The Antitumorigenic Function of EGFR in Metastatic Breast Cancer is Regulated by Expression of Mig6

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Abstract

Numerous studies by our lab and others demonstrate that epidermal growth factor receptor (EGFR) plays critical roles in primary breast cancer (BC) initiation, growth and dissemination. However, clinical trials targeting EGFR function in BC have lead to disappointing results. In the current study we sought to identify the mechanisms responsible for this disparity by investigating the function of EGFR across the continuum of the metastatic cascade. We previously established that overexpression of EGFR is sufficient for formation of in situ primary tumors by otherwise nontransformed murine mammary gland cells. Induction of epithelial-mesenchymal transition (EMT) is sufficient to drive the metastasis of these EGFR-transformed tumors. Examining growth factor receptor expression across this and other models revealed a potent downregulation of EGFR following EMT and metastasis. EGF stimulation changes from a proliferative to an apoptotic response in in situ versus metastatic tumor cells, respectively. Furthermore, overexpression of EGFR in metastatic MDA-MB-231 BC cells promoted their antitumorigenic response to EGF in three dimensional (3D) metastatic outgrowth assays. In line with the paradoxical function of EGFR through EMT and metastasis we demonstrate that the EGFR inhibitory molecule, Mitogen Induced Gene-6 (Mig6), is tumor suppressive in in situ tumor cells. However, Mig6 expression is absolutely required for prevention of apoptosis and ultimate metastasis of MDA-MB-231 cells. Further understanding of the paradoxical function of EGFR between primary and metastatic tumors will be essential for application of its targeted molecular therapies in BC.

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Abbreviations: Mig6, mitogen-induced gene-6; BC, breast cancer; EGFR, epidermal growth factor receptor; Mig6-EBR, EGFR binding region of Mig6; TGF-β, transforming growth factor-β; WT, wild type; NMuMG, normal murine mammary gland cells; NME, NMuMG cells transformed by EGFR overexpression; CCLE, Cell Line Encyclopedia; MET, mesenchymal-epithelial transition

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Introduction

Epidermal growth factor receptor (EGFR) is a well-established proto-oncogene. Indeed, we demonstrated that the overexpression of EGFR is capable of transforming mammary epithelial cells, and that transforming growth factor β (TGF-β)–mediated epithelial-mesenchymal transition (EMT) in concert with EGFR facilitates primary mammary tumor cell invasion and dissemination [1,2]. Along these lines, primary breast tumors expressing elevated levels of EGFR display decreased clinical prognosis [3]. These and other findings point to the targeting of EGFR as an effective means by which to combat breast cancer (BC) metastasis. However, the clinical application of EGFR-targeting agents to treat BC has been largely unsuccessful [4–6]. The molecular mechanisms that underlie this inherent resistance of BC to EGFR-targeted therapies remain undefined.

An emerging topic in the field of EGFR signaling is the role of inhibitory proteins that function to mitigate signaling following EGFR activation. One such protein is Mitogen Inducible Gene-6 (Mig6), also known as receptor-associated late transducer (RALT), Gene33, or ErbB receptor feedback inhibitor (ERRFI). Cells utilize Mig6 and other known as receptor-associated late transducer (RALT), Gene33, or ErbB receptor feedback inhibitor (ERRFI). Cells utilize Mig6 and other feedback inhibitory molecules to balance physiological signaling emanating from EGFR in terms of both time and intensity [7]. Mechanistically, Mig6 binds to the intracellular domain of EGFR via its EGFR binding Region (EBR) and inhibits downstream signaling to various effector molecules such as Erk1/2 and Akt [8,9]. Furthermore, Mig6 is genetically altered and transcriptionally silenced in lung cancer, that EGFR expression is diminished in fully metastatic BC cells as described previously [10]. Cellular depletion of Mig6 in MDA-MB-231 was accomplished by VSVG lentiviral transduction of pLKO.1 shRNA vectors as previously described (Thermo Scientific), sequences of shRNAs can be found in Supplementary Table 1 [2]. The human MCF10A parental cell line and its increasingly tumorigenic variants T1k, Ca1h and Ca1a were kindly provided by Dr. Fred Miller (Wayne State University) and were cultured as described previously [20]. A list of the chemical inhibitors used throughout the study can be found in Supplementary Table 2.

Immunoblotting and Immunofluorescent Analyses

For immunoblot assays, equal aliquots of total cellular protein were resolved by SDS-PAGE and transferred to PVDF membranes using standard methods as described [21]. Immunofluorescent assays were conducted using primary antibodies in combination with a biotinylated secondary antibodies (Jackson) and Texas-Red conjugated avidin (Vector) as described [1]. Antibody concentrations and suppliers are listed in Supplementary Table 3.

Cell Biological Assays

DNA synthesis was measured by [3H]thymidine incorporation as previously described [18]. Caspase 3/7 activity was quantified using the Caspase 3/7 Glo reagent (Promega) according to the manufacturer’s instructions. Visualization of the actin cytoskeleton was perform by staining fixed cells with FITC-conjugated Phalloidin according to the manufactures instructions (Thermo Scientific).

Three-Dimensional (3D) Organotypic Growth Assays

Ninety-six well plates were coated with Cultrex (50 μl/well) and cells were resuspended in DMEM supplemented with 10% FBS and 4% Cultrex (150 μl/well). Luciferase-expressing cells were seeded at a density of 1 × 10³ cells/well. Media containing the indicated inhibitors or growth factors was replaced every 4 days and organoid outgrowth was detected by the addition of D-luciferin potassium salt (Gold Biotechnology) to induce bioluminescence, which was quantified using a GloMax-Multi detection system (Promega).

Tumor Growth and Metastasis Analysis

Orthotopic NME tumors were established and tumor volume was quantified as previously described [2]. Pulmonary tumor development was assessed by injection of parental (scrambled shRNA) and Mig6-deficient cells into the lateral tail vein of nu/nu mice (1 × 10⁶ cells/mouse). Where indicated tumor growth and metastasis was monitored by in vivo bioluminescent imaging as previously described [1,2,18,20,22]. Bioluminescent images were captured on a Xenogen IVIS-200 (PerkinElmer). All animal procedures were performed in accordance to protocols approved by the Institutional Animal Care and
In Silico Analyses

The Cancer Cell Line Encyclopedia contains a repository of log2 expression data derived from Affymetrix U133+2.0 Arrays for 947 unique human cancer cell lines. GEO Dataset GSE3744 contains expression data using the Affymetrix U133+2.0 for clinical samples classified as basal-like and non-basal-like [23]. This dataset was analyzed using the NCBI curated dataset browser tool. The TCGA [24] dataset was accessed via cBioPortal and analyzed using R.

Statistical Analyses

Statistical analyses were carried out using an unpaired Student’s T-test. \( P \) values < 0.05 were considered statistically significant. \( P \) values for all experiments are indicated. Correlation coefficients and \( P \) values were generated using Prizm-Graph Pad.

Results

Mig6 is Tumor Suppressive in an EGFR-Driven In Situ Model of Breast Cancer

Mig6 has previously been described as a tumor suppressor [10], findings that are contingent upon EGFR acting as a protumorigenic molecule. To verify the ability of Mig6 to suppress EGFR-induced tumorigenesis, we sought to heterologously overexpress Myc-tagged Mig6 constructs in our in situ model of normal murine mammary gland (NMuMG) cells transformed by overexpression of EGFR (referred to here as NME cells) (Figure 1A). Importantly, expression of these constructs did not appreciably affect EGFR levels in our recombinant system (Figure 1A). However, when these cells were cultured on 2D plastic substrates under a mixture of extracellular matrix, expression of the EGFR-binding region (EBR) or WT Mig6 decreased the formation of filopodia (arrows), an event we have previously attributed to the function of EGFR in these cells (Figure 1B; [25]). Furthermore, when...
these cells were cultured under 3D organotypic conditions expression of EBR and WT Mig6 significantly enhanced the formation of hollow, acinar structures, characteristic of normal mammary epithelial cells (Figure 1, B and C). Furthermore, expression of EBR and WT Mig6 decreased the ability of EGF to induce phosphorylation of EGFR at Y845 (Figure 1D). We recently demonstrated that the ability of EGFR to transform NMuMG cells is dependent upon downstream activation of Stat3 [19]. Importantly, this critical signaling event was potently inhibited by expression of EBR and WT Mig6 (Figure 1D). To focus specifically on the ability of the EGFR-directed function of Mig6 to inhibit tumor initiation we engrafted the NME cells expressing GFP or Mig6-EBR onto the fat pad of mice and primary tumor formation was monitored by caliper measurements (Figure 1E). Mig-EBR dramatically reduced the ability of NME cells to form primary tumors (Figure 1, F and G). Collectively, these findings indicate that EGFR-initiated tumorigenesis can be normalized by the inhibitory actions of Mig6.

Metastatic Breast Cancer Cells Become Inherently Resistant to Targeted Inhibition of EGFR

NME tumors can be driven to metastasize by a transient induction of EMT via treatment with TGF-β1 prior to allograft onto the mammary fat pad [2]. Isolation of the resulting pulmonary metastases yielded the highly aggressive NME-LM cell line. This cell line undergoes robust primary tumor formation and spontaneous pulmonary metastasis upon mammary fat pad engraftment, independent of TGF-β pretreatment [2]. Isolation of these resultant pulmonary metastases yielded the NME-LM2 cell line. These isogenic cell lines now constitute a progression series of cells ranging from nontransformed (NMuMG) to carcinoma in situ (NMuMG cells transformed by EGRF overexpression, NME) to highly metastatic (NME-LM and NME-LM2) [2].

Given the ability of Mig6-EBR to prevent NME tumor formation we next sought to verify inhibition of primary tumor development via pharmacological inhibition of EGFR. Indeed, similar to expression of Mig6-EBR, daily oral administration of Erlotinib (50 mg/kg) to mice bearing orthotopic NME cell engraftments dramatically reduced tumor formation (Figure 2, A, C and D). In line with our previous report, no tumor recurrence or metastases were observed by these cells irrespective of EGFR-directed therapy (Figure 2A; [2]). In stark contrast to the NME cells, tumor formation by the NME-LM cells (described above) was only minimally delayed by Erlotinib treatment (Figure 2B), and no significant differences were observed in primary tumor weight at the time of primary tumor excision (Figure 2E). Also, following primary tumor excision, NME-LM tumor recurrence was actually enhanced by Erlotinib treatment (Figure 2B). Consistent with the role of EGFR in primary tumor cell dissemination [1,26], pulmonary dissemination of NME-LM cells was delayed by Erlotinib (Figure 2F), but at the time of necropsy robust pulmonary metastases were observed in NME-LM tumor bearing mice irrespective of treatment (Figure 2G). Taken together, these data strongly suggest that through the processes of EMT and metastasis even BC cells whose initial transformation is driven by EGFR are capable of inherently developing resistance to EGFR-targeted therapy.

**EGFR Expression is Diminished Following EMT and Metastasis**

Given the inherent resistance of the NME-LM cells to EGFR targeted therapy, we next sought to utilize this isogenic system to identify mediators of this process. As noted above, NME cells can be driven to metastasize via TGF-β–induced EMT prior to tumor cell engraftment [2]. Our previous *in vitro* studies demonstrate that EGFR is not modulated during induction of EMT by TGF-β1, but EGFR is

![Image](image_url)

**Figure 2.** EGFR transformed mammary epithelial cells become inherently resistant to Erlotinib following EMT-induced metastasis. (A and B) NME and NME-LM (1×10⁶) cells were engrafted onto the mammary fat pad of female nu/nu mice and 14 days thereafter mice were treated daily with Erlotinib (50 mg/kg). At the indicated time points (line on graph), primary tumors were surgically removed and mice were monitored for recurrent tumors. (C) Bioluminescent images showing the sensitivity (NME) and resistance (NME-LM) of the indicated tumors described in panels A and B to systemic Erlotinib treatment. (D) Ex-vivo visualization and (E) weight of indicated tumors from control and Erlotinib-treated mice. (F) Pulmonary dissemination of NME-LM tumors in control and Erlotinib-treated mice was determined by bioluminescence at the indicated time points following fat pad engraftment. For panel A–B and E–F data are the mean values ± SE (n = 4 mice per group) where * indicates P < .05. (G) Representative *ex vivo* bioluminescence images demonstrating robust metastasis in the lungs from control and Erlotinib-treated NME-LM tumor bearing mice.
diminished following withdrawal of exogenous TGF-β1 [18]. To examine this phenomenon in vivo, we conducted single cell analysis of cell surface EGFR levels in cells subcultured from control (NS) or TGF-β1 pretreated NME primary tumors. Figure 3A shows flow cytometry using an antibody specific for the human EGFR transgene (Figure 3A). These two ex vivo primary tumors displayed similar EGFR

Figure 3. EGFR expression is diminished following EMT-induced metastasis. (A) Following primary tumor removal, control (NS) and TGF-β1-pretreated (TGF-β) NME primary tumors were disassociated and subcultured in the presence of Puromycin (5 μg/ml). The resultant cultures were analyzed by flow-cytometry for cell surface EGFR. Isolation of the distinct EGFR-negative population within the post-EMT tumors by FACS analysis (denoted by the red box) resulted in a highly mesenchymal cell population. (B) Ex-vivo subcultured, post-EMT primary tumor cells were co-stained with antibodies against Ecad and EGFR (i), or co-stained with phalloidin and antibodies against EGFR (ii). Arrows denote cells with a highly mesenchymal phenotype (either Ecad negative or F-actin positive) that fail to express EGFR. (C) NME-LM cells and their parental NME counterparts were analyzed by flow cytometry for cell surface expression of EGFR. (D) NME and NME-LM cells were treated for 48 hours with EGF (50 ng/ml) or the EGFR inhibitor, AG1478 (1 μM). Afterward, whole cell lysates were analyzed by immunoblot for cleavage of PARP and expression levels of EGFR. (E) The outgrowth of NME and NME-LM cells grown under 3D-culture conditions in the presence of EGF or AG1478 was quantified by bioluminescence. Data are the mean (± SE) of 3 independent experiments completed in triplicate resulting in the indicated P values. (F) NME and NME-LM cells were serum starved for 6 hours and subsequently stimulated with the indicated concentrations of EGF for 30 minutes. Whole cell lysates were analyzed for phosphorylated EGFR (pEGFR). This blot was stripped and reprobed for total EGFR (tEGFR). Erk1/2 served as a loading control. Data in panels A-D and F are representative of at least two independent experiments yielding similar results.
profiles barring a small population of EGFR-negative cells that was only detectable in the TGF-β1 pretreated NME tumors (Figure 3A). In contrast to our in vitro data fluorescence-activated cell sorting for this ex vivo EGFR(−) population yielded a very mesenchymal cell population (Figure 3A; inset) that failed to thrive when cultured alone. Immunofluorescent staining verified the presence of a highly mesenchymal population of cells that lacked expression of Ecad and displayed a very filamentous actin cytoskeleton (Figure 3B). More importantly, although resistant to puromycin, EGFR was undetectable in this mesenchymal cell population (Figure 3B). Similar comparison of cell surface EGFR levels between the parental NME cells and their metastatic and isogenic NME-LM counterparts also displayed a marked reduction in cell surface EGFR levels (Figure 3C). Analysis of total and phosphorylated levels of EGFR similarly revealed a marked diminution in the NME-LM cells as compared to their parental NME cells (Figure 4D, F). Given that EGFR is recombinantly overexpressed in the NME system we sought to identify possible mechanisms of negative selection that could be altering the observed EGFR expression levels in NME-LM cells. Consistent with a negative selection against high level EGFR expression, prolonged stimulation of NME-LM cells with exogenous EGF results in apoptosis (Figure 3D) and drastic inhibition of outgrowth when cultured under 3D organotypic conditions (Figure 3E). These findings contrast sharply with those obtained in the parental NME cell line in which apoptosis and growth inhibition results upon treatment with the EGFR inhibitor AG1478 (Figure 3D and E). Furthermore, the maintenance of 3D growth of the NME-LM cells in the presence of AG1478 (Figure 3E) is consistent with our in vivo data (Figure 2), as well as with the clinical inability of EGFR-targeted therapies to effectively treat metastatic BC [4].

To verify that diminution of EGFR is not an artifact of our overexpression system, we assessed EGFR expression across the human MCF-10A BC progression series. Consistent with the results of our NME progression series, the fully metastatic MCF-Ca1a cells expressed very little EGFR (Figure 4A). Consistent with our previous studies in the NME cells, endogenous EGFR expression was not affected upon in vitro TGF-β1 stimulation of the MDA-MB-231, but was markedly lower in ex vivo cells that had metastasized from the mammary fat pad to axillary lymph nodes and lungs (Figure 4B). Expression of Mig6 mirrored that of EGFR and was similarly diminished in increasingly metastatic BC cells (Figure 4, A and B). Consistent with the notion that EGFR and Mig6 expression levels track together, the slight increase in EGFR expression observed in the NME-LM2 cells as compared to the NME-LM cells correlated with a corresponding increase in Mig6 (Figure 4C). Furthermore, Mig6 is maintained upon TGF-β treatment of the NME-LM2 cells, which is contrast to their nonmetastatic counterparts in which Mig6 is decreased during TGF-β-induced EMT (Figure 4C). The precise mechanisms that lead to the paradoxical function and diminution of EGFR following EMT and metastasis are likely to be highly complex. Nevertheless, our data clearly indicate that the processes of metastasis support the inherent resistance of BC to EGFR-targeted therapies via a shift in EGFR signaling from pro- to antitumorigenic.

**There is a Direct Relationship Between EGFR and Mig6 Expression in Basal-Like Breast Cancer**

To expand our findings beyond our in vitro model systems we sought to further examine the regulation of EGFR and Mig6 in clinical BC samples. Therefore, we analyzed the TCGA BC dataset for the expression relationship between EGFR and Mig6 [24]. Consistent with our findings in Figure 4, there was a significant direct correlation between EGFR and Mig6 expression (Figure 5A). Next, we analyzed the GEO data set GSE3744 in which patient BCs are classified as basal like and non-basal like as characterized by microarray analysis [23]. Consistent with EGFR being a major defining factor of basal-like BC [27] and there being a direct correlation between EGFR and Mig6 expression (Figure 5A). Furthermore, Mig6 is maintained upon TGF-β treatment of the nonmetastatic counterparts in which Mig6 is decreased during TGF-β-induced EMT (Figure 5A). The precise mechanisms that lead to the paradoxical function and diminution of EGFR following EMT and metastasis are likely to be highly complex. Nevertheless, our data clearly indicate that the processes of metastasis support the inherent resistance of BC to EGFR-targeted therapies via a shift in EGFR signaling from pro- to antitumorigenic.
and the EGFR-amplified MDA-MB-468 (MDA-468) cells expressed the second highest and lowest Mig6/EGFR expression ratio, respectively. In contrast to the MDA-231 cells, EGF stimulation of the MDA-468 cells is known to induce apoptosis[14]. Along these lines, EGF stimulation results in a progressive decrease in [3H]Thymidine incorporation in the MDA-468 cells, but not in the MDA-231 cells (Figure 5D). In an attempt to manipulate the Mig6/EGFR ratio in the MDA-231 cells we overexpressed EGFR (Figure 5E). However, consistent with all of the correlative data above, recombinant overexpression of EGFR in the MDA-231 cells lead to a concomitant increase in Mig6 expression levels (Figure 5E). To identify the role of Mig6 in empowering metastatic BCs with the ability to subvert growth inhibitory EGFR signaling, we utilized shRNAs to specifically deplete Mig6 in the MDA-231 cells (Figure 6A). Consistent with our previous data in Figures 4 and 5, depletion of Mig6 using two independent shRNAs led to a corresponding decrease in EGFR levels, however receptor levels were still readily detectable in these cells (Figure 6A). Depletion of Mig6 dramatically inhibited the 3D-outgrowth of MDA-231 cells independent of addition of exogenous EGF (Figure 6B and C). Examination of these 3D cultures revealed the appearance of several apoptotic bodies (arrows, Figure 6B). This observation was verified by increased levels of Caspase3/7 activity in Mig6-depleted MDA-231 cells as compared to their control scrambled shRNA-expressing counterparts (Figure 6D). Consistent with increased levels of apoptosis, we found that MDA-231 cells lacking Mig6 expression immediately failed to thrive within the pulmonary microenvironment after being injected into the lateral tail vein mice (Figure 6, E and F), thereby dramatically inhibiting the formation of pulmonary tumors by Mig6-deficient MDA-231 cells as compared to their Mig6 proficient counterparts (Figure 6, E–G).

**Discussion**

The current study delineates the evolution of EGFR function over the metastatic progression of BC. Our previous studies demonstrate the
transforming capabilities of EGFR overexpression in mammary epithelial cells and the ability of EMT to drive EGFR-mediated invasion and dissemination [1]. However, our findings herein have gone on to establish a proapoptotic switch in EGFR function in the later stages of metastatic outgrowth, a process that can be regulated by corresponding expression of Mig6.

Figure 6. Mig6 depletion induces apoptosis and prevents pulmonary metastasis of MDA-MB-231 cells. (A) MDA-MB-231 cells stably expressing a scrambled (sc) shRNA or three independent Mig6 targeted shRNAs were analyzed for Mig6 and EGFR expression by immunoblot. Actin served as a loading control. (B and C) Luciferase expressing, Mig6-depleted MDA-MB-231 cells were grown under 3D culture conditions and cellular outgrowth was quantified by bioluminescence. Photomicrographs in panel B are representative structures formed by control (scram) and Mig6-depleted (shMig6) cells. Arrows indicate apoptotic cell morphologies. Data in panel C are the average bioluminescent (± SE) values normalized to values measured immediately after plating (T0) from two independent experiments carried out in triplicate. (D) The floating cell fraction of control and Mig6-depleted MDA-MB-231 cells were collected and assayed for Caspase 3/7 activity. (E) Control (scram) and Mig6-depleted (shMig6) MDA-MB-231 cells were injected into the lateral tail vein of nu/nu mice. Longitudinal bioluminescent images from the same mice are shown immediately following injection (T0) and at the indicated time points thereafter. (F) Data are the mean (± SE, n = 5 mice per group) pulmonary bioluminescent values, normalized to the injected value (T0). (G) Representative ex vivo lungs isolated from mice 49 days after injection with control (scram) or Mig6-depleted (shMig6) MDA-MB-231 cells.
The notion that EGFR can play an anti-tumorigenic role in late-stage BC is supported by our recent study in which we observed loss of EGFR expression in metastatic outgrowth proficient D2.A1 BC cells as compared to their invasive but systemically dormant D2.OR counterparts [18]. We also previously demonstrated the progressive loss of EGFR expression in the NME cells during in vitro recovery from TGF-β-induced EMT [18]. This progressive down-regulation of EGFR during oncogenic mesenchymal-epithelial transition (MET) is further supported herein by our data utilizing the MCF-10A progression series where EGFR expression is diminished in Ca1a cells as compared to their Ca1h counterparts. Indeed, separate studies demonstrate a MET reaction characterizes the metastatic progression of these two cell lines [28]. Along these lines, EGFR is undetectable in the highly metastatic 4T1 model of BC (unpublished observation), a cell line we have recently characterized to undergo EMT:MET reactions during tumor growth and metastasis [2]. The importance of this bifurcated expression of EGFR is born out by clinical data that indicate BCs expressing very high or low levels of EGFR have similarly poorer outcomes as compared to BCs expressing intermediate levels of EGFR [29]. Our findings herein expand upon these primary tumor-derived data to suggest that even high-level EGFR expression is diminished during formation of the metastatic lesion. These data are consistent with clinical findings that have demonstrated downregulation of EGFR2 (Her2) in metastatic versus primary tumors [30]. Whether this discordance in receptor expression results due active downregulation of EGFR expression or selection of a preexisting EGFR™ subpopulation remains to be established.

In addition to deletion of EGFR, our data in Figures 4 and 5 suggest that metastatic BCs can also neutralize EGFR-driven apoptosis via Mig6. This exciting finding is contrasted sharply by the widely assumed notion that Mig6 functions solely as a tumor suppressor, a supposition that holds to the idea that EGFR signaling universally functions in an oncogenic manner [10,31]. However, it should be noted that several recent studies have established the ability of EGFR to induce apoptosis in BCs [15–17,32], while others have established the ability of Mig6 to protect human BC cells from apoptosis [33]. Indeed, genetic inactivation of Mig6 results in pulmonary endothelial cell apoptosis [13]. Likewise, a recent study demonstrated that EGF-induced apoptosis in MDA-468 cells, which scored the lowest Mig6:EGFR ratio in our 58 cell line analysis, occurs upon EGFR internalization [17]. Therefore, it is tempting to speculate that unbalanced expression levels of Mig6 with relation to EGFR leads to unabated activation of internalized EGFR, initiating a pro-apoptotic event. Why this does not occur in normal and premalignant cells in which overexpression of EGFR is strictly protumorigenic is a subject currently under investigation in our laboratory.

In summary, our study has delineated a paradoxical shift in which the potent growth promoting properties of EGFR are converted to antitumorigenic mechanisms during the later stages of metastatic progression. Furthermore, we show that the antitumorigenic properties of EGFR in metastatic BC cells are held at bay by the EGFR inhibitory molecule Mig6. This EGFR paradox presents a plausible explanation for the inherent resistance of metastatic BC to EGFR-targeted therapies. Understanding the evolution in EGFR and other RTK signaling across the metastatic cascade will be essential for the proper application of their respective targeted therapies for the treatment of metastatic BC.

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Appendix A. Supplementary Data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.11.009.

References
[1] Wende MK, Smith JA, and Schiemann WP (2010). Transforming growth factor-beta-induced epithelial-mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression. Oncogene 29(49), 6485–6498.
[2] Wende MK, Taylor MA, Schiemann BJ, Sossey-Alaoui K, and Schiemann WP (2014). Fibroblast growth factor receptor splice variants are stable markers of oncogenic transforming growth factor-beta1 signaling in metastatic breast cancers. Breast Cancer Res 16(2), R24.
[3] Tischkowitz M, Brunet J-S, Begin L, Huntsman D, Cheang M, Akled L, Nielsen T, and Foulkes W (2007). Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. BMC Cancer 7(1), 134.
[4] Dickler MN, Cobleigh MA, Miller KD, Klein PM, and Winer EP (2009). Efficacy and safety of erlotinib in patients with locally advanced or metastatic breast cancer. Breast Cancer Res Treat 115(1), 115–121.
[5] Dickler MN, Rugo HS, Eberle CA, Brogi E, Caravelli JF, Panageas KS, Boyd J, Yeh B, and Lake DE, et al (2008). A phase II trial of erlotinib in combination with bevacizumab in patients with metastatic breast cancer. Clin Cancer Res 14 (23), 7878–7883.
[6] Tredan O, Campone M, Jassem J, Vyzula R, Coudert B, Pacilio C, Prausova J, Hardy-Bessard AC, and Arance A, et al (2014). Ixabepilone Alone or With Cetuximab as First-Line Treatment for Advanced/Metastatic Triple-Negative Breast Cancer. Clin Breast Cancer [pii: S1526-8290(14)00162-1].
[7] Segato O, Anastasi S, and Alemà S (2011). Regulation of epidermal growth factor receptor signaling by inducible feedback inhibitors. J Cell Sci 124(Pt 11), 1785–1793.
[8] Anastasi S, Baietti MF, Frosi Y, Alemà S, and Segato O (2007). The evolutionarily conserved EBR module of RALT/MIG6 mediates suppression of the EGFR catalytic activity. Oncogene 26(57), 7833–7846.
[9] Zhang YW, Stall B, Su Y, Swiatek P, Zhao P, Cao B, Resau J, Sigler R, and Bronson R, et al (2007). Evidence that Mig-6 is a tumor-suppressor gene. Oncogene 26(22), 269–276.
[10] Ferby I, Reschke M, Kudlacek O, Knazyev P, Pante G, Amann K, Sommergruber W, Kraut N, and Ullrich A, et al (2006). Mig6 is a negative regulator of EGFR receptor-mediated skin morphogenesis and tumor formation. Nat Med 12(5), 568–573.
[11] Hopkins S, Linderoth E, Hanschel O, Suarez-Henriques P, Pilia G, Kendrick H, Smalley MJ, Superti-Furga G, and Ferby I (2012). Mig6 is a sensor of EGFR receptor inactivation that directly activates c-Abl to induce apoptosis during epithelial homeostasis. Dev Cell 23(3), 547–559.
[12] Xu J, Keeton AB, Wu L, Franklin JL, Cao X, and Messina JL (2005). Gene 33 inhibits apoptosis of breast cancer cells and increases poly(ADP-ribose) polymerase expression. Breast Cancer Res Treat 91(3), 207–215.
[13] Jin N, Cho SN, Raso MG, Wistuba I, Smith Y, Yang Y, Kurie JM, Yen R, and Evans CM, et al (2009). Mig-6 is required for appropriate lung development and to ensure normal adult lung homeostasis. Development 136(19), 3347–3356.
[14] Eitenberg SA, Rubinstein YR, Barnerjee P, Nau MM, Keane MM, and Lipkowitz S (1999). cbl-b inhibits EGF-receptor-induced apoptosis by enhancing ubiquitination and degradation of activated receptors. Mol Cell Biol Res Commun 2(2), 111–118.
[15] Hyatt DC and Ceresa BP (2008). Cellular localization of the activated EGFR determines its effect on cell growth in MDA-MB-468 cells. Exp Cell Res 314(18), 3415–3425.
[16] Reinehr R and Haussinger D (2012). CD95 death receptor and epidermal growth factor receptor (EGFR) in liver cell apoptosis and regeneration. Arch Biochem Biophys 518(1), 2–7.
Rush JS, Quinalty LM, Engelman L, Sherry DM, and Ceresa BP (2012). Endosomal accumulation of the activated epidermal growth factor receptor (EGFR) induces apoptosis. *J Biol Chem* 287(1), 712–722.

Wendt MK, Taylor MA, Schiemann BJ, and Schiemann WP (2011). Down-regulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer. *Mol Biol Cell* 22(14), 2423–2435.

Balanis N, Wendt MK, Schiemann BJ, Wang Z, Schiemann WP, and Carlin CR (2013). Epithelial-to-Mesenchymal Transition Promotes Breast Cancer Progression via a Fibronectin-Dependent Stat3 Signaling Pathway. *J Biol Chem* 288(25), 17954–17967.

Wendt MK, Smith JA, and Schiemann WP (2009). p130Cas is required for mammary tumor growth and transforming growth factor-beta-mediated metastasis through regulation of Smad2/3 activity. *J Biol Chem* 284(49), 34145–34156.

Wendt M and Schiemann W (2009). Therapeutic targeting of the focal adhesion complex prevents oncogenic TGF-beta signaling and metastasis. *Breast Cancer Res* 11(5), R68.

Wendt MK, Schiemann BJ, Parvani JG, Lee YH, Kang Y, and Schiemann WP (2013). TGF-beta stimulates Pyk2 expression as part of an epithelial-mesenchymal transition program required for metastatic outgrowth of breast cancer. *Oncogene* 32(16), 2005–2015.

Richardson AL, Wang ZC, De Nicole A, Lu X, Brown M, Miron A, Liao X, Iglehan JD, and Livingston DM, et al (2006). X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell* 9(2), 121–132.

TCGA (2012). Comprehensive molecular portraits of human breast tumours. *Nature* 490(7418), 61–70.

Balanis N, Yoshigi M, Wendt MK, Schiemann WP, and Carlin CR (2011). beta3 integrin-EGF receptor cross-talk activates p190RhoGAP in mouse mammary gland epithelial cells. *Mol Biol Cell* 22(22), 4288–4301.