Figure.S1,Li et al.

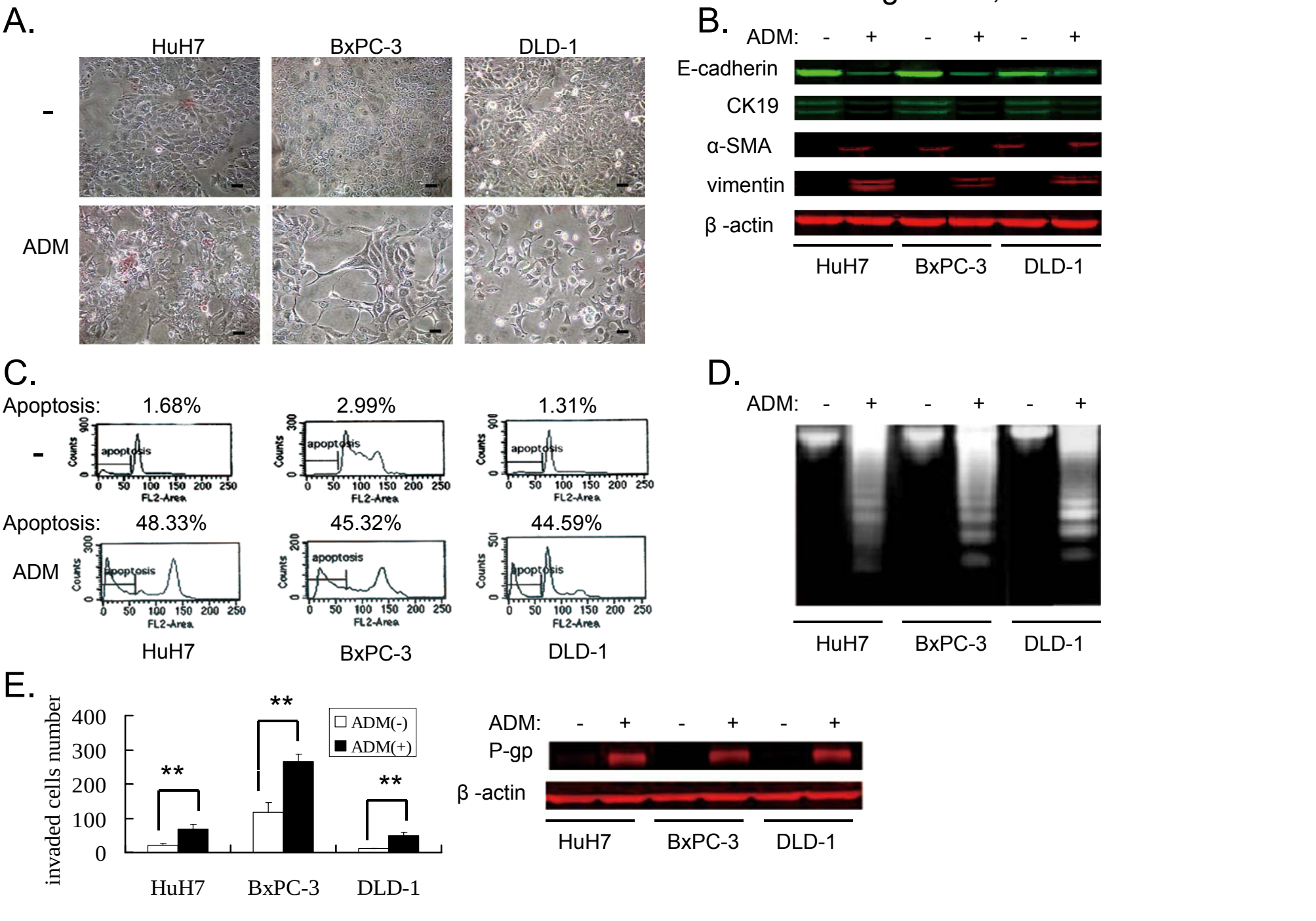


Figure.S3,Li et al.

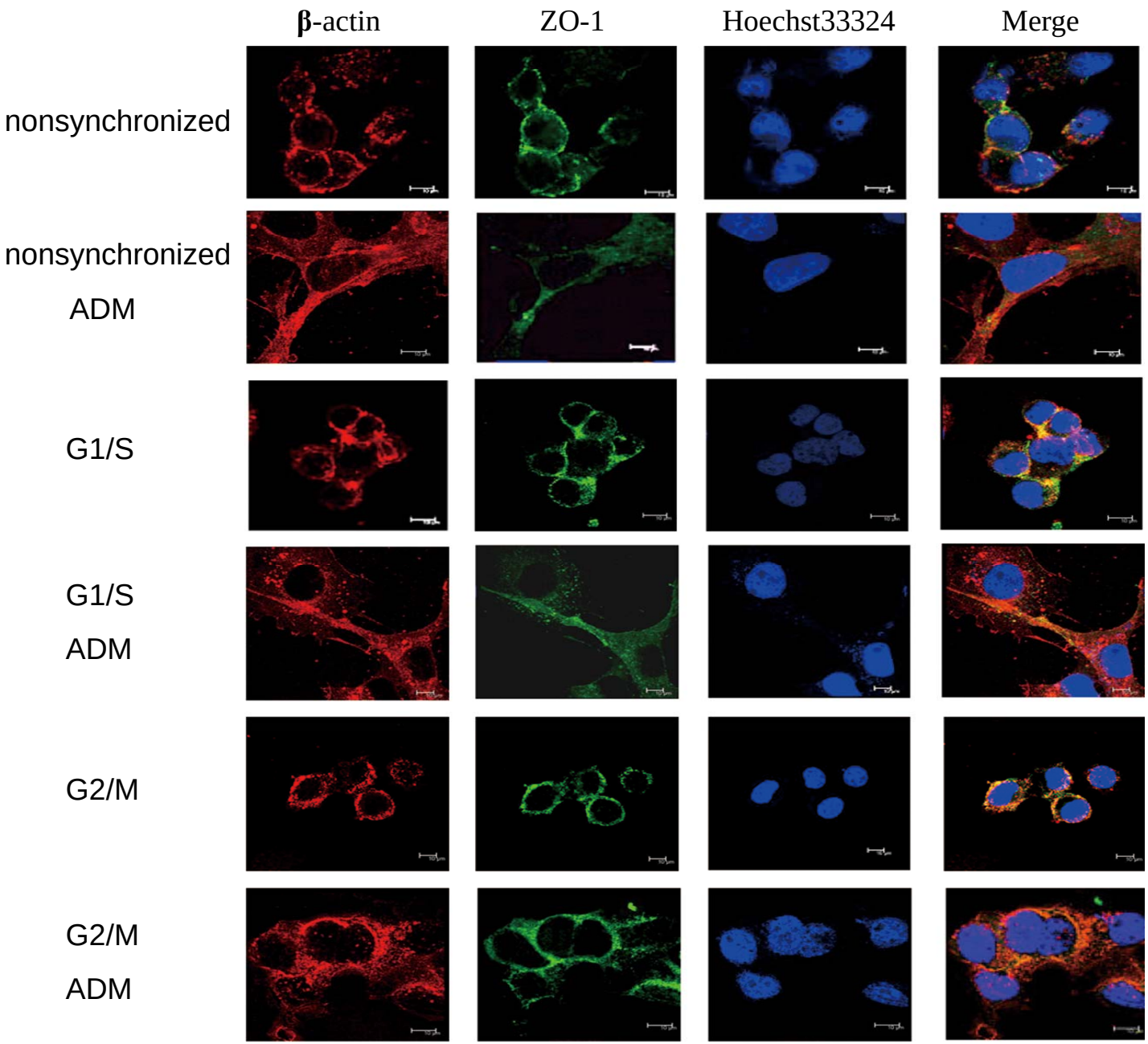
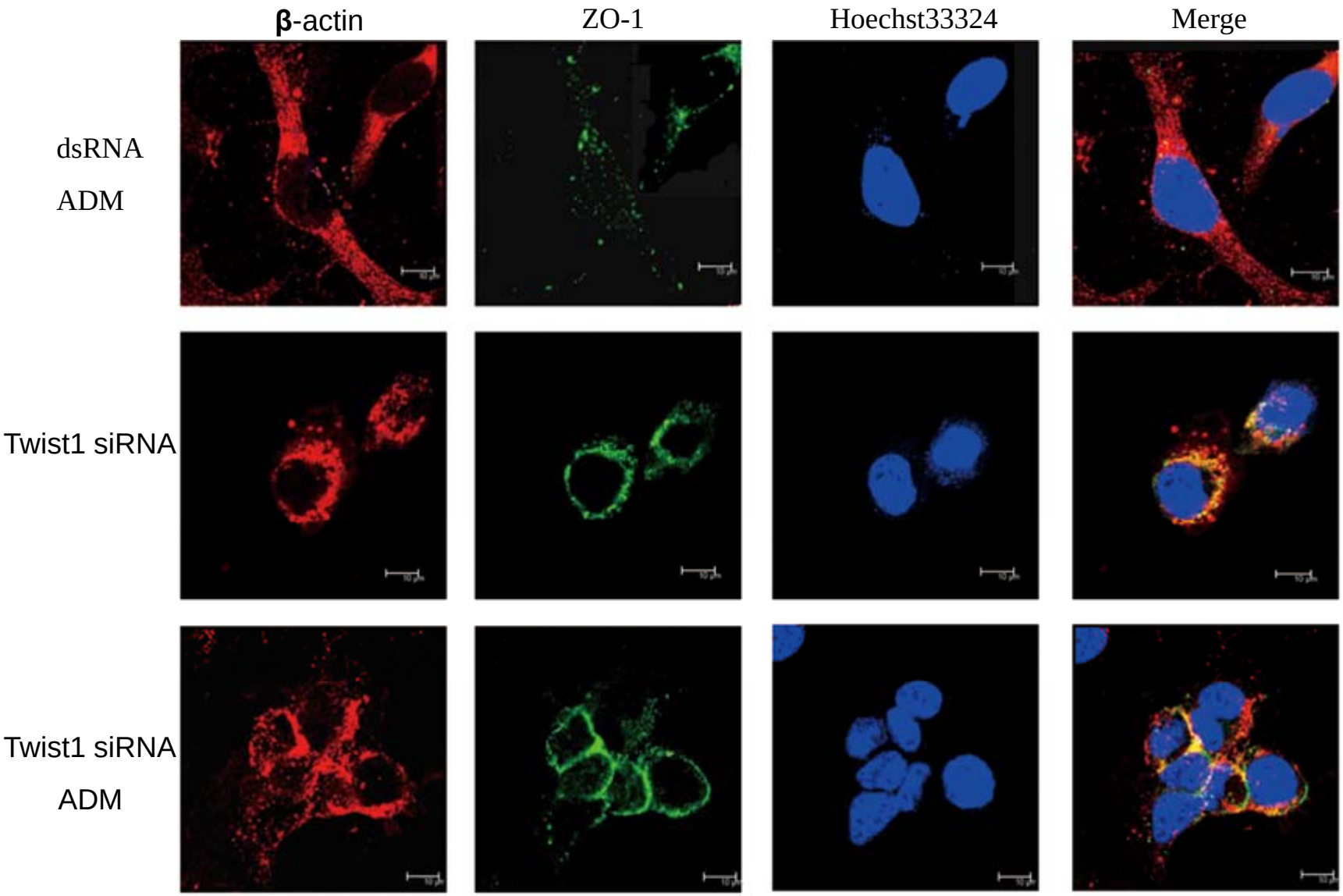


Figure.S3,Li et al.



Supplemental Data

Twist1 Mediated Adriamycin-induced Epithelial-Mesenchymal Transition (EMT) Relates to Multidrug Resistance and Invasive Potential in Breast Cancer Cells

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Supplemental Methods

Examination of morphological change. The morphological changes of the cells were observed under the inverted phases-contrast microscope (Olympus, Tokyo, Japan). The photographs were taken using a Nikon digital camera (Nikon, Tokyo, Japan).

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). For Western blots, 50 μg of protein extract/lane were electrophoresed, transferred to PVDF membranes, and incubated overnight with antibodies against E-cadherin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), CK19(Santa Cruz Biotechnology), α-SMA(Sigma), vimentin (Chemicon International, Temecula, CA), P-gp (Chemicon) respectively. Membranes were treated with the appropriate AlexaFluor700/800nm-conjugated secondary antibodies (Invitrogen, Carlsbad, CA), and analyzed using the Odyssey Infra-Red Imaging System (Li-Cor BioSciences, Lincoln, NE).

DNA fragmentation assay. Cells were lysed in solution containing 5mM Tris buffer (pH 7.5) 0.5% Triton X-100 and 20mMEDTA. Supernatants were separated by centrifugation and incubated with the same lysis buffer containing 0.1% RNase A for 30 min at 37 °C. Lysate were then further treated with 1 mg/ml of protease K for 30 min at 37 °C. DNA extracted from a phenol chloroform precipitation was then resuspended in TE buffer (Tris buffer, pH 7.2, plus 1mM EDTA and 0.5% SDS) and subjected to electrophoresis on 1.2% agarose gel containing 0.5 μg/ml of ethidium bromide for 2 h at 50 V.

Flow cytometry assay. Cells (1×10^6) were plated into 10cm tissue culture dishes 1 day before the treatment, and were then treated with different types of external stresses. After the treatment, floating and attached cells were harvested, washed with PBS, fixed in 70% ethanol overnight at 4°C and stained with propidium iodide (PI; Sigma) 50 mg/ml. The sub-G1 peak (DNA content less than 2 N) was measured with FACScan Flow Cytometry (Becton Dickinson Labware, Franklin Lakes, NJ) and analyzed by Cell Quest software.

Tumor cell *in vitro* invasion assays. Transwell plates (Corning Costar, NY) were coated with Basement Membrane Matrigel (0.8mg/ml, Becton Dickionson) for 4h at 37℃. Cells were detached with trypsin and washed with serum-containing medium. 1x 10^4 cells were added to the upper chamber in a total volume of 100μl of serum-free medium supplemented with adriamycin

(25μg/ml). The lower chamber contained 600μl conditioned medium (incubating NIH3T3 cells in serum-free RPMI 1640 medium for 24 h) as chemoattractant. After 48 hours, cells that migrated through the permeable membrane were fixed in methanol, stained with hematoxylin and eosin, and counted. Each assay was performed in triplicate and repeated for three times.

Immunofluorescence confocal microscopy analysis. Cells were grown on glass coverslips, and after a brief washing with PBS, were fixed at 4°C for 30 minutes with 2% formaldehyde/30mM sucrose followed by 20 minutes in methanol. The fixed cells were washed thrice with PBS and then incubated with 10% normal goat serum in PBS for 20 minutes to suppress nonspecific binding of IgG. After another washing with PBS, the cells were incubated with anti-ZO-1 and anti-β-actin antibody for 1 hour followed by 45 minutes incubation with FITC- or Cy3-conjugated secondary antibody (Invitrogen) respectively in PBS with 1.5% goat serum. The nuclei were stained with Hoechst33342 (Sigma). The labeled cells were then analyzed by laser scanning confocal microscopy (Leica Microsystems, Wetzlar, Germany). Images were analyzed with the MetaMorph software (Universal Imaging, West Chester, PA).

Supplemental Figure Legends

Figure S1. Cells were treated with or without adriamycin (25μg/ml) for 36 h for EMT analysis or 72 h for apoptotic analysis. (A), EMT was examined by phase-contrast photomicrographs (100×magnification, scale bar: 10μm). (B), Western blotting of E-cadherin, CK19, vimentin, α-SMA was assessed. (C), Cell apoptosis was evaluated by FCM. (D), DNA was extracted and analyzed by 1.5% agarose gel electrophoresis. (E), Endogenous P-gp expression was measured by western blotting and *in vitro* invasion assays were performed. Number of invaded cells was evaluated in three fields for each experimental group and averaged. Statistical analysis was performed with Dunnett's test. **: $p < 0.05$ versus control cells.

Figure S2. Nonsynchronized cells and cells synchronized at G1/S or G2/M phase were treated with adriamycin (25μg/ml) for 36h, EMT was examined by immunofluorescence staining of β-actin (red), ZO-1 (green) and nuclei (blue, Hoechst33342 staining).

Figure S3. Non-transfected cells and cells transfected with control dsRNA or small interference RNA specific for Twist were treated with adriamycin (25μg/ml) for 36h. EMT was examined by immunofluorescence staining of β-actin (red), ZO-1 (green) and nuclei (blue, Hoechst33342

staining).