Imaging and Targeting of the Hypoxia-inducible Factor 1-active Microenvironment

Shinae Kizaka-Kondoh¹,², Shotaro Tanaka² and Masahiro Hiraoka²

¹Innovative Techno-Hub for Integrated Medical Bio-imaging, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
²Department of Radiation Oncology and Image-applied Therapy, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Abstract: Human solid tumors contain hypoxic regions that have considerably lower oxygen tension than normal tissues. They are refractory to radiotherapy and anticancer chemotherapy. Although more than half a century has passed since it was suggested that tumour hypoxia correlates with poor treatment outcomes and contributes to recurrence of cancer, no fundamental solution to this problem has been found. Hypoxia-inducible factor-1 (HIF-1) is the main transcription factor that regulates the cellular response to hypoxia. It induces various genes, whose function is strongly associated with malignant alteration of the entire tumour. The cellular changes induced by HIF-1 are extremely important therapeutic targets of cancer therapy, particularly in therapy against refractory cancers. Therefore, targeting strategies to overcome the HIF-1-active microenvironment are important for cancer therapy. To Target HIF-1-active/hypoxic tumor cells, we developed a fusion protein drug, PTD-ODD-Procaspase-3 that selectively induces cell death in HIF-1-active/hypoxic cells. The drug consists of the following three functional domains: the protein transduction domain (PTD), which efficiently delivers the fusion protein to hypoxic tumor cells, the ODD domain, which has a VHL-mediated protein destruction motif of human HIF-1α protein and confers hypoxia-dependent stabilization to the fusion proteins, and the human procaspase-3 proenzyme responsible for the cytotoxic activity of the protein drug. In vivo imaging systems capable of monitoring HIF-1 activity in transplanted human cancer cells in mice are useful in evaluating the efficiency of these drugs and in study of HIF-1-active tumor cells. (J Toxicol Pathol 2009; 22: 93–100)

Key words: hypoxia-inducible factor 1 (HIF-1), tumour hypoxia, hypoxia responsive element (HRE), protein transduction domain (PTD), bioluminescence, in vivo imaging

Introduction

Most solid tumours contain a tumour-specific microenvironment that is completely different from that inside normal tissues (Fig. 1). The microenvironment of a solid tumour is characterised by low pO₂ and low pH, which are well below physiological levels¹–⁴. This is due to the generation of areas within the solid tumours that do not receive adequate nutrients and oxygen from blood vessels because of the uncontrolled growth of the tumour cells and disproportional and incomplete vascular structures during angiogenesis. Due to certain physical factors within these hypoxic areas, hypoxic tumour cells are resistant to cancer therapy¹–⁴. Since transport of anticancer agents via blood flow to hypoxic tumour cells, which are located distant from blood vessels, is inefficient, there is only a small chance that an anticancer agent reaches hypoxic tumour cells at an effective concentration. In addition, many anticancer agents target dividing cells and, thus, are ineffective in growth-arrested or slowly growing hypoxic tumour cells. Furthermore, radiation, which enhances cytotoxicity via oxygen molecules, and certain types of anticancer agents cannot exert their therapeutic effects sufficiently under hypoxic conditions. Therefore, there are cases in which hypoxic tumour cells survive after radiotherapy or chemotherapy, even though the surrounding well-oxygenated and proliferating cancer cells die, suggesting that they are the cause of poor treatment outcomes and recurrence of cancers.

At the same time, cells in hypoxic areas contribute to malignant alteration of cancers due to biological factors. Compared with the actively growing cancer cells surrounding them, which are exposed to an aerobic environment, they are ‘impaired cancer cells’ and are not normally considered as a serious target for cancer therapy. However, recent research has shown that these ‘impaired
cancer cells' increase the malignancy of the entire tumour. Although these hypoxic tumour cells are in a 'moribund state', they try to adapt to their poor environment. Hypoxia-inducible transcription factor (HIF-1) supports their adaptation. HIF-1, whose activity is barely detectable in cells under aerobic conditions, is immediately activated under hypoxic conditions. Moreover, it induces expression of genes that are related to glucose metabolism and glucose transport, produces angiogenic and growth factors and helps to improve the nutritional environment. HIF-1 attempts to prevent apoptosis and death by inducing expression of genes that induce mutations. At the same time, it induces expression of genes that are involved in metastasis and invasion. These chain-of-survival actions are linked to malignant alteration of the entire cancer. Therefore, extensive research is being conducted to identify genes whose expression is directly induced by HIF-1, and nearly a hundred such genes have been reported thus far.

HIF-1 Activity and Hypoxic Regions in Tumors

The binding of 2-nitroimidazole derivatives such as pimonidazole (Pimo), to cellular macromolecules increases dramatically below an oxygen concentration of 10 mmHg and is considered to indicate chronic hypoxia. Recently, it has been reported that the intratumour regions in which HIF-1α is expressed (HIF-1-active regions) hardly overlap Pimo-positive regions (Fig. 2); HIF-1-active regions are more closely distributed in blood vessels than Pimo-positive regions. Janssen et al. performed an extensive study of surgical specimens from patients and reported that while typical Pimo-positive regions at a distance from blood vessels with peaks of around 80 μm were observed, the HIF-1-positive regions were more variable, without clear peaks, and no correlation was observed between the percentage of positive tumour tissue for either markers; although the median values of the positive area were similar for both markers (5.8% vs. 5.6%), the median percentage of the regions positive was below 5% (range 0.2–2.3%) for both markers.

As gene clusters whose expression is induced by transcription factor HIF-1 exert functions that contribute greatly to the malignant alteration of a cancer, imaging and targeting of 'hypoxic cells with HIF-1 activity' has become important. In addition, while compounds such as Pimo function at absolute oxygen concentrations below 10 mmHg, the oxygen concentrations at which HIF-1 activity occurs differs among tissue cells, indicating that the abnormal oxygen concentrations necessary for HIF-1 activity and the oxygen concentrations required by cells differ in each tissue and cell type. For example, pulmonary cells are consistently exposed to relatively high oxygen concentrations, and thus, the oxygen concentrations that these cells perceive as abnormal are relatively high, while bone marrow cells, which usually exist under low oxygen concentrations, require no HIF-1 activity when they are kept under the same oxygen concentrations. Sensitive recognition of abnormal hypoxia with respect to tissue or cell type is not possible with compounds that respond to absolute oxygen concentrations. Therefore, bioprobes that respond to biological reactions in specific microenvironments are required.

Regulations of HIF-1

Structure of HIF-1 subunits

HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β. The β subunit (HIF-1β), also known as the aryl hydrocarbon receptor nuclear translocator (Arnt1), is a
constitutively expressed nuclear protein. The α subunit (HIF-1α) is regulated at the translational level and strictly controlled by post-translational modification (Fig. 3). HIF-1α translocates to the nucleus, forms a heterodimer with HIF-1β through protein-protein interactions via their PER-ARNT-SIM (PAS) domains and binds to hypoxia-responsive elements (HREs) of the target genes. Thus, HIF-1 activity depends on the degree of HIF-1α expression. Both HIF-1α and HIF-1β belong to the family of basic helix-loop-helix (bHLH) and PAS domain-containing transcription factors. The bHLH and PAS domains mediate DNA binding and dimerisation. The other HIF-1α domains include a unique oxygen-dependent degradation (ODD) domain and two transcription domains, N-terminal activation domain (NAD) and C-terminal activation domain (CAD; Fig. 4).

Modification of HIF-1α by proline hydroxylases

Control of HIF-1α via post-translational modification mainly occurs via oxygen-dependent proline hydroxylase. The ODD domain is responsible for the regulation of the oxygen-dependent degradation of the HIF-1α protein that ‘stabilizes in a hypoxic environment and degrades immediately under normal oxygen conditions (aerobic environment)’. The details of this control mechanism were clarified in 2001 on a molecular level by cloning three human proline hydroxylase genes. These genes encode PHD1, PHD2 and PHD3, which have closely related catalytic domains and belong to the superfamily of 2-oxoglutarate-dependent oxygenases. The PHDs contain Fe(II) in their catalytic centres, which is oxidised to Fe(III) during the hydroxylation reaction, and should be regenerated prior to another round of catalysis. The PHDs hydrate proline residues (P402 and P564) in the ODD domain (Fig. 4). Hydroxylated prolines enable specific recognition of HIF-1α by the von Hippel-Lindau (VHL) protein, which, in a complex with elongin B, elongin C and Cul2, functions as an E3 ubiquitin ligase for HIF-1α. Binding of hydroxylated HIF-1α by VHL is followed by rapid polyubiquitylation. The E2 ubiquitin-conjugating enzyme UbCH5 requires K532, K538, and K547 as acceptors for the VHL-mediated ubiquitylation of HIF-1α. Polyubiquitylated HIF-1α is then translocated to and degraded in the 26S proteasome (Fig. 3). This ODD control mechanism is strictly controlled, and when cells expressing HIF-1α are oxygenated, they degrade within a few minutes.

Modification of HIF-1α by FIH

Factor-inhibiting HIF (FIH) is an asparaginyl hydroxylase that hydroxylates asparaginyl residue N803 in the CAD domain at the N-terminal end. Oxygen-dependent hydroxylation of this single asparagine has been found to be sufficient to prevent the interaction of CAD with the essential transcriptional coactivator CBP/p300, thus silencing HIFs transcriptional activity. FIH has an absolute requirement for oxygen to maintain enzymatic activity, and thus has the potential to be a cellular oxygen sensor. The estimated Km values of FIH and the PHDs for molecular oxygen were first reported to be approximately 90 and 250 mM, respectively. Since FIH appears to have a higher affinity for O2 than the PHDs based on in vitro experiments, it follows that as the severity of hypoxia increases, the PHDs would be inactivated first, while FIH would require more oxygen.
severe hypoxia to lose activity (Fig. 2).

**HIF-1-Responsive Genes**

**Hypoxia responsive elements**

HIF-1 activity drastically changes cellular response and cell properties through the expression of HIF-1-responsive genes. HIF-1 also forms a unit with p300 and CBP to bind to the hypoxia responsive element (HRE), promoting transcription of many genes with various functions related to fundamental cell activities, such as cell proliferation, cell survival, apoptosis, adhesion, angiogenesis, glucose metabolism, iron metabolism, energy metabolism and amino-acid metabolism.

HREs are enhancer elements localized at various positions and orientations in the coding region of the HIF-1 target genes. HREs contain a consensus core sequence (A/G)CGT(G/C)(G/C), which is the HIF-1 binding site. Among them, the HREs in the erythropoietin (Epo), vascular endothelial growth factor (VEGF), and phosphoglycerate kinase 1 (PGK-1) genes have been extensively studied. The Epo HRE is located in the 3' untranslated region, while PGK-1 and VEGF HREs are located in the 5' flanking region. The HRE sequences isolated from HIF-1-responsive genes have been shown to selectively induce gene expression in response to hypoxia when placed upstream of a promoter. Thus, their promoters become a useful tool for selective gene therapy in the HIF-1-active microenvironment.

**Artificial HIF-1-dependent promoters**

Several constructs of HIF-1-dependent promoters have been reported. They contain tandem-repeats of HRE consensus core sequences of HIF-1-responsive genes, such as PGK-1, enolase, LDH-A, EPO and VEGF, and a minimum promoter isolated from various virus promoters, such as SV40, CMV and Adeno virus E1B. The number of copies of HRE consensus core sequences influences the induction levels of gene expression. In the case of promoters containing Epo-HRE, increasing the HRE copy number to three or six results in a 4-fold increase in basal gene expression under anoxic conditions compared with that observed under aerobic conditions, while the use of nine HRE copies results in an 11- to 18-fold increase in gene expression. A similar result has been reported for VEGF-HRE, however, it was observed that a saturation effect can occur for constructs containing more than five copies. Thus increasing the number of copies of HRE consensus core sequences appears to be a valuable option for promoting enhanced gene expression.

**Visualisation of the HIF-1-active Microenvironment**

**Reporter genes**

Visualisation of HIF-1 activity in vivo provides useful information about the tumour microenvironment because the presence of HIF-1 activity indicates the expression of HIF-1-responsive genes, which cause the aforementioned dynamic changes in the cells. To directly monitor HIF-1 activity in vivo as well as in vitro, several reporter genes were set under HIF-1-dependent promoters, which included β-galactosidase (β-gal), enhanced green fluorescent protein (EGFP) and firefly luciferase. These HIF-1-dependent reporters are useful for monitoring HIF-1 activity in vivo as well as in vitro.

**In vivo bioluminescence imaging of HIF-1 activity**

Optical in vivo imaging provides noninvasive collection of spatiotemporal information for biological activities in small animals. Among the HIF-1-dependent reporters, luciferase reporter genes are more suitable for quantitative monitoring of ongoing biological processes in vivo because bioluminescence, which is produced during luciferase-mediated oxidation of a molecular substrate, can be imaged as deep as several centimetres within tissues with low intrinsic bioluminescence. Thus, bioluminescence imaging with remarkably high signal-to-noise ratios can be obtained. We established human cancer cell lines that contain an integrated firefly luciferase reporter gene...
downstream of p5HRE (5HRE-Luc) (Fig. 5A). When these cells are grafted into nude mice, the luciferase protein that responds to the HIF-1-active microenvironment is expressed, and visible light is produced for a fixed period of time after administration of the luciferin substrate. Bioluminescence imaging can be performed using an in vivo imaging system (IVIS®) equipped with an ultra-sensitive cooled CCD camera (Fig. 5B). Immunohistochemical analysis demonstrated that the localization of the regions detected with anti-luciferase antibody and the Pimo-positive regions were similar and that both regions were located at the boundary areas between viable and necrotic regions (Fig. 5C). These results indicate that the cells expressing luciferase were certainly hypoxic and that we successfully imaged HIF-1 activity in vivo.34, 35.

Bioprobes Specific to HIF-1-active Microenvironment

Construction of PTD-ODD fusion protein

Because HIF-1 activity is a hallmark for malignant tumours as well as ischemic diseases, bioprobes specific to HIF-1-active cells have been desired. We previously reported that a VHL-mediated protein destruction motif of the human HIF-1α ODD domain produced hypoxia-dependent stabilization when a protein was fused with it (Fig. 6A)36, 37. We found that at least 18 amino acids are necessary to control the activity of an optional protein in an oxygen-dependent manner and that an ODD domain comprising approximately 50 amino acid sequences is necessary to achieve optimal ODD control. The ODD regulatory mechanism is dependent on the ubiquitin-proteasome system, which is an intracellular mechanism. Thus, an ODD fusion protein must enter cells to be subject to the ODD regulatory mechanism. We fused the ODD fusion protein to a protein transduction domain (PTD) that gives it a membrane penetrating activity. We confirmed the membrane penetration and ODD function of the PTD-ODD fusion protein with cultured cells; we were able to introduce PTD-ODD fusion protein into almost 100% of the cells and succeeded in regulating the stability of ODD fusion protein in an oxygen-dependent manner 36, 37. Furthermore, it has been reported that when mice are administered PTD fusion protein intraperitoneally, the protein can be delivered into the tissues and cells of the entire body, including the brain38. The PTD-ODD-β-gal fusion protein was intraperitoneally administered, and the biodistribution of this fusion protein and the β-gal activity were investigated. When PTD-β-gal was administered, protein and β-gal activities were observed in normal hepatic and all tumour tissues, whereas when PTD-ODD-β-gal protein was administered, no protein or β-gal activity was observed in normal tissues and only partial activity was observed in tumour tissues37. In brief, this suggests that most normal tissues and tumours are sufficiently exposed to oxygen and have no HIF-1 activity and that hypoxic HIF-1-active cells are not present throughout tumors. We stained tumour sections with Pimo for a further examination and found that the locations of the β-gal protein and Pimo-positive regions were similar (Fig. 6B). As expected, these findings demonstrated that the degradability of the PTD-ODD fusion protein is controlled in an oxygen-dependent manner, and its distribution and function have the same specificity as HIF-1-active cells. This makes specific imaging and targeting of HIF-1 active cells feasible when the functional domain of the PTD-ODD fusion protein (Fig 6A) possesses imaging and targeting functions, respectively.

Development of an imaging bioprobes specific to HIF-1-active cell microenvironment

Because PTD-ODD fusion proteins underlie the same ODD control as HIF-1α, they could serve as bioprobes for imaging of the diseases related to HIF-1. As mentioned above, PTD-ODD-β-gal fusion protein coexists in hypoxic cells that express HIF-1α and can be used as an imaging bioprobe by substituting β-gal with an imaging material. Therefore, we first designed a probe by labelling PTD-ODD-EGFP with near-infrared fluorescent dye Cy5.5, for use as a model protein. When testing the membrane permeability
and oxygen-dependent degradation control of this prototype probe using cultured cells, we found that it permeated cell membranes with high efficiency, and its stability was controlled in an oxygen concentration-dependent manner. When this bioprobe is administered to tumour-bearing mice, it is delivered to the whole body soon after administration, but it degrades immediately in cells that are under aerobic conditions, and this leads to immediate clearance of the probe. On the other hand, the probe accumulates in hypoxic cancer cells with HIF-1 activity, and in contrast to the surrounding cells that are under aerobic conditions, the hypoxic cancer cells with HIF-1 activity can be imaged (Fig. 7A). Currently, we are performing immunohistochemical analyses to investigate whether the probe accumulates locally in HIF-1-active cells. In addition, we are continuing our research into preparation of a probe with radioactive reagents for clinical PET/SPECT probes.

Development of a targeting bioprobe specific to the HIF-1-active cell microenvironment

To endow the PTD-ODD fusion protein with a targeting function, we fused it with an endogenous cytotoxic protein, procaspase-3, which is a major executioner protease located at the most downstream position in several apoptotic pathways that remains dormant until the initiator caspses activate it by direct proteolysis. It is activated specifically in hypoxic tumour cells because, as described above, the apoptotic pathway and, hence, initiator caspses are activated in hypoxic tumour cells, generating active caspase-3. The final fusion protein product, TAT-PTD-ODD-procaspase-3 (TOP3), was examined for its efficacy in tumour-bearing mice. Systemic administration of TOP3 significantly suppressed tumour growth and reduced tumour size without any apparent side effects in mice bearing human pancreatic tumour xenografts.

The hypoxic tumour cell-specific effect of TOP3 was further confirmed in a rat ascites model39. The fluid of rat ascites hepatoma cells has exceedingly low oxygen tension (less than 1% pO2), low pH, low glucose and high lactate concentrations. Intra-peritoneal administration of TOP3 elongated the life spans of rats that bore a significant volume of malignant ascites. The effect was so drastic that 60% of the treated animals were cured without recurrence of ascites. Our ultimate goal is to improve the effect of radiotherapy and chemotherapy on solid tumours by removing hypoxic tumour cells. In fact, we have recently shown that the combination of TOP3 with radiotherapy significantly suppresses long-term tumour growth and neovascularisation35, indicating that TOP3 targeted both radiation and hypoxia-dependent increases in HIF-1 and thus suppressed HIF-1-dependent expression of survival factors, such as proangiogenic and growth factors, in the treated tumours. Recently, we analyzed a well-characterized animal model of bone metastasis in MDA-MB-231 human breast cancer cells with TOP3, and the results indicated that hypoxia and HIF-1 expression contribute to the development of bone metastases40.

The HIF-1-active microenvironment becomes a driving force of metastasis in pancreatic cancer. We grafted SUIT-2/5HRE-Luc cells (a human pancreatic cancer cell line carrying 5HRE-Luc) orthotopically into the pancreas. The mice who received these grafts died due to peritoneal dissemination and ascites within 7 weeks of grafting. Without using in vivo imaging system, it is not possible to observe the presence of cancer until the animals are dissected. Bioluminescence imaging with IVIS allowed for real-time external observation of the proliferation, local invasion and metastasis of HIF-1-active cancer cells that were grafted into the pancreas (Fig. 7B, upper panels). Recently, we constructed POP33, a new version of TOP3, that has a novel PTD, PTD3, instead of TAT-PTD. PTD3 was five times more efficient in penetrating membranes than TAT-PTD. When POP33 was intraperitoneally injected into the aforementioned orthotopic mouse model of pancreatic cancer, it significantly suppressed local invasion and peritoneal dissemination (Fig. 7B) and significantly increased survival of the transplanted mouse. These results confirm that the HIF-1-active microenvironment plays a
crucial role in invasion and metastasis.

Conclusion

Although the significance of the tumor-specific microenvironment for cancer therapy has been recognized for a long time, many problems have been left unsolved. However, with the discovery of HIF-1, investigation at the molecular level eventually began on a large scale, and current research in this field is progressing with increasing speed and on a global scale. The hypoxia visualisation and capturing methods introduced in this report represent pioneering research in regard to functional application of HIF-1. Our current projects using PTD-ODD fusion protein comprise 1) development of the aforementioned hypoxia imaging probe, 2) development of anticancer agents that target hypoxic cancer cells and 3) development of a cell death-suppressing agent for hypoxic cells in ischemic disorders. In regard to development of a PO fusion protein-imaging probe for clinical use, we are currently evaluating the specificity and in vivo pharmacokinetics of a probe by optical imaging. The target diseases of this project include the three most prevalent disorders in Japan, namely, cancer, cardiac infarction and cerebral infarction, which are expected to become even more prevalent in the future. We hope to make a contribution to improvement of the outcomes of these diseases by developing diagnostic and therapeutic products specifically for the HIF-1-active microenvironment.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, Cancer, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study is part of joint research focusing on development of core technology for establishing a COE for nano-medicine carried out through the Kyoto City Collaboration of Regional Entities for Advancing Technology Excellence (CREATE) Project of the Japan Science and Technology Agency (JST).

References

1. Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. Seminars in Radiation Oncology. 14: 198–206. 2004.
2. CancerBrown JM and Wilson WR. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer. 4: 437–447. 2004.
3. Harris AL. Hypoxia—A key regulator factor in tumor growth. Nature Rev Cancer. 2: 38–47. 2002.
4. Kizaka-Kondoh S, Inoue M, Harada H, and Hiraoka M. Tumor hypoxia: a target for selective cancer therapy. Cancer Sci. 94: 1021–1028. 2003.
5. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 3: 721–732. 2003.
6. Raleigh JA, Chou SC, Arteel GE, and Horsman MR. Comparisons among pimonidazole binding, oxygen electrode measurements, and radiation response in C3H mouse tumors. Radiat Res. 151: 580–589. 1999.
7. Vordermark D and Brown JM. Endogenous markers of tumor hypoxia. Predictors of clinical radiation resistance? Strahlenther Onkol. 179: 801–811. 2003.
8. Sobhanifar S, Aquino-Parsons C, Stanbridge EJ, and Olive P. Reduced expression of hypoxia-inducible factor-1alpha in perinecrotic regions of solid tumors. Cancer Res. 65: 7259–7266. 2005.
9. Janssen HL, Haustermans KM, Balm AJ, and Begg AC. Hypoxia in head and neck cancer: how much, how important? Head Neck. 27: 622–638. 2005.
10. Jiang B, Semenza GL, Bauer C, and Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol. 271: C1172–C1180. 1996.
11. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor. Annu Rev Cell Dev Biol. 15: 551–578. 1999.
12. O’Rourke JF, Dachs GU, Gleadle JM, Maxwell PH, Pugh CW, Stratford IJ, Wood SM, and Ratcliffe PJ. Hypoxia response elements. Oncol Res. 9: 327–332. 1997.
13. Epstein AC, Gleadle JM, McNeil LA, Hewitson KS, O’Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhand A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, and Ratcliffe PJ. C. elegans and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 107: 43–54. 2001.
14. Knowles HJ, Raval RR, Harris AL, and Ratcliffe PJ. Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. Cancer Res. 63: 1764–1768. 2003.
15. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin WG Jr. HIFα targeted for VHL-mediated degradation by proline hydroxylation: implications for O2 sensing. Science. 292: 464–468. 2001.
16. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim AV, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, and Ratcliffe PJ. Targeting of HIF-1 to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science. 292: 468–472. 2001.
17. Maxwell PH, Wiesener M, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 399: 271–275. 1999.
18. Mohon PC, Hirota K, and Semenza GL. FIH-1: a novel protein that interacts with HIF-1 alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. 15: 2675–2866. 2001.
19. Lando D, Peet DJ, Whelan DA, Gorman JJ, and Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science. 295: 858–861. 2002.
20. Koivunen P, Hirsla M, Gunzler V, Kivinkko KI, and Myllyharju J. Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. J Biol Chem. 279: 9899–9904. 2004.
21. Hirsla M, Koivunen P, Gunzler V, Kivinkko KI, and Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor J.
100 HIF-1-active Microenvironment

22. Arany Z, Haung EL, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF, and Livingston DM. An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci USA. 93: 12969–12973. 1996.

23. Ruas JL and Poellinger L. Hypoxia-dependent activation of HIF into a transcriptional regulator. Semin Cell Dev Biol. 16: 514–522. 2005.

24. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, and Giallongo A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem. 271: 32529–32537. 1996.

25. Greco O, Patterson AV, and Dachs GU. Can gene therapy overcome the problem of hypoxia in radiotherapy? J Radiat Res. 41: 201–212. 2000.

26. Shibata T, Akiyama N, Noda M, Sasai K, and Hiraoka M. Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. Int J Radiat Oncol Biol Phys. 42: 913–916. 1998.

27. Shibata T, Brown JM, Shibata T, Giaccia AJ, and Brown JM. Development of a hypoxia-responsive vector for tumor-specific gene therapy. Gene Ther. 7: 493–498. 2000.

28. Ruan H, Su H, Hu L, Lamborn KR, Kan YW, and Deen DF. A hypoxia-regulated adeno-associated virus vector for cancer-specific gene therapy. Neoplasia. 3: 255–263. 2001.

29. Binley K, Askham Z, Martin L, Spearman H, Day D, Kingsman S, and Naylor S. Hypoxia-mediated tumour targeting. Gene Ther. 10: 540–549. 2003.

30. Liu J, Qu R, Ogura M, Shibata T, Harada H, and Hiraoka M. Real-time imaging of hypoxia-inducible factor-1 activity in tumor xenografts. J Radiat Res (Tokyo). 46: 93–102. 2005.

31. Cao Y, Li CY, Moeller BJ, Yu D, Zhao Y, Dreher MR, Shan S, and Dewhirst MW. Observation of incipient tumor angiogenesis that is independent of hypoxia and hypoxia-inducible factor-1 activation. Cancer Res. 65: 5498–5505. 2005.

32. Harada H, Kizaka-Kondoh S, Itasaka S, Shibuya K, Morinibu A, Shinomiya K, and Hiraoka M. The combination of hypoxia-response enhancers and an oxygen-dependent proteolytic motif enables real-time imaging of absolute HIF-1 activity in tumor xenografts. Biochem Biophys Res Commun. 360: 791–796. 2007.

33. Sadikot RT and Blackwell TS. Bioluminescence imaging. Proc Am Thorac Soc. 2: 537–540. 2005.

34. Harada H, Kizaka-Kondoh S, and Hiraoka M. Optical Imaging of Tumor Hypoxia and Evaluation of Efficacy of a Hypoxia-targeting drug in living animals. Mol Imaging. 4: 182–193. 2005.

35. Harada H, Kizaka-Kondoh S, Li G, Itasaka S, Shibuya K, Inoue M, and Hiraoka M. Significance of HIF-1-active cells in angiogenesis and radioreistance. Oncogene. 26: 7508–7516. 2007.

36. Harada H, Hiraoka M, and Kizaka-Kondoh S. Anti-tumor effect of TAT-ODD-Caspase-3 fusion protein specifically stabilized and activated in hypoxic tumor cells. Cancer Res. 62: 2013–2018. 2002.

37. Harada H, Kizaka-Kondoh S, and Hiraoka M. Mechanism of hypoxia-specific cytotoxicity of procaspase-3 fused with a VHL-mediated protein destruction motif of HIF-1α containing Pro564. FEBS Letter. 580: 5718–5722. 2006.

38. Schwarze SR, Ho A, Vocero-Akbani A, and Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. Science. 285: 1569–1572. 1999.

39. Inoue M, Mukai M, Hamanaka Y, Tatsuta M, Hiraoka M, and Kizaka-Kondoh S. Targeting hypoxic cancer cells with a protein prodrug is effective in experimental malignant ascites. Int J Oncol. 25: 713–720. 2004.

40. Hiraga T, Kizaka-Kondoh S, Hirota K, Hiraoka M, and Yoneda T. Hypoxia and Hypoxia-inducible factor-1 expression enhance osteolytic bone metastases of breast cancer. Cancer Res. 67: 4157–4163. 2007.