

Influences of the p53 Status on Hypoxia-induced Gene Expression

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p53/Hypoxia/Hypoxia-inducible factor-1/Vascular endothelial growth factor/Angiogenesis.

The *p53* tumor-suppressor gene is one of the most frequently mutated genes in human cancers, and its genetic alterations may play critical roles in oncogenesis, tumor progression, and angiogenesis. To clarify the influence of the *p53* status on hypoxia-inducible gene expression, we first performed transfection assays with a hypoxia-responsive vector carrying 5 hypoxia-responsive elements upstream of the human CMV minimal promoter driving transcription of the luciferase gene in various human tumor cell lines with wild-type (wt) or mutant (mut) *p53*. As a result, hypoxia responsiveness considerably varied between cell lines, and we could not obtain clear evidence that the hypoxia-inducible factor-1 (HIF-1) mediated gene expression in the wt-*p53* cells was lower than that in cells with mut-*p53*. It is interesting that SaOS2 cells (*p53* null) showed the highest luciferase activities under both aerobic and hypoxic conditions among tested cells. Next, to elucidate the effects of endogenous wt- and mut-*p53*s, a transfection assay and Northern blot analysis for VEGF transcription under hypoxia were performed by using isogenic variants of HT1080 cells differing in their *p53* status. The luciferase and the endogenous VEGF mRNA expression were apparently lower in a variant carrying mutations in both *p53* alleles than in a parental line harboring wt-*p53*, implying that some types of mutant *p53* constitutively accumulated in cells can decrease both the basal and the hypoxia-induced expressions in addition to wt-*p53*.

INTRODUCTION

Hypoxia has been recognized as a unique tumor-specific microenvironment.¹⁾ Several genes supporting tumor growth and angiogenesis are induced via hypoxia-inducible factor 1 (HIF-1)-mediated transcription under hypoxic conditions, including *vascular endothelial growth factor (VEGF)*, *erythropoietin (Epo)*, and glycolytic enzymes.²⁻⁵⁾ Recent clinical studies have shown that the overexpression of HIF-1 α is associated with an unfavorable prognosis in patients with various types of cancers.⁶⁻⁹⁾

As a tumor suppressor transcription factor, *p53* regulates the expression of genes involved in cellular proliferation, cell death, and mutagenesis.^{10,11)} Under normal conditions, *p53* protein is maintained at very low levels in cells because of its short half-life, and is stabilized and activated in response to various stress signals, including DNA damage and hypoxia.¹⁰⁻¹³⁾ In human solid tumors, the *p53* gene is often inactivated by

mutations or deletion. The loss of *p53* functions may cause a further acquisition of malignant tumor phenotypes, which could be promoted by tumor hypoxia.¹⁴⁾

So far, some questions remain about the roles of *p53* in HIF-mediated transcriptions under hypoxic conditions. The concept is generally believed that wt-*p53* negatively modulates HIF-mediated gene expression, such as the *VEGF* gene, as a part of its tumor suppressor function.¹⁵⁻¹⁸⁾ Evidence in published studies with transfection assays suggests that *p53* repressed HIF-stimulated transcription from VEGF- and Epo-promoter constructs¹⁶⁾ and that wt-*p53*, but not mutant *p53*, suppressed the expression of endogenous VEGF mRNA.¹⁵⁾ A transduction with an adenoviral vector containing the wt-*p53* gene could down-regulate VEGF expression in human colon cancer cell lines with *p53* mutations.¹⁷⁾ However, these *in vitro* experiments were performed without hypoxic culture conditions. In contrast, others have argued that there was no evidence for a casual relationship between the loss of *p53* activity and increased VEGF expression.¹⁹⁾ They showed that hypoxia could increase endogenous VEGF mRNA levels in either the presence or absence of functional *p53*. These controversial results led us to analyze the *p53* function in hypoxia-induced gene expressions in our experimental settings. Recent studies showed that HIF-1 α either directly bound to *p53*^{20,21)} or indirectly via binding to Mdm2,²²⁾ suggesting functional interactions between *p53* and HIF.

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Here we evaluated the influences of p53 status on an HIF-1 mediated gene expression by using an original hypoxia-responsive reporter, 5HRE/hCMVmp-luc, in a panel of various human tumor cell lines. We then used two isogenic human fibrosarcoma cell lines harboring endogenous wt- or mut- p53 and performed the Northern blot analysis for the endogenous VEGF gene under aerobic and hypoxic conditions as well as the transient reporter assays.

MATERIALS AND METHODS

Cell culture and hypoxic treatment

The human tumor cell line SaOS2 cells (an osteosarcoma), Hela (a cervical carcinoma), MCF7 (a breast cancer), and T98G (a malignant glioma) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in a well-humidified incubator with 5% CO₂ at 37°C. DLD-1, HCT15, WiDr, and HT29 (colon cancers) were also maintained in a PMRI-1640 culture medium with 10% FBS in the same incubation condition. All cells were purchased from the American Type Culture Collection, Rockville, MD. The p53 status in these cell lines was previously described.^{23–25} Isogenic clones of the human HT1080 cell line, P48 and 6TG, were kindly provided by Dr. E. J. Stanbridge (University of California, Irvine, CA) and maintained in DMEM with 10% heat-inactivated fetal bovine serum.²⁶ For transient transfection experiments, 4 × 10⁵ exponentially growing cells were plated overnight on a 6 cm culture dish. Hypoxic conditions were achieved with prewarmed aluminum hypoxic chambers by evacuation and gassing with 95% N₂/5% CO₂; the tightly sealed chambers were then incubated at 37°C. The oxygen concentration of 0.02% was used for hypoxic incubation as previously described.²⁷

Plasmid construction

A tetracycline-regulated plasmid, pTRE2wt-p53, was constructed as follows. Briefly, a cDNA fragment encoding a wild-type p53 gene was obtained by an RT-PCR method with a Superscript reverse transcription kit and with an Elongase enzyme mixture (Gibco BRL, Gaithersburg, MD) with the following paired primers: 5'-GATGGATCCAACACGACGGTGACACGCTTCCCT-3' and 5'-AGGCGGCCGCGAGGTGGGGAACAAGAAGTGGAGAATGTCA-3'. The PCR conditions for 35 cycles were 94°C, 1 min; 56°C, 1 min; and 68°C, 2 min. The amplified fragment was digested, subcloned into the sites of a pTRE2hyg expression vector (Clontech, Palo Alto, CA), and confirmed by a sequencing analysis (DNA Sequencer LR4200, Li-COR Inc., Lincoln, NE). A hypoxia-inducible luciferase vector, 5HRE/hCMVmp-luc, was reported previously.²⁷

Establishment of stable SaOS2 Tet-Off clones

SaOS2 cells were transfected with 5 µg of pTet-Off vector (Clontech) by using Superfect reagent (Qiagen, GmbH, Ger-

many). At 48 h after transfection, the cells were trypsinized and plated for a clonal selection of stable transfectants in 400 µg/ml G-418. To obtain good clones that showed a robust responsiveness to various concentrations of doxycycline, transient transfection assays with a pTRE2-luc vector were performed with a Dual Luciferase Assay system (Promega, Co., Madison, WI).

Transient transfection and reporter assay

To examine a hypoxia-inducible gene expression, we performed transfections of the 5HRE/hCMVmp-luc and a pRL-CMV into tested cells with Superfect reagent. Then, at 12 h after transfection, the cells were trypsinized and plated into four glass dishes, then incubated overnight at 37°C before the desired hypoxic or aerobic treatment. A reporter assay was performed with a Dual Luciferase Assay system. For the experiment with a tet-regulatable expression of wt-p53 in SaOS2 cells, we performed cotransfections of the pTRE2wt-p53 into tet-off clones of SaOS2 cells. After transfection, the cells were trypsinized and plated into four glass dishes with or without 1 µg/ml of Doxycycline.

To clarify the effects of endogenous p53 status on gene expression under hypoxic conditions, we cotransfected the 5HRE/hCMVmp-luc and the pRL-CMV vectors into P48 with wild-type p53 and 6TG clones with mutant p53. After transfection, the cells were trypsinized, plated in triplicate into the glass dishes, and incubated overnight. They were then treated under aerobic or hypoxic conditions for 6–18 h. After 2 h of reoxygenation, cell lysates were prepared with 400 µl of passive lysis buffer (Promega), and the luciferase activities were measured with a luminometer.

Western blotting

After a treatment of irradiation, aerobic, or hypoxic incubation, the cells were washed twice with PBS and harvested in a 2× SDS sample buffer on ice (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8). After centrifugation at 14,000 rpm at 4°C for 30 min, the supernatants were recovered and immediately stored at –80°C. Protein concentrations were measured with a Protein DC assay kit (Bio-Rad, Hercules, CA). Twenty micrograms of total proteins were separated on a 10% SDS-PAGE polyacrylamide gel, transferred to nitrocellulose membranes, and blocked with 5% nonfat milk in Tris-buffered saline (TBS) for 1 h. The membranes were then incubated with primary antibodies, followed by washing and incubation with goat antimouse IgG conjugated with alkaline phosphatase. The antibodies against p53, p21, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The chemifluorescence signals on membranes were developed with an ECF Western blotting kit (Amersham, Arlington Heights, IL) and scanned by a Fluorimager according to the manufacturer's instructions (Molecular Dynamics, Sunnyvale, CA).

Northern blot analysis

The total RNA was extracted from P48 and 6TG cells after aerobic or hypoxic incubation with an RNeasy Mini kit (Qiagen). Twenty μg of total RNA per lane were separated on 1% denaturing formaldehyde/agarose gels, transferred to Hybond nylon membranes by capillary elution, and cross-linked by means of a UV linker (Bio-Rad). A VEGF cDNA probe was prepared from an hVEGF expression vector, pCAG-BSD-VEGF.²⁸⁾ A cDNA probe for β -actin was purchased from Clontech. Each probe was labeled with an ALK-Phos DIRECT kit (Amersham). After 1 h of prehybridization, the membranes were probed for VEGF and β -actin overnight at 55°C. They were then washed twice at 55°C in a primary washing buffer for 10 min and twice in a secondary washing buffer for 5 min. The hybridization signals were detected with chemiluminescent CDP-Star (Amersham).

RESULTS

Hypoxia-inducible gene expression in transfection assay in a panel of human tumor cells

To examine the effects of p53 status on hypoxia-inducible gene expression, we have used the 5HRE/hCMVmp-luc reporter, which carries five hypoxia-responsive elements upstream of the human CMV minimal promoter driving transcription of the luciferase gene. The reporter assay was performed in 8 human tumor cell lines carrying wt- or mut-p53. SaOS2 cells are p53 null. HeLa, MCF-7, and DLD-1 cells harbor wt-p53. HT29 and WiDr cells have the same missense mutation, Arg-273 to His-273; HCT-15 cells have one wild-type allele and one mutant allele, Phe-153 to Ala-153; T98G cells have mutation, 237-Met to 237-Ile.²³⁻²⁵⁾ After 6 h of hypoxic incubations, the luciferase expression was significantly increased in all the tested cells. As an index of hypoxia-responsiveness, the hypoxic/aerobic (H/A) ratio was determined by a comparison of the luciferase activity for the cells treated under hypoxic conditions with that for the aerobic control.²⁹⁾ As shown in Fig. 1, the level of the expression under aerobic conditions varied considerably among the lines. Moreover, the H/A ratios ranged from 40 to more than 500 between cell lines. Thus we could not observe a consistent effect of the p53 status in either the basal or the hypoxia-induced gene expressions of the reporter. The cell lines used in the present study are very likely to carry a variety of mutations in a gene-regulating transcription besides mutations in the p53 gene. This could have been caused inconsistency for the reporter's expression in the cells.

Inhibition of gene expression by wt-p53 in transfection assay in SaOS2 cells

We found it interesting that SaOS2 cells (p53 null) showed the highest activities under both aerobic and hypoxic conditions and the highest H/A ratio among 8 cell lines (Fig. 1). To examine the effects by wt-p53 expression in SaOS2 cells, we

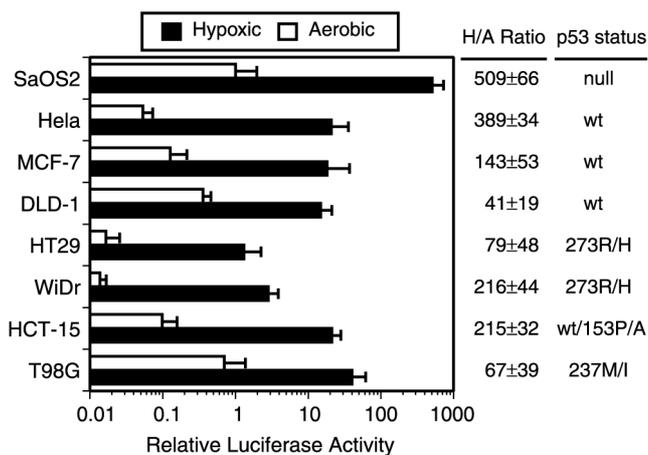


Fig. 1. Hypoxia-inducible gene expression in transfection assay in a panel of human tumor cells carrying wt- or mut-p53 genes. The cells were transfected with 5 μg of 5HRE/hCMVmp-luc and 0.1 μg of pRL-CMV. After 12 h of transfection, they were trypsinized and replated into four glass dishes overnight, followed by 6 h of incubations under aerobic and hypoxic conditions. The luciferase activities were shown as relative values normalized by the luciferase activity for the SaOS2 cell line treated under aerobic conditions. The data represent the mean and S.E. from 3–4 independent experiments. The p53 status for all cell lines was also indicated.

established clones with a stable transfection of a tet-off system, showing a low basal expression and a highly inducible expression (more than 350-fold) in the luciferase assay, with and without doxycycline, respectively. Cotransfected into the SaOS2 tet-off cells were a wt-p53 expression vector, pTRE2wt-p53; a hypoxia-responsive vector, 5HRE/hCMVmp-luc; and a plasmid, pRL-CMV. The 5HRE/hCMVmp-luc vector increased the luciferase expression more than 500-fold in response to hypoxia in SaOS2 cells. We found that the luciferase activity with wt-p53 expression in the absence of doxycycline was significantly lower than that in the presence of doxycycline, and that the increasing amounts of wt-p53 plasmid resulted in decreased luciferase activities under both aerobic and hypoxic conditions (Fig. 2). We should also note that even the activity of a control vector pRL-CMV was obviously decreased by wt-p53 under both hypoxic and aerobic conditions (data not shown). Thus it seems that the inhibitory effects by exogenous wt-p53 expression were not so specific to HIF-dependent promoters.

Responses to ionizing radiation and hypoxia of isogenic HT1080 cell lines harboring wt-p53 or mutant p53

The above results indicate that wt-p53 may function as a less specific repressor for the tested reporters under both hypoxic and aerobic conditions. Further analyses were performed to examine the effect of specific p53 mutations on a hypoxia induction of the reporter. To avoid the potential problems in the comparison of cell lines having various genetic features, we used an isogenic set of human fibrosarcoma cell

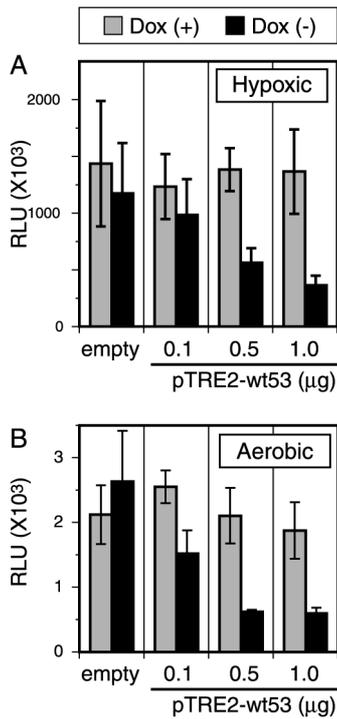


Fig. 2. Effects of wt-p53 on transcription from a hypoxia-responsive vector under hypoxic (A) and aerobic (B) conditions. An SaOS2 tet-off stable transfectant was cotransfected with 5 µg of 5HRE/hCMVmp-luc and 0.1 µg of pRL-CMV, together with different amounts of pTRE2wt-p53 expression vector or empty vector. The total amounts of the transfected DNA were adjusted by adding the empty vector. The cells were then treated in the growth medium with or without Doxycycline (1 µg/ml) for 24 h, followed by incubations under hypoxic or aerobic conditions for 6 h, then harvested for the dual luciferase assay. The 5HRE/hCMVmp-luc vector increased the luciferase activity more than 500-fold in response to hypoxia. The data represent the mean and S.E. from 4 independent experiments.

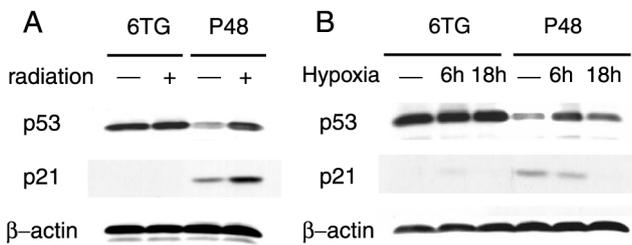


Fig. 3. Response to ionizing radiation and hypoxia of isogenic variants of HT1080 cells harboring wt-p53 or mutant p53. Western blot analysis for p53 and p21 proteins in P48 and 6TG cells. The samples were collected either after 4 Gy of irradiation (A) or after treatments under hypoxic conditions for 6 h or 18 h (B). The membranes were probed for p53, p21, and β-actin, as described in Materials and Methods.

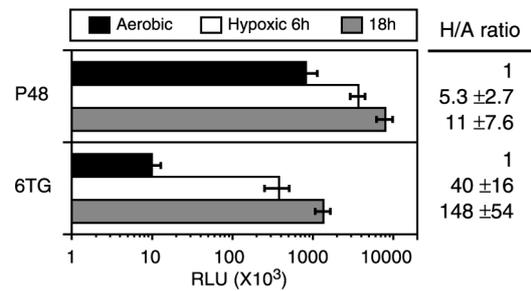


Fig. 4. Effects of endogenous p53 status on transcription from the 5HRE/hCMVmp vector under aerobic and hypoxic conditions. P48 cells or 6TG cells were cotransfected with 5 µg of 5HRE/hCMVmp-Luc and 0.1 µg of pRL-CMV. The transfected cells were divided into triplicate plates, followed by 6 h or 18 h of hypoxic or aerobic incubations. The luciferase activity was measured as described in Materials and Methods. The H/A ratio was determined by a comparison of the luciferase activity for the hypoxically treated cells with that for the aerobic control.

lines differing only in their p53 status. A parental line, P48 contains two wild-type p53 alleles and a variant, 6TG, has two mutant p53 alleles: Gly-245 to Arg-245 and Cys-277 to Phe-277, which are in the DNA binding domain of p53.²⁶ These cells are useful to elucidate the true effects of the p53 status on the hypoxia-induced gene expression because their p53 proteins are expressed from their own endogenous promoters, which is completely different from forced expression.

At first, we treated these cells with 4 Gy of irradiation to confirm their p53 status. In Fig. 3A, the p53 and p21 protein levels were increased only in P48 cells with wt-p53 in response to irradiation, but the mutant p53 proteins were constitutively accumulated, and no clear induction of p21 was observed in 6TG cells, suggesting that these mutations of p53 are transcriptionally inactive. We then also treated these cells under hypoxic conditions. After 6 h and 18 h of hypoxic incubations, the p53 protein levels were increased only in the P48 cells, and the p21 protein was not induced in either cell line, as shown in Fig. 3B. These results indicated that wt-p53 proteins induced under hypoxic conditions might be different from those induced by irradiation in terms of transcriptional activity.

Hypoxia-induced gene expression in isogenic HT1080 cells

We examined whether the difference in the p53 status in these isogenic cells could influence a hypoxia-induced gene expression under aerobic and hypoxic conditions. After a transfection of the 5HRE/hCMVmp-luc vector, these two cell lines were treated under hypoxic conditions for 6 h and 18 h; the luciferase assay followed this treatment. In Fig. 4, the H/A ratio in 6TG cells was about 10 times higher than that in the P48 cells. If we compare the H/A ratios, it seems that the wild-type, but not the mutant, p53 could repress a hypoxia-related gene expression. However, we should notice that the

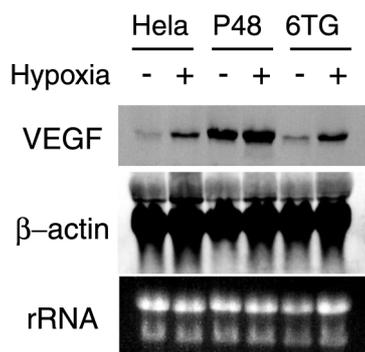


Fig. 5. Effects of the p53 status on hypoxia-induced VEGF expression. A Northern blot analysis for endogenous VEGF mRNA expression was performed in P48 and 6TG cells, harboring wt-p53 and mutant p53, respectively. HeLa cells were also used as a control. Total RNA was extracted from the cells after 18 h of hypoxic or aerobic incubations, separated on 1% denaturing formaldehyde/agarose gels, and transferred to nylon membranes. The membranes were hybridized with a human VEGF cDNA and β -actin probes. Photos of the gels stained by ethidium bromide were also shown.

luciferase expression levels are quite different in the two cell lines. As shown in Fig. 4, the luciferase activity in the P48 cells was much higher than that in the 6TG cells under both the aerobic and hypoxic conditions. These results suggest that mut-p53 that constitutively accumulated in the cells may also show transrepression under both aerobic hypoxic conditions.

Effect of the endogenous p53 status on endogenous VEGF transcription

A Northern blot analysis was performed for the expression of endogenous VEGF mRNA in P48 and 6TG cells under both aerobic and hypoxic conditions. As shown in Fig. 5, VEGF mRNA levels were markedly increased in response to hypoxia. It is interesting that 6TG cells harboring endogenous mut-p53 exhibited lower levels of transcriptions compared with P48 cells with endogenous wt-p53 under both aerobic and hypoxic conditions. These results suggest that some types of mut-p53 existing in 6TG cells might negatively influence endogenous VEGF expression.

DISCUSSION

Mutations or genetic alterations of the p53 tumor-suppressor gene are frequently observed in human cancer and might be associated with the angiogenic switch during tumor progression.^{30, 31} Using our 5HRE/hCMVmp-luc vector without the assistance of an HIF-1 α expression plasmid, we can now demonstrate potent hypoxia-inducible up-regulations in the luciferase assay in various human tumor cell lines. In our experiments, the wt-p53 cell lines exhibited no hypoxia-responsiveness lower than the cells with mut-p53, suggesting the potential difficulties in comparing the effects of the p53

status by the use of cell lines with different genetic backgrounds. Our data do not support the postulated concept that wt-p53 negatively modulates HIF-mediated gene expressions including the VEGF gene.

SaOS2 cells, whose p53 status is null, showed the highest activities under both aerobic and hypoxic conditions among the tested cells. This potentially implies that endogenous p53 genes, either wild type or even mutated, in the other 7 cell lines might retain some influence on HIF-1 mediated gene expression, at least when compared with the p53-null cells.

As shown in Fig. 2, we found that a cotransfection of a tet-regulatable wt-p53-expressing vector resulted in decreased luciferase activities in the SaOS2 cells. However, an expression of wt-p53 also similarly inhibited the luciferase activity from a control vector pRL-CMV. Thus it may be reasonable to speculate that the inhibitory effects by exogenous wt-p53 could be less specific to HIF-1-mediated expression, though several previous studies reported a suppression of gene expression by wt-p53 when using the forced expression system.¹⁵⁻¹⁸ In this sense the past studies that attribute repressor functions to p53 based on an analysis of cells transiently overexpressing this protein should be interpreted cautiously.¹⁹ In fact, several previous studies have showed that wt-p53 blocked the transcription from numerous promoters lacking p53-binding sites.^{16,32,33} Moreover, posttranscriptional modifications by the specific signals are quite essential for the functions and stability of p53 and of HIF-1 proteins. Nevertheless, early studies were performed only under aerobic conditions or with an ectopic overexpression of HIF-1 α .^{15,16,18-20} We wish to reemphasize the importance of our study performed under hypoxic conditions.

We should be aware that the p53 protein is usually kept at low levels in normal conditions and that it can be stabilized and accumulated only under cellular stress.³⁴ Therefore it is likely that the forced expression of p53 proteins may not reflect its true functions. To avoid these and other potential artifacts and to further elucidate the physiological effects of endogenous p53 under hypoxia, we next used two isogenic lines of HT1080 cells differing only in their p53 status.²⁶ As shown in Fig. 3, p53 proteins were clearly induced by ionizing radiation and hypoxia in P48 cells with wt-p53, but not in 6TG cells. It is interesting that despite accumulations of p53, hypoxia did not up-regulate a downstream target, p21, in contrast to ionizing radiation in P48 cells. These findings were consistent with those by Koumenis *et al.*,³⁵ that hypoxia-induced p53 failed to activate endogenous downstream effectors, suggesting that p53 is differentially regulated by radiation and hypoxia. Previous studies^{16,18-20} attempted to show the negative effects of wt-p53 on HIF-1-mediated expression via its induction by treatments with DNA-damaging agents, such as radiation and chemotherapeutic agents. However, these methods would not be relevant to hypoxic treatments because p53 should have different modifications between stresses.^{11,35}

Because the H/A ratio for 6TG cells was higher than for P48 cells, we might have considered that mutant p53 could augment the hypoxia responsiveness of the 5HRE/hCMVmp-luc vector, which is generally consistent with the postulated theory of repression by wt-p53 on VEGF gene.^{15–17)} However, our findings suggest that at least some types of mutant p53 proteins when constitutively accumulated in cells can repress the gene expression from the 5HRE/hCMVmp-luc vector, and they are partly in agreement with a recent report¹⁸⁾ that a panel of codon 175 p53 mutants showing well-characterized dominant-negative effects inhibited HIF-1-stimulated transcription with a cotransfection of expression vectors for HIF-1 α . Since the 6TG cells used here harbor two mutant p53 alleles within their DNA-binding domain, Arg-245 and Phe-277²⁶⁾, they may exhibit similar dominant-negative and transrepression effects in the transfection assay.

To clarify the true physiological relationship between p53 status and hypoxia-inducible gene expression, it is essential to examine endogenous *VEGF* gene expression in these isogenic cell lines. We found that *VEGF* mRNA in 6TG cells was obviously lower under both the aerobic and hypoxic conditions than that in P48 cells, as shown in Fig. 5. These findings are well correlated with those in the transfection assay (Fig. 4). Therefore it appears reasonable that at least mutant p53 constitutively accumulated in 6TG cells could strongly inhibit both a basal and a hypoxia-induced transcription for endogenous *VEGF*. A recent study with an isogenic set of human HCT116 cells harboring a wild-type or homozygous knockout of the *p53* gene has demonstrated that the loss of p53 resulted in an HIF-1-dependent transcriptional activation of the *VEGF* gene in response to hypoxia.³⁶⁾ It appears natural that *p53*^{-/-} cells should exhibit a higher response to hypoxia than parental *p53*^{+/+} cells, providing good complementary findings to ours.

On the mechanism for interactions of wt-p53 on HIF-1, some authors demonstrated that p53 directly binds with HIF-1 α ,^{20,21)} others that HIF-1 binds with Mdm2 but not p53,²²⁾ implicating an important role of HIF-1 α in the regulation of the p53-Mdm2 pathway in response to hypoxia. However, the interactions of mutant p53 proteins with HIF-1 α have not been elucidated.

In summary, we demonstrated that a robust hypoxia responsiveness in the luciferase assay in human tumor cell lines with a variety of the p53 status. Hypoxia responsiveness varied between cell lines, and we could not clearly show that the HIF-1-mediated gene expression in the wt-p53 cells was lower than that in cells with mut-p53. Indeed, an exogenous expression of wt-p53 in SaOS2 cells could result in a strong inhibition of transgene expression regardless of oxygen status. Furthermore, our findings with isogenic cell lines indicated that at least some mutant p53 when constitutively accumulated, can also decrease the HIF-1-dependent transcription of genes under both aerobic and hypoxic conditions, including the *VEGF* gene.

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