Supporting Information

ATP-responsive Mitochondrial Probes for Monitoring Metabolic Processes of Glioma Stem Cells in a 3D Model

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Materials and Methods

Materials

U251 cells were provided by Cell Resource Cencer (China). RPMI 1640, 10 % FBS, 1 % penicillin-streptomycin and Hoechst 33342 were purchased from Invitrogen. DMEM/F12 was purchased from Corning. The antibody of CD133 and Nestin were purchased from biorbyt and abcam respectively. The epidermal growth factor of EGF, FGF-β and TGF-β were from Pepro Tech. 2 % of B27 was purchased from Invitrogen. ATP aptamer, bPEI, PNA-TPP were from CP Biochem Co., Ltd. The Mito-Tracker Green and Lyso-Tracker Red were from Life technologies. ATP Assay Kit was purchased from Beyotime Biotechnology. ATP, ADP, GTP, and ATP were from Sigma-Aldrich (USA). Cell Titer-Glo Luminescent cell viability assay was purchased from Promega (USA).

Preparation of ATP-responsive mitochondrial probes

All PNA oligomers sequence were shown in **Table S1**. The sequence of PNA oligomers to prepare nanogold-labeled PNA was: H_2N-Cys-TTTTTTAGGT -Cys-CO_2H. PNA-TPP conjugates were synthesized as previously described.[1] Briefly, the reaction between cysteine residues of PNA and iodobutyltriphenylphosphonium (IBTP) can displace iodide and form stable TPP-PNA conjugates. PNA oligomers (1 μmol mL^{-1}) were mixed with 2-mercaptoethanol (50 μmol mL^{-1}) in 50 μL solution (10 mM HEPES, 1 mM EDTA, pH 8.0) at 40 ℃ for 1 h. After that, IBTP (500 nmol) in 200 μL solution (HEPES/EDTA:ethanol=4:1) was added and incubated for 4 h, followed by adding 250 nmol of 2-mercaptoethanol. The products were then purified by reverse phase high performance liquid chromatography. To form AMP, the PNA-TPP conjugates were mixed with ATP aptamer.[2] After incubation at 88 ℃ for 10 min, the reaction was slowly cooled to room temperature to form a hybridization complex as the TPP-targeted ATP indicator. The fluorescence of PNA-TPP conjugate was quenched when it was successfully hybridized to the ATP aptamer. The TPP-targeted ATP indicators were dissolved and diluted with phosphate buffer saline (PBS). Effective formation of bPEI/ATP indicator complex was completed by adding 1μg of

indicators into 100 μL of PBS buffer within 2 μL of bPEI. The complex was stand at room temperature for 20 min before diluted to 300 μL with culture medium. Cells were incubated with these bPEI/ATP indicator complexes for 2 h and stained with Mito-Tracker Green or Lyso-Tracker Red.

Performance of ATP detection

The ATP was provided at a concentration of 10^{-4} M. The fluorescence measurements were carried out with the addition of a series of concentrations of ATP at Ex=615 nm, Em=594 nm for Texas Red modified AMP. For specificity test, ATP and analogues (GTP, CTP and UTP) were added at the concentration of 1 μM. The fluorescence intensity was read out at 615 nm after incubation at 37 ℃.

Glioma stem cells culture and culture with pH treatment

Glioma stem cells (GSCs) were derived from U251 cells. GSCs were cultured and maintained in DMEM/F12 supplemented with 20 ng mL^{-1} of epidermal growth factor (EGF), 20 ng mL^{-1} of FGF-β, 2 % of B27. After culturing for 3-7 days, the GSCs neurospheres appeared (Fig. S1a). Nestin protein and CD133 proteins were detected as markers to identify GSCs. The second generation of GSCs was used in the following experiment. Immunofluorescent staining of GSCs was shown in Fig. S1b. All cells were cultured at 37 ℃ in a humidified incubator containing 5 % CO_2. To assess effects of low pH, media pH was altered with hydrochloric acid. Non-stem tumor cells were cultured in a DMEM/Neurobasal media at pH 7.5 or 6.5 containing 5 % FBS, 5 ng mL^{-1} FGF-β, and 5 ng mL^{-1} EGF and media were changed every other day for the indicated times. For stem cell maintenance studies, GSCs were cultured in Neurobasal adjusted to pH 7.5 or pH 6.5 containing 10 ng mL^{-1} FGF-β and 10 ng mL^{-1} EGF, and media was changed every other day.

Detection of mitochondrial membrane potential by flow cytometry

Cells were treated with AMP for 2 h before detection. Cells as positive control were addressed with 10 μM of CCCP for 6 h. Cells treated with equal volume of phosphate

buffer saline (PBS) buffer were taken as negative control. All groups of cells were treated with tyrisin and collected for JC-1 dyeing. After treatment of JC-1 for 45 min, all groups were washed and collected for flow cytometry.

Nanogold-labeled electron microscopy

Nanogold-labeled PNA was prepared by conjugating monomaleimido nanogold (Nanoprobes) to PNA oligomers according to the manufacturer's protocol. The final production was purified by HPLC. Adherent U251-MG cells were transformed with nanogold-labeled PNA for 4 h in the presence of bPEIs. Cells were then removed from the substrate, washed and prepared for transmission electron microscopy.[3,4] Briefly, the cells were fixed with 2.5% glutaraldehyde overnight, and then dehydrated in ethanol, embedded in Lowicryl white and polymerized under UV at 0 ℃. Ultrathin sections were mounted on nickel grids which were floated on drops of 0.1 M glutaraldehyde in PBS for 10 min, followed by goldGoldenhance EM (Nanoprobes) treatment.

3D microfluidic chip design and fabrication

The main straight part of three paralleled channel is 5 mm long (Fig. S3a). As shown in Fig. S3b, The side microchip channel is 1 mm wide and 0.17 mm high. The middle channel (0.6 mm wide, 0.17 mm high) is designed to fill with mixed matrix and is separated from the side channels by small pillars (0.1 mm). Channels of microfluidic device were fabricated using PDMS (Sylgard 184, Dow Corning) by soft lithography and replica molding techniques.[5] First, a negative photoresist SU-8 2050 (Microchem) was spin coated onto a cleaned silicon wafer that precleaned by piranha solution. Then, the wafer was pre-baked in the oven (5 min at 65 °C and then 4 min at 95 °C). After cooling down to room temperature, the wafer covered by a shadow mask containing channels was exposed to UV light (7 mW) for 1 min. After developing, the silicon wafer was post-baked at 65°C for 5 min. PDMS (Sylgard 184, Dow corning) with the weight rate of 10:1 between monomer and curing agent was poured into the silicon mold and baked in the oven at 80°C for 2 h. Then the PDMS was peeled off

from the wafer mold, and was treated with oxygen plasma (PDC-32G, Harrick Plasma, Ithaca, NY) for 90 s. After that, the PDMS with microchannels was irreversibly sealed with a glass slide.

Establishment of three-dimensional invasion model

The invasion assay was processed on a three-channel microfluidic chip. The middle channel was filled with Collagen I and Matrigel. GSCs were seeded at a density of 6×10^6 mL^{-1} in one side channel in DMEM medium. In the other side channel, the medium containing TGF-β (10 ng mL^{-1}) and was filled and flowed at a rate of 20 μL h^{-1} by Harvard PHD 22/2000 Advance Syringe Pump. After 7 days of flow culture, GSCs were transformed with AMP and stained with Mito-Tracker Green or Mito-Tracker Red. The images were gained by Zeiss780 inverted fluorescence microscope (Oberkochen, Germany) after cells were washed with PBS.

Table S1. Sequences of ATP-responsive mitochondrial probe.

Name	Sequence
ATP-responsive sequence	5'-ACCTGGGGGAGTATTGCGGAGGGAGGT-DABCYL-3' 5'-ACCTGGGGGAGTATTGCGGAGGGAGGT-BHQ2-3'
PNA reporter	H_2N-Cys-TTTTTTTCCA-Texas Red H_2N-Cys-TTTTTTTCCA-FITC H_2N-Cys-TTTTTTAGGT -Cys-CO_2H

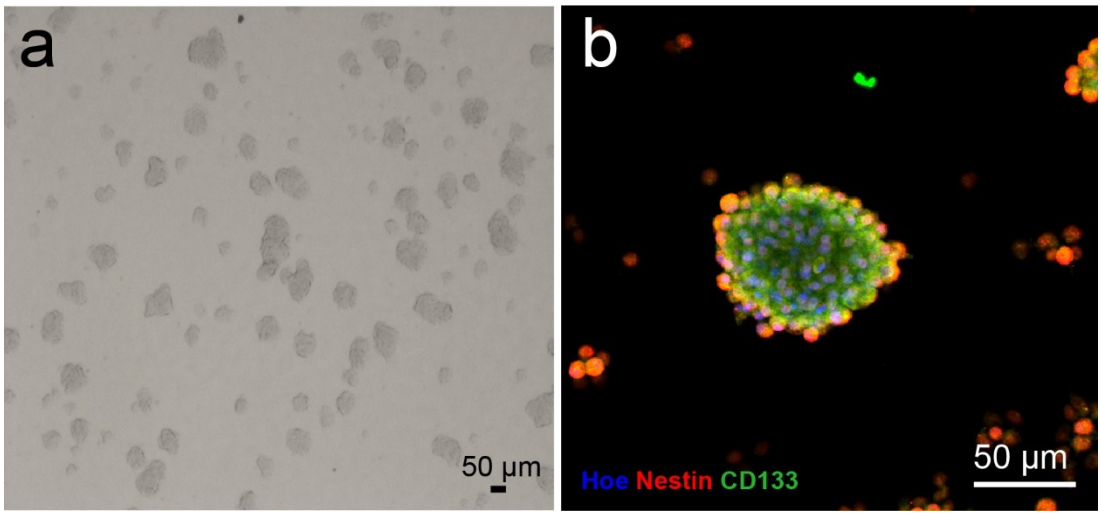

Fig. S1 Forming of glioma stem-like cells (GSCs) (a) U251 GSCs form neurospheres (>50 μm). (b) Immunofluorescence image of markers for GSCs derived from U251 cells.

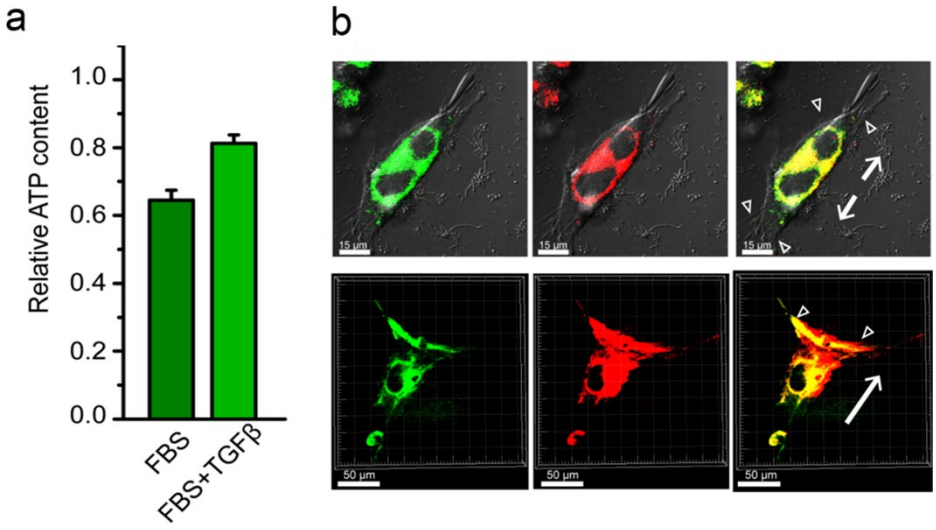

Fig. S2 Characterization of AMP. (a) The level of intracellular ATP in undifferentiated U251 cells in FBS and GSCs derived from U251 cells under the TGF-β treatment by AMP. (b) Fluorescence imaging of differentiated U251 GSCs. The hollow triangles indicate the lamellipodia, and white arrows indicate the migration direction. Mitochondria are stained by Mito-Tracker green, and red fluorescence is from AMP.

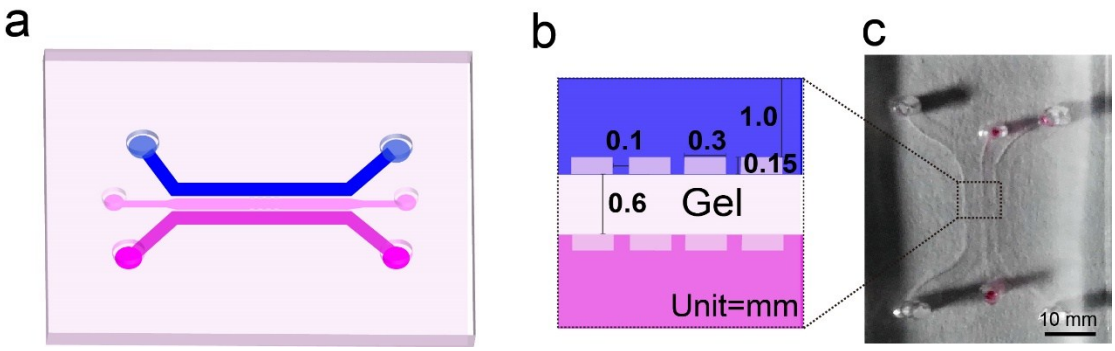

Fig. S3 Microfluidic chip design. (a) The 3D invasion model is established based on a three-channel PDMS microfluidic chip. (b) The size of channels in the chip. (c) Photo of prepared chip.

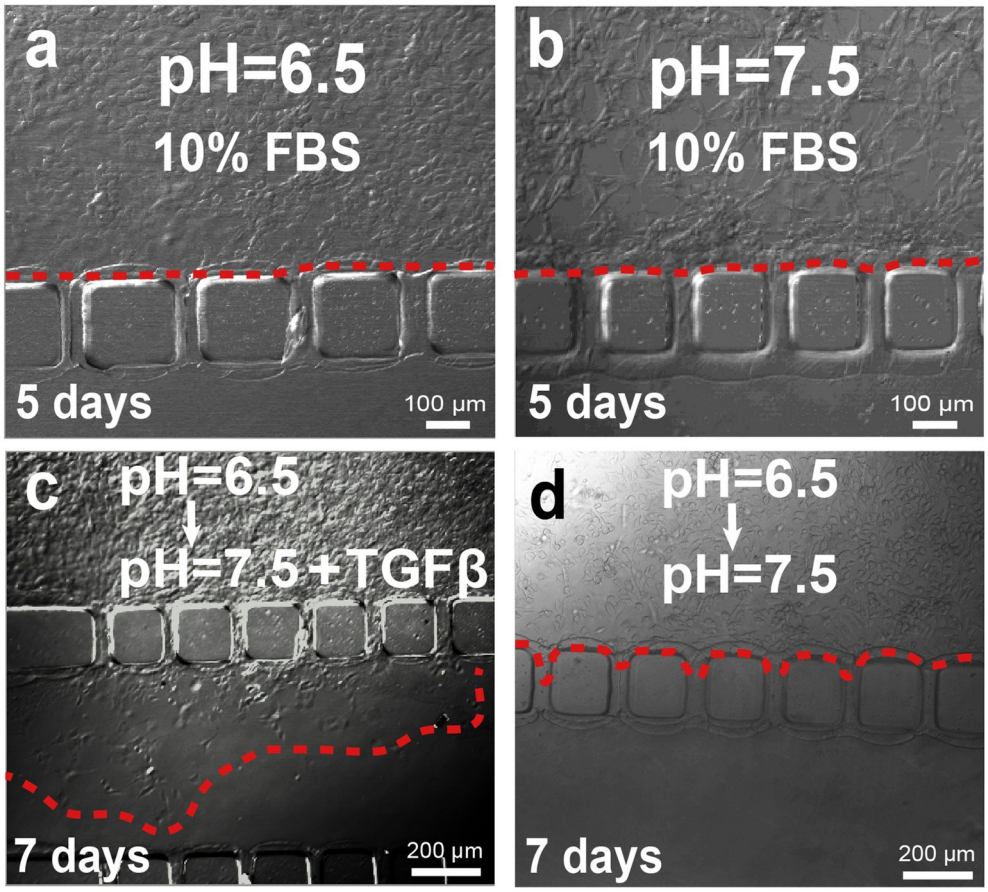

Fig. S4 The effect of pH 7.5 and 6.5 pH on migration of U251cells. (a) Bright-field microscopic images of U251 cells under normal (10 % FBS at pH 6.5) and (b) acidic stress (10 % FBS at pH 7.5) for 5 days. (c) Bright-field microscopic image showing a high malignant potential of U251 cells when replacing the culture medium from 2 % FBS at pH 6.5 to 10 % FBS with TGF-β (10 ng mL^{-1}) at pH 7.5, (d) and restoring pH to 7.5 without addition of TGF-β.

Reference

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