Supplemental Experimental Procedures

Chemicals and cytokines and neutralized antibodies

LY294002 (Cat.# 440202), JSH23 (Cat. #481408) JAK2 inhibitor (Cat. #420132) and Stattic (Cat. #573099) were purchased from Calbiochem (CA, USA). Cisplatin (Cat. # 1134357) was purchased from Sigma-Aldrich (MO, USA). Lapatinib (Cat. #S1028) was purchased from Selleckchem.com (TX, USA). Paclitaxel (Cat. #RS036) was purchased from TSZCHEM (MA, USA). PD0325901 (Cat. #13034), PP2 (Cat. #13198), IRAK1/4 inhibitor (Cat. #17540) and D-Luciferin (Cat. #14681) were purchased from Cayman Chemical Company (MI, USA).

Human IL-1α (Cat. 44-IL1α), IL-1β (Cat. 44-IL1β) and IL-6 (Cat. 44-IL6) were purchased from BioAbChem Inc. (SC, USA). Recombinant IL-1 soluble receptor type II (Cat. #263-2R) and neutralized antibody for IL6 (Clone 1936, Cat. #MAB2061) were purchased from R&D system (MN, USA).

Cell culture

HCC1954 was cultured in RPMI1640 with 10% fetal bovine serum (FBS). MCF10A-vector and HER2 cells was maintained in DMEM/F12 containing 5% horse serum, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone and 100 ng/ml cholera toxin. MDA-MB361 cells were cultured in DMEM medium containing 10% 10% fetal bovine serum (FBS).

Plasmids

To generate a luciferase reporter under the control of human IL1α or IL6 promoter, DNA fragments containing IL1α promoter (-1046 to +181) or IL6 promoter (-869 to +98) was amplified using genomic DNA. The PCR products were cloned into pGL3 promoter vector. The primers for IL1A promoter are: forward 5'- CAACAACCACCTTTGGGGAA -3'; and reverse 5'-

CTACAGAAGACCAGGCTTCT -3'. The primers for IL6 promoter are: forward 5'-TTTCTGCCCTGAACCAAGTG -3'; and reverse 5'- CGAAAGAGAAGCTCTATCTC -3'

PU.l expression plasmid pECE-PU.l was generously provided by. Dr. Richard Maki, (La Jolla Cancer Research Foundation, La. Jolla, CA). Plasmid pAH 9614 (anti-HER2/neu ScFv-TNF- α (S147Y), expressing HER2 agonist) was a gift from Dr. Sherie L. Morrison (University of California, Los Angeles, CA) .

Generation of *IL1A* and *IL6* knockout cell lines

We generated the lentiCRISPRv2 plasmids for human *IL1A* or *IL6* knockout by fully following Target Guide Sequence Cloning Protocol (http://genome-engineering.org/gecko/?page_id=15). Briefly, the target sequence was designed using Cas9 target design tools (http://genome-engineering.org). The target sequence for human *IL1A* is 5'-GGTAGTAGCAACCAACGGGA-3' (in exon 4) and the sequence for human *IL6* is 5'-AAAGATGGATGCTTCCAATC-3' (in exon 3). The synthesized oligos were cloned into vector lentiCRISPRv2 (a gift from Dr. Feng Zhang, AddGene, #52961). The recombinant plasmid was co-transfected into HEK293T cells with the packaging plasmids pVSVg (AddGene, #8454) and psPAX2 (AddGene, #12260) to produce virus. The lentiviruses were then used for infection of target cell lines MCF10A, MCF10A-HER2 and HCC1954. Single clones were selected by serial dilution of cells into 96-well plate and confirmed by sequencing. The supernatant of the positive clones were collected for measurement of IL-1α or IL-6 concentration by ELISA (Eve Technologies Corporation, Canada).

Preparation of HER2 agonist

The preparation of supernatant was modified from the protocol reported previously (3). Briefly, Plasmid encoding anti-HER2/neu ScFv-TNF-α(S147Y) and control plasmid were transfected to 293T cells using the lipofectamine 2000 (Invitrogen). The media were replaced with serum-free

fresh media after transfection for 24 hours. The supernatants were collected after another 48 hours, and concentrated with an Amicon stirred ultrafiltration cell (Amicon, Billerica, MA). Concentration of proteins was determined using a standard bicinchoninic acid (BCA) assay.

Real-time PCR analysis

RNA samples were prepared by using Trizol according to the standard protocol. Real-time RT-PCR was carried out using RT2 Fast SYBR® Green/ROXTM qPCR Master Mix (SABiosciences). Data analysis was performed using the 2– ΔCT method for relative quantification, and all sample values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression value (as the internal reference control).

Western blotting analysis

Protein samples were prepared by using M-PER protein extraction buffer (Cat # 78501, Thermo Scientific) added with 10% protease inhibitor, 2% Sodium Orthovanadate (1mM), 1% Sodium Floride (10mM). Immunoblotting was carried out as previously described (4).). All of the antibodies below were purchased from Cell Signaling. p-STAT3 (Tyr705, Cat. #9145); p-p65 (Ser536, Cat. #3033); p-ERK (Thr202/Tyr204, Cat. #4370); p-AKT (Ser473, Cat. #4058); p-IkB (Ser32, Cat. #2859); p-HER2 (Tyr1221/1222, Cat, #2243); p-Src (Tyr416, Cat. #2101); STAT3 (Cat. #4904); p65 (Cat. ##8242); ERK (Cat. #4695); AKT (Cat. #4691); IkB (Cat. #4814); HER2 (Cat. #4290); Src (Cat. #2109) and β-actin (Cat. #3700). GAPDH (Cat. #SC-25778) was purchased from Santa Cruz biotechnology.

FACS analysis

Fluorescence-activated cell sorting (FACS) analysis was performed using BD AccuriTM C6 flow cytometer (BD Biosciences, CA, USA) (4). Single cells were stained with the following

antibodies for 30 min on ice: FITC-conjugated anti-CD44 (Cat. #555478, BD Biosciences) and PE-conjugated anti-CD24 (Cat. #555428, BD Biosciences).

In vitro tumorsphere formation assay

Single cells were plated in ultra-low attachment 6-well plates (Corning, NY, USA) at a density of 10,000 cells/well in serum-free DMEM/F12 medium (Invitrogen, CA, USA) supplemented with 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 10 ng/ml basic fibroblast growth factor (bFGF, Sigma-Aldrich), 5μg/ml insulin (Sigma-Aldrich), 1×B27 supplement (Invitrogen) and 0.4% bovine serum albumin (BSA, Sigma-Aldrich). Cells were cultured under 5% CO2 at 37°C for a week. Spheres were counted and photographed, and sphere formation efficiency (SFE) was calculated (4).

Chromatin immunoprecipitation (ChIP) assay

CHIP was performed as previously reported 2. MCF10A-Vector and MCF10A-HER2 were treated with DMSO or PD0325901 for 1 day. The cells were cross-linked by 1% formaldehyde for 10 min at room temperature. Nuclear extracts were prepared and chromatin was sonicated to generate 200- to 1000-bp DNA fragments. Protein-DNA complexes were immunoprecipitated with PU.1 or p65 antibody and control IgG. The DNA-protein cross-links were reversed by heating at 65°C for 4 h. After phenol and chloroform extraction, DNA was purified by ethanol precipitation. To amplify the region from −72 to -63 in the *IL6* promoter, PCR was performed with forward primer 5`- TTAGCGCTAGCCTCAATGAC -3` and reverse primer 5'- TGTGGGATTTTCCCATGAGT -3'. To amplify the region from -749 to -740 in the *IL1A* promoter, PCR was performed with forward primer 5`- CTTTGGTGAACTGAGGCATT -3' and reverse primer 5'- CTTAGCAATAGCCCTCACAA -3'. To amplify the region from -96 to -82 in the *IL1A* promoter, PCR was performed with forward primer 5`- ATGCCATCACACCTAGTTCA -3' and reverse

primer 5'- CACCATTGAAGGCTCATATG -3'. A region in the GAPDH promoter was amplified to serve as a negative control with forward primer 5`-AAAAGCGGGGAGAAAGTAGG-3` and reverse primer 5`-AAGAAGATGCGGCTGACTGT-3`.

Luciferase assay

For in vitro luciferase assay, Dual-Luciferase® Reporter Assay System (Cat. #E1910, Promega, WI, USA) was used for measurement of the activity of IL1A or IL6 promoter . Briefly, 0.25 μg of *IL1A* or *IL6* promoter reporter, 0.25 μg of pcDNA3, pECE-PU.l (expressing PU.1), pcDNA3-C/EBPb (expressing C/EBPb) or pcDNA3-p65 and pcDNA3-p50 (expressing NFkB) and 10 ng of Renilla luciferase assay vector pRL-CMV were cotransfected into MCF10A-Vect and MCF10A-HER2 cells for 48 hours. The bioluminescence was measured by following the instruction of manufacturer.

Animal experiments

The animal study was approved by the USC committee for research in vertebrate animals. For the serial transplantation experiments, *IL1A* or *IL-6* knockout, *HER2* knockdown or parental HCC1954 cells were resuspended in 25 μl 1×PBS and mixed with 25 μl Matrigel (BD Biosciences) at a 1:1 ratio and held on ice. The entire 50 μl sample was injected into the fourth mammary glands of NOD/SCID mice anesthetized with isoflurane according to the animal protocol approved by the USC committee for research in vertebrate animals. Tumor sizes were measured weekly. Incidence of xenograft tumor formation was scored 8 weeks after injection.

For the drug treatment experiments, NOD/SCID mice (The Jackson Laboratory) were inoculated with 2×10^6 of HCC1954 cells mixed with Matrigel (1:1) orthotopically into the 4th mammary gland. 2 weeks later, tumor-bearing mice were randomized to treatment with saline, IRAK inhibitor (4 mg/kg/d 15 times, i.p.), paclitaxel (10 mg/kg/2d 6 times i.p.), or both drugs. Tumor

sizes were measured with calipers, and mice were weighed every 3 days. Tumor volume (in mm^3) was calculated by the following formula: volume = 1/2 length × $width^2$. Tumor-bearing mice were also photographed by using the IVIS® Spectrum in vivo imaging system at the beginning (0 day) and the end (18 days) of treatment. Prior to the in vivo imaging, the mice were anesthetized with isoflurane. D-luciferin solution was then injected intraperitoneally (150 mg/kg). The mice were imaged using an IVIS Spectrum. Bioluminescent signals were quantified using Living Image 3.0. Most of xenografts were harvested at day 18 and divided into two parts. One part was fixed in 10% formalin, followed by embedding in paraffin. The other part was rinsed in PBS, mechanically minced with sterile blades in DMEM/F12 with 5% FBS and 1% penicillin-streptomycin, and incubated with digestion medium (DMEM/F12 with 1.5mg/ml collagenase I, 0.1mg/ml hyaluronidase, 2% FBS and 1% penicillin-streptomycin solution) for 1-2 hours at 37°C with constant agitation. The dissociated cells were passed through 35-μm filters and then used for FACS analysis or mammosphere assays. Cell viability was verified by trypan blue exclusion assays.

Immunohistochemical (IHC) staining assay

Immunohistochemical staining of formaldehyde fixed, paraffin embedded human breast cancer tissues were carried out as previously reported (4). Double-immunostaining with antibodies for CD44 and CD24 was performed by using EnVision G|2 Doublestain System Rabbit/Mouse (DAB+/Permanent Red) (DAKO) according to the manufacturer's instructions (5). CD24 was detected with Permanent Red and CD44 was detected with diaminobenzidene (DAB). For HER2, IL-1α or IL6 single-immunostaining, stained slices were scored according to intensity of staining and percentage of positive tumor cells [0%, IHC level 0; 1%–30%, IHC level 1; 31% –70%, IHC level 2; and >70%, IHC level 3]. In the statistical analysis, samples with a score of 0 or 1 were regarded as negative staining, while 2 or 3 as positive staining.

Microarrays

RNA was extracted from MCF10A-HER2 and MCF10A–Vector cells with Trizol reagent, followed by clean-up and DNase I treatment with QIAGEN RNeasy mini kit in accordance with the prescribed protocol provided with the kit. Quality control was performed with Agilent Bioanalyser. Biotinylated cRNA were prepared with the Ambion MessageAmp kit for Illumina arrays. For each analysis, four biological replicates were hybridized to HumanHT-12 v4.0 BeadChip Illumina whole genome expression arrays.

The microarray data processing includes a normalization procedure utilizing quantile normalization method to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization and/or scanning. Hierarchical clustering and Principal Component Analysis were performed on the normalized signal data to assess the sample relationship and variability. Probes absent in all samples were filtered out in the downstream analysis. Differential gene expression between the different conditions was assessed by a statistical linear model analysis using the bioconductor package limma, in which an empirical Bayes method is used to moderate the standard errors of the estimated log-fold changes of gene expression, and results in more stable inference and improved power, especially for experiments with small numbers of microarrays. The model included a factor to account for the two experiment batches. The moderated t-statistic p-values derived from the limma analysis above were further adjusted for multiple testing by Benjamini and Hochberg's method to control false discovery rate (FDR). The lists of differentially expressed genes were obtained by the criteria of FDR<10% and fold change cutoff of > 1.5. The expressions of the differentially expressed genes were displayed with heatmap. Function analysis was performed on the differentially expressed gene lists using R bioconductor package topGO. GSEA

was applied to identify the gene sets enriched in the HER2-overexpresssing MCF10A cells compared to control cells. The gene sets are collected from the Molecular Signatures Database (MSigDB) v5.2. The raw and normalized microarray data have been deposited in the Gene Expression Omnibus database under accession number GSE95035.

Supplemental references

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