In Cultured Astrocytes, p53 and MDM2 Do Not Alter Hypoxia-inducible Factor-1α Function Regardless of the Presence of DNA Damage*

Received for publication, March 14, 2007. Published, JBC Papers in Press, April 9, 2007, DOI 10.1074/jbc.M702203200

David A. Rempe ‡, Katherine M. Lelli ‡, Grace Vangeison ‡, Randall S. Johnson ‡, and Howard J. Federoff ‡

From the ‡ Department of Neurology, Center for Aging and Developmental Biology, and the Interdepartmental Graduate Program in Neuroscience, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 and the ‡ Department of Biology, University of California, San Diego, California 92093

A principal molecular mechanism by which cells respond to hypoxia is by activation of the transcription factor hypoxia-inducible factor 1α (HIF-1α). Several studies describe a binding of p53 to HIF-1α in a protein complex, leading to attenuated function, half-life, and abundance of HIF-1α. However, these reports almost exclusively utilized transformed cell lines, and many employed transfection of p53 or HIF-1α plasmid constructs and/or p53 and HIF-1α reporter constructs as surrogates for endogenous protein activity and target expression, respectively. Thus, it remains an open and important question as to whether p53 inhibits HIF-1α-mediated transactivation of endogenous HIF-1α targets in nontransformed cells. After determining in primary astrocyte cultures the HIF-1α targets that were most dependent on HIF-1α function, we examined the effect of the loss of p53 function either alone or in combination with MDM2 on expression of these targets. Although p53 null astrocyte cultures resulted in markedly increased HIF-1α-dependent target expression compared with controls, this altered expression was determined to be the result of increased cell density of p53 null cultures and the accompanying acidosis, not loss of p53 protein. Although activation of p53 by DNA damage induced p53 target expression in astrocytes, it did not alter hypoxia-induced HIF-1α target expression. Finally, a combined loss of MDM2 and p53 did not alter HIF-1α target expression compared with loss of p53 alone. These data strongly suggest that p53 and MDM2 do not influence the hypoxia-induced transactivation of HIF-1α targets, regardless of p53 activation, in primary astrocytes.

Molecular responses to hypoxia provide a means of cellular adaptation that may enhance or decrease survival to this injurious stimulus depending on the cell type and cellular microenvironment. A primary molecular mechanism enhancing cellular responses to hypoxia is the transcription factor hypoxia-inducible factor 1α (HIF-1α). HIF-1α, the master regulator of the cellular response to hypoxia, is a transcription factor stabilized and activated during hypoxia (for reviews, see Refs. 1–4). HIF-1α is a basic helix-loop-helix protein in the PAS (Per-Arnt-Sim) transcription factor family. Under normoxic conditions, proline hydroxylation within the oxygen-dependent domain by prolyl hydroxylases allows for binding of HIF-1α to von Hippel-Lindau factor, an E3 ubiquitin ligase, and rapid elimination by ubiquitination and proteasomal degradation. During hypoxia, however, the activity of the prolyl hydroxylases is inhibited, leading to HIF-1α stabilization (5–7) and binding to its PAS binding partner HIF-1β (otherwise known as the aryl hydrocarbon receptor nuclear translocator) to form the dimeric HIF-1. HIF-1 binds to hypoxia-responsive cis-elements (HREs) present in the promoter region of several genes, including several glycolytic enzymes, glucose transporters, erythropoietin (EPO), vascular endothelial growth factor (VEGF), and the proapoptotic proteins Nip3 and Nix (1, 8, 9).

Several reports have suggested that p53, a transcription factor critically important in maintenance of cell cycle regulation and apoptosis, interacts with HIF-1α either directly or though binding to p300 or MDM2 (10–16). These interactions are thought to attenuate the activity, half-life, and abundance of HIF-1α (14–16). In contrast, HIF-1α is reported to stabilize p53 during hypoxia, increasing its abundance and enhancing its ability to transactivate its targets (10, 16). However, there is disagreement as to the conditions under which p53 and HIF-1α interact and to the molecular mechanisms by which p53 suppresses HIF-1 function. For example, recent work suggests that p53 does not alter HIF-1α protein abundance or function during hypoxia unless it is first activated by DNA damage (13), which contrasts with prior reports (14). Regarding the mechanism by which p53 may inhibit HIF-1α stabilization and function, some studies suggest that competition between p53 and HIF-1α for binding to their transactivator p300 was critical for altering transactivation of HIF-1 targets (12, 15). MDM2, an E3 ubiquitin ligase that targets p53 to the proteasome, is reported to reduce HIF-1α protein abundance by forming a complex with p53 (14). Yet, in contrast, MDM2 may also directly interact with HIF-1α under hypoxic conditions and enhance the transactivation of a HIF-1 reporter construct (17). Finally, direct interactions of HIF-1α and p53 were described using in vitro biophysical techniques (11). Although these studies illustrate possible molecular mechanisms and conditions by which p53 alters HIF-1α activity, they employed almost exclusively trans-
HIF-1α and p53 in Astrocytes

formed or cancerous cell lines, in which altered signal cascades relevant to HIF-1α and p53 function may be operative. Although these observations are important to determine the role of HIF-1α and p53 in cancer biology, they may not apply to nontransformed cell lines. Furthermore, prior work regularly employed transfection of cell lines, resulting in supraphysiologic levels of expressed protein. Finally, reporter constructs containing multiple copies of HREs were employed as surrogates for activation of endogenous HIF-1α targets, or HIF-1α targets were examined without first demonstrating the HIF-1 dependence of these targets within the cell line employed. Therefore, whether p53 attenuates the function of HIF-1α and impedes expression of endogenous HIF-1α targets in nontransformed cells with physiological levels of HIF-1α, p53, and MDM2 remains an open and important issue.

Putative interactions of p53 and HIF-1α in astrocytes could have important implications for the function of astrocytes during ischemia. Although the role of astrocytes during times of oxygen depletion, such as stroke, is debated, their ability to provide functions such as neurotrophic support, glutamate uptake, and metabolic substrates to neurons almost certainly alters neuronal survival during hypoxia (reviewed in Ref. 18). Interestingly, the HIF-1α and HIF-2α targets EPO and VEGF are neuroprotective and secreted by astrocytes, and they significantly contribute to the neuronal protection provided by hypoxic preconditioning (19, 20). Thus, a putative interaction between HIF-1α and p53 in astrocytes could alter the ability of astrocytes to provide neurotrophic support to neurons or could alter the ability of astrocytes to maintain their energy balance through glycolysis, ultimately impacting their own viability. Prior work in our laboratory expressed a dominant negative form of HIF-1α in neurons and demonstrated that it was neuroprotective. Since this protection was not observed in p53 null cells, this suggested that HIF-1α was contributing to neuronal death through a p53-dependent pathway (21). Moreover, in a recent report, which employed a neonatal rat model of ischemia, immunoprecipitations demonstrated interactions of p53 and HIF-1α (22). However, the influence of these interactions on HIF-1α function was not examined. The putative role of HIF and p53 in astrocytes remains unexplored. Since p53 protein is augmented in astrocytes in the stroke penumbra (23), we questioned if p53 and/or MDM2 may impair transactivation of endogenous HIF-1α target genes in cultured astrocytes. By measuring transcript abundance of a number of demonstrated HIF-1α-dependent targets, we examined putative HIF-1α and p53 interactions in the presence and absence of hypoxia, acidoisis, and/or DNA damage.

Using astrocyte cultures with loss of HIF-1α, p53, or p53 and MDM2 function, we demonstrate that HIF-1α targets vary in their dependence on HIF-1 function. Those encoding genes involved in glucose metabolism and proapoptotic proteins are more dependent on HIF-1α function compared with EPO and VEGF. Transcriptional targets of p53 are not induced by hypoxia, and their expression is not dependent on HIF-1α function. Astrocyte cultures derived from traditional p53 null mice had increased HIF-1α target expression. However, this finding was dependent on cell density and acidoisis rather than a direct p53-dependent effect, since it was eliminated when cell density was equivalent between p53 null and wild type cultures. Activation of p53 by camptothecin induced p53 target expression in astrocytes but did not alter hypoxia-induced HIF-1α target expression. Finally, a combined loss of MDM2 and p53 did not alter HIF-1α target expression compared with loss of p53 alone. Together, these data strongly suggest that p53 and MDM2 do not influence the hypoxia-induced transactivation of HIF-1α targets in cultured astrocytes even when p53 is activated by DNA damage. Therefore, the influence of p53 on HIF-1α protein abundance and function is probably a cell type-specific phenomenon and may occur most prominently in cancerous cells.

EXPERIMENTAL PROCEDURES

Astrocyte Culture Preparation—All methods were approved by our university committee devoted to the ethical use of animals in research. Primary astrocyte cultures were prepared from P0 neonatal mice. The brain was removed, and, using a dissecting microscope, the cortex and hippocampus were dissected away from the brain stem. Meninges were removed, and the telencephalon was placed into ice-cold Hanks’ balanced salt solution. The telencephalon was cut into 1-mm cubes and dissociated by repeated pipetting. The cells were pelleted and resuspended with glia minimal essential medium (minimal essential medium (11095-080; Invitrogen) supplemented with 6 g/liter glucose, 1 mM sodium pyruvate, 6% horse serum, and 4% fetal bovine serum). The cells were fed every 3–4 days until 100% confluent. In some cases, after the astrocytes were confluent, HEPES and AraC were added to maintain pH and prohibit microglial proliferation, respectively. All cultures were confluent at the time of the experiments. Using this protocol, greater than 85% of cells were GFAP(+) . In some cases, tamoxifen (1 μM) was added to these confluent cultures for 48 h and then removed 7 days prior to subsequent experiments. In some cultures, camptothecin (10 μM) was added to confluent cultures 2 h prior to 18 h of hypoxia (total exposure of 20 h). Hypoxia was accomplished by placing cultures into a triple gas incubator that replaces oxygen with nitrogen to achieve 0.5% oxygen (Thermo Electron Corp.).

RNA Purification and Synthesis of cDNA—RNA was isolated either using Trizol per the manufacturer’s (Invitrogen) recommendations or by using RNAeasy columns per the manufacturer’s (Qiagen) recommendations. In all cases, the samples were exposed to DNase treatment. The RNA pellet was stored at −80 °C until cDNA synthesis. Synthesis of cDNA was done either with avian myeloblastosis virus reverse transcriptase (Promega) or SuperScript III (Invitrogen) per the manufacturer’s recommendations. As template for first strand synthesis, we used 400–1000 ng of total RNA, and random hexamers were utilized for priming first strand synthesis. Control reactions were done without reverse transcriptase to control for genomic DNA contamination.

Real-time PCR Using Taqman—Primer-probe sets were designed with Primer-express III. To perform real-time PCR, 25-μl reactions contained 900 nm primers and 50 nm probe. An ABI prism 7700 or 7300 sequence detector real-time PCR thermocycler was utilized for measuring concentration of mRNA. All samples were measured in duplicate. Standard curves of known concentrations of product were utilized to calculate rel-
buffer and underwent three serial freeze/thaws on dry ice. Samples were centrifuged at 4°C, and the supernatant was saved. Protein concentration was determined using a modified Lowry method (Bio-Rad).

Western Blots—Nuclear or whole cell lysates were diluted into buffer containing SDS and β-mercaptoethanol to ensure denaturing conditions, and electrophoresis was performed using polyacrylamide gels. The protein was transferred to polyvinylidene difluoride membranes (Millipore) and placed in blocking buffer (5% dry milk, 0.1% Tween, 34 mM NaCl, Tris, pH 7.6) overnight at 4°C or left at room temperature for 1–2 h. The membranes were subsequently placed in blocking buffer with anti-HIF-1α antibody (1:500 dilution; Novus), anti-HIF-2α antibody (1:500 dilution; Novus), a p53 antibody (Ab1; Oncogene), a serine 15 phosphorylation-specific antibody for identification of activated p53 (Cell Signaling), or an antibody specific for β-actin (25). After exposure to a horseradish peroxidase-conjugated 1:2000 dilution of secondary antibody, the blots were developed with chemiluminescence (PerkinElmer Life Sciences) and exposed to X-Omat film (Eastman Kodak Co.). In some cases, membranes were stripped and probed with multiple antibodies.

Transgenic Mice—We utilized multiple lines of transgenic mice in these studies, the creation of which is detailed elsewhere. Transgenic mice included HIF-1α+/−/− (26), p53−/−/−, hGFAPcre (27), p53f+/−/− (28), MDM2−/−/− (29), and tamoxifen-regulated cre mice (EsrCre) (30). The HIF-1α+/−/− mice were obtained from Dr. Johnson, p53−/−/− mice were obtained from Taconic, hGFAPcre mice were obtained from Dr. Messing, p53+/+/- mice were from the NCI, National Institutes of Health, Mouse Models of Human Cancer Consortium, MDM2−/−/− mice were from Dr. Lozano, and EsrCre mice were from Jackson Laboratory. To achieve loss of HIF-1α in astrocyte cultures, HIF-1α+/−/− mice were bred with hGFAPcre mice, resulting in HIF-1α+/−/−::hGFAPcre mice. Loss of both HIF-1α and p53 function was achieved by breeding triple transgenic HIF-1α+/−/−::hGFAPcre::p53−/−/− mice. Conditional loss of p53 function in astrocyte cultures was achieved by preparing cultures from bigenic p53−/−/−::EsrCre mice and then treating the cultures with tamoxifen.

RESULTS

Loss of HIF-1α Function and Its Effect on HIF-1α Target Expression—Before examining the potential effects of p53 or MDM2 on the expression of HIF-1α targets in astrocyte cultures, we first characterized the dependence of hypoxia-induced expression of HIF-1α targets on HIF-1α function. Accordingly, only those targets determined to be dependent on HIF-1α function would be examined when p53 function was removed. To achieve loss of HIF-1α function, we utilized transgenic mice containing a recombinatorial substrate to allow for elimination of the HIF-1α function (26). These HIF-1α+/−/− mice contain two loxp sites, which flank exon 2 of HIF-1α. Upon exposure to cre recombinase, expression and function of HIF-1α is eliminated, as demonstrated in multiple studies (31–34).

Astrocyte cultures were prepared from sibling offspring of matings between HIF-1α+/−/− (no cre expression) and

---

**TABLE 1**

| Primer and probe sequences | Primers/Probes | Sequences |
|----------------------------|---------------|-----------|
| HIF exon 2                 | Forward        | T0AACCTCGAGAAAGGAGATTCAAGAGCAGCTC |
|                           | Reverse        | GCAACCTCGAGAAAGGAGATTCAAGAGCAGCTC |
| Glut-1                     | Forward        | TCTCCTGTCGCCCTTCGGCC |
|                           | Reverse        | GGTGCTCTGTTCAACCTTTGTTAGT |
| HKII                       | Forward        | GACTCGGAATTCTCGGAGATGAG |
|                           | Reverse        | AGGAGAAGTAGCGGACGAGAG |
| EPO                        | Forward        | TTGCTAGCTGTTTAGCTGAGG |
|                           | Reverse        | GCTACTGCTGCTGCTGAGAG |
| Nip3                       | Forward        | CACAGGCTCTGTTGATAGAG |
|                           | Reverse        | GACGGGAGTGGAGATGGAGAG |
| Nix                        | Forward        | ACTTCTGCTGCTGCTGAGAG |
|                           | Reverse        | GAGACTGGAAGCGGCACGAG |
| EGLN2                      | Forward        | GCTGCGCACTACATGCCTCA |
|                           | Reverse        | TGCCGCACTACATGCCTCA |
| Bax                        | Forward        | GAGGAGACCTGTTGAGAGG |
|                           | Reverse        | CAAAGAACCCTCTCTCTCAT |
| PUMA                       | Forward        | GAGCGCGGACGAGAGAA |
|                           | Reverse        | GAGATTTCTACAGCTCCTGAG |
| IGFPR3                     | Forward        | GCCGCGGACACGAGGAGAG |
|                           | Reverse        | TGGCATGGAGTGGAGATGGAG |
| Noxa                       | Forward        | GCCGCGGACACGAGGAGAG |
|                           | Reverse        | TGGCATGGAGTGGAGATGGAG |
| p53                        | Forward        | TTGAGAGCTGCTGCTGAGAG |
|                           | Reverse        | TTGAGAGCTGCTGCTGAGAG |
| MDM2                       | Forward        | ATCCCTCCTCCTCCACACCT |
|                           | Reverse        | CCCCCTATCTGCTGAGAGC |
|                           | Probe          | CAAAAGATGCTGGACCCTTCGTGAGAAC |

---

ative abundance of the genes of interest. Target abundance was normalized to a housekeeping gene (β-actin), which was used as an internal control. Preliminary experiments demonstrated that known dilutions of target cDNA were accurately predicted using this methodology. The nucleotide sequences of the primers and probes are listed in Table 1.

**Isolation of Nuclear Enriched and Whole Cell Protein Lysates—** We isolated nuclear and cytoplasmic proteins using a rapid method described by Andrews and Faller (24). Briefly, cultures were washed with ice-cold PBS, and to the dish, 400 μL of solution A (10 mM Tris-HCl, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Sigma)) was added to the cultures. The cells were scraped into a tube and placed on ice for 10 min. The samples were centrifuged at 10,000 rpm for 10 s, and the supernatant was removed (cytoplasmic component). The pellet was resuspended in 40 μL of solution C (420 mM KCl, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (Sigma)) and allowed to sit on ice for 20 min. The cellular debris was removed by centrifugation for 2 min at 4°C, and the supernatant was removed and stored at −80°C. Protein concentration was subsequently determined using a modified Lowry method per the manufacturer’s protocol (Bio-Rad). Whole cell protein lysate was collected into radioimmune precipitation

---

**RESULTS**

---

HIF-1α and p53 in Astrocytes

---
**A**

**HIF-1α Exon2**

- **Fold induction**
  - Norm: *****
  - Hypox: *****

**Glut-1**

- **Fold induction**
  - Norm: ****
  - Hypox: ****

**HKII**

- **Fold induction**
  - Norm: ****
  - Hypox: ****

**EPO**

- **Fold induction**
  - Norm: NS
  - Hypox: NS

**VEGF**

- **Fold induction**
  - Norm: ****
  - Hypox: ****

**Nip3**

- **Fold induction**
  - Norm: *****
  - Hypox: *****

**Nix**

- **Fold induction**
  - Norm: *****
  - Hypox: *****

**EGLN2**

- **Fold induction**
  - Norm: NS
  - Hypox: NS

---

**B**

**Astrocyte**

- HIF-1α
- HIF-2α
- β-actin
- Cob.
- Cre

**HN33**

- HIF-1α
- HIF-2α
- β-actin
- Cob.
- Cre
HIF-1α+/−/+::hGFAPcre (heterozygous for hGFAPcre allele) mice. From these matings, we obtained astrocyte cultures from neonatal mice with one of two genotypes: HIF-1α+/−/+ (control) or HIF-1α+/−/+::hGFAPcre (test). As expected, astrocyte cultures derived from HIF-1α+/−/+::hGFAPcre mice expressed markedly reduced HIF-1α exon 2 transcript abundance when measured by quantitative PCR (Fig. 1). Similarly, HIF-1α protein stabilization by cobalt, a hypoxia mimetic, was eliminated in HIF-1α+/−/+::hGFAPcre astrocytes. In contrast, the protein abundance of HIF-2α was not altered by loss of HIF-1α function. Similar to astrocyte cultures, HIF-1α was markedly induced in HN33 cultures, a cell line formed by fusion of hippocampal neurons with a neuroblastoma cell line. The amount of HIF-1α in the HN33 cell line was greater than that observed in astrocyte cultures, in agreement with the greater amount reported in primary neuronal cells (34).

When astrocyte cultures remained at 21% oxygen, reduced HIF-1α function did not alter HIF-1α target transcript abundance. In contrast, when astrocyte cultures were placed into hypoxia (0.5% oxygen) for 18 h, loss of HIF-1α function significantly reduced hypoxia-induced expression of several HIF-1α targets, including Glut-1, HKII, Nip3, and Nix (Fig. 1). Although hypoxia-induced VEGF expression also significantly declined with reduced HIF-1α function, it was less dependent on HIF-1α function for its hypoxic induction compared with HKII, Nip3, and Nix. Despite being a known HIF-1α target, EPO transcript abundance was not significantly attenuated by loss of HIF-1α function during hypoxia, in agreement with recent literature demonstrating the dependence of EPO induction on HIF-2α function (34, 35) (Fig. 1). Thus, in astrocytes, hypoxia-inducible expression of HIF-1α targets involved in glucose metabolism and apoptosis was more dependent on HIF-1α function compared with EPO and VEGF. Before examining the effect of loss of p53 function on the expression of these HIF-1α targets during hypoxia, we first decided to examine the impact of hypoxia on p53 target expression and their expression with the loss of p53 function.

Expression of p53 Targets Is Dependent on p53 Function in Astrocytes, but They Are Not Induced by Hypoxia—Before examining if loss of p53 function impacts expression of HIF-1α targets, we first wanted to examine if p53 transactivates its targets in astrocyte cultures in normoxia or hypoxia. Although transcriptional activity of p53 in primary astrocytes has not been examined in detail, one report suggests that p53 may induce astrocyte cell death independent of transcriptional activation (36). Therefore, we measured p53 target abundance in astrocyte cultures derived from control C57Bl/6 mice or traditional p53 null (knock-out) mice. For ease of reference, astrocyte cultures prepared from these mice will be referred to as p53 null astrocyte cultures to distinguish them from the conditional p53 floxed cultures (p53+/−/+ ) utilized in subsequent experiments. As predicted, p53 transcript abundance was largely eliminated in cultures derived from p53 null mice (Fig. 2). A p53 protein-specific band and p53 serine 15 phosphorylation was induced by camptothecin in control cultures but not observed in astrocytes derived from p53 null mice (Fig. 2, B and C). Although p53 protein is not appreciated under normoxic conditions, transcript abundance of several p53 targets, including p21, Bax, PUMA (p53 up-regulated mediator of apoptosis), IGFBP3, and Noxa, were markedly attenuated with loss of p53 function in normoxia and hypoxia. Therefore, p53 is transcriptionally active in these cells during normoxia and hypoxia. In all cases except PUMA, hypoxia did not transactivate p53 targets above base-line expression levels in agreement with other reports (13, 25, 37). Although the induction of PUMA by hypoxia was modest, it was significant and was observed in multiple experiments (see Figs. 3, 7, and 8). It should be noted that although some reports describe Noxa (38) and p21 (39) as targets of HIF-1α, in our cultures the expression of these transcripts was markedly dependent on p53 function. This illustrates the importance of defining the dependence of HIF-1α target expression on HIF-1α function, since it may be cell type-specific.

Expression of p53 Targets in Normoxia or Hypoxia Is Not HIF-1α-dependent—Since some reports suggest that HIF-1α can stabilize p53 and enhance its transcriptional activity (10, 16), we sought to examine if expression of p53 targets during hypoxia is altered in astrocytes by the loss of HIF-1α function. In the same manner as that described above, astrocyte cultures prepared from HIF-1α+/−/+ or HIF-1α+/−/+::hGFAPcre mice. Despite the fact that loss of HIF-1α markedly attenuated expression of multiple HIF-1 targets (Fig. 1), it did not alter expression of p53 targets under either normoxic or hypoxic conditions (Fig. 3). Thus, in these astrocyte cultures, HIF-1α function does not alter p53 target expression.

Astrocyte Cultures Derived from p53 Null Mice Have Increased HIF-1α Target Expression—To examine the effect of loss of p53 function on expression of HIF-1α targets, we compared the hypoxia-induced expression of HIF-1α targets in C57Bl/6 and p53 null cultures under normoxic and hypoxic conditions. Initial experiments, using p53 null cultures prepared from traditional p53 knock-out mice, examined hypoxia-induced expression of several HIF-1 targets. HIF-1 targets were significantly induced in p53 null cultures, which was not observed in a non-HIF-1 target (Fig. 4). In general, no such enhancement of HIF-1α target expression was observed in normoxia, except for HKII, which was not replicated in subsequent experiments (data not shown). Interestingly, both HIF-1α transcript and protein abundance were also increased in these cultures, suggesting that loss of p53 function induced a HIF-1-de-
HIF-1α and p53 in Astrocytes

A

**p53**

![Bar graph showing fold induction of p53 in Norm and Hypox conditions for C57Bl/6J and p53null (p53-/-) genotypes.]

**p21**

![Bar graph showing fold induction of p21 in Norm and Hypox conditions for C57Bl/6J and p53null (p53-/-) genotypes.]

**Bax**

![Bar graph showing fold induction of Bax in Norm and Hypox conditions for C57Bl/6J and p53null (p53-/-) genotypes.]

**PUMA**

![Bar graph showing fold induction of PUMA in Norm and Hypox conditions for C57Bl/6J and p53null (p53-/-) genotypes.]

**IGFBP3**

![Bar graph showing fold induction of IGFBP3 in Norm and Hypox conditions for C57Bl/6J and p53null (p53-/-) genotypes.]

**Noxa**

![Bar graph showing fold induction of Noxa in Norm and Hypox conditions for C57Bl/6J and p53null (p53-/-) genotypes.]

- **C57Bl/6J**
- **p53null (p53-/-)**

B

**p53**

![Western blot showing p53 expression in C57Bl/6J and p53null (p53-/-) genotypes with Camptothecin treatment.]

**β-actin**

![Western blot showing β-actin expression in C57Bl/6J and p53null (p53-/-) genotypes with Camptothecin treatment.]

C

**p53**

![Western blot showing p53 expression in C57Bl/6J and p53null (p53-/-) genotypes with Camptothecin treatment.]

**β-actin**

![Western blot showing β-actin expression in C57Bl/6J and p53null (p53-/-) genotypes with Camptothecin treatment.]

- **p53 ser15**
HIF-1α and p53 in Astrocytes

enhanced expression of HIF-1α targets in p53 null cultures was mediated primarily through HIF-1α-dependent mechanisms.

Not surprisingly, loss of p53 function was associated with an apparent increased cell proliferation. Despite plating equal cell numbers, p53 null cultures became confluent more quickly. Protein extracted from p53 null cultures typically was 2-fold greater than controls (Fig. 4D), as was the amount of RNA (data not shown). Because of the increased cell number, we noted that the p53 null cultures became more acidic during hypoxia (see below). Recent reports demonstrate that these different conditions could alter HIF-1α target expression independent of any putative interactions between p53 and HIF-1α (40, 41). Therefore, we also sought to examine HIF-1α target expression in astrocyte cultures in which p53 function is eliminated after the cultures become confluent. This approach provides equal cell numbers and eliminates potential confounds of altered proliferation, cell density, and the resultant acidosis.

Loss of p53 Function Does Not Alter Hypoxia-induced HIF-1α Target Expression in Astrocyte Cultures When Cell Numbers Are Equivalent—Using bigenic mice with a floxed p53 construct (p53<sup>f/f</sup>) (28) and a tamoxifen-regulated conditional cre recombinase (EsrCre), which is active only in the presence of tamoxifen (30), loss of p53 function was achieved after astrocyte cultures became confluent. In this way, the cultures were confluent prior to loss of p53 function, eliminating any confounds of differences in proliferation. Astrocyte cultures were prepared from siblings with one of two genotypes: p53<sup>f/f</sup> or bigenic p53<sup>f/f</sup>:EsrCre. After becoming confluent, the cultures were treated with AraC to eliminate further cell division and exposed to tamoxifen to activate cre recombinase activity. The presence or absence of p53 transcript, protein, and transactivation of p53 targets was evaluated 5–7 days later. As expected, camptothecin induced a robust Ser15 phosphorylation of p53, an indicator of activated p53, in p53<sup<f/f</sup> cultures (control) and p53<sup<f/f</sup>:EsrCre cultures not treated with tamoxifen (control) (Fig. 6). Similarly, total p53 protein abundance was also enhanced. Moreover, camptothecin
HIF-1α and p53 in Astrocytes

A. 

**Glut 1**

Fold Induction

- Norm
- Hypox

**HKII**

Fold Induction

- Norm
- Hypox

**Nip3**

Fold Induction

- Norm
- Hypox

**Nix**

Fold Induction

- Norm
- Hypox

**VEGF**

Fold Induction

- Norm
- Hypox

**Non-HIF target**

Fold Induction

- Norm
- Hypox

B. 

**C57Bl/6**

**P53-/-**

HIF-1α

β-actin

Hypoxia

- +
- +

C. 

**HIF-1α Exon2**

Fold Induction

- Norm
- Hypox

D. 

Protein abundance

- Wildtype 21
- p53-/-
induced the expression of the p53 target PUMA in these controls (Fig. 6). In contrast, in the presence of tamoxifen, transcript and protein abundance of p53 was markedly reduced in p53f<f/H11001/f/H11001::EsrCre cultures, and PUMA transcript abundance was reduced similar to that observed with the traditional p53 null cultures (compare Figs. 2 and 6). Thus, recombination and elimination of p53 protein was not observed in p53<sup>f<sup>f</sup>/H11001</sup>/f/H11001::EsrCre cultures unless tamoxifen was present, and tamoxifen-induced loss of p53 markedly attenuated camptothecin-induced p53 protein activation and PUMA transcript abundance. By employing this approach, we were able to achieve loss of p53 expression and function in astrocyte cultures without altering cell number.

To determine if loss of p53 function induces HIF-1α target expression in astrocyte cultures with equal cell numbers during hypoxia, p53f<f/H11001/f/H11001 and p53f<f/H11001/f/H11001::EsrCre cultures were subject to hypoxia in the presence and absence of tamoxifen, and HIF-1α targets were quantified. Unlike that observed in the traditional p53 null cultures (Fig. 4), there was no consistent increase in HIF-1α target expression in astrocyte cultures lacking p53 function (Fig. 7). In some cases, such as Glut-1, transcript abundance in p53<sup>f<sup>f</sup>/H11001/f/H11001::EsrCre cultures treated with tamoxifen was slightly increased. However, this small change was not replicated in subsequent experiments (data not shown).

**Activated p53 Induced with DNA Damage Does Not Alter Hypoxia-induced HIF-1α Target Expression in Astrocyte Cultures**—Since some reports suggest that p53 only alters HIF-1α abundance and function when p53 is activated (13), we treated cells with camptothecin in the presence and absence of hypoxia. In agreement with published reports (42), the astrocytes remained viable for prolonged times in camptothecin, and there was no significant cell death during the 20-h exposure (data not shown). Furthermore, camptothecin induced activation of p53, as measured by serine 15 phosphorylation, and

![FIGURE 5.](image-url) **The induction of HIF-1α targets in p53 null astrocyte cultures is primarily mediated by HIF-1α dependent mechanisms.** As in Fig. 3, p53<sup>−/−</sup> astrocyte cultures had enhanced hypoxia-induced expression of HIF-1α targets above controls (HIF-1α<sup>f<sup>f</sup>/H11001/f/H11001). In those cultures with a combined loss of HIF-1α and p53 function (HIF-1α<sup>f<sup>f</sup>/H11001::GFAPcre::p53<sup>−/−</sup>), hypoxia-induced HIF-1α target expression was only slightly greater than in cultures with loss of HIF-1α alone (HIF-1α<sup>f<sup>f</sup>/H11001::GFAPcre). This small increase may be due to a small increase in HIF-1α transcript observed in p53 null cultures. Regardless, the enhanced expression of HIF-1α targets seen in p53<sup>−/−</sup> astrocytes was primarily mediated through HIF-1α-dependent mechanisms.

![FIGURE 4.](image-url) **The expression of HIF-1α targets in p53 null astrocyte cultures.** A, hypoxic induction of HIF-1α targets is enhanced in the p53 null cultures, in which cell number was greater than controls. The enhanced hypoxia-induced expression was not observed with brain factor, a non-HIF-1α target. B, HIF-1α protein abundance was enhanced in astrocyte cultures derived from p53 null mice when exposed to hypoxia. C, similarly to protein, HIF-1α transcript abundance is induced in p53 null astrocytes. D, astrocyte cultures derived from p53 null mice proliferate at an enhanced rate. As such, the amount of total protein extracted from p53 null cultures was approximately twice that of controls (D). The data in A and C are representative examples of three independent experiments, whereas the data in D are an average of these three experiments.
induced the expression of the p53 target PUMA (Fig. 6). If, as in published reports (13), activated p53 suppresses HIF-1\(^{+/−}\) function, then the addition of camptothecin is predicted to decrease HIF-1 target expression during hypoxia. Furthermore, if this effect were observed, it would be predicted that loss of p53 function would reverse the decline in HIF-1\(^{+/−}\) target expression. In contrast to this postulate, the addition of camptothecin did not inhibit hypoxia-induced HIF-1\(^{+/−}\) target expression (Fig. 7). In fact, in some cases (Glut-1), the combination of hypoxia and camptothecin enhanced expression of the transcript above that observed with hypoxia alone. Furthermore, loss of p53 function did not enhance HIF-1\(^{+/−}\) target expression in the presence of camptothecin (Fig. 7). We conclude that p53 function, even in the presence of DNA damage, does not reduce expression of multiple HIF-1 targets in primary astrocyte cultures.

**Although the Combination of Hypoxia and Acidosis Enhances HIF Target Expression, Loss of p53 Function Does Not Enhance the Effect**—Not only did p53 null cultures have increased cell numbers, but they were also acidic compared with wild type. Potentially, the acidosis alone or in combination with loss of p53 function could account for alterations in HIF-1\(^{+/−}\) target expression seen in the p53 null cultures (Fig. 4) independent of cell number. To examine this question, p53\(^{+/−}\)::EsrCre astrocyte cultures were exposed to medium that was transferred from p53 null cultures that had been exposed to hypoxia for 24 h to produce acidic media (AM). These p53\(^{+/−}\)::EsrCre astrocyte cultures with AM were then subjected to hypoxia. The pH of the AM collected following p53 null cultures exposed to hypoxia was 7.1–7.2. This pH was more acidic than that measured in media from C57Bl/6 cultures exposed to hypoxia (pH 7.35) and C57Bl/6 that remained in normoxia (pH 7.6). Following exposure of p53 null cultures to hypoxia, the cells were equilibrated under normoxic conditions prior to their AM being transferred to p53\(^{+/−}\)::EsrCre cultures. In this way, the p53\(^{+/−}\)::EsrCre cultures were exposed to AM but did not have additional time in hypoxia compared with control. The p53\(^{+/−}\)::EsrCre cultures either remained in normoxia or were exposed to hypoxia (0.5% oxygen) for 24 h.

Exposure of astrocyte cultures to AM derived from hypoxic p53\(^{−/−}\) cultures did not change HIF-1 target expression under normoxic conditions (Fig. 8). However, the combination of AM and hypoxia did induce HIF-1 targets above that observed with hypoxia alone. Thus, the additive effects of acidosis and hypoxia on HIF-1 target expression at least partially accounted for some of the induction of HIF-1 targets observed in p53 null cultures (Fig. 4). However, loss of p53 function did not elicit a further induction of HIF-1 targets (Fig. 8). In fact, in some cases, such as Nix and Nip3, loss of p53 function attenuated expression of the
To confirm loss of p53 expression and function in these experiments, we also measured transcript abundance of p53 and its target PUMA. Significant loss of expression of p53 and PUMA was achieved in all conditions tested (Fig. 8).

It should also be noted that neither acidosis nor loss of p53 function induced HIF-1α transcript level. This is in contrast to the induction of HIF-1α transcript abundance seen in p53 null cultures (Fig. 4). Thus, the induction of HIF-1α transcript seen in the p53 null cultures is not due to acidosis or loss of p53 function.

Protein Abundance of HIF-1α and p53 Is Not Altered by Loss of the Other’s Function—Although loss of p53 or HIF-1α function did not alter hypoxic induction of the other’s target expression, it is still possible that protein stability of HIF-1α could be altered by loss of p53 expression or vice versa. Thus, we examined p53 protein abundance in conditions in which both HIF-1α and p53 are activated (i.e. during hypoxia and exposure to camptothecin). The protein abundance of p53 was not altered by the loss of HIF-1α function during hypoxia and camptothecin (Fig. 9B). Similarly, the loss of p53 function during hypoxia in the presence and absence of camptothecin did not alter HIF-1α protein abundance (Fig. 9C). Thus, the abundance of HIF-1α protein is not altered by loss of p53 or activation of p53 in these cultures.

Loss of MDM2 Does Not Alter HIF-1α Target Expression—MDM2 has been suggested to alter HIF-1α target expression by...
One of two mechanisms. First, it may decrease HIF-1α half-life through its interaction with p53 (14), or it may directly interact with HIF-1, potentially enhancing HIF protein abundance (17, 43). Although loss of MDM2 alone is embryonic lethal, mice with a combined loss of MDM2 and p53 are viable (29). To examine a putative direct effect of MDM2 on HIF-1α function, we utilized astrocytes with loss of p53 alone or a combined loss of MDM2 and p53 function. As anticipated, p53 transcript abundance was largely eliminated in both p53 null and double null p53\(^{-/-}\)::MDM2\(^{-/-}\) cultures, whereas loss of MDM2 transcript was only observed in the

expression was not HIF-1α-dependent (Fig. 3). However, the expression of Noxa is also dependent on p53 expression (49, 50), and the different contributions of these transcription factors is probably cell type-specific. Regardless, identifying the dependence of the hypoxia-inducible transcripts on HIF-1α function is important, since it validates using these targets to examine HIF-1α function. Moreover, it gives us the ability to examine endogenous targets known to be HIF-1α-dependent, which subserve diverse cellular functions, without use of HRE reporter constructs, which may or may not faithfully replicate the activation pattern of endogenous targets.
Recent work by Giaccia's group (37) has demonstrated that p53 stabilized by hypoxia is transactivation-deficient, although it is capable of suppressing gene expression. Our results suggest that hypoxia alone does not induce expression of p53 targets, since the p53 targets Bax, IGFBP3, and Noxa are not induced during hypoxia, whereas PUMA is modestly hypoxia-responsive (Fig. 2). However, loss of p53 function did result in a marked loss of expression of several p53 targets in normoxia and hypoxia, suggesting a basal level of p53 activity in astrocyte cultures. In fact, under control conditions, a minimal amount of phosphorylation of p53 serine 15 is detected (Fig. 6). These findings were unexpected, since in cancerous cell lines, activated p53 is typically not reported under control conditions (13). This basal activity of p53 could result from the treatment of the cultures with AraC, which is utilized to inhibit microglial proliferation. Regardless of the cause, this basal p53 activation may increase HIF-1α and p53 interactions and potentially cause suppression of HIF-1α targets under basal conditions even in the absence of camptothecin. However, since loss of p53 function did not alter expression of HIF-1α targets under these conditions (Fig. 7), this interpretation is flawed and does not alter our conclusions. Prior work by others suggested that p53 stabilization during hypoxia is dependent on HIF-1α (10), and HIF-1α augments the transactivation of a p53 reporter (16). However, other studies did not find a dependence of hypoxia-induced p53 activity on HIF-1 function (51), and similarly, loss of HIF-1α function in our cultures did not impact expression of endogenous p53 targets. These data suggest that HIF-1α function does not alter p53-dependent target expression during hypoxia in astrocyte cultures. We did not, however, examine if loss of HIF-1α function attenuates p53 transcriptional activity when p53 activity is more robust, such as is present with DNA damage. Thus, we cannot rule out this possibility.

Preliminary experiments suggested that loss of p53 function markedly altered expression of HIF-1α targets. This effect appeared HIF-1α-dependent, since HIF-1α transcript and protein were increased in p53 null cultures, and no increase was seen in non-HIF-1α targets in the p53 null cultures (Fig. 4). Moreover, a combined loss of HIF-1α and p53 in astrocytes largely abrogated the increase in HIF-1α target expression observed in p53 null cultures (Fig. 5), demonstrating the HIF-1α dependence of the effect. However, since the p53 null astrocytes had an increased proliferation rate, there was an increased cell density in p53 null cultures, and the media became more acidic during exposure to hypoxia. Changes in HIF-1α protein levels and targets are described for both cell density (41) and acidosis (40). In the case of cell density, a recent report demonstrates reduced binding of von Hippel-Lindau factor to HIF-1α with increasing cell density, an effect primarily mediated because of impaired hydroxylation of proline residue 564 of HIF-1α within the oxygen-dependent domain (41). During acidosis, nuclear sequestration of von Hippel-Lindau factor leads to increased HIF-1α protein abundance (40). Thus, either one of these factors could alter HIF-1α protein abundance and target expression in p53 null cultures. These concerns led us to examine HIF-1α target expression in astrocyte cultures in which loss of p53 function was achieved only after the cells were confluent, eliminating confounds of altered proliferation, cell density, or acidosis. When cell density was equivalent in cultures with loss or preserved p53 function, HIF-1α target expression was not altered by loss of p53 function. Interestingly, when cells were exposed to conditions of acidosis and hypoxia, enhanced expression of HIF-1α targets was observed. This demonstrates that acidosis can work in concert with hypoxia to enhance HIF-1 target expression. These results demonstrate that simply quantifying endogenous HIF-1α target expression, HIF-1α protein abundance,
or transactivation of HRE constructs when evaluating the dependence of HIF-1 activity on p53 status can easily be complicated by alterations in cell number and differences in pH among the different genotypes.

Since loss of p53 function does not modify hypoxic induction of HIF targets in astrocyte cultures with equivalent cell density, we wanted to evaluate if activation of p53 is required to observe a change in HIF-1α target expression as described in cancer cell lines (13). Treatment with the topoisomerase I inhibitor camptothecin induced a robust activation of p53 as judged by serine 15 phosphorylation and induced the expression of the p53 target PUMA (Fig. 6). Furthermore, loss of p53 function attenuated this camptothecin induction of PUMA. However, treatment with camptothecin did not inhibit hypoxia-induced HIF-1α target expression (Fig. 7). Furthermore, loss of p53 function did not induce HIF-1α targets in the presence of camptothecin. Interestingly, although not examined for camptothecin itself, some camptothecin analogs are described as inhibiting HIF-1α activity (52, 53). This, of course, could potentially alter HIF-1α function independent of p53 activity. However, camptothecin did not attenuate hypoxia-induced expression of HIF-1α targets in our hands, suggesting no direct role of camptothecin on HIF-1 function in our cultures. In subsequent experiments, we also employed mitomycin to activate p53 but saw no p53-dependent effects on HIF-1α target expression (data not shown). Thus, although DNA damage and p53 activation were clearly linked to a loss of HIF-1α targets expression (CAIX) and decreased HIF-1α protein half-life in cancer cell lines (13), they had no effect on HIF-1α target expression in our cells. It is difficult to reconcile these divergent results, but this discrepancy could be related to differences between transformed and nontransformed cell lines.

Currently, it is unknown if p53 and HIF-1α interact in the brain altering the transactivation of their respective targets. Prior work from our laboratory suggested a pathological role of HIF-1α mediated by p53 in neuronal cultures (21). However, this study employed a supra-physiological expression of a dominant negative form of HIF-1α, which could alter p53-dependent pathways by nonphysiologic mechanisms. Furthermore, neurons derived from p53 null cultures induced HIF-1α targets in hypoxia to levels comparable with wild type (data not shown). However, we have not examined this putative interaction in neurons in the presence of DNA damage. Prior work demonstrated a pathological role of HIF-1α in CA1 neurons following transient global ischemia (33), but any putative role of p53 in mediating this cell death remains unexplored. Finally, a recent report suggests an interaction of p53 with HIF-1α in the brain of neonatal rats following hypoxia-ischemia (22). The influence of this p53-HIF-1α interaction on HIF-1α target expression was not examined. Thus, its role in mediating cell death and the cell type in which it is expressed remain to be elucidated.
