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Hypoxia Suppression of Bim and Bmf Blocks Anoikis and Luminal Clearing during Mammary Morphogenesis

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Proper adhesion to extracellular matrix is critical for epithelial cell survival. Detachment from matrix signals results in apoptosis, referred to as anoikis. Selective apoptosis of cells that become detached from matrix is associated with the formation of a lumen in three-dimensional mammary epithelial acinar structures in vitro. Because early breast cancer lesions such as carcinoma in situ, characterized by ducts exhibiting lumens filled with cells, are often associated with hypoxic markers, we sought to examine the role of hypoxia in anoikis and lumen formation in mammary epithelial cells. Here, we show that hypoxic conditions inhibit anoikis and block expression of proapoptotic BH3-only family members Bim and Bmf in epithelial cells. Hypoxia-mediated anoikis protection is associated with increased activation of the epidermal growth factor receptor–mitogen-activated protein kinase kinase–extracellular signal-regulated kinase (Erk) kinase pathway and requires the hypoxia-activated transcription factor. Consistent with these data, hypoxic conditions inhibit luminal clearing during morphogenesis in human mammary epithelial acini when grown in three-dimensional cultures and are associated with decreased expression of Bim and Bmf as well as Erk activation. We show that hypoxia regulates specific cell survival pathways that disrupt tissue architecture related to clearing of luminal space during mammary morphogenesis and suggest that hypoxia-mediated anoikis resistance may contribute to cancer progression.

INTRODUCTION

Epithelial cell adhesion to extracellular matrix (ECM) is critical in dictating cell fate, including cell survival. Loss of adhesion of normal epithelial cells leads to apoptosis, referred to as anoikis (Gilmore, 2005). Anoikis plays a fundamental role in maintaining tissue homeostasis, because cells that lack cell matrix attachment or have incorrect ECM signals will undergo cell death to maintain tissue integrity (Gilmore, 2005). In vivo, anoikis is believed to play a role in sculpting of cavities in epithelial tissues during morphogenesis of mammary and salivary glands (Gilmore, 2005; Mailleux *et al*., 2008). Moreover, alteration in normal anoikis mechanisms is known to enhance oncogenesis, including tumor metastasis, because anoikis resistance allows cells to survive in inappropriate ECM environments.

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Abbreviations used: 3D, three dimensional; ADM, adrenomedullin; BH3, Bcl-2-homology domain 3, DCIS, ductal carcinoma in situ; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; HIF, hypoxia-inducible factor; HMEC, human mammary epithelial cell.

Three-dimensional (3D) epithelial culture systems that allow cells to organize into duct-like structures, recapitulating their in vivo architecture, have provided cell-based models that facilitate the study of anoikis in a more biologically relevant context compared with cell detachment assays (Schmeichel and Bissell, 2003). Normal mammary epithelial cells under 3D culture conditions proliferate and organize into spheroids, characterized by the presence of a centrally localized hollow lumen and the polarization of cells surrounding this lumen. During 3D morphogenesis of the spontaneously immortalized human mammary epithelial cell line MCF-10A, the hollow lumen is created via selective apoptosis of centrally located cells that are not in direct contact with ECM and therefore die via anoikis (Debnath *et al*., 2002). This morphogenetic process in vitro is similar to development of the hollow ductal system that occurs during mammary gland development in vivo (Reginato and Muthuswamy, 2006). Previous studies have demonstrated the requirement for Bcl-2-homology domain 3 (BH3)-only family proteins Bim (Reginato *et al*., 2003, 2005) and Bmf (Schmelzle *et al*., 2007) in MCF-10A anoikis and lumen formation during morphogenesis. In addition, Bim was found to be required for apoptosis during lumen formation in terminal end buds during mouse mammary gland ductal morphogenesis (Mailleux *et al*., 2007), confirming the role of Bim as critical regulator of mammary morphogenesis in vivo. Moreover, MCF-10A cells overexpressing oncogenes implicated in breast cancer, including the receptor tyrosine kinase ErbB2, block anoikis during 3D morphogenesis, in part, by inhibiting expression of Bim (Reginato *et al*., 2005) and Bmf (Schmelzle *et al*., 2007). This leads to disorganized acini structures containing filled lumens that resemble early premalignant breast cancer lesions, including ductal carcinoma

in situ (DCIS) (Muthuswamy *et al*., 2001). Because oncogenes found to be overexpressed in DCIS block anoikis, BH3-only proteins, and luminal clearing during mammary morphogenesis in vitro, we have examined other potential factors associated with early premalignant lesions in regulating anoikis resistance.

One critical prognostic indicator in early breast cancer lesions is decreased oxygen levels, known as hypoxia. Hypoxic cancers are generally associated with aggressive growth, metastasis, and poor response to radiation treatment and chemotherapy (Goonewardene *et al*., 2002; Harris, 2002). In early lesions of breast cancer, including DCIS, hypoxic markers such as hypoxia inducible factor (HIF)-1 are increased and correlate with poor architectural and cellular differentiation (Helczynska *et al*., 2003). Indeed, HIF-1α levels are increased from low levels in normal breast and ductal hyperplasias to high levels in the majority of DCIS and invasive cancers (Bos *et al*., 2001), implicating hypoxia as a critical event in breast cancer progression. More recently, studies have shown that HIF-1α expression predicts poor therapeutic response and clinical outcome in human breast cancers (Generali *et al*., 2006). Although the mechanism linking hypoxia to poor prognosis is unclear, cells expressing HIF-1 are able to escape hypoxia-induced cell death and acquire sustained resistance to apoptotic signals (Graeber *et al*., 1996).

Very little is known about what role, if any, hypoxia plays in regulating anoikis or changes in tissue architecture relating to breast cancer progression. In this study, we examine the effect of hypoxia on anoikis, related signaling, and use the 3D tissue culture system to examine how exposure to hypoxia influences mammary ductal architecture and lumen formation.

MATERIALS AND METHODS

Cell Culture and Materials

MCF-10A, MCF-7, and RWPE cells were obtained from the American Type Culture Collection (Mannassas, VA) and maintained according to American Type Culture Collection instructions. In brief, MCF-10A and RWPE cells were cultured in DMEM/F-12 (Invitrogen, Carlsbad, CA) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor ([EGF]Peprotech, Rocky Hill, NJ), 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 1 ng/ml cholera toxin (Sigma-Aldrich), 100 μg/ml hydrocortisone (Sigma-Aldrich), and 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen). MCF-7 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 50 U/ml penicillin and 50 μg/ml streptomycin. Human mammary epithelial cells (HMECs) were obtained from Cambrex (East Rutherford, NJ) and maintained according to the manufacturer's protocol. Poly-HEMA and methylcellulose were purchased from Sigma-Aldrich. Growth factor-reduced Matrigel was purchased from BD Biosciences (San Diego, CA). U0126, LY294002, and deferoxamine mesylate (DFOM) were purchased from Calbiochem (San Diego, CA). AG1478 was purchased from Invitrogen. All chemical compounds were added to attached cells 6–24 h after plating and added directly to cells placed in suspension. Anti-Bim was purchased from Assay Designs (Ann Arbor, MI). Anti-Bmf was purchased from Sigma-Aldrich. Anti-actin and anti-extracellular signal-regulated kinase (Erk) 2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HIF-1α and anti-integrin α6 (CD49f) were purchased from BD Biosciences. Anti-Akt, anti-cleaved caspase-3 (Asp175), anti-mitogen-activated protein kinase kinase (Mek)1/2, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-phospho-Mek1/2 (Ser217/221), anti-phospho epidermal growth factor receptor (EGFR; Y992), anti-phospho-RSK (Thr360/Ser364), and anti-RSK were purchased from Cell Signaling Technology (Danvers, MA). Anti-phospho-Erk1/2 (pTpY185/187) and anti-phalloidin Alexa-Flour 488 were purchased from Invitrogen. Anti-EGFR was purchased from BD Biosciences Transduction Laboratories (Lexington, KY). Anti-integrin α5 (CD49e) was purchased from BD Biosciences Pharmingen (San Diego, CA). Anti-E-cadherin and anti-β-catenin were purchased from BD Biosciences Transduction Laboratories.

Hypoxic Treatment

Attached and suspended cells were placed into a humidified hypoxic chamber (Coy Laboratory, Grass Lake, MI) preequilibrated to 1.0% O_2, 5.0% CO_2 at 37°C. Oxygen levels in the hypoxic chamber were verified using an independent oxygen sensor. Attached cells were put into chamber 6–24 h after plating and lysed in the chamber. Suspended cells were immediately placed into chamber then removed at indicated times, immediately pelleted, washed, and processed as required for each assay at indicated times. MCF-10A cells cultured in 3D were put into the hypoxic chamber 6 d after plating. To collect RNA and protein, cells were trypsinized in the chamber then removed, immediately pelleted, washed, and lysed at indicated times. For immunofluorescence staining, cells were removed from the hypoxia chamber at indicated times then immediately fixed. Any experimental chemical compounds were added to cells immediately before placement in hypoxic chamber.

Annexin V/Propidium Iodide (PI) Apoptosis Assay

Attached or suspended cells were cultured for 36–72 h and then stained using the Annexin V-FITC apoptosis detection kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. Cells were assessed for staining using a Guava PCA-96 flow cytometer (Millipore, Billerica, MA) and analyzed using Guava CytoSoft 5.3 software (Guava Technologies, Hayward, CA). Each sample was processed in duplicate, and each experiment was performed at least three independent times.

3D Morphogenesis Assay

Assays were performed as described previously (Haenssen *et al*., 2010). In brief, MCF-10A cells were resuspended in assay medium (DMEM/F-12 supplemented with 2% horse serum, 10 μg/ml insulin, 1 ng/ml cholera toxin, 100 μg/ml hydrocortisone, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5 ng/ml EGF). Individual wells of eight-well chamber slides (Falcon; BD Biosciences Discovery Labware, Bedford, MA) were each coated with 45 μl of Matrigel, and 5000 cells were plated in each well in assay medium supplemented with 2% Matrigel. Cells were fed with assay medium containing 2% Matrigel every 4 d. For investigations using chemical inhibitors, the assay was performed as described until the medium was replaced with assay medium supplemented with 2% Matrigel and dimethyl sulfoxide (1:1000) or U0126 (10 μM) for the indicated times. Structures were stained and subject to confocal analysis as described to obtain images. For quantification of percentage of filled acini or percentage of cleaved caspase-3–positive acini, a minimum of 100 MCF-10A acinar structures were counted per experiment, and each experiment was repeated three independent times. Filled acini were characterized as any structure with two or more cells present in the luminal space. Caspase positivity was defined as a structure with two or more cleaved caspase-3–positive cells.

Immunofluorescence and Image Acquisition

Acinar structures were prepared as described previously (Haenssen *et al*., 2010). In brief, acini were fixed with 4% Formalin for 20 min at room temperature. Fixed structures were washed with phosphate-buffered saline (PBS)-glycine (130 mM NaCl, 7 mM Na_2HPO_4, and 100 mM glycine) three times for 10 min. The structures were then blocked using IF buffer (130 mM NaCl, 7 mM Na_2PO_4, 7.7 mM NaN_3, 0.1% bovine serum, 0.2% Triton X-100, and 0.05% Tween 20) plus 2% goat serum for 1–1.5 h. Structures were then incubated with a secondary blocking buffer (IF buffer supplemented with 10% goat serum and 20 μg of goat anti-mouse F(ab')/ml) for 40 min. Structures were incubated with primary antibodies diluted in secondary blocking buffer overnight at 4°C. After three 20-min washes with IF buffer, structures were incubated with anti-mouse or anti-rabbit secondary antibodies coupled with Alexa-Fluor dyes (Invitrogen) diluted in IF buffer supplemented with 10% goat serum for 1 h at room temperature. After incubation with secondary antibodies, structures were washed three times with IF buffer then incubated with 0.5 ng/ml 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) or TO-PRO-3 iodide (642/661; Invitrogen) for 10 min at room temperature. Structures were washed again for 5 min with IF buffer before mounting with the antifade agent Prolong (Invitrogen). Confocal analysis was performed using a DM6000 B confocal microscope (Lecia Microsystems, Deerfield, IL). Images were generated using the confocal imaging software system (Leica Microsystems) and converted to Tiff format.

Detachment-induced Apoptosis Assay

MCF-10A cells were placed in suspension in growth media as described previously (Haenssen *et al*., 2010). In brief, tissue culture plates were coated with poly-HEMA (6 mg/ml), incubated at 37°C until dry, and washed with PBS before use. Cells were suspended in growth medium in the presence of 0.5% methylcellulose (to avoid potential survival effects caused by cell clumping) in suspension at a density of 500,000 cells/ml and plated on poly-HEMA–coated 60-mm plates for the indicated time. Cell suspension was then removed from the plate, and wells were washed with 1× PBS to collect any residual cells. Cells pellets were obtained through centrifugation then washed twice with 1× PBS and processed for specific assays.

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cells using the RNeasy mini kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Equal amounts of total

RNA (250 ng) were added to Brilliant II QRT-PCR master mix (Stratagene, La Jolla, CA) with primer/probe sets purchased from Applied Biosystems (Foster City, CA). PCR reactions were performed in a volume of 25 μl using an MX 3000 machine, and analysis was performed using the MxPro software according to the manufacturer's instructions (Stratagene). Gene and catalog numbers for the primer/probe sets are as follows: Bim (Hs00197982_m1), Bmf (Hs00372937_m1), HIF-1β/ARNT (Hs01121918_m1), adrenomedullin (ADM; Hs00181605_m1). Expression of the housekeeping gene cyclophilin A (Hs99999904_m1) was used as an internal loading control. Each reaction was performed at least three independent times. Data are represented as a fold change between samples.

Immunoblot Analysis

Cell lysates from 2 to 5×10^6 MCF-10A cells were harvested from attached or suspended conditions and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF, and 1 mM Na_3VO_4) supplemented with 1 μg each of pepstatin, leupeptin, aprotinin, and 200 μg/ml phenylmethylsulfonyl fluoride. Lysates were homogenized by passing through a 27-gauge needle then cleared by centrifugation ay 16,000 $\times$ *g* for 20 min at 4°C and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. Lysates were collected from 3D structures as described previously (Haenssen *et al*., 2010). In brief, acini were washed with PBS then incubated with RIPA lysis buffer for 15 min at 4°C. Lysates were homogenized and cleared as described above, and then proteins were analyzed by SDS-PAGE and autoradiography. Quantitation of bands from autoradiograms was performed by determining the density of the bands using EZQuant-Gel software (EZQuant, Tel-aviv, Israel). All bands were normalized to actin.

RNA Interference (RNAi)

Small interfering RNA (siRNA) transfections were carried out as described previously (Reginato *et al*., 2003). In brief, MCF-10A cells were seeded onto six-well plates at 200,000 cells/well. After 24 h, cells were transfected with double-stranded RNA-DNA hybrids at a final concentration of 1 μg of annealed oligonucleotides using Oligofectamine (Invitrogen) according to manufacturer's instructions. Cells were retransfected as described at 48 h. At 76 h after initial transfection, cells were placed in anoikis assays or processed for SDS-PAGE analysis at indicated times. Initial siRNA oligonucleotides (SMARTpool) were obtained from Dharmacon RNA Technologies (Lafayette, CO) for HIF-1α. Single siRNAs were identified with the highest level of knockdown and purchased from Dharmacon RNA Technologies: control, sense (luciferase) 5′-(GGCUCCCGCUGAAUUGGAAUU)d(TT)-3; and HIF-1α, sense 5′-(GGGUAAAGAACAAACACAUU)d(TT)-3′. siRNA targeting HIF-1β (ARNT) were purchased from Ambion (Austin, TX): HIF-1β, sense 5′-GGGCGUAUCCUGGAUCUAATT-3′.

Statistical Analysis

All data are reported as mean ± SEM. Continuous variables were compared using unpaired *t* tests.

RESULTS

Hypoxia Inhibits Anoikis of Normal Epithelial Cells and Blocks Expression of Bim and Bmf

For our experiments, we used 1% O_2 for hypoxic conditions because this level of oxygen has been found in tumor microenvironment (Hockel and Vaupel, 2001b). Exposure to the defined hypoxic parameters induces stabilization of HIF-1α (Figure 1A), a critical mediator of the cellular response to hypoxia. In addition, MCF-10A cells cultured at 1% oxygen exhibit a >10-fold induction of the established hypoxia-responsive gene and HIF-1 target ADM (Figure 1B)

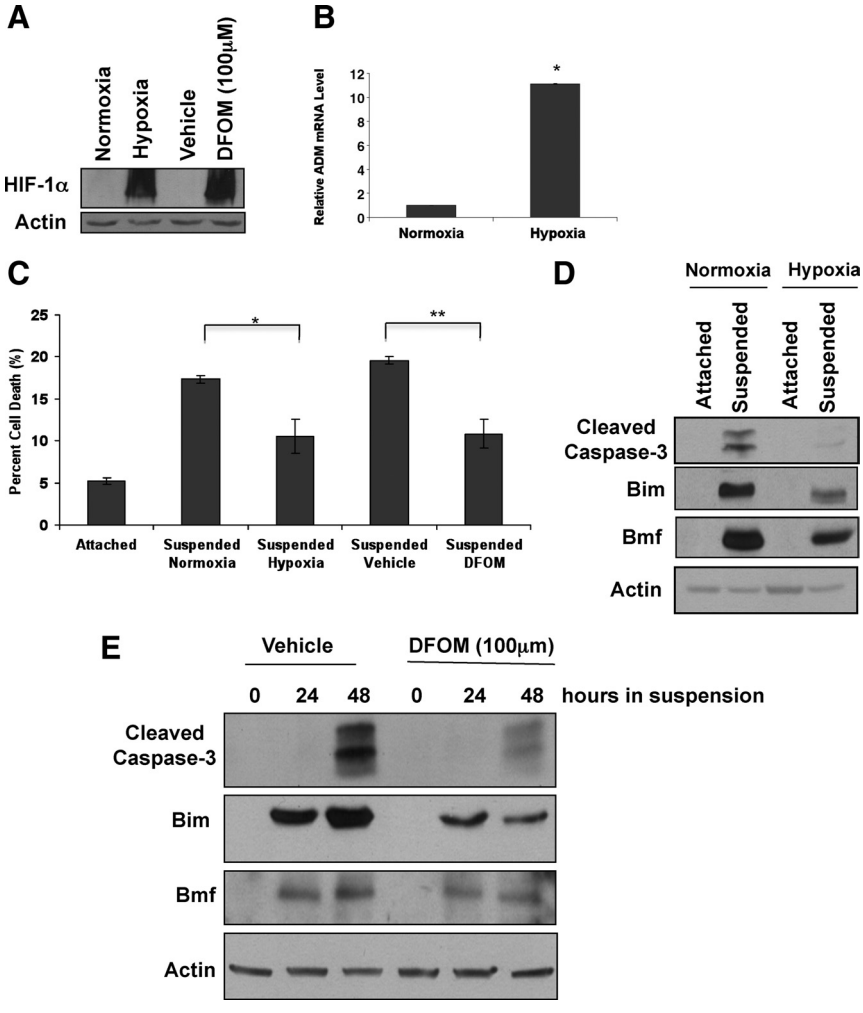

Figure 1. Hypoxia blocks anoikis and Bim and Bmf expression. (A) Attached MCF-10A cells were incubated at 20% (normoxia) or 1% O_2 (hypoxia) for 6 h and then lysed and subjected to immunoblot analysis to determine level of HIF-1α induction. (B) Attached MCF-10A cells were cultured under normoxic or hypoxic conditions for 24 h and then lysed for total mRNA. Equal amounts of total mRNA were analyzed by quantitative RT-PCR to determine relative levels of the hypoxic target ADM. (C) Attached or suspended MCF-10A cells were incubated under normoxic or hypoxic conditions or treated with vehicle or DFOM for 36 h and assessed by fluorescence-activated cell sorting analysis to determine level of cell death by Annexin V/PI labeling. Histogram represents data from three independent experiments. Error bars indicate SE. (D) Attached or suspended MCF-10A cells were incubated under normoxic or hypoxic conditions for 48 h, and equal amounts of protein lysate were subjected to immunoblot analysis and analyzed for cleaved caspase-3, Bim, and Bmf protein levels. Actin was used as a loading control. (E) Attached (0 h in suspension) or suspended MCF-10A cells were treated with vehicle or DFOM and then lysed at indicated times and assessed for levels of cleaved caspase-3, Bim, and Bmf by using immunoblot analysis. Actin was used as a loading control (*$p < 0.05$, **$p < 0.01$).

(Garayoa *et al*., 2000). We investigated the effects of hypoxia on apoptosis of MCF-10A cells after ECM detachment. We have shown previously that MCF-10A cells placed in suspension undergo apoptosis between 36 and 48 h (Reginato *et al*., 2003). Attached and suspended cells were either maintained in normoxic conditions (20% O_2) or placed in hypoxic chamber for 36–48 h. Suspended cells in hypoxic conditions had a twofold inhibition of apoptosis, as measured by Annexin V positivity (Figure 1C) compared with cells maintained in normoxic conditions. Consistent with this data, we found significant inhibition of caspase-3 cleavage in suspended cells incubated in hypoxic conditions compared with normoxic cells at 48 h (Figure 1D). In addition, we found that treatment of MCF-10A cells under normoxic conditions with DFOM, a hypoxia-mimetic that stabilizes the oxygen-sensitive HIF-α subunit under normoxic conditions (Figure 1A), also inhibits anoikis (Figure 1C) and blocks caspase-3 cleavage after detachment (Figure 1E). Because we and others have shown that Bim (Reginato *et al*., 2003) and Bmf (Schmelzle *et al*., 2007) are induced and required for anoikis of MCF-10A cells, we examined expression of these BH3-only proteins during cell detachment under hypoxic conditions. We find that MCF-10A cells placed under hypoxic conditions (Figure 1D) or after DFOM treatment (Figure 1E) significantly blocked Bim protein levels (at least twofold; Supplemental Figure 1, A and B) compared with respective controls. Hypoxia also blocked Bmf protein expression twofold (Figure 1D and Supplemental Figure 1A); however, although DFOM treatment reduced Bmf levels, this reduction was not to the extent as was seen with hypoxic treatment (Figure 1E). We further examined hypoxic suppression of anoikis and Bim/Bmf in MCF-10A cells over a period of 72 h in suspension. Although both caspase-3 cleavage and Bim and Bmf protein levels are significantly suppressed at 36, 48, and 72 h in suspension (Supplemental Figure 2A), there is a restoration of cell death by 72 h in suspension under hypoxic conditions (Supplemental Figure 2B), suggesting that cell death at this time point may occur via a apoptosis-independent pathway. Inhibition of Bim and Bmf by hypoxia during cell detachment is specific because we did not detect decreased expression of other Bcl2-family BH3-only proteins, including Bid and Bad or proapoptotic Bax (Supplemental Figure 3). To determine whether the hypoxic effect on anoikis and BH3-only proteins Bim and Bmf is limited to immortalized MCF-10A cells, we examined the effects of hypoxia on detachment-induced cell death in other epithelial cells. We observed a similar hypoxia-mediated decrease in the induction of Bim and Bmf during detachment of epithelial cells, including primary HMECs (Supplemental Figure 4A), the breast cancer cell line MCF-7 (Supplemental Figure 4B), and the human immortalized prostate epithelial cell line RWPE-1 (Supplemental Figure 4C). Thus, hypoxia inhibits anoikis of mammary epithelial cells, and this block is associated with decreased expression of proapoptotic BH3-only proteins Bim and Bmf.

Mek–Erk Activation Is Maintained in Detached Cells under Hypoxic Conditions

Cells undergoing anoikis exhibit decreased activation of various cellular signaling pathways that regulate cell growth and survival, including EGFR, Mek–Erk, and Akt pathways (Reddig and Juliano, 2005). We examined activation of these pathways in attached or suspended cells under both normoxic and hypoxic conditions. As expected, Mek, Erk, and Akt activation was significantly decreased in suspended MCF-10A cells (Figure 2A) under normoxic conditions; however, hypoxic treatment maintained Mek and Erk but not Akt (Figure 2A) activity in suspension. Attached cells after

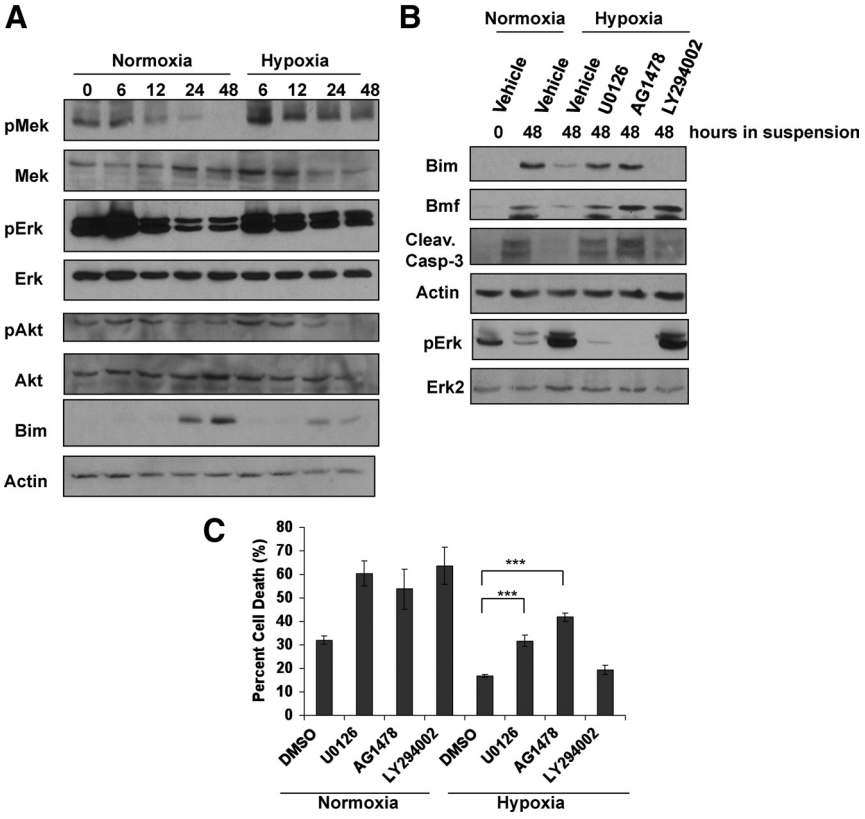

Figure 2. Mek–Erk signaling is maintained in suspended cells under hypoxia and is required for hypoxic inhibition of anoikis. (A) MCF-10A cells were cultured in suspension for the indicated times under normoxic or hypoxic conditions and then lysed for protein. Immunoblot analysis was used to determine levels of phospho- and total Erk, Mek, and Akt as well as level of Bim protein. Actin was used as a loading control. (B) Attached or suspended MCF-10A cells were pretreated with dimethyl sulfoxide (DMSO) or indicated inhibitor compounds then incubated at 20% or 1% O_2 for 48 h. Whole cell lysates were immunoblotted for indicated protein levels. Actin was used as a loading control. (C) Suspended MCF-10A cells were pretreated with DMSO or indicated inhibitors, grown in suspension for 36 h, and then subjected to Annexin V/PI staining and fluorescence-activated cell sorting analysis to determine level of cell death. Histogram represents data from three independent experiments. Error bars indicate SE (***$p < 0.005$).

48 h of exposure to hypoxic conditions also exhibited increased activation of EGFR (as measured by phosphorylation of Y992), Mek, Erk, and Akt compared with attached cells under normoxic conditions (Supplemental Figure 5). Although hypoxia activated these pathways in attached cells above control level, we did not detect increases in signaling pathways in detached states (Figure 2A), suggesting that hypoxia is able to maintain these signaling pathways during cell detachment. Activation of the Mek–Erk pathway also was maintained in suspended MCF-10A cells treated with DFOM under normoxic conditions (data not shown). Hypoxia treatment of HMEC, MCF-7, and RWPE-1 cells also increased Erk activation in the attached state and maintained Erk activity during matrix detachment compared with cells cultured under normoxic conditions (Supplemental Figure 4). Furthermore, to ensure that activation of Erk in attached or suspended cells under hypoxic conditions could activate downstream signaling, we examined phosphorylation of p90RSK at Thr360/Ser364, a well-characterized direct Erk phosphorylation site (Dalby *et al*., 1998). Indeed, hypoxia increased phosphorylation of p90RSK in attached cells and maintained signaling in suspended conditions (Supplemental Figure 5). We detected an increase in Mek, Erk, and p90RSK activation between two- and threefold in cells in suspension under hypoxic conditions compared with suspended normoxic cells (Supplemental Figure 6).

To determine which signaling pathways are required for hypoxia-mediated inhibition of Bim and Bmf expression and anoikis resistance in MCF-10A cells, we examined effect of inhibitors of the EGFR, Mek, and phosphatidylinositol 3-kinase [PI(3)K] signaling pathways. Incubation of MCF-10A cells for 48 h under hypoxic conditions in the presence of Mek inhibitor (UO126) or EGFR inhibitor (AG1478) fully reversed hypoxia-mediated Bim and Bmf inhibition as well as caspase-3 cleavage (Figure 2B). In contrast, pretreatment with PI(3)K inhibitor (LY294002) did not reverse hypoxic Bim inhibition, although it did fully reverse Bmf inhibition as well as partially reverse hypoxia-mediated inhibition of caspase-3 cleavage (Figure 2B). These data are consistent with previous work showing that Bim is specifically regulated by EGFR and Mek–Erk pathway, independent of PI(3)K signaling in MCF-10A cells (Reginato *et al*., 2003), whereas Bmf is coregulated by both Erk and Akt pathways in these cells (Schmelzle *et al*., 2007). We then examined which of these pathways could reverse the anoikis resistance mediated by hypoxia. Treatment of cells with EGFR inhibitor and Mek inhibitor reversed hypoxia-mediated anoikis resistance (Figure 2C). However, treatment with PI(3)K inhibitor was unable to fully reverse hypoxia-mediated anoikis resistance. Treatment of normoxic cells with these inhibitors all enhanced anoikis (Figure 2C), as expected, because these pathways contribute to cell survival. However, the effect of these inhibitors was not dominant because hypoxia was able to inhibit anoikis even in the presence of these inhibitors. In addition, DFOM-mediated anoikis resistance was significantly reversed in the presence of EGFR or Mek inhibitors, whereas inhibition of PI(3)K did not significantly alter DFOM-mediated anoikis suppression (Supplemental Figure 7). Thus, these data suggest that hypoxia activation of EGFR–Mek–Erk pathway contributes to inhibition of Bim, Bmf, and anoikis of MCF-10A cells. However, because EGFR or Mek inhibitors did not fully restore anoikis levels to those under normoxic conditions, hypoxia may regulate additional pathways involved in anoikis resistance.

HIF-1 Is Required for Hypoxia-induced Bim Inhibition and Anoikis Resistance

Because HIF-1 is a critical player in the cellular response to hypoxia and the transcription of hypoxia-regulated genes, we examined whether HIF-1 expression was required for hypoxia-mediated anoikis resistance. We targeted HIF-1α by using RNAi to determine how depletion of HIF-1α affects the anoikis sensitivity of MCF-10A cells cultured under hypoxia. MCF-10A cells transfected with siRNA oligonucleo-

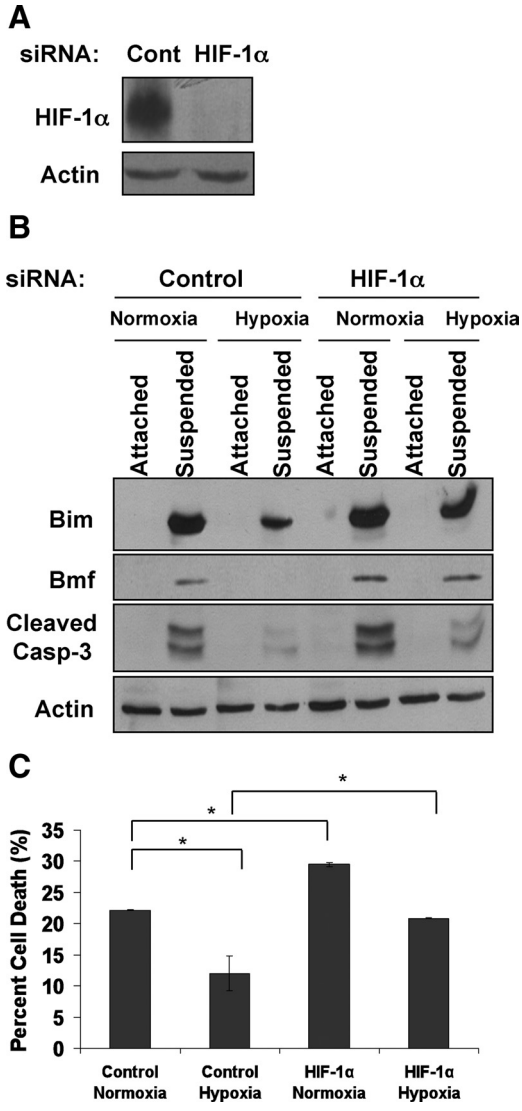

Figure 3. HIF-1α is required for hypoxia-mediated inhibition of Bim, Bmf, and anoikis. MCF-10A cells were transfected with control or HIF-1α–targeting siRNA oligonucleotides. (A) Attached cells were placed under hypoxic conditions for 6 h, and then cells were lysed and immunoblot analysis was used to assess HIF-1α knockdown. (B) After transfection, cells were placed in attached or suspended culture under normoxic or hypoxic conditions for 48 h. Cells were lysed and levels of Bim, Bmf, and cleaved caspase-3 were determined using immunoblot analysis. Actin was used as a loading control. (C) After transfection, cells were cultured in suspension for 48 h under normoxic or hypoxic conditions. Cells were then analyzed for level of cell death using Annexin V/PI staining and fluorescence-activated cell sorting analysis. Histogram represents data from three independent experiments Error bars indicate SE (*$p < 0.05$).

tides homologous to HIF-1α sequence, but not control siRNA, showed significantly reduced expression of HIF-1α after 6 h under hypoxic conditions (Figure 3A). Reducing HIF-1α expression was able to reverse hypoxia-mediated inhibition of Bim and Bmf expression as well as partially reverse caspase-3 cleavage during cell detachment compared with cells transfected with control RNAi (Figure 3B). Moreover, reducing HIF-1α expression also reversed hypoxia-mediated anoikis resistance (Figure 3C). To test specificity of RNAi oligonucleotides, we also targeted HIF-1β, the common dimerization partner of both HIF-1α and HIF-2α (Tian *et al*., 1997). Reducing HIF-1β levels with RNAi also reversed hypoxia-mediated inhibition of Bim, Bmf, and anoikis (Supplemental Figure 8). In addition, we noticed that reducing HIF-1α with RNAi increased Bim and Bmf levels, cleaved caspase-3 (Figure 3B), and anoikis under normoxic conditions compared with control RNAi (Figure 3C), suggesting that basal levels of HIF-1 in MCF-10A cells may provide some protection from anoikis. This increase in apoptosis was specific to anoikis because we did not detect changes in apoptosis in attached cells with reduced HIF-1α levels compared with control (data not shown). These data suggest that hypoxia-mediated Bim and Bmf inhibition and anoikis resistance partly requires HIF-1 expression.

Hypoxia Disrupts Acinar Organization, Inhibits Lumen Formation, and Blocks Bim and Bmf Expression during Morphogenesis of MCF-10A Cells

Single MCF-10A cells placed in 3D culture will form a solid cell cluster at days 4 to 5. Although the outer cells, in direct contact with ECM, develop an axis of apical–basal polarity and undergo growth arrest, the inner cells will undergo anoikis starting at days 7 and 8 (Debnath *et al*., 2002). Bim (Reginato *et al*., 2005) and Bmf (Schmelzle *et al*., 2007) expression is induced during this time and is required for luminal apoptosis and clearing. We examined whether exposure to hypoxia alters morphogenetic events leading to luminal clearing in MCF-10A cells undergoing acinar formation in 3D culture. MCF-10A cells were grown in 3D culture under standard tissue culture conditions (20% O_2, 5% CO_2) until day 6, and then cells were either kept under these conditions or placed under hypoxia (1% O_2, 5% CO_2). At day 10, after 4 d in hypoxia, MCF-10A cells cultured in 3D display a hypoxic response as evidenced by a more than threefold induction of the hypoxia-induced gene ADM (Supplemental Figure 9A). In terms of structural architecture, acini cultured under hypoxic conditions from day 6 through day 10 display filled lumens with very few caspase-3–positive cells located in luminal space compared with acini cultured under normoxic conditions (Figure 4A and Supplemental Figure 9B). In addition, unlike acini cultured under normoxic conditions, hypoxic acini are highly disorganized, as evidenced by the mislocalization of actin and integrin α5 as well as loss of β-catenin and E-cadherin staining at cell–cell junctions (Figure 4A). However, cells at periphery of filled acini under hypoxic condition still had basally localized integrin α6 (Figure 4A) and basally deposited collagen IV (data not shown), suggesting that hypoxia-treated acini retain some of their epithelial properties. We measured percent filled acini after 48, 72, and 96 h in hypoxia and found

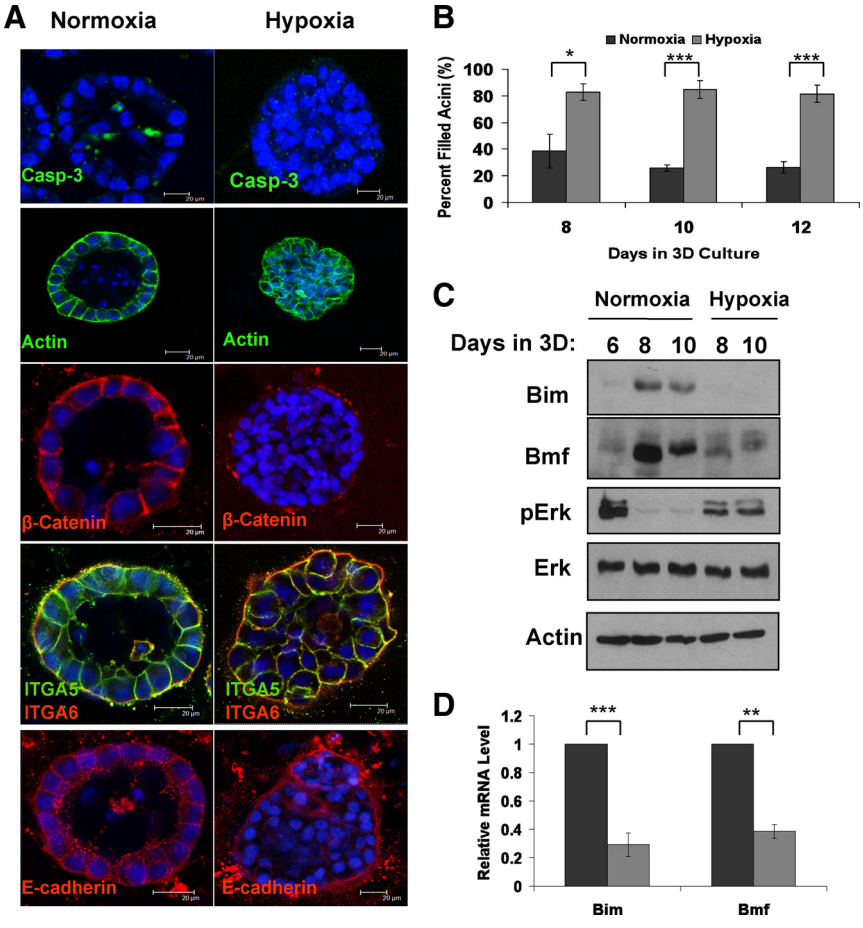

Figure 4. Hypoxia disrupts acinar organization, blocks luminal clearance and Bim and Bmf expression in 3D culture. MCF-10A cells were placed in a morphogenesis assay under standard tissue culture conditions (20% O_2, 5% CO_2) until day 6, and cells were then incubated under normoxic or hypoxic conditions for the duration of the experiment. (A) At day 10, structures were fixed and stained with antibodies directed against cleaved caspase-3, actin, β-catenin, integrin α5, integrin α6, and E-cadherin. For all images, blue indicates nuclear stain DAPI. (B) At days 8, 10, and 12, structures were fixed and stained with the nuclear stain DAPI, and the percentage of filled acini in the population was determined for each condition. Histogram represents percentage of filled acini under normoxic and hypoxic conditions for three independent experiments. Error bars indicate SE. (C) At days 6, 8, and 10, structures were lysed and subjected to immunoblot analysis and analyzed for Bim, Bmf, and phospho- and total Erk protein levels. Actin was used as a loading control. (D) At day 8, structures were lysed for total RNA. Equal amounts of total RNA were subjected to quantitative RT-PCR analysis to determine relative level of Bim and Bmf expression. Histogram represents data from at least three independent experiments. Error bars represent SE (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.001$).

at least a two- to threefold inhibition of luminal clearing under hypoxic conditions compared with normoxic acini (Figure 4B). Under hypoxia, 83% of day 12 acini remained filled compared with just 26% of acini cultured under normoxic conditions (Figure 4B). Consistent with decreased luminal clearing and apoptosis, we found that acini under hypoxic conditions also blocked Bim and Bmf induction (Figure 4C) at the level of five- and twofold, respectively (Supplemental Figure 1C). In addition, hypoxia-mediated block of Bim and Bmf in 3D culture occurred at the level of RNA, because we found a threefold and twofold reduction of RNA of Bim and Bmf, respectively, compared with normoxic conditions (Figure 4D). Thus, hypoxia disrupts epithelial architecture, blocks luminal clearing during mammary acinar morphogenesis and inhibits expression of critical anoikis regulators Bim and Bmf.

To test whether the Mek–Erk pathway was also responsible for hypoxia-mediated anoikis resistance in 3D culture, we examined levels of Erk activity in hypoxic acini. Under normoxic conditions Erk activity is significantly down-regulated between day 6 and day 8, which is consistent with elevation of both Bim and Bmf expression during this time (Figure 4C). In contrast, acini incubated under hypoxic conditions contained elevated Erk phosphorylation at days 8 and 10 compared with normoxic acini at same time points (Figure 4C), consistent with decreased Bim and Bmf levels. Moreover, treatment of acini with the Mek inhibitor U0126 or the EGF receptor inhibitor AG1478, but not the PI(3)K inhibitor LY294002, reversed hypoxia-mediated Bim inhibition at level of protein in 3D culture (Figure 5A) as well as RNA (data not shown). To test whether hypoxic activation of Erk was required for hypoxia-mediated inhibition of luminal apoptosis, we examined cleaved caspase-3 staining after Mek inhibitor treatment. Consistent with elevation of Bim and Bmf expression, treatment of hypoxic acini with Mek inhibitor induced a threefold increase in cleaved caspase-3–positive acini, thus reversing hypoxia-mediated anoikis resistance in 3D culture (Figure 5B).

Thus, we show for the first time that hypoxia, via HIF-1, can induce anoikis resistance in epithelial cells. Hypoxia suppresses anoikis in human epithelial cells by specifically inhibiting the expression of the anoikis-associated BH3-only proteins Bim and Bmf in a HIF-1–dependent manner. Hypoxia increases EGFR–Mek–Erk pathways under normal adhesion and can maintain activation of these pathways after cell detachment, leading to anoikis resistance. Furthermore, hypoxia-mediated anoikis suppression in a 3D context correlates with inhibition of luminal clearing and disruption of acinar organization during mammary morphogenesis.

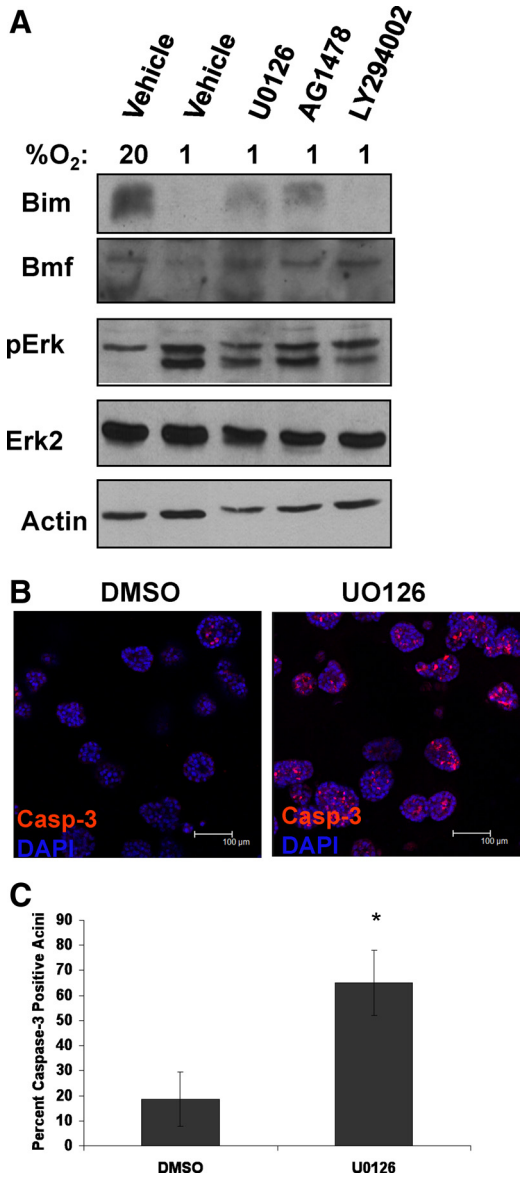

Figure 5. Erk activation in hypoxic acini is required for inhibition of expression of Bim and Bmf as well as luminal apoptosis. MCF-10A cells were placed in a morphogenesis assay under standard tissue culture conditions (20% O_2, 5% CO_2) until day 6, and then cells were incubated under normoxic or hypoxic conditions after pretreatment with indicated inhibitor compounds until day 8. (A) Structures were lysed and immunoblot analysis was used to determine the level of indicated phospho- and total proteins. Actin was used as a loading control. (B) Structures were fixed and stained with an antibody against cleaved caspase-3 as well as the nuclear stain DAPI. (C) Percentage of caspase-positive acini was determined in each population. Caspase-positive acini were defined as any structure with two or more caspase-3–positive cells present in the luminal space. Histogram represents data from three individual experiments as described in B. Error bars represent SE (*$p < 0.05$).

DISCUSSION

Oxygen levels are reduced in many human cancers compared with surrounding normal tissue. Reduced oxygen concentration induces HIF-1, a hypoxia-responsive transcription factor that regulates transcription of a number of genes implicated in many aspects of cancer biology, including energy metabolism, resistance to chemotherapy and metastasis (Hockel and Vaupel, 2001a). Here, we show for the first time that hypoxia via HIF-1 also can induce anoikis resistance in epithelial cells. Hypoxia suppresses anoikis in human epithelial cells by specifically inhibiting the expression of the anoikis-associated BH3-only proteins Bim and Bmf in a HIF-1–dependent manner. Hypoxia increases EGFR–Mek–Erk pathways under normal adhesion and can maintain activation of these pathways after cell detachment, leading to anoikis resistance. Furthermore, hypoxia-mediated anoikis suppression in a 3D context correlates with inhibition of luminal clearing and disruption of acinar organization during mammary morphogenesis.

Within the Bcl-2 family of proteins, both proapoptotic family members and antiapoptotic family members have been reported previously to be subject to hypoxic regulation (Greijer and van der Wall, 2004). Hypoxia can induce ex-

pression of BH3-only protein BNIP3 that has been linked to hypoxia-induced survival by inducing autophagy in fibroblasts (Zhang *et al*., 2008; Bellot *et al*., 2009). Our results provide evidence that induction of Bim and Bmf, two BH3-only Bcl-2 family members that are critical for anoikis in epithelial cells, is blocked by hypoxia after cell detachment and during morphogenesis. Although it is possible that hypoxia may regulate other Bcl-2 family members that contribute to decreasing anoikis of epithelial cells, we did not observe changes in expression of other proapoptotic Bcl-2 family members in hypoxic cells cultured in suspension, including Bid, Bad, and Bax. Thus, hypoxia may specifically target Bim and Bmf expression to suppress epithelial cell anoikis.

This study also provides evidence that suppression of anoikis under hypoxic conditions occurs in a HIF-dependent manner, because depletion of HIF-1 restores anoikis sensitivity and Bim and Bmf expression fully. Because HIF is a primary mediator of the cellular response to hypoxic stress, it is not surprising that hypoxic regulation of a variety of both proapoptotic and anti-apoptotic proteins has been shown to occur in a manner that is at least partially HIF dependent (Greijer and van der Wall, 2004). Hypoxia-mediated down-regulation of Bid also has been shown to be HIF-1–dependent and may be through HIF-1 binding to Bid promoter (Erler *et al*., 2004). It is not clear whether HIF can regulate Bim and Bmf directly at the level of transcription and/or indirectly via regulation of signaling pathways; however, because inhibition of hypoxia-induced signaling pathways, such as EGFR and Mek–Erk, was able to reverse Bim and Bmf inhibition, this suggests that HIF-1 regulation of these BH3-only proteins requires maintenance of signaling pathways. Although hypoxia can block anoikis and maintain activated signaling pathways in detached cells, we found that long-term hypoxia induces caspase-independent death pathways in detached cells. Thus, hypoxia treatment of immortalized cells alone may not contribute to anchorage independent growth unless cells already express certain oncogenes such as Akt (Bedogni *et al*., 2005). Collaboration of oncogenes with hypoxia in regulating anchorage independence will be furthered examined in future studies.

MCF-10A cells cultured in 3D under hypoxic conditions also exhibit suppression of Bim and Bmf expression. Consistent with the role of Bim and Bmf in anoikis during lumen formation of acini-like structures during mammary morphogenesis, hypoxia results in inhibition of luminal clearing and disorganized acinar structures. Interestingly, hypoxia-mediated disruption of acinar architecture and inhibition of luminal clearing is similar to phenotypes of MCF-10A acini-overexpressing oncogenes such as ErbB2 (Muthuswamy *et al*., 2001) and Mek (Reginato *et al*., 2003). Thus, it is possible that oncogenes that stabilize HIF may regulate HIF-dependent pathways that contribute to anoikis resistance. Consistent with this idea, recent studies have shown that reducing HIF-1α via RNAi in gastric cancer cells under normoxic conditions leads to increased anoikis and decreased anchorage-independent growth (Rohwer *et al*., 2008).

Although hypoxia has been shown to enhance both EGFR (Wang *et al*., 2007) and Erk activity (Minet *et al*., 2000), our results are the first to demonstrate such activation by hypoxia in suspended cells and in the context of 3D culture. It is well established that EGFR-mediated mitogen-activated protein kinase signaling acts as a negative regulator of Bim and Bmf expression in adherent MCF-10A cells (Reginato *et al*., 2003; Schmelzle *et al*., 2007). The mechanism through which hypoxia promotes anchorage-independent activation of EGFR and Erk signaling is not clear. Increased levels of EGFR at the level of protein (Franovic *et al*., 2007) have been reported in response to exposure to hypoxia in human cancer cells; however, we did not detect increase in total EGFR protein expression, but we have not ruled out that hypoxia may up-regulate EGFR surface expression, inhibit EGFR internalization in epithelial cells, or both, thereby potentiating EGFR-mediated Erk signaling during detachment. Hypoxic induction of amphiregulin (O'Reilly *et al*., 2006), an EGFR ligand, and tumor necrosis factor-converting enzyme/ADAM17 (Charbonneau *et al*., 2007), a sheddase whose activity releases the EGFR ligand TNF-α, have been also reported. This offers the possibility that hypoxia-mediated secretion or processing of an EGFR ligand may promote EGFR–Erk activation in an autocrine manner in suspended cells; however, hypoxic conditioned media was unable to activate EGFR–Erk pathways in normoxic MCF-10A cells (data not shown). Integrin-mediated EGFR transactivation is a well-documented phenomenon (Cabodi *et al*., 2004). Because hypoxia can enhance integrin α5 expression (Koike *et al*., 2004), and we and others have shown that integrin α5 can regulate ErbB2 (Haenssen *et al*., 2010) and EGFR signaling (Caswell *et al*., 2008), it is possible that EGFR activation under hypoxic conditions occurs via integrin-mediated transactivation. Recently, ECM remodeling and stiffening have been shown to drive tumor progression in myoepithelial cells, in part by activating lysyl oxidase (LOX), a known cross-linker of ECM (Levental *et al*., 2009). Lysyl oxidase is highly induced under hypoxic conditions (Postovit *et al*., 2008); thus, it would be interesting to determine whether LOX-mediated ECM remodeling contributes to altered signaling and tissue organization of acini under hypoxic conditions.

Hypoxic markers are often associated with early premalignant DCIS lesions (Wykoff *et al*., 2001); our results indicate that hypoxia may contribute to loss of epithelial organization in these lesions, in part by providing survival signals under states of low adhesion allowing mutilayering and contributing to luminal filling of ducts. Moreover, hypoxic tumors have been shown to correlate with poor patient prognosis, in part due to resistance to existing cancer chemotherapies. Because Bim (Sunters *et al*., 2003; Belloc *et al*., 2007; Gong *et al*., 2007) and to a lesser extent Bmf are required for apoptosis mediated by several chemotherapeutic agents, here we propose that hypoxic suppression these proteins may offer possible explanation as to why hypoxic tumors are not responsive to certain cancer therapies. Thus, elevation of Bim and Bmf levels in hypoxic tumors could be a potential strategy in the development of novel antitumor therapies to treat resistant hypoxic cancers.

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