Supplemental Methods

Isolation of Primary Fibroblasts and Cultures

We isolated CAFs from human invasive mammary ductal carcinomas obtained from mastectomies. All tissues were minced with scalpels and then enzymatically dissociated in mammary epithelial basal medium (Cat#: cc-3151, Lonza, USA) supplemented with 2% bovine serum albumin (Promega, USA), 10ng/mL cholera toxin (Cat#:c8052,Sigma-Aldrich, USA), 300 units/mL collagenase (Cat#: 17018-029, Invitrogen, USA), and 100 units/mL hyaluronidase (Cat#: H3506, Sigma-Aldrich) at 37℃ for 18 h. On the second day, the digested suspension was centrifuged at 700 rpm for 4 min to separate the epithelial and fibroblast cells. The supernatant was collected for centrifugation at 800 rpm for 10 min to pellet the fibroblasts followed by two washes with DMEM/F12 (GIBCO, USA) medium. The cell pellet was resuspended in DMEM/F12 medium supplemented with 5% fetal bovine serum (GIBCO) and 5μg/mL insulin (Cat#:3435, Tocris Bioscience, UK) and plated in cell culture flasks kept undisturbed for 2 to 5 days. All tissues were obtained from Ruijin Hospital with approval of the hospital ethical committee and by the patient's written informed consent (Shanghai, China).

In addition, we extracted non-cancer-associated fibroblasts (NAFs) from samples obtained from 3 reduction mammoplasties, in which only normal mammary tissue was detectable.

Collection of Fibroblasts Conditioned Media (CM)

CM were prepared from culturing 1×10^5 fibroblasts with 5ml serum-free DMEM/F12 medium at 37℃ for 48 hours and then centrifuged to remove dead cells.

Breast Cancer Cells Cultures and Multi-treated Analysis

All breast cancer cell lines applied in this work were tested and authenticated by DNA typing in Shanghai JiaoTong University Analysis Core (last test in April 2013)

and were cultured in variant nutrient solution according to the online instructions of the manufacturer from the American Type Culture Collection Website at 37℃ water-saturated 5% CO2 atmosphere.

Breast cancer cell lines (SK-BR3 and BT474) were cultured in 6-well plates (80–90% confluence) and serum-starved for overnight. Cells were then treated with 1% FBS medium or CM from CAFs alone or combined with pathway inhibitors for 1 h in pathway status or with 10% FBS treatment (as control) for 24 h. The protein of the treated cells was then collected for Western Blots analysis in pathway status and PTEN detection.

Cytokines and pathway inhibitors that applied in this procedure were shown below: rhIL-6 (Cat#: 206-IL) and human IL-6 neutralizing antibody (Cat#: MAB206) were purchased from R&D Systems (USA). STAT3 inhibitor S3I-201 (Cat#: S1155), PI3K inhibitors BKM120 (Cat#: s2247), BEZ-235 (Cat#: s1009) and MEK inhibitor U0126 (Cat#: S1102) were from Selleck Chem (Houston, TX, USA), NF-κB inhibitor Bortezomib (Cat#: 1351) was purchased from LC Laboratories (Woburn, MA, USA). Trastuzumab (Herceptin™, Genentech Inc., San Francisco, California) was purchased from pharmacy.. Human normal IgG was obtained from Jackson Immuno Research Laboratories, Inc. (West Grove, Pennsylvania).

Western Blots and Antibodies

Treated cells were lysed in RIPA buffer (Cat#: 89900, Thermo Scientific, Waltham, MA, USA) containing complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany), and protein concentration was measured by Pierce® BCA Protein Assays kit (Cat#23225, Thermo Scientific). The protein fractions were suspended in loading buffer and denatured at 100°C for 10 min. Proteins (20-50μg) were separated by electrophoresis on 8%-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel according to the molecular weight of detected protein bands and then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Immunoblotting was performed with primary antibodies

incubation by appropriate dilution overnight at 4℃ and incubated with HRP-conjugated secondary antibodies for 2 h at RT. Results were visualized by the use of Immobilon Western Chemiluminescent HRP Substrate (Millipore) and detected through ImageQuant LAS 4000 biomolecular imager.

The commercial antibodies of the following were used: anti-human p-HER2 (Tyr1221/1222)(Cat#:2243), p-HER2(Tyr877) (Cat#:2241), HER2(Cat#:2242), p-EGFR(Tyr845) (Cat#:2231), p-EGFR(Tyr1086) (Cat#: 2220), EGFR(Cat#:2232), p-STAT3(Tyr705) (Cat#: 4093), STAT3 (Cat#:4904), p-AKT(Ser 473) (Cat#: 9271), AKT (Cat#: 9272), p-ERK1/2 (Cat#: 9101), ERK1/2 (Cat#: 9102), p-NF-κB p65 (Cat#:3033), NF-κB p65 (Cat#:4764), and PTEN (Cat#:9552). These antibodies were purchased from Cell Signaling Technology (CST, Boston, USA) and diluted in 1:1000 in the Western blotting. Jagged-1 (Cat#: sc-11376), Hes-1 (Cat#: sc-25392) were purchased from Santa Cruz Biotechnology. Other commercial antibodies used were as follows: GAPDH (1:5000, Cat#: KC-5G4, Kangchen, Shanghai, China).

Proliferation Assays

After a 24h serum withdrawal, BT474 and SK-BR3 were digested and plated at 5000 cells/well into 96-well flat-bottomed cell culture plates with 5 replications in 100ul complete culture medium for a night. Media was exchanged with 1% of FBS containing CM from CAFs, NAFs and normal DMEM/F12. After at least 48 h different concentrations of trastuzumab or vehicle (IgG) or various combination of trastuzumab (10ug/ml), IL-6 neutralizing antibody (100ng/ml), STAT3 inhibitor (S3I-201,10μmol/L), PI3K inhibitors (BKM120, 1μmol/L, and BEZ-235, 1μmol/L), NF-κB inhibitor (Bortezomib, 10nmol/L), MEK inhibitor (U0126, 1μmol/L) were added into the CM. Five days later, 10 μl of cell counting kit-8 (CCK-8) (Cat#: CK04, Dojindo Molecular Technologies, Japan) reagent was added into each well and the plates were incubated in a 37℃ incubator with 5% CO2 for 3 h. Cell proliferation was determined by CCK-8 assay and read on multi-well scanning spectrophotometer (Thermo) at A450 for wavelength correction. All experiments were repeated at least three times.

Luciferase reporter assays

Constructs of the PTEN promoter region at -1986/+14 were generated from genomic DNA of SK-BR3 cells. The PTEN promoter was cloned into the pGL3 basic reporter gene vector and verified by sequencing.SK-BR3 and BT474 cells were maintained in RIPM 1640 containing 10% FBS and transfected by Lipofectamine™ 2000 Reagent (Invitrogen Corp, Carlsbad, CA) in 12-well plates. PTEN promoter constructs: Renilla plasmid DNA (200ng: 20ng) were co-transfected for 6−9 h and then incubated with IL6 (4ng/ml) for 36−48 h in fresh complete medium. Cells were then rinsed in cold PBS and lysed with the luciferase assay buffer. Luciferase activities were measured by using a dual luciferase assay kit (Cat#: E1960, Promega, Wisconsin, USA) with a Berthold chemiluminometer (Berthold Detection Systems GmbH). The results were expressed as ratio of firefly luciferase activity to Renillaluciferase activity. Data were expressed as the mean values and standard deviations from at least three independent transfections performed in triplicate.

Tumor sphere assay

Tumor sphere culture was performed as previously described [15; 16]. CM collected from differentially fibroblasts, or recombinant IL6 were added to ultralow attachment 6-well plates filled with 2 ml mammary epithelial growth medium (MEGM), and supplemented with B27 supplement (10 ng/ml EGF, 10 ng/ml bFGF, 10 μg/ml Insulin). (Corning; Corning, NY) containing freshly plated single BC cells at the beginning of the tumor sphere formation assay. On day 10, numbers of tumor spheres (diameter $\geqslant$100 μm) were counted, and tumor sphere efficacy was calculated based on the numbers of initially seeded cells.

For secondary sphere formation, first-passage tumor spheres from day 10 cultures were collected by gentle centrifugation (320g) and dissociated into single cells by incubation in trypsin-EDTA solution (Invitrogen; Carlsbad, CA), before plating into new wells. Similar analysis was done as mentioned in primary tumor sphere assay.

Cell Cycle Analysis

BT474 and SK-BR3 single-cell suspension were cultured in 6-well plate at a density of 10,000 cells per well for a night. Then media were exchanged for medium with 1% of FBS containing CM from CAFs, NAFs and Normal DMEM/F12 medium. At least 48h later, different concentrations of trastuzumab or vehicle (IgG) or various combination of trastuzumab (10ug/ml), IL-6 neutralizing antibody (100ng/ml), STAT3 inhibitor (S3I-201, 10μmol/L), PI3K inhibitors (BKM120, 1μmol/L, and BEZ-235, 1μmol/L), NF-κB inhibitor (Bortezomib, 10nmol/L), MEK inhibitor (U0126, 1μmol/L) were added. After 24 h treatment, all cells were harvested and washed twice with cooled PBS and fixed in 75% ethanol for 2 h at 4 °C. The fixed cells were washed with cooled PBS and were then stained with the staining solution containing 0.05 μg/mL PI (Sigma-Aldrich), 1 μg/mL DNase-free RNase (Sigma-Aldrich) for 30 min at RT. Ten thousand events were acquired using a FACSCalibur analyzer (Becton-Dickinson, San Jose, CA, USA), and cell cycle data were determined using Modfit software (Verity Software House, Topsham, ME, USA).

Apoptosis Analysis

All cells had the same treatment mentioned above in cell cycle analysis. After 24h treatment, cells were detached with EDTA-free trypsin and washed twice with cooled PBS. Cells were resuspended in 400 μL 1× loading buffer with 5 μL Annexin V and 5 μL PI (BD Pharmingen, San Diego, CA, USA) for 15 min on ice in dark. Analyses were performed at FACSCalibur analyzer (Becton-Dickinson)

RNA Extraction, Reverse Transcription and Real-Time PCR

Total RNA was extracted from cell lines using TRIzol regent (Cat#: 15596-026, Invitrogen, USA) and was reverse transcribed using the transcriptase cDNA synthesis kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions. Real-time PCR analysis was performed by SYBR Premix Ex TaqTM (Cat#: RR420A,

TaKaRa, Japan) in an Applied Biosystems 7500 Fast Real-Time PCR System (ABI, USA). The primers are used at a concentration of 0.5 μM to generate only one PCR product.

The total RNA (1 μg) was reversely transcribed into cDNA and equal volume of cDNA was used as PCR template. According to a melting point analysis, only one PCR product was amplified with the standard program: 30 s at 95℃ for initial denaturation, 5 s at 95℃ and 30 s at 60℃ for 40 cycles, followed by the thermal denaturing step to generate the dissociation curve to verify amplification specificity. Results were normalized with respect to the internal controls. All reactions were run in triplicate. The primers are used as follows:

Human PTEN:

Sense: 5'- TTGCTATGGGATTTCCTGC-3',

Anti-sense:5'- TGTGGTGGGTTATGGTCTT-3';

Human CD44:

Sense: 5'-CTGCCGCTTTGCAGGTGTA-3',

Anti-sense:5'-CATTGTGGGCAAGGTGCTATT-3';

Human CD24:

Sense: 5'-CTCCTACCCACGCAGATTTATTC-3'

Anti-sense:5'- AGAGTGAGACCACGAAGAGAC-3';

Human CD133:

Sense: 5'- ACATGAAAAGACCTGGGGG-3'

Anti-sense:5'- GATCTGGTGTCCCAGCATG -3';

Human ALDHA1:

Sense: 5'- GCACGCCAGACTTACCTGTC -3'

Anti-sense:5'- CCTCCTCAGTTGCAGGATTAAAG -3';

Human NANOG:

Sense: 5'- TTTGTGGGCCTGAAGAAAACT-3'

Anti-sense:5'- AGGGCTGTCCTGAATAAGCAG-3';

Human SOX2:

Sense: 5'- GCCGAGTGGAAACTTTTGTCG-3'

Anti-sense:5'- GGCAGCGTGTACTTATCCTTCT-3';

Human OCT4:

Sense: 5'- GACAACAATGAGAACCTTCAGGAGA-3′

Anti-sense:5’- CTGGCGCCGGTTACAGAACCA -3’

Human ABCG2:

Sense: 5’- CAGGTGGAGGCAAATCTTCGT-3’

Anti-sense:5’- ACCCTGTTAATCCGTTCGTTTT-3’;

Human GAPDH:

Sense: 5’-CGCTCCTGGAAGATGGTGAT-3’,

Anti-sense:5’-ACGGATTTGGTCGTATTGGG-3’.

Statistical analyses

Data are presented as mean ± SD. Values of $p < 0.05$ were considered statistically significant. qRT-PCR was analyzed using Student's t test, 2-tailed with Excel software.

References:

1. Malhotra R, Patel V, Vaque JP, Gutkind JS, Rusling JF. Ultrasensitive electrochemical immunosensor for oral cancer biomarker IL-6 using carbon nanotube forest electrodes and multilabel amplification. Analytical chemistry 2010, **82**(8)**:** 3118-23.