-E	*CTA CTA CCC CT* ATC ACG ATT AGC ATT AA *TCT CCA TCA TC*
	let-7a mimic	*AGA GGU AGU AG*G UUG UAU AGU U
Con_L-CTS	Con-E1[b]	*CTA CTA CCC CT* TCA AGC CTC TCC ATA AC *TCT CCA TCA TC*
	let-7a mimic	*AGA GGU AGU AG*G UUG UAU AGU U
Con_N-CTS	Con-E2[c]	*CTA CTA CCC CT* TCA AGC CTC TCC ATA AC *TAT ATA TAT AT*
	let-7a mimic	*AGA GGU AGU AG*G UUG UAU AGU U
Con_D-CTS	Con-E3[d]	*TCT TTT CTT TC* TCA AGC CTC TCC ATA AC *CT TTC TTT TCT*
	Con-7a[e]	*GAA AGA AAA GA*T AAT ATA ATA T
miR-155 mimic		UUA AUG CUA AUC GUG AUA GGG G
miR-155 inhibitor		CCC CUA UCA CGA UUA GCA UUA A
Sub_{7a}[1]		TGA TTT GCA AGG AAC TAC CTC C
DNA-21		TAG CTT ATC AGA CTG ATG TTG
DNA-122		TGG AGT GTG AGA ATG GTG TTT G
DNA-31		AGG CAA GAT GCT GGC ATA GCT
DNA-221		ACC AAC CTG GCA TAC AAT GTA GAT TTC AGG
DNA-205		ACC AGA TTT CAG TGG AGT GAA GTT CAG G
DNA-155		TTA ATG CTA ATC GTG ATA GGG G
mt 1		TTA ATG CGA ATC GTG ATA GGG G
mt 2		TTA ATG CGA ATC GTG AGA GGG G
mt 3		TTA ATG CGA ATC CTG AGA GGG G
hmga2 forward primer		GTC CCT CTA AAG CAG CTC AAA A
hmga2 reverse primer		CTC CCT TCA AAA GAT CCA ACT G
socs1 forward primer		AGA GCT TCG ACT GCC TCT TC
socs1 reverse primer		GAT GCG CTG GCG GCA CAG CT
β-Actin forward primer		CCT GGC ACC CAG CAC AAT
β-Actin reverse primer		GGG CCG GAC TCG TCA TAC

Continued

Oligomer or Triplex switch		Sequence (5'-3')
CTS-4	Anti-21-E-4	*CTA CTA CCT CA* TCA ACA TCA GTC TGA TAA GCT A*_CTC CAT CAT C*
	let-7a mimic-4	*UGA GGU AGU AG*G UUG UAU AGU U
CTS-5	Anti-21-E-5	*TTT ACA TTC CA* TCA ACA TCA GTC TGA TAA GCT A*_CCT TAC ATT T*
	miR-1 mimic	*UGG AAU GUA AA* GAA GUA UGG AG
CTS-6	Anti-155-E-6	*TTT ACA TTC CA* TAT CAC GAT TAG CAT TA A*CC T TAC ATT T*
	miR-1 mimic	*UGG AAU GUA AA* GAA GUA UGG AG

[a] For the sequences above, the loop of triplex triplex switch is shown underlined, the stem region of triplex triplex switch is shown in italic, the mismatched base in mutants of miR-155 is shown in red.

[b] For Con-E1, it is obtained by replacing the anti-155 sequence in Anti-155-E with arbitrary base composition, which can hybridize with let-7a mimic to form triple-helix stabilized Con_L-CTS.

[c] For Con-E2, it is obtained by replacing one arm sequence in Con-E1 with arbitrary base composition, which can hybridize with let-7a mimic to form double helix stabilized Con_N-CTS.

[d] For Con-E3, it is obtained by shuffling Anti-155-E composition.

[e] Con-7a is designed with arbitrary base composition instead of let-7a mimic sequence, which can hybridize with Anti- Con-E3 to form triple-helix stabilized Con_D-CTS.

Table S2. Quantification of hmga2 expression in Fig. 3A

Therapeutic agent	Relative hmga2 expression level	Error bar
Blank	1.0000	0.0822
CTS	0.4940	0.0188
Con_L-CTS	0.7779	0.0159
Con_D-CTS	0.8785	0.0564
Con_N-CTS	0.5370	0.0157

Table S3. Quantification of cell viability in Fig. 3C

Type of cell	Therapeutic agent	Cell viability (%)	Error bar
MCF-7	Blank	100.0000	6.9691
	CTS	52.7322	3.0624
	Con_L-CTS	88.7653	4.8516
	Con_D-CTS	92.4828	7.8846
	Con_N-CTS	77.8728	7.6893
MCF-10A	Blank	100.0000	2.7663
	CTS	96.4609	5.9168

Table S4. Quantification of hmga2, socs1 expression in Fig. 4A

Therapeutic agent	Relative hmga2 expression level	Error bar	Relative socs1 expression level	Error bar
Blank	1.0000	0.0173	1.0000	0.0830
let-7a mimic	0.5407	0.0864	1.0241	0.0621
Anti-155-E	0.9256	0.0439	1.7142	0.0643
CTS	0.6950	0.0414	1.8833	0.0541

Table S5. Quantification of cell viability in Fig. 4C

Incubation time	Cell viability with different treatments (%)							
	Blank	Error bar	let-7a mimic	Error bar	Anti-155-E	Error bar	CTS	Error bar
0	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
24	100.00	7.27	90.19	3.65	81.43	2.64	77.50	6.90
48	100.00	6.71	72.64	3.10	63.42	5.67	52.85	3.34
72	100.00	4.32	64.69	3.08	51.93	6.22	25.32	1.84

Table S6. Quantification of colony density in Fig. 4D

Therapeutic agent	Colony Density
Blank	6463.0000
let-7a mimic	4603.0000
Anti-155-E	3428.0000
CTS	2459.0000

Supplementary Figures

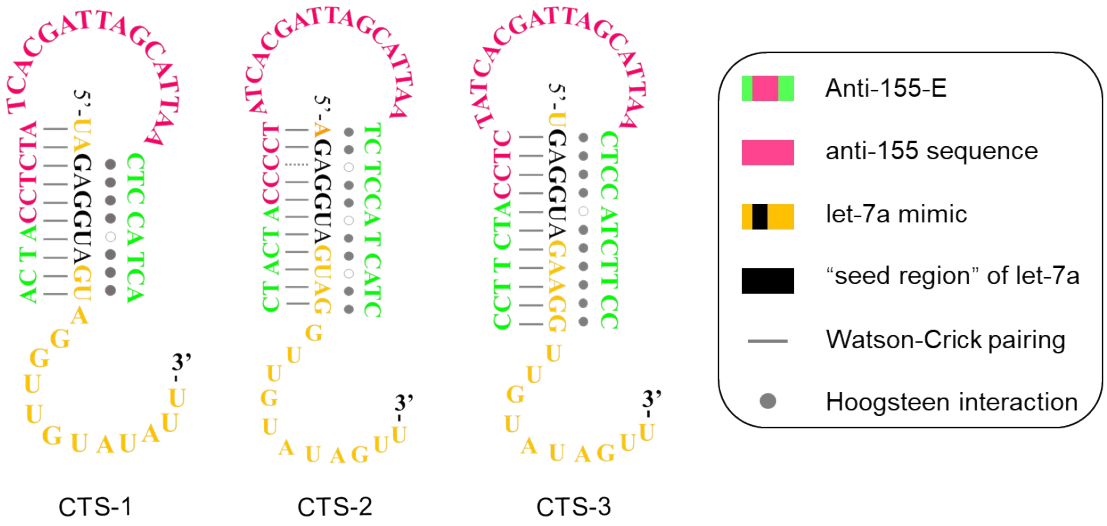


Fig. S1. The sequence details of CTS-1, CTS-2, CTS-3 with different triple-helix stem.

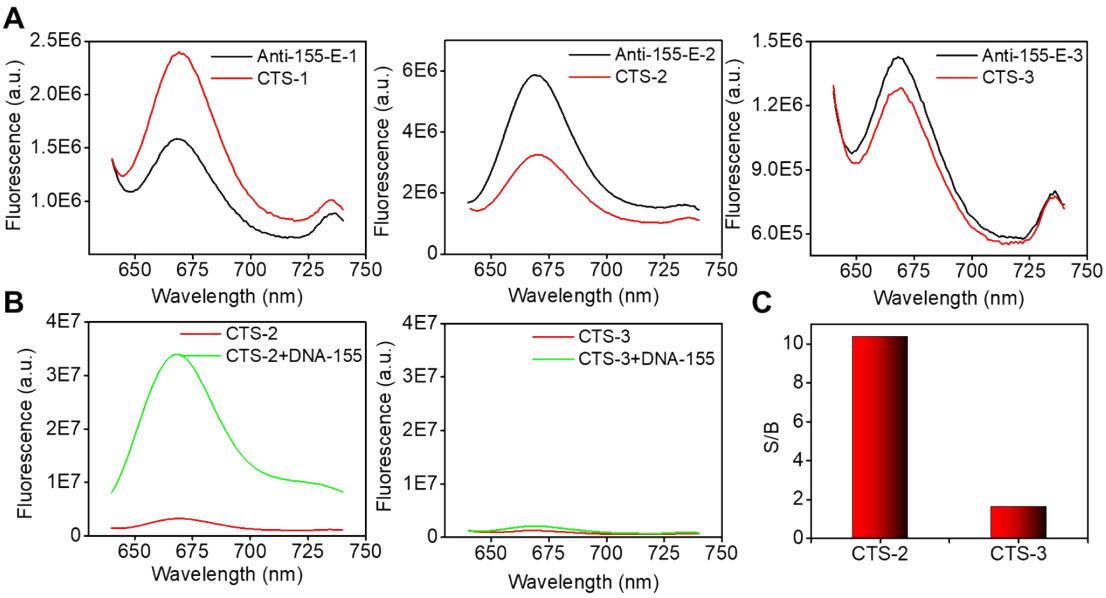


Fig. S2. (A) Fluorescence changes of Cy5 after adding let-7a mimic (100 nM) into different Anti-155-E strands (100 nM), respectively. The decreased fluorescence indicated formation of triple-helix stabilized CTS-2, CTS-3. For CTS-1, the length of triple-helix stem was too short to maintain its stability. (B) Fluorescence behaviors of CTS-2, CTS-3 in response to DNA-155 (100 nM), respectively. (C) The fluorescence ratios of (CTS+DNA-155) to CTS in (B). The higher S/B suggested CTS-2 had better responsiveness.

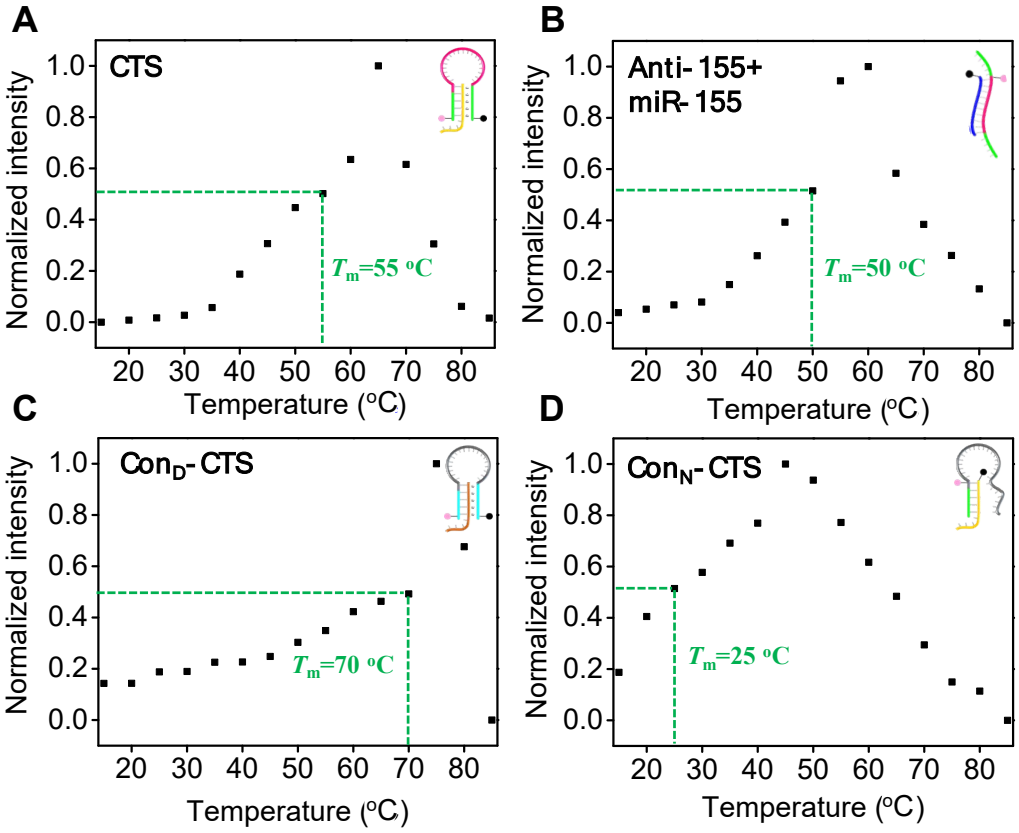
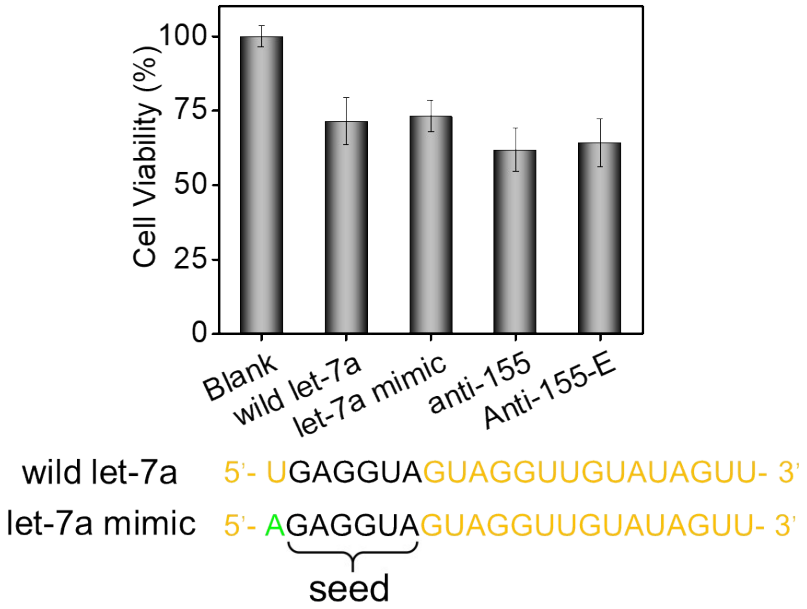


Fig. S3. The melting temperature (T_m) curves of (A) CTS, (B) hybrid of Anti-155-E and miR-155, (C) Con_D-CTS and (D) Con_N-CTS. Fluorescence emission was recorded at 670 nm with an excitation wavelength of 620 nm. Switches concentration: 100 nM.



wild let-7a 5'- UGAGGUAGUAGGUUGUAUAGUU- 3'

let-7a mimic 5'- AGAGGUAGUAGGUUGUAUAGUU- 3'

seed

Fig. S4. Viability of MCF-7 cells treated with wild let-7a, let-7a mimic, anti-155 and Anti-155-E at 37℃ for 48 h. Error bars represent standard deviations from five repeated experiments. The mutation is shown in green.

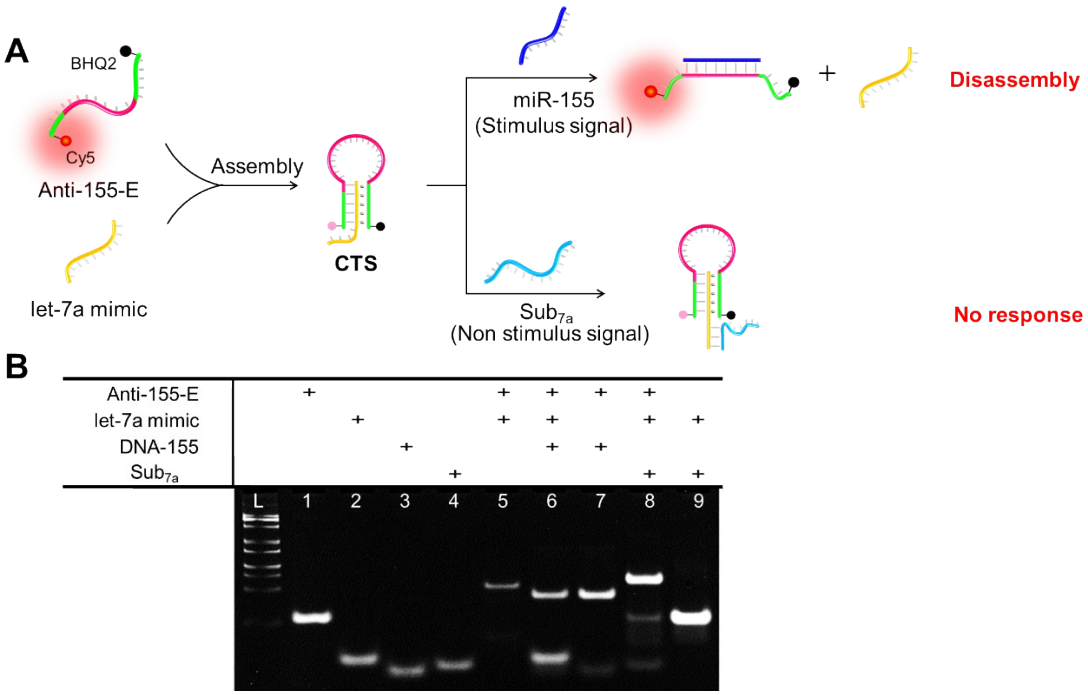


Fig. S5. Validation of CTS's assembly and disassembly in response to different oligonucleotides. (A) Illustration of the assembly and disassembly of CTS. (B) 15% PAGE analysis of the assembly and disassembly of CTS.

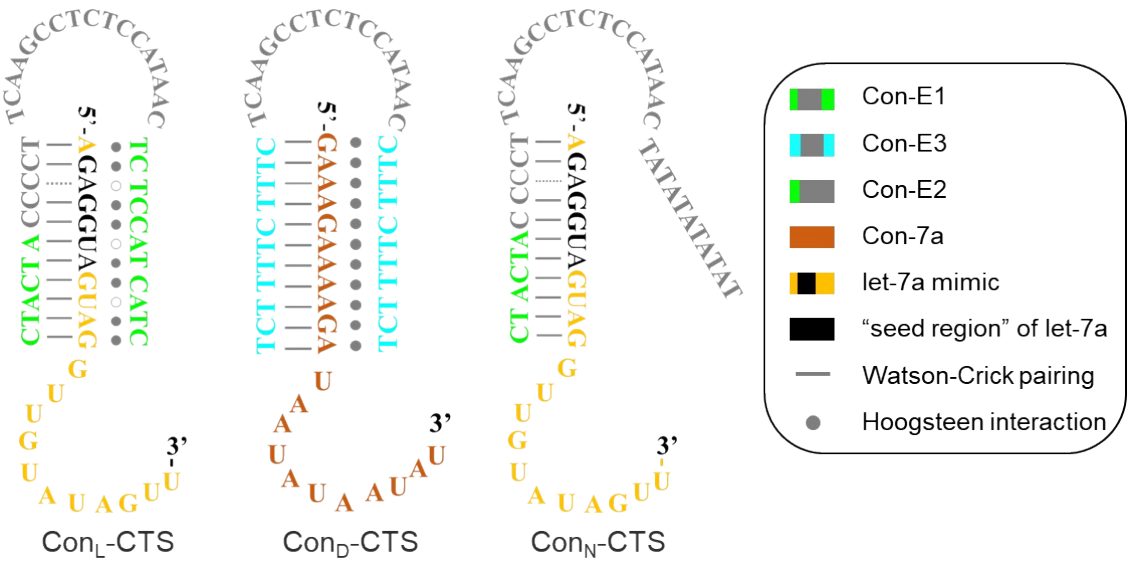


Fig. S6. The sequence detail of Con$_L$-CTS, Con$_D$-CTS and Con$_N$-CTS.

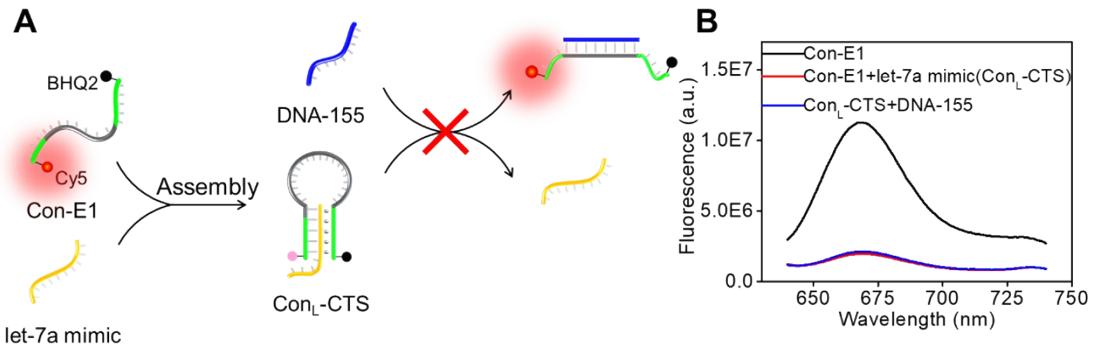


Fig. S7. (A) Illustration of the assembly of Con$_L$-CTS and its response behavior to DNA-155. (B) Fluorescence change of Con$_L$-CTS in response to DNA-155.

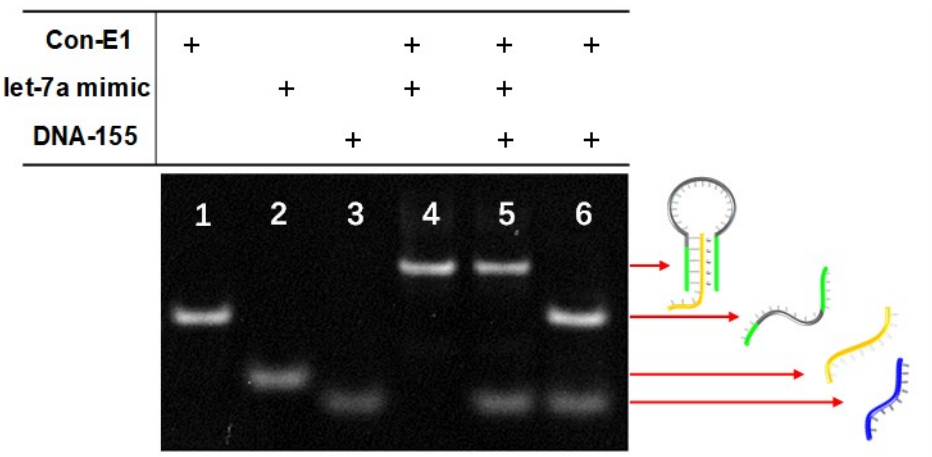


Fig. S8. 15% PAGE analysis the assembly and disassembly of Con_L-CTS.

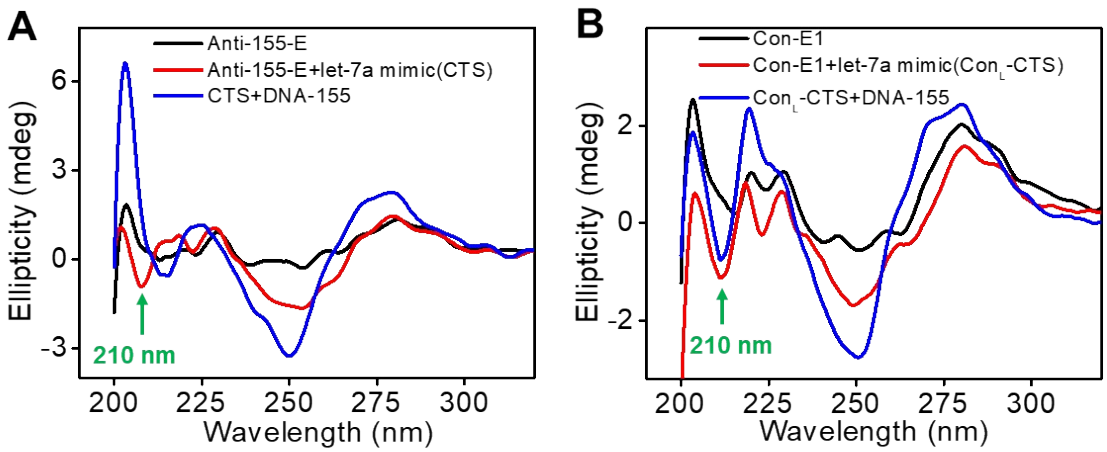


Fig. S9. Changes of circular dichroism (CD) spectra of (A) CTS and (B) Con_L-CTS in response to DNA-155, respectively. The CD spectral profile characterized with a negative band centered at ~210 nm indicates the formation of triple helix.[2,3]

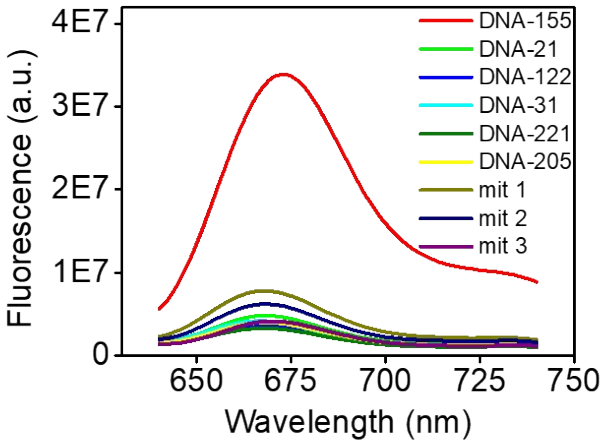


Fig. S10. The fluorescence intensity of CTS in the presence of different analytes. The detections were carried out in 10 mM Tris-HAc buffer after incubating CTS (100 nM) and different analytes (100 nM) at 37 °C for 2 h.

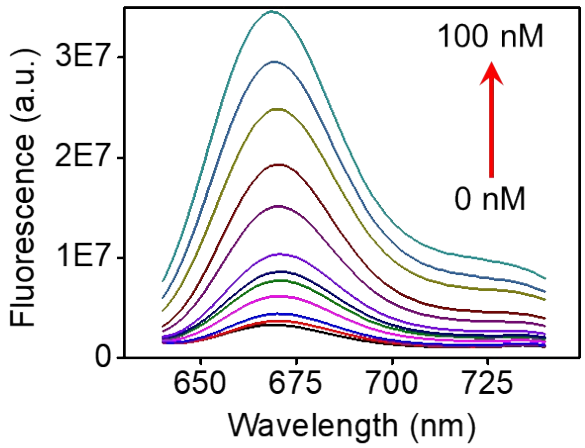


Fig. S11. Fluorescence recovery of CTS in response to DNA-155 with varying concentrations (0, 0.3, 1.0, 2.5, 7.5, 15.0, 30.0, 40.0, 50.0, 70.0, 90.0, 100.0 nM).

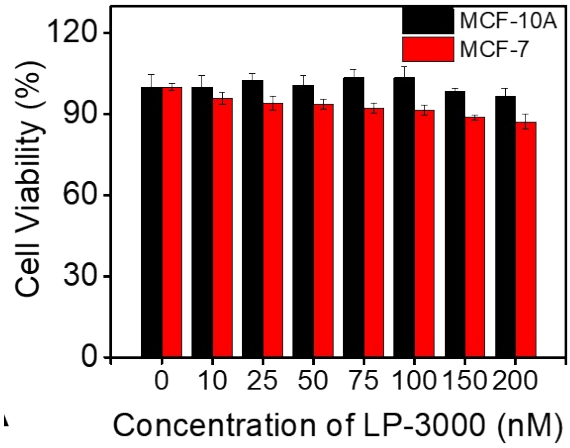


Fig. S12. Viability of MCF-7 cells treated with Lipofectamine 3000 (LP-3000) at different doses for 72 h. Error bars represent standard deviations from five repeated experiments.

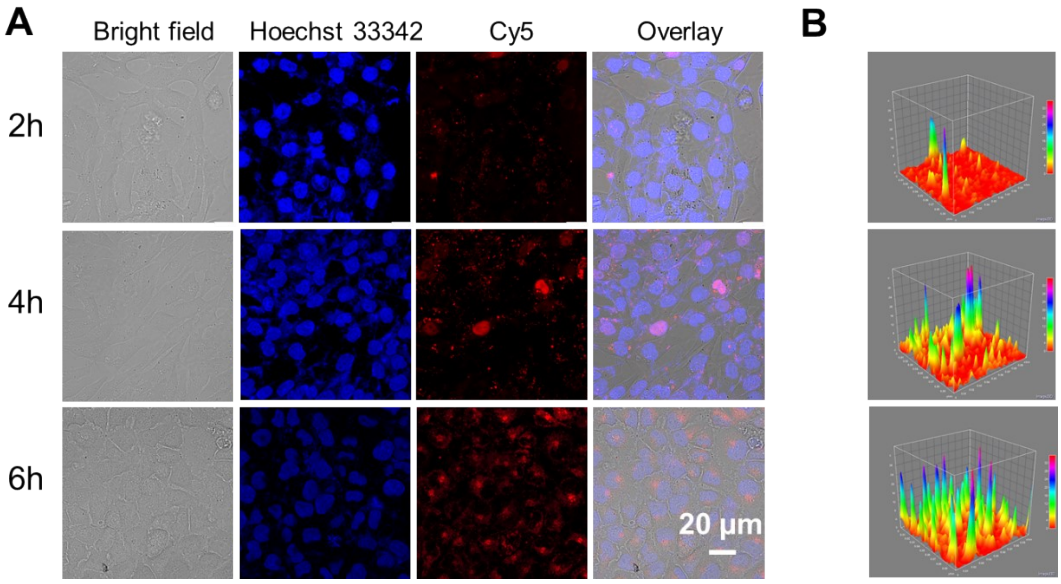


Fig. S13. (A) Confocal microscopy images of MCF-7 cells treated with CTS for different times. (B) Quantitative analysis of fluorescence intensity in (A) at the corresponding time.

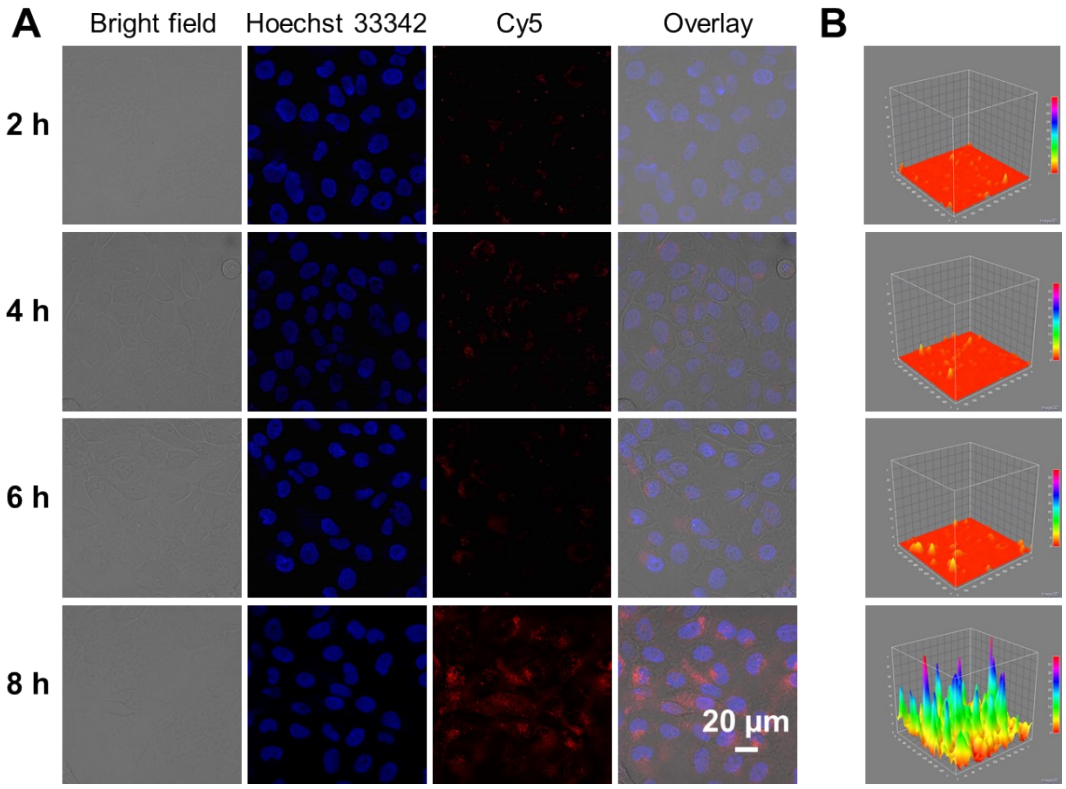


Fig. S14. (A) Confocal microscopy images of MCF-10A cells treated with CTS (100 nM) for different times. (B) Quantitative analysis of intracellular fluorescence intensity of CTS at the corresponding time. The results showed that most of CTSs in MCF-10A cells kept intact structure with faint fluorescence after 6 h incubation, due to lack of stimulus. When prolonging the incubation time to 8 h, non-specific disassembly for nuclease degradation was observed, accompanied by remarkably enhanced fluorescence.

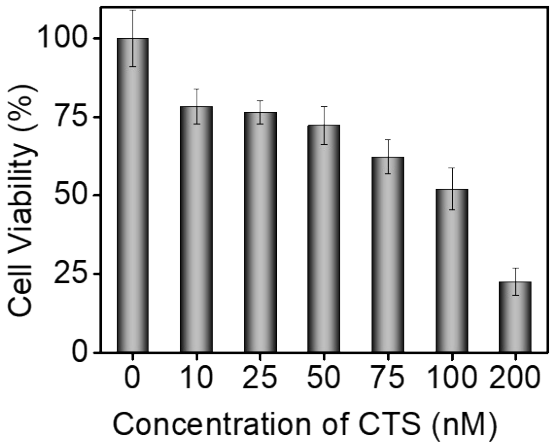


Fig. S15. Viability of MCF-7 cells treated with CTS at different doses for 48 h. Error bars represent standard deviations from five repeated experiments.

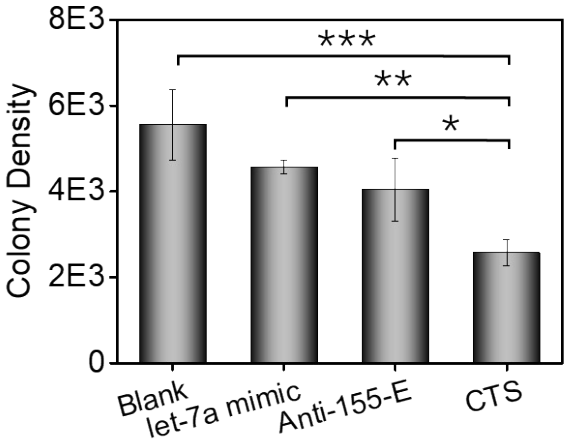


Fig. S16. Graph depicts the colony density of MCF-7 cells in Fig. 4D that calculated by Image J (n=3, statistical analysis performed with a two-tailed Student's t-test, ***P <0.001; **P <0.01; *P <0.05).

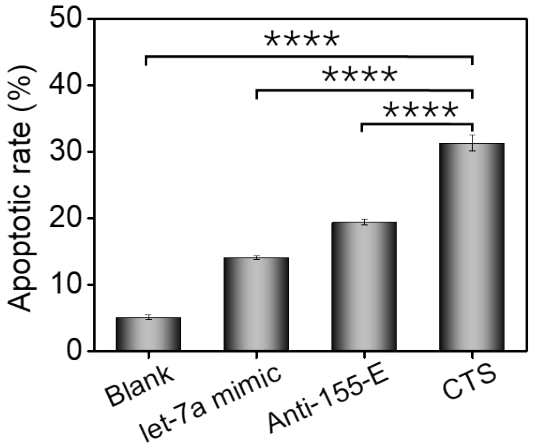


Fig. S17. Graph depicts the apoptotic rate in MCF-7 cells with different trearment in Fig. 4E (n=3, statistical analysis performed with a two-tailed Student's t-test, ****P<0.0001).

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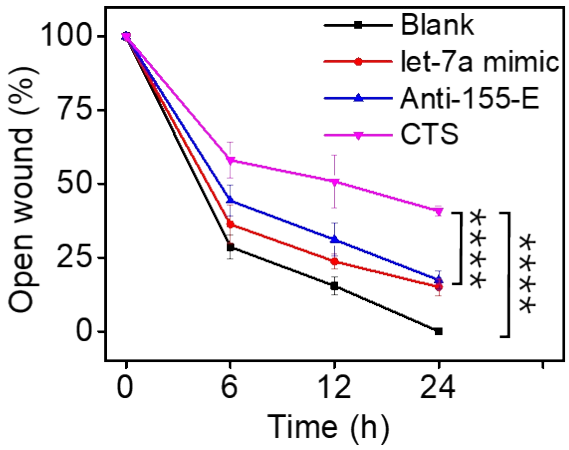

Fig. S18. Graph depicts the relative width of open wound in Fig. 4F (n=3, statistical analysis performed with a two-tailed Student's t-test, ****P<0.0001).

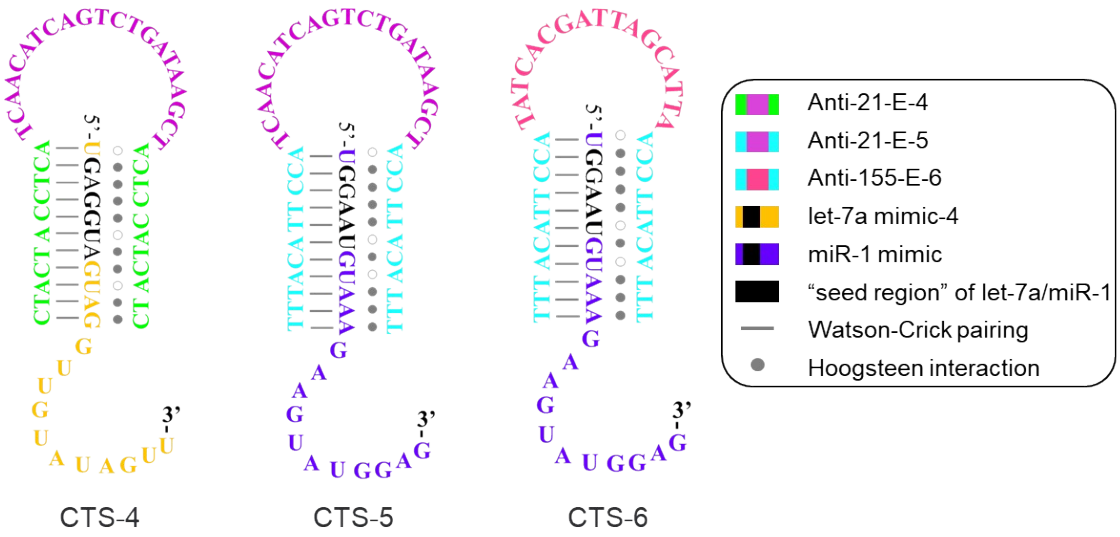

Fig. S19. A serial of clamp-like triplex switches, including CTS-4, CTS-5 and CTS-6 were designed to demonstrate the generality of CTS-based strategy. CTS-4 consists of a let-7a mimic-4 and an anti-21 sequence contained exterior strand (Anti-21-E-4). CTS-5 consists of a miR-1 mimic and an anti-21 sequence contained exterior strand (Anti-21-E-5). CTS-6 consists of a miR-1 mimic and an anti-155 sequence contained exterior strand (Anti-155-E-6).

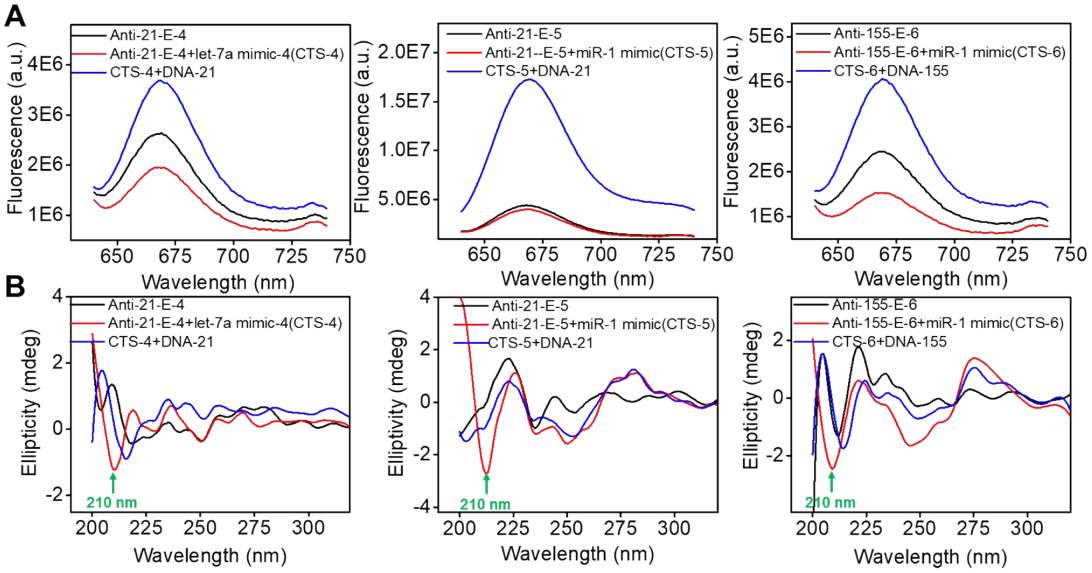

Fig. S20. (A) Fluorescence changes of Cy5 after adding miRNA mimic (100 nM) into different Anti-miR-E strands (100 nM), respectively. The decreased fluorescence (red lines) indicated formation of triple-helix stabilized CTS-4, CTS-5 and CTS-6. The recovered fluorescence (blue lines) suggested that triplex switches underwent conformation alters in response to the corresponding stimulus signal (100 nM). (B) Changes of CD spectra of CTS-4, CTS-5 and CTS-6 in response to the corresponding stimulus signal, respectively. The CD spectral profile characterized with a negative peak centered at ~210 nm indicates the formation of triple helix.

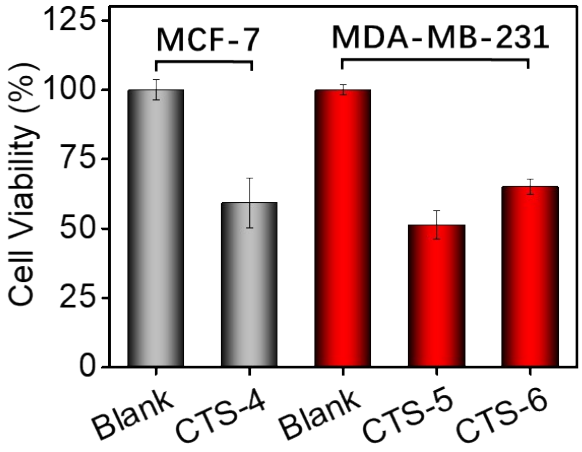

Fig. S21. Viability of MCF-7 and MDA-MB-231 cells treated with different triplex switches (100 nM) for 48 h. Error bars represent standard deviations from five repeated experiments.

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