Supporting Information

Cancer cell accurate identification and microRNA silencing induced therapy using tailored DNA tetrahedron nanostructure

Juan Su,[a] Hongping Xia,[b,] Yafeng Wu,[a,*] Songqin Liu[a,*]*

[a] Jiangsu Engineering Laboratory of Smart Carbon-Rich Materials and Device, School of Chemistry and Chemical Engineering, Southeast University, Nanjing 211189, China.

[b] Department of Pathology, School of Basic Medical Sciences & The Affiliated Sir Run Run Hospital & State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 21116, China.

Materials and Characterization Methods

Chemicals and Materials. Trihydroxymethyl aminomethane (Tris), magnesium chloride ($MgCl_2$) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from China National Pharmaceutical Group Corporation (Shanghai, China). siRNA-Mate and RNA oligonucleotides (Table S3) were received from GenePharma (Shanghai, China). DNA oligonucleotides (Table S3) were obtained from Sangon Biotech Co., Ltd (Shanghai, China). Cell culture products were purchased from Nanjing KeyGen Biotech. Inc (Nanjing, China). Human HCC cells (Huh7), human hepatoblastoma cells (HepG2), and human normal cells (HEK293) were obtained from Committee on Type Culture Collection of the Chinese Academy of Sciences (Beijing, China). All other chemicals were of analytical grade and used as received. Ultrapure water (18.2 MΩ·cm, Millipore Corp., Bedford, MA) was used throughout all experiments. The Tris-HCl buffer was prepared by mixing 20 mM Tris and 50 mM $MgCl_2$, then adjust pH to 8.0 with 0.1 M HCl.

Instruments. All fluorescence spectra were collected on a fluorescence spectrometer (Fluoromax-4, Horiba Jobin Yvon, Japan). Absorbance of MTT assays was recorded by a microplate reader (Multiskan™ FC, Thermo Scientific). Gel images were photographed by a gel imaging analyzer (KODAK Gel Logic 2200). Confocal fluorescence imaging was performed with Olympus FV3000 laser scanning confocal microscopy (Japan). Flow cytometry analysis was performed on BD FACSCalibur (BD Bioscience, USA). Agarose Gel Electrophoresis was carried out on DYY-6C Electrophoresis System (Beijing, China). The DNA assemblies were identified by 4%

Agarose Gel stained GelRed in TAE running buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at applied voltage of 90 V for 60 min. Then, the gels were visualized with a gel imaging analysis system. To verify nuclease degradation of DTNS in serum, DTNS (500 nM) were mixed with fetal calf serum (FBS, 10% in v/v) and incubated at 37 ^{o}C for 1, 2, 3 and 4 h. Then, agarose gel electrophoresis running and gel imaging were carried out. Atomic force microscopy (AFM) was performed with Bruker-Dimension Icon (Bruker Inc., USA). For sample preparation, 2 μL of as-prepared DTNS (1 μM) was deposited onto freshly cleaned silicon slice and dried overnight at 4 ^{o}C.

Experimental Details

Preparation of DNA tetrahedron nanostructures (DTNS). The preparation of DTNS was according to previous reported one-step thermal annealing method with slightly modification.[1] Briefly, seven strands: P1, P2, P3, P4, P5, P6 and P7, were respectively dissolved in ultrapure water to 50 μM as mother liquor. Then, 4 μL of each strand was simultaneously added to 172 μL of Tris-HCl buffer and heated to 95 ^{o}C for 5 min, followed storing at 4 ^{o}C ice box for more than 1 h to form 1 μM DTNS. On the same way, non-rigid DNA tetrahedron-based nanoprobes (n-DTNS) was obtained by mixing 2 μL of each strand except for P4 with 88 μL of Tris-HCl buffer, while 2 μL of each two strands: P1 and P5, P2 and P6, P3 and P7 were separately mixed with 30 μL Tris-HCl buffer, after thermal annealing treatement, re-mixed them together to obtain three DNA line nanostructures (DLNS). Besides that, another DNA tetrahedron-based nanoprobes (denoted DTNS-C) composed of P1, P2, P3, P4, P8, P6 and P7 strands was synthesized in the same way as DTNS. All the assembled DNA nanostructures were

stored at 4 °C.

Cell cytotoxicity and transfection. The cell cytotoxicity of DTNS was evaluated with MTT assay. In brief, 200 μL of HEK293 or Huh7 cells were seeded in 96-well plate (8000 cells/well) and incubated in a 5% CO_2 incubator at 37 ^oC for 24 h, replaced the culture medium with fresh RPMI-1640 or DMEM containing 100 nM DTNS and cultured for a series of time period (4, 12, 24, 36 and 48 h). Then cells in each well was rinsed with PBS and incubated with 200 μL MTT solution (500 μg/mL) for another 4 h. Finally, the MTT solution was carefully substituted with 150 μL DMSO and the absorbance at 490 nm was recorded using a microplate reader. The cell viability was determined by $(A_{test}/A_{control}) \times 100\%$.

For cell transfection, HepG2 cells were seeded in 24-well plate (10000 cells/well) or glass-bottom dishes and incubated in a 5% CO_2 incubator at 37 ^oC for 24 h. After replace the medium with fresh one, 100 μL of serum-free DMEM medium containing 20 nM transfection reagent (siRNA-mate) as control regent, or 20 nM siRNA-mate and 20 nM miRNA-194 mimic, or 20 nM siRNA-mate and 20 nM miRNA-194 inhibitor, were separately added in the wells or dishes with HepG2 cells and incubated for 24 h. Then, flow cytometric and confocal microscopy assays were performed to assess the cell transfection.

Flow cytometric and confocal microscopy assays. All the cells were cultured in 24-wells for flow cytometric assay or glass-bottom dishes for confocal microscopy analysis. Huh7, HepG2 cell lines were cultured in DMEM medium containing 10% FBS (v/v) and 100 IU/mL penicillin-streptomycin at 37 °C in a humidified atmosphere

containing 5% CO_2 whereas HEK293 cells in RPMI-1640. They were digested by trypsinization using 0.25% trypsin (m/v) in 10 mM pH 7.4 PBS during cell passage cultivation. For flow cytometric analysis, fluorescent of channel Cy5 (633 nm excitation) was collected by BD FACSCalibur Flow Cytometer. Also, the levels of miRNA-21 (FAM), miRNA-122 (TAMRA) and miRNA-194 (Cy5) in cells were imaged on a laser-scanning confocal microscopy system at the excitation/emission wavelengths of 488/500-545 nm for FAM, 561/570-670 nm for TAMRA, 635/650-750 nm for Cy5.

Wound-healing assay and *in vitro* cellular apoptosis assay. For cell wound-healing assay, 1×10^5 cells/well of HEK293 cells or Huh7 were seeded onto 12-well plate and were allowed to incubate until cells density to reach 90% confluence. Then, a straight wound was scratched with a 200 μL pipette tip in each well, following the wounded cells were gingerly washed with PBS for 3 times to remove the free cells. After that, the wounded cells were incubated with fresh medium containing DTNS-C or DTNS for another 48 h. Invading cells were photographed under Olympus CKX53 inverted microscope. For cellular apoptosis assay, the ability of DTNS induced apoptosis was determined by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (Sangon Biotech Co., China). The technology of Annexin V-FITC/PI double staining was operated according to the manufacturer's instruction. Briefly, after incubated with DTNS for 48 h, cells were collected, washed with cold PBS, suspended into 200 μL binding buffer. Then cells were stained in the dark for 15 minutes by 2.5 μL Annexin V-FITC and 5 μL PI. Finally, the stained cells were analyzed by flow cytometer.

Quantitative reverse transcriptase polymerase chain reaction analysis (qRT-PCR).

The levels of miRNA-21, miRNA-122 and miRNA-194 expression in cells were evaluated by qRT-PCR technique. Firstly, total cellular RNA was isolated by Trizol regents according to the conventional instructions. The quantity and quality of the extracted RNA were respectively evaluated by NanoDrop 2000 (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. Secondly, the complementary DNA (cDNAs) synthesis were manipulated by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) in terms of the manufacture's protocol. Finally, qRT-PCR analysis was carried out with SYBR Green I on ABI StepOne Plus Real-Time PCR thermal cycler. RNU6B (U6) was chosen as housekeeping gene, the specificity of PCR products was assessed *via* the melting curve analysis. The relative expression levels of miRNAs were normalized to the U6 using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used for qRT-PCR analysis were represented in Table S4.

Animal study in Huh7 liver tumor orthotopic xenograft model. Huh7-luciferase cells were subcutaneously injected into male BALB/c Nude mice (5 weeks of age). After the tumor size reached to 1 cm diameter, Hypnorm/Midazolam was used to anesthetize the mice and the tumor was cut into similar-sized pieces in the DMEM cell culture medium. Similar-sized tumoroids surgically transplanted into the liver of mice to set up the Huh7-luciferase orthotopic xenograft tumor models. After one week tumor transplantation, the mice were imaged and grouped according to equivalent signal intensity. The mice were then injected with DTNS-C or DTNS through the tail vain. The tumor models were imaged by bioluminescence and fluorescence imaging using the Xenogen IVIS Lumina system (Xenogen Corporation, Hopkinton, MA). In brief, after

anesthesia with 2% isoflurane, mice were intraperitoneally injected with D-Luciferin (100 mg/kg; Caliper Life Sciences, Inc, Hopkinton, MA) and received 1 min scans to assess the bioluminescent signal. Meanwhile, the fluorescence imaging was conducted using the FAM and TAMRA. The liver tumor tissues were collected for measuring the expression of miRNA-21, miRNA-122 and miRNA-194 by qRT-PCR analysis at the end of experiment.

Supplementary Tables

Table S1. The quenching efficiency determined according to Figure S2.

P5/P6/P7 : TDN	FAM	TAMRA	Cy5
1:1	76%	68%	78%
1:1.25	85%	98%	99%
1:1.5	91%	99%	99%
1:1.75	93%	99%	99%
1:2	92%	99%	99%

Table S2. Detection performance of RNA targets based on fluorescence off-on nanoprobes.

RNA Targets	Probe types	LOD	Reference
C-myc mRNA	DNA Tetrahedron	3.1 nM	[2]
TK1 mRNA		1.2 nM	
GalNAc-T mRNA		3.2 nM	
TK1 mRNA	DNA Octahedron	3.03 nM	[3]
GalNAc-T mRNA		1.09 nM	
mRNA	DNA Tetrahedron	0.33 nM	[4]
miRNA-21	DNA hexahedron	1.04 nM	[5]
miRNA-1246		0.79 nM	
TK1 mRNA	DNA Tetrahedron	7.6 nM	[6]
miRNA-21	DNA Tetrahedron	0.13 nM	This work
miRNA-122		0.64 nM	
miRNA-194		0.68 nM	

Table S3. Sequences of nucleic acid used in this work.

Name	Sequences of nucleic acid（5’→3’）
P1	*ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCC ATAGTA*TTTTTTTTTTACTGATGTTGA-**BHQ1**
P2	*TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGT CCAATAC*TTTTTTTTTTAATGGTGTTTG-**BHQ2**
P3	*TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCG GCTCTTC*TTTTTTTTTTCTCCATGTGGA-**BHQ2**
P4	*TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCT CGCAT*
P5	**FAM-**mU*mC*mAmAmCmAmUmCmAmGmUmCmUmGmAmU mAmAmG*mC*mU*mA*-**Chol**
P6	**TAMRA**-CAAACACCATTGTCACACTCCA
P7	**Cy5**-TCCACATGGAGTTGCTGTTACA
P8	**FAM**-TCAACATCAGTCTGATAAGCTA
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
miRNA-122	UGGAGUGUGACAAUGGUGUUUG
miRNA-194	UGUAACAGCAACUCCAUGUGGA
Mis-miRNA-21	UAGCU**G**AUCAG**G**CUGA**G**GUUGA
Mis-miRNA-122	UGGAG**G**GUGAC**G**AUGG**G**GUUUG
Mis-miRNA-194	UGUAA**G**AGCAA**G**UCCA**G**GUGGA
miRNA-194 inhibitor	mUmCmCmAmCmAmUmGmGmAmGmUmUmGmCmUmGmUUmA mCmA

Table. S4 Primers used in qRT-PCR experiments.

miRNA	Forward primer (5’ to 3’)	Reverse primer (5’ to 3’)
miRNA-21	TCGCCCGTAGCTTATCAGACT	CAGAGCAGGGTCCGAGGTA
miRNA-122	GATGCTCTGGAGTGTGACAATG	TATGGTTGTTCACGACTCCTTCAC
miRNA-194	GCCCGTGTAACAGCAACTC	CAGAGCAGGGTCCGAGGTA
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCATC

Supplementary Figures

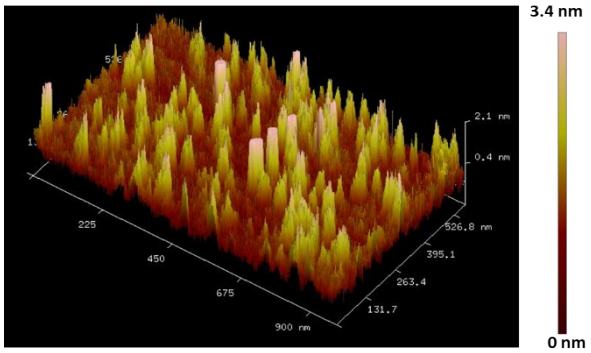

Fig. S1 Characterization of DTNS by atomic force microscopy (AFM), the prepared DTNS were vertebral-shape nanoparticles with diameter of ~3 nm.

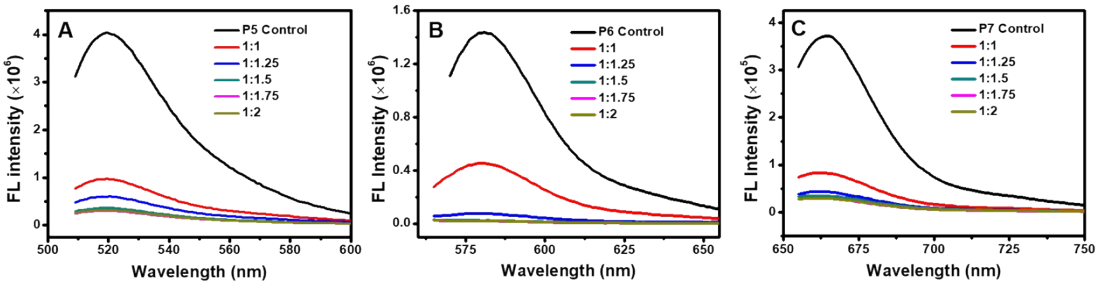

Fig. S2 The fluorescence spectra of P5, P6, P7 and DTNS assemblies that synthesized by adjusting hybridization ratio of P5/P6/P7 to TDN from 1:1 to 1:2. (A) P5 and P5-TDN; (B) P6 and P6-TDN; (C) P7 and P7-TDN.

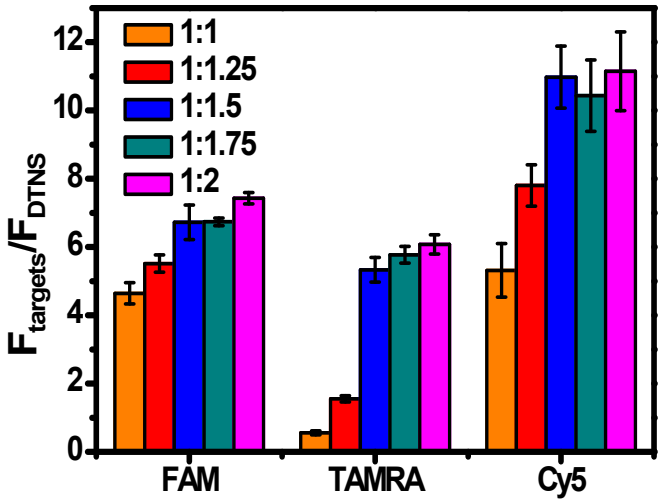

Fig. S3 The degree of fluorescence recovery of DTNS assemblies that prepared by adjustment of hybridization ratio between P5/P6/P7 and TDN. The fluorescence recovery degree was determined by comparison the fluorescence intensity of DTNS between with and without miRNA targets addition.

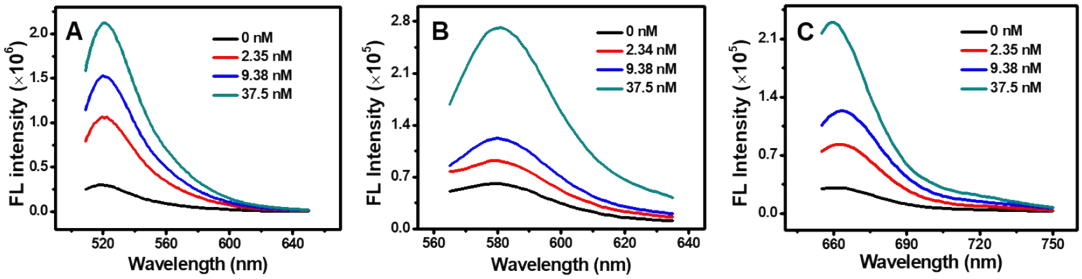

Fig. **S4** The fluorescence response of DTNS (37.5 nM) to miRNA-21 targets in the presence of miRNA-122 (375 nM) and miRNA-194 (375 nM) (A), to miRNA-122 targets in the presence of miRNA-21 (375 nM) and miRNA-194(375 nM) (B), to miRNA-194 targets in the presence of miRNA-21 (375 nM) and miRNA-122(375 nM) (C).

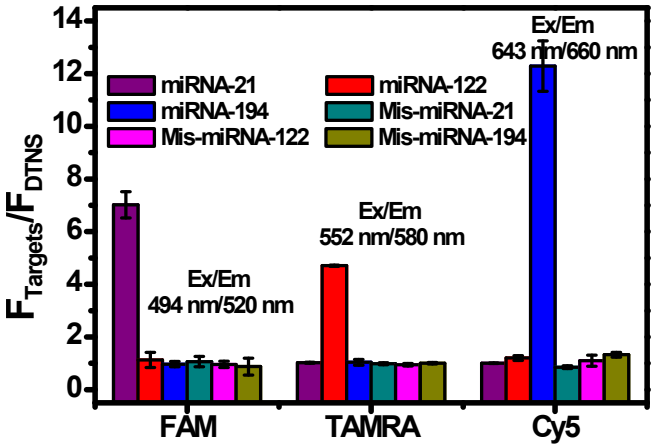

Fig. S5 The degree of fluorescence recovery of DTNS (37.5 nM) after adding 75 nM miRNA-21, miRNA-122, miRNA-194, Mis-miRNA-21, Mis-miRNA-122 or Mis-miRNA-194.

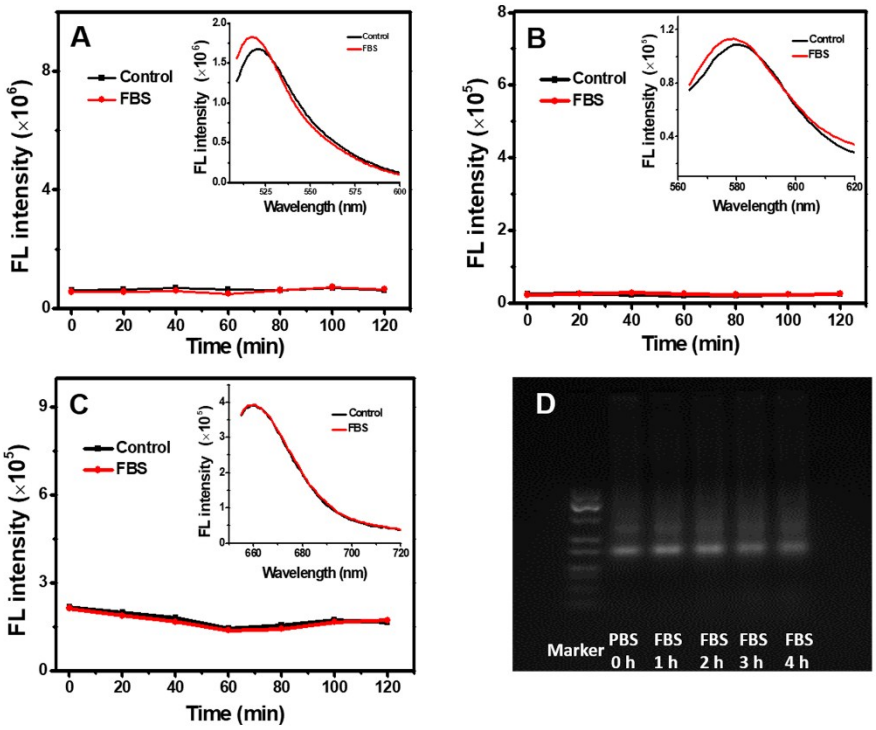

Fig. S6 The stability of the DTNS treated with FBS (10%, v/v), (A-C) Fluorescence changes of the DTNS treated with FBS (red trace) or with PBS (set as control, black trace) for 0-120 min. Insets: fluorescence spectra of the DTNS treated with FBS (red trace) and with PBS (set as control, black trace) after adding targets. (D) Agarose gel analysis of the DTNS treated with PBS or with FBS for a few hours at 37 ^{o}C.

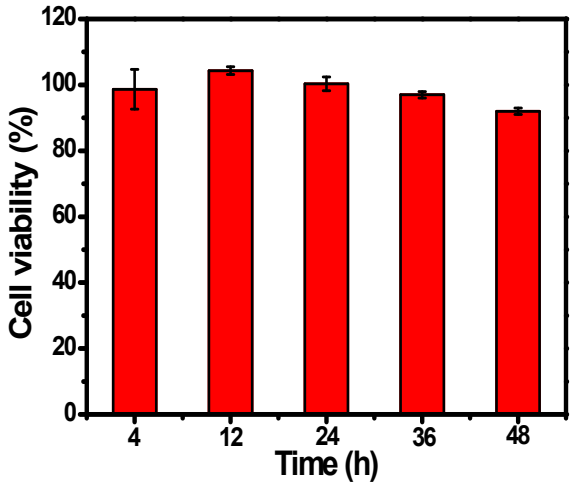

Fig. S7 Cell viability of HEK293 cells treated with 100 nM DTNS for different period of time.

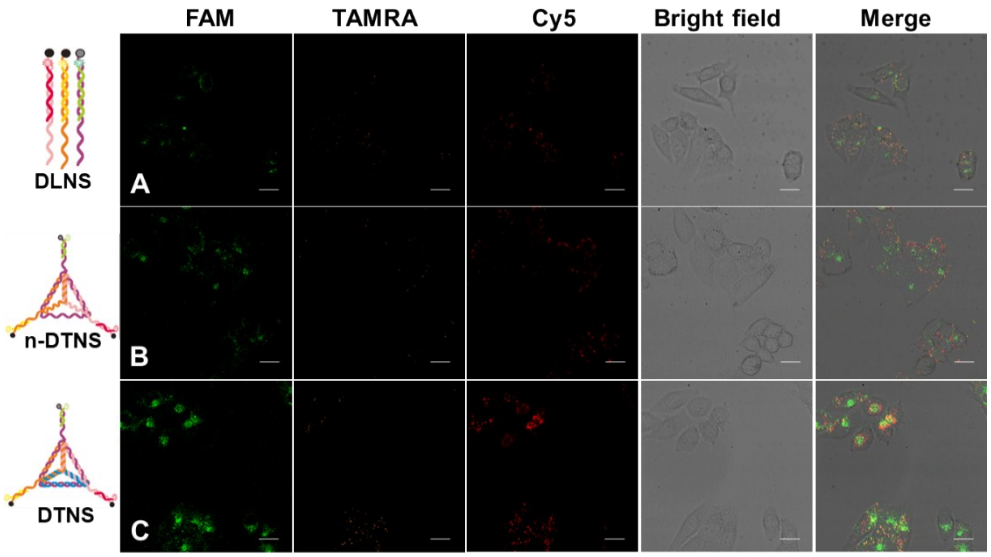

Fig. S8 Fluorescence images of miRNA-21, miRNA-122 and miRNA-194 in HepG2 cells incubated with DLNS, n-DTNS, and DTNS, respectively. Scale bar: 50 μm.

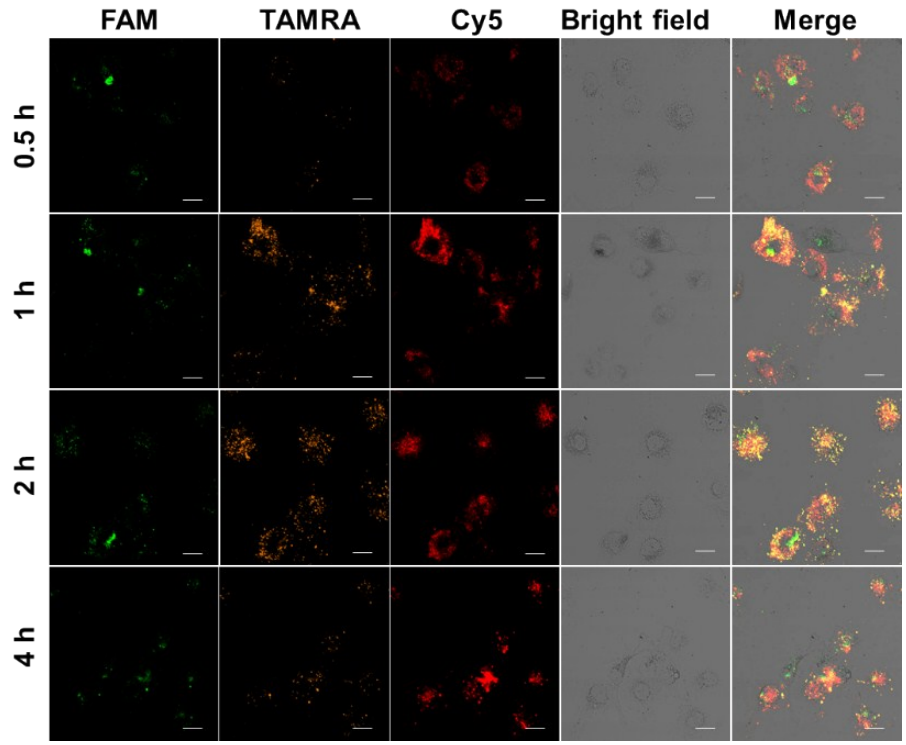

Fig. S9 Fluorescence confocal images of Huh7 cells after incubation with DTNS (100 nM) for different period times (0, 1, 2 and 4 h).

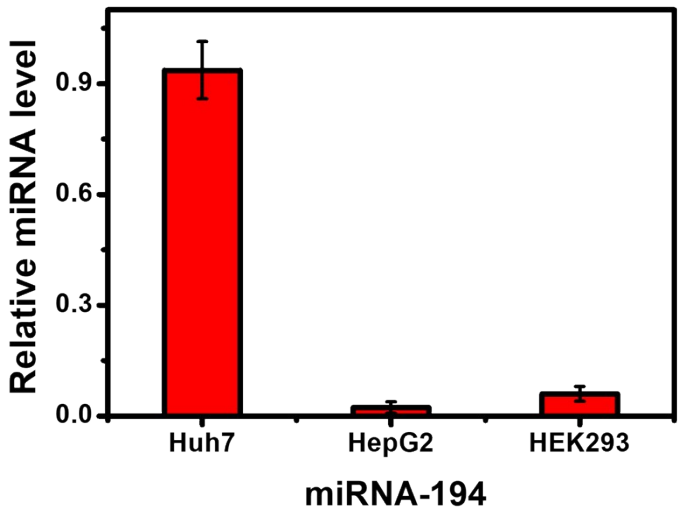

Fig. S10 qRT-PCR analysis of the relative miRNA-194 expression level in Huh7, HepG2 and HEK293 cells.

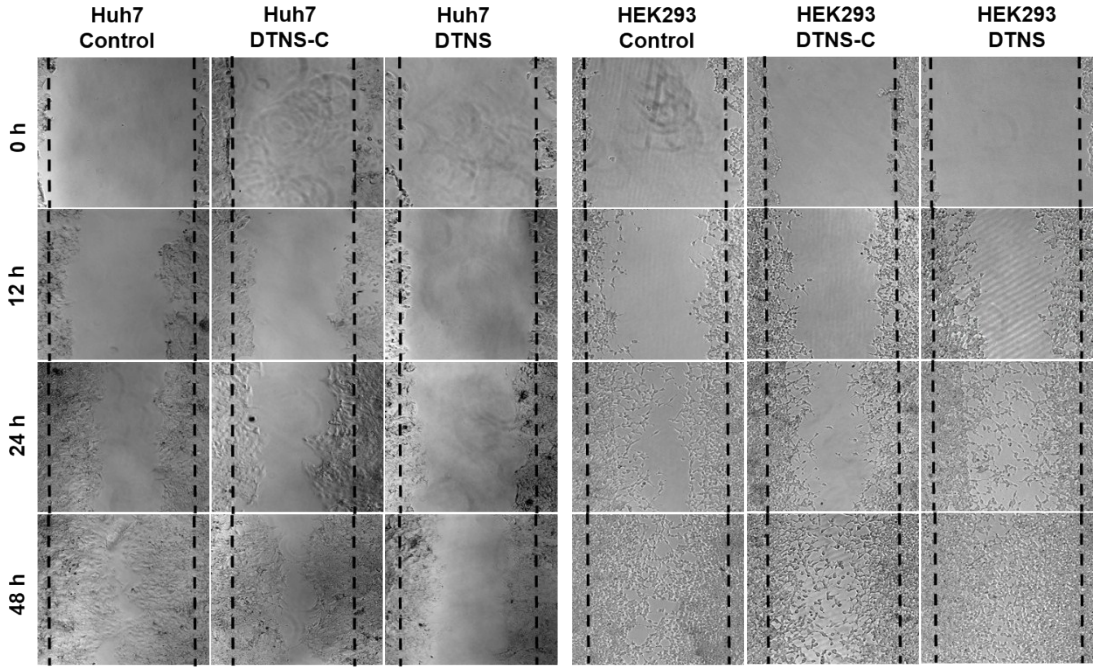

Fig. S11 Wound-healing analysis of Huh7 and HEK293 cells upon treatment with DTNS-C or DTNS (500 nM) for 12 h, 24 h, 48 h, respectively. The cell growth of HEK293 cells was almost unaffected when treated with DTNS-C and DTNS for a period of time. However, the cell migration and invasion of Huh7 cells was obviously inhibited in DTNS group while nearly insusceptible in DTNS-C group.

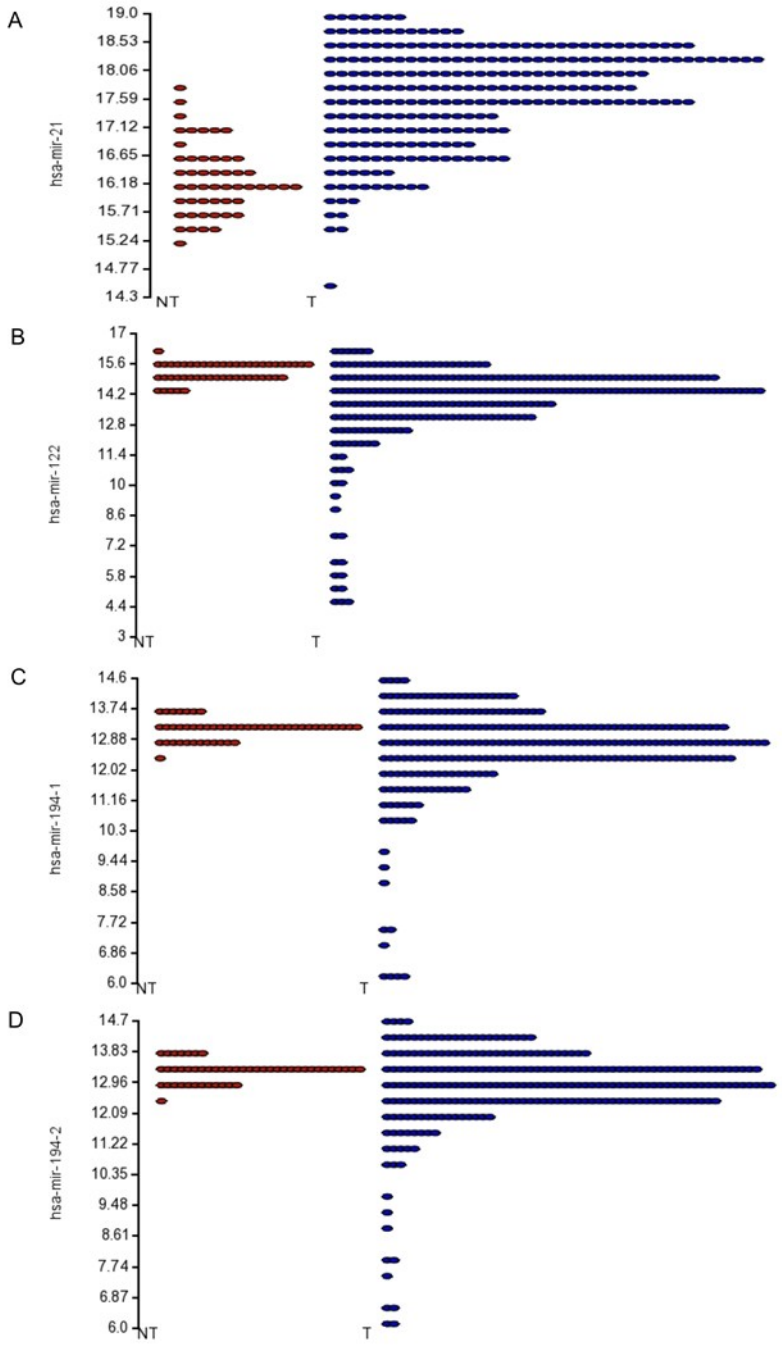

Fig. S12 The expression of miRNA-21, miRNA-122 and miRNA-194-1/2 in the clinical patients' liver cancer tumor (T) or non-tumor liver tissues (NT). The data showed that miRNA-21 was significantly overexpression in T, but miRNA-122 was decreased in T, the expression of miRNA-194-1/2 were not significantly different between T and NT.

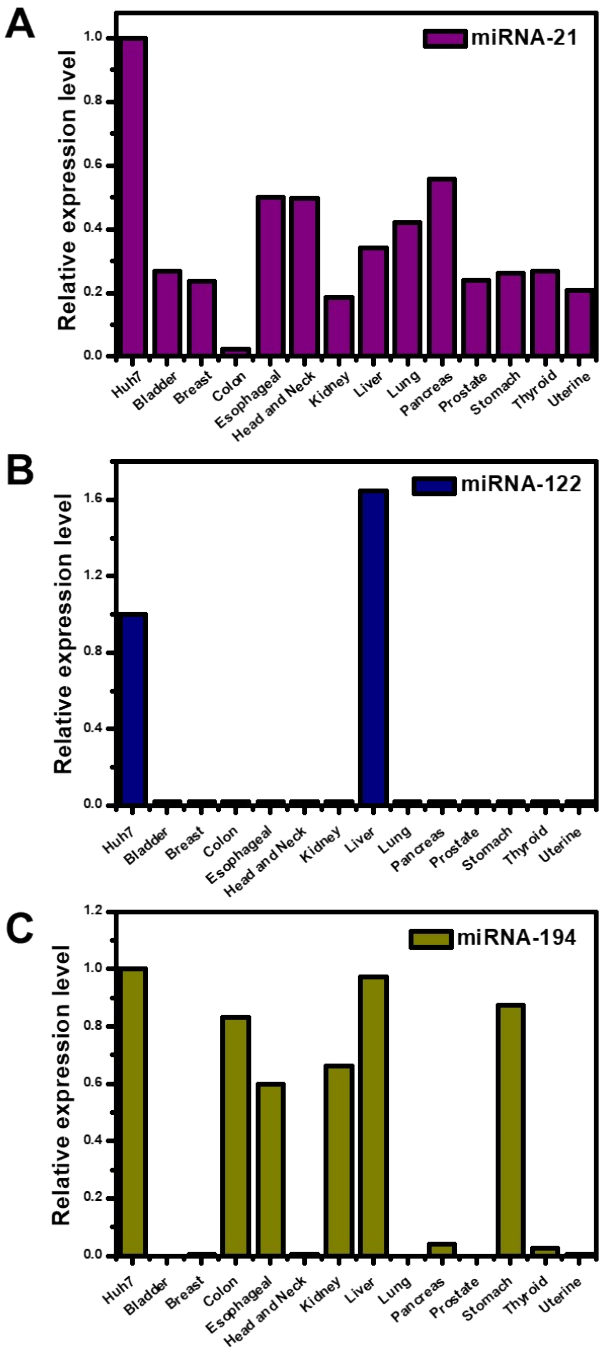

Fig. S13 The normalized relative expression of miRNA-21, miRNA-122 and miRNA-194 in the Huh7 cells compared with different tissues of mice. The data showed that the expression of miRNA-21 was higher in Huh7 compared to other mice tissues, miRNA-122 was mainly expressed in Huh7 cells and mice liver tissues, but not other tissues, miRNA-194 was expressed in Huh7, mice liver and other tissues like colon and stomach, etc.

References

1. N. Mitchell, R. Schlapak, M. Kastner, D. Armitage, W. Chrzanowski, J. Riener, P. Hinterdorfer, A. Ebner

and S. Howorka, *Angewandte Chemie-International Edition*, 2009, **48**, 525-527.
2. S. Wang, M. Xia, J. Liu, S. Zhang and X. Zhang, *ACS Sens*, 2017, **2**, 735-739.
3. L. Zhong, S. Cai, Y. Huang, L. Yin, Y. Yang, C. Lu and H. Yang, *Anal Chem*, 2018, **90**, 12059-12066.
4. L. He, D. Q. Lu, H. Liang, S. Xie, C. Luo, M. Hu, L. Xu, X. Zhang and W. Tan, *ACS Nano*, 2017, **11**, 4060-4066.
5. J. Dong, H. Dong, W. Dai, X. Meng, K. Zhang, Y. Cao, F. Yang and X. Zhang, *Anal Chim Acta*, 2019, **1078**, 176-181.
6. N. Xie, J. Huang, X. Yang, Y. Yang, K. Quan, M. Ou, H. Fang and K. Wang, *ACS Sensors*, 2016, **1**, 1445-1452.