Supplementary Methods

Article title: Metalloprotease-dependent activation of EGFR modulates the $CD44^{+}/CD24^{-}$ populations in triple negative breast cancer cells through the MEK/ERK pathway

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Methods

Reagents and antibodies

Antibodies for immunoblotting included anti-EGFR_pY1068 (clone D7A5), anti-total EGFR (clone D38B1), anti-ERK1/2_pT202/Y204 (clone D13.14.4E), anti-total ERK1/2 (clone 137F5), and anti-MEK1/2 (clone D1A5), all from Cell Signaling Technology. Anti-HA epitope tag antibody (clone 16B12) was from BioLegend, anti-α-Tubulin antibody (clone DM1A) was from Sigma, and anti-β1-integrin (clone 18) was from BD Biosciences. For flow cytometry, PE-conjugated anti-CD24 (clone ML5) and IgG2aκ isotype control (clone G155-178) were purchased from BD Biosciences. APC-conjugated anti-CD44 (clone IM7) and IgG2bκ isotype control (clone eB149/10H5) were from Affymetrix eBioscience. Reagents included erlotinib (Cell Signaling Technology), human recombinant epidermal growth factor (EGF) (Life Technologies), and selumetinib (Selleck Chemicals). Plasmids were obtained from Addgene: pBabe-puro (#1764, "EV") was a gift from Hartmut Land, Jay Morgenstern, and Bob Weinberg

[1], pBabe-puro-MEK-DD (#15268, "MEK1-DD") was a gift from William Hahn [2], pBabe-puro-HA-MEK1 (#53195, "HA-MEK1-WT") was a gift from Christopher Counter [3].

Cell culture

SUM149PT and SUM159PT breast cancer cell lines (Asterand) were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum (FBS), 10 mM HEPES, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. Human MCF10A mammary epithelial cells (ATCC) were cultured in DMEM/F12 (1:1) supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 20 ng/ml human EGF, 10 μg/ml insulin, 100 ng/ml cholera toxin, and 1% penicillin/streptomycin. EGF was omitted from the medium 24 h prior to ELISA assays or erlotinib treatment and Western blot analysis. Phoenix amphotropic (Ampho) cells (a gift of G. P. Nolan, Stanford University) were cultured in DMEM media containing 10% FBS. Cells were maintained at 37°C under humidified atmosphere containing 5% CO_2.

Retroviral transduction and generation of stable cell lines

Phoenix Ampho cells grown in 100-mm plates and supplemented with 25 μM chloroquine were transfected with 15 μg plasmid DNA using calcium phosphate precipitation method. After 48 h, media containing retroviral particles were collected, centrifuged, and applied to SUM159PT or SUM149PT, along with 5 μg/ml polybrene. Twenty-four hours after infection, media were replaced and, after an additional 24 h, stably transduced cells were selected using 2 μg/ml puromycin.

Flow cytometry

Cells were treated for 24 h with erlotinib, selumetinib, or DMSO, then 20 ng/ml EGF or vehicle alone was added for an additional 48 h. Culture media containing fresh additives were changed daily. Seventy-two hours after treatment, cells were analyzed by flow cytometry. After blocking for 20 min with either 5% FC block (BD Biosciences #564219) or 5% donkey serum, cells were stained for 30 minutes with PE-conjugated anti-CD24 and APC-conjugated anti-CD44 antibodies or their respective isotype antibody controls, and then evaluated on a BD LSR Fortessa X20 instrument. Data were analyzed with FCS Express 6 (DeNovo) software.

Immunoblotting

Immunoblotting was performed as previously described [4-6]. Lysis buffer was supplemented with phosphatase inhibitors (50 mM NaF, 2 mM Na_3VO_4, and 10 mM $Na_4P_2O_7$). Nitrocellulose membranes were incubated with primary monoclonal antibodies and HRP-conjugated secondary antibodies, followed by signal detection using SuperSignal West Pico or West Femto chemiluminescence detection kit (Pierce) and Azure c500 digital imaging system. Band intensities were quantified using ImageJ software.

ELISA assay

Cells were incubated for 48 h in culture media containing 1% FBS, in the presence of 10 μM BB-94 or DMSO. Conditioned media were then harvested, centrifuged for 10 min at 3,000 rpm to remove debris, and the amount of soluble amphiregulin was determined by ELISA using human amphiregulin Quantikine ELISA kit (R&D Systems) and Synergy H1 microplate reader (BioTek).

Calculation of EGFR and MEK activation scores

The top 100 genes whose expression was most significantly changed upon stable expression of ligand-activatable EGFR or constitutively active MEK in MCF-7 cells, compared to control MCF-7 cells adapted for long-term estrogen-independent growth [7], were retrieved from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), using accession number GSE3542. The expression values for these EGFR- or MEK-regulated genes in $CD44^+/CD24^-$ and $CD44^-/CD24^+$ subpopulations of MCF10A cells [8] were then extracted from GEO using accession number GSE15192. The EGFR and MEK scores were calculated as:

$$s = \sum_i w_i x_i / \sum_i |w_i|$$

where w is the weight +1 or –1, depending on whether the gene was upregulated or downregulated in the signature, and x is the normalized gene expression level.

TCGA data mining

Expression values for MEK-regulated genes (mRNA expression z-scores, measured by Agilent microarrays) and the EGFR phosphorylation status at Y992, Y1068, and Y1173 (protein expression z-scores, measured by reverse-phase protein arrays) for basal and claudin-low tumors were retrieved from The Cancer Genome Atlas (TCGA) (Nature 2012 dataset) [9] via the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/) [10, 11]. The *DUSP4* copy number status was determined by the Genomic Identification of Significant Targets in Cancer (GISTIC) algorithm. GISTIC copy numbers "-2" (a deep loss) and "-1" (a shallow loss) for *DUSP4* were considered homozygous and heterozygous deletions of *DUSP4*, respectively. Tumors for which *DUSP4* GISTIC copy numbers were ≥ 0 were assumed not to harbor *DUSP4*

deletion. Pearson *r* correlation coefficient and two-tailed *P* values were calculated using GraphPad Prism 6.0 software.

References

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