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Intermittent Induction of HIF-1α Produces Lasting Effects on Malignant Progression Independent of Its Continued Expression

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Abstract

Dysregulation of hypoxia-inducible transcription factors HIF-1α and HIF-2α correlates with poor prognosis in human cancers; yet, divergent and sometimes opposing activities of these factors in cancer biology have been observed. Adding to this complexity is that HIF-1α apparently possesses tumor-suppressing activities, as indicated by the loss-of-function mutations or even homozygous deletion of *HIF1A* in certain human cancers. As a step towards understanding this complexity, we employed 8-week intermittent induction of a stable HIF-1α variant, HIF1α(PP), in various cancer cell lines and examined the effects on malignant progression in xenografts of immunocompromised mice in comparison to those of HIF2α(PP). Although 8-week treatment led to eventual loss of HIF1α(PP) expression, treated osteosarcoma U-2 OS cells acquired tumorigenicity in the subcutaneous tissue. Furthermore, the prior treatment resulted in widespread invasion of malignant glioma U-87 MG cells in the mouse brain and sustained growth of U-118 MG glioma cells. The lasting effects of HIF-1α on malignant progression are specific because neither HIF2α(PP) nor β-galactosidase yielded similar effects. By contrast, transient expression of HIF1α(PP) in U-87 MG cells or constitutive expression of HIF1α(PP) but not HIF2α(PP) in a patient-derived glioma sphere culture inhibited tumor growth and spread. Our results indicate that intermittent induction of HIF-1α produces lasting effects on malignant progression even at its own expense.

Introduction

Malignant tumors encounter conditions of low oxygen and nutrient deprivation as they progress. These adverse conditions, albeit detrimental to tumor growth, are associated with tumor progression and resistance to chemo- and radiotherapies. Since its initial discovery as a nuclear factor that binds to the human erythropoietin gene [1], the hypoxia-inducible transcription factor HIF-1 has been recognized as a major regulator that enables cells to overcome the severe microenvironmental stress in tumor development [2–9].

HIF-1 is a heterodimer consisting of HIF-1α and ARNT (aryl hydrocarbon receptor nuclear translocator) [10], and its activation depends primarily on the oxygen-sensitive HIF-1α subunit [11,12], which is degraded through the ubiquitin—proteasome pathway upon recognition by the von Hippel-Lindau (VHL) protein as part of the E3 ubiquitin ligase [13–17]. The VHL protein binds to HIF-1α and its paralog HIF-2α by recognizing two highly conserved, hydroxylated proline residues (HIF-1α Pro-402 and Pro-564, and HIF-2α Pro-405 and Pro-531) for polyubitylation [18–20]. Hypoxia inhibits prolyl hydroxylation, thereby preventing HIF-1α degradation. Subsequently, stabilized HIF-1α and HIF-2α undergo nuclear translocation, dimerization with ARNT, and recruitment of the transcription coactivators p300/CBP, resulting in transcriptional activation of a series of genes for angiogenesis, metabolism, and survival.

Whereas HIF-1α is ubiquitously expressed, HIF-2α expression seems restricted to certain tissues in development and physiology [21,22]. The abundance of HIF-1α as well as HIF-2α is frequently detected in the vast majority of human cancers [2–7,23]. Although these transcription factors were initially thought to share overlapping functions in tumor progression, each seems to possess unique and sometimes opposing activities through specific target gene activation and differential interactions with other proteins [24–26]. Specifically, their opposing activities have been shown in the regulation of cell cycle and DNA repair: Whereas HIF-1α inhibits cell-cycle progression and DNA repair by antagonizing c-Myc activities, HIF-2α does the reverse by enhancing c-Myc activities [26–28].

Furthermore, the roles of HIF-1α and HIF-2α in cancer seem context dependent. Whereas HIF-2α acts as a tumor suppressor in glioma, non-small cell lung cancer, and hepatocellular carcinoma [29–31], it drives tumorigenesis and growth of VHL-deficient renal clear-cell carcinoma [32]. In keeping with this, *EPAS1* (encoding HIF-2α) polymorphisms have been identified as one of the two susceptibility loci in renal cell carcinoma [33]. In addition, somatic, gain-of-function mutations in HIF-2α have been linked to the development of paraganglioma and somatostatinoma in patients [34]. Likewise, HIF-1α has been implicated as a tumor suppressor especially in kidney cancer [35], even though substantial evidence in the literature support a critical role of HIF-1α in progression and metastasis [7]. The tumor-suppressing activity of HIF-1α is strongly indicated by the genetic evidence that focal, homozygous deletions of *HIF1A* gene are found in many VHL-deficient renal clear-cell carcinoma cell lines and the functional evidence that HIF-1α inhibits cell proliferation and tumor growth [35]. All these studies suggest complex roles for HIF-1α and HIF-2α in cancer.

As a step towards understanding the complexity of cancer biology, we employed intermittent induction of HIF-1α and HIF-2α in various cancer cell types and investigated their differential effects on malignant progression in immunodeficient mice.

Materials and Methods

Plasmid construction and viral production

An oxygen-resistant HIF-1α, HIF1α(PP), with P402A and P564A substitutions [36], was cloned into pLenti6.3/TO/V5-DEST through homologous recombination reactions (Invitrogen, Carlsbad, CA, USA). Similarly, HIF-2α(PP) with P405A and P531A substitutions and a 3xFLAG at the amino terminus was cloned into the same vector. To produce lentiviruses, 293FT cells (Invitrogen) derived from a human embryonic kidney cell line were transfected with a lentiviral vector and Virapower packaging mix (Invitrogen) using Lipofectamine 2000. Lentiviral supernatant was harvested 3 days after transfection and filtered through a 0.45-μm sterile syringe filter (VWR, Radnor, PA, USA). The filtered virus was aliquoted and stored at −80°C. Viral titers were determined according to the manufacturer's instruction.

Lentiviral transduction

To establish tetracycline-regulated stable cell lines, we used the ViraPower HiPerform T-REx Gateway Vector kit (Invitrogen) following the manufacturer's instruction. Tetracycline regulation in the T-REx system is based on the binding of tetracycline to the tetracycline repressor and derepression of the promoter controlling expression of either HIF1α(PP) or HIF2α(PP). Cells were infected at 5 multiplicity of infections (MOIs) with the lentivirus expressing tetracycline repressor and selected with geneticin at 500 μg/ml for U-2 OS, 200 μg/ml for U-87 MG, and 400 μg/ml for U-118 MG. These cells were then infected at 2 MOIs with a lentivirus derived from any of the pLenti6.3/TO/V5-DEST constructs and selected with blasticidin at 5 μg/ml for U-2 OS and U-118 MG, and 2 μg/ml for U-87 MG. Selected cells were pooled and used for further studies.

Cell culture and intermittent induction with tetracycline

U-2 OS, U-87 MG, and U118MG were purchased from the American Type Culture Collection (Rockville, MD, USA). U-2 OS and U-118 MG cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. U-87 MG cells were maintained in minimum essential medium with supplements as above. Cell culture conditions were maintained at 37°C and 5% CO_2. For intermittent induction of the gene of interest, tetracycline was administered weekly at 1 μg/ml on day 1 and removed on day 4 for a total of 8 weeks.

Western blot

Cell extract was prepared in a lysis buffer [11] which contains 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol freshly supplemented with 0.5 mM DTT and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were determined by using Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). Antibodies used for Western blotting were mouse anti-human HIF-1α (#610959, BD Bioscience, San Jose, CA, USA), rabbit anti-V5 antibody, and anti-β-tubulin (#V8137 and #T0198, Sigma-Aldrich, St. Louis, MO, USA). Signals were developed using Super Signal West Pico chemiluminescent substrate (Cat#34018, Thermo Scientific, Rockford, IL, USA).

Gene expression, cell proliferation, and anchorage-independent growth

For reporter assays, 293T cells were seeded in 24-well plates and transiently cotransfected with 400 ng pEpoE-luc [11] and 200 ng pLenti-HIF1α(PP) or pLenti-HIF2α(PP), as well as 50 ng pCMV-EGFP for normalization. pLenti-LacZ was used as a control. Twenty-four hours after transfection, cells were lysed and assayed for reporter activity in the Bright-Glo Luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Reagents for TaqMan gene expression were purchased from Invitrogen and real-time PCR reactions were performed according to the manufacturer's instructions. Assays for cell proliferation and anchorage-independent growth were described previously [37].

Tumor transplantation

All animal studies were performed according to the protocol (13–09003) approved by the University of Utah Institutional Animal Care and Use Committee. There were six mice in each group or otherwise indicated. For subcutaneous injections, treated U-2 OS (5×10^6) cells were suspended in 100 μl of phosphate-balanced saline (PBS) per injection and grafted into the flanks of 6–8-week-old non-obese diabetic/severe-combined immunodeficient IL-2Rg-null

(NOD/SCID gamma (NSG)) male mice. Mice were sacrificed and tumor was extracted when the diameter reached 1 cm. For intracranial implantation, treated U-87 MG or U-118 MG (7×10^5) cells in a total volume of 5 μl were mixed with BD Matrigel basement membrane matrix (BD Biosciences). NSG mice were anesthetized with isoflurane, and injections were positioned at 2 mm right to the bregma and 1 mm anterior to the coronal suture, with 2 mm depth. Approximately 5–7 weeks after injection, mice were sacrificed and the brain was extracted for histological examination.

Bioluminescent imaging

Mice were injected intraperitoneally with a 50 mg/kg D-luciferin and 100 mg/kg ketamine/10 mg/kg xylazine (Sigma) mixture. Images were acquired 10 min after injection with the IVIS 100 or 200 imaging system (Xenogen, Alameda, CA, USA). Quantitative analysis of bioluminescent intensity from the images was performed using LivingImage software (Xenogen).

Glioma Stem Cell Culture, Transduction, and Intracranial Implantation

U-87 MG, U-118 MG, and patient-derived glioma sphere culture cells, GSC20 [38], were maintained in DMEM/F12 medium supplemented with B27, 20 ng/ml of EGF and 20 ng/ml of bFGF (Invitrogen). To maintain spheric growth, fresh medium with bFGF and EGF at ~10–20% of the total volume was added twice per week. Spheres were triturated with Accutase (Invitrogen, Cat: A11105-01) and fed with fresh culture medium with supplement. Lentiviral transduction was performed after trituration at 1 MOI. Intracranial implantation of GSC20 was performed with 2×10^5 cells per injection.

Statistical analysis

Statistical differences between groups of data were determined in a t-test of two tails. n = 3 or greater as indicated was used for each data set. Statistical significance was indicated as *, p-value < 0.05; **, p-value < 0.01; and ***, p-value < 0.001.

Results

Regulated HIF-α expression in various cancer cell lines

To better investigate the role of HIF-α in cancer, we developed a tetracycline-regulated gene expression system through lentiviral transduction (Fig 1A) of the cancer cell lines used below. Prolyl hydroxylation sites (Pro-402 and Pro-564) in HIF-1α [18–20] were replaced with alanine to generate a stable HIF-1α variant, HIF1α(PP). The HIF-2α equivalent, HIF2α(PP), was generated similarly. Human cancer cell lines including U-2 OS of osteosarcoma and U-87 MG and U-118 MG of glioblastomas were used for HIF-α expression. As a control, a lentivirus expressing β-galactosidase (β-gal) was included. Cells were pooled after selection and analyzed for gene expression.

As expected, the addition of tetracycline resulted in robust induction of HIF1α(PP), HIF2α(PP), and β-gal in all the cell lines examined by Western blot analysis (Figs 1B and S1). Furthermore, both HIF1α(PP) and HIF2α(PP) were transcriptionally active in stimulating an erythropoietin reporter gene, *EPO-luc*, by five- and sixfold, respectively (Fig 1C). Moreover, induction of HIF1α(PP) upregulated target genes *CA9* (carbonic anhydrase IX) [39] and *PGK1* (phosphoglycerate kinase 1) [40] (3- and 6-fold in U-118 MG and 2- and 5-fold in U-87 MG) (Fig 1D). Similarly, HIF2α(PP) also increased expression of *VEGFA* (vascular endothelial growth factor A) [41] and *LOX* (lysyl oxidase) [42] by 4- and 16-fold, respectively, in U-2 OS (Fig 1E).

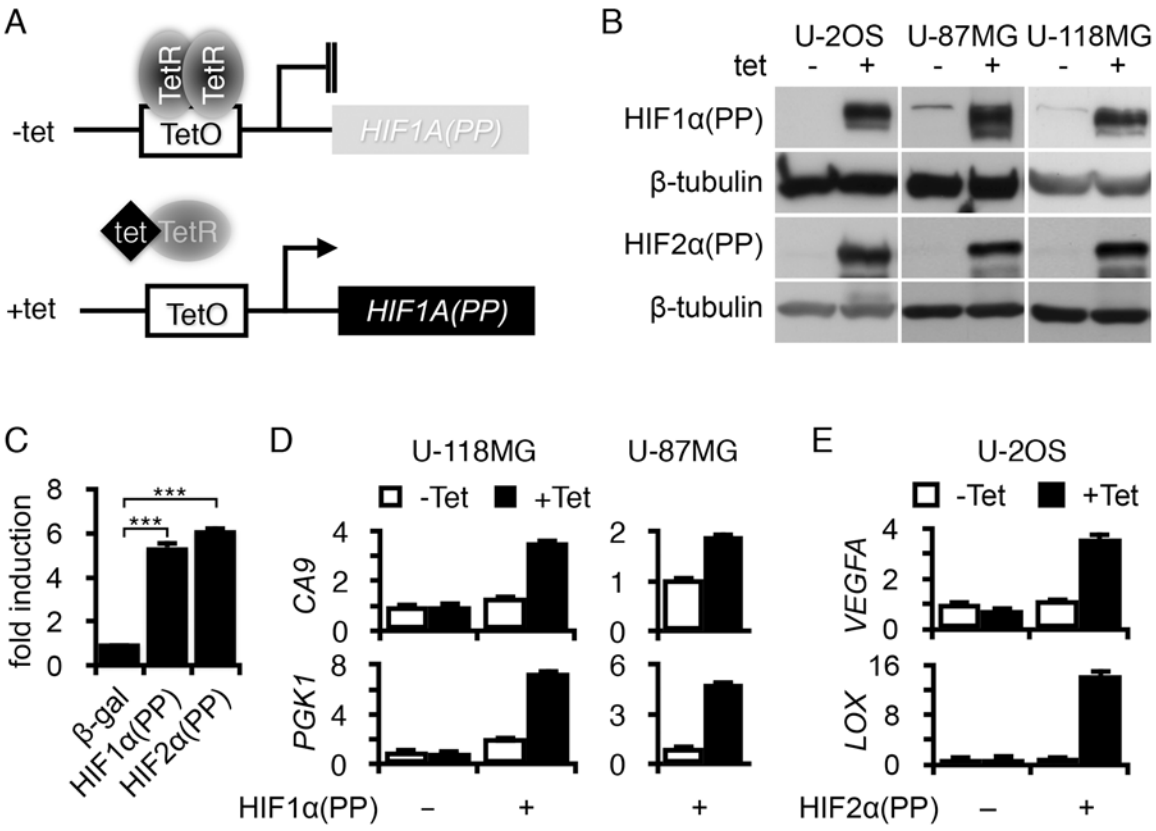

Fig 1. Tetracycline regulation of HIF1α(PP) and HIF2α(PP) expression and transcriptional activity. (A) Tetracycline regulation is diagrammed where the addition of tetracycline (tet) results in dissociation of tetracycline repressor (TetR) from the tetracycline operon (TetO) and, in turn, gene activation. (B) Western blot analysis of transduced cell types, as indicated, for the expression of HIF1α(PP) and HIF2α(PP) after 2-day treatment with tetracycline. (C) Transcriptional activities of HIF1α(PP) and HIF2α(PP) were tested in a reporter assay in reference to β-galactosidase (β-gal). ***, *p*-value < 0.001. (D, E) The expression of HIF target genes (*PGK1*, *CA9*, *VEGFA*, and *LOX*) was analyzed in specified cell lines by using real-time PCR after 2-day treatment with tetracycline.

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Intermittent induction of HIF-1α transgene leads to eventual loss of expression

Although hypoxia has long been known to enhance cancer metastatic potential [43,44], intermittent hypoxia, defined as repeated cycles of hypoxia and reoxygenation, seems more effective than prolonged hypoxia in enhancing spontaneous metastasis [45]. Recent studies indicate intermittent hypoxia is a key regulator of the interplay between cancer cell and endothelial cell for tumor angiogenesis and growth and resistance to chemo- and radiotherapy [46]. Furthermore, HIF-1α levels seem well maintained during the cycling [47].

To reproduce intermittent hypoxia in cancer, we elected to treat the transduced cells with tetracycline for 3 days and without for 4 days every week for 8 weeks (Fig 2A). Interestingly, at the end of the treatment most of these treated cells no longer or barely responded to tetracycline (Fig 2B). Specifically, HIF1α(PP) expression was essentially lost at protein levels in all cell lines, while various degrees of HIF2α(PP) attenuation were observed. However, β-gal expression remained inducible (S1 Fig). The loss at protein levels correlated with that at transcript levels. To exclude the possibility of slow recovery, we continued to culture these cells for additional weeks in the absence of tetracycline and found no recovery of HIF1α(PP) expression. Although the underlying mechanism requires further investigation, we reasoned that the loss of

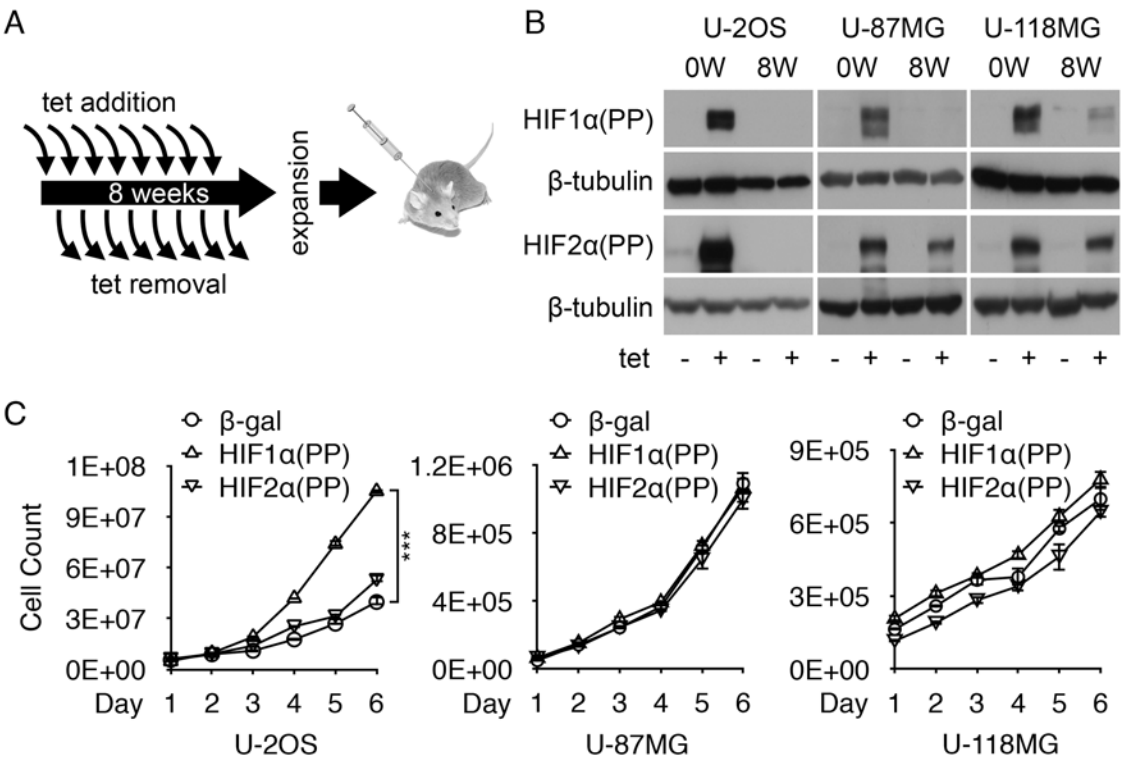

Fig 2. Intermittent induction resulted in loss of of HIF1α(PP) expression. (A) Intermittent induction involves the administration of tetracycline into cell culture each week on day 1 and removal on day 4 each week for a total of 8 weeks. Afterwards, cells were allowed to expand for further analyses and injections. (B) After intermittent induction (8W), different types of cells as indicated were induced again with tetracycline for 2 days and analyzed by Western blotting in reference to those without intermittent induction (0W). (C) Cell proliferation was determined by cell counting after intermittent induction. ***, *p*-value < 0.001.

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HIF1α(PP) induction would help us determine the consequence of intermittent induction, rather than *de novo* expression, of HIF1α(PP) on malignant progression.

U-2 OS cells acquire tumorigenicity in the absence of continued expression of HIF-1α variant

We first observed that the HIF1α(PP)-induced U-2 OS cells grew twofold faster than the β-gal control without further induction, whereas only a modest increase was detected in the HIF2α(PP)-induced cells (Fig 2C). Importantly, the former but not the latter became tumorigenic when injected subcutaneously into the flanks of NSG mice; all 6 injections with the HIF1α(PP) cells resulted in tumor formation, whereas none of the HIF2α(PP) cells did at the contralateral sites (Fig 3A and 3B). Of note, none of the β-gal controls became tumorigenic. Moreover, the HIF1α(PP) tumors grew at an exponential pace (Fig 3C). Histological examination confirmed the malignant growth of these tumors, as indicated by hypercellularity and necrosis, increased mitosis, and invasion into the dermis and the striated muscle layers (Fig 3D). Similar data were obtained when CD1 nude mice were used. Thus, we conclude that intermittent induction of HIF1α(PP), but not HIF2α(PP), programs the non-tumorigenic U-2 OS cells for malignant progression independent of its continued expression.

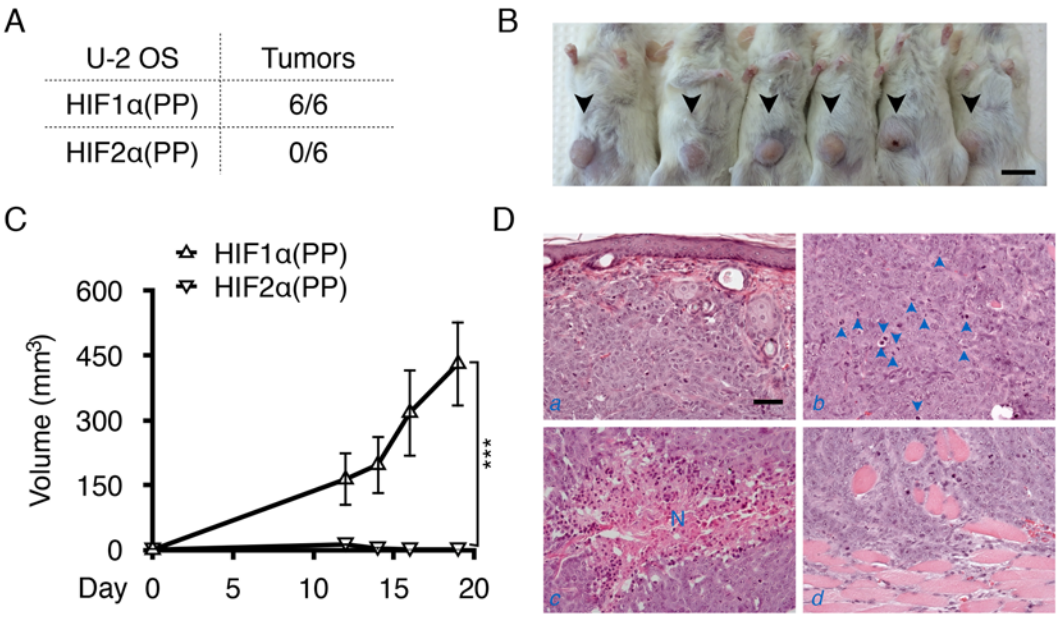

Fig 3. U-2 OS cells acquired tumorigenicity after intermittent induction of HIF1α(PP). (A) Tumor incidence is shown in 6 NSG mice after bilateral, subcutaneous injections of the 8-week HIF1α(PP) and HIF2α(PP) cells. (B) Only injections of the HIF1α(PP) cells produced tumors, as indicated by arrowheads. Scale bar, 1 cm. (C) Tumor volume was calculated based on measurements and plotted as a function of time. ***, *p*-value < 0.001. (D) Hematoxylin and eosin staining of the tumor specimens reveals invasion of the dermal layer (*a*), numerous mitoses (arrowheads) (*b*), necrosis (N) (*c*), and invasion into the striated muscle layer (*d*). Scale bar, 100 μm.

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HIF-1α promotes invasion of glioma cells in the mouse brain

To extend this original observation, we focused on glioma progression and employed an orthotopic tumor model through intracranial injections. Unlike their U-2 OS counterpart, the U-87 MG and U-118 MG cells with prior HIF1α(PP) induction exhibited a similar growth rate in culture as their controls (Fig 2C), suggesting variation of HIF1α(PP) effects after intermittent induction. Furthermore, HIF1α(PP)-induced U-87 MG cells showed ~10-fold increase in the development of tumor spheres but no increase in anchorage-independent growth (S2 Fig). Yet, a decrease in tumor sphere formation and anchorage-independent growth was observed in HIF1α(PP)-induced U-118 MG cells.

Original U-87 MG cells are tumorigenic after intracranial transplantation. Interestingly, during a ~5-week monitoring of tumor growth using bioluminescent imaging, we found that the increase in tumor volume from the β-gal-induced cells was equivalent to, if not greater than, that from the HIF1α(PP)-induced cells (Fig 4A and 4B). By contrast, tumors derived from the HIF2α(PP) cells grew poorly and failed to expand during the experimental period (Fig 4C and 4D). The lack of tumor growth from the HIF2α(PP) cells appears consistent with a previous report that HIF-2α acts as a tumor suppressor in glioma [29].

Although prior intermittent induction of HIF1α(PP) had hardly any effect on U-87 MG cell proliferation in culture and *in vivo*, it is noticeable that the bioluminescent signals from the derived tumors permeated broadly beyond the frontal lobes of the brain (Fig 4A), indicative of spreading of tumor cells. Indeed, tumor lesions from the HIF1α(PP) cells were numerous foci throughout the brain (Figs 4E and S3B); in addition to the cerebral cortex, invasions were identified in the hypothalamus, midbrain, hindbrain, and even cerebellum. By contrast, tumor lesions derived from the β-gal control tended to be singular, large, and localized to the cerebral cortex (Fig 4F), even though very few developed more than single lesions with distal invasions

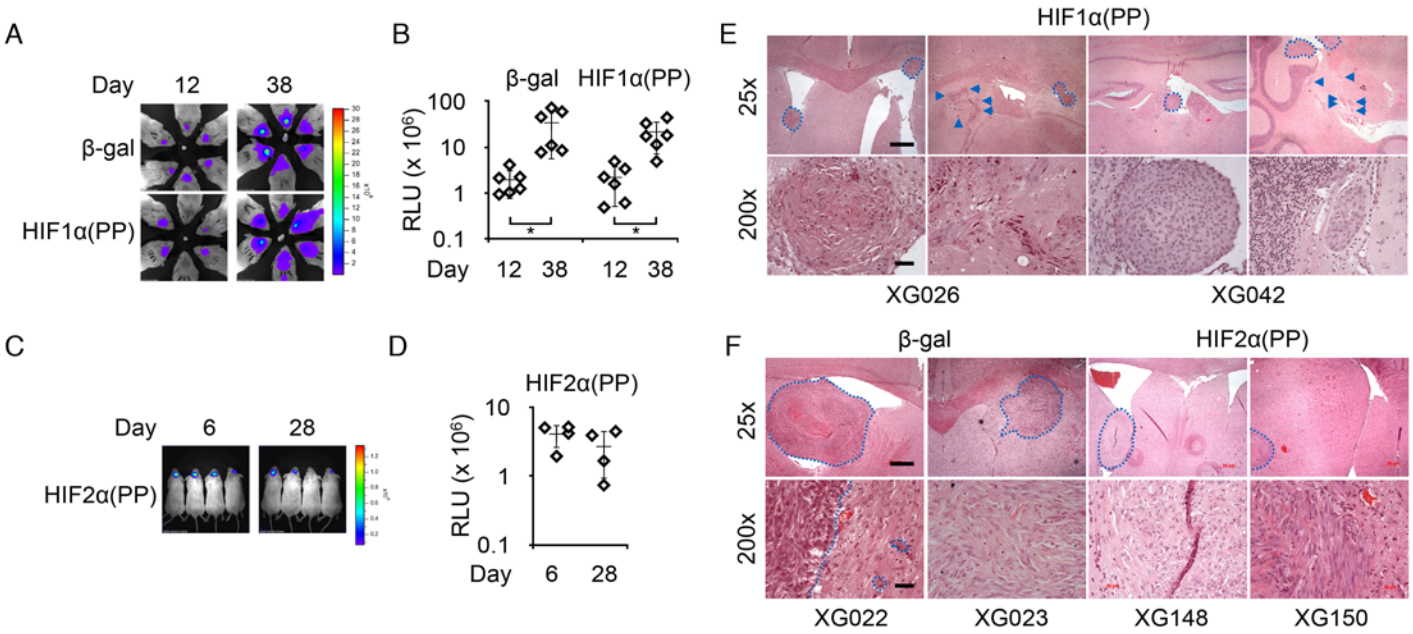

Fig 4. Intermittent induction of HIF1α(PP) promoted intracranial spread of U-87 MG cells. Bioluminescent imaging analysis of β-gal and HIF1α(PP)-derived intracranial tumors (A) and HIF2α(PP)-derived intracranial tumors (C). The respective tumor volumes were calculated based on the relative luminescent units (RLU) and plotted in a log scale (B and D). *, *p*-value < 0.05. (E) HIF1α(PP)-derived tumors had small yet numerous lesions invading the Ammon's horn of the hippocampal region (XG026) and hindbrain and cerebellum (XG042), as indicated by arrowheads. (F) β-gal- and HIF2α(PP)-derived tumors were large and often singular in the cerebral cortex. Tumor lesions are demarcated in dash lines. Hematoxylin and eosin—stained images are presented at 25× and 200× magnifications, with scale bars of 1 mm and 100 μm, respectively.

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(S3A Fig). Furthermore, only small, singular lesions were found in all HIF2α(PP)-derived tumors (Fig 4F). Histological examination at high magnification revealed aggressive invasion of round cells in the HIF1α(PP)-derived tumors, whereas relatively low-density, spindle-shaped tumor cells were frequently observed interwoven with fibrous tissues in β-gal and HIF2α(PP) tumors (Fig 4E and 4F). However, all three cell types appeared indistinguishable in culture.

With respect to U-118 MG, 8-week intermittent induction of HIF1α(PP) failed to result in discernible morphological differences in culture but apparently helped tumor maintenance (Fig 5A and 5B), as indicated by the preserved bioluminescent signals throughout a 7-week period of observation. In contrast, the signals from the β-gal control were diminished progressively, resulting in a statistically significant drop in tumor volume (Fig 5B). Tumor incidence of the HIF1α(PP) cells was 5/6, similar to that of the control (4/6). Furthermore, tumors from the HIF1α(PP) cells often had multiple lesions, in contrast to mostly single lesions in the β-gal control group. Therefore, these results further corroborate the long-lasting effects of HIF-1α on malignant progression and the differential observations between cell culture and animal models.

Transient induction of HIF-1α inhibits glioma growth

To test whether transient induction of HIF1α(PP) would produce similar effects *in vivo*, we analyzed tumor growth of U-87 MG cells that had been treated with tetracycline in culture for only 2 days before intracranial transplantation. Fig 6A shows that transient induction of HIF1α(PP) retarded tumor growth significantly in the brain in reference to that of β-gal. To extend this finding, we employed a patient-derived, mesenchymal glioma sphere culture, GSC20 [38]. These cells were allowed for constitutive expression of β-gal, HIF1α(PP), or HIF2α(PP) after lentiviral infection and grown in sphere culture medium for adequate expansion prior to

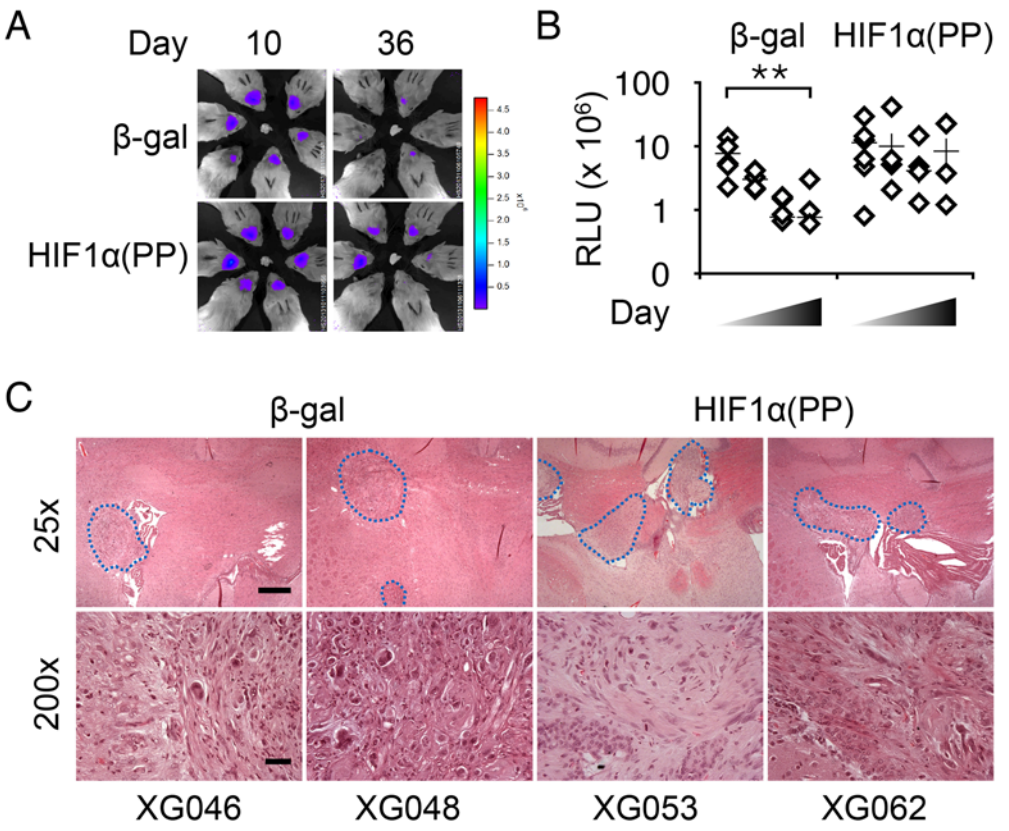

Fig 5. Intermittent induction of HIF1α(PP) facilitated intracranial tumor growth of U-118 MG cells. (A) Bioluminescent imaging analysis of intracranial tumors derived from 8-week treated cells as indicated. (B) Tumor volumes, plotted in a log scale, from β-gal cells decreased significantly during the course of 49 days, but those from HIF1α(PP) did not. **, *p*-value < 0.01. (C) Representative tumor lesions are shown in hematoxylin and eosin staining from 2 individual mice of each group at 25× and 200× magnifications, with scale bars of 1 mm and 100 μm, respectively.

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intracranial injections. It is noteworthy that while the HIF1α(PP) population expanded slightly faster than the β-gal control and more readily formed tumor spheres, the HIF2α(PP) multiplied at an extremely slow rate (Fig 6B).

Contrary to these *in vitro* findings, injection of the HIF1α(PP) cells led to the development of only small, primarily singular tumors in the mouse brain, whereas the other two cell types yielded much larger, multiple lesions and invasions (Figs 6C and S4). Tumor incidence was slightly decreased for HIF1α(PP) cells (3/5 vs 4/5 for the other two cell types). Strikingly, tumors derived from the β-gal and HIF2α(PP) cells manifested a multitude of mitoses and multinuclear giant cells, both of which were markedly diminished in HIF1α(PP)-derived tumors (Figs 6C and S4). Furthermore, vascular proliferation and necrosis, common features of glioblastomas [48], were present in tumors derived from the β-gal and HIF2α(PP) cells but not the HIF1α(PP) cells (S4 Fig). Taken together, these results suggest that HIF-1α can also inhibit glioma growth and progression.

Discussion

We provide evidence in this study that intermittent induction of HIF1α(PP) *in vitro* produced lasting effects on malignant progression of different cancer cell lines, unexpectedly independent of continued expression of the transgene. All these cells eventually lost HIF1α(PP)

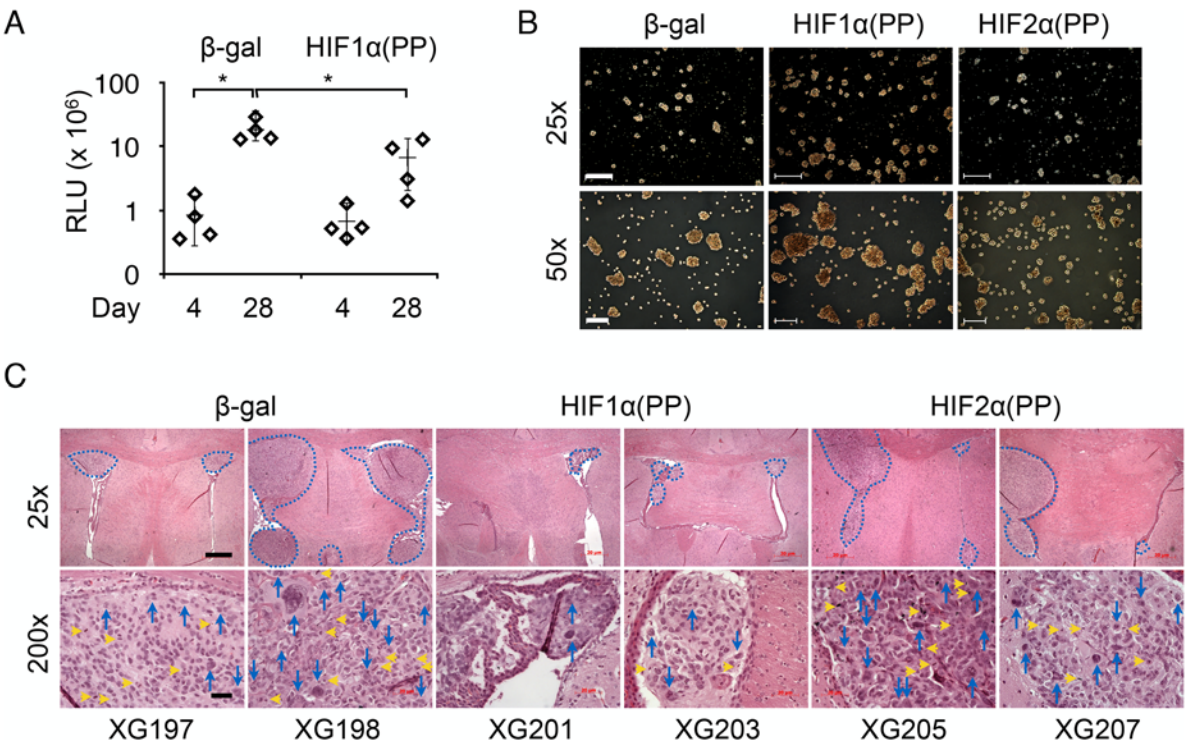

Fig 6. Transient expression of HIF1α(PP) inhibited intracranial tumor growth. (A) U-87 MG cells were induced for 2 days prior to intracranial injections. Tumor volumes, plotted in a log scale, decreased significantly from HIF1α(PP) in reference to β-gal. (B) After infection with lentiviruses expressing gene products as indicated, GSC20 cells were grown in neurobasal-A medium and images of tumor spheres were captured at 25× and 50× magnifications, with scale bars of 1 mm and 400 μm, respectively. (C) Representative tumor lesions from 2 individual mice of each group are presented at 25× and 200× magnifications, with scale bars of 1 mm and 100 μm, respectively. Tumor lesions are demarcated in dash lines. Mitoses are indicated by yellow arrowheads and multi-nucleation by blue arrows.

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expression after continuous culture, yet retained the acquired malignant traits in the *in vivo* setting. These results indicate that repeated activation of HIF-1α can program cancer cells to acquire perpetual signaling possibly through feed-forward biochemical/metabolic loops or genetic/epigenetic changes, even though the underlying mechanism requires further investigation. The study also suggests that HIF-1α can promote malignant progression at its own expense, which might account for HIF-1α inactivation in human cancer.

Although repeated cycles of hypoxia and reoxygenation have long been known to promote tumor progression [45], our experimental system allows us to directly interrogate HIF-1α and HIF-2α, excluding other possible aspects such as changes in reactive oxygen species, which may also contribute to malignant progression. It should be noted that the cancer cells treated for 8 weeks in our study, although they no longer expressed HIF1α(PP), did maintain endogenous HIF-1α expression under hypoxia, a potential contributing factor to the process and a disadvantage of our experimental system. Interestingly, knockdown of endogenous HIF-1α by short-hairpin RNA in the cancer cells treated for 8 weeks impeded tumor growth but failed to prevent invasion, suggesting that intermittent induction of HIF1α(PP) in culture is key to programming glioma cells for invasion whereas endogenous HIF-1α potentially facilitates tumor growth. This interpretation is consistent with our results that tumors from the control groups, β-gal and HIF2α(PP), exhibited much less invasion despite endogenous HIF-1α expression; however, we cannot exclude the possibility that endogenous HIF-1α was required for programming cancer

cells during intermittent induction of HIF1α(PP). This possibility will be better addressed in HIF-1α-deficient cancer cells or conditional genetic models.

It is interesting to note that GSC20-derived tumor cells in the control group featured rampant mitoses and numerous giant-sized, hyperchromatic nuclei, indicative of rapid cell proliferation and aberrant DNA replication; however, HIF1α(PP) expression not only retarded tumor growth but also markedly reduced mitosis of tumor cells and diminished multinucleation. These effects are in agreement with the inhibitory roles of HIF-1α, but not HIF-2α, in cell-cycle progression and DNA replication upon transient induction [49–51]. Although these findings are reminiscent of previous reports that HIF-1α retards tumor growth [52,53], our data collectively indicate that HIF-1α has pleotropic effects that are context dependent.

Our study also indicates that HIF-1α is a potent inducer of glioma invasion. This is particularly interesting because investigations of glioma invasion have been hampered by the scarcity of representative experimental models [54]. Although we have not identified the signaling pathway leading to glioma invasion, it appears to be independent of the major signaling pathways such as PI(3)K-Akt and MAPK after intermittent induction. Glioma aggressiveness is associated with a mesenchymal phenotype that is regulated by the C/EBPβ and STAT3 transcription factors [55]. In addition, CHI3L1 (chitinase 3-like 1), also known as BRP-39/HCGP39/YKL-40 [56], is considered a reliable gene expression marker for the mesenchymal subclass and local invasiveness of glioblastomas [57–60]. However, we observed no increase in STAT3 phosphorylation in HIF1α(PP)-induced U-87 MG cells. Furthermore, we detected decreased CHI3L1 expression in the invasive lesions by immunohistochemistry. Therefore, further studies are warranted to understand the mechanism by which intermittent induction of HIF-1α drives malignant progression.

Targeting HIF-1 for cancer therapy was based originally on the critical roles of HIF-1α in cancer biology, the association of HIF-1α overexpression with increased patient mortality in various cancer types, and the marked effects on tumor growth by inhibiting HIF-1α activity in preclinical studies [3–5,61]. Numerous small-molecule inhibitors therefore have been identified in preclinical studies to inhibit HIF-1α via targeting various signaling pathways that regulate HIF-1α expression, degradation, dimerization, DNA binding, and transactivation [62]. Despite these advances, caution must be exercised for the use of these inhibitors as a potential therapeutic strategy owing to the complex role of HIF-1α and HIF-2α in cancer [63]. Therefore, an in-depth understanding of complex hypoxia biology in cancer will be key to precision targeting and therapeutic efficacy [64].

Supporting Information

S1 Fig. Loss of HIF1α(PP) expression after 8-week intermittent induction. U-87 MG variants, as specified, were induced with tetracycline for 2 days and analyzed by Western blotting with antibodies against V5 and β-tubulin. HIF1α(PP) cells post intermittent induction (8W) no longer responded to tetracycline induction in contrast to those prior to intermittent induction (0W).
(TIF)

S2 Fig. Analysis of U-87 MG and U-118 MG cells treated for 8 weeks. HIF1α(PP)-induced cells were assayed for tumor sphere formation in reference to the control (Con) (A). Scale bar, 200 μm. Tumor spheres > 200 μm were quantified and plotted (B). Soft agar assays were performed for *in vitro* tumorigenicity (C). Scale bar, 200 μm. Colonies > 200 μm were quantified and plotted (D).
(TIF)

S3 Fig. Intracranial tumor lesions derived from U-87 MG variants after intermittent induction. (A) Invasive lesions were seen occasionally in tumors derived from β-gal cells (XG021), presented at 25× and 200× magnifications, with scale bars of 1 mm and 100 μm, respectively. (B) Additional lesions of widespread invasion derived from HIF1α(PP) cells are shown (XG042). Scale bar, 100 μm. Tumor lesions are demarcated in dash lines.
(TIF)

S4 Fig. Additional malignant features of intracranial tumors derived from transduced GSC20. Invasive lesions were identified in the hindbrain of NSG mice injected with β-gal-transduced GSC20 (XG198) and HIF2α(PP)-transduced GSD20 (XG205). Tumor lesions also contained necrosis (XG198) and invasion in the forth ventricle (XG205, arrowhead). Vascular proliferation and multi-nucleation were observed commonly in these tumor lesions. Images are presented at 25× and 200× magnifications, with scale bars of 1 mm and 100 μm, respectively.
(TIF)

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Author Contributions

Conceived and designed the experiments: H. Choi LEH. Performed the experiments: H. Choi DLG SB CR SC JG LEH. Analyzed the data: H. Choi DLG SB CR SC JG H. Colman RLJ LEH. Contributed reagents/materials/analysis tools: H. Colman. Wrote the paper: H. Choi DLG SB CR SC JG H. Colman RLJ LEH.

References

1. Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci USA 1991; 88:5680–4. PMID: 2062846
2. Maxwell PH, Pugh CW, Ratcliffe PJ. Activation of the HIF pathway in cancer. Curr Opin Genet Dev 2001; 11:293–9. PMID: 11377966
3. Harris AL. Hypoxia—a key regulatory factor in tumour growth. Nat Rev Cancer 2002; 2:38–47. PMID: 11902584
4. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003; 3:721–32. PMID: 13130303
5. Giaccia A, Siim BG, Johnson RS. HIF-1 as a target for drug development. Nat Rev Drug Discov 2003; 2:803–11. PMID: 14526383
6. Pouysségur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 2006; 441:437–43. PMID: 16724055
7. Bertout JA, Patel SA, Simon MC. The impact of O2 availability on human cancer. Nat Rev Cancer 2008; 8:967–75. doi: 10.1038/nrc2540 PMID: 18987634
8. Rice C, Huang LE. From antiangiogenesis to hypoxia: current research and future directions. Cancer Manag Res 2010; 3:9–16. doi: 10.2147/CMR.S14812 PMID: 21407995
9. Tiburcio PD, Choi H, Huang LE. Complex role of HIF in cancer: the known, the unknown, and the unexpected. Hypoxia 2014; 2:59–70.
10. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA 1995; 92:5510–4. PMID: 7539918
11. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 1996; 271:32253–9. PMID: 8943284

12. Pugh CW, O'Rourke JF, Nagao M, Gleadle JM, Ratcliffe PJ. Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. J Biol Chem 1997; 272:11205–14. PMID: 9111021
13. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997; 272:22642–7. PMID: 9278421
14. Huang LE, Gu J, Schau M, Bunn HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 1998; 95:7987–92. PMID: 9653127
15. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999; 399:271–5. PMID: 10353251
16. Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol 2000; 2:423–7. PMID: 10878807
17. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. EMBO J 2000; 19:4298–309. PMID: 10944113
18. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 2001; 292:464–8. PMID: 11292862
19. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001; 292:468–72. PMID: 11292861
20. Yu F, White SB, Zhao Q, Lee FS. HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci USA 2001; 98:9630–5. PMID: 11504942
21. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev 1997; 11:72–82. PMID: 9000051
22. Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, et al. Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J 1999; 18:1905–14. PMID: 10202154
23. Rankin EB, Giaccia AJ. The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ 2008; 15:678–85. doi: 10.1038/cdd.2008.21 PMID: 18259193
24. Hu C-J, Wang L-Y, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 2003; 23:9361–74. PMID: 14645546
25. Wang V, Davis DA, Haque M, Huang LE, Yarchoan R. Differential gene up-regulation by hypoxia-inducible factor-1alpha and hypoxia-inducible factor-2alpha in HEK293T cells. Cancer Res 2005; 65:3299–306. PMID: 15833863
26. Keith B, Johnson RS, Simon MC. HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer 2011; 12:9–22. doi: 10.1038/nrc3183 PMID: 22169972
27. Huang LE. Carrot and stick: HIF-alpha engages c-Myc in hypoxic adaptation. Cell Death Differ 2008; 15:672–7. doi: 10.1038/sj.cdd.4402302 PMID: 18188166
28. Huang LE. How HIF-1α Handles Stress. Science 2013; 339:1285–6. doi: 10.1126/science.1236966 PMID: 23493703
29. Acker T, Diez-Juan A, Aragonés J, Tjwa M, Brusselmans K, Moons L, et al. Genetic evidence for a tumor suppressor role of HIF-2alpha. Cancer Cell 2005; 8:131–41. PMID: 16098466
30. Mazumdar J, Hickey MM, Pant DK, Durham AC, Sweet-Cordero A, Vachani A, et al. HIF-2alpha deletion promotes Kras-driven lung tumor development. Proc Natl Acad Sci USA 2010; 107:14182–7. doi: 10.1073/pnas.1001296107 PMID: 20660313
31. Sun H-X, Xu Y, Yang X-R, Wang W-M, Bai H, Shi R-Y, et al. Hypoxia inducible factor 2 alpha inhibits hepatocellular carcinoma growth through the transcription factor dimerization partner 3/ E2F transcription factor 1-dependent apoptotic pathway. Hepatology 2013; 57:1088–97. doi: 10.1002/hep.26188 PMID: 23212661
32. Kaelin WG. Von Hippel-Lindau disease. Annu Rev Pathol Mech Dis 2007; 2:145–73. PMID: 18039096
33. Purdue MP, Johansson M, Zelenika D, Toro JR, Scelo G, Moore LE, et al. Genome-wide association study of renal cell carcinoma identifies two susceptibility loci on 2p21 and 11q13.3. Nat Genet 2011; 43:60–5. doi: 10.1038/ng.723 PMID: 21131975

34. Zhuang Z, Yang C, Lorenzo F, Merino M, Fojo T, Kebebew E, et al. Somatic HIF2AGain-of-Function Mutations in Paraganglioma with Polycythemia. N Engl J Med 2012; 367:922–30. doi: 10.1056/NEJMoa1205119 PMID: 22931260

35. Shen C, Beroukhim R, Schumacher SE, Zhou J, Chang M, Signoretti S, et al. Genetic and functional studies implicate HIF1α as a 14q kidney cancer suppressor gene. Cancer Discov 2011; 1:222–35. doi: 10.1158/2159-8290.CD-11-0098 PMID: 22037472

36. Kageyama Y, Koshiji M, To KK-W, Tian Y-M, Ratcliffe PJ, Huang LE. Leu-574 of human HIF-1alpha is a molecular determinant of prolyl hydroxylation. FASEB J 2004; 18:1028–30. PMID: 15084514

37. Hayashi M, Yoo Y-G, Christensen J, Huang LE. Requirement of evading apoptosis for HIF-1alpha-induced malignant progression in mouse cells. Cell Cycle 2011; 10:1–9. PMID: 21191187

38. Bhat KPL, Salazar KL, Balasubramaniyan V, Wani K, Heathcock L, Hollingsworth F, et al. The transcriptional coactivator TAZ regulates mesenchymal differentiation in malignant glioma. Genes Dev 2011; 25:2594–609. doi: 10.1101/gad.176800.111 PMID: 22190458

39. Dayan F, Roux D, Brahimi-Horn MC, Pouysségur J, Mazure NM. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. Cancer Res 2006; 66:3688–98. PMID: 16585195

40. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 1994; 269:23757–63. PMID: 8089148

41. Mazure NM, Chen EY, Yeh P, Laderoute KR, Giaccia AJ. Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. Cancer Res 1996; 56:3436–40. PMID: 8758908

42. Erler JT, Bennewith KL, Nicolau M, Dornhöfer N, Kong C, Le Q-T, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 2006; 440:1222–6. PMID: 16642001

43. Young SD, Marshall RS, Hill RP. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci USA 1988; 85:9533–7. PMID: 3200838

44. Rofstad EK, Danielsen T. Hypoxia-induced metastasis of human melanoma cells: involvement of vascular endothelial growth factor-mediated angiogenesis. Br J Cancer 1999; 80:1697–707. PMID: 10468285

45. Cairns RA, Kalliomaki T, Hill RP. Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. Cancer Res 2001; 61:8903–8. PMID: 11751415

46. Toffoli S, Michiels C. Intermittent hypoxia is a key regulator of cancer cell and endothelial cell interplay in tumours. FEBS J 2008; 275:2991–3002. doi: 10.1111/j.1742-4658.2008.06454.x PMID: 18445039

47. Martinive P, Defresne F, Bouzin C, Saliez J, Lair F, Grégoire V, et al. Preconditioning of the tumor vasculature and tumor cells by intermittent hypoxia: implications for anticancer therapies. Cancer Res 2006; 66:11736–44. PMID: 17178869

48. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. 2007; 114:97–109. PMID: 17618441

49. Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE. HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. EMBO J 2004; 23:1949–56. PMID: 15071503

50. Hubbi ME, Kshitiz, Gilkes DM, Rey S, Wong CC, Luo W, et al. A Nontranscriptional Role for HIF-1 as a Direct Inhibitor of DNA Replication. Sci Signal 2013; 6:ra10. doi: 10.1126/scisignal.2003417 PMID: 23405012

51. Gordan JD, Bertout JA, Hu C-J, Diehl JA, Simon MC. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell 2007; 11:335–47. PMID: 17418410

52. Raval RR, Lau KW, Tran MGB, Sowter HM, Mandriota SJ, Li J-L, et al. Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol 2005; 25:5675–86. PMID: 15964822

53. Mack FA, Patel JH, Biju MP, Haase VH, Simon MC. Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. Mol Cell Biol 2005; 25:4565–78. PMID: 15899860

54. Louis DN. Molecular pathology of malignant gliomas. Annu Rev Pathol Mech Dis 2006; 1:97–117. PMID: 18039109

55. Carro MS, Lim WK, Alvarez MJ, Bollo RJ, Zhao X, Snyder EY, et al. The transcriptional network for mesenchymal transformation of brain tumours. Nature 2010; 463:318–25. doi: 10.1038/nature08712 PMID: 20032975

56. Lee CG, Da Silva CA, Cruz Dela CS, Ahangari F, Ma B, Kang M-J, et al. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu Rev Physiol 2011; 73:479–501. doi: 10.1146/annurev-physiol-012110-142250 PMID: 21054166

57. Nigro JM, Misra A, Zhang L, Smirnov I, Colman H, Griffin C, et al. Integrated array-comparative genomic hybridization and expression array profiles identify clinically relevant molecular subtypes of glioblastoma. Cancer Res 2005; 65:1678–86. PMID: 15753362

58. Ku BM, Lee YK, Ryu J, Jeong JY, Choi J, Eun KM, et al. CHI3L1 (YKL-40) is expressed in human gliomas and regulates the invasion, growth and survival of glioma cells. Int J Cancer 2011; 128:1316–26. doi: 10.1002/ijc.25466 PMID: 20506295

59. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 2006; 9:157–73. PMID: 16530701

60. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 2010; 17:98–110. doi: 10.1016/j.ccr.2009.12.020 PMID: 20129251

61. Jensen RL. Hypoxia in the tumorigenesis of gliomas and as a potential target for therapeutic measures. Neurosurg Focus 2006; 20:E24. PMID: 16709030

62. Rapisarda A, Melillo G. Overcoming disappointing results with antiangiogenic therapy by targeting hypoxia. Nat Rev Clin Oncol 2012; 9:378–90. doi: 10.1038/nrclinonc.2012.64 PMID: 22525710

63. Young RM, Simon MC. Untuning the tumor metabolic machine: HIF-α: pro- and antitumorigenic? Nat Med 2012; 18:1024–5. doi: 10.1038/nm.2865 PMID: 22772556

64. Huang LE. Targeting HIF-α: when a magic arrow hits the bull's eye. Drug Discov Today 2004; 9:869. PMID: 15493075