Enhancing Cell Membrane Phase Separation for Inhibiting Cancer Metastasis with a Stimuli-responsive DNA Nanodevice

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Experimental Procedures

Materials

All DNA oligonucleotides presented in Table S1 were purchased from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China). Anti-E-cadherin, anti-N-cadherin, anti-Vimentin, α-Tubulin antibody, GAPDH antibody, FITC labeled CD44 Monoclonal antibody, FITC labeled CD59 Monoclonal antibody, FITC labeled transferrin (TfR) Monoclonal antibody and anti-rabbit immunoglobulins/HRP were purchased from Cell Signaling Technology (CST). Cell lysates (1% Triton X-100, 1% deoxycholate, 0.1% SDS), Enhanced BCA Protein Assay Kit, Cell Counting Kit-8 and lactate dehydrogenase (LDH) release assay kits were purchased from Beyotime biotechnology. (Jiangsu, China). Culture medium, fetal bovine serum (FBS), trypsin-EDTA solution and Alexa647 labeled Cholera Toxin Subunit B were purchased from Thermo Fisher Scientific. Cholera Toxin Subunit B without labeled was purchased from Absin. Cell culture inserts (8.0 μm PET), Adenosine Triphosphate (ATP), methyl-beta-cyclodextrin (MβCD), formaldehyde and crystal violet were purchased from Sigma-Aldrich.

Experimental Section

Fluorescence spectroscopy to interrogate the ATP initiated HCR in solution.

To characterize the HCR in solution, 1 μM of hairpin1 (H1), 1 μM of hairpin 2 (H2) and 200 nM initiator I strands were annealed at 95 °C to room temperature and kept at 4 °C for 1 h. Then H1, H2, initiator I and ATP were prehybridized for 1 h. The incubation concentration of ATP was 5 mM. The molar ratio of H1, H2 and initiator I was 5:5:1. The fluorescence spectroscopy was measured by Edinburgh Spectrofluorometer FS5. The excitation wavelength was 554 nm, and the emission wavelength was collected from 560 to 800 nm. FRET ratio generated from the emission intensity of Cy5 at 662 nm to the Cy3 at 565 nm (Cy5/Cy3).

Agarose gel electrophoresis to verify the formation of DNA nanodevice in solution.

The structure of DNA nanodevice was validated by gel electrophoresis performing with 5% agarose (dissolved in 1× TBE buffer). 5 μL sample was loaded in the gel and electrophoresis separation was performed with a Bio-Rad electrophoresis system at 100 V for 90 min. After that, the agarose gel was stained with GelRed solution for 15 min and washed with 1× TBE buffer. The gel was analyzed on a GelDoc™ EZ Imager System (BIO-RAD, USA).

Cell Culture

Hep G2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO). B16 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium. Both mediums contain 10% FBS, 1% Penicillin-Streptomycin Solution (100 μg/mL), 1% sodium pyruvate (100 mM). Cells were maintained in tissue culture dish with 9 mL of medium and were incubated in an incubator with 5% CO₂ at 37 °C in humidified atmosphere. Cells were free of mycoplasma contamination. Cells were routinely passaged once every 2 days with fresh medium.

DNA anchor at Cell membrane through specific interaction of CTxB

1×10⁵ cells (Hep G2 cells or B16 cells) were plated in 35 mm glass bottom dishes and cultured at 37 °C with 5% CO₂ for 24 h and grown to around 80% confluence prior to experiments. Then the cells were washed with PBS for three
times. Cells were treated with 200 µL 2 µg/mL Alexa647-CTxB at 4 °C for 30 min. Cells in control group were treated with 200 µL PBS at the same condition. Afterward, cells were washed with PBS for three times and treated with 200 µL 2 µM DNA anchor at 4 °C for 30 min. After the cells were washed with PBS three times, cells were captured with confocal florescence microscopy (Olympus, FV31-HSD) with 488 nm excitation for Alexa488 dye and 647 nm excitation for Alexa647. The fluorescence images were collected from 500 to 560 nm of Alexa488 channel and 660 to 700 nm of Alexa647 channel, respectively.

**ATP stimuli-responsive HCR on live cell membrane**

For Hep G2 cells (or B16 cells), 1×10^5 Hep G2 cells were plated in 35 mm dishes with glass bottom and cultured at 37 °C with 5% CO₂ for 24 h and grown to around 80% confluence prior to experiments. Then cells were washed with PBS for three times and treated with 200 µL 2 µg/mL CTxB at 4 °C for 30 min. Then they were washed with PBS for three times and treated with DNA anchor strand containing CTxB aptamer sequence at 4 °C for 30 min. After washed with PBS for three times, cells were treated with hairpin1, hairpin 2 and initiator I strands with 5 mM ATP in advance at 4 °C for 30 min. Finally, cells were washed with PBS three times for confocal imaging. 561 nm laser was used to excite Cy3. The fluorescence images were collected from 580 to 620 nm of Cy3 channel and 660 to 700 nm of Cy5 channel, respectively.

**Microdomain associated proteins recruitment into DNA nanodevice regions**

For Hep G2 cells, 1×10^5 Hep G2 cells were plated in 35 mm dishes with glass bottom and cultured at 37 °C with 5% CO₂ for 24 h and grown to around 80% confluence prior to experiments. After washed with PBS for three times, cells were incubated with CTxB for 30 min. And then the mixture of the consistent of DNA nanodevice and FITC functioned acceptor proteins antibodies were added into the dishes. After incubated at 4 °C for 30 min, cells were washed with PBS three times before imaging. 488 nm laser was used to excite FITC and 561 nm laser to excite Cy3. The fluorescence images were collected from 500 to 550 nm of FITC channel, 580 to 620 nm of Cy3 channel and 660 to 700 nm of Cy5 channel, respectively.

**Preparation of total cell lysates for characterizing expression of migration associated proteins**

For cell lysate preparation, Hep G2 lysates were prepared as previous reports. 2×10^5 Hep G2 cells were plated in the 6-well plates and cultured at 37 °C with 5% CO₂ for 24 h and grown to around 80% confluence. After washing with ice-cold PBS three times, cells were treated with CTxB and DNA strands as illustrated in confocal laser scanning microscopic imaging for HCR on cell membrane. Cells without any treatments were used as a control group and cells treated with HCR strand but no initiator strand were used as a positive control group. Cells only treated with CTxB also used as a positive control group. Then the cells were treated with ice-cold RIPA lysis buffer with an addition of 1×PMSF. Protein quantification of cell lysate was determined with BCA assay.

**Changing of the expression of migration associated proteins induced by enhanced phase separation.**

Equal amounts of proteins (30 µg) in cell lysates were mixed with equal volume of 2× Laemilli buffer and boiled at 95°C for 10 min. Then the protein samples were separated on 8% SDS page gels and transferred to polyvinylidene difluoride (PVDF) membrane with transfer buffer. After blocked in TBST supplemented with 5% skim milk powder at room temperature for 2 h, membranes were incubated with primary antibodies at 4°C overnight, followed by the
incubation of Goat anti-rabbit horseradish peroxidase-conjugated antibodies (1:10,000) for 1 h at room temperature. Membranes were washed three times during 10 min per washing prior to each step. The secondary antibodies used included: GAPDH, α-Tubulin, anti-CD44, anti-E-cadherin, anti-N-cadherin, anti-Vimentin. The labeled bands were subsequently detected using enhanced chemiluminescence system ECL (Adansta, USA). The gel images were captured and analyzed by using GelDoc™ EZ Imager System (BIO-RAD, USA) and ImageJ program, respectively.

Toxicity measurements

Hep G2 cells seeded in 96-well plates (2,000 cells/well) were allowed to attach overnight and incubated with various treatments. 10 μL CCK-8 solutions were subsequently added to each well, followed by incubation at 37°C for 1 h. Absorbance values were recorded using a microplate reader at 450 nm (LabServ™K3, Part of Thermo Fisher Scientific). The results from each well were averaged, and cell viability was calculated as a percentage of the untreated group. Each experiment was performed in triplicate, and the experiment was repeated three times.

LDH release measurement for investigating the permeability change of cell membrane

The release amount of LDH was quantitatively measured according to LDH Cytotoxicity Assay Kit. Hep G2 cells seeded in 96-well plates (2,000 cells/well) were allowed to attach overnight and incubated with various treatments. After treatment, Hep G2 cells were incubated at 37°C for 24 h for maximum release and spontaneous release of LDH. Cells without any treatments were lysed by addition of lysis solution before detection, followed by incubation at 37°C for 1 h. 140 μL supernatants of sample from each well were transferred to a 96-well assay plate with addition of 60 μL of LDH reaction solution. The reaction was allowed to proceed for 30 min at room temperature and kept in dark. After the reaction was stopped, the plate was read at 490 nm using a plate reader. The cellular damage (%) was determined by calculating % cytotoxicity as [Experimental Value A490 – Spontaneous Release A490]/ [Max Release A490 – Spontaneous Release A490] ×100%. Data represent the percentage of LDH released relative to maximum released.

HCR-induced inhibition of cell migration in vitro

In in vitro scratch assay, 1×10^5 Hep G2 cells were seeded in a 24-well plate with a grid etched onto the bottom and cultured for 24 h to achieve 100% confluence in starvation with serum-free DMEM. A 10 μL sterile pipette tip was used to make a scratch in the cell monolayer. Cells were washed with fresh PBS three times to remove floating cells and then treated with different treatments. For HCR regulation, cells were treated with CTxB, DNA anchor, hairpins and initiator with ATP per washing after each step. Cells treated with CTxB, without initiator and ATP, and without any treatment were used as control groups. Cells were then incubated in fresh medium without serum for different times (0, 6, 12, 18 and 24 h) at 37 °C in a 5% CO₂ incubator. The scratch gap width at each time point in each treatment group was observed at six different positions and compared with the gap width at 0 h, which was set as 1. Quantification of wounds area was determined using Image J.

Transwell migration assay was performed using Millipore transwell chambers (8 μm pore size, Millipore) as previously reported. Hep G2 cells (2 × 10^4 in each well) with various treatments were seeded in the upper chambers of the 12 well plate (Corning, USA) in 500 μL serum-free medium. For HCR regulation, cells were treated with CTxB, DNA anchor, hairpins and initiator with ATP per washing after each step. Cells treated with CTxB, without initiator and ATP and without any treatment were used as a control group. The lower chambers were filled with 1 ml medium containing 10% FBS. The chamber was incubated at 37 °C with 5% CO₂ in a humidified incubator for 24 h. At the end
of incubation, the cells in the upper chamber were removed by wiping the upper side of the membrane with a cotton swab. Cells in lower chamber were migration cells and fixed with 4% methanol and stained with crystal violet. The images were captured with inverted microscopy and analyzed using ImageJ software.

**Stimuli-responsive inhibition of tumor metastasis in vivo**

Female C57 mice (6 weeks old) with body weights ranging from 18 to 20 g [East China Normal University (ECNU) Laboratory Animal Centre] were depilated and implanted in the right flank region with B16 cells \((1.0 \times 10^6\) cells). 1 week following implantation, the mice with tumor sizes > 100 mm\(^3\) were selected and randomly divided into four groups \((n = 6\) per group), termed as blank, CTxB, CTxB - HCR and CTxB + HCR groups. These groups were treated with PBS, 50 µL CTxB solution (5 µg/mL), CTxB - HCR (5 µg/mL CTxB, 5 µM H1 and 5 µM H2) and CTxB + HCR (5 µg/mL CTxB, 5 µM H1, 5 µM H2 and 1 µM initiator I). The body weights and tumor sizes were measured every two days and the tumor volumes were calculated according to the following formula: \(\text{width}^2 \times \text{length} \times 0.5\). All experimental animals were treated according to the protocols approved by the ECNU Animal Care and Use Committee (protocol ID: m20210402). All sample or data collection procedures used are carried out in accordance with the provisions of the Ministry of Science and Technology of the People’s Republic of China on Animal Care Guidelines. All mice were euthanized after the experiments.

To determine the impact of DNA nanodevice on tumor cell metastasis, the number of the B16 tumor nodules on the whole surface of the lungs were counted. Furthermore, H&E stains of sequential tissue samples were used to confirm the location of B16 tumor nodules. For details, the lung was isolated, fixed, paraffin-embedded and coronally sliced into 4 µm thicknesses. The tissue sections were stained with H&E. Images were obtained on an invert microscopy.
Table S1. The sequence of DNA nanodevices employed in this work.

| Sequence Name       | DNA Sequence (5’ to 3’)                                                                 |
|---------------------|------------------------------------------------------------------------------------------|
| CT916-spacer-toehold (Anchor) | AATAATAATAATAATCATCCGTCACACCTGCTCGGCAAAAGGATTGCCAGGTCGTGCTAGCCGGATTCGGTGTTCGGTCCCGGTATC-Alexa 647 |
| Hairpin 1           | ATTATTATTATTATTATTTCTCCTGTTTGGCTTTTCTTTGTTACCCAGGTAAACAAGAAAGCCAAACC-Cy3                 |
| Hairpin 2           | TAACAAGAAAGCCAAACCGAGAT/Cy5/GGGTTTGGCTTTTCTTTGTTACCTGGGTTTTTATTATTATTA                   |
| Initiator I         | CCCAGGTAACAAGAAAGCCAAACCTCTTTGTTACCTGGGAGTGATTGCCGAGGAAGGT                             |
| Strand A            | ATTATTATTATTTGTATTTTCGTGTGATG-FAM                                                        |
| Strand B            | Cy3-AAGCGTGATCCCATGTTGCAATACATTATTATTATTATT                                            |
| Trigger             | TAMRA-CTACACACGAAGACACATGGGATACACGGCTT-Cy5                                              |
**Figure S1.** Stimuli-responsive HCR in solution. (A) Fluorescence profile of the ATP stimuli-responsive HCR in solution. No FRET signals were observed for mixtures of H1 (green), H2 (blue), H1+H2+I (grey violet) or H1+H2 (purple) upon the addition of the initiator. When the mixture was mixed with ATP (5 mM), the Cy5 fluorescence increased concomitantly (red). (B) Gel-electrophoresis assays characterizing the success of HCR. Only the hairpins without initiator or without ATP in channel H1, H2, H1+H2+I, H1+H2 showed only one band. Several bands emerged in channel H1+H2 with initiator and ATP indicating HCR polymeric products with different weight.
**Figure S2.** Specific combination of DNA anchor with cell membrane. (A) Upper: schematic illustration of cells only treated with Alexa488 functionalized anchor. Lower: Confocal microscopy images of Hep G2 cells upon the above-mentioned treatment. Scale bar: 20 μm. (B) Upper: schematic illustration of Hep G2 cells treated with Alexa647 functionalized CTxB and Alexa488 functionalized anchor. Lower: Confocal microscopy images of Hep G2 cells upon the above-mentioned treatment. Scale bar: 20 μm.
**Figure S3.** Stability of the HCR nanodevice on Hep G2 cell membrane without endocytosis. The Hep G2 cells were treated with HCR in 35 mm dishes and incubated for 24 h in cell culture condition before imaging. There was no obvious fluorescence detected in Hep G2 cell intracellular. Scale bar: 10 μm.
Figure S4. The hairpins indeed hybridization with the anchor on Hep G2 cell membrane. (A) Schematic illustration of treating Hep G2 cells with hairpin1 and hairpin2, which can hybridize with anchors on cell membrane. (B) Confocal microscopy images of Hep G2 cells upon the treatment mentioned in (A). Scale bar: 5 μm.
Figure S5. Colocalization of CD59 and DNA nanodevice on Hep G2 cell membrane. Upper: scheme of the distribution of CD59 and DNA nanodevice. The CD59 was labelled with FITC. Lower: Confocal fluorescence microscopy images show the distribution of Cy5/Cy3 and FITC on cell membrane. Scale bar: 5 μm.
Figure S6. Non-colocalization of TfR and DNA nanodevice on Hep G2 cell membrane. Upper: scheme of the distribution of TfR and DNA nanodevice. The TfR was labelled with FITC. Lower: Confocal fluorescence microscopic images show the distribution of Cy5/Cy3 and FITC on cell membrane. Scale bar: 5 μm.
Figure S7. No significant changes in cell morphology after HCR-treatment for 24 h. Microscopic images show that the cells seeded in 24-well plate were close to the scratch. Scale bar: 50 μm.
Figure S8. Schematic illustration of the different steps of transwell migration assay. Cells were seeded on the upper side of the transwell membrane. In the upper compartment, DMEM medium without FBS was added. In the lower compartment, DMEM medium with 10% FBS was added as a chemoattractant. Within 24-hour incubation, cells migrated to the basal chamber through the porous membrane and were quantified via crystal violet staining.
Figure S9. Schematic illustration of DNA nanodevice guiding phase separation enhanced on cell membrane generating and blocking interaction of MMP with CD44. CD44 can be sequentially cleaved by membrane matrix metalloprotease. Cleavage produced extracellular domain (ECD) fragment, transmembrane domain (TMD) and CD44 intracellular domain (ICD) fragment. CD44-ICD translocated to the nucleus to activate transcription of CD44 expression target genes. DNA nanodevice generated and blocked interaction of MMP with CD44. The combination of CD-ICD with CD44 target gene was cut off and the expression of CD44 was inhibited.
Figure S10. The specific combination of DNA strands making up the dimer on Hep G2 cell membrane. Upper: schematic illustration of treating Hep G2 cells with DNA strand A functionalized with Cy3 and strand B functionalized with FAM, which can hybridize with anchors on cell membrane. Lower: Confocal microscopy images of Hep G2 cells upon the treatment mentioned in upper. Scale bar: 20 μm.
Figure S11. No cluster emerged induced by DNA dimer on Hep G2 cell membrane. Upper: schematic illustration of treating Hep G2 cells with DNA trigger strand which can hybridize with strand A and strand B. Lower: Confocal microscopy images of Hep G2 cells upon the treatment mentioned in upper (Scale bar, 20 µm). The enlarged confocal microscopy images represent the enlarged cell region in cells treated with DNA dimer. Scale bar: 5 µm.
Figure S12. The impact of DNA dimer on Hep G2 cells migration. (A) The impact of DNA dimer on cell migration in scratch healing analysis at 0 h and 24 h. Scale bar: 100 μm. (B) Healing area ratio of Hep G2 cells treated under different conditions in scratch healing analysis at 24 h.
Figure S13. The GM1 distribution of B16 cells. Upper: Confocal microscopy images of Hep G2 cells treated with CTxB functionalized with Alexa647. Scale bar: 20 μm. The enlarged images of cells in upper. Scale bar: 10 μm.
Figure S14. HCR initiated on the membrane of B16 cells. Confocal microscopy images of Hep G2 cells treated without or with HCR. Scale bar: 20 μm. The enlarged images of cells are in lower. Scale bar: 10 μm.
Figure S15. CD44 membrane acceptors distribution on B16 cell membrane. Upper: Confocal microscopy images of Hep G2 cells treated with antiCD44 functionalized with FITC. Scale bar: 20 µm. The enlarged images of cells are in upper. Scale bar: 10 µm.
Figure S16. Measurement of function of cell membrane integrity and cell viability of B16 cells under different treatment. (A) LDH release from the cells treated with different conditions for 1h, and the results are shown as means ± SEM, n = 6. (B) Cell viability measured using CCK-8 assay after cells being incubated under different conditions for 24 h.
Figure S17. The impact of DNA nanodevice on B16 cells migration. (A) The impact of DNA nanodevice on cell migration in scratch healing analysis at 0 h and 24 h. Scale bar: 100 μm. (B) Healing area ratio of B16 cells treated under different conditions in scratch healing analysis at 24 h.
Figure S18. Quantitative size statistics of cluster. After HCR, the homogeneously distributed Cy5 fluorescence was gradually transformed onto cluster with aggregates ranging from 0.707-10.5 μm.
References

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