SUPPORTING INFORMATION

In situ programmable DNA circuit-promoted electrochemical characterization of stemlike phenotype in breast cancer

Ya Cao[§, #], Xiaomeng Yu[§], Bing Han[§], Langjian Dong[§], Jingjing Xu[§], Yuhao Dai[§], Genxi Li[§, #, *], Jing Zhao[§, *]

[§]Center for Molecular Recognition and Biosensing, School of Life Sciences, Shanghai University, Shanghai 200444, P. R. China

[#]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210023, P. R. China

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1. Experimental Materials and Procedures

Material. All DNA probes were synthesized and purified by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Their sequences were listed in Table S1. FITC-labeled anti-CD44 (anti-CD44/FITC), anti-CD24, PE-labeled anti-CD24 (anti-CD24/PE), anti-NCL, FITC-labeled mouse IgG1 (isotype control), and PE-labeled mouse IgG1 (isotype control) were purchased from Abcam (Shanghai, China). Anti-CD44 was purchased from Sino Biological Inc. (Beijing, China). FITC-labeled anti-NCL (anti-NCL/FITC) and FITC-labeled rabbit IgG (isotype control) was purchased from Biosynthesis Biotechnology Inc. (Beijing, China). Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), sodium ascorbate, 3-(N-morpholino)-propane sulfonic acid (MOPS), N-hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethlcarbodiimide hydrochloride (EDC), chlorpromazine, methyl-β-cyclodextrin, and methylcellulose were purchased from Sigma-Aldrich (Shanghai, China). Copper sulfate ($CuSO_4$) and nitric acid (HNO_3) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dihydrochloride (DAPI), doxorubicin, metformin hydrochloride (MET), and salinomycin sodium salt (SAL) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Bovine serum albumin (BSA) and 0.1% crystal violet staining solution were purchased from Coolaber Science & Technology (Beijing, China). Fetal bovine serum (FBS), 4% paraformaldehyde fix solution, basic fibroblast growth factor (FGF-basic), epidermal growth factor (EGF), and 3,3′-dioctadecyloxacarbocyanine perchlorate (Dio) were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Cell lines. MCF-7, BT-474, MCF-10A, and MDA-MB-231 cells were purchased from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China). MCF-7 and BT-474 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% FBS in a humidified atmosphere with 5% CO_2 at 37°C, while MCF-10A cells were grown in mammary epithelial cell medium (MEpiCM) (ScienCell) and MDA-MB-231 cells were grown in RPMI 1640 (Gibco) with 10% FBS under the same culture conditions. These cells were collected at the end of the log phase and used for the following experiments. eBCSCs were enriched by serum-free culture of MCF-7 cells. To this end, MCF-7 cells were harvested at the end of the log phase using 0.25% trypsin digestion, and then

centrifuged at 500 rpm for 5 min to separate from the supernatant. After that, the cells were cultured in serum-free DMEM/F12 (Gibco) with the addition of 10 ng/mL of FGF-basic, 20 ng/mL of EGF, and 0.4% B-27 supplement (Gibco). After 36 h, eBCSCs were enriched and dispersed in a sterile PBS for the following experiments.

Flow cytometry analysis. For FCM analysis, detached cells were washed with ice-cold PBS (containing 1% FBS and 1% sodium azide) and re-suspended in 1-mL volume at a number of 2×10^6 cells. Afterward, fluorophore-conjugated antibodies were added to the cell suspension at concentrations recommended by the manufacturers and incubated at 4°C in the dark. After 1 h, the cells were washed with ice-cold PBS for three times and centrifuged at 500 rpm for 5 min to remove unbounded antibodies. The following antibodies were used for FCM analysis: anti-CD44/FITC, anti-CD24/PE, anti-NCL/FITC, FITC-labeled mouse IgG1 (isotype control), PE-labeled mouse IgG1 (isotype control), and FITC-labeled rabbit IgG (isotype control). Stained cells were finally brought to analysis on a MoFlo XDP flow cytometer (Beckman Coulter, USA) and data were analyzed using FlowJo software (version 10.0.7r2).

Immunofluorescence analysis. Cells were seeded in confocal dishes for 24 h, then fixed with 4% paraformaldehyde solution for 15 min (except for NCL analysis), and incubated with PBS containing 0.1% Tween 20 and 1% BSA for 30 min. After that, the cells were immunostained at 4°C with fluorophore-conjugated antibodies. After 4 h, the cells were further stained with DAPI for 15 min and subsequently washed twice with PBS. The following antibodies were used for immunofluorescence analysis: anti-CD44/FITC, anti-CD24/PE, and anti-NCL/FITC. Finally, stained cells were imaged using a LSM 710 confocal laser scanning microscope (Zeiss, Germany) equipped with a 40×/1.30 oil lens objective. DAPI, FITC, and PE were excited by 405, 488 and 633 nm lasers, respectively. ZEN lite software (ZEN 3.2, blue edition) was used for image processing.

Mammosphere-forming assay. Formation of mammospheres were carried out with reference to the literature.[1] In detail, one thousand cells were plated per well in low-adherence 96-well plates (Corning) and grown in a serum-free medium supplemented with 20 ng/mL EGF, 10 ng/mL FGF-basic, 0.4% B-27, and 0.5% methylcellulose. After being cultured for 7 days in a humidified atmosphere with 5% CO_2 at 37°C, the cells were brought to be counted and photographed.

Drug resistance assay. Cells were seeded at a number of 1×10^5 cells per well in

96-well plates and cultured in medium for one day. After that, the cells were treated with 250 ng/mL doxorubicin for an additional 72 h, and were then collected. Viability of the cells was determined with the use of Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's recommended instructions.

Preparation of anti-CD44-functionalized magnetic beads (MB-CD44) and cell enrichment. To prepare MB-CD44, 0.2 M EDC and 0.1 M NHS was first mixed in 1 mL solution and used to activate the carboxylated magnetic beads (Invitrogen) at 25°C for 30 min. Then, 20 μL of 0.16 mg/mL activated beads were incubated with 480 μL of PBS that contained 5 μL of anti-CD44 at 4°C for 2 h. After being blocked with 0.1% BSA, MB-CD44 were successfully obtained. For cell enrichment, 50 μL of MB-CD44 were incubated with 500 μL of cell suspensions at 25°C for 1 h. After magnetic separation, the captured cells were stained with Dio for 15 min. The fluorescent images were obtained by Zeiss Axio Imager M2 fluorescent microscopy (Zeiss, Germany).

Gel electrophoresis analysis. In brief, 10 μL of different DNA samples (A_1A, A_1T, B_1B, B_1F, A_1B_1T, B_1B incubated with T and F, A_1A incubated with B_1B, T, and F) were prepared in PBS with a final concentration of 1 μM. Afterward, 10 μL of the samples together with 2 μL of SYBR Green I and 2 μL 6× loading buffer were loaded onto a 20% non-denaturing polyacrylamide gel. Electrophoresis separation was then carried out in 1×Tris-boric acid-EDTA (TBE) at 120 V for 90 min. Imaging of the resulting gel was performed using a Gel Doc XR^+ System (Bio-Rad, USA).

Conjugation of DNA probes to antibodies. Antibody-DNA probe conjugates were prepared using sulfo-SMCC as the crosslinking reagent. Briefly, 1.3 μM antibody and 10 μM sulfo-SMCC were reacted in 100 μL of PBS at 25°C for 2 h. Then, the solution was centrifuged in an ultrafiltration centrifuge tube (MWCO 30 K) at 14,000 rpm for 10 min. The upper unfiltered substance was re-dissolved in 100 μL of PBS and further incubated with 10 μM DNA probes at 25°C. After 2 h, the reacted mixture was transferred to an ultrafiltration centrifuge tube to be centrifuged at 14,000 rpm for 10 min. Finally, the upper unfiltered antibody-DNA probe conjugates were collected and re-dissolved in 200 μL of PBS for further use. The following antibodies were used for the preparation of antibody-DNA probe conjugates: anti-CD24 and anti-NCL.

Confocal microscopic analysis of in situ PDC at cell surfaces. For confocal imaging analysis, two kinds of antibody-DNA probe conjugates (A_1A@Anti-CD24 and FAM-B_1B@Anti-NCL) were prepared. After being seeded in confocal dishes and

cultured overnight, cells were pretreated with 50 μM chlorpromazine and 5 mM methyl-β-cyclodextrin at 4°C for 30 min. Then, the cells were washed twice, and desired amounts of cells were re-suspended in 200 μL of ice-cold PBS containing 1% FBS and 1% sodium azide. Afterward, the cells were immunostained with A_1A@Anti-CD24 and FAM-B_1B@Anti-NCL at 4°C for 1 h, and further stained with DAPI for 15 min. After being washed with ice-cold PBS, the cells were incubated with 500 μL of ice-cold PBS containing 1% sodium azide and 1.5 μM T and F at 4°C to sustain PDC on cell surfaces. During 3-h incubation, the cells were subjected to imaging by a LSM 710 confocal laser scanning microscope equipped with a 40×/1.30 oil lens objective. DAPI and FAM were excited by 405 and 488 nm lasers, respectively. ZEN lite software (ZEN 3.2, blue edition) was used for image processing.

Preparation of B_1-templated copper nanoparticles (CuNPs-B_1) and B_1B@Anti-NCL. CuNPs-B_1 were synthesized using probe B_1 as the template because it contained a 30-mer poly T sequence. The synthesis was performed by adding 10 μL of 30 mM sodium ascorbate and 10 μL of 2 mM $CuSO_4$ into 80 μL of 10 μM B_1 in MOPS buffer (20 mM MOPS, 300 mM NaCl, pH 7.5). After being reacted for 15 min at 25°C, CuNPs-B_1 were formed. Then, CuNPs-B_1 were hybridized with probe B, and cross-linked with anti-NCL using sulfo-SMCC, resulting in the formation of B_1B@Anti-NCL.

Electrochemical phenotyping method toward eBCSC determination. A typical experiment for electrochemical phenotyping was performed by firstly incubating cells with immunomagnetic beads MB-CD44. After repeatedly magnetic separation and washing, the enriched cells were treated with chlorpromazine and methyl-β-cyclodextrin for 30 min, followed by being re-suspended in ice-cold PBS containing 1% sodium azide. Then, the cells were incubated with 200 μL of A_1A@Anti-CD24 and B_1B@Anti-NCL at 4°C for 1 h. Thereafter, the cells were separated from the remaining free antibody-DNA probe conjugates with the help of a magnetic field, and were thoroughly washed with ice-cold PBS. Then, the cells were mixed with 200 μL of ice-cold PBS containing 1% sodium azide and 1.5 μM T and F at 4°C to sustain the PDC process. 2 h later, the cells were magnetically separated again and treated with 200 μL of 0.5 M HNO_3 to dissolve CuNPs loaded on cell surface. The resulting solution was mixed with 3.8 mL of 0.5 M acetic acid-sodium

acetate buffer, and used for electrochemical measurements to record the stripping signal of CuNPs. Electrochemical measurements were performed on CHI 660C workstation (Shanghai, China) with a three-electrode system, including a platinum wire as the auxiliary electrode, a saturated calomel electrode as the reference electrode, and a glassy carbon electrode as the working electrode. The procedure for electrochemical measurements involved a 480-s electrodeposition at -1.2 V, followed by a DPV scanning conducted from 0 to 0.5 V with a 50-mV amplitude.

Xenograft mouse model of tumorigenesis. To establish a xenograft mouse model of tumorigenesis, female Balb/c nude mice (5 to 6 weeks) were purchased from GemPharmatech Co. Ltd. (Nanjing, China), and acclimated in the Specific Pathogen Free animal facility for at least 7 days before experiments. Then, the mice were subcutaneously inoculated with 5×10^6 MDA-MB-231 cells (suspended in a mixture of medium and Matrigel) or MCF-7 cells and housed under aseptic conditions. Body and tumor weights of the mice were measured once per 5 days. After 6 weeks of inoculation, the mice were sacrificed, and the tumor issues were collected for electrochemical phenotyping, FCM, and immunohistochemistry experiments. All mice were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee, and the experiment protocol was approved by the Ethical Committee of the Shanghai University.

Statistical analysis. Two-tailed Student's *t*-test was used for evaluating statistical mean differences between two groups. All statistical analyses were performed using the GraphPad software (Prism 7), and $P < 0.05$ was regraded statistically significant.

REFERENCE

(1) Gupta, P. B.; Onder, T. T.; Jiang, G.; Tao, K.; Kuperwasser, C.; Weinberg, R. A.; Lander, E. S. Identification of selective inhibi-tors of cancer stem cells by high-throughput screening. *Cell* **2009**, *138*, 645-659.

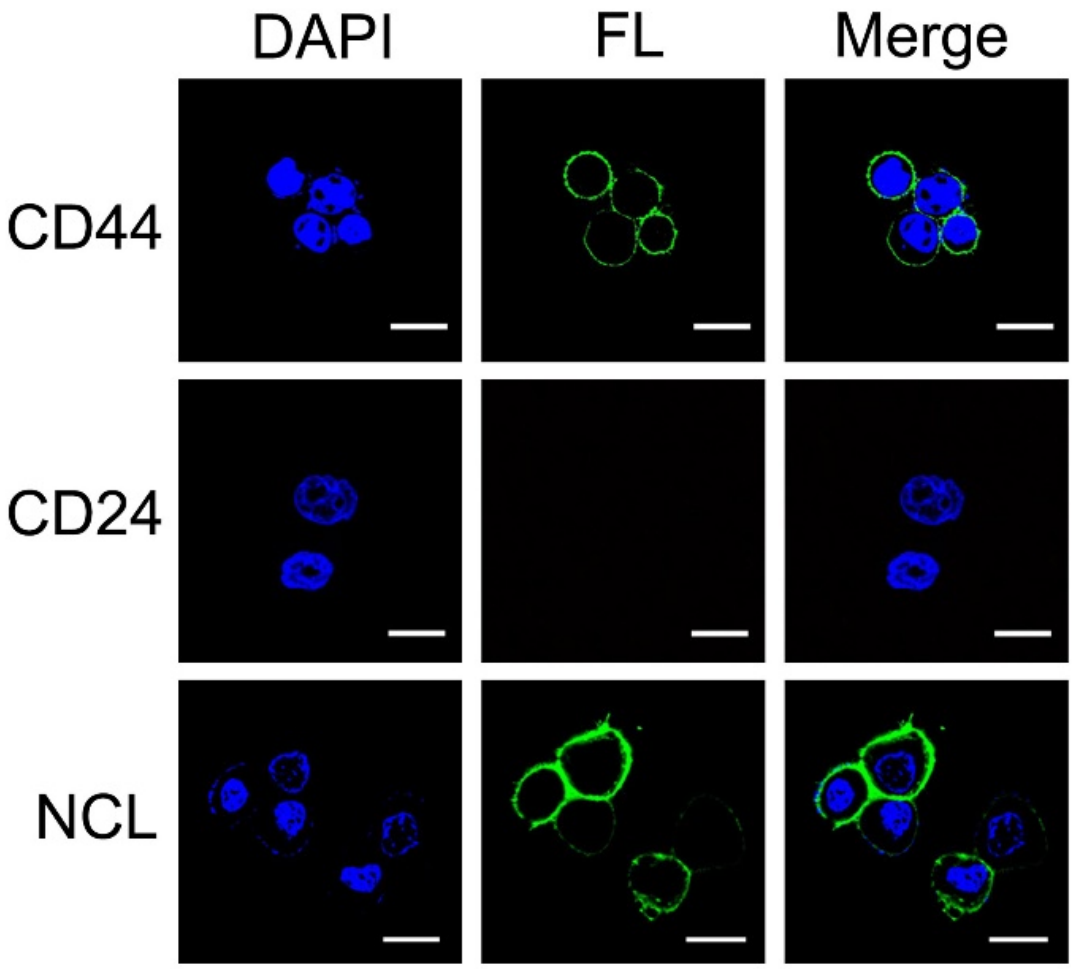

Figure S1. Immunofluorescence staining of eBCSC. DAPI (blue) was used to highlight nuclei. Anti-CD44/FITC (green), anti-CD24/PE (red), and anti-NCL/FITC (green) were used to reveal the membrane location of CD44, CD24 and NCL in eBCSC. Scale bars, 10 μm.

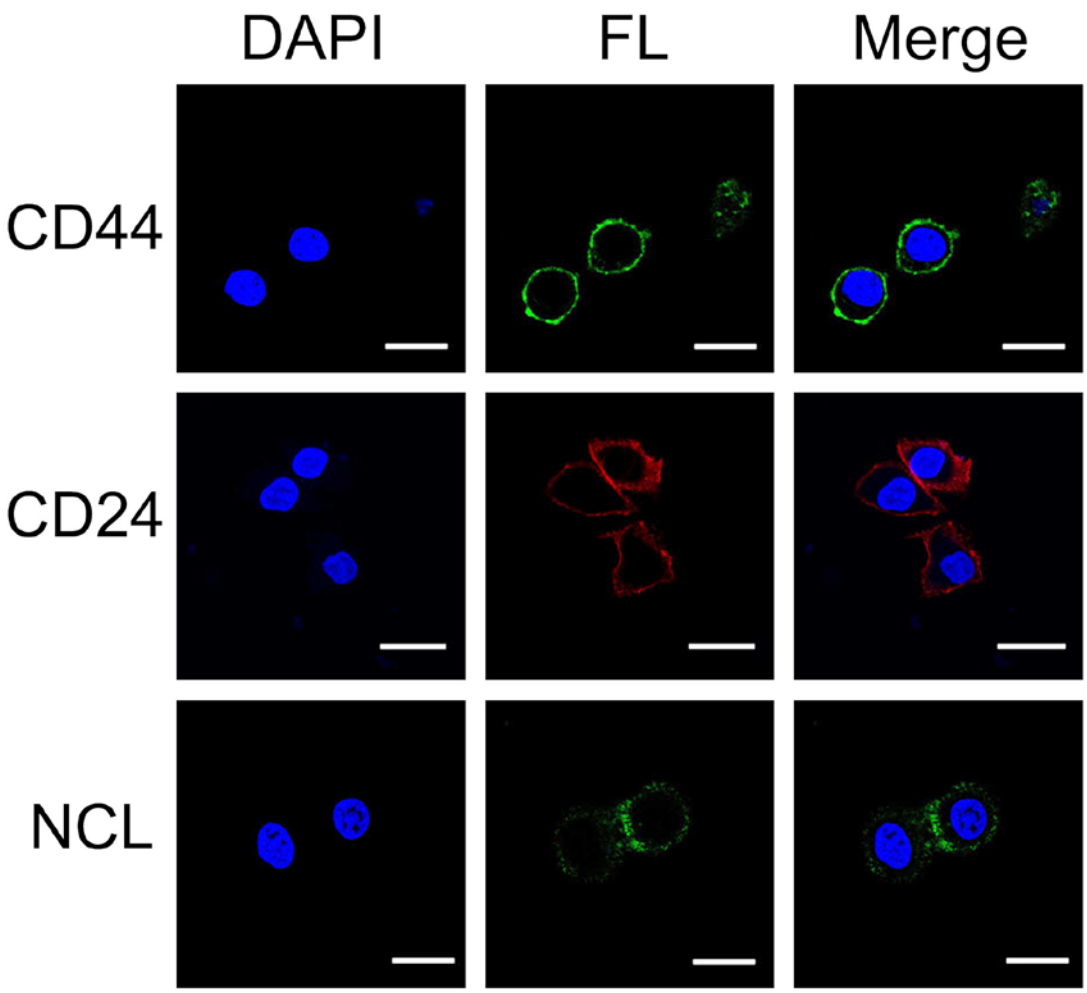

Figure S2. Immunofluorescence staining of MCF-7 cell. DAPI (blue) was used to highlight nuclei. Anti-CD44/FITC (green), anti-CD24/PE (red), and anti-NCL/FITC (green) were used to reveal the membrane location of CD44, CD24 and NCL in MCF-7 cell. Scale bars, 20 μm.

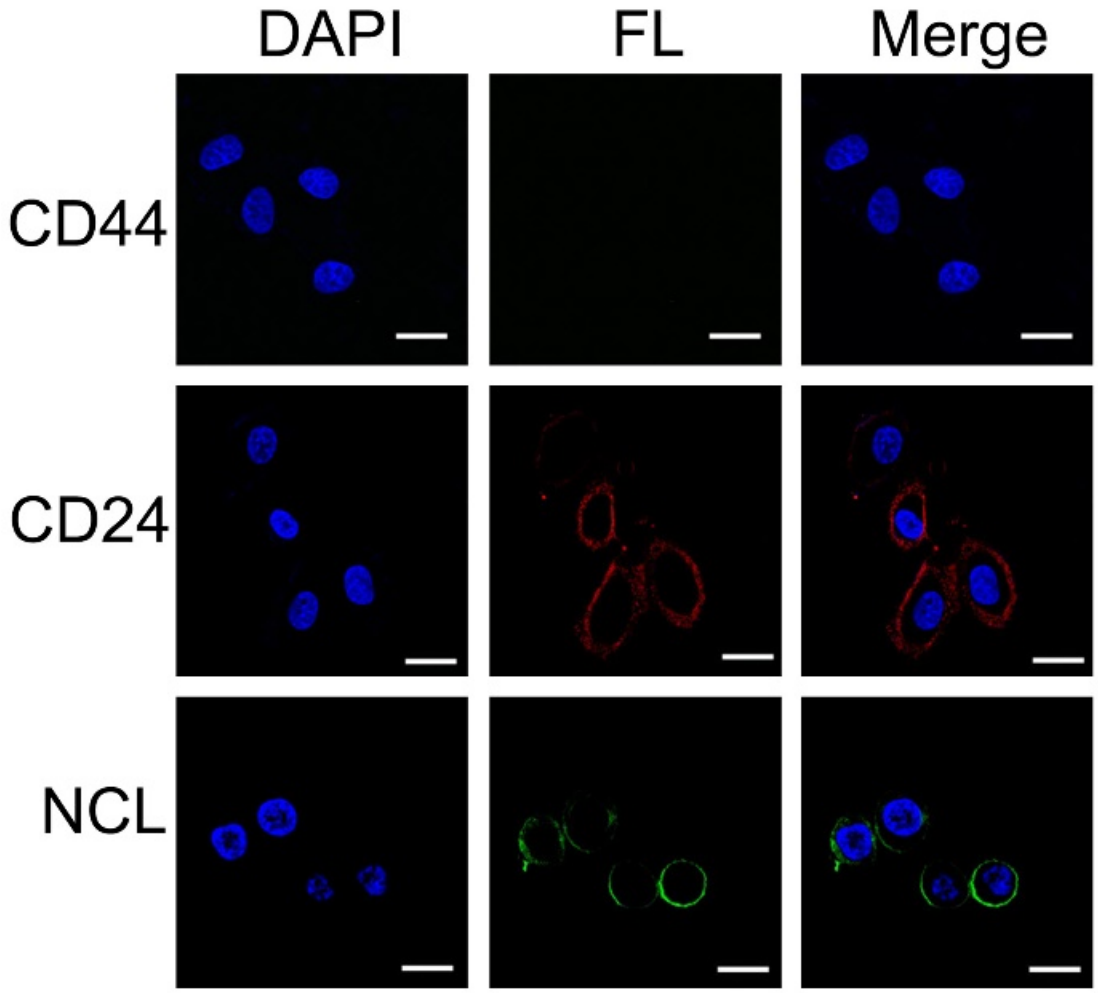

Figure S3. Immunofluorescence staining of BT-474 cell. DAPI (blue) was used to highlight nuclei. Anti-CD44/FITC (green), anti-CD24/PE (red), and anti-NCL/FITC (green) were used to reveal the membrane location of CD44, CD24 and NCL in BT-474 cell. Scale bars, 20 μm.

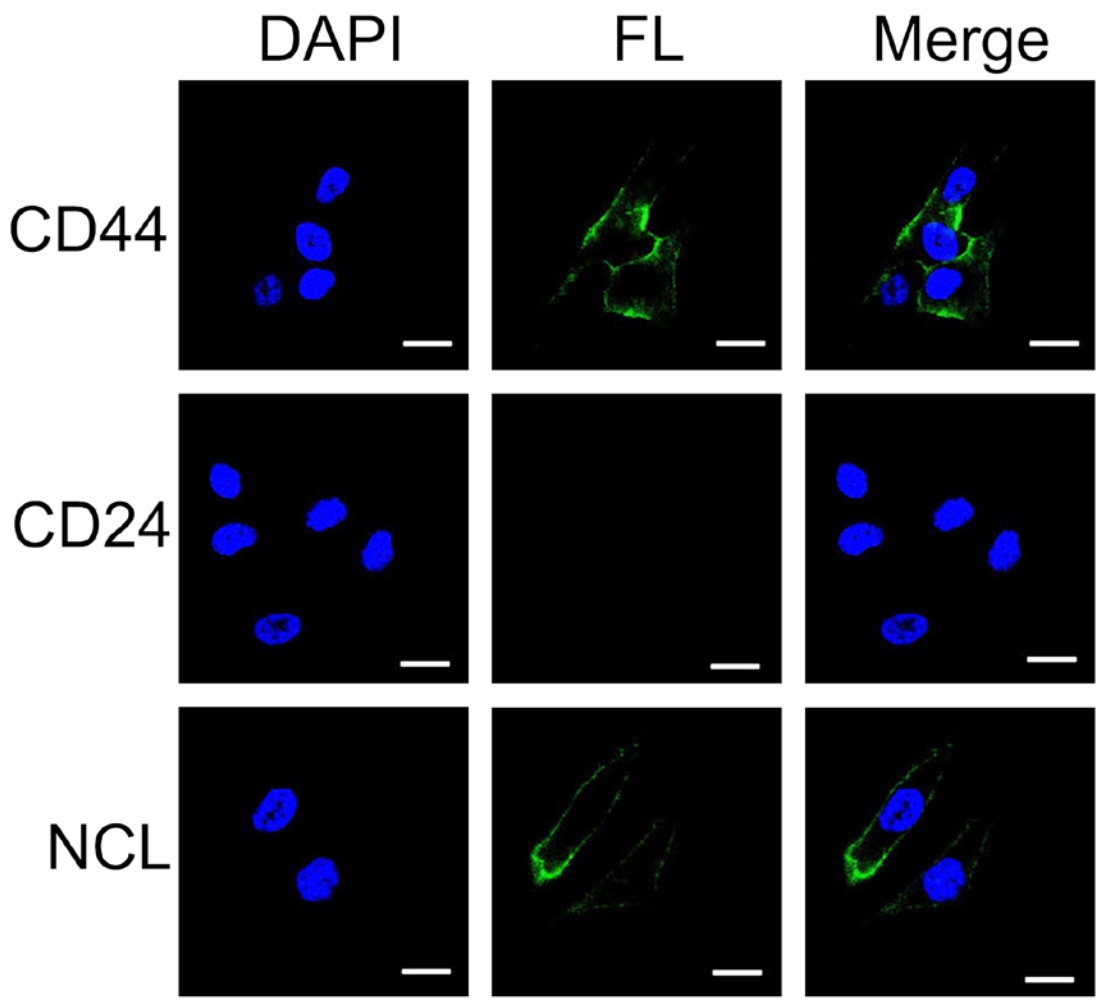

Figure S4. Immunofluorescence staining of MDA-MB-231 cell. DAPI (blue) was used to highlight nuclei. Anti-CD44/FITC (green), anti-CD24/PE (red), and anti-NCL/FITC (green) were used to reveal the membrane location of CD44, CD24 and NCL in MDA-MB-231 cell. Scale bars, 20 μm.

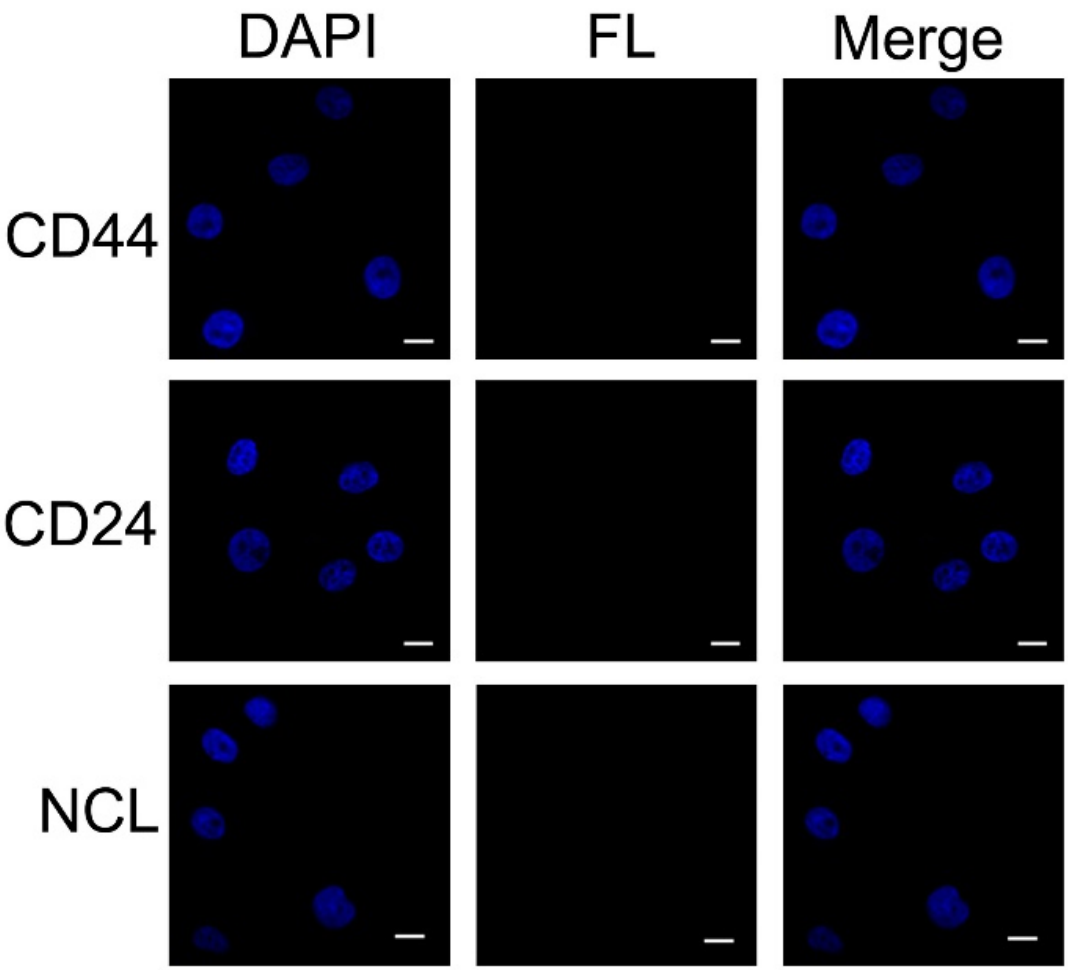

Figure S5. Immunofluorescence staining of MCF-10A cell. DAPI (blue) was used to highlight nuclei. Anti-CD44/FITC (green), anti-CD24/PE (red), and anti-NCL/FITC (green) were used to reveal the membrane location of CD44, CD24 and NCL in MCF-10A cell. Scale bars, 20 μm.

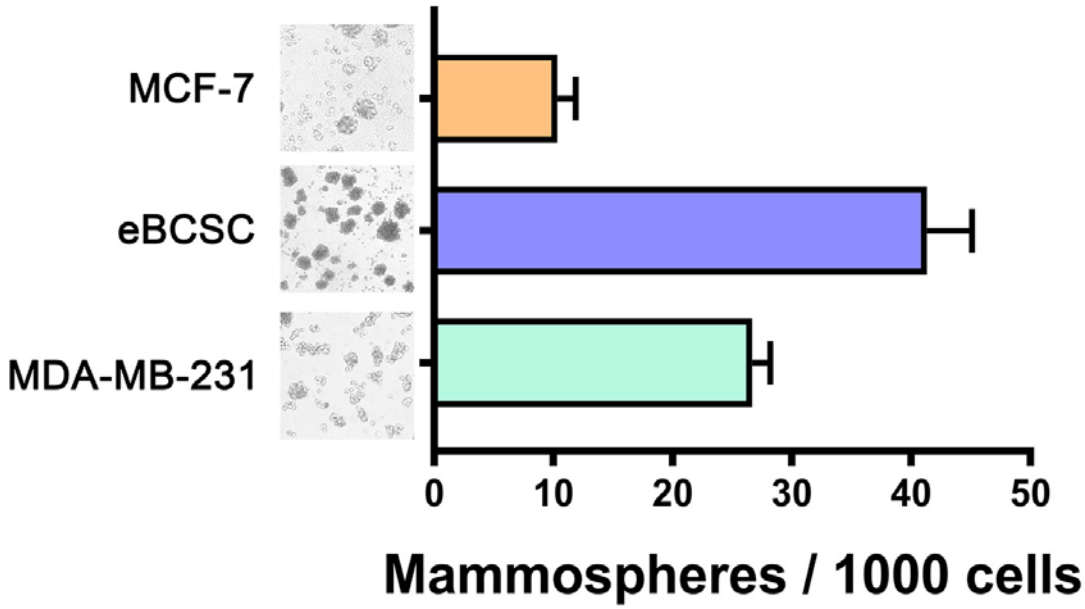

Figure S6. Imaging and quantification of mammospheres formed by MCF-7, eBCSC, and MDA-MB-231 cells. The quantification data were reported as the number of mammospheres formed per 1000 plated cells.

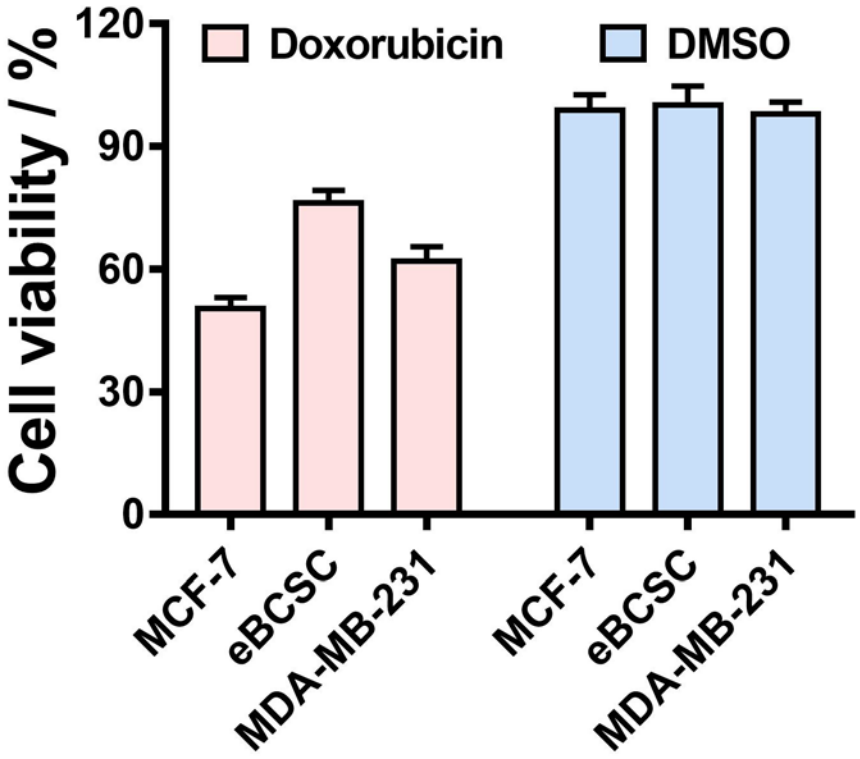

Figure S7. Viability of MCF-7, eBCSC, and MDA-MB-231 cells treated with 250 ng/mL doxorubicin and DMSO (as the control), respectively.

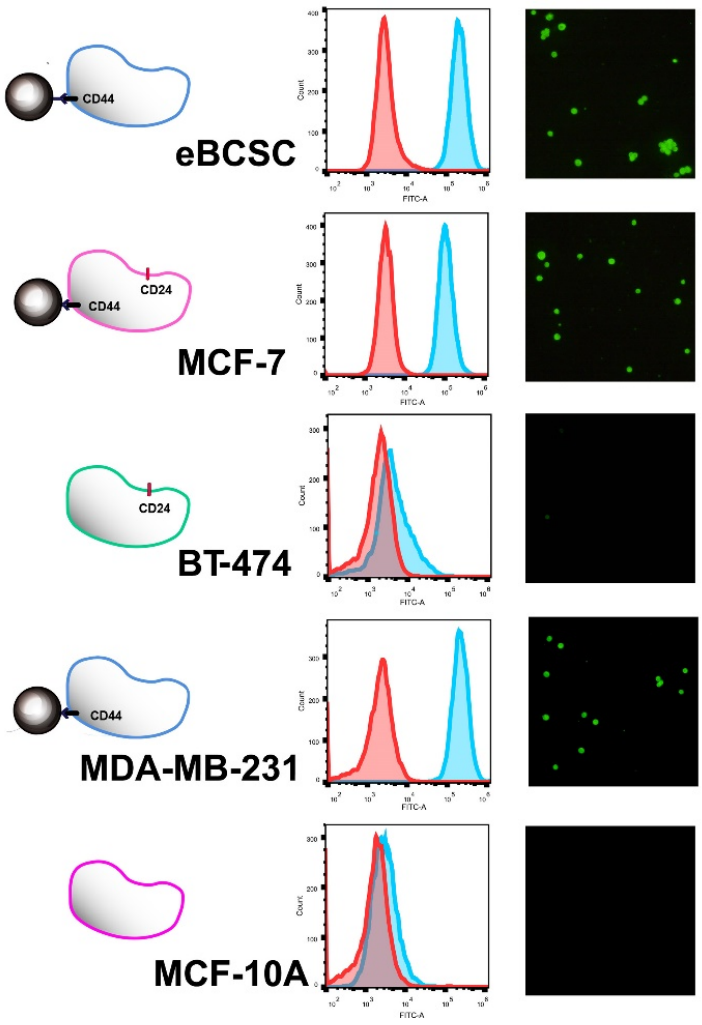

Figure S8. Flow cytometry verification and fluorescent microscopic observation of CD44-MB after incubation with eBCSCs, MCF-7 cells, BT-474 cells, MDA-MB-231 cells, and MCF-10A cells. The enriched cells were stained with Dio (green). Controls (red) in flow cytometric results were carried out using CD44-MB without cells.

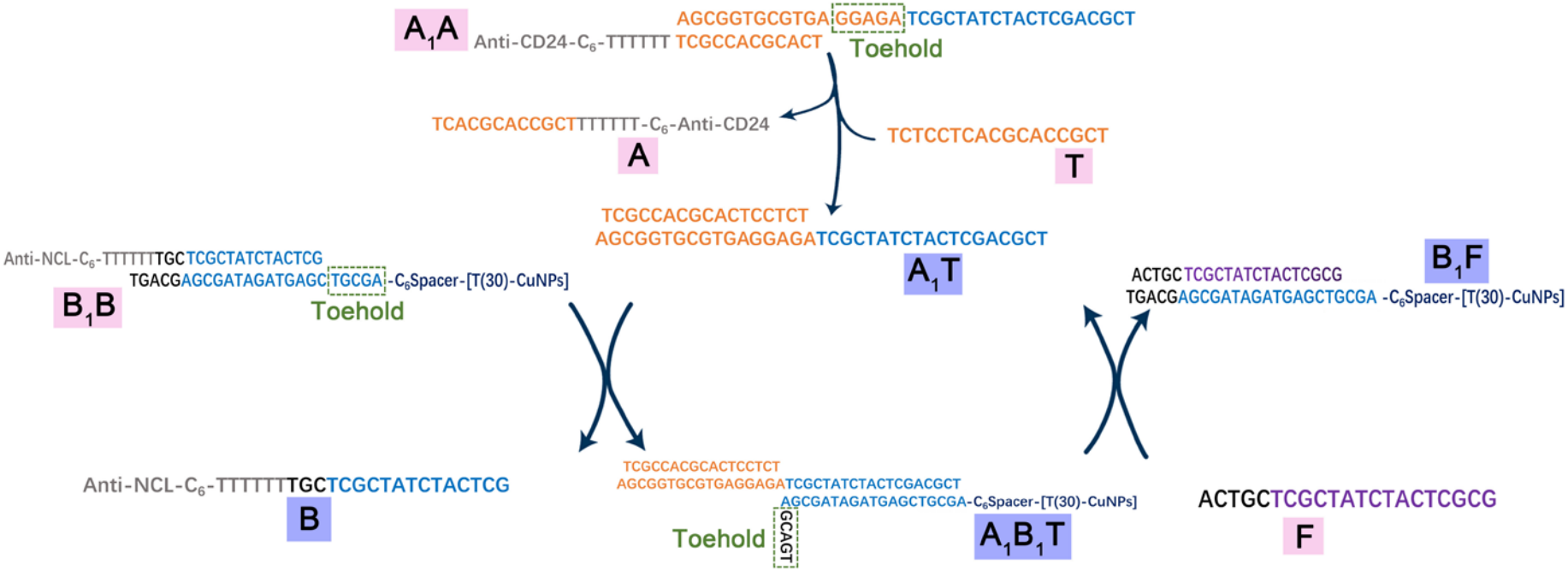

Figure S9. Domain level diagram of PDC.

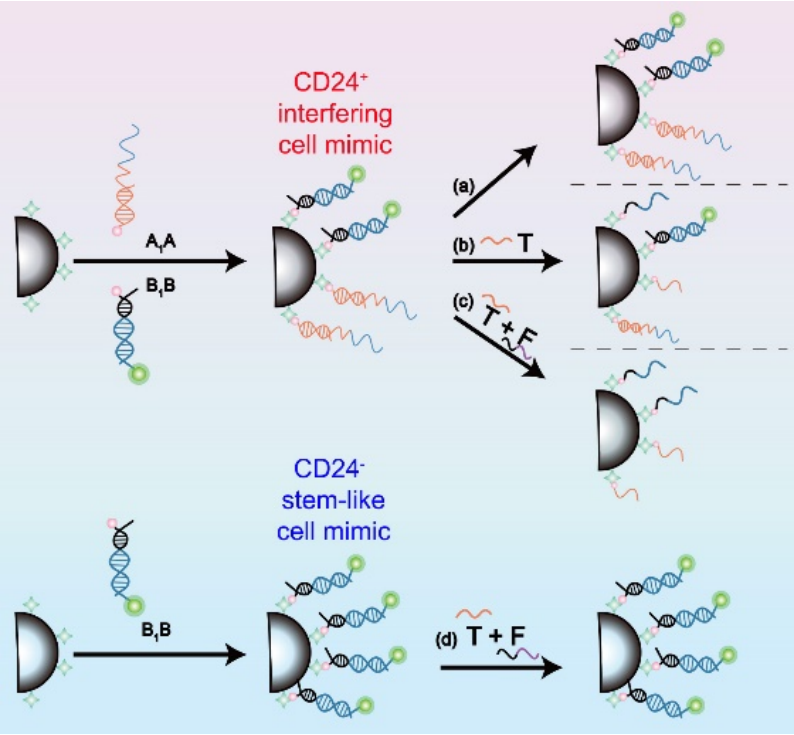

Figure S10. Schematic illustration of fluorescence verification of the feasibility of PDC on interface. In this case, B_1B together with or without A_1A were immobilized at magnetic bead surface through biotin-streptavidin interaction, to mimic $CD24^+$ interfering cells and $CD24^-$ stemlike cells. B1 was labeled with FAM. Fluorescence signal was collected from the supernant after occurrence of PDC and magnetic separation.

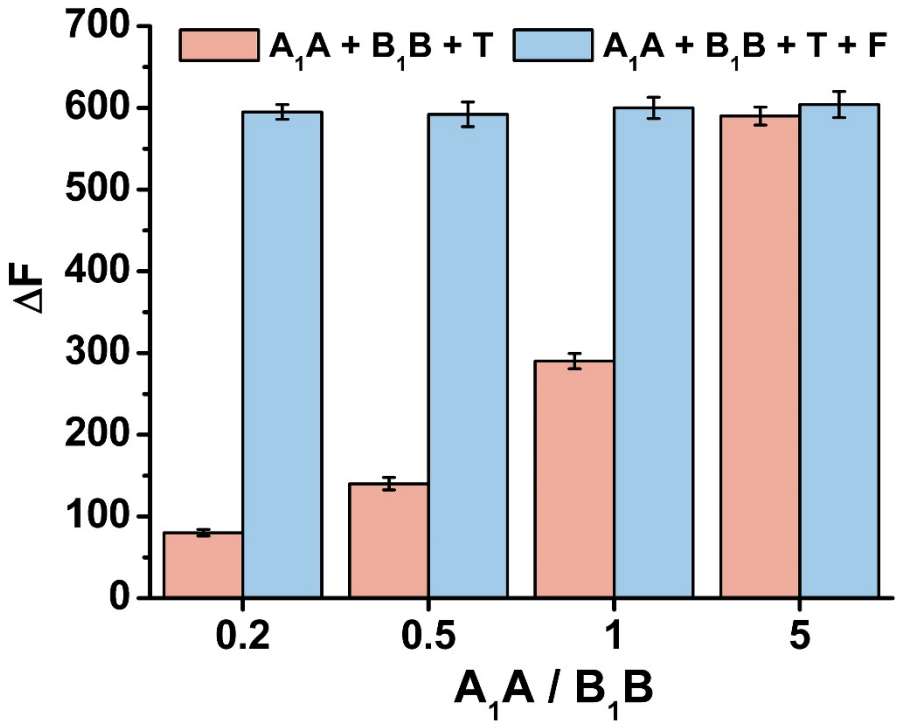

Figure S11. Fluorescence investigation of PDC with variable proportions of A_1A and B_1B. Increase of fluorescence intensity (*ΔF*) obtained with the input of T or T and F at different A_1A/B_1B ratios after the immobilization of A_1A and B_1B at the magnetic bead surface through biotin-streptavidin interaction.

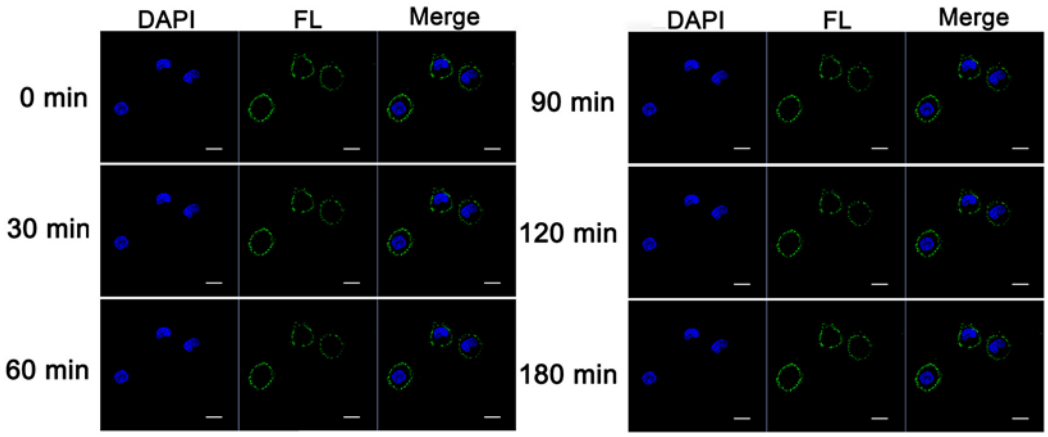

Figure S12. Confocal microscopic recording of the fluorescence of FAM-B_1B@Anti-NCL anchored at the surface of MCF-7 cells for different time. Scale bars, 20 μm.

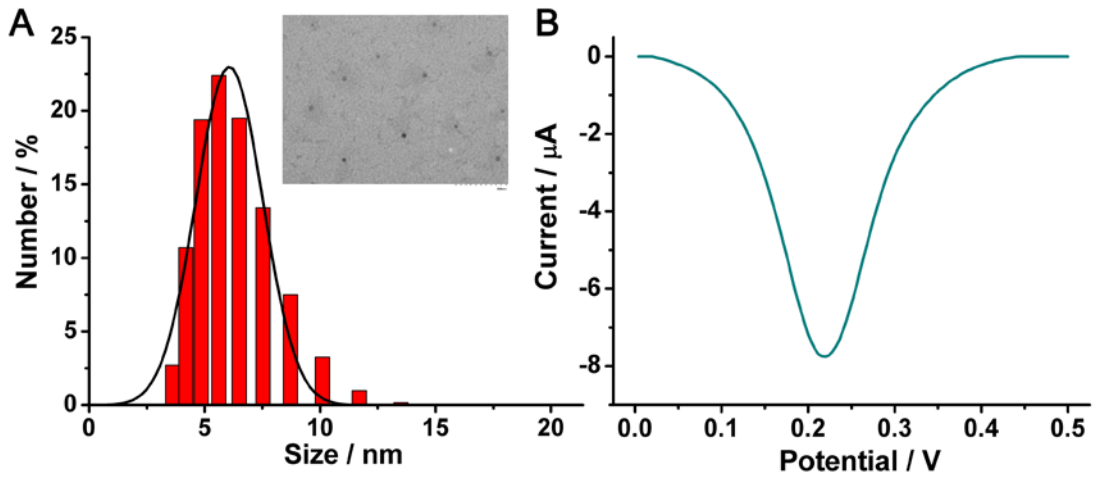

Figure S13. Characterization of CuNPs-B_1. (A) Dynamic light scattering analysis and transmission electron microscopy image (inset) of CuNPs-B_1. (B) DPV current obtained the acid dissolution of CuNPs-B_1.

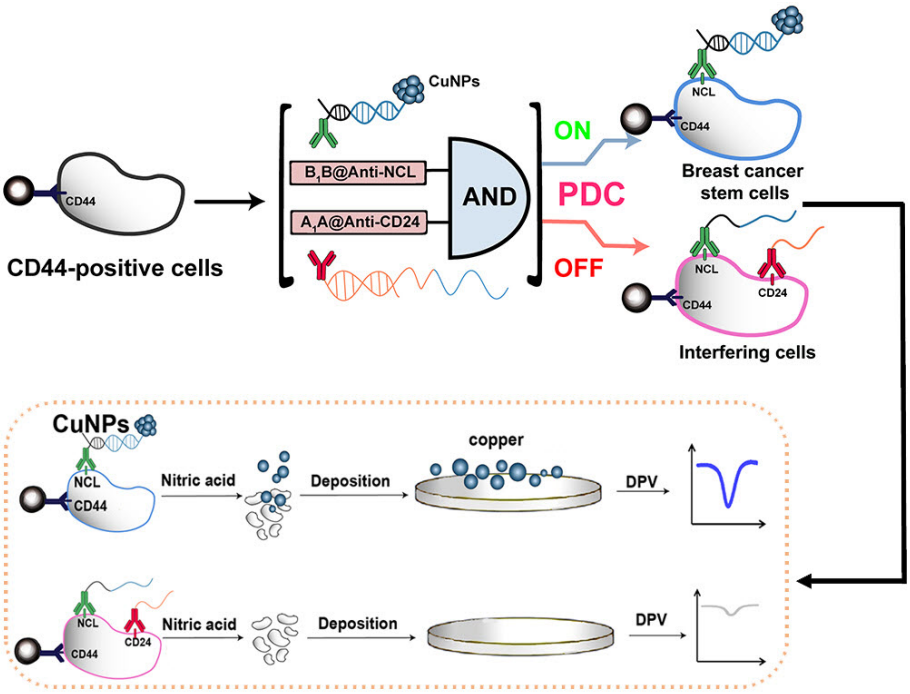

Figure S14. A cartoon with detailed steps to perform electrochemical measurement of the cells.

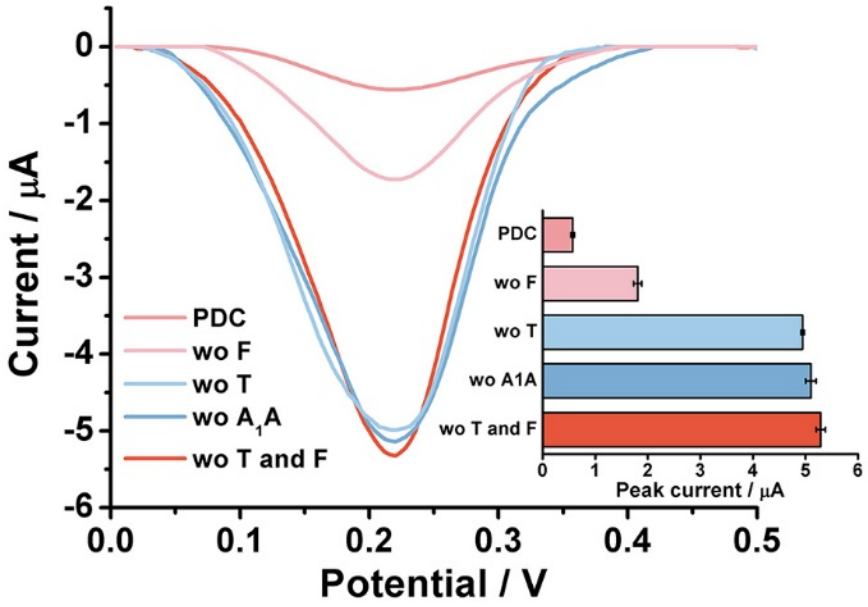

Figure S15. Electrochemical study of in situ PDC at MCF-7 cells. Peak currents obtained for MCF-7 cells after PDC and in the cases that were lack of F, T, A_1A or T and F, respectively.

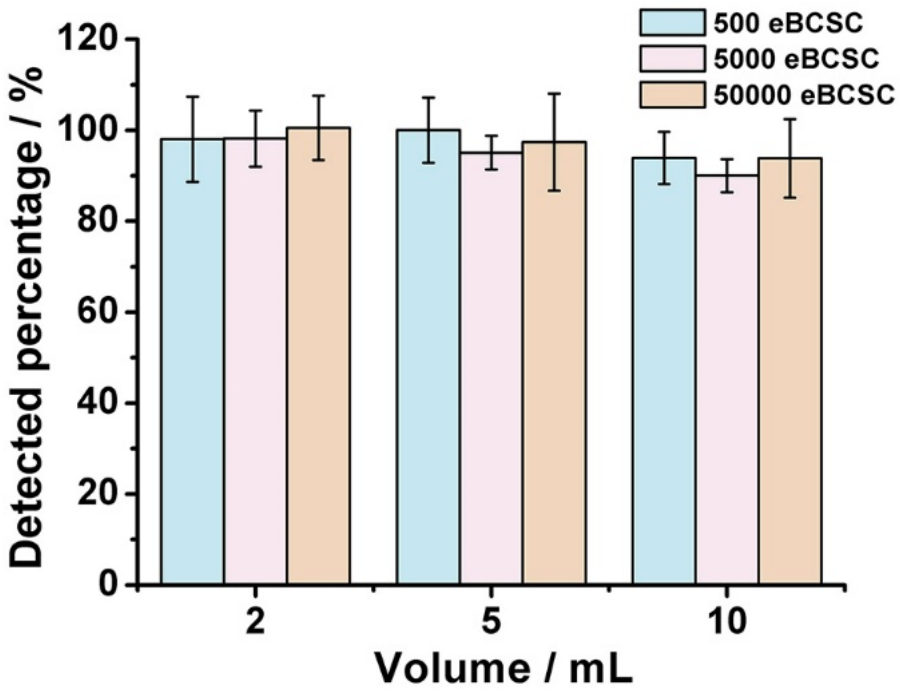

Figure S16. Performance of electrochemical phenotyping method in serum samples. Detected percentage (recovery) obtained after adding different numbers of eBCSCs (500, 5000 and 50000 cells) in different volumes of serum, including 2, 5, and 10 mL. The detected percentage was calculated using the ratio of the detected cell number to the added cell number.

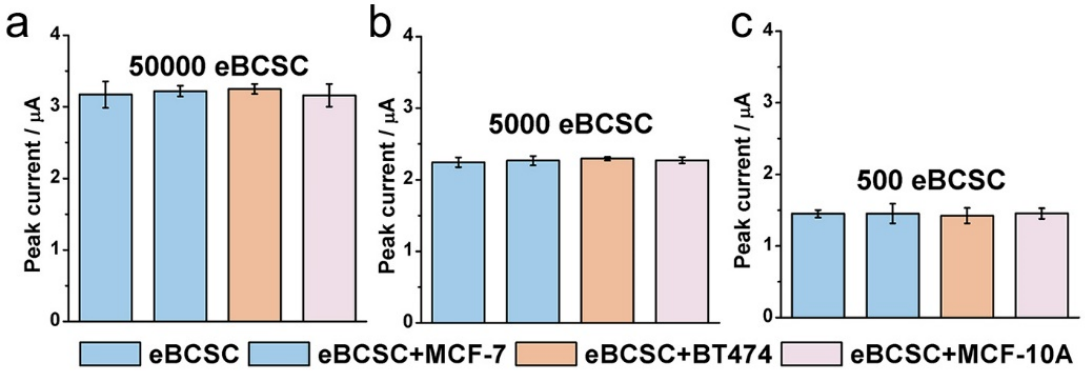

Figure S17. Performance of electrochemical phenotyping method in mixed samples. Peak currents obtained when mixing different numbers of eBCSCs (50000, 5000, and 500 cells) with 5×10^4 of the control cells, including MCF-7, BT474 and MCF-10A.

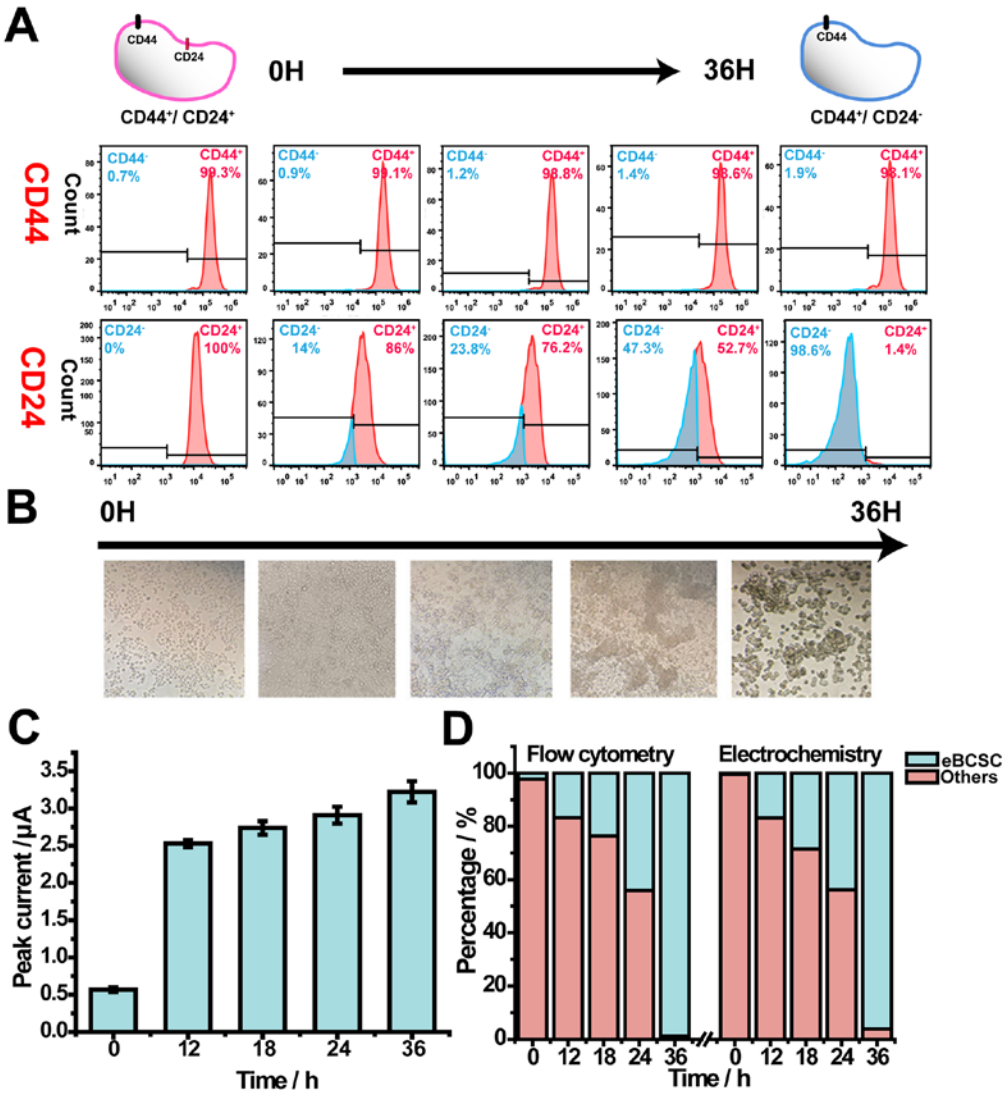

Figure S18. Phenotypic analysis during the switching from MCF-7 cell to eBCSC. (A) Flow cytometry plots of CD44 and CD24 expressions during mammosphere culture of MCF-7 cells within a period of 36 h (from left to right: 0, 12, 18, 24, and 36 h). (B) Microscopic examination of cellular morphology during mammosphere culture of MCF-7 cells (from left to right: 0, 12, 18, 24, and 36 h). (C) Peak currents obtained upon analyzing fixed amounts (50000 cells) of cancer cells at varying culture time. (D) Flow cytometric and electrochemical measurements of eBCSC ratios at different culture time. The flow cytometric results were obtained by dual staining of CD44 and CD24.

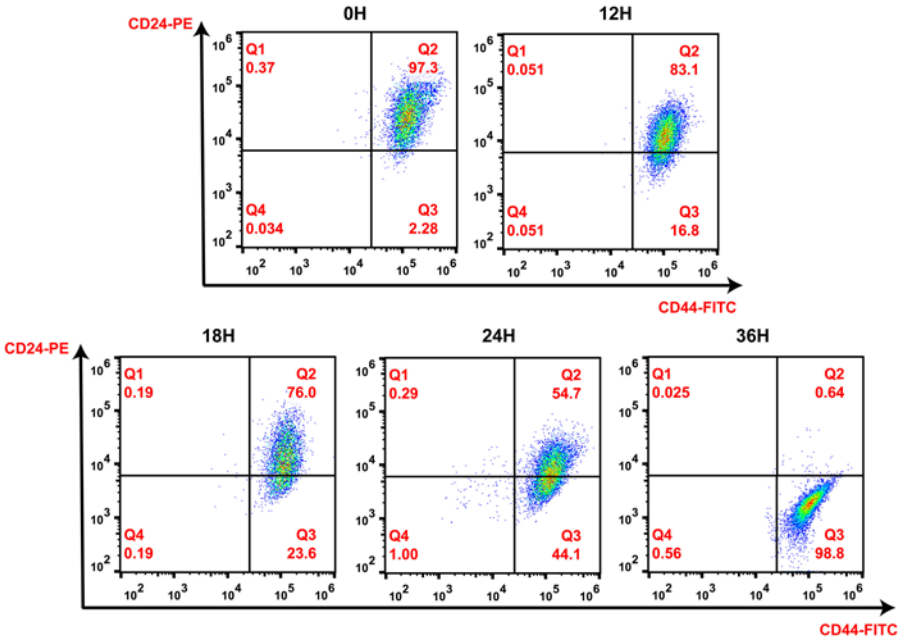

Figure S19. Flow cytometric recording during the switching from MCF-7 cell to eBCSC. Flow cytometric recording of CD44 and CD24 expressions during mammosphere culture of MCF-7 cells within a period of 36 h.

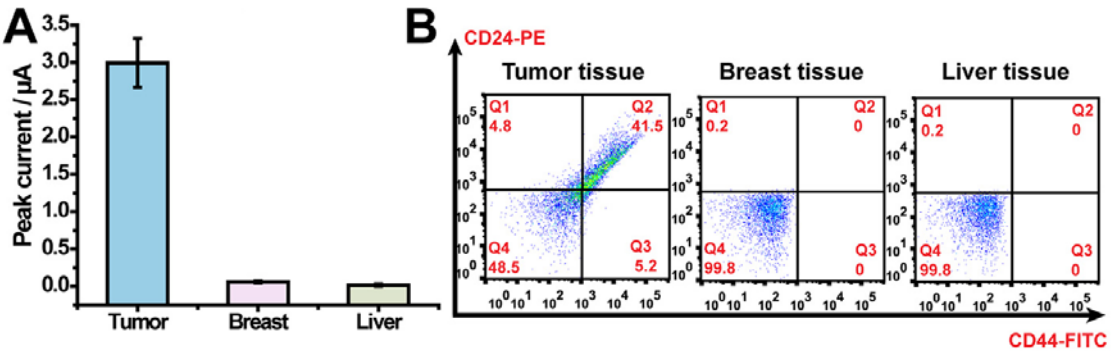

Figure S20. Characterization of stemlike phenotype in tumor-bearing mice that were implanted with MCF-7 cells. (A) Peak currents obtained in different tissues (tumor, breast and liver) excised from the tumor-bearing mice. (B) Flow cytometric recording of CD44 and CD24 expressions in the tumor, breast and liver tissues.

Table S1. Sequences of DNA probes used in the work.

Probe	Sequence (from 5' to 3')
A_1	AGCGGTGCGTGAGGAGATCGCTATCTACTCGACGCT
A	TCACGCACCGCTTTTTTT-C_6-SH
A-Biotin	TCACGCACCGCTTTTTTT-Biotin
B_1	T(30)-C_6 Spacer-AGCGTCGAGTAGATAGCGAGCAGT
FAM-B_1	FAM-AGCGTCGAGTAGATAGCGAGCAGT
B	SH-C_6-TTTTTTTGCTCGCTATCTACTCG
B-Biotin	Biotin-TTTTTTTGCTCGCTATCTACTCG
T	TCTCCTCACGCACCGCT
F	ACTGCTCGCTATCTACTCGCG