The Opposing Effect of Hypoxia-Inducible Factor-2α on Expression of Telomerase Reverse Transcriptase

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Abstract

Hypoxia-inducible factor-1α (HIF-1α) has been implicated in the transcriptional regulation of the telomerase reverse transcriptase (hTERT) gene expression and telomerase activity, essential elements for cellular immortalization and transformation. However, controversial results were obtained in different studies. Moreover, it is totally unclear whether HIF-2α, the paralog of HIF-1α, plays a role in regulating hTERT expression. In the present study, we found that hypoxic treatment enhanced hTERT mRNA expression and telomerase activity in three renal cell carcinoma (RCC) cell lines with different genetic backgrounds. Both HIF-1α and HIF-2α were capable of significantly increasing the hTERT promoter activity in these cells. Moreover, depleting HIF-2α led to a down-regulation of hTERT mRNA level in RCC A498 cells expressing constitutive HIF-2α. It was found that HIF-2α bound to the hTERT proximal promoter and enhanced the recruitment of the histone acetyltransferase p300 and histone H3 acetylation locally in A498 cells treated with hypoxia. Increased levels of hTERT mRNA were observed in two of three hypoxia-treated malignant glioma cell lines. However, HIF-1α stimulated whereas HIF-2α inhibited the hTERT promoter activity in these glioma cell lines. Ectopic expression of HIF-2α resulted in diminished hTERT expression in glioma cells. Collectively, HIF-1α activates hTERT and telomerase expression in both RCC and glioma cells, and HIF-2α enhances hTERT expression in RCC cells, whereas it represses the hTERT transcription in glioma cells.

Introduction

Activation of telomerase, a ribonucleoprotein enzyme responsible for the stabilization of telomere length at the chromosome termini, is a critical event for immortalization and transformation of human cells (1-4). The core telomerase complex consists of two components: the rate-limiting, catalytic subunit telomerase reverse transcriptase (hTERT) and ubiquitously expressed RNA template hTER (3). It has been established that most normal human cells lack telomerase activity due to the stringent transcriptional repression of the hTERT gene, and during the oncogenic process, induction of hTERT expression is, in general, required for telomerase activation (3-6). To elucidate the mechanism(s) underlying the acquisition of telomerase activity by tumor cells, numerous studies have therefore focused on the transcriptional regulation of the hTERT gene. Significant progresses have been made in the last years; however, it remains incompletely understood how exactly the hTERT transcription is de-repressed in malignant cells.

In response to low oxygen tension (hypoxia), mammalian cells undergo the transcriptional activation of a series of genes that are involved in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation and survival (7-9). The hypoxia-inducible factor (HIF), an oxygen-sensitive transcriptional activator, is the essential element that mediates such a response. HIF is a heterodimer composed of a constitutively expressed subunit HIF-1α and an oxygen-regulated subunit HIF-1β (or its paralogs HIF-2α and HIF-3α; refs. 7-9). By promoting angiogenesis, HIF-1 plays important roles in progression, invasiveness, or metastasis of malignant diseases (7, 8). Furthermore, recent studies have suggested that hypoxic treatment or overexpression of HIF-1α affects hTERT and telomerase expression (10-16). Most observations showed that the incubation of human cancer cells at low levels of oxygen enhanced hTERT mRNA expression and telomerase activity via HIF-1α–mediated trans-activation of the hTERT gene. However, a number of issues remain to be delineated. (a) Koshiji et al. observed that hypoxia-induced HIF-1α significantly inhibited hTERT expression in human colon cancer cells, in sharp contrast to other reports (11). (b) Different...
mechanisms, including transcriptional and post-transcriptional approaches, have been implicated in hypoxia-regulated hTERT expression (10, 12-14, 16). It remains to be defined whether these regulatory pathways contribute to the enhanced hTERT expression or telomerase activity in all human cancer cells. (c) More importantly, in certain malignant cells such as renal cell carcinoma (RCC) cells, because the HIF-1α gene is mutated, or silent due to many reasons, its paralog HIF-2α is constitutively expressed or induced as the predominant form of oxygen-sensitive transcriptional activator in response to hypoxia (17). It is totally unclear whether HIF-2α plays any roles in regulating hTERT expression. HIF-1α and HIF-2α were shown to have opposite effects in different normal and cancer cells, including RCCs and malignant gliomas (17-21). It is thus likely that their activities may differ in controlling hTERT and telomerase expression. In the present study, we sought to answer these questions by examining how HIF-1α and HIF-2α regulate hTERT mRNA expression and telomerase activity in RCC and glioma cells.

Results

Hypoxic Treatment Up-Regulates Overall hTERT mRNA Expression and Telomerase Activity Concomitant with Induction of HIF-1α and/or HIF-2α in RCC Cells with Different Genetic Backgrounds

Compared with other types of cancers, loss of function of von Hippel-Lindau disease tumor suppressor (VHL) gene, an E3 ubiquitin ligase responsible for the degradation of the α-subunit of HIF, frequently occurs in RCCs, which results in constitutive HIF-1α and/or HIF-2α expression even under normal levels of oxygen (9, 22, 23). Thus far, there have been no data available regarding hTERT mRNA expression in RCC cells under a hypoxic condition. To address this, we chose three RCC cell lines in the study: A498 cells with VHL inactivation and constitutive HIF-2α expression and Caki-2 and TK-10 cells with functional VHL. As shown in Fig. 1A, A498 cells expressed HIF-2α protein under physiologic oxygen levels and underwent further up-regulation of this protein in response to hypoxia. Both TK-10 and Caki-2 cells expressed HIF-1α and HIF-2α only when cultured under hypoxic conditions. In all three cell lines treated with hypoxia, the expression of vascular endothelial growth factor (VEGF) mRNA, an established HIF target gene (7, 8), significantly increased (Fig. 1A), which indicates the presence of an intact hypoxia-HIF-target gene signal pathway in these cells. hTERT expression was then examined using a conventional reverse transcription-PCR (RT-PCR) approach, and we observed a significantly higher level of overall hTERT mRNA in these three RCC cell lines following hypoxic treatment (Fig. 1A). A real-time quantitative PCR (RTQ-PCR) was further applied for analyses of hTERT mRNA in these same sets of the samples. The hypoxia-treated Caki-2 and A498 cells exhibited 2.3- and 3-fold increases [normal oxygen level (N) versus hypoxia (H); P = 0.008 for Caki-2, and P < 0.001 for A498, a two-tailed, unpaired t test] in their hTERT mRNA expression, respectively (Fig. 2B). Although TK-10 cells displayed higher levels of hTERT mRNA, a hypoxic treatment still resulted in almost 2-fold up-regulation of hTERT mRNA expression (N versus H, P = 0.02; Fig. 2B).

Consistent with alterations in hTERT transcripts, telomerase activity increased substantially in these same cells (Fig. 1C).

It is known that the alternative splicing of hTERT transcription participates in controlling telomerase activity (5, 24-31), and that a recent study suggests a transcriptional switch to the full-length hTERT mRNA in ovary cancer cells in response to hypoxia (10). Therefore, we asked whether this was the case in RCC cells by determining the expression profile of hTERT mRNA variants. As shown in Fig. 1A, all the spliced variants of hTERT transcripts increased simultaneously in the hypoxia-treated A498, Caki-2, and TK-10 cells, which suggests that the RCC cells under hypoxia, unlike ovary cancer cells, exhibit enhanced expression of overall hTERT mRNA variants rather than a specific increase in full-length hTERT transcripts in our experimental settings.

Malignant Glioma Cells Exhibit Differential Alterations of hTERT mRNA Expression and Telomerase Activity in Response to Hypoxic Treatment

We then selected three glioma cell lines (U251, U373, and U563) for analyses of their hTERT mRNA and telomerase expression under a hypoxic condition. All these cell lines exhibited a strong induction of HIF-1α in response to hypoxia (Fig. 1D). HIF-2α was simultaneously induced in U251 cells but not in the other two cell lines (Fig. 1D). Consistently, VEGF mRNA was up-regulated in these glioma cells (Fig. 1D), hTERT mRNA abundance increased 2.6-fold in U373 and 3.4-fold in U563 cells following their exposure to hypoxia, respectively (N versus H, P < 0.001 for both U373 and U563; Fig. 1E), as shown by RTQ-PCR analyses. Unexpectedly, we observed negligible changes of hTERT expression in U251 cells (Fig. 1D and E). Moreover, the substantially increased hTERT mRNA variants and telomerase activity occurred only in hypoxia-treated U373 and U563 cells but not in U251 cells (Fig. 1D and F).

Depletion of HIF-2α Expression Leads to Diminished Levels of hTERT mRNA in A498 Cells Expressing Constitutive HIF-2α

As described above, A498 cells express only HIF-2α, which provides a good model to study the effect of HIF-2α on hTERT expression (23). We inhibited HIF-2α expression using small interfering RNA (siRNA) specifically targeting HIF-2α mRNA in A498 cells and then determined hTERT mRNA level 48 h later. As shown in Fig. 2A, a significant decrease (two-thirds) in hTERT mRNA abundance was observed in HIF-2α siRNA-treated A498 cells compared with the control cells (control versus HIF-2α siRNA, P = 0.01). Furthermore, knocking down HIF-2α substantially blocked hypoxia-induced up-regulation of hTERT expression in these cells. Thus, HIF-2α is required for constitutive and hypoxia-inducible hTERT expression in A498 cells.

Overexpression of HIF-2α Inhibits hTERT mRNA Expression in Glioma Cell Lines

HIF-2α is required for hTERT expression in A498 cells, but we noticed that the inability to up-regulate hTERT mRNA expression by hypoxia occurred concomitantly with HIF-2α
induction in hypoxia-treated U251 cells. Therefore, we further determined whether HIF-2α was capable of inhibiting endogenous hTERT mRNA expression in glioma cells. U251 and U373 cells were transfected with a HIF-2α expression plasmid, selected with G418, and harvested for hTERT mRNA analyses. Western blot analyses showed the expression of HIF-2α protein in the cells transfected with the HIF-2α expression vector (Fig. 2B). Compared with the control cells, HIF-2α–expressing U251 and U373 cells displayed lower levels of hTERT mRNA as revealed by both conventional and RTQ-PCR assessments (control versus HIF-2α, P < 0.01 for both U251 and U373 cells), which indicates that HIF-2α inhibits hTERT expression in glioma cells (Fig. 2B).

Both HIF-1α and HIF-2α Enhance the hTERT Promoter Activity in RCC Cells

The hTERT gene has previously been identified as a direct target of HIF-1α (13, 16). We therefore sought to further determine the effect of both HIF-1α and HIF-2α on the hTERT promoter activity in RCC cells. It is known that p181, a reporter construct with an insert of the core hTERT promoter sequence, carries the hypoxia-responsive element (HRE), and that HIF-1α activates the hTERT transcription by binding this HRE (13, 16). When p181 was cotransfected with either HIF-1α or HIF-2α expression plasmids into A498 and Caki-2 cells, we observed substantial increases in luciferase activity driven by the hTERT core promoter in a dose-dependent manner.
(Fig. 3A). HIF-1α and HIF-2α at 0.5 μg stimulated the hTERT promoter activity ~400% and 250% higher, respectively, in A498 cells, whereas they were 100% and 60% higher in Caki-2 cells (Fig. 3A).

**HIF-1α Stimulates whereas HIF-2α Suppresses the hTERT Promoter Activity in Glioma Cell Lines**

We then asked how HIF-1α and HIF-2α affected the hTERT transcription in glioma cells. To this end, we introduced p181 and HIF-1α or HIF-2α expression plasmids into U251 and U373 glioma cells and then assessed luciferase activity in these cells. Similarly to those found in RCC cells, HIF-1α dramatically enhanced hTERT promoter activity in a dose-dependent manner in both U251 and U373 cells (Fig. 3B). In striking contrast, however, HIF-2α significantly inhibited the hTERT promoter activity in these cells (Fig. 3B). Moreover, HIF-1α–mediated activation of the hTERT promoter was substantially attenuated in the presence of HIF-2α (Fig. 3B).

**Increased Binding of HIF-2α to the hTERT Promoter Occurs Concomitantly with Enhanced Recruitment of Histone Acetyltransferase p300 and Histone H3 Acetylation in A498 Cells under Hypoxic Conditions**

To gain mechanistic insights into the opposing effect of HIF-2α on the hTERT expression between RCC and glioma cells, we sought to determine whether HIF-2α bound to the hTERT promoter and, if so, which changes would in turn occur locally in A498 and U251 cells under hypoxic conditions. Using a chromatin immunoprecipitation (ChIP) assay, we showed the presence of HIF-2α in the hTERT proximal promoter (the region a) in A498 cells, consistent with the previous observation that the region harbors functional HREs (Fig. 4A; refs. 13, 16). Following a hypoxic treatment, the accumulation of HIF-2α led to the down-regulation of hTERT mRNA in control A498 cells, as assessed by RTQ-PCR, was set as 1 (right). B. Overexpression of HIF-2α led to the down-regulation of hTERT mRNA in U251 and U373 glioma cells. β2-M, β2-microglobulin. Left, conventional PCR assays. Right, RTQ-PCR results. The amount of hTERT mRNA in untransfected control cells, as assessed by RTQ-PCR, was set as 1. Columns, relative levels of the overall hTERT mRNA; bars, SD. H and N, the cells were cultured under hypoxic and normal oxygen conditions, respectively.

**Discussion**

Solid tumors frequently experience various degrees of oxygen deprivation through which HIF expression is induced (7-9). It is, in general, believed that HIF’s function as oncogenic promoting factors by targeting their downstream genes that govern multiple cell functions such as angiogenesis, metabolism, invasion/metastasis, and apoptosis/survival (8, 9). However, evidence has recently accumulated that the role of HIFs in oncogenesis is more complicated and may depend on cell types...
and specific settings. One striking example is HIF-2α, the protein that is absolutely required for tumor formation of RCCs with the inactivation of VHL while it acts as a tumor suppressor in malignant gliomas (19, 21, 32-34). Interestingly, the effect of HIF-1α and HIF-2α on certain target gene expression has been shown to differ completely between RCC cells and other types of cancer cells (17). These observations thus promoted us to examine how HIF-1α and HIF-2α regulate the expression of hTERT, a critical element for cellular immortalization and transformation, in different types of human cancers.

Based on the studies described above, we addressed our questions using RCC and glioma cells. Hypoxic treatment strongly induced HIF-1α and HIF-2α expression accompanied by increased levels of hTERT mRNA and telomerase activity in Caki-2 and TK-10 RCC cells with functional VHL. To discriminate the effect between HIF-1α and HIF-2α, we further did the following analyses. (a) A498 cells, lacking HIF-1α while constitutively expressing HIF-2α (23), were triggered to exhibit a strong induction of hTERT and telomerase expression when incubated under hypoxic conditions. More importantly, depletion of HIF-2α significantly attenuated hypoxia-mediated hTERT expression. This indicates that HIF-2α is involved in controlling hTERT transcription. (b) We indeed observed the HIF-2α occupancy on the hTERT promoter and its accumulation accompanied by enhanced recruitment of p300 and histone H3 acetylation in A498 cells treated with hypoxia. (c) Ectopic expression of either HIF-1α or HIF-2α strongly stimulated the hTERT promoter activity in A498 and Caki-2 cells. It is thus evident that both HIF-1α and HIF-2α up-regulate hTERT transcription in RCC cells with and without VHL inactivation.

Compared with RCC cells, glioma cells responded differently to HIF-1α and HIF-2α. Unexpectedly, HIF-2α was shown to inhibit the hTERT promoter activity and gene expression and to attenuate HIF-1α–induced up-regulation of hTERT transcription. In accordance with these results, simultaneous induction of both HIF-1α and HIF-2α in hypoxia-treated U251 cells failed to enhance hTERT mRNA expression. Indeed, HIF-1α and HIF-2α have been shown to have opposite effects on many other downstream target genes in RCC and other types of cancers (17). It is currently unclear how HIF-1α and HIF-2α function differently in regulating the transcription of hTERT and other target genes in different types of cancer cells. It is even more intriguing to document an opposing effect of HIF-2α on hTERT expression between RCC and glioma cells. We found that the enhanced accumulation of HIF-2α at the hTERT promoter occurred concomitantly with increased histone acetyltransferase p300 and H3 acetylation in hypoxia-treated A498 cells. Although HIF-2α was also found recruited to the hTERT promoter in U251 cells under a hypoxic condition, there were no detectable changes in p300 and histone H3 acetylation. It has been well defined that histone acetylation at the hTERT promoter plays key roles in the activation of the hTERT gene transcription (6, 35-38). Therefore, the differential capability to recruit histone acetyltransferases and thereby to induce histone acetylation by HIF-2α between A498 and U251 cells may provide a plausible explanation for different functional readouts of HIF-2α effects. However, it remains unclear why this paradoxical scenario happens. Further studies are certainly required to elucidate the issue.

When HIF-1α and HIF-2α were separately introduced into U251 cells, the former stimulated whereas the latter inhibited hTERT expression (Figs. 2B and 3B), which seems contradictory to a failure to enhance hTERT expression in U251 cells under a hypoxic environment (Fig. 1). However, HIF-1α and HIF-2α were both induced in the hypoxia-treated U251 cells, under which conditions HIF-1α and HIF-2α may counteract each other, and the stimulatory and inhibitory effects on the hTERT expression are thus neutralized. Consistent with this model, we indeed found that HIF-1α–mediated activation of the hTERT promoter was attenuated in the presence of HIF-2α in glioma cells, and moreover, p300 recruitment and histone H3 acetylation at the hTERT promoter region neither increased nor decreased when HIF-1α and HIF-2α were simultaneously induced by the hypoxic treatment in U251 cells.

In addition to their effect at transcriptional levels, hypoxia or HIF-1α is capable of enhancing telomerase activity via other pathways (10-14). Alternatively spliced hTERT mRNA contributes to telomerase regulation under certain settings, and it has been established that the full-length hTERT transcripts,
a was also found to interact with HIF-1 codon (ATG). The putative HREs in region a are indicated (16). Region b and locations of the ChIP PCR amplicons relative to the translational start codon (ATG). The putative HREs in region a are indicated (16). Region b and locations of the ChIP PCR amplicons relative to the translational start codon (ATG).

FIGURE 4. HIF-2α occupancy on the hTERT promoter, recruitment of p300, and changes in histone H3 acetylation in A498 and U251 cells under hypoxic conditions. A. Schematic presentation of the hTERT promoter and locations of the ChIP PCR amplicons relative to the translational start codon (ATG). The putative HREs in region a are indicated (16). Region b was also found to interact with HIF-1α according to Anderson et al. (10). B. The ChIP assay for the presence of HIF-2α, p300, and histone H3 acetylation at the hTERT promoter in hypoxia-treated A498 and U251 cells. The precipitated DNA was subject to PCR analysis using the primer pairs specific to the hTERT promoter regions a and b. Input is sonicated chromatin without immunoprecipitation, but reverse cross-linked and purified together with ChIP samples. HRE, hypoxia-responsive element; H and N, the cells were cultured under hypoxic and normal oxygen conditions, respectively. Ab-., no antibody controls. H3-Ac, the antibody against acetylated histone H3.

rather than any other variants, is translated into a functional protein required for active telomerase (5, 6, 24-30). The recent study has indicated that hypoxia drives preferential expression of the full-length hTERT mRNA in ovary cancer cells (10). In contrast to that finding, we observed an up-regulation of all the hTERT mRNA variants in both RCC and glioma cells in response to hypoxic treatment. Taken together, post-transcriptional regulation of hTERT expression is not a general mechanism behind hypoxia-mediated telomerase activation in tumor cells.

Hypoxia or HIF-1α has been shown to have opposite effects on hTERT expression in different cancer cells (10, 11, 13). Our present finding adds a further layer of complexity of the relationship between hypoxia and telomerase expression. The different roles for HIF-1α and HIF-2α in regulating hTERT expression in different types of cancers may reflect their general functions in these malignancies. HIF-2α has been shown to act as a tumor suppressor in malignant gliomas by promoting apoptosis of the tumor cells (19). Interestingly, it has been found that a decline in hTERT expression leads to apoptotic death of glioma cells (39). Therefore, it is likely that HIF-2α-mediated inhibition of hTERT expression may directly or indirectly contribute to apoptosis occurring in HIF-2α-overexpressing glioma cells. On the other hand, RCC cells with VHL inactivation require HIF-2α for their oncogenesis (21, 32-34). Given an essential role for telomerase in cell immortalization and transformation (1, 2), it seems reasonable that HIF-2α induces rather than inhibits hTERT expression in RCC cells. Our findings may have clinical implications in relation to antiangiogenesis for cancer therapy.

Materials and Methods

Cell Culture and Treatment

The cells used in the present study included renal cell carcinoma cell lines A498, TK10, and Caki-2 and malignant glioma cell lines U251, U373, and U563. The cells were maintained at 37°C in RPMI 1640 (Life Technologies) supplemented with 10% FCS, 2 mmol/L l-glutamine, and antibiotics (100 units/mL penicillin and streptomycin) under 5% CO2-95% air. For hypoxic treatment, the cells were either incubated under 1% O2 or treated with 150 μmol/L desferrioxamine (Sigma) for 8 to 10 h.

RNA Extraction and RT-PCR

We used the ULTRASPEC-II RNA kit (Biotec Lab-www) to isolate total RNA from all the cell lines. cDNA was synthesized using random primers (N6; Pharmacia). Expression of hTERT mRNA was analyzed by RT-PCR using different primers. The PCR primer sequences specific for all the variants of hTERT mRNA (accession no. AF015950) were 5’-CGGAAGAGTGTCAGAGGCAAA’-3’ and 5’-GGATGAAAGCGGATCTGGA-3’ (24, 29), and the resultant amplicon was 145 bp long. The amplification of the alternatively spliced hTERT mRNA was done with the primers 21645 (5’-GCCCTGAGCTGTACCTTGTGCA-3’) and 2620AS (5’-CGCAAACAGCTTCTCCATGTG-3’; refs. 24, 25, 29). This single primer set allowed detection of all differently spliced variants, including the full-length hTERT transcript simultaneously (25). All the above primer pairs cross intron/exon boundaries in the hTERT gene, and thus, the resultant PCR products do not represent genomic DNA contamination. RT-PCR for VEGF mRNA was done by running 24 cycles (95°C, 15 s; 62°C, 45 s; and 72°C, 45 s) with the following specific primer pair (forward: 5’-TTTCTCAGGTCTGCTT-3’; and reverse: 5’-TCTGCAATGTTGATGTGGGATC-3’). The primers for HIF-2α were forward 5’-AGGGGACGGATCATCAACC-3’; and reverse 5’-ATGGCCCTTGGCCATGGCAG-3’, and PCR conditions were 25 cycles with 95°C, 15 s; 62°C, 45 s; and 72°C, 45 s. The PCR products were subjected to electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized under UV light. The expression of β2-microglobulin mRNA was amplified in parallel as control for RNA loading and RT-PCR efficiency.

RTQ-PCR Analysis

RTQ-PCR was done using cDNA samples generated as described above, and the ABI Prism 7700 Sequence Detection System and ABI SYBR green PCR core reagents kit according to the manufacturer’s instructions (Applied Biosystems; ref. 40). The hTERT primers specific to the all mRNA variants as described above were used. PCR conditions for 40 cycles were 95°C for 10 min, 95°C for 15 s, and an annealing temperature of 60°C for 1 min. hTERT mRNA expression levels were calculated based on cycle threshold (Ct) values and normalized using the endogenous reference β2-microglobulin. For all the
RTQ-PCR analyses, the hTERT mRNA expression in controls or untreated cells was set as 1.

Assessment of Telomerase Activity
The cells with different treatments were subjected to CHAPS extraction, and a commercial Telomerase PCR ELISA kit (Roche Diagnostics Scandinavia AB) was applied for telomerase activity determination according to the manufacturer’s protocol. For each assay, 0.5 μg of protein was used, and 26 PCR cycles were done following the telomerase-primer elongation reaction. Under these conditions, the PCR amplification was maintained in a linear range, and levels of telomerase activity could be semiquantitatively determined based on our earlier studies (41).

Plasmids and Transfection
The HIF-1α and HIF-2α expression plasmids encoding constitutively active proteins, kindly provided by Dr. M.C. Simon (University of Pennsylvania School of Medicine, Philadelphia, PA), were described (18, 42). U251 and U373 glioma cells were transfected with either HIF-2α plasmid or the empty control vector using LipofectAMINE 2000 (Invitrogen) according to manufacturer’s protocol. After 48 h, the transfected cells were selected by incubating them in G418-containing medium for 10 days and then harvested for hTERT mRNA analyses.

RNA Interference
Chemical modified Stealth siRNA targeting HIF-2α and control siRNA were purchased from Invitrogen. The sequence used was 5'-CAGCAUCUUUGAUAGCAGU-3' as described (17). RNAi transfection was done on six-well plates using LipofectAMINE 2000.

Luciferase Activity Assay
The hTERT luciferase reporter construct (p181), harboring the core promoter sequence of the hTERT 5'-flanking region, was described previously (43) and kindly provided by Dr. S. Kyo (Kanazawa University, Japan). p181 and HIF-1α or HIF-2α expression vectors or pcDNA were transfected into the cells cultured in 24-well plates by using LipofectAMINE 2000. A *Renilla reniformis* luciferase-containing plasmid, which is under the control of the thymidine kinase (TK) promoter, was cotransfected to monitor the transfection efficiency. Luciferase activity in the cell lysates was determined by using a dual luciferase reporter assay system (Promega) 48 h post-transfection, and the hTERT promoter-driven firefly luciferase activity was normalized to the TK *Renilla* activity.

Western Blot
The cells with different treatments were harvested, and total cellular proteins were extracted with SDS lysis buffer (29). About 30 μg of the proteins were resolved in SDS-PAGE and transferred to an intracellulose membrane. The membranes were probed with the mouse monoclonal antibody against HIF-1α or HIF-2α rabbit polyclonal antibody (Novus Biologicals), followed by a secondary anti-mouse or rabbit horseradish peroxidase–conjugated immunoglobulin G, and developed with the enhanced chemiluminescence method (Amersham).

ChIP
ChIP assay was carried out as described previously (44, 45). The cells with different treatments were cross-linked by incubating them in 1% (vol/vol) formaldehyde-containing medium for 10 min at 37 °C and then sonicated to make soluble chromatin with DNA fragments between 200 and 1,000 bp. The antibodies against HIF-2α (Novus Biologicals), p300 (Santa Cruz Biotechnology), and acetylated histone H3 (Upstate) were used to precipitate DNA fragments bound by their corresponding elements. The omission of antibodies served as a negative control. The protein-DNA complex was collected with protein A-Sepharose beads (Upstate), eluted, and reverse cross-linked. Following a treatment with Protease K (Sigma), the samples were extracted with phenol-chloroform and precipitated with ethanol. The recovered DNA was resuspended in TE buffer and used for the PCR amplification with the following primer pairs: the hTERT proximal promoter (hTERT-a; refs. 44, 45): 5'CCAGGCGGGGCTCCACGATG-GAT-3' (forward) and 5'GGCTTCACAGCGGCGAGA3- (reverse); and upstream (hTERT-b) regions: 5'TCCCGTTACACGTCCTCCGGATT-3' (forward) and 5'CGTCTGTTGCCCGGAATCCA3- (reverse). The primers for glycer-aldehyde-3-phosphate dehydrogenase were described elsewhere (44, 45).

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References


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