Supporting Information

A Tumor mRNA-Dependent Gold Nanoparticle–Molecular Beacon Carrier for Controlled Drug Release and Intracellular Imaging

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Experimental Section

***Synthesis of Oligonucleotides*:** DNA oligonucleotides were synthesized and HPLC purified by TaKaRa Biotechnology Co., Ltd (Dalian, China). Sequences used to prepare and test Cyclin D1 target were given in Table S1. The oligonucleotides were first reduced with TCEP·HCl (Tris(2-carboxyethyl) phosphine hydrochloride, Pierce) before they were added to the gold nanoparticle (AuNP).

***Preparation of AuNP*:** The 20 nm AuNP was synthesized using the sodium citrate reduction of gold ions developed by Michael J. Natan et al.[1] In the preparations, all glassware was cleaned in aqua regia (HCl/HNO_3, 3:1), rinsed with triply distilled H_2O, and then oven-dried prior to use. The 100 mL of 0.01% $HAuCl_4$ ($HAuCl_4.4H_2O$, >99.999% purity, Sigma), was brought to a boil with vigorous stirring, and 1.5 mL of 1% trisodium citrate was added under stirring. The solution turned blue within 25 s, the final color change to red-violet occurred 70 s later. Boiling continued for an additional 10 min, the heating source was removed, and the colloid was stirred for another 15 min. Then the solution was set aside to cool to room temperature. The prepared AuNP was stored at 4 °C.

***Synthesis of AuNP–MB*:** The procedure for coating AuNP with MB oligonucleotides was adapted from the previously reported protocol.[2,3] This protocol is based on the Au–S interaction between the gold lattice and thiolfunctionalized oligonucleotides. The 5 μM MB was added to 20 nm AuNP. The conjugate was slowly aged for 24–48 h with increasing salt

concentration (until 1× PBS concentration is reached). The solution containing the oligonucleotide-coated AuNP was centrifuged (13,000 rpm, 30 min) and washed in 100 mM PBS solution (pH 7.0) three times. It finally was resuspended in the buffer (10 mM Tris·HCl, pH 8.0, 100 mM KCl and 1 mM $MgCl_2$) for subsequent experiments. The concentration of AuNP–MB was determined via the absorption of AuNP (UV-1700, Shimadzu, Japan).

***MB Structure*:** The potential secondary structure of MB was shown by using mfold (www.idtdna.com). It indicated that the "stem and loop" conformation has the binding sites of Dox.

***Binding Studies of Dox and MB*:** To confirm that the fluorescence of Dox could be quenched by MB, a fixed concentration of Dox (0.2 μM) was incubated with an increasing concentration of MB (concentration of 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 μM) for 30 min, and the fluorescence spectrum of Dox (520–700 nm) was measured at 490 nm excitation.

***Synthesis of AuNP–MB(Dox)*:** The purified AuNP-MB conjugate (1 nM) was suspended in the Tris-HCl buffer (10 mM Tris·HCl, pH 8.0, 100 mM KCl and 1 mM $MgCl_2$). After addition of Dox (1 μM), the solution was mixed by vortexing for 30 min, and then was washed with the buffer three times. It finally was concentrated 10-fold and resuspended in the buffer (10 mM Tris·HCl, pH 8.0, 100 mM KCl and 1 mM $MgCl_2$) by centrifugation at 6000 rpm for 15 min for subsequent experiments.

***Quantitation of Dox loaded on the AuNP–MB complex*:** AuNP–MB(Dox) (1 nM) was first dissolved in mercaptoethanol and the mixture solution was incubated overnight with shaking at room temperature. The released MB(Dox) was then separated via centrifugation. After addition of perfectly matched DNA target (overdose), the solution was mixed for 30 min. DNA was precipitated by adding anhydrous ethanol and separated via centrifugation. Then the fluorescence intensity (λex=490nm, λem=590 nm) of the supernatant was obtained with a fluorescence spectrometer (Varian Cary Eclipse Fluorescence Spectrophotometer, Varian, Inc.

America). The fluorescence signals were converted to molar concentrations of Dox by interpolation from a standard linear calibration curve was generated using the known concentrations of Dox under the identical condition. By dividing molar concentrations of Dox by the original AuNP concentration, we calculated that there was 174 Dox per AuNP–MB complex.

***Stability of AuNP–MB(Dox) Conjugate*:** The fresh synthesized and purified AuNP–MB(Dox) (1 nM) was stored at room temperature, and the fluorescence intensity of Au–MB(Dox) was measured with a 490-nm excitation wavelength at 0, 5, 10, 20, 30, 60 day, respectively. The percent of leakage was calculated with 0 day as a reference. The perfectly matched DNA target of 1 μM was employed, and the fluorescence intensity was recorded.

***Release of Dox from AuNP–MB(Dox) Induced by DNA Target*:** The obtained AuNP–MB(Dox) conjugate probes were diluted to a concentration of 1 nM in buffer (10 mM Tris·HCl of pH 8.0, 100 mM KCl, 1 mM $MgCl_2$), and treated with a complementary DNA target or single-base mismatched DNA target with an increasing concentration (concentration of 1, 1.5, 2, 2.5, 3, 5, 10, 20, 50, 100 and 200 nM). After 30 min incubation, the fluorescence spectra were obtained in the range from 520 to 700 nm by use of the maximal excitation wavelength at 490 nm. In kinetic studies, DNA target of 200 nM was employed. The kinetic study was performed at 37 ºC, and the fluorescence intensity was measured with 490 nm excitation wavelength.

***Nuclease Assay*:** Two groups of AuNP–MB(Dox) were diluted to a concentration of 1 nM in buffer (10 mM PBS, pH 7.4, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$) and placed in a 96-well fluorescence microplate at 37 ºC. After allowing the samples to equilibrate (10 minutes), 1.3 μL of DNase I in assay buffer (2 U/L) as added to one group. The fluorescence of these samples was monitored for 60 min. Then DNA target of 200 nM was respectively delivered into two groups with incubation for 30 min. When cooled slowly to room temperature, the fluorescence was measured with 490 nm excitation and 590 nm emission wavelength.

***Cell Culture*:** The breast cancer cell line SK-BR-3 and normal immortalized human mammary epithelial cell line MCF-10A were cultured in Dulbecco's modified Eagles medium (DMEM, Gibco, 1% antibiotics penicillin/streptomycin, 100 U/ml) plus 10% fetal bovine serum (FBS, Gibco) and maintained at 37 ^{o}C in 5 % CO_2.

***Cellular Cytotoxicity of AuNP–MB(Dox)*:** Cells were grown in 96-well plates for 24 h. Cells were washed with prewarmed PBS buffer and incubated with prewarmed fresh media for 30 min before the addition of AuNP–MB (0.05 μM), Dox alone (10 μM) or AuNP–MB(Dox) (0.05 μM). Cells were incubated with them for 4 h at 37 °C, washed three times with PBS (1 mL per well), and further incubated for 24 h. Cell viability was assessed colorimetrically with the MTT reagent (ATCC) following the standard protocol provided by the manufacturer. The absorbance was measured with a microplate reader at 570 nm.

***Intracellular Testing the Release of Dox from AuNP–MB(Dox) under a Confocal Microscope*:** Cells were plated on chamber slides for 24 hours. One group of SK-BR-3 cells was treated with cartilage polysaccharide for 24 hours. Then 1 nM AuNP–MB(Dox) was delivered into the treated SK-BR-3 cells and the other untreated group SK-BR-3 and MCF-10A cells in Opti-MEM at 37 °C for 90 minutes. The images were obtained using a confocal laser scanning microscopy with a laser (488 nm) (Leica TCS SPE, Germany).

***Real-time Reverse Transcription–PCR*:** Total RNA from sorted cells was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA synthesis was performed using an iScript kit (Bio-Rad). Real-time PCR was carried out with SYBR Green Ⅰ (Qiagen) on an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). The primer pairs for detecting the expression of cyclin D1 gene were cyclin D1 forward 5'-AGAAGCTGTGCATCTACACCGACAACTCCATCCGGC- 3' and cyclin D1 reverse 5'-GGTTCCACTTGAGCTTGTTCACAA-3'. GAPDH was used as an internal control. The relative expression of cyclin D1 gene was analyzed using the $2^{-\triangle\triangle CT}$ method.

***Flow Cytometry*:** Cells were treated with 1 nM AuNP–MB(Dox). After treatment, cells were detached from culture flasks using trypsin. Flow cytometry was performed using a Beckman Coulter Epics XL, exciting at 488 nm.

***AuNP–MB(Dox)-induced Apoptosis of Cancer Cell According to Different Stages of Tumor Progression*:** The apoptotic cell death induced by AuNP–MB(Dox) was examined with DAPI stained SK-BR-3 cells cultured in 12-well plates. After treatment with 10 nM of AuNP–MB(Dox) for 12 and 24 h respectively, the cells were incubated with a fixative solution (Tissue Fixative, OH, US) for 15 min. Then the fixative solution was replaced with 1 ml DAPI solution (final concentration 10 μg/ml) for each well. The DAPI staining solution was removed and the cells were washed with PBS twice before coverslips were mounted on the slides with Permount. To investigate the ability of AuNP–MB(Dox)-induced apoptosis according to different stages of tumor progression, cells were incubated with 100μg/ml of cartilage polysaccharide (a cyclin D1 mRNAs inhibitor) for 24 h before adding AuNP–MB(Dox), followed by adding 10 nM of AuNP–MB(Dox) and incubation for 24 h. The following procedures were the same as described above.

References

1 K. C. Grabar, R. G. Freeman, M. B. Hommer and M. J. Natan, *Anal. Chem.*, 1995, **67**, 735.

2 R. Elghanian, J. J. Storhoff, R. C. Mucic, R. L. Letsinger and C. A. Mirkin, *Science*, 1997, **277**, 1078.

3 C. A. Mirkin, R. L. Letsinger, R. C. Mucic and J. J. Storhoff, *Nature*, 1996, **382**, 607.

Table S1. Sequences of MB and DNA targets

Oligonucleotides	sequences
MB	5′-GCTCG GAG TTG TCG GTG TAG AT CGAGCAAA AAA-$(CH_2)_3$-SH-3′
DNA target 1 (perfectly matched)	5′-ATC TAC ACC GAC AAC TC-3′
DNA target 2 (single-base mismatched)	5′-ATC TAC ACT GAC AAC TC-3′

[a]Underlined letters represent the stem sequence; [b]Letters in red reprent the mismatched site.

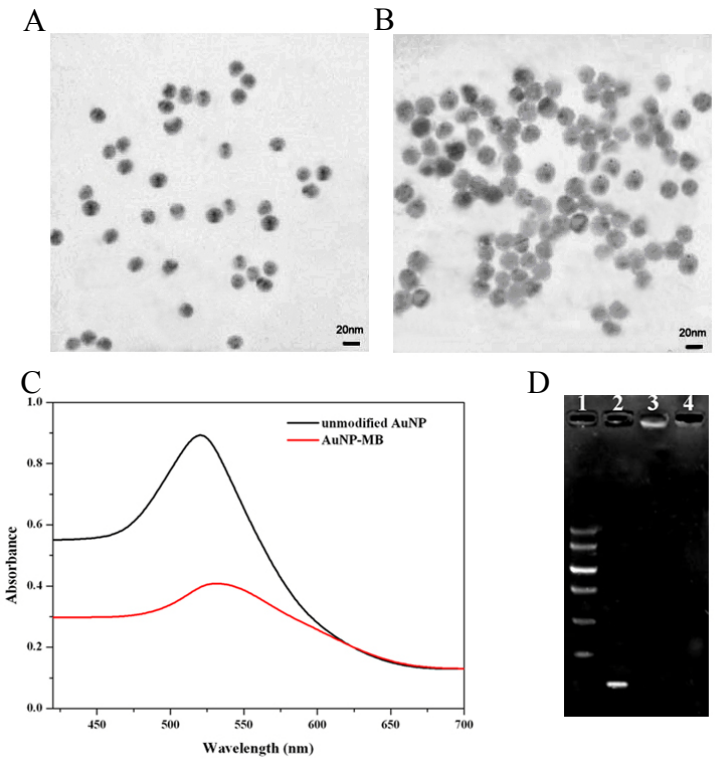

Fig. S1 (A) TEM image of AuNP. (B) TEM image of AuNP after modification with MB. (C) Comparison of UV-vis spectra for AuNP (20 nm) and AuNP–MB. (D) Gel electrophoresis results of AuNP–MB after staining with ethidium bromide. Lanes 1, 2, 3, and 4 represent the DNA Marker Ⅰ, MB, AuNP–MB, and AuNP alone, respectively.

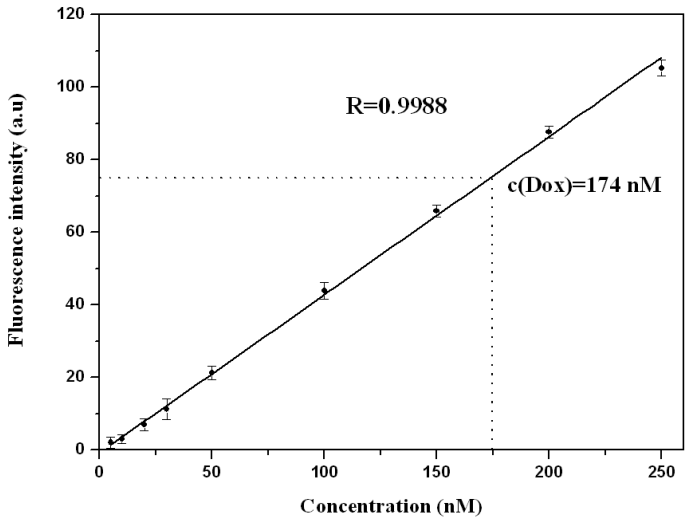

Fig. S2 Standard linear calibration curve of Dox.

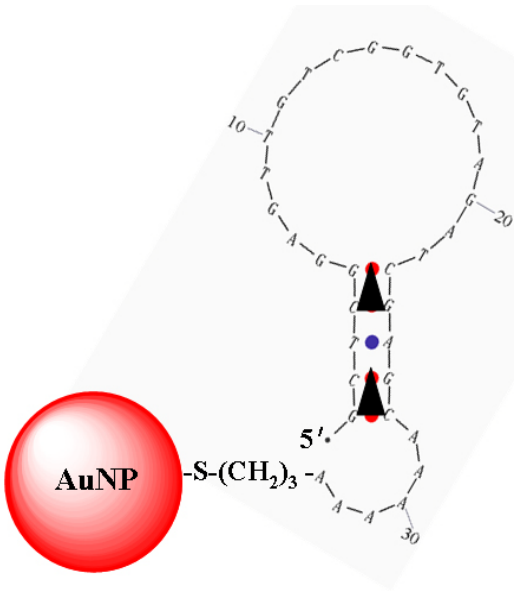

Fig. S3 The two-dimensional structure of AuNP–MB(Dox). It showed Dox physical intercalation sites in AuNP–MB(Dox).

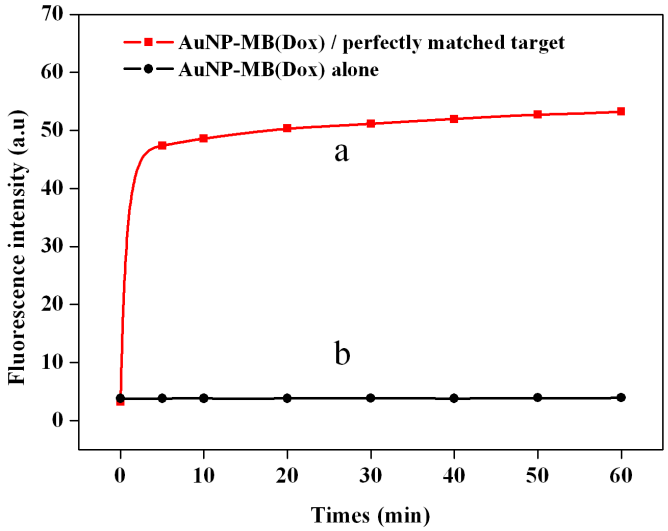

Fig. S4 Kinetic studies of AuNP–MB(Dox) (1 nM) in response to perfectly matched DNA target (concentration of 200 nM) (trace a) and AuNP–MB(Dox) alone (trace b). The fluorescence intensity was measured at 37 ºC with 490 nm excitation wavelength.

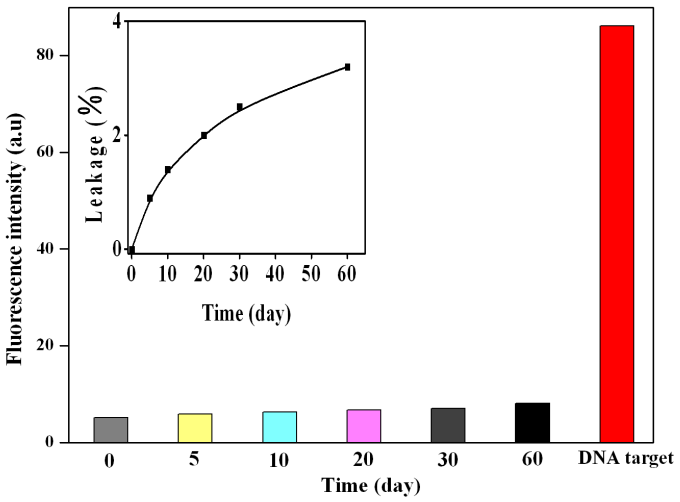

Fig. S5 Variation of the fluorescence intensity of 1 nM AuNP–MB(Dox) over a time profile measured with 490 nm excitation wavelength at 0, 5, 10, 20, 30, 60 day, respectively. The perfectly matched DNA target of 1 μM was employed (red). The inset was the percentage of leakage of Dox which is calculated with 0 day as a reference.

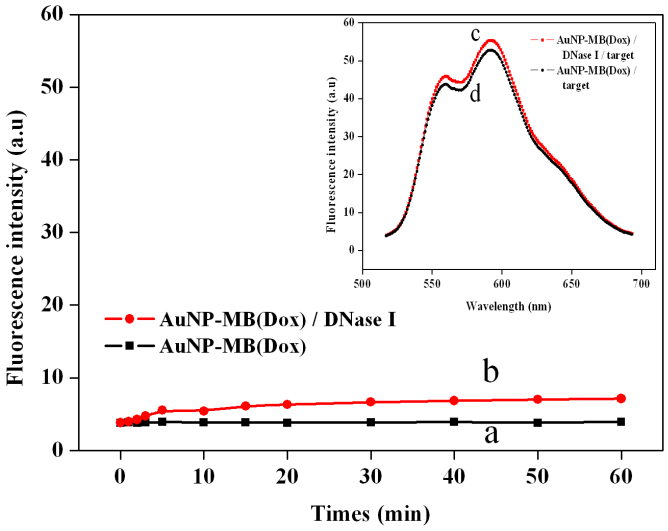

Fig. S6 The nuclease stability of AuNP–MB(Dox) in the absence or presence of DNase I. Fluorescence curve of 1 nM AuNP–MB(Dox) in buffer without DNase I (trace a), in the presence of DNase I (trace b). Fluorescence spectrum after hybridization AuNP–MB(Dox) with DNA target in the presence of DNase I (trace c, inset) and absence of DNase I (trace d, inset). The fluorescence intensity was measured with 490 nm excitation wavelength.

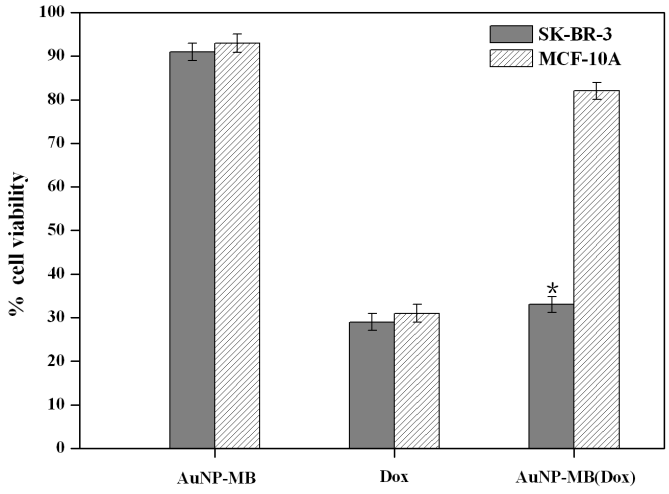

Fig. S7 Growth inhibition assay (MTT). SK-BR-3 (cyclin D1+) and MCF-10A (cyclin D1-) were incubated with AuNP–MB (0.05 μM), Dox alone (10 μM) or AuNP–MB(Dox) (0.05 μM) for 4 h, and the cells were washed and further incubated for 24 h. Asterisk indicated significant differences between SK-BR-3 and MCF-10A cells.

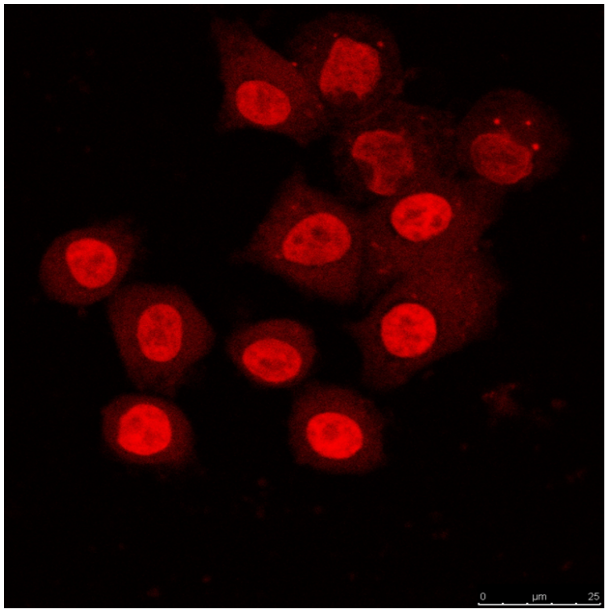

Fig. S8 The distribution of fluorescence in SK-BR-3 cells incubated with AuNP–MB(Dox). Scale bar was 25 μm.

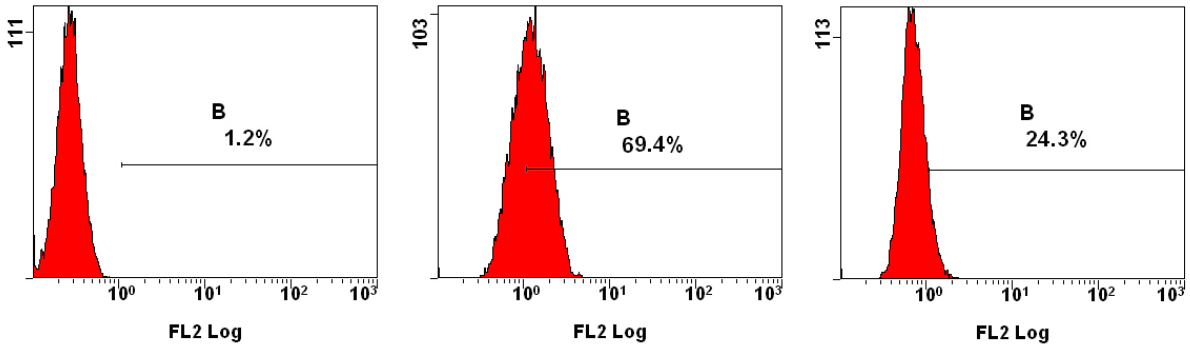

Fig. S9 Flow cytometry data were collected from the SK-BR-3 cells alone (A), the untreated SK-BR-3 cells incubated with AuNP–MB(Dox) (B) and the treated SK-BR-3 cells incubated with AuNP–MB(Dox) (C).

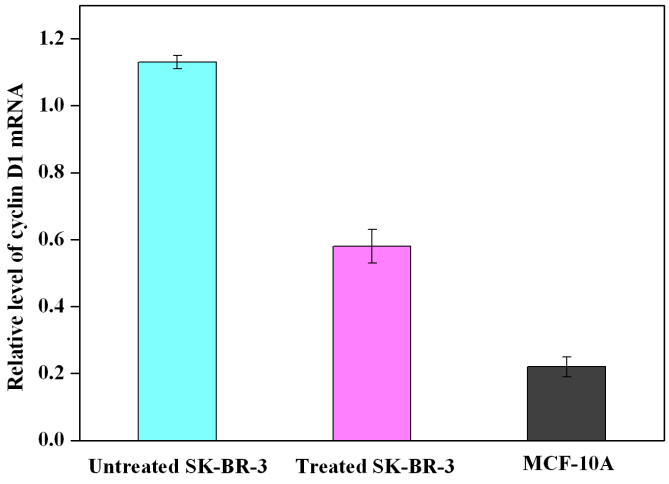

Fig. S10 Detection of the level of cyclin D1 mRNA by real-time RT-PCR. Relative level of cyclin D1 mRNA was calculated from the quantity of cyclin D1 PCR products and the quantity of GAPDH PCR products. The SK-BR-3 cells (left), the cartilage polysaccharide treated SK-BR-3 cells (middle) and the MCF-10A cells (right).