HER-2/neu Overexpression Increases the Viable Hypoxic Cell Population within Solid Tumors without Causing Changes in Tumor Vascularization

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Abstract
The effects of HER-2/neu overexpression on the tumor microenvironment in an aggressive breast cancer xenograft model were investigated. These studies focused on tumors derived following the subcutaneous injection of MDA-MB-435/LCC6 cells transfected with human c-erbB2 (LCC6HER-2) into SCID-Rag2M mice. LCC6HER-2 tumors were more viable (H&E-stained tumor sections) than isogenic vector control tumors (LCC6Vector). Correspondingly, a 2.7-fold increase in trypan blue−excluding cells (P = 0.000056) and a 4.8-fold increase in clonogenic cells (P = 0.001146) were noted in cell suspensions derived from disaggregated LCC6HER-2 versus LCC6Vector tumors. Tumor sections stained with the antibody detecting 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3-pentafluoropropyl)-acetamide (EFS), a marker of hypoxia, showed a greater fraction of hypoxic tissue in LCC6HER-2 tumors compared with control tumors. Flow cytometric analyses based on viable tumor cells (DNA content ≥ 2N) in cell suspensions from disaggregated tumors confirmed that there were significantly more EFS-positive cells (i.e., hypoxic) in LCC6HER-2 than in LCC6Vector tumors (16.41 ± 8.1% and 5.96 ± 4.1%, respectively; P = 0.0015). Protein levels of phosphorylated (Ser536) nuclear factor-κB p65 were significantly elevated in LCC6HER-2 tumors (P = 0.00048), and a trend in increased hypoxia-inducible factor-1α protein levels was observed in LCC6HER-2 compared with LCC6Vector tumors. Despite the substantial viable hypoxic cell fraction and a 1.7-fold increase of vascular endothelial growth factor protein (P = 0.05) in LCC6HER-2 tumors, no significant differences were found (P > 0.05) between LCC6HER-2 and LCC6Vector vasculature (CD31 staining and Hoechst 33342 perfusion). These results suggest that HER-2/neu overexpression may be linked with overall increased tumor viability and a significant increase in the population of viable hypoxic cells, which is not due to differences in tumor vascularization. (Mol Cancer Res 2004;2(11):606−19)

Introduction
HER-2/neu (ErbB2), together with epidermal growth factor receptor, HER-3, and HER-4, belongs to the epidermal growth factor receptor family of receptor tyrosine kinases, which play an important role in the pathogenesis of cancer (1). Approximately 25% to 30% of human breast cancer tumors amplify the c-erbB2 gene, which results in overexpression of the HER-2/neu protein. Overexpression of HER-2/neu is associated with poor disease outcome (2) and is implicated in chemoresistance (3, 4), radioresistance (5), and resistance to tamoxifen (6, 7).

The role of HER-2/neu in cancer progression and resistance to treatment modalities is complex and is currently the subject of many biological investigations. These studies generally focus on molecular events related to the gene and changes in the response of downstream interrelated signaling pathways that are implicated in cell survival strategies. For example, activated oncogenic pathways such as phosphatidylinositol 3-kinase/Akt (4, 8-10), Ras-mitogen-activated protein kinase (11) and Src family kinase pathways (12), overexpression of c-Myc (13), and increased levels and activation of transcription factors such as nuclear factor-κB (NF-κB; refs. 8, 9) and hypoxia-inducible factor-1α (HIF-1α; ref. 10) have been found in HER-2/neu-overexpressing cells. Furthermore, the production of angiogenic factors such as vascular endothelial growth factor (VEGF) was found to be elevated in HER-2/neu-overexpressing cells and tumors (10, 14, 15), resulting in enhanced tumor vascularization (15). Coexpression of epidermal growth factor receptor, HER-3, and HER-4 was shown to have a significant impact on the nature and scale of HER-2/neu-associated cellular signaling (15-18) and to contribute to cell survival and resistance to treatment modalities (19).

In contrast to these molecularly driven studies, our group is interested in the broader consequences of HER-2/neu overexpression on the tumor microenvironment. In solid tumors, changes engendered by overexpression of a gene at the cellular level will ultimately have an impact on the tumor microenvironment and how these tumor cells interact with, are affected by, and adapt to changes in the microenvironment. These
interactions often result in emergence of tumor cells able to withstand (a) adverse conditions known to be present within regions of tumor growth (e.g., poor oxygenation and changes in pH) or (b) external therapeutic assaults (chemotherapy and radiotherapy). In this regard, the development of tissue hypoxia (reduced oxygen levels) in tumors is of particular importance, because its selective environmental pressure will lead to the manifestation of biologically distinct tumor cell populations within a tumor. These cells respond to environmental challenges and specific treatment modalities in different ways than their well-oxygenated counterparts (20-25). Hypoxia in solid tumors results from uncontrolled growth and/or inadequate/dysfunctional vascularization (20), that is, the balance between the tumor oxygen requirements and the supply of oxygen is no longer in equilibrium. The radioresistance of hypoxic tumors is well established (20), and there is evidence that such tumors are also resistant to chemotherapy (20, 23). Other clinical studies now indicate that tumor hypoxia is considered a negative clinical prognostic factor in various malignancies (20, 26, 27).

The relationship between HER-2/neu overexpression and hypoxia in tumors has not been examined extensively, and we endeavored to characterize how HER-2/neu overexpression in breast cancer tumors may influence oxygenation status as determined using 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)-acetamide (EF5), a label for viable but hypoxic cells. Because the tumor microenvironment, particularly the presence of hypoxia in viable tumor tissue, may significantly influence therapeutic outcomes (20, 23, 28), a thorough characterization of the environment including mapping its heterogeneous components would aid in the development of rational treatment strategies. Using the LCC6 cell line stably transfected with human c-erbB2, we established a reproducible xenograft LCC6HER-2 tumor model in SCID-Rag2M mice; the biological traits of this tumor were then compared with matched isogenic LCC6Vector control tumors. We report that HER-2/neu overexpression in LCC6 tumor xenografts resulted in a drastically altered tumor microenvironment, characterized by a higher degree of overall viability and a concomitant increase in viable hypoxic cell populations in tumor tissue, changes that occurred independently of tumor vascularization. HER-2/neu overexpression was also associated with elevated levels of phosphorylated (Ser536) NF-κB p65 (NF-κB p65\(^{\text{P}}\))/RelA and a trend toward higher levels of HIF-1α. We hypothesize that HER-2/neu-overexpressing tumor cells have superior adaptive mechanisms and therefore can withstand unfavorable tumor microenvironments (e.g., hypoxia) compared with cells that express reduced HER-2/neu levels. Thus, the increased viability and survival of hypoxic cell populations may result from a HER-2/neu-dependent adaptive response. In turn, this would significantly enhance the aggressiveness of HER-2/neu-overexpressing breast cancers.

### Results

#### In Vitro Characterization of the LCC6\(^{\text{HER-2}}\) Cell Line

The first objective of these studies was to establish that the LCC6\(^{\text{HER-2}}\) cell line, prepared by introduction of the pREP9-HER-2/neu plasmid into LCC6 cells, behaved consistently with what is already known about HER-2/neu-positive breast cancer cell lines. The results in Fig. 1A indicate that \(~4.5 \times 10^5\) HER-2/neu molecules were expressed in the transfected cell line as compared with \(1.5 \times 10^4\) for the control LCC6\(^{\text{Vector}}\) cell line. HER-2/neu receptor number was assessed by a mean Antibody Binding Capacity assay described in Materials and Methods. The HER-2/neu expression was stable, and the transfected cell line expressed protein levels that were three to five times lower than those determined for the standard breast cancer cell lines SKBR3 (\(1.55 \times 10^6\) HER-2/neu molecules) and BT474 (\(2.2 \times 10^5\) HER-2/neu molecules; data not shown). Others have shown that introduction of the HER-2/neu gene can alter the expression levels of other EGRF protein-tyrosine kinase family members.

**FIGURE 1.** Cell surface expression of HER-2/neu, secreted VEGF, and Western blot analysis of HIF-1α and NF-κB p65\(^{\text{P}}\) in cultured breast cancer cell lines. A. HER-2/neu cell surface expression in LCC6\(^{\text{HER-2}}\) and LCC6\(^{\text{Vector}}\) cells determined by Neu 24.7-FITC Antibody Binding Capacity (ABC) and flow cytometry. B. Secreted VEGF levels in LCC6\(^{\text{HER-2}}\) and LCC6\(^{\text{Vector}}\) cultures. Columns, mean (\(n = 3\)); bars, SD. \(P = 0.0008\). C. Western blot analyses of HIF-1α, NF-κB p65\(^{\text{P}}\), and total NF-κB p65 protein levels in LCC6\(^{\text{HER-2}}\) (HER-2) and LCC6\(^{\text{Vector}}\) (Vector) cultured cells.
Inoculation of LCC6 HER-2 Cells

Characteristics of Tumors Arising Following s.c. Inoculation

In the absence of DFO, were considerably different.

The growth of both cell lines in serum-containing culture medium (Fig. 2A) was comparable. Small differences in the cell density, as indicated by absorbance assessments, were noted on days 7 and 8, where the LCC6HER-2 cells exhibit an increase (~ 25% higher) in viable cells compared with the LCC6Vector cells. To assess growth responses of LCC6HER-2 and LCC6Vector cells to stress conditions in vitro, these cells were cultured in the absence of serum or in the presence of deferoxamine mesylate (DFO; Fig. 2B). In the absence of serum, the viability (trypan blue exclusion assay) of LCC6HER-2 and LCC6Vector cells evaluated following 6 days in culture was 58.8 ± 5.3% and 35.9 ± 2.8%, respectively. These differences, which were determined relative to the viability of control cells grown in the presence of 10% fetal bovine serum (FBS), were statistically significant (P = 0.0027). In the presence of DFO (4 days), the viability of LCC6HER-2 and LCC6Vector cells was 70.9 ± 7.9% and 54.8 ± 8.2%, respectively (P = 0.062; Fig. 2B). These values, again normalized to the viability of cells cultured in the absence of DFO, were considerably different.

In Vivo Characteristics of Tumors Arising Following s.c. Inoculation of LCC6HER-2 Cells

The in vitro data summarized above suggest that LCC6 cells overexpressing HER-2/neu also express increased levels of HER-3, VEGF, HIF-1α, and NF-κB p65. Therefore, additional studies were conducted with LCC6HER-2 and LCC6Vector cells cultured in DMEM + 10% FBS and DMEM + 10% FBS + DFO (260 μmol/L; P = 0.062). Viability was assessed with a trypan blue exclusion assay and normalized to that of control cultures (DMEM + 10% FBS). Columns, mean (n = 3); bars, SD.

A

B

FIGURE 2. In vitro growth rates of LCC6HER-2 and LCC6Vector cells and their responses to environmental stress conditions in culture. A. In vitro growth rates of cells cultured in DMEM + 10% FBS measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. This pattern of growth was consistent in three experiments. B. Viability of LCC6HER-2 and LCC6Vector cells cultured for 6 days in serum-free DMEM (0% FBS; P = 0.0027) and for 4 days in DMEM + 10% FBS + DFO (DFO: 260 μmol/L; P = 0.062). Viability was assessed with a trypan blue exclusion assay and normalized to that of control cultures (DMEM + 10% FBS). Columns, mean (n = 3); bars, SD.
paraffin-embedded, and H&E-stained sections of LCC6 tumors were evaluated using a method that overlaid fluorescent (Fig. 4A) and bright-field (Fig. 4B) images. When illuminated with 488 nm light, areas exhibiting necrotic cells emitted a green fluorescence (Fig. 4A). Using this approach, as shown in the representative photomicrographs in Fig. 4, it was evident that LCC6HER-2 tumors exhibited larger areas of viable tumor tissue (Fig. 4C and D) when compared with micrographs of LCC6Vector tumors (Fig. 4E and F). As suggested by the images provided, the extent of tumor tissue viability/necrosis seemed to be independent of tumor size, that is, images obtained from tumors exhibiting 2-fold differences in mass (e.g., 0.28 versus 0.54 g) were comparable. To more quantitatively assess tumor cell viability, tumors were disaggregated and the percentage of viable and dead cells was determined using a trypan blue exclusion assay and flow cytometry. These results have been summarized in Fig. 5A and B, respectively. The percentage of trypan blue–excluding cells in tumor cell suspensions derived from LCC6HER-2 (24.49 ± 10.6%) was significantly (P = 0.00056) greater than that obtained for LCC6Vector (8.98 ± 4.7%) tumors. These results are consistent with the images shown in Fig. 4. Flow cytometric analyses of ethanol-fixed and propidium iodide–stained cells derived from disaggregated tumors (Fig. 5B) suggest that LCC6HER-2 tumors, when compared with the LCC6Vector controls, contained more cells with intact DNA content (≥2N), representative of viable cells (30.6 ± 8.9% and 17.2 ± 4% for LCC6HER-2 and LCC6Vector tumors, respectively), and correspondingly fewer necrotic/apoptotic cells in the sub-G1/G0 phase (<2N DNA content; Fig. 5B, arrows).

A clonogenic assay evaluating viability in cell suspensions derived from disaggregated tumors was also completed (Fig. 5C) and these data suggest that the plating efficiency of trypan blue–excluding cells derived from LCC6HER-2 tumors was higher (30.6 ± 8.9% and 17.2 ± 4% for LCC6HER-2 and LCC6Vector tumors, respectively) compared to the LCC6Vector tumors (8.98 ± 4.7%). These results are consistent with the images shown in Fig. 4. Flow cytometric analyses of ethanol-fixed and propidium iodide–stained cells derived from disaggregated tumors (Fig. 5B) suggest that LCC6HER-2 tumors, when compared with the LCC6Vector controls, contained more cells with intact DNA content (≥2N), representative of viable cells (30.6 ± 8.9% and 17.2 ± 4% for LCC6HER-2 and LCC6Vector tumors, respectively), and correspondingly fewer necrotic/apoptotic cells in the sub-G1/G0 phase (<2N DNA content; Fig. 5B, arrows).

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was increased significantly \( (P = 0.0015) \) in comparison with LCC6\text{Vector}-derived cells. For the LCC6\text{HER-2} tumors, plating efficiencies of 29.56 ± 4.12% were obtained in comparison with 16.77 ± 4.63% for the LCC6\text{Vector} tumors, which constitutes a 1.8-fold difference. Because LCC6\text{HER-2} tumors contained 2.7-fold greater percentage of trypan blue–excluding cells, the overall percentage of clonogenic cells was 4.8-fold higher in these tumors than in LCC6\text{Vector} controls.

**Analyses of Hypoxia and Vascularization in LCC6\text{HER-2} Tumors**

The data presented in Figs. 4 and 5 clearly show that the viable cell fraction obtained from LCC6\text{HER-2} tumors is greater than that from LCC6\text{Vector} tumors. These results are also consistent with in vitro data suggesting that LCC6\text{HER-2} cells are better able to survive under stress such as nutrient removal. Based on these results, it was not unreasonable to suggest that increased viability may be a consequence of improvements in blood supply and increases in vascular permeability (as would be expected for cell lines that overproduce VEGF) and/or it could be a consequence of the ability of tumor cells to survive better within the stressful environments typically observed within tumors. Based on these rationales, studies assessing \( a \) regions of hypoxia, as determined by EF5 labeling, and \( b \) tumor vascularization, as determined by CD31 staining and Hoechst 33342 vascular perfusion, were completed.

The nitroimidazole EF5 (31), which localizes in viable hypoxic cells, was used to evaluate hypoxic regions in LCC6 tumors. Immunofluorescent staining of frozen tumor tissue sections with ELK3-51-CY3 antibody (anti-EF5-CY3) from animals exposed to EF5, shown in representative photomicrographs in Fig. 6, clearly suggested that there was an increase in hypoxic areas and much brighter anti-EF5-CY3 staining in LCC6\text{HER-2} tumor sections (Fig. 6A) as compared with LCC6\text{Vector} tumor sections (Fig. 6B). To quantify these differences, tumors were disaggregated and tumor cell suspensions were stained with the anti-EF5-CY3 antibody and counterstained with the DNA dye 4',6-diamidino-2-phenylindole (DAPI). These cells were then analyzed with flow cytometry and the results have been summarized in Fig. 7. Primary gating variables were determined based on time-of-flight and DAPI staining (Fig. 7A), where time-of-flight allowed for the exclusion of cell aggregates and DAPI identified tumor cells with intact DNA content \( (\geq 2N) \). These cells were considered viable and this approach excluded necrotic/apoptotic human tumor cells as well as host (mouse) cells, which had lower DNA content \( (\leq 2N) \) than viable tumor cells. A threshold used for calculating the percentage of hypoxic cells was established based on the fluorescence intensity of tumor cells subjected to hypoxic conditions in the presence of EF5 (100 \( \mu \)mol/L) and later stained with anti-EF5-CY3 antibody (Fig. 7B). Using this methodology to calculate the percentage of viable hypoxic cells (Fig. 7C and D), flow cytometric analyses (summarized in Fig. 7E) revealed that the percentage of viable hypoxic cells was significantly \( (P = 0.0015) \) greater in cell suspensions derived from LCC6\text{HER-2} \( (16.41 \pm 8.11\% ) \) compared with LCC6\text{Vector} \( (5.96 \pm 3.13\% ) \) tumors. These data were consistent with the stained sections represented in Fig. 6.

Because the hypoxia levels in viable tumor tissue seemed to be significantly higher in LCC6\text{HER-2} xenografts, changes in HIF-1α protein levels in LCC6\text{HER-2} tumors were determined. A 1.27-fold higher expression of mean HIF-1α protein levels, when normalized to actin, was found in LCC6\text{HER-2} compared with LCC6\text{Vector} tumors (Fig. 8A and B). These differences, albeit consistent with the in vitro data shown in Fig. 1C, were not statistically significant \( (P = 0.267) \). Given studies from other investigators suggesting that increases in survival of tumor cells in hypoxia may be due to NF-κB expression, total and phosphorylated levels of NF-κB p65 in LCC6\text{HER-2} and LCC6\text{Vector} tumors were determined. The results in Fig. 8C and D indicated that the ratio of NF-κB p65\( ^{\text{N}} \) to total NF-κB p65 protein levels was significantly higher \( (27.5\text{-fold}) \) in LCC6\text{HER-2} versus LCC6\text{Vector} tumors \( (P = 0.00048) \). These differences were consistent with in vitro results (Fig. 1C); however, the in vivo increase in the ratio of NF-κB p65\( ^{\text{N}} \) to total NF-κB p65 protein levels was 15-fold higher than in vitro. Finally, it is interesting to note that tumors derived from a subcutaneous injection of LCC6\text{HER-2} cells would express higher levels of VEGF, as indicated in studies from other laboratories and as shown in Fig. 1B, it was prudent to assess expression levels of
VEGF protein in established tumors. The results in Fig. 8E and F were as expected and the LCC6HER-2 tumors exhibited significantly higher levels of VEGF (1.7-fold higher) compared with that found in LCC6Vector tumors ($P = 0.05$).

The results summarized in Figs. 6 and 7 clearly suggest that HER-2/neu expression is associated with increases of viable hypoxic tumor cell populations, although the cells produce greater levels of VEGF (Figs. 1B and 8E and F), which should correlate to enhance tumor vascularization. To resolve this dilemma, the association between hypoxia and vascularization needed to be established. Several blood vessel variables were quantitated using an automated image analysis system described in detail in Materials and Methods. The percentage of tumor vasculature calculated from CD31-positive staining events (pixels) in viable regions of tumor tissue was determined from images (representative images of LCC6HER-2 and LCC6Vector tumor tissue are provided in Fig. 9A-D). Data obtained from numerous tumors, where the tumor area occupied by blood vessels is represented as a percentage of CD31 or percentage of CD31/Hoechst 33342 (an indicator of perfused blood vessels)–positive pixels, are summarized in Fig. 9E. There were no significant differences ($P = 0.919$) in the percentage of CD31-positive pixels (endothelial cells) and CD31-positive pixels that

FIGURE 6. Immunofluorescent staining of representative LCC6HER-2 and LCC6Vector tumor tissue sections with anti-EF5-CY3 antibody. Frozen tumor sections were fixed and stained with anti-EF5-CY3 antibody. Single color images (>40) of CY3 (yellow/orange, hypoxic tissue) and DAPI (blue, not hypoxic tissue) fluorescence were captured and overlaid using Adobe Photoshop. A. LCC6HER-2 tumor: hypoxic tumor cells are residing at the border of viable and necrotic tissue; viable tumor tissue exhibits well organized, confluent structure, and necrotic tumor tissue located outside hypoxic areas has distorted and loose structure. B. LCC6Vector tumor: hypoxic tumor tissue exhibits much lower intensity of anti-EF5-CY3 staining and is not organized in any particular tissue pattern; a significant portion of the tumor appears to consist of loose necrotic tissue structure. This pattern of staining was consistent for six tumors examined for each group.

FIGURE 7. Flow cytometric analysis of viable hypoxic cells in cell suspensions derived from disaggregated LCC6HER-2 and LCC6Vector xenografts. A. Calculations of the percentage of hypoxic cells were done only on populations with $2N$ DNA content after exclusion of cell aggregates (boxed area). B. Mixture of control LCC6 cells cultured with EF5 (100 μmol/L) under air (negative control, lower cell population) and under hypoxic conditions (positive control, upper cell population). Boxed area, a threshold used to calculate the percentage of hypoxic cells based on differences in anti-EF5-CY3 staining intensity between positive and negative control cells measured in PMT3/FL2 channel and differences in green autofluorescence signal of negative control cells measured in PMT2/FL1 channel. C and D. Representative tumor cell suspensions from a single disaggregated tumor xenograft stained with anti-EF5-CY3 antibody, gated as shown in A: LCC6HER-2 tumor cell suspension (C) and LCC6Vector tumor cell suspension (D). Numbers in boxed areas, percentage of hypoxic cells calculated using a threshold indicated in B. E. Summary of flow cytometric analyses of the percentage of hypoxic cells among tumor cell populations with $2N$ DNA content derived from disaggregated LCC6HER-2 ($n = 9$) and LCC6Vector tumors ($n = 8$). Columns, mean; bars, SD. $P = 0.0015$. 

were also Hoechst 33342 positive \((P = 0.947)\) in viable tumor regions of sections obtained from LCC6\(^{HER-2}\) tumors when compared with LCC6\(^{Vector}\) tumors. An analysis of the median distance of viable tumor tissue (all pixels in a viable tissue) to the nearest CD31-stained vessel, reflective of tumor microvessel density, is summarized in Fig. 9F, and these data indicated that there were no significant differences in microvessel density between LCC6\(^{HER-2}\) and LCC6\(^{Vector}\) tumors \((P = 0.1502)\). When microvessel density was calculated using a random Chalkley grid in three hotspots selected in each tumor based on Hoechst 33342 perfused microvessels (Fig. 9G), again no significant differences were found between LCC6\(^{HER-2}\) and LCC6\(^{Vector}\) tumors \((P = 0.1274)\).

**Discussion**

Considerable effort has been made to control HER-2/neu-overexpressing breast cancers by using specific agents targeted against HER-2/neu such as trastuzumab (Herceptin; refs. 32, 33). The suppression of HER-2/neu alone transiently reduces tumor burden. However, only 15% to 30% of patients with metastatic disease benefit from treatment with single agent Herceptin based on standard response criteria (34, 35). Our group has thus taken an integrated approach to examine what impact changes at the molecular level in a cell, such as HER-2/neu overexpression, have on the biological properties of the tumor and how tumor cells react or adapt to these changes. Such studies could ultimately help to explain the observed aggressiveness of HER-2/neu-positive tumors, and this information would be essential for the rational design of novel treatment strategies to improve the management of this disease. The results reported here provide new insights into the complex effects of HER-2/neu overexpression on the tumor biology in a clinically relevant LCC6\(^{HER-2}\) tumor xenograft model. Our data indicate that LCC6 xenografts overexpressing...
HER-2/neu contain a significant population of viable hypoxic cells likely due to the enhanced ability of the cells to adapt to hostile microenvironments (e.g., hypoxia and increased pH). The presence of a viable hypoxic population in these tumors would certainly have a negative impact on radiation therapy and some forms of chemotherapy and perhaps contribute to the development of a more aggressive tumor phenotype. In the discussion, we emphasize the following points: (a) relevance of the LCC6HER-2 model to breast cancer and (b) impact of HER-2/neu on the tumor microenvironment (i.e., viability, hypoxia and vascularization, and improved adaptability).

**FIGURE 9.** Analyses of vascularization in LCC6HER-2 and LCC6Vector xenografts. A-D. Images of frozen tumor tissue sections from representative LCC6HER-2 (A and B) and LCC6Vector (C and D) tumors perfused in vivo with Hoechst 33342 and subsequently stained with PECAM/CD31 antibody. Black, CD31 staining; blue, Hoechst 33342 perfusion; dark gray, viable tissue; light gray, necrotic tissue. B and D. Enlarged boxed regions in A and C show functional CD31-positive and Hoechst 33342 perfused tumor blood vessels (blue arrows) and nonfunctional CD31-positive but not Hoechst 33342 perfused tumor blood vessels (black arrows). E. Percentage of CD31-stained and Hoechst 33342 perfused tumor vasculature in viable tumor tissue. Columns, mean (n = 8 tumors); bars, SD. Total CD31-stained vessels (CD31+), P = 0.919; CD31-stained and Hoechst 33342 perfused vessels (CD31+/H33342+), P = 0.947. F. Median distance of the viable tumor tissue to the nearest CD31-stained vessel. Columns, mean (n = 8 tumors); bars, SD. P = 0.1502. G. Hoechst 33342 perfused vessels were used to calculate microvessel density from three “hotspots” for each tumor using a random Chalkley grid. Columns, mean (n = 8 tumors); bars, SD. P = 0.1274.
The aggressive estrogen receptor–negative breast cancer tumor model MDA-MB-435/LCC6 (LCC6; ref. 36) is a p53-mutated cell line (37). The origins of the parental cell line (MDA-MB-435), however, remain controversial (38). Unfortunately, the handful of estrogen receptor–negative and HER-2/ neu-overexpressing breast cell lines available, such as SKBR3, grow poorly or not at all as subcutaneous xenografts. In contrast, LCC6 xenografts grow in an exceptionally reproducible manner and respond well to cytoxic drugs used in the treatment of breast cancer patients (36, 39). Moreover, our results suggest that the LCC6HER-2 cell line behaves consistently with what is known about HER-2/neu-positive breast cancer tumor cell lines, such as enhanced survival under stress (3, 4, 6, 19, 24), overproduction of VEGF (15), and expression of activated NF-κB (9, 40). Finally, LCC6HER-2 tumors are sensitive to Herceptin treatment (Fig. 3E). Thus, it seems reasonable to use the LCC6HER-2 cell line as a relevant model for aggressive breast cancer.

In this study, the biological traits of the LCC6HER-2 tumor, including its microenvironment, were compared with matched isogenic LCC6Vector control tumors. The expression of HER-2/neu, as measured with flow cytometry, increased 30-fold in LCC6HER-2 cells and tumor xenografts compared with LCC6Vector cells/tumors (Figs. 1A and 3A and B). This overexpression of HER-2/neu in LCC6HER-2 tumors was confirmed in formalin-fixed and paraffin-embedded tissue sections stained with HercepTest (Fig. 3D).

Although HER-2/neu expression was increased in LCC6HER-2 compared with LCC6Vector cells, the growth rates of both cell lines in vitro were similar under normal culture conditions. When LCC6Vector and LCC6HER-2 cells were grown in serum-free medium, a greater percentage of LCC6HER-2 cells were viable (Fig. 2B). The ability of LCC6HER-2 cells to withstand these stress conditions are not surprising; it has been reported that HER-2/neu overexpression in estrogen receptor–positive MCF-7 breast cancer and LNCaP prostate cancer cells was associated with enhanced survival in estrogen-depleted and androgen-depleted media, respectively (6, 41). In the presence of DFO, an iron chelator, the LCC6HER-2 cells showed a strong trend in enhanced viability compared with LCC6Vector cells (Fig. 2B). Iron chelators are known to exert broad range of biological effects, including inhibition of DNA synthesis and accumulation of HIF-1α protein in cells (42), thereby mimicking to a certain extent the accumulation of HIF-1α arising under atmospheric hypoxia (43, 44). Others have also shown that MCF-7 breast cancer cells overexpressing HER-2/neu are more viable in cultures treated with DFO than non-Her-2/neu-overexpressing cells (24).

Because in vitro survival of LCC6HER-2 cells under stress conditions is enhanced compared with LCC6Vector cells, we also compared in vivo viability of tumors derived from these cell lines. Solid tumors generally have poor and/or dysfunctional vasculature (20); the supply of oxygen levels and nutrients can be restricted and these factors impose stresses on the tumor cells, which can contribute to cell necrosis, apoptosis, and inhibition of proliferation. These, in turn, help to explain the presence of necrotic regions within the tumor core or at certain distances from tumor blood vessels. Perhaps most importantly, however, the stresses created within the tumor can lead to the creation of a population of nondividing but viable tumor cells. These cells will be less affected by commonly employed cancer drugs, may exhibit an increased potential for resistance, and will certainly contribute to tumor repopulation. The percentage of viable cells in LCC6HER-2 tumors was significantly higher than in size-matched LCC6Vector tumors (Fig. 5A and B), and this finding corresponded well with the significantly improved clonogenic potential of cells from LCC6HER-2 tumors (Fig. 5C).

As part of our examination of the tumor microenvironment, the levels of hypoxia in the two LCC6 tumor types were evaluated. The hypoxia marker EF5, which is reduced only in viable cells and binds covalently to proteins only under hypoxic conditions (31), was used. Frozen tumor sections stained with the anti-EF5-CY3 antibody showed that there was more hypoxic tissue in LCC6HER-2 than in LCC6Vector tumors (Fig. 6). In accordance with these data, flow cytometry analyses of cell suspensions derived from disaggregated tumors showed that LCC6HER-2 xenografts contained ~3 times more viable hypoxic cells compared with LCC6Vector xenografts (Fig. 7).

Recently, we have shown that treatment of LCC6HER-2 tumors with Herceptin results in a small decrease of EF5-positive (hypoxic) cell fraction in these tumors, possibly indicating a trend in reversing HER-2/neu–associated hypoxic phenotype (45). Importantly, a study by Bartosova et al. (46) have shown that there is statistically strong association of HER-2/neu expression in primary breast cancers with carbonic anhydrase IX, a surrogate marker of tumor hypoxia. In contrast, a recent report by Blackwell et al. (47) indicated an inverse correlation between HER-2/neu gene amplification and hypoxia in primary breast tumors, but this study used a relatively small number of HER-2/neu-positive cases. It will be very important to pursue additional studies of this nature to definitively determine the association between molecular markers of aggressive HER-2/neu–positive breast cancers and formation of hypoxic regions within these tumors. The relationship between hypoxia and increased HIF-1α expression in tumor cells has been studied intensively (23, 25, 43, 44). Because the LCC6HER-2 tumors contained a higher percentage of hypoxic cells, HIF-1α levels in LCC6HER-2 and LCC6Vector cells and tumors were compared. Both LCC6HER-2 and LCC6Vector cells grown in vitro under normoxic culture conditions express HIF-1α, with LCC6HER-2 cells expressing about twice as much HIF-1α as the vector-transfected LCC6 cells (Fig. 1C). Production of HIF-1α in the absence of hypoxia may seem anomalous; however, recent studies have shown that a host of other factors can increase the levels of HIF-1α in normoxic conditions including the inactivation of tumor suppressor genes, the activation of oncogenic pathways (43) including HER-2/neu (10), the Warburg effect (aerobic glycolytic metabolism; ref. 48), and the presence of cytokines, nitric oxide, and transition metals such as Co2+ (49). Thus, the expression of HIF-1α in LCC6 cells cultured in vitro is not particularly surprising. The in vivo data for HIF-1α indicated that LCC6HER-2 tumors did not express significantly higher levels of this protein than LCC6Vector tumors (Fig. 8A and B), although the former contained on average ~3 times more hypoxic viable cells than the latter (Fig. 7E). Our data suggest that perhaps the degree of HIF-1α protein expression does not simply reflect the presence of viable hypoxic cells within a tumor. Alternatively, the levels
of HIF-1α in control LCC6Vector tumors might be elevated as a result from the responses of cells to its particular tumor microenvironment and/or higher levels of aerobic glycolysis (43, 48). Interestingly, in clinical studies, no significant correlation between HIF-1α and HER-2/neu expression in breast cancer tumors has been found (50, 51).

VEGF levels and the vasculature in both tumor types were evaluated to determine their role (if any) in the presence of hypoxic cell population in LCC6HER-2 tumors. LCC6HER-2 cells and tumors had significantly higher VEGF protein levels (compared with LCC6Vector control tumors; Figs. 1B and 8E and F), a result that is consistent with previous reports showing increased VEGF production in HER-2/neu-overexpressing cells and xenografts (10, 14, 15). Some studies have shown that levels of VEGF are directly related to vascularization of xenografts (15) and the vascular density of breast cancers (52). However, this is not always the case—other studies show the lack of any relationship between VEGF levels and increased vascularization (53, 54), and it was suggested that tumor vascularity may not reflect tumor angiogenesis and/or hypoxia (55, 56). Our data indicate that the higher levels of VEGF in LCC6HER-2 tumors did not increase tumor vascularization (Fig. 9); therefore, VEGF levels in the LCC6 tumors did not seem to correspond to tumor angiogenesis or, indeed, hypoxia. Detailed quantitative analyses of CD31-stained and Hoechst 33342 perfused microvessels, as well as Chalkley counts in tumor vascular hotspots, indicated that there were no statistically significant differences in vascularization between LCC6HER-2 and LCC6Vector tumors. In addition, the median distance of viable tissue from the blood vessel in images of the tumor sections was similar in both LCC6HER-2 and LCC6Vector tumors (Fig. 9F). These data indicate that hypoxic tissue in LCC6HER-2 tumors was closer to blood vessels than in LCC6Vector tumors. This can be explained by suggesting that oxygen consumption was higher in the HER-2/neu tumors, which may be the result of HER-2/neu signaling effects on cellular metabolic pathways. Indeed, altered metabolism has been implicated as a reason for changes in tumor oxygen consumption. For example, the inhibition of Ras pathway in glioblastoma U87 xenografts (57) and epidermal growth factor receptor pathway in HER-2/neu-overexpressing MCF-7 breast cancer xenografts (45, 58) decreased tumor hypoxia, although the tumor vessel density decreased and the tumor size was not a factor.

It is concluded that the higher percentage of viable cells in the LCC6HER-2 tumors cannot be attributed to improved vascularization. Further, LCC6HER-2/neu-overexpressing tumors contain a significant proportion of viable hypoxic cells despite their appropriate proximity to blood vessels. The viability of these hypoxic cells could not be attributed to elevated levels of HIF-1α. This apparent paradox leads us to speculate that LCC6HER-2 tumor cells have superior adaptive characteristics allowing them to remain viable in regions within tumor microenvironment, including hypoxia. These adaptive characteristics may be related to other survival factors, such as NF-κB, the expression of which was established previously in HER-2/neu-overexpressing cells (8, 9). The role of NF-κB in adaptation and cell survival in adverse conditions, including hypoxia, has been implicated in many studies (25, 59–62). Our data show that both LCC6HER-2 and LCC6Vector cells express significant levels of total NF-κB p65 (regardless of phosphorylation status) and NF-κB p65P (Fig. 1C). However, in vivo, we showed a dramatic 27.5-fold increase in NF-κB p65P when normalized to total NF-κB p65 (Fig. 8C and D). These data may also help explain the elevated clonogenic potential of LCC6HER-2 tumor cells demonstrated in our study (Fig. 5C), as activation of NF-κB has been shown to play a role in enhanced clonogenicity in cancer cells (63).

The value of this study—examining the tumor microenvironment of the LCC6 tumor models in an integrated manner—is clear because we can now identify novel treatment strategies or combinations based on the characteristics of the cell populations present. We propose that anti-HER-2/neu treatment strategies may be more effective when combined with antihypoxia drugs such as the hypoxia-activated topoisomerase II inhibitor tirapazamine. Therapeutic regimens based on combining inhibitors of NF-κB and glycolysis together with the anti-tyrosine kinase receptor antibodies may also be useful. Based on the data presented, it is argued that HER-2/neu-overexpressing breast cancer cells have a high degree of adaptability in vivo. It is also important to emphasize that tumor microenvironments can be influenced significantly by different treatments (28, 45, 57, 58, 64–67). A thorough characterization of changes in tumor microenvironment during treatment would provide an increase in understanding of tumor repopulating cells that survived attempts to control tumor growth. It may soon be possible to monitor tumors noninvasively (e.g., using magnetic resonance imaging or positron emission tomography with appropriate contrast and radioactive tracer agents, respectively), to evaluate changes in the tumor environment, and subsequently to apply treatment modalities or combination strategies (as suggested above) based on what cell populations or conditions are present in the tumor.

Materials and Methods

Cell Lines and Culture

MDA-MB-435/LCC6 (LCC6) cells were a gift from Dr. Robert Clarke (Georgetown University, Washington, DC). SKBR3 and BT474 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM/high glucose supplemented with l-glutamine (2 mmol/L; DMEM and l-glutamine from Stem Cell Technologies, Vancouver, British Columbia, Canada) and 10% FBS (HyClone, Logan, UT). LCC6 cells were transfected via electroporation with the mammalian expression vector pREP9 (Invitrogen, Grand Island, NY) containing the 4.3-kb full-length human c-erbB2 cDNA to yield LCC6HER-2 or with the empty vector pREP9 to yield LCC6Vector cells. The c-erbB2 cDNA was a generous gift from Dr. Ming Tan (University of Texas M.D. Anderson Cancer Center, Houston, TX). Transfected cells expressing HER-2/neu and vector control cells were selected by resistance to G-418 (500 μg/mL; Mediatech, Inc., Herndon, VA). LCC6Vector cells were expanded in DMEM-10% FBS supplemented with G-418 and cell aliquots were frozen for future use. Expanded LCC6HER-2 cells were harvested with EDTA (2.5 mmol/L), stained with anti-HER-2/neu antibody (Neu 24.7-FITC, BD Biosciences, San Jose, CA), and sorted several times for the top 5% HER-2/neu expressors. Sorted cells
were expanded in DMEM-10% FBS supplemented with G-418, and cell aliquots were frozen for future use. Transfected cells were grown for 1 week in DMEM-10% FBS without G-418 prior to use for in vitro and in vivo studies.

To determine responses to environmental stress, LCC6HER-2 and LCC6Vector cells were cultured in the absence of serum and in the presence of DFO (260 μmol/L, Sigma-Aldrich, St. Louis, MO) added to the medium. Experiments were carried out in triplicate in six-well plates (3-mL medium, 50,000 cells per well). After 6 or 4 days (serum starvation and DFO treatment experiments, respectively), both nonadherent and adherent cells were harvested and cell numbers and viability were established by counting trypan blue diluted cells with a hemocytometer.

**In Vitro Secreted VEGF Assay**

Supernatant from LCC6HER-2 and LCC6Vector cultures (~80-90% confluent) grown for 3 days was collected and assayed with Quantikine Human VEGF Immunoassay specific for recombinant human VEGF165 and recombinant human VEGF121 (R&D Systems, Minneapolis, MN). The results were normalized to 107 cells/mL of cell culture supernatant.

**Western Blotting**

Frozen tumor pieces were sonicated in lysis buffer (150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 2.5 mmol/L EDTA, 0.1% SDS, Mini protease inhibitor cocktail tablets from Roche Diagnostics, Mannheim, Germany), and all protein lysates were equilibrated to 7.5 mg protein/mL. Total protein (50-70 μg) was separated on 10% SDS-PAGE gels. All samples, except those detected with anti-VEGF antibody, were run under reduced conditions (0.37% β-mercaptoethanol). Protein was transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) and blocked in 5% skim milk powder in TBS-T [150 mmol/L NaCl, 50 mmol/L Tris, 0.1% Tween 20 (pH 7.4)] for 2 hours. VEGF was detected with goat anti-human recombinant human VEGF antibody (R&D Systems), HIF-1α was detected with mouse anti-human monoclonal antibody (BD Transduction Laboratories, San Jose, CA), and NF-αB and NF-κB p65γ were detected with rabbit anti-human polyclonal antibodies (Cell Signaling Technology, Beverly, MA). Horseradish peroxidase–conjugated secondary antibodies were purchased from Promega (Madison, WI). Signals were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and visualized with autoradiography film (X-OMAT blue XB-1, Eastman Kodak Co., Rochester, NY). Films were scanned with a Personal Densitometer SI (Molecular Dynamics, Piscataway, Eastman Kodak Co., Rochester, NY). Films were scanned with a Personal Densitometer SI (Molecular Dynamics, Piscataway, NJ). The absorbance of specific protein bands in a square region of interest surrounding each band, after background subtraction, was normalized to actin bands measured in the same way.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Cells were grown in triplicates in 96-well plates over a period of 9 days in DMEM-10% FBS. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (25 μg/well) was added to each well and plates were incubated for 3 hours at 37°C. The colored formazan product was dissolved using DMSO (200 μL). Plates were read using the microtiter plate reader (Dynex Technologies, Inc., Chantilly, VA) at 570 nm.

**Animal Tumor Models**

LCC6 cells were harvested in the exponential growth phase; 5 × 106 cells were injected subcutaneously on the back of female SCID-Rag2M mice. Different batches of LCC6Vector and LCC6HER-2 cells, expanded from frozen aliquots, were used to initiate tumors. Tumor growth was monitored twice a week; tumor sizes were calculated using the formula: 0.5 length (cm) × width (cm)². Herceptin (Hoffman-La Roche, Mississauga, Ontario, Canada) was provided by the British Columbia Cancer Agency pharmacy. Herceptin treatment of LCC6 tumors was initiated on day 17 and continued twice a week for 5 weeks (1 mg/kg) delivered i.p. Tumors with various weights (0.06-0.054 g) were harvested for analyses. Animal protocols were approved by the University of British Columbia Animal Care Committee, and these studies were done in accordance with guidelines established by the Canadian Council of Animal Care.

**Tumor Disaggregation and Processing**

Single cell suspensions from solid tumors were obtained using a disaggregation procedure that resulted in efficient tumor cell recovery (mean ± SD, 3.4 ± 1.2 × 106 cells/g; combined viable and necrotic cells, n = 31). In this disaggregation procedure, tumors were digested by collagenases followed by mechanical disaggregation in a Medimachine. This is in contrast to disaggregation methods that use a mixture of harsh enzymes such as DNase, trypsin, and Pronase or using a Medimachine alone, which causes an extensive damage to tumor cells and results in poor cell recovery (refs. 68, 69; data not shown). Viability and cell surface expression of HER-2/neu in LCC6HER-2 and LCC6Vector cultured cells was not altered when these cells were subjected to the same enzymatic digestion and mechanical procedure used for the disaggregation of solid LCC6 tumors. Because there were some cell losses during tumor disaggregation, extensive efforts were made to correlate flow cytometry data to histology and immunohistochemistry assessments of tumor sections. The majority of cells from disaggregated tumors were found to be of tumor origin, with a maximum 5% attributed to mouse cells in LCC6HER-2 tumors and up to 8% in LCC6Vector tumors.

Three hours prior to tumor harvest, animals were injected i.v. and i.p. with EF5 (30 mg/kg, a gift from Dr. Cameron Koch, University of Pennsylvania, Philadelphia, PA) in PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 2 mmol/L KH2PO4), and Hoechst 33342 (100 μL, 16 mg/mL, Sigma-Aldrich) in PBS was injected i.v. 20 minutes prior to tumor harvest. Harvested tumors were rinsed in HBSS (Stem Cell Technologies) containing 0.4% bovine serum albumin and divided as follows: one fourth was fixed in 10% buffered formalin and later embedded in paraffin, one fourth was immediately frozen on dry ice in embedding medium, and the remainder was chopped with scalpels in ice-cold HBSS 0.4% bovine serum albumin. For Western blotting, parts of tumors...
were frozen in liquid nitrogen. Chopped tissue was transferred to 14-mL tubes with disaggregation mixture (HBSS, 1% bovine serum albumin fraction V, Calbiochem, San Diego, CA; collagenase type 2 and 4, final concentration 250 units/mL, Worthington Biochemical Corporation, Lakewood, NJ) and rotated at 37°C for 2 hours. The resulting suspensions were placed in Medicines (50 μM, BD Biosciences) and run (3 times, 1 minute each) through a Medimachine (BD Biosciences) with extensive PBS-0.1% bovine serum albumin (PBSB) washes after each run. Collected cells were washed once with PBSB, and pellets were resuspended in EDTA (2.5 mmol/L) and incubated at 37°C for 5 minutes to reduce cell clumping. Lastly, cells were washed, resuspended in PBSB, and stored on ice for subsequent processing. Tumor cell suspensions were diluted in 0.1% trypan blue in PBS and cells were counted using a hemocytometer. Fragments of cells, erythrocytes, and cells with two times erythrocyte volume or smaller were excluded. For clonogenic assays, 500 trypan blue–excluding cells from disaggregated tumors were plated in quadruplicates (from two independent dilutions) in 3-mL wells in DMEM-10% bovine serum albumin supplemented with l-glutamine (2 mmol/L) and incubated at 37°C for 2 hours. The resulting suspensions were placed in Medicines (50 μM, BD Biosciences) and run (3 times, 1 minute each) through a Medimachine (BD Biosciences) with extensive PBS-0.1% bovine serum albumin (PBSB) washes after each run. Collected cells were washed once with PBSB, and pellets were resuspended in EDTA (2.5 mmol/L) and incubated at 37°C for 5 minutes to reduce cell clumping. Lastly, cells were washed, resuspended in PBSB, and stored on ice for subsequent processing. Tumor cell suspensions were diluted in 0.1% trypan blue in PBS and cells were counted using a hemocytometer. Fragments of cells, erythrocytes, and cells with two times erythrocyte volume or smaller were excluded. For clonogenic assays, 500 trypan blue–excluding cells from disaggregated tumors were plated in quadruplicates (from two independent dilutions) in 3-mL wells in DMEM-10% bovine serum albumin supplemented with l-glutamine (2 mmol/L) and cultured for 14 to 17 days. After removal of medium from wells, colonies were stained with 0.2% malachite green and counted. Plating efficiency was defined as the percentage of trypan blue–excluding cells that formed colonies of >50 cells.

Flow Cytometry

All flow cytometric analyses were done using the EPICS ELITE ESP flow cytometer (Beckman-Coulter, Miami, FL) equipped with INNOVA Enterprise 621 laser (Coherent, Santa Clara, CA). HER-2/neu cell surface expression was detected using the Neu 24.7-FITC antibody. Analyses of HER-1, HER-2, HER-3, and HER-4 expression were done with primary antibodies against extracellular domains of epidermal growth factor receptor (Ab-5), HER-2/neu (Ab-2), HER-3 (Ab-4), and HER-4 (Ab-1) from NeoMarkers (Fremont, CA) and detected with goat anti-mouse phycoerythrin (Tago, Inc., Burlingame, CA). Isotypic control antibodies were purchased from Caltag Laboratories (Burlingame, CA). Briefly, 5 × 10^5 cells were stained with a saturating amount of antibody for 30 minutes on ice. Cells were washed twice with PBSB and resuspended in PBSB plus propidium iodide (0.5 μg/mL, Sigma-Aldrich) to differentiate live from dead cells. Quantitation of HER-2/neu surface expression and the mean Antibody Binding Capacity calculations were done using Quantum 24 or 25 FITC premixed beads and Simply Cellular beads as standards (Bangs Laboratories, Fishers, IN) according to the protocol described previously (70). Briefly, the mean fluorescence intensity of a sample stained with Neu 24.7-FITC was converted to molecules of equivalent soluble fluorochrome and divided by the effective F/P ratio of the Neu 24.7-FITC antibody to give Antibody Binding Capacity values.

Histology and Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor sections were cut at 5-μm thickness. Sections were stained with H&E. Several images per tumor section were captured under low magnification (4× objective) using both bright-field and fluorescent microscopy (450-490/515 filter cube). Some tumor sections were stained with HercepTest (A0485 antibody). One batch of frozen tumor sections (8-μm thickness) was fixed in ice-cold acetone for 5 minutes, air-dried, and stained with ELK3-51-CY3 antibody to detect EF5 adducts according to procedures described elsewhere (31). Sections were counterstained with DAPI (0.1 mg/mL) and mounted in 90% glycerol. Images were acquired with low-power magnification (4× objective) using 515-560/590 (CY3) and 340-380/425 (DAPI) filter cubes. Digital images of H&E, HercepTest, and ELK3-51-CY3 stained sections were captured with a Leica microscope system equipped with a DC 100 digital camera and Image Database software (Leica, Bensheim, Germany). A second batch of frozen tumor sections (10-μm thickness) was air-dried for 24 hours, fixed in acetone/methanol, and blocked with 3% H2O2. Tumor vasculature was stained using PECAM/CD31 antibody (BD PharMingen, San Jose, CA) and biotinylated goat anti-rat secondary antibody (BD PharMingen) followed by extravidin horseradish peroxidase (Sigma-Aldrich) and metal enhanced 3,3’-diaminobenzidine substrate (Pierce, Rockford, IL). Slides were then counterstained with hematoxylin, dehydrated, and mounted using Permount (Fisher Scientific, Fair Lawn, NJ).

Image Analysis

Frozen sections were imaged prior to immunostaining for Hoechst 33342 fluorescence using a 360 nm excitation filter and a 450 nm long-pass emission filter. The same sections were imaged in bright-field after CD31/hematoxylin staining. The imaging system consisted of a fluorescence microscope (Zeiss III RS, Oberkochen, Germany); a cooled, monochrome CCD video camera (model 4922, Cohu, San Diego, CA); frame grabber (Scion, Frederick, MD); a custom-built motorized x-y stage; and customized NIH Image software, public domain program developed at the NIH (http://rsb.info.nih.gov/nih-image). The motorized stage allowed for tiling of adjacent
microscope fields of view (6.3 × objective). Using this system, images of entire tumor sections (25-50 mm²) were captured at a resolution of 1 pixel per µm². With the NIH Image software application and user-supplied algorithms, images of Hoechst 33342 fluorescence and CD31 tissue staining from each tumor section were overlaid and areas of necrosis and staining artifacts were removed. On the fluorescence image, Hoechst 33342–positive regions were identified by selecting all pixels that were 12 SD above the tissue background. On the bright-field images, CD31 positive staining was identified by selecting pixels that were 5 SD above tissue background levels. Threshold levels were kept constant for all sections. Counting the number of positive pixels divided by the total number of pixels (<40 µm²) were considered artifacts and were removed from the distance analysis. CD31-positive vessels were considered perfused if >2% pixels of each CD31-positive object were also Hoechst 33342 positive. This countered registration errors up to 5 µm between images. Frozen sections imaged for Hoechst 33342 fluorescence (4 × objective) were also used to count microvessel density in tumor tissue using the Chalkley grid (72). Results were expressed as numbers of vessel hitting dots in the grid in three hotspots for each tumor (excluding areas of necrosis and hemorrhage).

Statistical Analyses
All statistical analyses were performed with ANOVA comparisons of means using Statistica software. Differences were considered significant at P ≤ 0.05.

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HER-2/neu Overexpression Increases the Viable Hypoxic Cell Population within Solid Tumors without Causing Changes in Tumor Vascularization

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