Anoxia Is Necessary for Tumor Cell Toxicity Caused by a Low-Oxygen Environment

Ioanna Papandreou, Chaya Krishna, Fiona Kaper, Deli Cai, Amato J. Giaccia, and Nicholas C. Denko

Department of Radiation Oncology, Division of Radiation and Cancer Biology, Stanford University School of Medicine, Stanford, California

Abstract

Cells exposed to oxygen deprivation *in vitro* have been shown to reduce proliferation and/or engage in programmed cell death. There is considerable controversy in the literature as to the role of hypoxia-inducible factor-1 (HIF-1) and HIF-1 target genes in initiating these responses. We therefore examined the oxygen dependence and the role of the hypoxia-responsive transcription factor HIF-1 in making the cellular death decision. Oxygen concentrations as low as 0.5% did not alter the growth of HIF-1–proficient or HIF-1–deficient murine fibroblasts, or human tumor cells, despite the appropriate induction of HIF-1 target genes. Severe hypoxia (<0.01% oxygen) did induced apoptosis, resulting in decreased colony formation, chromatin condensation, DNA fragmentation, and caspase activation but also independent of HIF1α status. Transcriptional induction of HIF-1–dependent genes putatively involved in cell death like *BNip3* and *BNip3L* was therefore disassociated from hypoxia-dependent toxicity. Likewise, forced overexpression of a nondegradable form of HIF-1α in several human tumor cell lines was not sufficient to induce apoptosis under normoxic conditions. Taken together, these findings indicate that additional molecular events are triggered by anoxia in a HIF-1–independent manner, and these changes are necessary for cell death observed in low-oxygen environments. (Cancer Res 2005; 65(8): 3171-8)

Introduction

Reduced oxygen tension is a common feature of many pathophysiologic conditions including myocardial infarction, stroke, and cancer. Hypoxia in solid tumors is associated with radioresistance and chemoresistance, increased metastatic potential, and poor outcome (1). Following the identification of the oxygen sensitivity of the transcription hypoxia-inducible factor-1 (HIF-1α), a great effort has been put in the study of its role during development and in pathologic conditions where oxygen deprivation occurs.

Cultured normal cells grow in reduced oxygen concentrations *in vitro* and even display extended replicative life span (2) when grown at 3% oxygen. Oncogenically stimulated tumor cells exposed to hypoxia, however, can activate a programmed cell death pathway both *in vitro* and *in vivo* (3–5). The molecular trigger for this program is not known, but the intrinsic apoptotic pathway has been implicated (6, 7), with the tumor suppressor p53 sensitizing cells to hypoxic conditions (3).

Mitochondrial signaling is pivotal to the execution of apoptosis by oxygen deprivation. Genetic removal of cytochrome *c*, Apaf-1, or caspase 9 confers almost complete resistance to hypoxia-induced apoptosis (7, 8). The central role of the mitochondria has lead investigators to focus on the hypoxic regulation of Bcl-2 family members. Overexpression of the prosurvival members of the Bcl-2 family or ablation of the proapoptotic members Bax and Bak protects cells from oxygen deprivation–induced death (6, 9, 10). Other proposed contributors include the BH3-only molecules PUMA (11), Noxa (12), Bax, and Bid (13). Conversely, induction of antiapoptotic proteins ORP150 (14) and the caspase inhibitor IAP-2 (15) have been reported to be induced by hypoxia/ischemia.

The role for HIF-1α in hypoxic cell death is not clear. The putative BH3 only proapoptotic genes *BNip3* and *BNip3L* are induced by hypoxia through a HIF-dependent mechanism (16, 17). Transient overexpression of BNip3 has also been shown to be cytotoxic in a number of tumor cell lines (18–25), making it an attractive candidate mediator of hypoxia-induced apoptosis. BNip3 can form homodimers and heterodimers with antiapoptotic proteins, including Bcl-2 and E1B19K, and it contains domains that resemble a BH3 motif and a transmembrane motif necessary for mitochondrial localization (19, 20, 25). Toxicity by BNip3 however seems to be mechanistically distinct from other Bcl-2 family members, with characteristics of a necrotic or an authophagic type of death (24, 26). The role of the BNip3-related protein BNip3L is even more controversial, with reports of both positive (18, 19, 22) and negative (23) effects on the initiation of cell death.

Given the confusing state of the literature, this work uses genetically defined model tumor cells to determine the role for HIF-responsive genes in the death induced by oxygen deprivation. We show that there is no alteration in the hypoxic sensitivity of murine fibroblasts ablated for HIF activity. Second, we show that the candidate apoptotic mediators BNip3 and BNip3L are not necessary or sufficient for physiologically appropriate, hypoxia-induced death. Third, we find that forced expression of HIF target genes is not sufficient to induce normoxic death in a wide variety of human tumor cell lines.

Materials and Methods

Cell culture. Wild-type and HIF-1α knockout mouse embryonic fibroblasts (MEF) were a gift from R. Johnson (University of California at San Diego). RCC4 and RCC4-VHL were provided by P. Ratcliffe (John Radcliffe Hospital, Oxford, United Kingdom). MCF7, RKO, HeLa, and HT1080 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were grown in DMEM with 10% fetal bovine serum and 20 mmol/L HEPES. Transfected HeLa clones were grown in 2 μg/mL puromycin. For moderately hypoxic conditions (0.5-2.0%

oxygen), cells were incubated either in a humidified hypoxic workstation (Invivo₂, Ruskinn Technologies, Bridgend, United Kingdom) or a three gas incubator (2.0%; Forma Scientific, Marietta, OH). Severe hypoxia was generated in an anaerobic working station with a palladium catalyst (Sheldon Co., Cornelius, OR). Final oxygen concentrations were confirmed using a Clark-type polarographic electrode (Animas, Frazer, PA).

Cell viability and death assays. Cell death was measured by harvesting all cells, double staining with 5 μg/mL Hoechst 33342 and 5 μg/mL propidium iodide and microscopic scoring. For colony formation, 200 cells were plated in 60-mm dishes, and 6 hours later placed in the indicated environment. Seven to 11 days later, cells were stained with crystal violet and colonies (>50 cells) were scored.

Expression vectors. BNip3-T7, BNip3L-HA, tBid-HA, and Bax-HA open reading frames were generated by PCR using primers containing the indicated epitope tags, from appropriate IMAGE clones. PCR product was cloned into either pcDNA3.1 for transient transfection, or pEF2aIRESpuro[1] for the establishment of stable clones. The Hif-1α double proline mutant (P402A/P564G) expression vector has been described (27).

Generation of polyclonal antibodies. Antisera against amino acids 97 to 115 of human BNip3L and 98-116 of BNip3L were raised by immunizing rabbits with peptides conjugated to keyhole limpet hemocyanin (Open Biosystems, Huntsville, AL). Crude antisera were affinity purified using peptide immobilized to Sulfolink resin (Pierce, Rockford, IL). Purified antibody recognized unique bands corresponding to the appropriately sized, hypoxia-inducible proteins (Figs. 3 and 6; data not shown).

Western blotting. Cells were harvested and lysed in radioimmunoprecipitation assay buffer, protein was quantitated, 20 micrograms were separated on Tris-tricine gels and transferred onto Hybond-P membranes (Amersham, Piscataway, NJ). Signal was visualized with ECF substrate (Amersham) and scanned on a Storm Imager (Molecular Dynamics, Sunnyvale, CA).

Antibodies. The antibodies used were: mouse anti-T7·Tag (Novagen, Madison, WI) 1:1,000, mouse anti-HA (BabCO, Richmond, CA) 1:500, mouse anti-bcl-2 (Dako, Glostrup, Denmark) 1:500, rabbit anti-BNip3 1:100, rabbit anti-BNip3L 1:500, mouse anti-tubulin (Research Diagnostics, Flanders, NJ) 1:1,000, anti-HIF-1α (BD Transduction Laboratories, San Diego, CA) 1:1,000, secondary Alexa Fluor 594 donkey anti-mouse and Alexa Fluor 488 goat anti-mouse (Molecular Probes, Eugene, OR), 1:500 Secondary AP conjugated horse antimouse and goat antirabbit antibodies were from Vector Laboratories, Inc. (Burlingame, CA).

RNA isolation and Northern blotting. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Five micrograms of RNA were denatured with glyoxal/DMSO, fractionated on an agarose gel and transferred to Nytran(+) membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH). Following cross-linking, the membrane was stained with methylene blue solution to visualize 18 S rRNA. Hybridizations were done in 0.5 mol/L Na_2PO_4 (pH 7.4), 7% SDS, and 1 mmol/L EDTA. The random primed probes were generated from appropriate I.M.A.G.E. clones (ATCC).

Transient transfection, immunocytochemistry and small interfering RNA. Cells in 8-chamber slides (Fisher Scientific, Pittsburgh, PA), were transfected using Fugene 6 reagent (Roche, Indianapolis, IN), fixed in 4% paraformaldehyde, and probed in PBS buffer, 0.2% Tween20, and 3% nonfat dry milk. Nuclei were stained with 10 μg/mL Hoechst 33342, mitochondria with 25 nmol/L MitoTracker Red CMXRos (Molecular Probes) for 20 minutes before fixation. Activated caspases were detected with APO LOGIX, carboxyfluorescein FAM-VAD-FMK kit (Cell Technology, Mountain View, CA), positive cells were detected by microscopic excitation at 488 nm. siRNA oligos were obtained from Dharmacon (Lafayette, CO), and directly transfected into cells using Oligofectamine (Invitrogen) as recommended by manufacturer. Oligos were obtained from Dharmacon as the annealed duplex, desalted 2′hydroxyl form and used directly in transfection.

Small interfering (siRNA, target) sequences used are hBNip3 AAGGAA-CACGAGCGUCAUGAA, mBNip3 AAGAAACACAAGCGUUAUGAA, BNip3L AAAUAGCUCUCAGUCAGAAGA, and mBNip3L AAAAACGACUGAGCA-CACCUU.

Reporter assays. Cells were transfected in triplicate with HIF expression constructs and the 5× HRE-luc reporter with pSVβ-gal for normalization. Thirty hours post-transfection, cells were lysed, and reporter activity measured using a β-gal reporter gene assay kit (Roche) and Luciferase activity assay reagent (Promega, Madison, WI). All measurements were taken with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The data shown in the graphs are normalized for β-gal activity values.
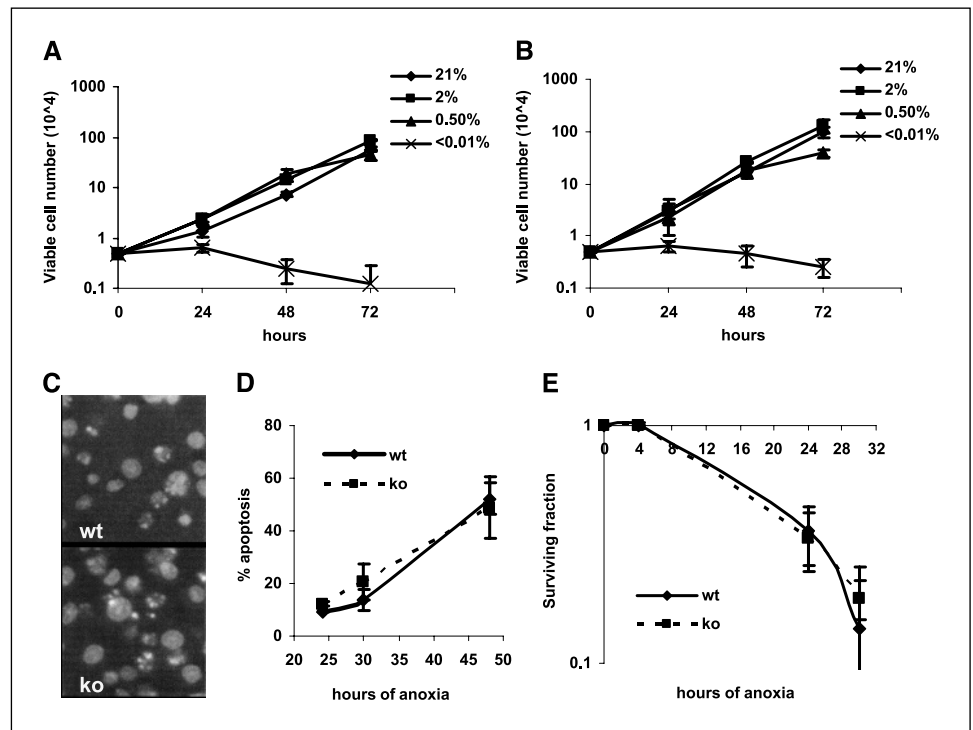
Results

Moderate hypoxia does not kill cells *in vitro*, regardless of hypoxia-inducible factor-1 status. To study any possible contribution of HIF-1 in the death of tumor cells *in vitro*, we measured the survival of fibroblasts derived from control animals or HIF-1α knockout mice grown in various levels of oxygen. These model tumor cells were immortalized with SV40 large T antigen to make them functionally p53 negative and focus our findings on HIF effects and not p53 effects. Cells were plated at low density in high glucose, buffered media and placed in environments with regulated oxygen concentrations. At 24, 48, and 72 hours, cells were trypsinized, treated with trypan blue, and viable cells counted from triplicate wells. Note that cells in 21%, 2%, and 0.5% continued to proliferate with undiminished kinetics, irrespective of HIF-1 status. Cells treated with extreme hypoxia (<0.01%) showed a block to proliferation by 24 hours and a progressive loss of viable cells down to 20% to 30% at 72 hours, again irrespective of HIF-1 status (Fig. 1A and B). Severe hypoxia has been reported to lead to either an apoptotic or a necrotic cell death. To determine the mechanism for the loss of viability, cells were exposed to severe hypoxia, harvested by trypsinization, and the nuclear morphology determined by staining with Hoescht 33342 and propidium iodide. Both wild-type and knockout cells showed a similar time-dependent increase in cells showing an apoptotic morphology (Fig. 1C and D). These cells stained bright with Hoechst and presented the characteristic chromatin condensation and DNA fragmentation of apoptotic death (Fig. 1C). We also used colony formation assay as an alternative measure to quantitate cell death from severe hypoxia. Cells were plated at very low density, treated with severe hypoxia over the same time course, and then reoxygenated to allow for colony formation in the surviving cells. The loss in viability was identical between the wild-type and knockout cells, demonstrating that anoxia-dependent killing by any mechanism is independent of HIF-1 status in cells treated *in vitro*.

To confirm the findings that intermediate hypoxia did not lead to toxicity in the short-term viability assays shown in Fig. 1, we asked if long-term exposure to intermediate hypoxia could inhibit colony formation. We first plated wild-type and knockout MEFs in 60-mm dishes at very low density in buffered, high glucose media and incubated them in oxygen concentrations of 21%, 2%, and 0.5% for colony growth. After 7 days of continuous culture in the hypoxic conditions, the plates were fixed in 70% ethanol and stained with crystal violet to detect colonies. Consistent with the short-term assays, moderate hypoxia (2% and 0.5% oxygen) did not affect the colony formation of either the HIF-proficient, or the HIF-deficient cells (Fig. 2A).

To determine if growth in moderate hypoxia was somehow unique to immortalized murine cells, we tested a series of human

Figure 1. Moderate hypoxia supports the proliferation of murine fibroblasts, but severe hypoxia leads to cellular toxicity, independent of HIF-1α status. Immortalized cells from wild-type (*A*) or HIF-1α knockout embryos (*B*) were plated at low density in high glucose, buffered medium in the indicated oxygen concentrations, and viable cell number determined every 24 hours by trypsinization and microscopic examination after trypan blue exclusion. Growth rate is not diminished in either cell type until oxygen concentration falls below 0.5%. *C*, photomicrograph of cells displaying classic apoptotic morphology after treatment in severe hypoxia (<0.01%) for 48 hours and staining of nuclear morphology using Hoescht and propidium iodide. *D*, identical induction kinetics of the apoptotic nuclear morphology in the percentage of wild-type or knockout murine fibroblasts treated with severe hypoxia for the indicated times. *E*, identical loss of colony forming ability in wild-type and HIF-1α knockout MEFs treated transiently in anoxia and removed to normoxic culture for recovery and colony formation (7 days).

tumor cells for growth in reduced oxygen. We also found results similar to the murine cells in which growth in moderate hypoxia did not reduce survival of a series of HIF-proficient human tumor cells (Fig. 2*B*). The plating efficiency of HT1080 fibrosarcoma, RKO colon carcinoma, HeLa cervix carcinoma, and RCC4 renal clear cell carcinoma was not reduced by growth in either 2% or 0.5% oxygen (Fig. 2*B* and *C*). Additionally, an RCC4 subclone where the tumor suppressor von Hippel-Lindau (VHL) has been reintroduced and HIF-1 returned to normal control (RCC4-VHL) showed the same reproductive viability as the parental RCC4 line.

Hypoxia-inducible factor–dependent and hypoxia-inducible factor–independent transcriptional changes in hypoxic fibroblasts. The growth of fibroblasts in moderately reduced oxygen is not influenced by the cell's HIF-1 status. To confirm the HIF-responsiveness of target genes and identify possible HIF-independent mediators of the apoptotic response, MEFs were treated with reduced oxygen and RNA and protein was isolated to examine changes in gene expression (Fig. 3). As expected, induction of previously identified HIF-1 target genes glucose transporter *Glut1*, *BNip3L*, and *BNip3* occurred in response to any reduction oxygen, but only in the wild-type cells. Neither PUMA nor Noxa transcripts were expressed at detectable levels in these cells (data not shown). Detecting robust induction of BNip3 and BNip3L proteins at 2% and 0.5% (Fig. 3*B*), we conclude that the transcriptional activation of BNip3 and BNip3L by moderate hypoxia can be dissociated from the anoxic induction of apoptosis in these cells.

In addition to the induction of HIF-1 target genes, HIF-1–independent transcriptional changes occurred in both cell types. We have recently shown that extreme hypoxia leads to pronounced activation of the genes in the unfolded protein response (UPR) pathway (28). We show here that extreme hypoxia leads to the activation of the *Epr99* gene, the murine orthologue of the human ER chaperone GRP94 (29) in a HIF-independent manner (Fig. 3*A*). These extreme conditions also lead to a down-regulation of the
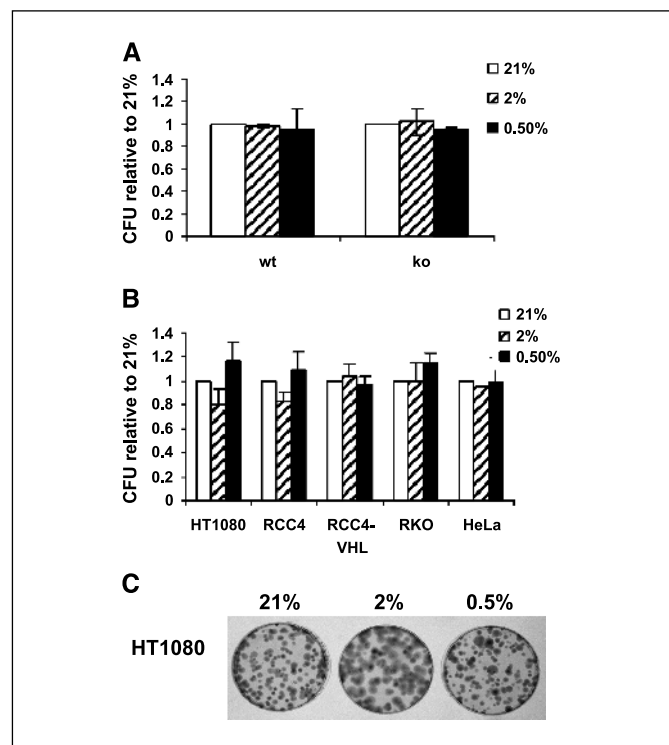
Figure 2. Long-term growth of tumor cells is not affected by moderately reduced oxygen *in vitro*. *A*, wild-type and HIF-1α knockout MEFs were plated at low density and grown under continuous culture in atmospheres of 21%, 2%, and 0.5% oxygen. Colony formation at 7 days was determined by staining with crystal violet and did not diminish in either cell line in moderately reduced oxygen. *B*, no diminishment of colony forming ability of human HT1080, RCC4, RCC4-VHL (parental RCC4 with VHL reintroduced), RKO, or HeLa tumor cell lines grown in the same moderately hypoxic conditions of 2% or 0.5% oxygen. *C*, photograph of HT1080 colonies that had been fixed and stained with crystal violet following growth in the indicated oxygen conditions.
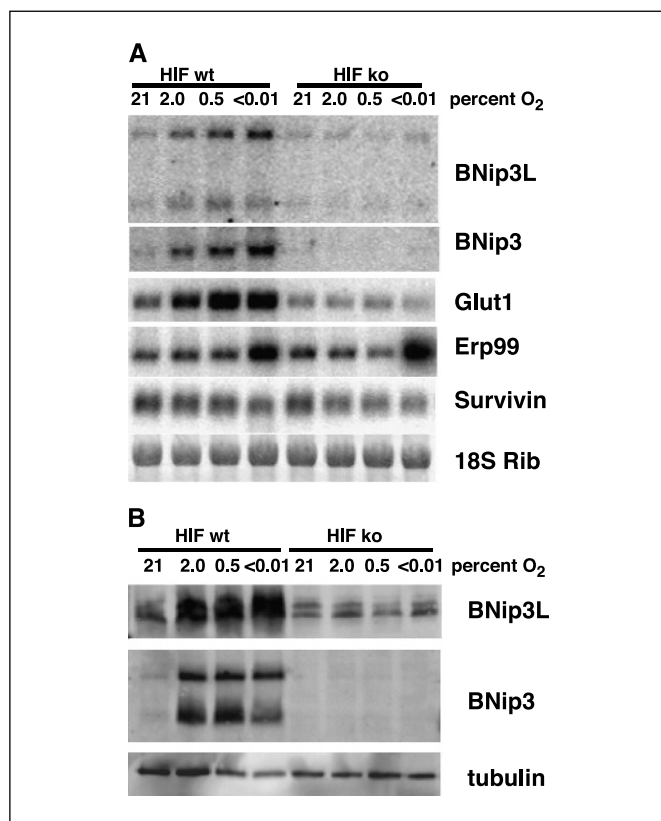
Figure 3. Gene expression changes induced by oxygen deprivation. Wild-type and HIF-1α knockout MEFs were incubated under reduced oxygen for 24 hours and (*A*) the mRNA levels of HIF-responsive (BNip3, BNip3L, Glucose transporter 1) and HIF-independent (Erp99, Survivin) genes determined by Northern blotting. Note 18S ribosomal RNA loading control. *B,* Western blot of hypoxic induction of BNip3 and BNip3L proteins in extracts from similarly treated wild-type and HIF1α knockout cells. Note tubulin as loading control.

apoptosis but its potency relative to Bax is low. Conversely, expression of BNip3L showed only background apoptosis. To determine if the HA-tag had altered BNip3L activity, we also found HA-BNip3L had no toxicity (data not shown). The difference in apoptotic potential of these closely related proteins suggests that they may have additional or alternative activities.

Although initial studies of the mechanism of BNip3's cytotoxic effects implicated the intrinsic apoptotic pathway (19, 20), more recent studies suggest that the cell death may be necrotic or
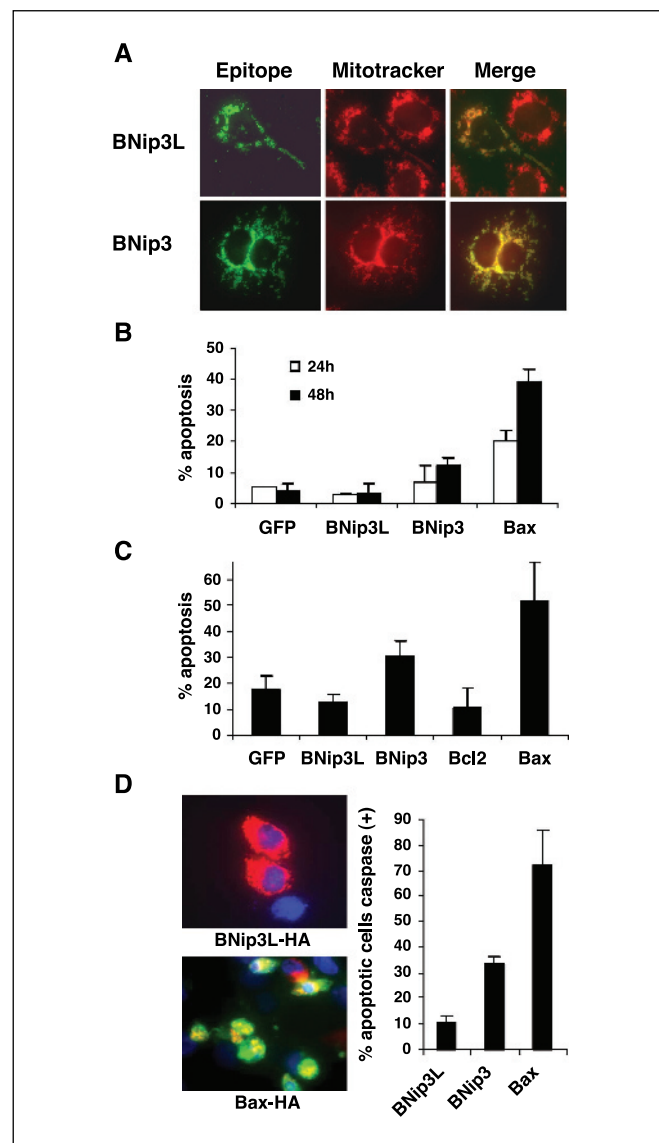

Figure 4. Epitope tagged BNip3-T7 and BNip3L-HA are poorly apoptogenic. *A,* immunodetection of epitope tags in transiently transfected wild type MEFs stained with anti epitope antibody (Alexa 488, green secondary) and MitoTracker (CMXRos, red). Colocalization results in yellow image. *B,* apoptosis in MEF cells transfected with the indicated expression plasmid. After 24 or 48 hours, cells were fixed and transfected proteins detected by immunorecognition of the epitope tags. Immunopositive cells were scored for apoptosis by nuclear morphology. *C,* apoptosis in transfected MCF7 cells treated similarly to the MEF cells. *D,* activation of caspases in MEFs following transfection with either BNip3L, BNip3, or bax expression plasmids. Immunopositive cells (Alexa 594, red) were scored for caspase activation following treatment with FAM-VAD-FMK (green). *E,* quantitation of percentage of apoptotic cells that activated caspases following BNip3 and BNip3L expression compared to Bax.

antiapoptotic gene *survivin* (30). These HIF-1–independent transcriptional changes confirm the HIF-1α knockout cells do not have an overall defect in transcriptional responses to low oxygen. Furthermore, it provides some candidate pathways that may be involved in the execution of HIF-1–independent phenotypes *in vitro*.

BNip3/BNip3L overexpression is not sufficient to induce death. HIF target genes *BNip3* and *BNip3L* have been reported in the literature to be toxic in transient overexpression studies. The ability of the HIF-1α knockout cells to apoptose under severe hypoxia, however, indicated that BNip3 and BNip3L are not absolutely necessary for this response (Fig. 1). To determine if BNip3 or BNip3L are sufficient to induce apoptosis, we transiently overexpressed epitope tagged BNip3-T7 and BNip3L-HA into either wild-type MEFs or MCF7 cells under normoxic conditions and evaluated apoptosis by nuclear morphology. Figure 4*A* shows that the localization of the T7 and HA epitope tags in the transfected MEFs localizes with mitochondrial Mitotracker staining.

BNip3 and BNip3L were compared with Bax (positive control) and pEGFPN1 and Bcl-2 (negative controls) in their apoptotic potential. Immunopositive cells were scored for nuclear changes consistent with apoptosis (Fig. 4*B* and *C*). As expected, EGFP and Bcl-2 showed minimal toxicity in both the MEFs (Fig. 4*B*) and MCF7 (Fig. 4*C*) cells, whereas Bax was the most potent. In agreement with previous reports, BNip3 was able to initiate

autophagic (24, 26). We therefore looked for caspase activation in the transfected cells using the cell-permeable fluorescent caspase indicator carboxyfluorescein FAM-VAD-FMK, which binds irreversibly to a broad range of activated caspases and can be detected microscopically as fluorescent cytoplasm. HIF-1α knockout fibroblasts transfected with BNip3L-HA, BNip3-T7, or Bax-HA plasmids were stained with FAM-VAD-FMK and scored for transfection, nuclear morphology and caspase activation. Scoring only the apoptotic cells revealed that 72.3% of the Bax cells stained for activated caspases (Fig. 4*D*). In contrast, only 33.4% of the apoptotic BNip3 cells showed caspase activation and 11% of the BNip3L cells. The inability of overexpressed BNip3L to activate either the caspase network or morphologic nuclear changes leads us to conclude that any activity is different than prototypical BH3-containing molecules.

Constitutive overexpression of BNip3 or BNip3L is not toxic. To investigate possible nonapoptotic mechanisms of toxicity by both BNip3 and BNip3L, we attempted to make stable over-expressing clones. If either of these molecules is toxic by any means, then the ability to generate stable clones should be greatly reduced. We constructed bicistronic, internal ribosomal entry site (IRES)–based expression vectors expressing first the test genes and then the puromycin resistance gene for drug selection. We found the drug-resistant colony formation reduced in plasmids expressing toxic controls Bax-HA and tBid-HA but not in control Bcl2 as well as test BNip3-T7 and BNip3L-HA. Because of the variability in these replicate experiments, we chose to analyze the pool of primary colonies for expression of the target protein. Figure 5*A* is a Western blot comparing the expression of the transfected protein in a pool of stable clones to the expression of the endogenous gene in hypoxia-treated parental cells. We find stable, long-term expression of either BNip3-T7 or BNip3L-HA that is even higher than that of the hypoxia-induced endogenous gene, whereas the tBID-HA transfected cells that grew show no expression of the epitope-tagged protein. The ability to efficiently make these high expressing clones implies that neither of these proteins is inherently toxic by any means, apoptotic, autophagic, or necrotic.

It is possible that BNip3-T7 or BNip3L-HA may require additional, HIFs to activate a death signal under physiologically relevant conditions. We formally tested this possibility by testing plating efficiencies of the stable pool of clones in normoxic and hypoxic conditions. We find that growth of stable pools in 2% oxygen (where there is robust HIF response, Fig. 3) did not alter the plating efficiency or size of colonies, suggesting that additional hypoxia-inducible effects were unable to "activate" a latent form of either of these molecules (Fig. 5*B*).

BNip3 or BNip3L induction is not necessary for anoxia-induced apoptosis. After determining that these two genes are not sufficient to induce death, we next used siRNAs to determine if they were necessary for anoxia-induced death. We synthesized siRNA oligonuceotides that were chosen based on the Dharmacon siRNA predictive program (http://dharmacon.com) and tested their ability to block anoxic induction of the appropriate target gene and the apoptotic response. Figure 6*A* shows that transfection with these oligos into either wt MEFs, HeLa, or RKO cells for 12 hours does not alter the apoptotic response to an additional 24 hours of <0.01% oxygen. Figure 6*B* shows the Western blot indicating that the oligos are indeed successful in selectively blocking the hypoxic induction of these target proteins. The siRNA oligos therefore show a very specific repression of either BNip3 or BNip3L but no effect on the anoxia-induced apoptosis. Note that in the RKO cells, only

BNip3L shows hypoxic induction, so that when it is inhibited by siRNA, neither of the two proteins is induced. Even in this "double knockdown," there is still robust apoptosis in response to severe hypoxia (Fig. 6*A*).

Forced overexpression of hypoxia-inducible factor-1α under normoxia is not acutely cytotoxic. The experiments presented above showed that physiologic activation of HIF-1 by oxygen deprivation and activation of the *BNip3* and *BNip3L* target genes is not necessary for anoxia-induced apoptosis. We next asked if there were any other HIF-1 target genes that could directly result in cell death by forced HIF-1 activation under normoxia. To test this possibility, we transfected a constitutively active form of Hif-1α into a panel of human tumor cell lines and quantitated the apoptotic response.

HIF-1α has a very short half-life under normoxic conditions; thus, we used a mutant form of HIF-1 where the two proline residues, P402 and P564, have been mutated to arginine and glycine, respectively, so that HIF-1 is stable and active under normoxia (31). To confirm the functionality of this molecule, we transiently transfected the expression plasmids for wild-type HIF-1α or the P402A/P564G form along with a 5× HRE-luc reporter plasmid and pSV-βgal and measured luciferase activity. Figure 7*A* shows the fold activation in luciferase activity, following normalization for transfection efficiency. Overexpression of wild-type HIF-1α or the double proline mutant in normoxia was able to induce HIF target gene activation by between 30- and 1,000-fold. The double proline mutant was even more potent, likely because of its enhanced stability (Fig. 7*A*).
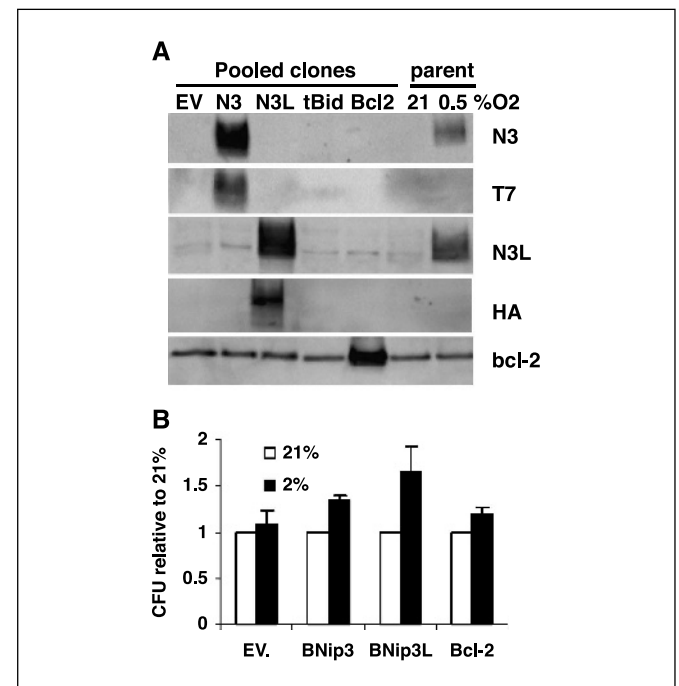

Figure 5. BNip3-T7- and BNip3L-HA stable overexpressing cells. HeLa cells were transfected with bicistronic plasmids expressing the indicated proteins and the puromycin resistance gene. Colonies were established by survival in 2 μg/mL puromycin. *A*, normoxic pools of >25 clones were assayed for protein expression using anti-epitope antibodies (T7, HA), or anti-protein antibodies as indicated. For comparison, extracts from parental HeLa cells grown in either 21% or 0.5% O₂ for 23 hours were run in parallel. Note endogenous bcl2 as loading control. *B*, colony survival of the stable transfected cells under hypoxia. Indicated cells were plated at very low density and grown in 21% or 2% O₂ for 10 days. Cells were stained with crystal violet and colonies >50 cells counted.
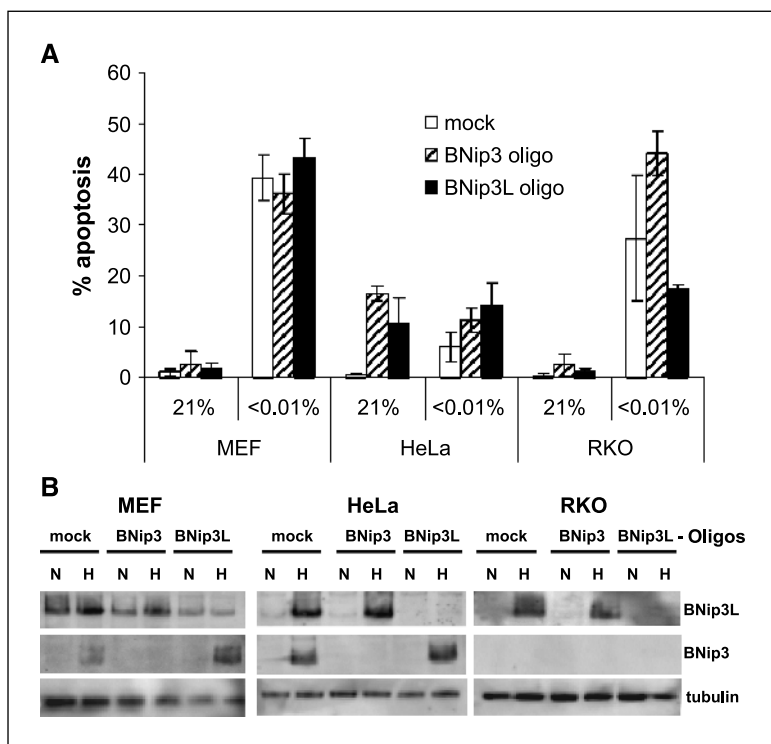
Figure 6. siRNA knockdown of BNip3 or BNip3L does not inhibit anoxia-induced apoptosis. *A,* apoptotic counts (by nuclear morphology) in cells transfected with the indicated siRNAs after 36 hours. 12 hours after transfection, the cells were placed in either 21% or <0.01% O_2 for the remaining 24 hours. No effect of specific gene knockdown is seen in anoxia-induced apoptosis in either MEF, Hela, or RKO cells. *B,* Western blot confirmation of successful siRNA block to hypoxic induction of the indicated proteins. Note lack of BNip3 expression in RKO cells such that when BNip3L is blocked by siRNA that there is the loss of both proteins. Oligonucleotide (*top*) and protein immunoblotted (*right*).

Having established that the P42A/P564G HIF-1α expression plasmid was a potent inducer of HRE activity under normoxia, we then determined its cytotoxicity. Cells were transiently transfected, 30 hours later immunostained for nuclear HIF-1α, and positive cells were scored for an apoptotic nuclear morphology. For comparison, nontransfected cells were treated with extreme hypoxia for the same time period. Whereas 30 hours of hypoxic incubation in buffered, nutrient-rich medium caused a small but reproducible level of apoptosis in the tumor cell lines, overexpression of functional HIF-1a under normoxic conditions failed to induce cell death (Fig. 7*B* and *C*). This implies that the activation of HIF-1 alone is not sufficient to drive cells into apoptosis, and additional biochemical and/or molecular requirements are met only under conditions of severe hypoxia.

Discussion

Animal models have shown the utility of studying HIF-regulated expression changes, especially as they pertain to development. Our data from HIF-1a knockout cells however show that *in vitro* death of MEFs under reduced oxygen does not need HIF-1 activated genes but does need anoxia-specific molecular changes. Furthermore, the plating efficiency of a wide panel of human tumor-derived cell lines in moderate hypoxia also shows that tumor cell survival is unaffected by the induction of HIF target genes. Finally, deletion of the VHL tumor suppressor gene and constitutive HIF activation does not necessarily stop the growth of either ES (32) or renal cancer cells (33).

Several reports suggest that HIF-1 target genes could contribute to hypoxia-induced apoptosis (11, 12, 16, 17, 34). These studies use a variety of experimental conditions, most often combining reduced oxygen conditions with additional apoptogenic conditions such as glucose and/or serum deprivation. Two of the best studied
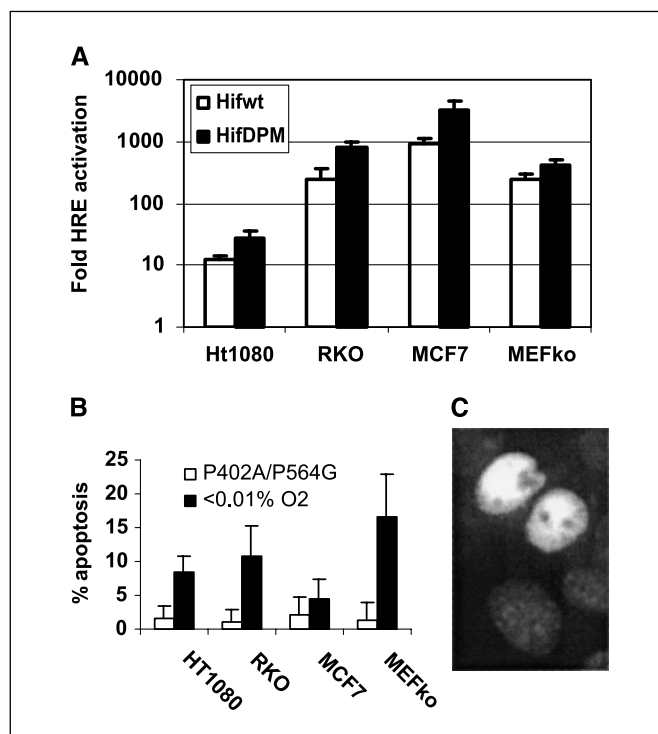

Figure 7. Forced overexpression of stable HIF-1α is not acutely apoptogenic. *A,* normoxic activation of 5× HRE reporter gene in cells cotransfected with either HIF-1α (*wt*) or a double proline mutant (P402A/P564G) expression plasmids. *B,* comparison between HIF-induced and anoxia-induced apoptosis. Normoxic cells transfected with HifDPM were fixed and stained 30 hours post-transfection with an anti-HIF-1α antibody and Alexa 594 secondary antibody (*red*) and Hoescht (*blue*). Apoptosis was scored in the immunopositive cells by nuclear morphology. For comparison, cells were placed under severe hypoxia (<0.01% oxygen) for the same time period and cell death was also measured by Hoechst staining. *C,* photomicrograph showing nuclear localization of the transfected HIF-1α (*light grey*) in normoxic MCF7 cells (*dark grey*).

candidate mediators of HIF-induced cell death are BNip3 and BNip3L. In agreement with the literature (7, 16, 33), we find BNip3 and BNip3L induced in response to HIF-1 activation. However, in our system, induction of BNip3 and BNip3L was disassociated from the physiologic induction of death in several ways. First, both molecules accumulated at significant levels even at very moderately hypoxic conditions where there is no cell death. Second, the lack of BNip3 and BNip3L induction in the HIF-1α knockout cells did not confer resistance to anoxia-induced apoptosis. Third, the siRNA-mediated knockdown of BNip3 and BNip3L also failed to confer resistance to tumor cell lines treated with anoxia.

Additional environmental conditions may contribute to hypoxia-induced killing *in vivo*. For example, BNip3 expression in cardiac myocytes requires acidosis to initiate cell death (21). Whereas BNip3 has also been suggested to activate an atypical cell death (24, 26), our stable overexpressing cells show no evidence for any type of toxicity. BNip3L shares high homology with BNip3 (22), but both proapoptotic and antiapoptotic functions have been described *in vitro* in the literature (19, 22, 23). *In vivo*, BNip3L has been proposed to be an effector of heart failure (35), but the possible role of hypoxia and/or acidosis as an activating signal for this response is not known.

The anoxia-dependent death-inducing signals are therefore initiated through a non-HIF mechanism. We find transcriptional changes in survival genes occurred in anoxic cells irrespectively of their HIF-1 status such as down-regulation of IAP family member survivin and induction of the molecular chaperone Erp99. Repression of survivin could contribute to hypoxic apoptosis as its down-regulation has been reported in the context of p53 activation and DNA damage–induced apoptosis (36, 37). Erp99 is the mouse homologue of GRP94, which facilitates the proper assembly and folding of newly synthesized proteins (38). ER chaperones exhibit antiapoptotic properties (39–41), and their hypoxic induction can be seen *in vivo* (42) through presumed activation of the UPR (28).

In vivo studies have shown very convincingly that areas of tumors that stain with hypoxia markers such as pimonidozole and EF5 show reduced proliferation, and increased fraction of apoptotic cells (43). These colocalization findings have prompted many groups to look for hypoxia-responsive mediators of apoptosis. One difficulty in translating the *in vivo* findings to *in vitro* work relates to the question what exactly are the microenvironmental stresses that exist in these regions. For example, oxygen tensions have been measured *in situ* from near normal levels to virtually zero in regions of necrosis. Additionally, areas of tumors that have poor perfusion and reduced delivery of oxygen could also suffer from other stress such as low glucose or low pH (44). These *in vitro* findings show that hypoxia alone is not sufficient to result in cellular death, but does not exclude the possibility that hypoxia contributes to death in regions of the tumor suffering from added stresses such as glucose deprivation or acidosis.

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