The Oncogene HER2/neu (ERBB2) Requires the Hypoxia-inducible Factor HIF-1 for Mammary Tumor Growth and Anoikis Resistance*§

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Background: Oncogene HER2 (ERBB2) regulates breast cancer growth and anoikis resistance.

Results: ERBB2 requires hypoxia-inducible factor (HIF)-1 for breast cancer growth in vivo and anoikis resistance in vitro.

Conclusion: HIF-1 plays a critical role in ERBB2-positive breast cancers.

Significance: Genes co-regulated by ERBB2 and HIF-1 may be novel therapeutic targets for treating breast cancer.

ERBB2, a receptor tyrosine kinase amplified in breast cancer, is a well established regulator of tumor growth in vivo and anoikis resistance leading to disruption of architecture in three-dimensional mammary epithelial acinar structures in vitro. ERBB2 promotes anoikis resistance by maintaining signaling pathways and by rescuing metabolic defects and thus inhibiting accumulation of deleterious reactive oxygen species. Recent evidence suggests that hypoxia, via hypoxia-inducible factors (HIFs), can inhibit anoikis; thus, we hypothesized that HIF-1 may play a role in ERBB2-mediated anoikis resistance and oncogenesis. Indeed, tumors isolated from MMTV-Neu mice contain elevated HIF-1α levels and tumor cells created from MMTV-Neu mice harboring deletion of Hif1α alleles reduced primary tumor growth in vivo. ERBB2 overexpressing cancer cells stabilize HIF under normoxic conditions and require HIF-1 for ERBB2-mediated anchorage-independence, three-dimensional culture growth and anoikis resistance. HIF-1 reduction in ERBB2 cells was associated with induction of the pro-anoikis protein BIM and decreased ERK and AKT signaling during cell detachment. ERBB2-mediated inhibition of metabolic defects, including decreased reactive oxygen species generation in suspension, required HIF-1 expression that was critical for ERBB2-mediated oncogenesis. Gene expression profiling of hypoxic three-dimensional acinar structures identified a number of genes elevated in response to hypoxia that are known ERBB2 targets, suggesting that hypoxic conditions and ERBB2 overexpression share both phenotypic and genetic components via HIF-1 regulation. Thus, our data demonstrate that ERBB2 requires HIF-1 for tumor growth and suggest that HIF is a major downstream regulator of ERBB2 that protects cells from anoikis and metabolic stress caused by decreased matrix adhesion.

Adhesion to extracellular matrix (ECM) is critical for epithelial cell survival as cells that lose matrix engagement undergo apoptosis, referred to as anoikis (1). Anchorage-mediated cell survival serves to maintain normal tissue homeostasis by ensuring that cells only survive in the proper environmental context (2). In vivo, anoikis is believed to be critical in cavitation during development of the salivary and mammary glands. Moreover, alterations in ECM-mediated cell survival are common in epithelial-derived cancer cells where resistance to anoikis promotes oncogenesis, particularly metastasis, by allowing cells to survive in the absence of proper ECM cues (3).

ERBB2 (also known as HER2, Neu) is a receptor tyrosine kinase found to be amplified in 20–30% of human breast cancers (4). Overexpression of ERBB2 is known to promote anoikis resistance (5) and cause luminal filling as well as disruption of polarity in acinar-like structures of mammary epithelial cells cultured using three-dimensional basement membrane cultures (6). ERBB2-mediated anoikis resistance is associated with maintained ERK and AKT signaling in the absence of proper cell adhesion, resulting in suppression of the Bcl2-homology domain 3 (BH3)-only BCL2 family member BIM (5) and BMF (7) as well sustained glucose transport and ATP production that inhibits ROS accumulation (8), allowing for survival under conditions of reduced cell-matrix attachment. However, it is not clear how ERBB2 can regulate signaling pathways and maintain metabolic homeostasis in the absence of adhesion.

HIF-1 is a heterodimeric transcription factor consisting of a constitutively expressed, nuclear HIF-1α subunit (also known as aryl hydrocarbon nuclear receptor translocator) and an oxy-

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The abbreviations used are: ECM, extracellular matrix; HIF, hypoxia-inducible factor; ROS, reactive oxygen species; ITGA5, integrin α5; MNK2, MAPK-interacting serine/threonine kinase 2; ANGPTL4, angiopoietin-like 4; BNIP3L, Bcl2/adenosine E18 protein-interacting protein 3-like; ADM, adrenomedullin; MTEC, mammary tumor epithelial cell; BH3, Bcl2-homology domain 3.
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gen-labile HIF-1α subunit (9) that modulates the expression of a variety of genes as an integral part of the cellular response to hypoxia. HIF-1 regulated genes include those involved in survival, angiogenesis, migration, and invasion of which are associated with tumorigenesis, thus, it is not surprising that HIF-1α expression is a common feature of many solid tumors. Indeed, HIF-1α expression predicts poor clinical response and clinical outcome in human breast cancer (10). Although HIF-1α stabilization primarily occurs in response to decreased oxygen tension, hypoxia-independent stabilization of HIF-1α has been reported in response to growth factor stimulation, oncogenic activation, and loss of tumor suppressor function (11). ERBB2-mediated stabilization of HIF-1α has been reported to occur under normoxic conditions through AKT/mammalian target of rapamycin-dependent increase in HIF-1α protein synthesis (12) as well as through AKT-mediated enhancement of HIF-1α/HIF-1β heterodimerization (13). However, the role of HIF-1 in ERBB2-mediated oncogenesis is not known. We have recently shown that exposure to hypoxia suppresses anoikis in mammary epithelial cells in a manner that is dependent upon HIF-1α expression leading to inhibition of luminal clearance and development of disorganized acinar structures (14, 15). Furthermore, HIF-1, like ERBB2, acts at the ERK-BIM axis to promote cell survival in the absence of adhesion (14). This intersection between HIF-1- and ERBB2-mediated regulation of anoikis coupled with the fact that oncogenes, including ERBB2, have been shown to stabilize HIF-1α under normoxic conditions (13) led us to hypothesize that HIF-1α may be involved in ERBB2-mediated oncogenesis.

Here, we show that mammary tumors from MMTV-Neu transgenic mice express more HIF-1α levels as compared with the normal mammary gland and that tumors require HIF-1α for efficient growth in vivo. Similarly, breast cancer cell lines and MCF-10A cell overexpressing ERBB2 cells contain elevated HIF levels and require HIF-1α for anchorage independence, three-dimensional growth and anoikis resistance. Reduction of HIF-1α in ERBB2 cells was associated with induction of the pro-apoptosis protein BIM and decreased ERK and AKT signaling during cell detachment. Furthermore, we show ERBB2-mediated suppression of oxidative stress during cell detachment requires HIF-1 activity and treatment of HIF-1α-depleted ERBB2-overexpressing cells with antioxidants rescues anchorage-independent and three-dimensional growth. Gene expression profiling of normoxic versus hypoxic acini identified that 25% of differentially expressed targets are known ERBB2-regulated genes. We show that a subset of these ERBB2-regulated genes require HIF for expression. These data suggest that oncogenes such as ERBB2 may co-opt HIF-mediated signaling under normoxic conditions to maintain cell signaling that blocks induction of anoikis, as well as reprograms metabolic pathways that prevent ROS accumulation, thereby promoting cell survival in the absence of proper matrix adhesion and facilitating tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Animals**—Mice harboring two alleles of exon 2 of Hif1a flanked byloxP sites (double-floxed, DF) (16) were backcrossed to the FVB/Nj strain (Jax Labs) for 11 generations prior to breeding to MMTV-Neu (unactivated) transgenic mice (FVB/N) (Jackson Labs, Bar Harbor, ME, stock 002376). Hif1a DF; MMTV-Neu + females were constitutively bred to accelerate mammary tumorigenesis, with the average time to tumor onset occurring near 1 year of age. All animal procedures were approved by Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center.

Establishing HIF-1 Wild-type (WT) and Knock-out (KO) Mammary Tumor Epithelial Cells (MTECs)—Several late-stage mammary carcinomas (>500 mm3) were isolated and pooled from Hif1a DF; Neu+ bigenic females. Tumors were mechanically and enzymatically digested as described in Ref. 17. Organoids were plated and at passage 6, MTECs were transduced with either Adenovirus β-gal or Cre at a multiplicity of infection of 80 pfu/cell to generate WT and KO MTECs, respectively. Adenoviral transduction was repeated 3 times and the deletion efficiency between WT and KO MTECs was confirmed by Western blotting. Additional information is available under supplemental Materials and Methods.

**MTEC Transplant into FVB Recipients**—MTECs dissociated to single cells with 0.05% trypsin/EDTA were counted by hemocytometer, diluted into Hank’s balanced salt solution and kept on ice until injection. One million cells/Hank’s balanced salt solution were transplanted into each inguinal mammary fat pad of 3-week-old female FVB/Nj recipients (Jax Labs) using a 26-gauge PT2 needle mounted to a Hamilton syringe (10 μl volume), followed by clearing of the endogenous epithelium. Recipients (n = 8 mice/genotype) were palpated 2 times per week for up to 62 days post-tumor cell injection, and the outgrowths were measured with digital calipers to calculate tumor volume, as described in Ref. 17.

**Tissue Histology, Immunostaining, and Image Acquisition**—Tumors were harvested and processed as previously described (17). Additional information is available in supplemental Materials and Methods.

**Cell Culture**—MCF-10A and SK-BR-3 were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to ATCC instructions. Briefly, MCF-10A were cultured in DMEM/F-12 (Invitrogen) supplemented with 5% horse serum, 20 ng/ml of EGF (Peprotech, Rocky Hill, NJ), 10 μg/ml of insulin (Sigma), 1 ng/ml of cholera toxin (Sigma), 100 μg/ml of hydrocortisone (Sigma), 50 units/ml of penicillin, and 50 μg/ml of streptomycin (Invitrogen). SK-BR-3 cells were cultured in McCoy’s 5A supplemented with 10% fetal bovine serum.

**Reagents and Antibodies**—All reagents and antibodies used are listed under supplemental Materials and Methods.

**Three-dimensional Morphogenesis Assay**—Assays were performed as previously described (18) with additional information available under supplemental Materials and Methods. For quantification of percent cleaved caspase-3 positive acini, a minimum of 50 MCF-10A-ERBB2 acinar structures were counted per experiment and each experiment was repeated three independent times. Caspase positivity was defined as a structure with 2 or more cleaved caspase-3-positive cells.

**Immunofluorescence of Acini and Image Acquisition**—Acinar structures were prepared as previously described (18). Addi-
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ERBB2 Requires HIF-1 for Tumor Development in Vivo—The MMTV-Neu transgenic mouse is a widely used model of HER2-dependent breast cancer. Carcinomas develop from focal lesions, which after long latency (several months) metastasize to the lung (20). A HIF-dependent transcriptional core signature was recently identified as up-regulated in HER2+ breast tumors as compared with luminal tumors (21), suggesting that HIF activity is enriched in HER2+ tumors. As expected, based on prior reports that HIF-1α protein expression is not detected in the normal breast, but is increased in late-stage metastatic breast cancers (22), expression of HIF-1α was up-regulated in Neu+ hyperplasias compared with the normal mammary gland and was further increased in tumors relative to hyperplasias. Likewise, HIF-2α was not detected in the normal mammary gland, and expression among hyperplastic and tumor samples varied by less than 2-fold. In general, as compared with HIF-1α expression, lower levels of HIF-2α were detected in Neu+ tumors, suggesting that HIF-1 is the primary mediator of the hypoxic response in the breast.

To determine whether HIF-1α was essential for tumor growth, HIF-1α wild type (WT) and knock-out (KO) MTECs were prepared from Hif1α ΔF; MMTV-Neu+ bigenic females. Efficient deletion of the HIF-1α protein was confirmed by Western blotting (Fig. 1B). Of interest, the expression of HIF-2α was increased in HIF-1 KO MTECs cultured at hypoxia, but not at normoxia. WT and KO cells were then transplanted to each cleared inguinal mammary fat pad of FVB/Nj syngeneic recipients to generate WT or KO tumors. The onset of palpable tumors occurred by 35 days post-injection in both genotypes. WT tumors grew faster than KO tumors; the mean tumor volume between genotypes was statistically significant by two-way analysis of variance analysis at days 55 and 60 post-transplant (Fig. 1C). The final mean tumor volume was also calculated by ex vivo caliper measurements (Fig. 1D), confirming that loss of HIF-1 activity reduced final tumor size. Sections from end-stage tumors were immunostained with either Ki-67 or active caspase-3 antibodies to evaluate changes in proliferation or apoptosis. The mean percentage of proliferating tumor cells decreased from 10.5% in WT tumors to 6.1% in KO tumors (Fig. 1D). The percentage of apoptotic cells decreased from 11.4% of cells for WT tumors to 7.9% in KO tumors (supplemental Fig. S1). Thus, HIF-1α is required for ERBB2-mediated tumorigenesis in vivo.

ERBB2-mediated Stabilization of HIF-1 Is Required for Oncogenesis in Vitro—We next sought to verify in vitro whether ERBB2 overexpression results in increased HIF-1α protein expression under normoxic (20% O2) and hypoxic (1% O2) conditions. Indeed, MCF-10A cells expressing constitutively active...
ERBB2 as well as the ERBB2 positive breast cancer line SK-BR-3 demonstrate increased HIF-1α and HIF-2α protein levels in normoxic conditions as compared with control MCF-10A cells (Fig. 2A). Under hypoxic conditions (1% O₂, 6 h) there was also an increase in HIF-1α levels between MCF-10A-ERBB2 and control MCF-10A cells (Fig. 2A). In addition, because cancer cells also stabilize HIF-2α (23), we examined expression of HIF-2α and found elevated levels of HIF-2α at normoxia in MCF-10A-ERBB2 cells compared with parental controls, with little increase in normoxic SK-BR-3 cells (Fig. 2A). However, under hypoxic conditions, all three cell lines expressed similar levels of HIF-2α (Fig. 2A). Next, to assess whether HIF-1 may play a role in ERBB2-mediated transformation in vitro we depleted HIF-1 subunits using RNAi. Members of the HIF family of transcription factors require heterodimerization between a typically oxygen-labile α subunit and an oxygen-independent β subunit (24), thus depleting cells of either subunit will negatively impact the HIF-1 hypoxic transcriptional response. After confirming knockdown efficiency and specificity of siRNA oligonucleotides targeting HIF1α (Fig. 2B, supplemental Fig. S2A) or HIF1β (Fig. 2C), we examined the effect of depleting HIF-1α levels on anchorage independent growth. HIF-1 depletion decreased soft agar colony formation in MCF-10A-ERBB2 cells 3-fold (Fig. 2D) and in SK-BR-3 breast cancer cells 2-fold

Figure 1. MMTV-Neu-derived tumors requires HIF-1 expression for tumor growth in vivo. A, expression of HIF-1α and HIF-2α proteins was compared by Western blotting of HS-WCE (10 µg/lane) prepared from mammary glands of non-transgenic, mature virgin FVB/N mice, or hyperplastic and late-stage carcinomas (tumors 1 and 2, each originating in two independent Hif1a ΔF; MMTV-Neu+/ transgenic females). Blots are representative of 3 normal FVB glands, 3 hyperplasias, and 5 tumors. Tumor 1 contained the least amount of HIF-1α and Tumor 2 the most HIF-1α. The two tumors shown in this blot were included in the pooled tumor material utilized to prepare WT and KO Neu+/ MTECs. WT cells were cultured at hypoxia (HYP) to serve as positive controls for HIF-1α (6 h, 0.5% O₂) and HIF-2α (24 h, 0.5% O₂). Approximately equivalent loading is indicated by a Ponceau S-stained membrane, which showed the hyperplastic sample appears to be overloaded relative to other tissue samples. B, deletion of HIF-1α protein in cultured KO MTECs was confirmed by Western blotting of H5-WCE (10 µg/lane) prepared from cells (80% confluent) cultured in 2% FBS serum/DMEM/F-12 and exposed to 0.5% O₂ for 6 (for HIF-1α) or 24 h (HIF-2α) prior to harvest. Loading is indicated by β-tubulin. C, mean tumor volume of WT and KO tumors plotted over time after transplantation of one million cells to each inguinal mammary fat pad of FVB/Nj recipients. Differences in mean tumor volume between genotypes were significant at days 55 and 60 post-transplant by two-way analysis of variance analysis (*, p < 0.05) (n = 8 recipients/genotype). D, comparison of mean tumor volume derived from ex vivo tumor measurements following tumor harvest on day 62 post-transplant (*, p < 0.05, Student’s t test). E, representative Ki-67 immunostaining of HIF-1α WT and KO end-stage tumors (×630 magnification). The percentage of Ki-67+ tumor cells was determined as described under “Experimental Procedures” (n = 7 tumors/genotype, p < 0.05 Student’s t test).
indicating that HIF-1 expression is critical for ERBB2-mediated anchorage-independent growth. MCF-10A cells overexpressing ERBB2 form multiacinar structures characterized by filled lumens (6, 19) that mimic histological phenotypes of breast cancer lesions in vivo (25). We examined the impact of HIF-1 depletion on three-dimensional acinar growth in ERBB2-overexpressing cells. In the three-dimensional culture, HIF-1 depletion significantly decreased growth of MCF-10A-ERBB2 cells (Fig. 2E) and SK-BR-3 cells (supplemental Fig. S3, A and B) compared with control cells. Thus, HIF-1α levels are elevated in a ERBB2-dependent manner at normoxia and are required for ERBB2-mediated oncogenesis in vivo and in vitro.

**FIGURE 2.** HIF-1 is required for anchorage independent and three-dimensional growth in ERBB2-overexpressing cells. A, MCF-10A, MCF-10A-ERBB2, and SK-BR-3 cells were cultured in normoxic conditions for 24 h or hypoxic conditions (1% O2) for 6 h. Equal amounts of protein were analyzed for levels of HIF-1α, HIF-2α, and ERBB2 through immunoblot analysis. Actin was used as a loading control. B, MCF-10A-ERBB2 cells were transfected with control siRNA oligos or oligos targeting HIF-1α or HIF-1β. 72 h after the initial transfection, equal amounts of protein were assayed for levels of HIF-1α through immunoblot analysis. Actin was used as a loading control. C, total mRNA from cells in B were subjected to qRT-PCR analysis to determine the relative levels of HIF-1α and HIF-1β compared with control RNAi-transfected cells. Cyclophilin A was used as an internal control. D, cells in B were cultured in soft agar colony formation assay and allowed to grow for 14 days at which point colonies were stained and counted. Inset, representative images of colony growth. E, after 8 days of growth in three-dimensional culture, cells were trypsinized and counted. Inset, representative images of cells at ×5 magnification. All histograms represent data from at least three independent experiments. Error bars indicate S.E. (*, p < 0.05).
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We also note that whereas HIF-1α-depleted cells show no change in cell death in the attached state, depletion of HIF-1β in ERBB2-overexpressing cells increases cell death regardless of the state of cell attachment compared with control and HIF-1α-depleted cells (Fig. 3A). Because HIF-1β is a common dimerization partner of both HIF-1α and HIF-2α (24), depletion of this subunit would negate both HIF-1- and HIF-2-mediated survival signaling that occurs during attached conditions in the normoxic state. Similarly, HIF-1α depletion induces cell death and BIM expression in attached SK-BR-3 cells (supplemental Fig. S2, D and E). SK-BR-3 cells weakly express stabilized HIF-2α under normoxic conditions (Fig. 2A) thus, it is possible that depletion of HIF-1α alone is sufficient to impair HIF-mediated survival signaling that contributes to survival in the attached state in this cell line. Importantly, cell death occurring in attached cells as a result of HIF-1β depletion in MCF-10A-ERBB2 cells and HIF-1α depletion in SK-BR-3 cells is significantly reduced compared with the corresponding levels of cell death under detached conditions (Fig. 3A, supplemental Fig. S2E). This suggests that whereas loss of HIF influences cell survival in the presence of cell-ECM engagement, HIF is of particular importance for survival of ERBB2-overexpressing cancer cells in the absence of adhesion.

Decreased three-dimensional growth also correlates with enhanced apoptosis as evidenced by a 2-fold increase in the number of caspase-3-positive acinar structures in MCF-10A-ERBB2 cells depleted of HIF-1 (Fig. 3, C and D). Confocal analysis of acinar structures suggest that apoptosis predominantly occurs in HIF-1-depleted cells without contact to ECM (Fig. 3D) consistent with the idea that loss of HIF-1 sensitizes to anoikis. In addition, loss of HIF-1 significantly decreases cell proliferation in acini as measured by Ki-67 positivity (supplemental Fig. S4), indicating that HIF-1 loss inhibits survival and growth in the context of ERBB2 overexpression.


![Graph](https://example.com/graph.png)

**FIGURE 3. HIF-1 depletion in ERBB2-overexpressing cells promotes anoikis and apoptosis in three-dimensional culture.** MCF-10A-ERBB2 cells were transfected with control siRNA oligos or oligos targeting HIF-1α or HIF-1β. 72 h after the initial transfection, cells were used for anoikis assay or cultured in three-dimensional morphogenesis assay. A, cells were cultured in attached or suspended conditions for 48 h then stained for Annexin V/propidium iodide and assessed for relative level of cell death by FACS analysis. B, cells were cultured in attached or suspended conditions for 48 h, then lysed. Equal amounts of proteins were assessed for levels of BIM. Actin was used as a loading control. C, after 4 days of growth, cells were fixed and stained for cleaved caspase-3 (green) and DAPI (blue). For each population, the percent of cleaved caspase-3 positive acini was determined. D, representative images of cells described in C at ×20 magnification. All histograms represent data from at least three independent experiments. Error bars represent S.E. (*, p < 0.05; **, p < 0.01).
(supplemental Fig. S3C), perhaps because of a lack of HIF-2-mediated survival signals that may serve to attenuate cell death in the absence of HIF-1. We also compared activation of the ERK pathway in wild type (WT) versus HIF-1α KO tumors by conventional (Fig. 4C) and infrared-based, quantitative (Fig. 4D) Western blotting. Likewise, the activation of pERK1/2 is decreased in MMTV-Neu KO tumors compared with WT tumors (Fig. 4, C and D). In addition, using the same samples as

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**FIGURE 4.** HIF-1 is required for ERBB2-mediated ERK and AKT oncogenic signaling. A, MCF10A-ERBB2 overexpressing cells were transfected with control siRNA oligos or oligos targeting HIF1A or HIF1B. 72 h after initial transfection, cells were assayed as indicated. MCF-10A-ERBB2 cells were placed in attached (Att) or suspended cultures (48) for 48 h, at which point, cells were lysed and analyzed by immunoblot analysis for levels of the indicated phospho- and total proteins. B, MCF-10A-ERBB2 cells were cultured in a three-dimensional morphogenesis assay until day 4 when cells were lysed and assayed for levels of the indicated phospho- and total proteins through immunoblot analysis. C, the levels of pERK1/2 were compared in WT and HIF-1 KO Neu end-stage tumors by Western blotting; two tumors per genotype are shown, and the blot is representative of 5 tumors blotted per genotype. /Tubulin is included as a loading control. D, the decreased expression of pERK1/2 was validated by quantitation following infrared Western blotting after normalization to β-tubulin levels. The mean normalized intensity of pERK1 (p44) and pERK2 (p42) among tumors was significantly decreased in KO tumors as compared with WT tumors (n = 4 WT and 5 KO tumors, Student’s t test). E, the enrichment of pERK1/2 in HIF-1 WT tumors is not rescued by compensatory increases in HIF-2α protein observed in the majority of KO tumors. The same extracts used in D were blotted and probed for HIF-1α, HIF-2α, and β-tubulin.
profiled for pERK1/2 levels, we observed that the majority of KO tumors up-regulated HIF-2α in response to HIF-1 deletion (Fig. 4E), therefore, we conclude that ERBB2-mediated pERK1/2 activity is predominantly HIF-1-dependent. Overall, HIF-1α is required for optimal ERBB2-mediated signaling.

**ERBB2-mediated Metabolic Regulation during Detachment Requires HIF-1**—In addition to loss of cell signaling and induction of BIM expression, recent studies have indicated that metabolic defects arise in nontransformed epithelial cells during cell detachment including decreased glucose uptake, ATP levels, and elevated ROS levels and must be overcome for cells to survive (8). In MCF-10A cells, apoptosis induced by low adhesion can be rescued by introduction of oncogenes, such as ERBB2 (5), or with antioxidant treatment alone, which suppresses the accumulation of ROS that accompanies cell detachment (8). As HIF-1 is a well known regulator of ROS and metabolic stress (26), we investigated the effect of HIF-1 depletion on ERBB2-mediated suppression of ROS and maintenance of metabolic pathways during ECM detachment. Indeed, depletion of HIF1A or HIF1B reverses ERBB2-mediated ROS inhibition in cells cultured in suspension, restoring ROS levels to those similar to detached MCF-10A control cells (Fig. 5A). Moreover, loss of HIF-1 also inhibited ERBB2-mediated glucose uptake (supplemental Fig. S5A) and ATP maintenance (supplemental Fig. S5B) in cells detached from ECM. In light of the HIF-1-dependent nature of ERBB2-mediated ROS suppression, we next questioned what role this suppression plays in ERBB2-mediated transformation. Treatment of HIF-1-depleted MCF-10A-ERBB2 cells with the ROS scavenger compound Trolox was able to restore both anchorage-independent (Fig. 5, B and C) and three-dimensional growth (Fig. 5, D and E), highlighting the significance of the antioxidant capacity of HIF-1 in promoting ERBB2-mediated oncogenesis.

**Identification of Genes Up-regulated by ERBB2 in a HIF-dependent Manner**—MCF-10A cells cultured under normoxic conditions form hollow, well organized structures in the three-dimensional culture with localization of integrin α6 on the basal surface of cells and integrin α5 at sites of cell-cell contact...
In contrast, MCF-10A cells exposed to hypoxia exhibit a lack of proper lumen formation as well as mislocalization of integrin α5 staining (Fig. 6A), a phenotype resembling MCF-10A-ERBB2 cells (Fig. 6A). This phenotypic similarity is complemented by the similar signaling patterns for anoikis-resistant, hypoxic MCF-10A cells and MCF10A-ERBB2 cells, both of which maintain MEK/ERK signaling under conditions of low adhesion, resulting in suppression of the pro-anoikis BH3-only protein BIM and cell death (5, 14).

Although ERBB2-mediated MEK/ERK signaling, BIM inhibition, and subsequent anoikis suppression during mammary morphogenesis has been established to inhibit luminal clearance (18), the signals that drive hypoxia-mediated alterations in mammary morphogenesis are not clear. In an effort to identify factors involved in hypoxia-mediated disruption of morpho-
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genesis, we performed a microarray analysis comparing gene expression profiles of normoxic and hypoxic acinar structures. We found 102 genes up-regulated and 240 genes down-regulated at least 2-fold (supplemental File S1). When analyzing the top 44 genes induced at least 3-fold in response hypoxia, we identified a subset of genes (25% of total genes) that have previously been linked with ERBB2 expression. These genes include ITGA5 (integrin α5) (19), ANGPTL4 (angiopoietin-like 4) (27), MNK2 (MAP kinase-interacting serine-threonine kinase protein 2) (28), CAR9 (carbonic anhydrase IX) (29), LDHA (lactate dehydrogenase A) (30), NDRG1 (N-Myc down-stream regulated gene 1), ET2 (etothelin 2), LGALS1 (galectin 1), VIM (vimentin) (31), LOX (lysyl oxidase), and LOXL2 (lysyl oxidase-like 2) (32) (supplemental Table S1).

Two of the well established ERBB2 targets, ITGA5 and ANGPTL4, displayed >5- and 50-fold induction in normoxic MCF-10A-ERBB2 cells as compared with parental controls (Fig. 6B), therefore, we analyzed expression levels of additional genes induced by hypoxia in MCF10A parental cells that were also induced in response to ERBB2 overexpression at normoxia. Indeed, RNA levels of MNK2, BNIP3L, ADM, and HIF1A were also significantly enhanced in MCF-10A-ERBB2 cells as compared with parental MCF-10A controls (Fig. 6B). Interestingly, HIF2A was not up-regulated at the RNA level in cells overexpressing ERBB2 (data not shown) suggesting differential regulation of HIF1A and HIF2A by ERBB2. We then tested whether the induction of these target genes in MCF-10A-ERBB2 cells is dependent upon HIF-1 activity. ITGA5 (33), ANGPTL4 (34), BNIP3L (35), and ADM (adromedullin) (36) have each previously been established as hypoxia-inducible genes. Up-regulation of these genes in response to ERBB2 overexpression occurs in a HIF-1-dependent manner under normoxic conditions because deletion of either HIF1A or HIF1B reduced gene expression (Fig. 6C). Moreover, the expression of both ANGPTL4 and MNK2 were almost exclusively dependent upon HIF-1 activity because deletion of either HIF1A or HIF1B blocked expression. In contrast, down-regulation of ADM was more prominent for HIF1B than for HIF1A deletion, suggesting a predominant role for HIF-2α in regulation of ADM in MCF10A-ERBB2 cells. In addition, we confirmed that ADM, BNIP3L, ITGA5, and ANGPTL4 are regulated by HIF-1α in SK-BR-3 cells as RNAi to HIF1A reduced expression of all four genes (data not shown). We identify BNIP3L and ADM as novel ERBB2-regulated genes (Fig. 6B) and MNK2 as a novel HIF-1-regulated gene co-regulated by ERBB2 (Fig. 6C). These data suggest a significant role for HIF-1 in transcriptional gene modulation in the context of ERBB2 overexpression and suggest that either hypoxia or the overexpression of ERBB2 may exhibit similar phenotypic and transcriptional responses in mammary epithelial cells via HIF-1 activity.

DISCUSSION

Oncogenic ERBB2 is a well known mediator of anoikis resistance in mammary epithelial cells; however, the molecular mechanisms underlying the ability of ERBB2-positive cells to survive in the absence of proper ECM signals have yet to be fully elucidated. Here, we implicate HIF-1 as a critical regulator of anoikis resistance in the context of ERBB2 overexpression. Although accumulating evidence suggests a role for HIF-1 in blocking detachment-induced cell death in both the hypoxic (14) and normoxic states (37), a clear mechanism linking HIF-1 to anoikis-associated signaling has not previously been defined. Our data support a model wherein ERBB2 regulation of HIF-1α expression facilitates cell survival in the absence of adhesion in at least two ways: 1) by allowing sustained MEK/ERK signaling and subsequent inhibition of the pro-apoptotic BH3-only protein BIM, and 2) by maintaining metabolic flux, thus suppressing induction of ROS, to maintain the cellular redox state. Therefore, we propose hypoxia-independent oncogenic stabilization of HIF-1 in breast cancer lesions is a mechanism through which tumor cells hijack signaling pathways inherent to cell survival during detachment to become anoikis resistant, thereby supporting both early tumor development and malignant progression.

Maintenance of MEK/ERK signaling downstream of integrin and growth factor receptors serves as a negative regulator of BIM expression in mammary epithelial cells in the attached state (5). Both hypoxia and oncogenic ERBB2 act to promote anoikis resistance through maintenance of MAPK signaling and suppression of BIM expression under conditions of low adhesion (14, 18). Here, we demonstrate that ERBB2-mediated anchorage-independent survival, much like that mediated by hypoxia, requires expression of HIF-1α. We have previously shown that hypoxia via induction of HIF-1α can maintain ERK signaling in suspension, block BIM and Bmf expression, and induce anoikis resistance in immortalized mammary epithelial cells (14). Taken together, these findings implicate HIF-1 as a positive modulator of ECM-mediated cell survival signaling, specifically at the level of the ERK-BIM axis, in mammary epithelial cells, regardless of oxygen and transformation status.

The mechanism of MAPK maintenance by HIF in mammary epithelial cells in the context of hypoxia and ERBB2 overexpression is yet to be determined. However, the microarray analysis in hypoxic cells, coupled with HIF depletion studies in the context of ERBB2 overexpression, has uncovered several potential mechanisms. Here, we demonstrate that ANGPTL4 is an ERBB2 target that is also up-regulated in a HIF-dependent manner. This adipocyte-secreted serum hormone has recently been implicated as a mediator of HIF-1-dependent primary tumor growth and breast tumor metastasis through regulation of endothelial cell attachment (34, 38). Convergence of ANGPTL4- and ERBB2/HIF-mediated anoikis resistance signaling pathways as well as the greater than 50-fold induction of ANGPTL4 in response to ERBB2 overexpression, which appears to be entirely HIF-dependent, presents ANGPTL4 as an attractive candidate for further studies as a potential regulator of ERBB2-mediated anchorage-independent survival. Moreover, as both ANGPTL4 (39) and ITGA5 (19) have been independently linked to anoikis resistance, targeting both of these pathways may be a novel way to treat metastatic progression.

We also report that the serine/threonine kinase MNK2 is a novel HIF-1 target that displays enhanced expression in response to ERBB2 overexpression. Although the specific role of MNK2 in breast cancer progression is not fully understood, increased phosphorylation and activity of MNKs correlate with
ERBB2 overexpression in breast cancer cells (28) and inhibiting MNK2 along with the closely related kinase MNK1 blocks colony formation in ERBB2-positive breast cancer cells (28). We are the first to report MNK2 as a HIF-1-regulated gene although future studies must be done to determine whether the regulation is through direct HIF-1 binding to the MNK2 promoter. In addition, our data suggests ADM may be predominantly regulated by HIF-2α as reducing HIF-1β levels in ERBB2-expressing cells significantly reduced expression of ADM compared with HIF-1α. Understanding differential regulation of gene expression between HIF-1α and HIF-2α in the context of ERBB2 may reveal novel therapeutic targets for therapy.

Resistance to existing cancer therapies is characteristic of both ERBB2-positive (40, 41) and hypoxic tumors (22, 42), the latter of which is proposed to occur in a HIF-dependent manner (43). Given the fact that HIF-1α and HIF-2α are stabilized in a wide variety of cancers in both hypoxia-dependent and hypoxia-independent manners, targeting of these molecules therapeutically has been the source of much investigation (24). In the case of ERBB2, targeted therapies including Hereceptin have been shown to display initial clinical efficacy in tumor treatment; however, multiple modes of resistance have been established to occur with prolonged treatment. One study asserts that Hereceptin treatment exhibits many of its anti-tumoral effects by abolishing HIF-1 levels (12). Our data suggests that the interplay between ERBB2, HIF-1, and BIM may be important in chemoresistance. ERBB2 is a known regulator of BIM in mammary epithelial cells (5, 18) and our data suggest that HIF-1 plays a role in ERBB2-mediated inhibition of BIM in cancer cells. Recent evidence showing that BIM expression predicts responsiveness to kinase inhibitors in HER2-amplified cancers (44) suggests that increased HIF-1 expression in these tumors may provide protection against kinase inhibitors in normoxic and also hypoxic conditions. However, it is clear that HIF-2α may, in some cases, compensate for chronic loss of HIF1α as we show levels of HIF-2α elevated in HIF-1A KO MTEC (Fig. 4E). This compensation may explain why we detect differences in survival between in vivo and in vitro reduction of HIF-1α in cancer cells. Nevertheless, reducing HIF-1α in both cases blocks ERBB2-mediated oncogenesis thus HIF-1α is a dominant isoform for ERBB2-driven tumors.

Recent evidence suggests that oncogenes such as ERBB2 must rescue metabolic defects caused by lack of matrix attachment including maintaining glucose uptake that allows production of antioxidant NADPH through the pentose phosphate pathway leading to inhibition of ROS levels and maintenance of ATP levels in suspension (8). Our results indicate that HIF-1 is, in part, required for ERBB2-mediated metabolic changes during detachment as suppression of HIF-1 reversed the ERBB2-mediated maintenance of glucose uptake, ATP, and ROS levels in suspension. Interestingly, PI3K activation is required for ERBB2-mediated metabolic rescue under conditions of low adhesion (8), which is consistent with the ability of ERBB2 via AKT to stabilize HIF-1 levels in the cancer cell (12). Moreover, as HIF-1 is well known to promote aerobic glycolysis in cancer cells (45) we propose that ERBB2 may stabilize and require HIF-1 for this metabolic switch that is required for survival and growth in the absence of ECM.

The role of oxidative stress in carcinogenesis is complex as many tumors exhibit enhanced ROS levels that are believed to promote tumor initiation and maintenance through genetic instability, oncogenic activation, proliferation, angiogenesis, metastasis, and immune cell invasion. The induction of ROS in tumor cells also correlates with cell death through apoptosis, necrosis, and autophagy as well as reversal of chemoresistance (46). Survival during detachment in ERBB2-overexpressing mammary epithelial cells involves suppression of intracellular ROS (8), which our data establish as being, in part, HIF-1-dependent. Moreover, our data support the idea that transformation in the context of ERBB2 overexpression is heavily reliant on HIF-mediated maintenance of cellular metabolism and oxidative homeostasis in states of low adhesion. Regulation of ROS by HIF-1 in MEFs is attributed to the direct up-regulation of PDK1 (47), which aids in inhibition of oxidative phosphorylation and BNIP3 (48), a BH3-only protein that is critical for hypoxia-driven autophagy (49). Interestingly, we identify BNIP3L, a BH3-only protein that has also been established as a HIF-1 target and acts in concert with BNIP3 to stimulate autophagy under hypoxic conditions (49), as up-regulated in ERBB2-overexpressing cells in a HIF-1-dependent manner. This observation suggests a possible role for BNIP3L in mediating ERBB2-dependent autophagy and anokis resistance.

In summary, we identify HIF-1 as a novel regulator of ERBB2-mediated oncogenesis, anokis resistance, and maintenance of cellular redox and metabolic status under conditions of cell detachment. A key question that remains to be addressed is the direct mechanism through which HIF-1 promotes activation of ERK signaling to block anokis induction in the absence of adhesion. Moreover, it will be interesting to examine the possibility that genes identified as targets of both HIF-1 and ERBB2 may serve as novel therapeutic targets for the treatment of HIF-positive and ERBB2-positive breast cancers, independent of oxygen tensions in the tumor microenvironment.

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