ERBB1 and ERBB2 Have Distinct Functions in Tumor Cell Invasion and Intravasation

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Abstract

Purpose: The epidermal growth factor receptor (ERBB1) and related family member HER-2/*neu* (ERBB2) are often overexpressed in aggressive breast cancers and their overexpression is correlated with poor prognosis. Clinical studies using ERBB inhibitors have focused on tumor growth effects, but ERBBs can contribute to malignancy independent of their effects on tumor growth. Our studies were designed to evaluate the effect of ERBB inhibition on tumor cell motility and intravasation *in vivo* using clinically relevant small-molecule inhibitors.

Experimental Design: Using *in vivo* mouse models of breast cancer, we test the effects of ERBB1 and ERBB2 inhibitors AC480 and lapatinib, ERBB1 inhibitor gefitinib, and ERBB2 inhibitor AG825 on *in vivo* tumor cell invasive properties in mammary fat pad tumors.

Results: ERBB1 and ERBB2 inhibition rapidly (within 3 h) inhibits both tumor cell motility and intravasation. Using gefitinib, ERBB1 inhibition rapidly inhibits tumor cell motility and invasion but not intravasation, whereas ERBB2 inhibition by AG825 rapidly blocks intravasation.

Conclusions: ERBB1 and ERBB2 inhibition can rapidly block tumor cell invasive properties. In addition, we differentiate for the first time the contributions of ERBB1 and ERBB2 to the key metastatic properties of *in vivo* tumor cell invasion and intravasation. These experiments temporally and molecularly separate two key stages in tumor cell entry into blood vessels: invasion and intravasation. These results indicate that ERBB inhibition should be considered for blocking other tumor cell malignant properties besides growth.

Metastatic spread is complex, requiring stromal invasion, intravasation (entry of cells into the vasculature), arrest at a metastatic site, and growth of a metastasis. The development of therapies that target specific steps in the cascade is growing, with the current therapeutic armamentarium focused on inhibiting growth (1). The epidermal growth factor (EGF) receptor (ERBB1) and related family member HER-2/*neu* (ERBB2) are often overexpressed in aggressive breast cancers and their overexpression is correlated with poor prognosis (2–4). In addition to their well-characterized contributions to cell proliferation and survival, ERBB1 and ERBB2 also contribute to other characteristics of aggressive tumors such as local invasion and intravasation potentially independent of their effects on growth (5–7). Important for the optimization of anti-ERBB treatments in cancer is a clear *in vivo* identification of the specific tumor properties that are dependent on ERBB1 and ERBB2.

The interpretation of studies that use stable, long-term alteration of ERBB1 or ERBB2 expression is limited by the time (weeks to months) required to produce a tumor or metastasis. During that time, the altered ERBB expression can cause dramatic changes in gene expression within the tumor cells, which may in turn induce changes in the surrounding tumor stroma. The availability of drugs targeted to ERBBs that rapidly act to inhibit ERBB activity provides a novel opportunity to examine cellular processes that are more directly dependent on ERBB activity. In this article, we make use of ERBB-targeted drugs to rapidly inhibit ERBB function to dissect the contributions of ERBB1 and ERBB2 to invasion and intravasation at the primary tumor site. We find that ERBB1 is important for local stromal invasion, whereas ERBB2 is more directly important for intravasation.

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Translational Relevance

The epidermal growth factor receptor family (ERBB) is overexpressed in a wide variety of tumor types and is correlated with poor prognosis. Consequently, there has been a significant investment of resources in the development of drugs to target these molecules. However, clinical trials of these drugs have shown limited efficacy using tumor size or growth as an endpoint. Previous studies of ERBB function using either altered expression levels of ERBBs or tumor growth are long-term and do not differentiate the direct action of ERBBs on cell behavior from downstream effects of altered gene expression. We show for the first time *in vivo*, using small-molecule inhibitors to rapidly inhibit ERBB1 and/or ERBB2 within 3 h, that tumor cell motility, invasion, and intravasation are directly dependent on ERBB function in the primary tumors of three different breast cancer models. We therefore propose that these drugs may have potential for inhibition of tumor cell invasion independent of their effects on tumor growth.

Materials and Methods

Cell culture. MTLn3 cells expressing GFP and human ERBB1 were generated (MTLn3E) and propagated as described previously (6). Leibowitz L-15 medium supplemented with 0.3% bovine serum albumin was used as serum-free starvation medium. MDA-MB-231-4173 cells (*in vivo* selected lung metastatic MDA-MB-231 cells) generously provided by Joan Massague (8) were transduced with a GFP-expressing lentivirus and GFP-expressing transductants selected by fluorescence-activated cell sorting. MDA-MB-231 cells were cultured in DMEM high-glucose supplemented with 10% fetal bovine serum. 1R, 5R, and control (pBabe) vectors for down-regulation of surface ERBB1 and ERBB2 expression, respectively, were used as described previously (9).

Inhibitors. Gefitinib (Iressa), lapatinib (GW572016), and AC480 [also described as BMS-599626 (Ambit Biosciences)] were kindly provided by AstraZeneca, GlaxoSmithKline, and Bristol Myers Squibb, respectively. AG825 was purchased from Tocris.

Tumor formation and drug treatment. One million MTLn3E or MDA-MB-231 cells were injected under the second nipple from the rear of 4- to 6-week-old severe combined immunodeficient mice. For polyoma middle T tumors, mice carrying the polyoma middle T oncogene under the control of the MMTV promoter and expressing GFP in the mammary gland (10) were used. For all tumors, analysis was done when tumor diameters were between 1.5 and 2 cm (~35-40 days for MTLn3E or 50-57 days for MDA-MB-231). Mice were treated with carrier alone (0.5% hydroxypropylmethylcellulose, 0.1% Tween 80 for gefitinib or 50% propylene glycol for AC480 and lapatinib) or carrier containing the inhibitor (100 mg/kg). AG825 treatment was administered via intraperitoneal injection in 10% DMSO at 20 mg/kg. To test the effects of drug treatment on cell viability, cells were seeded at low density on 10 cm plates and allowed to attach. To mimic 3 h treatment by oral gavage, the medium was changed to one containing 10 μmol/L drug or DMSO control for 3 h and then replaced with fresh medium. Cells were allowed to grow and form colonies for several days and the number of colonies was counted.

In vivo *imaging*. For a detailed protocol, see ref. 11 (Unit 19.7). Mice were treated with carrier or drug 3 h before the start of the imaging session. Multiple fields were imaged for each animal and the numbers of moving cells per field were counted and compared. For each field, a 30 min z-stack time-lapse series was collected and analyzed.

In vivo *invasion and intravasation.* MTLn3E tumor-bearing mice were treated via oral gavage with the appropriate carrier compound or drug for 3 h before beginning of the needle collection assay. The *in vivo* invasion and intravasation assays were done as described previously (6, 10).

Tumor histology and immunohistochemistry. Sections from formalin-fixed paraffin-embedded samples were cut and processed for H&E or immunohistochemistry. Serial sections were incubated with either anti-phospho-ERBB1 (Tyr^{845}; Cell Signaling Technology) or anti-phospho-ERBB2 (pNeu-1248; Santa Cruz Biotechnology) and stained using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate detection method (Vector Laboratories). Samples were then dehydrated and mounted, and for each tumor, the same area on all sections was imaged on a Zeiss Axioscop2 light microscope under identical imaging conditions.

Fluorescencectivated cell sorting analysis. Cells were detached with PBS + 2 mmol/L EDTA and then incubated in the cold with primary antibodies against ERBB1 or ERBB2 (both from Neomarkers) in PBS + bovine serum albumin. Primary antibody binding was detected using PE-labeled goat anti-mouse secondary antibody (Jackson Immunoresearch).

Results

We first evaluated the effect of the ERBB1 and ERBB2 inhibitor AC480 (previously published as BMS-599626; ref. 12) on the highly metastatic mammary adenocarcinoma MTLn3E cells (6, 13). Consistent with *in vitro* studies with other cell lines (12), concentrations in the 1 μmol/L range were sufficient *in vitro* to block EGF-induced phosphorylation of ERBB1 and ERBB2 (Supplementary Fig. S1A), lamellipod extension, chemotaxis, and invasion (Supplementary Fig. S1B-E), whereas inhibition of proliferation required higher concentrations (Supplementary Fig. S1F). To determine the effects of ERBB1 and ERBB2 inhibition on cell behavior *in vivo*, mice bearing MTLn3E xenograft tumors (6) were given 100 mg/kg AC480 via oral gavage for 3 h (12). Using immunohistochemistry with phospho-ERBB antibodies, we confirmed that both ERBB1 and ERBB2 are phosphorylated in vehicle-treated primary tumors and that inhibition of endogenous ERBB1/2 phosphorylation in the tumor was complete by 3 h after oral gavage with AC480 (Fig. 1A), consistent with pharmacodynamic data indicating that plasma concentrations reach >1 μmol/L after 3 h (12). We therefore performed further *in vivo* analyses at this time point.

To evaluate whether the endogenous motility and invasiveness of cancer cells in the primary tumor was dependent on ERBB activity, we used intravital multiphoton microscopy (14) to directly image cells in tumors generated by GFP-expressing tumor cells. Individual cells were followed in time-lapse z-series by GFP fluorescence. In the tumors of animals treated with carrier alone, >10 moving cells per field were observed on average, often invading along extracellular matrix fibers (Fig. 1B and C; Supplementary Movie S1). AC480 treatment resulted in an 80% reduction in the number of cells moving per field in the tumors (Fig. 1B and C; Supplementary Movie S1). Thus, in parallel with reduced ERBB1 and ERBB2 phosphorylation, AC480 inhibited endogenous breast tumor cell motility in the primary tumor. Studies with a second aggressive breast cancer model, the transgenic polyoma middle T model (10), confirmed the importance of ERBB signaling

for endogenous tumor cell motility and invasiveness (Fig. 1C; ref. 10). To extend these findings to human cells, we used MDA-MB-231 cells. The measurement of *in vivo* motility in the primary tumors using intravital imaging revealed that treatment of animals with AC480 dramatically reduces the numbers of moving cells in this model as well (Fig. 1C). Although the motility of MDA-MB-231 cells was five-fold higher than that of MTLn3E cells, the relative decrease in motility was similar. In summary, blockade of ERBBs resulted in inhibition of *in vivo* motility in both rat and human xenograft tumor models as well as in a transgenic mouse model.

Inhibition of *in vivo* responses to direct EGF stimulation was confirmed by measuring *in vivo* tumor cell invasion into microneedles filled with Matrigel and EGF (10). Treatment with AC480 reduced EGF-induced *in vivo* invasion to background levels (Fig. 2A). An important consequence of tumor cell invasion and motility is the ability to enter tumor blood vessels or intravasate (15). Intravasated tumor cells can then be transported to

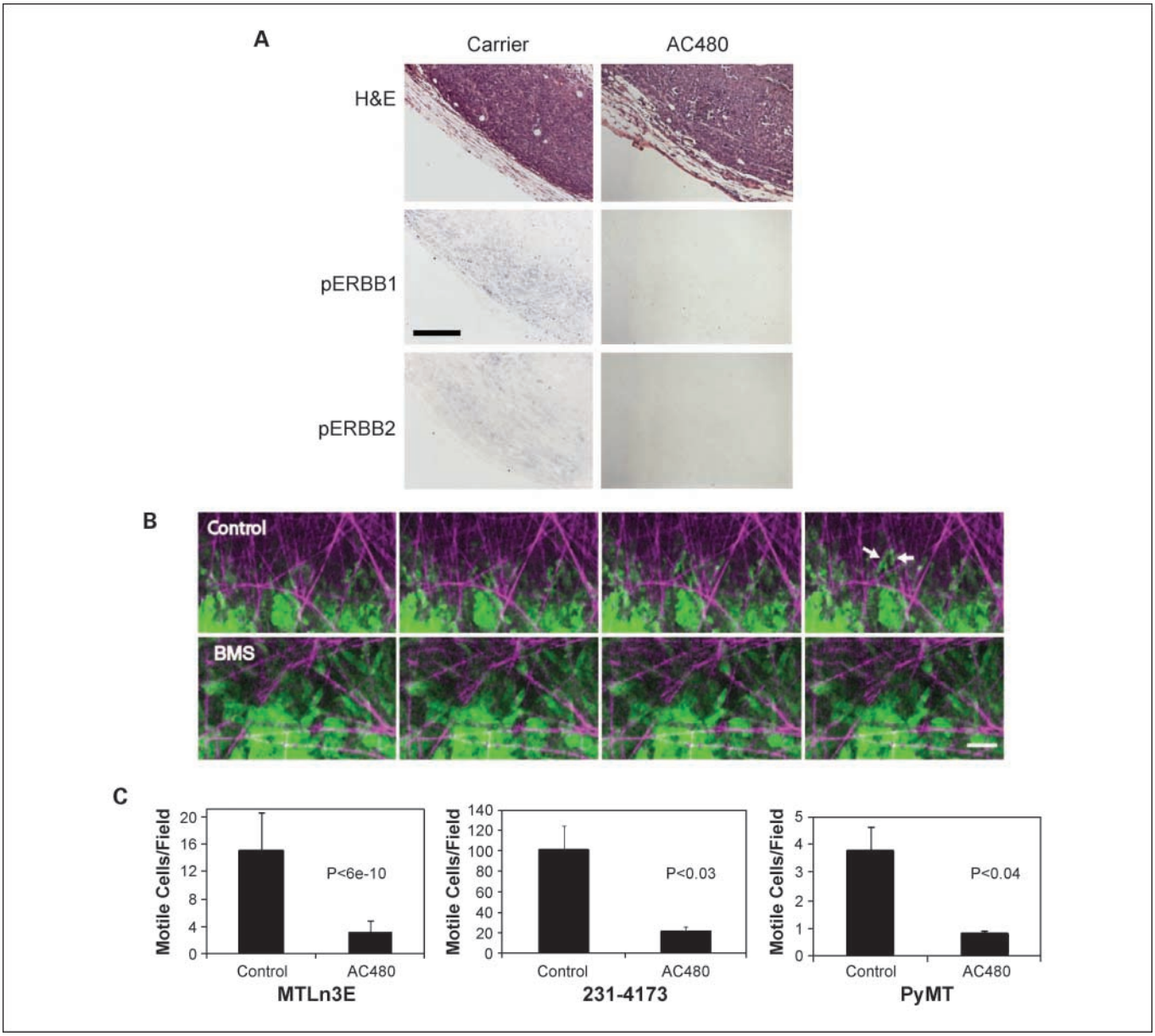

Fig. 1. Inhibition of ERBB signaling blocks *in vivo* motility of tumor cells. *A,* AC480 inhibits phosphorylation of ERBB1 and ERBB2. Serial sections from MTLn3E tumors from animals treated with 100 mg/kg AC480 or carrier by oral gavage were stained with H&E, for phospho-ERBB1 or phospho-ERBB2, as described in Materials and Methods. Representative of samples stained from 10 AC480-treated and 10 carrier-treated animals. Bar, 100 um. *B,* representative motility images from Supplementary Movie S1 show MTLn3E tumor cell movement in a carrier-treated animal (Control), with several cells (*green*) moving on matrix fibers (*purple*). *White arrows,* moving cells. Movement in the tumor of an AC480-treated animal (BMS-599626) is rarely seen and nonmotile clusters of cells arrested on matrix fibers are often observed. Images at 9 min intervals are shown. Bar, 42 um. *C,* quantitation of *in vivo* motility of cells in MTLn3E xenografts, MDA-MB-231 xenografts, and polyoma middle T transgenic tumors. The tumors of AC480-treated or carrier-treated animals were imaged using intravital microscopy as in *B.* Data were acquired in z-series time-lapse format at 1 min intervals and analyzed as described in Materials and Methods. Mean and SE of 13 measurements from 4 animals (MTLn3E), 16 measurements from 5 animals (polyoma middle T), and 12 measurements from 4 animals (MDA-MB-231).

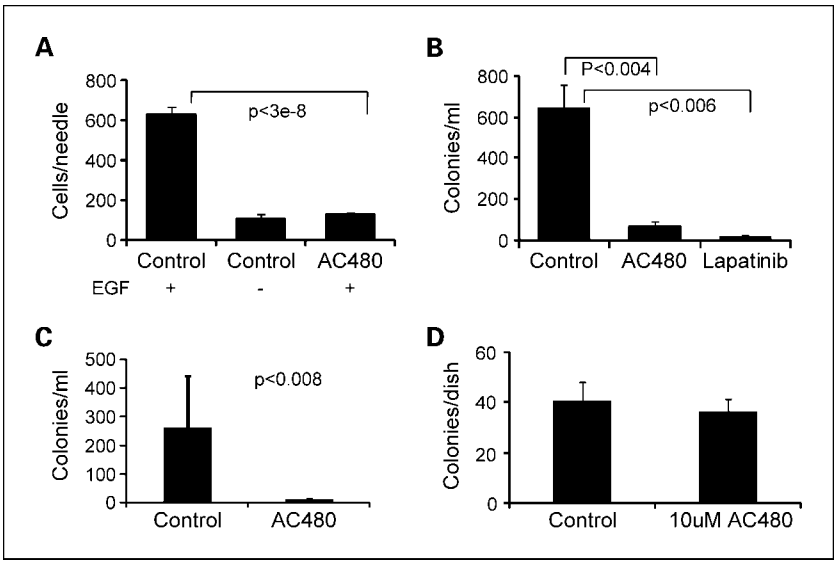

Fig. 2. Inhibition of ERBB signaling blocks *in vivo* invasion and intravasation. *A,* AC480 blocks EGF-induced *in vivo* invasion. Tumor cell *in vivo* invasion in MTLn3E tumors in response to EGF or buffer in carrier-treated or AC480-treated animals was measured as described in Materials and Methods. Mean and SE of 11 measurements from 4 animals for controls and 8 measurements from 3 animals for AC480. *B,* intravasation of MTLn3E tumor cells is reduced by two different ERBB1 and ERBB2 inhibitors. Mean and SE of measurements from 18 carrier-treated animals, 17 AC480-treated animals, and 9 lapatinib-treated animals. *C,* intravasation of MDA-MB-231 cells is reduced by treatment of tumor-bearing animals with AC480 compared with carrier-treated animals. Averages and SE for 12 control and 7 AC480-treated animals. *D,* AC480 treatment does not affect cell viability during the intravasation measurement. Cells were treated as described in Materials and Methods, and colonies of AC480-treated cells were counted and plotted in comparison with DMSO-treated controls (Control). Mean and SE of 5 separate experiments.

distant organs, resulting in the formation of metastases that can lead to patient mortality. To test the ability of AC480 to block intravasation, blood from the right atria of animals carrying MTLn3E or MDA-MB-231 xenograft tumors was collected and the numbers of tumor cells per milliliter were scored (6). We found that AC480 treatment resulted in >80% decrease in the number of intravasated MTLn3E (Fig. 2B) or MDA-MB-231 (Fig. 2C) cells. Cells exposed to AC480 for 3 h showed similar survival post-treatment to DMSO controls (Fig. 2D), showing that the effect of AC480 on intravasation was not due to altered cell survival. To confirm that the observed effects of AC480 treatment are caused by ERBB inhibition and not by off-target effects, we treated tumor-bearing animals with a different ERBB1 and ERBB2 inhibitor, lapatinib (GW572016; ref. 16). Lapatinib treatment also significantly reduced intravasation of tumor cells (Fig. 2B), indicating that the inhibition of intravasation reflects inhibition of ERBB signaling. To determine if there were individual contributions of ERBB1 and ERBB2 to these *in vivo* tumor cell properties, we next evaluated the effects of selective ERBB1 or ERBB2 inhibition.

Gefitinib (Iressa), a highly selective inhibitor for the ERBB1 kinase activity (17), blocks EGF-stimulated ERBB1 and ERBB2 phosphorylation, lamellipod extension, chemotaxis, and invasion of MTLn3E cells *in vitro* at lower concentrations than proliferation (Supplementary Fig. S2). The effect on EGF-stimulated ERBB2 phosphorylation is a result of inhibition of ERBB1 kinase activity but not a direct effect on ERBB2 (18). *In vivo*, immunostaining for phosphorylated forms of ERBB1 and ERBB2 showed that gefitinib treatment strongly inhibited ERBB1 phosphorylation, with partial inhibition of ERBB2 phosphorylation (Fig. 3A). The *in vivo* motility of tumor cells in the primary tumors was significantly reduced by gefitinib treatment, showing that the endogenous *in vivo* motility is ERBB1 dependent (Fig. 3B, *top*). In addition, the number of cells invading *in vivo* in response to EGF was reduced to levels similar to the buffer control group in gefitinib-treated animals (Fig. 3B, *middle*), confirming that the gefitinib treatment was fully blocking *in vivo* responses to EGF. As an alternative method for evaluating the role of ERBB1 in cell motility, we suppressed the surface expression of ERBB1 using a single-chain antibody that retains ERBB1 in the endoplasmic reticulum (9). MDA-MB-231 cells transduced with the 1R anti-ERBB1 single-chain Fv showed a 90% reduction in cell surface ERBB1 compared with cells transduced with the pBabe empty vector control. Suppression of surface expression of ERBB1 reduced motility by 70% (Fig. 3B, *bottom*, and C; Supplementary Movie S2), confirming that cell surface ERBB1 is important for spontaneous cell motility in the primary tumor site. Thus, ERBB1 signaling is critical for endogenous motility and invasion in the primary tumor. However, although ERBB1 phosphorylation, endogenous motility, and EGF-induced *in vivo* invasion were blocked, there was not a statistically significant inhibition of intravasation 3 h after gefitinib treatment (Fig. 3D). To intravasate, tumor cells must invade the neighboring stroma and approach blood vessels. Given that *in vivo* motility and invasion were inhibited by gefitinib, we hypothesized that gefitinib might be able reduce the efficiency of approach to blood vessels while not affecting intravasation directly. To test this hypothesis, we extended the treatment time to 9 h, which resulted in significantly reduced intravasation efficiency (Fig. 3D), consistent with ERBB1 being important for invasion from the primary tumors toward blood vessels before intravasation but not for the intravasation event itself.

Because the ERBB1 and ERBB2 inhibitors were able to inhibit intravasation rapidly, whereas gefitinib did not, this suggested that ERBB2 could be more directly involved in intravasation than ERBB1. We therefore evaluated the effect of selectively inhibiting ERBB2 function using the ERBB2 inhibitor AG825 (19). AG825 had no effect on *in vitro* invasion or proliferation of MTLn3B1 cells at concentrations up to 10 μmol/L (data not shown). Treatment of animals with AG825 resulted in strong inhibition of ERBB2 phosphorylation with limited effects on ERBB1 phosphorylation (Fig. 4A), consistent with a selective *in vivo* inhibition of ERBB2 signaling. Correlated with the inhibition of ERBB2 was a strong inhibition of intravasation (Fig. 4B), showing that ERBB2 contributes directly to intravasation. As an alternative approach, we reduced the levels of ErbB2 on the cell surface of MDA-MB-231 cells by expressing a gene encoding a single-chain antibody that binds specifically to the extracellular portion of ErbB2 and prevents its transit through

the endoplasmic reticulum (20). Fluorescence-activated cell sorting analysis showed >90% decrease in cell surface ERBB2 in cells expressing the 5R single-chain Fv compared with cells transduced with the pBabe empty vector control. The number of circulating tumor cells in mice with orthotopic xenografts of MDA-MB-231 cells expressing this 5R single-chain antibody was significantly reduced compared with the empty vector (pBabe) controls (Fig. 4C).

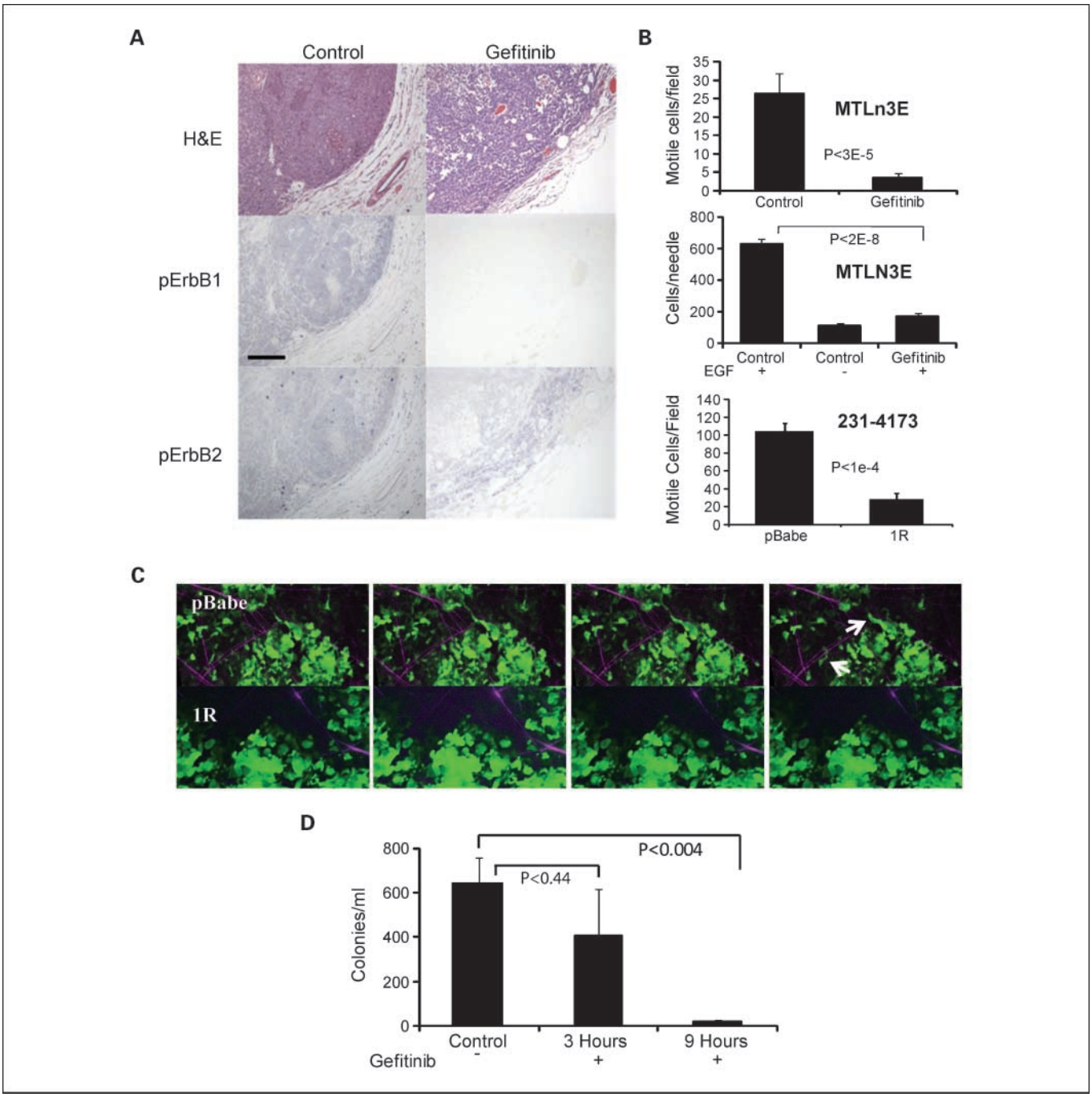

Fig. 3. ERBB1 inhibition distinguishes *in vivo* motility and invasion from intravasation. *A,* gefitinib (100 mg/kg) completely inhibits ERBB1 phosphorylation but not ERBB2 phosphorylation. MTLn3E tumors from animals treated with 100 mg/kg gefitinib or carrier by oral gavage were stained with H&E, for phospho-ERBB1 or phospho-ERBB2, as described in Materials and Methods. Representative of samples from 9 gefitinib-treated animals and 10 carrier-treated animals. Bar, 100 μm. *B, in vivo* motility (*top*) and invasion (*middle*) of cells in MTLn3E xenografts treated with gefitinib and *in vivo* motility of cells in MDA-MB-231 xenografts (*bottom*) with intracellular retention of ERBB1. Mean and SE of at least 15 fields from 3 animals (*top*), 10 measurements from 6 animals, and 7 fields from 4 animals. *C,* representative motility images from Supplementary Movie S2 show MDA-MB-231 cell movement in empty vector MDA-MB-231 cells (pBabe) and 1R-expressing animals (1R) with cells (*green*) moving toward vessels (dark areas on top for both the pBabe and the 1R images). *Arrows,* moving cells. Individual frames are 5 min apart. Bar, 42 μm. *D,* gefitinib requires longer treatment to block intravasation. Intravasation was measured in gefitinib-treated animals after 3 or 9 h (with treatments 9 and 5 h before measurement). Mean and SE of measurements from 34 control animals (Control) and 9 (3 h) and 13 (9 h) gefitinib-treated animals.

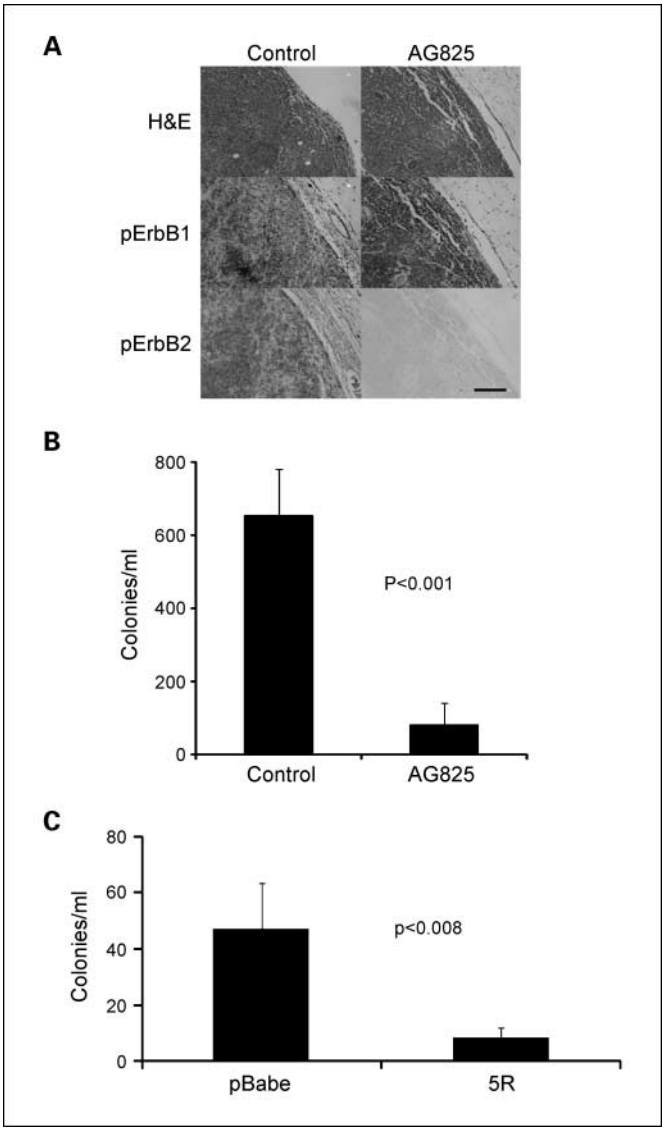

Fig. 4. Inhibition of ERBB2 blocks intravasation. *A,* treatment with AG825 reduces ERBB2 phosphorylation with limited effects on ERBB1 phosphorylation. One hour after intraperitoneal injection of 20 mg/kg AG825 or carrier (Control), tumors were stained for H&E, phospho-ERBB1, or phospho-ERBB2 as described in Materials and Methods. Representative of samples from 6 AG825-treated and 10 carrier-treated animals. Bar, 100 μm. *B,* 20 mg/kg intraperitoneal administration of AG825 resulted in a significant reduction in the number of intravasating cells compared with carrier treatment (Control). Mean and SE for 6 AG825-treated and 34 control animals. *C,* intravasation of MDA-MB-231 empty vector control cells (pBabe) and 5R-expressing ErbB2 down-regulated cells (5R). Mean and SE for 8 animals in each group. $P < 0.008$.

Discussion

In this article, we have examined the roles of ERBB1 and ERBB2 in invasion and intravasation at the primary tumor. Because these processes can be extremely sensitive to changes in tumor structure and microenvironment, we have used both drugs and stable retention in the endoplasmic reticulum to inhibit ERBB1 and/or ERBB2 *in vivo* in the primary tumor. Both approaches show that ERBB1 makes a major contribution to spontaneous tumor cell motility in the primary tumor microenvironment. Our work complements studies using alteration of ERBB expression to show a role for ERBB1 and ERBB2 in tumor cell invasion, intravasation, and metastasis (5–7, 13, 21, 22). The direct imaging of spontaneous motility and invasion shows an important role for ERBB1 in *in vivo* invasion and motility. The rapid change in motility following inhibition of ERBB1 using both ERBB1 and ERBB2 inhibitors as well as the ERBB1-selective drug gefitinib supports a direct role for ERBB1 rather than indirect effects on tumor microenvironment due to altered gene expression. If ERBB1 plays a direct role in stromal invasion toward blood vessels, invasion could be stimulated by endogenous gradients of EGF, and consistent with this possibility, we find cellular sources of EGF in the stroma (data not shown).

Although ERBB1 inhibition does block both spontaneous tumor cell motility and *in vivo* invasion in response to an applied gradient of EGF, it does not directly block intravasation. Longer treatment with gefitinib (9 h) was needed to produce a significant reduction in intravasation. This temporal difference between the effects of gefitinib on motility and intravasation suggests that intravasation occurs after, and depends on, ERBB1-mediated invasion. Such a temporal sequence suggests that tumor cells must transit the loose connective tissue stroma before intravasation. This is consistent with the physical arrangement of the tumor microenvironment; the primary tumor mass is separated from the vasculature by loose connective tissue barriers of varying thickness.

In contrast to the indirect dependence of intravasation on ERBB1 function, we find that ERBB2 is more directly involved in the intravasation process. Two ERBB1 and ERBB2 inhibitors, AC480 and lapatinib, blocked intravasation within 3 h of oral gavage. This conclusion was further reinforced by intraperitoneal injection of AG825, an ERBB2-specific inhibitor, which was found to inhibit intravasation with 1 h of treatment. ERBB2 phosphorylation in the primary tumor was strongly inhibited, whereas significant ERBB1 phosphorylation remained, consistent with a requirement for ERBB2 activation during intravasation. The importance of surface ERBB2 for intravasation was confirmed using retention of ERBB2 in the endoplasmic reticulum. Although the analyses using intracellular antibodies argue for the importance of ERBB1 and ERBB2 signaling in the tumor cells, it is also possible that the drugs are affecting other cells in the tumor microenvironment, such as endothelial cells, and through them affecting either invasion or intravasation.

The distinct contributions of ERBB1 and ERBB2 to invasion and intravasation may reflect different microenvironments stimulating intravasation and invasion. ERBB2 has been shown to be important for chemotaxis to a variety of chemoattractants including EGF and heregulin (6, 23). Consistent with the *in vitro* data, we find that AG825 inhibits *in vivo* invasion in response to EGF (data not shown). Thus, ErbB2 activation contributes to both invasion and intravasation, and there is no direct evidence that different intracellular pathways are activated by ErbB2 under these two conditions. Rather, other ligands that do not act via ERBB1, such as heregulin (via ERBB3; ref. 5), which can be present in serum and around blood vessels, could stimulate intravasation via ERBB2 in the absence of ERBB1 activation (or in the presence of gefitinib).

These studies have clinical implications because inhibition of invasion and intravasation could have significant effects on the ability of tumor cells to spread and metastasize without necessarily affecting proliferation. On the order of 30% of ERBB1- or ERBB2-expressing tumors have shown reduction in tumor size

in response to ERBB inhibition (24). Our results suggest that clinical trials directly evaluating tumor invasion and spread might reveal an additional patient population whose tumor aggressiveness might be reduced independent of effects on tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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