Stromal EGF and IGF-I Together Modulate Plasticity of Disseminated Triple-Negative Breast Tumors

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ABSTRACT

The causes for malignant progression of disseminated tumors and the reasons recurrence rates differ in women with different breast cancer subtypes are unknown. Here, we report novel mechanisms of tumor plasticity that are mandated by microenvironmental factors and show that recurrence rates are not strictly due to cell-intrinsic properties. Specifically, outgrowth of the same population of incipient tumors is accelerated in mice with triple-negative breast cancer (TNBC) relative to those with luminal breast cancer. Systemic signals provided by overt TNBCs cause the formation of a tumor-supportive microenvironment enriched for EGF and insulin-like growth factor-I (IGF-I) at distant indolent tumor sites. Bioavailability of EGF and IGF-I enhances the expression of transcription factors associated with pluripotency, proliferation, and epithelial–mesenchymal transition. Combinatorial therapy with EGF receptor and IGF-I receptor inhibitors prevents malignant progression. These results suggest that plasticity and recurrence rates can be dictated by host systemic factors and offer novel therapeutic potential for patients with TNBC.

SIGNIFICANCE: Currently, processes that mediate progression of otherwise indolent tumors are not well understood, making it difficult to accurately predict which patients with cancer are likely to relapse. Our findings reveal novel mechanisms of tumor phenotypic and gene expression plasticity that are mandated by microenvironmental factors, identifying novel therapeutic targets for patients with TNBC.

INTRODUCTION

Breast cancer is categorized into histopathologic subtypes based on estrogen (ER) and progesterone (PR) hormone receptor status and HER2/ERBB2 expression levels. Triple-negative breast cancer (TNBC), which is considered the most malignant form of breast cancer, does not express ER or PR and lacks HER2/ERBB2 amplification. Women with TNBC are at the greatest risk of early recurrence compared, for instance, with women with ER-positive or luminal breast cancer (LBC; ref. 1), but the reasons for these differences in recurrence rates are unclear. Patients who present with distant metastases at the time their primary tumor is detected are diagnosed with stage IV disease. Other patients who do not have detectable metastases at the time of diagnosis will eventually recur with disease in distant organs. For women with metastatic TNBC, intensive cytotoxic chemotherapy is currently the only treatment approach, even though it is not curative. Furthermore, therapies designed to target primary tumors are not as successful against recurrent disease (2).

The fact that disease recurs after primary breast tumor removal indicates that tumor cells were disseminated before surgical resection, but remained indolent and undetected before progressing to symptomatic disease (3, 4). Hence, in women with recurrent or stage IV disease, the primary tumor and a number of disseminated tumors coexist for an indefinite period of time. A growing body of clinical and experimental evidence supports the concept that coexisting tumors in a patient with clinically silent metastases can interact with the host environment to modulate overall disease progression (reviewed in ref. 5). These interactions arise from a host response involving circulating cytokines, immune cells, and bone marrow–derived cells that instruct formation of tumor-supportive microenvironments (reviewed in ref. 6). The tumor microenvironment regulates primary tumor growth, homeostasis, and progression (7); however, the means by which systemic and microenvironmental processes facilitate malignancy of otherwise indolent disseminated tumors have been unclear. We report here that bioavailability of EGF and insulin-like growth factor-I (IGF-I), provided by the tumor microenvironment, modulates phenotypic plasticity, gene expression, and the recurrence rate of certain TNBC tumors. Combinatorial therapy with EGF receptor (EGFR) and IGF-I receptor (IGF-IR) inhibitors prevents disease progression by interrupting paracrine interactions between TNBC tumor cells and their microenvironment.

RESULTS

Malignancy of Indolent Tumors Is Accelerated in Hosts with TNBC

To understand if systemic processes might explain the differences in relapse rates associated with different breast cancers, we used a human tumor xenograft model that represents situations in which a patient has either coexisting primary and distant metastases (i.e., stage IV disease) or multiple disseminated metastatic foci (i.e., recurrent disease) and allows us to precisely...
tumors formed after a latency period of approximately 50 days (Fig. 1B and C and not shown). In contrast, responding tumors stained for DAPI were predominantly necrotic.

Systemic environment determines growth kinetics and histopathology of responsive tumors. A, scheme of bilateral human tumor xenograft implantation system used for data represented in figure. For these experiments, we used triple-negative HMLER hygro-H-RasV12 (HMLER-HR) tumor cells (46) as responders, oncotype-matched BPLER tumor cells (47) as TNBC instigators, and MCF7Ras tumor cells (48) as LBC instigators. B, growth kinetics of responding tumors in mice bearing Matrigel (Mg; n = 6), TNBC (TNBC-BPLER; n = 4), or LBC (LBC-MCF7Ras; n = 3). Data are represented only for cases in which the contralateral instigating tumors grew; incidence of responding tumor formation is indicated on graph. C, hematoxylin and eosin (H&E) stains of responding tumors from indicated cohorts; ×60 magnification; arrows indicate mitotic tumor cells. D, merged photomicrographs of responding tumors stained for α-SMA. E, quantification of responding tumors stained for the indicated malignancy profile factors. The number of positively stained cells is represented as percentage of the total number of 4′,6-diamidino-2-phenylindole (DAPI)-positive cells per random field; three fields per tumor (n = 3 Matrigel; n = 12 TNBC; n = 9 LBC). F–I, merged photomicrographs of responding tumors stained for the indicated malignancy profile factors. Scale bars: for panel C, scale bar = 50 μm; for panels D, F–I, scale bar = 100 μm. BV, blood vessels.

Figure 1. Systemic environment determines growth kinetics and histopathology of responsive tumors. A, scheme of bilateral human tumor xenograft implantation system used for data represented in figure. For these experiments, we used triple-negative HMLER hygro-H-RasV12 (HMLER-HR) tumor cells (46) as responders, oncotype-matched BPLER tumor cells (47) as TNBC instigators, and MCF7Ras tumor cells (48) as LBC instigators. B, growth kinetics of responding tumors in mice bearing Matrigel (Mg; n = 6), TNBC (TNBC-BPLER; n = 4), or LBC (LBC-MCF7Ras; n = 3). Data are represented only for cases in which the contralateral instigating tumors grew; incidence of responding tumor formation is indicated on graph. C, hematoxylin and eosin (H&E) stains of responding tumors from indicated cohorts; ×60 magnification; arrows indicate mitotic tumor cells. D, merged photomicrographs of responding tumors stained for α-SMA. E, quantification of responding tumors stained for the indicated malignancy profile factors. The number of positively stained cells is represented as percentage of the total number of 4′,6-diamidino-2-phenylindole (DAPI)-positive cells per random field; three fields per tumor (n = 3 Matrigel; n = 12 TNBC; n = 9 LBC). F–I, merged photomicrographs of responding tumors stained for the indicated malignancy profile factors. Scale bars: for panel C, scale bar = 50 μm; for panels D, F–I, scale bar = 100 μm. BV, blood vessels.

trace the growth kinetics of individual tumors (Fig. 1A). On the basis of previously defined functional properties of various tumor cells in this xenograft system (8, 9), we use the term “instigator” to define tumors that elicit a protumorigenic host systemic response and “responder” to define tumors that are otherwise indolent, but can respond to systemic stimuli to form overt tumors. We injected responding and instigating TNBC cells into anatomically distinct sites in nude mice, using Matrigel as a vehicle control for the instigators in another group of mice. We also injected the same responder cell population into hosts bearing LBC tumors, which we previously determined can stimulate responding tumor growth (8).

Only one of the six mice injected with Matrigel formed a distant responding tumor, which was predominantly necrotic (Fig. 1B and C and not shown). In contrast, responding tumors formed after a latency period of approximately 50 days in 100% of the mice with LBC (Fig. 1B). These responders were highly mitotic without forming α-smooth muscle actin (SMA)–rich desmoplastic stroma (Fig. 1C and D). In mice with TNBC, responding tumors formed with 100% penetrance following a latency period of only approximately 35 days, after which they maintained a constant rate of growth (Fig. 1B). These responders showed a spectrum of pathologic grades from atypical/high grade to differentiated/low grade, were moderately mitotic with no observable necrosis, and were highly desmoplastic (Fig. 1C and D). Importantly, responding tumors were devoid of instigating tumor cells and were comprised exclusively of the responding tumor cells (Supplementary Figs. S1A–S1C). In both cases, responding tumor histopathology was consistent with breast adenocarcinomas observed in the clinic (10). Moreover, the latency and growth kinetics with which responding tumors formed
in hosts bearing different breast cancer subtypes reflected the relative rates of recurrence that are observed in patients with the respective breast cancer subtype (1).

Specific cytokeratin expression is frequently used to stratify normal epithelium and for tumor diagnosis (11). Responding tumors that formed in mice with Matrigel or LBC expressed both the luminal cytokeratin CK18 (∼42% and 55%, respectively) and the basal cytokeratin CK14 (∼15% and 8%, respectively; Fig. 1E and F). Conversely, in mice with TNBC, responding tumors were significantly enriched for CK14 (50%), whereas only approximately 10% were CK18 positive (Fig. 1E and F).

Expression of transcription factors that modulate proliferation and pluripotency has been correlated with high-grade breast cancers and poor clinical outcome (12–15). In particular, Oct4 maintains pluripotency and self-renewal (16), whereas c-Myc, which is often amplified in lethal metastases of unamplified primary tumors (17), regulates proliferation, differentiation, apoptosis, and self-renewal (18). Tumor-forming capacity has also been associated with cells that undergo an epithelial–mesenchymal transition (EMT; ref. 19). Specifically, expression of the EMT-inducing transcription factor Zeb1 is correlated with early relapse (20–22). Immunohistochemical analysis revealed that in mice with TNBC, responding tumors were enriched with cells expressing Oct4 (∼47%) and c-Myc (∼42%) relative to counterpart tumors in control Matrigel-bearing mice (∼8% each; Fig. 1E and G–I). In responding tumors from mice with TNBC, the majority of c-Myc was localized to the cytoplasm, in agreement with clinical studies showing that approximately 95% of Myc-amplified breast tumors display cytoplasmic c-Myc localization (23). Zeb1 was expressed in the nucleus of approximately 67% of TNBC-responsive tumor cells, as opposed to only approximately 10% of responder cells in the cancer-free mice (Fig. 1E and I). We obtained similar results using another responding cell line, BT549 (Supplementary Fig. S2), and another TNBC instigator cell line, MDA-MB-231 (data not shown), showing that response to the TNBC-induced systemic environment was not oncotype-dependent. In contrast, only 2% of the responding tumor cells in mice with LBC expressed Oct4, approximately 17% expressed c-Myc, and approximately 19% expressed nuclear Zeb1 (Fig. 1E and G–I). These expression levels were significantly lower than those of responding tumors in the mice with TNBC and not significantly different from those in the control mice (Fig. 1E), supporting our earlier report that LBC systemic tumor promotion operates via different mechanisms than that of TNBC (8).

Together, these results established for the first time that the same population of otherwise indolent xenografted tumor cells forms tumors with dramatically different growth kinetics and resultant tumor cell phenotypes depending on the breast tumor subtype borne by the host. In particular, enhanced expression of the same pluripotency and EMT-inducing transcription factors that we observed in the mice with TNBC has been observed in tumors from women with TNBC (12–20).

**Brief Exposure to TNBC Is Sufficient for Responder Tumors to Progress Independently**

To understand whether malignant conversion, defined by enhanced proliferation and the collective expression of Oct4, c-Myc, and Zeb1, was an early event in hosts with TNBC, we injected GFP+ responding tumor cells into mice that were either cancer-free or bearing TNBC instigator tumors and recovered tumor tissues after 8 days (Fig. 2A). At this time point, tissue plugs from cancer-free and TNBC mice were comparable in size and contained responding tumor cells (Figs. 2B and Supplementary Fig. S1C).

Immunofluorescence analysis of resulting tissues revealed that α-SMA–positive myofibroblasts were abundant in the responding tumors in mice with TNBC but not in those with Matrigel, confirming that a hallmark of systemic tumor promotion, stromal desmoplasia, had already been initiated (Fig. 2C). Luminal and basal cytokeratins were equivalently expressed in responding tumors from both cohorts (Fig. 2D), indicating that enrichment for CK14, which occurred after 90 days, was not an early event in the TNBC-mediated response. However, responding tumors from mice with TNBC were significantly enriched for expression of malignancy profile factors after only 8 days (Fig. 2D).

To validate these observations, we sorted GFP+ tumor cells from the tissues by fluorescence-activated cell sorting (FACS; Fig. 2E) and analyzed expression by quantitative PCR (qPCR). All three malignancy factors were elevated in responding tumor cells exposed to TNBC: Oct4A (a splice variant of Oct4; ∼29-fold), c-Myc (∼threefold), and Zeb1 (∼93-fold; Fig. 2F). Expression of the EMT marker vimentin (Vim) was also significantly elevated (∼397-fold) in the responding tumors from mice with TNBC (Fig. 2F). We confirmed these results using another responding TNBC cell line, BT549, which acquired a proliferative advantage and displayed the malignant profile after only 8 days in mice with TNBC as compared with cancer-free controls (Supplementary Fig. S2A–S2C).

To determine whether early acquisition of the malignant phenotype was relevant to disease progression, we surgically removed responder plugs after 8 days of exposure to the TNBC-induced environment, immediately transplanted them into secondary hosts bearing either Matrigel or a TNBC instigator tumor, and allowed them to progress (Fig. 3A). After 5 weeks, the percentage of Ki67–positive proliferative cells and the average mass of responding tumors was the same in both secondary cohorts (Fig. 3B and C). Responding tumors from both cohorts were also nearly identical on the histopathologic level (Fig. 3D) and maintained an activated stroma, characterized by the presence of α-SMA–positive myofibroblasts (Fig. 3E). Likewise, transplanted responding tumors maintained equivalent expression of Oct4, c-Myc, and Zeb1 in both cohorts (Fig. 3F–H). Hence, the tumor microenvironment and the malignant conversion that occurred during the initial phases of instigation by TNBC were sufficient to maintain malignancy, even in the absence of the initiating TNBC tumor.

**Microenvironmental Factors that Mediate TNBC Progression**

We and others have shown that systemic signals impinge upon the microenvironment of disseminated tumors to facilitate their outgrowth (reviewed in ref. 6). To identify candidate factors that mediated malignant conversion in hosts with TNBC, we interrogated our gene expression data that had been generated from different components of the responding
tumor microenvironment in hosts with TNBC or Matrigel control (9), including protumorigenic bone marrow–derived cells (GEO GSE25620) and two different types of cancer-associated fibroblasts (GEO GSE25619). From each dataset, we generated a list of the most differentially expressed genes that met the following criteria: (i) protein products that are secreted; (ii) cytokines known to regulate self-renewal, transdifferentiation, and EMT; and (iii) factors that mediate recruitment of bone marrow–derived cells. This resulted in a combined list of genes representing the collective responding tumor microenvironment in hosts with TNBC or Matrigel control (9).
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**Figure 3.** Brief exposure to the TNBC environment is sufficient for responding tumors to progress independently. **A,** scheme of responding tumor transplantation system used for data represented in figure. **B,** mass of responding tumors from mice with TNBC-BPLER 36 days following their surgical transplantation into secondary hosts bearing either Matrigel or TNBC-BPLER. Incidence of tumor formation is shown above data bars (n = 3 mice per group); differences were not statistically significant (n.s.).

**C,** transplants from indicated cohorts stained for the proliferation marker Ki67 (red), and cell nuclei (blue) under indicated conditions. Graph represents number of Ki67–positive cells as a percentage of the total number of cells per field; n = 6 Matrigel (Mg) images; n = 9 TNBC images.

**D,** hematoxylin and eosin (H&E) stains of responder tumors that had been transplanted into indicated secondary hosts; scale bar = 50 μm.

**E–H,** merged immunofluorescent images (**E–G**) and corresponding quantification (**H**) of transplanted responding tumors stained for the indicated malignancy profile factors (n = 6 Matrigel images; n = 9 TNBC images). Scale bars: for panel C, scale bar = 40 μm; for panel D, scale bar = 50 μm; for panels E–G, scale bar = 40 μm.

tumor microenvironment (Supplementary Fig. S3A). We then sorted the GFP + stromal cells from responding tumors recovered from cancer-free or TNBC-bearing mice (Fig. 4A) and analyzed the relative expression of 13 of these genes. In this analysis, we included IGF-I due to the high expression levels of activating IGF-binding proteins and reduced expression of IGF-inactivating binding proteins (24) revealed from our meta-analysis (Supplementary Fig. S3A).

CXCL1, EGF, IGF-I, Wnt3, and TGFβ1 were significantly upregulated in the responding tumor microenvironment of mice with TNBC relative to that of control mice (Fig. 4B). Expression levels of CCL8, CSF1R, FRZB, ACTB, IL-6, SPP1, and TXLNA (IL-14) were not significantly different, and IGF-II expression was significantly downregulated in the responding tumor microenvironment of mice with TNBC relative to controls (Fig. 4B).
Figure 4. Identification of responding tumor stromal-derived factors. A, top, scheme of tumor subfractionation into GFP+ HMLER-HR responding tumor cell and GFP− stromal cell constituents after 8 days of exposure to mice with either Matrigel or TNBC-BPLER. Bottom, FACS plot of dissociated responding tumors that were separated into GFP+ (responder tumor cells) and GFP− (tumor stroma) subfractions. B, heatmap representing expression levels of indicated genes in the GFP− stromal cells from the responding tumors of mice with TNBC-BPLER relative to those with Matrigel (Mg). C, photomicrograph of agarose gel to visualize indicated qPCR products; RNA was prepared from GFP-negative tumor-associated stromal cells after 8 days of exposure to mice with either Mg or TNBC-BPLER. D, merged immunofluorescent images showing activated forms of EGF (phospho-Tyr1068; P-EGFR) and IGF/Ins (phospho-Tyr1161/Tyr1185; P-IGF1R/IR) receptors on responding tumor cells that had grown for 8 days in indicated mice. Responding HMLER-HR tumor cells express the simian virus 40 large-T oncoprotein (LgT; green). Scale bar = 25 μm. E, experimental scheme for implanting bone marrow cells (BMC) with responding tumor cells. F, mass of responding HMLER-HR tumors 12 weeks following injection of indicated bone marrow cell admixtures (n = 10 tumors per group). G, quantification of malignancy profile factors in responding HMLER-HR tumor cells under indicated conditions. The number of cells stained positively for each factor is represented as a percentage of DAPI-positive tumor cells per random field (n = 9 fields per group; three images quantified from each of three tumors per group). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
We concentrated on two growth factors that were highly upregulated in the TNBC-induced microenvironment, EGF and IGF-I (Fig. 4B and C). EGF ligands are found in 50% to 90% of tumors from patients with poor prognosis, and the majority of TNBCs express the EGF receptor (1, 25, 26). In certain contexts, EGF induces c-Myc expression to reduce breast tumor latency (27) and has been shown to enhance Zeb1 expression in breast tumor cells (28). High levels of phosphorylated IGF-I receptor and its ligands are present in malignant human breast tissues and are associated with poor patient prognosis (29–31). IGF-I signaling has been shown to induce Oct4 expression during cellular reprogramming (32).

The responding HMLE-TR tumor cells expressed both the EGF and IGF receptors (EGFR and IGF-IR) in vitro and did not express either of the ligands (Supplementary Fig. S3B–S3D). Moreover, in the absence of growth factor supplements, EGF and IGF-I receptors were not activated (Supplementary Fig. S3D), indicating that these cells would depend on paracrine sources of EGF and IGF-I to activate the cognate receptors. Responding BT-549 cells also expressed both receptors, and expression of the EGF ligand was similar to that of the responding HMLE-TR cells; however, IGF-I levels were approximately 3,000-fold higher in BT-549 cells relative to HMLE-TR cells (Supplementary Fig. S2B–S2F), and IGF-IR was activated in vitro during cellular reprogramming (32).

EGF and IGF-I Bioavailability Modulates Responder Tumor Cell Plasticity and Malignancy

We conducted a series of in vitro experiments to determine the effects of EGF and IGF-I on malignancy of both HMLE-TR and BT-549 responder cells. When HMLE-TR responder cells were maintained in medium deprived of ligands (see Methods and Supplementary Fig. S5A), EGF induced expression of Zeb1 and c-Myc (Fig. 5A), but was not sufficient to convert responder cells to a malignant state, as determined by colony formation in vitro (Supplementary Fig. S5B). EGF was sufficient for malignant conversion of BT-549 cells (Fig. 5A and Supplementary Fig. S5B), which express IGF-I in an autocrine fashion. Addition of IGF-I induced expression of Oct4 in both cell lines (Fig. 5A), but only promoted modest colony formation (Supplementary Fig. S5B). When both EGF and IGF-I were bioavailable, responder cells converted to malignancy and Oct4, c-Myc, and Zeb1 were significantly upregulated (Figs. 5A and Supplementary Fig. S5B). In all cases, we monitored cell proliferation and apoptosis (Supplementary Fig. S6A and S6B) and confirmed that cognate receptors were activated (Supplementary Fig. S3D).

As a control, we tested the effects of TGF-β, another growth factor we identified in the TNBC-instigated stroma. TGF-β induced Oct4 expression in BT-549 responder cells but otherwise did not induce expression of the other malignancy profile genes (c-Myc and Zeb1) in either responder tumor cell line (Fig. 5A). In both cell lines, proliferation was moderately but significantly reduced upon TGF-β treatment (Supplementary Fig. S6A and S6B).

Under conditions in which responder cells were deprived of EGFR and IGF-IR ligands (see Methods and Supplementary Fig. S5A), malignancy profile factor expression was significantly reduced in vitro and in vivo, and cells failed to form colonies in vitro (Fig. 5B and Supplementary Figs. S5B and S6C). Loss of EGF resulted in a reduction of Zeb1 and c-Myc expression, whereas IGF-I loss modulated a reduction in Oct4 expression (Fig. 5B).

Collectively, these results suggest a model in which both EGFR and IGF-IR activation together modulate interconversion of responsive tumor cell populations between indolent and malignant states (Fig. 5C).

EGFR and IGF-IR Inhibition Prevents Malignant Conversion of Responder Tumor Cells in Hosts with TNBC

Prompted by these results and clinical findings indicating that EGFR and IGF-IR activation are both associated with poor prognosis (29–31, 37–39), we tested whether EGFR and IGF-IR dual inhibition would prevent outgrowth of responding tumors in mice with TNBC. Mice bearing responding tumors in the context of TNBC or Matrigel control were treated with either dimethyl sulfoxide (DMSO) control or a combination therapy of the EGFR inhibitor erlotinib plus the IGF-IR inhibitor BMS-754807. Treatment was administered once per day for 8 days, after which tissues of equivalent mass were recovered from all cohorts (Fig. 6A and B). We confirmed that activation...
EGF and IGF-I modulate indolent and malignant states in vitro. A, expression levels of indicated malignancy profile genes in responder tumor cells (HMLER-HR and BT549) after 4 days in indicated culture conditions relative to those maintained in depleted medium (D.M.). See Supplementary Fig. S5A for experimental scheme. n = 6, where each sample was run in duplicate in three separate experiments. B, expression levels of indicated malignancy profile genes in responder tumor cells (HMLER-HR) after 4 days in indicated culture conditions relative to those maintained in complete media (C.M.). n = 6; each sample run in duplicate in three separate experiments. C, model representing interconversion of responsive tumor cells between malignant and indolent states depending upon EGF and IGF-I ligand availability.

The fact that recruitment of reactive stroma was not affected in hosts with TNBC under this treatment regimen suggested that EGFR/IGF-I dual inhibition prevented paracrine interactions between responding tumor cells and their systemically mandated microenvironment.

Primary Tumors from Patients with TNBC Accelerate Malignant Conversion of Otherwise Indolent Tumors

In an effort to understand whether primary tumors from patients with breast cancer establish similar protumorigenic environments, we analyzed the effects of two different TNBC primary tumor specimens (designated as hBrCa TNBC-I and hBrCa TNBC-II) on responding tumor outgrowth. For comparison, we tested a tumor from a woman with LBC (designated as hBrCa-LBC; Fig. 7A). After a 20-day period of equivalent tumor growth of primary tumor specimens (Supplementary Fig. S8A and S8B), responding tumor cells recovered from the mice with hBrCa TNBC-I, hBrCa-TNBC-II, and hBrCa-LBC were significantly more proliferative than those of EGFR and IGF-IR/IR were both significantly attenuated in the drug-treated cohorts, but not in control cohorts (Supplementary Fig. S7A and S7B). Instigating TNBC tumor mass was not affected by dual inhibitor treatment during the course of this dosing regimen (Supplementary Fig. S7C).

With respect to cancer-free mice, responding tumor cells in the vehicle-treated mice with TNBC were significantly more proliferative (~46% vs. ~18% Ki67-positive, respectively), formed with a desmoplastic stroma, and maintained expression of the malignancy profile factors, Oct4, c-Myc, and Zeb1 (Fig. 6C–E). Dual EGFR/IGF-I inhibition resulted in an approximately 60% decrease in Ki67-positive responder cells in mice with TNBC (Fig. 6C). These responders showed evidence of a myofibroblast-rich, reactive stroma; however, the percentage of cells expressing Oct4, c-Myc, and Zeb1 were significantly reduced relative to the vehicle-treated controls (~79%, ~87%, and ~73% reductions, respectively; Fig. 6D and E). Responding tumor cell expression profile and proliferation in the Matrigel-bearing control mice (~18%) were unaffected by drug treatment (~19%; Fig. 6C and Supplementary Fig. S7D).

Figure 5. EGF and IGF-I modulate indolent and malignant states in vitro. A, expression levels of indicated malignancy profile genes in responder tumor cells (HMLER-HR and BT549) after 4 days in indicated culture conditions relative to those in depleted medium (D.M.). See Supplementary Fig. S5A for experimental scheme. n = 6, where each sample was run in duplicate in three separate experiments. B, expression levels of indicated malignancy profile genes in responder tumor cells (HMLER-HR) after 4 days in indicated culture conditions relative to those maintained in complete media (C.M.). n = 6; each sample run in duplicate in three separate experiments. C, model representing interconversion of responsive tumor cells between malignant and indolent states depending upon EGF and IGF-I ligand availability.
Figure 6. EGFR/IGF-IR dual inhibition prevents malignant progression. A, scheme of pharmacologic targeting of TNBC progression for data represented in figure. Mice were treated with vehicle DMSO or both EGFR inhibitor (erlotinib; 100 mg/kg) and IGF-I inhibitor (BMS-754807; 50 mg/kg) once per day for 8 days by oral gavage. B, mass of responder HMLER-HR tumors plugs after 8 days in indicated mice, with indicated drug or control treatment. Incidence of tumor formation is shown above data bars (n = 5 mice per group). Differences were not significant (n.s.). C, indicated responding HMLER-HR tumors stained for LgT antigen (green; expressed only by responder cells), Ki67 (red), and cell nuclei (blue). Scale bar = 45 μm. Quantification of Ki67-positive cells as a percentage of the total number of LgT+ responder cells per field (n = 9; three random fields for each of three tumors per group). D and E, merged photomicrographs (D) and quantification (E) of malignancy profile factors in responding tumor cells under indicated conditions. The number of cells stained positively for each of the indicated factors is represented as a percentage of the total number of DAPI-positive nuclei of LgT+ (indicated) tumor cells (n = 9; three random fields for each of three tumors per group). DAPI, 4′,6-diamidino-2-phenylindole.
Figure 7. Human tumor specimens establish tumor-supportive systemic environments that influence disease malignancy. A, implantation scheme of human breast cancer surgical specimens from two different patients with TNBC (hBrCa TNBC-I and hBrCa TNBC-II) and one patient with LBC (hBrCa LBC). Each tumor specimen was minced and divided into equal portions that were surgically implanted with Matrigel (Mg) beneath the skin of three different nude mice per cohort. Mice injected with Matrigel were used as a control. HMLER-HR responder cells were injected contralaterally 20 days later and analyzed after 8 days. B, responding tumors stained for the proliferation marker Ki67 (red) and cell nuclei (blue) under indicated conditions. Graph represents number of Ki67–positive cells as a percentage of the total number of cells per field; n = 6 for Matrigel controls; n = 6 for the LBC cohort; n = 9 each for TNBC-I and TNBC-II cohorts. C, representative merged immunofluorescent images of responding tumors from indicated cohorts. D, quantification of malignancy profile factor expression in responding tumor cells under indicated conditions. The number of cells stained positively for each of the indicated factors is represented as a percentage of the total number of DAPI-positive nuclei or LgT+ (indicated) responder tumor cells per field. A minimum of three fields were quantified per responder tumor for each group; n = 6 for Mg controls, n = 9 for TNBC-I, n = 9 TNBC-II, n = 6 LBC. E, TNBC systemic instigation model. The host reaction to certain TNBC tumors (“instigators”) establishes a systemic environment that supports the outgrowth of otherwise indolent disseminated tumors (“responders”). Instigating TNBC tumors secrete osteopontin (OPN) to mobilize tumor-supportive bone marrow cells which are subsequently recruited to responding tumor sites, creating a protumorigenic tumor microenvironment (9, 36). This microenvironment is enriched for the growth factors EGF and IGF-I. Combinatorial treatment with EGFR and IGF-IR inhibitors prevents malignant conversion of the incipient TNBCs. Scale bars: panel B, scale bar = 40 μm; panel C, scale bar = 20 μm; DAPI, 4′,6-diamidino-2-phenylindole.
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from the control Matrigel-bearing mice (Fig. 7B). Responding tumors from the hBrCa TNBC-I and hBrCa TNBC-II cohorts were significantly enriched for Oct4, c-Myc, and Zeb1 relative to those from cancer-free and hBrCa-LBC cohorts (Fig. 7C). Correspondingly, positive staining for activated EGFR and IGF-IR was highest in the two hBrCa-TNBC cohorts (Fig. 7C and D).

Collectively, these results support a model in which the body’s response to an overt TNBC creates a cascade of systemic events that impinge upon plasticity and the recurrence rate of responsive disseminated tumors (Fig. 7E).

**DISCUSSION**

Previous ideas about why patients with TNBC relapse earlier than patients with other types of breast cancer focused on tumor cell-intrinsic properties. Our results support a novel idea that the host systemic environment also determines recurrence rates and the phenotype of the resultant tumors. We do not yet know whether systemically induced tumor plasticity is a consequence of a selection process (i.e., cell sub-populations that are able to respond to instigating tumors), or whether systemic signaling cascades serve to reprogram individual responding tumor cells. Nevertheless, in hosts with TNBC, distant tumor cell populations convert between indolent and malignant states depending on the bioavailability of EGF and IGF-I. This observed plasticity also suggests that the state in which tumor cell populations metastasize from a primary tumor, or the state in which they exist during a period of indolence in a foreign tissue, might not be reflected in the resulting tumor once it is detected. Indeed, striking clinical findings indicate that the molecular and histopathologic characteristics of recurrent breast cancer often do not reflect those of the primary tumor from which it was derived (17, 40).

Targeting features of malignancy and the signaling pathways that drive them appears to be a strategy that could prevent recurrence of certain TNBCs. A recent study showed that pretreatment of various TNBC cell lines in vitro with the EGFR inhibitor erlotinib rendered a subset of these lines more sensitive to DNA damage-mediated cell death by rewiring cell signaling networks (41). Interestingly, in the study, EGFR inhibition did not synergize with cytotoxic agents in some tumor cell populations, including BT549 cells. In fact, in phase II clinical trials of patients with breast cancer with advanced disease, less than 10% of patients responded to EGFR-targeted therapy, and resistance to treatment seemed to be a primary contributor to patient demise. It has been suggested that signaling through other tyrosine kinase receptors may confer resistance to EGFR inhibition. Our results support this conclusion and provide an explanation for why EGFR inhibition alone does not program some tumor cells to the indolent state; i.e., tandem inhibition of IGF-IR is also required.

A number of experimental studies have highlighted the importance of EGF and IGF-I during tumor evolution (42, 43). Our results expand upon these earlier findings by addressing the source of bioavailable ligands in vivo—the tumor-associated stroma. Bone marrow-derived cells, at least in part, provide these growth factors in hosts with TNBC. Indeed, xenografting human breast cancer cells and primary tumor specimens into nude mice revealed important information about malignant progression that is independent of mature lymphocytes. How lymphocytes play a role in the process will be an important aspect of future studies. Our studies also reveal the somewhat surprising fact that, ultimately, availability of EGF and IGF-I is determined by systemic processes that confer a malignant phenotype upon cells that would otherwise remain indolent in a foreign environment. While it may seem easier to target tyrosine kinase receptor signaling pathways that program malignancy rather than the transcription factors that do so, a recent study of pancreatic cancer revealed that resveratrol inhibited pluripotency and EMT factors, including Oct4, c-Myc, and Zeb1 (44). Therefore, valuable insights might also be gained by testing resveratrol in our preclinical model or others like it.

Our results suggest that an appropriately selected subset of patients with breast cancer would benefit from dual EGFR/IGF-IR inhibition and emphasize the need for focused preclinical and clinical trials. A phase II clinical trial using dual inhibition for both IGF-IR (BMS-754807) and EGFR (cetuximab) in patients with advanced colorectal cancer and squamous cell cancers is currently under way (Clinical Trials.gov Identifier NCT00908024). Our results reveal novel aspects of simultaneously targeting both EGFR and IGF-IR and advocate for similar trials in patients diagnosed with TNBC, for which the mainstay of current therapy is cytotoxic chemotherapy. Continued understanding of systemic processes that promote disease progression and the identity of otherwise indolent disseminated tumor cells that are capable of responding to systemic and microenvironmental cues should make it possible to treat patients with TNBC at a time when recurrent disease might yet be prevented.

**METHODS**

**Cell Lines**

HMLER hygro-H-RasV12 (HMLER-HR), BPLER, and MCF7-Ras human breast tumor cells were a generous gift from Dr. Robert A. Weinberg’s laboratory (Whitehead Institute and Massachusetts Institute of Technology, Cambridge, MA) and have been previously described (45–48). Expression of cytokeratins and introduced oncogenes was validated for these studies. All cell lines were validated as mycoplasma-negative. No additional authentication was conducted by the authors.

**Animals and Tumor Xenografts**

Female nude mice were purchased from Taconic. All experiments were carried out in accordance with the regulations of Harvard Medical School Committee on Animal Care (protocol #09-12-1566). Tumor cells were prepared in 20% growth factor-reduced Matrigel (BD Biosciences) in their respective growth medium. For BPLER cells, 5 × 10^4 cells in 0.1 mL Matrigel were injected per mouse, and 2 × 10^5 cells in 0.1 mL Matrigel were injected per mouse for HMLER-HR or BT549 cells. In all cases, cells were injected subcutaneously into nonirradiated mice. Tumor diameter was measured on the flanks of live nude mice using digital calipers; volume was calculated as \( \frac{1}{2} \times \text{length} \times \text{width}^2 \).

**Real-Time PCR**

RNA was extracted from cells or snap-frozen tissues using Trizol reagent following the manufacturer’s instructions (Invitrogen). RNA was retrotranscribed with the ProtoScript AMV First Strand cDNA Synthesis Kit (New England BioLabs). PCR amplification was conducted...
on an ABI Prism 7900 sequence detector using SYBR Green (Applied Biosystems). Analysis was done using the ΔΔt method, normalizing first to glyeraldehyde-3-phosphate dehydrogenase. See Supplementary Table S1 for primer sequences.

**Immunohistochemistry and Image Analysis**

Dissected tissues were fixed in 4% (w/v) paraformaldehyde for 24 hours, stored in 70% ethanol for 24 hours, embedded in paraffin, and sectioned onto ProbeOn Plus slides (Fisher Scientific) for immunohistochemistry using Vectastain Elite ABC kits (Vector Laboratories) as previously described (36). See Supplementary Table S2 for antibodies and dilutions. Images were captured under indicated magnification with identical exposure and gain for any given experiment using a Nikon Eclipse 90i microscope. Staining was quantified using ImageJ software.

**Human Breast Tumor Specimens**

Primary breast tumors were collected in compliance with a protocol approved by the Brigham and Women’s Hospital Institutional Review Board 93-085). Each tumor was analyzed for receptor (ER/PR/HER2) status and used for these studies without any patient identifiers. Shortly after resection, tumor specimens were cut into 3 to 4 mm pieces, washed in RPMI, and frozen in RPMI + 10% DMSO. For xenografts, tumor specimens were quickly thawed at 37°C, washed three times in RPMI, and minced finely into less than 1 mm organoids to ensure homogeneity of viable tumor tissue or nontumor areas. Organoids were divided into equal portions, transferred to individual wells of a 96-well plate, covered with 50% Matrigel in RPMI media, and incubated for 10 minutes at 37°C. Organoids were surgically implanted beneath the skin of nude mice following sterile surgical procedure.

**EGFR/IGF-IR Inhibitors**

For each administration, the EGFR inhibitor erlotinib (LC Laboratories, 100 mg/kg) and the IGF-IR inhibitor BMS-754807 (ActiveBiochem, 50 mg/kg) were freshly dissolved in 80% DMSO in PBS. Drugs or DMSO/PBS vehicle control were administered by oral gavage once daily for a period of 7 days. All mice were monitored on a daily basis during the course of drug treatment and found to be healthy. Of note, the mice developed a skin rash, which has also been reported for patients treated with erlotinib (49).

**Statistical Analysis**

Data are expressed as mean ± SEM. Data were analyzed by Student t test and were considered statistically significant if P ≤ 0.05.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Stromal EGF and IGF-I Together Modulate Plasticity of Disseminated Triple-Negative Breast Tumors
