Reactive oxygen species-mediated cyclin D1 degradation mediates tumor growth retardation in hypoxia, independently of p21^{cip1} and hypoxia-inducible factor

Ji-Hong Lim,[1] Yoon-Mi Lee,[1] Yang-Sook Chun[2,3] and Jong-Wan Park[1,3,4]

Departments of [1]Pharmacology and [2]Physiology, [3]Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea

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Cell growth arrest is an adaptation process for tumor survival in hypoxic environments. As proliferation is a very complicated and dynamic process, hypoxic growth arrest is not considered to be simply determined by a few molecules. Recently, several research groups have demonstrated that hypoxia-inducible factor (HIF)-1α plays a crucial role in hypoxia-induced cell-cycle arrest by inhibiting c-Myc and subsequently inducing p21^{cip1} expression. However, we found that hypoxic growth arrest could occur even in p21-null cancer cells, and addressed the p21-independent process of cell-cycle arrest. We show that cyclin D1 was downregulated in various cancer cell lines under hypoxic conditions, which was independent of p21 and HIF-1 and -2α expression. It was also found that cyclin D1 was destabilized by the ubiquitin–proteasome system and this degradation process was highly activated by hypoxia. Moreover, antioxidants prevented the hypoxic degradation of cyclin D1 and hydrogen peroxide destabilized cyclin D1 in normoxia. Finally, we demonstrated that ectopic expression of cyclin D1 rescued hypoxic growth arrest in both p21$^{+/+}$ and p21$^{-/-}$ HCT116 cells. Given the results, we here propose that reactive oxygen species-mediated cyclin D1 degradation contributes to tumor growth retardation in hypoxic environments. (*Cancer Sci* 2008; 99: 1798–1805)

Eukaryotic cell cycle progression is regulated tightly at various biological checkpoints by cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors.[1] Among the cyclin families, cyclin D1 plays an important role in regulating G_1–S transition. During G_1 phase, cyclin D1 is synthesized and bound with CDK4 and CDK6 in response to growth factor stimulation, thereby generating an active complex that phosphorylates and inactivates retinoblastoma protein. In terms of cyclin D1 regulation, early studies demonstrated that cyclin D1 expression is regulated at the transcriptional level. In addition, cyclin D1 expression is also controlled at the post-translational step, as cyclin D1 protein turnover is regulated by its ubiquitination and subsequent degradation through the proteasomes.[2–4] Indeed, several studies demonstrated that osmotic and oxidative stresses induce cell cycle arrest by destabilizing cyclin D1.[5,6]

Hypoxia not only stimulates angiogenesis and glycolysis, but also induces tumor growth retardation.[7] Previous studies have suggested that hypoxia inhibits cell entry into S phase to arrest cells at G_1 phase. G_1 arrest arises from hypoxia-inducible factor (HIF)-1α-mediated inhibition of c-Myc and subsequent induction of p21^{cip1}.[8,9] However, other studies have shown that hypoxia-induced cell-cycle arrest can occur even in p21-null cells,[10,11] and suggested that cell-cycle arrest could be mediated by mechanisms other than the HIF-1α–Myc–p21 axis. Cyclin D1 is a potential candidate responsible for hypoxic growth arrest because it was found to be downregulated under hypoxic conditions in human prostate and lung cancer cells.[12,13] However, little is known about the molecular mechanism underlying the hypoxic suppression of cyclin D1.

Our goal in the present study was to elucidate the regulatory pathways responsible for p21-independent cell-cycle arrest in hypoxia. In particular, we focused on the mechanism by which cyclin D1 is downregulated during hypoxia. We demonstrated that the ubiquitin–proteasome-dependent degradation of cyclin D1 and reactive oxygen species (ROS) production was stimulated concomitantly under hypoxic conditions. The hypoxic degradation of cyclin D1 was prevented by antioxidants and stimulated by pro-oxidants. We also confirmed that cyclin D1 degradation was responsible for p21-independent cell-cycle arrest in hypoxia. Based on these results, we propose that the ROS–cyclin D1 pathway partly contributes to tumor growth retardation in hypoxic environments.

Materials and Methods

Materials. MG132 were purchased from Alexis Biochemicals (Lausen, Switzerland), and lactacystin, *N*-acetylcystein (NAC), catalase, and 2′-7′-dichrolodihydrofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Culture media, fetal calf serum, and antibiotics were purchased from Invitrogen (Carlsbad, CA, USA). Anti-HIF-1α antiserum was generated in rabbits against amino acids 418–698 of human HIF-1α, as described previously.[14] Cyclin D1, β-tubulin, ubiquitin, hemagglutinin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HIF-2α and Flag antibodies were purchased from Novus Biologicals (Littleton, CO, USA) and Sigma-Aldrich, respectively.

Cell culture and hypoxic treatment. H1299 and A549 lung cancer cell lines and PC3 and LNCap prostate cancer cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). HCT116 (p21$^{-/-}$) and HCT116 (p21$^{+/+}$) colon cancer cell lines were generous gifts from Dr Deug Y. Shin (Dankook University College of Medicine, Cheonan, Korea). H1299, A549, PC3, and LNCap cells were cultured in RPMI-1640 medium and HCT116 cells in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, in a 5% CO_2 humidified atmosphere at 37°C. The oxygen tension in the incubator (Vision Sci, Seoul, Korea) was 20% (normoxic) or 0.5% (hypoxic).

[4]To whom correspondence should be addressed. E-mail: parkjw@snu.ac.kr

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Small interfering RNA, plasmids, and transfection. The synthesized small interfering RNA (siRNA) duplexes were obtained from Invitrogen. The siRNA sequences corresponded to nucleotides 360–384 (the coding region) of HIF-1α (Genbank accession no. NM_001530). cDNA of HIF-1α, HIF-2α (NM_001430), and cyclin D1 (NM_053056) were cloned by reverse transcription (RT)–polymerase chain reaction (PCR) using Pfu DNA polymerase, and inserted into the pcDNA-HA (hemagglutinin), Flag, or pcDNA expression vector by blunt-end ligation. The cyclin D1 promoter–luciferase reporter plasmid was constructed as described previously.[15] All plasmids were transfected into the cancer cell lines using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

Reporter assays. Cells were cotransfected with reporter plasmids and cytomegalovirus-β-gal plasmid using Lipofectamine 2000. The transfected cells were then allowed to stabilize for 48 h before being used in experiments. The cells were lysed and assayed for luciferase activity, and β-gal assays were carried out for normalization of transfection efficiency.

Semiquantitative RT-PCR. To quantify mRNA levels, we used a highly sensitive, semiquantitative RT-PCR method, as described previously.[15] Total RNA of hypoxia-treated lung cancer cells were isolated using Trizol (Invitrogen, Carlsbad, CA, US). Total RNA (1 μg) was reverse transcribed and the cDNA obtained was amplified over 18–20 PCR cycles in a reaction mixture containing 185 kBq [α-^{32}P]dCTP. PCR products were electrophoresed on a 4% polyacrylamide gel, and dried gels were autoradiographed. The sequences of the cyclin D1, vascular endothelial growth factor (VEGF)-A, aldolase-A, and β-actin primers were as described previously.[14,15]

Immunobloting and immunoprecipitation. Total proteins (30 μg) were separated on sodium dodecylsulfate–polyacrylamide gels, and blots were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TTBS) and then incubated overnight at 4°C with primary antibodies, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and visualized using an enhanced chemiluminescence reagent (GE Healthcare Bio-Science, Piscataway, NJ, USA). For immunoprecipitation, cell lysates of 1 mg were incubated with 1 μg anticyclin D1 and further incubated with 20 μL protein A/G-sepharose beads (GE Healthcare Bio-Sciences) for 4 h. Precipitated immunocomplexes were eluted in 2% sodium dodecylsulfate sample buffer and then subjected to immunoblotting.

Cell proliferation assay. Bromodeoxyuridine (BrdU) incorporation assays were done using a fluorescein isothiocyanate (FITC) BrdU flow kit purchased from BD PharMingen (San Diego, CA, USA). Briefly, after normoxic or hypoxic incubation, cells were treated with bromodeoxyuridine (BrdUrd) for 30 min at 3°C. After fixation and permeabilization, cells were treated with DNase and incubated with FITC-conjugated anti-BrdU (BD PharMingen). Total DNA was stained with 7-amino-actinomycin D (7-AAD). FITC and 7-AAD were detected using a FACStar flow cytometer (BD Biosciences, San Jose, CA, USA).

Determination of intracellular ROS levels. To measure intracellular ROS level, 30 μmol/L DCF-DA was used as a fluorescent probe.[16] Immediately after culture dishes were displaced from the normoxic or hypoxic chamber, this probe was loaded onto the dishes with Hank's balanced salt solution. To minimize changes in oxygen tension and temperature, the Hank's solution had been preincubated at 37°C in the normoxic chamber for normoxic or reoxygenated cells, or in the hypoxic chamber for hypoxic cells. After 20 min incubation with DCF-DA, cells were detached in a trypsin–ethylenediaminetetraacetic acid solution, pelleted by centrifugation, and resuspended in phosphate-buffered saline for FACS analysis. The oxidized form of DCF-DA, fluorescent dichlorofluorescein, was excited at 488 nm and detected at 530 nm.

Statistical analysis. Results are expressed as means and standard deviations using Microsoft Excel 2002 software (Microsoft, Redmond, WA, USA), and Student's *t*-test was used to compare BrdU incoporation, ROS levels, and reporter activities. All statistical tests were two-sided and *P*-values < 0.01 were considered to be significant.

Results

p21^{cip1}-independent cell growth inhibition in hypoxia. Under hypoxic conditions, p21 is upregulated at the transcriptional level due to c-Myc inhibition, leading to cell-cycle arrest.[8,9] To understand how much p21 contributes to the hypoxic growth arrest, we compared the cell proliferation of p21$^{+/+}$ and p21$^{-/-}$

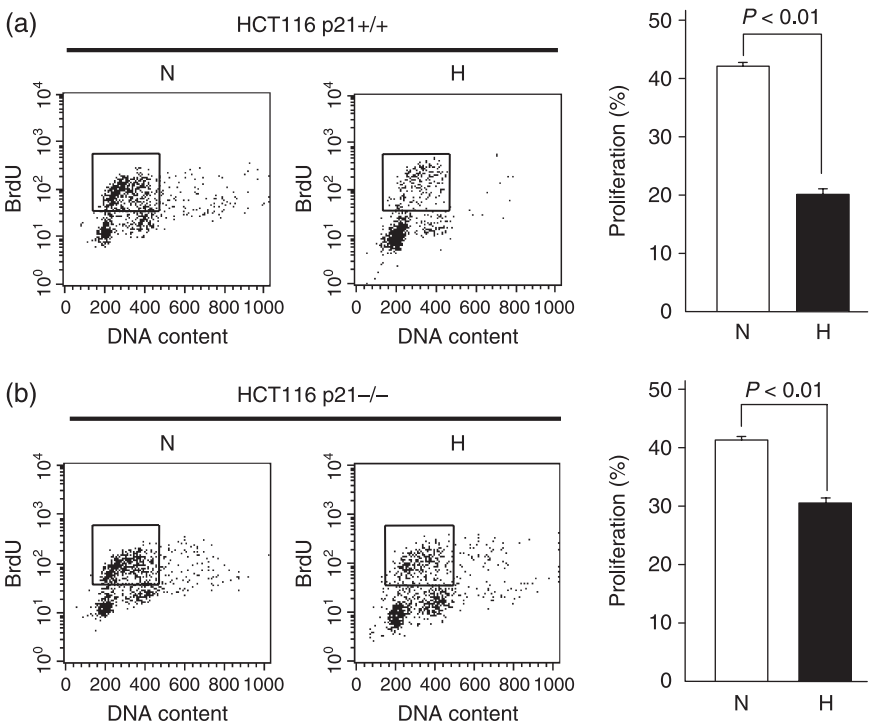

Fig. 1. p21-dependent and -independent cell growth inhibition in hypoxia. (a) HCT116 (p21$^{+/+}$) and (b) HCT116 (p21$^{-/-}$) cells were incubated under normoxic (N) or hypoxic (H) conditions for 24 h, and then treated with 10 μmol/L bromodeoxyuridine (BrdU) for 30 min. The incorporated BrdU was reacted with a fluorescein isothiocyanate-conjugated anti-BrdU antibody and total DNA was stained with 7-amino-actinomycin D. BrdU incorporation and DNA contents were analyzed by flow cytometry. (Left panel) Bivariate distributions of BrdUrd incorporation (*y*-axis) versus DNA contents (*x*-axis) were plotted. The boxes indicate BrdU-positive cells in S-phase. (Right panel) Cell proliferation was expressed as the percentage of BrdU-positive S-phase population to total cell number (means ± SD of five experiments).

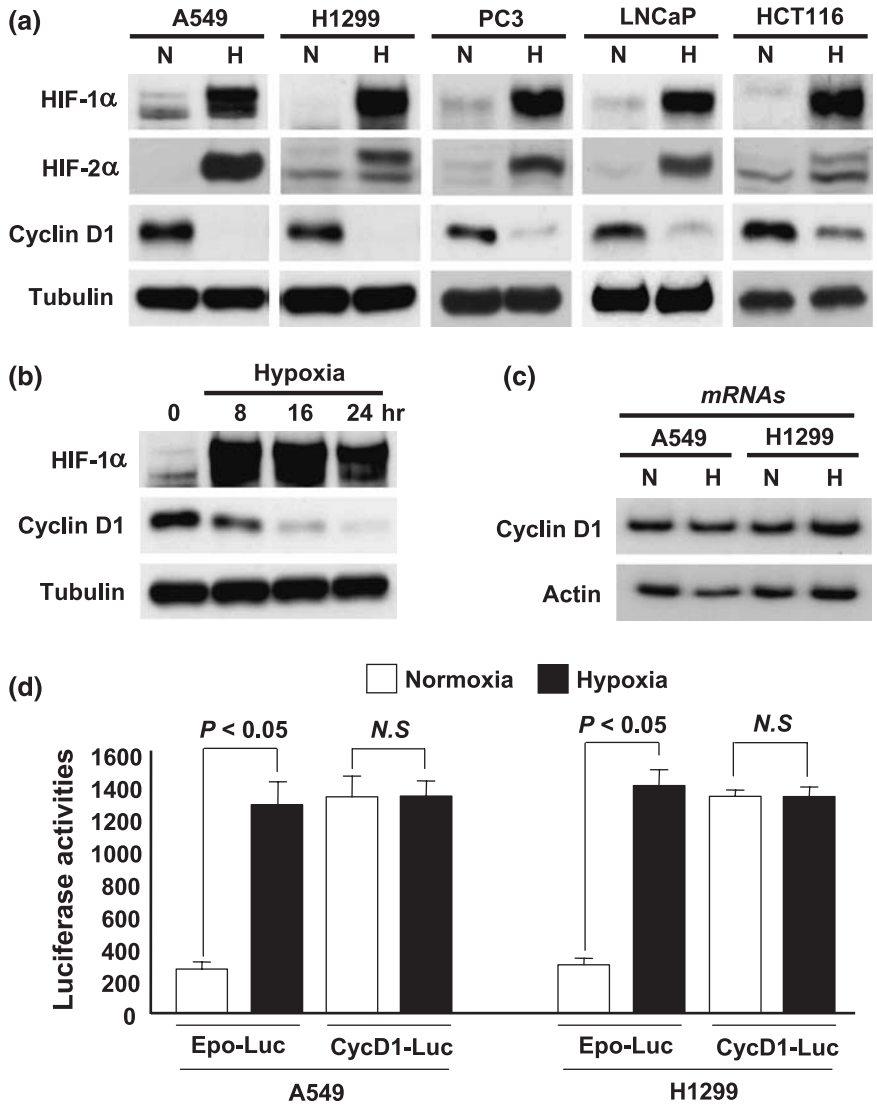

Fig. 2. Cyclin D1 is regulated post-transcriptionally by oxygen tension. (a) Hypoxia induces cyclin D1 downregulation. Five cell lines were incubated under normoxic (N) or hypoxic (H) conditions for 24 h. Hypoxia-inducible factor (HIF)-1α, HIF-2α, cyclin D1, and β-tubulin levels were analyzed by immunoblotting. (b) Time course of cyclin D1 expression. HIF-1α and cyclin D1 levels were analyzed in H1299 cells exposed to hypoxia for the indicated times. (c) No changes in cyclin D1 transcription. A549 and H1299 cells were incubated for 24 h and cyclin D1 mRNA levels were determined by reverse transcription–polymerase chain reaction and autoradiography. (d) No changes in cyclin D1 promoter activity. A549 and H1299 cells were transfected with 0.25 μg cycD1-Luc or 0.5 μg Epo-Luc plasmid. After 24 h-incubation in normoxia or hypoxia, luciferase activities were measured using a luminometer, and β-gal assays were done to normalize transfection efficiencies. Bars represent means ± SD of six experiments. NS, no significant difference in statistics.

HCT116 cells. In $p21^{+/+}$ cells, the population of proliferating cells was reduced to less than 50% of the normoxic control after 24 h hypoxia (Fig. 1a). In $p21^{-/-}$ cells, the proliferating cell population in hypoxia was partially recovered, but still decreased significantly compared to that in normoxia (Fig. 1b). Besides p21-mediated cell-cycle arrest, other mechanisms appear to contribute to hypoxia-induced cell growth inhibition. Next, we addressed p21-independent growth inhibition.

Cyclin D1 is downregulated under hypoxic conditions. As cyclin D1 is known as a key regulator of G_1–S transition, we analyzed the cellular levels of cyclin D1 in five different cancer cell lines. Under hypoxic conditions, both HIF-1α and HIF-2α were highly induced, which verifies that the cells normally responded to hypoxia. In contrast, cyclin D1 levels were noticeably diminished by hypoxia in all cell lines examined (Fig. 2a). We next examined the time course of the hypoxic suppression of cyclin D1 and found that cyclin D1 expression was reduced by >50% as early as 8 h after hypoxia (Fig. 2b). To examine whether cyclin D1 is downregulated transcriptionally by hypoxia, we analyzed cyclin D1 mRNA levels in two cell lines, and found that its levels were not affected by hypoxia (Fig. 2c). To further rule out the transcriptional regulation of cyclin D1, we analyzed the transcriptional activity of the cyclin D1 promoter using a luciferase reporter plasmid. As expected, the cyclin D1 promoter activity was not affected by hypoxia, whereas the erythropoietin enhancer activity (a marker for hypoxic gene induction) was strongly stimulated by hypoxia (Fig. 2d). These results suggest that cyclin D1 is downregulated by hypoxia but this is not attributable to transcriptional repression of the cyclin D1 gene.

Cyclin D1 is degraded via the ubiquitin–proteasome pathway under hypoxic conditions. Cyclin D1 expression is also known to be regulated by proteolysis via the ubiquitin–proteasome pathway.[17,18] To investigate whether hypoxia affects cyclin D1 protein stability, we examined the effects of the proteasome inhibitors MG132 and lactacystin, on cyclin D1 expression. In the presence of either MG132 or lactacystin, cyclin D1 did not decrease in hypoxia; rather, it increased (Fig. 3a,b), suggesting that hypoxia facilitates the proteasomal degradation of cyclin D1. In addition, we showed that cyclin D1 was strongly polyubiquitinated under hypoxic conditions (Fig. 3c). From these results, hypoxia is likely to stimulate cyclin D1 degradation through the ubiquitination–proteasome system.

Hypoxic downregulation of cyclin D1 is not related to HIF. We next examined whether cyclin D1 destabilization is dependent on HIF-α, because HIF-α governs a variety of hypoxic responses as the master protein.[19] Specifically, HIF-α has been reported to drive G_1 arrest by transactivating or derepressing the CDK inhibitor genes.[20] Despite successful knockdown of HIF-1α and HIF-2α,

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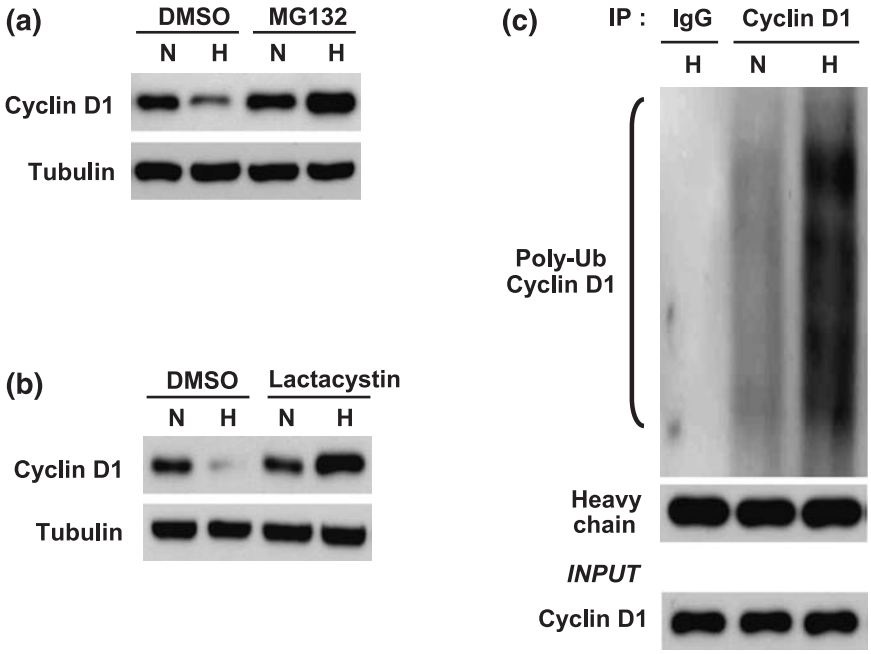

Fig. 3. Cyclin D1 is degraded by the ubiquitin–proteasome pathway. (a,b) The hypoxic downregulation of cyclin D1 is prevented by proteasome inhibition. H1299 cells were pretreated with proteasome inhibitor MG132 (30 μmol/L) or lactacystin (30 μmol/L) for 1 h, and then incubated under normoxic (N) or hypoxic (H) conditions for 24 h. hypoxia-inducible factor-1α, cyclin D1, and β-tubulin levels were determined by immunoblotting. (c) Cyclin D1 is ubiquitinated under hypoxic conditions. H1299 cells were pretreated with MG132 (30 μmol/L) for 1 h, and then incubated under normoxic or hypoxic conditions for 24 h. After cell lysates were immunoprecipitated with non-immunized rabbit serum or cyclin D1 specific antibody, ubiquitinated and total cyclin D1 were identified using ubiquitin (top) and cyclin D1 (bottom) antibodies. The heavy chain of IgG (middle) was used as a loading control. UB, ubiquitin.

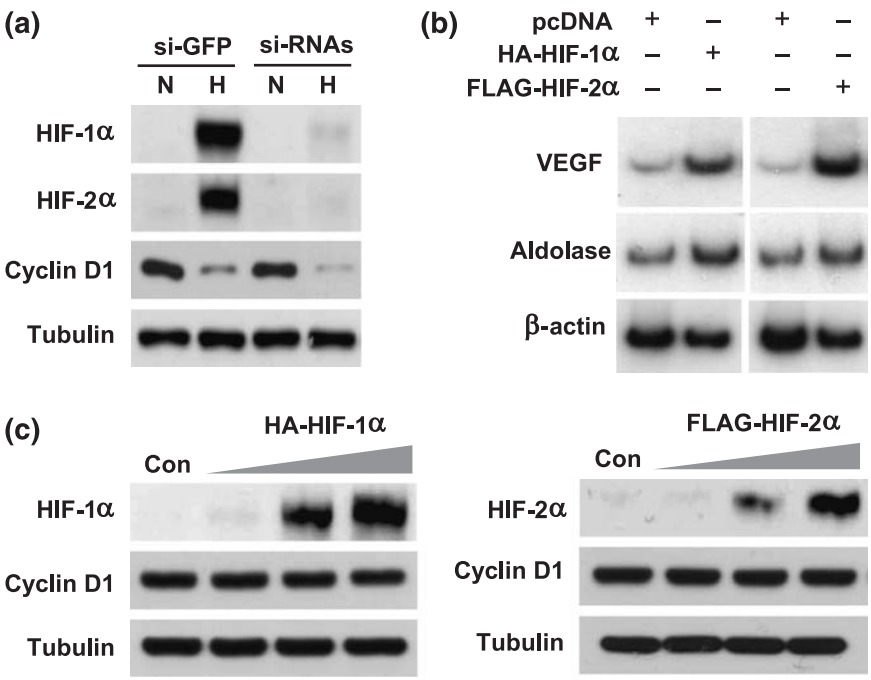

Fig. 4. Hypoxic downregulation of cyclin D1 is independent of hypoxia-inducible factor (HIF)-1α and HIF-2α. (a) Cyclin D1 expression in HIF-α knocked-down cells. H1299 cells were cotransfected with 40 nmol/L small interfering RNA (siRNA) against HIF-1α and HIF-2α. After 48 h, the cells were incubated under normoxic (N) and hypoxic (H) conditions for 24 h. The gene-silencing effects and hypoxic responses were evaluated by immunoblotting. (b) Functional activities of expressed HIF-1 and -2α. After transfection with 0.1 μg hemagglutinin (HA)-HIF-1α or Flag-HIF-2α plasmid, H1299 cells were incubated under normoxic conditions for 24 h. To validate whether expressed proteins were functional, vascular endothelial growth factor (VEGF)-A, aldolase-A, and β-actin mRNA were analyzed by semiquantitative reverse transcription–polymerase chain reaction and autoradiography. (c) Cyclin D1 expression in HIF-α overexpressing cells. H1299 cells were transfected with 0.1, 0.5, and 1 μg of plasmid (HA-HIF-1α or Flag-HIF-2α) and then incubated under normoxic conditions for 24 h. The gene-expression effects and hypoxic responses were evaluated by immunoblotting. GFP, green fluorescent protein; Con, control.

cyclin D1 was still suppressed under hypoxia (Fig. 4a). To confirm that the hypoxic suppression of cyclin D1 is independent of HIF-α, we examined whether cyclin D1 expression is regulated by expressed HIF-α. Given that both HA-HIF-1α and Flag-HIF-2α expression induced VEGF and aldolase mRNA, it is verified that both proteins are functional in gene transcription (Fig. 4b). Also, even when these proteins were expressed, cyclin D1 levels were not altered (Fig. 4c). Therefore, the hypoxic degradation of cyclin D1 is unlikely to be mediated by HIF-1α or HIF-2α at least in our experimental settings.

Reactive oxygen species mediate the hypoxic downregulation of cyclin D1. We next examined the possibility that ROS mediates the hypoxic downregulation of cyclin D1, because oxidative stress is known to cause growth arrest in various mammalian cells. Indeed, it was recently reported that oxidative stress causes cyclin D1 destabilization in human endothelial cells.[6] Moreover, it was also reported that hypoxia stimulates mitochondria to generate ROS, subsequently activating signaling pathways that promote cancer cell survival and tumor growth.[21] To induce oxidative stress in A549 and H1299 cells, we administered various concentrations of H_2O_2 to the culture media and found that cyclin D1 was downregulated by oxidative stress (Fig. 5a). Moreover, in both HCT116 ($p21^{+/+}$) and HCT116 ($p21^{-/-}$) cells, cyclin D1 was found to be downregulated by hypoxic or oxidative challenge (Fig. 5b). In addition, the DCF fluorescence peak was found to be right-shifted by hypoxia, indicating that intracellular ROS levels increased during hypoxia (Suppl. Fig. S1A and B). The ROS-dependent fluorescence of DCF-DA was verified using the antioxidant NAC. The differences in ROS levels are summarized in Figure 5c. Furthermore, compared to hypoxia alone,

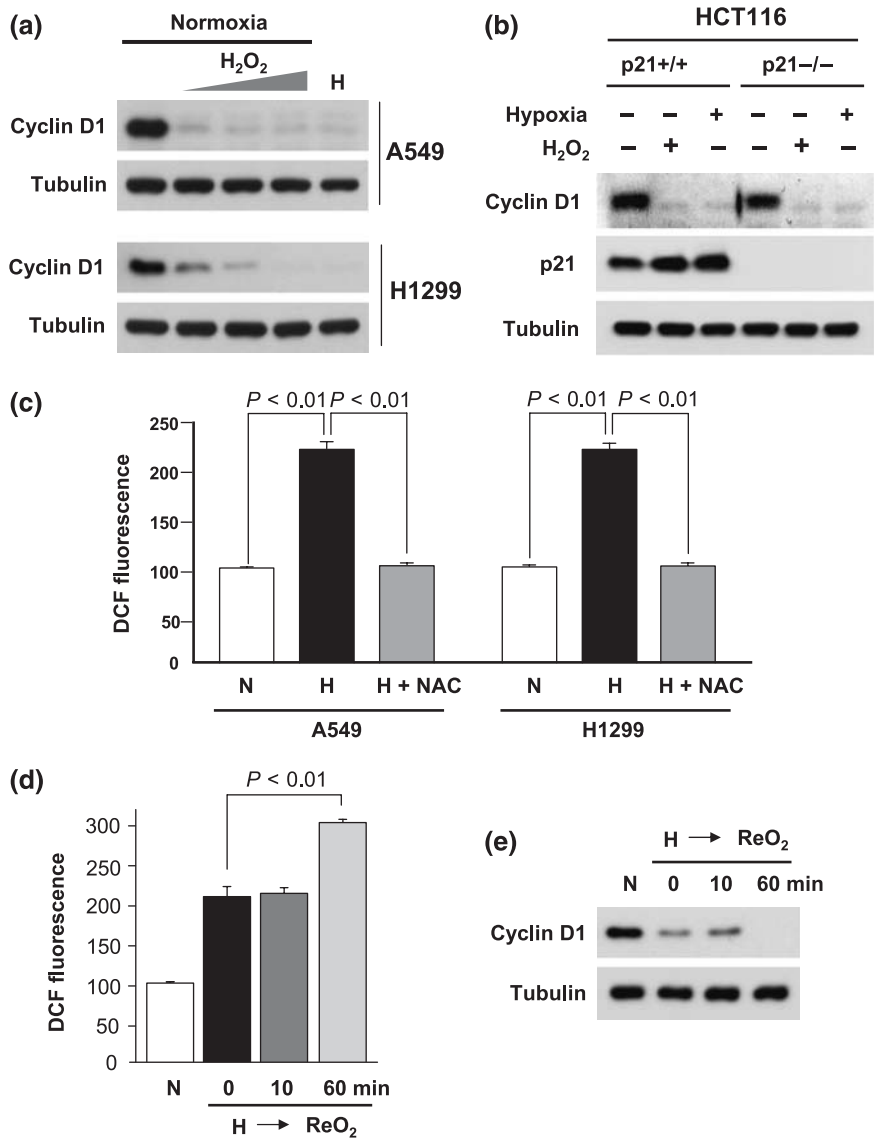

Fig. 5. Reactive oxygen species (ROS) mediate hypoxic cyclin D1 downregulation. (a) Cyclin D1 reduction by oxidative stress. H1299 and A549 cells were treated with H_2O_2 (50, 100, and 200 μmol/L) in a serum-free medium for 24 h. Cyclin D1 and β-tubulin proteins levels were determined by immunoblotting. (b) p21-independent regulation of cyclin D1. HCT116 ($p21^{+/+}$) and HCT116 ($p21^{-/-}$) cells were subjected to hypoxia or oxidative stress (H_2O_2) for 24 h, and cyclin D1 and p21 expressions were determined by immunoblotting. (c) Intracellular ROS levels in hypoxia. H1299 and A549 cells were incubated under normoxic (N) or hypoxic (H) conditions for 24 h, or pretreated with *N*-acetylcystein (NAC) (5 mmol/L) 1 h prior to hypoxic incubation. Cells were treated with DCF-DA for 20 min, and harvested to determine ROS levels by using flow cytometry. Intracellular ROS levels were determined by measuring the fluorescence intensity at Ex = 485 nm and Em = 530 nm. Intracellular ROS levels (means ± SD of five experiments) were expressed as percentages of DCF fluorescence intensity to total cell numbers. (d) Intracellular ROS levels after reoxygenation. After 24 h hypoxia, H1299 cells were transferred to the 20% O_2/5% CO_2 incubator for reoxygenation challenge. The cells were harvested at the indicated time points to analyze ROS levels. (e) Cyclin D1 levels after reoxygenation. H1299 cells were subjected to hypoxia and reoxygenation, as described in (d), and harvested to immunoblot cyclin D1.

posthypoxic reoxygenation further stimulated ROS production (Fig. 5d; Suppl. Fig. S1C) and suppressed cyclin D1 expression (Fig. 5e).

Antioxidants rescue cyclin D1 expression in hypoxia. To test the hypothesis that ROS destabilize cyclin D1, we examined the effects of NAC and catalase on cyclin D1 expression in hypoxic H1299 or HCT116 ($p21^{-/-}$) cells. Both antioxidants prevented the hypoxic downregulation of cyclin D1 in a dose-dependent manner (Fig. 6a). Furthermore, we found that hypoxia-induced polyubiquitination of cyclin D1 was prevented by NAC (Fig. 6b). These results suggest that cyclin D1 is ubiquitinated and degraded under hypoxic conditions via a ROS-mediated pathway, independently of p21.

Cyclin D1 is involved in growth arrest during hypoxia. What is the implication for the hypoxic suppression of cyclin D1? It is expected that cyclin D1 suppression is linked with cell-cycle inhibition in hypoxia, and that this cell-cycle inhibition is irrelevant to p21 induction. To test this possibility, we examined the effects of cyclin D1 expression on cell proliferation in $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells (Fig. 7a). As a result, cyclin D1 compensation significantly rescued cell proliferation inhibited by hypoxia in both cell lines (Fig. 7b). Similar findings were observed in A549 cells, as shown in Supplementary Figure 2. These results suggest that cyclin D1 suppression contributes to p21-independent growth inhibition under hypoxic conditions. However, it should be emphasized that hypoxia-induced growth retardation was not fully recovered by cyclin D1 overexpression. This also suggests that hypoxic growth retardation is mediated via complicated processes, including p21 induction and cyclin D1 suppression.

Discussion

Hypoxia-induced growth arrest is considered an essential response for cell survival in hypoxia because it gives cells a break to repair damaged DNA and to adapt to metabolic changes. Therefore, elucidating its molecular mechanisms might be important in understanding tumor biology. Previously, it was reported that hypoxia inhibits G_1–S transition by inducing p21 through c-Myc inhibition and by inhibiting E2F through retinoblastoma dephosphorylation.[7] However, several reports have shown that hypoxic G_1 arrest can occur even in p21-null cells, and indeed we here confirmed p21-independent cell-cycle arrest using a HCT116 ($p21^{-/-}$) colon cancer cell line. Furthermore, we found that ROS mediate the ubiquitination and proteasomal degradation of cyclin D1 under hypoxic conditions and in turn induce G_1 arrest irrespective of p21 induction.

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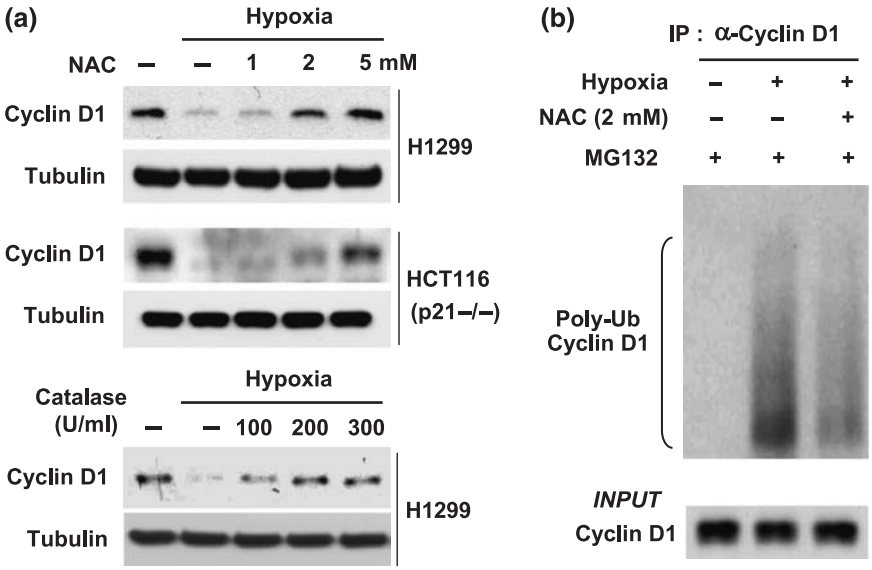

Fig. 6. Cyclin D1 recovery by antioxidants. (a) Effects of antioxidants on cyclin D1 expression. H1299 or HCT116 ($p21^{-/-}$) cells were pretreated with the antioxidants *N*-acetylcystein (NAC) (1, 2, and 5 mmol/L) and catalase (100, 200, and 300 units/mL) for 1 h, and then incubated under normoxic or hypoxic conditions for 24 h. (b) Effect of NAC on cyclin D1 ubiquitination. H1299 cells were pretreated with NAC (2 mmol/L) and MG132 (10 μmol/L) for 1 h, and then incubated under normoxic or hypoxic conditions for 24 h. After cell lysates were immunoprecipitated with cyclin D1-specific antibody, ubiquitinated and total cyclin D1 were identified using ubiquitin (upper panel) and cyclin D1 (lower panel) antibodies. Ub, ubiquitin.

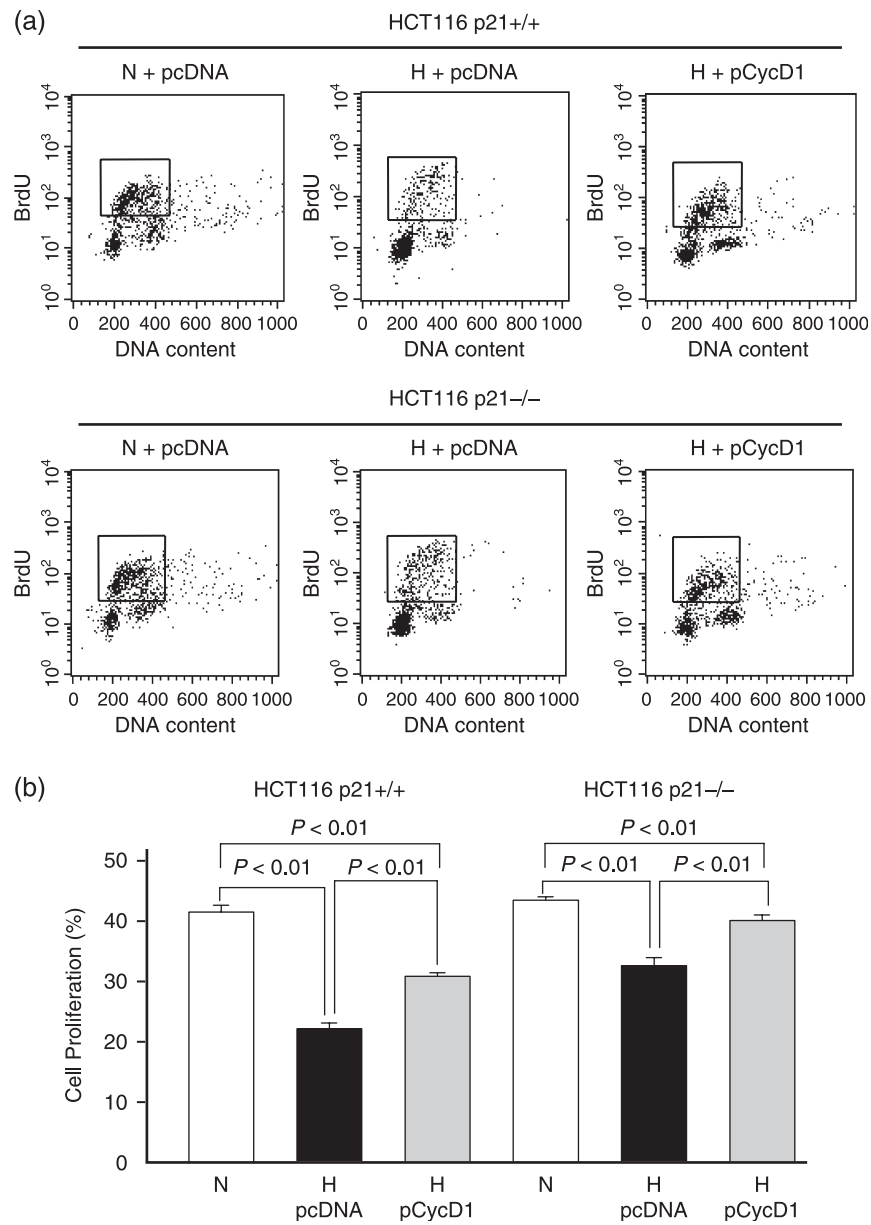

Fig. 7. Cyclin D1 expression rescues cell proliferation in hypoxia. (a) HCT116 ($p21^{+/+}$) and HCT116 ($p21^{-/-}$) cells were transfected with 2 μg empty vector (*pcDNA*) or cyclin D1 expression vector (*pCycD1*). After a 48-h stabilization period, the cells were incubated under normoxic (N) or hypoxic (H) conditions for 24 h. Cell proliferation was analyzed by bromodeoxyuridine (BrdU) incorporation. (b) Cell proliferations are expressed as percentages of BrdU-positive S-phase populations to total cell numbers. Bars represent means ± SD of five experiments.

Under hypoxic conditions, HIF-1α is induced acutely and inhibits the cell cycle-promoting activity of c-Myc. Mechanistically, HIF-1α disorganizes the β-catenin–Tcf4 complex, which determines transcription of the *c-Myc* gene, by interacting directly with β-catenin, resulting in downregulation of c-Myc.[9] Furthermore, HIF-1α disorganizes the c-Myc–Max complex by interacting directly with Max, resulting in inactivation of c-Myc.[8] Therefore, c-Myc can no longer repress the p21 promoter under hypoxic conditions, resulting in p21 upregulation, which in turn induces G_1 arrest. In addition to c-Myc, cyclin D1 could be involved in the cell-cycle arrest induced by HIF-1α. As the β-catenin–Tcf4 complex also participates in cyclin D1 expression, HIF-1α can reduce cyclin D1 levels under hypoxic conditions by inhibiting Tcf4.[9] Briefly, hypoxic G_1 arrest is initiated by HIF-1α stabilization and mediated by p21 induction and cyclin D1 suppression. Therefore, even in $p21^{-/-}$ cells, the cell cycle can be arrested by cyclin D1 suppression. In the present study, we examined whether or not cyclin D1 expression is regulated by HIF-1α. However, cyclin D1 expression was neither rescued by HIF-1α knockdown nor inhibited by HIF-1α overexpression. Moreover, cyclin D1 expression was not affected by HIF-2α knockdown or overexpression. Therefore, HIF-α proteins are unlikely to be responsible for the hypoxic suppression of cyclin D1, at least in our experimental settings. Instead, we here propose the ROS-dependent cyclin D1 degradation pathway as the mechanism underlying the p21-independent, HIF-independent cell-cycle arrest in hypoxia.

Cyclin D1 is present at low levels in quiescent cells, but highly induced transcriptionally by c-Myc, activator protein-1 (AP-1), or nuclear factor-κB activation.[22,23] Cyclin D1 levels are regulated dynamically, depending on the particular phase of the cell cycle.[22] That is, its levels begin to rise early in G_1, continue to accumulate during G_1, and then decline rapidly in the G_1–S transition phase. The cyclin D1 decline is initiated by glycogen synthase kinase (GSK)3-dependent phosphorylation and nuclear export, and developed by proteasomes in the cytoplasm.[2] Irrespective of the cell cycle, cyclin D1 degradation is also induced by various stresses, including growth factor deprivation,[4] osmotic stress,[5] ultraviolet irradiation,[24] and oxidative stress.[6] In this respect, hypoxia is another stress to induce cyclin D1 degradation. In terms of the mechanism underlying cyclin D1 degradation, we here propose that hypoxia-induced ROS stimulate the proteasomal degradation of cyclin D1. This conclusion is drawn from the results showing cyclin D1 degradation by H_2O_2, reduced cyclin D1 ubiquitination and degradation by antioxidants, and ROS enhancement in hypoxia. As to the extent of our knowledge, the role of ROS in cyclin D1 degradation has seldom been investigated; we therefore expect that such findings are original. However, we did not clarify the exact mechanism by which ROS stimulates the ubiquitination of cyclin D1.

Because dissolved O_2 is the essential substrate for ROS generation, it is expected that ROS levels are reduced in anoxia. However, ROS production in hypoxia seems to be up to the oxygen tension. Indeed, there have been many reports demonstrating that ROS production is stimulated during hypoxia.[25,26] Mechanistically, hypoxia alters the lipid–protein structures of mitochondrial inner membranes, which in turn induces the leakage of electrons from semiubiquinones in mitochondrial complex III. The leaked electrons produce ROS by reducing O_2 directly and this non-enzymatic process can occur even at low levels of O_2.[27] Likewise, intracellular ROS levels were elevated in A549 and H1299 cells exposed to hypoxia. Furthermore, we found that hypoxia-generated ROS mediated cyclin D1 ubiquitination and subsequent degradation through the proteasomes. Next, we examined the effects of reoxygenation on ROS production and cyclin D1 expression. It is well known that ROS production is stimulated at an early phase of reoxygenation after hypoxia.[28] As expected, ROS levels in H1299 cells were further increased at reoxygenation phase versus hypoxia phase. Inversely, cyclin D1 levels were further decreased after reoxygenation. These results also support our hypothesis regarding ROS-dependent cyclin D1 reduction.

How do ROS induce the ubiquitination and degradation of cyclin D1? Based on two recent reports, it is speculated that ROS provoke Ca^{2+} release from the endoplasmic reticulum, and then activate calcium- and calmodulin-dependent kinases to phosphorylate cyclin D1, leading to the ubiquitination of cyclin D1.[6,29] The report that cyclin D1 ubiquitination is regulated by phosphorylation of Thr286 and Thr288 residues supports this possibility.[18] However, it is emphasized that all of the previous results were normoxic events. The mechanism by which ROS mediate the hypoxic ubiquitination of cyclin D1 remains to be investigated in future studies.

In conclusion, ROS-mediated cyclin D1 degradation is likely to contribute to cell-cycle arrest under hypoxic conditions. During hypoxia, many proteins required for blood circulation, energy supply, and survival are induced. Forced cell proliferation in hypoxia might ultimately result in maladaptation and cell death. In this respect, the cell-cycle control mechanism may provide tumor cells with sufficient time for adaptation. Also, tumors that successfully adapt to hypoxia become more aggressive and produce poor clinical outcomes. Hence, our results may provide a better understanding of hypoxia-induced resistance to cancer therapy, and furthermore give information on useful targets for cancer therapy.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. FACS analyses for ROS levels. A549 (A) and H1299 (B) cells were incubated under normoxic or hypoxic conditions for 24 h, or pretreated with NAC (5 mM) 1 h prior to hypoxic incubation. For reoxygenation, H1299 cells subjected to 24 h hypoxia were transferred to the aerobic chamber and incubated for 10 or 60 min (C). Cells were treated with DCFH-DA for 20 minutes, and harvested to determine ROS levels by using flow cytometry. Intracellular ROS levels were determined by measuring the fluorescence intensity at Ex = 485 nm and Em = 530 nm.

Fig. S2. Cyclin D1 expression rescues A549 cell proliferation in hypoxia. (A) A549 cells were transfected with 2 mg of empty vector (*pcDNA*) or cyclin D1 expression vector (*pCycD1*). After a 48 h stabilization period, the cells were incubated under normoxic or hypoxic conditions for 24 h. Cell proliferation was analyzed by BrdU incorporation. (B) Cell proliferations are expressed as percentages of BrdU-positive S-phase populations to total cell numbers. Bars represent means±SDs of 5 experiments.