Stromal EGF and IGF1 Together Modulate Plasticity of Disseminated Triple Negative Breast Tumors

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines and cell culture conditions

BT549 human breast tumor cells were obtained from Dr. Kornelia Polyak´s Lab (no additional authentication was conducted by the authors).

Standard medium for each cell line is as follows: HMLER-HR cells were maintained in DMEM-F12 (1:1) medium, supplemented with 5% calf serum, 10 ng/ml human recombinant EGF (Sigma E9644), 10 μg/ml insulin (Sigma I9278), 1 μg/ml hydrocortisone (Sigma H0888) and 1% penicillin-streptomycin. BPLER cells were maintained in WIT Medium (StemGent), and MCF7-Ras cells in DMEM media (Thermo Scientific) supplemented with 10% heat inactivated FBS (Gibco), and 1% penicillin-streptomycin. BT549 cells were maintained in RPMI medium supplemented with 10% FBS and 10 μg/ml insulin. For proliferation, apoptosis and malignant profile signature analysis, HMLER-HR or BT549 were cultured for 7 days in depleted medium (DMEM/F12 supplemented with 5% calf serum and 1% penicillin-streptomycin for HMLER-HR, or RPMI with 10% FBS for BT549 cells). After deprivation, cells were either maintained or changed to complete medium (C.M.) composed by D.M. + 2.0 ng/ml human recombinant EGF + human recombinant 100 ng/ml of IGF1 (R&D). For ligand availability experiments, cells were incubated for 4 days with D.M. and 2.0 ng/ml human recombinant EGF, human recombinant 100 ng/ml of IGF1 (R&D), or 5 ng/ml

recombinant human TGF-β1 (R&D). For ligand depletion experiments cells were incubated with C.M. or C.M. deprived of EGF or IGF1 (see Fig. S5A).

Flow Cytometric Analysis

Freshly harvested tissues were digested in DMEM:F12 (1:1) with 1 mg/ml collagenase A (Roche), 1mg/ml Hyaluronidase (Roche) for 30 min at 37ºC with continuous rotation. Resulting cell suspensions were dispersed with a 21 g needle, washed with resuspension buffer (2% heat-inactivated fetal calf serum in sterile HBBS), and filtered through 70 um nylon mesh. Tissue cells and BMCs were prepared for flow cytometry by suspension in PBS containing 2% FCS, labeled with appropriate antibodies for 30 min at 4ºC, and analyzed on a FACSCanto II (FACSDiva software 5.02; BD Bioscience). Dead cells were excluded using Live/Dead Fixable Aqua cell stain (Invitrogen). See Table S2 for antibodies and dilutions.

Bone Marrow Cells and Assays

BMCs were harvested and tested for functional activity as previously described (McAllister et al., 2008). In vitro instigation assays were conducted by co-culturing $1x10^4$ GFP-positive responder tumor cells with $1x10^6$ BMCs harvested from mice bearing Matrigel, TNBC or LBC Instigators. Co-cultured cells were maintained for 4 days in BMDC medium (DMEM, 5%FBS, 5% horse serum, 10^{-6} M hydrocortisone), adding fresh medium on day 2.

Soft agar Tumorigenesis assays

For assays performed in depleted medium (D.M.) $5x10^3$ BT-549 or HMLER-HR tumor cells were plated in a 6-well plate and incubated with D.M. (RPMI 10% heat inactivated bovine serum or DMEM/F12 with 5% heat inactivated calf serum, respectively) for 7 days. For assays performed in completed medium (C.M.) BT-549 or HMLER-HR tumor cells were kept in their C.M. (RPMI 10% heat inactivated bovine serum + 10 μg/ml of Insulin for BT549 cells or DMEM/F12 with 5% heat inactivated calf serum + 20ng/ml EGF, and + 10 μg/ml of Insulin for HMLER-HR). Media was replaced every two days. After 7 days, cells were tripsinized and dispersed with a 21 g needle. Positive controls were generated using $1x10^4$ BPLER instigator cells in their complete medium. $1x10^3$ of responder cells incubated in C.M. or in D.M. for 7 days were dispersed with a 21 g needle were embedded in 0.4% soft agar dissolved in DMEM/F12 Media with 5% heat inactivated calf serum (in HMLER-HR soft agar experiments) or RPMI with 10% heat inactivated bovine serum (in BT549 soft agar experiments) ± 20ng/ml EGF, and ± 10 μg/ml of Insulin. 0.4% soft agar containing cells was deposited onto a basement of 0.6% soft agar, dissolved in the same medium. Cells were incubated for two weeks at 37ºC. To analyze number of colonies, MTT 1mg/ml dissolved in acetone/PBS (1:1), was added into each well, incubated for 4 hours and the reaction stopped with DMSO. Colonies were photographed after 14 days and counted using ImageJ software.

Apoptosis and proliferation Analysis

Proliferation was analyzed with Cell Titer 96 Aqueous kit (Promega) following manufacturer´s instructions. Apoptosis was determined staining the cells with PI (Calbiochem) for 5 mins. Stained cells were analyzed in the flow cytometer within 1 hr.

Gene Expression Array and Computational Analysis

Gene expression array analyses were previously performed on cell types known to comprise or represent stromal components of responding tumors exposed to the TNBC instigating systemic environment. These data were previously published (Elkabets et al., 2011) and deposited into GEO, and heatmaps were generated from these data. The first data set includes expression analysis of cancer-associated fibroblasts isolated from human mammary tumor xenografts (CAF), granulin-treated human mammary fibroblasts (PRGRN) analyzed relative to PBS-treated human mammary fibroblast controls (CTRL) (GEO GSE25620). The second data set includes gene expression analysis of Sca1+cKit- BMCs from mice bearing instigating TNBC tumors (instigator) relative to matrigel control (matrigel) (GEO GSE25619). Smyth's moderated t-test had been used to identify differentially expressed genes; to test for enrichments of higher- or lower-expressed genes in data sets, we had used RenderCat program, which implements a threshold-free technique with high statistical power based on Zhang C statistic (Elkabets et al., 2011). Our current analyses were motivated by

interrogation of these data sets for genes that met the following criteria: 1. secreted protein products, 2. cytokines known to regulate malignancy profile factors, 3. factors involved in recruitment of BMCs.

SUPPLEMENTAL FIGURE LEGENDS

Supplementary Figure 1. Instigating Tumors Facilitate Responder Growth in the Absence of "Self-seeding".

(**A**) Tumor sections stained for GFP (red) and SV-40 LgT (green) confirmed that GFP-positive instigator cells had not metastasized to the contralateral sites of GFP-negative responder cell injection (top row), and were not present in the bone marrow cell (BMC) preparations used in admixture experiments (bottom row). Cell nuclei are counterstained with DAPI (blue). (**B**) Merged inmunofluorescence images of responding tumor cells growing contralaterally to Matrigel or Instigators (described in Figure 1), in order to visualize the LgT Antigen (green), which is expressed by responder tumor cells; nuclei counterstained with DAPI (blue). Scale bar= 25 μm. (**C**) Merged immunofluorescent images representative of responding tumors after eight days in mice with either control matrigel (Mg, left) or TNBC (right). Tumors were stained for LgT Antigen (green; expressed only by responding tumor cells), and nuclei stained with DAPI (blue).

Supplementary Figure 2. Response to the Pro-Tumorigenic TNBC

Environment is Not Oncotype Specific.

(**A**) Final mass of responder BT549 tissues after 8 days in mice with Matrigel or TNBC-BPLER (protocol shown in Figure 2**A**). Incidence of tumor formation is shown above data bars; differences were not significant. (**B**) BT549 responding tumors stained for the proliferation marker Ki67 (red), and cell nuclei (blue) under indicated conditions. Graph represents number of Ki67+ cells as a percentage of the total number of cells per field; n=9; 3 random fields from 3 tumors per group. (**C**) Representative inmunofluorescence of BT549 responding tumors after 8 days in mice with Matrigel (Mg) or TNBC-BPLER. Graph represents the number of cells stained positively for each of the indicated factors as a percentage of the total number of DAPI-positive nuclei or human-Mitochondria (hMit), as indicated; n=9; 3 random fields from 3 tumors per group. (**D**) Growth kinetics of BT549 responding tumor cells during a 40 day time course in mice with control Matrigel or TNBC-BPLER (protocol shown in Figure 1A). Data represented only for cases in which the contralateral instigating tumors grew. Incidence of tumor formation is shown next to data lines. (**E**) Representative flow cytometric density plots for IGFR and EGFR (right) cell surface expression on BT549 responder tumor cells in culture. (**F**) Expression of EGF, and IGF-1 in the responder BT549 cells relative to that of HMLER-HR responding tumor cells (n=3). On (**A**), (**B**), (**C**), (**D**) and (**F**) data represents the mean ± s.e.m. P value was obtained by Student t-test analysis.

Supplementary Figure 3. Activation of EGF and IGF1 Receptors.

(**A**) Combined heat map showing the most variable individual genes based on our criteria (see Methods) for analysis of responding HMLER-HR tumor microenvironmental components: cancer-associated fibroblasts isolated from human mammary tumor xenografts (CAF) and granulin-treated human mammary fibroblasts (PGRN) relative to PBS-treated human mammary fibroblast controls (CTRL) (GEO GSE25620 (9)); and pro-tumorigenic BMCs from mice bearing instigating TNBC-BPLER tumors (instigator) relative to matrigel controls (matrigel) (GEO GSE25619 (9)). Genes are ranked by differential expression of CAF vs CTRL (See Methods). (**B**) Flow cytometry density plots representing the EGFR/IGFR profile of responding tumor cells and instigating TNBC-BPLER cells in culture. (**C**) Expression of EGF and IGF1 in the instigating TNBC-BPLER cells (Inst) relative to that of responding tumor cells HMLER-HR (Resp). Data are expressed as mean ± s.e.m. (n=3) *, $p<0.05$, **, $p<0.01$. Inset represents qPCR products run in 2% agarose gel. (**D**) Immunoflourescence analysis of phosphorylated EGF receptor and IGF1 receptor on HMLER-HR and BT549 responder cells in the presence of the indicated growth factors. Cell nuclei stained with DAPI (blue). Images for each given stain were taken under identical exposure and gain.

Supplementary Figure 4. Tumor Microenvironment of Hosts with TNBC Expresses EGF and IGF-1 and Supports Responding Tumor Cell Malignancy.

(A) Characterization of phosphorylated EGFR and IGFR on responder HMLER-HR tumor cells growing in mice bearing Matrigel (Mg) or TNBC-BPLER after 60 days (left panels, described in Figure 1), or after 36 d (right panels, described in Figure 3). Responding HMLER-HR tumors were stained for the LgT Antigen (green) and phosphorylated Tyr1068 EGFR (red) (Top panels). The same responding tumors were stained for phosphorylated Tyr1161/Tyr1185 IGFR (red) (Bottom panels). In all cases, nuclei were counterstained with DAPI (blue). Size of scale bars is indicated on each panel. (**B)** EGF and IGF-1 expression levels in bone marrow cells from mice bearing TNBC (BMC-TNBC) relative to control BMCs (BMC-C) (n=4 per group). Bottom, photomicrograph of agarose gel to visualize indicated Q-PCR products of mRNA prepared from BMC-TNBC and BMC-C. (**C**) Scheme of in vitro co-culture experiments with GFP-positive responder HMLER-HR tumor cells and bone marrow cells from mice bearing: Matrigel control (BMC-C), or triple-negative breast cancers BPLER (BMC-TNBC). (**D**) Gene expression levels in GFP+ responder HMLER-HR tumor cells that had grown in co-culture for 4 days with BMC-TNBC (BPLER) relative to those under identical co-culture conditions with BMC-C, as determine by qRT-PCR (n=7 per group). Data represents the mean ± s.e.m; p value was obtained by Student t-test analysis. (**E**) Merged immunofluorescent images of responding tumors resulting from comixture of tumor cells with BMCs from mice bearing Matrigel control (BMC-C), or triple-negative breast cancers (BMC-TNBC). Tumors were analyzed for indicated proteins. Images for each given stain were taken under identical exposure and gain.

Supplementary Figure 5. EGF and IGF-1 Together Modulate Tumorogenicity of Responder Cells in vitro.

(**A)** Experimental scheme for ligand bioavailability and depletion experiments. (**B**) Left graph: Number of soft agar colonies formed by responder HMLER-HR tumor cells in the presence of their standard medium (S.M.) or S.M. devoid of the indicated growth factors: insulin (Ins), epidermal growth factor (EGF), or hydrocortisone (Hydr). Data represent average of 2 independent experiments, where each condition was assessed in triplicate (n=6). Right graph: Number of colonies formed by responding BT549 tumor cells cultured in soft agar in either depleted medium (D.M.) or D.M. with epidermal growth factor (EGF) (n=3). Table indicates phosphorylation status of EGFR and IGF1R under each condition.

Supplementary Figure 6. EGF and IGF-1 Together Modulate Viability and Expression Profile of Responder Cells.

(A) Responder HMLER cells were cultured in the presence of the indicated growth factors for 4 days. Cell viability was determined based on absorbance at 450nm (Abs) in a standard MTT assay (n=5), and apoptosis was based on analysis of 10,000 total events by flow cytometry based on propidium iodide (PI) incorporation (n=2). Cells treated with detergent for 1 minute served as positive control. (**B**) Responder BT549 cells were cultured in the presence of the indicated growth factors for 4 days. Cell viability was determined based on

absorbance at 450nm (Abs) in a standard MTT assay (n=5). Data represent the mean ± s.e.m.; p values were obtained by Student's t-test. (**C**) Gene expression profile of GFP-positive HMLER-HR responder cells sorted from responder tumors grown *in vivo* in either control Matrigel (Mg) or TNBC bearing mice, relative to responder cells from culture prior to injection. Data are expressed as mean ± SEM; p values above data bars represent Student's t-test when comparing cells from the tumor to those in culture. p values above connectors represent Student's t-test when comparing responding tumor cells from tumors in the Matrigel control mice to those in the mice with TNBC.

Supplementary Figure 7. EGFR/IGFR Inhibitors do not Affect the Phenotype of Responding Tumor Cells in Animals Bearing Control Matrigel.

(**A, B**) Immunoflourescence staining for phosphorylated EGF and IGF1 receptors in responding tumors after 8 days of treatment with EGFR and IGFR inhibitors in vivo. Responding tissues were stained for the LgT+ Antigen (green), and phosphorylated Tyr1068 EGFR (red) (A) or phosphorylated Tyr1161/Tyr1185 IGF1R (red) (B). Scale bars indicated on images. (**C**) Final mass of TNBC instigators after 5 weeks of growth in mice that were treated during the last 8 days with either vehicle DMSO or the combined EGFR inhibitor (erlotinib; dose 100 mg/kg/day) and IGFR inhibitor (BMS-754807; dose 50 mg/kg/day) (n=5 mice per group). Data represent the mean ± s.e.m.; p values were obtained by Student's t-test. Differences were not statistically significant (n.s.). (**D**) Responding tumors were analyzed by immunofluorescence staining for indicated

malignancy profile factors. Scale bars indicated on image.

Supplementary Figure 8. Identification of Instigating Human Breast Tumor Surgical Specimens.

(**A**) Left, growth kinetics of TNBC or LBC human tumor specimens (hBrCa TNBC-I, hBrCa TNBC-II, hBrCa LBC) used to instigate responding tumors (n=3 mice per tumor specimen). Right, average final mass of TNBC or LBC hBrCa instigator tumors 28 days after injection; values were not statistically significantly different. Data represent the mean ± s.e.m. P value was obtained by Student t-test analysis. (**B**) Hematoxylin and eosin stains to visualize histopathology of resulting responder tumors/tissues (top panels) and indicated human tumor specimens (bottom panels); scale bar = 100 μm.

Supplemental Table 1: PCR Oligonucleotide Primers

Name	Sequence	Size amplified
H-Zeb1 (Pena C et al., 2008)	F: GCCAATAAGCAAACGATTCTG R: TTTGGCTGGATCACTTTCAAG	100 bp
H-VIM (Mani et al., 2008)	F: GAGAACTTTGCCGTTGAAGC R: GCTTCCTGTAGGTGGCAATC	162 bp
H-OCT4 (Peng S et al., 2010)	F: GTGGAGGAAGCTGACAACAA R: GCCGGTTACAGAACCACACT	203bp Transcript 1, 2 or 3
H-OCT-4A (Wang Wi et al., 2009)	F: AGCAAAACCCGGAGGAGT R: CCACATCGGCCTGTGTATATC	113 bp
H-cMyc (Wang J et al., PlosOne 2008)	F: TCAAGAGGCGAACACACAAC R: GGCCTTTTCATTGTTTTCCA	109 bp
H-EGF (Patsialou A. et al., Cancer Research 2009)	F: CAATGCAACCAACTTCATGG R: AAGCTTCGCTCCATTACCTG	120 bp
H-IGF1	F: GTGACATTGCTCTCAACATCTCCCA R: GCGAGGAGGACATGGTGTGCA	187 bp
H-GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	225 bp
H-βactin	F: ACTATGACTTAGTTGCGTTACAC R: GCCATGCCAATCTCATCTTG	75 bp
M-EGF (Christopher R et al., 1999)	F: AATAGTTATCCAGGATGCCC R: ACGCAGCTCCCACCATCG TA	158 bp
M-IGF1 (Yu S. et al., Prostate 2011)	F: GGTGGATGCTCTTCAGTTC R: TTTGTAGGCTTCAGTGGG	173 bp
M-IL6	F: GCTGGAGTCACAGAAGGAGTGGCT R: GGCATAACGCACTAGGTTTGCCGA	117 bp
M-CCL8	F: AGGCTCCAGTCACCTGCTGCT R: ACCACAGCTTCCATGGGGCAC	109 bp
M-CXCL1	F: GAGCTGCGCTGTCAGTGCCT R: CAAGGCAAGCCTCGCGACCA	142 bp
M-CSF1R (Patsialou A et al., Cancer Research 2009)	F: TGGTGCACCCCCTAGTTCTCT R: GGCCACTCCTGTGAGCTTAG	201 bp
M-WNT3	F: TGAGTCCCGAGGCTGGGTGG R: GTCCCTCTCGGTGGGTGGCT	70 bp
M-IL14	F: CGGCAGGAGCACCCATCGAC R: GCCCGGCTTGGTTCTCGGTT	87 bp
M-SPP1	F: TCGGAGGAAACCAGCCAAGGACT R: AAGCTTCTTCTCCTCTGAGCTGCCA	129 bp

M-FRZB	F: CCTGAGGCCATCGTCACCGC R: GCAACGTTCGCTGCTTGCCC	87 bp
M-IGF2 (Dong/Myung Shin et al., Leukemia 2009)	F: TGGTCCCAGAGAGGTTTTAGGTGG R: ACTTGCTCCCGCCTGATGTAAC	222 bp
M-βactin (Ohtani et al., PNAS 2007)	F: GTATGGAATCCTGTGGCATC R: AAGCACTTGCGGTGCACGAT	283 bp
M-GAPDH	F: GGTGAAGGTCGGTGTGAACG R: CTCGCTCCTGGAAGATGGTG	233 bp

(H: human, M: mouse)

Supplemental Table 2: Antibodies

Specific Antibodies for Inmunofluorescence

Antibody	Dilution	Company
Rabbit polyclonal anti-GFP	1:1000	Abcam (ab290)
Rabbit polyclonal anti-Oct4	1:100	Chemicon AB3209
Rabbit polyclonal anti-ZEB1	1:50	Santa Cruz sc-25388
Mouse monoclonal anti-CK14	1:20	Leica (NCL-L-LL002)
Rabbit polyclonal anti-CK18	1:100	Abcam (ab32118)
Rabbit polyclonal anti-Ki67	1:200	Thermo scientific (9106)
Mouse monoclonal anti-SMA	1:50	Vector (VP-S281)
Mouse monoclonal anti-SV40 LgT	1:75	Santa cruz (sc-147)
Mouse monoclonal anti-c-myc	1:100	Millipore (MAB 8864)
P-IGFR/IR	1:50	Abcam (ab39398)
P-EGFR	1:600	Cell Signaling (3777)
Alexa Fluor 488 goat anti-mouse	1:200	Invitrogen (A11001)
Alexa Fluor 594 goat anti-rabbit	1:200	Invitrogen (A11012)
Alexa Fluor 647 goat anti-rat	1:200	Invitrogen (A21247)

Specific Antibodies for FACS

Antibody	Dilution/Concentration	Company
CD16/CD32 $F_c\gamma$ III/II receptor	250 ng/10^6 cells	BD Pharmingen
APC-humanIGF1R (clone147)	1:20	eBiosciences
PE-humanEGFR	1:20	BD Pharmingen